

Article

Acute Depletion of Cited2 in Embryonic Stem Cells Disrupts Gene Networks Controlling Self-Renewal, Homeostasis, and Early Cell Fate Commitment

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Abstract

Cited2 is a transcriptional regulator essential for embryonic development and cellular homeostasis. Studies in vertebrate models highlight its critical roles in heart, placental, neural tube, and hematopoietic development. In humans, CITED2 variants are associated with congenital heart disease. Functionally, Cited2 interacts with the transcriptional co-regulators p300/CBP and modulates the activity of multiple transcription factors. In embryonic stem cells (ESC), Cited2 supports pluripotency, self-renewal, and differentiation potential. Here, we performed comparative transcriptomic analysis after acute Cited2 depletion in mouse ESC to define its role in maintaining self-renewal, lineage competence, and cell survival. Loss of Cited2 rapidly destabilized the pluripotency network and induced aberrant activation of developmental gene programs. Nodal/Activin pathway targets, including key regulators of mesoderm, cardiac, and neural development, were markedly downregulated, consistent with Cited2-null embryonic phenotypes. Cited2 depletion also altered the expression of genes involved in DNA damage response, immune signaling, and apoptosis, correlating with the increased γ H2AX accumulation and decreased cell viability at least in part involving p53. Comparison with p300-, CBP-, and Cited2-depletion datasets revealed only partial overlap between affected gene sets. These results position Cited2 as a core regulator preserving ESC identity, genomic stability, and proper lineage engagement during early differentiation.

Keywords: Cited2; embryonic stem cells; pluripotency and cell fate regulation; p300 and CBP



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1. Introduction

The Cited2 (CBP/p300-interacting transactivator with ED-rich tail 2) is a transcriptional regulatory factor critical for the integration of multiple signaling pathways necessary to ensure proper embryonic development, and physiological function across various tissues

and organs in vertebrates. Cited2 modulates the activity of transcription factors in response to various external and internal cellular conditions, including cytokines, growth factors, hypoxia, and oxidative stress. Through these pathways, Cited2 plays a pivotal role in regulating the expression of genes necessary for cell adaptation to its environmental and intracellular stressors, thus maintaining homeostasis and promoting cell survival. Importantly, functional studies in mouse and zebrafish models have emphasized the essential role of Cited2 in embryonic survival and the development of the placenta, heart, adrenal glands, neural tissues, liver, lungs, hematopoietic system, coronary and placental vasculature, and thymus, among other processes [1–17]. Additionally, mutations in human Cited2 have also been extensively associated with congenital heart defects and other pathologies including cancer [17], further underscoring its pivotal role in maintaining normal physiological functions.

At the molecular level, Cited2 interacts with high affinity with p300 and CBP (p300/CBP), which are transcriptional coactivators and acetyltransferases. p300/CBP acetylate histones, such as histone H3 at the lysine 27 (H3K27) and other several residues, and non-histone transcription factors such as p53, the p65 subunit of NF- κ B, and SMAD proteins among others, modulating their DNA-binding affinity, transactivation capacity and/or nuclear accumulation [18]. Through competitive binding, Cited2 inhibits the interaction of transcription factors with p300/CBP, which for hypoxia-induced factor-1 α (HIF1 α) decreases the cellular response to hypoxia [19,20]. In addition to HIF1 α , CITED2 also negatively regulates the activity of other factors interacting with p300/CBP, such as NF- κ B, STAT2, p53, ETS-1, IRF1 and STAT1, therefore modulating responses to inflammation, viral infection and interferon (IFN) signaling and cancer therapies [20–26]. In addition to the competitive binding to p300/CBP, CITED2 reduces p53 and NF- κ B transcriptional activity by interfering with their acetylation by p300/CBP [21,22,27]. CITED2 also interacts with the acetyltransferase GCN5 and under fasting conditions, it promotes PGC-1 α activation in response to glucagon by inhibiting its acetylation by GCN5 [28]. Conversely, CITED2 act as a co-activator for TFAP2 transcription factors, LHX2/3, SMAD2/3, PPAR α/γ , Estrogen receptors, HNF4 α , WT1 and ISL1, which are critical for embryonic development and are involved in some pathologies when dysfunctional [29].

In mouse ESC, Cited2 and p300/CBP play important and complex roles in balancing the maintenance of pluripotency and self-renewal, as well as directing cell fates upon differentiation [30–35]. p300/CBP perform their roles in ESC by establishing long-range chromatin interactions that promote the expression of core pluripotency genes, such as Oct4 and Nanog, and through histone acetylation [36]. p300 also supports Nanog expression acting directly at its promoter and modulating the expression of other genes involved in the transition of mouse ESC from pluripotent to a differentiated state [32,34]. Other studies have nuanced the influence of p300 and CBP in gene regulation in mouse ESC, suggesting that their functions and target genes are not redundant [34]. For instance, the Wnt/ β -catenin signaling pathway promotes self-renewal of mouse ESC through the interaction of activated β -catenin with CBP to drive the transcription of pluripotency-related genes, while β -catenin/p300-driven transcription is associated with differentiation and a more restricted proliferative capacity of ESC [33]. In mouse ESC, p300/CBP may also be involved in maintaining the balance between histone modifications, such as H3K27 methylation and acetylation, which are involved in gene transcriptional repression and activation, respectively. H3K27 acetylation driven by p300/CBP has been proposed to act, at least in part, through counteracting the repressive effects of Polycomb group (PcG) proteins, such as Polycomb Repressive Complex 2 (PRC2), which mediate the methylation of H3K27 [37]. However, under certain conditions, H3K27 acetylation is dispensable for the activity of enhancer regulatory elements in mouse ESC [38], implying that alternative

mechanisms or modifications, such as H3K27 demethylation or other histone marks, may sufficiently support enhancer activity and gene regulation in those cells. Thus, the role of histone acetylation and methylation is likely influenced by the specific genomic context and the combination of regulatory factors involved [38]. These observations further suggest that histone acetylation, while important, might not always be the key factor in enhancer activity or transcriptional regulation across all genes or cell types, and other factors like methylation and protein interactions likely play significant roles.

Overexpression of *Cited2* in mouse ESC is sufficient to sustain self-renewal in the absence of Leukemia Inhibitory Factor (LIF) [30]. Conversely, acute loss of *Cited2* in these cells, when cultured in media that supports pluripotency and self-renewal, results in impaired proliferation or survival, accompanied by spontaneous differentiation [35]. Furthermore, the generation of induced pluripotent stem cells (iPSC) from mouse *Cited2*-null mouse fibroblasts fails to be efficiently initiated, highlighting further its importance in establishing pluripotency [35]. The decreased expression of *Nanog*, and to some extent of *Tbx3* and *Klf4*, upon acute *Cited2* depletion in mouse ESC, is likely involved in the phenotypes observed. Additionally, *Nanog*, *Tbx3* and *Klf4* promoter activity and expression were shown to be directly regulated by *Cited2* in those cells. *Nanog* overexpression enhanced *Cited2* expression and its promoter activity, indicating a positive feedback loop [35]. Thus, *Cited2* is involved in self-renewal and survival of mouse ESC, at least in part through the regulation of the expression of core pluripotency gene regulator network. Furthermore, when *Cited2* is depleted in mouse ESC at the onset of differentiation, the expression of early mesodermal and cardiac transcription factors is reduced, leading to impaired cardiac differentiation [15,39].

Therefore, *Cited2* has a dual and critical role in mouse ESC, promoting their self-renewal while undifferentiated, and simultaneously safeguarding their full potential for differentiation. Moreover, the cellular processes affected by *Cited2* depletion in pluripotent cells, may be highly relevant for proper embryonic development of vertebrates. To investigate the role of *Cited2* in ESC, we performed a microarray-based transcriptional profiling of mouse ESC depleted of *Cited2* cultured under conditions that support self-renewal and pluripotency. Consistent with the essential role of *Cited2* in embryonic development, several genes downregulated upon *Cited2* depletion are important for processes failing in *Cited2*-null mouse embryos, such as heart and brain development, as well as neural tube formation [1,2,4,8,12–14,16]. These observations support the role for *Cited2* to maintain transcriptional programs relevant to early developmental processes. Notably, *Cited2* depletion also resulted in the downregulation of genes encoding amino acid transporters, components of antiviral and interferon-associated responses, and factors involved in DNA damage sensing and repair. These processes and pathways are increasingly recognized as critical for ESC homeostasis, genome integrity, and the ability of pluripotent cells to appropriately respond to differentiation cues. In contrast, genes upregulated in *Cited2*-depleted ESC were enriched for stress-responsive, inflammatory, and p53-associated pathways, suggesting that loss of *Cited2* shifts ESC toward a stress-primed transcriptional state rather than directly inducing apoptosis. A comparative analysis with gene expression changes upon p300 or CBP knockdown in mouse ESC revealed that most of *Cited2*-responsive genes are not shared with global p300/CBP-regulated targets. However, a subset of genes co-regulated by *Cited2* and p300 or CBP was identified, which include genes associated with mesoderm specification, neurogenesis, and cardiogenesis. Conversely, overlapping upregulated genes were enriched for inflammatory and immune-related functions, as well as p53-mediated stress pathways. Overall, our study suggests that *Cited2* does not function as a global transcriptional amplifier in ESC, but instead contributes to a selective transcriptional buffering program that supports developmental competence. By sustaining metabolic, genome-protective, and signaling-associated gene expression while restraining

stress and inflammatory responses, *Cited2* enables pluripotent cells to initiate and stabilize lineage-specific transcriptional programs during early differentiation. Disruption of this buffering function provides a mechanistic framework for understanding the developmental defects observed upon *Cited2* loss of function.

2. Materials and Methods

2.1. Animals

Cited2 floxed mice [40] were obtained from Dr. Sally Dunwoodie and rederived by Jackson Laboratory (Bar Harbor, ME, USA) using JR 664 C57BL/6 oocytes. *Emx1*-Cre mice [41] were obtained from Jackson Laboratory (RRID:IMSR_JAX:005628). E15.5 embryos were generated by crossing *Cited2*^{fl/fl} females with *Cited2*^{fl/+}; *Emx1*-cre⁺ males. The morning of the day of the appearance of the vaginal plug was defined as E0.5 and cortices were dissected from embryos on E15.5. Genotypes were assessed by PCR on genomic DNA using those described in Supplemental Table S7.

2.2. Embryonic Stem Cells and Culture Conditions

C2^{fl/fl}[Cre] and E14TG2A mouse ESC lines were previously described [35]. In brief, ESC were cultured on gelatine-coated plates in undifferentiating medium supplemented with LIF. C2^{fl/fl}[Cre] ESC Harbor the exon 2 of *Cited2* flanked by LoxP sites, and express CreERT2 which consists of Cre recombinase fused to a mutant estrogen receptor ligand-binding domain (ERT2). Upon treatment with 1 µM of 4-hydroxytamoxifen (4HT; Sigma Aldrich, H7904, St. Louis, MO, USA), the CreERT2 can translocate into the nucleus of C2^{fl/fl}[Cre] ESC and excise the exon 2 [35].

For the microarray studies, all *Cited2*-related samples used in the present work were generated, treated, harvested, and processed in parallel with those previously described [15], using identical experimental conditions, microarray platforms, and data-analysis pipelines. In brief, C2^{fl/fl}[Cre] ESC maintained under undifferentiated conditions were treated with 1 µM 4HT or ethanol (vehicle) for 48 h, then were collected. In parallel cultures of C2^{fl/fl}[Cre] ESC treated similarly were subsequently differentiated up to 4 days using the hanging drop method, as described previously [15].

For in vitro inhibition of p53 activity by pifithrin-α, 5 × 10⁵ per well of C2^{fl/fl}[Cre] ESC were seeded in 6-well plates coated with gelatine and cultured in medium supporting the undifferentiated state. The following day, cells were treated for 48 h with either ethanol with DMSO, 1 µM 4HT with DMSO, or 1 µM 4HT combined with increasing concentrations of pifithrin-α (0–10 µM). After treatment, ESC colony morphology and cell counts were assessed. Ethanol and DMSO were used as vehicles for 4HT and pifithrin-α (Sigma Aldrich, 506132, St. Louis, MO, USA), respectively. Cell proliferation was quantified by direct cell counting at 48 h post-treatment and analyzed by ordinary one-way ANOVA followed by Dunnett's multiple comparisons test (GraphPad Prism version 10.6.1), comparing each 4HT plus pifithrin-α condition to the 4HT/DMSO control (family-wise α = 0.05).

2.3. Microarray Analysis

All samples used in the present study were generated, treated, harvested, and processed in parallel with those described in [15], using identical experimental conditions, microarray platforms, and data-analysis pipelines. *Cited2*-depleted ESC samples (C2^{fl/fl}[Cre] ESC treated with 4HT for 48 h) were prepared, hybridized, and analyzed simultaneously with the datasets reported in [15], but were not included in that study and are presented here for the first time. RNA preparation, hybridization to Clariom S Mouse Arrays (Affymetrix), quality control, background correction, normalization, and principal component analysis (PCA) to assess transcriptomic variability following ethanol or 4HT treatment were per-

formed as previously described [15], with PCA also incorporating previously published datasets from C2^{fl/fl}[Cre] ESC differentiated for 4 days by hanging-drop embryoid body formation. Cited2-related microarray data discussed in this publication are deposited in NCBI's Gene Expression Omnibus (GEO; [42]) under GEO Series accession GSE320002 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE320002>, accessed on 6 February 2026) and will be made publicly available upon publication. Differential expression was determined through application of an empirical Bayes linear model, and the significance of the changes was adjusted using Benjamini–Hochberg method, with adjusted $p < 0.05$ considered statistically significant. After standardization of signal values to a mean of 0 and standard deviation (SD) of 1 significant probe sets were analyzed by k -mean clustering based on Euclidian distance measurement and $k = 7$ using the Cluster 3.0 tool. Gene ontology (GO) and transcription factor enrichment analyses (ChEA 2022) of differentially expressed genes (DEG) were performed using Enrichr [43,44], (<https://maayanlab.cloud/Enrichr/>, accessed on 6 February 2026). Over-representation in GO or ChEA 2022 datasets was assessed relative to the genomic background using the hypergeometric test implemented on the platform, and p -values were adjusted for multiple testing using the Benjamini–Hochberg false discovery rate (FDR), with adjusted p -values < 0.05 considered significant.

For cross-comparison of commonly affected genes between Cited2, p300, and CBP depletion, lists of up- and downregulated genes from our study (Supplementary Table S1) were directly compared to previously published gene sets provided in Additional File 10 (<https://link.springer.com/article/10.1186/s12860-020-00296-9>, accessed on 6 February 2026) of [34]. This analysis was performed at the level of individual genes using Excel.

2.4. Quantitative Real-Time PCR

TRIzol (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was used to extract RNA from E15.5 cortices, and cDNA was synthesized using qScript Ultra cDNA SuperMix (Quanta Biosciences, Beverly, MA, USA). qRT-PCR was performed on a CFX Connect Real-Time System (Bio-Rad Laboratories, Berkely, CA, USA) with PerfeCTa SYBR Green FastMix (Quanta Biosciences). Primer pairs were designed in intron-spanning regions or exon–exon junctions to prevent amplification of genomic DNA. Three technical replicates of each sample were averaged and normalized to the mean expression of *Gapdh* and *S16*. Total RNA was also isolated from ESC using a commercial kit (Nzytech, MB13402, Lisbon, Portugal) and used to synthesize complementary DNA with the reverse transcriptase kit (Nzytech, MB13405). Quantitative real-time PCR (qRT-PCR) assays were carried out in CFX96 or CFX-384 (Bio-Rad, Laboratories, Berkely, CA, USA) thermocyclers using SsoFast EvaGreen Supermix (Bio-Rad, Laboratories, Berkely, CA, USA). Three biological replicates were analyzed. For each biological replicate, three technical replicates were performed, averaged, and normalized to the mean expression of *Gapdh*. The relative quantification analysis was performed as follows: $\Delta Cq = Cq$ of gene of interest—geometric mean of Cq of reference genes; $\Delta\Delta Cq = \Delta Cq$ —mean of ΔCq of wild-type samples; fold change = $2^{-\Delta\Delta Cq}$. Melt curve analysis was also performed to verify specificity of the amplicons. qRT-PCR primers are listed in Supplemental Table S7.

2.5. Immunocytochemistry

Immunocytochemistry was performed with C2^{fl/fl}[Cre] ESC cultivated on coverslips coated with 0.2% gelatine type-A, before being washed with phosphate buffered saline (PBS; Sigma, St. Louis, MO, USA), fixed in 2% formaldehyde (Sigma, P6148, St. Louis, MO, USA) for 15 min, permeabilized in 0.5% Triton X-100 diluted in PBS (Sigma, T9284, St. Louis, MO, USA) for 15 min on ice, and blocked at room temperature with a 5% (w/v) bovine serum albumin (BSA; Nzytech, Lisbon, Portugal) in PBS for 1 h. C2^{fl/fl}[Cre] ESC were

also treated with 100 μ M hydrogen peroxide for 30 min to induce DNA damage. Samples were then incubated either with an anti- γ H2AX monoclonal antibody (Millipore, 05-636, Merck Millipore, Burlington, MA, USA; at 1:500 or 1:1000 dilution) diluted in blocking solution either at room temperature for 1 h or overnight at 4 °C. The coverslips were then washed three times 10 min in PBS and incubated for 1 h at room temperature with the AlexaFluor-488 donkey anti-mouse antibodies (Thermo Fisher Scientific, Waltham, MA, USA) used at 1:5000 dilution in blocking solution. The coverslips were then washed three times with PBS and placed onto slides using Fluoromount-G-DAPI (Invitrogen, 00-4959-52, Thermo Fisher Scientific, Waltham, MA, USA) mounting medium containing DAPI. Fluorescence microscopy was performed a 63 $\times$ magnification using an LSM710-Airsyscan (Carl Zeiss, Oberkochen, Germany) super-resolution to obtain four slices of 0.15 μ m thickness from which the maximum intensity orthogonal projection was obtained. γ H2AX speckles were quantified using *CellProfiler* (v4.1.3, www.cellprofiler.org) following the developer's guidelines. Negative controls were used to set up exposure conditions for detection of a specific signal. The analysis involved the following: (1) feature enhancement with a 5 pixels (px) speckle size; (2) nucleus segmentation using an Otsu threshold (three classes, 65 px adaptive window) with shape-based declumping; (3) nucleus measurement (area and shape); (4) filtering based on Feret diameter (58.5–97.5 px) and form factor (0.5–1); (5) speckle segmentation using an Otsu threshold (three classes, 10 px adaptive window), with size constraints (5–20 px) and intensity-based declumping; (6) speckle measurement (area and shape); (7) size filtering (1–66 px); (8) intensity measurement; and (9) establishment of parent-child relationships between nuclei and speckles. Segmentation images and quantification tables were exported.

Western blotting assays were performed using 20 μ g of whole cell lysates prepared from the indicated mouse ESC as previously described [35]. Mouse monoclonal anti- γ H2AX antibody was used at 1:1000 dilution (Millipore, 05-636, Merck Millipore, Burlington, MA, USA). The membrane was then probed for total protein quantification with No-Stain whole protein labelling (Thermo Fisher, Scientific A44717, Waltham, MA, USA) according to the manufacturer's instructions. A semi-quantification of the bands obtained by western blotting was achieved using a densitometric approach through Fiji built-in function Gels [45].

2.6. Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) assays were conducted as previously described [35], with the exception of the sonication step, which was performed in a Bioruptor Pico (Diagenode, Liège, Belgium). Sonication was carried out in three cycles of 30 s on/30 s off, followed by four additional cycles of 30 s on/30 s off. Histones–chromatin complexes immunoprecipitations were performed with 5 μ g mouse monoclonal anti-H3K27me3 (Abcam, ab6002, Cambridge, UK) and rabbit polyclonal anti-H3K27ac (Abcam, ab4729, Cambridge, UK) antibodies, respectively. A mouse monoclonal anti-FLAG antibody (Sigma, F1804, St. Louis, MO, USA) for control immunoprecipitations of anti-H3K27me3 antibody, while a rabbit IgG-ChIP grade (AB46540, Abcam, Cambridge, UK) was used for control immunoprecipitations of anti-H3K27ac. The co-immunoprecipitated DNA was purified by phenol:chloroform:isoamyl extraction and precipitation. The enrichment of target genomic elements was determined by qRT-PCR as previously described [35], using primers listed in Supplemental Table S7. ChIP-qRT-PCR data were normalized using a two-step strategy to account for IP efficiency and input chromatin variability. First, Ct values for each target locus in the IP samples were normalized to *Gapdh* (housekeeping control locus with constitutive promoter activity) within the same IP sample: $\Delta\text{Ct}_{\text{IP}} = \text{Ct}(\text{target}_{\text{IP}}) - \text{Ct}(\text{Gapdh}_{\text{IP}})$. Second, ΔCt values were normalized to the corresponding input chromatin (1/10 dilution of sonicated chromatin

pre-IP) using the %Input method: $\%Input = 100 \times 2^{[\Delta Ct_{input} - \Delta Ct_{IP} - \log_2(10)]}$, where $\Delta Ct_{input} = Ct(target_input) - Ct(Gapdh_input)$ and $\log_2(10) \approx 3.32$ corrects for the 1/10 input dilution.

2.7. Transient Transfection Assays

Transient transfection assays E14TG2A or C2^{fl/fl}[Cre] mouse ESC were performed as previously described [35]. In brief, cells were plated in gelatine-coated 24-well plates at a density of 1.25×10^4 cells per well and transfected the following day using Lipofectamine 2000 (Invitrogen, 11668-019, Thermo Fisher Scientific, Waltham, MA, USA), as previously described [35]. Cells were transfected with 400ng per well of pNanog-L, a plasmid harboring a ~6 kb DNA segment from the Nanog promoter driving *luciferase* expression described elsewhere [46], gift from Lingyi Chen (Division of Pediatric Hematology/Oncology, Children's Hospital Boston, Dana Farber Cancer Institute, Boston). Additionally, 100 ng of each plasmid expressing flag-CITED2 (pPyCAGIP-flagCITED2), p300 (CMV-p300), and CBP (CMV-CBP) or their control vectors described elsewhere [35,47], were co-transfected. The reporter plasmid (ARE)3-lux (200 ng per well) and the expression vectors pCMV5b-flag-Smad2 (expressing the flag-tagged Smad2), pCMV5b-flag-mFast (expressing the flag-tagged mouse Foxh1) or their control vectors at 100 ng per well. The 3xHRE-Luc reporter (100 ng per well) was also transfected to assess HIF1 α activity. CMV-lacZ (125 ng per well) was included in all transfections as an internal control. Luciferase and β -galactosidase activities were measured two days post-transfection, as previously described [35,48], and the ratio of luciferase to β -galactosidase was calculated to correct for variations in transfection efficiency. For Activin A stimulation, cells were supplemented with Activin A (Sigma, SRP6057, St. Louis, MO, USA) at a final concentration of 30 ng/mL, 24 h after transfection. For Cited2 depletion C2^{fl/fl}[Cre] cells were treated with 1 μ M 4HT for 24 h, 24 h after transfection and control cells were treated with ethanol.

2.8. Histone Acetylation Assay

For immunoprecipitation assays, C2^{fl/fl}[Cre] ESC cells were plated in gelatine-coated 6-well plates with a density of 1.5×10^4 cell/cm² and treated with 1 μ M 4HT for 48 h or ethanol. Next, whole cell extracts were prepared with lysis buffer (50 mM TRIS pH 8.0, 0.5% NP-40 and 150 mM NaCl, supplemented with protease inhibitors (Roche, cOMplete tablets 04293159001, Basel, Switzerland), 1 mM PMSF (Sigma, P7626, St. Louis, MO, USA), 0.2 μ M sodium orthovanadate (Sigma, S65081) and 1 mM DTT (Sigma, D9799, St. Louis, MO, USA). The lysate was pre-cleared with 50% unblocked slurry of Protein-G sepharose beads (Cytiva, 17-0618-01, Marlborough, MA, USA) in IP-buffer (50 mM TRIS pH 8.0, 0.5% NP-40 and 150 mM NaCl, with protease inhibitor and 1 mM PMSF) for 30 min with agitation at 4 °C. Subsequently, 1/10 of the total lysate volume was retained as 'input', while the remaining lysate was equally divided for immunoprecipitation with either 5 μ g of mouse monoclonal anti-p300 antibody (Millipore, 05-257, Merck Millipore, Burlington, MA, USA) or 5 μ g of mouse monoclonal anti-FLAG antibody (Sigma, F1804, St. Louis, MO, USA) overnight at 4 °C. Next, 1/10 of the volume of a 50% slurry of blocked beads (pre-blocked with 5% (*w/v*) BSA in IP buffer) was added to each sample and incubated with agitation at 4 °C for 45 min. Beads were then washed three times with excess IP buffer and pelleted by centrifugation. Immunoprecipitated proteins were eluted using elution buffer (0.1 M glycine-HCl, pH 3.5) for 5 min with gentle shaking, followed by the addition of 1/10 of the volume of neutralizing buffer (0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl). The final volume of eluted samples was 55 μ L. The histone acetyltransferase activity of immunoprecipitated p300 was tested using the fluorometric Histone Acetyltransferase Activity Assay Kit (Abcam, ab204709, Cambridge,

UK) following the manufacturer's instructions. For this assay, 5 μ L of the IP-p300 eluted was used, and 2 μ L of the IP-input samples.

2.9. Intracellular Reactive Oxygen Species Assessment

Reactive oxygen species (ROS) levels were assessed using 2',7'-Dichlorofluorescein Diacetate (DCF-DA) as previously described [49]. In brief, mouse C2^{fl/fl}[Cre] ESC were treated for 24 h either with ethanol or 1 μ M 4HT, followed by incubation with 10 μ g/mL DCF-DA (Sigma-Aldrich, 35845, St. Louis, MO, USA) in PBS at 37 °C for 20 min, followed by 2 PBS washes, before trypsinization without phenol red. Cells were suspended in PBS containing 5% FBS to inactivate the trypsin and washed and centrifuged in PBS. Fluorescence was measured using a FACSCalibur flow cytometer (Becton Dickinson; excitation: 492 nm, emission: 530 nm). Mean fluorescence intensity was used to quantify ROS levels.

2.10. Statistical Analysis

Statistical analyses were performed using Student's *t*-test, with differences considered significant at $p < 0.05$, except for the viability rescue assays with Pifithrin- α , one-way ANOVA was used as described in Section 2.2. Specific tests are indicated in figure legends.

3. Results

3.1. *Cited2* Depletion Disrupts Pluripotency-Associated Transcription and Stress-Protective Programs in Undifferentiated ESC

We have previously demonstrated that the acute depletion of *Cited2* in mouse ESC induced spontaneous differentiation and cell death under culture conditions that support pluripotency and self-renewal [35]. To elucidate the molecular mechanisms underlying the cellular anomalies caused by *Cited2*-depletion in ESC, we performed a microarray-based transcriptional profiling to compare gene expression profiles of mouse C2^{fl/fl}[Cre] ESC cultured in conditions that support self-renewal, after treatment for 48 h either with 4-hydroxytamoxifen (4HT) to deplete *Cited2* or ethanol (vehicle) serving as control cells expressing normal levels of *Cited2*, as previously described [15,35]. For text simplification these conditions will be referred to as day 0 (D0) ethanol or 4HT. Gene expression patterns were also compared to previously reported DEG in *Cited2*-depleted cells at day 4 (D4) of differentiation [15], which were generated concurrently under the same conditions as the D0 samples. The principal component analysis (PCA) revealed that the gene expression profile of cells treated with 4HT (*Cited2*-depleted cells) at D4 of differentiation clustered more closely with undifferentiated control ESC than with control cells at D4 of differentiation (Figure 1A). Interestingly, *Cited2*-depleted cells cultured in conditions that support self-renewal at D0, previously shown to differentiate spontaneously [35], displayed gene expression profiles distinct from those of differentiated control cells at D4 (Figure 1A). Thus, *Cited2* is required for proper transcriptional trajectory during ESC differentiation, and its absence leads to aberrant gene expression states that are neither fully pluripotent nor appropriately differentiated. We analyzed gene expression profiles by focusing on transcripts exhibiting significant differential expression (adjusted p -value < 0.05) and a log2 fold-change > 1 or < -1 . DEG were analyzed by comparing control ESC with 4HT-treated cells cultured under self-renewal culture conditions (Tables 1 and S1). In *Cited2*-depleted cells, 168 genes were upregulated, and 231 genes were downregulated relative to control ESC (Figure 1B).

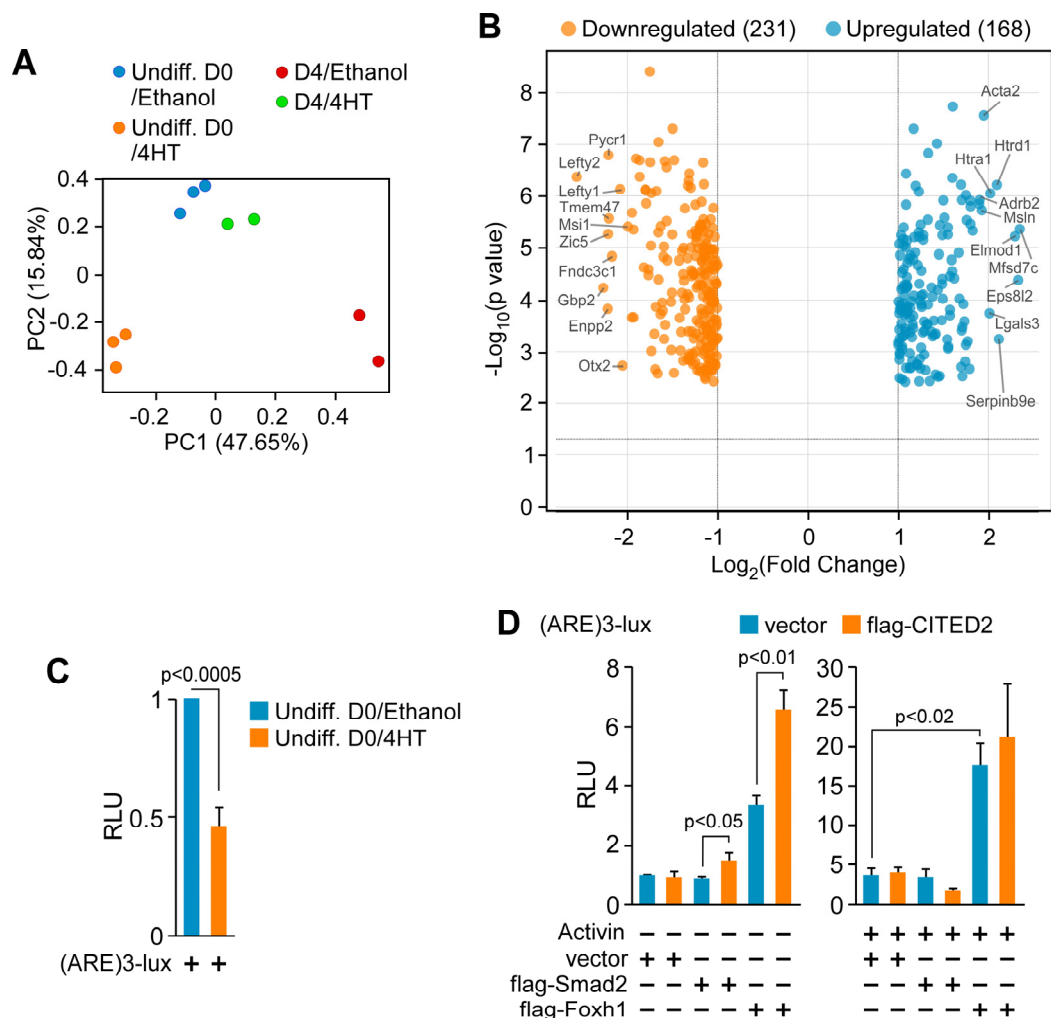


Figure 1. *Cited2* depletion alters gene expression and modulates the activin/nodal signaling pathway. (A) Principal Component Analysis (PCA) of the entire normalized array datasets. After normalization of the entire transcriptome dataset obtained from undifferentiated $C2^{fl/fl}[Cre]$ ESC treated with ethanol (Undiff. D0/Ethanol), 4HT for 48 h (Undiff. D0/4HT), and differentiated for 4 days upon treatment with ethanol (D4/Ethanol) or 4HT (D4/4HT) for the first 48 h. Each circle represents an individual sample. PC1 shows the main variability among the transcriptome differences and PC2 the second largest variability. (B) Volcano plot of differential gene expression in control $C2^{fl/fl}[Cre]$ ESC treated with ethanol (vehicle) or 4HT for 48 h. Only the genes with a significant differential expression (adjusted p value < 0.05) and a positive or negative $\log_2$ fold-change > 1 with a $\log_2$ were plotted. Each point represents the average value of one transcript in three biological independent experiments. Names of the top ten downregulated and upregulated genes are indicated. (C) Activity of the luciferase reporter (ARE)3-lux in $C2^{fl/fl}[Cre]$ ESC treated with ethanol (Undiff. D0/Ethanol), 4HT for 48 h (Undiff. D0/4HT). Gene expression in Undiff. D0/Ethanol ESC was set to 1. Results are presented as mean $\pm$ SEM from three independent biological replicates, each measured in technical duplicate. (D) Luciferase activity of the (ARE)3-lux reporter in E14TG2A mouse ESC co-transfected with either pPyCAGIP-flagCITED2 (orange bars) or pPyCAGIP control vector (blue bars), with or without pCMV-flag-Smad2 or pCMV-flag-FoxH1. Cells were either left unstimulated or stimulated 24 h post-transfection with 30 ng/mL Activin A for 24 h. Reporter activity in cells transfected with (ARE)3-lux and control vectors, without Activin A stimulation, was set to 1. Data represent the mean $\pm$ SEM from three independent biological experiments, each measured in technical duplicate.

Table 1. Microarray analysis of *Cited2* top-ranked target genes. Fold changes (log₂) are indicated in parentheses.

	Downregulated Genes	Upregulated Genes
Transcription factors and modulators	<i>Otx2</i> * (2.0), <i>Pitx2</i> ** (1.9), <i>T/Brachyury</i> ** (1.8), <i>Olig1</i> * (1.8), <i>Zfp521</i> * (1.7), <i>Dppa3/Stella</i> (1.7), <i>Olig2</i> *(1.6), <i>Camta1</i> * (1.5)	<i>Gadd45g</i> (1.9), <i>Creb3</i> (1.7), <i>Fhl2</i> * (1.5)
Signaling pathways	<i>Lefty2</i> ** (2.6), modulates TGF- β signaling, left-right axis patterning, organogenesis <i>Gbp2</i> ** (2.3), immune response during development <i>Enpp2</i> (2.2), regulation of adenosine signaling pathways, vascular development <i>Tmem47</i> * (2.2), signaling in cellular differentiation and neural/epithelial development <i>Lefty1</i> */** (2.1), modulates TGF- β signaling pathway involved in mesodermal patterning, organogenesis <i>Pim2</i> (2.0), modulates PI3K/Akt and mTOR pathways <i>Rgs9bp</i> */** (1.9), G protein signaling, DNA damage response <i>Sfrp1</i> (1.8), negative regulator of Wnt/ β -catenin signaling <i>Rragb</i> (1.8), mTOR crucial for cell growth, differentiation, and survival <i>Irgm1</i> (1.7), immune response during inflammation <i>Ifitm1</i> (1.7), related to inflammation and immune responses, particularly in response to viral infections <i>Klk1</i> (1.7), protease signaling related to inflammation and tissue remodeling <i>Pde3b</i> (1.6), phosphodiesterase regulating cAMP signaling pathways <i>Gbp7</i> (1.6), mediator of immune responses, particularly in macrophages <i>Gbp3</i> (1.6), immune response, during inflammation <i>Rad9b</i> * (1.5), DNA damage response, cell cycle regulation	<i>Gadd45g</i> ** (1.9), modulates stress-activated signaling pathways, including the p38 MAPK pathway <i>Gpr50</i> * (1.8), G-protein coupled receptor <i>Itgb4</i> ** (1.8), integrin, cell adhesion and migration <i>Tnfrsf23</i> (1.7), tumor necrosis factor receptor family involved in cell survival signaling <i>Cyp1a1</i> ** (1.7), xenobiotics metabolism <i>Dusp8</i> */** (1.6), modulates MAPK signaling <i>Il24</i> */** (1.6), inflammation and immune responses <i>F3</i> ** (1.6), coagulation factor involved in cell signaling during thrombosis and inflammation <i>Anxa2</i> */** (1.6), membrane trafficking and cytoskeletal organization <i>Nckap1l</i> ** (1.6), cytoskeletal dynamics and signaling related to cell migration <i>Arf2</i> (1.5), small GTPase involved in vesicle trafficking

* and ** indicate genes expressed and/or involved in neural and cardiovascular development, respectively.

Gene set enrichment analysis of downregulated genes in *Cited2*-depleted cells at D0 was performed using Enrichr [43,44]. Downregulated genes (Supplementary Table S1) were tested against transcription factor target gene sets from ChEA 2022 in Enrichr. Many of the downregulated genes were significantly enriched for targets of the core pluripotency transcription factors Oct4, Sox2, and Nanog, as well as auxiliary factors including Klf2, Klf4, Klf5, Smad3, and Tcf3 (Supplementary Table S2A). These observations indicate that *Cited2* depletion preferentially affects genes associated with pluripotency regulatory networks, consistent with our previous study showing that *Cited2* is necessary for the maintenance of self-renewal in mouse ESC and promotes Nanog expression through recruitment to Nanog, Tbx3, and Klf4 regulatory regions [35].

Moreover, the top-ranking downregulated genes include *Lefty1* and *Lefty2* (Figure 1B and Supplementary Table S1), that play crucial roles in maintaining the balance between ESC pluripotency and differentiation, left-right patterning and embryonic heart and neural development [50–52]. Additionally, *T/Brachyury* that encodes a pan-mesodermal marker that we have showed to be downregulated in *Cited2*-depleted cells during ESC differentiation [15,53], and *Pitx2*, a *Cited2* direct target gene in mouse embryos, which encodes a transcription factor that encodes a transcription factor [2], are also among the most downregulated genes in *Cited2*-depleted ESC (Tables 1 and S1). *Lefty1*, *Lefty2* and *Pitx2* are

well-established direct targets of Nodal/Activin signaling pathway, activated through the transcription factors Smad2, Smad3, and in cooperation with Foxh1 [54].

In addition, *Cited2* is known to function as a transcriptional co-activator for Smad2/Smad3 [55], suggesting a potential role in the modulation of the transcriptional output of this pathway in mouse ESC. Based on these observations, we hypothesized that *Cited2* depletion impairs the transcriptional responsiveness of ESC to Nodal/Activin signaling, rather than the upstream activation of the pathway itself.

To assess whether *Cited2* is involved in Nodal/Activin pathway activity via the co-activation of Smad2/Smad3 and Foxh1, we performed a transient transfection assay in *Cited2*-depleted and control ESC using the (ARE)₃-lux luciferase reporter [56]. This reporter contains the luciferase gene under the control of three tandem repeats of activin-responsive elements derived from the promoter of an activin-immediate early response gene in *Xenopus*. (ARE)₃-lux is specifically designed to measure Smad2/Smad3-Foxh1-dependent transcriptional activity downstream of TGF- β /Activin/Nodal pathway. Notably, (ARE)₃-lux activity was significantly reduced in *Cited2*-depleted cells (Figure 1C), indicating compromised Smad/Foxh1-dependent transcriptional output in the absence of *Cited2*.

To further investigate the contribution of TGF- β /Activin/Nodal pathway components to this transcriptional response, we performed co-transfection assays in E14TG2A mouse ESC. Co-transfection of (ARE)₃-lux with expression plasmids encoding either flag-Smad2 or flag-Foxh1 resulted in the reporter activation, with Foxh1 exerting a particularly strong effect (Figure 1D), consistent with its role as a key mediator of Smad2/Smad3-dependent transcription in ESC. Importantly, co-expression of flag-CITED2 together with either flag-Smad2 or flag-Foxh1 further enhanced reporter activity, supporting a role for CITED2 as a transcriptional co-activator within this context. Addition of exogenous Activin A robustly increased (ARE)₃-lux activity in all co-transfection conditions, consistent with strong pathway activation. Under these conditions, the individual contributions of exogenous Smad2 and CITED2 were no longer evident, likely reflecting saturation of Smad2/Smad3-dependent transcriptional complexes at target loci, as previously reported for maximal Activin/Nodal signaling in pluripotent stem cells [57]. Collectively, these findings suggest that *Cited2* is required for efficient transcriptional activation of Smad2/Smad3 target genes downstream of Nodal/Activin pathway, rather than for initiation of the signaling cascade itself. Reduced *Cited2* expression in mouse ESC, therefore, limits the productive transcriptional output of Smad2/Smad3 at target loci, leading to decreased expression of canonical targets such as *Lefty1*, *Lefty2*, and *Pitx2*.

Building on our previous findings that *Cited2* is crucial for mesoderm and cardiac cell specification during ESC differentiation, we analyzed the expression of key lineage markers (*Mesp1*, *Kdr*, *Isl1*, *Dkk1* and *Cdx2*) both at D0 and D4 by qRT-PCR (Figure 2A). These genes were notably upregulated in *Cited2*-depleted cells at D0, indicating a premature or dysregulated activation of mesodermal and early lineage markers, aligning with our previous observations [35]. Thus, unlike the orderly progression observed in embryoid body (EB)-mediated differentiation, where lineage markers are activated in a tightly controlled sequence, *Cited2* depletion may lead to spontaneous and uncoordinated differentiation. Specifically, the acute loss of *Cited2* may result in aberrant upregulation of mesodermal markers (*Mesp1*, *Kdr*, *Isl1*) and early lineage regulators (*Dkk1*, *Cdx2*) in undifferentiated conditions, highlighting a distinct differentiation trajectory compared to the EB process differentiation [15,35,39]. This premature expression of lineage markers reinforces the breakdown of pluripotency gatekeeping, as the loss of *Cited2* leads to the downregulation of core transcription factors, including Nanog, Klf4 and Tbx3, which are central to maintaining the pluripotent state [35]. Moreover, as the microarray analysis indicated many of the downregulated genes in *Cited2*-depleted cells at D0 are also targets of other

pluripotency-associated regulators, such as Oct4, Sox2, Nanog, Klf2, Klf4, Klf5, Smad3, and Tcf3, further underscoring the broad impact of *Cited2* on sustaining the pluripotency network. Additionally, genes involved in IFN responses (*Bst2*, *Ifitm1*, *Gbp2*, *Gbp3*, *Gbp7* and *Parp12*) were also downregulated (Supplementary Table S1). Recent studies reported that CITED2 acts as a negative regulator of interferon-stimulated genes (ISG) by inhibiting IRF1 and STAT1, which are key mediators of type I and type II IFN-activated signaling pathways [25,26]. Of note, NF- κ B and STAT2, modulated by CITED2, also play a central role in IFN- β and ISG transcription, respectively.

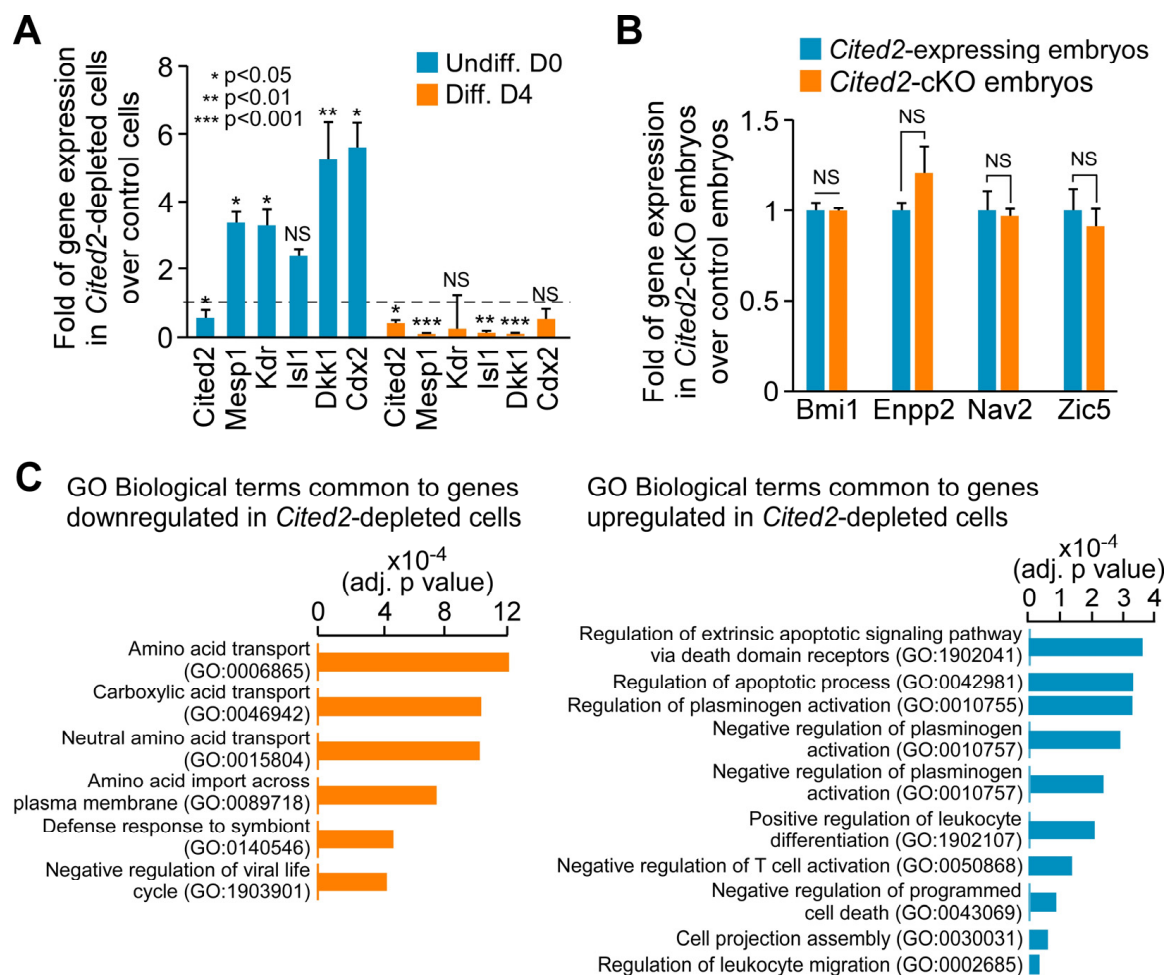


Figure 2. Identification of biological processes affected in *Cited2*-depleted cells. (A) Relative expression of the indicated genes determined by qRT-PCR in *C2f/f*[Cre]ESC treated either with ethanol or 4HT for 48 h (Undiff. D0) and in cells differentiated for 4 days upon treatment with ethanol or 4HT for the first 48 h (Diff. D4). Gene expression in ethanol/vehicle conditions was set to 1. Bars represent mean $\pm$ SEM of three independent biological triplicates, each measured in technical duplicate. NS means “not significant”. (B) Relative expression of *Bmi1*, *Enpp2*, *Nav2* and *Zic5* genes by qRT-PCR in *Cited2* conditional-null (cKO) in neural progenitor-stage of embryos at E15.5, as previously described [6]. Bars represent mean $\pm$ SEM of at least three independent biological triplicates, each measured in technical duplicate. NS means “not significant”. (C) Top gene ontology biological process terms for the genes downregulated (left) and upregulated (right) by *Cited2* depletion at D0 of differentiation determined using Enrichr [43,44].

Interestingly, *Mycn*, *Zic2*, *Enpp2*, *Otx2*, *Zic5*, and *Rad9b* were among the top down-regulated genes in *Cited2*-depleted cells at D0 (Tables 1 and S1). *Enpp2*, *Nav2*, *Zic5* and *Bmi1* play essential roles in neural crest and neural development, including neural patterning, neurogenesis, and neural stem cell maintenance [58–63]. Given their developmental

significance, we analyzed the expression of these genes in the neocortex at embryonic day (E)15.5 by qRT-PCR in a forebrain-specific *Cited2* conditional-null (*Cited2*-cKO) depleting *Cited2* at the neural progenitor-stage using the *Emx1-Cre*, as described elsewhere [6]. However, no differences in expression were observed between *Cited2*-cKO and control embryos (Figure 2B).

Thus, the downregulation of *Enpp2*, *Zic5*, *Bmi1* and *Nav2* observed in ESC following *Cited2* loss is likely an indirect consequence of spontaneous differentiation and the exit of the pluripotent state rather than a result of direct transcriptional regulation by *Cited2*. In the developing embryo, gene expression is likely to be governed by more complex and redundant regulatory networks, with compensatory mechanisms likely buffering the impact of *Cited2* loss on these genes.

Interestingly, the Gene Ontology (GO) Biological Process enrichment analysis using Enrichr revealed the downregulation of several amino acid transporters (Figure 2C and Supplementary Table S2B), including *Slc6a15*, *Slc43a1*, *Slc6a14*, *Slc1a4*, *Slc1a5*, *Slc38a3*, and *Slc7a3*. The expression of amino acid transporters is highly relevant for ESC pluripotency, self-renewal, and differentiation, as well as blastocyst implantation, neural and placental development and fetal growth [64–66], and remains to be investigated in the context of embryo development. In addition, genes involved in DNA integrity maintenance and repair, and homologous recombination, including *Neil3*, *Rad51b*, *Rad51c*, *Rad9b*, *Fancd2*, *Prkdc*, *Mdc1*, *Rpa3*, *Poli*, *Brca2*, *Dtx3l*, *Parp9*, *Usp28*, and *Mcm8* also exhibited reduced expression in *Cited2*-depleted cells (Supplementary Table S2B). The downregulation of *Rad51c* and *Mdc1* expression in *Cited2*-depleted cells was confirmed by qRT-PCR, while in the same conditions *Rad9b* expression was also decreased but failed to reach statistical significance (Section 3.5). Together, these observations argue that the loss of *Cited2* destabilizes the pluripotent transcriptional network and induces a dysregulated, non-physiological differentiation-associated gene expression state.

3.2. *Cited2* Depletion in ESC Increases the Expression of Genes Associated with Apoptosis and Differentiation Programs

The enriched GO analysis of biological processes among genes upregulated in *Cited2*-depleted cells cultured under self-renewal conditions revealed that several of these genes are involved in apoptosis modulation (*Anxa1*, *Tnfrsf12a*, *Plk2*, *Htra1*, *Fhl2*, *Plaur*, *Serpinh9*, *Vtn1*, *Ets1*, *Thbs1*, *Gadd45g*, *Lgals3*, *Sh3rf2*, *Lgals1*, *Krt18*, *Nckap1l*, *Phlda1*, *Sqstm1*, *Cryab*, *Phlda3*, *Bcl2l1*, *Nqo1*, *Hmox1*, *Tmbim1*, *F3*) (Figure 2C and Supplementary Table S3A). Additionally, many of the upregulated genes are predicted targets of transcriptional regulators in mouse ESC, including Jun, Lef1, p53, and Suz12, based on Enrichr ChEA-2022 analysis (Supplementary Table S3B). The enrichment of Lef1 target genes, a key mediator of Wnt signaling, supports a possible overactivation of the Wnt canonical pathway, which is in line with previous observations showing that canonical Wnt inhibitors such as *Dkk1*, *Wnt5a*, and *Wnt11* were downregulated in *Cited2*-depleted cells [15]. The upregulation of genes which are targets of Runx2 in mouse fibroblasts, a critical driver of bone development and related processes, including extracellular matrix remodeling, cell differentiation, and bone marrow function, likely reflects ectopic activation of differentiation-associated transcriptional programs that are normally repressed in the pluripotent state, rather than a bias toward osteogenic lineage commitment. The upregulation in *Cited2*-depleted cells of *Depdc7*, *Krt18*, *Itgb4*, *Fbxw9*, *Casq2*, *Htra1*, *Adrb2*, *Parvb*, *Sbsn*, *Scn3b*, *Gadd45g*, associated with p53 pathway, further supports changes in apoptosis regulation, although p53 is not essential for apoptosis in mouse ESC.

Notably, Suz12, a subunit of PRC2, plays a key role in maintaining pluripotency by repressing differentiation-associated genes in undifferentiated ESC [67]. Therefore, the expression of normally repressed Suz12 target genes may contribute to spontaneous

differentiation by destabilizing the repression of lineage-associated gene programs in undifferentiated ESC [15,35,39]. Overall, these observations suggest that loss of *Cited2* disrupts the transcriptional restraint and stress buffering in ESC, leading to deregulated activation of differentiation-associated and survival pathways that compromise developmental competence.

3.3. Comparison and Analysis of Genes Affected by *Cited2*, p300 and CBP Depletion in Undifferentiated Mouse ESC

Both p300 and CBP are essential transcriptional co-activators in mouse ESC, regulating pluripotency and lineage specification through histone acetylation, including histone H3 acetylation at lysine 27 (H3K27ac), and interacting with transcription factors to facilitate enhancer-promoter interactions [32,33,35,36]. *Cited2* interacts functionally with p300/CBP and has been implicated in transcriptional responses downstream of multiple signaling pathways, suggesting both shared and context-specific roles in ESC biology [68]. To examine how *Cited2*-dependent transcriptional programs relate to those regulated by p300 or CBP in mouse ESC, we compared genes differentially expressed upon *Cited2* depletion with published transcriptomic datasets from p300- or CBP-depleted mouse ESC [34]. Using consistent gene symbol harmonization and comparable thresholds for differential expression, we observed limited overlap between the gene sets under these experimental conditions. For instance, only 1.8% of genes upregulated upon *Cited2* depletion were also upregulated in p300 or CBP depleted ESC, and 6% of downregulated genes were shared across all three conditions (Table 2). These shared downregulated genes include *Lefty1* and *Lefty2*, canonical Nodal/Activin pathway targets and key regulators of early developmental patterning, which are among the most strongly affected transcripts in *Cited2*-depleted ESC. Notably, genes commonly downregulated upon *Cited2* and p300 or CBP depletion include targets of core pluripotency-associated transcription factors such as *Tcf3*, *Nanog*, *Smad3*, *Oct4*, and *Klf2/4/5* based on Enrichr ChEA-2022 analysis (Supplementary Table S4), suggesting convergence on selected regulatory nodes rather than global co-regulation.

Table 2. Genes commonly affected by *Cited2*, p300, and CBP depletions in mouse ESC. Genes indicated in blue are modulated solely by *Cited2* and p300, while genes presented in orange are modulated solely by *Cited2* and CBP.

	Downregulated Genes	Upregulated Genes
Cited2 p300 CBP	<i>Lefty2</i> *, <i>Gbp2</i> **, <i>Lefty1</i> *, <i>Dppa3</i> , <i>Gstm3</i> , <i>Irgm1</i> **, <i>Tmem63a</i> , <i>Zfp521</i> *, <i>Atp10a</i> , <i>Pde1b</i> ***†, <i>Myb</i> **, <i>Ifitm3</i> **, <i>Ttpa</i> , <i>Ppp1r1a</i> */***, <i>Trim12c</i> **	<i>Ptges</i> **, <i>Phlda1</i> , <i>Prosl</i> *
Cited2 p300	<i>Lefty2</i> *, <i>Gbp2</i> **, <i>Enpp2</i> , <i>Fndc3c1</i> , <i>Zic5</i> */***, <i>Lefty1</i> *, <i>Pim2</i> , <i>Pla2g1b</i> , <i>Nav2</i> */***, <i>Igsf11</i> , <i>Mtap7d3</i> , <i>Stc2</i> , <i>Dppa3</i> *, <i>Gstm3</i> , <i>Ifitm1</i> **, <i>Irgm1</i> **, <i>Tmem63a</i> , <i>Zfp521</i> */***, <i>Atp10a</i> , <i>Pde1b</i> */***†, <i>Ltb</i> **, <i>Csn3</i> , <i>Gabrb3</i> *, <i>Lpar4</i> , <i>Zic2</i> */***, <i>Mycn</i> */***, <i>Alg13</i> , <i>Aass</i> , <i>Dtx1</i> , <i>Ephx2</i> , <i>Foxr2</i> , <i>Myb</i> **, <i>Ifitm3</i> **, <i>Prkar2b</i> */***, <i>Psmb8</i> **, <i>Ttpa</i> , <i>Igsf10</i> *, <i>Ppp1r1a</i> */***, <i>Trim12c</i> **	<i>Msln</i> , <i>Lgals1</i> ‡, <i>Scn3b</i> , <i>Ano3</i> , <i>Parvb</i> , <i>Ptges</i> **, <i>Vamp8</i> **/†, <i>Rhox6</i> , <i>Tagln</i> , <i>Col4a2</i> , <i>Rhox9</i> , <i>Slc4a11</i> , <i>Pla2g7</i> **/†, <i>Procr</i> , <i>Phlda1</i> **, <i>Tmem150a</i> , <i>Wnt7b</i> */***, <i>Prosl</i> */**, <i>Fbp2</i> , <i>TBtg2</i>
Cited2 CBP	<i>Lefty2</i> *, <i>Gbp2</i> **, <i>Lefty1</i> *, <i>Dppa3</i> , <i>Gstm3</i> , <i>Irgm1</i> **, <i>Tmem63a</i> , <i>Zfp521</i> *, <i>Atp10a</i> , <i>Pde1b</i> ***, <i>Myb</i> **, <i>Ifitm3</i> **, <i>Ttpa</i> , <i>Ppp1r1a</i> */***, <i>Trim12c</i> **, <i>Irf1</i> **	<i>Anxa1</i> */†, <i>Nckap1</i> ‡, <i>Ccser1</i> , <i>Ptges</i> **, <i>Lyn</i> */†, <i>Phlda1</i> **, <i>Itipr12</i> , <i>Prosl</i> */**

*, ** and *** indicate genes associated with mesodermal commitment and neural development, IFN signaling and the response to infection, and hippocampal dentate gyrus formation, respectively. † indicates genes involved in the positive regulation of the inflammatory response and hematopoietic processes.

Since p300 and CBP exhibit both overlapping and distinct regulatory functions, we aimed to refine the list of genes that are potentially co-regulated by *Cited2* and either p300 or CBP. To achieve this, we first compared the expression profiles of genes in *Cited2*- and

p300-depleted ESC and found that 16% of the genes downregulated in *Cited2*-depleted cells (39 out of 231) were also downregulated in p300-depleted cells (Table 2). This gene set includes *Ifitm1*, *Ifitm3* and *Gbp2* which are involved in responses to type I and II IFN, and many of these genes are targets of core pluripotency transcription factors network, such as Tcf3, Nanog, Smad3, Sox2, Oct4, and Klf2/4/5 in mouse ESC, based on Enrichr GO and ChEA-2022 analysis (Supplementary Table S5A,B). We also compared the gene expression profiles of *Cited2*- and CBP-depleted ESC. Twenty-six genes were downregulated in both conditions (Table 2), representing 11% of the genes downregulated in *Cited2*-depleted cells. Similar to the overlap observed with p300, GO analysis of biological processes by Enrichr for genes commonly affected by *Cited2* and CBP identified *Lefty1*, *Lefty2*, *Ifitm3*, *Irf1*, and *Gbp2*, but the enrichment did not reach statistical significance (Supplementary Table S6A). Commonly downregulated genes in *Cited2* and CBP-depleted cells are also targets core pluripotency transcription factors, including Nanog, Smad3, Oct4, Klf2/4/5 and Sox2 among others (Supplementary Table S6B). Additionally, a subset of these shared genes is involved in inflammatory responses and hematopoietic processes (Table 2).

Genes downregulated in both *Cited2*- and p300- or CBP-depleted cells were mainly associated with IFN response pathways, although statistical significance was observed only for the *Cited2*-p300 overlap. Notably, this shared gene set includes *Lefty1*, *Lefty2*, and *Zic2*, key regulators of early embryonic patterning, mesoderm specification, and neurogenic competence. These observations argue against broad transcriptional equivalence and instead indicate that *Cited2* intersects with p300/CBP activity at a restricted set of developmentally relevant genes, consistent with a role in fine-tuning or stabilizing p300/CBP-dependent transcription at specific regulatory nodes rather than acting as a global co-activator of pluripotency-associated programs.

Accordingly, the overlap among upregulated genes was minimal and largely restricted to stress- and damage-related pathways. Specifically, 12% of genes upregulated upon *Cited2* depletion were also upregulated following p300 depletion (Table 2). This shared gene set includes *Vamp8*, *Lgals1*, and *Pla2g7*, which are associated with inflammatory responses, as well as *Vamp8*, *Procr*, and *Btg2*, which participate in p53-mediated regulatory pathways. A smaller overlap (4.7%) was observed with CBP depletion and included genes implicated in immune and hematopoietic regulation, such as *Anxa1* and *Lyn* (Table 2). These observations are consistent with the established roles of *Cited2* in immune regulation and hematopoietic stem cell maintenance [8,69], and suggest that *Cited2* loss may predispose ESC toward stress- and immune-associated transcriptional states. While Nckap1 is best known for its role in actin cytoskeleton organization, emerging evidence suggests that it may also participate in immune-related regulatory processes (Table 2).

To further assess potential cooperation (or lack of) between CITED2, p300, and CBP, we performed transient transfections in E14TG2A ESC using a luciferase reporter plasmid driven by ~6 kb of the *Nanog* promoter (pNanog-L), together with expression plasmids for human FLAG-CITED2, p300, CBP, or control vectors, as previously described [35,46,47]. The individual co-transfection of either flag-CITED2, p300 or CBP expression plasmids significantly increased pNanog-L activity by approximately 2-folds in comparison to control empty expression vectors (Figure 3A), confirming results previously reported for CITED2 [35], and p300 and CBP [32,34,36]. However, the co-transfection of either flag-CITED2 with p300 or flag-CITED2 with CBP expression vectors did not result in additive or synergistic effects on *Nanog* promoter activity, supporting a model in which CITED2, p300, and CBP can each enhance *Nanog* promoter activity, but without evidence of cooperative activation. However, this result does not exclude cooperation at other endogenous *loci* or chromatin contexts, which is consistent with our transcriptomic cross-analyses, which revealed limited overlap between genes dysregulated by *Cited2* depletion and those by

p300 or CBP knockdown. Overall, these observations indicate that *Cited2* depletion induces a transcriptional program largely distinct from published p300- and CBP-depletion signatures in mouse ESC. However, cross-study comparisons may be influenced by differences in experimental design. Therefore, the limited overlap should be interpreted cautiously and requires experimental validation.

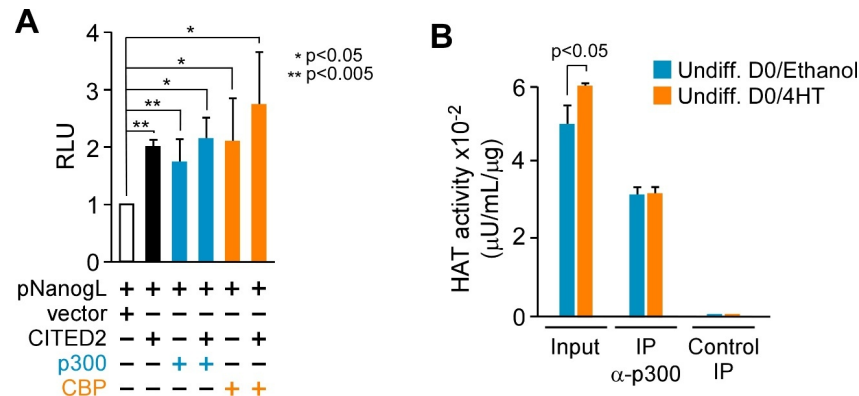


Figure 3. *Cited2* and p300/CBP exhibit limited functional overlap in mouse ESC. **(A)** pNanog-L activity in E14TG2A ESC co-transfected with pPyCAGIP-flagCITED2 (black bar) or pPyCAGIP (white bar), with or without pcDNA3-p300 (blue bars) or pcDNA3-CBP (orange bars). Gene expression in cells transfected with pNanog-L and control vector was set to 1. Results are presented as the mean $\pm$ SEM of three biological experiments, each measured in technical duplicate. **(B)** *Cited2*-depletion in ESC modestly increased global histone acetyltransferase activity. *C2^{f/f}[Cre]* ESC maintained in undifferentiating conditions, treated with ethanol (Undiff. D0/Ethanol) or 4HT (Undiff. D0/4HT) for 48 h. Cell extracts were subjected to immunoprecipitation using either an antibody specific to p300 (IP α -p300) or an anti-Flag antibody as a control (Control IP). Immunoprecipitated proteins were used to assess the histone acetyltransferase (HAT) activity, which were normalized to the total protein content. 40% of the total whole lysate used for IP (Input) was used for HAT assay. Results are shown as mean $\pm$ SEM from at least two biological replicates, each measured in technical duplicate.

3.4. *CITED2* Regulates Chromatin Acetylation Capacity Beyond p300 HAT Activity

Given the established role of p300 as the dominant H3K27 acetyltransferase (HAT) in mouse ESC [34], and previous reports indicating that *CITED2* can inhibit p300 HAT activity [70], we assessed, whether *Cited2* depletion alters p300-dependent acetylation in ESC. To this end, whole protein extracts from ESC cultured in a medium supporting self-renewal, treated for 48 h with either 4HT to deplete *Cited2* or ethanol as a control. The extracts were immunoprecipitated with either anti-p300 or control antibodies to assess HAT activity in vitro, which was then compared to the HAT activity in crude whole-protein extracts (Figure 3B).

The results showed a modest but significant increase in H3 acetylation in crude extracts from *Cited2*-depleted cells compared to controls. However, no difference in HAT activity was observed between p300-immunoprecipitated extracts from *Cited2*-depleted and control cells. Thus, the increased histone acetylation capacity observed upon *Cited2* depletion occurs in the absence of measurable changes in p300 HAT activity under the conditions tested. Alternatively, the lack of detectable change in p300 immunoprecipitates may reflect locus-specific or chromatin-dependent regulation not captured by the in vitro assay.

3.5. *Cited2* Loss Induces Stress-Associated Transcriptional Programs and Compromises Genome Maintenance

The transcriptomic analysis of genes dysregulated upon *Cited2*-depletion in ESC revealed that genes involved in DNA repair and homologous recombination, such as

Neil3, *Rad51c*, *Fancd2*, *Prkdc*, *Rpa3*, *Poli* and *Brca2*, also exhibited reduced expression (Supplementary Table S2B), alongside the upregulation of several apoptosis-regulating genes (Supplementary Tables S3B). Notably, *Bcl2l1*, *Hmox1*, *Thbs1*, *Krt18*, *Plk2*, *Htra1*, and *Gadd45g* are direct targets of p53, suggesting an increase of p53 activity in *Cited2*-depleted cells compared to control cells. To validate the microarray data, qRT-PCR were performed to assess the expression of genes upregulated in *Cited2*-depleted ESC, including *Fhl2*, *Casq2*, *Lin28a*, and *Lmna*. Additionally, we examined genes associated with apoptosis and up-regulated by p53 (*Bmi1*, *Hmox1*, *Ndr1*, *Parp1*, *Perp*, *Ptges*, *Plk2*, *p53*, *Rbm24*, and *p21* as a positive control), as well as genes involved in DNA repair (*Rad51c*, *Rad9b*, and *Mdc1*). *Grp50*, a gene unaffected by *Cited2* depletion in the microarray data, was also included as a control (Figure 4A). The qRT-PCR confirmed the enhanced expression of *Fhl2* and *Lmna* upon depletion of *Cited2* in ESC, while the expression of *Gpr50* was not significantly altered.

Interestingly, the expression of most p53-target genes was significantly increased by 2- to 5-folds in *Cited2*-depleted cells. The expression of *Rad51c* and *Mdc1*, genes related to DNA repair, was significantly decreased by 1.5- to 2-folds in *Cited2*-depleted cells, suggesting that DNA repair processes may be impaired in these cells (Figure 4A), which would be consistent with the spontaneous differentiation and increased apoptosis previously detected in *Cited2*-depleted mouse ESC [35]. *Cited2* has been shown to maintain low levels of reactive oxygen species (ROS) production in hemopoietic stem cells (HSC) by promoting glycolysis and suppressing mitochondrial respiration [69]. *Cited2* depletion in these cells leads to increased ROS production and activation of p53 activation which triggers cell cycle arrest and apoptosis, contributing to the exhaustions of HSC pool [69]. Interestingly, ESC treated with 4HT for 24 h to deplete *Cited2* exhibited increased general ROS production compared to ethanol-treated control cells (Supplementary Figure S1A), suggesting a higher level of oxidative stress in *Cited2*-depleted cells, which potentially may lead to DNA damage and activation of the p53 pathway, also suggested by the upregulation of p53 target genes (Figure 4A). Therefore, we assessed the number of nuclear γ H2AX foci (phosphorylated histone H2AX at Ser139, a marker activated by DNA double-strand breaks (DSB) recruited to DNA break sites) in control and *Cited2*-depleted ESC (Figure 4B–D, and Supplementary Figure S1D). No significant difference was observed in the number of γ H2AX foci present in the nuclei of both cell types (Figure 4C). However, a modest but significant increase in the intensity of γ H2AX foci was evidenced in *Cited2*-depleted cells (Figure 4D and Supplementary Figure S1D). In addition, the expression of γ H2AX detected by western blotting was also elevated upon 4HT and H_2O_2 treatment compared to control conditions (Figure 4E and Supplementary Figure S1C). Overall, these observations suggested that *Cited2*-depleted cells may not accumulate more DSB than control cells. However, the increased intensity of γ H2AX foci argues that *Cited2*-depleted cells experience greater stress or reduced efficiency in repairing existing DSB, which is consistent with the downregulation of genes associated to repair in those cells (Figure 4A).

To assess whether p53 overactivation contributes to the phenotype of *Cited2*-depleted ESC, *C2^{fl/fl}*[Cre] cells were treated with 4HT to induce *Cited2* depletion in the presence of increasing concentrations of the selective p53 inhibitor, pifithrin- α . As previously described [35], 4HT treatment (with DMSO as vehicle) caused increased of culture cellular debris and reduced colony and cell numbers compared to ethanol- and DMSO-treated controls (Figure 4F). One-way ANOVA revealed a significant effect of pifithrin- α on cell proliferation, $F_{(4,40)} = 3.194$, $p = 0.0229$, and post hoc Dunnett's tests (4HT/DMSO as control) showed that 0.5 μ M and 1 μ M pifithrin- α significantly increased cell counts at 48 h (mean differences -30.33 and -31.89 cells, 95% CIs $[-57.79, -2.88]$ and $[-59.34, -4.44]$, adjusted $p = 0.026$ and 0.018), whereas 5 μ M and 10 μ M had no significant effect (adjusted $p = 0.38$ and 0.79 ; Figure 4F, right panel). The elevated p53 activity observed in *Cited2*-depleted ESC,

together with increased apoptosis and spontaneous differentiation, is in line with previous reports describing the interplay between p53 and Cited2 in other cell types [35]. The treatment with pifithrin- α at 0.5–1 μ M which partially improved cell survival and colony morphology (Figure 4F, left panels), suggests a potential contribution of p53-associated pathways to the observed phenotypes of Cited2-depleted cells. However, as pifithrin- α can have off-target effects and dose-dependent toxicity, these results support a potential involvement of p53 without establishing direct dependence.

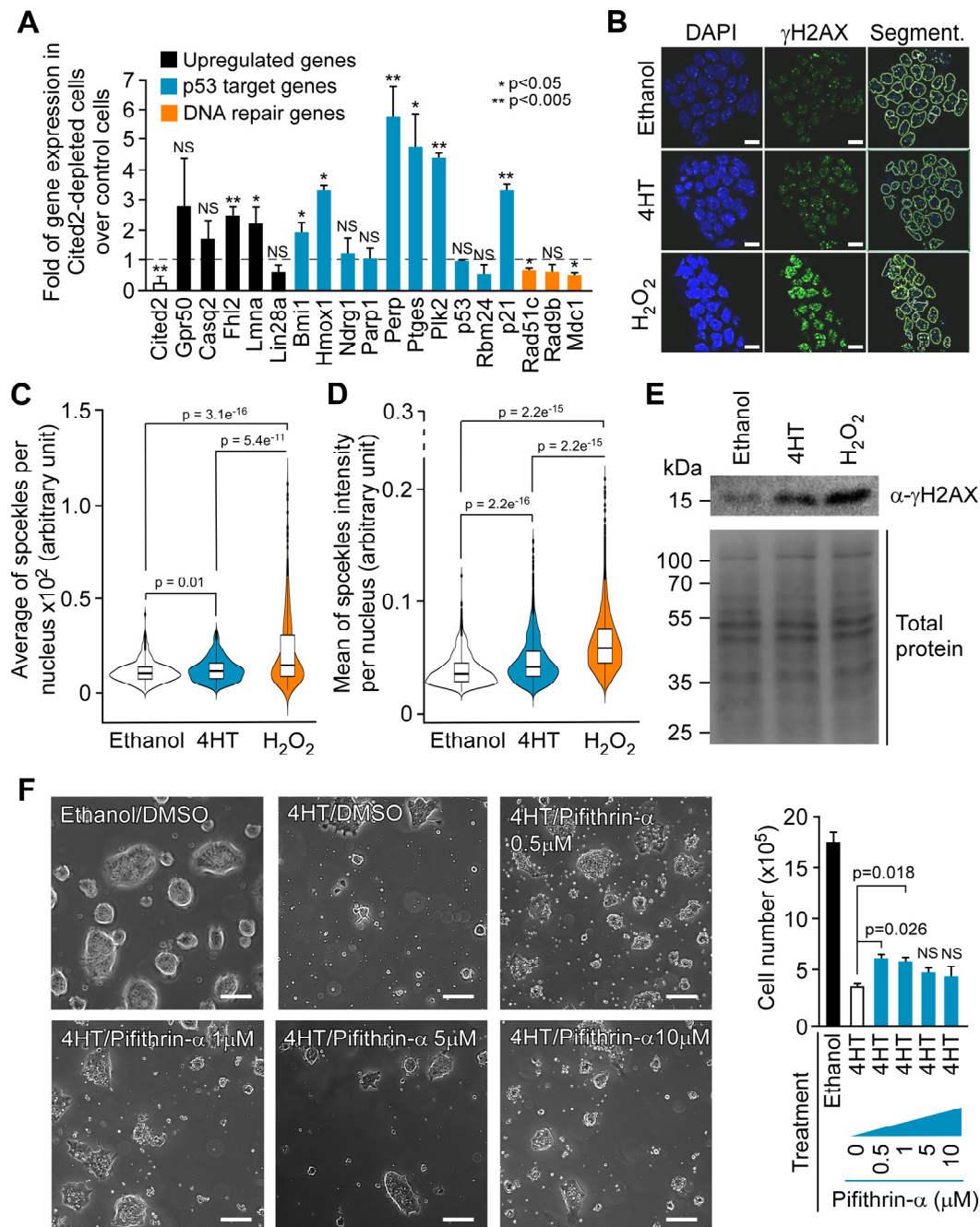


Figure 4. *Cited2* depletion in ESC increases the risk for DNA damage. (A) Relative expression of p53-target and other upregulated genes and DNA repair related genes determined by qRT-PCR in C2^{fl/fl}[Cre]ESC treated either with treated ethanol or 4HT for 48 h. Gene expression in ethanol/vehicle conditions was set to 1. Means were compared with the control condition using a *t*-test considering the *p* value threshold of 0.05 for significance. Bars represent mean $\pm$ SEM of three independent biological triplicates, each measured in technical duplicate. NS means “not significant”. (B) Detection of γ H2AX

nuclei in C2^{fl/fl}[cre] cells cultured in undifferentiating conditions treated with 1 μ M 4HT (4HT) for 16 h, 30 min with 100 μ M hydrogen peroxide (H₂O₂) or ethanol as a control, using an anti- γ H2AX antibody (green). Nuclei were stained with DAPI (blue). Nuclei and the γ H2AX speckles were determined (Segment.) using the segmentation pipeline in Cell Profiler. Scale bars represent 10 μ m. (C) Images acquired as described in B, were used to count the number of γ H2AX speckles per nucleus. (D) The mean of the intensity of the γ H2AX speckles per nucleus was also determined. Means were compared with the control condition using a *t*-test considering the *p* value threshold of 0.05 for significance. The results are from three independent biological experiments, and 321 to 411 nuclei were analyzed per condition. (E) γ H2AX protein levels determined by western blotting in protein extracts from cells treated as in B. Bottom panel: Total protein transferred on the PVDF membrane stained with *No-Stain whole protein labelling* (Thermo Fisher, A44717) used to control for loading. (F) Proliferation of C2^{fl/fl}[Cre]ESC treated either with treated ethanol and DMSO vehicle (Ethanol/DMSO), 4HT and DMSO (4HT/DMSO) or 4HT plus increasing concentrations of pifithrin- α (4HT/pifithrin- α). Left panel: representative images of the colonies at 48 h after treatment. Scales bars, 250 μ m. Right panel: Cell counts at 48 h (mean $\pm$ SEM; *n* = 9 wells per group, from three independent biological triplicates, each measured in technical triplicate). Data were analyzed by one-way ANOVA followed by Dunnett's test versus 4HT/DMSO ($F_{(4,40)} = 3.194$, *p* = 0.0229). NS means "not significant".

In addition to canonical p53 targets, several genes upregulated upon CITED2 depletion, including *Ndr1*, *Hmox1*, *Bcl2l1*, and *Thbs1*, are established direct HIF-1 α targets [71–73], whereas others, such as *Plk2*, *Krt18* and *Gadd45g*, are regulated in a hypoxia- and HIF1-dependent manner under specific stress contexts. Given that CITED2 negatively regulates HIF1 α activity through competition for p300/CBP binding [19,20], we hypothesized that Cited2 depletion in mouse ESC enhances HIF1 α -mediated transcription. To evaluate this, we performed transient transfection assays in C2^{fl/fl}[Cre] ESC treated or untreated with 4HT using the 3xHRE-Luc reporter in to quantify HIF-dependent transcriptional activity [48]. We observed a significant increase in 3xHRE-Luc in Cited2-depleted cells (Supplementary Figure S1E), indicating elevated HIF activity in the absence of Cited2. Notably, sustained or excessive HIF1 α activation has been reported to trigger stress responses and apoptosis in mouse ESC under hypoxic or stress conditions, indicating that tight regulation of HIF1 α activity is critical for ESC survival [74,75]. Together, these results raise the possibility that enhanced HIF1 α activity contributes to apoptosis following Cited2 depletion, a hypothesis that will require further mechanistic validation.

3.6. Impact of Cited2 Depletion on H3K27 Methylation and Acetylation Status in ESC

As outlined by the transcriptomic analysis, the depletion of Cited2 in ESC resulted in the modulation of the expression of Suz12-target genes (Supplemental Table S3B). Since Suz12 is a core component of the PRC2 complex, which represses transcriptional activity via H3K27 methylation, we performed chromatin immunoprecipitation (ChIP) assays to assess the H3K27 trimethylation (H3K27me3) status at the promoters of *Mesp1*, *Isl1*, *Dkk1*, *Wnt5a* and *Kdr* genes, which are critical for early differentiation commitment and previously identified as Cited2 target genes [15,39]. Extracts for ChIP were harvested from ESC maintained under conditions supporting self-renewal (termed D0, for text simplification) and cells at D4 of differentiation (Figure 5A). As expected, all tested regulatory elements showed a decreased recruitment of H3K27me3 at D4 of differentiation compared to D0, which is consistent with their early expression upon the initiation of differentiation. Although the regulatory regions of the *Mesp1*, *Kdr*, *Dkk1*, and *Wnt5a* promoters exhibited a reduced occupancy by H3K27me3 in Cited2-depleted cells compared to their respective controls at D0, this reduction only reached statistical significance for *Dkk1* (Figure 5A). The tendential decrease in H3K27me3 recruitment to *Mesp1*, *Dkk1* and *Kdr* is consistent with the increased transcript expression observed for these genes under these conditions (Figure 2A). Given the observed increase in overall acetyltransferase activity in crude whole

cell extracts from *Cited2*-depleted cells (Figure 3B), we assessed the acetylation status of H3K27, a marker of transcriptionally active chromatin, at the same promoters. Depletion of *Cited2* did not significantly affect the acetylation status of the promoters tested at the examined time points, except for the *Wnt5a* promoter, which exhibited reduced H3K27ac recruitment at day 4 of differentiation (Figure 5B). This observation is in agreement with previous observations of decreased *Wnt5a* transcript and protein expression, as well as reduced extracellular secretion, during ESC differentiation [15].

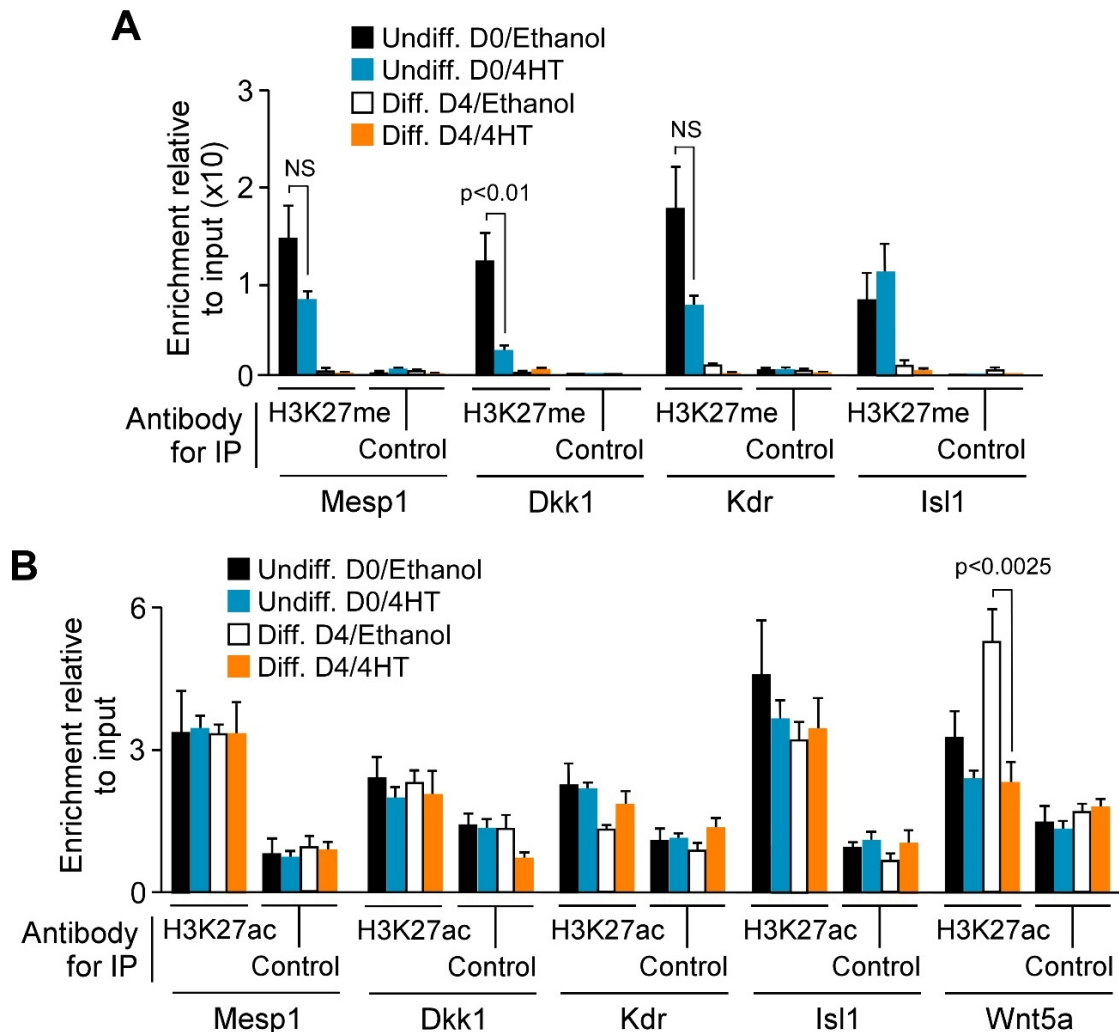


Figure 5. *Cited2* depletion decreases the H3 acetylation status of *Wnt5a* promoter in ESC. (A) Chromatin immunoprecipitation (ChIP) from $C2^{fl/fl}[Cre]$ ESC with treated ethanol (Undiff. D0/Ethanol) or 4HT for 48 h (Undiff. D0/4HT) and in cells differentiated for 4 days upon treatment with ethanol (Diff. D4/Ethanol) or 4HT (Diff. D4/4HT) for the first 48 h of differentiation, using an antibody against trimethylated histone H3 on the lysine 27 (H3K27me) and a control antibody to the flag epitope (Control). The enrichments relative to the input (10% of the extracts used for ChIP) are presented. Results are shown as mean $\pm$ SEM from three independent biological experiments, each measured in technical duplicate. No statistically significant (NS) differences were observed for *Mesp1* ($p = 0.56$) or *Kdr* ($p = 0.075$). (B) ChIP performed as in A, using a polyclonal antibody against acetylated histone H3 on the lysine 27 (H3K27ac) and a control polyclonal antibody against rabbit-IgG (Control). T-test considering the p value threshold of 0.05 for significance was applied and revealed only a statistical significance for the promoter of *Wnt5a* between the conditions D4/Ethanol and D4/4HT.

Collectively, these findings support a model in which *Cited2* is not required for the global establishment or removal of H3K27me3 or H3K27ac, but instead contributes to the

fine-tuning of chromatin states at a restricted set of developmental regulatory *loci*. The selective reduction of Polycomb-mediated repression at genes such as *Dkk1*, coupled with largely preserved promoter acetylation, suggests that loss of *Cited2* creates a chromatin environment that is permissive yet improperly configured for productive lineage engagement. This imbalance may underlie the uncoupling of pluripotency maintenance from developmental competence observed in *Cited2*-depleted ESC.

4. Discussion

Cited2 is a multifunctional transcriptional co-regulator that integrates developmental signaling, transcriptional control, and cellular stress responses across multiple tissues, including the placenta, heart, and nervous system [1–11,29,76]. In this study, we investigated the mechanistic role of *Cited2* in mouse ESC by analyzing the immediate transcriptional and functional consequences of its acute depletion. Our observations revealed that loss of *Cited2* rapidly destabilizes the pluripotent state, perturbs key signaling pathways, compromises genome integrity, and induces spontaneous and dysregulated differentiation programs. A striking feature of *Cited2* depletion is the rapid transcriptional response, with substantial gene expression changes detected within 48 h. This prompt effect underscores the central role of *Cited2* in sustaining ESC identity and survival. Acute *Cited2* loss led to downregulation by widespread changes in the expression of genes controlled by the core pluripotency transcriptional network, which is consistent with previous observations [35]. These findings reinforce the concept that *Cited2* is not a peripheral modulator but a critical component of the transcriptional circuitry that maintains ESC self-renewal.

Concomitant with the loss of pluripotency-associated gene expression, *Cited2*-depleted ESC exhibited premature activation of differentiation-associated transcriptional programs. A substantial fraction of the genes upregulated upon *Cited2* depletion are targets of *Suz12*, a core component of the PRC2 complex that represses developmental regulators in undifferentiated ESC [67]. These observations indicate that *Cited2* is required not only to sustain the expression of core pluripotency factors, but also to the restraint of the expression of differentiation-associated genes, thereby preventing spontaneous and aberrant differentiation as well as increased cell death in mouse ESC. This dual regulatory function highlights the role of *Cited2* in preserving the balance between self-renewal and differentiation in pluripotent stem cells. Importantly, our chromatin immunoprecipitation analyses do not support a global disruption of Polycomb-mediated repression, but they rather point to a selective and locus-specific relaxation of repressive chromatin states at a subset of developmental genes. Consistently, modest reductions in H3K27me3 at promoters such as *Dkk1*, *Mesp1*, and *Kdr*, together with largely preserved H3K27 acetylation, suggest that *Cited2* contributes to the fine-tuning of chromatin states that govern transcriptional competence rather than directly controlling global epigenetic marks. This selective chromatin permissiveness is likely to predispose ESC to inappropriate transcriptional activation without supporting orderly lineage commitment. Thus, the increased expression of *Runx2* target genes in *Cited2*-depleted ESC should not be interpreted as evidence of osteogenic fate commitment, but rather be viewed as further evidence of ectopic activation of differentiation-associated transcriptional programs following loss of pluripotency control. Similarly, several genes critical for neural development were downregulated in ESC, including *Otx2*, *Zic2*, *Zic5*, and *Enpp2* [1,6,16], but remained unchanged in *Cited2*-cKO embryos, in which *Cited2* was specifically depleted at the neural progenitor stage using *Emx1*-Cre [6], indicating that many transcriptional effects observed in vitro reflect secondary consequences of pluripotency exit rather than direct regulatory roles in later developmental contexts.

Although endogenous Activin/Nodal signaling in naïve mouse ESC is near saturation under standard culture conditions [57], our data indicate that *Cited2* is important for the full transcriptional output downstream of this pathway. Indeed, *Cited2* depletion led to the dysregulation of Activin/Nodal-activated genes with *Lefty1*, *Lefty2*, and *Pitx2* among the most strongly downregulated, in agreement with reduced Smad2/3- and Foxh1-dependent transcriptional activity. These findings are in line with in vivo observations, as reduced expression of *Lefty* genes and *Pitx2* has been reported in *Cited2*-null embryos [2,35,77]. Notably, *Cited2*-depleted ESC simultaneously upregulated markers associated with early mesodermal and extraembryonic programs, including *Mesp1*, *Kdr*, *Dkk1*, and *Cdx2*. Rather than reflecting directed lineage commitment upon *Cited2* depletion, this transcriptional pattern is more consistent with ectopic activation of differentiation-associated programs resulting from the loss of pluripotency gatekeeping. This interpretation is further supported by the observation that, during controlled embryoid body at day 4 of differentiation, these same genes are downregulated [15,39]. Indeed, self-renewal and differentiation in ESC are tightly interconnected, and defects in the pluripotency network can predispose cells to inappropriate differentiation responses. *Cited2* depletion has been shown to impair self-renewal through dysregulation of core pluripotency factors such as Nanog, Oct4, Tbx3, and Klf4 [35,53], suggesting that the altered differentiation patterns observed are likely secondary to compromised pluripotency rather than directed lineage commitment. In the context of *Cited2*-depleted cells, the attenuation of Activin/Nodal signaling combined with an overactivation of canonical Wnt/ β -catenin signaling suggested by our observations may create a permissive environment for premature differentiation rather than instructing specific lineage programs. These signaling imbalances are predicted to weaken pluripotency gatekeeping, promote premature exit from pluripotency, and bias subsequent cell fate decisions, in agreement with previous studies linking such pathway perturbations to altered ESC fate outcomes [78–80]. Together, these observations highlight the context-dependent role of *Cited2*, which is required for proper lineage engagement during differentiation, whereas its loss under self-renewal conditions triggers uncoordinated and non-physiological transcriptional activation.

Beyond differentiation defects, *Cited2* depletion in ESC profoundly affects genome maintenance and cell survival by activating stress-associated transcriptional programs. Loss of *Cited2* led to early increases in γ H2AX foci intensity, indicative of DNA damage stress, along with downregulation of DNA repair genes such as *Rad51c* and *Mdc1*. Concurrently, p53 target genes, including *Plk2*, *Hmox1*, and *Gadd45g*, were upregulated, reflecting an enhanced p53 activity. Mechanistically, *Cited2* is known to interact with p300/CBP and modulate p53 transactivation, providing a pathway through which its loss impairs DNA repair and cell-cycle control [81]. Notably, genomic regions in mouse ESC that retain H3K27ac after p300 depletion are enriched for p53-binding sites [34], supporting convergence on stress-responsive regulatory circuits in *Cited2*- and p300-depleted mouse ESC. Elevated ROS in *Cited2*-depleted ESC and HSC may further exacerbate DNA stress, promoting activation of p53 and apoptosis [69,82]. Pharmacological inhibition of p53 with pifithrin- α partially rescued colony morphology and proliferation, indicating that hyperactive p53 contributes to, but does not fully account for, the phenotype. Together, these findings support a model in which *Cited2* safeguards genome integrity and differentiation by maintaining DNA repair gene expression, limiting oxidative stress, and restraining p53 activation. In addition to p53 signaling, our data are consistent with altered HIF-responsive transcription following *Cited2* depletion. Several genes upregulated in *Cited2*-depleted ESC are established or context-dependent HIF1 α targets, and concordantly, HIF-dependent transcriptional activity, as assessed using a reporter assay, was increased those cells. Although these observations do not establish a direct functional role for HIF1 α in the reduced

survival and increased stress response of Cited2-depleted ESC, they are notably in light of in vivo genetic evidence that has shown that partial reduction of HIF1 α levels ameliorates key developmental defects in Cited2-deficient mouse embryos [83]. Together, these observations suggest that elevated HIF-responsive transcription represents a reproducible consequence of Cited2 loss across experimental contexts, demanding further investigation into its potential contribution to stress responses in ESC.

Given the well-established interaction between CITED2 and p300/CBP, we compared the transcriptomic consequences of Cited2 depletion with those reported upon p300 or CBP knockdown in mouse ESC [34]. Surprisingly, the overlap in dysregulated genes was limited, and functional assays revealed no additive or synergistic activation of the *Nanog* promoter upon co-expression of CITED2 with p300 or CBP. Furthermore, a modest increase in global histone acetylation capacity was observed in extracts from Cited2-depleted cells, with no measurable change in p300 activity. Although Cited2 depletion did not affect HAT activity of p300, the acetylation status of *Wnt5a*, a Cited2-target gene, was decreased in Cited2-depleted cells [15]. It would be of interest to understand the molecular mechanisms that lead to the regulation of *Wnt5a* by Cited2, since the Cited2–*Wnt5a* axis appeared to play critical roles in cellular and embryonic functions [8,15]. Notably, early supplementation with recombinant *Wnt5a* rescued cardiac defects and lethality in Cited2-depleted mouse ESC and zebrafish embryo [15]. Overall, these observations suggest that Cited2 depletion elicits a transcriptional program that is largely distinct from p300/CBP depletion signatures, with convergence restricted to a subset of developmental regulators.

Our study also uncovered unexpected links between Cited2 and metabolic and immune-related gene networks. Indeed, upon Cited2 loss in mouse ESC, several amino acid transporters essential for ESC maintenance, blastocyst development, placental function, fetal growth and metabolic adaptation to the intrauterine environment [84–86], were significantly downregulated. In parallel, genes involved in antiviral and interferon-responsive pathways were also reduced, including *Ifitm1* and *Ifitm3*, which have emerging roles in trophoblast function and embryonic immune protection [87–89]. Interestingly, these genes can be expressed independently of IFN stimulation [90,91], which could explain the inconsistency with previous reports showing that Cited2 acts as a negative regulator of ISG by inhibiting *Irf1* and *Stat1*, key mediators of type I and type II IFN-activated signaling pathways [25,26]. In addition, the reactivation of endogenous retrovirus HERVK (HML-2) during early human development, driven by DNA hypomethylation and OCT4 activation, has been showed to enhance *IFITM1* expression which may confer protection to early embryonic cells from viral infections [92]. It would be important to investigate whether CITED2 mutations impair immune and antiviral responses during pregnancy, resulting in a combined detrimental effect on embryonic development and potentially compromising viability. Altogether, these findings suggest that Cited2 may contribute to developmental robustness by coordinating transcriptional programs that support nutrient uptake, stress resistance, and immune defense during early embryogenesis.

5. Conclusions

In conclusion, our findings establish Cited2 as a key regulator of ESC identity that integrates pluripotency maintenance, signaling pathway output and stress response control. Loss of Cited2 destabilizes the pluripotent state, compromises genome integrity, and allows inappropriate activation of developmental programs, leading to spontaneous differentiation and cell death. Importantly, many Cited2-regulated genes identified in ESC are directly linked to cardiac, neural, and placental development, providing a mechanistic bridge between ESC phenotypes and the complex developmental defects observed in Cited2-null embryos. These insights not only advance our understanding of Cited2 function in early

development but also have broader implications for congenital disease, stem cell biology, and stress-associated pathologies.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/cells15050450/s1>, Figure S1: Acute *Cited2* depletion increases ROS production and increases γ H2AX protein levels in ESC; Table S1: DEG in control and depleted mouse ESC; Table S2: Analysis of genes downregulated in *Cited2*-depleted cells; Table S3: Analysis of genes upregulated in *Cited2*-depleted; Table S4: Analysis of genes downregulated in *Cited2* p300 and CBP depleted ESC; Table S5: Analysis of genes affected *Cited2* p300 depleted ESC; Table S6: Analysis of genes affected *Cited2* CBP depleted ESC; Table S7: Primers used for qRT-PCR, ChIP qRT-PCR and genotyping. Refs. [8,31,39,93–118] were cited in Supplementary Materials.

Author Contributions: Conceptualization, J.B. and L.M.-S.; methodology, J.B., L.M.-S., S.M.B., J.M.A.S., R.M., M.T., L.R., A.L.E., A.S. and J.L.M.; validation, L.M.-S., M.T. and S.M.B. formal analysis, J.B., L.M.-S., S.M.B., and J.L.M.; investigation, J.B., L.M.-S., S.M.B., J.M.A.S., R.M., M.T., L.R., A.L.E., A.S. and J.L.M.; writing—original draft preparation, J.B. and L.M.-S.; writing—review and editing, J.B., L.M.-S., S.M.B., J.M.A.S., R.M., M.T., L.R., A.L.E., A.S. and J.L.M.; funding acquisition, J.B. and J.L.M. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ESC	Embryonic stem cells
<i>CITED2</i>	CBP/p300-interacting transactivator with ED-rich tail 2
H3K27	Histone H3 at the lysine 27
HIF1 α	Hypoxia-Induced Factor-1 α
HSC	Hemopoietic stem cells
IFN	Interferon
ISG	Interferon-stimulated genes
PcG	Polycomb group

PRC2	Polycomb Repressive Complex 2
LIF	Leukemia Inhibitory Factor
iPSC	induced pluripotent stem cells
4HT	4-hydroxytamoxifen
PCA	Principal component analysis
DEG	Differentially expressed genes
GO	Gene ontology
EB	Embryoid body
DSB	DNA double-strand breaks
ChIP	Chromatin immunoprecipitation
NS	Not statistically significant

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