A NOTCH feed-forward loop drives reprogramming from adrenergic to mesenchymal state in neuroblastoma

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Transition between differentiation states in development occurs swift but the mechanisms leading to epigenetic and transcriptional reprogramming are poorly understood. The pediatric cancer neuroblastoma includes adrenergic (ADRN) and mesenchymal (MES) tumor cell types, which differ in phenotype, super-enhancers (SEs) and core regulatory circuitries. These cell types can spontaneously interconvert, but the mechanism remains largely unknown. Here, we unravel how a NOTCH3 intracellular domain reprogrammed the ADRN transcriptional landscape towards a MES state. A transcriptional feed-forward circuitry of NOTCH-family transcription factors amplifies the NOTCH signaling levels, explaining the swift transition between two semi-stable cellular states. This transition induces genome-wide remodeling of the H3K27ac landscape and a switch from ADRN SEs to MES SEs. Once established, the NOTCH feed-forward loop maintains the induced MES state. In vivo reprogramming of ADRN cells shows that MES and ADRN cells are equally oncogenic. Our results elucidate a swift transdifferentiation between two semi-stable epigenetic cellular states.
Development of the embryonic neuroepithelium generates a variety of specialized cell types from pluripotent stem cells. Experimental models of induced pluripotent stem cells (iPSCs) or direct conversion of lineage-committed cells have provided a wealth of information on signaling molecules, gene transcription, and chromatin states that underlie the reprogramming of cellular fate. Lineage transdifferentiation is also observed in malignant cells.

An increasing number of human cancers appears to consist of phenotypically divergent tumor cell types, which recapitulate stages of normal development. We and others recently showed that neuroblastoma is composed of two cell types that reflect developmental stages of the adrenergic lineage. These cell types resemble neural crest-derived precursor cells, while adrenergic-type (ADRN) cells are committed to the adrenergic lineage. Both cell types can spontaneously interconvert, providing neuroblastoma with a high transcriptional plasticity. Chemotherapy might select for the MES cell type, as suggested by enrichment of these cells in post-treatment samples and in relapses. Glioblastoma, melanoma, and oligodendroglioma include heterogeneous populations of tumor cells. Both in glioblastoma and neuroblastoma, the more undifferentiated cell types have mesenchymal phenotypes and are more drug resistant, supporting the concept that lineage fate decisions are important drivers of therapy resistance in cancer.

The distinct cell populations detected in glioblastoma and neuroblastoma have unique enhancer and super-enhancer (SE) landscapes. These SEs are associated with expression of lineage transcription factors (TFs) that constitute the Core Regulatory Circuitry (CRC) for each cell type. This core set of SE associated TFs were postulated to impose lineage identity. These TFs bind to their own SE and to SEs of the other CRC TFs. This creates a strong feed-forward loop driving high levels of CRC TF expression and thereby impose lineage identity. In neuroblastoma, we identified a MES CRC of 20 TFs and an ADRN CRC of 18 TFs. Several ADRN TFs are indeed proven to bind each other’s SEs. Overexpression of PRRX1, a MES-specific CRC TF, was found to reprogram the transcriptional- and epigenetic landscapes of ADRN cells towards a MES state. This shows that CRC TFs are potent inducers of lineage identity.

The CRC of MES cells included NOTCH2 and MAML2 that are transcriptional activators of the NOTCH pathway. The NOTCH signaling cascade is an evolutionarily conserved cell to cell signaling pathway that imposes cell identity switches during development and can induce a motile phenotype in neuroblastoma cells. Ligands of the Delta-like (Dil) or Jagged families activate full-length NOTCH receptors on neighboring cells, resulting in proteolytic cleavage of NOTCH and generation of an intracellular NOTCH-IC domain. NOTCH-IC translocates to the nucleus, where it forms a transcriptional complex with a mastermind-like (MAML) co-activator and the DNA-binding protein CSL. This complex regulates expression of Notch target genes, including lineage-specific TFs to instruct cell fate decisions.

Here, we investigate how a robust cancer cell type can undergo a fast and nearly complete transdifferentiation to an alternative cellular state. Expression of an inducible NOTCH-IC transgene activates an endogenous feed-forward loop among NOTCH receptors and results in transcriptional and epigenetic reprogramming of ADRN cells to a MES state. Our findings reveal how a semi-stable cancer cell type is susceptible to reprogramming by a feed-forward cascade of core lineage TFs.

**Results**

**The CRC of MES cells includes NOTCH pathway genes.** The MES CRC consists of 20 TFs, including NOTCH2 and MAML2. ChIP-sequencing analysis of H3K27ac revealed SEs within the gene bodies of NOTCH2 and MAML2 that were specific for MES-type neuroblastoma cells (Fig. 1a). The same super-enhancers of NOTCH2 and MAML2 were observed in neural crest cells, corroborating the idea that MES-type neuroblastoma cells are neural crest-like (Fig. 1a). The SEs were associated with strong transcription of NOTCH2 and MAML2 mRNA. In addition, we observed MES-specific expression of NOTCH1 and NOTCH3 receptors as well as the NOTCH target gene HES1 (Fig. 1b).

NOTCH1, NOTCH3, and HES1 lack a MES-specific SE. The non-canonical inhibitory ligand DKK1 was associated with an ADRN SE and showed ADRN-specific expression (Fig. 1b and see Methods). Neither of the NOTCH ligands JAG1, JAG2, DLL1, DLL3 and Dlk1 were associated with a cell-type specific SE. There was no MES- or ADRN-specific expression pattern of these ligands. We analyzed isogenic MES and ADRN cell line pairs that were derived from the same patient for NOTCH signaling activity. Activation of NOTCH receptors requires sequential proteolytic cleavages. Immunoblot analysis of membrane- and nuclear fraction from MES and ADRN cells revealed full-length and transmembrane NOTCH proteins in the membrane fraction and NOTCH-IC proteins in the nucleus of MES cells (Fig. 1c), indicating ligand-induced activation of NOTCH. In addition, MES cells had higher protein levels of NOTCH2, MAML2, and HES1 (Fig. 1c). We conclude that MES-specific SEs are associated with NOTCH2 and MAML2 and that mesenchymal neuroblastoma cells have transcriptional activation and signaling of the NOTCH pathway.

**NOTCH induces mesenchymal reprogramming of ADRN cells.** Core Regulatory Circuitries of lineage TFs are proposed as master regulators of cell fate decisions in development and cancer. The inclusion of NOTCH in the MES CRC urged us to test the reprogramming potential of NOTCH paralogues in the transdifferentiation of ADRN to MES lineage identity. ADRN-type SH-SY5Y cells were equipped with inducible expression of either NOTCH1-IC, NOTCH2-ICFLAG or NOTCH3-IC paralogs (Fig. 2a, see Methods). All NOTCH-IC transgenes induced expression of HES1, confirming activation of the NOTCH pathway. Expression of each NOTCH paralog induced the MES marker proteins FN1 and SNAI2, while protein levels of ADRN markers PHOX2A, PHOX2B, and DBH were decreased (Fig. 2a). Since MES-type neuroblastoma cells are highly migratory, we assessed cell motility and observed that each NOTCH-IC paralog increased migration in transwell migration assays (Fig. 2b). Thus, each of the NOTCH1-IC, NOTCH2-IC, and NOTCH3-IC paralogs induces a MES-like transition of ADRN cells.

We selected NOTCH3-IC for further in-depth analysis as it showed the strongest repression of ADRN lineage markers (Fig. 2a). In two additional ADRN neuroblastoma cell lines, we confirmed that NOTCH3-IC induced a MES marker profile and a migratory phenotype (Supplementary Fig. 1a–c). To test the kinetics of NOTCH3-IC-induced mesenchymal reprogramming, we performed a time course gene expression analysis of up to 21 days of NOTCH3-IC induction. RNA was harvested at time points of 1, 7, 14, and 21 days of NOTCH3-IC induction and analyzed by Affymetrix gene expression profiling. We calculated scores for MES and ADRN signatures (see ref. 1 and Methods for calculation of MES and ADRN signature scores) to follow the cell fate of SH-SY5Y cells with NOTCH3-IC expression. After 1 day of NOTCH3-IC expression cells had markedly reduced the ADRN score, but still grouped with a panel of ADRN cell lines (Fig. 3a). After 7 days the cells had achieved a strong MES score, maintained a reduced ADRN score and grouped with a panel of MES-type cells. Thus ADRN-type SH-SY5Y cells undergo a...
step-wise transition to an induced MES state after 7 days of NOTCH3-IC expression (Fig. 3a and Supplementary Data 1). Principal Component Analysis of these cell lines using all expressed genes instead of MES and ADRN signatures, showed that principal components 1 and 2 resolved the pattern of MES expressed genes instead of MES and ADRN signatures, showed that principal components 1 and 2 resolved the pattern of MES expressed genes instead of MES and ADRN signatures, showed that principal components 1 and 2 resolved the pattern of MES expressed genes instead of MES and ADRN signatures, showed that principal components 1 and 2 resolved the pattern of MES expressed genes instead of MES and ADRN signatures, showed that principal components 1 and 2 resolved the pattern of MES expressed genes instead of MES and ADRN signatures, showed that principal components 1 and 2 resolved the pattern of MES expressed genes instead of MES and ADRN signatures, showed that principal components 1 and 2 resolved the pattern of MES expressed genes instead of MES and ADRN signatures, showed that principal components 1 and 2 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NOTCH3-IC. These results indicated that a NOTCH3-IC transgene driven induction of full-length NOTCH3 as well as NOTCH1 and NOTCH2 receptors appeared to be induced by the NOTCH3-IC transgene within 24 h (Fig. 5a). In addition, NOTCH3-IC induced transcription of the NOTCH-ligand JAG1, the co-factor MAML2 and the NOTCH target gene HES1 (Fig. 5a). We tested whether the induced NOTCH receptors were activated. Western blot analysis confirmed induction of full-length NOTCH1 and NOTCH3 proteins by NOTCH3-IC (Fig. 3c). Ligand-induced activation of full-length NOTCH receptors results in transmembrane- and intra-cellular domain proteins of lower molecular weight. We observed increased levels of transmembrane protein epitopes of NOTCH1 and NOTCH3 receptors as well as NOTCH2-IC proteins, indicating that NOTCH receptors were activated by ligands and processed to signaling proteins (Fig. 3c). This activation of NOTCH receptors was accompanied by an induction of JAG1, MAML2, and HES1 (Fig. 3c), the latter confirming the transcriptional activity of NOTCH3-IC. These results indicated that a NOTCH3-IC transgene can start a positive feed-forward loop in the NOTCH

**Fig. 2** NOTCH paralogues induce a mesenchymal phenotype in ADRN neuroblastoma cells. a Western blot analysis of cell lysates of SH-SY5Y with inducible overexpression of NOTCH1-IC, NOTCH2-IC\(^\text{FLAG}\) or NOTCH3-IC. Immunoblots show NOTCH1-IC, NOTCH2-IC\(^\text{FLAG}\) or NOTCH3 proteins as well as protein of the known NOTCH target gene HES1 to confirm induction of each transgene. Lysates are analyzed for MES-markers (FN1 and SNAI2) and ADRN-markers (PHOX2A, PHOX2B, and DBH). \(\beta\)-actin was used as loading control. IC intracellular domain, TM transmembrane domain, dox doxycycline. Source data are provided as a Source Data file. b Transwell migration assay of SH-SY5Y cells with inducible overexpression of NOTCH1-IC, NOTCH2-IC\(^\text{FLAG}\) or NOTCH3-IC. Cells were allowed to migrate for 48 h. dox, doxycycline. Box plots show the number of migrated cells per High-Power Field (HPF). Whiskers denote the interval within 1.5 times the interquartile range (box edges) of the median (center line). Two-sided Student’s \(t\)-test assuming equal variance was used to calculate statistical difference, *** \(p < 0.001\). Source data are provided as a Source Data file
Fig. 3 NOTCH3-IC is a master regulator of ADRN-to-MES reprogramming. a Analysis of mRNA signature scores for MES and ADRN cells in a panel of neuroblastoma cell lines and a time series of NOTCH3-IC induction. Control (-dox) SH-SYSY-NOTCH3-IC cells are depicted in blue; SH-SYSY cells with induction of NOTCH3-IC (+dox) are shown in red. Time of NOTCH3-IC induction is indicated in days. Dashed circles group cell lines of MES- or ADRN-type as previously determined1. b Z-score mRNA expression of core super-enhancer associated transcription factors specific for ADRN or MES cells. Time of NOTCH3-IC induction (in days) is indicated above. dox, doxycycline. c Western blot analysis showing proteins of ADRN-markers (PHOX2A, PHOX2B, DBH, and TFAP2B), MES-markers (FN1, SNAI2, YAP1, and SOX9) and members of the NOTCH pathway (JAG1, NOTCH1, NOTCH2, NOTCH3, MAML2, and HES1) in SH-SYSY with inducible overexpression of NOTCH3-IC. Time of NOTCH3-IC induction is indicated above the western blots. Protein lysates from 691-MES and 691-ADRN cells are loaded on the same blot as a reference for MES and ADRN states, respectively. β-actin was used as loading control. dox, doxycycline. Source data are provided as a Source Data file.

We examined the epigenetic mechanisms of this transcriptional feed-forward. Analysis of H3K27ac revealed that de novo SEs were established at NOTCH2 and MAML2 genes, while the promoter regions of JAG1, NOTCH1, NOTCH3, and HES1 became marked by H3K27ac after induction of NOTCH3-IC (Fig. 4c). Thus, a single reprogramming factor induces a transcriptional feed-forward cascade of NOTCH from endogenous loci that is associated with epigenetic remodeling.

To test whether this transcriptional amplification is a general property of NOTCH paralogues, we overexpressed NOTCH1-IC, NOTCH2-ICFLAG or NOTCH3-IC in SH-SYSY cells and analyzed the endogenous transcriptional regulation of each of these NOTCH paralogues by qRT-PCR (see Methods). Each transgenic NOTCH-IC paralogue induced expression of the corresponding endogenous full-length NOTCH receptor, showing an autoregulatory feed-forward loop (Supplementary Fig. 4). Furthermore, each NOTCH-IC transgene induced transcription of the paralogous NOTCH genes from endogenous loci (Supplementary Fig. 4), starting a transcriptional feed-forward cascade among NOTCH receptor paralogues. In addition, JAG1 and MAML2 were induced (Supplementary Fig. 4). The full-length NOTCH receptors were processed to NOTCH transmembrane proteins of lower molecular weight, indicative of their activation (Fig. 5b). To test whether this NOTCH feed-forward cascade is required for ADRN-to-MES reprogramming, we applied the gamma-secretase inhibitor RO4929097 to SH-SYSY cells during NOTCH3-IC induction. RO4929097 attenuated formation of NOTCH1-IC and diminished induction of HES1. Western blot analysis for a selected set of marker proteins showed that treatment with RO4929097 attenuated the induction of MES markers and prevented silencing of ADRN markers (Fig. 5c). Gene expression profiling revealed that RO4929097 prevented the ADRN-to-MES transdifferentiation induced by NOTCH3-IC (Fig. 5d). We conclude that a reciprocal feed-forward loop generates a NOTCH cascade with high signaling levels that is required for mesenchymal reprogramming.

Transient NOTCH3-IC induces commitment to a MES lineage. The generation of a strong feed-forward loop implies that a temporary activation of NOTCH signaling may trigger a full-scale transition. We therefore tested whether transient activation of the NOTCH3-IC transgene can result in a lasting activation of the feed-forward loop and mesenchymal transition. In cultures of SH-SYSY-NOTCH3-IC, the NOTCH3-IC transgene was induced for 0 to 7 days, after which all cultures were followed until day 14 and harvested. Western blot analysis showed from 5 days of transient NOTCH3-IC expression onwards a strong induction of JAG1, full-length- and transmembrane NOTCH1 and NOTCH3, and intracellular domain of NOTCH3 (Fig. 6a). The strength of
NOTCH3-IC elicits an endogenous NOTCH feed-forward loop and remained induced and core ADRN TFs remained repressed by PCR (Fig. 6c and Supplementary Fig. 5). Also core MES TFs were previously shown to be associated with MES or ADRN cells in neuroblastoma. Shown are changes of H3K27ac signal in SH-SY5Y cells with induced expression of NOTCH3-IC. Shown are examples of ADRN-specific SE-associated TF genes (ASCL1, PHOX2A) and ADRN lineage genes (DBH, DDC). In c, examples of MES-specific SE-associated TF genes (SMAD3, ELK4, NOTCH2, MAML2) are shown. SEs associated with ADRN- and MES cells are indicated by blue and orange horizontal bars, respectively. Gene color depicts orientation on sense (green) and anti-sense (red) DNA strands. d, Motif analysis on regions with increased H3K27ac signal. Statistical significance of enrichment was calculated using Fisher’s exact test with Bonferroni correction.

**Fig. 4** NOTCH3-IC induces epigenetic remodeling of super-enhancer landscapes. a Analysis of H3K27ac marked super-enhancer regions (n = 1662) that were previously shown to be associated with MES or ADRN cells in neuroblastoma. Shown are changes of H3K27ac signal in SH-SY5Y cells with induced expression of NOTCH3-IC (T = 7 days of induction). Red (increased) or blue (decreased) H3K27ac signal of the super-enhancers is depicted by horizontal bars on the genomic regions. Expanded views show the super-enhancer (SE) regions that distinguish MES and ADRN lines (indicated by orange and blue vertical bars, respectively) (right). b, c H3K27ac profiles in SH-EP2, SH-SY5Y and in SH-SY5Y cells with (NOTCH3-IC on) or without (NOTCH3-IC off) 7 days of doxycycline-induced expression of NOTCH3-IC. Shown are examples of ADRN-specific SE-associated TF genes (ASCL1, PHOX2A) and ADRN lineage genes (DBH, DDC). In c, examples of MES-specific SE-associated TF genes (SMAD3, ELK4, NOTCH2, MAML2) are shown. SEs associated with ADRN- and MES cells are indicated by blue and orange horizontal bars, respectively. Gene color depicts orientation on sense (green) and anti-sense (red) DNA strands. d, Motif analysis on regions with increased H3K27ac signal. Statistical significance of enrichment was calculated using Fisher’s exact test with Bonferroni correction.

This induction increased until 7 days of transient NOTCH3-IC induction (Fig. 6a). After 5–7 days of transient NOTCH3-IC expression, the MES markers FN1 and YAP1 remained induced and the ADRN markers PHOX2A, PHOX2B, DBH, and GATA2 remained repressed until day 14 (Fig. 6a). Evidently, five days of transient NOTCH3-IC activation is sufficient to initiate and maintain the feed-forward cascade until day 14, albeit that continued NOTCH3-IC induction for 14 days shows stronger protein signals (Fig. 6a).

We then designed an experiment to analyze the extent of ADRN-to-MES transition after transient NOTCH activation. NOTCH3-IC was transiently induced for 7 days followed by 7 days without NOTCH3-IC induction (+7/-7). As controls, the NOTCH3-IC transgene was not induced (-14), or induced for 7 days (+7) or 14 days (+14). mRNA profiling showed that the +7/−7, the +7 and the +14 cells all reached a comparable ADRN-to-MES transition (Fig. 6b). NOTCH pathway genes remained upregulated in the +7/−7 as compared to the +14 cells, albeit at slightly reduced levels as revealed by mRNA profiling and quantitative RT-PCR (Fig. 6c and Supplementary Fig. 5). Also core MES TFs remained induced and core ADRN TFs remained repressed (Fig. 6d). We conclude that transient expression of exogenous NOTCH3-IC elicits an endogenous NOTCH feed-forward loop and is sufficient to maintain reprogramming of the induced MES state.

ADN-to-MES reprogramming is tumorigenic in vivo. We previously found that FACS-sorted ADRN and MES cells are both oncogenic in vivo and generated heterogeneous tumors. We now investigated whether neuroblastoma tumors remained oncogenic after massive trans-differentiation to the MES phenotype. SY5Y-NOTCH3-IC cells were inoculated in immunodeficient mice. When palpable tumors reached about 200 mm3 in size, doxycycline was added to the drinking water to induce NOTCH3-IC expression. Tumors with NOTCH3-IC induction maintained equally aggressive growth kinetics as control tumors (n = 3 mice per group, Fig. 7a).

We analyzed the transcriptome of ADRN SH-SY5Y tumors after 7 or 14 days of NOTCH3-IC induction in vivo. The MES and ADRN signatures were used to test an ADRN-to-MES transition. Tumors with NOTCH3-IC expression showed downregulation of the ADRN signature and induction of the MES signature comparable to the effect of NOTCH3-IC in vitro (Fig. 7b). ADRN lineage markers, such as DBH, DDC, and TH were repressed, while MES lineage markers like FN1, SNAI2, YAP1, and various NOTCH pathway genes, were induced (ANOVA, p < 0.001; Supplementary Fig. 6a, c). Moreover, NOTCH3-IC downregulated expression of 12 out of 18 expressed TFs from the ADRN CRC, including PHOX2A, PHOX2B, and ASCL1 (ANOVA, p < 0.001; Supplementary Fig. 6b).
To follow the transition of ADRN-to-MES reprogramming in vivo, we used NCAM1 and MAML2 as SE-associated marker genes of ADRN and MES cells, respectively (Supplementary Fig. 6a, c and Supplementary Fig. 7a–c, Figs. 3c, 4b). Non-induced control tumors expressed NCAM1 protein, but hardly expressed MAML2 (Fig. 7c). In strong contrast, tumors with NOTCH3-IC expression were largely devoid of NCAM1 but induced MAML2, indicating a homogeneous pattern of ADRN-to-MES transition (Fig. 7c).

We determined the relation of NOTCH gene expression and the MES state in a series of 498 primary human neuroblastomas. The MES signature was significantly correlated to expression of genes from the NOTCH feed-forward cascade that included NOTCH1, NOTCH2, NOTCH3, JAG1, MAML2, and HES1 (ANOVA, p-values between $10^{-65}$ to $10^{-125}$ for all correlations tested, Supplementary Fig. 8a–d). We conclude that NOTCH pathway genes are co-regulated with a MES state in human neuroblastoma and that an induced mesenchymal reprogramming of neuroblastoma cells does not affect the tumorigenicity of neuroblastoma cells in vivo.

**Discussion**

Neuroblastomas are pediatric tumors that originate from the developing peripheral sympathetic nervous system. They occur in the adrenal medulla and at various sites along the routes of migrating neural crest cells. During normal development, lineage-specific enhancers regulate the cell-type specific transcriptomes and mark phenotypically divergent states of cellular differentiation. These epigenetic mechanisms allow cells to differentiate via remodeling of their regulatory landscapes. Differentiation follows a unidirectional hierarchy, although in exceptional cases lineage-committed cells can revert to a stem-like state. Lineage differentiation hierarchies of normal development show parallels in cancer heterogeneity. Neuroblastoma tumors include a majority of lineage-committed ADRN tumor cells and a minority of MES tumor cells that resemble neural-crest cells. These cell types can spontaneously interconvert. However, the potential for transdifferentiation and the driving mechanisms remain largely unexplored.

Here, we identified the NOTCH pathway as a driver of a dedifferentiated MES identity. Many NOTCH receptors and cofactors are expressed in MES neuroblastoma cells. Some of these, i.e. NOTCH2 and MAML2, are part of the CRC of lineage TFs in MES cells. NOTCH2 and MAML2 are highly expressed, probably as a result of their associated SE. Together, these genes drive high levels of NOTCH signaling, supported by detection of NOTCH-IC signaling domains and expression of the NOTCH target gene HES1. The different NOTCH paralogues can regulate unique target genes as well as a shared set of genes. The concurrent activation of NOTCH1, NOTCH2, and NOTCH3 paralogues may therefore exert broad control on induction and/or maintenance of the MES state. Reprogramming experiments using paralogous NOTCH-IC transgenes revealed that all three NOTCH paralogues initiated transdifferentiation, while NOTCH3-IC is the strongest inducer of reprogramming ADRN...
reprogramming may be in the background of tumors or cancer cell lines.

Fig. 6 A transient phase of NOTCH3-IC expression induces commitment to a MES phenotype. a Western blot analysis of transient NOTCH3-IC induction. Time of doxycycline (dox) induction is indicated above the blots. Induction was followed by doxycycline wash-out and all samples were harvested after $T = 14$ days. Immunoblots show NOTCH pathway proteins (JAG1, NOTCH1, and NOTCH3), MES-markers (FN1 and YAP1) and ADRN-markers (PHOX2A, PHOX2B, DBH, and GATA2). As a positive reference, a sample of continued NOTCH3-IC induction for $T = 14$ days was run on the same gel (right). β-actin was used as loading control. Source data are provided as a Source Data file. b Summed z-scores of mRNA signatures for MES and ADRN genesets. Non-induced control (−dox) cells are shown in dark blue. Persistent NOTCH3-IC induction of $T = 7$ and $T = 14$ days are shown in pink and red, respectively. A transient phase of NOTCH3-IC induction ($T = 7$ days + doxycycline, followed by $T = 7$ days - doxycycline wash-out) is shown in light blue. As a reference, various neuroblastoma cell lines are shown in gray dots and grouped by dashed lines according to MES and ADRN phenotypes (see ref. 7). c, d mRNA analysis (z-score of expression) of C) NOTCH pathway genes and D) core transcription factors of MES and ADRN lineages. + and − indicate culture conditions with or without doxycycline, respectively. Time of doxycycline treatment is indicated in days. For color-coding of samples, see panel c.

A core set of stem-cell TFs can induce pluripotent stem cells from differentiated fibroblasts, albeit at low frequency and with slow kinetics. During reprogramming of neuroblastoma, we observed a swift and rather homogeneous phenotypic transition upon expression of a single NOTCH-IC transgene. This suggests that the majority of cells retained the competence for reprogramming to a dedifferentiated MES state. The potential for reprogramming may be influenced by genetic or epigenetic events that occur in specific backgrounds of tumors or cancer cell lines. For instance, an H3K27ac NOTCH2 enhancer in SH-SY5Y may cooperate with NOTCH3-IC in the induction and/or maintenance of the MES state. At the genetic level, chromosomal gains or losses may affect essential genes that facilitate reprogramming. Nevertheless, the transcriptional states of MES and ADRN cell lines represent a general phenomenon that can exist across different genetic backgrounds. The kinetics of reprogramming revealed a step-wise transition from the ADRN to a MES state, characterized by initial repression of ADRN genes, followed by induction of MES gene expression within a week. In search for a mechanism explaining this rapid transition, we found that various NOTCH-IC paralogues induced feed-forward expression of the corresponding endogenous NOTCH gene. Moreover, each NOTCH-IC cross-activated paralogous full-length NOTCH receptors. The reciprocal feed-forward loop among NOTCH paralogs can rapidly amplify the transcription of NOTCH receptors. This may explain the co-regulated expression of paralogous NOTCH receptors in MES cells, while only NOTCH2 is associated with a SE. The NOTCH ligand JAG1 is a target of NOTCH3-IC in ovarian cancer and in neuroblastoma (ref. 31 and this work), suggesting that NOTCH
feed-forward cascades exist across different tumor backgrounds. While the NOTCH cascade in ovarian cancer includes regulation of RBPJ, this gene is not induced by NOTCH3-IC in neuroblastoma. The concurrent induction of the JAG1 ligand and the MAML2 co-factor can concomitantly activate NOTCH signaling and induce an ADRN-to-MES transition. Such feed-forward loops may represent a general mechanism in which transcriptional cascades rapidly activate a signaling pathway among a population of cells, ultimately leading to repression of lineage-specific transcriptional states and induction of an alternative reprogrammed state.

Phenotypically divergent cancer cell types may cooperate to drive tumor malignancy. Small-cell lung cancer (SCLC) includes neuro-endocrine (NE) and non-NE cells. The latter endowed NE drive tumor malignancy. Small-cell lung cancer (SCLC) includes reprogramming of cells, ultimately leading to repression of lineage-specific cascades rapidly activate a signaling pathway among a population

**Methods**

**Cell culture.** Patient-derived neuroblastoma cell lines were derived and cultured in neutral stem cell (NSC) medium as described. Serum-cultured cell lines SH-EP2 and SH-SY5Y were maintained as described. Cell line authenticity was verified using Short Tandem Repeat (STR) analysis. Cells were routinely checked for the presence of mycoplasma using MycoAlert detection kit (Lonza). The gamma-secretase inhibitor RO4929097 (Selleckchem) was added to the culture medium at a final concentration of 10 µM.

**Generation of transgenic cell lines with inducible NOTCH-IC.** Polyclonal SH-SY5Y Tet repressor (TetR) cells were established by lentiviral transduction of a TetR construct (Invitrogen) and selected with 3 µg/ml blasticidin. These SH-SY5Y-TetR cell-lines were subsequently transduced with pLenti vectors that contain cDNAs encoding human NOTCH1-IC, NOTCH2-ICFLAG or NOTCH3-IC. Stable polyclonal cell lines were established using puromycin selection (4 µg/ml). All constructs were transduced at a multiplicity of infection (MOI) of 3. Transgenes were induced by addition of doxycycline to the culture medium at a final concentration of 100 ng/ml. For constitutive overexpression in 691-ADRN and in 700T-ADRN cell lines, pLent6-NOTCH3-IC, and pLent6-Luciferase2 (LUC2) constructs were packaged and transduced at an MOI of 3.

**Gene expression profiling and analysis of micro-array data.** mRNA expression profiles of SH-EP2, SH-SY5Y, 691-MES, 691-ADRN, 700-MES, 700-ADRN, 717-MES, and 717-ADRN are available from GEO (GSE90805). Total RNA from SH-SY5Y-TetR-NOTCH3-IC cells was harvested at 1, 7, 14, and 21 days of doxycycline induction and isolated using Trizol reagent (Invitrogen). RNA quality was verified on a Bioanalyzer (Agilent). RNA was hybridized on Affymetrix HG U133A plus2.0 gene chips and scanned data were normalized using the MASS5.0 algorithm. Expression data is available from GEO (GSE116893). Bio-informatic analysis of microarray data was conducted using R2 ([http://R2.amc.nl](http://R2.amc.nl)). RNA-sequencing analysis of 498 primary human neuroblastoma had been published previously and is available from GEO (GSE62564) and in R2. Expression of NOTCH receptors and is therefore not measured using Affymetrix probeset 203238_s_at that is located in the NOTCH3 3’UTR.

**MES and ADRN gene signature scores.** The gene expression signatures for MES and ADRN cells were calculated as previously described. In short, these gene signatures were derived from mRNA expression data of four MES and ADRN cell
line pairs of isogenic origin. From each cell line pair, genes with (1) significantly different expression in at least three cell line pairs, (2) with a minimum expression difference of 100%, and (3) present in 3 transwell assays were selected and merged. This identified 485 MES-specific and 369 ADRN-specific genes. Gene signatures were converted in a single value per sample using the following method: Genes were rank-ordered according to their expression level and the percentile was calculated relative to the full list. The average of the percentiles for all MES and ADRN signature genes was used as a signature score and can be compared across samples.

**qRT-PCR analysis.** Analysis of the NOTCH feed-forward cascade was performed by qRT-PCR on oligo-dT generated cDNA. Oligos for qRT-PCR were required to span intron-exon boundaries, if possible. Oligos for the detection of NOTCH receptor fusions were designed in exons that were exclusively present in the full-length NOTCH mRNA. Oligos were designed from the transcribed NOTCH IC constructs, to allow detection of transcription of NOTCH receptors from the endogenous loci. Forward (F) and reverse (R) oligo sequences were NOTCH1-F (5′-CGCGTCCAGGCACGCAACCTGAGT-3′), NOTCH1-R (5′-CTGAGCACTGATTCTGCTCGTATG-3′), NOTCH2-F (5′-ACAGCCTCTTCGACGTCCTGGA-3′), NOTCH2-R (5′-ACTGTCCTGTCATTTGGCATCA-3′), NOTCH3-F (5′-CGCGTGCTTTCCCTTACTG-3′), NOTCH3-R (5′-TTGTCAGCACTGGCCAAA-3′), HES1-F (5′-ATAAACAAAAGACGCTATCGACG-3′), HES1-R (5′-GTTCGGAAGTGTCCTGACCTCAT-3′), MAML2-F (5′-GTGGGTAAGAGGACGAGCAGCA-3′), MAML2-R (5′-TTGGTTTATTTGGAGGCACACGG-3′), MAML2-F (5′-GGCGTGGCTTCTTTCTACT-3′), MAML2-R (5′-GTGGGTAAGAGGACGAGCAGCA-3′), MAML2-F (5′-GGCGTGGCTTCTTTCTACT-3′), MAML2-R (5′-GTGGGTAAGAGGACGAGCAGCA-3′).

**Motif-enrichment analysis.** RSEG diff (using default arguments) was used to select regions with increased H3K27ac in SY5Y-NOTCH3-IC cells treated with 10 mM Tris-1 mM EDTA pH 9.0 and sections were incubated overnight at 4 °C with rabbit monoclonal NCAM1 (CD56) antibody (MRQ-42, Cell Marque, 1:100 dilution) or rabbit polyclonal MAML2 antibody (N4618, Cell Signaling Technology, 1:100). This was followed by incubation with secondary Brightview poly-anti-Rabbit IgG antibody (Immunologic). Stainings were developed using Bright DAB fluorescent detection, membranes were washed with PBS, pre-fixed for 10 min in paraformaldehyde, then fixed in steps of 50% and 100% methanol and stained with 0.1% crystal violet. The number of migrated cells per high-power microscopic field was determined. For each experimental condition, a minimum number of 3 transwells were assayed and 3–4 high-power microscopic fields per transwell were quantified. Two-sided Student’s t-test assuming equal variance was used to calculate statistical difference.

**In vivo tumorigenicity and histological analysis.** For tumor growth assays, 2.5 × 10^6 SH-SYSY-TetR-NOTCH3-IC cells were suspended in 200 μl of a 50% Matrigel (BD, 354234) solution in PBS and subcutaneously injected in female nu/nu nude mice (Crlnu-Foxnu1m, 6–8 weeks old, 20–30 g) (Charles Rivers). After tumor outgrowth to around 200–250 mm³, mice were randomized to control or treatment groups. Doxycycline was added to the drinking water at a final concentration of 200 μg/ml to induce expression of NOTCH3-IC. Tumor size was measured using a caliper. Two-sided Student’s t-test assuming equal variance was used to calculate statistical difference in size of control and doxycycline-treated tumors. Mice were sacrificed at day 7 or day 14 after start of doxycycline treatment, the tumors were isolated and divided in two parts. One tumor piece was fresh frozen in liquid nitrogen and RNA was isolated using Trizol reagent. The other tumor piece was fixed in 4% (v/v) buffered formaldehyde (Klinipath) and embedded in paraffin for histological analyses. In vivo experiments were conducted after ethical approval from the animal experiments committee of the AMC was obtained.

**Data availability** The Affymetrix gene expression data and ChIP-seq data generated in this study are available from GEO (accession number GSE116893). Previously generated mRNA expression data sets of MES and ADRN cells are available from GEO (accession numbers GSE90805). RNA-seq data from 498 primary neuroblastomas is available from GEO (accession number GSE25646). The source data underlying Figs. 1c, 2a, b, 3c, 5b, c, 6a and 7a are provided as a Source Data file. Requests for material can be send to J. NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-019-09470-w | www.nature.com/naturecommunications

**Received:** 19 July 2018 Accepted: 14 March 2019

**Published online:** 04 April 2019

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