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Transcriptional control of DNA replication licensing by Myc

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The c-*myc* protooncogene encodes the Myc transcription factor, a global regulator of fundamental cellular processes. Deregulation of c-*myc* leads to tumorigenesis, and c-*myc* is an important driver in human cancer. Myc and its dimerization partner Max are bHLH-Zip DNA binding proteins involved in transcriptional regulation of target genes. Non-transcriptional functions have also been attributed to the Myc protein, notably direct interaction with the pre-replicative complex (pre-RC) controlling the initiation of DNA replication. A key component of the pre-RC is the Cdt1 protein, an essential factor in origin licensing. Here we present data suggesting that the *CDT1* gene is a transcriptional target of the Myc-Max complex. Expression of the *CDT1* gene in v-*myc*-transformed cells directly correlates with *myc* expression. Also, human tumor cells with elevated c-*myc* expression display increased *CDT1* expression. Occupation of the *CDT1* promoter by Myc-Max is demonstrated by chromatin immunoprecipitation, and transactivation by Myc-Max is shown in reporter assays. Ectopic expression of *CDT1* leads to cell transformation. Our results provide a possible direct mechanistic link of Myc's canonical function as a transcription factor to DNA replication. Furthermore, we suggest that aberrant transcriptional activation of *CDT1* by deregulated *myc* alleles contributes to the genomic instabilities observed in tumor cells.

The *myc* oncogene was originally discovered as the transforming principle in the genome of avian acute leukemia virus MC29[1], representing a transduced retroviral allele (v-*myc*) derived from the chicken cellular protooncogene c-*myc*[2,3]. The Myc protein product, initially identified as a Gag-Myc hybrid protein specified by MC29[4], is a transcription factor with strong oncogenic potential[2,3,5,6]. Myc is a bHLH-Zip protein, forms heterodimers with the Max protein, binds to specific DNA sequence elements (E-boxes), and is the central hub of a global transcriptional regulator network[7,8]. Myc and Max homologs with conserved basic functions were found in primitive metazoans[9], and there is even evidence for a premetazoan evolutionary origin of Myc and Max proteins[10]. The human Myc transcription factor network controls thousands of genes involved in fundamental cellular processes including growth, proliferation, differentiation, metabolism, and apoptosis[5,6,11–13]. The basic function of the Myc-Max transcription factor complex is transcriptional activation of distinct target genes, but Myc has also been implicated in transcriptional repression of specific genes[5,6,11]. The target genes transcriptionally activated by Myc-Max are related to important pathways of cell growth and metabolism, including protein synthesis, ribosomal biogenesis, glycolysis, mitochondrial function, and cell cycle progression[5,6,11,14]. The genes repressed by Myc are typically involved in cell cycle arrest, cell adhesion, and cell-to-cell communication[5,6,11], or encode inhibitors of Myc-induced cell transformation[15]. The discovery of rearrangements and transcriptional deregulation of the human *MYC* gene in Burkitt's lymphoma was the first indication that the cellular homolog of the retroviral v-*myc* oncogene is involved in human tumorigenesis[16], and it is now established that *MYC* is one of the crucial drivers in many, if not most human cancers[3,5,6,17].

Myc has also been associated with non-transcriptional functions, possibly not even requiring dimerization with Max[5,6]. An important example is non-transcriptional control of DNA replication by Myc[18], providing a possible link to genomic instability typically observed in cells with deregulated Myc expression[19–21]. Genetic instabilities, including changes at the nucleotide level, aneuploidy, chromosome translocations, and gene amplification, are a hallmark of many human cancers[22,23]. It has been proposed that the non-transcriptional control of DNA replication involves direct interaction of the Myc protein with components of the pre-replicative complex (pre-RC)[18,24]. Eukaryotic DNA replication is tightly regulated both spatially and temporally to ensure correct copying of the entire genome only once in every cell cycle. To prevent rereplication, licensing of specific replication origins in the G1 phase of the cell cycle is achieved by the assembly of the pre-RC onto chromatin, starting with

recruitment of the origin recognition complex (ORC), followed by loading of the minichromosome maintenance complex (MCM) mediated by the Cdc6 and Cdt1 proteins, and additional replication proteins[25,26]. The Cdt1 protein, originally identified in yeast and then in insects and vertebrates[26–29], promotes the loading of MCM and is the key factor in the licensing process. In higher eukaryotes, Cdt1 activity is therefore strictly regulated by ubiquitin-dependent degradation and binding of the specific inhibitor geminin to ensure temporal confinement of licensing to the G1 phase[30,31]. Here we report that the *CDT1* gene is a transcriptional target of the Myc-Max complex and that deregulated Myc expression in transformed cells leads to increased expression of the essential DNA replication factor Cdt1. Our results suggest a direct implication of Myc's fundamental function as a transcriptional regulator in genomic instabilities observed in tumor cells.

Results

Activation of *CDT1* in *myc*-transformed cells. Using a conditional cell transformation system in which expression of the MC29 v-*myc* allele is controlled by doxycycline[32], several partial cDNA clones representing candidate *myc* target genes were isolated by representational difference analysis (RDA), a polymerase chain reaction (PCR)-based subtractive hybridization procedure. One of these clones was of particular interest since it proved to be derived from the gene encoding the DNA replication licensing factor Cdt1, providing a possible link of Myc transcription factor function with DNA replication. The tight correlation of v-*myc* and *CDT1* mRNA levels was demonstrated in the conditional cell transformation systems Q/tM$_{ON}$ and Q/tM8 in which v-*myc* expression is controlled by a doxycycline-dependent or a doxycycline-inhibited transactivator, respectively[32]. In time course experiments, expression of *CDT1* mRNA closely parallels that of the conditional v-*myc* alleles in both cell systems, and every activation/deactivation of the oncogene by addition or removal of the drug, even repeatedly, is precisely reflected in concurrent changes in *CDT1* expression (Figure 1a and Supplementary Figure 1). Notably, the *CDT1* expression pattern parallels that of the specific *myc* target *WS5* (also called *Mmp115* in the chicken genome) encoding a protein related to human melanoma glycoproteins[33], and is exactly opposite to the expression pattern of *BASP1*, a specific target suppressed by Myc and shown to be an inhibitor of Myc's transforming function[15]. Comparative expression analyses were also performed with normal quail embryo fibroblasts (QEF) or quail cell lines transformed by v-*myc* (Q8, QEF/MC29, QEF/Rc-Myc), v-*jun* (VJ, VCD), v-*src* (R(-)3), or by a chemical carcinogen (QT6). All rapidly growing transformed cells contain elevated levels of *CDT1* mRNA, but the highest levels were found in v-*myc*-transformed cells (Figure 1b). Expression of the *myc* target *WS5* was used as a control. Simultaneous activation of *CDT1* and the *WS5/Mmp115* control gene was also observed in chicken embryo fibroblasts (CEF) transformed by the MC29 v-*myc* allele (Figure 1c). Furthermore, expression analysis using the human leukemia cell lines K-562, MOLT-4, and HL-60, and the colon carcinoma line SW-480 revealed a strong correlation of elevated c-*myc* mRNA levels with *CDT1* expression (Figure 1d), indicating that deregulated c-*myc*, like v-*myc*, is possibly involved in *CDT1* activation. To test if *CDT1* activation by v-*myc* is also detectable at the protein level, a 726-bp fragment of quail *CDT1* cDNA (see below) was cloned into prokaryotic expression vector pET-21a and used for the production of a truncated Cdt1 recombinant protein. The purity and identity of recombinant Cdt1(200–441) was verified by mass spectrometry and fragment ion mapping (Supplementary Figure 2), and the protein was used for the generation of a polyclonal antiserum. Using this serum, a translational product with an apparent M_r of 74,000 (p74^{CDT1}) was detected in the Q8 and QEF/MC29 cell lines which are transformed by the p110 Gag-Myc hybrid protein, but not in normal QEF (Figure 1e).

Structural organization of the *CDT1* gene and protein. A full-length 2,162-bp *CDT1* cDNA clone was assembled from overlapping clones obtained by screening a cDNA library from the MC29-transformed quail cell line Q8[4] with a *CDT1* RDA fragment (see above), and from four overlapping 5′ RACE products. The deduced 588-amino acid quail Cdt1 protein has a calculated M_r of 64,690 and an isoelectric point of 9.94. An alignment of the amino acid sequences of the quail Cdt1 protein and the chicken, mouse, and human homologs revealed extensive sequence similarities (Supplementary Figure 3), particularly in the binding domains for geminin and the MCM complex[34,35]. To determine the structural organization of the *CDT1* gene, the nucleotide sequence of a 5,677-bp quail genomic DNA segment hybridizing with a *CDT1* cDNA probe was determined. The quail *CDT1* gene contains 10 exons and displays a similar architecture (Figure 2a) like its chicken ortholog (contig NC_006098). The nucleotide sequences of the quail and chicken *CDT1* promoter regions are highly conserved and contain two canonical Myc binding sites (CACGTG) immediately upstream of the transcriptional start sites (Figure 2b). Comparison of the avian *CDT1* promoters with those from mouse and human (Figure 2c) showed that the mammalian regulatory regions also contain several canonical and non-canonical E-boxes within 2 kbp upstream of the transcription start sites. A further common feature of the avian and mammalian promoters is the presence of a consensus E2F binding site in close proximity to the transcription start site (Figure 2c).

Myc binds to the *CDT1* promoter. To test if Myc binds to the *CDT1* promoter *in vivo*, chromatin immunoprecipitation (ChIP) analysis was performed using chromatin from normal QEF and from QEF transformed by MC29 encoding the p110 Gag-Myc hybrid protein[4], or by the Rc-Myc construct encoding a p52 v-Myc protein not fused to viral structural proteins[15]. Both QEF/MC29 and QEF/Rc-Myc contain high levels of *CDT1* mRNA (cf. Figure 1b). Antisera directed against Myc or Max, and NRS as a control, and PCR primers flanking the canonical E-boxes in the *CDT1* promoter (cf. Figure 2b) or primers flanking the Myc binding sites in the bona fide Myc target *WS5*[33] were used (Figure 3a). Strikingly, the principal ChIP pattern obtained with the *CDT1* primers is nearly identical to that obtained for the *WS5* promoter. In normal QEF, the *CDT1* and *WS5* promoters are mainly occupied by Max which also forms stable homodimers, and by only low amounts of endogenous c-Myc protein. In the v-*myc*-transformed cells, both promoters are bound by equal amounts of Myc and Max, presumably Myc-Max heterodimers. This was fully confirmed when occupation of the chicken *CDT1* promoter was tested in normal CEF and CEF/MC29 (Figure 3b). Again, the ChIP patterns for *CDT1* and *WS5* were very similar. As an additional control, primers from an unrelated gene region (*BASP1*) lacking E-boxes were used in the ChIP analysis. Further ChIP analyses included the chemically transformed cell line QT6 and the Q8 cell line transformed by MC29 (Figure 3c). To directly prove that viral Myc protein products occupy the *CDT1* promoter in v-*myc*-transformed cells, an α-Gag serum was also used. The binding pattern of the *CDT1* promoter in chemically transformed cells was similar to that in normal QEF. Occupation of the promoter in cells transformed by the p110 Gag-Myc hybrid protein (QEF/MC29, Q8) was demonstrated not only with Myc and Max antisera, but also with the Gag antiserum, whereas ChIP on cells transformed by a v-Myc protein unlinked to viral structural proteins (QEF/Rc-Myc) was positive only with the Myc and Max antisera (Figure 3c). In order to quantify binding of Myc and Max to the *CDT1* promoter, real-time quantitative PCR was performed on the ChIP products (Figure 3d). The results are in complete agreement with the qualitative analysis. A strong increase of Myc binding to the *CDT1* promoter was observed in the v-*myc*-transformed cell lines QEF/MC29 and QEF/Rc-Myc compared to normal QEF, whereas Max binding is observed in normal and transformed cells.

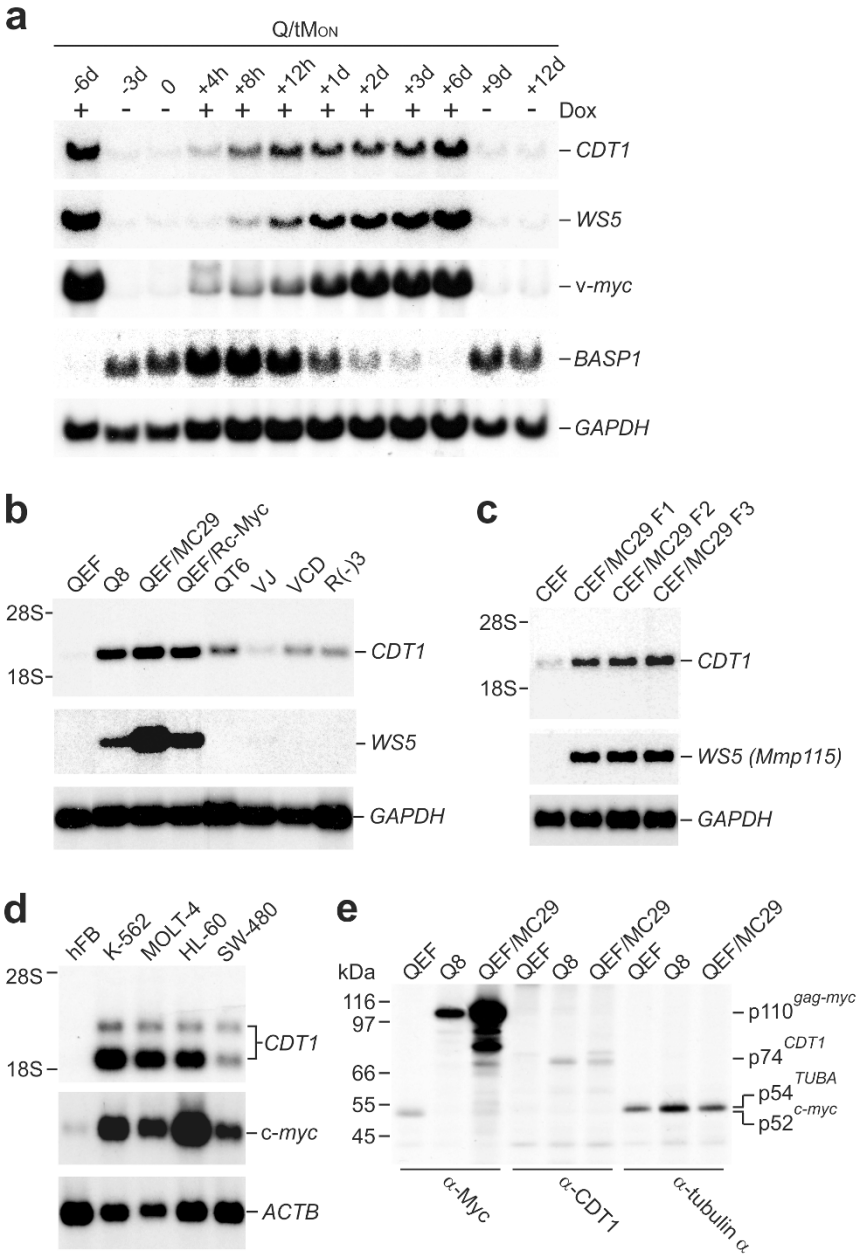

Figure 1 | ***CDT1* activation in cells with elevated v-*myc* or c-*myc* expression.** (a) Kinetics of *CDT1* activation monitored by Northern analysis using total RNAs from the quail cell line Q/tM$_{ON}$ conditionally transformed by a v-*myc* allele controlled by a doxycycline-dependent transactivator[32]. Cells were first grown continuously in the presence (+) of doxycycline, at day −6 the drug was removed, re-added at day 0, removed again after 6 days, and the cells were incubated for further 6 days. RNAs were isolated before removal or addition of the drug, and at the time points indicated. (b) Northern analysis of poly(A)$^+$-selected RNAs (2.5 μg) from normal quail embryo fibroblasts (QEF), or the quail cell lines Q8, QEF/MC29, QEF/Rc-Myc transformed by v-*myc*, QT6 chemically transformed by methylcholanthrene, VJ and VCD transformed by v-*jun*, or R(-)3 transformed by v-*src*. Filters in (a) and (b) were hybridized with ^{32}P-labeled cDNA probes as indicated: quail *CDT1*, *WS5*, and *GAPDH*, chicken *BASP1*, and MC29 v-*myc*. Sizes of the mRNAs were: *CDT1*, 2.5 kb; *WS5*, 2.8 kb; *BASP1*, 2.0 kb; v-*myc*, 1.9 kb; *GAPDH*, 1.4 kb. (c) Northern analysis of poly(A)$^+$-selected RNAs (5.0 μg) from normal chicken embryo fibroblasts (CEF), or clonal cultures of CEF transformed by MC29 (v-*myc*). Filters were hybridized with probes as in (a) and (b). Sizes of the mRNAs were: *CDT1*, 2.5 kb; *WS5* (*Mmp115*), 2.6 kb; *GAPDH*, 1.4 kb. (d) Northern analysis of poly(A)$^+$-selected RNAs (2.5 μg) from normal human fibroblasts (hFB), or from the human cancer cell lines K-562 (chronic myeloid leukemia), MOLT-4 (T-cell leukemia), HL-60 (acute myeloid leukemia), and SW-480 (colon adenocarcinoma). The filter was hybridized with ^{32}P-labeled cDNA probes specific for the human *CDT1*, c-*myc*, and β-actin (*ACTB*) genes. Sizes of the mRNAs were: *CDT1*, 2.7 kb and 2.0 kb; c-*myc*, 2.4 kb; *ACTB*, 2.0 kb. (e) SDS-PAGE (10% wt/vol) analysis of v-Myc (Gag-Myc), c-Myc, Cdt1, and tubulin α (TUBA) proteins using metabolically [^{35}S]methionine-labeled lysates from QEF, or the v-*myc* transformed quail cell lines Q8 and QEF/MC29. Aliquots (1×10^7 cpm) of the lysates were immunoprecipitated with the antisera indicated. Full-length gels and blots are included in the supplementary information.

Electrophoretic mobility shift analysis (EMSA) was employed to test *in vitro* binding of a recombinant Myc-Max heterodimer complex[36] to DNA probes derived from the *CDT1* promoter region containing either the proximal or the distal E-box (Supplementary Figure 4a). The purified Myc-Max complex (Supplementary Figure 4b) bound to double-stranded DNA containing the *CDT1* consensus Myc binding sites with high affinity (Supplementary Figure 4c and d). Application of DNA probes in which the E-boxes were either

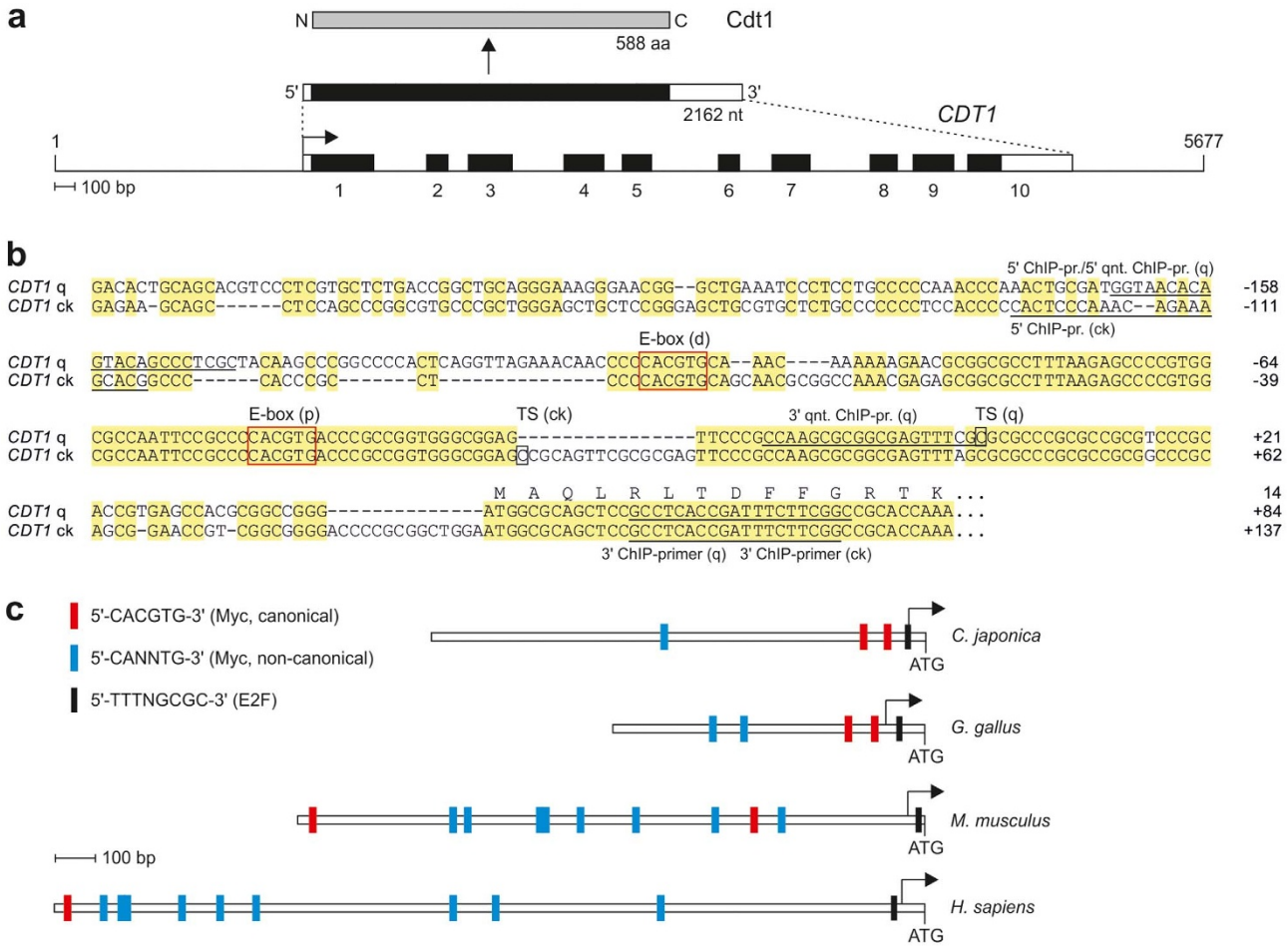

Figure 2 | Structure of the *CDT1* gene. (a) In the schematic diagram of the quail *CDT1* gene, the ten exons are depicted as boxes with the coding region shown in black. The structure of a cDNA clone encoding the 588-amino acid Cdt1 protein is shown above. (b) Nucleotide sequence alignment of the quail (q) and chicken (ck) *CDT1* 5′ regulatory regions (accession nos. KF494239, NC_006098.2). Identical nucleotides are shaded in yellow, the conserved distal (d) and proximal (p) canonical Myc binding sites (E-boxes) are boxed in red, the transcription start (TS) sites in black. The positions of the 5′- and 3′-primers (pr.) used for ChIP and quantitative (qnt.) ChIP are underlined. (c) Schematic diagram of the quail (*Coturnix japonica*), chicken (*Gallus gallus*), mouse (*Mus muculus*) (accession no. NT_078575), and human (*Homo sapiens*) (accession no. AC092384) *CDT1* promoters. Transcription start sites (arrows), and the positions of possible binding sites for Myc (canonical, non-canonical) or E2F are indicated.

mutated or deleted (Supplementary Figure 4a) led to significantly reduced binding. As controls, DNA probes containing a consensus E-box (USF E) for Myc binding[36] or no E-box (control), and a recombinant Cdt1 protein fragment instead of the Myc-Max complex were used. To quantify the binding affinities, increasing amounts of Myc-Max proteins were added to constant amounts of DNA in EMSA experiments (Supplementary Figure 5a), the ratios of bound to total DNA were determined, and the dissociation constants (K_d) for the protein-DNA complexes were calculated (Supplementary Figure 5b). The K_d values for protein-DNA complexes formed by Myc-Max and DNA containing the proximal or distal E-box of the *CDT1* promoter were 1.9 and 2.2 nM, respectively, similar to the K_d determined previously for the USF E control probe[36]. The residual albeit strongly reduced binding of the mutated DNA probes may be due to the nonspecific E-box-independent DNA binding capacity of Myc[5,13].

Transcriptional activation of the *CDT1* promoter by Myc. To determine whether Myc is indeed involved in transcriptional activation of the *CDT1* gene, the quail *CDT1* promoter region encompassing nucleotides −217 through +42 (cf. Figure 2) was cloned into a luciferase reporter plasmid to yield pGL3-CDT1. For comparison, the reporter plasmid pGL3-WS5 containing the promoter region of the Myc target *WS5*[15,33] was analyzed in parallel. The analysis revealed that the *CDT1* promoter was efficiently activated in v-*myc*-transformed QEF/Rc-Myc or QEF(CEF)/MC29 similar to the *WS5* promoter, and both promoters showed only basal activity in normal QEF or CEF (Figure 4a). Furthermore, efficient transactivation of the pGL3-CDT1 and pGL3-WS5 promoter constructs was observed in normal QEF or CEF that were transiently co-transfected with a pRc/RSV-derived expression vector (pRc-Myc) encoding the p52 v-Myc protein, as compared to cells co-transfected with the empty pRc vector (Figure 4b). Promoter analysis of pGL3-CDT1 mutants in which the two E-boxes had either been mutated (*) or deleted (Δ) revealed that intact E-boxes are required for efficient transactivation (Figure 4b and Supplementary Figure 6). Expression of ectopic v-Myc and endogenous c-Myc proteins in transfected cells was monitored by immunoblotting. To analyze the contributions of the proximal and distal canonical E-boxes in transactivation of the *CDT1* promoter, reporter constructs were created in which either one or both of the binding sites were mutated (Supplementary Figure 7). Co-transfection with pRc-Myc and luciferase analysis revealed that mutation of the proximal E-box was sufficient to abrogate the activation observed for the wild-type promoter, whereas mutation of the distal site allowed residual promoter activation. We note that the mutational analyses of

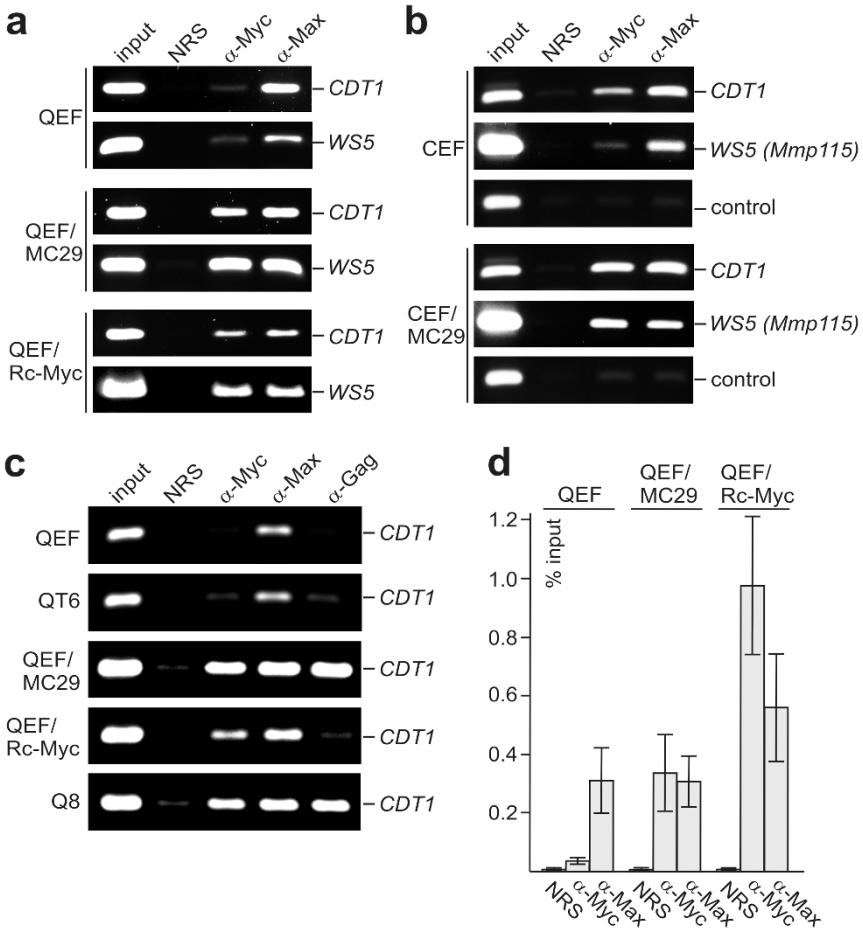

Figure 3 | ***In vivo*** **binding of Myc to the quail and chicken *CDT1* promoters.** (a) ChIP analysis using chromatin from normal QEFs, or from QEF/MC29 and QEF/Rc-Myc transformed by the v-Myc protein. (b) ChIP using chromatin from normal CEF and from CEF/MC29. (c) ChIP using chromatin from normal QEF, from the chemically transformed quail cell line QT6, or from the v-*myc*-transformed cell lines QEF/MC29, QEF/Rc-Myc, and Q8. The QEF(CEF)/MC29 cells and the Q8 cell line are transformed by the p110 Gag-Myc hybrid protein, whereas the QEF/Rc-Myc cells are transformed by a v-Myc protein not fused to viral structural protein sequences. Antisera directed against Myc, Max, or Gag were used for immunoprecipitation, followed by PCR amplification of DNA from the quail *CDT1* (241 bp fragment) or chicken *CDT1* (253 bp fragment) regulatory regions both containing two canonical Myc binding sites, or of DNA from the quail *WS5* (290 or 273 bp fragment) and chicken *WS5* (*Mmp115*) (293 bp fragment) promoters both containing four Myc binding sites[33]. Primers specific for a chicken *BASP1* exon 2 fragment (197 bp) containing no E-boxes (control), and reactions with normal rabbit serum (NRS) or 1.6% of total chromatin (input) were used as controls. Fragments were analyzed by agarose (1.5% wt/vol) gel electrophoresis. (d) ChIP-quantitative PCR (ChIP-qPCR) using chromatin from normal QEF, or from QEF/MC29 and QEF/Rc-Myc. Immunoprecipitated DNA was analyzed in triplicate by qPCR using primers to amplify a 167-bp segment of the regulatory region from the quail *CDT1* promoter containing the two canonical Myc binding sites. Signals were normalized to input chromatin and shown as % input. Full-length gels are included in the supplementary information.

the *CDT1* promoter suggest that the transcriptional regulation by the Myc-Max complex is a direct mechanism.

Deregulated *CDT1* expression induces cell transformation. The strong activation of *CDT1* in v-*myc*-transformed cells prompted us to test if overexpression of *CDT1* by itself would induce parameters of the transformed phenotype. Therefore, the entire *CDT1* coding sequence was inserted into the replication-competent retroviral pRCAS vector (Figure 5a), and the pRCAS-CDT1 construct was transfected into QEF. For control and comparison, QEF were also transfected with the empty pRCAS vector or with pRCAS-MC29[15]. Expression of the proteins encoded by the retroviral constructs was verified by immunoprecipitation and SDS-PAGE. In QEF transfected with pRCAS-CDT1 or pRCAS-MC29, the ectopic p74 Cdt1 and p110 Gag-Myc proteins, as well as increased levels of endogenous p74 Cdt1 in cells transformed by pRCAS-MC29 were detected (Figure 5b). Cells transfected with the MC29 construct showed the typical morphology and capacity for anchorage-independent growth of fully v-*myc*-transformed cells[4]. QEF transfected with pRCAS-CDT1 were able to grow in semi-solid medium at relatively high numbers, although the colonies were significantly smaller than those induced by transformation with pRCAS-MC29 (Figure 5c). Similar results were obtained when CEF were used (Supplementary Figure 8). CEF were transfected with the empty pRCAS vector, pRCAS-CDT1, pRCAS-MC29, or pRCAS-WS5[33]. The overexpressed p74 Cdt1 protein from RCAS-CDT1-transfected CEF co-migrated with *in vitro* translated Cdt1 in the SDS-PAGE, and the MC29 Gag-Myc hybrid protein and the p118 WS5 protein[33] were efficiently expressed from the retroviral vectors (Supplementary Figure 8a). MC29-transfected CEF showed full transformation, and *WS5* overexpression led to partial cell transformation and colony formation as reported previously[33]. Ectopic expression of *CDT1* led to morphological changes and colony forming capacity in CEF, although the numbers of soft agar colonies were significantly lower both for *CDT1* and *WS5* compared to those obtained with the MC29 v-*myc* oncogene (Supplementary Figure 8b).

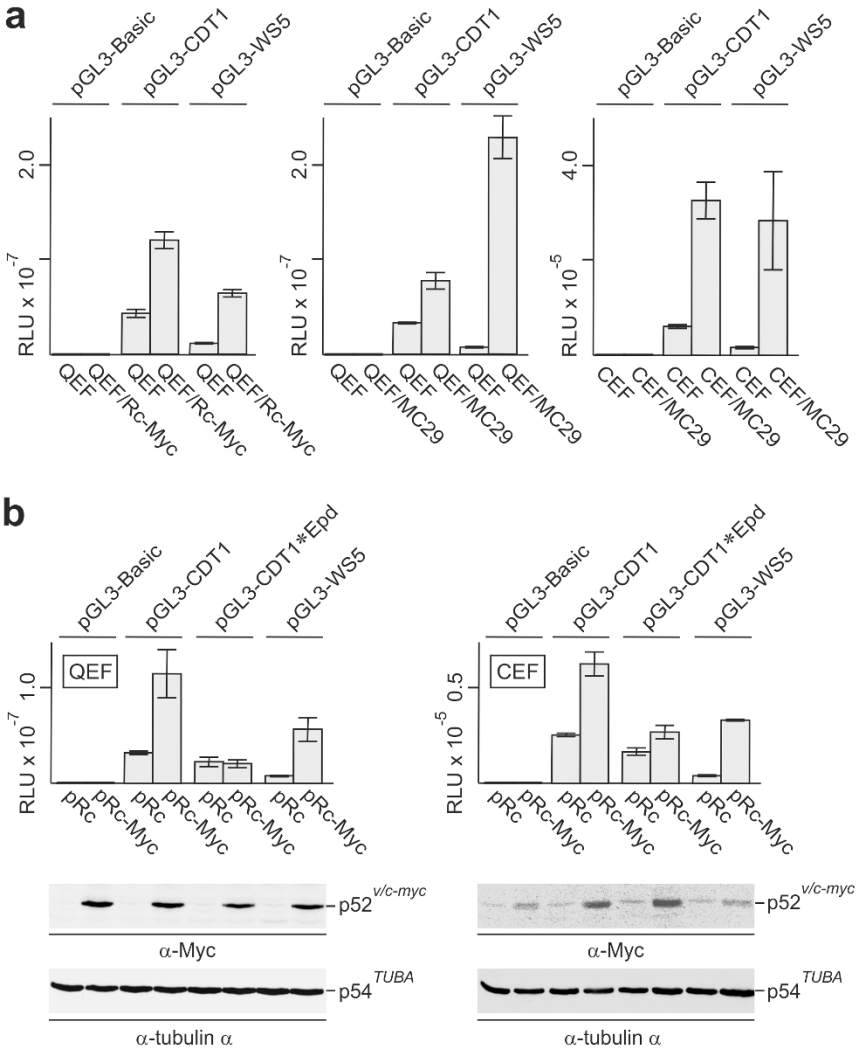

Figure 4 | Transcriptional transactivation of the quail *CDT1* promoter. (a) Aliquots (2.0 μg) of the pGL3-Basic vector, or of the pGL3-CDT1 and pGL3-WS5 reporter constructs were transfected together with 1.0 μg of the pSV-β-galactosidase plasmid into equal numbers (8×10^5) of normal QEF or CEF, or of the v-*myc*-transformed cell lines QEF/Rc-Myc, QEF/MC29, and CEF/MC29. Luciferase activities in relative light units (RLU) normalized to β-galactosidase activities were determined for 10-μl aliquots of cell extracts prepared 24 h after transfection. (b) Aliquots (2.0 μg) of the pGL3-Basic vector, or of the reporter constructs pGL3-CDT1, pGL3-WS5, or pGL3-CDT1*Epd (in which both Myc binding sites have been mutated) were cotransfected with 2.0-μg aliquots of a pRc-derived expression vector encoding the v-Myc protein (pRc-Myc) or of the empty expression vector (pRc), together with the pSV-β-galactosidase plasmid (1.0 μg) into 8×10^5 normal QEF or CEF. Luciferase assays were performed as in (a). For control of protein expression, equal amounts of cell extracts (30 μl for QEF, 60 μl for CEF) were analyzed by SDS-PAGE (10% wt/vol), and v-Myc, c-Myc and tubulin α proteins were detected by immunoblotting. Full-length blots are included in the supplementary information.

Discussion

Extensive investigations into Myc biology and biochemistry over more than 30 years have revealed that the *myc* gene is essential for normal cell function, but also one of the major driving forces in human cancer[3,6]. The physiological role and the oncogenic capacity of the Myc protein are largely based on its principal biochemical function as a regulator of gene expression, exhibiting both transcriptional activating and repressing potential. Elucidation of the cellular pathways affected by Myc therefore requires identification of the relevant target genes. The unambiguous definition of distinct targets and their detailed mechanistic link to Myc's important biological functions is substantially complicated by the vast number of genes apparently controlled by the Myc-Max network. Combinations of microarray screening, methylation marking procedures, global chromatin immunoprecipitation, high throughput sequencing, and expression profiling have been used to identify genome-wide binding sites and putative targets for Myc[12,13,37–41]. Obviously, for the majority of the thousands of putative regulatory targets identified, over 7000 alone in Burkitt lymphoma cells[40], detailed analyses of their transcriptional regulation is not available, and not all of the binding sites may mediate transcriptional regulation[5]. Despite the enormous complexity of the Myc-directed transcriptome, previous detailed analyses of the regulation and biochemical function of distinct Myc targets[6,11], and the overlapping patterns of the recent high throughput approaches have provided an emerging picture of major cellular processes controlled by Myc[5,6]. Myc regulates energy metabolism by controlling a large number of genes involved in glycolysis, glutamine metabolism, and mitochondrial biogenesis[5,6,11,14,42,43]. Furthermore, Myc controls genes involved in protein synthesis and ribosome biogenesis, key processes in biomass accumulation prior to cell division[5,6]. Myc is also involved in cell cycle regulation, induces G1-S progression, and directly activates the genes encoding cyclin D2 and CDK4[6,44–46].

In this report, we provide a possible direct molecular link of the canonical transcriptional function of Myc to DNA replication, another fundamental cellular process. We show that deregulated

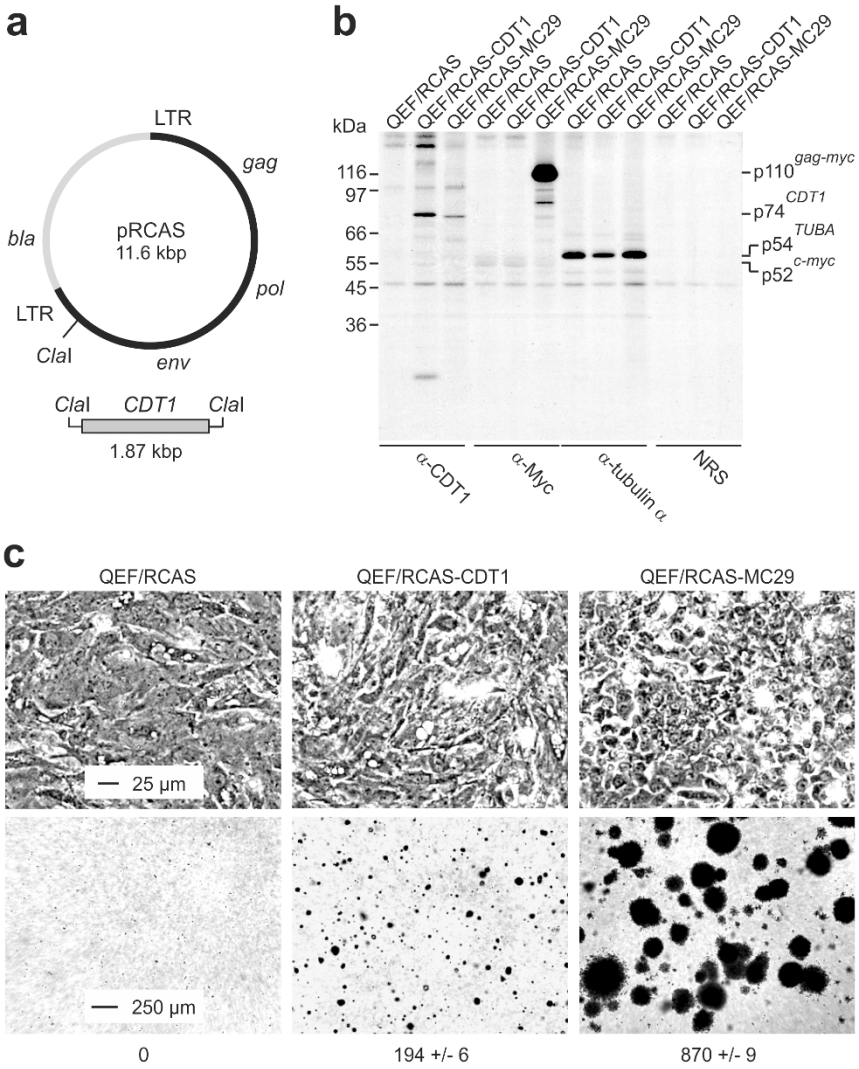

Figure 5 | Transforming potential of ectopically expressed *CDT1*. (a) Structure of the retroviral pRCAS vector with a *Cla*I cloning site. Insertion of adapter DNA carrying a cDNA fragment containing the entire *CDT1* coding region yielded plasmid construct pRCAS-CDT1 specifying a replication-competent retrovirus encoding the quail Cdt1 protein. LTR, long terminal repeat; *gag*, *pol*, *env*, essential retroviral genes; *bla*, β-lactamase. (b) SDS-PAGE (10% wt/vol) analysis of v-Myc (Gag-Myc) and Cdt1 proteins ectopically expressed after transfection of QEF with the replication-defective construct pRCAS-MC29 together with pRCAS as a helper virus construct, with the replication-competent construct pRCAS-CDT1, or with pRCAS as a control. Aliquots (1×10^7 cpm) of lysates from cells metabolically labeled with [^{35}S]methionine were immunoprecipitated with the antisera indicated. (c) Upper panels: phase-contrast micrographs of QEF transfected with vector DNA (pRCAS), or with the retroviral constructs pRCAS-CDT1 or pRCAS-MC29/pRCAS. Lower panels: agar colony formation by cells transfected with pRCAS-CDT1 or pRCAS-MC29/pRCAS. Equal numbers of cells (1×10^5) were seeded in soft agar. pRCAS-transfected QEF were used as controls. Bright-field micrographs were taken after 3 weeks. Numbers of colonies/1000 cells seeded are indicated.

Myc expression leads to transcriptional activation of the *CDT1* gene, encoding the key DNA replication licensing factor Cdt1 in eukaryotes. Through late mitosis and G1, replication origins become licensed for DNA replication in the S phase by loading MCM proteins in order to assemble the pre-RC complex[25,47]. The Cdt1 protein, together with ORC and Cdc6, is responsible for recruitment of the MCM complex. To ensure that chromosomal DNA is replicated only once per cell cycle, origin licensing is restricted to late mitosis and G1. Metazoans prevent licensing during S phase and G2 mainly by down-regulation of Cdt1 activity by ubiquitin-dependent proteolysis and activation of the Cdt1 inhibitor geminin[30,31,47–49]. Our results provide a comprehensive molecular definition of the *CDT1* gene as a transcriptional target of Myc, shown by correlation of *CDT1* expression with conditional or non-conditional Myc expression and by extensive promoter analyses including ChIP, EMSA and reporter assays. Notably, in normal non-transformed cells the *CDT1* promoter is mainly occupied by the Max protein, possibly homodimeric or in complex with another E-box binding factor, and to a lesser degree by c-Myc. In *myc*-transformed cells, the ChIP analyses show equal occupation by Myc and Max, as expected for the heterodimeric structure of the Myc-Max complex. It has been postulated that deregulated Myc expression leads to a switch in the regulation of E-box genes that are normally controlled by other E-box transcription factors and that this may be an important mechanism contributing to Myc-induced oncogenesis[6]. In addition to canonical and non-canonical Myc binding sites, all *CDT1* promoter regions compared (cf. Figure 2) contain a binding site for E2F transcription factors near the transcription start site. Based on the genome-wide analysis of Myc binding sites in the human B-cell system, it was found that many of the candidate Myc targets also contain E2F binding sites, suggesting that Myc targets may require additional regulators of gene expression[6,12,13]. It was indeed reported that human *CDT1* expression is regulated by

E2F[50]. Interestingly, based on genome-wide high throughput technologies genes encoding other components of DNA replication licensing, notably MCM proteins, were listed among putative Myc-regulated genes[12,13,38,41]. In a single study based on a microarray approach, *CDT1* was also listed as an initial candidate target, but then, in contrast to our current results, classified as a gene not regulated by Myc and the mechanism of its transcriptional regulation was not analyzed further[51]. Although direct transcriptional activation of *CDT1* by Myc ist the most plausible explanation for the results of several independent experimental approaches as reported here, we would like to point out that indirect mechanisms like changes in *CDT1* expression due to altered cell cycle distribution by deregulated Myc or due to activation of E2F by Myc[52] cannot be ruled out.

Recent reports have demonstrated that Myc has a non-transcriptional role in the control of DNA replication[18,53]. Intriguingly, this involves direct interaction of the Myc protein with components of the pre-RC, including MCM proteins and Cdt1. It was shown that overexpression of Myc causes increased replication origin activity and subsequent DNA damage[18], and it was proposed that genomic instability observed in cancer cells with high Myc expression is related to deregulation of DNA replication by Myc - pre-RC interactions[18,24]. Our current results demonstrate that the *CDT1* gene encoding the major regulator of DNA replication and important safeguard against rereplication in metazoans is a transcriptional target of Myc, providing a mechanistic link of Myc's canonical function as a transcription factor to DNA replication licensing, in addition to its non-transcriptional function. Addition of Cdt1 to replicated nuclei does indeed stimulate rereplication[48], and we (Figure 5 and Supplementary Figure 8) and others[54] have shown that deregulated *CDT1* exhibits oncogenic potential. Our results suggest that aberrant transcriptional activation of the *CDT1* gene by constitutive Myc expression in tumor cells leads to genomic instabilities observed not only in *myc*-transformed cells[19–21], but considered as a major hallmark of human cancer[22,23]. Further analyses are required to assess in detail the putative role of *CDT1* as an effector in Myc-induced carcinogenesis.

Methods

Cells and retroviruses. Cell culture, DNA transfection, and transformation of quail or chicken embryo fibroblasts (QEF, CEF) were performed as described[4,15]. The established quail cell lines Q8, QEF/MC29, QEF/Rc-Myc, QT6, VJ, VCD, R(-)3, and the cell lines Q/tM8 and Q/tM$_{ON}$ conditionally transformed by v-*myc* were used[4,15,32,33]. To generate CEF freshly transformed by the MC29 retroviruses, primary cells were transfected with pRCAS-MC29 as described[15]. The human cancer cell lines K-562, MOLT-4, HL-60, and SW-480 which are derived from chronic myeloid leukemia cells in blast crisis, acute lymphoblastic leukemia T lymphoblasts, acute myeloid leukemia cells, or colon adenocarcinoma cells, respectively, and human immortalized fibroblasts (hFB) were provided by J. Troppmair (Medical University Innsbruck). The retroviral expression vector pRCAS-WS5 has been described[33]. For construction of pRCAS-CDT1, a 1767-bp cDNA fragment containing the *CDT1* coding region was inserted into the *Cla*I site of the pRCAS vector as described[15]. DNA transfection and colony assays of transfected cells in 0.3% (wt/vol) agarose were done as described[4,33].

DNA cloning and nucleic acid analysis. Molecular cloning, DNA sequencing, Northern analysis, and 5′-rapid amplification of cDNA ends (RACE) have been described[15,33]. Subtractive probe generation and representational difference analysis (RDA) were done as described[55] using RNAs isolated from Q/tM8[32] grown in the absence of doxycycline, and from the same cells 48 h after addition of doxycycline for generation of tester and driver cDNAs, respectively. For the isolation of cDNA clones representing the quail *CDT1* gene, a Q8-specific cDNA library was screened as described[33] using an RDA fragment as a probe. 5′-RACE was applied to obtain a 2,162-bp full-length cDNA sequence. For determination of the *CDT1* gene structure, clones were isolated from a quail genomic DNA library as described[33]. Bioinformatic analyses of nucleotide and amino-acid sequences of the *CDT1* gene were performed as described[15,33,55]. For multiple sequence alignments, the ClustalW algorithm (DS/Gene, Accelrys, San Diego, CA) was used. The nucleotide and deduced amino-acid sequences have been deposited in the GenBank database (accession nos. HQ840726, KF494239). Hybridization probes for Northern analysis included cDNA fragments derived from quail *CDT1*, *WS5*, *GAPDH*, and v-*myc* as described previously[33]. To obtain DNA probes specific for human *CDT1* and β-actin, PCR was performed on cDNAs derived from MOLT-4 or human bronchial epithelial cells (HBEpC), respectively, using gene specific primers. A human c-*myc* probe specifying a region from c-*myc* exon 3 was amplified using genomic DNA as a template.

Chromatin immunoprecipitation analyses. ChIP analyses were carried out as described by using sheared extracts derived from formaldehyde-treated normal QEF or CEF, and from the transformed cell lines QT6, Q8, QEF/MC29, QEF/Rc-Myc, and CEF/MC29[15,33]. Immunoprecipitations were performed with specific antibodies followed by PCR amplification of fragments from the quail *CDT1* (241 bp) or chicken *CDT1* (253 bp) regulatory regions, or from the quail *WS5* (QEF, QEF/MC29: 290 bp; QEF/Rc-Myc: 273 bp) and chicken *WS5*/*Mmp115* (293 bp) promoters using the primer pairs 5′-GGTAACACAGTACAGCCCTCGC-3′/5-GCCGAAGAAATCGG TGAGGC-3′, 5′-CACTCCCAAACAGAAAGCACG-3′/5′-CCGAAGAAATCGGT GAGGC-3′, 5′-GGTCCCTATATGAACGTGCC-3′/5′-AGAAGGGACCCTCTTT ACATAACC-3′, 5′-CCGCAGCACCATCGCTGTGC-3′/5′-GTGCTCCGACTCC GGGAGAGG-3′, 5′-AGACCCTCACGGAGGTCTCAC-3′/5′-CTGCTGTGCCG TGACCAG-3′, respectively. A primer pair specific for a 197-bp chicken *BASP1* exon 2 segment (5′-AGGAGCAGCAACTGAGGAAGAG-3′/5′-GTTCTGCTTTCTGG GCTTCTTC-3′), reactions with normal rabbit serum (NRS), or with 1.6% of total chromatin (input) were used as controls. The antisera α-Myc, α-Max, and α-Gag have been described[33]. For ChIP - real-time quantitative PCR (ChIP-qPCR), immunoprecipitated DNAs were analyzed on a Step One Real Time PCR System (Applied Biosystems, Carlsbad, CA) using the primer pair 5′-GGTAACACAGTA CAGCCCTCGC-3′/5′-GCGAAACTCGCCGCGCTTGG-3′, amplifying a 167-bp segment from the quail *CDT1* promoter adjacent to the transcription start site. Signals were normalized to input chromatin and shown as % input. The raw cycle threshold (Ct) values of the 16.6% input (dilution factor: 6.02) was adjusted to 100% by calculating raw Ct – $\log_2$6.02. To calculate the % input of the immunoprecipitations, the equation $100 \times 2^{[Ct\ (adjusted\ input\ to\ 100\%)\ -\ Ct\ (IP)]}$ was applied.

Transactivation analysis. The reporter construct pGL3-WS5 (pLUC-WS5) containing the quail *WS5* promoter has been described[15]. To construct pGL3-CDT1, a 1246-bp fragment encompassing nucleotides −1204 to +42 from the quail *CDT1* promoter region was first inserted into the *Bgl*II and *Hind*III sites of the pGL3-Basic vector (Promega, Madison, WI) yielding pGL3-CDT1-L. Deletion of a 996-bp *Sma*I/*Pst*I fragment from this construct yielded pGL3-CDT1 encompassing nucleotides −217 to +42 from the quail *CDT1* promoter. To create derivatives of pGL3-CDT1 in which the proximal, the distal, or both canonical E-boxes (5′-CACGTG-3′) were either mutated to 5′-GCCGTG-3′ or deleted, site-directed mutagenesis or overlapping PCR were performed as described[15,33]. The expression vector pRc-Myc, DNA transfer into cells using the calcium phosphate method, and transcriptional transactivation analysis using the luciferase reporter system have been described[15].

Electrophoretic mobility shift assay (EMSA). Protein-DNA binding reactions were performed as described[9]. Protein-DNA complexes were resolved by native 6% (wt/vol) polyacrylamide gel electrophoresis (PAGE), and radioactive signals were quantified using a PhosphorImager (GE Healthcare, Little Chalfond, UK) as described[9]. A recombinant Myc/Max protein complex was expressed in *Escherichia coli* and purified as described[36].

Purification of recombinant quail Cdt1(200–441) protein and antiserum preparation. A 726-bp cDNA fragment encoding amino acid residues 200–441 of quail Cdt1 flanked by start/stop codons and cloning sites provided by the PCR primers was amplified and cloned into the *Nde*I and *Eco*RI restriction sites of the prokaryotic expression vector pET-21a (Novagen, Darmstadt, Germany), yielding the pET-qCDT1(200–441) expression construct. The plasmid was transformed into *Escherichia coli* strain BL21 (DE3) CodonPlus-RIL (Stratagene, Santa Clara, CA). Induction of protein expression, cell lysis, and protein purification were essentially done as described[9]. Recombinant Cdt1(200–441) protein was used for polyclonal antiserum preparation as described[15].

Mass spectrometry (MS) of Cdt1 recombinant protein. The recombinant Cdt1(200–441) protein was desalted for MS as described[9]. The final protein concentration of the electrospray ionization (ESI) solution (H_2O : MeOH 1 : 1, 1% vol/vol acetic acid) was 1 μM. ESI MS using a 7 Tesla Fourier transform ion cyclotron resonance (FT-ICR) instrument (Bruker Austria) for top-down experiments using collisionally activated dissociation (CAD) was performed as described[9].

Protein analysis. *In vitro* translation, immunoprecipitation of L-[^{35}S]methionine-labeled proteins, SDS-PAGE, and immunoblotting were done as described using antisera directed against Myc and WS5[15,33], Cdt1 (this report), and tubulin α (Sigma-Aldrich, St. Louis, MO). To construct pET-CDT1 used for *in vitro* translation of full-length Cdt1 protein, a 1767-bp cDNA fragment encompassing the *CDT1* coding region was inserted into pET-21a.

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Author contributions

M.H. and Klaus Bister designed research, T.V., M.H., M.S., P.R., Kathrin Breuker, and T.D.-M. performed experimental work, T.V., M.H., M.S., P.R., Kathrin Breuker, T.D.-M. and Klaus Bister analyzed data, T.V., M.H. and Klaus Bister wrote the manuscript.

Additional information

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