Discovery, characterization and potential roles of a novel NF-YAx splice variant in human neuroblastoma

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Abstract

Background: Identification of novel cancer-associated splice variants is of potential diagnostic, prognostic and therapeutic importance. NF-Y transcription factor is comprised of NF-YA, NF-YB and NF-YC subunits, binds inverted CCAAT-boxes in ≈70% of gene promoters, regulates > 1000 cancer-associated genes and proteins involved in proliferation, staminality, differentiation, apoptosis, metabolism and is subject to component alternative splicing. RT-PCR evaluation of alternative NF-YA splicing in primary human neuroblastomas (NBs), led to discovery of a novel NF-YAx splice variant, also expressed during mouse embryo development and induced by doxorubicin in NB cells. Here, we report the discovery and characterisation of NF-YAx and discus its potential roles in NB.

Methods: NF-YAx cDNA was RT-PCR-cloned from a stage 3 NB (provided by the Italian Association of Haematology and Paediatric Oncology, Genova, IT), sequenced and expressed as a protein using standard methods and compared to known fully-spliced NF-YA and exon B-skipped NF-YAs isoforms in: EMSAs for capacity to form NF-Y complexes; by co-transfection, co-immunoprecipitation and Western blotting for capacity to bind Sp1; by IF for localisation; in AO/EtBr cell-death and colony formation assays for relative cytotoxicity, and by siRNA knockdown, use of inhibitors and Western blotting for potential mechanisms of action. Stable SH-SYSY transfectants of all three NF-YA isoforms were also propagated and compared by RT-PCR and Western blotting for differences in cell-death and stem cell (SC)-associated gene expression, in cell-death assays for sensitivity to doxorubicin and in in vitro proliferation, substrate-independent growth and in vivo tumour xenograft assays for differences in growth and tumourigenic capacity.

Results: NF-YAx was characterized as a novel variant with NF-YA exons B, D and partial F skipping, detected in 20% of NF-YA positive NBs, was the exclusive isoform in a stage 3 NB, expressed in mouse stage E11.5–14 embryos and induced by doxorubicin in SH-SYSY NB cells. The NF-YAx protein exhibited nuclear localisation, competed with other isoforms in CCAAT box-binding NF-Y complexes but, in contrast to other isoforms, did not bind Sp1. NF-YAx expression in neural-related progenitor and NB cells repressed Bmi1 expression, induced KIF1Bβ expression and promoted KIF1Bβ-dependent necroptosis but in NB cells also selected tumourigenic, doxorubicin-resistant, CSC-like sub-populations, resistant to NF-YAx cytotoxicity.

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Conclusions: The discovery of NF-YAx in NBs, its expression in mouse embryos and induction by doxorubicin in NB cells, unveils a novel NF-YA splice mechanism and variant, regulated by and involved in development, genotoxic-stress and NB. NF-YAx substitution of other isoforms in NF-Y complexes and loss of capacity to bind Sp1, characterises this novel isoform as a functional modifier of NF-Y and its promotion of KIF1Bβ-dependent neural-lineage progenitor and NB cell necroptosis, association with doxorubicin-induced necroptosis and expression in mouse embryos coinciding with KIF1Bβ-dependent sympathetic neuroblast-culling, confirm a cytotoxic function and potential role in suppressing NB initiation. On the other hand, the in vitro selection of CSC-like NB subpopulations resistant to NF-YAx cytotoxicity not only helps to explain high-level exclusive NF-YAx expression in a stage 3 NB but also supports a role for NF-YAx in disease progression and identifies a potential doxorubicin-inducible mechanism for post-therapeutic relapse.

Keywords: NF-Y, NF-YA, Neuroblastoma, Alternative splicing, Necroptosis, KIF1Bβ, Cancer stem cells, Genotoxic stress

Background

Alternative gene splicing is a fundamental physiological mechanism for the differential expression of proteins from the same gene coding sequence and is largely responsible for the increased proteomic complexity of higher organisms that cannot be explained by differences in individual gene numbers alone [1]. Aberrant alternative splicing has been reported in cancer and is promoted by stress within the tumour microenvironment, oncogenic viral infection, gene translocation and oncogene de-regulation of splice factor expression. Cancer-associated alternative splicing has been shown to inactivate onco-suppressors and activate oncogenes, making the identification of novel cancer-associated splice isoforms of potential diagnostic, prognostic and therapeutic importance [1–5].

The ubiquitous transcription factor NF-Y binds inverted CCAAT-boxes (5′-ATTGG-3′) in ≥70% of gene promoters, recruits other transcription factors and proteins to promoters and regulates > 1000 cancer-associated genes involved in proliferation, stemness, differentiation, apoptosis, miRNA expression and metabolism [6–9]. NF-Y also regulates chromatin de-condensation during mitotic bookmarking and post-mitotic transcriptional re-activation, and acts as a bi-directional histone-like transcription factor, switching promoter histone methyl-marks from positive to negative. NF-Y is necessary for development, its inactivation is embryonically lethal and alterations in NF-Y activity have wide-ranging effects on cell behaviour [6–10]. The involvement of NF-Y in cancer is underpinned by its role in proliferation, the presence of inverted CCAAT-boxes in the promoters of many cancer-associated genes and interaction with and regulation of cancer-related proteins, including c-fos, wild type and mutant p53, p73, ΔNp63α, p21, Ash2L, BRCA-1, cMyc, Sox9, lamin, ZHX1/2 and in particular the ubiquitous cancer-related transcription factor Sp1 [6–11].

NF-Y is a hetero-trimer composed of NF-YA, NF-YB and NF-YC subunits, all of which are required for DNA binding and transcriptional activity. NF-YB complexes with NF-YC prior to binding NF-YA, which confers DNA binding and transcriptional activity to the NF-Y complex [6–8, 12–14]. The 29.44 kb NF-YA gene localises to chromosome 6p21, is organized into 9 exons [15] and is predominantly expressed as a fully-spliced 42 kDa, 347 amino acid (aa) long-form NF-YA with glutamine-rich, S/T-rich transactivation, subunit-interaction and DNA-binding domains or an alternative exon B-spliced 40 kDa, 318 aa short-form NF-YAs, deleted of glutamine-rich aa’s 26–54 [15, 16]. Minor NF-YA isoforms also include a 3 βp aa 27 deletion-variant, a 18 βp aa 548–565 deletion-variant and L2-L6 alternative splice variants deleted in glutamine-rich and S/T domains, driven by nucleotide 79, 154 and 548 deletions at A/B, B/C and E/F splice junctions [15, 17].

Alternative splicing of the NF-YA gene has been implicated in the regulation of cell staminality, differentiation, apoptosis and transformation. NF-YAs forms part of the stem cell (SC) transcriptional circuitry, predominates in embryonic SCs and is lost upon SC differentiation. In contrast, NF-YA/β promotes differentiation and loss of NF-YA expression induces senescence or apoptosis. Alternative NF-YAs splicing is promoted by the oncogenic polyomavirus SV40 and by v-ras oncogene and converts tumor-suppressing, differentiation-promoting NF-Y complexes predominated by NF-YA into tumor and CSC promoting complexes predominated by NF-YAs [8, 18–23].

Neuroblastomas (NB) are aggressive embryonic tumours of neural crest origin, derived from immature sympathetic neuroblasts [24]. These primitive tumours initiate under conditions that impair sympathetic neuroblast culling during development, reported to depend upon either loss of the KIF1B gene associated with chromosome 1p36-deletion, germline KIF1B mutations or Nmyc amplification [25–33]. NF-Y involvement in NB pathogenesis and progression, however, has received scant attention. In the few existing reports, NF-Y has been shown to be critical for expression of soluble guanylyl cyclase in NB cells required for cGMP production and differentiation [34] and is involved in elevated cGMP expression in NBs [35]. NF-Y and Sp1 transcription factors combine to promote tetramethylpyrazine-induced neuronal differentiation of
NB cells [36] and regulate expression of the α3 Na+, K+-ATPase subunit, essential for maintaining electrochemical gradients across cell membranes [37]. Suboptimal NF-Y function in NB cells has also been implicated in de-regulating the matrix metalloproteinase and tissue inhibitor of metalloproteinase equilibrium, resulting in invasion [38] and increased expression of the NF-YA subunit has been reported to differentiate between aggressive stage 4 NBs and stage 4S NBs that exhibit spontaneous regression [39].

Considering the relative absence of studies of NF-Y expression in NB, combined with reports associating fully spliced NF-YA with cellular differentiation and reduced malignancy and associating alternative exon B spliced NF-YAs with cellular staminality and increased malignancy [18–23], we initiated a study of NF-YA and NF-YAs expression in human primary NBs. This led to the unexpected discovery of a novel NF-YAx splice variant, with NF-Y functional modifying activity, which forms the subject of this report.

Materials and methods
Aim, design and setting
The aim of this study was to report the discovery and characterization of the novel alternative NF-YAx splice variant, discovered as the exclusive NF-YA isoform expressed in an advanced stage 3 NB, expressed during E8.5–E18.5 mouse embryo development and induced in NB cells by doxorubicin, and to provide insights into its potential function in NB pathogenesis and progression. Experimental design included cloning and sequence characterization, expression vector construction, protein expression and characterization, transient and stable transfection and biological characterizations in terms of growth, cytotoxicity and tumorigenicity.

Characteristics of participants and materials
RNAs from primary human stage 1, 2, 3 and 4 NBs (International NB Staging System) were kindly provided by the tissue bank of the Italian Association of Haematology and Pediatric Oncology (Genova, Italy). Patient age, sex or gender was not provided. RNAs from stage 4 progressive stage 4 NBs and stage 4S NBs that exhibit spontaneous regression [39].

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NF-YA variant cDNA cloning and sub-cloning
NF-YAx coding cDNA was cloned from the primary human stage 3 NB, exhibiting exclusive NF-YAx expression. NF-YAj, NF-YAs, NF-YB, and NF-YC cDNAs were cloned from SH-SY5Y mRNA using primers 5′-CTCTCTGATT GGGTTTCGAGT-3′ and 5′-GGGTTTACGACT CGGATGT-3′ spanning fully-spliced human NF-YAj cDNA (NM_002505.5); primers 5′-GGTCTTCGACTGA-3′ and 5′-GGTCTTCGACTGA-3′ spanning fully-spliced human NF-YB cDNA (NM_006166) and primers 5′-GGACTCCTGA CAGCAGCAT-3′ and 5′-GGGCGAGTCGAC TGAATTC-3′ spanning fully-spliced human NF-YC cDNA (HSU78774). RT-PCR products were agarose gel purified, sub-cloned into the TA cloning vectors (Invitrogen, Thermo-Fisher Scientific, Waltham, MA) and sequenced in an ABI prism automated DNA-sequencer. Full length NF-YAj, NF-YAs, NF-YAx, NF-YB and NF-YC cDNA clones were then sub-cloned into mammalian pcDNA3.1Zeo (+) and pAc insect expression vectors (Thermo-Fisher Scientific, Waltham, MA), together with cDNA for full-length Sp1, available in our laboratory. NF-YAdv expression vector was from Dr. R. Mantovani (University of Milan, Italy).

SiRNA knockdown
SiRNA knockdown was achieved using a TriFECTa Dicer-Substrate RNAi kit and three KIF1B-specific Dicer-Substrate siRNA duplexes (hs.Ri.KIF1B.13: 5′-UCCACUGAGAAGGUGAUAUAACGGACU-3′ and 5′-UGAUUUGGACUGACGAA-3′; hs.Ri.KIF1B.13: 5′-CAAGGUAUCAACGUGUGUGA-3′ and 5′-CU GAAUGAUCUCCGAGUAAUAAUA-3′; hs.Ri.KIF1B.13: 5′-AGCUUAUGAGAAGGUA-3′), as described (Integrated DNA Technologies, Bologna, Italy). Briefly, 1 × 10^5 cells/ml HEK-293 cells on 24 well plates were grown overnight to ≈80% confluence and co-transfected with either 50 nM of negative control siRNA duplex (5′-CGUUAACCGGUAUAACCGGUAT-3′ and 5′-AUACGGGUAUAACGGAUUAACAG-3′) or 50 nM of a mix of KIF1B-specific siRNA duplexes, plus 1 μg/ml of pcDNA3.1 NF-YAx expression plasmid or empty pcDNA control plasmid, using TransIT-X2 Dynamic Delivery System, as directed (Mirus Bio, www.mirusbio.com/6000). Sham-transfected controls received transfection reagent alone. Knockdown of NF-YAx-induced KIF1B expression was confirmed at 24 h by RT-PCR and the effect of knockdown assessed on NF-YAx-induced cell death at 48 h, by phase contrast and AO/EtBr cell death assays. Knockdown experiments were performed in duplicate and repeated (n = 4). Transfection efficiency was confirmed using HPRT-S1 DS positive control and validated using a negative control duplex (NC1), as directed (Integrated DNA Technologies, Bologna, Italy).

Immunoprecipitations and Western blotting
Cell proteins, extracted in lysis buffer (PBS containing 0.5% sodium deoxycholate, 1% NP40, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM PMSF, 1 μg/ml of peptatin A and Aprotinin), were analysed by reducing SDS-PAGE/Western blotting, as previously described [3]. For immunoprecipitations, cell extracts (200–500 μg), pre-cleared with IgG and Protein A Sepharose (Fast flow, Sigma-Aldrich, St Louis, MI), were incubated overnight with primary antibody (0.1–1.0 μg), with rotation at 4 °C, then 20 μl of Protein A Sepharose in lysis buffer was added and incubated for 30 min at 4 °C. Protein A Sepharose/IgG conjugates, collected by centrifugation (10,000 x g for 5 min), were washed in lysis buffer and examined by reducing SDS-PAGE/Western blotting.

Stable SH-SY5Y transfection
SH-SY5Y cells were stable-transfected with pcDNA 3.1 NF-YAj, NF-YAs or NF-YAx or empty pcDNA3.1Zeo expression vectors, as previously described [3]. Zeocin-resistant colonies were isolated upon appearance, clonally expanded and characterized by RT-PCR and Western blot.

Transient transfections
Cells at 1 × 10^5/ml were grown overnight to ≈80% confluence on 6 or 24 well plates, transfected with NF-YA variant or empty plasmid DNA (1 μg/ml) in Fugene HD, as directed (Promega, Madison, WI), washed at 6 h, grown in complete medium, monitored at 6, 12, 24 and 48 h for evidence of cytotoxicity and photographed. At 48 h, suspension and adherent cell populations were separated and either analysed in AO/EtBr cell-death assay, used for RNA purification and RT-PCR analysis or extracted for Western blotting. Transfection efficiency was estimated using pEGFP-N1 green fluorescent protein reporter plasmid (Clontech, Mountain View, CA).
Fig. 1 (See legend on next page.)
Southern blotting
Southern blots of NF-YA and GAPDH RT-PCR products were prepared, as previously described [3]. Briefly, RT-PCR products from primary human NB RNAs (1 μg) and doxorubicin-treated SH-SY5Y mRNAs (100 ng), resolved by 1.5% agarose gel electrophoresis, were in-gel denatured doxorubicin-treated SH-SY5Y mRNAs (100 ng), resolved PCR products from primary human NB RNAs (1 μg) and hybridized with P32-labeled probes for either NF-YA exon C (5′-CAAGGGCCAGCAATTAATGGTGCAGGTGTCAGTGGAGGCCAGCTAATACATCACTGAACTGGAACCAACCCA TCATG-3′), NF-YA exon D (5′-AGGGCCAGCAGGCCGACAGTGACAGATCAATCTCATGACAGGAGCGGT AGCTGTACCTGCGCAGAGA-3′) or GAPDH and visualized by autoradiography.

MTS proliferation and thymidine incorporation assays
In MTS proliferation assays (Promega, Madison, WI), 5 × 10^3 cells in 96-well microtiter plates were grown for 0–4 days. MTS reagent (20 μl) was added at 24-h intervals to individual wells and colorimetric conversion monitored at 492 nm every 30 min for 4 h in a microtiter plate reader. Assays were performed in duplicate and repeated three times (n = 6). For thymidine incorporation assays, 5 × 10^3 cells seeded on 96-well microtiter plates were grown for 0–4 days. At 24-h intervals, 1 μCi of tritiated [3H] thymidine (Amersham International, Little Chalfont, UK) was added and incubated for 4 h. Cultures were then washed in pre-warmed PBS, trypsinized, vacuum transferred onto 3MM Whatman paper filters and counted in a β-Scintillation counter (Becton, Dickinson and Company, Franklyn Lakes, NJ). Assays were performed in duplicate and repeated 3 times (n = 6).

In vitro protein translation
NF-YAl, NF-YAs, NF-YAx, NF-YB and NF-YC proteins were translated in vitro from linearized cDNAs (1 μg), in the presence of 35S labelled methionine, for 90 min at 37 °C, in a rabbit reticulocyte TnT T7 Quick Coupled Transcription/translation System, as directed (Promega, Madison, WI). Translated proteins were resolved by reducing SDS-PAGE autoradiography. For EMSAs, 35S-labelled methionine was omitted from in vitro translation reactions.

In vitro Colony formation assays
Cell suspensions (1 × 10^5/ml) in 6 well culture plates were grown overnight to ~80% confluence, transfected with empty pcDNA3.1 or pcDNA3.1 NF-YAl, NF-YAs or NF-YAx expression vectors (2 μg/ml) in Fugene, as directed (Promega, Madison, WI), and grown for a further 48 h. Zeocin (200 μg/ml) was then added, replaced every 3 days and colonies grown for 21 days, fixed in 100% Methanol, stained in 0.5% w/v crystal violet, 1% Formaldehyde, 1x PBS, 1% methanol, washed in tap water, air dried, photographed and counted. Assays were performed in triplicate and repeated 3 times (n = 9).

Neural stem cell sphere growth assays
Cells seeded at 1 × 10^5/ml in vertical T75 culture flasks (NUNC) were grown in DMEM/F1, containing 0.6% glucose, 1 x B27 supplement (Gibco, Thermo-Fisher Scientific, Waltham, MA), 1 x N2 supplement (Gibco), antibiotics (1 x Pen/Strep), glutamine, EGF (40 ng/ml) and FGF (40 ng/ml), and monitored for formation of neurospheres consisting of > 50 cells. Assays were performed in duplicate and repeated (n = 4).

Direct RT-PCR sequencing
Agarose gel-purified RT-PCR product sequencing was performed using BigDye Direct Cycle Sequencing kit, as directed (Thermo-Fisher Scientific, Waltham, MA). Briefly, cDNAs (final concentration 4 ng/ml) were added to reaction mixtures containing forward or reverse primer (0.8 μM), BigDye direct PCR master mix, deionized water in a final volume of 20 μl and subjected to 35 PCR cycles (94 °C and for 10 min and 96 °C for 10 s, 55 °C for 5 min and 60 °C for 4 min). PCR reactions were mixed with 2 ul of NaAc and 50 μl absolute ethanol, incubated for 10 mins, spin-dried at 120000 rpm for 20 mins and sequenced in a mono-capillary sequencer, as directed (ABI PRISM 310, Life Technologies, Monza, IT).

Tumor growth in soft agar
Single-cell suspensions (passed through a gauge ×18 syringe needle) of 5 × 10^4 cells were mixed in a 33% solution of agar (BiTec; Difco) in RPMI containing 5% FCS at 37 °C and layered onto a solid 0.6% agarose substrate prepared in the same growth medium. Following agar solidification, complete medium was added, replaced
Fig. 2 (See legend on next page.)
every 2 days and growth monitored over 14 days. Assays were performed in duplicate and repeated (n = 4).

Tumor growth in NGS mice
Tumorigenesis in vivo was performed as previously described [3]. Stable-transfected SH-SY5Y cells, prepared as a single-cell suspension in PBS without Ca\(^{2+}\) and Mg\(^{2+}\), were injected subcutaneously into the flanks of anesthetized, 6-week-old female NGS mice (Charles River, Calco, Italy), at a concentration of 1 × 10\(^7\) cells in 200 μl per site. Tumor initiation was recorded at a minimum volume (tumor length × [tumor width]\(^2\) × 0.44) of 12 mm\(^3\) and animals sacrificed at 21 days. Groups consisted of 5 animals and the assay performed once (n = 5). All experiments were performed in accordance with Italian and Rome University guidelines.

Nuclear extracts and EMSAs
Nuclear extracts and EMSAs were performed as previously described [3]. Briefly, binding reactions, performed at room temperature for 20 min, contained \(^{32}\)P-5′ end-labelled, double-stranded oligonucleotide probes, 2 μg of poly-deoxyinosinic-deoxy-cytidilic acid, 5 μg of nuclear extract and additional competitor DNAs or antibodies as specified in the figure legends. The oligonucleotides used were as follows: inverted CCAAT-box: 5′-GGGAGACCGTACGT GAATTGTTAATCTCTT-3′ and non-specific: 5′-GGTCA TGATGGGATTACGCTG-3′ oligonucleotides. All oligonucleotides were double stranded, the complementary affinity strands were not indicated. Assays were repeated 3 times (n = 3).

Cell death assays
Cell death assays were as previously described [43]. Suspension or adherent cells, detached in ice cold PBS containing 1 mM EDTA, were transferred into sterile tubes, pelleted at 1000 g at 4 °C, washed in ice cold PBS, re-pelleted, re-suspended in 25 μl of PBS containing 2 μl of acridine orange/ethidium bromide solution (100 μg/ml acridine orange and 100 μg/ml ethidium bromide in PBS), mounted onto glass slides, examined immediately under a Zeiss “Axioplan-2” fluorescence microscope, digitally photographed and dead (orange/red nuclei) and live cells (green nuclei) counted. Assays were performed in duplicate and repeated 3 times (n = 6).

Indirect immunofluorescence
Cells grown on Nunc glass chamber slides (Sigma-Aldrich, St Louis, MI) were washed in PBS, fixed and permeabilized in 100% ice cold methanol (−20 °C), incubated for 1 h in blocking solution (1% bovine serum albumin in PBS-0.03% TX100), incubated for 2 h with primary antibody diluted in blocking solution at room temperature, washed, incubated with secondary fluorochrome-conjugated antibody diluted in blocking solution, for 1 h at room temperature, mounted with VectaMount (Vector Laboratories, Berlingame, CA) and observed using a Zeiss Axioplan 2 fluorescence microscope with digital camera and Leica M500 Image Manager software.

Statistical analysis
Data were analysed by Student’s t-test (https://www.graphpad.com/quickcalcs/ttest1.cfm) and statistical significance associated with probabilities of ≤0.05.

Results
This study, originally designed to evaluate fully-spliced NF-YA/B and alternatively exon B spliced NF-YAs mRNA expression in primary human NBs, resulted in the unexpected discovery of a novel NF-YA/B splice variant.
Fig. 3 (See legend on next page.)
NF-YAx cloning and sequencing

NF-YA (1284 bp) and NF-YAs (1197 bp) coding cDNAs from SH-SY5Y cells and full-length NF-YAx cDNA (947 bp) from a stage 3 NB were sequenced, and NF-YAx characterized as a novel splice variant exhibiting in-frame exons B (87 bp, amino acids (aa) 26–54), D (132 bp, aa 103–146) and partial F (18 bp, aa 182–188) skipping (Fig. 1a) [15].

NF-YAx is expressed in NBs and mouse embryos

RT-PCR of NB RNAs using primers spanning NF-YA exons A-F, generated 575 bp (NF-YAI), 488 bp (NF-YAs) and 338 bp (NF-YAx) products that hybridized to NF-YA exon C probe in Southern blots (Fig. 1b). The 338 bp product, characterized as NF-YAx by direct PCR sequencing (not shown), was the exclusive isoform expressed in a stage 3 NB and a minor isoform, together with NF-YAI and NF-YAs, in two stage 2 NBs but not detected in either stage 1 or 4 NBs. RNAs from SK-N-MC, SK-N-SH, SH-SY5Y, KCNR, IMR-32 and SK-N-Be NB cell lines, HEK293 embryonic neuronal-lineage kidney cells and human neonatal brain stem cells (SCs) exhibited predominant NF-YAs but not constitutive NF-YAx expression; CHP-126, LAN-1 and LAN-5 NB cell lines exhibited equivalent NF-YAs and NF-YAI but not NF-YAx expression and SHEP NB cells exhibited predominant NF-YAI but not NF-YAx expression. Predominant NF-YAs with minor NF-YAx expression characterized ST14A embryonic striatal neuronal progenitors under progenitor-maintaining (33°C) and differentiation-inducing (39°C) conditions [40] (Fig. 1c and d). Western blots confirmed predominant NF-YAs protein expression in SH-SY5Y cells, predominant NF-YAI expression in SHEP cell extracts (Fig. 1e). NF-YA isoforms translated in vitro were characterized by SDS-PAGE with approximate molecular masses of 46 kDa (NF-YAI), 42 kDa (NF-YAs) and 35 kDa (NF-YAx) (Fig. 1f).

Samples were not available for NF-YAx protein analysis in primary NBs.

In mouse embryo RNAs, NF-YAs mRNA expression was predominant in stage E8.5 embryos, NF-YAI mRNA expression predominant in stage E9.5-E18.5 embryos and NF-YAx mRNA expression, confirmed by direct sequencing (data not shown), was detected in main body (M) but not head (U) or lower limbs (L) RNAs from stage E12.5, 13.5 and 14.5 embryos (Fig. 2a).

Doxorubicin induces NF-YAx expression in SH-SY5Y cells

Doxorubicin (10 μM) induced NF-YAx mRNA expression in SH-SY5Y cells at times of maximal >95% cell death (Fig. 2b), which in Southern blots exhibited time and concentration-dependent induction kinetics at concentrations of 5 and 10 μM, with NF-YAx identified by hybridization to the NF-YA exon C (Exon C) but not exon D (Exon D) probe (Fig. 2c). NF-YAx protein expression was also detected in SH-SY5Y nuclear extracts at 24 and 48 h, following Doxorubicin (10 μM) treatment (Fig. 2d). Necrostatin-1 (100 μM) significantly reduced cell death induced by 10 μM doxorubicin from 94 ± 7.5% to 74 ± 8.7% (p < 0.002, n = 12), at 48 h, (Fig. 2e) and doxorubicin-induced cell death was also characterised by vacuolation, swelling and lysis, consistent with a proportion of necroptotic cell-death (Fig. 2e). NF-YAx expression in SH-SY5Y cells was not induced by treatment with either DTT (5 mM), thapsigargin (10 ng/ml), tunicamycin (1 μM), TPA (10 ng/ml), dibutyryl cAMP (0.5 mM), retinoic acid (10 μM) or CoCl2 (150 μM) over 48 h and was not detected in either stable TrkT3 or TrkAIII SH-SY5Y-transfectants [3] (Fig. 2f).

NF-YAx forms CCAAT-box binding NF-Y complexes

In EMSAs, in vitro-translated NF-YAI, NF-YAs and NF-YAx all formed specific CCAAT-box binding complexes.
Fig. 4 (See legend on next page.)
with NF-YB and NF-YC, confirmed by competition EMSAs in which excess un-labelled CCAAT-box oligonucleotide (S-oligo), anti-NF-YA and anti-NF-YB antibodies but not non-specific oligonucleotide (NS-oligo) or pre-immune IgG abrogated binding (Figs. 1f and 3a and b). EMSAs performed with constant NF-YA, NF-YB and NF-YC and increasing NF-YAx levels (0–4 μl), or constant NF-YAx, NF-YB and NF-YC and increasing NF-YAI levels (0–4 μl), revealed reciprocal NF-YAx/NF-YAI substitution, confirmed by changes in electrophoretic mobility (Fig. 3b). EMSAs also detected specific CCAAT-box binding NF-Y complexes, containing NF-YAI, NF-YAs and NF-YAX, in nuclear extracts from stable NF-YA isomorph SH-SY5Y-transfectants, exhibiting similar levels of nuclear-localized NF-YAI, NF-YAs and NF-YAx expression (Fig. 3c, d and e). NF-YAx-specific antibodies, however, are not available to confirm NF-YAx recruitment to gene promoters in vivo by chromatin immunoprecipitation assay.

NF-YAx does not bind Sp1
NF-YAI and NF-YAs bind Sp1 and regulate Sp1 recruitment to promoters [11, 44]. IP/Western blots confirmed Sp1 pull-down of NF-YAI and NF-YAs but not NF-YAx in Sp1-negative SL2 cells [45], co-transfected with pAc Sp1 and NF-YAI, NF-YAs and NF-YAX insect expression vectors (Fig. 3f). In stable SH-SY5Y-transfectants evidence for Sp1 pull down of NF-YAI and NF-YAs but not NF-YAx was also supported by augmented immunoreactivity overlapping non-specific (NS1 and NS2) bands, consistent with Sp1 binding of NF-YAI and NF-YAs but not NF-YAX (Fig. 3g).

NF-Yx is cytotoxic to embryonal neural-lineage progenitors and NB cells and inhibits colony formation
Transient 48-h transfection of ST14A, HEK-293 and SH-SY5Y with pEGFP-N1 reporter gene, resulted in transfection-efficiencies of 28.3 ± 8.2%, 55.9 ± 4.7% and 30.3 ± 6.5% (mean ± SD) (n = 6, for all three), respectively (not shown). Transient 48-h ST14A, HEK-293 and SH-SY5Y transfection with empty pcDNA, NF-YAI, NF-YAs, NF-YAX or NF-YAdn vectors, resulted in similar levels of NF-YA isoform expression (Fig. 4a, shown for HEK-293 cells only).

In ST14A cells, NF-YAx and NF-YAdn transfection significantly increased the percentage of suspension cells at 48 h, from 7.5 ± 2.25% (n = 6) in pcDNA, 8.5 ± 2.1% (n = 6) in NF-YAI and 8 ± 5.2% (n = 6) in NF-YAs-transfectants, to 25 ± 3.1% (n = 6) in NF-YAx and 27 ± 4.2% (n = 6) (means ± SD) in NF-YAdn-transfectants (p < 0.0001 for both NF-YAx and NF-YAdn vs controls), with 98 ± 5.5% (n = 6) of NF-YAdn-induced suspension cells and 95 ± 7.6% (n = 6) (means ± SD) of NF-YAdn-induced suspension cells confirmed dead by AO/EtBr assay (Fig. 4b and c). In SH-SY5Y cells, NF-YAx and NF-YAdn also significantly increased the percentage of suspension cells at 48 h, from 4.25 ± 2.25% (n = 6) in pcDNA, 6.25 ± 2.1% (n = 6) in NF-YAI and 5 ± 5.2% (n = 6) in NF-YAs-transfectants, to 26 ± 3.1% (n = 6) in NF-YAx and 26.75 ± 4.2% (n = 6) in NF-YAdn-transfectants (means ± SD) (p < 0.0001 for NF-YAx and NF-YAdn vs controls), with 88 ± 5.5% (n = 6) of NF-YAx-induced suspension cells and 93 ± 5.6% (n = 6) (means ± SD) of NF-YAdn-induced suspension cells confirmed dead by AO/EtBr assay (Fig. 4b and c). In HEK-293 cells, NF-YAx and NF-YAdn also significantly increased the percent of suspension cells, from 5.25 ± 2.22% (n = 6) in pcDNA, 8.75 ± 1.9% (n = 6) in NF-YAI and 11.25 ± 4.8% (n = 6) in NF-YAs-transfectants, to 40.75 ± 3.1% (n = 6) in NF-YAx and 39.6 ± 4.3% (n = 6) in NF-YAdn-transfectants (means ± SD) (p < 0.0001 for NF-YAx and NF-YAdn vs controls), with 92 ± 6.5% (n = 6) of NF-YAx-induced suspension cells and 94 ± 7.8% (n = 6) (means ± SD) of NF-YAdn-induced suspension cells confirmed dead by AO/EtBr assay (Fig. 4b and c). In contrast, NF-YAI, NF-YAs or NF-YAx did not induce the death of SH-SY5Y cells expressing TrkAIII oncoprotein (Fig. 4d). NF-YAx-induced death was not detected prior to 16 h, was maximal at 48 h, was not prevented by z-VAD-fmk (20 μM) but was significantly inhibited by necrostatin-1 (100 μM) [46], from 29 ± 3.1% (n = 6) to 12 ± 1.6% (n = 6) in SH-SY5Y cells and from 30 ± 4.5% (n = 6) to 15 ± 1.5% (n = 6) in HEK-293 cells (means ± SD) (P < 0.001, for both) (Fig. 5a).
Fig. 5 (See legend on next page.)
In colony formation assays, sham-transfected SH-SY5Y, HEK-293 and ST14A cells did not form colonies and pcDNA-transfected counterparts formed maximum colony numbers. Compared to respective pcDNA controls, in SH-SY5Y cells, NF-YA\textsubscript{I}-transfectants formed 22.6 ± 7.5% fewer colonies \((p < 0.001, n = 9)\), NF-YA\textsubscript{S}-transfectants 39.5 ± 2.1% fewer colonies \((p < 0.0001, n = 9)\), NF-YA\textsubscript{X}-transfectants 91.1 ± 5.5% fewer colonies \((\text{means } ± \text{ SD}) \text{ (p < 0.0001, n = 18)}\) and NF-YA\textsubscript{dn}-transfectants did not form any colonies \((p < 0.001, n = 18)\). In ST14A cells, NF-YA\textsubscript{I} transfectants formed 23.3 ± 7.5% fewer colonies \((P < 0.0001, n = 9)\), NF-YAs 27.3 ± 2.1% fewer colonies \((P < 0.001, n = 9)\) whereas NF-YAx and NF-YA\textsubscript{dn}-transfectants did not form colonies \((p < 0.0001, n = 9 \text{ for both})\). In HEK-293 cells, NF-YA\textsubscript{I}-transfectants formed 28 ± 9.1% fewer colonies and NF-YA\textsubscript{Sx}-transfectants 38.6 ± 8.5% fewer colonies \((p < 0.0001 \text{ for both}, n = 9 \text{ for each})\), whereas NF-YAx and NF-YA\textsubscript{dn}-transfectants did not form any colonies at all (Fig. 5b).

**NF-YA\textsubscript{X} induces Necroptosis**

NF-YA\textsubscript{X}-induced ST14A, SH-SY5Y and HEK293 cell-death was characterized by vacuolation, swelling and necrotic-lysis but not by apoptotic-body formation (Fig. 6a) or chromatin condensation (Fig. 4f). Western blots confirmed NF-YA\textsubscript{X} expression in dead suspension SH-SY5Y and HEK-293 cells (Fig. 6b) but did not detect caspase-3, caspase-9 or PARP-cleavage, enhanced JNK phosphorylation, reduced AIP/Alix expression or altered Bcl-xL, Bcl2, Mcl1 and EglN3 expression (Fig. 6c and d). In both SH-SY5Y and HEK-293 cells, NF-YA\textsubscript{X} but not NF-YAI reduced Bmi1 expression, induced KIF1B\textbeta expression but did not alter EglN3 expression (Fig. 6c and d). In HEK-293 cells, siRNA knockdown of NF-YA\textsubscript{X}-induced KIF1B\textbeta expression significantly reduced NF-YA\textsubscript{X}-induced death from 24.6 ± 7.05% \((n = 6)\) without siRNAs and 27.2 ± 1.2% \((n = 6)\) with scrambled siRNAs to 5.4 ± 0.61% \((n = 6)\) with KIF1B-specific siRNAs \((p < 0.0001 \text{ versus siRNA controls})\) (Fig. 6e). Western blots also detected a significant 187.08 ± 10.36% \((n = 3)\) increase in ratio of ubiquitinated proteins to β-actin and significant 148.2 ± 8.6% \((n = 3)\) increase in the ratio p62 to β-actin \((p < 0.036 \text{ for all comparisons})\) in extracts from HEK-293 cells transfected with NF-YAx compared to pcDNA, NF-YAI or NF-YAs transfected counterparts (Fig. 6f).

**NF-YA\textsubscript{X} selects tumorigenic, doxorubicin-resistant cancer SCs**

Duplicate stable SH-SY5Y NF-YA\textsubscript{I}-transfectants were established and compared to stable control, NF-YAI and NF-YAs-transfectants. Stable NF-YAI, NF-YAs, NF-YAx1 and x2-transfectants expressed similar levels of NF-YAI, NF-YAs and NF-YA\textsubscript{X} (Fig. 3c) and did not differ significantly in either proliferation or mitotic rates, assessed by MTS and thymidine-incorporation assays (Fig. 7a). NF-YAI, NF-YAs and NF-YAx stable transfectants all formed neuro-spheres in neural stem cell assays in vitro (Fig. 7b) and similar numbers of similar sized tumor spheroids in soft-agar tumorigenesis assays in vitro (Fig. 7c). In xenograft tumorigenesis assays in NSG mice, pcDNA, NF-YAs, NF-YAx1 and x2-transfectants formed sub-cutaneous tumours of similar dimensions, significantly larger than tumours formed by stable NF-YAI-transfectants (>2 fold smaller, \(p < 0.049\) compared to all other stable-transfectants, \(n = 5 \text{ per group})\) (Fig. 7d). In cell death assays, doxorubicin induced similar levels of stable pcDNA, NF-YAI and NF-YAs-transfectant cell death at all concentrations at 6, 24 and 48 h. In contrast, stable NF-YAx1 and x2-transfectants exhibited significantly enhanced survival at 6 but not 24 or 48 h \((p < 0.0001, n = 6 \text{ for both})\) in the presence of 10 μM doxorubicin, at 6 and 24 but not 48 h \((p < 0.0001, n = 6 \text{ for both})\) in the presence of 5 μM doxorubicin and at all time points in the presence of 0.01, 0.1 and 1 μM doxorubicin \((p < 0.0001, n = 6 \text{ for both at all time points and doses})\) (Fig. 7e).

In RT-PCR assays NF-YAx1 and x2 transfectants exhibited higher levels of p75\textsuperscript{NTR}, Nanog, Nestin and EglN3 expression than NF-YAI and NF-YAs-transfectants, similar levels of Sox-2, CD133 and CD117 expression nor induce KIF1B\textbeta expression but did not express KIF1B\textbeta. In contrast, NF-YAI-transfectants exhibited lower levels of p75\textsuperscript{NTR} and Nanog than the other transfectants. Bcl2, Mcl1, Bcl-xL, PUMA, BAD and Bax expression levels did not differ between stable transfectants (Fig. 8a, b and c). RNAs purified from xenograft tumours exhibited a similar pattern of CSC gene expression to corresponding cell cultures (Fig. 8d). PTC-209 (10 μM for 24 h) abrogated proliferation of all stable transfectants (Fig. 9a) but did not reduce Bmi1 expression nor induce KIF1B\textbeta mRNA expression in NF-YAx1 or x2-transfectants (Fig. 9b).
Fig. 6 (See legend on next page.)
Discussion

We report a novel development and genotoxic stress-regulated alternative splice mechanism for promoting embryonic neural-lineage progenitor and NB cell-death, characterized by a switch to alternative NF-YA splicing and expression of a novel cytotoxic NF-YAx extra short-form variant. This novel isoform, originally discovered in human primary stage 2 and stage 3 NB RNAs, was the exclusive NF-YA isoform expressed at a high-level in an advanced stage 3 NB and was characterised as a novel NF-YA splice variant, exhibiting in-frame exon B, D and partial F skipping, responsible for truncating NF-YA transactivation domain sequence. NF-YAx readily competed with fully-spliced NF-YA/ in CCAAT-box binding NF-Y complex formation but in contrast to NF-YA/ and NF-YAs isoforms did not bind Sp1 and, therefore, represents a functional modifier of one of the more important physiological and cancer-associated transcription factors. In mouse embryos, NF-YAx expression coincided with the reported phase of neurotrophin-regulated KIF1Bβ-dependent sympathetic neuroblast-culling, unrestrained NF-YAx expression induced KIF1Bβ-dependent necroptosis in neural-lineage progenitors and NB cells and association between doxorubicin-induced NF-YAx expression and necroptosis in NB cells, supports a pro-neurotrophic cytotoxic function for NF-YAx and a potential role in KIF1Bβ-dependent suppression of NB initiation, during development. On the other hand, propagation through selection of tumorogenic, doxorubicin-resistant CSC-like stable NF-YAx SH-SY5Y transfectants, resistant to NF-YAx cytotoxicity, not only helps to explain the high-level exclusive NF-YAx expression detected in an advanced stage 3 NB but also supports an additional potential role for NF-YAx, within the tumour context, in disease progression and identifies a potential mechanism for doxorubicin-induced post-therapeutic relapse, through CSC selection.

NF-YAx cDNA was cloned from a stage 3 NB and sequence characterised as a novel NF-YA splice variant with in-frame exons B, D and partial F sequence skipping, adding to existing NF-YA, NF-YAs and NF-YA L2–6 variants [8, 12, 13, 17], with potential implications for NB pathogenesis and progression. NF-YAx expression, detected in 20% (3/15) NF-YA-positive NB RNA samples, was the exclusive high-level NF-YA mRNA isoform expressed in a stage 3 NB but was not detected in stage 1 and 4 NBs, human neonatal neural stem cells, HEK-293 human neural-lineage embryonic kidney cells and 14 human NB cell lines. Although samples were not available for NF-YAx protein detection in primary NBs, expression of the endogenous NF-YAx protein was confirmed in SH-SY5Y cells, following treatment with doxorubicin, corroborating the characterisation of NF-YAx cDNA as a complete, in-frame, non-mutated NF-YA splice variant that is readily translated into the NF-YAx protein in vitro and in vivo. Considering the relatively small number of NB samples analysed in this study, however, the possibility that NF-YAx mRNA expression is restricted to localized NB disseminated at most to local lymph nodes (Stages 2 and 3) [47] and may differentiate stage 3 from other disease stages, must await confirmation in a future larger NB cohort study.

NF-YAx mRNA was also expressed during mouse stage E12.5–E14.5 embryo development, consistent with identical exon organization of human (NCBI: NM_002505.5) and mouse (NCBI: NM_001110832.1) NF-YA genes. NF-YAx expression during stage E12.5–E14.5 embryo development coincides with the reported phase of neurotrophin-regulated EgfN3/KIF1Bβ-dependent sympathetic neuroblast-culling, required for sympathetic nervous system development and suppressing NB initiation [25–28, 48–55]. Furthermore, unrestrained NF-YAx expression in neural-related progenitors and NB cells reduced expression of the KIF1B repressor Bmi1 [29], induced KIF1Bβ expression, promoted KIF1Bβ-dependent necroptosis and abrogated colony forming capacity in vitro. This suggests that NF-YAx expression
NF-YA expression did not kill NB cells expressing TrkAIII oncoprotein [3]. This bears similarity to NGF-activated TrkA protection of sympathetic neuroblast against KIF1Bβ-dependent death [26, 27] and suggests that TrkAIII, which associates with NB, may facilitate NB initiation by preventing KIF1Bβ expression, however, did not associate with changes in EglN3 expression, suggesting that this novel death-mechanism may act downstream of EglN3 through NF-YA reduction of Bmi1 expression. Whether EglN3 promotion of alternative splicing [56], extends to NF-YAx remains to be determined. NF-YAx cytotoxicity to neural-related progenitors and NB cells was not associated with caspase 3, caspase 9 or PARP cleavage nor with changes in Bcl-2, BclXL, Mcl-1, NOXA, PUMA, BAX, BAD and AIP1/Alix expression or JNK-phosphorylation, was not inhibited by the pan-caspase inhibitor z-VAD-fmk but was characterized by cell vacuolation, swelling and necrotic-lysis and inhibited by the RIPK-1 inhibitor necrostatin-1 [46], consistent with necroptosis rather than apoptosis [57, 58]. NF-YAx also increased the levels of ubiquitinated and p62 proteins in HEK-293 cells, suggesting reduced autophagic flux, consistent with reports that both Bmi1 inhibition and KIF1Bβ expression promote lysosomal/autophagosome uncoupling and necroptosis [59, 60], which may explain the NB suppressing function of KIF1Bβ [25, 32, 33, 54, 61, 62]. Although KIF1Bβ-induced apoptosis [32, 33, 57, 62, 63] was not detected in this study, we do not exclude potential roles for KIF1Bβ-induced ROS-mediated mitophagy or Drp1-dependent mitochondrial-fission in this death mechanism [33, 60, 64–66].

NF-YAx was significantly more cytotoxic to neuronal-related progenitors and NB cells than either NF-YA1 or NF-YAs, contrasting with a previous report that unrestrained NF-YA1 expression is highly cytotoxic and induces p53-dependent apoptosis [9]. This discrepancy can be explained by fact that p53 is compromised by SV40 large T-antigen in ST14A cells [40], by adenovirus-5 in HEK-293 cells [41, 42] and by different mechanisms in SH-SY5Y cells [67–69], suggesting that this novel NF-YA-dependent necroptosis mechanism may be restricted to genotoxic-stress under p53 compromised conditions. Furthermore, the sensitivity of ST14A neural progenitors, which exhibit low-level constitutive NF-YA expression, to NF-YA-induced necroptosis, confirms that this mechanism depends upon predominant NF-YAx expression. Unrestrained NF-YAdn expression, which contains a DNA binding-domain mutation that prevents NF-Y binding and transcription [70], also induced HEK-293 and SH-SY5Y necroptosis. This indicates that survival can switch to necroptosis in these cell types when either NF-Y binding is prevented (i.e. with NF-YAdn) or when a change in NF-Y function reaches a critical level (i.e. with NF-YAx). Similar necroptotic-like neuronal death has also been reported during development [25, 48, 54, 62, 71, 72]. A pro-necroptotic role for NF-YAx was also supported by the association between doxorubicin-induced NF-YAx expression and SH-SY5Y cell-death, which was also characterised by vacuolation, swelling and cell lysis and significantly inhibited by the necroptosis inhibitor necrostatin-1 [46], consistent with a percentage of doxorubicin-induced necroptosis. Confirmation of the role of NF-YAx in this necroptotic proportion of doxorubicin-induced death, however, must await development of reagents specific for NF-YAx depletion.

Doxorubicin induction of alternative NF-YAx splicing not only implicates the DNA damage-associated alternative splice mechanism [73] and a role for NF-YAx in the response to genotoxic stress but also identifies NF-YAx as a potential biomarker of response to genotoxic therapy, suggesting that NF-YAx expression in primary NBs could also reflect neo-
Fig. 8 (See legend on next page.)
adjuvant genotoxic chemotherapy [74]. Furthermore, the propagation of tumorigenic, doxorubicin-resistant, CSC-like stable NF-YAx SH-SY5Y transfectants, resistant to NF-YAx cytotoxicity, unveils an additional potential role for NF-YAx in genotoxic drug-induced, post-therapeutic relapse through CSC selection and maintenance. Moreover, the fact that NF-YAx expression was not induced by agents that promote ER-stress, hypoxic-stress, differentiation or malignant behaviour, suggests that this alternative splice mechanism may be relatively restricted to conditions of genotoxic stress in NB cells.

NF-YAx substitution of NF-YAl in DNA-binding NF-Y complexes and the capacity of NF-YAl and NF-YAs but not NF-YAx to bind Sp1 (this study, [11, 44, 75]), characterises NF-YAx as a potential modifier of both NF-Y and NF-Y-dependent Sp1 function. NF-YAx loss of Sp1 binding results from truncation of the aa 55–139 binding site for Sp1 in NF-YAl and NF-YAs to aa 55–103 in NF-YAx (this study, [44, 76]). This truncation may also compromise NF-Y interaction with ZHX transcriptional repressors that bind NF-YA aa’s 31–140, with potential to also alter NF-Y/ZHX-regulated genes expression, including MDR-1 chemotherapeutic-cytotoxicity-regulator and polo kinase-1 mitosis-regulator [77–81]. Transactivation-domain truncation may also weaken NF-Y function and interaction with other factors and the reduced size of NF-YAx, particularly in complexes with smaller NF-YB and NF-YC variants [82], may de-regulate transcription from promoters with precisely-spaced NF-Y-dependent transcriptional domains, e.g. ER-stress response gene promoters [83].

Although, NF-YAx-specific antibodies are not yet available for confirmation, by chromatin immunoprecipitation assay, that NF-YAx is recruited to gene promoters in vivo, the fact that NF-YAx contains an intact DNA binding domain, competes with other isoforms in NF-Y complex formation and binds double stranded inverted CCAAT-box oligonucleotides in NF-Y complexed-form in vitro with similar kinetics to other NF-YA isoforms, strongly supports this probability. Although, we are only beginning to understand how NF-YAx may influence transcription, transient NF-YAx expression reduced Bmi1 and induced KIF1Bβ expression in neural-lineage progenitors and NB cells, and stable NF-YAx expression was associated with enhanced p75NTR, SOX2, Nestin, Nanog, CD117, CD133 and EglN3 expression.

Despite the acute cytotoxicity of NF-YAx to SH-SY5Y cells, stable SH-SY5Y transfectants exhibiting predominant NF-YAx expression were eventually propagated. These transfectants did not exhibit altered Bcl2, Bcl-xL or Mcl1 expression nor constitutive KIF1Bβ expression, suggesting selection based upon KIF1Bβ repression but not Bcl2-regulated mitochondrial apoptosis. Stable NF-YAx transfectants also exhibited high constitutive Bmi1 expression, consistent with resistance to NF-YAx-induced KIF1Bβ-dependent cytotoxicity. This indicates that the SH-SY5Y cell line contains sub-populations that are sensitive and resistant to acute NF-YAx cytotoxicity, characterised by differences in NF-YAx regulation of Bmi1 and KIF1Bβ expression. The Bmi1 inhibitor PTC-209 [84], however, failed to induce KIF1Bβ expression in stable NF-YAx-transfectants, indicating that Bmi1 alone is not responsible for KIF1Bβ repression in the resistant subpopulations. PTC-209 did, however, abrogate the proliferation of all SH-SY5Y-transfectants, consistent with the role of Bmi1 in proliferation and reports that PTC-209 induces G1/S checkpoint cell-cycle arrest [84–89]. We are currently investigating whether KIF1B repression in stable NF-YAx SH-SY5Y transfectants, as a potential selection mechanism, may involve inhibition EglN3 prolyl hydroxylase activity, required for KIF1Bβ expression and regulated by oxygen and high/low-order complexing [55, 90–92] and/or changes in NB-associated NF-Y and Sp1-regulated microRNA expression [93–95].

Stable NF-YAx SH-SY5Y transfectants were more CSC-like than stable control, NF-YAl or NF-YAs transfectants and were characterized by higher levels of p75NTR, Nestin, Nanog and EglN3 expression compared to all other stable transfectants, similar levels of SOX2, CD133 and CD117 expression to NF-YAs-transfectants elevated over other transfectants, high-level Bmi1 expression and KIF1Bβ repression, consistent with reports that elevated Bmi1 and EglN3 expression associated with
Fig. 9 (See legend on next page.)
embryonic staminality [84–89, 92, 95, 96], elevated p75NTR expression characterises neural crest and SH-SY5Y SCs [97–99] and KIF1Bβ-repression characterises undifferentiated aggressive NBs with 1p36 deletion or Nm3c amplification or that NBs that initiate in KIF1Bβ−/− mice [25, 26, 29–33, 100]. Stable NF-YAx SH-SY5Y transfectants were tumorigenic both in vitro and in vivo, confirming that acute NF-YAx cytotoxicity selects a tumorigenic NB CSC-like subpopulations resistant to NF-YAx-induced KIF1Bβ-dependent necroptosis. This provides an explanation for high-level exclusive NF-YAx expression in the advanced stage 3 NB and supports a role for NF-YAx, within the tumour context, in disease progression and suggests that doxorubicin induction of NF-YAx expression may also promote CSC selection in a potential mechanisms for post-therapeutic relapse, enhanced drug-resistance, increase adaptive plasticity and metastatic behaviour (this study, [101, 102]).

Conclusions

The discovery of NF-YAx mRNA in primary stage 2 and 3 NBs, its expression as the exclusive isoform in an advanced stage 3 NB, expression in stage E12.5 to E14.5 mouse embryos and induction by doxorubicin in NB cells, unveils a novel NF-YA splice mechanism and variant that is both regulated by and involved in NB, development and conditions of genotoxic-stress. NF-YAx substitution of other isoforms in NF-Y complexes and loss of Sp1 binding capacity characterises this novel isoform as a functional modifier of one of the more important physiological and cancer-associated transcription factors. NF-YAx induction of KIF1Bβ-dependent embryonic neural-related progenitor and NB necroptosis, association with doxorubicin-induced necroptosis and expression in murine embryos at times corresponding to the phase of neurotrophin-regulated KIF1Bβ-dependent sympathetic neuroblastum culling, supports a predominant pro-necrotic cytotoxic function for NF-YAx in neural progenitors and NB cells, with potential to suppress NB initiation during development. On the other hand, propagation by selection of tumorigenic, doxorubicin-resistant CSC-like stable NF-YAx expressing SH-SY5Y transfectants, resistant to NF-YAx cytotoxicity, not only helps to explain the exclusive high-level NF-YAx expression in an advanced stage 3 primary NB but also suggests that NF-YAx expression within the tumour context may promote disease progression and provides a possible doxorubicin-inducible mechanism for post-therapeutic relapse, through CSC selection and maintenance, with potential to enhance survival within stressful chemotherapeutic tumour microenvironments [62, 103].

Abbreviations

Bmi-1: B cell-specific Moloney murine leukaemia virus integration site 1; CSC: cancer stem cell; KIF1Bβ: kinesin family member 1B; NB: neuroblastoma; NF-YAx: long-form nuclear transcription factor YA; NF-YAβ: short-form nuclear transcription factor YA; NF-YAγ: extra short-form nuclear transcription factor YA; NSG mice: NOD severe combined immunodeficient gamma mice; Sp1: specificity protein 1

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Authors’ contributions

LC, ARF and ARM conceptualized, designed, supervised this study and take responsibility for data integrity and accuracy of analysis; LC, ARF, LD, PI, DD, MS and ARM acquired, analysed, interpreted the data and provided materials and technical support; ARM wrote this manuscript; LC, ARF and ARM revised and edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data sets used and/or analysed during this study are either included in this published article or are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Written consent was obtained for the use of all patient NB RNA samples by the tissue bank of the Italian Association of Haematology and Pediatric Oncology (Genova, Italy). Animals experiments were performed at University of Rome (La Sapienza) in accordance with Italian National and Rome university regulations.

Consent for publication

No identifying patient details are contained within this manuscript.

Competing interests

The authors declare that they have no competing interests.

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References

1. Modrek B, Lee C. A genomic view of alternative splicing. Nucleic Acids Res. 2000;30:1303–9.

2. Kalnin Z, Zayakin P, Silina K, Line A. Alterations in pre-mRNA splicing in cancer. Genes Chromosomes Cancer. 2005;42:342–57.

3. Taccorielli A, Farina AR, Cappabianca L, DeSantis G, Tessitore A, Vetuschi A, et al. TrkA alternative splicing: a regulated tumor-promoting switch in human neuroblastoma. Cancer Cell. 2004;6:347–60.

4. Cappabianca L, Guadagni S, Macaronne R, Sebastiano M, Chionnito A, Farina AR, Mackay AR. A pilot study of alternative TrkAIII splicing in Merkel cell carcinoma: a potential oncogenic mechanism and novel therapeutic target. J Exp Clin Cancer Res. 2019;38:424. https://doi.org/10.1186/s13049-019-1425-3.

5. Wang B-D, Lee NH. Aberrant splicing in Cancer and drug resistance. Cancers (Basel). 2018. https://doi.org/10.3390/cancers10010143.

6. Mantovani R. Nuclear factor Y in development and disease. Biochim Biophys Acta Gene Regul Mech. 2017;1860:524–34.

7. Maity SN. NF-Y (CBF) regulation in specific cell types and mouse models. Biochim Biophys Acta Gene Regul Mech. 2017;1860:598–603.

8. Li G, Zhao H, Wang L, Wang Y, Guo X, Xu B. The animal nuclear factor Y: an enigmatic heterotrimetric transcription factor. Am J Cancer Res. 2018;8:106–25.

9. Gurner A, Manni I, Paggio G. NF-Y in cancer: impact on cell transformation of a gene essential for proliferation. Biochim Biophys Acta Gene Regul Mech. 1860;2017:604–16.

10. Zambelli F, Pavesi G. Genome wide features, distribution and correlations of NF-Y binding sites. Biochim Biophys Acta Gene Regul Mech. 2017;1860:581–9.

11. Suske G. NF-Y and SP transcription factors-New insights in a long-standing liaison. Biochim Biophys Acta Gene Regul Mech. 2017;1860:590–7.

12. Maity SN, de Crombrugghe B. Biochemical analysis of the B subunit of the heterotrimeric CCAAT-binding factor. J Biol Chem. 1992;267:8286–92.

13. Country F, Maity SN, de Crombrugghe B. Studies on transcription activation by the multimeric CCAAT-binding factor CBF. J Biol Chem. 1995:270:648–75.

14. Romier C, Cocchiarella F, Mantovani R, Moras D. The NF-YB/NF-YC structure gives insight into DNA binding and transcription regulation by CCAAT factor NF-Y, Biochim Biophys Acta. 2003:278:1336–45.

15. Li X-Y, Hoft van Huisjesduinen R, Mantovani R, Benoist C, Mathis D. Intron-exon organisation of the NF-Y genes. J Biol Chem. 1992;267:9894–90.

16. Dolfini D, Gatta R, Mantovani R. NF-Y and the transcriptional activation of CCAAT promoters. Crit Rev Biochem Mol Biol. 2012:47:29–49.

17. Ge Y, Jensen TL, Matherly LH, Taub JW. Synergistic regulation of the heterotrimeric CCAAT-binding factor. J Biol Chem. 1992;267:8286–92.

18. Zhu J, Zhang Y, Joe GJ, Pompetti R, Emerson SG. NF-YA activates multiple isoforms and Sp1. Biochim Biophys Acta. 2002;1579:73–84.

19. Cattaneo E, Conti L. Generation and characterization of embryonic striatal neurons. Biochim Biophys Acta Gene Regul Mech. 2017;1860:581–90.

20.印刷者 fugnn 右侧の文献に記載されているものを読み取ります。
49. Mesner PW, Winters TR, Green SH. Nerve growth factor withdrawal-induced cell death in neuronal PC12 cells resembles that in sympathetic neurons. J Cell Biol. 1992;119:1669–80.

50. El Shamy WM, Linnarsson S, Lee K-F, Jaenisch R, Emrns P. Prenatal and postnatal requirements for sympathetic neuroblast survival and innervation of specific targets. Development. 1996;122:491–500.

51. Pinon LGP, Minichiello L, Klein R, Davies AM. Timing of neuronal death in trkA, trkB and trkC mutant embryos reveals developmental changes in sensory neuron dependence on Trk signaling. Development. 1996;122:3255–61.

52. Lipscomb EA, Sarmiere PD, Crowder RJ, Freeman S. Expression of the SM-20 gene promotes death in nerve growth factor-dependent sympathetic neurons. J Neurochem. 1999;73:429–32.

53. Zhao C, Takita J, Tanaka Y, Seto M, Nakagawa T, Tekada S, et al. Charcot-Marie-tooth disease type 2A caused by mutation in a microtubule motor KIF1Bbeta. Cell. 2001;105:587–97.

54. Sommer L, Rao M. Neural stem cells and regulation of cell number. Prog Neurobiol. 2002;66:1–18.

55. El Shamy WM, Linnarsson S, Lee K-F, Jaenisch R, Ernfors P. Prenatal and postnatal requirements for sympathetic neuroblast survival and innervation of specific targets. Development. 1996;122:491–500.

56. Schlisio S. Neuronal apoptosis by prolyl hydroxylation: implication in neuroblastoma. Sci Reports. 2017;7:16867.

57. El Shamy WM, Linnarsson S, Lee K-F, Jaenisch R, Ernfors P. Prenatal and postnatal requirements for sympathetic neuroblast survival and innervation of specific targets. Development. 1996;122:491–500.

58. Sperandio S, de Belle J, Breiden DE. An alternative, nonapoptotic form of programmed cell death. Proc Natl Acad Sci U S A. 2000;97:14376–81.

59. Sperandio S, Poksay K, de Belle I, Lafuente MJ, Liu B, Nasir J, et al. Parapoptosis: mediation by MAP kinases and inhibition by AIP-1/Alk. Cell Death Diff. 2004;11:1066–75.

60. Dey A, Mustafi SB, Saha S, Kumar Dhar Dwivedi S, Mukherjee P, Battacharya R. Inhibition of Bmi1 induces apoptosis-mediated necrosis. Autophagy. 2012;6:1265–99.

61. Fricker M, Tolkovsky AM, Borutaita V, Coleman M, Brown GC. Neural cell death. Physiol Rev. 2018;98:813–80.

62. Schlisio S. Neuronal apoptosis by prolyl hydroxylation: implication in neuronal death. Physiol Rev. 2018;98:813–80.

63. Nappi LGP, Minichiello L, Klein R, Davies AM. Timing of neuronal death in the nervous system tumours and the Warburg conundrum. J Cell Mol Med. 2009;3:4104–23.

64. Miyazaki M, Otomo R, Matsushima-Hibiya Y, Suzuki H, Nakajima A, Abe N, et al. Toh WH, Logette E, Corcos L, Sabapathy K. Tap73 -paraptosis-dependent mechanism. Sci Reports. 2018;8:9566.

65. Nakayama K, Yamada K, Shou Z, Mizutani T, Yazawa T, Yoshino M, et al. Zinc-fingers and homeoboxes (ZH1 X), a novel member of the ZHX family functions as a transcription factor. Biochem J. 2003;373:747–57.

66. Hirano S, Yamada K, Kawata H, Shou Z, Mizutani T, Yazawa T, et al. Rat zinc-fingers and homeoboxes 1 (ZH1X), a novel member of the ZHX family functions as a transcription factor. Biochem J. 2003;373:747–57.

67. Nazio F, Bordi M, Cianfanelli V, Locatelli F, Cecconi F. Autophagy and cancer stem cells: molecular mechanisms and therapeutic applications. Cell Death Differ. 2017;24:1326–38.

68. El Shamy WM, Linnarsson S, Lee K-F, Jaenisch R, Ernfors P. Prenatal and postnatal requirements for sympathetic neuroblast survival and innervation of specific targets. Development. 1996;122:491–500.

69. Chen J, Crutchley J, Zhang D, Oozvar K, Kastan MB. Identification of a DNA damage-induced alternative splicing pathway that regulates p53 and cellular senescence markers. Cancer Disc. 2017;7:766–81.

70. Appelhoff RJ, Tian YM, Raval RR, Turley H, Harris AL, Pugh CW, et al. The miR-17-92 microRNA cluster regulates multiple components of the TGFβ-pathway in neuroblastoma. Mol Cell. 2009;37:8455–65.

71. Lessard J, Baban S, Sauvageau G. Stage-specific expression of polycomb group genes in human bone marrow cells. Blood. 1998;91:1216–24.

72. Mestdagh P, Bostrom A-K, Impens F, Fredlung E, Van Peer G, De Antonellis M. shRNA knockdown of Bmi-1 reveals a critical role for p21-Rb pathway in NSC self-renewal and neural development but not mouse growth and survival by repressing the Ptenlina and p19Arf senescence pathways. Genes Dev. 2005;19:1432–7.

73. Jin M, Rao E, Ramachandrareddy H, Shen Y, Jiang C, Chen J, et al. The miR-17-92 microRNA cluster regulates multiple components of the TGFβ-pathway in neuroblastoma. Mol Cell. 2010;39:762–73.

74. Mestdagh P, Bostrom A-K, Impens F, Fredlung E, Van Peer G, De Antonellis M. shRNA knockdown of Bmi-1 reveals a critical role for p21-Rb pathway in NSC self-renewal and neural development but not mouse growth and survival by repressing the Ptenlina and p19Arf senescence pathways. Genes Dev. 2005;19:1432–7.

75. Fasano CA, Dimos JT, Ivanova NB, Lowry N, Lemischka IR, Temple S. shRNA knockdown of Bmi-1 reveals a critical role for p21-Rb pathway in NSC self-renewal and neural development but not mouse growth and survival by repressing the Ptenlina and p19Arf senescence pathways. Genes Dev. 2005;19:1432–7.

76. Lessard J, Baban S, Sauvageau G. Stage-specific expression of polycomb group genes in human bone marrow cells. Blood. 1998;91:1216–24.

77. Mestdagh P, Bostrom A-K, Impens F, Fredlung E, Van Peer G, De Antonellis M. shRNA knockdown of Bmi-1 reveals a critical role for p21-Rb pathway in NSC self-renewal and neural development but not mouse growth and survival by repressing the Ptenlina and p19Arf senescence pathways. Genes Dev. 2005;19:1432–7.

78. Fasano CA, Dimos JT, Ivanova NB, Lowry N, Lemischka IR, Temple S. shRNA knockdown of Bmi-1 reveals a critical role for p21-Rb pathway in NSC self-renewal and neural development but not mouse growth and survival by repressing the Ptenlina and p19Arf senescence pathways. Genes Dev. 2005;19:1432–7.

79. Fasano CA, Dimos JT, Ivanova NB, Lowry N, Lemischka IR, Temple S. shRNA knockdown of Bmi-1 reveals a critical role for p21-Rb pathway in NSC self-renewal and neural development but not mouse growth and survival by repressing the Ptenlina and p19Arf senescence pathways. Genes Dev. 2005;19:1432–7.
97. Achilles A, Trainor PA. Neural crest stem cells: discovery, properties and potential for therapy. Cell Res. 2012;22:288–304.
98. Biagiotti T, D’Amico M, Marzi I, Di Gennaro P, Arcangeli A, Wanke E, Oliotto M. Cell renewing in neuroblastoma: electrophysiological and immunocytochemical characterization of stem cells and derivatives. Stem Cells. 2005;24:443–53.
99. Ross RA, Spengler BA. Human neuroblastoma stem cells. Semin Cancer Biol. 2007;17:241–7.
100. Li S, Fell SM, Surova O, Smedler E, Wallis K, Chen ZX, et al. The 1p36 tumor suppressor KIF1Bβ is required for calcineurin activation controlling mitochondrial fission and apoptosis. Dev Cell. 2016;36:164–78.
101. Pandian V, Ramraj S, Khan FH, Azim T, Aravindan N. Metastatic neuroblastoma cancer stem cells exhibit flexible plasticity and adaptive stemness signaling. Stem Cell Res Ther. 2015;6:2. https://doi.org/10.1186/s13287-015-0002-8.
102. Garner EF, Beierle EA. Cancer stem cells and their interaction with the tumour microenvironment in neuroblastoma. Cancers. 2016; 8:doi: https://doi.org/10.3390/cancers8010005.
103. Zheng X, Naiditch J, Czurylo M, Jie C, Lautz T, Clark S, et al. Differential effect of long term drug selection with doxorubicin and virinostat on neuroblastoma cells with cancer cell characteristics. Cell Death Dis. 2013;4:e740.

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