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Cdt1 overexpression drives colorectal carcinogenesis through origin overlicensing and DNA damage

Michalis Petropoulos[1,2‡], Spyridon Champeris Tsaniras[1], Sofia Nikou[3], Styliani Maxouri[2†], Vasilis S. Dionellis[4†], Argyro Kalogeropoulou[2], Angeliki Karamichali[4], Konstantinos Ioannidis[2], Iosif-Rodolfos Danalatos[2], Mandy Obst[5], Ronald Naumann[6], George J. Delinasios[7], Vassilis G Gorgoulis[8,9,10], Vassilis Roukos[11], Konstantinos Anastassiadis[5], Thanos D. Halazonetis[4], Vasiliki Bravou[3], Zoi Lygerou[2*], Stavros Taraviras[1*]

1. Department of Physiology, Medical School, University of Patras, Patras, Greece
2. Department of General Biology, Medical School, University of Patras, Patras, Greece
3. Department of Anatomy-Histology-Embryology, School of Medicine, University of Patras, Patras, Greece
4. Department of Molecular Biology, University of Geneva, Geneva, Switzerland
5. Stem Cell Engineering, Biotechnology Center, Center for Molecular and Cellular Bioengineering, University of Technology Dresden, Dresden, Germany
6. Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany
7. International Institute of Anticancer Research, Kapandriti, Greece
8. Department of Histology and Embryology, School of Medicine, National Kapodistrian University of Athens, Athens, Greece
9. Biomedical Research Foundation of the Academy of Athens, Athens, Greece
10. Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Science Centre, Manchester, UK

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11. Institute of Molecular Biology (IMB), Mainz, Germany

‡Present address: Department of Molecular Biology, University of Geneva, 1205 Geneva, Switzerland.

†These authors contributed equally

***Correspondence to: Z Lygerou,** Department of General Biology, Medical School, University of Patras, Asklipiou Street 1, Rio 26500, Patras, Greece. E-mail: lygerou@med.upatras.gr or **S Taraviras,** Department of Physiology, Medical School, University of Patras, Asklipiou Street 1, Rio 26500, Patras, Greece. E-mail: taraviras@med.upatras.gr

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Abstract

Chromatin licensing and DNA replication factor 1 (CDT1), a protein of the pre-replicative complex, is essential for loading the minichromosome maintenance complex (MCM) helicases onto the origins of DNA replication. While several studies have shown that dysregulation of CDT1 expression causes re-replication and DNA damage in cell lines, and *CDT1* is highly expressed in several human cancers, whether CDT1 deregulation is sufficient to enhance tumorigenesis *in vivo* is currently unclear. To delineate its role *in vivo*, we overexpressed *Cdt1* in the mouse colon and induced carcinogenesis using azoxymethane/dextran sodium sulfate (AOM/DSS). Here, we show that mice overexpressing *Cdt1* develop a significantly higher number of tumors with increased tumor size, and more severe dysplastic changes (high grade dysplasia), compared to control mice under the same treatment. These tumors exhibited an increased growth rate, while cells overexpressing Cdt1 loaded greater amounts of Mcm2 onto chromatin, demonstrating origin over-licensing. Adenomas overexpressing *Cdt1* showed activation of the DNA damage response, apoptosis, formation of micronuclei, and chromosome segregation errors, indicating that aberrant expression of *Cdt1* results in increased genomic and chromosomal instability *in vivo*, favoring cancer development. In line with these results, high level expression of *CDT1* in human colorectal cancer tissue specimens and colorectal cancer cell lines significantly correlated with increased origin licensing, activation of the DNA damage response and microsatellite instability (MSI).

Keywords: Cdt1, Geminin, MCMs, origin licensing, DNA replication, colorectal cancer, microsatellite instability

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Introduction

DNA replication is a highly regulated process that results in genome duplication during the S-phase of the cell cycle. Initiation of replication occurs with the formation of the pre-replicative (pre-RC) complex at the origins of replication. The latter consists of the Origin Recognition Complex (ORC) and MCM multi-subunit proteins, as well as cell division cycle 6 (CDC6) and CDT1, which are essential for loading the MCMs onto the origins. Following origin firing, the pre-RC is inactivated, and its components are negatively regulated by multiple mechanisms. In the case of CDT1, this includes ubiquitin-mediated proteolysis by two E3 ubiquitin ligases as well as additional inhibition by its interacting protein Geminin [1].

Re-licensing of origins within the same cell cycle results in replication stress and re-replication; which has been linked with the accumulation of DNA damage and perturbations in genome stability. In line with this, it has been shown that stabilization of CDT1 in S-phase induces DNA re-replication, triggers a DNA damage response together with p53 upregulation, which leads to activation of apoptosis or a senescence pathway [2]. So far, a limited number of *in vivo* studies have assessed the role of CDT1 deregulation in tumorigenesis, mainly focusing on the synergistic effect of multiple licensing factors by using prone-to-tumorigenesis mouse genetic backgrounds. For example, Liontos *et al* have reported that overexpression of CDT1 in premalignant papilloma cells resulted in their transformation and tumor formation upon injection into nude mice [3], while Seo *et al* have shown that mice lacking p53 and overexpressing *Cdt1* in thymocytes develop lymphoblastic lymphomas [4]. More recently, Muñoz *et al* showed that combined overexpression of *Cdc6* and *Cdt1*, but not of *Cdt1* alone, caused lethal tissue dysplasias in mice [5].

Those studies, however, have not shown whether *Cdt1* deregulation is sufficient to accelerate tumorigenesis *in vivo*. Here, we report the generation of transgenic mice conditionally overexpressing *Cdt1*, which were used to study tumor formation in the colon. Our results

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collectively demonstrate that *Cdt1* overexpression is sufficient to enhance colorectal tumorigenesis through origin over-licensing and increased genomic instability *in vivo*.

Materials and methods

Conditional overexpression of *Cdt1* and ablation of *Geminin* was carried out in $Cdt1^{LSL/LSL}$ and $Geminin^{FL/FL}$ mice, respectively. Overexpression of *Cdt1* and ablation of *Geminin (Gmnn)* in $Lgr5^{+}$ intestinal stem cells was performed using the *Lgr5-EGFP-Ires-CreERT2* allele [6]. Azoxymethane (AOM) and dextran sodium sulfate (DSS) were used for the induction of colorectal carcinogenesis (Figure 1A). All experiments were performed according to the regulations of the Medical School of the University of Patras and were approved by the regional veterinary authority (118009/575, Prefecture of Western Greece). Following euthanization, mouse colons were removed, and macroscopic measurements were performed. The colons were then fixed in 10% formalin and embedded in paraffin; sections were cut at 4–10-μm thickness and used for hematoxylin/eosin staining (H/E), immunohistochemistry (IHC) /immunofluorescence (IF) analyses and Feulgen staining. RNA was extracted from normal and tumor tissues using TRIzol and cDNA synthesis and qPCR were then performed. Isolation of crypts, adenomas and F for chromatin bound Mcm2 were performed as described previously [7]. Expression of CDT1 and of other genes was also evaluated by IHC in formalin-fixed paraffin-embedded (FFPE) tissue samples from 35 primary human colorectal carcinomas (CRC) that were retrieved from the archives of the Department of Pathology, 'Agios Andreas' General Hospital of Patras, Greece (291/15-7-2022 and 367/08-08-2022). Evaluation of immunostaining was performed with the H-score system in a blinded-fashion by a certified pathologist. Ethical approval was obtained from the Committee on Research and Ethics and the Scientific Committee of the University Hospital of Patras, Greece. EdUseq-HU, sequencing read alignment and data processing were performed as described in detail previously [8]. Detailed materials and methods are available in the Supplementary materials and methods section.

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Results and Discussion

To delineate the role of deregulated *Cdt1* expression *in vivo*, we generated mice conditionally overexpressing *Cdt1*. A CMV-loxP-stop sequence-loxP-*CDT1*-IRES-EGFP cassette was targeted to the ROSA26 locus by homologous recombination (supplementary material, Figure S1A); the insertion was verified by Southern blotting and PCR (supplementary material, Figure S1B,C). Heterozygous and homozygous mice are referred to as $Cdt1^{LSL/WT}$ and $Cdt1^{LSL/LSL}$, respectively. The latter were then crossed with *Lgr5-Cre* mice, targeting Cre recombinase expression in $Lgr5^{+}$ cells of the intestine, upon induction with tamoxifen (hereafter referred to as *Lgr5* [6]). Selective overexpression of *Cdt1* and ablation of *Gmnn* in the colonic tissue was then verified with qPCR with no off-target expression detected in other tissues (supplementary material, Figure S1D).

We then sought to investigate colorectal tumorigenesis using the AOM/DSS protocol in mice with *Cdt1* overexpression and/or *Geminin* ablation. The AOM/DSS protocol is a colon carcinogenesis model initiated by the carcinogen AOM and driven by DSS-induced colitis, a condition akin to inflammatory bowel disease (IBD). Mutations induced by AOM coupled with colonic inflammation, aggravated by repeated cycles of DDS administration, accelerate tumor development in as little as 7–10 weeks. The observed tumors are mostly adenomas and metastatic tumors rarely develop within the normal experimental timespan [9]. Following chemical-induced carcinogenesis, the tumor number, size, and burden in mice overexpressing *Cdt1* and/or lacking *Geminin* were all significantly higher compared to control mice under the same treatment (Figure 1A and supplementary material, Figure S2A). The survival time of $Lgr5;Cdt1^{LSL/LSL}$ mice was also found to be significantly reduced compared to controls (supplementary material, Figure S2B). We have previously shown that *Geminin* ablation enhances colorectal tumorigenesis [10] but, interestingly, *Cdt1* overexpression seems to have a more profound phenotype. $Lgr5;Cdt1^{LSL/LSL}$ mice had a significantly higher tumor number compared to $Lgr5;Geminin^{FL/FL}$ mice (Figure 1A) while a shorter colon length was evident in $Lgr5;Cdt1^{LSL/LSL}$ and $Lgr5;Cdt1^{LSL/LSL};Geminin^{FL/FL}$

mice, (supplementary material, Figure S2C) in combination with significant weight loss (supplementary material, Figure S2D). We also observed a significantly higher number of adenomas in the small intestine of *Cdt1* overexpressing mice compared to controls (supplementary material, Figure S2E). However, we did not detect any metastases macroscopically, in the liver or lungs of control and experimental mice.

Mice with deregulated expression of *Cdt1* and/or *Geminin* showed a significantly higher number of colonic adenomas with high grade dysplasia as revealed by histopathological analysis (Figure 1B), and higher expression of the proliferation markers Ki67, Pcna and Mcm2 (Figure 1C). Considering that high Ki67, PCNA and MCM2 expression in human CRC have been shown to correlate with adverse histologic features, vascular invasion, metastases, and poor survival [11,12], our data indicate that overexpression of Cdt1 and/or lack of Geminin elevates the proliferation rate of colorectal adenomas through impairment of origin licensing. These characteristics were also confirmed *in vitro,* as adenoma cells derived from mice overexpressing *Cdt1* alone or in combination with *Gmnn* depletion (supplementary material, Figure S3A), developed a higher number of spheroids with larger diameter, as compared to adenoma cells from control mice (Figure 1D and supplementary material, Figure S3B).

Overall, these findings show that deregulated expression of *Cdt1* and/or *Geminin* enhances colorectal carcinogenesis *in vivo* and this effect seems to be more pronounced in the case of *Cdt1*. Interestingly, colons of both *Ctrl* and *Lgr5;Cdt1*$^{LSL/LSL}$ mice showed only a mild to moderate infiltration of lamina propria by chronic inflammatory cells with no erosions. We did not observe any differences in inflammation by H&E or CD3 IHC in both tumors and adjacent non-neoplastic colon between control and *Cdt1* overexpressing mice (supplementary material, Figure S3C,D). Moreover, throughout the course of the AOM/DSS treatment and before sacrifice, we did not observe any differences in gastrointestinal disorders among the different genotypes. These data

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suggest that Cdt1 overexpression does not enhance colorectal carcinogenesis through inflammation, pointing to a different underlying mechanism.

We therefore sought to investigate the mechanism by looking into origin licensing abnormalities *in vivo*. DNA replication licensing was visualized by immunostaining for DNA-bound Mcm2-7 complexes. To distinguish between soluble and DNA-bound forms of Mcm2-7 complexes, we briefly extracted tissues with nonionic detergent to remove soluble forms and quantified the remaining Mcm2, which marks cells with licensed origins (supplementary material, Figure S3E,F). By performing pre-extraction in isolated adenoma cells, adenomas from mice with deregulated *Cdt1* and/or *Geminin* expression were shown to load greater amounts of Mcm2 onto chromatin, compared to controls (Figure 2A). This was also the case in the adjacent normal intestinal crypts (Figure 2A). Therefore, *Cdt1* overexpression and/or *Geminin* ablation induces origin overlicensing both in tumor and normal tissues. Overlicensing has been linked to replication stress and re-replication [1]; in line with this, increased DNA content was identified in both adenomas and adjacent normal tissue of *Lgr5; Cdt1*$^{LSL/LSL}$ compared to controls (Figure 2B), suggesting DNA re-replication.

We further evaluated this *in vitro*, by generating an HCT116 colorectal cancer cell line overexpressing *CDT1* upon doxycycline (DOX) induction (supplementary material, Figure S4A,B). Following addition of DOX, CDT1 overexpression resulted in increased nuclear area and DNA content (supplementary material, Figure S4C,D), suggesting DNA re-replication. To examine whether CDT1 overexpression deregulates origin firing apart from re-replication, both induced and non-induced HCT116 cells were collected by mitotic shake-off and allowed to progress through the cell cycle for 14 h the presence of 5-ethynyl-2′-deoxyuridine (EdU) and hydroxyurea (HU). EdU-labeled DNA was then isolated and subjected to high-throughput sequencing (EdUseq-HU, supplementary material, Figure S5A,B). Analysis of the sequencing data at a genomic bin resolution of 10 kb, did not reveal any severe difference in the profile of

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origin firing upon CDT1 overexpression (supplementary material, Figure S5C,D). This result shows that CDT1 overexpression does not induce firing of novel sets of origins during early S-phase (*i.e.* ectopic origins) and therefore indicates that re-replication is driven by the same set of origins used during normal DNA replication, which is in line with a recent study [13]. However, given the limitations of the EdUseq-HU technique, we cannot exclude the possibility that following CDT1 overexpression, more origins may fire in the late replicating regions or that early origins may re-fire during late S-phase.

Re-replication induced by CDT1 overexpression or Geminin depletion has been reported to result in double-strand breaks (DSBs), to activate the DNA damage response (DDR) and induce senescence and/or apoptosis in cancer cell lines. To evaluate this *in vivo*, we examined the expression of different DDR markers by immunofluorescence and qPCR. Increased phosphorylation of H2AX was evident in adenomas overexpressing *Cdt1* as compared to controls, in combination with an increased number of apoptotic cells (Figure 2C). Activation of the DDR through increased phosphorylation of H2AX and a larger number of 53BP1 foci were also verified in HCT116 CDT1 overexpressing cells (supplementary material, Figure S6A,B). In line with this, transcriptional activation of *Rad51* and *Blm*, which function in the homologous recombination pathway for the repair of DSBs [14], were all upregulated in *Cdt1*-overexpressing adenomas (Figure 2C). This was also the case for the Fanconi-Anemia repair pathway proteins *Fanca* and *Fanci*, of which the latter has been reported to regulate dormant origin firing following replication stress [15] (Figure 2C). However, we did not observe any signs of senescence in the normal or cancer intestinal epithelium of mice overexpressing Cdt1 compared to control mice, by immunohistochemistry for p16 (supplementary material, Figure S6C). DNA replication origin reactivation with DNA damage has been linked to genomic instability. In line with this, a greater number of micronuclei and chromatin bridges during mitosis was evident in *Lgr5;Cdt1*$^{LSL/LSL}$ adenomas compared to controls, indicating increased chromosomal instability and segregation

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errors (Figure 2D). Overall, these findings show that Cdt1 overexpression *in vivo* induces origin overlicensing, replication stress, DNA breaks, activation of DNA repair pathways and apoptosis; eventually resulting in increased genomic instability favoring cancer development.

Several studies have previously reported increased *CDT1* expression in human cancers [16,17]. Using TCGA bulk RNA-seq expression data, very high *CDT1* levels can be seen in human colorectal cancer compared to other cancer types (supplementary material, Figure S7A), while gene array data in TNMplot [18] show that *CDT1* levels significantly increase from normal to tumor to metastatic colorectal cancer tissue, in that order (supplementary material, Figure S7B). High *CDT1* expression is also associated with poor survival in cancer patients (supplementary material, Figure S7C), based on TCGA and GTEx data [19]. To address the links to DNA damage and genomic instability, we analyzed recently published human single cell RNA-seq data from ~370,000 cells of colorectal tumors and adjacent normal tissues from 62 patients [20]. These data show that high *CDT1* expression is mainly found in the stem/transit-amplifying (TA)-like cell clusters of both normal and tumor cells (E02 and E03) (Figure 3A and supplementary material, Figure S8). In these clusters, *CDT1* is co-expressed with genes participating in origin licensing and DNA replication (*GMNN*, *MCM2*, *MCM7*, *POLD1*), DDR (*H2AFX*, *TP53*, *RAD51*, *RAD54L*) as well as with the colon cancer stem cell markers *PROM1* (*CD133*) and *CD44* (Figure 3B). These correlations were also identified in bulk RNA-seq data from CRC patients, human CRC cell lines (supplementary material, Figure S9A,B) and by qPCR analysis of mouse adenomas overexpressing *Cdt1* (Figure 3C). We then performed IHC for CDT1, γH2AX, phospho-ATM/ATR, p53, MCM7 and Geminin in human CRC specimens. Tumors overexpressing CDT1 displayed significantly higher expression levels of all the above markers and showed increased numbers of micronuclei (Figure 3D and supplementary material Figure S10A,B).

Colorectal cancers are often characterized by microsatellite instability (MSI) [21], mutations [22], and copy number alterations [23], which are all segments of genomic instability [24]. By

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analyzing genomic and transcriptomic data from CRC patients and CRC human cells lines, we identified a strong association of high *CDT1* expression levels with MSI (Figure 4A). Moreover, we identified that deletions of *WWOX*, a common fragile site (CFS) gene product that regulates ATM/ATR signaling and DDR [25] and *AGBL4*, a large gene encoding for a metallocarboxypeptidase, are significantly more frequent in CRC patients with high *CDT1* expression levels (Figure 4B). Similarly, human CRC cell lines with high expression levels of *CDT1* contain a higher number of mutations compared to cell lines with normal *CDT1* levels (Figure 4C). Both CFS deletions and mutations are linked to DNA replication stress [26,27], revealing a strong association of high *CDT1* expression with increased re-replication, DNA damage and genomic instability in human colorectal cancer.

Overall, our data demonstrate that *CDT1* shares oncogenic properties, as its overexpression enhances colorectal carcinogenesis *in vivo*. *Cdt1* overexpression in the Lgr5+ intestinal stem cells altered the normal replication licensing program, resulting in activation of the DDR and chromosomal instability (Figure 4D). Previous work in cellular systems, has shown that loss of origin licensing control, through overexpression of CDT1, induces DNA lesions and chromosomal abnormalities [2,3,13] which can fuel malignant cell transformation. In addition, the intestinal epithelium is characterized by daily epithelial renewal driven by small populations of $Lgr5^+$ adult stem cells that reside in the intestinal crypts, which give rise to ‘‘transit-amplifying’’ progenitors [6]. Cells with a high proliferative rate, such as embryonic stem cells or cancer cells, rely mainly on Geminin to suppress abnormal origin licensing mediated by CDT1 [28,29]. Given the high proliferative rate of the $Lgr5^+$ stem cells, Cdt1 overexpression seems to be sufficient to promote origin overlicensing, even in the presence of functional Geminin. The aggressive phenotype observed upon *Cdt1* overexpression *in vivo* may be reflected by the high rate of genomic and chromosomal instability, which in turn is associated with tumor evolution, drug resistance and poor outcome of most cancers [30]. Moreover, we uncovered the clinical

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significance of CDT1, as its high expression levels correlates with DNA damage and MSI in CRC. Given the fact that CDT1 is highly expressed in different cancer cell lines and human cancers [3,16,17], CDT1 could be a potential prognostic and therapeutic target especially in CRC colorectal cancer. Notably, stabilizing CDT1 activity or inactivating Geminin has been shown to induce genotoxic stress in cancer-derived cell lines in contrast to non-transformed cells [28]. Therefore, development of inhibitors stabilizing CDT1 activity, as the neddylation inhibitor MLN4924 [31], or chemical compounds inhibiting Geminin, or its interaction with CDT1 [32], may serve as specific anticancer agents in CRC. Interestingly, it has recently been reported that mismatch repair-deficient stage II or III rectal adenocarcinomas are highly sensitive to single-agent programmed death 1 (PD-1) blockade [33]. Given that high CDT1 expression is associated with MSI, CDT1-overexpressing tumors could also respond well to PD-1 inhibition, opening new therapeutic windows.

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Author contributions statement

MP, SCT, ZL and ST conceived the study and planned the experiments. MP, SCT, SN, AK, AK, KI and IRD carried out experiments. SCT, MO, RN and KA contributed to the generation of the *Cdt1*^{LSL} transgenic mouse. MP, SCT, SM, VSD, VG, VR, TDH, VB, ZL and ST analyzed the data and SCT, SM, VSD and GJD performed the bioinformatics analyses. MP wrote the manuscript and prepared the figures with the help of SCT. ZL and ST finalized the manuscript. All authors read, commented, and approved the final version of the manuscript.

Data availability statement

Computer codes and data files utilized for processing and plotting the data are available upon request. The raw fastq files and processed BigWig files have been deposited in NCBI GEO (Gene Expression Omnibus) database under the accession number: GSE212710.

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Figure Legends

Figure 1. *Cdt1* overexpression and/or *Geminin* ablation enhances colorectal carcinogenesis.

(A) (Upper Left) Schematic representation of the model of murine colon carcinogenesis. (Upper Right) Representative macroscopic images of intestinal adenomas from *Ctrl* (*Lgr5*), *Lgr5;Geminin*$^{FL/FL}$, *Lgr5;Cdt1*$^{LSL/LSL}$ and *Lgr5;Geminin*$^{FL/FL}$*;Cdt1*$^{LSL/LSL}$ mice; all groups were treated with AOM/DSS. (Bottom) Graphical representation of the number and size of intestinal adenomas, as well as tumor burden of *Lgr5;Geminin*$^{FL/FL}$ (n=5), *Lgr5;Cdt1*$^{LSL/LSL}$ (n=6),

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Lgr5;Geminin$^{FL/FL}$*;Cdt1*$^{LSL/LSL}$ (n=7) and *Ctrl* (n=16). (B) Histopathological analysis in the murine colon of AOM/DSS treated mice. (Left) Representative images of colorectal sections stained with H&E depicting adenomas with low- and high-grade dysplasia. (Right) Graphical representation of the number of lesions from *Lgr5;Geminin*$^{FL/FL}$ (n=4), *Lgr5;Cdt1*$^{LSL/LSL}$ (n=5), *Lgr5;Geminin*$^{FL/FL}$*;Cdt1*$^{LSL/LSL}$ (n=4) and *Ctrl* (n=8). Low-grade lesions include adenomas with low-grade dysplasia; high-grade lesions include adenomas with high-grade dysplasia and intramucosal carcinomas. Analysis was performed along the entire proximal–distal axis. (C) Adenomas overexpressing *Cdt1*, lacking *Geminin* or having combination of both genetic alterations, show higher proliferation rate compared to controls. (Left) Representative images of immunohistochemistry in sections with colorectal adenomas for Ki67, Pcna and Mcm2 from *Ctrl*, *Lgr5;Gem*$^{FL/FL}$, *Lgr5;Cdt1*$^{LSL/LSL}$ and *Lgr5;Cdt1*$^{LSL/LSL}$*;Geminin*$^{FL/FL}$ mice. (Right) Graphical representation of H-Score for Ki67^{+}-, Pcna^{+}- and Mcm2^{+}- cells. *n*=3 mice per genotype. (D) Cells derived from adenomas overexpressing *Cdt1*, lacking *Geminin*, or having combination of both, developed a higher number and larger size of spheroids *in vitro*. (Left) Graphical representation of the number of spheroids formed, following the plating of 10^3 cells derived from colorectal adenomas. (Right) Graphical representation of the size of spheroids formed. *n*=3 mice per genotype. In all cases, data were analyzed using one-way ANOVA with Bonferroni correction. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001. Error bars indicate ± SEM. Scale bars, 20 μm

Figure 2. Increased loading of Mcms, DNA damage and genomic instability upon *Cdt1* overexpression *in vivo*. (A) (Upper left) Representative images of adenoma cells derived from *Ctrl*, *Lgr5;Geminin*$^{FL/FL}$, *Lgr5;Cdt1*$^{LSL/LSL}$ and *Lgr5;Geminin*$^{FL/FL}$*;Cdt1*$^{LSL/LSL}$ AOM/DSS treated mice, immunostained for DNA-bound Mcm2. Nuclei were stained with DAPI. Scale bar: 7 μm. (Upper Right) Scatter plot depicts quantification of DNA-bound mean Mcm2 intensity per cell. At least 500 cells were quantified per mouse. *n*=3 mice per genotype. (Lower left) Representative images of isolated intestinal crypts derived from *Ctrl*, *Lgr5;Geminin*$^{FL/FL}$, *Lgr5;Cdt1*$^{LSL/LSL}$ and

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Lgr5;Geminin$^{FL/FL}$*;Cdt1*$^{LSL/LSL}$ AOM/DSS treated mice. Crypts were immunostained for DNA-bound Mcm2. Nuclei were stained with DAPI. Scale bar, 7 μm. (Lower right) Scatter plot depicts the quantification of DNA-bound mean Mcm2 intensity per cell. At least 1000 cells were quantified from 20 crypts per mouse. *n*=3 mice per genotype. Data were analyzed using one-way ANOVA with Bonferroni correction. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001. (B) Increased DNA content upon *Cdt1* overexpression *in vivo*. (Left) DAPI-stained nuclei of adenoma- and crypt- cells from colon tissue of *Ctrl* and *Lgr5;Cdt1*$^{LSL/LSL}$ AOM-DSS treated mice. Scale bars: 20 μm. (Right) Box plots depict DAPI integrated density. More than 1000 crypt- or adenoma-cells were analyzed per mouse. *n*=3 mice per genotype. (C) Increased incidence of double strand breaks and apoptosis in colorectal adenomas with *Cdt1* overexpression. (Left) Representative images of immunohistochemistry for γH2AX and Caspase3 (Cc3) in colorectal adenomas from *Ctrl* and *Lgr5;Cdt1*$^{LSL/LSL}$ mice. Nuclei stained with DAPI. Scale bars, 30 μm. (Center) Quantification of γH2AX expression using H-Score and % percentage of Caspace3^{+} cells, from *Ctrl* (*n*=6) and *Lgr5;Cdt1*$^{LSL/LSL}$ (*n*=5) mice. (Right) Transcriptional activation of genes participating in DNA damage response and repair in adenomas overexpressing *Cdt1*. Real time RT PCR assessment of *Rad51*, *Fanca*, *Fanci*, and *Blm* expression levels in adenomas derived from *Lgr5;Cdt1*$^{LSL/LSL}$ (n=3) and *Ctrl* (n=3) mice. (D) Elevated signs of genomic instability in colorectal adenomas overexpressing *Cdt1*. (Left) Representative images of adenomas derived from *Ctrl* and *Lgr5;Cdt1*$^{LSL/LSL}$ stained with Feulgen. Arrows indicate micronuclei and chromatin bridges. Scale bars: 10 μm. (Right) Quantification of % percentage of micronuclei and chromatin bridges from *Ctrl* (*n*=6), and *Lgr5;Cdt1*$^{LSL/LSL}$ (*n*=5) mice. Scale bars, 10 μm. Data were analyzed using Mann–Whitney *U*-tests. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001. Error bars indicate ± SEM.

Figure 3. CDT1 is overexpressed in human colorectal cancer and its expression correlates with increased proliferation and DDR. (A) Single cell RNA-seq data from the dataset generated

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using Broad Institute's single cell RNA-seq data portal. t-SNE plot of cell clusters of epithelial cells from colorectal cancer and adjacent normal tissue (top) together with the t-SNE plot showing CDT1 gene expression (bottom). (B) Dotplot based on the data in (A), showing average expression level and percentage of expressing cells for *CDT1*, *Geminin*, *MCM2*, *MCM7*, *CHK1*, *POLD1*, *H2AFX*, *TP53*, *RAD51*, *RAD54L*, *LGR5*, *PROM1* and *CD44* across cell clusters. (C) High level expression of genes implicated in origin licensing, DNA replication and DDR in adenomas overexpressing *Cdt1*. Real time qPCR assessment of *Mcm2*, *Mmc7*, *Orc1*, *Cdc6, Pold1, Lgr5, Cd133, Cd44* expression levels in adenomas derived from *Lgr5;Cdt1*$^{LSL/LSL}$ (n=3) and *Ctrl* (n=3) mice. Data were analyzed using Mann–Whitney *U*-test. **p*<0.05, **p*<0.01. Error bars indicate ± SEM. (D) Representative images of γH2AX, phospho-ATM/ATR and P53 expression, detected by immunohistochemistry, and Feulgen staining, in human colorectal cancer tissue samples with low (H-score<30) and high expression (H-score>90) of CDT1, respectively. Scale bars, 20 μm. Graphs indicate expression of γH2AX, phospho-ATM/ATR and P53 (H-Score), and percentage of cells with micronuclei, in tissue samples with low (H-score<30) and high expression (H-score>90) of CDT1. Data were analyzed using Mann–Whitney *U*-test. ***p*<0.01, ****p*<0.001, *****p*<0.0001. Error bars indicate ± SEM.

Figure 4. CDT1 overexpression is associated with genomic instability in colorectal cancer. (A) High expression levels of *CDT1* correlate with MSI in CRC patients (left) and in human CRC cell lines (right). Data were analyzed using Mann–Whitney *U*-tests. ***p*<0.01, ****p*<0.001. Error bars indicate ± SEM. (B) High expression levels of CDT1 correlate with deletion of *WWOX* and *AGBL4* genes in CRC patients. Data were analyzed using Fisher's exact test. **p*<0.05, ***p*<0.01. (C) High expression levels of CDT1 associate with elevated number of mutations in human CRC cell lines. Data were analyzed using Mann–Whitney *U*-tests. ***p*<0.01. (D) Proposed model of CDT1-enhanced colorectal carcinogenesis.

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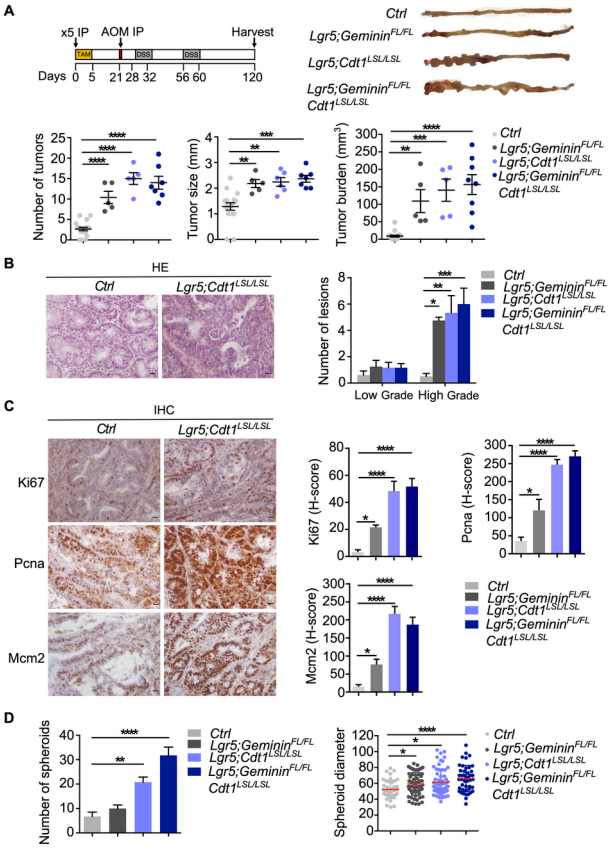

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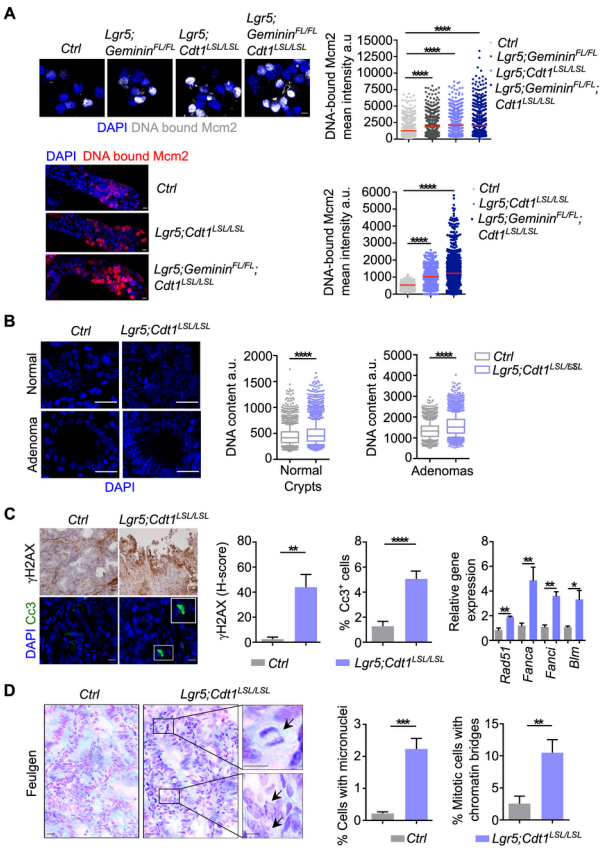

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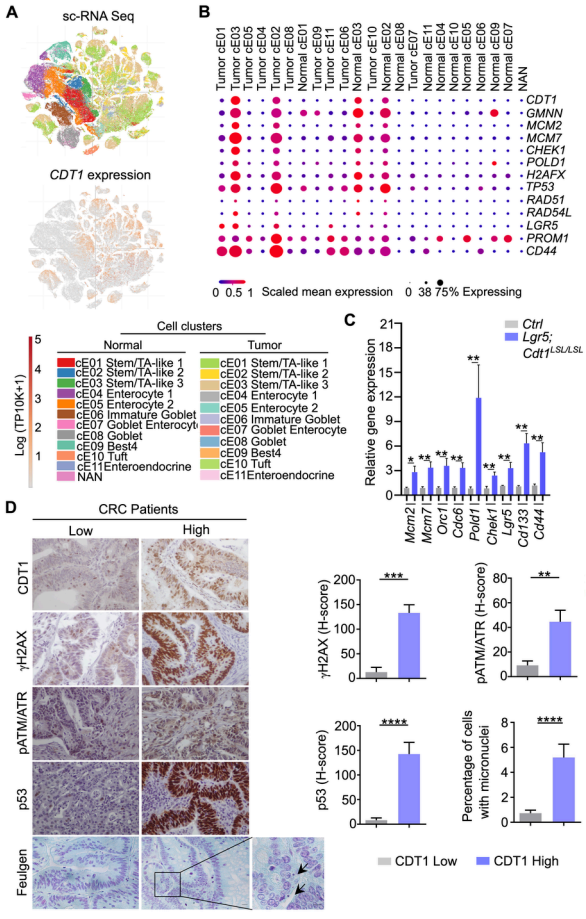

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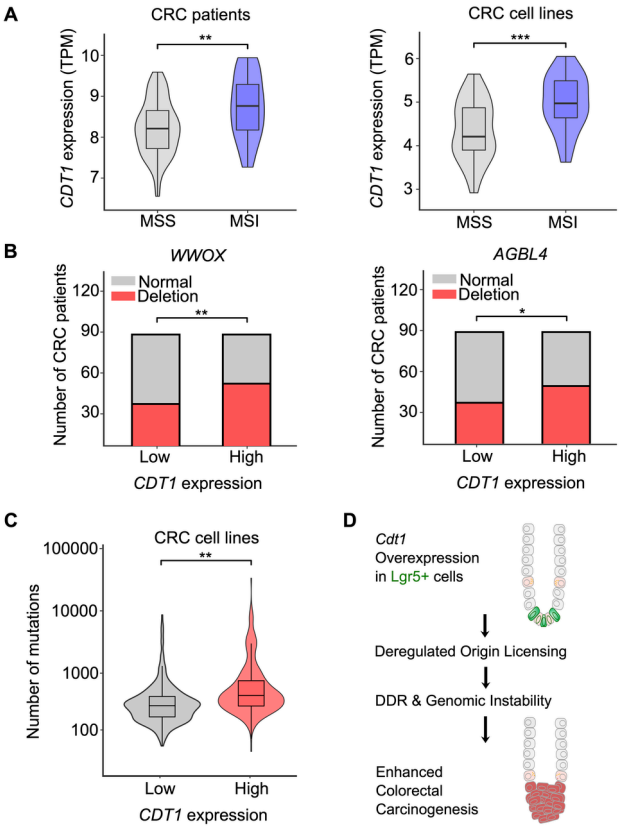

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