**MYCN mediates cysteine addiction and sensitizes neuroblastoma to ferroptosis**

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Aberrant expression of MYC transcription factor family members predicts poor clinical outcome in many human cancers. Oncogenic MYC profoundly alters metabolism and mediates an antioxidant response to maintain redox balance. Here we show that MYCN induces massive lipid peroxidation on depletion of cysteine, the rate-limiting amino acid for glutathione (GSH) biosynthesis, and sensitizes cells to ferroptosis, an oxidative, non-apoptotic and iron-dependent type of cell death. The high cysteine demand of MYCN-amplified childhood neuroblastoma is met by uptake and transsulfuration. When uptake is limited, cysteine usage for protein synthesis is maintained at the expense of GSH triggering ferroptosis and potentially contributing to spontaneous tumor regression in low-risk neuroblastomas. Pharmacological inhibition of both cystine uptake and transsulfuration combined with GPX4 inactivation resulted in tumor remission in an orthotopic MYCN-amplified neuroblastoma model. These findings provide a proof of concept of combining multiple ferroptosis targets as a promising therapeutic strategy for aggressive MYCN-amplified tumors.

Many human cancers rely on aberrant expression of MYC transcription factor family members to allow unhindered growth and proliferation; high expression levels are predictive of poor clinical outcome. Aberrant MYC oncoprotein levels lead to gross transcriptional changes with hundreds, if not thousands, of upregulated and downregulated genes, which together drive various hallmark features of malignant cells. Pharmacological approaches to target aberrant MYC have largely failed. Therefore, MYC synthetic lethal interactions have been exploited for the development of therapeutic concepts to specifically target MYC-driven tumors, yet with limited success. Remarkably, in view of MYC's oncogenic activity, its potential to promote cell death apart from cell proliferation is paradoxical. Transgenic mouse models support a role for MYC for both tumor development but also spontaneous cell death depending on tissue type and context. Both may ultimately be related to MYC's profound influence on various aspects of cellular metabolism, with their interdependencies still poorly understood.

Childhood neuroblastoma, an embryonic tumor derived from progenitors of the sympathetic nervous system, is a paradigmatic
model for MYC-driven cancers. Amplified MYCN identifies a highly aggressive subtype associated with malignant progression and poor outcome despite intensive multimodal treatments. However, a substantial proportion of low-risk neuroblastomas with elevated MYCN expressed from a normal MYCN locus, particularly those arising in children younger than 18 months, regress spontaneously (stage 4S disease) by unknown mechanisms even when the disease is metastatic. High-risk neuroblastomas lacking amplified MYCN harbor rearrangements of other MYC gene family members, TERT or alternative mechanisms of telomere lengthening (ALT) often associated with ATRX gene mutations, the latter subtype being incompatible with high MYCN or MYC expression \(^{11,12}\) (later referred to as MYCN(N)). Beside these alterations linked to telomere maintenance mechanisms, mutations in ALK or other developmental genes lead to stalled differentiation and tumors composed of heterogeneous cell types resembling different states of the normal neuroendocrine differentiation trajectories. The spectrum of cell types ranges from differentiated over undifferentiated adrenergic-to-mesenchymal cell types (triggered by adrenergic-to-mesenchymal transition), where malignant progression, therapy resistance and disease relapse are strongly associated with undifferentiated cell types.\(^{8,9}\)

MYCN-amplified neuroblastoma cells, like other MYC-driven cancer cells, have been found to be addicted to the amino acid glutamine (Gln), the absence of which causes growth arrest or apoptosis\(^{10}\). More recently, reports showed that neuroblastoma cells are also addicted to iron and are sensitized to ferroptosis\(^{11,12}\), a new iron-dependent oxidative form of cell death associated with lipid peroxidation and insufficient capacity to eliminate lipid peroxides\(^{11}\). Whereas the apoptosis pathway of regulated cell death is fronted with accumulating reactive oxygen species (ROS). In this study, by performing single amino acid deprivations in high MYCN and low MYCN neuroblastoma cells, we discovered strong dependency of high MYCN cells on the amino acid cysteine. Using functional MYCN synthetic lethal metabolic and genetic screens, we further identified cyst(e)ine deprivation and glutathione peroxidase 4 (GPX4) inhibition as selective liabilities in MYCN-amplified neuroblastomas. Combined targeting of cystine uptake, cysteine synthesis via transsulfuration and GPX4 in an orthotopic neuroblastoma model strongly reduced tumor growth in vivo. Multi-omics profiling identified multiple cell type-specific and MYCN-regulated mechanisms inhibiting ferroptosis in adrenergic and mesenchymal neuroblastoma cells. Taken together, our study uncovered mechanisms crucial to ferroptosis escape in MYCN-amplified neuroblastomas; simultaneous inhibition of those mechanisms led to tumor regression in vivo.

### Results

**Cystine deprivation induces MYCN-dependent ferroptosis.** First, we analyzed the interplay of oncogenic MYC activity with amino acid metabolism. Downregulating MYCN in the MYCN-amplified IMR5/75 neuroblastoma cell model\(^{13}\) (approximately 65% reduction; Fig. 1a) slowed cell proliferation without inducing cell death (Extended Data Fig. 1a,b) and reduced the intracellular pools of all amino acids (Fig. 1b). Most prominently, cysteine was reduced nearly tenfold (Extended Data Fig. 1c). Inhibiting MYC binding to Myc-associated factor X (MAX) using 10058-F4 (ref. \(^{19}\)) yielded similar results (Fig. 1b and Extended Data Fig. 1d). These data show that high MYCN levels are associated with high levels of cellular cysteine, probably mediated by increased synthesis and/or uptake from the microenvironment. Systematic depletion of individual amino acids from the growth medium impaired cell viability in both high MYCN and low MYCN cells in most cases (Fig. 1c).

However, in line with recent reports\(^{11,12}\), cells with high MYCN expression exhibited stronger dependency on cystine imported by cystine/glutamate-exchange transporter \(x_7^-\) and readily reduced to two cysteine molecules intracellularly. Cystine deprivation caused robust cell death in high MYCN cells, which was largely prevented by downregulation of MYCN expression (Fig. 1c) or inhibition of MYCN–MAX binding (Fig. 1d). Overexpressing MYCN in MYCN diplod cells (Tet21N neuroblastoma cell model\(^{15}\)), rendered these cells highly vulnerable to cystine deprivation (Fig. 1e).

Neuroblastoma cell lines with intermediate MYCN or MYC levels caused by gene translocations are known to exist. We inferred MYCN(N) activity by target gene expression score, which agrees with transcript and protein MYCN(N) levels (Extended Data Fig. 1e,f). Cell death after cystine deprivation increased with MYCN(N) activity score, being virtually absent in cell lines immortalized by alternative telomere lengthening and lacking MYCN(N) aberrations and peaking in MYCN-amplified cell lines with the highest activity scores (Fig. 1f). These data demonstrate that oncogenic MYCN(N) expression is associated with cysteine addiction, with cysteine reduction resulting in massive cell death in an MYCN(N)-dependent manner.

Cell death in the cystine-deprived high MYCN cells was not abrogated by the inhibition of (1) caspases to prevent apoptosis, (2) lysosomal function to downregulate autophagy or (3) receptor-interacting serine/threonine-protein kinase 1 (RIPK1) to prevent necroptosis (Extended Data Fig. 1g). Availability of cysteine/cysteine is the rate-limiting step in GSH synthesis counteracting ROS\(^{15}\), which prevents the execution of ferroptosis, an oxidative, non-apoptotic and iron-dependent form of regulated cell death caused by ROS-mediated lipid peroxidation\(^{13,16}\). Indeed, ferrostatin-1 (Fer-1), a specific inhibitor of ferroptosis, or a lipophilic antioxidant or an intracellular iron chelator inhibited lipid peroxidation in cystine-deprived high MYCN cells (Fig. 1h). Hence, when deprived of cysteine, high MYCN neuroblastoma cells exhibited lipid peroxidation and died via ferroptosis.

Addition of GSH to the cystine-free medium also prevented lipid peroxidation in MYCN-amplified cells similar to Fer-1 (Fig. 1i) and rescued cell viability similar to Fer-1 (Fig. 1j) and Extended Data Fig. 1i), suggesting that high GSH levels protect against ferroptosis. Consistent with their higher sensitivity to ferroptosis, MYCN-amplified neuroblastoma cells had lower baseline GSH and cysteine levels compared to cell lines with lower MYCN(N) activity scores (Extended Data Fig. 1j,k). However, on downregulation of MYCN in MYCN-amplified cells, intracellular GSH levels were reduced threefold, the reduced-to-oxidized GSH ratio was halved and intracellular ROS levels increased (Fig. 1k), suggesting that high MYCN expression increases GSH synthesis and ROS clearance. Overall, these results indicate that although oncogenic MYCN activates the production of GSH, it is maintained at a low steady state level due to its rapid consumption in fast-proliferating cells.

The only other amino acid showing a selective dependency on MYCN was Gln, confirming previous reports\(^{12,13}\), although this was significantly less pronounced (Fig. 1c). Interestingly, cysteine and Gln (when converted to glutamate) are two important GSH precursors. Depleting Gln in addition to cysteine partially restored GSH and cysteine levels compared to cell lines with lower MYCN(N) activity scores (Extended Data Fig. 1j,k). However, on downregulation of MYCN in MYCN-amplified cells, intracellular GSH levels were reduced threefold, the reduced-to-oxidized GSH ratio was halved and intracellular ROS levels increased (Fig. 1k), suggesting that high MYCN expression increases GSH synthesis and ROS clearance. Overall, these results indicate that although oncogenic MYCN activates the production of GSH, it is maintained at a low steady state level due to its rapid consumption in fast-proliferating cells.

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Fig. 1 | Cystine addiction in MYCN-expressing neuroblastoma cells. a. Representative western blot of IMR5/75 neuroblastoma cells on MYCN knockdown using Dox (expression: –Dox, high; +Dox, low); the experiment was replicated three times. b. Intracellular amino acid quantification after MYCN inhibition for 96 h (+Dox, n = 5 samples and –Dox, n = 6 samples, or 10058-F4, inhibiting MYCN–MAX binding, n = 4 samples and dimethyl sulfoxide (DMSO)-treated, n = 5 samples). Data represent the mean ± s.e.m. The experiment was replicated three times. c. Standardized viability of IMR5/75 after single amino acid depletions (48 h). Data represent the mean ± s.e.m.; n = 3 samples. The experiment was replicated three times. d, e, Cellular responses to Cys2 deprivation in high MYCN (–Dox) and low MYCN (+Dox) state in IMR5/75 (d) and Tet21N (e) cells; the mean viability of cells was standardized to untreated (full medium) and representative western blot of neuroblastoma Tet21N cells. Data represent the mean ± s.e.m.; n = 3 samples. The experiment was replicated three times. Images of the cells are shown on the left. Scale bar, 50 μm. f. Sensitivity to Cys2 deprivation versus level of MYC(N) activity in a panel of neuroblastoma cell lines. g. Relative viability (survival of compound-treated cells divided by survival of vehicle-treated cells) of IMR5/75 cells after Cys2 deprivation for 72 h in the presence or absence of fer-1. Data represent the mean ± s.e.m.; n = 3 samples. The experiment was replicated three times. Images of the cells are shown on the left. Scale bar, 50 μm. h, i. Analysis of lipid peroxidation in Cys2-deprived high or low MYCN IMR5/75 cells (n = 3 samples; the experiment was replicated 3 times). j. Analysis of lipid peroxidation in Cys2-deprived high or low MYCN IMR5/75 cells in the presence or absence of Trolox, CPX, Lip-1, GSH or fer-1. The experiment was replicated three times. k. Relative viability of SK-N-DZ, IMR5/75, SK-N-FI and normal human dermal fibroblasts (NHDF) after Cys2 deprivation in the presence or absence of GSH and fer-1, n = 4 samples. The experiment was replicated three times. Quantification of total intracellular GSH (n = 8 samples) levels and the reduced GSH/GSH disulfide (GSSG) ratio in IMR5/75 cells (n = 3 samples). Analysis of intracellular ROS levels using CellROX staining and flow cytometry in IMR5/75 cells in high MYCN (–Dox) and low MYCN (+Dox) state (n = 3 samples). Data represent the mean ± s.e.m. The experiment was replicated three times. Statistical analysis was performed using a two-tailed Student’s t-test.
Fig. 2 | Gln is required for ferroptosis in high MYCN cells. a–c, Cysteine (a), total GSH levels (b) and cell viability (c) on Cys2 and Gln deprivation. Data represent the mean ± s.e.m.; n = 3 samples. The experiment was replicated three times. d, RNA-seq of IMR5/75 cells on treatment with the conditions indicated in the figure. n = 3 samples. e, siRNA-mediated GLSKGA/GAC knockdown (72 h) on Cys2 deprivation (24 h) in IMR5/75 cells. Representative western blots of each isoform. Data represent the mean ± s.e.m.; n = 4 samples. The experiment was replicated three times. f, Expression of glutaminolysis genes (mitochondrial GLS, GAC; cytosolic GLSKGA and GLS2) compared with MYCN-regulated genes (CBS, AHCY, GSR) in high MYCN and low MYCN IMR5/75 cells. TPM, transcripts per million. Data represent the mean ± s.e.m. The statistical analysis was performed using a two-tailed Student’s t-test.
Identification of ferroptosis genes in MYCN-amplified cells.

To systematically identify the cellular vulnerabilities associated with high MYCN expression, we performed an unbiased synthetic lethal small interfering (siRNA) screen using high/low MYCN IMR5/75 cells (Fig. 3a). Among the top hits that reduced the survival of high MYCN cells were enzymes involved in GSH metabolism that detoxify lipid peroxides (Fig. 3b,c, Extended Data Fig. 2a and Supplementary Table 1). Namely, inhibition of GSR, GPX4, GPX6 or GSTM1, GSTM5 and GSTK1 (Fig. 3b and Extended Data Fig. 2a) caused a selective reduction in viability in high MYCN cells. Knockdown of either of the two enzymes catalyzing GSH biosynthesis, glutamate–cysteine ligase catalytic subunit (GCLC) and glutathione synthetase (GSS) also showed synthetic lethality with high MYCN state (Fig. 3b and Extended Data Fig. 2a).

One of the screening hits, GPX4, is an anti-ferroptotic selenoprotein that protects against lipid peroxidation using GSH in vitro, siRNA-mediated GPX4 knockdown induced ferroptosis in high MYCN cells, which was rescued by Fer-1 (Fig. 3d and Extended Data Fig. 2b). Similarly, we observed reduction of viability using the GPX4 inhibitor RSL3, enhanced by supplementing iron and rescued by Fer-1 or GSH (Fig. 3e,f) or simultaneous MYCN and MYCN–MAX inhibition. The dependency of cell viability on GPX4 activity increased with MYCN activity across our broader cell line panel (Fig. 3g,h and Extended Data Fig. 2d,e) unlike GCLC or GSR, which exhibited no dependency (Extended Data Fig. 2f). Next, we studied whether the effect of GPX4 knockdown was linked to cystine uptake and metabolic gene expression in 348 cancer cell lines. Cells dependent on GPX4 showed low expression levels of SLC7A11 of the x− system, import cystine in exchange for glutamate (Extended Data Fig. 2g). Neuroblastoma cell lines were, after ovarian cancer cell lines, the second most dependent on GPX4 (Extended Data Fig. 2h).

To therapeutically investigate the role of GPX4 inhibition in vivo, we used an inducible CRISPR–Cas9 system to eliminate GPX4 (GPX4 knockout). In the MYCN-amplified SK-N-DZ three-dimensional (3D) cell culture model, GPX4 knockout induced ferroptotic cell death. This was rescued by simultaneously treating cells with the 10058-F4 MYC–MAX inhibitor (Fig. 3i). We then generated an orthotopic mouse model for human neuroblastoma by transplanting these SK-N-DZ neuroblastoma cells orthotopically into the adrenal gland and allow the cells expressing native GPX4 levels to develop into small tumors (Fig. 3j). Subsequently, the CRISPR–Cas9 GPX4 knockout was induced by doxycycline (Dox) treatment 7 d after transplantation. Although this resulted in a significant reduction in tumor weight compared to controls (Fig. 3k), it was not sufficient for tumor eradication as also shown by others. Ferroptosis markers such as CHAC1 and TFRC increased despite only partial reduction of GPX4 transcripts, suggesting activation of ferroptosis in vivo (Fig. 3l). In summary, our data show that GPX4 partially protects high MYCN neuroblastoma cells from ferroptosis in vitro and in an orthotopic neuroblastoma mouse model.

MYCN induces transsurfusion and prevents ferroptosis.

Cellular cysteine can also be produced in some cell types by transsulfuration. In this process, homocysteine (Hcy), an intermediate of the methionine cycle, and serine are combined to form cystathionine (Cysta), which is further converted to cysteine (Fig. 5a). GPX4 dependency in cancer cell lines was associated with enhanced expression of cystathionine beta-synthase (CBS), the rate-limiting enzyme for transsulfuration (Extended Data Fig. 2g, with neuroblastoma being among the cancer entities with the

Fig. 3 | Inhibition of GPX4 is synthetic lethal with high MYCN. a, MYCN synthetic lethal druggable genome-wide siRNA screening approach in IMR5/75 cells. b, Effects of individual siRNAs (gray dots): high MYCN versus low MYCN, including key players (median of two or three siRNAs) of the top MYCN synthetic lethal hits (single star symbol) of GSH metabolism (black) and biosynthesis (green). c, MYCN effects on lipid peroxide formation and intracellular amino acid levels (fold changes shown in red), the x− system (Cys3 uptake), the two-step biosynthesis of GSH and GSH metabolism; the single star symbol marks the top MYCN synthetic lethal hits of GSH metabolism, with an FDR of 0.2. The action of ferroptosis inhibitors (CPX, Fer-1, Lip-1, Trolox and 10058-F4), class I (erastin, IKE, sulfasalazine), class II ferroptosis inducers (RSL3, ML-210) and the GSH biosynthesis inhibitor buthionine sulfoximine as indicated. d, siRNA GPX4 knockdown in the presence or absence of Fer-1. Data report the mean ± s.e.m.; n = 4 samples. The experiment was replicated three times. e, Relative viability (survival of compound-treated cells divided by survival of vehicle-treated cells) of the KELL Y cell line after GPX4 inhibition in vivo, ± s.e.m.; n = 4 samples. The experiment was replicated three times. f, Relative viability of IMR5/75 cells treated with RSL3 in the presence or absence of Fer-1 or GSH, ± s.e.m.; n = 4 samples. The experiment was replicated three times. g, DRIVE database (RSA values, Pearson’s correlation, P = 0.01; filled circle, MYCN-amplified; white circle, MYCN-non-amplified). h, Cellular responses of neuroblastoma cell lines to 72 h of RSL3 treatment: cells with MYCN amplification (black symbols), moderate MYCN expression (white circle) and lack thereof (white triangle). Data report the mean ± s.e.m.; n = 3 samples. The experiment was replicated three times. i, Dox-inducible GPX4 CRISPR–Cas9 knockout in a 3D model with MYCN-amplified SK-N-DZ cells in the presence or absence of MYCN–MAX inhibition. Data report the mean ± s.e.m. Right: representative western blot; n = 4 samples. The experiment was replicated three times. j, Orthotopic mouse neuroblastoma model allowing CRISPR–Cas9-mediated GPX4 deletion. Panel created with BioRender. k, Tumor weight after GPX4 knockout (+Dox) (n = 5 mice per group). A representative western blot for CRISPR–Cas9-mediated GPX4 deletion is shown. l, Elevated messenger RNA expression of the ferroptosis markers CHAC1 and TFRC. Data report the mean ± s.e.m.; n = 4 samples from each group. Statistical analysis was performed using a one-tailed Student’s t-test for the in vivo experiments and a two-tailed Student’s t-test for the in vitro experiments. Box plots: the center line indicates the median value, the lower and upper hinges represent either the 25th and 75th percentiles or the minimum and maximum and the whiskers denote 1.5x the interquartile range (IQR). Each dot corresponds to one sample; one-sided Student’s t-test; P values as indicated.
highest CBS expression (Extended Data Fig. 2m,l). We hypothesized that transsulfuration provides a cysteine source for neuroblastoma cells preventing ferroptosis in cystine deprivation conditions. Cystathionine gamma-lyase (CTH), converting Cysta to cysteine, and S-adenosyl-L-homocysteine hydrolase (AHCY), synthesizing Hcy for transsulfuration, show synthetic lethality with high MYCN (Fig. 5b,c and Extended Data Fig. 3a), as are two methyltransferases that feed into Hcy production (Extended Data Fig. 3b).
Supplementing cysteine-deprived cells with either Hcy or Cysta prevented ferroptosis in all adrenergic neuroblastoma cell lines tested with high or intermediate oncogenic MYCN expression, but not in the less common mesenchymal neuroblastoma lines (Fig. 5d and Extended Data Fig. 3c). Pharmacologically inhibiting CTH using propargyglycine (PPG) sensitized adrenergic, but not mesenchymal, high MYCN cell lines to either erastin- or imidazole ketone erastin (IKE)-induced cell death (Fig. 5c). Knockdown of AHCY in adrenergic high MYCN but not mesenchymal neuroblastoma cells impaired colony formation, which was associated with reduced GSH levels and reduction of GSH reduced-to-oxidized ratios (Fig. 5f, g). In summary, these data indicate that transsulfuration provides an internal cysteine source for GSH biosynthesis protecting high MYCN adrenergic neuroblastoma cells from ferroptosis.

Intracellular cysteine is required for two rate-limiting cellular processes: GSH-mediated ROS clearance and production of building blocks in the synthesis of proteins, nucleotides and lipids (Fig. 5a). In line with this, protein synthesis inhibition with cycloheximide increased cysteine and total GSH levels (Extended Data Fig. 3d). Notably, reduction/deprivation of cysteine (Cys) in the medium drastically reduced intracellular cysteine and GSH in the high MYCN state before ferroptosis (Fig. 2a,b) but did not affect cell cycle progression and only moderately reduced protein synthesis (Extended Data Fig. 3e,f). MYCN protein levels were not affected on cystine deprivation compared to methionine- or Gln-deprived cells (Extended Data Fig. 3g). These data suggest robust cysteine channeling into protein synthesis under limited cysteine supply when GSH production is already diminished.

To investigate this further, we performed transcriptomic analysis of IMR5/75 cells cultured in cystine-free medium or inhibiting the cystine system with erastin. This revealed activation of a stress response before ferroptosis that channeled cysteine into protein synthesis by inducing CARS and cysteine recycling from GSH (Fig. 2d and Extended Data Fig. 3h–j). HMOX1 was also activated in these cells, suggesting that the free iron pool is increased before inducing ferroptosis (Fig. 2d and Extended Data Fig. 3h–j). Inhibiting CARS prevented ferroptosis in the cystine-deprived high MYCN state, highlighting the competition for intracellularly synthesized cysteine between protein synthesis and redox balance (Fig. 5h). Taken together, transsulfuration supplies cysteine for both protein and GSH synthesis in adrenergic MYCN-amplified neuroblastoma cells but prioritizes cysteine for protein synthesis at the expense of GSH and redox balance when cysteine uptake is limited, thus triggering ferroptosis.

Next, we asked how cysteine metabolism and redox homeostasis are affected by oncogenic MYCN activity in the adrenergic (active transsulfuration) or mesenchymal (inactive transsulfuration) neuroblastoma subtypes. Adrenergic cells upregulated three key enzymes in transsulfuration, CBS, AHCY and D-3-phosphoglycerate dehydrogenase (PHGDH), in the high MYCN state, while the x− system (SLC7A11) controlling cysteine uptake was unaffected by changes in MYCN (Extended Data Fig. 4a). In a panel of 32 neuroblastoma cell lines, MYCN or MYC amplification or translocation in adrenergic subtypes was accompanied by upregulated CBS expression, while MYC translocation/activation in mesenchymal cell lines was not (Fig. 5i). Ectopically expressing MYCN in mesenchymal Tet21N cells left transsulfuration via its rate-limiting enzyme CBS
unchanged but induced SLC7A11, GSR, GCLC and thioredoxin reductase 1 (TXNRD1), the last one being significantly expressed in MYCN-amplified primary neuroblastomas (Extended Data Fig. 4b,c)7. At the gene regulatory level, we found that the CBS locus harbored both activating (H3K27ac, H3K4me3) and silencing (H3K27me3) histone modifications, with the former being increased and the latter decreased in the presence of amplified MYCN in adrenergic cells compared to non-amplified or mesenchymal cells (Fig. 5) and Extended Data Fig. 5. In primary neuroblastomas, differences in CBS expression correlated with histone modifications and methylation of intragenic CpGs dependent on genomic MYCN status (Fig. 5k and Extended Data Fig. 6). Together, this suggests that transsulfuration is active in the adrenergic state and regulated at the epigenetic level in high MYCN cells.

We found that the levels of CBS and AHCY were associated with poor patient survival (Fig. 6a). Global gene expression profiles from 498 primary neuroblastomas36 confirmed elevated AHCY and CBS in MYCN-amplified neuroblastomas (Fig. 6b,c). Higher HMOX1 and lower SLC3A2 and TXNRD1 expression were found in stage 4S tumors (Fig. 6d), which tend to regress spontaneously. CBS, AHCY and PHGDH expression was also elevated in mass spectrometry-based global proteomes from MYCN-amplified neuroblastoma tumors (Fig. 6e). SLC7A11 antiporter expression did not correlate with MYCN amplification or other risk factors for poor patient outcomes (Fig. 6c). In addition, genes involved in Gln (that is, SLC38A5, SLC1A5), methionine (that is, SLC7A5) and iron uptake (TFRC) had higher expression in MYCN-amplified or MYCN-overexpressed neuroblastomas; TFRC was recently associated with ferroptosis in neuroblastomas31 (Fig. 6c,d and Extended Data Fig. 4c). Genes associated with GSH synthesis/metabolism (that is, GCLC, GSR) were also upregulated both at the transcript and protein level (Fig. 6c,e,f). Higher expression of the mitochondrial glutaminase (GLS2mit) and lower expression of the cytoplasmatic glutaminase (GLS2cyt) isoforms were observed in MYCN-amplified tumor transcriptomes and proteomes (Fig. 6c), suggesting dependence on mitochondrial glutaminolysis in these neuroblastomas. Together, our results show that MYCN-amplified neuroblastomas increase transsulfuration activity, iron import, glutaminolysis and GSH production through coordinated changes in gene expression thereby increasing the susceptibility to ferroptosis.

Cyst(e)ine and GPX4 inhibition as therapeutic targets. To exploit our findings in vivo, we first tested simultaneous inhibition of cysteine uptake and transsulfuration in vitro using IKE, an erasist analog with acceptable pharmacokinetic properties40,41, and PPG39 (Fig. 7a). We observed a strong synergistic effect with the two drugs in only MYCN-amplified adrenergic cells (Fig. 5e). We then injected mice orthotopically with SK-N-DZ neuroblastoma cells and treated them for two 5-d cycles with a combination of IKE 45 mg kg d−1 and PPG 45 mg kg d−1 (Fig. 7b). When combining these two drugs, we observed a 60% reduction in tumor growth in MYCN-amplified SK-N-DZ-driven tumors (Fig. 7c). These data suggest that MYCN-amplified neuroblastoma cells with very high levels of MYCN are sensitive to a reduction of the intracellular cysteine pool by simultaneous inhibition of cysteine import and cysteine synthesis via transsulfuration.

Next, we combined this protocol for reducing cellular cysteine with genetic targeting of GPX4 activity (Fig. 7b). We observed a robust effect with complete remission in most animals (Fig. 7d,e). Transcriptional profiling of residual small tumors revealed induction of ferroptosis markers after combined inhibition of cysteine uptake/cysteine synthesis and GPX4 compared to tumors treated with vehicle control (Fig. 7f). These data provide strong in vivo evidence that concomitant reduction of cysteine uptake, transsulfuration and GPX4 activity can be utilized as a new therapeutic strategy for high-risk, MYCN-amplified neuroblastomas.

Discussion

We demonstrated that oncogenic MYCN sensitizes neuroblastoma cells to ferroptosis when intracellular cysteine availability for GSH synthesis and the cysteine/cysteine redox cycle are limited. A high MYCN state in neuroblastoma cells sensitizes them to lipid peroxidation, which in combination with acute intracellular cysteine reduction triggers massive ferroptotic cell death. Our study shows transsulfuration and GSH redox activity to be crucial to escape ferroptosis, whereas Gln import and glutaionolysis are required for ferroptosis in neuroblastoma cells with oncogenic MYCN (Fig. 7a). Expression levels of key genes for these processes are correlated with MYCN expression in high-risk tumors. The recently identified adrenergic and mesenchymal subtypes2 also appear to determine how cysteine is maintained in neuroblastoma cells, with transsulfuration only being activated by oncogenic MYCN(N) in adrenergic cells, where CBS is accessible and not epigenetically repressed in mesenchymal cells.

In this study, we describe metabolic rewiring in MYCN-amplified adrenergic neuroblastoma cells, where high consumption of cysteine used for the synthesis of cellular building blocks at the expense of GSH synthesis and ROS clearance creates a new MYCN-dependent liability. To prove that this liability can be exploited as a new therapeutic concept, we established and optimized an orthotopic model for neuroblastoma using intra-adrenal gland tumor cell transplantation. This model allowed robust in vivo testing of
ferroptosis induction: we obtained remarkable tumor remission in high MYCN neuroblastoma by combining inhibition of (1) cysteine import using IKE and (2) transsulfuration using PPG together with (3) CRISPR–Cas9-mediated GPX4 deletion. Among the different parameters tested, this was the most effective strategy revealing almost complete tumor remission. Currently there are no GPX4 inhibitors for in vivo use, hence future improvements of this therapeutic strategy would involve the development of potent GPX4 inhibitors with optimal pharmacokinetics and pharmacodynamics.
In addition, we demonstrated that the iron chelator CPX rescues ferroptotic cell death under cystine deprivation conditions (Extended Data Fig. 7a) reflected on the protein level (Fig. 6e). Similarly, we observed HMOX1 upregulation, involved in increasing the labile iron pool, in cystine-deprived neuroblastoma cells before ferroptosis induction. Together, this highlights a prominent role for iron metabolism in promoting ferroptosis in neuroblastoma cells.

We have described a detailed mechanism that explains how cysteine deprivation triggers ferroptosis in MYCN-amplified tumors, which depends on the (epi)genetic context of adrenergic or mesenchymal subtypes, the latter having transsulfuration silenced. These results differ from Floros et al., who suggested that high MYCN mediates upregulation of SLC7A11 in neuroblastoma cells. In contrast, our results indicate that in most, if not all, adrenergic neuroblastoma cells (>95% of neuroblastomas) high MYCN fails to boost cysteine uptake via xCAT-2. Instead, MYCN strongly activates transsulfuration, the intracellular conversion of methionine to cysteine. In general, neuroblastoma cells have very low transsulfuration, the intracellular conversion of methionine to cysteine. In general, neuroblastoma cells have very low SLC7A11 expression compared to other cancer types, which may explain the remarkable sensitivity of neuroblastoma cells to ferroptosis stimuli. Our results demonstrate that the uptake of cysteine via xCAT-2 is not regulated on tumor progression in adrenergic MYCN-amplified cells.

Intriguingly, our data may also explain spontaneous neuroblastoma regression, the mechanisms of which have remained elusive. In low-risk, metastatic neuroblastomas (stage 4S), which are likely to become relevant in the relapse scenario, which is associated with elevated proportions of mesenchymal cells.

Recent studies highlighted the role of iron in MYCN-dependent neuroblastoma and ferroptosis. The TFRC gene, involved in iron uptake, is activated by MYCN in several cell types enhancing cellular proliferation. In line with these studies, we showed a synergistic effect of iron in drug-induced ferroptosis in MYCN-amplified cells. In addition, we demonstrated that the iron chelator CPX rescues ferroptotic cell death under cystine deprivation conditions. In general, neuroblastoma cells have very low SLC7A11 expression compared to other cancer types, which may explain the remarkable sensitivity of neuroblastoma cells to ferroptosis stimuli. Our results demonstrate that the uptake of cysteine via xCAT-2 is not regulated on tumor progression in adrenergic MYCN-amplified cells.
(Extended Data Fig. 6a); (3) HMox1 upregulation to increase free iron pools; and (4) SLC3A2 and TXNRD1 downregulation (Fig. 6d). Spontaneous regression may be the physiological resolution of this cellular state sensitive to ferroptosis. Unlike low-risk tumors, MYCN-amplified neuroblastomas appear to metabolically adapt to survive events that deplete intracellular cysteine, such as high systemic glutamate present in the first two years of life. The cysteine requirement of cancers dependent on oncogenic MYCN activity creates a previously unknown Achilles’ heel that could be exploited to selectively induce ferroptosis for treatment. Our findings identify cysteine, transulfuration and the lipid peroxidation-specific scavenging system as vulnerabilities in cancer cells driven by oncogenic MYCN(N) activity such as MYCN-amplified neuroblastomas.

Methods

Ethics. All patients with neuroblastoma were enrolled in the German Neuroblastoma Trial (NB87, NB90/4, NB 2016) and approved by the ethic committee of the University of Cologne. All studies involving mice and experimental protocols were conducted in compliance with German Cancer Research Center guidelines and approved by the governmental review board of the state of Baden-Württemberg.

Experimental in vitro procedures. Cell culture. Human neuroblastoma cells (IMR5/75, KELLY, SiMa, NBL-S, SK-N-FL, SH-SYSY, NB69, SK-N-DZ, SH-EP GI-ME-RN) were cultivated at 37 °C with 5% CO2 in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) supplemented with 10% fetal calf dylalyzed serum (Gibco) and penicillin/streptomycin (AppliChem). The KELLY and SiMa cell lines were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen. SK-N-FL cells were purchased from ATCC. NBL-S and Tet21N (SH-EP) cells were provided by G.M. Brodeur and W. Lutz, respectively. Tunable cell lines, IMR575 MYCN short hairpin RNA (shRNA) and SH-EP MYCN transgene (Tet21N) cells were generated and cultured as described previously. Cell line identity/unique single-nucleotide polymorphism profiles were confirmed by the Multiplexin Multiplex Cell Authentication service as described recently. The purity of cell lines was validated using the Multiplex Cell Contamination Test (Multiplexion) as described recently. No Mycoplasma, squirrel monkey retrovirus or interspecies contamination was detected. To assess the effects of amino acid deprivation, cells were cultivated using modified amino acid-free DMEM powder (PAN-Biotech) supplemented with individual amino acids (Sigma-Aldrich) as indicated, at final concentrations used in standard DMEM. For the 3D culture experiments, we used the hanging drop method. Twenty thousand cells were placed in hanging drop culture and incubated under physiological conditions until they form 3D spheroids.

Analysis of cell viability and proliferation. The impact of various treatments on cellular proliferation/viability was assessed using a sulforhodamine B (SRB) or CellTiter-Blue (Promega Corporation) cell assay. To determine changes in cellular proliferation, approximately 2 × 104 cells seeded per well were cultured for 96 well format for the SRB assay and 96-well format for the CellTiter-Blue assay in full medium. After 24 h, cells were washed with PBS, fed with the chosen medium and treated as indicated. Cell viability was analyzed in full or Cys-, free medium cotreated with 1058 F4 (30 µM, catalog no. F3680; Sigma-Aldrich), Z-VAD-FMK (30 µM, catalog no. sc-3067; Santa Cruz Biotechnology), baflomycin A1 (30 µM, catalog no. sc-201550; Santa Cruz Biotechnology), necrostatin-1 (2 µM, catalog no. N9037; Sigma-Aldrich), Fer-1 (5 µM, catalog no. SML0583; Sigma-Aldrich), Trolox (100 µM, catalog no. 238813; Sigma-Aldrich), aminooxyaceticate (500 µM, Sigma-Aldrich), dimethylformylglycine, N-(methyloxoxoacetyl)-glycine methyl ester (5 mM, Sigma-Aldrich), CPX (1 µM, catalog no. sc-204688; Santa Cruz Biotechnology), D9 (synthesized and provided by Bayer Pharma AG), erastin (Cay17754; Biomol), IGEK (MedChemExpress), 2-mercaptoethanol (50 µM; Sigma-Aldrich), glutamine inhibitors BPTES (catalog no. SML0691; Sigma-Aldrich) and compound 968 (catalog no. sc-204688; Santa Cruz Biotechnology). Protein lysates were extracted with 0.1 ml lysis buffer per well containing 1× Lysis buffer (0.04 ml per well; Thermo Fisher Scientific) and 0.01 µM L-proline. Western blot analysis was performed using methods previously described. Blots were probed with antibodies directed against MYCN (1:1,000 dilution, catalog no. sc-53993; Santa Cruz Biotechnology), c-MYC (1:1,000 dilution, catalog no. ab23072; Abcam), CTbI (1:1,000 dilution, catalog no. ab85473; Abcam), SAAH (A-11) (AHY antibody) (1:1,000 dilution, catalog no. sc-271389; Santa Cruz Biotechnology), GPX4 (1:1,000 dilution, catalog no. ab41787; Abcam), CARS (1:1,000 dilution, catalog no. ab216714; Abcam), glutaminase 1 (1:40,000 dilution, catalog no. ab156876; Abcam), vinculin (1:1,000 dilution, catalog no. sc-73614; Santa Cruz Biotechnology) or housekeeping peroxidase-conjugated anti-Î±-actin (1:5,000, catalog no. ab20272; Abcam). Peroxidase-AffiniPure goat anti-mouse IgG (H+L) (1:1,000 dilution, catalog no. 115-035-033; Dianova) or peroxidase AffiniPure goat anti-rabbit IgG (H+L) (1:1,000 dilution, catalog no. 111-035-144; Dianova) antibodies were used as secondary antibodies. Proteins were visualized using enhanced chemiluminescence detection reagents (GE Healthcare) and a chemiluminescence reader (VILBER). Protein quantification was performed with ImageJ (https://imagej.net).

Flow cytometry. Analysis of intracellular ROS levels and lipid peroxidation. Low MYCN populations were established by incubating cells with 1 µM dox at least 48 h before further treatment. Cells were then fed either with full or cysteine-free medium and cotreated with Fer-1 (5 µM), lipotatin-1 (1-1 µM) catalog no. SML1414; Sigma-Aldrich), CPX (1 µM), Trolox (100 µM) or GSH (2 mM, catalog no. G4251; Sigma-Aldrich) for 20 h. Lipid peroxidation was analyzed with the C11-BODIPY BD FACSaria III cell sorter. Total intracellular ROS levels were determined using CellROX (Thermo Fisher Scientific). Gating strategy is shown in Extended Data Fig. 7.

MYCN synthetic lethal screen. Large-scale drugable genome siRNA screen. For high-throughput screening, a Silencer Select siRNA custom library (catalog no. 4404034; Ambion) was used encompassing 51,242 unpoled sirRNAs targeting 10,414 genes (3 siRNAs per gene). Lipofectamine RNAiMax Transfection Reagent (Thermo Fisher Scientific) only and ON-TARGETPlus Non-targeting siRNA no. 1 (Dharmacon) served as negative transfection controls; PLK1 (Silencer Select siRNA no. 1; Ambion) served as positive control. Liquid reverse transfection was performed in 384-well plates (2,100 cells per well) using a Freedom EVO 200 robotic platform. Two treatment conditions were screened in triplicate: (1) cultured in medium only (IMR5/75 high MYCN); and (2) plus Dox (1 µg/ml) final concentration to induce the shRNA targeting MYCN (IMR5/75 low MYCN). 96h after transfection, cells were fixed with 11% glutaraldehyde and subsequently Hoechst-stained (10 mg/ml stock in 1x PBS, 1,250,000; Invitrogen). The number of Hoechst-positive cell nuclei was determined using an OPERA fluorescence microscope (Thermo Fisher Scientific). In order to ensure the identification of viable cells, they were treated with 0.2% Triton X-100 in a mixture of RNAiMax (0.04 µl per well; Thermo Fisher Scientific) and 0.01 µM per well of siRNA according to the manufacturer’s instructions. siRNA sequences are listed in Supplementary Table 2.
Generation of inducible Cas9 neuroblastoma cell lines. Molecular cloning. The CRISPR–Cas9-mediated inducible knockout experiments were performed using the lentiviral pCW-Cas9-EGFP plasmid. The plasmid was obtained as follows: the lentiviral pCW-Cas9-EGFP plasmid (plasmid no. 50661; Addgene) was subcloned by cutting it with the restriction enzymes HincII and XbaI (New England Biolabs). Next, a gBlock DNA fragment (Integrated DNA Technologies) encoding enhanced green fluorescent protein (eGFP) with complementary overhangs was cloned into the plasmid backbone thus replacing the puromycin resistance gene and generating the pCW-Cas9-EGFP plasmid. Guided by CRISPR knockout experiments were designed with Benchmarking (https://www.benchmarking.com) using the human reference genome GRCh38. Oligonucleotides were ordered (Sigma-Aldrich) with complementary overhangs to the lentiviral delivery plasmid backbone pLKO5.8gRNA.EFS.IRF6F572 and listed in Supplementary Table 2.

Lentivirus production. Lentivirus production was performed using a second-generation lentiviral system and a Calcium Phosphate Transfection Kit (Invitrogen) in HEK 293T cells. Briefly, early passed HEK 293T cells were cotransfected with the lentiviral transfer plasmid, a packaging plasmid (psPAX2; plasmid no. 12260; Addgene), and with a plasmid coding for the VSV-G envelope (pMDG; plasmid no. 12259; Addgene). All experimental procedures for lentivirus production were performed in a biosafety level 2 laboratory. The SK-N-DZ cell line was previously transduced with lentiviral particles carrying the luciferase reporter plX-puro-Luc at a multiplicity of infection (MOI) of 0.3. After recovery, resistant cells were transduced with pCW-Cas9-EGFP lentiviral particles at an MOI of 0.3. Polyclonal cell lines were maintained in DMEM supplemented with 10% tetracycline-free FCS (Clontech Laboratories). Next, cells expressing eGFP and the luciferase reporter were transduced with lentiviral particles carrying the gRNA targeting GPRX4 at an MOI of 0.3. To generate monoclonal CRISPR cell lines, cells were individualized based on eGFP and RFP657 expression using FACS. In vitro Cas9 expression was induced supplementing the culture medium with 1 µg/ml Dox (Sigma-Aldrich).

In vivo orthotopic mouse experiments. All studies involving mice and experimental protocols were conducted in compliance with German Cancer Research Center guidelines and approved by the governmental board of the state of Baden-Württemberg, Karlsruhe District Council, under authorization no. G-176/19, according to German legal regulations. The mouse strains used in the study were NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (stock no. 005557; The Jackson Laboratory). Female mice (3–4 months old) were used for the experiments. Mice enrolled in the study were NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (stock no. 005557; The Jackson Laboratory). Female mice (3–4 months old) were used for the experiments. Mice were housed in individually ventilated cages under temperature and humidity control. Animal treatment) were blinded during the experiments and outcome assessment. Data are presented as the mean ± s.e.m. Statistical analyses were performed using Prism 7.

Quantitative PCR with reverse transcription. Complementary DNA was synthesized using the SuperScript IV Reverse Transcriptase Kit (catalog no. 18090-200; Invitrogen) according to the manufacturer’s instructions. Quantitative PCR with reverse transcription (RT–qPCR) was performed for the genes of interest and two housekeeping genes using the Platinum SYBR Green qPCR Supermix UDG kit (catalog nos. 1173308/11733-046; Invitrogen). Primers are listed in Supplementary Table 2.

Epigenetic characterization of tumors and cell lines. Chromatin immunoprecipitation followed by sequencing analysis of histone modifications in neuroblastoma primary tumors and cell lines. Formaldehyde cross-linking of cells, cell lysis, sonication, chromatin immunoprecipitation and library preparation were performed as described previously7, starting with approximately 4 × 10^6 cells (1 × 10^6 cells per individual immunoprecipitation). Direct cell lysis for each sample was achieved by 30 min incubation on ice in 95 µl radioimmunoprecipitation assay buffer using approximately 30 mg of fresh-frozen tumor tissue per individual chromatin immunoprecipitation followed by sequencing (ChiP–seq) experiment. Library preparation was performed using the NEBNext Ultra DNA Library Prep Kit (New England Biolabs) according to the manufacturer’s protocol. Samples were mixed in equal molar ratios and sequenced on an Illumina sequencing platform. ChiPmentation of MYCN transcription factor in neuroblastoma cell lines. Formaldehyde cross-linking, cell lysis, sonication and chromatin immunoprecipitation were performed as described previously7, adding the ChiPmentation module by Schmedt et al. with the following changes: a Bioruptor Pico Plus with automated cooling (4°C) was used for high-intensity sonication (20–30 min each with 30 s on and 30 s off intervals) and 10 µg MYCN antibody and 100 µl cells for ChiP. The tagmentation reaction (Illumina Nextera DNA library Prep Kit) was performed at 37°C for 1 h with the bead-bound chromatin sample or 5 ng purified input DNA for normalization. After de-cross-linking, purified samples were amplified using the Nextera Index Kit and 1 × 125 PCR cycles. Illumina libraries were purified and pooled. ChiPmentation libraries were sequenced (50 single-end bases) on the Illumina sequencing platform (German Cancer Research Center Core Facility).

Data analysis of ChIP–seq and ChiPmentation. Single-end reads were aligned to the hg19 genome using Bowtie2 v2.3.0, keeping uniquely aligned reads only. The BAM files of aligned reads were further processed using the deepTools suite v3.0 (ref. 8). Input files were subtracted from the treatment files using the bamCompare tool v3.0, applying the simple exponential smoothing method to normalize signal to noise. The resulting signals were normalized to an average 1× coverage to produce signal (bigWig) files. Peaks were called using the MACS v2.1 tool using default parameters. Data are available at the Gene Expression Omnibus (GEO) under accession no. GSE189174.

DNA methylation analysis. DNA methylation and gene expression data from 105 primary neuroblastomas assayed in Infinitum HumanMethylation450 BeadChips and 44K Agilent oligonucleotide microarrays (GEO accession no. GSE73518) were analyzed for candidate loci. The R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl) was used to visualize the expression/methylation of selected gene–CpG pairs.

Tumor proteome analysis. Tumor proteome data were generated as part of a previous study8 and was reanalyzed for this study. Briefly, tumor samples were lysed in SDS, homogenized, split into replicates, reduced, alkylated and purified by Vessel-Flujuge precipitation. Samples were then digested by LysC and trypsin and fractionated by strong cation exchange before being measured by reversed phase liquid chromatography–mass spectrometry (LC–MS) on Q Exactive Plus instruments (Thermo Fisher Scientific). Proteins quantified in less than 50% of MYCN high- or low-risk cases were excluded. Data were imputed by random draw from a normal distribution with default parameters: 0.3 width and 1.8 downshift. A two-sided Welch’s t-test was used to calculate the P values for the differential protein expression analysis using multiple testing correction by the Benjamini–Hochberg method was applied.

Statistics and reproducibility. No statistical method was used to predetermine sample size. No data were explicitly excluded from the analyses unless they were of poor quality as determined by standard sequencing quality control metrics, or experiments were not randomized and the investigators were not blinded to allocation during the experiments and outcome assessment. Data are presented as the mean ± s.e.m. Statistical analyses were performed using Prism 7.
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Author contributions
A.F.E and H.A. found the link between MYCN and ferroptosis. A.F.E., H.A. and F.W. designed the experiments. The metabolic assays and viability studies were conducted and analyzed by H.A.; A.F.E.; L.M.B.; M. Shahkarami, M.L.-P. E.M.W.; J.K.; K.G. and S.K. U.Y.; H.A. and R.W. generated/validated the CBISPR knockout cell lines. H.A., A.F.E and A.T. designed and analyzed the in vivo experiments. H.A. performed the in vivo orthotopic experiments with support from C.K., J.M. and P.Z. H.A. and A.F.E. performed the flow cytometry assays. S.K. performed the MYCN synthetic lethal siRNA screen. M.G. helped with the MYCN synthetic lethal screen optimization. C. Shao, S.A.S., A.F.E., D.J.O., N.I., M. Schlesner and C.J.H. assisted with the bioinformatic analyses. M.G. and E.P. performed and analyzed the ChIP–seq and ChIPmentation experiments. M.N.-H., M.Z., S.A.S. and M. Selbach provided the tumor proteomic data. E.B., M.G., E.M.W. and J.K. conducted the RNA-seq experiments. RT–qPCR was performed by E.M.W. Regulatable cell models were established and colony formation assays performed by J.K. The western blot was performed by S.K., E.M.W. and J.K. G.P.; J.B. and M.B. implemented and analyzed the LC–MS measurements. K.-O.H. contributed to the primary tumor RNA expression and DNA methylation analyses. B.N., C. Stresmann, J.H.R., M.F., I.A. and S.W. contributed reagents, materials and analysis tools. The manuscript was prepared by F.W., T.H., A.T., H.A., A.F.E., L.M.B. and S.K. with support from K.-O.H. H.A., A.F.E., S.K. and L.M.B. contributed equally to the study. All authors approved the final version of the manuscript.

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Competing interests
The authors declare no competing interests.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Cell lines phenotypic data. a, Doubling time calculation from exponential growth curve quantified by FACS and impedance measured using the RCTA xCELLigence system for high-MYC (-Dox) and low-MYC (+Dox) IMR5/75 cells. Cell proliferation curves of exponentially growing cells (n = 3 samples, experiment replicated 3 times). b, Viability quantification using FACS and propidium iodide staining in exponentially growing cells: high MYCN (-Dox) vs. low MYCN (+Dox) (n = 3 samples, experiment replicated 3 times). c, d, Fold changes of intracellular amino acid levels after MYCN inhibition (+Dox or 10058-F4, inhibiting MYCN/MAX binding, 96 h). e, MYC(N) RNA, protein expression (experiment replicated 3 times) and f, MYC(N) activity score for a panel of adrenergic neuroblastoma cell lines. MYC(N) activity score of 32 neuroblastoma (NB) cell lines (CLs) having different MYC(N) genetic status (t = translocation; amp = amplification; act = activated due to unknown mechanism). g, Relative viability of IMR5/75 cells after Cys2 deprivation in the presence or absence of Inhibitors of apoptosis (z-VAD FMK, n = 5 samples), autophagy (Bafilomycin A1, Baf-A1, n = 4 samples,) and necroptosis (RIPK1 inhibitor, Necrostatin-1, Nec-1, n = 3 samples) and h, Trolox, or intracellular iron chelator ciclopirox olamine (CPX); n = 3 samples, experiment replicated 3 times. i, Relative viability of neuroblastoma cell lines after Cys2 deprivation in the presence or absence of ferrostatin-1 (Fer-1) or glutathione (GSH); n = 4 samples for SK-N-AS, NB69 and SH-SY5Y cell lines and n = 6 samples for GI-ME-N cell line; experiment replicated 3 times. j, k, Total GSH and cysteine (Cys) levels in neuroblastoma cells with amplified MYCN, MYC(N) translocations or normal MYC(N) status; Wilcoxon rank-sum test; center line indicates the median value, lower and upper hinges represent the 25th and 75th percentiles, respectively, and whiskers denote 1.5x interquartile range. Each dot corresponds to one sample; p-values as indicated.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Additional screen targets and expression data. a, MYCN synthetic lethal screen hits. MYCN effects on the two-step biosynthesis of GSH and GSH metabolism (*top MYCN synthetic lethal hits of GSH metabolism, false discovery rate of 0.2) (median of 2-3 siRNAs). b, Western Blot and quantification of siRNA-mediated GPX4 knockdown (96 h) in Tet21N cells with doxycycline-regulatable MYCN (experiment replicated 3 times). c, Relative viability (survival of compound-treated cells divided by survival of vehicle-treated cells) of IMR5/75 cell line after treatment with RSL-3 in the presence or absence of MYC inhibitor (10058-F4) (n = 4 samples, experiment replicated 3 times) and d, Response to GPX4 inhibitors (RSL-3, ML-210) (CTRPv2 database55); H = high and L = low MYC(N) activity; Wilcoxon rank-sum test. e, Sensitivity to RSL-3-induced ferroptosis in MYCN-amplified neuroblastoma cell lines (n = 4 samples, experiment replicated 3 times). f, MYC(N) activity correlation with GCLC or GSR dependency in nine neuroblastoma cell lines (DRIVE database55). g, Low expression of SLC7A11 and high expression of CBS associated with GPX4 dependency in 384 cancer cell lines (DRIVE database55). TXNRD1 expression dependent on GPX4 knockdown sensitivity in 384 cancer cell lines (Wilcoxon rank-sum test). h, GPX4 dependency in 348 cancer cell lines grouped according to cancer entities (DRIVE database55). i, Relative viability of NB69, SH-SY5Y and SK-N-DZ cell lines after treatment with erastin. n = 3 samples, experiment replicated 3 times. j, l, m, Expression of SLC7A11 or CBS in cell lines (CCLE database, n = 91755) or tissues (R2 database56). Box plots: center line indicates the median value, lower and upper hinges represent the 25th and 75th percentiles, respectively, and whiskers denote 1.5× interquartile range. Each dot corresponds to one sample; Wilcoxon rank-sum test; p-values as indicated.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Quantification of transsulfuration activity. a, Western Blot and quantification of CTH or AHCY protein expression upon siRNA-mediated knockdown in IMR5/75 high MYCN or low MYCN cells. Experiment replicated 3 times. b, Two methyltransferases that may indirectly increase Hcy levels are synthetic lethal with high MYCN (MYCN siRNA screen; *false discovery rate of 0.2); center line indicates the median value of 2-3 siRNAs, lower and upper hinges represent the 25th and 75th percentiles, respectively, and whiskers denote 1.5× interquartile range. c, Relative viability (survival of compound-treated cells divided by survival of vehicle-treated cells) of adrenergic and mesenchymal neuroblastoma cell lines after Cys2 deprivation in the presence or absence of Cysta or Hcy. n = 4 samples, experiment replicated 3 times. d, Impact of protein synthesis inhibition by cycloheximide (CHX) on cysteine (n = 5 samples) and glutathione level (n = 6 samples and CHX, 100 ng/ml, 24 h and experiment replicated 3 times). e, Cell cycle analysis of MYCN-amplified IMR5/75 cells (24 h) after Cys2 depletion (representative measurement of 3 replicates). f, Analysis of protein synthesis in MYCN-amplified IMR5/75 cells (24 h) after low levels or complete depletion of Cys2, methionine (Met) or glutamine (Gln) in the culture medium (n = 3 samples, experiment replicated 3 times). g, MYCN protein expression (by western blot) upon MYCN-MAX inhibition (10058-F4), protein synthesis inhibition by cycloheximide (CHX, 100 ng/ml) and low levels or complete depletion of Cys2, methionine (Met) or glutamine (Gln) in the culture medium after 8 h. Experiment replicated 3 times. h, i, j, RNA-seq and RT-qPCR of IMR5/75 cells 24 h following Cys, Gln depletion and Glu excess or erastin treatment and regulation of ferroptosis-related genes (n = 6 samples in h, n = 3 samples in i; experiment replicated 3 times). Statistical analysis was performed using Student’s two-tailed t-test.
Extended Data Fig. 4 | Expression profiles of neuroblastoma cell lines. a, Time-resolved gene expression profiles during cell cycle progression and cell cycle analysis distinguish pervasive MYCN functions from indirect effects related to cell proliferation rate in high MYCN and low MYCN IMR5/75 cells: MYCN gene expression profile reveals two cell cycle-related peaks (P1 before G1-S transition and P2 before S-G2/M). b, Fold change (log2) of gene expression in mesenchymal TET21N (SH-EP-ectMYCN) cells harboring an inducible MYCN transgene upon MYCN induction. TET21N and parental SH-EP cells have low CBS expression and inactive transsulfuration. MYCN induction has a significant effect on AHCY, GCLC, GSR, SLC7A11 and TXNRD1 but not on CBS expression; experiment replicated 3 times. c, Expression of transsulfuration, amino acid uptake/metabolism and glutathione biosynthesis/metabolism/degradation genes in 32 neuroblastoma cell lines (RNA-seq): MYCN-amplified (amp) vs. MYCN/MYC-translocated/activated (t/a) cell lines, MYC(N)-non-expressors (n) (Wilcoxon rank-sum test; NS = not significant). Center line indicates the median value, lower and upper hinges represent the 25th and 75th percentiles, respectively, and whiskers denote 1.5× interquartile range. Each dot corresponds to one sample; p-values as indicated.
Extended Data Fig. 5 | ChIP-seq data for histone modifications. RNA-seq normalized reads and input normalized read counts of ChIP-seq experiments for histone modifications at the CBS gene locus in adrenergic MYCN-amplified IMR5/75, MYCN-translocated NBL-S, and MYC-translocated NB69 cells with high CBS expression and active transsulfuration, mesenchymal MYCN-normal diploid SH-EP, GI-M-EN, and SK-N-AS cells with elevated MYC expression due to translocations or unknown mechanism (GI-M-EN) and very low/absent CBS expression and inactive transsulfuration.
Extended Data Fig. 6 | Methylation profiles and CBS expression in tumor samples. Genomic position of CBS-annotated CpGs whose methylation is significantly associated with CBS expression and patient risk (p < 0.01, Wilcoxon rank-sum statistics and p < 0.05, Fisher exact test). DNA methylation assessed by Infinium Human Methylation 450 BeadChips, and CBS expression assessed by 44k customized Agilent oligonucleotide microarrays in 105 primary neuroblastomas (GEO accession GSE73518\(^\text{\textregistered}\)). For all CBS CpGs whose methylation significantly correlated with CBS expression and patient risk, hypomethylation was associated with CBS upregulation and high-risk disease. R2 Genomics Analysis and Visualization Platform (http://r2.amc.nl) was used for data visualization.
Extended Data Fig. 7 | Gating strategy for flow cytometry data. For all samples, an initial manual gate in SSC-A by FSC-A was set to identify live cells and exclude debris. From the live cells, a rectangular gate was set on FSC-H by FSC-A to exclude doublets, meaning cells off the diagonal. If DNA staining was carried out, an additional gate was set on DNA (Violet channel)-W by DNA-A to exclude additional cell debris or DNA doublets.
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a  Confirmed

☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

☐ The statistical test(s) used AND whether they are one- or two-sided

Only common tests should be described solely by name; describe more complex techniques in the Methods section.

☐ A description of all covariates tested

☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons

☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

Give P values as exact values whenever suitable.

☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐ Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection  NA

Data analysis

Software used in the study:
R (version 3.4.0)
Image J (version 1.51)
FlowsJo version 10.6.0
Microarray analysis (as described in Klaus & Reisenauer, F1000Res 2016)
Bioconductor - limma (version 3.46)
BHC in-house software (developed and owned by BAYER Health care, intellectual property)

RNAseq:
STAR (version 2.5.3a)
R (version 4.3.0 & 3.4.3)
R - edgeR (version 3.20.9)
R - golists (version 3.1.1)

ChIPseq:
trimgalore (version 0.4.3)
bowtie2 (version 2.3)
deeptools (version 3.0)
macs2 (version 2.1)
Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

All information about data availability is provided in the data availability statement of the article:

Proteome data of neuroblastoma tumors was previously published by [Hartlieb, S. A. et al. Nat Commun 2021] and all data is deposited at the European Genome-Phenome Archive (EGA) as dataset EGAD00001006737 as part of the study EGAS00001004349. Data is available upon request by contacting Frank Westermann. RNA seq data of 498 primary neuroblastoma patients was previously published [Lutjé, W. et al. 1996] and is available at Gene Expression Omnibus (GEO) under the accession number GSE62564. DNA methylation data of primary neuroblastoma tumors was previously published [Cassago, A. et al. 2012] and is available at GEO under the accession number GSE73518. Time-course RNAseq profiling of IMR5/75 MYCN-high and MYCN-low cells was previously published [Muth, D. et al. 2010].

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [ ] Life sciences
- [ ] Behavioural & social sciences
- [ ] Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size: For in vivo studies, sample size was calculated with the help of a biostatistician using R version 3.4.0. Assumptions for power analysis were as follows: α error, 5%; β error, 20%. Values for standard deviations and differences between experimental groups were based on previous experiments (whenever a similar data type was available); for in vitro studies we used at least 3 biological replicates for each experiments.

Data exclusions: no data were excluded

Replication: in vitro experiments were repeated at least three times with similar results.

Randomization: For in vivo experiments mice were randomized into treatment groups prior to treatment. In case animals had to be sacrificed before the pre-defined endpoint (due to weight loss or other termination criteria), they were excluded from any downstream analyses. For the in vitro experiments, all samples were analyzed equally with no subsampling; therefore, there was no requirement for randomization

Blinding: For in vivo experiments all animal experiments [except during animal treatment] were blinded during entire experiments or follow up assessment. For in vitro studies, data collection and analysis were not performed blinded to the conditions of the experiments.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.
Materials & experimental systems

Methods

Antibodies

- MYCN (clone B8.4.8, sc-53993, Santa Cruz, Lot. B2316, 1:1000)
- c-MYC (clone Y69, ab32072, Abcam, Lot. 625369, 1:1000)
- CTH (ab54573, Abcam, Lot. GR3260298-2, 1:1000)
- SAHH [A-11] (AHCC antibody, clone A-11, sc-271389, Santa Cruz, Lot. C2111, 1:1000),
- GPX4 (ab41787, Abcam, Lot. GR56784-1, 1:1000),
- CAIX (clone EP7212, ab126714, Abcam, Lot. Y0818190S, 1:1000)
- Glutaminase I (clone EP7212, ab156876, Abcam, Lot. GR249636-29, 1:40,000)
- Loading control: vinculin (clone 746, sc-73614, Santa Cruz, Lot. A2319, 1:1000) or β-actin-conjugated (ab20272, Abcam, Lot. GR3418697-2, 1:5000)
- Secondary Ab: HRP–peroxidase labeled anti-mouse (115-035-003, Dianova, 1:1000) or anti-rabbit (111-035-144, Dianova, 1:1000)

- ChIP-seq: For all ChIP-seq experiments 3ug of antibody were used per ChIP.
- H3K27me3; rabbit polyclonal; Active Motif 39155; Lot. 31014017
- H3K36me3; rabbit polyclonal; Abcam ab9050; Lot. GR723250-1
- H3K9me3; rabbit polyclonal; Abcam; ab8898; Lot. GR148830-2
- H3K27ac; rabbit polyclonal; Abcam; ab4729; Lot. GR183919-2

Validation

All antibodies used in this study were validated by manufactures for the species and specific application. Relevant validation results can be found in the website of each manufacture. A protein size marker was run on every western blot and the size of the assessed bands was compared to the manufactures information (See source data)

Eukaryotic cell lines

Policy information about cell lines

- Cell line source(s)
  - Human neuroblastoma cell lines: IMR5/75, KELLY, SiMa, NBL-S, SK-N-FI, SH-SYSY, NB69, SKNDZ, SH-EP, GI-ME-N.
  - SK-N-FI, SK-N-DZ, SH-SYSY cells were purchased from ATCC.
  - KELLY, SiMa and GI-ME-N were purchased from DSMZ.
  - NB69 were kindly provided by Larissa Savelyeva
  - NBL-S and TET21N (SH-EP) were provided by G.M. Brodeur and W. Lutz, respectively.
  - Tunable cell lines, IMR5/75 MYCN shRNA, and SH-EP MYCN transgene (Tet21N) were generated and cultured as described previously (please see Methods section)

- Authentication
  - Cell line identity/unique SNP profiles were confirmed by the Multiplexion Multiplex Cell Authentication service (Heidelberg, Germany). The purity of cell lines was validated using the Multiplex cell Contamination Test by Multiplexion (Heidelberg, Germany).

- Mycoplasma contamination
  - All cell lines tested negative for mycoplasma contamination as noted in ‘Materials and Methods’

Animals and other organisms

Policy information about studies involving animals, ARRIVE guidelines recommended for reporting animal research

- Laboratory animals
  - Mouse strains used in the study: NOD.Cg-PkdcsclidI2rgtm1Wjl/SzJ (NSG, JAX stock #005557). Female mice, 3 – 4 months of age, were used for experiments. Mice were housed in individually ventilated cages under temperature and humidity control. Cages contained an enriched environment with bedding material.
Wild animals

No wild animals were used in the study.

Field-collected samples

No wild animals were used in the study.

Ethics oversight

All studies involving mice and experimental protocols were conducted in compliance with German Cancer Center Institute guidelines and approved by the governmental review board of the state of Baden-Württemberg, Regierungspräsidium Karlsruhe, under the authorization number G-176/19, followed the German legal regulations. Animals' health were monitored daily and mice were euthanized as soon as they reached abortion criteria defined in the procedure.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics

Tumor samples are from patients enrolled in the German Neuroblastoma Trials of the GPOH (NB97, NB2004, NB2016). Detailed patient specific information (Age, Status, Gender etc) is provided in the previous publication by Gartiguber et al, Nature Cancer 2020.

Recruitment

Almost all childhood neuroblastoma in Germany (>99%) are enrolled in a clinical trial, informed consent is given for the use of tumor material for research purposes.

Ethics oversight

All neuroblastoma patients were enrolled in the German Neuroblastoma Trial (NB97, NB2004, NB 2016) approved by the Ethics Committee of the University of Cologne and informed written consent was obtained from the patients' parents.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration

not applicable

Study protocol

not applicable

Data collection

not applicable

Outcomes

not applicable

Dual use research of concern

Policy information about dual use research of concern

Hazards

Could the accidental, deliberate or reckless misuse of agents or technologies generated in the work, or the application of information presented in the manuscript, pose a threat to:

No ❏ Yes ❏

Public health

National security

Crops and/or livestock

Ecosystems

Any other significant area
Experiments of concern

Does the work involve any of these experiments of concern:

| No | Yes |
|----|-----|
| ☒  | ☐   |
|   | Demonstrate how to render a vaccine ineffective |
|   | Confer resistance to therapeutically useful antibiotics or antiviral agents |
|   | Enhance the virulence of a pathogen or render a nonpathogen virulent |
|   | Increase transmissibility of a pathogen |
|   | Alter the host range of a pathogen |
|   | Enable evasion of diagnostic/detection modalities |
|   | Enable the weaponization of a biological agent or toxin |
|   | Any other potentially harmful combination of experiments and agents |

ChIP-seq

Data deposition

☒ Confirm that both raw and final processed data have been deposited in a public database such as GEO.

☒ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links

ChIP-seq data has been uploaded to GEO under the accessions GSE189174.

Files in database submission

| GSM5695680 | GI-ME-N H3K27ac |
| GSM5695681 | GI-ME-N H3K27me3 |
| GSM5695682 | GI-ME-N H3K36me3 |
| GSM5695683 | GI-ME-N H3K4me1 |
| GSM5695684 | GI-ME-N H3K4me3 |
| GSM5695685 | GI-ME-N H3K9me3 |
| GSM5695686 | GI-ME-N input DNA |
| GSM5695687 | IMR575 H3K27ac |
| GSM5695688 | IMR575 H3K27me3 |
| GSM5695689 | IMR575 H3K36me3 |
| GSM5695690 | IMR575 H3K4me1 |
| GSM5695691 | IMR575 H3K4me3 |
| GSM5695692 | IMR575 H3K9me3 |
| GSM5695693 | IMR575 input DNA |
| GSM5695694 | Kelly H3K27ac |
| GSM5695695 | Kelly H3K27me3 |
| GSM5695696 | Kelly H3K36me3 |
| GSM5695697 | Kelly H3K4me1 |
| GSM5695698 | Kelly H3K4me3 |
| GSM5695699 | Kelly H3K9me3 |
| GSM5695700 | Kelly input DNA |
| GSM5695701 | NB69 H3K27ac |
| GSM5695702 | NB69 H3K27me3 |
| GSM5695703 | NB69 H3K36me3 |
| GSM5695704 | NB69 H3K4me1 |
| GSM5695705 | NB69 H3K4me3 |
| GSM5695706 | NB69 H3K9me3 |
| GSM5695707 | NB69 input DNA |
| GSM5695708 | NBL-S H3K27ac |
| GSM5695709 | NBL-S H3K27me3 |
| GSM5695710 | NBL-S H3K36me3 |
| GSM5695711 | NBL-S H3K4me1 |
| GSM5695712 | NBL-S H3K4me3 |
| GSM5695713 | NBL-S H3K9me3 |
| GSM5695714 | NBL-S input DNA |
| GSM5695715 | SH-EP H3K27ac |
| GSM5695716 | SH-EP H3K27me3 |
| GSM5695717 | SH-EP H3K36me3 |
| GSM5695718 | SH-EP H3K4me1 |
| GSM5695719 | SH-EP H3K4me3 |
| GSM5695720 | SH-EP H3K9me3 |
| GSM5695721 | SH-EP input DNA |
| GSM5695722 | SK-N-FI H3K27ac |
| GSM5695723 | SK-N-FI H3K27me3 |
| GSM5695724 | SK-N-FI H3K36me3 |
| Genome browser session (e.g. UCSC) | No longer applicable. |
|-----------------------------------|----------------------|
| Replicates                         | For each tumor and cell line one biological replicate was done due to the limited amount of material. |
| Sequencing depth                   | ChIP sequencing was done using Illumina HSeq2000 50 bp single end sequencing. |
| Antibodies                         | H3K27me3; rabbit polyclonal; Active Motif 39155; Lot.31014017  
| H3K36me3; rabbit polyclonal; Abcam ab9050; Lot.GR273250-1  
| H3K9me3; rabbit polyclonal; Abcam; ab8898; Lot.GR148830-2  
| H3K27ac; rabbit polyclonal; Abcam; ab4729; Lot.GR183919-2 |
| Peak calling parameters            | Peak calling was performed with the MACS2 algorithm, both with the narrowPeak and broadPeak parameters; the FDR cutoff for peak calling was set to 1%. |
| Data quality                       | Only uniquely aligned reads are considered, and duplicates are removed.  
| Data quality                       | Data quality was ensured both by visual inspection of reference region in the genome browser. Moreover, we assessed the enrichment signal using the fingerprint method developed as part of the DeepTools package (Diaz et al.), and using criteria recommended by the ENCODE consortium such as the PCR bottleneck coefficient and the FRIP (fraction of reads in peaks). |
| Software                           | The ChIP-seq processing was performed using a custom pipeline written in Snakemake; the steps involve (1) read trimming using TrimGalore, (2) alignment using Bowtie2 on the hg19 genome with standard parameters, (3) merger of replicates if available, (4) peak calling using MACS2, (5) QC using the fingerprint and FRIP method, (6) SE5 normalization and bigwig generation by subtracting normalized input from IP. |

**Flow Cytometry**

**Plots**

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

**Methodology**

**Sample preparation**

MYCN-low populations were established by incubating cells with 1 μg/ml doxycycline at least 48 h prior to further treatment. Cells were harvested and lipid peroxidation was analyzed, using C11-BODIPY BD of 4 μM final concentration in Hanks’ Balanced Salt Solution (HBSS). Cells were incubated at 37°C for 15 min and signal intensity was measured. Total intracellular ROS levels were determined using CellROX+ (according to Thermo Fisher Scientific instruction).

**Instrument**

MACSQuant VYB, model number: 130-096-116, SN:3050  
BD FACSAria™ cell sorter IIu, model number: 355119, SN: P0087

**Software**

FlowJo software version 10.6.0 (commercial standard software for analysis of flow cytometric data).

**Cell population abundance**

In this manuscript, only one pure population at the time was analyzed (neuroblastoma cell lines).

**Gating strategy**

For all samples, an initial manual gate in SSC-A by FSC-A was set to identify live cells and exclude debris. From the live cells, a rectangular gate was set on FSC-H by FSC-A to exclude doublets, meaning cells off the diagonal. If DNA staining was carried out, an additional gate was set on DNA [Violet channel] W by DNA-A to exclude additional cell debris or DNA doublets (Extended Data Figure 7))

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.