Transcriptional control of DNA replication licensing by Myc

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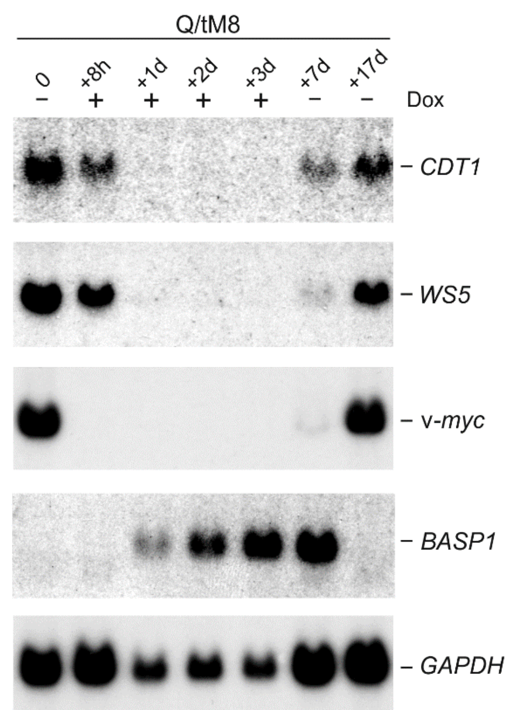
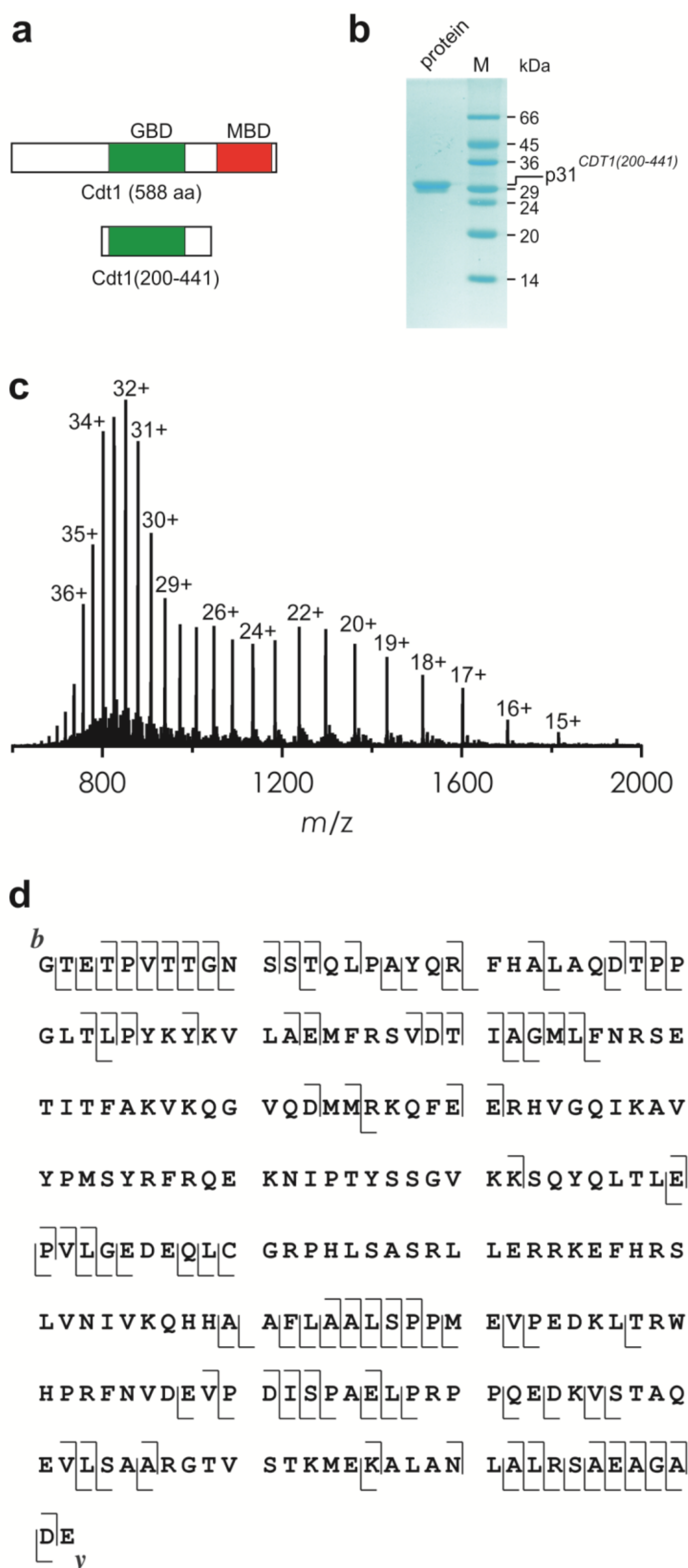
SUPPLEMENTARY INFORMATION



Supplementary Figure 1. Northern analysis of *CDT1* expression in the cell line Q/tM8 conditionally transformed by a v-*myc* allele controlled by a doxycycline-inhibited transactivator.[32] Cells were first grown continuously in the absence (-) of doxycycline, at day 0 the drug was added (+), removed again after 3 days, and the cells were incubated for further 14 days. RNAs were isolated before addition or removal of the drug, and at the time points indicated. Filters were hybridized with the indicated ^{32}P-labeled cDNA probes: 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. In the Q/tM8 cell line, inactivation of the transforming v-*myc* allele by doxycycline leads to retarded cell growth and downregulation of the glycolytic control gene *GAPDH* as observed previously.[32] Equal RNA loading is verified by ethidium bromide staining.



Supplementary Figure 2. Recombinant quail Cdt1(200-441) protein. (**a**) Schematic structure of the 242-amino acid recombinant Cdt1(200-441) protein in comparison to the 588-amino acid full-length quail Cdt1 protein (GBD, geminin binding domain; MBD, MCM binding domain). (**b**) SDS-PAGE (15% wt/vol) analysis of 1.5 µg of bacterially expressed and purified Cdt1(200-441) stained with Coomassie brilliant blue. (**c**) Electrospray ionization (ESI) mass spectrum of the Cdt1(200-441) protein recorded on a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. (**d**) Map of fragment ions from collisionally activated dissociation (CAD), illustrating 40% sequence coverage. Using the y_2 to y_{10} fragment ions from CAD of $(M + 32H)^{32+}$ protein ions as internal calibrants (error 0.5 ppm), a value of 27217.01 Da was deduced for the mass (most abundant isotopic peak) of the Cdt1(200-441) protein (theoretical mass: 27217.04 Da).

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Cdt1 q    MAQLRLTDFFGRTKTAVSITGKHGTRAHKAAILTPPGSRNDGAVPTPPASPRTPTRSAAPQIPRVAGRKRSRREMGEETPGGERGGKSARKRLELPRDAG  100
Cdt1 ck   MAQLRLTDFFGRTKAAVSLPAKHGARGLKAALVAPPEPRNHGAVSPPPASPRTPTRSAAPAVPRMAGRKRSRREMEAETPGGERGGKSARKRLELPRDAG  100
Cdt1 m    MAQSRVTDFYACRRPG------LTTPRAKSICLTPSPGGLVAPAFTRSSSRKRARPPAEPGSDQPAPLARRRLRLPGLDSCPSSLPEPSSPAEPSPPADP   94
Cdt1 h    MEQRRVTDFFARRRPGP--PRIAP-PKLACRTPSPARPALRAPASATSGSRKRARPPAAPGRDQARPPARRRLRLS--------VDEVSSP---------   80

Cdt1 q    PEPASPPSLAPLQPPTASRTCIPSLEQNSGTTPRLEQEELAALQSRLQRMRMKPPPTQAPPVPAGVGAELRSRLQSLRGLEQRLRAKAAGNGTGQHGVTG  200
Cdt1 ck   PEPASPTSLGPPQPPIASRLCTPSPEQDSGTTPRLEQEELATLQSRLQRMRMKPP-TQPPPIPAGVGAELRSRLQSLRGLELRLRAKAAGSGTGQPGVTG  199
Cdt1 m    SPPADPGSPVCPSPVKRTKSTTVYVGQQPGKIPSED--SVSELQSCLRRARKLGAQARALRARVQENAVEPSTPDAKVPTEQPCVEKAP-----------  181
Cdt1 h    STPEAPDIPACPSPGQKIKKSTPAAGQPPHLTSAQDQDTISELASCLQRARELGARVRALKASAQD-AGESCTPEAEGRPEEPCGEKAP-----------  168

Cdt1 q    TETPVTTGNSSTQLHAYQRFHALAQDTPPGLTLPYKYKVLAEMFRSVDTIAGMLFNRSETITFAKVKQGVQDMMRKQFEERHVGQIKAVYPMSYRFRQEK  300
Cdt1 ck   TEAPIAAQN-STQLHAYQRFHALAQDTPPGLTLPYKYKVLAEMFRSVDTIAGMLFNRAETITFAKVKQGVQDMMRKQFEERHVGQIKAVYPTSYRFRQEK  298
Cdt1 m    --------------AYQRFHALAQPGLPGLVLPYKYQVLVEMFRSMDTIVSMLHNRSETVTFAKVKQGVQEMMRKRFEERNVGQIKTVYPTSYRFRQEC  266
Cdt1 h    --------------AYQRFHALAQPGLPGLVLPYKYQVLAEMFRSMDTIVGMLHNRSETPTFAKVQRGVQDMMRRRFEERNVGQIKTVYPASYRFRQER  253
                                                       geminin binding
Cdt1 q    NIPTYSSGVKKSQYQLTLEPVLGEDEQLCGRPHLSASRLLERRKEFHRSLVNIVKQHHAAFLAALSPPMEVPEDKLTRWHPRFNVDEVPDISPAELPRPP  400
Cdt1 ck   NIPTYSSGVKKSQYQLTLEPVLGEDEQLCGRPHLSASRLLERRKEFHRSLVNIVKQHHAAFLAALSPPMEVPEDKLTRWHPRFNVDEVPDISPAELPQPP  398
Cdt1 m    NVPTFKDSIKRSDYQLTIEPLLGQEA-G-GATQLTATCLLQRRQVFRQNLVERVKEQHKVFLASLNPPMAVPDDQLTRWHPRFNVDEVPDIEPAELPQPP  364
Cdt1 h    SVPTFKDGARRSDYQLTIEPLLEQEADG-AAPQLTASRLLQRRQIFSQKLVEHVKEHHKAFLASLSPAMVVPEDQLTRWHPRFNVDEVPDIEPAALPQPP  352

Cdt1 q    QEDKVSTAQEVLSAARGTVSTKMEKALANLALRSAEAG------ADEPALSKAASPASTSSALKGVSQALLERIRAKEARRLQVLMTRAVEQEERLAMMG  494
Cdt1 ck   QEDRVSTAQEVLSAARGTMSTKMEKALANLALRSAEAG------VEEPALSKAASPASTSSALKGVSQELLERIRAKEARRLQALMTREMGQEERLAMLG  492
Cdt1 m    VTEKLTTAQEVLARARSLMTPKMEKALSNLALRSAEPGSPGTSTPPLPATPPATPPAASPSALKGVSQALLERIRAKEVQKQLARMTRCPEQELRLQRLE  464
Cdt1 h    ATEKLTTAQEVLARARNLISPRMEKALSQLALRSAAPSSPGSPRPALPATPPATPPAASPSALKGVSQDLLERIRAKEAQKQLAQMTRCPEQEQRLQRLE  452
                                                                                  MCM binding
Cdt1 q    RLPAMARILRGVFVAEKKPALPMELLCARMVDSYPTEMAAGEMEKHLRLLAELLPDWVTFHAIRADTYMKLDKNTDLSLVTERLEKAAKEAEAH        588
Cdt1 ck   RLPAMARILRGVFVAEKKPALPMELACARMADSFPTQMAAGEMEKHLRLLAELLPDWVTIHALRTDTYMKLDKNVDLGLVTERLEKAAKEAEAL        586
Cdt1 m    RLPELARVLRNVFVSERKPALTMEVVCARMVDSCQTALSPGEMEKHLVLLAELLPDWLSLHRIRTDTYVKLDKAVDLAGLTARLAHHVHAE-GL        557
Cdt1 h    RLPELARVLRSVFVSERKPALSMEVACARMVGSCCTIMSPGEMEKHLLLLSELLPDWLSLHRIRTDTYVKLDKAADLAHITARLAHQTRAEEGL        546
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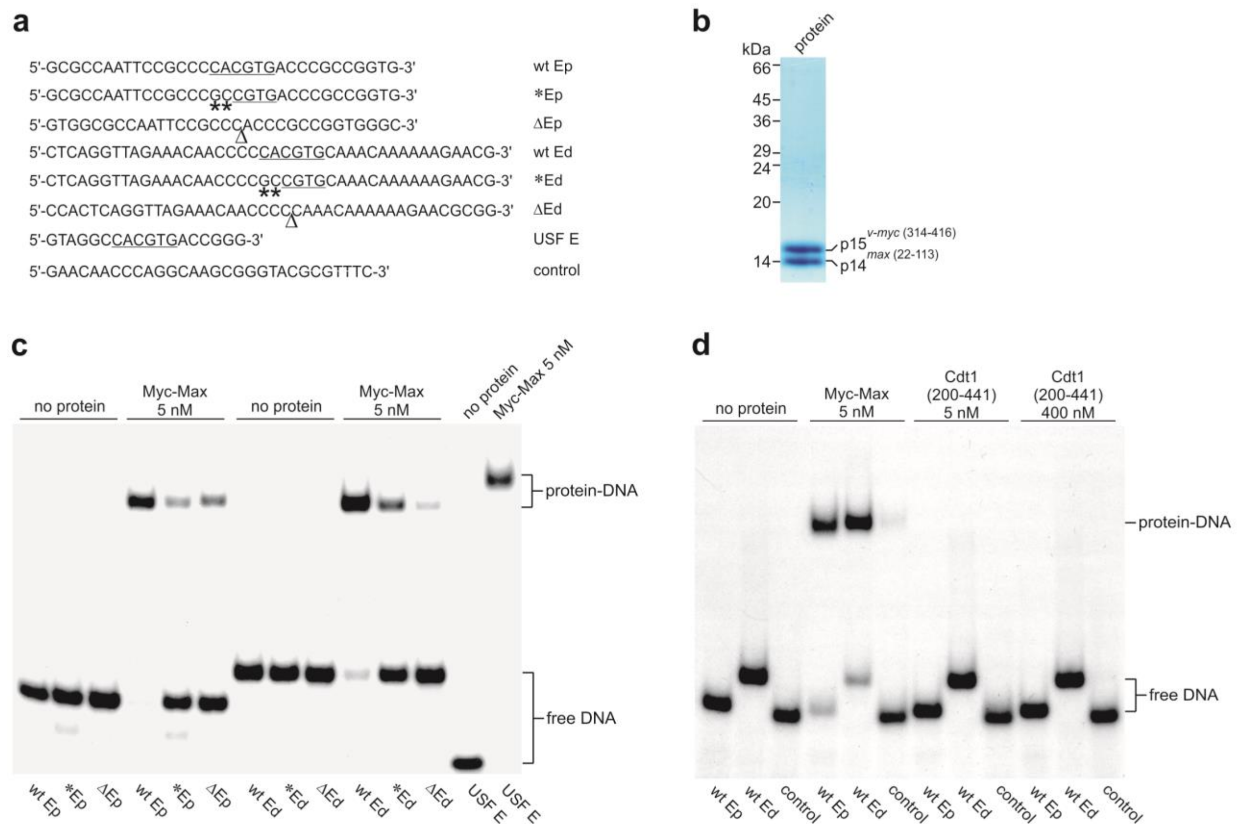
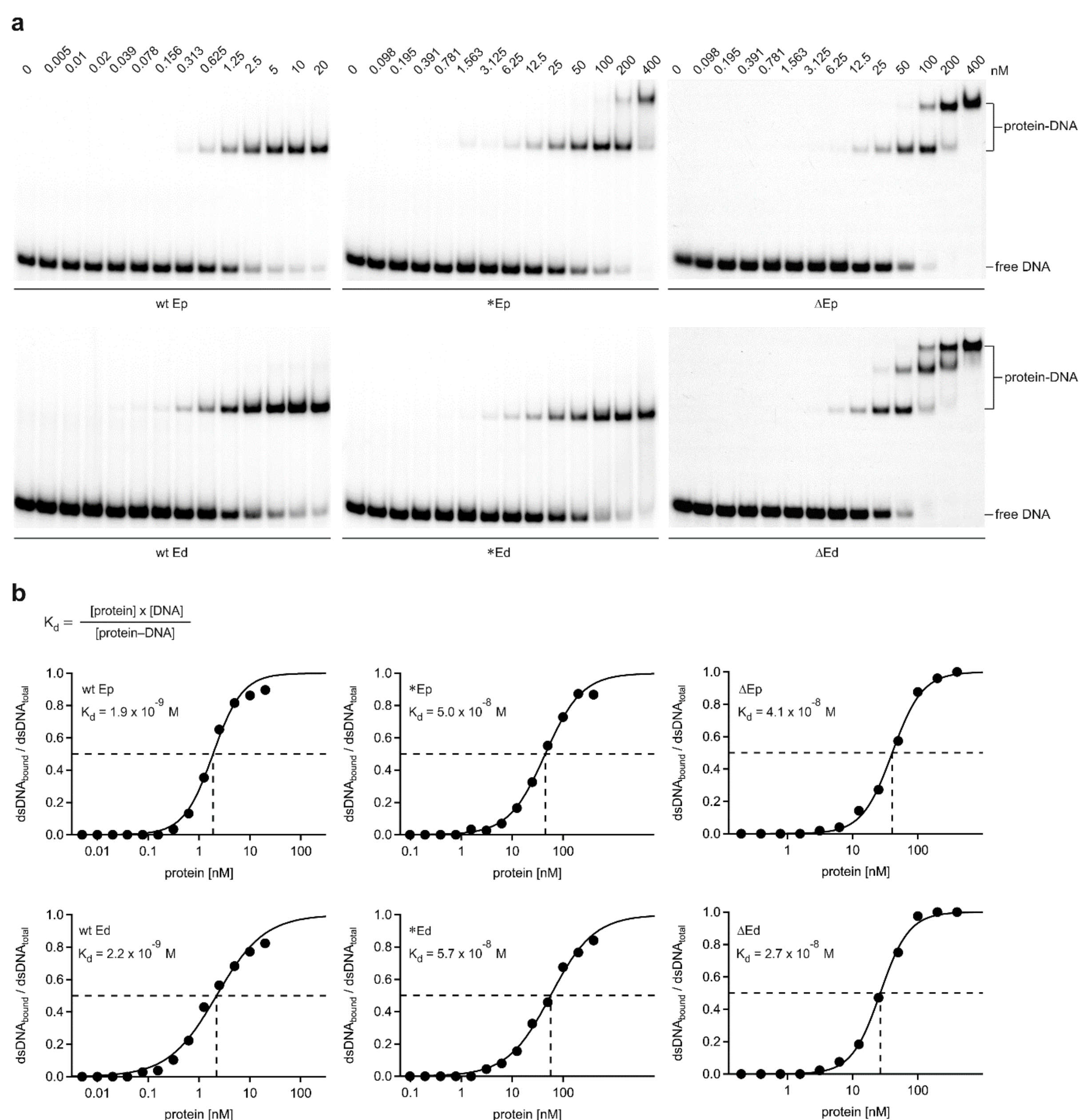
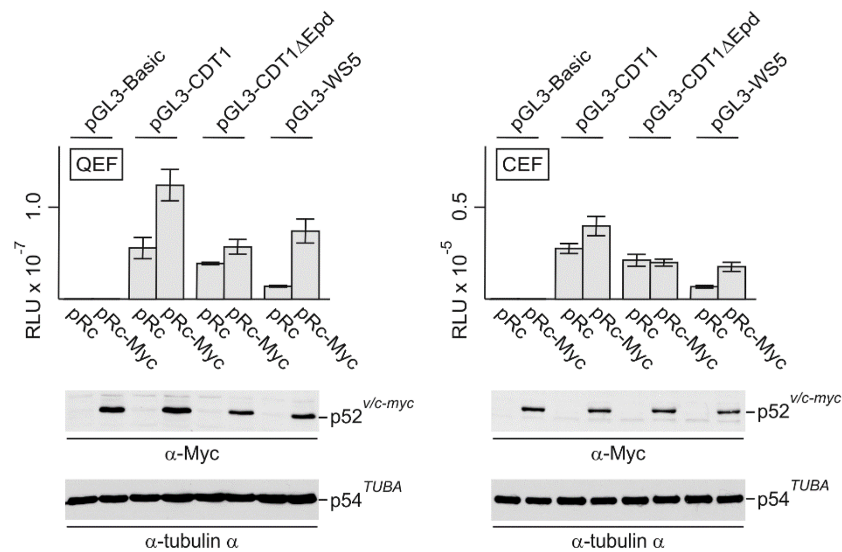
Supplementary Figure 3. Amino acid sequence of the quail Cdt1 protein and alignment with its chicken (ck), mouse (m), and human (h) orthologs (GenBank accession nos.: q Cdt1, ADX62069; ck Cdt1, XP_423919; m Cdt1, NP_080290; h Cdt1, BAB61878). Identical residues are shaded in blue, gaps are indicated by dashes. The binding domains for geminin and the minichromosome maintenance (MCM) complex are indicated by green and red boxes, respectively. The alignment was generated using the ClustalW algorithm with additional manual adjustments.



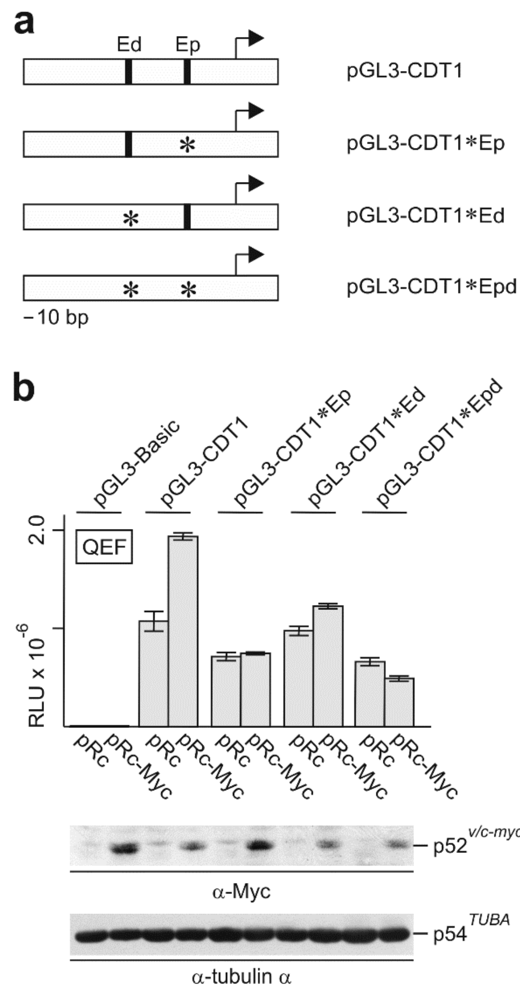
Supplementary Figure 4. *In vitro* DNA binding of recombinant Myc-Max proteins to *CDT1* promoter fragments. (**a**) Sequences of the upper strands of double-stranded oligodeoxynucleotides containing the wild type (wt) proximal (p) or distal (d) canonical E-box (E) motifs (CACGTG) present in the quail *CDT1* promoter (cf. Figure 2), or of mutant derivatives in which the E-box motifs were mutated (∗) or deleted (Δ). Oligodeoxynucleotides containing a consensus Myc/Max binding site (USF E), or a random sequence (control) were used as controls. (**b**) SDS-PAGE (14% wt/vol) of a 2-µg aliquot of purified recombinant chicken Myc p15 in complex with chicken Max p14 stained with Coomassie brilliant blue. (**c**, **d**) Electrophoretic mobility shift assays (EMSA) using the Myc-Max protein complex (5 nM) shown in **b** and 0.1-ng (25000 cpm) aliquots of the [^{32}P]-radiolabeled double-stranded oligodeoxynucleotides shown in **a**. The recombinant quail Cdt1(200-441) protein (5 nM, 400 nM) was used as a control. Electrophoresis was on native polyacrylamide gels (6% wt/vol).



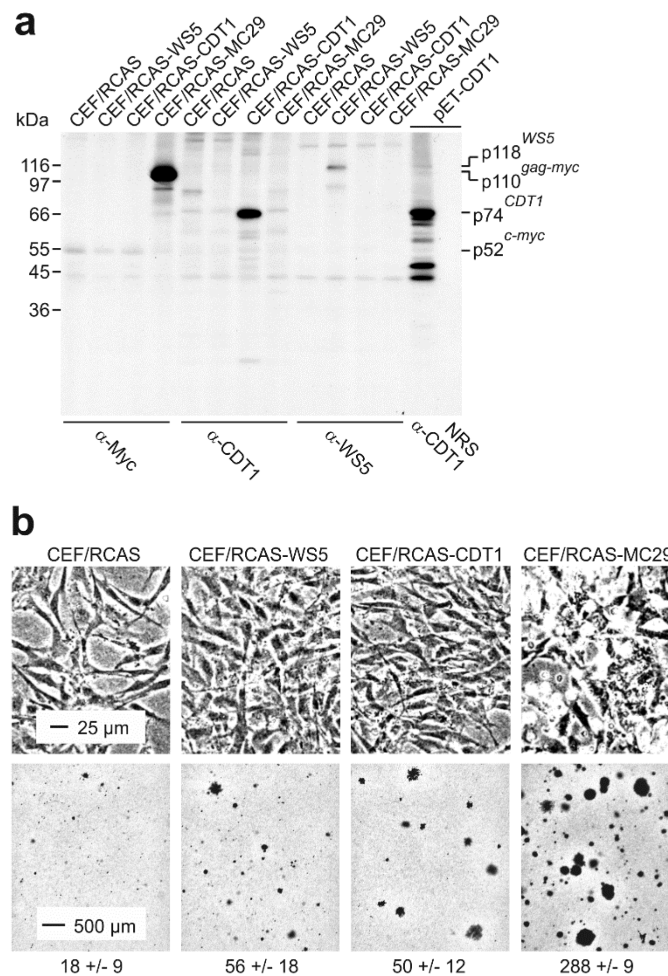
Supplementary Figure 5. Determination of the dissociation constants (K_d) of protein-DNA complexes by EMSA analysis. (**a**) Titration of 0.1-ng (25000 cpm) aliquots of [^{32}P]-labeled double-stranded (ds) oligodeoxynucleotides described in Supplementary Figure 4 with increasing amounts of the chicken Myc p15/Max p14 complex. Total protein concentrations are indicated at the top. (**b**) The ratio of bound dsDNA to total dsDNA was determined by phosphorimaging and plotted versus protein concentration. The sigmoidal fit function f (x) = 1/{1 + exp [(a-x)/b]} was used to generate the binding curves. Calculated K_d values for the protein-DNA binding reactions are indicated.



Supplementary Figure 6. Deletion analysis of the *CDT1* promoter. Aliquots (2.0 µg) of the pGL3-Basic vector, or of the reporter constructs pGL3-CDT1, pGL3-WS5, or pGL3-CDT1ΔEpd (in which both Myc binding sites have been deleted) 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 x 10^5 normal QEF or CEF. Luciferase assays were performed as in Figure 4. For control of protein expression, equal amounts of cell extracts (30 µl for QEF, 60 µl for CEF) were separated by SDS-PAGE (10% wt/vol), and v-Myc, c-Myc and tubulin α proteins were detected by immunoblotting.



Supplementary Figure 7. Mutational analysis of the *CDT1* promoter. (**a**) Structure of the *CDT1* promoter fragments cloned into the luciferase reporter plasmid. The proximal (p), distal (d), or both (pd) canonical E-boxes (CACGTG; cf. Figure 2) were mutated (∗) to GCCGTG. (**b**) Aliquots (2.0 µg) of the pGL3-Basic vector, or of the reporter construct pGL3-CDT1 and its mutant derivatives 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 x 10^5 normal QEF. 24 h after transfection, luciferase activities were determined in triplicate from 10-µl aliquots of cell extracts. For control of protein expression, equal amounts of cell extracts (30 µl) were separated by SDS-PAGE (10% wt/vol), and v-Myc, c-Myc and tubulin α proteins were detected by immunoblotting.



Supplementary Figure 8. Transforming activity of ectopically expressed *CDT1* in CEF. (**a**) SDS-PAGE (10% wt/vol) analysis of v-Myc (Gag-Myc), Cdt1, and WS5 proteins ectopically expressed after transfection of CEF with the replication-defective construct pRCAS-MC29 together with pRCAS as a helper virus construct, with the replication-competent constructs pRCAS-CDT1 and pRCAS-WS5, or with pRCAS as a control. Aliquots (1 x 10^7 cpm) of lysates from cells metabolically labeled with [^{35}S]methionine were immunoprecipitated with the antisera indicated. *In vitro* translated [^{35}S]methionine-labeled and immunoprecipitated quail Cdt1 protein encoded by the plasmid pET-CDT1 was used as size control. (**b**) Upper panels: phase-contrast micrographs of CEF transfected with vector DNA (pRCAS), or with the retroviral constructs pRCAS-WS5, pRCAS-CDT1, and pRCAS-MC29/pRCAS. Lower panels: agar colony formation by cells transfected with pRCAS-WS5, pRCAS-CDT1 or pRCAS-MC29/pRCAS. Equal numbers of cells (5 x 10^4) were seeded in soft agar. pRCAS-transfected CEF were used as controls. Bright-field micrographs were taken after 3 weeks. Numbers of colonies/1000 cells seeded are indicated.

Fig. 1a

Fig. 1b

Fig. 1c

Fig. 1d

Fig. 3a

Fig. 4b

Supplementary Figure 9. Full-length blots and gels from the key data in Figures 1, 3, and 4.