

A CIC::DUX4 Sarcoma Model Reveals Oncogene-Driven MHCI-Dependent Immune Evasion

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Abstract

CIC::DUX4 sarcoma (CDS) is an exceptionally aggressive cancer characterized by very few available treatment choices. In this work, we describe a doxycycline-inducible CIC::DUX4 fusion mouse model and its derived cancer cell line, imChCDS, which accurately reproduces the molecular, histological, and immunological traits of human CDS. Our results establish that CIC::DUX4 expression alone is sufficient to initiate tumor development in susceptible soft connective tissue lineages. The imChCDS line preserves the transcriptional signature of its mesenchymal progenitor cells, readily forms metastatic tumors in hosts with functional immunity, and shows strong reliance on the P300/CBP transcriptional co-activators. Significantly, we reveal that CIC::DUX4/P300/CBP-driven repression of MHC class I (MHCI) constitutes a critical pathway for CDS immune evasion. Genetic disruption of CIC::DUX4 or pharmacological blockade of P300/CBP causes cell-cycle arrest in tumor cells, reinstates MHCI surface expression, and elicits potent anti-tumor immunity. This conversion changes the typically “cold” CDS tumor microenvironment into an inflamed “hot” state, ultimately promoting tumor regression. Collectively, these models provide a flexible and biologically faithful platform for exploring CDS disease mechanisms, dissecting immune escape mechanisms, and assessing novel therapies, particularly those targeting the CIC::DUX4/P300/CBP oncogenic pathway.

Keywords: CIC::DUX4, Sarcoma, Immune evasion, Experimental models, MHCI

Introduction

CIC::DUX4 sarcoma (CDS) constitutes a highly aggressive and uncommon variant among small round blue cell tumors [1, 2]. It is defined genetically by a chromosomal rearrangement that links 19q13.1 to either 4q35 or 10q26.3, resulting in a fusion between the CIC and DUX4 genes [3-6]. The resulting CIC::DUX4 fusion oncoprotein retains the DNA-binding domain of CIC while acquiring a strong transcriptional activation domain from DUX4 [7-10]. Consequently, this change

converts CIC from its normal role as a transcriptional repressor into an abnormal activator of genes that regulate the cell cycle and promote cancer development [9, 11]. In patients, CDS manifests as a fast-growing neoplasm with substantial metastatic capability and an exceptionally unfavorable outlook, frequently showing resistance to conventional chemotherapy approaches derived from Ewing sarcoma treatment protocols [12-15].

Cell lines established from patient tumors have played an essential role in delineating the transcriptional networks driven by CIC::DUX4 [16-22]. Nevertheless, these lines do not fully reflect the genetic, epigenetic, and cellular heterogeneity present in primary tumors. Prolonged in vitro propagation frequently leads to genetic alterations and selective outgrowth of certain clones, thereby diminishing their value for preclinical studies [23]. More sophisticated systems, including patient-derived

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organoids (PDOs) and patient-derived xenografts (PDXs), offer greater accuracy but are constrained by the scarcity of CDS cases and limited availability of clinical specimens [24]. In living organisms, CDS research has mainly relied on transplanting human cells into immunodeficient mice [11, 19, 20, 25, 26]. While useful for examining oncogenic signaling, these approaches cannot replicate key aspects, such as tumor initiation, native tissue microenvironments, or an intact immune system—elements that are vital for exploring immune dynamics and testing immunotherapeutic interventions. Addressing these shortcomings necessitates the creation of additional model systems that more comprehensively represent CDS biology and facilitate translation to clinical applications [11, 12].

Emerging evidence indicates that the C-terminal portion of DUX4 promotes immune evasion in various cancers and muscle-derived cells by downregulating MHC class I (MHCI) antigen presentation and inhibiting interferon-responsive pathways [27-29]. Since the CIC::DUX4 fusion preserves this C-terminal domain, it is hypothesized to foster an immunologically “cold” tumor microenvironment in CDS, hindering lymphocyte infiltration and activation [29-31]. Despite this, the precise processes governing immune escape in CDS and their influence on disease advancement in hosts with competent immunity are not well characterized, primarily because appropriate *in vivo* models have been unavailable. This deficiency restricts the ability to clarify immune-related mechanisms and slows progress toward effective immune-based therapies for CDS.

Earlier efforts to produce transgenic mice with tissue-restricted CIC::DUX4 expression via *Lox/Cre* technology demonstrated that the fusion protein can induce malignant conversion [31]. However, those models suffered from unexpected *Cre*-independent, unregulated CIC::DUX4 activity and an inability to achieve germline transmission of the transgene. Additionally, persistent CIC::DUX4 expression in the resulting cell lines restricted their usefulness in experiments [31].

To address these constraints, we generated a doxycycline (dox)-inducible chimeric mouse model capable of expressing CIC::DUX4 and developing soft tissue sarcomas that closely mimic human CDS. From this model, we derived a dox-regulated murine CDS cell line designated imChCDS, which mirrors the molecular signature of human CDS and readily forms tumors in both immunodeficient and immunocompetent recipients.

Using syngeneic transplantation experiments, we show that the CIC::DUX4/P300/CBP transcriptional axis functions as the primary oncogenic driver in CDS, supporting sustained tumor cell growth while inhibiting MHCI display. This sustains a non-inflamed tumor microenvironment and enables immune evasion. The new model system thus serves as a powerful resource for examining CDS tumor biology, tumor–immune crosstalk, and potential therapies, thereby advancing novel treatment options.

Materials and Methods

Cell culture

Mouse embryonic stem (ES) ZX1 cells were grown on irradiated mouse embryonic fibroblasts (MEFs) in DMEM containing 15% fetal bovine serum (FBS), penicillin/streptomycin (P/S; Gibco), 2 mM GlutaMAX (Invitrogen), nonessential amino acids, 0.1 mM β -mercaptoethanol, and 100 U/mL leukemia inhibitory factor (LIF; PeproTech). CIC::DUX4 was inserted at the HPRT locus of ZX1 cells to produce iCIC::DUX4 mouse ES lines as previously reported [32]. In brief, the CIC::DUX4 fusion sequence described by Yoshimoto *et al.* [33] was inserted into the *NheI* site of the p2Lox plasmid (Plasmid #34635, Addgene) and introduced into ZX1 cells by Lipofectamine (Invitrogen) transfection. Neomycin selection began two days later and persisted until colonies formed. Clones were isolated, and those showing tight, non-leaky regulation were chosen for chimera production. Doxycycline-inducible mouse CIC::DUX4 sarcoma (imChCDS) cells were maintained in DMEM (HyClone) with 10% FBS (Peak Serum, Ps-FB3, lot 293Q16), GlutaMAX, and P/S. The TRE-driven CIC::DUX4 transgene was induced with 500 ng/ μ L doxycycline (dox; Sigma-Aldrich, CAS No. 564-25-0) in PBS. All cultures were incubated at 37 °C with 5% CO₂ and humidity. Population doubling times were determined by regular passaging and cell counting. For spheroid cultures, 2.5×10^4 imChCDS cells were plated in ultra-low attachment 96-well plates (Corning) in DMEM plus 10% FBS. Spheroid diameters were quantified from 10 $\times$ images using ImageJ. GFP lentivirus was generated in 293T cells through co-transfection of pSAM-GFP with packaging (psPAX2) and envelope (pMD2.G) plasmids. Supernatants harvested at 48 and 72 h were filtered and applied to target cells. GFP-positive cells were FACS-sorted two days post-transduction and expanded for subsequent use.

*In vitro assays**Cell viability assay*

cells viability was evaluated using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions. Cells were plated at 1×10^5 per well in 96-well plates 24 hours before applying treatments.

Extracellular ATP assay

Release of extracellular ATP was determined using the RealTime-Glo™ Extracellular ATP Assay (Promega) as instructed. In brief, 1×10^5 cells were seeded, and dox was removed at the 24-, 48-, and 72-hour time points. Doxorubicin treatment (0.5 μ M for 24 h) served as a positive control. Detection reagents were added to quantify ATP in the supernatant at 4-hour intervals. Culture media from these conditions were also transferred to fresh plates for ATP measurement via CellTiter-Glo® assay. Luminescence was recorded using a POLARstar Optima Microplate Reader (BMG Labtech).

HMGB1 release assay

Secreted HMGB1 levels were measured using the Lumit® HMGB1 (Human/Mouse) Immunoassay (Promega) per the manufacturer's protocol. Cells (1×10^5) were plated, and dox was withdrawn for 24, 48, or 72 h, with doxorubicin (0.5 μ M for 24 h) as a positive control. Reagents were added directly to the wells, and bioluminescence signals were captured. Concentrations were calculated using the HMGB1 Positive Control Kit (Promega, W140A-C).

Histological analysis, immunostaining and western blotting

Tumor specimens were embedded in OCT medium and rapidly frozen in liquid nitrogen. Sections (10–12 μ m) cut on a cryostat were mounted and processed for hematoxylin and eosin (H&E) staining using conventional methods. For immunofluorescence, samples were fixed in 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 (30 min), and blocked in 3% BSA (1 h, room temperature). Primary antibodies in 3% BSA were incubated overnight at 4 °C, followed by fluorophore-conjugated secondary antibodies for 1 h at room temperature. DAPI (1:5000; Sigma) was used for nuclear counterstaining. β -galactosidase staining involved 1 h incubation at 37 °C with spider- β -Gal solution (Dojindo, SG02-01). For

immunoblotting, cells were lysed in RIPA buffer plus protease inhibitors (Complete, Roche). Proteins were separated by 7.5–10% SDS-PAGE, transferred to PVDF membranes, blocked in 5% skim milk/TBST, and probed with primary antibodies overnight at 4 °C or 1 h at room temperature. HRP-secondary antibodies were applied for 1 h, and bands were visualized with Pierce ECL substrate (Thermo Scientific). The following antibodies were used: GAPDH-HRP (1:5000, Proteintech 60,004), rabbit anti-DUX4 [E5.5] (1:500, Abcam ab124699), mouse anti-ETV4 (1:250, Santa Cruz D2720), rabbit anti-WT1 (1:500, Cell Signaling 83,535), rabbit anti-H3K18Ac (1:500, Abcam ab1191), rabbit anti-H3K27Ac (1:500, Abcam ab1791, lot GR3297878-1), rabbit anti-Ki-67 (1:250, Cell Signaling 9129T), anti-mouse CD45 (1:100, BioLegend 30-F11), anti-mouse F4/80 (1:500, BioLegend BM8), anti-mouse CD8 (1:400, Thermo Fisher 2.43), Alexa Fluor 488/555/647 goat secondaries (1:1000, Invitrogen), HRP anti-rabbit (1:5000, Jackson 111-035-003, lot 149393), and HRP anti-rabbit/mouse (1:2500, Novus NBP1-75130, lot 58-173-090418).

In vivo experiments and tumor formation assays

Animal procedures were carried out at the University of Minnesota Research Animal Resources under IACUC-approved protocol #2209A-40422. NSG, C57BL/6J, and 129P3/J strains were sourced from Jackson Laboratory. Chimeric CIC::DUX4 mice were produced at the University of Minnesota Mouse Genomics Laboratory. CIC::DUX4 was induced by administration of 625 mg/kg dox chow (Envigo) to chimeric animals or to mice receiving imChCDS transplants in both immunocompetent (C57BL/6J $\times$ SV129) and NSG hosts. Subcutaneous injections used 5×10^5 imChCDS cells mixed 1:1 with Matrigel into the flank. Intravenous injections delivered 2×10^4 cells in 100 μ L PBS via the tail vein. For orthotopic tibial injection, 2×10^5 cells in 20 μ L PBS were introduced into the right tibia of anesthetized NSG mice using an insulin syringe needle. Tumors were measured every 2–3 days and volume computed as $0.5 \times \text{length} \times \text{width}^2$. Mice were tagged numerically, and personnel were blinded during collection, weighing, and photography. For therapeutic studies, mice with $\sim 1 \text{ cm}^3$ tumors were randomized to vehicle or iP300w (5.2–6.1 mg/kg twice daily i.p.). iP300w was prepared as a 20 mM stock in DMSO and diluted in PBS.

Tumor dissociation

Dissected tumors were washed in ice-cold PBS and finely minced. Tissue pieces were enzymatically digested in high-glucose DMEM containing 1 mg/mL collagenase II and 1% P/S for 45 min at 37 °C with agitation. Cells were pelleted twice at 1500 RPM in Ham's F-10. A follow-up digestion used 0.1 mg/mL collagenase II plus 0.5 mg/mL Dispase for 20 min. The suspension was passed through 18G needles, filtered (40 µm), and washed twice more. Single-cell preparations were used for culture or flow cytometry.

FACS analyses

Tumor suspensions were treated with ACK lysis buffer for 10 min at room temperature and washed twice. Fc receptors were blocked with anti-CD16/32 (0.1 µg/100 µL) for 15 min on ice. Antibody staining was performed in PBS + 1% FBS for 60 min at 4 °C in the dark. Cells were washed twice and analyzed on a BD FACS Symphony S6 in PBS + 10% FBS containing propidium iodide—GFP-marked imChCDS cells. MHC I (H2Db, β2m) was assessed on GFP+ live cells. Immune populations were defined as: T cells (CD45+, TCRβ+, CD4+ or CD8+), Tregs (CD45+, TCRβ+, CD4+, FOXP3+), NK cells (CD45+, NK1.1+), macrophages (CD45+, CD11b+, F4/80+), M2 macrophages (CD45+, CD11b+, F4/80+, CD206+), dendritic cells (CD45+, CD11b+, CD11c+, MHCII+). Analysis used FlowJo. Antibodies: PE-Cy7 H2Db (KH95), BV396 β2m (S19.8), BV605/APC CD45 (30-F11), BV421 TCR-β (H57-597), PE-Cy7 CD4 (RM4-5), APC CD8a (53-6.7), PE CD11b (M1/70), PE-Cy7 F4/80 (BM8), BV421 CD206 (HK1.4), PE-Cy7 NK1.1 (PK136), AF647 FOXP3 (MF/14), Percp5.5 MHCII (M5/114.15.2), Percp/Cy5.5 Ki67 (11F6), PE HLA-ABC (W6/32), APC β2-microglobulin (2M2). Viability was assessed using propidium iodide or Zombie NIR™. Intracellular staining was performed using Cyto-Fast Fix/Perm and Annexin V/7-AAD kits (BioLegend).

RNA isolation, quantitative real-time RT-PCR (RT-qPCR), and RNA-seq

RNA was purified using Zymo kits. cDNA synthesis from 0.5 µg of RNA was performed using the Verso cDNA Synthesis Kit with oligo(dT) primers. qPCR was performed with Premix Ex Taq or SYBR Green. Normalization was to Gapdh or Actin via Δthe CT method. RNA-seq libraries from 500 ng RNA were sequenced as 2 × 150 bp paired-end reads (20 million pairs/sample) by Azenta.

RNA-seq data analyses

Processing followed the University of Minnesota CHURP pipeline. Alignment used STAR (v2.7.10a) to GRCh38 or GRCm39 with Ensembl 110 annotations; counts via featureCounts. Spearman correlations on log-normalized data included chimeric tumors, Kitra/X1 lines, and public datasets (GSE60740 CDS, GSE270004 ES, GSE222045 LMS, GSE39058 OS, GSE40021 SS). Heatmaps generated with pheatmap in R. Differential expression (DOXON vs DOXOFF) used $|\log_2FC| > 1$ and adj. $p < 0.05$ thresholds; volcano plots via ggplot2. CDS targets (157 orthologs from Okimoto *et al.* [11]) and a 137-gene senescence set (SenMayo [34] + SASP [35]) were highlighted. Lineage analysis used markers from a germ-layer teratoma scRNA-seq study [36], converted to mouse orthologs, and displayed as $\log_2(\text{expression} + 1)$ heatmaps.

Statistics

Analyses were performed in GraphPad Prism unless stated otherwise. Sample sizes were powered based on previous experiments. Group variances were comparable. Differences were assessed by one- or two-way ANOVA with Tukey post-hoc tests. Statistical significance was set at $P \leq 0.05$.

Results and Discussion

CIC::DUX4 is sufficient to drive malignant transformation in chimeric mice

The main objective of our study was to establish tissue-specific CIC::DUX4 transgenic mouse models that could act as dependable platforms for exploring CDS development and testing therapeutic approaches. As a first step, we aimed to establish whether CIC::DUX4 could be precisely controlled in vivo and whether its expression alone would be sufficient to cause malignant transformation. To explore this question, we created dox-inducible CIC::DUX4 transgenic mouse embryonic stem cells. We employed them to produce chimeric animals in which the inducible cells are distributed among diverse cell populations in the host. This strategy bypasses the need for germline transmission and allows CIC::DUX4 to be expressed in a supportive cellular and stromal environment, thereby enhancing the chance of tumor formation.

We generated dox-inducible CIC::DUX4 mouse embryonic stem cells (iCD4 mESCs) through targeted insertion of the p2Lox-CIC::DUX4 construct into the

HPRT locus of ZX1 cells—an enhanced derivative of A2Lox.cre cells—following our previously reported methodology [32, 37]. In addition to the Lox/Cre system at the HPRT site, ZX1 cells constitutively express the reverse tetracycline transactivator (rtTA) driven by the Rosa26 promoter [32]. Clones resistant to neomycin were isolated and propagated for additional characterization. Inducibility of CIC::DUX4 was tested by exposing the clones to 500 ng/mL dox for 24 hours. Successful induction was verified by immunofluorescence staining (**Figure 1a**). At the same time, upregulation of established CIC::DUX4 target genes such as *Etv1* and *Etv4* was measured using RT-qPCR (**Figure 1b**). Three clones displaying robust, tightly regulated, and non-leaky CIC::DUX4 induction were chosen and injected into C57BL/6 blastocysts to produce chimeric mice (**Figure 1c**). These chimeras successfully transmitted the transgene through the germline.

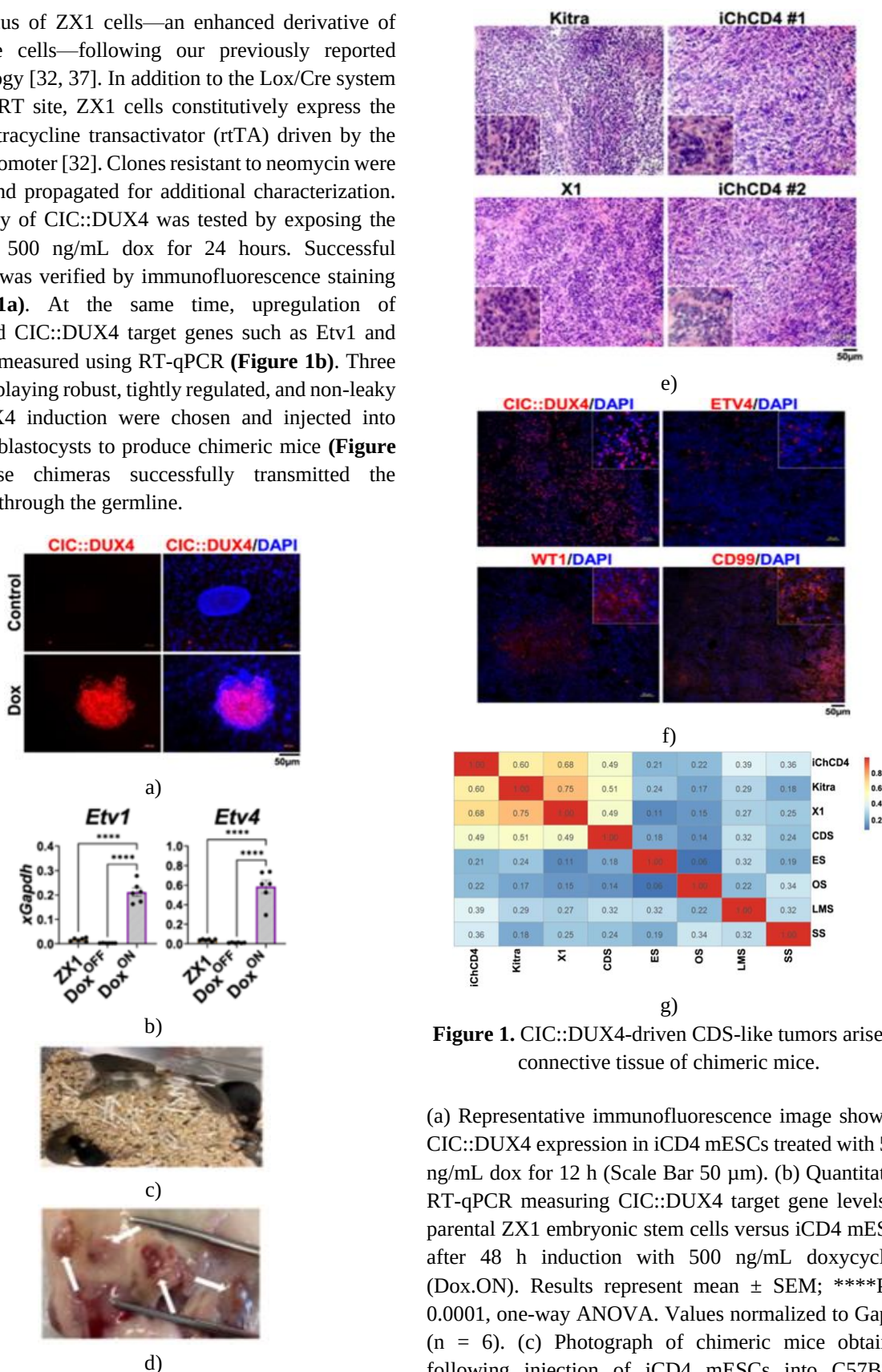


Figure 1. CIC::DUX4-driven CDS-like tumors arise in connective tissue of chimeric mice.

(a) Representative immunofluorescence image showing CIC::DUX4 expression in iChCD4 mESCs treated with 500 ng/mL dox for 12 h (Scale Bar 50 µm). (b) Quantitative RT-qPCR measuring CIC::DUX4 target gene levels in parental ZX1 embryonic stem cells versus iChCD4 mESCs after 48 h induction with 500 ng/mL doxycycline (Dox.ON). Results represent mean ± SEM; ****P < 0.0001, one-way ANOVA. Values normalized to Gapdh (n = 6). (c) Photograph of chimeric mice obtained following injection of iChCD4 mESCs into C57BL/6

blastocysts. (d) Gross appearance of multiple soft connective tissue tumors developing subcutaneously (arrows) in iChCD4 mice after 4 weeks on a 625 mg/kg dox-supplemented diet. (e) Typical H&E-stained sections of tumors from iChCD4 mice alongside those generated by subcutaneous injection of human CDS lines Kitra-SRS (Kitra) and NCC-X1-CDS (X1) into NSG mice. Scale bar: 50 μ m. (f) Immunofluorescence detection of CIC::DUX4, ETV4, WT1, and CD99 in tumor sections from iChCD4 mice. Scale bar: 50 μ m. (g) Heatmap of Spearman correlation coefficients illustrating the similarity of a 165-gene CDS signature across iChCD4 mouse tumors, Kitra and X1 cell line tumors, patient CDS samples (CDS), Ewing's Sarcoma (ES), Osteosarcoma (OS), Leiomyosarcoma (LMS), and Synovial Sarcoma (SS).

Six-month-old retired CIC::DUX4 chimeric breeders (iChCDS, $n = 10$) displaying different degrees of chimerism received 625 mg/kg dox-containing chow. Within 4 weeks of continuous induction, 6 animals (60%) formed visible, palpable subcutaneous tumors in soft connective tissue (**Figure 1d**). Two mice died for undetermined reasons, and the remaining two showed no tumor development. Microscopic examination indicated that these tumors shared strong morphological similarity with xenografts derived from human CDS cell lines Kitra-SRS (Kitra) and NCC-CDS-X1 (X1) (**Figure 1e**). The iChCD4 tumors displayed classic CDS features, such as small-to-medium neoplastic cells growing in nodules demarcated by thick collagenous stroma (**Figure 1e**) [2]. Immunostaining confirmed the CDS identity through prominent CIC::DUX4 positivity in the bulk of tumor cells, along with characteristic patterns of ETV4 (diffuse), WT1 (nuclear/cytoplasmic in subsets), and focal CD99 expression (**Figure 1f**), accompanied by sparse immune cell presence.

Global gene expression profiling was carried out to determine how closely the chimeric tumors matched human CDS and other sarcomas. Spearman rank correlation demonstrated high similarity between the transcriptional profiles of iChCD4 tumors and both Kitra/X1-derived tumors and clinical CDS biopsies. In comparison, the profiles diverged substantially from Ewing sarcoma (ES), osteosarcoma (OS), leiomyosarcoma (LMS), and synovial sarcoma (SS) (**Figure 1g**).

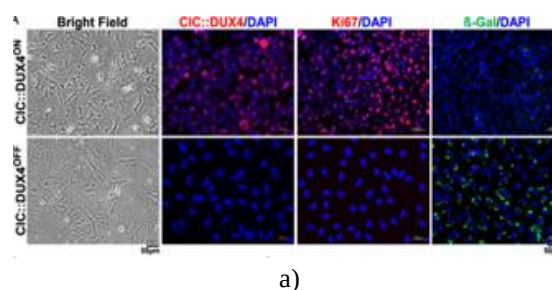
In summary, these observations indicate that CIC::DUX4 expression is sufficient to provoke malignant conversion in living animals, producing tumors that arise in

subcutaneous connective tissue and mirror the essential microscopic and molecular traits of human CDS. This supports the utility of the chimeric mouse model for research on CIC::DUX4-mediated sarcoma development.

CIC::DUX4-driven murine cancer cell line imChCDS

We next derived a doxycycline-controllable murine CDS cell line from the chimeric tumors to enable studies in immunocompetent hosts via syngeneic grafts. Tumors from chimeric mice were dissociated and re-transplanted subcutaneously into NSG mice using the same protocol previously applied to the X1 line [38]. Animals were maintained on 625 mg/kg dox chow to sustain CIC::DUX4 levels. Tumors became detectable by day 45 and grew to the ethical size limit (2 cm^3) within the following 8 days.

The secondary tumors exhibited histology indistinguishable from the primary chimeric neoplasms and highly comparable to human CDS. Enzymatic dissociation of these tumors yielded single cells that were expanded in 500 ng/ μ L dox-containing medium, yielding the inducible murine cell line imChCDS. Initial cultures showed mixed cellular morphology, but by passage 9 they stabilized into a uniform population of small polygonal cells (**Figure 2a**). Proliferation and viability of imChCDS cells were strictly CIC::DUX4-dependent; dox withdrawal rapidly induced cell-cycle arrest, loss of Ki67, and senescence (evidenced by β -galactosidase staining), (**Figure 2a**). Doubling time under dox was 50.1 h. In contrast, dox-deprived cells remained non-proliferative over 12 days (**Figure 2b**). The cells readily formed spheroids in low-attachment 3D culture (**Figure 2c**), and dox removal similarly caused growth arrest and spheroid regression (**Figures 2c and 2d**). Western blotting confirmed that CIC::DUX4 triggered analogous molecular responses in the murine system as seen in human CDS [17], including upregulation of ETV4 and WT1 as well as increased H3K18 and H3K27 acetylation (**Figure 2e**).



a)

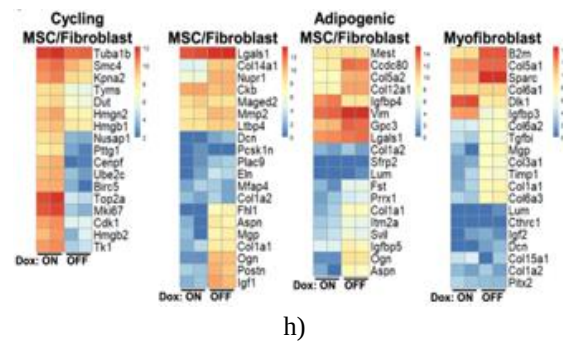
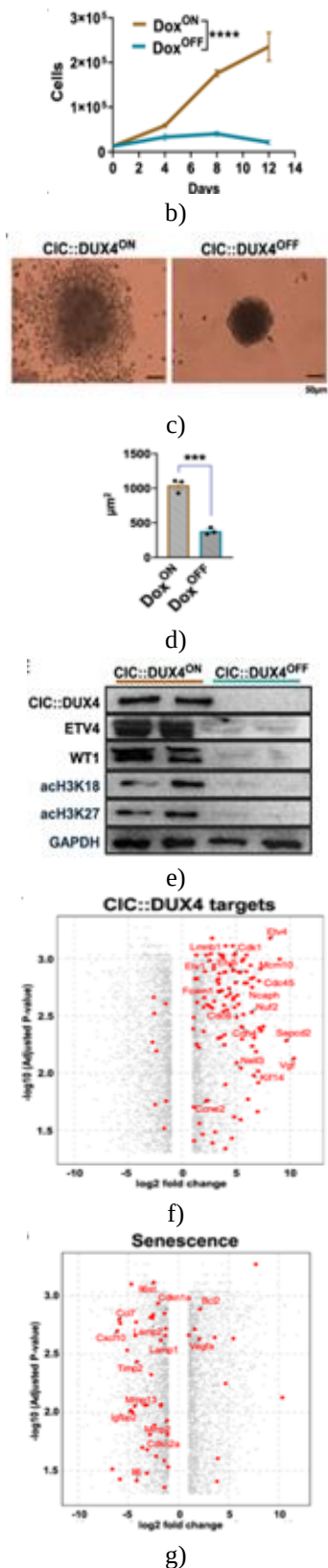


Figure 2. CIC::DUX4-inducible murine imChCDS cells closely resemble patient-derived CDS cell lines.

(a) Representative brightfield microscopy and immunofluorescence images depicting CIC::DUX4, Ki67, and Spider- β -gal in imChCDS cells 72 hours following dox removal. Scale bar: 50 μ m. (b) Proliferation curves of imChCDS cells in the presence (CIC::DUX4ON, 500 ng/mL dox) or absence (CIC::DUX4OFF) of induction over 12 days. Results expressed as mean $\pm$ SEM; **** p < 0.0001, two-way ANOVA (n = 3). (c) Brightfield images illustrating spheroid appearance in imChCDS cultures six days after dox withdrawal. Scale bar: 50 μ m. (d) Quantified spheroid size in imChCDS cells after six days without dox. Data shown as mean $\pm$ SEM; *** P < 0.001, t -test (n = 3). (e) Immunoblot analysis of CIC::DUX4 protein, its targets ETV4 and WT1, and histone acetylation marks (acH3K27, acH3K18) in imChCDS cells 72 hours post-dox withdrawal. (f) Volcano plot displaying differentially expressed CIC::DUX4 target genes (red) [11] between CIC::DUX4ON and CIC::DUX4OFF states at 72 hours in imChCDS cells. Thresholds: log₂ fold change > 1 and adjusted P -value < 0.05. (g) Volcano plot of senescence-associated genes (red) from the SenMayo collection of 125 genes [34] plus 54 SASP genes reported by Coppe *et al.* [35] upregulated in imChCDS cells 72 hours after dox removal. Criteria: log₂ fold change > 1 and adjusted P -value < 0.05. (h) Heatmap of lineage signatures for cycling MSC/fibroblasts, MSC/fibroblasts, adipogenic MSC/fibroblasts, and fibroblasts in imChCDS cells with dox (DoxON) versus three days after dox withdrawal (Dox.OFF). Additional transcriptomic profiling showed that dox-treated imChCDS cells upregulated 116 of 157 established human CIC::DUX4 target genes [11] (**Figure 2f**). Separate validation experiments confirmed that dox administration alone did not affect these target genes in human CDS lines, eliminating any direct influence of the

compound. Seventy-two hours after dox removal, CIC::DUX4 and its regulated targets were downregulated, coinciding with senescence-like cellular changes (**Figure 2a**) and induction of 59 out of 137 senescence markers (**Figure 2g**). Notably, CIC::DUX4 shutdown did not promote significant apoptosis or immunogenic cell death pathways, according to Annexin V analysis and undetectable release of ATP or HMGB1. Lineage tracing via gene expression revealed that imChCDS cells preserved the identity of their progenitor. When compared against 23 cell populations from all three germ layers (ectoderm, endoderm, mesoderm) [36], the cells preferentially expressed mesoderm-linked genes, with strongest enrichment in mesenchymal and fibroadipogenic programs (**Figure 2h**). In the proliferative dox-present state, they displayed a cycling progenitor-like signature. After dox withdrawal, the transcriptional state shifted toward a differentiated mesenchymal stem cells (MSC) and multiple fibroblast populations (**Figure 2h**).

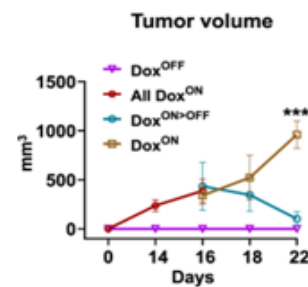
Overall, these results establish imChCDS as a reliable murine model of CIC::DUX4 sarcoma that faithfully reproduces essential molecular and phenotypic properties of human CDS while retaining the original mesenchymal transcriptional heritage.

imChCDS cells exhibit CIC::DUX4-dependent tumorigenic characteristics

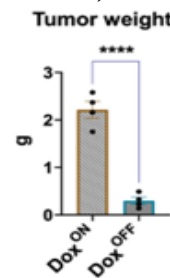
The *in vivo* tumor-forming ability of imChCDS was assessed by subcutaneous injection of 5×10^5 cells into NSG mice ($n = 12$). Eight animals received dox-supplemented chow (625 mg/kg; All DoxON group), while four were maintained without dox. Tumors developed rapidly within 14 days only in the DoxON cohort, with none observed in the DoxOFF group (**Figure 3a**). This demonstrated the essential role of CIC::DUX4 in tumor initiation. To test its requirement for sustained growth, dox was removed from a subset of tumor-bearing mice for an additional 8 days (Dox ON > OFF). Continuous dox exposure led to ongoing tumor expansion, whereas dox withdrawal caused marked tumor shrinkage (**Figures 3a–3c**).

Pathological examination by H&E staining indicated that DoxON tumors preserved the typical small round cell histology of CDS with dense cellular packing. Tumors after dox withdrawal, however, showed decreased cell density and prominent extracellular matrix accumulation (**Figure 3d**). Immunostaining confirmed abundant CIC::DUX4 protein and active proliferation (Ki67)

specifically in DoxON tumors (**Figure 3e**), together with expression of hallmark CDS markers ETV4, WT1, and CD99 (**Figure 3e**). Gene expression profiling verified selective activation of CIC::DUX4 targets exclusively in DoxON tumors (**Figure 3f**). Functional pathway analysis of genes changing upon dox withdrawal primarily implicated alterations in metabolic processes and cell cycle control (**Figure 3g**).



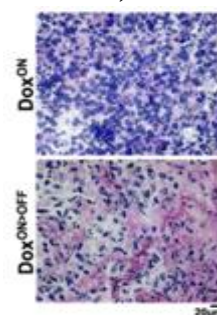
a)



b)



c)



d)

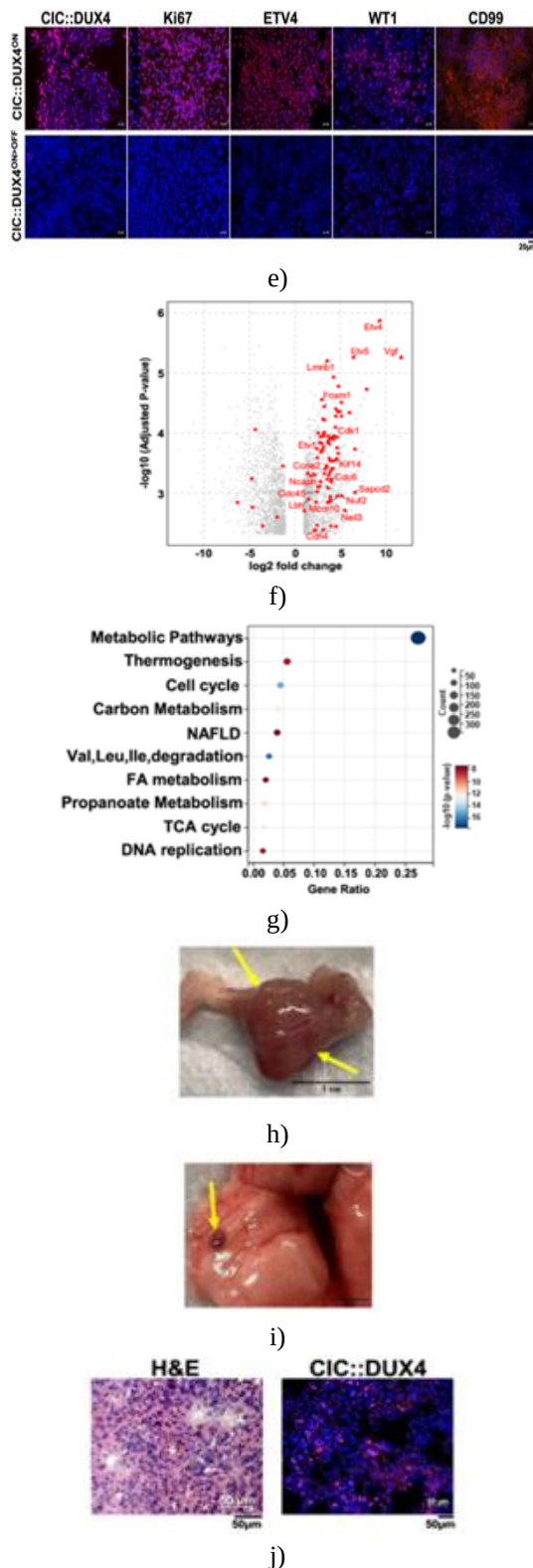


Figure 3. imChCDS cell line develops CDS-like tumors in NSG recipient.

(a) Tumor volume kinetics measured across 22 days. Results displayed as mean $\pm$ SEM; *** P < 0.001 via two-way ANOVA (n = 4). DoxOFF control animals were never exposed to dox. The other cohort received a 625 mg/kg dox diet for 16 days (All Dox). At day 16, dox was stopped in half of the tumor-bearing mice (DoxON > OFF), whereas the remaining mice continued on dox until the study concluded (DoxON). Representative gross photographs of tumors produced by imChCDS injection are included. (b) Final tumor mass at day 22 for the DoxON and DoxON > OFF cohorts. Data presented as mean $\pm$ SEM; **** P < 0.0001, t-test (n = 4). (c) Macroscopic view of imChCDS-generated tumors harvested at experimental endpoint (day 22). (d) Typical H&E histology of tumor sections from continuous dox-treated mice (DoxON) compared with those experiencing dox cessation over the last 6 days (DoxON > OFF). Scale bar: 20 μ m. (e) Immunofluorescent detection of CIC::DUX4, ETV4, WT1, and CD99 proteins in the tumors depicted in panel C (Scale bar: 20 μ m). (f) Volcano plot indicating CDS target genes that are differentially expressed (red) between DoxON and DoxON > OFF tumors 72 h after dox removal. Shown are genes satisfying Log2FC > 1 and adjusted P-value < 0.05. (g) KEGG pathway analysis highlighting enriched biological processes among differentially expressed genes from DoxON versus DoxON>OFF conditions. (h) Example photograph of a primary tumor resulting from intra-tibial imChCDS injection in NSG mice on day 24. Scale bar: 1 cm. (i) Lung photograph revealing visible surface metastatic foci after intra-tibial delivery. (J) H&E histology together with CIC::DUX4 immunofluorescence (red) of metastatic deposits in lung tissue from the orthotopic model. Scale bar: 50 μ m. Homing and metastatic properties of imChCDS cells were examined through subcutaneous, intra-tibial orthotopic, and intravenous transplantation approaches in NSG mice [39]. To facilitate post-engraftment tracking, the cells were transduced with a GFP-expressing lentivirus. Subcutaneous grafts progressed to the maximum permitted volume by day 24. No overt metastatic nodules were observed in distant organs, such as the lungs, liver, or kidneys. Nevertheless, rare CIC::DUX4-expressing cells were detected by immunofluorescence in liver and lung parenchyma. Organ-derived mononuclear cells cultured under dox conditions yielded GFP-positive populations within 4–5 days. Initial cultures contained fewer than 1% GFP-positive cells at passage 1 across all

six mice. These cells proliferated vigorously, comprising roughly 80% of the culture by passage 3. Dox-inducible CIC::DUX4 protein expression in the expanded cells was verified through immunofluorescence.

Intra-tibial injection produced local bone tumors (**Figure 3h**) and widespread macroscopic lung metastases in every animal ($n = 4$), with additional liver involvement in one mouse (**Figure 3i**). Both H&E and immunofluorescence confirmed that the secondary lesions displayed typical CDS architecture and retained CIC::DUX4 expression (**Figure 3j**). Intravenous delivery resulted in rapid multi-focal tumor development restricted to lungs and liver in all recipients ($n = 7$) by day 16, revealing clear tissue tropism.

In aggregate, imChCDS cells demonstrate robust, CIC::DUX4-dependent tumor-forming ability in NSG hosts and accurately mirror the molecular and tissue-level characteristics of human CDS when grown in vivo.

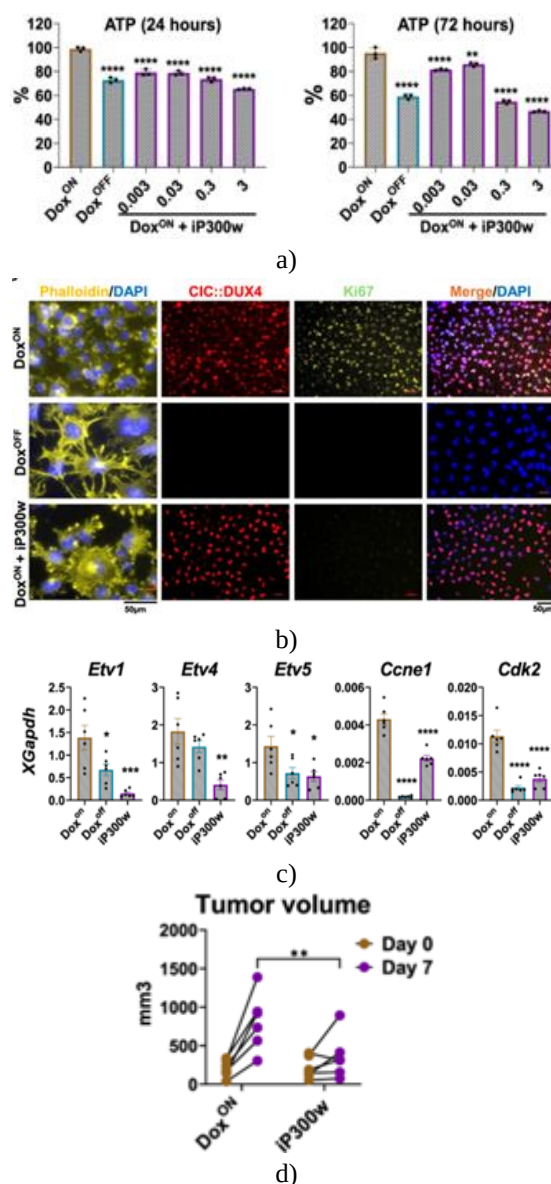
imChCDS cell viability depends on the CIC::DUX4/P300/CBP transcriptional axis

Earlier investigations established that CIC::DUX4 promotes malignancy largely by partnering with the co-activators P300 and CBP [17]. We tested whether this dependency exists in imChCDS cells using our P300/CBP inhibitor iP300w across a dose range [17, 40, 41], benchmarking results against direct CIC::DUX4 withdrawal. iP300w produced clear dose-responsive and time-dependent declines in viability (**Figure 4a**). Peak activity occurred at 0.3 μM , aligning with concentrations found effective against human CDS cell lines [17]. Inhibitor treatment triggered morphological alterations comparable to those induced by dox withdrawal (DoxON > OFF), shifting cells from compact polygonal forms to enlarged, flattened senescent phenotypes (**Figure 4b**). This was paralleled by sharply decreased Ki67 staining, signifying proliferation arrest (**Figure 4b**). Consistent with these changes, iP300w strongly repressed CIC::DUX4 target gene transcription, demonstrating successful disruption of the fusion protein's transcriptional program through P300/CBP blockade (**Figure 4c**).

The therapeutic potential of P300/CBP inhibition was further explored in vivo. Following subcutaneous implantation of imChCDS cells into NSG mice ($n = 6$) and establishment of palpable tumors on dox chow after 7 days, iP300w was dosed at 6.1 mg/kg twice daily. Seven days of treatment produced notable decreases in tumor dimensions and overall volume (**Figures 4d–4f**).

Flow cytometric evaluation showed reduced GFP-positive tumor cell fractions and lowered proliferative (Ki67) activity specifically within the GFP-labeled population (**Figures 4g and 4h**).

These data indicate that imChCDS proliferation and survival are critically reliant upon the CIC::DUX4/P300/CBP axis. Chemical blockade of P300/CBP not only corroborates its essential role in CIC::DUX4 oncogenic signaling but also underscores the utility of the imChCDS system for screening and advancing targeted agents against this driver pathway.



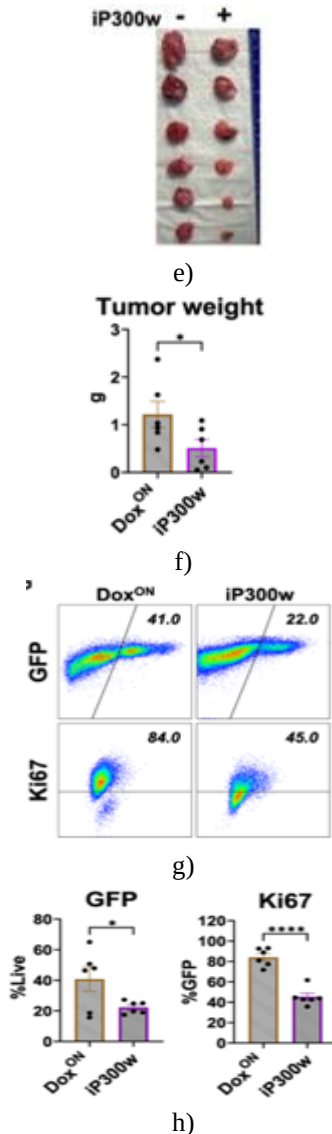


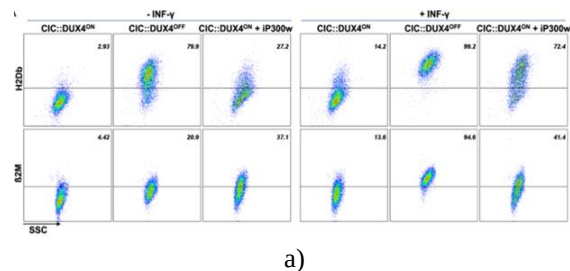
Figure 4. ImChCDS cell viability depends on P300/CBP activity.

(a) An ATP-based viability assay was performed on imChCDS cells exposed to multiple doses of iP300w over 24 h and 72 h. Cultures maintained at 500 ng/mL dox continuously served as the DoxON group, while dox was withdrawn during inhibitor exposure for the DoxOFF group. Values expressed as mean $\pm$ SEM relative to DoxON controls; **P < 0.01, ***P < 0.0001, one-way ANOVA (n = 3). (b) Representative immunofluorescence panels for Phalloidin (F-actin), CIC::DUX4, and Ki67 in imChCDS cells after 48 h under DoxON conditions, DoxOFF conditions, or DoxON plus 0.3 μ M iP300w. Scale bar: 50 μ m. (c) RT-qPCR quantification of CIC::DUX4 downstream target genes

following 24 h in DoxON, DoxOFF, or DoxON + 0.3 μ M iP300w settings. Data are mean $\pm$ SEM; *P < 0.05, **P < 0.01, ***P < 0.001 by two-way ANOVA, normalized to Gapdh (n = 6). (d) Measured tumor volumes before treatment start (day 0) and at the end of 7 days of therapy. NSG mice (n = 6) bearing subcutaneous imChCDS tumors were kept on 625 mg/kg dox chow for 14 days prior to iP300w dosing (6.1 mg/kg i.p. twice daily). Results shown as mean $\pm$ SEM; **P < 0.01, one-way ANOVA. (e) Dissected tumor photographs from the experiment. (f) Recorded tumor weights upon harvest. Data presented as mean $\pm$ SEM; *P < 0.05 by t-test (n = 6). (g) Flow cytometry examples illustrating the fraction of GFP-positive imChCDS tumor cells and Ki67 positivity within the GFP-positive subset in vehicle control versus iP300w-treated mice. (h) Statistical summary of FACS quantification from panel G. Data are mean $\pm$ SEM; *P < 0.05, ***P < 0.001 using Student's t-test.

CIC::DUX4 suppresses MHCI expression

Prior research has proposed that DUX4 contributes to immune evasion mechanisms by reducing MHC class I antigen display, possibly via disruption of IFN- γ -triggered gene programs [27, 28]. We therefore evaluated whether the CIC::DUX4/P300/CBP complex exerts analogous repressive effects on MHCI in imChCDS cells. Surface expression of MHCI components was tracked by flow cytometry following CIC::DUX4 inactivation through dox withdrawal or pharmacological P300/CBP blockade, with or without concurrent IFN- γ exposure (**Figures 5a and 5b**). Active CIC::DUX4 expression led to profound downregulation of both H2Db and β 2M on the cell surface. Upon dox removal, H2Db and β 2M levels rose substantially within 24 hours, independently of IFN- γ , and continued to increase through 72 hours. IFN- γ cotreatment produced even stronger H2Db upregulation, achieving broad, uniform surface expression across virtually all cells (**Figures 5a and 5b**).



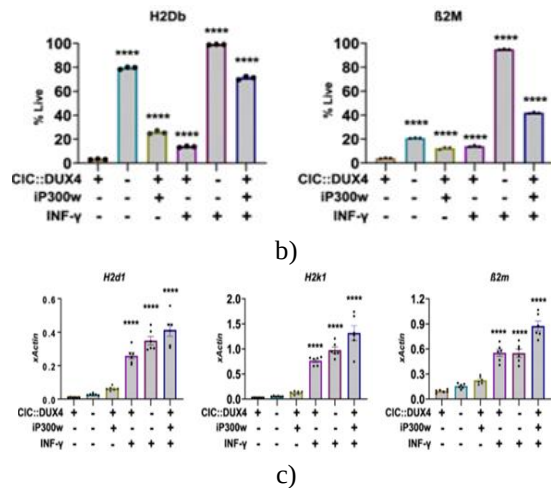


Figure 5. CIC::DUX4/P300/CBP complex suppresses MHC expression.

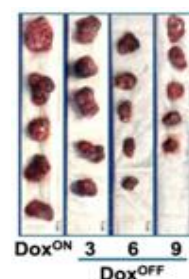
(a) Typical flow cytometry profiles of surface H2Db and β2M MHC markers on imChCDS cells. CIC::DUX4ON cells were kept under continuous 500 ng/mL dox exposure. In the CIC::DUX4OFF group, dox was withdrawn for 72 h. Cells in the CIC::DUX4ON + iP300w group were maintained with dox while treated with 0.3 μM iP300w for 72 h. IFN-γ (200 ng/mL) was added for the last 24 h where specified. (b) Summary quantification of H2Db and β2M surface levels from panel A, calculated within the live cell gate. (c) RT-qPCR data for MHC-associated genes (H2d1, H2k1, β2m) across CIC::DUX4ON, CIC::DUX4OFF, and iP300w-treated conditions. Results shown as mean ± SEM; ****P < 0.0001 by one-way ANOVA relative to CIC::DUX4ON (n = 3), normalized to Gapdh. P300/CBP inhibition with 0.3 μM iP300w likewise restored surface MHC components over time (**Figures 5a and 5b**). Transcriptional studies by RT-qPCR verified direct CIC::DUX4-mediated repression of key MHC genes including H2d1, H2k1, and β2m (**Figure 5c**). We extended these findings to human cells by applying iP300w to patient-derived CDS lines Kitra and X1. Within 48 h, the combination of iP300w and IFN-γ nearly fully restored MHC surface expression in X1 cells and partially restored it in Kitra cells. Upregulation of MHC pathway genes at the mRNA level was also observed. These experiments collectively show that the CIC::DUX4/P300/CBP transcriptional unit inhibits MHC expression at both the transcriptional and protein levels, thereby likely supporting immune escape in tumors driven by this fusion oncogene.

CIC::DUX4 mediates CDS immune evasion

Because imChCDS cells originated from 129P2 embryonic stem cells, they can be transplanted into compatible 129-background mice to model CIC::DUX4 sarcoma progression within an intact immune system using a syngeneic approach. GFP-tagged imChCDS cells were injected into 24 F1 hybrid recipients (129P3/J × C57BL/6) following the same procedure used in immunodeficient NSG hosts. Dox chow was provided to all animals from the day of transplantation onward. Palpable masses developed in 21 of the 24 mice by day 30.

A staggered dox withdrawal design was used in tumor-bearing animals to examine the relationships among CIC::DUX4 activity, MHC status, and immune cell recruitment. Mice were divided into four cohorts: Group 1 experienced continuous dox removal throughout the 9-day study; Group 2 had dox withdrawn beginning on day 3 (total 6 days off); Group 3 received dox withdrawal only during the final 3 days; and Group 4 remained on dox as untreated controls.

Longer durations of dox deprivation led to progressively greater reductions in tumor size, with significant regression evident after just 3 days without dox (**Figures 6a-6c**). Microscopic evaluation indicated that tumors from the continuous dox control group preserved typical CIC::DUX4 sarcoma architecture, which became increasingly altered following loss of the fusion protein (**Figure 6d**). Staining for lineage markers confirmed high levels of CIC::DUX4, ETV4, WT1, and CD99 specifically in DoxON tumors. Marker expression dropped sharply after 3 days off dox and was absent after 9 days of withdrawal. Importantly, CD45-positive leukocyte infiltration became prominent by day 3 after dox removal (**Figure 6d**) and increased further by day 9. Parallel rises in F4/80-positive macrophages and CD8-positive cytotoxic T cells were documented, consistent with mounting anti-tumor immunity (**Figure 6d**).



a)

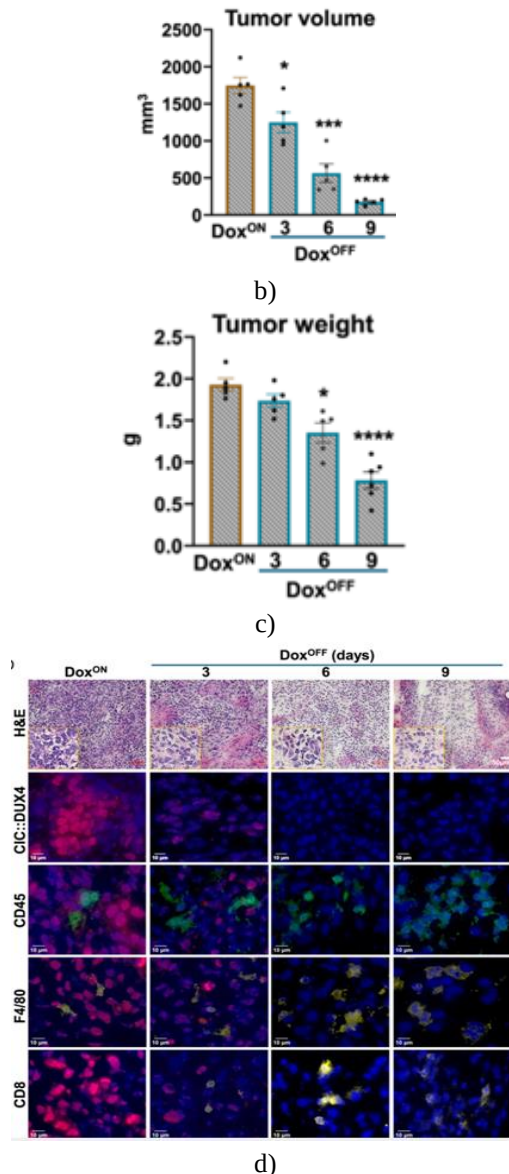


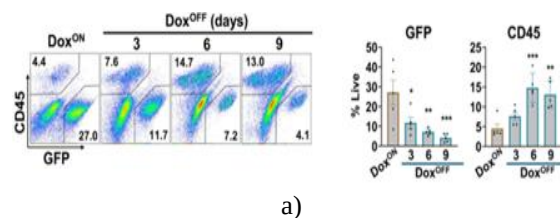
Figure 6. imChCDS cells develop CDS-like “cold” tumors in immunocompetent mice.

(a) Tumor development following imChCDS transplantation into immunocompetent hosts. Animals in the DoxON cohort remained on 625 mg/kg dox-supplemented chow throughout the study, while DoxOFF groups had dox removed for the final 3, 6, or 9 days. (b) Quantification of tumor volumes at the study endpoint (day 31) comparing DoxON and DoxOFF conditions. Data presented as mean $\pm$ SEM; * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ by one-way ANOVA ($n = 5$). (c) Comparison of tumor weights between DoxON and DoxOFF experimental arms. (d) Typical H&E images illustrating tumor morphology along with

immunofluorescence panels for CIC::DUX4 (red) and immune cell markers CD45 (green), F4/80 (yellow), and CD8 (yellow). Nuclei counterstained with DAPI (blue). Scale bar: 10 μ m.

Flow cytometry was used to precisely measure MHCII levels and track the dynamics of immune cell recruitment. Within 3 days of dox withdrawal, a substantial drop in GFP-positive imChCDS tumor cells occurred alongside a sharp rise in CD45-positive hematopoietic cells (**Figure 7a**). Extended periods without dox led to further reductions in the tumor cell fraction and progressively greater immune cell infiltration (**Figure 7a**). Notably, only 3 days of CIC::DUX4 inactivation was adequate to restore MHCII surface expression on a portion of remaining GFP-positive tumor cells, with levels continuing to increase over time (**Figure 7a**). This MHCII recovery coincided with rapid and extensive T cell recruitment (**Figure 7c**). In DoxON tumors, the T cell population was mostly CD4-positive, but dox withdrawal prompted a clear shift favoring CD8-positive cytotoxic T cells (**Figure 7c**). In addition, immunosuppressive CD4⁺FOXP3⁺ regulatory T cells were nearly eliminated within three days of dox removal (**Figure 7d**). Macrophage numbers also rose, transitioning from a tumor-supportive M2-dominant state in DoxON tumors to an anti-tumor M1 phenotype following CIC::DUX4 shutdown (**Figure 7e**). Dendritic cells expressing CD11b and MHCII followed a similar infiltration pattern, becoming significantly elevated after six days without dox (**Figure 7f**). NK cell recruitment reached its highest point at day 3 post-withdrawal before decreasing as MHCII and CD8 T cell responses strengthened, dropping below baseline DoxON levels by day 9 (**Figure 7g**).

Taken together, these results reveal that CIC::DUX4 actively inhibits MHCII-dependent immune recognition in sarcoma cells. Its inactivation slows tumor expansion, reinstates MHCII presentation, and elicits strong anti-tumor immunity featuring prominent CD8 T cell influx, macrophage repolarization toward M1, and temporary NK cell engagement.



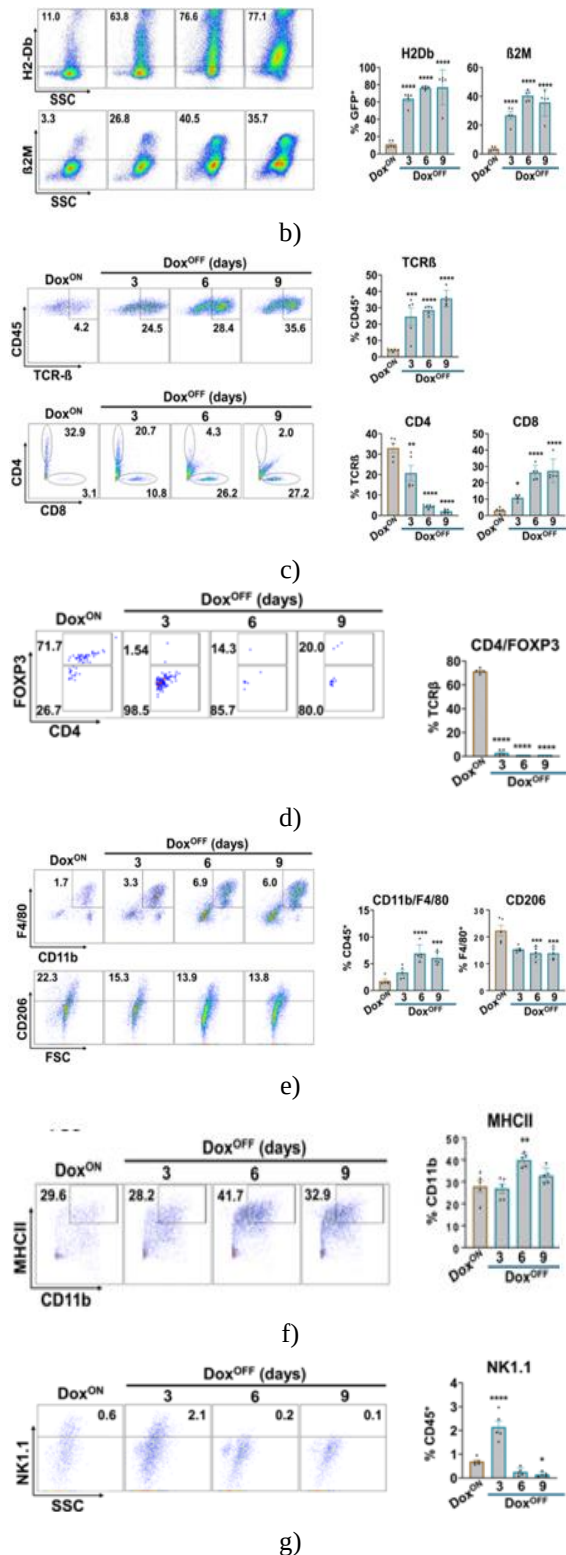


Figure 7. CIC::DUX4 inactivation restores MHCII expression and promotes immune cell infiltration in imChCDS tumors.

(a) Flow cytometry quantification of GFP-tagged imChCDS tumor cells alongside CD45-expressing leukocytes recovered from tumors of animals maintained on continuous dox (Dox.ON) or after dox cessation during the last 3, 6, or 9 days. (b) Analysis of MHCII components H2Db and β 2M displayed on the surface of GFP-positive imChCDS cells. (c) Levels of tumor-infiltrating CD4⁺ helper and CD8⁺ cytotoxic T cells following dox withdrawal. (d) Frequency of CD4⁺FOXP3⁺ regulatory T (Treg) cells present in the tumor microenvironment. (e) Assessment of tumor-associated macrophages identified as CD11b⁺F4/80⁺. Use of the CD206 marker allowed separation into M1 (anti-tumor) versus M2 (tumor-supporting) subsets. (f) Quantification of CD11b⁺MHCII⁺ dendritic cell populations. (g) NK cell presence measured as CD45⁺NK1.1⁺ events. Data shown as mean $\pm$ SEM; *P < 0.05, ***P < 0.001, ****P < 0.0001 by one-way ANOVA (n = 5).

P300/CBP inhibition restores MHCII expression and promotes a transition to an immunologically “hot” CDS microenvironment

We further examined if pharmacological suppression of P300/CBP activity could achieve similar MHCII recovery in vivo and drive productive immune responses against the tumors. GFP-labeled imChCDS cells were introduced into syngeneic immunocompetent F1 mice (129P3/J $\times$ C57BL/6, n = 6) and permitted to establish tumors on a dox diet for 21 days until reaching about 1 cm³ in size. Tumor-bearing animals were subsequently allocated to control or iP300w treatment groups for a 4-day course. Tumors collected at the end of the experiment were evaluated for MHCII levels and immune composition using methods identical to those following CIC::DUX4 deactivation. Short-term iP300w exposure for only 4 days resulted in a clear decrease of GFP-positive cancer cells coupled with strong accumulation of CD45-positive leukocytes (**Figures 8a and 8b**). During this interval, H2Db and β 2M surface abundance more than doubled (**Figure 8c**). Parallel MHCII restoration on tumor cells was documented in NSG hosts after 7 days of iP300w. This MHCII reappearance coincided with prompt recruitment of immune populations, most prominently CD8⁺ cytotoxic T lymphocytes (**Figure 8d**) as well as macrophages and dendritic cells (**Figure 8e**).

Consequently, inhibiting P300/CBP recapitulated the effects of CIC::DUX4 loss by reactivating MHCII

expression and shifting the CDS tumor environment from an immunologically “cold” to a “hot” inflammatory state.

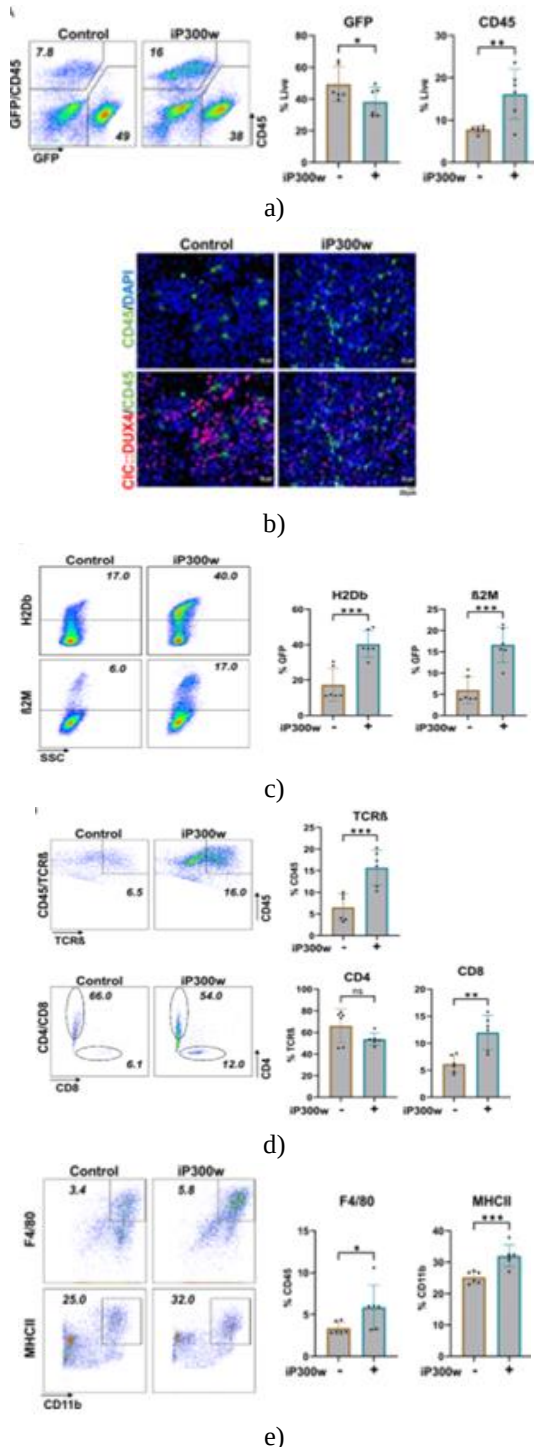


Figure 8. Pharmacological P300/CBP inactivation induces MHC I expression and promotes an immunologically active tumor microenvironment.

(a) Flow cytometric evaluation of GFP-positive imChCDS tumor cells and CD45-positive infiltrating leukocytes in tumors from mice treated with IP300w (5.2 mg/kg) for 4 days. C57BL/6J × SV129 mice were kept on dox-containing chow (625 mg/kg) for 2 weeks until tumors reached ~1 cm³, after which IP300w treatment was started while mice remained on dox. (b) Representative immunofluorescence images of CD45 (green) and CIC::DUX4 (red) in tumors from IP300w-treated animals. Nuclei counterstained with DAPI (blue). Scale bar: 20 μm. (c) Surface expression of MHC I markers H2Db and β2M on GFP-positive imChCDS cells analyzed by FACS. (d) Quantification of tumor-infiltrating CD4⁺ and CD8⁺ T cells after treatment. (e) Levels of macrophages (CD11b⁺, F4/80⁺) and CD11b⁺MHCII⁺ dendritic cells within the tumors. Data are presented as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001 by Student's t-test (n = 5).

In this study, we introduce a doxycycline-inducible chimeric mouse model, imChCDS, and its derived cell line, which accurately mirror the molecular, histological, and immunological properties of human CIC::DUX4 sarcoma (CDS). These new resources overcome major shortcomings of current preclinical systems by enabling research in immunocompetent settings and permitting precise temporal control over CIC::DUX4 levels. Collectively, they provide a robust experimental framework for dissecting sarcomagenesis, immune escape mechanisms, and potential treatment targets. Our work shows that CIC::DUX4 expression by itself can trigger and sustain malignant conversion in appropriate cell lineages, yielding tumors that match human CDS in microscopic appearance, biomarker profile, and gene expression patterns. We further establish that the CIC::DUX4/P300/CBP transcriptional complex downregulates MHC I to avoid immune detection, and that disrupting this axis reinstates MHC I levels and unleashes potent anti-tumor immunity.

We created an inducible chimeric model of CDS that bypasses problems encountered in earlier transgenic efforts, including leaky or unregulated CIC::DUX4 activity and difficulties with germline transmission [31]. The chimeric approach was selected because it supplies a natural, developmentally appropriate tissue context that favors tumor initiation. This platform is particularly valuable for exploring cancer onset, advancement, and interactions with the surrounding stroma in living organisms [42-46]. It also allows assessment of CIC::DUX4 oncogenic capacity independently of extra-

genetic changes or predefined progenitor populations. Validation of the doxycycline-regulated transgene not only supports the validity of our method but also justifies pursuing more refined tissue-specific transgenic lines in the future. This chimeric strategy further reduces breeding demands relative to standard germline models, offering an effective and practical option for initial disease modeling. Moreover, the inducible mouse embryonic stem cells produced here may be used in subsequent studies to control transgene activation during differentiation, facilitating detailed analysis of which embryonic lineages are permissive to CIC::DUX4 transformation.

Our experiments confirm that CIC::DUX4 expression is sufficient to induce sarcoma formation, consistent with reports of a generally low mutational load in CDS [47]. Prior investigations have likewise shown that CIC::DUX4 can drive malignant conversion on its own in mouse embryonic mesenchymal stem cells, osteochondrogenic progenitors, zebrafish, and other mouse systems [22, 31, 33]. Although chimerism occurred broadly, tumors arose specifically in subcutaneous soft connective tissues. This observation emphasizes the critical influence of cellular and epigenetic context, indicating particular vulnerability of mesenchymal lineages to CIC::DUX4 [33]. Beyond promoting proliferation and oncogenic reprogramming, CIC::DUX4 also confers the ability to evade immune surveillance, allowing tumor maintenance despite a competent immune system [29].

Tumors arising in the chimeric mice were histologically and transcriptionally equivalent to patient CDS specimens [1, 7, 31, 32, 37], validating the physiological relevance of this model. From these lesions, we isolated the imChCDS cell line, which faithfully reproduces key features of human CDS. It is important to note that many engineered oncogene-expressing lines proliferate well in culture but lose in vivo tumorigenic or metastatic capabilities. When tumors form, xenografted cells frequently accumulate extraneous mutations under selection pressure [48-50]. Hence, experimental tumors must both phenocopy the original disease and maintain a gene expression profile closely matching that of primary tumors [51-53]. A major strength of imChCDS is its capacity to generate metastatic disease upon transplantation into immunocompetent hosts, creating an excellent system for dissecting tumor-immune dynamics under natural conditions. Both the chimeric model and imChCDS cells also allow adjustable, time-controlled

CIC::DUX4 expression that approximates the physiological levels observed in patients. Importantly, imChCDS retains not only core CDS oncogenic programs but also the transcriptional imprint of its mesenchymal ancestry, consistent with established human CDS lines. Finally, we established that imChCDS tumorigenicity relies on the CIC::DUX4/P300/CBP axis, identifying P300/CBP as a druggable target in this cancer [16, 17]. The concentration-dependent sensitivity to P300/CBP inhibitors (iP300w, most effective at 0.3 μ M) matches previous findings in human cells, supporting cross-species conservation of vulnerabilities and the model's translational value [17]. Overall, these attributes position imChCDS as a highly pertinent tool for CDS research and preclinical therapeutic evaluation in both culture and animal settings [54-58]. The line meaningfully expands currently available CDS model systems [19, 26, 35].

A central discovery from this work is that the CIC::DUX4/P300/CBP pathway represses MHCI, creating an immunosuppressive milieu that aids immune evasion in CDS. Many cancers evade T cell recognition by downregulating MHCI, thereby impairing both natural and therapy-induced cytotoxic responses [59-62]. Experiments in culture and in animals demonstrated that CIC::DUX4 inhibits transcription and surface display of MHCI molecules, including H2Db and β 2M. Recent literature has linked the DUX4 C-terminal domain to immune suppression by interfering with IFN- γ -driven MHCI upregulation during development and malignancy [28, 29, 63]. This repression likely underlies the characteristically "cold" tumor microenvironment of CDS. Syngeneic transplantation studies in immunocompetent mice provide direct proof that eliminating CIC::DUX4 activity or inhibiting P300/CBP unleashes immune attack. Both dox withdrawal and iP300w administration led to rapid tumor shrinkage, MHCI recovery, and infiltration by CD8⁺ T cells, macrophages, and dendritic cells. Concurrently, regulatory T cells were rapidly cleared within three days of CIC::DUX4 inactivation, while macrophages transitioned from M2 to M1 polarization. NK cells showed transient early infiltration that subsided as MHCI and CD8 T cell responses strengthened. These observations are consistent with studies showing that MHCI restoration enhances tumor recognition by cytotoxic T cells [64, 65]. Therefore, targeting CIC::DUX4 directly or through P300/CBP blockade offers a promising dual-action approach for CDS that

simultaneously halts proliferation and sensitizes tumors to immune clearance. Such strategies may also enhance responsiveness to immune checkpoint blockade. Our data support clinical exploration of these combinations in human CDS.

Conclusion

In summary, the doxycycline-inducible chimeric mouse model and imChCDS cell line constitute valuable resources for investigating CDS development, immune escape mechanisms, and treatment responses. By uncovering the role of CIC::DUX4/P300/CBP in MHCI repression and immune evasion, we identify a key driver of CDS aggressiveness. These insights open avenues for therapies targeting P300/CBP inhibitors or approaches to restore MHCI, while emphasizing the need for immunocompetent models to advance research on rare cancers such as CDS.

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Ethics Statement: None

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