



## NF-YA splice variants have different roles on muscle differentiation



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### ABSTRACT

The heterotrimeric CCAAT-binding factor NF-Y controls the expression of a multitude of genes involved in cell cycle progression. NF-YA is present in two alternatively spliced isoforms, NF-YAs and NF-YAI, differing in 28 aminoacids in the N-terminal Q-rich activation domain. NF-YAs has been identified as a regulator of stemness and proliferation in mouse embryonic cells (mESCs) and human hematopoietic stem cells (hHSCs), whereas the role of NF-YAI is not clear. In the muscle system, NF-YA expression is observed in proliferating cells, but barely detectable in terminally differentiated cells *in vitro* and adult skeletal muscle *in vivo*. Here, we show that NF-YA inactivation in mouse myoblasts impairs both proliferation and differentiation. The overexpression of the two NF-YA isoforms differentially affects myoblasts fate: NF-YAs enhance cell proliferation, while NF-YAI boosts differentiation. The molecular mechanisms were investigated by expression profilings, detailing the opposite programs of the two isoforms. Bioinformatic analysis of the regulated promoters failed to detect a significant presence of CCAAT boxes in the regulated genes. NF-YAI activates directly Mef2D, Six genes, and p57<sup>kip2</sup> (Cdkn1c), and indirectly the myogenic regulatory factors (MRFs). Specifically, Cdkn1c activation is induced by NF-Y binding to its CCAAT promoter and by reducing the expression of the lncRNA Kcnq1ot1, a negative regulator of Cdkn1c transcription. Overall, our results indicate that NF-YA alternative splicing is an influential muscle cell determinant, through direct regulation of selected cell cycle blocking genes, and, directly and indirectly, of muscle-specific transcription factors.

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### 1. Introduction

Skeletal myogenesis, i.e. the formation of skeletal muscle, depends on multiple myogenic regulatory factors, in particular sequence-specific transcription factors, some of which are important for muscle fate commitment; others are involved in terminal differentiation. During development, the coordinated activity of combinatorial transcription factors is fundamental to elicit the proper *spatio-temporal* expression of genes throughout the complex process of myogenesis [1, 2]. The family of myogenic regulatory factors (MRFs), composed by MyoD, Myf5, Myogenin, and Mrf4, has a crucial role in muscle cell specification and differentiation [3]. Other transcription factors cooperate with MRF proteins in regulating target gene expression, such as Mef2 [4] and Six family members [5]. In vertebrates, the Mef2 family consists

of four proteins (Mef2A–D), all of which act as key players in the activation of the differentiation program [6]. The mammalian Six family is composed by six sequence-specific members (Six1–6), which have an important role in controlling the development of various organs and tissues [7]. In particular, Six1 and Six4 are involved in skeletal muscle development through direct and indirect transcriptional regulation of myogenic genes, such as Myog, Myf5, Pax3, MyoD, and Pbx [5, 8–10].

Skeletal muscle differentiation is strictly associated to cell cycle exit, orchestrated by cyclin-dependent kinase inhibitors (CDKIs) belonging to the CIP/Kip family: p21<sup>cip1/waf1</sup>, p27<sup>kip1</sup>, and p57<sup>kip2</sup>, encoded by Cdkn1a, Cdkn1b, and Cdkn1c genes, respectively. These CDKIs inhibit cyclin-CDK complexes modulating the activities of cyclin D-, E-, A-, and B-CDK complexes [11]. p21<sup>cip1/waf1</sup> and p57<sup>kip2</sup> control differentiation of skeletal muscle and their loss severely affects fiber formation: they have a key role not only in inducing cell cycle exit but also in triggering a muscle-specific transcriptional program [12]. MyoD transcriptionally controls both Cdkn1a and Cdkn1c expression [13–15]. The Cdkn1c gene is regulated by MyoD through indirect mechanisms, consisting in the up-regulation of the intermediate factors p73, Sp1, and Egr1, and directly by interacting with a distant *cis*-element located

Abbreviations: HSC, Hematopoietic stem cell; MES, Mouse embryonic stem cells; s.d., Standard deviation.

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within a differentially methylated region, KvDMR1, sited about 150 kb downstream of *Cdkn1c* [14,16,17]. KvDMR1, which is located within intron 10 of the *Kcnq1* gene and surrounds the promoter that drives for non-coding antisense transcript *Kcnq1ot1*, plays a role in the allele-specific expression pattern of different flanking genes belonging to the same imprinted cluster, composed by *Cdkn1C*, *Kcnq1*, *Ascl2*, *Tssc4*, *Tssc3*, and *Slc22a11* [18]. The *Kcnq1ot1* promoter is characterized by multiple CCAAT elements, required for its activity and bound by the NF-Y complex [19,20].

The transcription factor NF-Y, composed by NF-YA, NF-YB, and NF-YC subunits, plays a key role in cell proliferation and differentiation. NF-YA mediates sequence-specific CCAAT-binding [21]; it is regulated during cell cycle progression [22] and down-regulated in some cell types undergoing terminal differentiation, including C2C12 myoblasts [23–25]. NF-YA is not expressed in adult muscle tissues and consequently, NF-Y binding activity to CCAAT boxes is lost in skeletal muscle and heart [26]. However, NF-YA is detected in muscle of *mdx* mice, an animal model for human Duchenne and Becker muscular dystrophy [27]. In these mice, skeletal muscle undergoes cycles of degeneration and regeneration, associated to robust proliferation of satellite cells (SCs) [28]. In addition, the development of a transgenic mouse model harboring a NF-Y-dependent Cyclin B2 promoter driving a luciferase reporter, linked NF-Y activity in living organisms to muscle regeneration following acute hind limb ischemia, a repair process carried out by SCs [29,30].

These data support previous evidence showing a major role of NF-YA regulation in stem cell biology, through the transcriptional activation of “stemness” pathways [31–34]. The NF-YA gene encodes two spliced transcripts, NF-YA1 (long) and NF-YAs (short), which differ by retention of exon 3, coding for 28 aminoacids in the N-terminal Q-rich activation domain. In transcriptional activation experiments, the two isoforms showed similar activation potential [35–37], hence they were long thought to be functionally equivalent. However, NF-YAs transcript is highly expressed in hematopoietic stem cells (HSCs), declining with differentiation [34]. Its overexpression in HSCs enhances self-renewal [33], improving bone marrow transplantations *ex vivo*. In mouse ES cells (mES), a switch between NF-YAs and NF-YA1 occurs upon differentiation [31,33]: NF-YAs maintains stemness by direct transcriptional activation of key stem cells genes and by helping core stem transcription factors to their target genes [31,33]. Differently, the specific role of NF-YA1 remains elusive.

In this study, we evaluated the activity of the two NF-YA splice variants on proliferation and differentiation of skeletal muscle cells. Overall, our data highlight for the first time the differential role of NF-YA isoforms in modulating the myogenic gene expression program.

## 2. Materials and methods

### 2.1. Cell line and cultures

C2C12 myoblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum (GM = growth medium), avoiding cells becoming >70% confluent. To allow myoblasts differentiation, cells were grown to complete confluence and then the medium was switched to DMEM containing 2% horse serum (DM = differentiation medium). Stable-expressing NF-YA cells were obtained by lentiviral infection of C2C12 cells with pSIN-NF-YAs or pSIN-NF-YA1 particles for 48 h. Infected cells were then selected using puromycin (3 µg/ml) and maintained in medium supplemented with puromycin (1 µg/ml)

### 2.2. Plasmids

The cDNA of NF-YAs and NF-YA1 were cloned *via* EcoRI and BglII sites into EcoRI and BamHI pSIN-EF2-Pur vector backbone. The resulting constructs were then sequenced and used to obtain stable NF-YA-overexpressing cell lines. For transient transfections, pSG5 NF-YAs and

NF-YA1 plasmids were cotransfected together with pGL3 basic human *Kcnq1ot1* luciferase vector (kindly provided by P.D. Sadowski, University of Toronto) [19] and pGL3 basic human *Cdkn1c* luciferase vector (kindly provided by L. Guerrini, University of Milan) [38].

### 2.3. Transfection

Experiments were performed in triplicate in NIH3T3 cells. For each point, 100,000 cells were seeded in 24-well plates and transfected after 24 h with a Metafectene Pro reagent (Biontex) with 100 ng of pCMV β-Galactosidase and 200 ng of luciferase constructs. Cells were harvested 48 h post-transfection and resuspended in lysis buffer (1% Triton X-100, 25 mM glycil-glycine, 15 mM MgSO<sub>4</sub>, 4 mM EGTA) for luciferase activities. β-Galactosidase was assayed to control transfection efficiency.

### 2.4. Lentivirus transduction

Lentiviral supernatant expressing empty vector, NF-YAs and NF-YA1 were prepared by transfecting HEK293T packaging cells as previously described [39]. Briefly, pSIN vector plasmids (20 µg) and second-generation packaging plasmids (5 µg of pMD2-VSVG and 15 µg of pCMV-dR8.74) were cotransfected into HEK293T cells. Lentivirus-containing supernatant was collected 24 h after transfection, centrifuged at 3000 rpm to remove cell debris for 5 min, 0.45 µm filtered and frozen at –80 °C until use. Viral stock solutions were titered by puromycin selection and colony counting.

### 2.5. RNA interference

Third-generation short hairpin RNA pEGFP-C-sh Lenti scrambled (TR30021), murine shRNA NF-YA, targeting 5'UTR (AGCTTCAGGACTCTTAACGTGGCCG) were purchased by Origene. Lentiviral production was prepared as described for pSIN-NF-YA vectors. The supernatants were collected after 48 h from the transfection, 0.45 µm-filtered, ultracentrifugated at 19,000 rpm for 2 h and 30 min and frozen at –80 °C in medium without serum. Virus titer was determined by counting of GFP-positive cells. 7000 C2C12 cells/well (35 mm) were plated and infected by double spinoculation (1800 rpm for 45 min at 30 °C) at MOI 100 and MOI 50. Cells were then harvested for cell cycle analysis, total extracts and RNA extraction, 72 h after the second infection. For the analysis of cellular differentiation, 50,000 cells/well (35 mm) were infected as above, and after 72 h from the second infection were induced to differentiate in DM medium for additional 3 days.

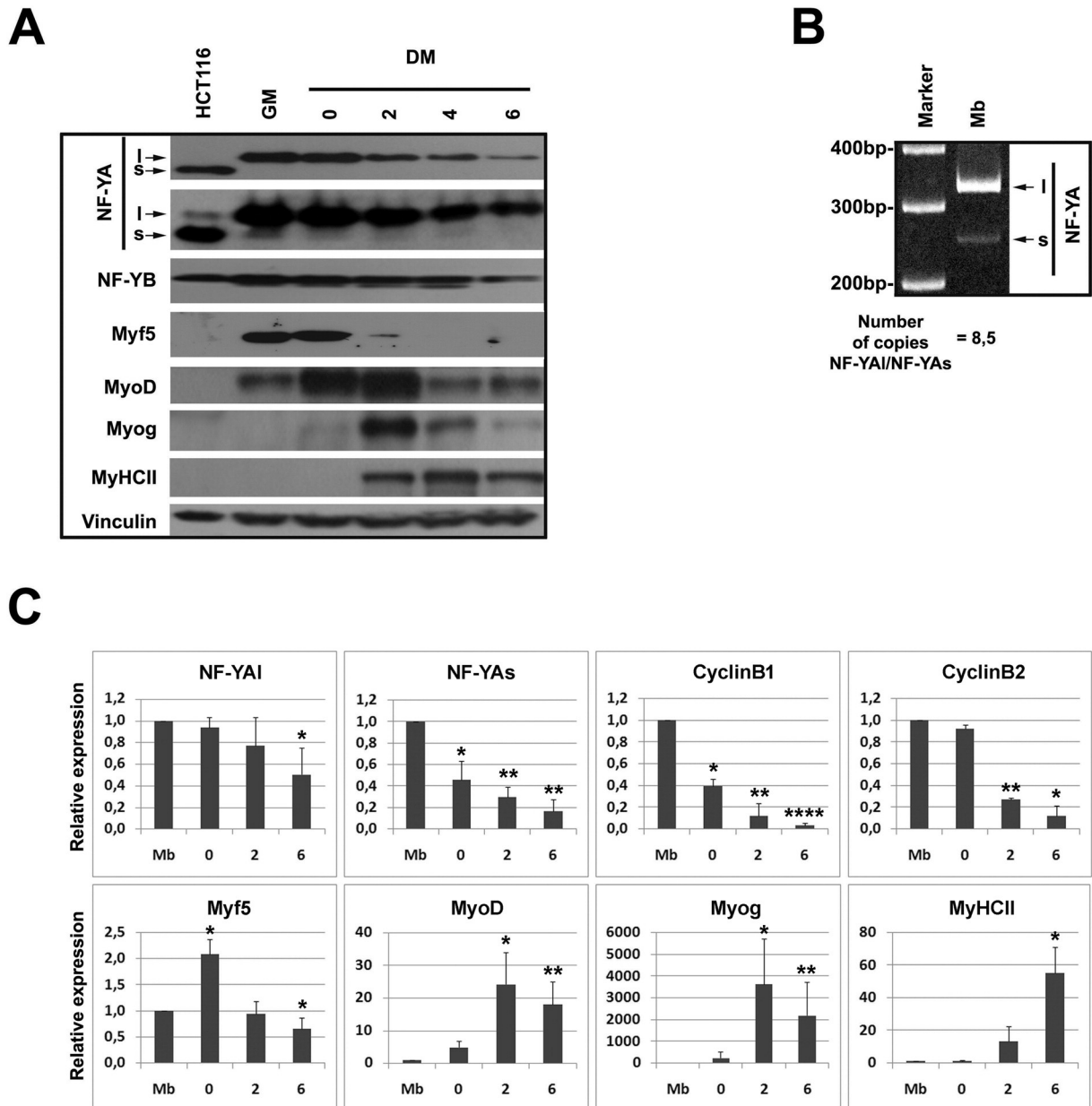
### 2.6. Cytofluorimetric cell cycle analysis

Monoparametric DNA distribution analysis of C2C12 cells was performed by Propidium-Iodide (PI) staining for flow cytometry (Epics cytofluorimeter, Beckman Coulter).

DNA content was analyzed using bivariate flow cytometry, as previously described [39]. BrdU pulsed cells were incubated with mouse anti-BrdU antibody (#317902 BioLegend) for 1 h at 4 °C, and then with FITC anti-mouse (#F0313 Dako) for 1 h at 4 °C. Cells were then counterstained with PI-solution (Propidium-Iodide 25 µg/ml, Na-Citrate 3.4 mM, NaCl 9.65 mM, NP-40 0.03%) for 15 min and analyzed by cytofluorimeter.

### 2.7. Proliferation assay

Cell proliferation of stably infected C2C12 cells was assessed by colometric MTT assay. Briefly, stable-expressing NF-YA cells were seeded into 24-well plates at a density of  $1 \times 10^5$  per well. Four plates were prepared in order to evaluate proliferation at 24, 48, 72, and 96 h. MTT solution (5 mg/ml Thiazolyl Blue Tetrazolium Bromide in PBS) was added to each well at a final concentration of 0.5 mg/ml per well and the plates



**Fig. 1.** NF-YA transcriptional modulation during differentiation of C2C12 cells. **A.** Analysis of NF-YA, NF-YB, Myf5, MyoD, Myogenin (Myog), MyHCII protein levels in C2C12 myoblasts maintained in growth medium (GM) and induced to differentiate for 0, 2, 4, and 6 days in differentiation medium (DM). HCT116 cell extract was used as a control of NF-YAs molecular weight. For NF-YA, two blots with different amounts of protein extracts and different exposure time are shown. Vinculin was used as loading control. **B.** Polyacrylamide gel electrophoresis of semi-quantitative RT-PCR products obtained by amplification with primers common to NF-YAs and NF-YAI transcripts in C2C12 myoblasts (Mb). The relative abundance of NF-YAI versus NF-YAs, performed by absolute quantification in qRT-PCR with isoform specific primers, is indicated as number of copies NF-YAI/NF-YAs. **C.** Real-time RT-PCR analysis of the indicated transcripts in proliferating myoblasts (Mb) and upon differentiation for 0, 2, and 6 days. mRNA levels are indicated as relative expression in differentiated cells versus proliferating myoblasts (arbitrarily set at 1). Data are mean of four independent experiments  $\pm$  s.d.

were incubated at 37 °C for 2 h. Then the medium containing the unconverted MTT was removed and 1 ml of MTT solvent (4 mM HCl, 0.1% NP-40 in isopropyl alcohol) was added to each well. The plate was gently rotated on an orbital shaker in the dark for 15 min before the measurements of absorbance at 570 nm.

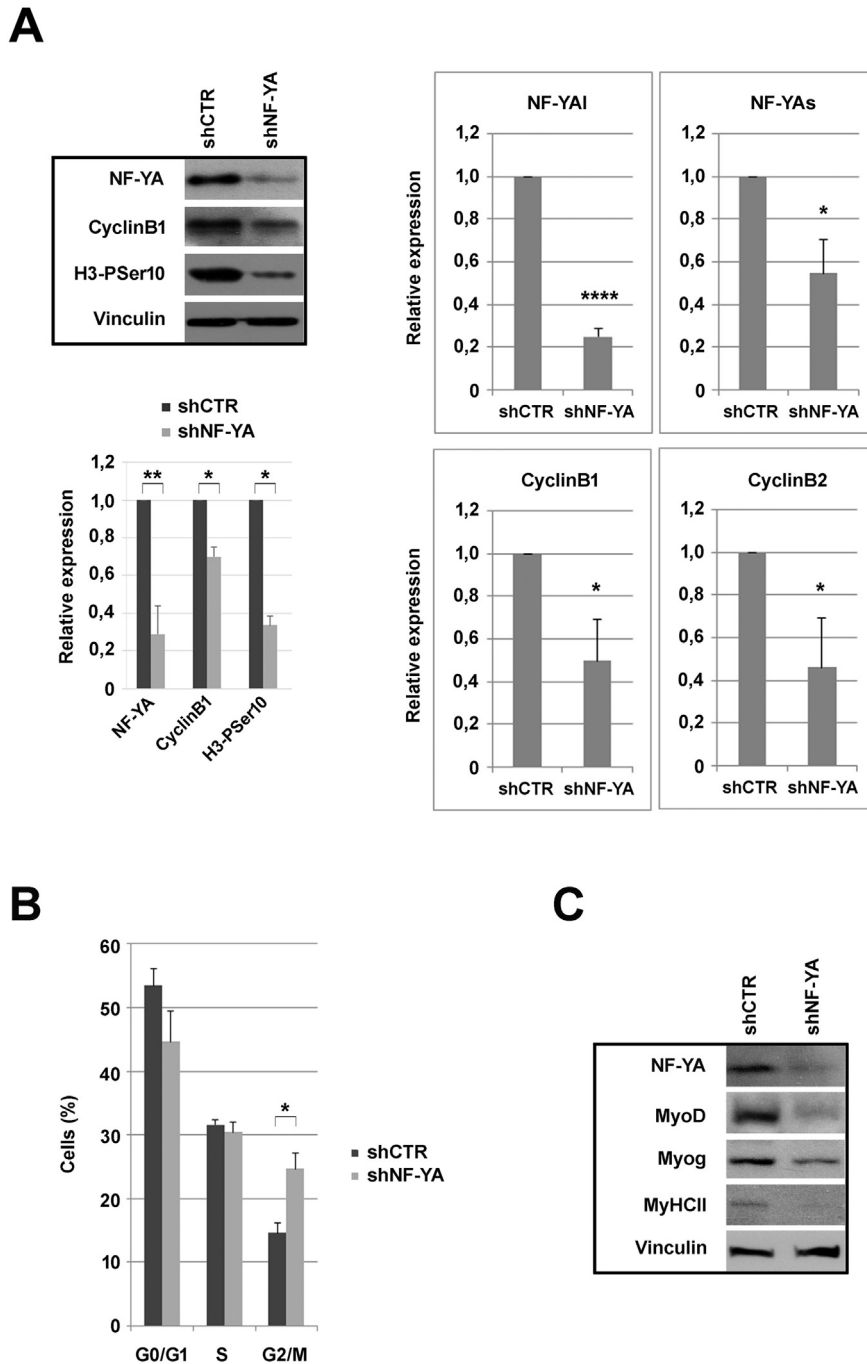
### 2.8. Western blot analysis

Whole cell protein extracts were prepared by cell lysis into 1X SDS sample buffer (25 mM TrisHCl pH 6.8, 1.5 mM EDTA, 20% glycerol, 2% SDS, 5%  $\beta$ -mercaptoethanol, 0.0025% Bromophenol blue). For immunoblotting, equivalent amounts of cellular extracts were resolved by SDS-PAGE, electrotransferred to PVDF membrane (GE Healthcare), and immunoblotted with the following primary antibodies diluted 1:1000

in TBS 1X-BSA 1  $\mu$ g/ $\mu$ l: anti-NF-YA (sc-10779, Santa Cruz), anti-NF-YB (GeneSpin), anti-MyoD C-20 (sc-304, Santa Cruz), anti-MyHCII MF20 (Developmental Studies Hybridoma Bank, IA, USA), anti-Myogenin M-225 (sc-304, Santa Cruz), anti-Vinculin (V4504, Sigma Aldrich), anti-Cyclin B1 (V152) (Gene Tex), and anti-phospho-Histone H3 (Millipore). Chemiluminescent detection reagent has been purchased from Millipore (Luminata Classico and Forte Western HRP).

### 2.9. Immunofluorescence staining

Immunofluorescence staining was carried out on C2C12 cells using anti-MyHCII MF20 (Developmental Studies Hybridoma Bank, IA, USA) and anti-NF-YA (sc-10779, Santa Cruz) antibodies, as described [40].

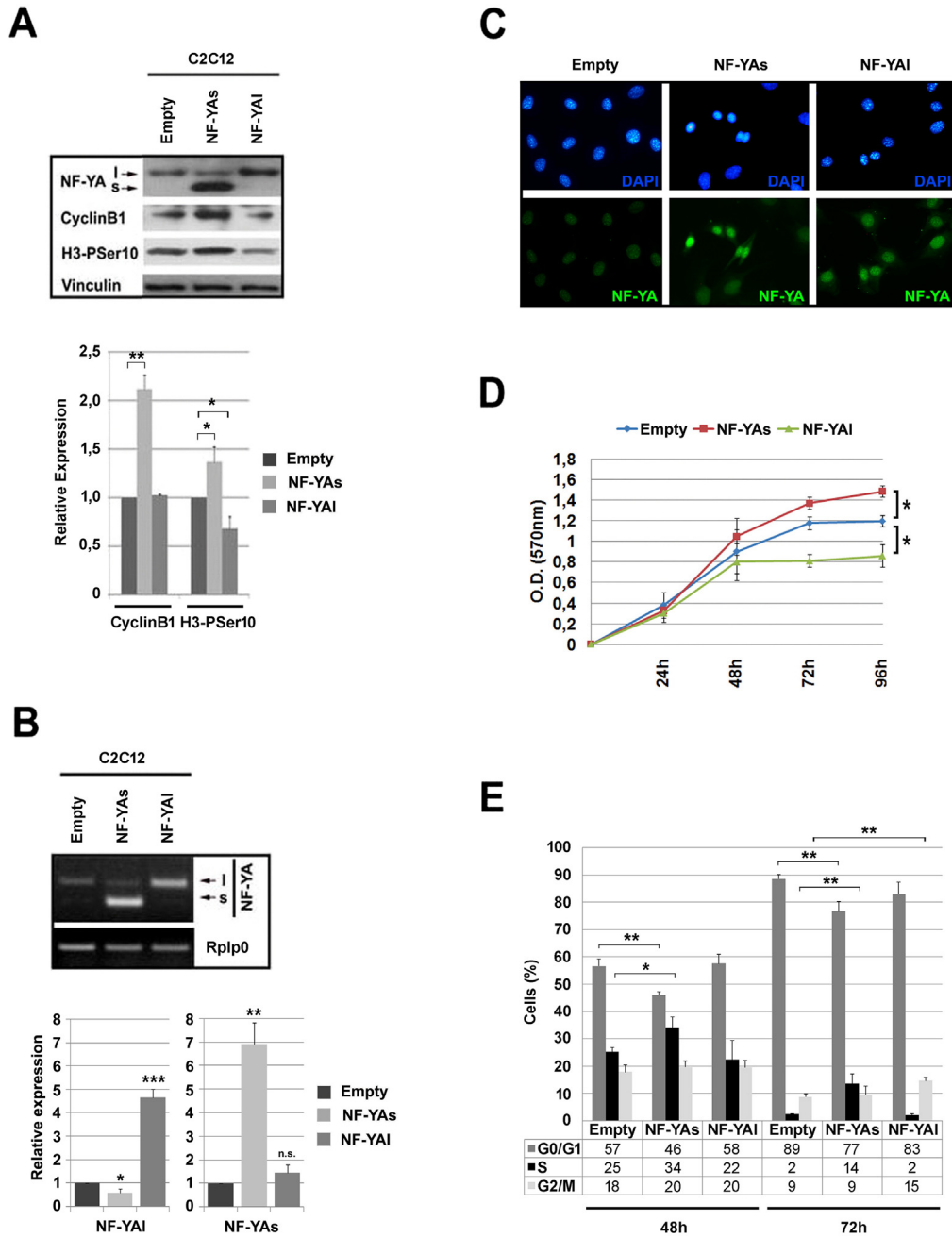


**Fig. 2.** NF-YA knock-down in C2C12 cells affects their proliferation and differentiation. **A.** Analysis of the expression levels (upper panel) and relative quantification (lower panel) versus Vinculin of NF-YA, CyclinB1, and H3-Pser10 in control (shCTR) and NF-YA-inactivated (shNF-YA) cells. Data are mean of four independent experiments  $\pm$  s.d. Right panel: qRT-PCRs of the indicated transcripts in control and NF-YA knocked-down cells. The bars represent the mean of four independent experiments  $\pm$  s.d. **B.** Cytofluorimetric cell cycle analysis of shCTR and shNF-YA cells. Error bars indicate s.d. of three biological replicates. **C.** Western blot analysis of NF-YA and myogenic factors MyoD, Myogenin, and MyHCII in shCTR and shNF-YA cells induced to differentiate for 3 days.

### 2.10. Gene expression profiling and data analysis

Gene expression profilings were performed using Affymetrix technology on RNA extracted from two biological samples of empty, NF-YAs and NF-YAI overexpressing cells induced to differentiate for 3 days (C3-DM). Total RNA was purified using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. Disposable RNA chips (Agilent RNA 6000 Nano LabChip kit) were used to determine the concentration and purity/integrity of RNA samples using the Agilent 2100 bioanalyzer. cDNA synthesis, biotin-labeled target synthesis, Affymetrix GeneChip Mouse 430 2.0 array (Affymetrix, Santa Clara,

CA) arrays hybridization, staining, and scanning were performed according to the standard protocol supplied by Affymetrix. Probe levels data were normalized and converted to expression values using the Robust Multiarray Average (RMA) procedure or the DChip procedure (invariant set). Quality control assessment was performed using Bioconductor packages (R-AffyQC Report, R-Affy-PLM, R-RNA Degradation Plot) and only arrays passing the quality controls were processed to the next step for differential gene expression analysis. Differentially expressed genes were selected for having a differential fold change above 2 with respect to the control sample (Empty). Gene Ontology of up-regulated genes and down-regulated genes was analyzed with

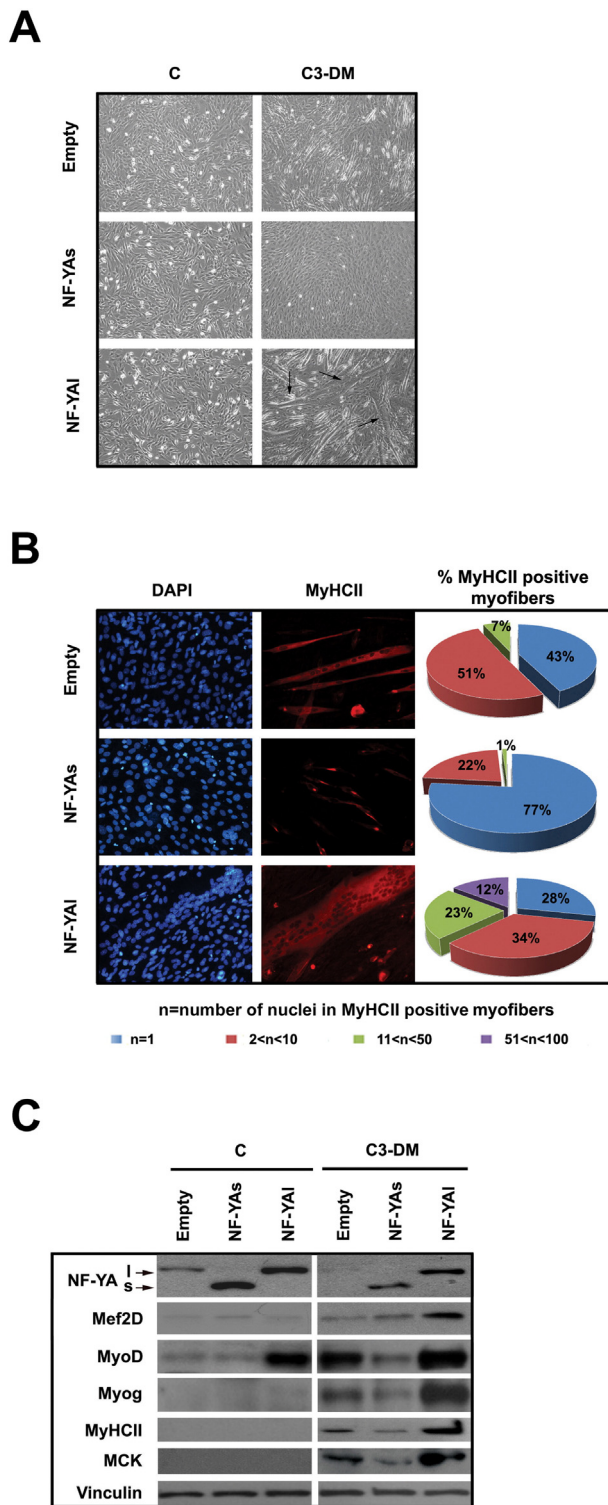


**Fig. 3.** NF-YAs forced expression in C2C12 stimulates cell proliferation. **A.** Upper panel: Western blot analysis of NF-YA, CyclinB1, and H3-PSer10 in NF-YAs and NF-YAI overexpressing C2C12 cells, compared to empty cells. Lower panel: quantification of CyclinB1 and H3-PSer10 protein levels in NF-YAs/I stably infected cells versus empty cells (arbitrarily set at 1) of three independent experiments. **B.** Upper panel: expression levels of NF-YA transcript variants in stably infected C2C12 cells using common primers. Rplp0 was used as internal control. Lower panel: Analysis of the levels of NF-YA transcripts in C2C12 NF-YAs- and NF-YAI-overexpressing cells compared to endogenous levels (empty cells) by *real-time* RT-PCRs with isoform-specific primers. The bars represent the mean of four independent experiments  $\pm$  s.d. **C.** Immunofluorescence analysis of NF-YA expression in empty, NF-YAs, and NF-YAI C2C12 cells. Nuclei were stained with DAPI. **D.** MTT cell proliferation assay of NF-YAs and NF-YAI overexpressing cells versus empty cells at the indicated time points from cells seeding. Data are the mean of six independent experiments  $\pm$  s.d. **E.** BrdU/PI cytofluorimetric analysis of empty and NF-YAs/I overexpressing cells 48 and 72 h from cells seeding. Data are the mean of three independent experiments  $\pm$  s.d.

the DAVID tool with default setting. GSEA was used to identify differentially expressed gene sets in C2C12 NF-YAI cells with respect to C2C12 NF-YAs cells. Affymetrix CEL files were processed with the GenePattern tool using the rma normalization option. The gct file was uploaded into GSEA v1.0 and gene set enrichment analysis was performed using the gene set database c5.all.v5.1.symbols (Gene Ontology) with the following setting: 1000 permutation, gene-set as permutation type, weighted as enrichment statistic, ratio of classes as metric for ranking genes. The GSEA results were ranked according to the nominal p-value ( $<0.005$ ) and FDR ( $\geq 0.01$ ).

### 2.11. RT-PCRs analysis

RNA was extracted from C2C12 cells using the Purelink RNA mini kit (Invitrogen, Life Technology) according to the manufacturer's protocol and 3  $\mu$ g of RNA were retro-transcribed with a Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Promega). *Real-time* PCRs were performed with oligonucleotides designed to amplify 100–200 bp fragments: sequences are available upon request. The housekeeping gene Rplp0 was used as a loading control. The relative sample enrichment was calculated with the formula  $2^{-\Delta\Delta Ct}$ , where



**Fig. 4.** NF-YAI overexpression increases differentiation of C2C12 cells. **A.** Light microscopy analysis of C2C12 stably infected cells. C = confluent cells, C3-DM = confluent cells maintained for additional 3 days in differentiating medium. The arrows indicate large myofibers. Images are representative of ten independent experiments. **B.** MyHCII immunofluorescence analysis of empty, NF-YAs, and NF-YAI C2C12 cells. The pie chart represents the percentage of multinucleated myofibers; different colors are indicative of the number of nuclei per MyHCII-positive myofiber. Percentages are the mean of three independent experiments. **C.** Western blot analysis of the indicated proteins in C and C3-DM empty and NF-YAs/I stably infected cells. Images are representative of at least three independent experiments.

$\Delta\Delta Ct = [(Ct_{\text{sample}} - Ct_{\text{Rplp0}})x - (Ct_{\text{sample}} - Ct_{\text{Rplp0}})y]$ , x = infected sample (Empty, NF-YAs, or NF-YAI) and y = Empty proliferant sample.

For generation of the absolute standard curves, cDNA of NF-YAs and NF-YAI transcripts were obtained by PCR. PCR products were run on a 1% TAE agarose gel. The fragments were excised and eluted using Promega Wizard® SV Gel and PCR Clean-Up System. The concentrations of the PCR products were measured using Nanodrop 1000. Micrograms of DNA were then converted to picomoles. Equimolar dilutions of both PCR fragments were used to generate the standard curves of seven orders of magnitude.

### 2.12. Chromatin Immunoprecipitations (ChIP)

Chromatin immunoprecipitations were performed as previously described [41,42]. 5 µg of anti-NF-YA (sc-10779, Santa Cruz) and anti-NF-YB (GeneSpin) were added to each IP and incubated over-night at 4 °C on a rotating wheel. DNAs were resuspended in TE buffer and quantitative real-time PCR was performed using SYBR green reagent (ThermoFisher) in the LightCycler Roche PCR machine. The relative sample enrichment was calculated with the following formula:  $2^{\Delta\Delta Ct_x} - 2^{\Delta\Delta Ct_y}$ , where  $\Delta Ct_x = Ct_{\text{input}} - Ct_{\text{sample}}$  and  $\Delta Ct_y = Ct_{\text{input}} - Ct_{\text{control Ab}}$ . Data have been shown as means of four independent experiments. Semiquantitative PCRs were performed to amplify the Cdkn1c promoter with primers described in Ref. [16].

### 2.13. Statistical analysis

All statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). One sample *t*-test was used for gene expression analysis presented in Figs. 1C, 2A (right panel), 3B (lower panel), 5B, 6A (left panel), 6D (left panel) and for Western blot quantification analysis shown in Figs. 2A (left panel), 3A, 6A (right panel). One sample *t*-test was also performed to analyze transfection and ChIP data of Figs. 6B (right panel), 6C (right panel), and 6D (middle and right panel). *P* values of <0.05 were considered to be statistically significant (\*), <0.01 (\*\*), <0.001 (\*\*\*), and <0.0001 (\*\*\*\*). Unpaired *t*-test (two-tailed) was used for Figs. 2B, 3E, 5C (lower panel), 5D and 6C (middle panel). *P* values of <0.05 were considered to be statistically significant (\*), <0.01 (\*\*), <0.001 (\*\*\*), and <0.0001 (\*\*\*\*). To compare means across empty and NF-YA-overexpressing cells, in Fig. 3D, one-way ANOVA analysis was utilized. *P* values of <0.05 were considered to be statistically significant (\*). The number of biological or technical replicates used for the calculation of the mean, s.d. and statistical analysis was indicated in figure legends.

## 3. Results

### 3.1. NF-YA inactivation impairs cell proliferation and differentiation

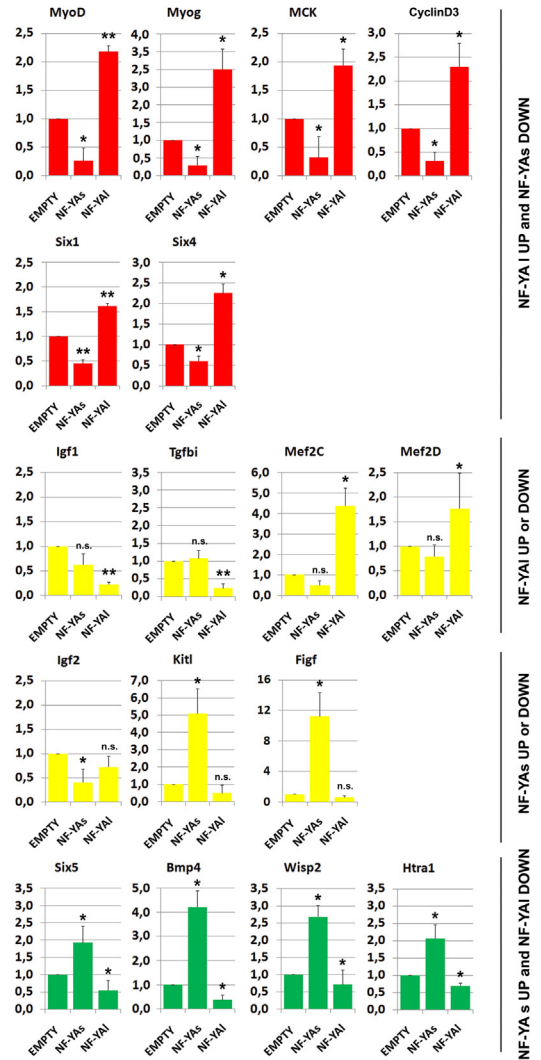
To investigate the mechanisms by which NF-YA affects myogenic progression, we used the mouse C2C12 cell line, immortalized adult post-injury-derived cells, able to recapitulate the process of adult myogenesis [43]. These cells were shown to express NF-YAI, whose levels decrease following differentiation [26] (Fig. 1A). To a lower extent, however, NF-YAs protein and mRNA are detected in C2C12, and lost after differentiation (Fig. 1A–C). Absolute quantifications of NF-YAs and NF-YAI mRNA levels, based on a standard curve, confirmed that NF-YAI was the prevalent transcript in proliferating myoblasts, being about 8.5-fold more abundant than NF-YAs (Fig. 1B).

As expected, differentiated cells showed a drop in the expression of Myf5 and an increase of MyoD, followed by Myogenin and Myosin Heavy Chain II (MyHCII) (Fig. 1A). qRT-PCRs highlighted that both NF-YA isoforms, as well as Myf5, MyoD, Myogenin, and MyHCII, are in part regulated at the transcriptional level (Fig. 1C). CyclinB1 and CyclinB2 levels were down-regulated following differentiation, as expected (Fig. 1C).

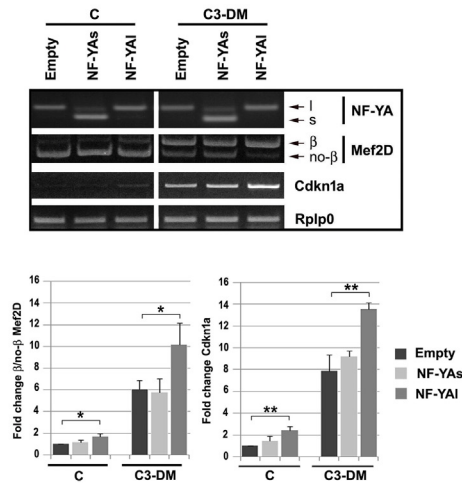
**A**

PValue	Term	PValue
NF-YA1 UP		NF-YAs DOWN
3,99E-19	GO:0043292~contractile fiber	4,18E-34
4,84E-19	GO:0030017~sarcomere	2,64E-30
1,44E-18	GO:0030016~myofibril	5,59E-34
3,73E-18	GO:0044449~contractile fiber part	4,87E-32
3,36E-15	GO:0016529~sarcoplasmic reticulum	9,67E-15
3,91E-15	GO:0031674~I band	4,16E-21
1,05E-14	GO:0016528~sarcooplasm	2,52E-14
6,24E-14	GO:0030018~Z disc	2,74E-15
7,94E-09	GO:0008092~cytoskeletal protein binding	4,60E-08
3,46E-08	GO:0006936~muscle contraction	9,37E-11
3,64E-08	GO:0007517~muscle organ development	3,10E-11
	GO:0007517~muscle organ development	3,10E-11
	GO:0014706~striated muscle tissue development	1,59E-10
	GO:0055002~striated muscle cell development	2,92E-10
	GO:0003012~muscle system process	4,55E-10
	GO:0060537~muscle tissue development	5,30E-10
	GO:0055001~muscle cell development	1,49E-09
NF-YA1 DOWN		NF-YAs UP
8,29E-28	GO:0005576~extracellular region	7,54E-10
1,05E-23	GO:0044421~extracellular region part	1,02E-09
3,88E-17	GO:0005578~proteinaceous extracellular matrix	
1,20E-16	GO:0031012~extracellular matrix	
4,80E-10	GO:0005520~insulin-like growth factor binding	
2,05E-09	GO:0005615~extracellular space	
5,06E-09	GO:0030247~polysaccharide binding	
5,06E-09	GO:0001871~pattern binding	
	GO:0007155~cell adhesion	1,44E-08
	GO:0022610~biological adhesion	1,55E-08
	GO:0001944~vasculature development	5,02E-08

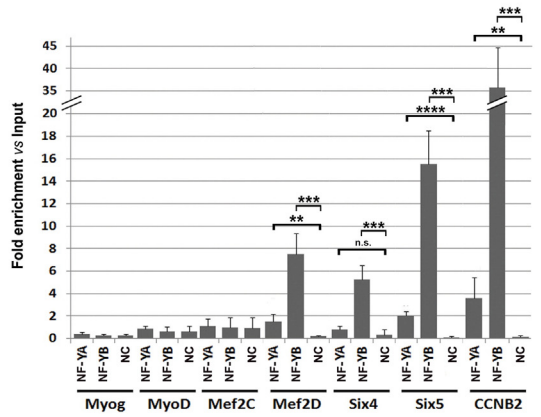
**B**



**C**



**D**

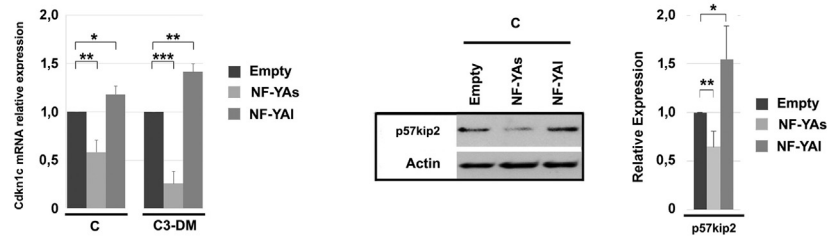


**Fig. 5.** Transcriptional effects of NF-YAs and NF-YA1 overexpression in C2C12 cells. **A.** Gene Ontology (GO) terms displaying a statistically significant over-representation in the sample-set (up- and down-regulated genes in NF-YAs and NF-YA1 cells versus control cells). P values refer to the over-representation (see Materials and methods). Only GO terms with  $p < 1.00E-08$  were displayed and ranked by decreasing NF-YA1 P-value. **B.** Real-time RT-PCR analysis of the indicated transcripts in C2C12 stably infected cells maintained in differentiating conditions for 3 days (C3-DM). mRNA levels of the mean of three biological replicates are indicated as relative expression compared to empty myoblasts (arbitrarily set at 1). **C.** Upper panel: semi-quantitative RT-PCR analysis of the splice variants of NF-YA (s and l) and Mef2D ( $\beta$  no- $\beta$ ), and of Cdkn1a in the indicated cell culture conditions. C = confluent cells and C3-DM = confluent cells maintained for additional 3 days in differentiating medium. Lower panel: Quantification of  $\beta$ /no- $\beta$  ratio and Cdkn1a mRNA levels in stably infected cells. The values of three independent experiments are indicated as fold change versus empty confluent cells  $\pm$  s.d. **D.** ChIP analysis showing NF-YA and NF-YB recruitment to regulatory regions of the indicated genes in C2C12 cells. Values are represented as % of Input DNA  $\pm$  s.d. Statistical analysis (unpaired t-test) was performed to compare the enrichment of NF-YA/NF-YB versus negative control antibody (NC). P values  $< 0.01$  were considered to be statistically significant (\*),  $< 0.001$  (\*\*), and  $< 0.0001$  (\*\*\*\*).

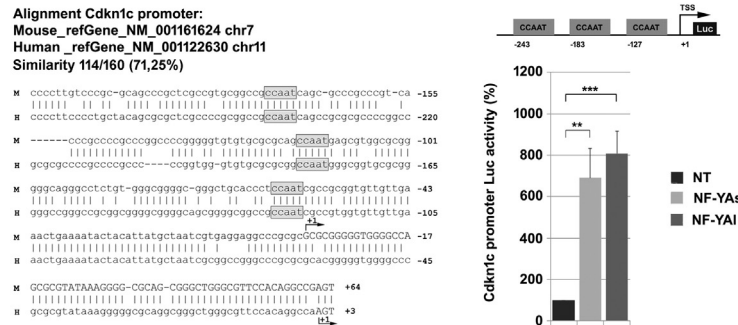
To assess the role of NF-YA in myoblasts proliferation and differentiation, we infected C2C12 cells with shRNA lentiviral particles targeting NF-YA. Fig. 2A shows a significant decrease in the protein levels of NF-YA1, but qRT-PCR indicates that both NF-YA transcripts were down-regulated by RNAi (Fig. 2B). Also the levels of the NF-Y target genes CyclinB1 and CyclinB2 were transcriptionally down-regulated upon NF-YA inactivation, as expected (Fig. 2B). Cell proliferation was affected

by NF-YA loss and an increase in G2/M population, compared to control cells (shCTR), was observed (Fig. 2A). Indeed, lowered CyclinB1 and phospho-Ser10H3 (H3-PSer10) levels are consistent with a G2 arrest (Fig. 2A). Moreover, we induced these cells to differentiate by serum withdrawal and analyzed the levels of differentiation markers: the expression of MyoD, Myogenin, and MyHCII were robustly reduced in shNF-YA with respect to shCTR cells (Fig. 2C).

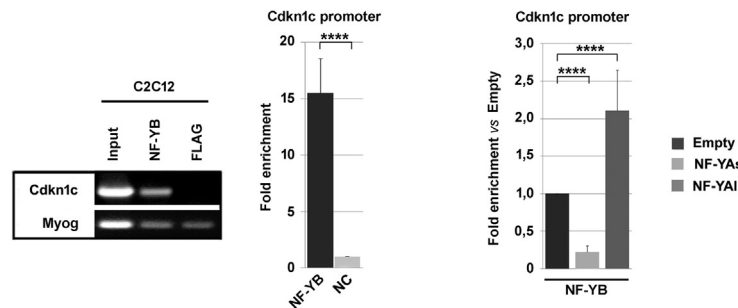
A



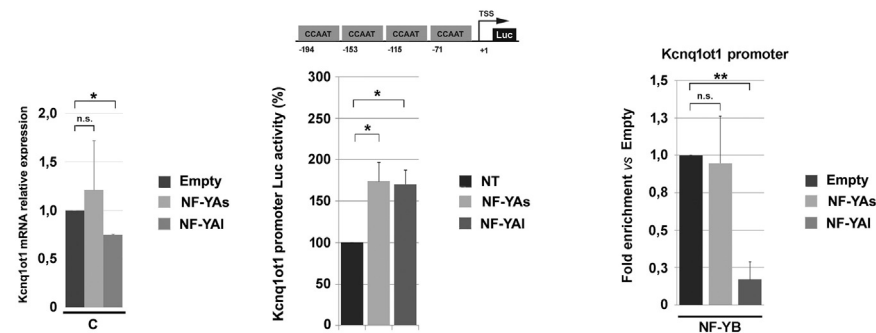
B



C



D





These data confirm that NF-YA expression is necessary for myoblasts proliferation and highlight a potential activity of NF-Y in myogenic differentiation.

### 3.2. Role of NF-YAs and NF-YA1 in proliferation of C2C12 cells

To study the activity of NF-YA isoforms in myoblasts proliferation, we stably overexpressed NF-YAs and NF-YA1 in C2C12 cells. Overexpression and nuclear localization of NF-YA proteins were confirmed by Western blot, RT-PCRs, and immunostaining (Fig. 3A–C). Note that endogenous NF-YA1 decreased in NF-YAs-overexpressing cells both at protein and mRNA level (Fig. 3A and B).

We then compared the effects of NF-YA forced expressions on proliferation. A significant difference was observed from 48 h post-cells seeding: empty cells reached the plateau phase in 72 h, NF-YA1 cells stopped growing 1 day before control cells, while NF-YAs cells continued proliferating even in confluence (Fig. 3D). The increase of the proliferation markers CyclinB1 and H3-PSer10 corroborated NF-YAs positive effect on cell growth (Fig. 3A).

PI/BrdU biparametric cell cycle cytofluorimetric analysis was performed on C2C12 myoblasts 48 and 72 h after seeding (Fig. 3E). Compared to control cells, at 48 h NF-YAs cells did show an increase of about 30% of BrdU positive cells, and a reduction of G0/G1 events. In contrast, NF-YA1 induced a 20% decrease in the proportion of S phase cells and a 10% increase in G0/G1 events. At 72 h, the effect of NF-YAs on DNA replication was even more evident, as BrdU incorporating cells increased from 3% to 16%, further suggesting that NF-YAs significantly stimulates cell proliferation.

### 3.3. NF-YA1 overexpression increases myogenic differentiation of C2C12 cells

We then analyzed the effects of NF-YA isoforms overexpression on C2C12 differentiation. Light microscopy analysis did not show differences between empty, NF-YAs, and NF-YA1 in cells reaching confluence (C). On the other hand, in confluent cells maintained for additional 3 days in differentiation medium (C3-DM), NF-YAs cells showed a decrease in myotubes formation compared to control cells, while NF-YA1 overexpression generated multiple and large myofibers, hinting that NF-YA1 could stimulate cell differentiation (Fig. 4A). To validate this hypothesis, we immunostained C3-DM cells with anti-MyHCII antibody: NF-YA1 overexpression led to large multinucleated MyHCII+ myotubes and increased the number of nuclei per myofiber; on the contrary, NF-YAs forced expression led to the formation of few MyHCII+ myotubes, the majority of which were mononucleated (Fig. 4B). In accordance with the morphology, higher levels of Mef2D, MyoD, Myogenin, MyHCII, and MCK were observed by Western blot in C3-DM NF-YA1-cells, and a decrease was detected in NF-YAs-cells (Fig. 4C). Note that an increase of MyoD expression was detected already in NF-YA1 confluent cells (C), hinting that this isoform could be a positive regulator of MyoD or could induce early differentiation in confluent cells. In summary, overexpression of the two NF-YA isoforms leads to a remarkable difference in the behavior of differentiating myoblasts.

### 3.4. NF-YAs and NF-YA1 differentially regulate the transcriptional program governing skeletal myogenesis

To gain insight into the role of NF-YA isoforms during muscle differentiation, we performed gene expression profilings of C2C12 stably overexpressing the two isoforms, induced to differentiate for 3 days (C3-DM). We found that 799 and 476 genes were differentially regulated (with fold change  $\geq 2.0$ ) in NF-YAs and NF-YA1 cells, compared to empty cells. Specifically, 374 and 210 genes were down-regulated, 425 and 266 genes were up-regulated in NF-YAs and NF-YA1-cells, respectively. Remarkably, Gene Ontology (GO) analysis highlighted that NF-YAs down-regulated categories were a nearly perfect mirror of NF-YA1 up-regulated terms, and vice versa (Fig. 5A). GO terms associated to muscle structure and function, specifically contractile fiber, myofibril, contractile fiber part, sarcomere, I band, Z disc, sarcoplasmic reticulum/sarcoplasm are up-regulated by NF-YA1 and repressed by NF-YAs. Differently, NF-YAs up-regulated categories were mainly associated to growth-factor binding and extracellular proteins, mostly down-regulated in the NF-YA1 cohort. Gene set enrichment analysis (GSEA) comparing NF-YA1 with respect to NF-YAs expression data highlighted that gene sets associated to muscle structure and function were significantly up-regulated in NF-YA1 cells compared to NF-YAs cells, in agreement with GO analysis and phenotypic differences between the two NF-YA-overexpressing cell lines (Suppl. Table 1).

Real-Time RT-PCRs on empty-, NF-YAs-, and NF-YA1 cells were performed to validate the microarray data (Fig. 5B). A first group includes genes up-regulated by NF-YA1 and down-regulated by NF-YAs, such as MyoD, Myogenin, and CyclinD3, key players in early cell differentiation, and MCK, marker of late differentiation. We also analyzed the expression of Six genes, which cooperate with MRFs in the myogenesis regulatory network [5]. The mRNA levels of Six1 and Six4, both required for myoblast differentiation, were doubled by NF-YA1 and halved by NF-YAs (Fig. 5B).

A second group consists of genes affected mostly by a specific isoform, such as Igf1, Tgfb1, Mef2C, Mef2D, Cdkn1a, Igf2, Kitl, and Figf. In particular, Cdkn1a, Mef2C, and Mef2D transcripts were up-regulated only upon NF-YA1 overexpression (Fig. 5B and C). Transcripts of the Mef2 family are subject to alternative splicing, which can affect their activation potential: inclusion of the  $\beta$ -exon occurs during muscle differentiation and leads to a more robust activation of Mef2-target genes [44]. The increase of the Mef2D  $\beta$ /no- $\beta$  ratio was indeed enhanced by NF-YA1 overexpression in differentiation conditions (C3-DM) (Fig. 5C). A third group is composed of genes up-regulated by NF-YAs and down-regulated by NF-YA1, such as Six5, Bmp4, Wisp2, and Htra1.

We then analyzed the genes for the presence of TFBS (Transcription Factors Binding Sites) in their promoters, using PSCAN and the JASPAR database (Suppl. Fig.1): many matrices were present in the activated and repressed cohorts analyzed—particularly E2Fs, GC-rich KLFs, Sp1/2, and TPAP2—with similar p values, making it unlikely that these transcription factors are involved in differential regulation. Surprisingly, the CCAAT box matrix was not enriched, suggesting that NF-YA overexpression might not affect the mRNA levels of the majority of differentially expressed genes through binding to the promoters and direct transcriptional regulation. Instead, MRF matrices (MyoD, Myogenin) were

**Fig. 6.** NF-YAs and NF-YA1 differentially regulate *Cdkn1C* transcription. **A.** Left panel: qRT-PCR of *Cdkn1C* transcript in empty, NF-YA1- and NF-YAs-overexpressing confluent (C) or differentiated (C3-DM) cells. The bars represent the mean of four experiments  $\pm$  s.d. Middle and right panels: protein levels and relative quantification of p57<sup>KIP2</sup> in NF-YA-overexpressing cells compared to empty cells. Protein levels of control cells have been arbitrarily set at 1 and the bars indicate the mean of five values. **B.** Left panel. Sequence alignment of *Cdkn1C* human and mouse promoters. Conserved CCAAT elements have been framed. Right panel: relative luciferase activity following transient co-transfections of the *Cdkn1C*-LUC vector with 100 ng NF-YAs and NF-YA1 expressing plasmids. Data represent means  $\pm$  s.e.m. of three independent transfections (two technical replicates for each transfection). **C.** Left panel: ChIP analysis of NF-Y binding to the *Cdkn1C* promoter in C2C12 confluent cells. Middle panel: the bars indicate fold enrichment of NF-Y immunoprecipitated with the NF-YB antibody versus negative control antibody (NC) and are the mean of three technical replicates of three independent experiments  $\pm$  s.d. Right panel: relative enrichment of NF-Y binding to the *Cdkn1C* promoter in NF-YA-overexpressing cells compared to empty cells (arbitrarily set at 1). Data are the mean of two independent experiments with five technical replicates  $\pm$  s.d. **D.** Left panel: qRT-PCR of *Kcnq1ot1* transcript in empty and NF-YA-overexpressing confluent cells (C). Transcript levels of empty cells have been arbitrarily set at 1 and the values are the mean of three experiments  $\pm$  s.d. Middle panel: *Kcnq1ot1* promoter-driven luciferase activity following NF-YAs or NF-YA1 transient overexpression. The bars represent the mean of three technical replicates of three different transfections  $\pm$  s.e.m. Right panel: qChIP analysis of NF-Y binding in empty, NF-YAs and NF-YA1 confluent cells. Relative enrichment of NF-Y binding to the *Kcnq1ot1* promoter in NF-YA-overexpressing cells has been compared to empty cells (arbitrarily set at 1). NC = negative control antibody. Data are the mean of three experiments.

enriched in NF-YAI up-regulated and NF-YAs down-regulated cohorts, in keeping with the observed morphological effect. This result suggests that, at least in part, the NF-YAI effect could be due to increased expression of myogenic transcription factors, which could then initiate the muscle-specific gene programs. The regulatory regions of MyoD, Mef2C, Mef2D, and Six genes indeed contain one or more CCAAT elements. ChIP analysis showed NF-Y binding to Mef2D and Six regulatory regions, but not to Mef2C or MyoD promoters (Fig. 5D). The promoter of CyclinB2, a known NF-Y target gene, was used as positive control, while the CCAAT-less Myogenin was used as negative control.

In summary, specific changes in C2C12 differentiation programs are affected by the NF-YA isoforms, mostly, but not necessarily exclusively, through an impact in some of the muscle-specific transcription factors.

### 3.5. *Cdkn1c* is a novel NF-Y-regulated target gene

Among genes up-regulated by NF-YAI and down-regulated by NF-YAs, *Cdkn1c* is particularly relevant, because of its dominant role in pushing differentiation. qRT-PCR and Western blot confirmed the different effect on gene expression in NF-YA stable-expressing cells (Fig. 6A). Three conserved CCAAT elements are located within the core promoter of the murine and human gene (Fig. 6B); we decided to investigate whether the two NF-YA isoforms could differently affect *Cdkn1c* transcription. Transient co-transfections of a *Cdkn1c*-LUC vector with NF-YAs and NF-YAI plasmids showed a robust increase in activity, equally induced by the two isoforms (Fig. 6B). This could be dissimilar *in vivo*, where the promoters are assembled into chromatin: we therefore performed ChIP analysis of NF-Y binding in C2C12 control and NF-YA overexpressing cells. NF-Y was clearly associated to the promoter of *Cdkn1c* in confluent C2C12 (Fig. 6C, left and middle panel), and an enrichment in binding is observed in NF-YAI cells compared to empty and NF-YAs (Fig. 6C, right panel), reflecting the increased transcription levels observed with former isoform (Fig. 6A).

*Cdkn1c* transcription is regulated by the lncRNA *Kcnq1ot1*. In particular, increased *Kcnq1ot1* levels are associated to reduced  $p57^{kip2}$  expression and NF-Y binding was demonstrated to be important for the regulation of *Kcnq1ot1*. We investigated the effects of NF-YAs and NF-YAI overexpression on this transcript: qRT-PCRs showed a significant decrease in *Kcnq1ot1* RNA levels in confluent shNF-YAI cells, consistent with increased *Cdkn1c* transcription (Fig. 6D, left panel). The two splice variants did not show differences in activation of a Luciferase reporter in transfection assays (Fig. 6D, middle panel), but again, ChIP analysis showed lower NF-Y binding to the *Kcnq1ot1* promoter only in NF-YAI overexpressing cells (Fig. 6D, right panel).

All together, these results provide evidence that NF-Y controls an important pro-differentiation gene *via* a lncRNA, hinting at a differential behavior of NF-YAs and NF-YAI *in vivo*, with diverse effects on specific CCAAT promoters.

## 4. Discussion

We report on the dual function of the transcription factor NF-Y in the regulation of proliferation and differentiation of skeletal muscle cells. Opposite activities are played by the two NF-YA splice variants, which activate different transcriptional and cell fate programs. These data are consistent with previous reports in HSCs and mouse mESCs showing that NF-YAs is the splice variant with pro-proliferative activity [31,34]. We describe here a novel role for NF-YAI to favor the commitment to differentiation of myoblasts.

We overexpressed NF-YA isoforms in C2C12 immortalized myoblasts, in which NF-YAI is the major variant: forced expression of NF-YAs results in increased proliferation, while higher levels of NF-YAI stimulate myoblasts fusion into large multinucleated myotubes. These results confirm NF-YAI modulation during C2C12 differentiation [26] and show a novel positive role in muscle differentiation, which was unnoticed before. Previous experiments performed in C2C12 through NF-

YAI transient overexpression showed that NF-YA down-regulation allows cells to exit the cell cycle and induces terminal differentiation program [26]. The different technical approaches used in our present work and in previous studies [26] likely account for the differences in the obtained results: stable infection of C2C12 cells shows moderate increase in protein levels compared to control cells, whereas transfections with NF-YAI considerably, but transiently, raise its expression [26]. Thus, it is possible that “chronic” *versus* “acute” overexpression of NF-YAI could activate different molecular and phenotypic effects. Note that myogenic conversion experiments by transient overexpression show that both NF-YA isoforms interfere with the myogenic program (data not shown), suggesting that not only the levels but also the timing of NF-YA overexpression is important for muscle differentiation.

The functional difference between the two NF-YA isoforms, presented for the first time here, is striking. Since its original discovery [45], different sets of data relegated the issue of NF-YA splicing into an oblivion: (i) various balanced levels of NF-YAI/NF-YAs are present in different cell types, with little apparent logic; (ii) the DNA binding affinity of trimers composed of NF-YAs or NF-YAI are similar *in vitro*; (iii) the spliced domain doesn't show significant differences in the aminoacids composition—high Q and hydrophobics content—with respect to the large N-term activation domain where it resides [35–37,46], which is reflected in a similar trans-activating behavior, at least in artificial activation assays. It is now clear that they are part of different, even opposite, transcriptional programs. In addition, our findings might also bear implications on the splicing of NF-YC, since there is a good correlation between NF-YAs and NF-YC-50, and between NF-YAI and NF-YC-37, at least in the cell lines tested so far [46].

NF-Y is a pioneer factor [47–49], known to activate the genes of many “activator/effector” transcription factors responsible for switching on specific transcriptional pathways. In most cases, the specific activator/effector protein teams up with NF-Y to jointly activate genes of that pathway: it is the case of SREBP1/2 (cholesterol/fatty acids), HSF1 (heat shock), CHOP and XBP1 (ER stress), E2F1 (cell cycle) [50]. These factors require the pioneering role of NF-Y in pre-setting the chromatin state of the targeted promoters before activation. Because of the transcriptional effects of NF-YAI overexpression, obvious activator transcription factors candidates for the pro-differentiation effects were MyoD, which harbors a perfect CCAAT in the promoter, and Myogenin. However, ChIPs were negative, proving that the effect on these genes is indirect. Instead, direct NF-Y binding is observed on the genes of two other transcription factors important in muscle differentiation, Mef2D and Six family members. The analysis of ENCODE genome-wide locations of Mef2A/C in several cell lines indicates a lack of intersections with NF-Y [47] (DD, RM, G. Pavesi, submitted). Unless Mef2 proteins bind to substantially different sets of genes in muscle cells, we consider a global synergistic effect of NF-Y on Mef2 unlikely. Rather, because members of the Mef2 family are known to activate MyoD and Myogenin, we favor a scenario in which NF-YAI triggers a CCAAT-less genes program mediated by Mef2D, and, subsequently, MyoD and Myogenin. Note that Mef2D, but not Mef2C, is targeted: although functional redundancy was described within Mef2 family members, this suggests that different mechanisms are involved in the control of Mef2 genes expression. NF-YAI ectopic expression not only increases Mef2D gene expression but also modulates its splicing pattern.

As for the homeobox-containing Six family members, Six1/4/5 are important for muscle differentiation [5,51,52]: they share the same binding consensus and directly control the expression of Myogenin *via* MEF3 sites, both in cultured cells and *in vivo* [9,53]. Direct NF-Y binding to the promoters and activation of Six genes upon NF-YAI overexpression points to a second branch of “differentiation” genes activated by the latter. Interestingly, CCAAT boxes are enriched in Six5 peaks of ENCODE data, with a very robust overlap of nearby peaks in different cell lines (DD, RM, G. Pavesi, submitted). Six5 peaks are heavily enriched in binding sites of Znf143 [54], an important cell cycle regulator [55], which are also found near NF-Y. Thus, it is possible that NF-Y and Six

transcription factors cooperate closely through NF-Y-Znf143 combinations. This hypothesis is worth validating with genomic studies in the future.

Among genes directly regulated by NF-Y, we identified Cdkn1c, encoding for the CDKI p57<sup>kip2</sup>. Differently from p21<sup>Cip1/Waf1</sup>, which participates to myogenesis only through cell growth arrest, p57<sup>kip2</sup> has also an active role in supporting MyoD function [56,57]. Following NF-YAL overexpression, NF-Y increases p57<sup>kip2</sup> levels through a double mechanism: in physiological chromatin context, it binds and up-regulates the Cdkn1c CCAAT promoter, and, at the same time, reduces its association to the regulatory region of Kcnq1ot1, whose expression is therefore reversely correlated with the expression of Cdkn1c. These data hint that the composition of the NF-Y heterotrimer could affect the hierarchy of target genes, with a selection exerted through the different splice variants of the CCAAT-binding subunit. This hierarchy could reflect the opposite role played by NF-YAs and NF-YAL, with NF-YAs binding to genes promoting cell proliferation or inhibiting cell differentiation (such as Kcnq1ot1), and NF-YAL favoring NF-Y recruitment to genes that arrest cell cycle progression (among which Cdkn1c) or participate to the differentiation program (such as Mef2D, Six1, and Six4). In addition, the two NF-YA isoforms could initiate different transcriptional programs because of specific combinatorial efficiencies in protein–protein interactions of the Q-rich activation domain: when NF-YAs and NF-YAL are bound to CCAAT, different coactivator complexes could be associated through specific interactions with the isoforms, triggering diverse transcriptional and physiological responses. This matter should be thoroughly evaluated in the future, by developing appropriate reagents, such as isoforms-specific antibodies and shRNAs.

## 5. Conclusions

In conclusion, we demonstrated that post-transcriptional regulation of NF-YA expression is important in determining myoblasts fate and provides evidence of the role of alternative splicing in proliferation *versus* differentiation programs. Our data corroborated the proliferative activity of NF-YAs and identified a different, not previously appreciated, role for NF-YAL in favoring the commitment to differentiation.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbaggm.2016.02.011>.

## Competing interests

The authors declare no competing interests.

## Transparency document

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

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