MYCN-amplified neuroblastoma maintains an aggressive and undifferentiated phenotype by deregulation of estrogen and NGF signaling

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Neuroblastoma (NB) is a remarkably heterogenic childhood tumor of the sympathetic nervous system with clinical behavior ranging from spontaneous regression to poorly differentiated tumors and metastasis. *MYCN* is amplified in 20% of cases and correlates with an undifferentiated, aggressive phenotype and poor prognosis. Estrogen receptor alpha (ERα) and the nerve growth factor (NGF) receptors TrkA and p75^{NTR} are involved in neuronal differentiation and survival. We have previously shown that MYCN, via miR-18a, targets ERα in NB cells. Here, we demonstrate that interference with miR-18a or overexpression of ERα is sufficient to induce NGF signaling and to modulate both basal and NGF-induced neuronal differentiation in *MYCN*-amplified NB cells. Proteomic analysis confirmed an increase of neuronal features and showed that processes linked to tumor initiation and progression were inhibited upon ERα overexpression. Indeed, ectopic ERα expression was sufficient to inhibit metabolic activity and tumorigenic processes, including glycolysis, oxidative phosphorylation, cell viability, migration, and anchorage independent growth. Importantly, ERα overexpression reduced tumor burden in NB mouse models and high ERα levels were linked to improved survival in patients. In addition to ERα, several other nuclear hormone receptors (NHRs), including the glucocorticoid and the retinoic acid receptors, correlated with clinical markers for favorable and low-stage NB disease. Our data suggest that MYCN targets ERα and thereby NGF signaling to maintain an undifferentiated and aggressive phenotype. Notably, we identified the estrogen–NGF crosstalk, as well as a set of other NHRs, as potential prognostic markers and targets for therapeutic strategies against NB.

neuroblastoma | MYCN | differentiation | NGF | estrogen receptor alpha

Neuroblastoma (NB), the most common solid malignant extracranial childhood tumor, develops from sympathetic precursor cells of neural crest origin. The etiology is unknown and the disease has a very heterogeneous clinical pattern ranging from spontaneous regression or maturation to widespread aggressive incurable disease. Neuroblastoma accounts for about 8–10% of all cases of childhood cancer and is the cause of 12–15% of cancer-related childhood mortality (1–3). About half of the affected children have a localized low-risk disease while the other half is diagnosed with a metastatic high-risk NB (3–5). Interestingly, there is a special group, 4S, of metastatic NB in some children below the age of 12 mo, which is characterized by an increased incidence of spontaneous regression and high survival (1, 2, 6). However, even today, metastatic high-risk NB is difficult to cure despite multimodal therapy, resulting in a 5-y survival rate of around 50% (1, 2). Genomic amplification of *MYCN* is the genetic aberration most consistently associated with poor outcome and is detected in ∼20% of all NB cases (1, 2). This strongly correlates to an undifferentiated phenotype as well as to high-risk disease and poor prognosis (7, 8). MYCN is a member of the MYC family of transcription factors, which are key regulators of a broad range of fundamental cellular processes, including survival, proliferation, and differentiation, many of which are linked to tumor initiation and progression (9, 10). During normal development, high *MYCN* expression is restricted to embryogenesis and to the forebrain, hindbrain, and kidneys in newborn mice. In contrast, its expression levels are generally very low in tissues of adult mice except in developing B cells (11). In high-risk NB without *MYCN* amplification, expression of MYC or MYC target genes is frequently enhanced (12), underlining the important role of MYC family signaling during NB tumorigenesis.

A relatively high number of low-risk NBs show a notable ability to spontaneously differentiate or regress (9, 13). Because of this, a considerable research effort has been made to find differentiation-inducing agents for NB cells. Retinoic acid is currently used as a maintenance therapy to treat minimal residual disease for high-risk patients resulting in significantly improved event-free survival (EFS) (14, 15). Importantly, interference with MYCN signaling results in the inhibition of proliferation and in the induction of terminal differentiation of neuronal cells (16, 17). In line with this, retinoic acid-induced differentiation is preceded by the down-regulation of MYCN and induction of nerve growth factor (NGF) receptors (17). NGF is a well-known and powerful mediator of neuronal differentiation and is up-regulated during maturation of neurons (18).

Significance

High-risk neuroblastoma (NB), a cancer of the sympathetic nervous system, is challenging to treat. *MYCN* is frequently amplified in high-risk NB and is linked to an undifferentiated phenotype and poor prognosis. Estrogen and nerve growth factor (NGF) are inducers of neural differentiation, a process associated with a favorable disease. We show that MYCN suppresses estrogen receptor alpha (ERα) and thereby NGF signaling and neural differentiation. ERα overexpression is sufficient to interfere with different tumorigenic processes and tumor growth. In patients with NB, *ERα* expression correlates with several clinical markers for good prognosis. Importantly, not only ERα but also the majority of other nuclear hormone receptors are linked to favorable NB, suggesting a potential prognostic and therapeutic value for these proteins.

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Additionally, expression of the NGF receptors, TrkA and $p75^{NTR}$, is linked to a good prognosis as well as to spontaneous differentiation and regression in NB and negatively correlates with *MYCN* amplification (19–21). Despite the fact that estrogen and/or its receptors have the ability to promote tumorigenesis in several other cancer types, including breast carcinoma (22–26), we have previously shown that estrogen receptor alpha (ERα) gene expression is associated with improved survival in patients with NB (27). ERα is one of 48 members of the human nuclear hormone receptor (NHR) family of transcription factors, which are activated by a broad range of different lipophilic ligands, e.g., steroids and thyroid hormones (28, 29). Once ERα is activated by its ligand estradiol, it shuttles to the nucleus to regulate gene expression by binding to specific estrogen response elements (EREs). In addition to regulating gene expression, ERs are able to directly modulate the activity of several signaling pathways by modifying proteins involved in, e.g., AKT (30) or β-catenin (31) signaling.

Our group has previously demonstrated that *MYCN*-amplified (MNA) NB cells in part maintain their undifferentiated phenotype by the up-regulation of microRNAs (miRNAs) of the miR17~92 cluster (27). We further showed that miR-18a and/or other members of this miRNA family interfere with the expression of ERα as well as with additional NHRs. Interestingly, we found that knockdown of miR-18a as well as ectopic expression of the ERα or glucocorticoid receptor (GR) is sufficient to induce neural differentiation in MNA NB cells (27, 32). In line with this, ERα is known to act as a neuroprotective factor and an inducer of differentiation in neuronal cells (17). We therefore hypothesized that ERα is important for the induction of a neuronal-like phenotype in NB cells and that this increased differentiation promotes a phenotype closer to low-risk NB. This study aimed to elucidate the effect of ectopic ERα expression on functional processes typically involved in progression and maintenance of MNA NB.

Results

MiR-18a Interference or Ectopic Expression of ERα Enhances NGF-Mediated Neuronal Differentiation by Up-Regulation of $p75^{NTR}$ and TrkA. We have previously shown that knockdown of miR-18a is sufficient to increase expression of ERα and to induce profound neuronal differentiation in the MNA NB cell line SK-N-BE(2) when cultured in medium containing phenol red and 10% normal FBS. In addition, we observed similar effects after ectopic expression of the miR-18a target ERα (27). Normal FBS contains several cytokines and growth factors, including the ERα ligand 17-β-estradiol (E2), and phenol red has structural similarities to estrogen and is a weak activator of ERα (33). Therefore, the cells in this study were maintained in medium without phenol red supplemented with charcoal-stripped FBS (containing reduced levels of growth factors and cytokines). These culture conditions circumvent nonspecific activation and allow for a controlled activation of the studied pathways. Under these improved conditions, we observed a marked increase in neuronal differentiation in SK-N-BE(2) cells with stable miR-18a knockdown [BE(2) α-miR-18a] compared with the scrambled control cells (*SI Appendix*, Fig. S1*A*). This was accompanied by a significant increase in the expression of the gene encoding ERα, *ESR1* (hereafter called *ERα*) (*SI Appendix*, Fig. S1*B*). Furthermore, we found that the mRNA levels of the NGF receptors *NTRK1* and *NGFR* (hereafter called *TrkA* and $p75^{NTR}$, respectively) were significantly up-regulated in cells with suppressed miR-18a levels (*SI Appendix*, Fig. S1*C*). Therefore, we activated the ERα and NGF receptors with their respective ligands E2 and NGF and found that the combined treatment potentiated the observed induction of neuronal differentiation in BE(2) α-miR-18a cells, but not in the scrambled control cells (*SI Appendix*, Fig. S1*A*). No significantly altered expression of $p75^{NTR}$ or *TrkA* was seen upon treatment with E2 and/or NGF (*SI Appendix*, Fig. S1*C*). Similarly, compared with SK-N-BE(2) cells transduced with an empty vector [BE(2) EV], untreated ERα-overexpressing cells [BE(2) ESR1 #1] showed a mildly differentiated phenotype as observed by neurite outgrowth (Fig. 1*A*). We further found that the neuronal differentiation was potentiated by NGF alone or in combination with E2, while BE(2) EV cells were not affected by the treatment. The NGF receptors $p75^{NTR}$ and TrkA are markers for neuronal differentiation (17). Levels of $p75^{NTR}$ protein and mRNA were not altered by overexpression of ERα alone, but were induced by E2 addition (Fig. 1 *B* and *C*). The up-regulation of $p75^{NTR}$ was concentrated in a subpopulation of cells (*SI Appendix*, Fig. S2*A*), in which $p75^{NTR}$ was spread over the whole cytoplasm, indicating active signaling. In the BE(2) EV control cells on the other hand, $p75^{NTR}$ was localized close to the nucleus. Moreover, down-regulation and inhibition of ERα with its antagonist fulvestrant (also called ICI 182, 780) (34) interfered with E2-induced up-regulation of $p75^{NTR}$ (*SI Appendix*, Fig. S2*B*), indicating that active estrogen signaling is essential for $p75^{NTR}$ induction. Independent of the treatment, *TrkA* expression was strongly up-regulated in BE(2) ESR1 #1 cells in comparison with the control cells (Fig. 1*C*). Together our data revealed the ability of ERα to induce NGF signaling in NB.

These results were confirmed using cells derived from tumors of homozygous *TH-MYCN* mice, which overexpress MYCN in neural crest cells and develop NB in sympathetic ganglia. This in vivo model resembles MNA human NB based on histological and pathological features (35, 36). When isolated, *TH-MYCN* tumor cells can be maintained in a proliferative state as floating tumor spheres or cultured in differentiation conditions to obtain sympathetic neurons (32). Similar to the BE(2) ESR1 #1 cells, we observed a profound E2-dependent increase in $p75^{NTR}$ on protein and mRNA level upon induction of differentiation, which was absent in the proliferating conditions (Fig. 1 *D* and *E*). Inhibition of ERα with fulvestrant interfered with this increase in $p75^{NTR}$ expression. The differentiated sympathetic neurons were further characterized by reduced *Mycn* expression, elevated *Trka* levels, as well as an E2-dependent increase in *Erα* expression (Fig. 1*E*). In summary, our findings suggest a crosstalk between estrogen and NGF signaling in the induction of NB cell differentiation.

Processes Linked to Tumorigenesis Are Down-Regulated in ERα Overexpressing Cells as Shown by Quantitative Mass Spectrometry-Based Proteomics. Our in vitro data indicated that overexpression of ERα is sufficient to induce a more differentiated phenotype in MNA SK-N-BE(2) NB cells. Well-differentiated tumor cells are usually linked to a less aggressive phenotype in cancer. We therefore performed a high-resolution quantitative proteomics analysis (*SI Appendix*, Fig. S3*A*) to obtain a more detailed molecular phenotype of the observed differences between the BE(2) EV and BE(2) ESR1 #1 cells and to identify promising candidates for further analysis. In total, 9,711 proteins were identified and quantified (Dataset S1), of which 1,395 were significantly up-regulated (>1.2-fold change; $P < 0.05$) and 1,542 were significantly down-regulated (<0.833-fold change; $P < 0.05$) in BE(2) ESR1 #1 cells compared with the EV control (*SI Appendix*, Fig. S3*A* and Dataset S2). The proteomic data were validated using Western blot analysis for ERα and six other proteins, all of which showed a similar regulation as found in the proteomic data (*SI Appendix*, Fig. S3 *A* and *B*). The gene ontology (GO) term enrichment analysis revealed several relevant GO terms for up-regulated (Fig. 2 and *SI Appendix*, Fig. S4*A*) as well as down-regulated (*SI Appendix*, Fig. S4 *B* and *C*) proteins in BE(2) ESR1 #1 cells, as highlighted. Among the up-regulated hits were the GO terms "axon guidance," "asymmetric synapse," and "neuron projection" as well as several processes which suggest reduced metabolic activity and an interference with tumorigenic processes, such as proliferation and cell motility. These findings supported our observation that overexpression of ERα is sufficient to induce neuronal differentiation in SK-N-BE(2) cells. The induction of neural differentiation by ectopic expression of ERα was confirmed in a second ERα-overexpressing clone, BE(2) ESR1 #2 (*SI Appendix*, Fig. S5*A*), and similar to BE(2) ESR1 #1, E2 treatment resulted in an up-regulation of $p75^{NTR}$ (*SI Appendix*, Fig. S5*B*). Furthermore, the levels of the six different proteins analyzed for the validation of the proteomics were similar in both BE(2) ESR1 clones (*SI Appendix*, Fig. S3*B*). A variety of NB cell lines, including SK-N-BE(2), consist of different subtypes,

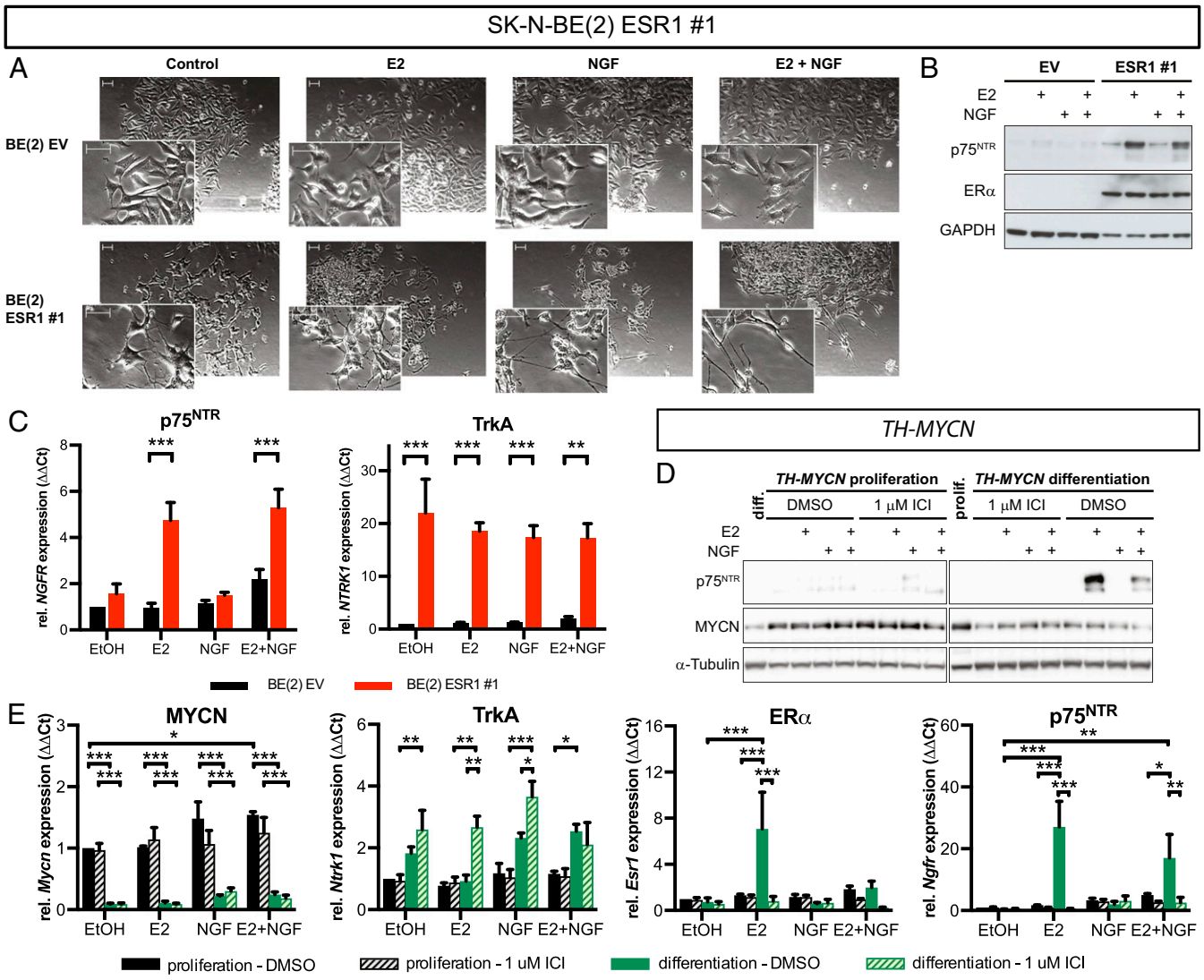

Fig. 1. Enhanced estrogen signaling induces expression of NGF receptors and NGF-mediated neuronal differentiation. SK-N-BE(2) cells overexpressing ERα [BE(2) ESR1 #1] or transduced with an empty vector as control [BE(2) EV] were treated with EtOH, E2, NGF, or the combination of E2 and NGF. (*A*) Neuronal differentiation was assessed using phase contrast microscopy at a magnification of 200× after 2 wk of treatment. (*B*) Western blot analysis of $p75^{NTR}$, ERα, and GAPDH after 24 h of treatment. (*C*) Relative mRNA expression levels of the NGF receptors *$p75^{NTR}$* and *TrkA* were quantified using SybrGreen real-time PCR with β2-microglobulin (*B2M*) as reference gene after 24 h of treatment. (*D* and *E*) *TH-MYCN* mouse-derived tumor cells were cultured in proliferation (spheres) or differentiation condition and treated with E2 and/or NGF, alone or in combination with the ERα inhibitor fulvestrant (here ICI) for 2 and 4 d, respectively. (*D*) Western blot analysis of $p75^{NTR}$, MYCN, and α-tubulin as loading control. (*E*) Taqman real-time PCR analysis of *MYCN*, *TrkA*, *ERα*, *$p75^{NTR}$*, and *B2M* as reference gene. Real-time PCR results are shown as mean ± SEM of (*C*) four and (*E*) three independent experiments and significances were determined using a two-way ANOVA. Significances are highlighted with $*P < 0.05$, $**P < 0.01$, or $***P < 0.001$. Western blots are representatives from three independent experiments.

substrate (S), neuronal (N), and intermediate (I) type (which has characteristics of both subtypes). N-type and I-type cells can transdifferentiate into the other subtypes (37, 38). We observed that in comparison with the BE(2) ESR1 #1 and #2 cells, the BE(2) EV control cells were flatter and more tightly attached (Fig. 1*A* and *SI Appendix*, Fig. S5*A*), indicating that they have a larger population of substrate adherent (S type) cells, which are also described as glial or Schwann cell-like cell types. This observed morphological shift from S to N type in BE(2) ESR1 cells was confirmed by real-time PCR analysis, showing increased gene expression of the neural differentiation markers neuropeptide Y (*NPY*) and tyrosine hydroxylase (*TH*) (Fig. 3*A*) and decreased levels of the glial cell markers S100 calcium binding protein B (*S100B*) and vimentin (*VIM*) (Fig. 3*B*). Furthermore, using immunofluorescence, we observed an increased percentage of cells positive for the neural differentiation marker TH and a reduction in the number of cells positive for the glial differentiation markers VIM and S100A6 (Fig. 3*C*), which was reflected in similar regulations in the proteomic data (*SI Appendix*, Fig. S5*C*).

ERα Interferes with Cell Viability but Potentiates Prosurvival Stimuli by NGF. We have previously shown that ectopic expression of ERα interferes with basal proliferation of SK-N-BE(2) cells (27). In line with these findings, the enrichment analysis of the proteomic data presented in this study revealed a significant probability for up-regulated proteins belonging to the GO terms "positive regulation of programmed cell death" and "negative regulation of cell proliferation." We thus analyzed the effect of ERα overexpression on the cell viability of MNA NB cells. BE(2) ESR1 #1 exhibited noticeably slower growth dynamics than their BE(2) EV counterpart (*SI Appendix*, Fig. S6*A*), resulting in a more than fourfold significant decrease in cell number after 7 d in culture. Inhibition of ERα with fulvestrant led to significantly increased cell viability in both BE(2) ESR1 clones compared

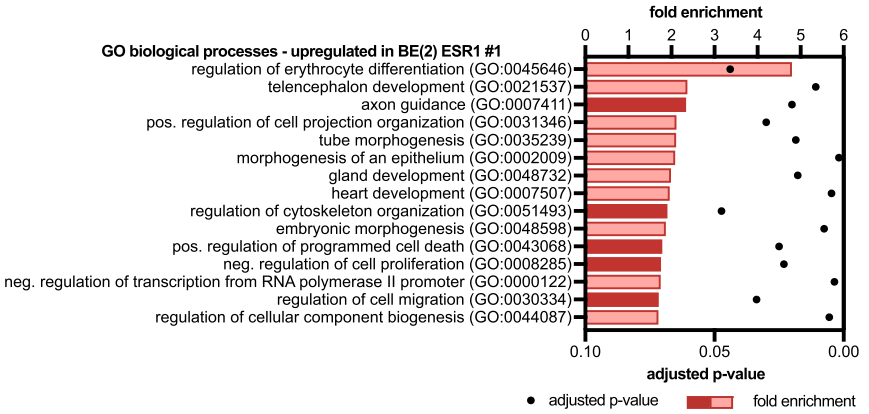

Fig. 2. Quantitative mass spectrometry-based proteomics indicate a deregulation of processes linked to tumorigenesis in NB cells overexpressing ERα. Cells were seeded and incubated for 48 h before harvesting and preparation of proteins for proteomic analysis. Gene ontology (GO) enrichment analysis (www.geneontology.org/page/go-enrichment-analysis) was used to identify GO biological processes and for up-regulated (>1.2, $P < 0.05$) proteins in BE(2) ESR1 #1 cells compared with BE(2) EV cells. Fold enrichment of GO terms compared with all genes identified in the proteomic analysis is shown as bars, adjusted *P* values as black dots. The dark colored bars highlight processes, which were subsequently analyzed in more detail. Enrichment of other up-regulated and down-regulated GO terms in BE(2) ESR1 #1 cells are shown in *SI Appendix*, Fig. S4.

with the EV control cells (Fig. 4*A*). In accordance with the cell counting experiment (*SI Appendix*, Fig. S6*A*), overexpression of ERα resulted in a significantly reduced cell viability of BE(2) ESR1 #1 (Fig. 4*B*) and #2 (*SI Appendix*, Fig. S6*B*) cells, compared with BE(2) EV cells as analyzed by WST-1 assay. Treatment with NGF alone or in combination with E2 significantly increased cell viability of BE(2) ESR1 #1 cells, whereas the EV control and BE(2) ESR1 #2 cells only showed a minor response to the treatments (Fig. 4*B* and *SI Appendix*, Fig. S6*B*). EdU incorporation and a cell death ELISA showed that proliferation and cell death rates of BE(2) EV and ESR1 #1 cells approached similar levels when treated with E2 and/or NGF (*SI Appendix*, Fig. S6 *C* and *D*). This was due to a weak but significant increase in proliferation and a concomitant decrease in cell death of BE(2) ESR1 #1 cells. Together, our results demonstrate that ectopic ERα expression is sufficient to markedly reduce cell viability, which in part can be rescued by treatment with NGF and E2.

Overexpression of ERα Inhibits Functional Features Typically Involved in Tumorigenesis and Malignant Transformation. A differentiated cell morphology is usually linked to a less aggressive phenotype and an improved prognosis in several cancer types. This, together with our findings from the proteomic analysis, led us to ask whether ectopic expression of ERα and its crosstalk with NGF signaling interferes with cellular processes, which contribute to malignant transformation and tumor aggressiveness. We indeed found that ERα strongly interfered with the ability of SK-N-BE(2) cells to grow anchorage independently, which was echoed by a robust decrease in both colony number and size of BE(2) ESR1 cells (Fig. 5*A* and *SI Appendix*, Fig. S7 *A–C*) in comparison with the control BE(2) EV cells. Treatment with NGF and/or E2 did not alter the colony formation capacity of the BE(2) EV cells significantly. In contrast, BE(2) ESR1 cells showed increased colony numbers (*SI Appendix*, Fig. S7 *A* and *C*) and, in the case of clone 1, also formed larger colonies (*SI Appendix*, Fig. S7 *D* and *E*)

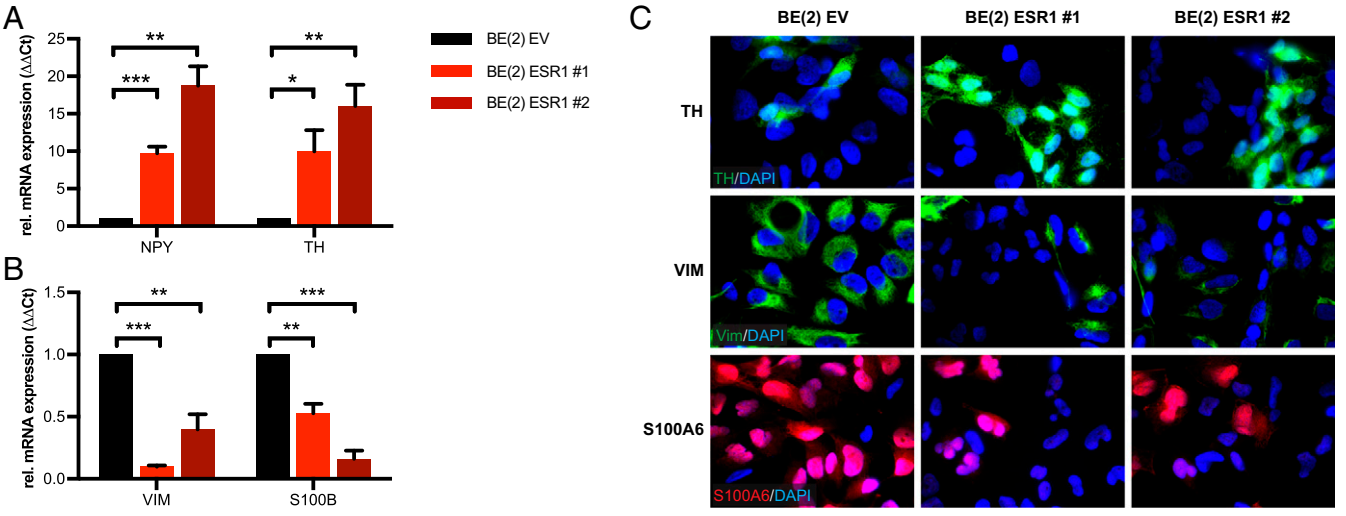

Fig. 3. Enhanced estrogen signaling induces a shift toward neuronal like cells. SK-N-BE(2) cells overexpressing ERα [BE(2) ESR1 #1] or transduced with an empty vector as control [BE(2) EV] were treated with EtOH, E2, NGF, or E2 and NGF. (*A*) Neuronal differentiation (*NPY* and *TH*) and (*B*) glial cell (*S100B* and *VIM*) markers were assessed after 9 d in culture using real-time PCR analysis with *B2M* as reference gene. (*C*) Immunofluorescence analysis was used to stain the neuronal differentiation marker TH (green) and the glial cell markers vimentin (green) and S100A6 (red) after a 3-d incubation. The nuclei where visualized using DAPI (blue). Real-time PCR results are shown as mean ± SEM of three independent experiments and significances were determined using a Student's *t* test. Significances are highlighted with $*P < 0.05$, $**P < 0.01$, or $***P < 0.001$. Microscopy pictures are representatives from three independent experiments.

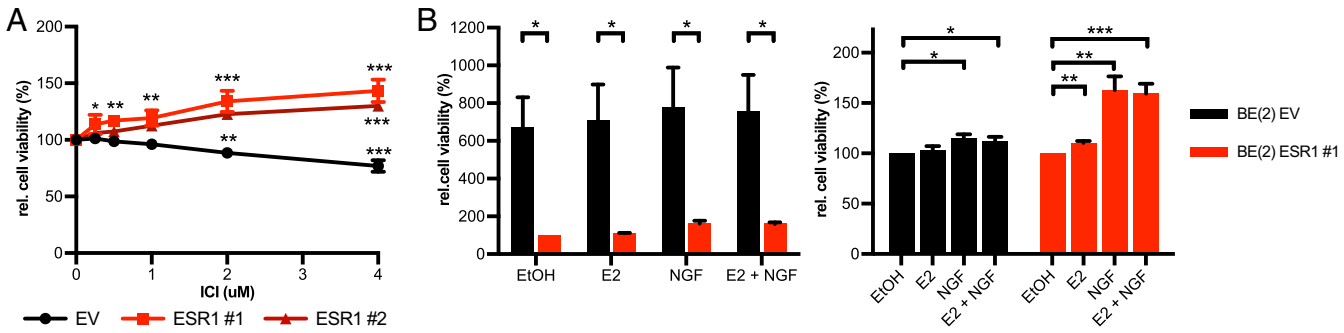

Fig. 4. Ectopic expression of ERα reduces basal cell viability. (*A*) BE(2) EV and BE(2) ESR1 #1 and #2 cells were treated with the indicated concentrations of the ERα inhibitor fulvestrant (here ICI) for 6 d. Cell viability was assessed using a WST-1 assay. (*B*) BE(2) ESR1 #1 and EV cells were treated with EtOH, E2, and/or NGF for 6 d. Cell viability was determined using the WST-1 assay. Results are shown as normalized to BE(2) ESR1 #1 untreated cells (*Left*) or to the corresponding BE(2) EV and BE(2) ESR1 untreated cells (*Right*). All data are shown as mean ± SEM of three independent experiments and significances were determined using a two-way ANOVA (*A*) or a Student's *t* test (*B*) and highlighted with $*P < 0.05$, $**P < 0.01$, or $***P < 0.001$.

when treated with NGF and/or E2 compared with control treatment, albeit not reaching the levels of the BE(2) EV cells (*SI Appendix*, Fig. S7*A*).

Cell migration and invasion are crucial processes during metastasis and strongly contribute to the malignant transformation of a tumor cell. The first step during these processes is the detachment of the tumor cell from the cell network. Interactions with components from the extracellular matrix provide anchorage for cell motility and invasion (39). A transwell migration assay revealed that the BE(2) EV control cells showed a significantly higher ability to migrate compared with the ERα-overexpressing cells (Fig. 5*B* and *SI Appendix*, Fig. S7*G*). Treatment with E2 resulted in a minor inhibition of cell migration in both control as well as in BE(2) ESR1 #1 cells (Fig. 5*B*). Furthermore, invasion was decreased in cells overexpressing ERα compared with control cells (Fig. 5*C*); however, when calculated as percentage of migration, it was similar in both cell lines (*SI Appendix*, Fig. S7*F*).

The Glycolytic Rate and Oxidative Phosphorylation Are Reduced in MNA Neuroblastoma Cells Overexpressing ERα. Metabolic processes are frequently altered in cancer to provide sufficient energy and building blocks for rapidly dividing tumor cells (9). The data analysis of our quantitative mass spectrometry-based proteomics (*SI Appendix*, Fig. S4*B*) indicated that several metabolic processes as well as mitochondrial organization were deregulated in BE(2) ESR #1 cells. In addition, we have previously demonstrated that neural differentiation and metabolic changes are linked in NB (40). Therefore, we next analyzed the two main

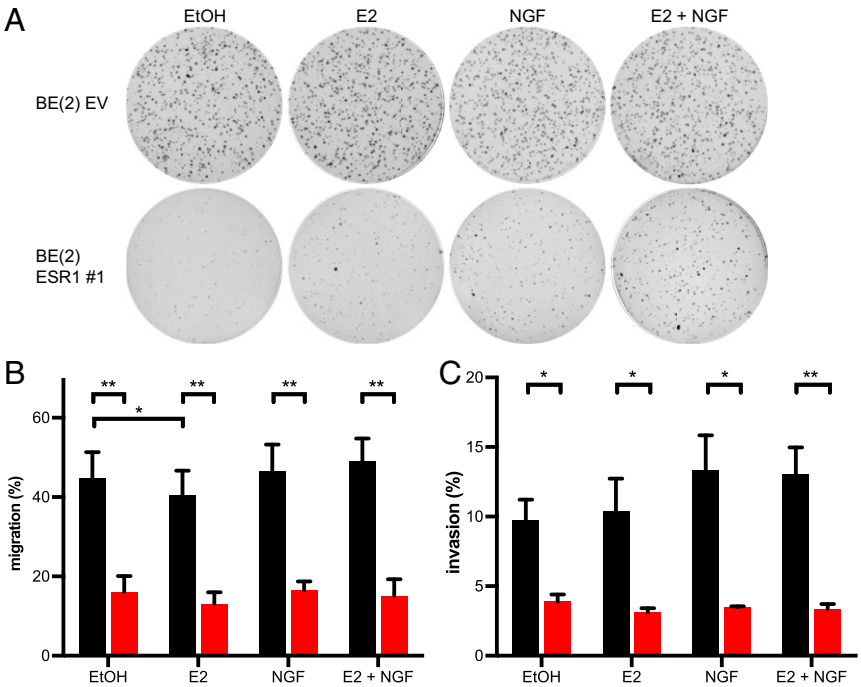

Fig. 5. ERα inhibits anchorage-independent growth and cell motility in SK-N-BE(2) cells. (*A*) BE(2) ESR1 #1 and EV control cells were cultured in soft agar and treated with E2, NGF, E2 and NGF, or left untreated. After a 14-d incubation, pictures were taken of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)-stained colonies, as shown here for representative wells from three independent experiments (see *SI Appendix*, Fig. S7 for quantification and analysis of the size of the soft agar colonies). (*B* and *C*) BE(2) EV and BE(2) ESR1 cells (represented by black and red bars, respectively) were pretreated with E2, NGF, or a combination of both for 48 h. Cell migration (*B*) and invasion through a basement membrane extract (BME) layer (*C*) was evaluated using a transwell assay with fibronectin as chemoattractant. All experiments are shown as mean ± SEM. $n = 4$ for *A*, $n = 3$ for *B* and *C*. Significances were calculated using a Student's *t* test and are shown as $*P < 0.05$, $**P < 0.01$, or $***P < 0.001$.

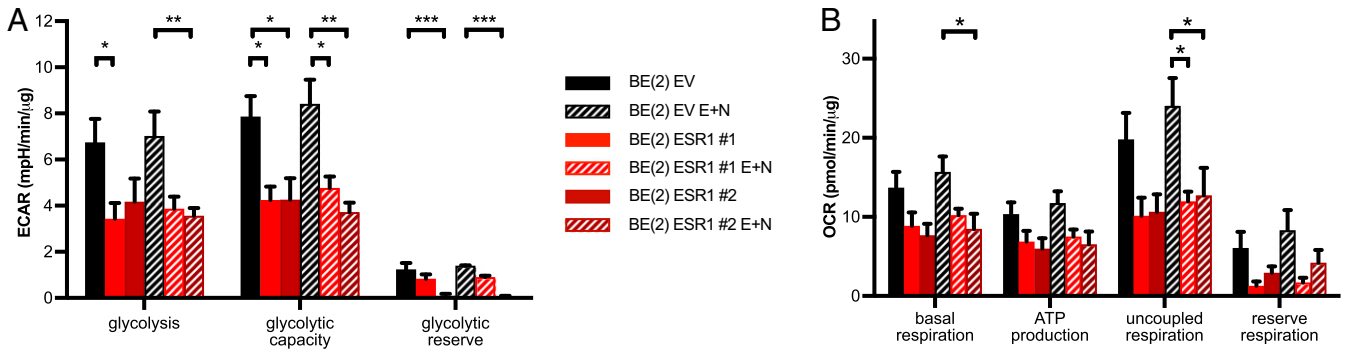

Fig. 6. Important energy producing metabolic processes are down-regulated in SK-N-BE(2) cells overexpressing ERα. BE(2) EV and BE(2) ESR1 #1 and #2 cells were left untreated or incubated with E2 and NGF (E+N) for 24 h. (*A*) The extracellular acidification rate (ECAR) was analyzed to assess the glycolytic activity using different glycolytic parameters. (*B*) The oxygen consumption rate (OCR) is used as readout for respiration as analyzed with different respiratory parameters. The results are shown as average ± SEM of three independent experiments and significances were calculated using a two-way ANOVA and are highlighted with $*P < 0.05$, $**P < 0.01$, or $***P < 0.001$.

energy-producing cellular processes, glycolysis and oxidative phosphorylation (OXPHOS), using the Seahorse XFe96 extracellular flux analyzer. Several glycolytic parameters can be identified through the reintroduction of glucose following a starving period before treatment with two specific inhibitors, oligomycin (an ATP synthase inhibitor, therefore inhibiting OXPHOS) and 2-deoxyglucose (2-DG), a glycolysis inhibitor. Interestingly, glycolysis, maximal glycolytic capacity, and the glycolytic reserve were all significantly reduced in cells overexpressing ERα and treatment with E2 and NGF had no additional effect on any of these parameters (Fig. 6*A* and *SI Appendix*, Fig. S8*A*). To study OXPHOS functionality, oligomycin, FCCP (which uncouples the respiratory chain from the ATP synthase), and the respiratory chain complex I and III inhibitors rotenone and antimycin A, respectively, were used. We found that independent of treatment, all respiratory parameters were reduced in both BE(2) ESR1 clones compared with control cells (Fig. 6*B* and *SI Appendix*, Fig. S8*B*). Considering the clear effect in the functional assays, we refined the analysis of our proteomic data to identify significantly ($P < 0.05$) up- or down-regulated proteins in these processes (Dataset S3). We focused on glycolysis, the citric acid cycle (*SI Appendix*, Fig. S9*A*), an important process to deliver energy equivalents to the electron transport chain (ECT), and the respiratory chain composed of the ECT and OXPHOS (*SI Appendix*, Fig. S9*B*). Importantly, the majority of affected proteins were down-regulated in all three processes. In addition, the enzymes in the first steps of fatty acid degradation were also down-regulated in BE(2) ESR1 #1 cells (*SI Appendix*, Fig. S9*C*). This process provides acetyl-CoA for the citric acid cycle and therefore serves as an important energy source. In accordance with our proteomic data (*SI Appendix*, Fig. S9*C*) and with our previous observations in NB cells differentiated by, e.g., interference with *MYCN* (40), we observed an accumulation of lipid droplets in BE(2) ESR1 cells (*SI Appendix*, Fig. S9*D*), suggesting a reduced utilization of fatty acids.

Our in vitro data demonstrated that overexpression of ERα is sufficient to induce neuronal like differentiation and to interfere with tumorigenic processes in *MYCN*-amplified NB cells in vitro. To further validate these results, we performed key experiments in a second *MYCN*-amplified NB cell line, IMR32, transduced with a vector expressing the *ERα* cDNA (IMR32 ESR1) or with an empty control vector (IMR32 EV). Similar to our data shown above, IMR32 cells with ectopic ERα expression were characterized by induction of differentiation, which was further enhanced by activation of estrogen signaling (*SI Appendix*, Fig. S10*A*). Importantly, as in BE(2) ESR1 cells, E2 treatment resulted in ERα dependent up-regulation of $p75^{NTR}$ in IMR32 ESR1 but not in the control IMR32 EV cells (*SI Appendix*, Fig. S10*B*). Finally, ERα expression significantly reduced cell viability in both untreated and especially in E2-treated cells and this effect could be inhibited by the addition of the ERα inhibitor fulvestrant (*SI Appendix*, Fig. S10*C*). Notably, cell migration was also significantly inhibited by E2 (*SI Appendix*, Fig. S10*D*).

ERα Reduces Tumor Burden in Vivo. To investigate whether our in vitro results were transferrable to in vivo conditions, we inoculated BE(2) EV and BE(2) ESR1 #2 cells into the groin fat pad of nude

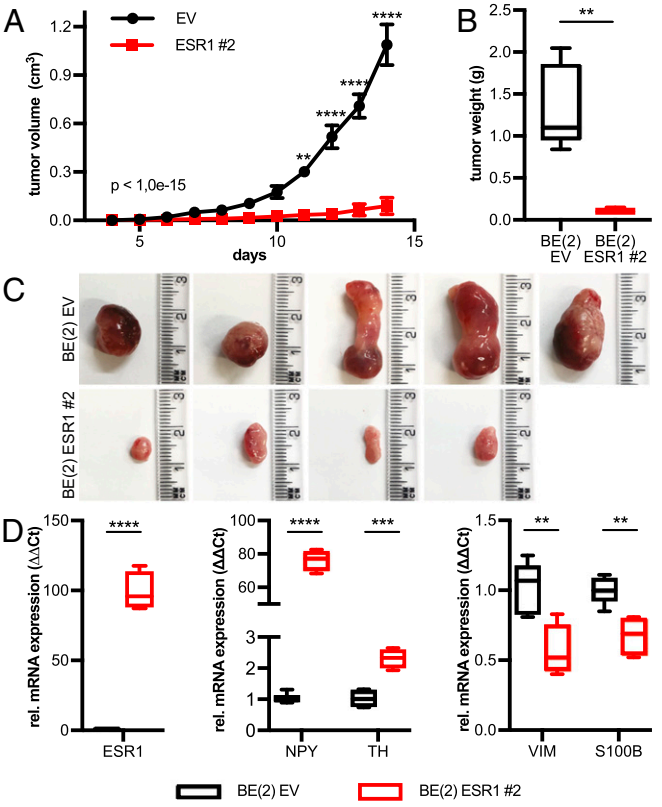

Fig. 7. ERα reduces tumor burden in vivo. BE(2) EV or BE(2) ESR1 were injected together with NIH3T3 fibroblasts into the groin fat pad of male Naval Medical Research Institute (NMRI)-*Foxn1*$_{nu}$ nude mice. (*A*) Tumor volume is shown for days 4–14 after inoculation. (*B*) Tumor weight and (*C*) pictures of the tumors at final day 14. (*D*) Expression of *ERα* and the neuronal differentiation (*NPY* and *TH*) or glial cell (*S100B* and *VIM*) markers were assessed using real-time PCR analysis with *18S* as reference gene. The results in the graphs are shown as average ± SEM of five BE(2) EV and four BE(2) ESR1 #2 tumors, respectively. Significances were calculated using a (*A*) two-way ANOVA or (*B* and *D*) Student's *t* test and are shown as $*P < 0.05$, $**P < 0.01$, or $***P < 0.001$.

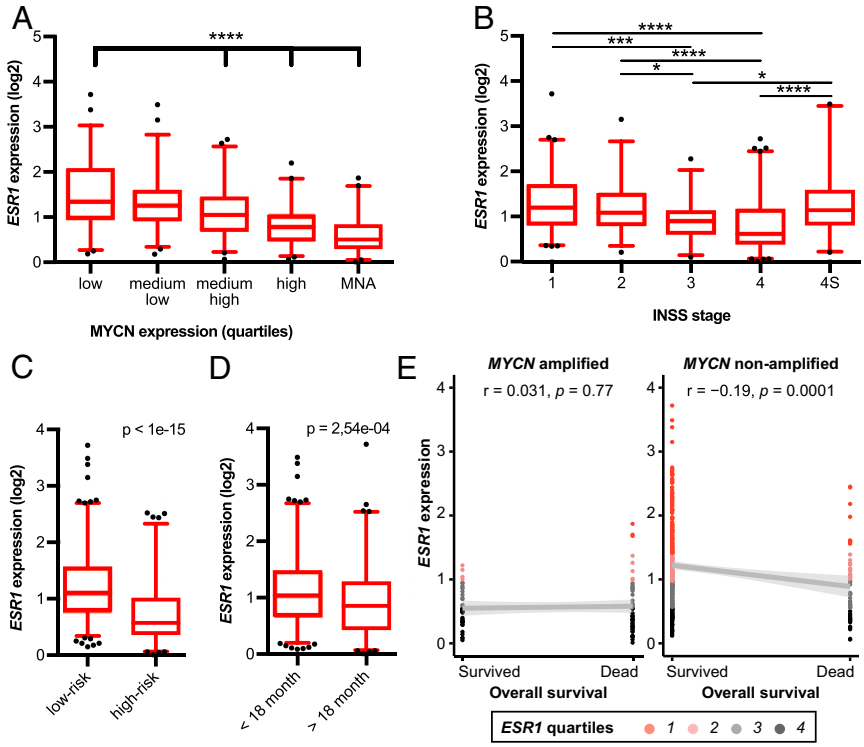

Fig. 8. ERα expression correlates with beneficial clinical parameters in NB patients. Analysis of a patient cohort comprising RNA-sequencing expression data from 498 patients with NB (r2.amc.nl/; Tumor Neuroblastoma - SEQC - 498 - RPM - seqcnb1). (*A*) *ERα* expression (*ESR1*) in *MYCN*-expression quartiles of non-MNA NB cases and in MNA tumors. (*B*) INSS stage, (*C*) risk status, and (*D*) age. (*E*) Correlation between survival and *ERα* expression in NB samples with or without *MYCN* amplification (see also *SI Appendix*, Fig. S12*D*). Significances were calculated using a one-way ANOVA (*A* and *B*), a Mann–Whitney (*C* and *D*), and a Spearman correlation (*E*) and are shown as $*P < 0.05$, $***P < 0.001$, or $****P < 0.0001$.

mice. Here, we demonstrate that overexpression of ERα indeed robustly inhibited tumor growth of *MYCN*-amplified NB cells in vivo (Fig. 7*A*), which was reflected in significantly reduced tumor weight and size (Fig. 7 *B* and *C*). Further, *ERα* overexpression was confirmed (Fig. 7*D*) and in line with our in vitro data (Fig. 3), the neuronal differentiation markers *NPY* and *TH* were up-regulated, while the glial cell markers *VIM* and *S100B* were suppressed in ERα-overexpressing tumors (Fig. 7*D*).

To validate that ERα inhibits neuroblastoma growth in vivo, we performed a second xenograft experiment using SK-N-BE(2) cells containing a Tet-inducible ERα expression system (*SI Appendix*, Fig. S11). Under cell culture conditions, doxycycline induced overexpression of ERα, whereas no expression was observed in BE (2) TetEV cells (*SI Appendix*, Fig. S11*A*). However, when analyzing the resulting tumors from the xenograft experiment, we noticed that the Tet-inducible system was leaky in vivo, as similar ERα expression levels were seen in mice with BE(2) TetESR1 cells whether untreated or induced with doxycycline. Mouse *Erα* levels were consistently low in all four groups (*SI Appendix*, Fig. S11*A*). Since human *ERα* levels were similar in doxycycline-treated versus untreated tumors, we pooled the two groups of mice bearing TetESR1 or TetEV tumors, respectively. Our results show that ERα also reduced tumor growth in this model (*SI Appendix*, Fig. S11*B*), albeit less efficiently than in the BE(2) ESR1 model (Fig. 7).

Expression of *ERα* and Other *NHRs* Correlates with Favorable Prognosis in Patients with NB. Our in vitro and in vivo data demonstrated that overexpression of ERα was sufficient to induce neuronal differentiation and to interfere with tumorigenesis. In our previous study, we showed that *ERα* mRNA expression correlates with improved EFS in patients with NB and that ERα is down-regulated by MYCN in MNA NB cells in vitro (27). *MYCN*-amplified NBs are classified as high-risk tumors. Here we extended our analysis and found an inverse correlation between *MYCN* expression quartiles (*SI Appendix*, Fig. S12*A*) and *ERα* mRNA levels, being lowest in MNA NB in a cohort of 498 patients with NB (Fig. 8*A*). Moreover, *ERα* levels were significantly reduced in tumors of the international NB staging system (INSS) stages 3 and 4 compared with the favorable stages 1 and 2, as well as 4S (Fig. 8*B*). Additionally, overall survival (OS) and EFS of patients with NB significantly decreased with reduced *ERα* expression (*SI Appendix*, Fig. S12 *B* and *C*), and low-risk patients with NB exhibited higher *ERα* mRNA levels than high-risk patients (Fig. 8*C*). Furthermore, *ERα* expression was reduced in children with NB above the age of 18 mo (Fig. 8*D*), age being another independent prognostic factor (1, 2). We next compared *ERα* expression levels in MNA versus non-MNA tumor samples with regard to survival (Fig. 8*E* and *SI Appendix*, Fig. S12*D*). *ERα* levels were generally lower in MNA patients but there was no difference between surviving versus diseased patients. Interestingly, *ERα* mRNA expression was significantly lower in patients who died of non-MNA NB compared with those still alive. This decreased expression was similar to the levels found in MNA tumor samples. These data suggest that MYCN suppresses ERα expression to a level at which it cannot exert its antitumorigenic effects. Finally, based on our observation that ERα can induce $p75^{NTR}$ expression in vitro (Fig. 1 *B*–*E* and *SI Appendix*, Fig. S10*B*), we analyzed expression of the genes encoding these proteins in the NB patient dataset. Notably, we observed a strong positive Spearman correlation between $p75^{NTR}$ and *ERα* mRNA gene expression (*SI Appendix*, Fig. S12*E*), and $p75^{NTR}$ was further linked to a favorable INSS stage (*SI Appendix*, Fig. S12*F*).

Taken together, our in vitro, in vivo, and patient data analyses suggest that ERα exerts antitumorigenic effects in NB, which are suppressed by MYCN. We recently demonstrated that MYCN apart from ERα, directly targets an additional five members of the NHR family and that high mRNA expression of these genes was linked to a favorable overall survival (32). Importantly, we showed that, similar to ERα (27) (Figs. 1 and 3 and *SI Appendix*,

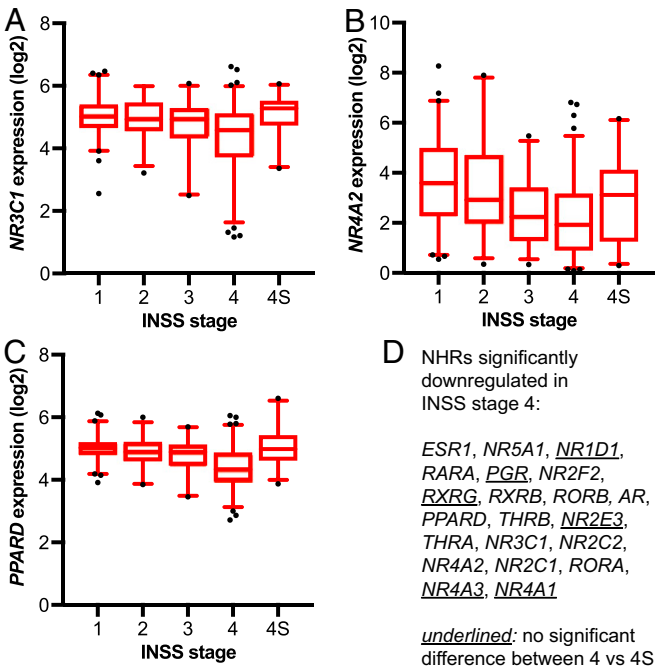

Fig. 9. Expression of different NHRs correlates with favorable INSS stage in patients with NB. Analysis of a patient cohort comprising RNA-sequencing expression data from 498 patients with NB (r2.amc.nl/; Tumor Neuroblastoma - SEQC - 498 - RPM - seqcnb1). The nuclear hormone receptor genes (*NHRs*) can be grouped into five different groups according to their expression levels in the different INSS stages. Group 1 contains 21 NHRs, which are significantly down-regulated in stage 4. The data for *GR* (*NR3C1*) (*A*), *NR4A2* (*NURR1*) (*B*), and *PPARD* (*C*) are shown and all genes in this group are summarized in *D*. See *SI Appendix*, Fig. S13 *A*–*C* and Dataset S4 for the other groups. Statistics and significances can be found in Dataset S4.

Figs. S5 and S10), expression of the GR is also linked to a more differentiated phenotype in NB (32). However, the effects of these two NHRs alone on neuronal-like differentiation are only partial, as not all cells in our model systems are differentiated. Our previous findings, together with the data presented in this study, therefore led us to hypothesize that several NHRs may act in concert to promote neuronal differentiation. This in turn prompted us to extend our previous patient data analysis by surveying the clinical and prognostic importance of the entire NHR family. We found that the genes encoding all 48 human NHRs can be subdivided into five different subgroups according to expression levels correlating to INSS stage (Fig. 9, *SI Appendix*, Fig. S13, and Dataset S4). The largest group is composed of 21 *NHRs*, among them *ERα*, *GR*, *NURR1*, *PPARD*, and the gene encoding the retinoic acid receptor alpha (*RARA*), for which expressions were significantly lower in INSS stage 4 compared with stage 1 and in most cases, also for stage 4S (Fig. 9). Group 2 has 10 members, including the genes encoding the peroxisome proliferator activated receptor alpha (*PPARA*) and the retinoid X receptor alpha (*RXRA*), which are specifically overexpressed in INSS stage 4S (*SI Appendix*, Fig. S13*A*). In contrast, a small number of *NHRs* were up-regulated in stage 4 (group 3; *SI Appendix*, Fig. S13*B*) or down-regulated in 4S (group 4; *SI Appendix*, Fig. S13*C*) while 12 *NHRs* did not show any significant changes (group 5; see Dataset S4 for all five groups). Notably, the majority of *NHRs* in group 1, which were correlated to favorable INSS stage, also showed a positive correlation to low age (<18 mo) and were linked to low-risk NB (Dataset S5). The nuclear receptor coactivator (NCOA) family members are important coactivators of different NHRs (41–44). Since we previously found that *NCOA1* is targeted by MYCN via miR-17~92 and that low expression is linked to poor survival (32), we included this protein family in our analysis. Similar to the *NHRs* in group 1 (Fig. 9), *NCOA1* was linked to favorable disease according to INSS (*SI Appendix*, Fig. S13*D*), age, and risk status (Dataset S5). The latter two criteria also correlated to *NCOA2*, while *NCOA3* did not show any relation to these prognostic groups (Datasets S4 and S5). Collectively, our patient data analysis clearly links high expression of *ERα* and a large set of the other *NHRs* to low-stage NB with favorable outcome.

Discussion

Neuroblastoma is a highly heterogenic childhood tumor with limited treatment strategies and low survival rates for high-risk patients (5). *MYCN* amplification (7, 8) but also hyperactive MYC signaling (12) correlates to an undifferentiated and more aggressive tumor type and to decreased survival. Here, we extend our previous findings showing that *MYCN*-amplified NBs, via the miR-17~92 cluster, maintain an undifferentiated phenotype by interference with the expression of ERα (27) (Fig. 1 and *SI Appendix*, Figs. S1 and S5).

We demonstrate that ERα-induced differentiation (Figs. 1 and 2) is reflected in a shift from a glial- (S type) to a neural-like (N type) phenotype (Fig. 3) and in increased expression of *TrkA* and $p75^{NTR}$ (Fig. 1). The NGF receptors $p75^{NTR}$ and TrkA are neuronal differentiation markers with the latter being a powerful indicator for a good prognosis (17) and believed to be a major factor during spontaneous regression in patients with NB (13). We further demonstrate that ERα interfered with the undifferentiated phenotype of the MNA NB cell line SK-N-BE(2) by promoting NGF-induced neuronal differentiation (Fig. 1). We hypothesize that this observation is TrkA dependent, since this effect is also prominent in BE(2) ESR1 cells treated with NGF alone. Our results are in line with other studies, which suggest that estrogens are able to modulate the synthesis and regulation of TrkA and $p75^{NTR}$ and their ligand NGF in sensory neurons (45–49). Furthermore, the effects of estrogen in inducing differentiation as well as neurotrophic and neuroprotective effects in various different neuronal cells (50–54) and in two NB cell lines (55–57) are documented. Activated estrogen signaling has also been shown to enhance NGF-induced neuronal outgrowth in PC12 pheochromocytoma cells (58, 59). However, until the present study, little was known about possible collaborative effects of estrogen and NGF signaling in NB.

MYC family members regulate various normal cellular processes, and when activated, are involved in different hallmarks of cancer (60). We addressed whether the observed differentiation induction in ERα-overexpressing SK-N-BE(2) cells resulted in a less malignant phenotype. Indeed, our proteomic data demonstrated that ERα overexpression interfered with processes linked to tumor initiation and malignant transformation (Fig. 2 and *SI Appendix*, Fig. S4). Resistance to apoptotic stimuli (survival) and induction of continuous proliferation are important and early steps of carcinogenesis (60). Functional assays confirmed increased basal cell death rates as well as inhibition of proliferation (Fig. 4 and *SI Appendix*, Fig. S6) in BE(2) ESR1 cells. NGF exerts neuroprotective functions (17, 18) and, as expected, increased cell viability in BE(2) ESR1 cells. The less neuronal-like BE(2) EV control cells, on the other hand, did not respond to NGF treatment. Together, the MNA NB cells overexpressing ERα seem to mimic low-stage NBs, which show enhanced cell viability and induction of terminal differentiation upon treatment with NGF (61).

We next investigated whether the reduced cell viability and the enhanced basal and NGF-induced differentiation in BE(2) ESR1 cells had any effect on cellular processes involved in tumor progression. The ability to grow anchorage independently is a good indicator for the tumorigenic and metastatic potential (62) and we therefore performed in vitro migration and anchorage-independent growth assays. Ectopic expression of ERα was sufficient to interfere with both colony formation as well as motility (Fig. 5 and *SI Appendix*, Fig. S7), supporting our hypothesis that up-regulation of ERα interferes with a more malignant phenotype. However, treatment with NGF resulted not only in antitumorigenic effects such as induction of differentiation, but also in weak potentially protumorigenic effects, as observed in slightly increased cell viability and anchorage-independent growth. Both NGF and ERα are

known to promote neuronal differentiation and to exert neuroprotective as well as neurotrophic functions, thereby enhancing survival and proliferation of neuronal cells (see discussion above). We hypothesize that the observed increase in survival in NGF-treated BE(2) ESR1 cells is due to a more neuronal like phenotype and thus potentially less aggressive NB cells.

Recently, increasing evidence has suggested that tumors can have different bioenergetic phenotypes with OXPHOS moving into the spotlight of cancer metabolism. While some tumors mainly rely on aerobic glycolysis or OXPHOS, others have been shown to be able to adjust their metabolic program according to their corresponding needs (63). The observed changes in the morphological and functional phenotype of SK-N-BE(2) cells with ectopic expression of ERα were accompanied by a reduced activity and decreased overall levels of proteins involved in the two main energy-generating cellular processes, glycolysis and OXPHOS (Fig. 6 and *SI Appendix*, Fig. S8). While this could be a consequence of a reduced demand of energy and metabolic building blocks, BE(2) ESR1 cells seemed to be closer to their maximal capacity in general, as reflected by low glycolytic and, especially, respiratory reserves. This is in agreement with our previous findings that inhibition of MYCN results in impaired glycolysis, TCA cycle, respiratory chain as well as fatty acid β-oxidation, which in turn can be directly linked to an accumulation of lipid droplets in the cytoplasm (40).

Altogether, our in vitro data demonstrate that ERα overexpression is sufficient to interfere with classical processes linked to tumorigenesis. Our key findings in BE(2) ESR1 cells were confirmed in a second MNA NB cell line, IMR32, in which ERα also exerted antitumorigenic effects (*SI Appendix*, Fig. S10). Importantly, we emphasized the biological importance of our in vitro findings in two different in vivo NB xenograft models (Fig. 7 and *SI Appendix*, Fig. S11).

Overall, our data suggest that ectopic expression of ERα and its crosstalk to NGF signaling can push MNA NB cells from an aggressive phenotype to one resembling low-risk NB. Intriguingly, those findings can be related to NB patient data: firstly, we found an inverse correlation between *ERα* and *MYCN* and a positive correlation between *ERα* and $p75^{NTR}$ mRNA expression in patients with NB (Fig. 8*A* and *SI Appendix*, Fig. S12 *A* and *E*), which was in accordance with our in vitro data showing that MYCN via miR-18a down-regulates ERα, which in turn can induce $p75^{NTR}$ expression. Secondly, we showed that ERα can be linked to a good prognosis, as established by higher *ERα* expression levels in low INSS stages, higher age, and low-risk NB (Fig. 8). The results of our study extend prior findings from our group, which identified ERα and GR and four other NHRs (27, 32) as direct targets of MYCN and as proteins associated with improved survival in NB. Intriguingly, both ERα and GR are linked to induction of differentiation and to decreased cell viability. Since the observed effects were only partial, we hypothesized that several NHRs act in concert to promote neuronal differentiation and thereby interfere with tumorigenesis. In support, we found that high expression levels of the majority of *NHR*s can be linked to low INSS NB stages and/or to the favorable 4S stage (Fig. 9, *SI Appendix*, Fig. S13*A*, and Dataset S4) and that high mRNA expression for most of the 21 *NHR* members in group 1 (favorable INSS stage, Fig. 9) were linked to lower age and low-risk status (Dataset S5). Additionally, the high mRNA levels of the NHR coactivators NCOA1 and NCOA2 also correlated to a favorable NB disease (Datasets S4 and S5) potentially by increasing the activity of NHRs, which may contribute to neural differentiation. Importantly, several human NHRs [e.g., NR1D1 (64), NR2E1 (65), NR5A1 (66), as well as RAR and RXR (67)] are implicated in neurogenesis, maintenance, and a functional neuronal system. The significance of NHRs is further highlighted by the fact that 7 of 18 NHRs are linked to neuronal remodeling in *Drosophila melanogaster* (68). This in turn indicates that at least some of their functions as protectors and modulators of the nervous system are evolutionally conserved. Further studies are needed to fully understand the role of NHRs in NB, to evaluate whether a NHRs score can be used as prognostic marker, and if this information can help in developing novel differentiating strategies for NB treatment.

In summary, our data suggest a mechanism which contributes to an undifferentiated phenotype in *MYCN*-amplified NB cells: MYCN-induced miR-18a down-regulates ERα and thereby interferes with estradiol and NGF-stimulated neuronal differentiation. We discovered that ERα overexpression is partly sufficient to overcome the malignant phenotype associated with MYCN overexpression both in vitro and in vivo. In addition, ERα enhances the expression of the NGF receptors TrkA and, after activation, $p75^{NTR}$, which are both crucial for NGF-induced differentiation. Importantly, we found that not only ERα but also several other NHRs, including GR and RARA as well as the coactivators NCOA1 and NCOA2, are linked to a favorable NB disease.

Together, our data suggest that MYCN down-regulates several NHRs in concert to suppress their cumulative effect on neuronal differentiation. In support, we identified a large group of NHRs, including ERα, with potential prognostic relevance. Importantly, this study provides insights into the ERα–NGF cross-talk and suggests that activation of ERα and/or NGF receptors could be a strategy to treat certain subtypes of NB.

Materials and Methods

Cell Culture. BE(2) EV and BE(2) ESR1 cells were maintained in DMEM:Nutrient Mixture F-12 medium (with L-glutamine, without phenol red) (Thermo Fisher Scientific) supplemented with 10 mM Hepes, 1% penicillin/streptomycin, 1% nonessential amino acids (all from HyClone), 0.5× GlutaMax, and 10% charcoal-stripped FBS (Thermo Fisher Scientific) in a humidified environment at 37 °C and 5% CO_2.

Analysis of Patient Data. For survival and correlation analysis, an NB patient dataset with RNA sequencing expression data (Tumor Neuroblastoma - SEQC - 498 - RPM - seqcnb1) from 498 patients placed on the R2 platform (https://hgserver1.amc.nl/) was used. Clinical and expression data of the genes of interest were extracted from the database and analyzed using GraphPad Prism software.

In Vivo Xenograft Experiments. The experimental procedures, housing, treatments, and analysis of the mice were in accordance with the guidelines of Karolinska Institutet and the ethical permit approved by the Swedish ethical committee Stockholms Norra Djurförsöksetiska Nämnd (ethical permit N71/15).

Statistical Analysis. If not stated otherwise, data are presented as the mean ± SEM of at least three independent experiments. Statistically significant differences of in vitro data were identified with Student *t* tests, one-way or two-way ANOVA (with Bonferroni's multiple comparisons test), as indicated in the figure legends. Patient data were analyzed as indicated in the figure legends. Significances are highlighted with $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and, for patient data, $****P < 0.0001$.

Further information regarding cell culture, extracellular flux assay, Western blot, immunofluorescence staining, quantitative real-time PCR, basal cell viability assay, WST-1 cell viability assay, anchorage-independent growth, neural differentiation assay, transwell migration assay, Oil Red O staining of lipids, quantitative mass spectrometry-based proteomics, in vivo xenograft experiments, and analysis of patient data are described in *SI Appendix*.

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