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An autoregulatory loop controls the expression of the transcription factor NF-Y

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ABSTRACT

The heterotrimeric NF-Y complex is a pioneer factor that binds to CCAAT-genes and regulates their transcription. NF-Y cooperates with multiple transcription factors and co-regulators in order to positively or negatively influence gene transcription. The recruitment of NF-Y to CCAAT box is significantly enriched in cancer-associated gene promoters loci and positively correlates with malignancy. NF-Y subunits, in particular the DNA-binding subunit NF-YA and the histone-fold subunit NF-YC, appear overexpressed in specific types of cancer.

Here we demonstrate that NF-Y subunits expression is finely regulated through transcriptional and post-translational mechanisms thus allowing control over basal expression levels. NF-Y negatively regulates the transcription of the genes encoding for its subunits. DNA pull-down/affinity purification assay coupled with Mass Spectrometry identified putative co-regulators, such as Lamin A, involved in NF-YA gene transcription level. We also evidentiate how the stability of the complex is severely affected by the absence of one subunit.

Our results identified for the first time one of the mechanisms responsible for NF-Y expression, which may be involved in the aberrant expression and activity observed in tumor cells and other pathological conditions.

1. Introduction

The mammalian CCAAT-box transcription factor (TF) NF-Y is composed by three subunits, NF-YA, NF-YB and NF-YC. The heterodimerization of NF-YB and NF-YC histone-fold subunits is a prerequisite for NF-YA association into the complex. The whole complex is able to bind specifically to CCAAT-boxes, which occur at 30% of eukaryotic promoters and enhancers, prevalently in tissue specific ones [1,2].

NF-Y subunits are evolutionarily strongly conserved in all eukaryotes [[3,4]. Although NF-Y is considered a ubiquitous TF, NF-YA has a tissue specific expression pattern and its mRNA levels are relatively constant in proliferating and differentiated cells [5,6], with the exception of adult skeletal muscle and heart tissues [7,8]. Consistently, NF-YA is present in proliferating myoblasts, but its expression is progressively lost during cells differentiation [7]. In contrast, NF-YA is not expressed in freshly isolated monocytes but it is synthesized during the maturation process [9]. NF-YA displays differential expression throughout the cell cycle, with high levels at the onset of S phase decreasing in G2/M [5]. The modulation of NF-YA protein expression is mediated by the ubiquitin–proteasome degradation system: p300-mediated acetylation at the C-terminus prevents, in part, its ubiquitylation and ubiquitin-dependent proteolysis [10].

Two major alternative splice variants of NF-YA have been described NF-YAs and NF-YAl. The short isoform (NF-YAs) lacks a 28-amino acid region within the NF-YA Glutamine-rich domain [11]. Relative NF-YAs and NF-YAl expression levels vary in different cell types. A switch in their abundance was documented during the differentiation of mouse ES cells and during somatic cells reprogramming into pluripotent stem cells [12,13]. Noticeably, while NF-YAl increases through mouse and human embryonic cell differentiation, NF-YAs levels are reduced. Also in human hematopoietic stem cells, NF-YAs has a demonstrated role in the maintenance of stemness [14].

Similarly, NF-YC subunit has different isoforms arising from alternative spliced transcripts translated into 37, 48 and 50 kDa polypeptides. The expression levels of specific NF-YA and NF-YC isoforms are strongly correlated, suggesting the presence of variously composed NF-

Abbreviations: TF, transcription factor; MS, Mass Spectrometry; ChIP, Chromatin ImmunoPrecipitation; shRNA, Short Hairpin RNA

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Y complexes [15].

NF-Y activity is essential for cell proliferation and viability, thus the deletion of both NF-YA alleles triggers early mouse embryo lethality [16]. NF-Y controls the expression of numerous cell cycle-regulated and pro/anti-apoptotic genes by binding to their proximal promoter elements [17–19]. The activity of NF-Y has been associated to the transformation process: systematic studies of deregulated pathways in different types of cancers identified NF-Y as one of the transcription factors orchestrating key transcriptional changes [20–22]. Significant association between NF-Y target genes expression and poor prognosis has been described in breast, lung, multiple myeloma and renal cancers [23]. In low grade epithelial ovarian cancers (EOCs), the pattern of NF-YAs isoform expression and Lamin A correlates with tumor aggressiveness [24]. In addition, NF-YC has been recently listed among potential oncogenes since NF-YC inactivation completely abolished choroid plexus carcinoma (CPC) initiation and maintenance [25] and it is differentially expressed in human glioma samples, where it activates cell proliferation [26].

We previously demonstrated that specific inactivation of NF-Y subunits leads to different cell cycle defects: NF-YA loss triggers S-phase defects and apoptosis, while NF-YB and NF-YC depletion delays the G2/M progression [18]. We also observed that NF-YA inactivation results in increased NF-YB expression, therefore we decided to investigate the role of NF-Y in the transcriptional and non-transcriptional regulation of its own subunits.

In this study, we show that a negative transcriptional feedback controls the expression of genes encoding for NF-Y subunits. NF-Y appears directly associated to NF-YA, NF-YB and NF-YC genes promoters and works as a transcriptional repressor. We used Mass Spectrometry (MS) to identify proteins isolated on the NF-YA regulatory region by a DNA pull-down strategy, and we demonstrate that Lamin A acts as a negative regulator of NF-YA gene transcription. Ultimately, we observed that the association of the NF-YB/NF-YC subunits is fundamental for the complex stability against degradation.

Overall, our data highlight the importance of controlled expression of NF-Y subunits for a proper regulation of cell proliferation and identify mechanisms potentially involved in NF-Y complex aberrant activation in cancer cells.

2. Materials and methods

2.1. Cell culture, treatments and lentiviral transduction

Human colon carcinoma HCT116 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM), supplemented with 10% fetal calf serum (FCS). Human hepatic fetal epithelial WRL-68 cells, immortalized human keratinocyte HACAT cells, human cervical cancer Hela cells, human colorectal adenocarcinoma HT-29 and SW-480 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% FCS. Immortalized prostatic epithelial PNT1A cells were cultured in RPMI medium additioned with 10% FCS. Protein degradation was inhibited through administration to cell culture media of 1 μM *Z*-Leu-Leu-Leu-H (MG132; Sigma-Aldrich), 5 μM N-Ac-Leu-Leu-norleucinal (LLnL; Sigma Aldrich) and 50 μM chloroquine (CQ) for 18 h.

NF-YA, NF-YB and NF-YC inactivation was obtained by lentiviral infection of the above-mentioned cells with pLKO.1 shRNA lentiviral particles (MOI = 4) and harvested 48 h post-infection, as previously described [18,27]. siRNA transfections were performed as previously reported [28]. NF-YA-DN overexpression was induced by lentiviral delivery of pCCL-NF-YA-DN particles (MOI = 4) for 48 h [8].

2.2. Immunoblots

Whole cell protein extracts were prepared by resuspending cells into 1× SDS sample buffer (25 mM TrisHCl pH 6.8, 1.5 mM EDTA, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.0025% Bromophenol blue). Cytoplasmic and chromatin-enriched extracts were prepared as previously described [27,29]. For immunoblotting equivalent amounts of extracts were resolved by SDS-PAGE, electrotransferred to PVDF membrane (GE Healthcare) and immunoblotted with the following primary antibodies: anti-H3 (sc-8654, Santa Cruz), anti- tubulin (T6074, Sigma-Aldrich), anti-actin (sc-1616, Santa Cruz), anti-NF-YA (A302–105, Bethyl lab), anti-NF-YB (Pab001, GeneSpin), anti-NF-YC (gift of Prof. R.Mantovani), anti- LaminA/C (sc-7292×, Santa Cruz).

2.3. RNA extraction and qRT-PCR analysis

RNA was extracted from cells using RNeasy kit (Qiagen) and reversed transcribed with a Moloney murine leukemia virus reverse transcriptase (Promega). Quantitative real-time PCRs were performed using DyNAmo ColorFlash SYBR Green Master mix (Thermo Scientific) on a Roche Light Cycler 480 II Real-time PCR instrument, with the following oligonucleotides: h-gACTIN (F gccaacagagagaagatgactc; R agaggcgtagagggacagc), hNF-YA (F ggaggccagctaatcacatc; R gccgagactcatgcaggtat), hNF-YB (F aggtgccatcaagagaaact; R tgttgttgaccgtctgtggt), hNF-YC (F agtggcactggacagaccat; R cctgatacaggctgggctaa). Relative fold change enrichments of transcripts were calculated with the formula 2–(ΔΔCt), where –(ΔΔCt) = –[(Ct*target* − Ct*gACTIN*) shNFY − (Ct*target* − Ct*gACTIN*) shCTR].

2.4. Plasmids and transfections

For each point, 150.000 HCT116 cells were seeded into 24-well plates and transfected after 24 h using Lipofectamine 2000 (Invitrogen), according to the protocol provided by the manufacturer. Cells were recovered 24 h after transfection and resuspended in lysis buffer (1% TritonX 100, 25 mM GlyGly pH 7.8, 15 mM MgSO4, 4 mM EGTA pH 8) for detection of luciferase activity. The results were normalized to protein concentration (Bradford reagent, Sigma Aldrich) and % GFP positive cells (measured by flow cytometric analysis). Three independent transfections were performed. The minimal human NF-YA promoter reporter construct, which contains the region spanning from −272 to +189 from NF-YA transcription start site, was generated by digestion of the previously described pGL3b-NF-YA promoter vector [15] with *Sma*I and *Pvu*II and subsequent re-legation. For transient overexpression of the NF-Y heterotrimer, 50 ng of each pSG5 expression vector encoding for murine NF-YA, NF-YB and NF-YC 37 kDa subunits were co-transfected with 150 ng of the minimal NF-YA promoter luciferase construct and 100 ng of pCMV-GFP. For the overexpression of the NF-YA dominant negative mutant, 100 ng of pSG5-NF-YA-DN vector was transfected together with NF-YA promoter and pCMV-GFP. All the pSG5 encoding vectors have been previously described [15,30]. Mouse NF-YA cDNA was cloned into pSG5 via *Eco*RI/*Bgl*II, NF-YB via EcoRI/BglII, NF-YC via *Kpn*I/*Bam*HI restriction enzymes. pCMV3-LMNA expression plasmid was purchased from Sino Biological Inc. (HG12058) and the corresponding pCMV3-Empty vector was generated by removing the coding region via *Hin*dIII and *Xba*I digestion. Transient protein overexpression versus basal levels was verified by western blot.

2.5. Chromatin immunoprecipitation (ChIP)

Chromatin was prepared 48 h post-shRNA infection and ChIPs were performed as previously described [18]. 4 μg of the following antibodies were added to each IP and incubated overnight at 4 °C: anti-NF-YA (A302–105, Bethyl lab), anti- LaminA/C (sc-7292×, Santa Cruz), anti-Histone H4Ac (06–866, Millipore), anti-NF-YB (Pab001, GeneSpin), anti-Histone H3K4me3 (39,159, Active Motif), anti-Histone H3K56ac (Active Motif, 39,281), anti-TFIID (TBP) (sc-204, Santa Cruz). Immunoprecipitated DNA was resuspended in TE buffer, and real-time PCRs were performed using DyNAmo ColorFlash SYBR Green Master mix (Thermo Scientific,) on a Roche Light Cycler 480 II Real-time PCR instrument, with the following primers: hNF-YA (F tccccttttgttcgggttc; R

attggctcctcacactcacc), hNF-YB (F gttccttcgcagccatttt; R gcgagacacaaacctccaat), hNF-YC P1 (F gacctggcaccttattggac; R cctctcttccccttaaagc), hSAT CHR11 as control for non-specific interactions (F ggcgaccaatagccaaaaagtgag; R caattatcccttcggggaatcgg). Results are presented as % of immunoprecipitated DNA or as Relative fold change of immunoprecipitated DNA in shNF-YA versus shCTR cells. The % of immunoprecipitated DNA was calculated with the formula 2-(ΔCt), where $-\Delta Ct = -(CtIP - CtINPUT)$ and the Relative fold change was calculated with the formula 2-(ΔΔCt), where $-\Delta\Delta Ct = -[(CtIP - CtINPUT)shNFYA - (CtIP - CtINPUT)shCTR]$.

2.6. DNA pull-down/affinity purification assay

A biotinylated 477 bp proximal hNF-YA promoter probe was obtained by PCR with Phusion High-Fidelity DNA Polymerase (F530, Thermo Scientific) and a 5′-biotinylated primer (IDT, Integrated DNA Technologies). The amplification was performed using a 2-step thermocycling protocol, as indicated by the manufacturer, and a typical 50 µl reaction containing 1 ng of pGL3-NFYA promoter plasmid as template, 1 U Phusion HighFidelity (HF) DNA Polymerase, 0.3 mM of dNTPs, 1 × HF PCR Buffer and 0.5 µM each of the following primers: F cacttggaaaggggtgggca; R [biotin]gcgagacccgccaatcgg. Multiple reactions were performed to obtain the required amount of probe, which was then purified using a QIAquick PCR Purification Kit (Qiagen) and eluted in water. shNF-YA and shCTR HCT116 cells were grown to 70–80% confluence, washed with PBS, scraped and centrifuged. Nuclear proteins were then extracted with a two-step method: nuclei were isolated by incubation for 10 min in buffer A (10 mM HEPES–KOH pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 10% glycerol, 0.1% triton X-100, 0.5 mM DTT, 1 × PIC (Sigma), 1 mM PMSF and phosphatase inhibitors) on ice and centrifuged at 4000 g for 10 min. Nuclei were lysed by incubation for 20 min on ice in 1 × Binding buffer (7 mM TrisHCl pH 7.5, 81 mM NaCl, 5 mM MgCl2, 10% glycerol, 1 mM DTT, 1 × PIC (Sigma), 0.5 mM PMSF and phosphatase inhibitors) and disrupted by using a syringe with a 25 gauge needle. Nuclear extracts were then centrifuged at 14000 rpm, 4 °C for 30 min and the supernatant was transferred to a new tube. The pellet was further extracted with 1 × Binding buffer containing 400 mM NaCl: after centrifugation at 14000 rpm, 4 °C for 15 min, NaCl concentration was adjusted to 81 mM and this extract was added to the previous one. Protein concentration was determined by the Bradford assay (Sigma).

Streptavidin-coated agarose beads (S1638, Sigma) were washed twice in 1 × Binding Buffer and pre-cleared for 30 min with 1 µg/µl BSA and 200 µg/ml salmon sperm DNA in 1 × binding Buffer. After two washes in 1 × Binding Buffer, pre-cleared beads were associated to double-stranded biotinylated oligonucleotides (200 pmol/mg of beads) by incubation for 1 h at 4 °C in 1 × Binding Buffer. 1 mg of nuclear extract was then incubated with 100 µl of pre-cleared streptavidin-coated agarose beads previously associated with the double-stranded biotinylated NF-YA promoter probe and 100 µg/ml salmon sperm DNA (D9156, Sigma) for 1 h, 4 °C, on a rotating wheel. Beads were washed three times in 500 µl 1 × Binding Buffer and proteins bound to the probe were isolated by elution in high-salt buffer (20 mM TrisHCl pH 7.5, 5 mM MgCl2, 1 M NaCl). Eluted proteins were then resolved on Bolt™ 4–12% Bis-Tris Plus Gels (NW04120BOX, Life Technologies) and stained with Coomassie blue. For GeLCMS sample analysis, 1D-SDS PAGE lanes were cut in 7 portions and in-gel digested with Sequencing Grade Modified Trypsin (V5111, Promega) following a standard procedure [31]. All peptide extracts for each gel portion were pooled, dried under vacuum and then submitted for analysis to the MS Facility (CIGS, University of Modena, Italy).

2.7. Mass spectrometry analysis and protein identification

MS analysis was performed on an ESI-Q-TOF Accurate-Mass spectrometer (G6520A, Agilent Technologies, Santa Clara, CA, USA), controlled by MassHunter software (v. B.04.00) and interfaced with a CHIP-cube to an Agilent 1200 nano-pump. Two biological replicates were performed for each sample. Raw data, converted from the vendor's data format into mascot generic format using MassHunter Qualitative Analysis (v. B.05.00), were searched against Swiss-Prot (v. 2013_04, 20253 entries for *Homo sapiens*) for peptide sequences and C-RAP (ftp://ftp.thegpm.org/fasta/cRAP) for contaminants with MASCOT (Version 2.4, Matrix Science, London, UK). The obtained protein list was further analyzed with DAVID software in order to point out only entries relevant for transcriptional regulation. Only proteins identified in both biological replicates were considered. The *ratio* between the protein in shNFYA versus shCTR, normalized according to emPAI (exponentially modified Protein Abundance Index) method [32], was then calculated. For further details, see Supplementary information.

2.8. Statistical analysis

At least three independent biological experiments have been performed. The values represented in the histograms are the average of the biological replicates and the bars indicate the Standard Error of the Mean (SEM). Statistical significance was analyzed with GraphPad software using independent Student's *t*-test between the indicated samples or between test sample and control when not differently specified. ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$).

3. Results

3.1. Loss of NF-Y activity increases the expression levels of its subunits

Following the inactivation of the NF-YA subunit through shRNA lentiviral delivery in human colon-carcinoma HCT116 cells, we compared the levels of the three NF-Y subunits in total protein extracts from control (shCTR) and NF-YA-abrogated cells (shNF-YA). Both NF-YB and NF-YC histone-fold subunits were significantly increased in shNF-YA infected cells (Fig. 1A). The same effect was observed in other human cancer and immortalized cell lines (Suppl. Fig. 1A). Despite NF-YB and NF-YC levels were higher in shNF-YA cells, NF-YA depletion led to NF-YB and NF-YC down-regulation in chromatin enriched extracts, demonstrating that NF-YA loss is sufficient to abrogate the DNA binding ability of the complex (Fig. 1A).

Similarly to shRNA delivery, siRNA-mediated NF-YA knock down induced an increase of NF-YB and NF-YC protein levels (Fig. 1B). Additionally, we analyzed the levels of NF-Y subunits following transient expression of a dominant negative NF-YA mutant, which titrates the endogenous NF-YB/NF-YC heterodimer and forms NF-Y complexes unable to bind DNA [33]. As shown in Fig. 1C, the formation of inactive NF-Y complexes led to increased NF-YB and NF-YC protein levels.

To determine whether NF-YA was also subject to a negative regulatory feedback, NF-YB or NF-YC were inactivated through shRNA delivery. Fig. 1D shows that both shNF-YB and shNF-YC were able to silence the targeted protein, but in contrast, the expression of the other non-targeted histone-fold subunit was down-regulated. This suggests that lack of either NF-YB/NF-YC dimer subunits affects the levels of the other. Furthermore, we tested whether this behavior could depend on a corresponding increase in protein degradation and, indeed, the co-treatment with proteasome and lysosomes activity inhibitors rescued the expression of the non-targeted histone-like partner in shNF-YB and shNF-YC cells (Fig. 1D, middle and right panels). Despite NF-YB or NF-YC knock down does not alter basal NF-YA protein expression, NF-YA levels increase following protein degradation inhibition in NF-YB and NF-YC-inactivated cells.

These results highlight that reduced NF-Y activity caused by the loss of the NF-YA DNA binding subunit, triggers an increase in the levels of NF-YB/NF-YC proteins. In contrast, the impossibility to form the NF-YB/NF-YC heterodimer, as the result of NF-YB or NF-YC abrogation, hampers the stability of the histone-fold partner.

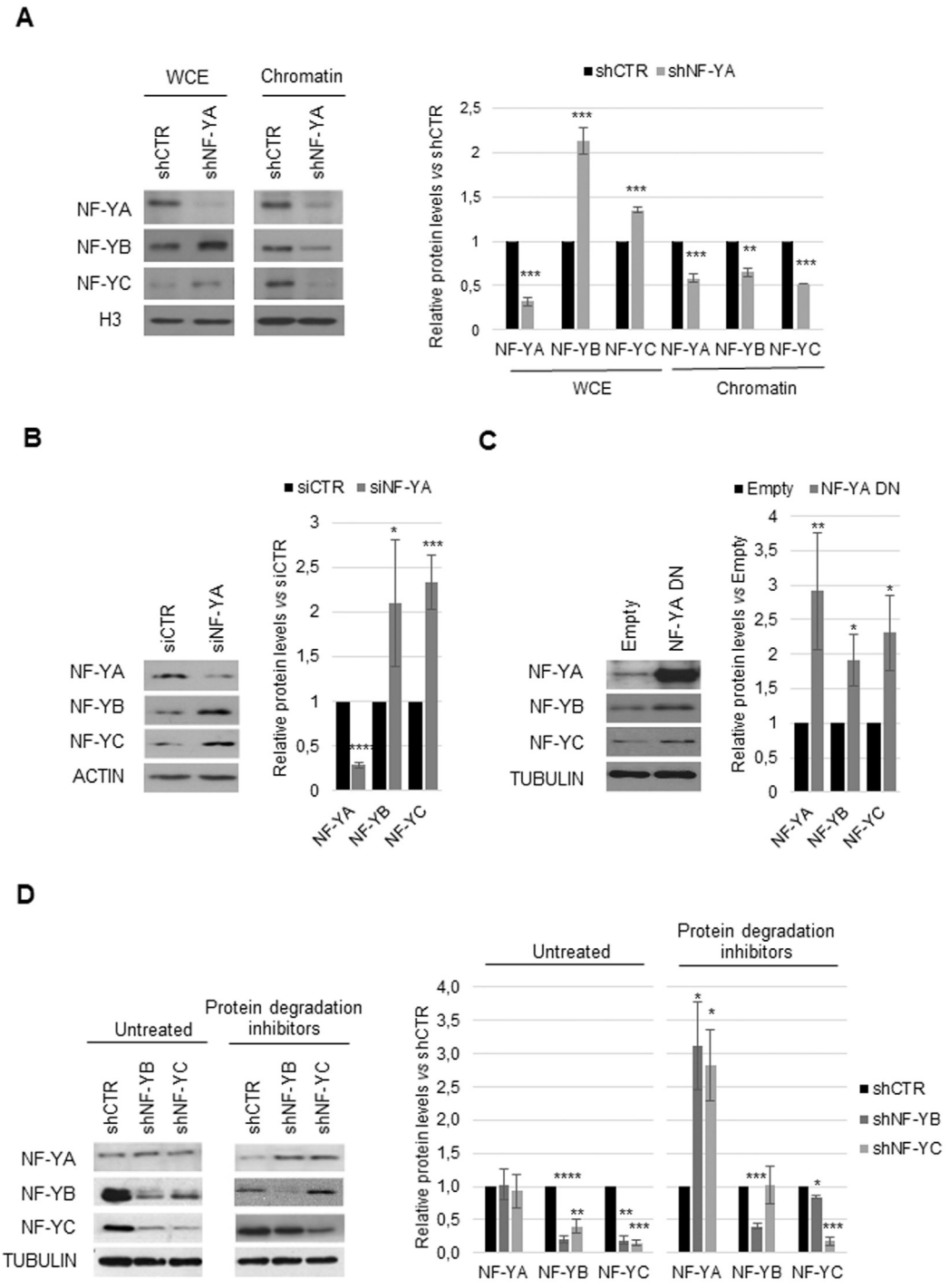

Fig. 1. The modulation of individual NF-Y subunits impacts on the expression of all the proteins of the complex. A. Western blot analysis performed on whole cell extracts (WCE) and chromatin-enriched extracts (chromatin) of control (shCTR) and NF-YA-inactivated (shNF-YA) HCT116 cells (left panel). Proteins expression was quantified normalized to total histone H3 and reported as fold change of shNF-YA versus shCTR levels, arbitrarily set at 1 (right panel). *p* values: *** < 0.001, ** < 0.01. B. Protein expression analysis (left panel) of NF-Y subunits in total extracts of HCT116 cells transfected with control and NF-YA-targeting siRNAs. Quantification of the expression levels of the three NF-Y subunits normalized to actin was shown as fold change versus siCTR, arbitrarily set at 1 (right panel). C. Western blot analysis of NF-YA, NF-YB and NF-YC expression following transient overexpression of NF-YA-DN mutant in HCT116 cells. Relative quantification of NF-YA, NF-YB and NF-YC, normalized to actin, was reported as fold change versus cells transfected with the empty vector, arbitrarily set at 1 (right panel). D. Left panel: Protein expression levels of NF-Y subunits in whole cell extracts of control (shCTR) and NF-YB (shNF-YB) or NF-YC (shNF-YC) HCT116 cells treated or not with inhibitors of protein degradation. Right panel: Relative quantification of NF-Y expression levels in NF-YB- or NF-YC-inactivated cells versus shCTR. Proteins expression was quantified normalized to tubulin and reported as fold change versus shCTR levels, arbitrarily set at 1. *p* values: * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

3.2. NF-Y binds to and regulates NF-YA, NF-YB and NF-YC promoters

To elucidate the mutual regulation between the three NF-Y subunits, we analyzed the effects of the abrogation of NF-Y activity on the mRNA levels of its subunits. NF-YA loss significantly increased both NF-YB and NF-YC transcripts in HCT116, as well as in other human cell lines (Fig. 2A, left panel, and Suppl. Fig. 1B–C). We analyzed the three NF-YC splice variants by RT-PCRs with specific primers and related quantification: NF-YA depletion induced the rise of 48 kDa and 37 kDa NF-YC transcripts (Fig. 2B), with the last one being the main translated isoform

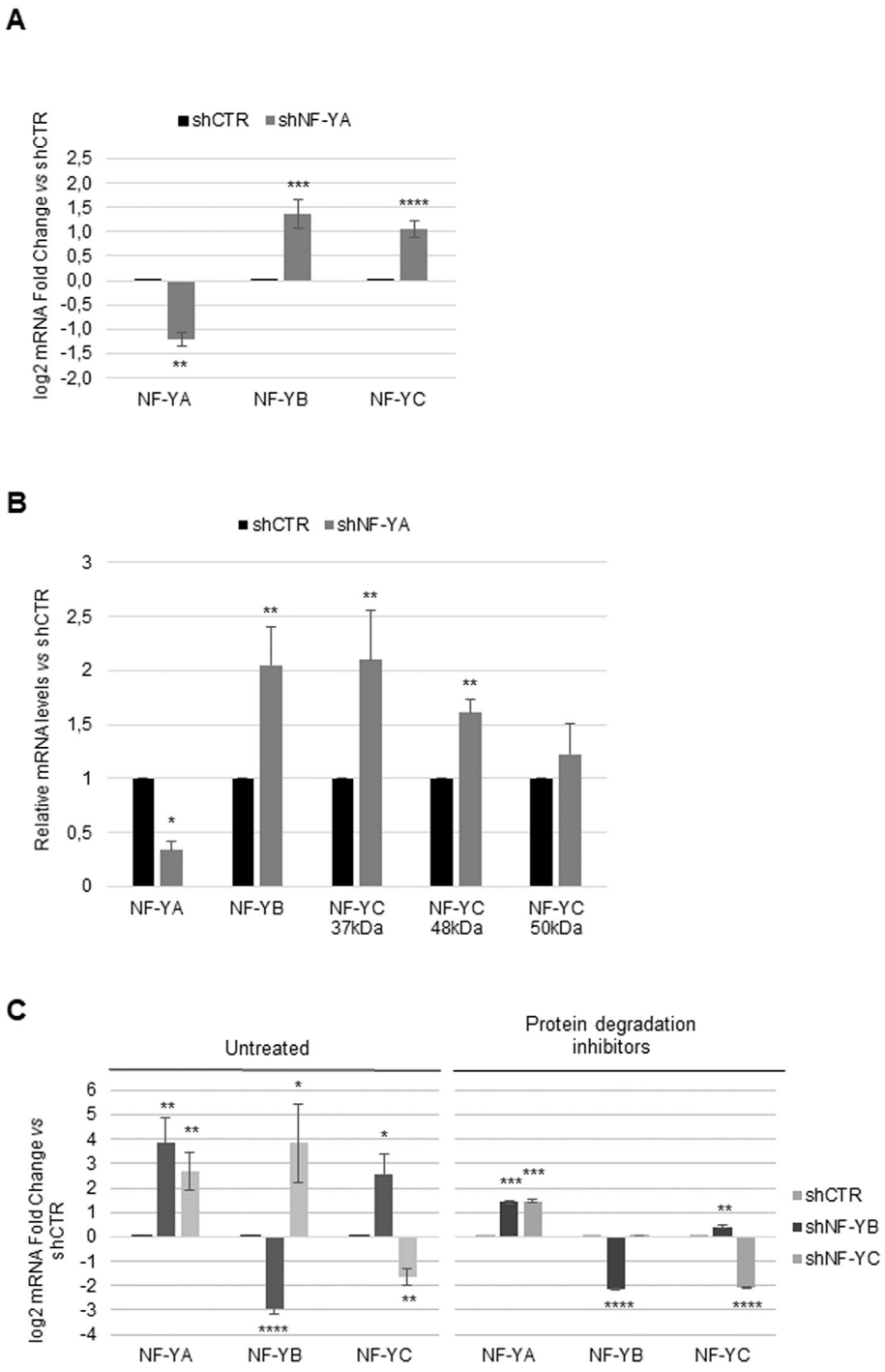

Fig. 2. Individual knock down of NF-Y subunits upregulates the transcription of the non-targeted ones. A. qRT-PCR analysis of NF-YA, NF-YB and NF-YC transcripts in shNF-YA and shCTR cells. Data are presented as log2 mRNA fold change in NF-Y-inactivated versus control cells. B. Relative quantification of RT-PCR-amplified transcripts of NF-YC splice variants normalized to γactin, in control (shCTR) and NF-YA-inactivated (shNF-YA) cells. The expression levels of NF-YA, NF-YB and NF-YC transcripts in shCTR cells were arbitrarily set at 1. C. Analysis of mRNA levels of NF-Y subunits through qRT-PCR in control (shCTR), NF-YB (shNF-YB) and NF-YC (shNF-YC) inactivated HCT116 cells treated or not with protein degradation inhibitors. Data are presented as log2 mRNA fold change in NF-Y-inactivated versus control cells. p values: * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

in HCT116 cells (Suppl. Fig. 1D). NF-YB inactivation was able to induce both NF-YA and NF-YC transcription, while NF-YC loss increased NF-YA and NF-YB mRNA levels (Fig. 2C). Interestingly, treatments with protein degradation inhibitors reduced the increase in NF-Y genes transcription, in particular of NF-YB and NF-YC transcripts. This result is consistent with protein expression analysis (Fig. 1D) and clarifies why the administration of proteasome/lysosomes inhibitors can only restore the expression of NF-YB and NF-YC to basal levels.

Overall, these data suggested that NF-Y could be a negative regulator of its own expression. Regulatory regions of encoding genes were consequently investigated. UCSC Genome browser was used to identify H3K27ac enriched regions, as active regulatory elements in NF-YA, NF-YB and NF-YC genes. Several NF-Y-binding sites are located in the NF-YA and NF-YB promoters regions (Fig. 3A). As for NF-YC gene, both the constitutive active P1 promoter and the inducible downstream P2 promoter contain CCAAT boxes [15] (Fig. 3A). Encode ChIP-seq data and previous results demonstrated that NF-Y binds to NF-YA, NF-YB and NF-YC promoters [15]. We corroborated these data in HCT116 cells through ChIP experiments with anti-NF-YA and anti-NF-YB antibodies. NF-Y binding is enriched at all the NF-Y promoters (Fig. 3B), concurrently with TBP and H4ac, H3K56ac, H3K4me3 positive-histone marks (Fig. 3C).

The abrogation of NF-YA through RNAi strongly impaired NF-Y binding to the identified regulatory regions (Fig. 4A). In an opposite way, an increase in positive histone marks and TBP binding was observed in NF-YA, NF-YB and NF-YC P1 promoters (Fig. 4B), hinting that

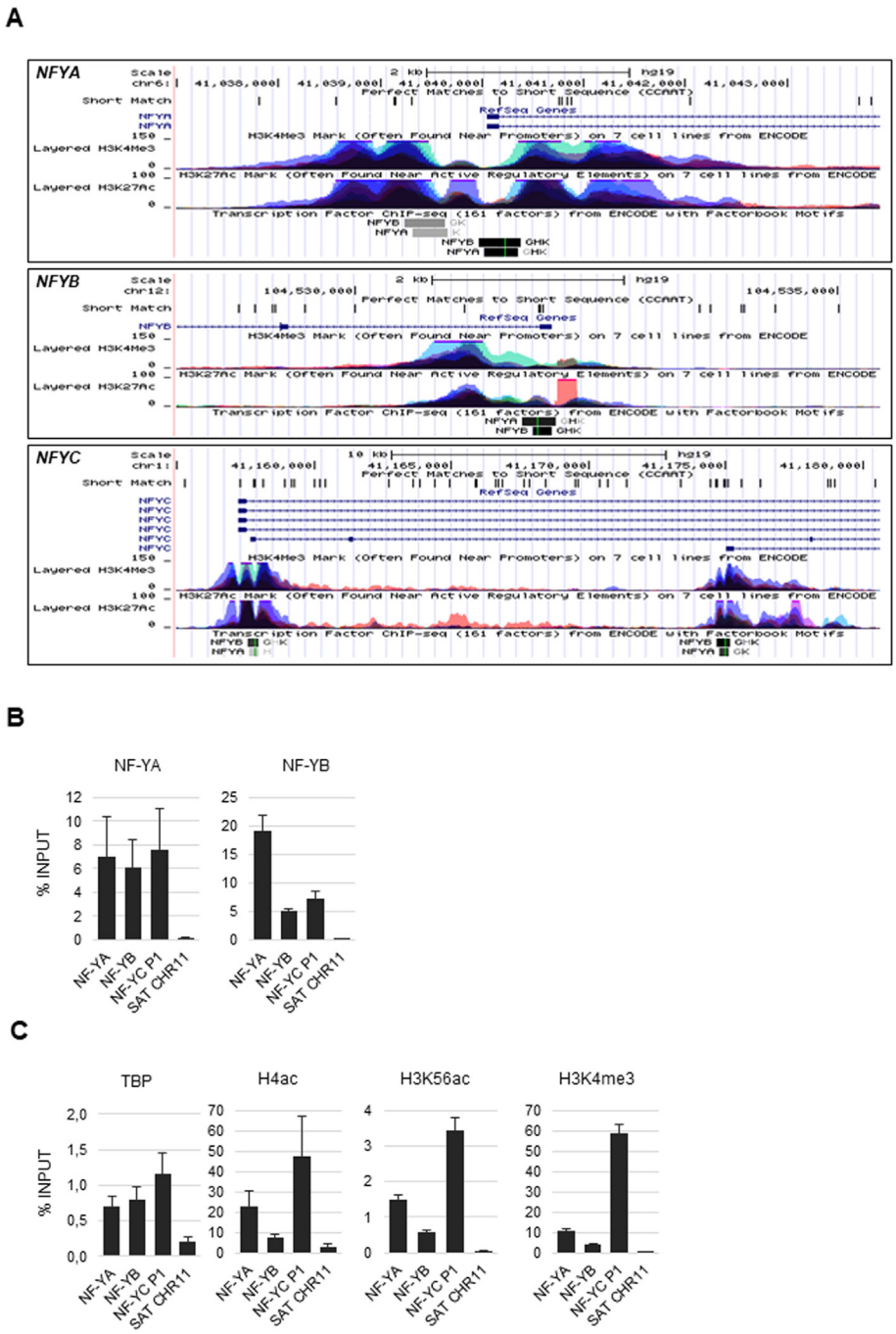

Fig. 3. NF-Y bounds to open chromatin regulatory regions of NF-YA, NF-YB and NF-YC genes. A. The images are screenshots that link ENCODE NF-YA and NF-YB data in the UCSC genome browser. Short matches represent direct or inverted CCAAT elements and promoter regions are identified by H3K4me3 and H3K27Ac peaks. B. qChIP analysis of NF-YA, NF-YB and TBP binding on NF-YA, NF-YB and NF-YC P1 regulatory regions in HCT116 cells. The satellite DNA-rich heterochromatin region of chromosome 11 (Sat Chr11) has been used as negative control region. C. Enrichment of H4ac, H3K56ac and H3K4me3 chromatin marks in HCT116 cells on NF-YA, NF-YB and NF-YC P1 promoters. Data are presented as % INPUT ± SD.

NF-Y release from its binding sites alters chromatin accessibility. These results support the hypothesis that NF-Y negatively regulates the expression of its subunits at the transcriptional level.

3.3. Mass spectrometry (MS) identified transcription regulators associated to NF-YA promoter

Since NF-YA function involves DNA binding, appears to be the complex limiting subunit and its increased expression has been observed in some tumors [34], we aimed to better characterize its transcriptional regulation machinery. In order to identify which proteins can contribute to the maintenance of basal NF-YA mRNA levels or trigger NF-YA transcriptional activation following the loss of NF-Y activity, shCTR and shNF-YA nuclear extracts were incubated with a PCR-amplified biotin-labelled NF-YA proximal promoter DNA probe, containing the four CCAAT elements. DNA-protein complexes were adsorbed to streptavidin beads and bound proteins were eluted in high-salt buffer, resolved through SDS-PAGE and subsequently analyzed by MS (Fig. 5A). Proteins corresponding to sequenced peptides were identified with Mascot, filtered based on their annotation in transcription-related GO category and then quantified according to the normalized emPAI (exponentially modified Protein Abundance Index)

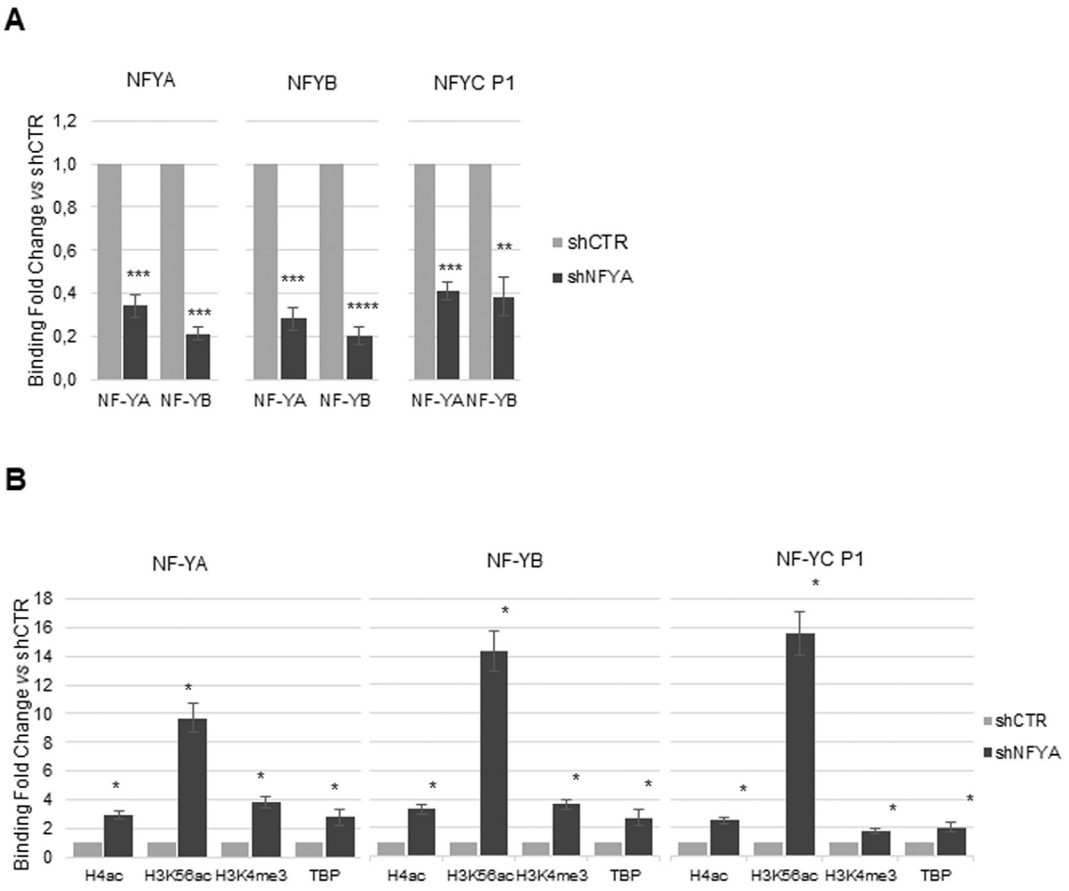

Fig. 4. Effects of NF-Y loss on chromatin status of NF-Y regulatory regions. A. Binding of NF-YA and NF-YB on regulatory regions of NF-Y genes in control and NF-YA-inactivated cells by qChIPs. Bars represent NF-Y binding as fold change versus control (shCTR), arbitrarily set at 1. B. qChIP analysis of H4ac, H3K56ac, H3K4me3 and TBP enrichment on NF-Y promoters in control and NF-YA knocked down cells. Data are presented as binding fold change versus control (shCTR), arbitrarily set at 1. *p* values are indicated: * < 0.05, ** < 0.01, *** < 0.001**** < 0.0001.

method [32]. Fig. 5B provides the list of selected transcriptional regulators differentially recruited to the NF-YA probe in the presence or absence of NF-YA. As expected, NF-YA, NF-YB and NF-YC were only identified in shCTR sample. The expression profiles analysis was performed with three different cell lines, therefore differences in peptide counts in control and NF-YA-inactivated cells are unlikely to depend on different expression levels of the corresponding proteins (Fig. 5C).

3.4. Lamin A is a negative regulator of NF-YA gene transcription

The majority of proteins recruited to the NF-YA promoter in the absence or presence of NF-YA showed a decrease in the shNF-YA/shCTR *ratio* with some proteins detected only when NF-Y was bound to the promoter (Fig. 5B). This suggests that NF-Y could negatively control the expression of its subunits through the recruitment of co-repressors. Among transcriptional regulators exclusive to shCTR extracts, we identified Lamin A, component of the nuclear lamina with a key role in chromosome organization and transcriptional regulation [35]. Direct interaction between NF-Y and Lamin A has been recently described by the group of G. Piaggio, which showed how an NF-Y-dependent enrichment of Lamin A in promoters of CCAAT-cell cycle genes is able to counteract NF-Y transcriptional activity [36]. ChIP analysis in HCT116 cells supported the binding of Lamin A on the NF-YA promoter in comparison to a negative control region (Fig. 6A). We monitored as a positive control the TOP2A regulatory region containing a CCAAT-promoter regulated through NF-YA/Lamin A complex binding [36]. The same behaviour was observed in Hela cells, where Lamin A binds the NF-YA promoter together with NF-Y (Suppl. Fig. 2). The decrease in NF-Y binding induced by shNF-YA corresponded to a lowering in Lamin A chromatin recruitment, suggesting that NF-Y abrogation may impair Lamin A association on NF-YA promoter (Fig. 6B). Similarly, NF-YB knock down by shRNA delivery reduced the binding of Lamin A on NF-YA promoter (Suppl. Fig. 2B–C).

We then performed transient luciferase assays in HCT116 cells, co-transfecting the NF-YA 477 bp promoter, cloned upstream to the luciferase gene, together with the expression vectors for NF-YA/NF-YB/NF-YC (NF-Y trimer) or NF-YA dominant negative mutant (NF-YA-DN), and Lamin A (Fig. 6C). As expected, NF-Y was able to decrease NF-YA promoter activity, while the overexpression of NF-YA-DN increased luciferase expression. Lamin A overexpression reduced of about 40% NF-YA promoter activity and co-transfections of Lamin A with NF-YA-DN rescued promoter transcriptional activity, thus corroborating that the repressive role of Lamin A is mediated by NF-Y binding to CCAAT boxes.

4. Discussion

Despite a multitude of NF-Y-regulated genes are overexpressed in cancer cells, mediating cell growth and cancer metabolism, increased expression levels of NF-Y subunits have been observed only in a handful of tumors and cancer cells [25,26,34,39,40]. In this study, we showed that NF-Y subunits are indeed under a transcriptional control played by NF-Y itself, which could NF-Y relatively stable expression cancer cells.

We demonstrated that the loss of NF-Y complex binding to NF-Y genes CCAAT-regulatory elements triggers their transcriptional upregulation, also linked to increased chromatin accessibility (Fig. 4). Similarly, in vivo NF-YA knock out in post-mitotic mouse neurons, via deletion of exons 3–8 out of 9 total coding exons, induced a significant

A

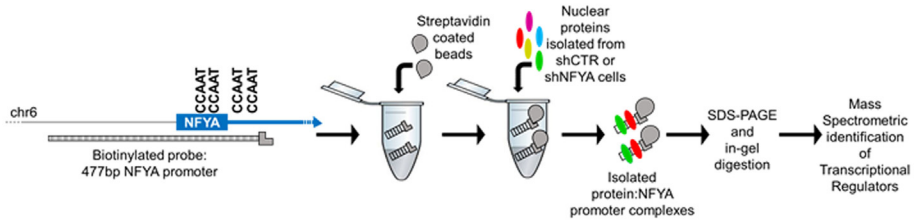

Fig. 5. Identification of putative regulators of NF-YA gene transcription. A. Schematic representation of the DNA pull down assay used to identify putative transcriptional regulators of the NF-YA gene by MS. B. Synopsis of MS-identified proteins associated to transcription-related GO category in shCTR and shNF-YA cells. For proteins identified in both samples, the arrows indicate whether protein binding to the NF-YA probe is unchanged (↔), decreased (↓, shNF-YA/shCTR *ratio* ≤ 0.8) or increased (↑, shNF-YA/shCTR *ratio* ≥ 1.2) following NF-YA loss. The term "ONLY CTR" indicates proteins exclusive to shCTR extracts. C. The table represents the expression levels of the indicated genes as fold change of shNF-YA versus shCTR. Data have been retrieved by our published gene expression profiles [18,52].

B

UniProtKD/ Swiss-Prot Entry	Acronym	Protein Name	shNF-YA/ shCTR
P60709	ACTB	Actin, cytoplasmic 1	↑
Q9UFW8	CGBP1	CGG triplet repeat-binding protein 1	↓
P16220	CREB1	Cyclic AMP-responsive element-binding protein 1	ONLY CTR
P68104	EF1A1	Elongation factor 1-alpha 1	↔
P78347	GTF2I	General transcription factor II-I	↓
P11142	HSP7C	Heat shock cognate 71 kDa protein	↓
P02545	LMNA	Prelamin-A/C	ONLY CTR
P23511	NFYA	Nuclear transcription factor Y subunit alpha	ONLY CTR
P25208	NFYB	Nuclear transcription factor Y subunit beta	ONLY CTR
Q13952	NFYC	Nuclear transcription factor Y subunit gamma	ONLY CTR
P35232	PHB	Prohibitin	↓
Q99623	PHB2	Prohibitin-2	↓
Q09028	RBBP4	Histone-binding protein RBBP4	ONLY CTR
Q9Y265	RUVB1	RuvB-like 1	↑
P08047	SP1	Transcription factor Sp1	↔
Q02447	SP3	Transcription factor Sp3	↓
Q12800	TFCP2	Alpha-globin transcription factor CP2	ONLY CTR

C

	Fold Change shNF-YA/shCTR		
	HCT116	H322	HELA
ACTB	NA	NA	0,98
CGBP1	0,99	0,97	1,21
CREB1	0,84	0,94	0,86
EF1A1	NA	NA	1,16
GTF2I	1,20	1,31	0,83
HSP7C	1,04	0,98	0,70
LMNA	1,20	1,11	1,30
PHB	0,95	0,89	0,91
PHB2	0,95	1,05	0,98
RBBP4	0,78	0,94	0,72
RUVB1	0,90	1,04	0,79
SP1	0,93	1,03	1,21
SP3	0,92	0,86	0,74
TFCP2	1,00	1,04	1,08

increase in NF-YC and mutant NF-YA mRNA [41]. Moreover, in the Huntington disease R6/2 mouse model, characterized by NF-YA and NF-YC localization in nuclear inclusions and mutant huntingtin localization in cortex and striatum, NF-YA and NF-YC mRNA levels were increased [42]. These patterns clearly suggest the existence of a negative transcriptional feedback acting as a compensatory system allowing the fine tuning of NF-Y activity and counteracting functional impairment of NF-Y in vivo.

We focused our work on the transcriptional regulation of NF-YA subunit, as it contains the DNA binding domain and is the limiting subunit of the NF-Y complex [5,7,43]. MS analysis of proteins associated to the NF-YA CCAAT-promoter in control cells and in NF-YA-inactivated cells identified several putative transcriptional regulators, the majority of which are released when NF-Y is lost. This result suggests that the NF-Y complex may repress the NF-YA gene by recruiting transcriptional co-repressors. Among these of particular interest is Lamin A, component of nucleoplasmic structures with a demonstrated role in chromatin organization and transcriptional regulation [44]. The association of NF-Y and Lamin A has been well described by Cicchilitti and coworkers, which showed that Lamin A directly interacts with NF-Y and inhibits NF-Y-dependent gene expression [36]. Our results demonstrate that the regulatory region of NF-YA, containing multiple CCAAT elements, is among NF-Y target genes and is modulated through NF-Y/Lamin A interaction. Transient transfection experiments with Lamin A and the dominant negative NF-YA mutant showed that Lamin A repressive activity on NF-YA promoter is mediated by NF-Y binding to CCAAT boxes (Fig. 6).

Other transcriptional regulators have been identified among NF-YA promoter associated proteins in concurrence with NF-Y binding, such as CREB1, RBBP4 and SP3. CREB1 is a ubiquitous TF, member of the leucine zipper family, which binds the cAMP-responsive element (CRE) to modulate gene transcription. Despite CREB1 binding to regulatory regions has been widely associated to activation of gene transcription, when recruited through other TFs, it can also exert a transcriptional activity repressive function [45]. ENCODE Chip-seq data available through UCSC Genome Browser support our MS results: CREB1 binds, together with NF-YA and NF-YB, not only to the NF-YA gene CCAAT-promoter, but also to NF-YB and NF-YC-P1 regulatory regions. Since

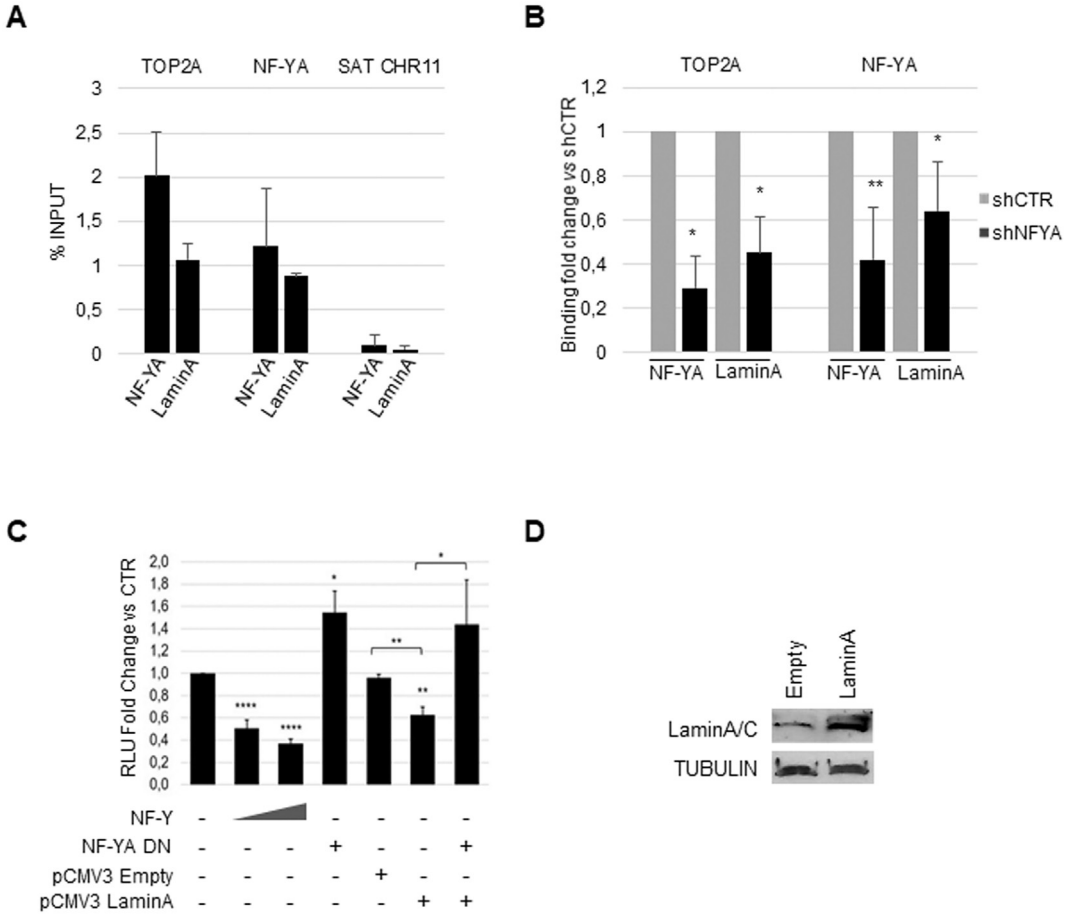

Fig. 6. Lamin A binds to and represses NF-YA promoter activity. A. qChIP analysis of the binding of NF-YA and Lamin A on the regulatory region of the NF-YA gene. Data are reported as %INPUT ± SD. Topoisomerase IIα promoter and satellite region of chromosome 11 (Sat Chr11) have been used as positive and negative control regions, respectively. B. Effects of NF-YA abrogation on chromatin binding of NF-YA and Lamin A, determined by qChIP analysis in HCT116 cells. Data are presented as binding fold change in the absence of NF-YA (shNF-YA) versus control cells (shCTR), arbitrarily set at 1. C. Transient luciferase assay performed in HCT116 cells: the NF-YA promoter-LUC vector has been co-transfected with the NF-Y heterotrimer (composed by NF-YAs isoform, NF-YB and NF-YC 37 kDa isoform) or the NF-YA dominant negative mutant (NF-YA-DN), Lamin A or its respective empty vector (pCMV3). Data are presented as RLU fold change versus the basal activity of the promoter (CTR). D. Western blot analysis of Lamin A expression in HCT116 cells transiently transfected with empty- or Lamin A-expressing vector. Tubulin was used as loading control.

CRE motives are not present under CREB1 peaks in NF-YA promoter, NF-Y could mediate its chromatin recruitment. RBBP4 is a component of the Mi-2 complex with a role in chromatin remodeling and transcriptional repression [46]. SP3 interacts with HDAC1 and HDAC2, and, when sumoylated, promotes the recruitment of corepressors, among which Mi-2 and histone methyltransferases (SETDB1/ESET and SUV4-20H), thus inhibiting gene transcription [47,48]. Future studies will address whether and how these predicted regulators control the transcription of NF-Y genes.

Since NF-YA and NF-YC expression has been found to be up-regulated in some solid tumors [20,21,27], it would be important to determine the underlying mechanism leading to NF-Y subunits overexpression in these cancer cells despite the existing regulatory negative feedback to preserve basal physiological levels. Two scenarios are possible: i) the transcriptional regulators of NF-Y genes, such as co-repressors, may be aberrantly expressed in cancer cells and therefore alter NF-Y expression or ii) the previously described auto-regulatory mechanism may be only active in response to acute transient modulation of NF-Y expression and could be disrupted in chronic conditions. As for the first hypothesis, deregulated Lamin A/C expression has been observed in various cancers. In particular, colon cancer, basal cell carcinoma, gastric carcinoma, breast cancer, neuroblastoma and gastrointestinal neoplasms present decreased Lamin A/C expression, while its overexpression was observed in prostate cancer and hepatocellular carcinoma (for a review see [49]). It would be therefore of interest to investigate whether a correlation between NF-YA and Lamin A/C expression exists in normal versus cancer cells and tissues.

In addition to the transcriptional mechanism analysis, our results demonstrate that also protein stability has an important role in controlling NF-Y expression. In facts, NF-YB and NF-YC subunits are degraded when their histone-fold partner is down-regulated. We already described that the subcellular localization of NF-YB and NF-YC depends on their interaction. Specifically, we showed that nuclear localization of NF-YC is regulated via heterodimerization with NF-YB [50], therefore NF-YB knock down likely impairs NF-YC nuclear shuttling and redirects it towards degradation. Similarly, NF-YC protects NF-YB from degradation. While the post-translational mechanisms controlling NF-YA stability have been described [10], NF-YB and NF-YC post-translational processes remain to be investigated. We may speculate that the NF-YB/NF-YC heterodimer assembly may be able to mask specific residues involved in proteasome or lysosome degradation.

5. Conclusions

Our data support the existence of an autoregulatory loop controlling the expression of the transcription factor NF-Y, based on mutual transcriptional and post-translational control of its subunits expression. Taking into consideration the key role of NF-Y in multiple physiological and pathological processes, we believe this knowledge will be instrumental when analyzing cell transformation processes and cancer-

related processes where NF-Y activity is altered.

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Transparency document

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

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