Cell resistance to the Cytolethal Distending Toxin involves an association of DNA repair mechanisms

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The Cytolethal Distending Toxin (CDT), produced by many bacteria, has been associated with various diseases including cancer. CDT induces DNA double-strand breaks (DSBs), leading to cell death or mutagenesis if misrepaired. At low doses of CDT, other DNA lesions precede replication-dependent DSB formation, implying that non-DSB repair mechanisms may contribute to CDT cell resistance. To address this question, we developed a proliferation assay using human cell lines specifically depleted in each of the main DNA repair pathways. Here, we validate the involvement of the two major DSB repair mechanisms, Homologous Recombination and Non Homologous End Joining, in the management of CDT-induced lesions. We show that impairment of single-strand break repair (SSBR), but not nucleotide excision repair, sensitizes cells to CDT, and we explore the interplay of SSBR with the DSB repair mechanisms. Finally, we document the role of the replicative stress response and demonstrate the involvement of the Fanconi Anemia repair pathway in response to CDT. In conclusion, our work indicates that cellular survival to CDT-induced DNA damage involves different repair pathways, in particular SSBR. This reinforces a model where CDT-related genotoxicity primarily involves SSBs rather than DSBs, underlining the importance of cell proliferation during CDT intoxication and pathogenicity.

The Cytolethal Distending Toxin (CDT) is a virulence factor produced by many pathogenic bacteria1. CDT is a tripartite holotoxin generally composed of two regulatory subunits (CdtA and CdtC) and one catalytic subunit (CdtB)2. As an exception, CdtB from the typhoid toxin, identified in Salmonella enterica serovar Typhi, is associated with another catalytic subunit (PltA) and regulatory subunits (PltB)3. Sequences and structures of the different CdtB subunits are highly conserved4 and the CdtB virulence properties have been documented in many cases5,6. Indeed, mice infected with Helicobacter hepaticus developed hepatic dysplasic nodules, whereas mice infected with the CdtB-deficient strain did not5. Moreover, many of the acute phase symptoms of typhoid fever can be reproduced in mice by systemic administration of the typhoid toxin, but not with a catalytically-dead mutant toxin3. This highlights the importance of understanding the mode of action of CdtB on host cells.

CdtB shares structural and functional homology with DNase I and displays nuclease activity, observed in vitro by plasmid digestion or in mammalian cells by chromatin fragmentation7,8. As CdtB induces DNA double-strand breaks (DSBs), intoxication of human cells with CDT is accompanied by DSB signaling through the ATM-dependent phosphorylation of H2AX (referred to as γH2AX) and the recruitment of DSB-processing factors to damaged sites, including the MRN complex components and 53BP19–12. The CDT-dependent activation of the ATM pathway promotes cell cycle arrest and eventually apoptotic cell death when the cell encounters excessive damage13,14.

However, several evidence challenges the model of direct DSB induction by CdtB. First, plasmid digestion by CdtB predominantly results in single-strand breaks (SSBs)9,15. Furthermore, we have shown that decreasing the CDT concentration to moderate doses (less than 1 ng/ml) induces primary DNA lesions, presumably SSBs, before DSB formation during S-phase12. These replication-dependent DSBs accumulate over time in proliferating cells,

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in contrast to the massive and rapid DSB induced by high doses of CDT (over 1 μg/ml) on both proliferating and non-proliferating cells. Thus, we hypothesized that these two dose-dependent modes of CDT-induced DSB formation may activate different cellular pathways.

As mammalian cells experience thousands of DNA lesions each day, they have evolved DNA repair mechanisms to maintain genomic integrity. While being partly interconnected, each repair pathway responds to specific types of DNA lesions (Table 1). Altered bases are processed by base excision repair (BER) while bulky adducts are repaired through the nucleotide excision repair (NER). SSBs, arising directly by disintegration of the oxidized sugar or indirectly as intermediates of BER, are repaired by SSB repair (SSBR). DSB management involves two major mechanisms: Non-homologous end joining (NHEJ), active throughout the cell cycle, directly ligates two double-stranded DNA ends without any sequence homology requirement, whereas Homologous recombination (HR) restores DNA integrity through homology search on an undamaged template. As sister chromatid is generally used as the homologous template, HR is restricted to S and G2 cells, and, contrary to NHEJ, allows the restart of collapsed replication forks. Finally, interstrand crosslink (ICL) is processed by the Fanconi Anemia (FA) pathway, which is also involved in replication fork stability.

To understand which repair pathways are involved in response to CDT, a genetic screen was performed in budding yeast and identified HR as the only mechanism able to repair the CdtB-induced DNA lesions. In human cells, the HR role was confirmed, particularly in replication-dependent DSBs, while resistance to CDT-induced direct DSBs involved another repair pathway, presumably NHEJ. Here, we adapted the Multicolor Competition Assay (MCA), based on human cells depleted in each of the main DNA repair pathways (Table 1), to study the repair mechanisms involved in the cellular response to genotoxic treatments. The implication of each DNA repair mechanism involved in CDT resistance was confirmed with classical genetic models of DNA repair pathways. Our data substantiate the importance of the DSB repair pathways for cells to survive CDT intoxication, and demonstrate for the first time the role of SSBR. Moreover, we analyzed the functional relationship between HR, NHEJ and SSBR to respond to CDT genotoxicity. Finally, we underline the importance of the replicative stress response and identify the FA pathway as being essential following a CDT treatment. Altogether, these findings depict a global view of the DNA repair pathways involved in the resistance to CDT-mediated genotoxicity, enabling a deeper understanding of the CdtB mode of action.

### Results

**HR processing of CDT-mediated DSBs is confirmed by the Multicolor Competition Assay.** To decipher which DNA repair mechanisms are required to survive CDT intoxication, we established fluorescent human stable cell lines deficient for each repair pathway from the HCT116 p53−/− background. The p53 deficiency confers a slight resistance to CDT but prevents the DNA-damage induced G1 block. Then, we developed a test based on the Multicolor Competition Assay (MCA). Basically, fluorescent DNA repair defective cells are co-cultured with the non-fluorescent parental control cells and subjected to CDT intoxication. The ratio of fluorescent cells is compared to the untreated condition, used as a control for the relative cell growth. If CDT exposure induces DNA damage, one or more cell line will present a proliferation defect.

As HR deficient cells are hypersensitive to CDT, we decided to validate the MCA strategy by confirming the role of HR. The shRNA depletion of PALB2 (Fig. S2A), an essential HR factor, led to a dose-dependent reduction of the fluorescent ratio with MMC (Fig. 1A), a chemical agent known to induce ICL and HR processing. We next performed MCA on shPALB2 cells treated with an active toxin (CDT) or a catalytically-dead mutant (CDTΔTrt) as a control. The fluorescence ratio decreases in CDT-treated cells, demonstrating that the impairment of HR, through PALB2 down-regulation, leads to CDT sensitization. CDTΔTrt exposure did not induce any proliferation defect. Thus, our results show that PALB2 is important to respond to CDT, confirming that HR can repair CDT-mediated DSBs and validating the MCA approach.

MCA was then conducted on isogenic cell lines deficient for NHEJ (shXRCC4), SSBR (shXRCC1), NER (shXPA), FA pathway (shFANCC) or replicative stress signaling (shATR). The shRNA-mediated gene knockdown has been confirmed for all cell lines (Fig. S2). Except for shXPA cells, all deficient cell lines exhibit a weaker

| Depleted protein | Pathway (DNA lesion) | Protein function | shRNA target sequence |
|------------------|----------------------|------------------|-----------------------|
| PALB2            | HR (DSB; ICL)        | Forms a complex with BRCA1 and BRCA2; HR mediator | GATGACATTGATGATCTCTTA |
| XRCC4            | NHEJ (DSB)           | Interacts with Lig1 and XLF to form the DSB end ligation complex | GCTCAGGAGAATCAGCTTCAA |
| XPA              | NER (bulky lesions)  | Binds and stabilizes the open pre-incision complexes; recruits ERCC1-XPF endonuclease | CAGATGATTGGACCAAGAGGA |
| XRCC1            | SSBR (SSB)           | Scaffold protein; stabilizes and stimulates multiple enzymatic components during SSBR | CATGTCGCTGAGAAGATA |
| ATR              | Replicative stress signaling | Serine/threonine kinase involved in the replicative stress signaling | GCTAATTCGTTGAGGCTT |
| FANCC            | Fanconi Anemia (ICL) | Subunit of the FA core complex involved in FANCD2/FANCC mono-ubiquitylation | CACGATCATTGCCGTTCTT |

Table 1. Summary of the DNA repair proteins down-regulated in HCT116 cells. This table depicts the down-regulated proteins, the repair pathways associated (and the processed DNA lesions), their function and the sequence targeted by the shRNA.
growth rate compared to their parental counterpart, after CDT<sup>wt</sup> but not after CDT<sup>H153A</sup> (Fig. 1B). Moreover, chronic exposure to sublethal dose of CDT<sup>wt</sup> induces micronucleus formation in all DNA repair defective cells apart from shXPA, indicative of enhanced genetic instability (Fig. 1C). These results indicate that cell survival after CDT treatment necessitates multiple repair mechanisms, but not NER, and involves replicative stress signaling.
NHEJ involvement in response to CDT-induced DSBs. NHEJ constitutes the primary DSB repair pathway in mammalian cells\(^2\), with XRCC4 implicated in the ligation core complex\(^2\). To strengthen the MCA data obtained on shXRCC4 cells, XRCC4\(^{-/-}\) MEFs were tested for CDT sensitivity (Fig. 2A). XRCC4\(^{-/-}\) MEFs are around 20-fold more sensitive than control cells when exposed to 250 pg/ml of CDT\(^{wt}\). These effects depend on the CdtB nuclease activity, as MEF cells intoxicated with CDTH\(^{153A}\) are as viable as untreated cells. DSB formation induces XRCC4 phosphorylation, illustrating an activation of NHEJ\(^3\). Compared to untreated or CDTH\(^{153A}\) treated cells, HeLa cells exposed to the DSB-inducing agent calicheamicin-\(^{\gamma1}\) (Cali), or for 8 hours with 250 pg/ml of CDT\(^{wt}\). Extracts were treated or not with Shrimp Alkaline Phosphatase (rSAP) for 30 minutes at 37°C. NT: non-treated cells. L indicates the long (phosphorylated) forms and S the short form of XRCC4. Lamin A is shown as a loading control. Full-length blots are presented in Fig. S8. (C) Representative images of 53BP1 immunostaining in XRCC4\(^{+/+}\) and XRCC4\(^{-/-}\) non-treated (NT) MEFs, treated with 25 pg/ml of CDT\(^{wt}\) for 24 h or for 3 h followed by a 21 h recovery time (repair 21 h). Scale bar = 20 \(\mu\)m. (D) Quantification of XRCC4\(^{+/+}\) and XRCC4\(^{-/-}\) MEFs positive for 53BP1 foci formation, after CDT\(^{wt}\) exposure for the indicated doses and times. NT: non-treated cells. Data are expressed as the mean ± SD of at least 3 independent experiments. Statistics were calculated by unpaired Student’s t-test (\(*P < 0.05; **P < 0.01; ***P < 0.001\)).

Figure 2. NHEJ is necessary to survive CDT-induced DSBs. (A) Clonogenic survival of XRCC4\(^{+/+}\) and XRCC4\(^{-/-}\) MEFs exposed to CDT\(^{wt}\) or CDTH\(^{153A}\). Results present the mean ± SD of at least 3 independent experiments. Statistics were calculated by unpaired Student’s t-test (\(*P < 0.05; **P < 0.01; ***P < 0.001\)). Graph in linear scale is presented in Fig. S9. (B) XRCC4 immunoblots of soluble extracts from HeLa cells treated for 1 hour with 5 nM of Calicheamicin-\(^{\gamma1}\) (Cali), or for 8 hours with 250 pg/ml of CDT\(^{wt}\). Extracts were treated or not with Shrimp Alkaline Phosphatase (rSAP) for 30 minutes at 37°C. NT: non-treated cells. L indicates the long (phosphorylated) forms and S the short form of XRCC4. Lamin A is shown as a loading control. Full-length blots are presented in Fig. S8. (C) Representative images of 53BP1 immunostaining in XRCC4\(^{+/+}\) and XRCC4\(^{-/-}\) non-treated (NT) MEFs, treated with 25 pg/ml of CDT\(^{wt}\) for 24 h or for 3 h followed by a 21 h recovery time (repair 21 h). Scale bar = 20 \(\mu\)m. (D) Quantification of XRCC4\(^{+/+}\) and XRCC4\(^{-/-}\) MEFs positive for 53BP1 foci formation, after CDT\(^{wt}\) exposure for the indicated doses and times. NT: non-treated cells. Data are expressed as the mean ± SD of at least 3 independent experiments. Statistics were calculated by unpaired Student’s t-test (\(*P < 0.05; **P < 0.01; ***P < 0.001\)).

SSBR is important to respond to CDT-related genotoxicity. We then aimed to clarify the consequences of a SSBR defect after CDT treatment. MCA pointed out the crucial role of XRCC1, an essential SSBR protein\(^3\), during repair of CDT-mediated DNA lesions (Fig. 1B). To strengthen this observation, CDT sensitivity was monitored in the XRCC1\(^{-/-}\) CHO cell line (EM9) compared to the corresponding control (AA8) (Fig. 3A), or in MEFs knocked-out for PARP1, another SSBR key protein (Fig. 3B)\(^3\). Cells deficient for XRCC1 or PARP1 displayed a higher sensitivity than their wild-type counterpart, for all tested CDT\(^{wt}\) concentrations, but not for CDTH\(^{153A}\). Therefore, SSBR clearly contributes to survival following CDT-induced DNA damage, favoring the assumption that SSBSs are the primary lesions induced by CDT.
Then, an alkaline Comet assay was performed on shXRCC1 HCT116 cells to reveal the overall DNA damage rate, including SSBs and DSBs. No significant change was observed between the control and the XRCC1-depleted cells exposed to CDT H153A or left untreated (Fig. 3C), showing that neither XRCC1 knockdown nor CDT H153A induces detectable DNA lesions. Treatment with 25 ng/ml of CDT wt provokes a time-dependent increase of tail-DNA level in both cell populations, which seems to reach a plateau after 48 h. This suggests that the toxin remains active for at least two days, as stated earlier and confirmed here (Fig. S4). However, shXRCC1 cells exhibit more tail-DNA level, as soon as 24 h of CDT wt treatment (22.6%), compared to control cells (7.4%).
cells lacking XRCC1 accumulate more damage, which may illustrate a lower repair capacity of CDT-lesions in SSBR deficient cells. Next, XRCC1−/− EM9 and AA8 control cells were subjected to different doses of CDT, for 24 h or for 2 h followed by 22 h of recovery time, and DSB accumulating cells have been quantified through 53BP1 immunofluorescence (Fig. 3D,E). In absence of treatment, EM9 cells exhibit a slight but statistically significant increase in 53BP1-positive cells compared to AA8, demonstrating that impairment of XRCC1-dependent repair of endogenous DNA lesions can result in DSB. As shown earlier (Fig. 3C), CDTγH2AX exposure does not lead to DSB formation. After 24 h of CDTγH2AX, the ratio of 53BP1-positive cells is not statistically different between AA8 and EM9 in our experimental settings (Fig. 3E). However, when cells are allowed to repair the CDT-induced DNA lesions for 22 h, EM9 cells exhibit more 53BP1-positive cells for all tested doses of CDTγH2AX, suggesting that the repair defects caused by XRCC1 knockout result in DSB persistence. Altogether, these data illustrate the consequence of SSBR deficiency in the accumulation of DSB after CDT.

As CDT-exposed XRCC1-deficient cells suffer more unrepaired DSBs, the DNA damage-dependent cell cycle arrest should be more important compared to control cells. Indeed, after 24 h of CDTγH2AX intoxication, the accumulation of G2/M cells is significantly more pronounced for shXRCC1 HCT116 cells, suggesting that the magnitude of G2 arrest in response to CDT reflects the level of unrepaired DNA lesions (Fig. 3F). Taken together, these data demonstrate that defective SSBR aggravates the cellular outcome of CDT exposure, with an accumulation of unrepaired DSBs associated with enhanced cell cycle arrest. In conclusion, our findings establish for the first time the crucial role of SSBR for cells to survive CDT-induced genotoxicity.

**Interplay between SSBR, NHEJ and HR in response to CDT.** To evaluate the relative importance of SSBR, NHEJ and HR to survive CDT-mediated genotoxicity, HeLa cells were impaired for one, two or three pathways through siRNA-mediated depletion of XRCC1, XRCC4 and/or RAD51 (Fig. 4A). First, a clonogenic assay has been performed to assess CDT-sensitivity (Fig. 4B). At 25 or 250 pg/ml, control cells are statistically less sensitive to CDT than cells depleted for one or more repair pathways, corroborating the MCA results. Interestingly, down-regulation of XRCC1, XRCC4 or RAD51 alone induces a less pronounced survival defect after 25 pg/ml of CDT than when all three proteins are depleted, pointing out the cumulative effect of SSBR, NHEJ and HR impairment on CDT-mediated genotoxicity. At 2500 pg/ml, RAD51 deficiency does no longer sensitize HeLa cells to CDT, which is in agreement with our previous report showing that RAD51 is not recruited to DSBs induced at 2500 pg/ml of CDTγH2AX, suggesting that HR is not essential to repair direct DSBs.

Then, we investigated the consequences of XRCC1, XRCC4 and/or RAD51 knockdown on DSB induction through γH2AX accumulation after a 250 pg/ml treatment of CDT for 24 h (Fig. 4C). CDT exposure induces γH2AX accumulation that is greater when XRCC4 is down-regulated, alone or in combination. Therefore, the global DSB level induced by CDT seems more particularly regulated by NHEJ. Next, γH2AX induction has been monitored by immunofluorescence after exposure to 250 pg/ml of CDT for 24 h or for 3 h followed by 21 h of recovery (Fig. 4D). After 24 h of CDT, control and XRCC1 deficient cells display around 50% of γH2AX positive cells (Fig. 4E). The proportion of damaged cells increases up to 80 and 65% after depletion of XRCC4 and RAD51, respectively. XRCC1 loss does not enhance the bulk of DSB-accumulating cells in XRCC4 and RAD51 deficient cells. Furthermore, RAD51 or XRCC1 knockdown does not aggravate the phenotype of XRCC4 deficient cells. Thus, depletion of XRCC4, and to a lesser extent depletion of RAD51, induces an augmentation of DSB-accumulating cells after a 24 h CDT exposure, and these two responses are not cumulative. Alternatively, XRCC1 knockdown does not induce more γH2AX signal. In order to estimate the defects caused by XRCC1, XRCC4 and/or RAD51 deficiencies in the repair of the CDT-mediated DSBs, the decrease of the γH2AX positive-cells population has been calculated between the long exposure (24 h) and the short exposure to CDT (3 h) followed by 21 h of recovery (Fig. 4E). In control cells, 46% of the γH2AX positive-cells from the long exposure condition do no longer accumulate γH2AX signal 21 h after the short exposure. We infer that 46% of the damaged cells have repaired the CDT-mediated DSBs during the recovery time. Interestingly, only 14% of the XRCC1 deficient cells have lost the γH2AX staining between the long and short exposure to the toxin, implying that this lower repair capacity could explain the CDT sensitivity (Fig. 4B). The repair rate in the absence of XRCC4 is not statistically different from the control cells, suggesting that the DSB accumulation caused by NHEJ impairment can be repaired through other pathways (Fig. 4F). Indeed, depleting XRCC1 and/or RAD51 significantly decreases the repair capacity of the XRCC4 deficient cells. On the other hand, RAD51 depletion results in a drastic loss of the CDT-induced DSB repair capacity, as the proportion of γH2AX positive-cells is similar between the long and short CDT-exposures. This strongly supports that DSBs directed to HR processing cannot be repaired by a compensatory mechanism. To summarize, the XRCC1-, XRCC4- and RAD51-dependent pathways play different roles in the management of the CDT-mediated DSBs. The global DSB accumulation induced by CDT is mainly hampered by NHEJ, but these lesions can be repaired through XRCC1- or RAD51-dependent mechanisms. Alternatively, other CDT-mediated DSBs are strictly processed by HR.

The Fanconi anemia pathway is activated in response to CDT. The requirement of the FA pathway to survive CDT has first been validated by MCA (Fig. 1B) on cells depleted for FANCC, component of the FA core complex. Similarly, FANCD2-deficient fibroblasts (PD20) displayed reduced clonogenic survival relative to their FANCD2-complemented counterpart (PD20-D2) after CDTγH2AX, and not CDTγH2AX (Fig. 5A). Moreover, FANCD2 knockdown sensitizes HeLa cells to CDT (Fig. 5S). This indicates the involvement of the FA pathway to overcome the replicative stress induced by the CDT-mediated DNA damage.

The core complex-dependent monoubiquitylation of FANCD2 is a marker of FA pathway activation. When HeLa cells are intoxicated with CDTγH2AX or with MMC, FANCD2 monoubiquitylation is observed by Western blot (Fig. S6), through the increase of the FANCD2 L/S form ratio (Fig. 5B,C). In contrast, CDTγH2AX does not impact the FANCD2 L/S ratio. FANCD2 L form accumulation is severely impaired in cells lacking FANCC, showing...
Figure 4. Epistatic studies of XRCC1, XRCC4 and RAD51 in response to CDT. (A) HeLa cells were transfected with scramble (ctrl), XRCC1 (X1), XRCC4 (X4) and/or RAD51 siRNA and soluble cell extracts were prepared to assess the level of indicated proteins with specific antibodies by Western blot analyses. β-Actin is shown as a loading control. Full-length blots are presented in Fig. S8. (B) Clonogenic survival of HeLa cells transfected with scramble (ctrl), XRCC1 (X1), XRCC4 (X4) and/or RAD51 siRNA and exposed to CDT. Data represent the mean ± SD of at least three independent experiments. Statistics were calculated by one-way ANOVA ($P < 0.05; **P < 0.01; ***P < 0.001). (C) Western blot analyses of soluble extracts from Hela cells transfected with scramble (ctrl), XRCC1 (X1), XRCC4 (X4) and/or RAD51 siRNA and treated or not with 250 pg/ml of CDT for 24 h. Full-length blots are presented in Fig. S8. (D) Representative images of γH2AX immunostaining in HeLa cells transfected with scramble (ctrl), XRCC1 (X1), XRCC4 (X4) and/or RAD51 siRNA, and left non-treated (NT) or treated with 250 pg/ml of CDT for 24 h or for 3 h followed by a 21 h recovery time (repair 21 h). Scale bar = 20 μm. (E) Quantification of HeLa cells from (C) positive for γH2AX foci formation, non-treated (NT) or treated with 250 pg/ml of CDT for 24 h. Data are expressed as the mean ± SD of at least 3 independent experiments. Statistics were calculated by one-way ANOVA (**P < 0.01). Conditions with the same letter at the bottom of the bars are not statistically different. (F) Quantification of the repair efficiency in HeLa cells from (C), calculated as the percentage of the decrease in the proportion of γH2AX positive cells from the condition CDT 3 h + repair 21 h compared to the condition CDT 24 h. Data are expressed as the mean ± SD of at least 3 independent experiments. Statistics were calculated by one-way ANOVA (**P < 0.01). Comparison among data were done using Tukey’s HSD Post-hoc test.
that the CDTr induced FANCD2 monoubiquitylation requires an intact FA pathway. Thus, CDTr-induced DNA lesions promote FA pathway activation through FANCD2 monoubiquitylation.

Once monoubiquitylated, FANCD2 is recruited to chromatin damaged loci. To investigate FANCD2 mobilization to the CDTr-induced DSBs, that can be either direct (high doses) or replication-dependent (moderate doses), HeLa cells were subjected to two treatments (Fig. 5D). When exposed to 7.5 ng/ml of CDTr for 6 h to induce direct DSBs, 65% of cells accumulate γH2AX foci (Fig. 5E). However, the proportion of FANCD2-positive cells did not increase compared to untreated cells. In contrast, a 40 h treatment with 0.25 ng/ml of CDTr results in 50% of γH2AX positive cells, these DSBs being mostly cell cycle-dependent and thus generated during
replication, as we previously reported. Indeed, 55% of cells also accumulate FANCD2 foci, indicative of replicative stress signaling. In control cells, almost all γH2AX positive cells also accumulate FANCD2 foci, demonstrating that spontaneous DSB are mainly formed during S-phase (Fig. 5F). In the same way, 80% of cells that accumulate DSB in response to moderate dose of CDT are FANCD2 positive. In comparison, only 52% of cells undergoing CDT-induced direct DSBs exhibit FANCD2 foci. Thus, replication-dependent DSBs are managed by the FA machinery, whereas direct DSBs are not.

We previously reported that RAD51 is recruited to the replication-dependent DSBs induced by CDT. To get more insight in the role of the FA pathway at these lesions, RAD51 localization was investigated in PD20 cells after a 24 h exposure to CDT 125 pg/ml (Fig. 5G). Under these conditions, PD20 and PD20-D2 control cells display comparable proportion of γH2AX-accumulating cells (Fig. 5H), representing the replication-dependent DSBs that must be processed through HR. However, the proportion of RAD51-positive cells is significantly higher in FANCD2 deficient cells (Fig. 5G,H), suggesting that in absence of a functional FA pathway, there is an over-accumulation of the CDT-mediated DSBs that require RAD51 to be repaired.

**CDT related DNA damage induces the replicative stress response.** The ATR signaling pathway being crucial to face off replicative stresses, we depicted the consequences of ATR depletion on the CDT-induced replication-dependent DSBs. Compared to control, ATR knockdown HeLa cells are hypersensitive to CDT, but not to CDTH153A (Fig. 6A,B), confirming MCA results (Fig. 1B) and indicating that replicative stress signaling is required to respond to CDT-mediated DNA damage. Next, we asked whether ATR deficiency impedes the signaling and repair of the CDT-mediated DSBs occurring during S phase, by analyzing the formation of γH2AX, RAD51 and FANCD2 foci after a 24 h CDT-exposure (Fig. 6C,D). In HeLa cells exposed to CDT, ATR knockdown significantly reduces the accumulation of cells positive for γH2AX and RAD51, and totally abolishes

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**Figure 6.** ATR requirement in the signaling and repair of the CDT-mediated DNA damage. (A) HeLa cells were transfected with scramble (ctrl) or ATR siRNA and soluble cell extracts were prepared to assess the ATR protein level by Western blot analyses. Lamin C is shown as a loading control. Full-length blots are presented in Fig. S8. (B) HeLa cells transfected with scramble (ctrl) or ATR siRNA were exposed for 5 days to CDT or CDTH153A and cell viability was analyzed by Crystal violet staining. Results represent the mean ± SD of at least three independent experiments. Statistics were calculated by unpaired Student’s t-test (*P < 0.05; **P < 0.01). (C,D) Representative images of γH2AX and RAD51 (C) or FANCD2 (D) immunostaining in HeLa cells transfected with scramble (ctrl) or ATR siRNA, non-treated (NT) or treated for 24 h with 250 pg/ml of CDT. Scale bars = 20 μm. (E) Quantification of HeLa cells transfected with scramble (ctrl) or ATR siRNA positive for γH2AX and RAD51 foci formation from (C) or positive for FANCD2 foci formation from (D). Results represent the mean ± SD of at least three independent experiments. Statistics were calculated by unpaired Student’s t-test (*P < 0.05; ***P < 0.001).
the CDT-related increase of the proportion of FANCD2 positive cells (Fig. 6E). Thus, these experiments further reinforce the concept that ATR-dependent replicative stress signaling is crucial for cells to respond to the CDT genotoxic potential.

Discussion

CDT producing bacteria have been implicated in numerous diseases, including cancer. In many cases, the CdtB catalytic activity has been shown to drive the bacterial pathogenic potential through induction of DNA damage. To better characterize the cellular processes involved in the repair of the CdtB-induced DNA lesions, we have depicted the importance of the major mammalian repair mechanisms in response to CDT. Our results compete with the classical posture supporting that resistance to CDT genotoxicity exclusively relies on the DSB repair machinery.

To deal with the different lesions that genomic DNA may suffer, cells display a battery of repair pathways. The primary aim of this work was to study the importance of each of the major repair mechanisms after CDT exposure. The strategy developed here derived from MCA, successfully used to identify genes involved in DNA repair. HCT116 cells were modified with shRNA to downregulate target genes (Table 1) rather than being knocked-out by endonuclease-driven gene inactivation, because knockout of essential genes is lethal at the cellular level, as previously shown for ATR. The decreased repair capacity of shRNA-depleted cells still induces a proliferation defect when the DNA damage level exceeds a certain threshold after genotoxic insults. Indeed, even a modest reduction of PALB2 expression level in the shPALB2 HCT116 cells was sufficient to induce a dose-dependent reduction in proliferation, after MMC or CDT, detectable by MCA. The cell viability loss of the DNA repair defective cells lines exposed to CDT has been related to enhanced genetic instability, revealed by micronucleus assay. Moreover, except for ATR that is an essential gene, all the MCA positive results were confirmed by clonogenic survival in knockout models. To conclude, the MCA strategy developed here allowed for an unambiguous overview of the repair processes required to survive CDT-induced genotoxicity, and could be used –in the future- for the screening of genotoxic compounds of unknown DNA damaging properties.

Before this study, information concerning the repair systems involved in response to CDT was limited. Two screens performed in yeast concluded that CdtB-induced DNA lesions are exclusively processed by HR. Evidence of HR implication in CDT-intoxicated human cells was likewise demonstrated after RAD51 depletion and in a human pancreatic adenocarcinoma cell line deficient for BRCA2. The data presented here further illustrates the role of HR by reporting the importance of PALB2 after CDT exposure, and by revealing that a subset of the CDT-related DSBs necessitates HR to be repaired. HR involvement was expected since the CDT-induced DSB formation in human cells is highly documented. However, the role of NHEJ, the second canonical DSB repair system, was still controversial. If data from yeast showed that NHEJ mutants were not hypersensitive to CdtB expression, one has to remind that DSB repair in yeast predominantly relies on HR processes. Our results clearly show that XRCC4, and thus NHEJ, is implicated in the resistance to CDT-induced genotoxicity by preventing DSB over-accumulation. Taken together, these data support that the two major DSB repair mechanisms, HR and NHEJ, are implicated in the resistance to the CDT-mediated DSBs.

Several evidences suggest that CdtB does not induce direct DSBs. First, comparison between yeast mutant strains hypersensitive to CdtB and direct DSB inducing agents (such as ionizing radiation or HO and EcoRI endonucleases) indicates that the CdtB-induced DSBs are atypical. Different types of DNA lesions, such as SSBs or bulky lesions, hinder the progression of replication forks and cause fork collapse and DSB formation. According to MCA data, inhibition of XPA, an important player of global NER, does not impact the viability of CDT-treated cells, strongly supporting that CDT-mediated DNA damage does not involve bulky lesions. Therefore, CDT-induced primary DNA lesions may be SSBs, as supported by several anterior works (see ref. 44 for detail). To test this hypothesis, we studied the XRCC1-depleted cells after CDT treatment. XRCC1 is a scaffold protein that interacts with different SSBR components to stabilize and/or stimulate them. As expected, human and rodent XRCC1 deficient cells were hypersensitive to CDT. Besides, yeast data showed that DNA glycosylases involved in early steps of BER are not required to survive CDT intoxication, implying that the toxin does not generate base modifications. Thus, these findings strongly suggest that CdtB, through its nuclease activity, only induces DNA strand breaks.

Alkaline comet assays showed that CDT-treated cells accumulate DNA strand breaks over time, and this effect is aggravated by reduced SSBR capacity in XRCC1-deficient cells. This indicates that once internalized, CdtB continuously generates SSBs, some of which are repaired by SSBR. The un-repaired SSBs sustain the CdtB-mediated genotoxicity, underpinning the well-characterized cellular consequences of CDT intoxication that are cell cycle arrest, cell distension and cell death. Indeed, shXRCC1 HCT116 cells accumulate un-repaired SSBs, and exhibit proliferation defects, as observed by MCA, and cell distension (data not shown) compared to control cells. The CDT-mediated cell cycle arrest and apoptosis depends on ATM. This implies that the CdtB-induced SSBs should be converted to DSBs to activate the ATM-dependent pathway, even if we cannot rule out the possibility that SSBs may partially mediate ATM activation. CdtB induces BER in CHO cells accumulate more DSBs than control cells during a continuous exposure to CDT, and the subsequent DSB repair after a short treatment is attenuated by XRCC1 depletion, even in knockdown HeLa cells. This effect may result from the impairment of the XRCC1-dependent alternative DSB repair pathway. Otherwise, it could reflect the defective repair of the SSBs generated after the pulse treatment by CDT, which may degenerate into DSB during S-phase, as CDT remains active in the cell for more than 48 h post-exposure and induces more DNA breaks in shXRCC1 than in control cells.

CDT can directly induce DSBs when two SSBs face each other, or indirectly during S-phase, associated with the presence of single-stranded DNA coated by RPA. The later condition implicates the activation of the ATR-dependent replicative stress response. CDT was shown to induce a rapid phosphorylation of ATR and CHK1 that increases over time. Here, we observed a hypersensitivity to CDT in two different ATR-depleted
especially in pre-malignant backgrounds56,57. Concerning this aspect, our data suggest that the cumulative defi-

The human PD20 cell line and the FANCD2 complemented cell line (PD20 D2) were maintained in Opti-MEM 
fibroblasts cell lines. Moreover, ATR deficiency results in a diminished accumulation ≠H2AX, RAD51 and 

To further characterize the cellular response to the CDT-induced replicative stress, attention has been drawn 

In conclusion, we have demonstrated that cellular survival to CDT-mediated genotoxicity involves a battery 

Figure 7. Model for repair of CDT-induced DNA damage. Moderate CDTwt doses induce SSBs, repaired 

Human colon cancer cells (HCT116), HeLa cells and Mouse Embryonic Fibroblasts (MEFs) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Life technologies). The human PD20 cell line and the FANCD2 complemented cell line (PD20 D2) were maintained in Opti-MEM (Gibco, Life technologies). Finally, AA8 and EM9 cell lines were maintained in a mixture of 50% DMEM media
and 50% F-12 media. All mediums were supplemented with 10% heat-inactivated calf serum, and 0.5 mg/ml Penicillin/Streptomycin (P/S). Cells lines were grown in a humidified incubator at 37 °C with 5% CO₂.

The Cytotoxic Distending Toxins (CDT₄ or CDT₁₅₃₃₃₅₃) were produced and purified as described previously12. When needed, HCT116 cells were treated with Mitomycin C (MMC) (Sigma-Aldrich), and HeLa cells were treated with 10 μM of calicheamicin-γ₁ (Pfizer) or with 20 μM of Wortmannin (Sigma-Aldrich).

Small-hairpin RNA (shRNA) knockdown. Stable cell lines defective in the different repair pathways (see Table 1 for details) were generated in HCT116 p53⁻/⁻ cells24 according to standard protocols using Mission short hairpin RNA (shRNA) lentiviral particles bearing the pLKO.1-puro vector either containing the turboGFP, TagCFP or TagRFP gene (Sigma-Aldrich). Targeted genes and shRNA sequences are listed in Table 1. Briefly, HCT116 cells were infected with lentiviral particles for 24 h, then puromycin was applied to select for lentiviral transduction. Puromycin resistant cells were diluted (limit-dilution), and fluorescent clones were selected, amplified and screened for the shRNA-mediated depletion efficiency by Western blot and RT-qPCR analysis.

Cytometry analyses. Cell cycle analysis was done as previously reported12. For Multicolor Competition Assay, control cells and fluorescent shRNA cells were counted, mixed with a 1:1 ratio, and were left untreated or treated either with CDT₄ or CDT₁₅₃₃₃₅₃. After 6 days of culture, cells were collected and the fluorescent rate was analyzed by flow cytometry with a Miltenyi MACSQuant Analyser 10 cytometer. Analysis was made with VenturyOne software (Applied Cytometry). Relative survival of shRNA-untreated cells was set to 1.

RNA interference. Gene silencing was performed by transfection of siRNA (Sigma-Aldrich) in HeLa cells with INTERFERin® according to the manufacturer's instructions (Polyplus). Briefly, siRNA were mixed with Interferin in Opti-MEM, and incubated 10 minutes at room temperature. Then, HeLa cells were transfected with the negative control siRNA (CAUGAUCAGUGCAACUC-dTdT), or with siRNA directed against XRCC1 (GGAGAUAUGACACAUUGAG-dTdT), XRCC4 (AAUCUGGGAAGAAGAA-dTdT), RAD51 (CCAGAUCUGUCUACGCA-dTdT), FANCDD2 (AACACGCAUGGUAACACUGA-dTdT), or ATR (CCUCGGUGAUUGGCUGUA-dTdT). After 24 h of incubation, cells were counted and plated for further analysis.

Crystal violet viability assay. 24 h after siRNA transfection, 6000 HeLa cells were grown for 24 h in 24-well plates before being treated with CDT for 6h. Cells were washed and released in fresh media for 5 days. After a PBS wash, cells were fixed for 10 minutes with 10% (vol/vol) methanol/10% (vol/vol) acetic acid at room temperature. Cells were then stained for 10 minutes with 1% (vol/vol) crystal violet (Sigma-Aldrich) in methanol, washed in water and the absorbed dye was released by incubation with agitation for 1 h at room temperature in 500 mM NaCl and 0.5% NP40) containing the Halt Protease & Phosphatase inhibitor cocktail (Thermo Scientific) and sonicated on a VibraCell 72434 (Bioblock Scientific). Membranes were incubated with the primary antibody for a period of 1–16 hours. H2AX antibody was purchased from Epitomics (3522-1), γH2AX antibody from Merck/Millipore (05-636), ATR (SAB4200348), Lamin A/C (SAB4200236), β-actin (sc-47778) antibodies from Santa Cruz, XRCC1 (X0629), XRCC4 (HPA006801), XPA (SAB1406599-50UG) antibodies from Sigma, FANCDD2 (NB100-182) antibody from Novus Biologicals, and GAPDH ( GTX100118) antibody from GeneTex. Secondary fluorescent antibodies (CFTM 770
Goat anti-rabbit (20078) or anti-mouse (20077) and CFTM680 anti-rabbit (20067) or anti-mouse (20065), purchased from BIOTUM, were visualized on the membrane using an Odyssey Infrared Imaging Scanner (Li-Cor ScienceTec). Lamin, β-actin or GAPDH were used as internal control to normalize the protein level.

**Immunofluorescence analysis.** Cells were grown on glass coverslips. After at least 24 h of culture, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and stained with primary antibodies overnight at 4°C. 53BP1 (NB100-304) and FANCID2 (NB100-182) antibodies were purchased from Novus Biologicals, γH2AX antibody from Merck/Millipore (05–636), and RAD51 antibody from Santa Cruz biotechnology (sc-8349). Cells were washed three times with PBS Tween 0.1% and incubated with the secondary antibodies for 1 h (Rhodamine Red X (R6394) and AlexaFluor 488 Goat anti-mouse (A11017) or anti-rabbit (A11070), purchased from Invitrogen). DNA was stained with 4.6-diamino-2-phenyl indole (DAPI). Cells were counted positive for foci formation when >10 foci/nuclei were detected.

**Comet assay.** Alkaline comet assay was performed as previously described with minor modification. Cells were embedded in 0.7% low melting point agarose and laid on CometAssay® HT Slide (Trevigen). Fifty cells per slide and 2 slides per sample were analyzed. The extent of DNA damage was evaluated for each cell through the measurement of intensity of all tale pixels divided by the total intensity of all pixels in head and tail of comet. The median from these 100 values was calculated, and named % tail DNA.

**Statistical analysis.** When not specified, results are expressed as the mean of three independent experiments. The error bars represent the standard deviation (SD). For clonogenic and crystal violet viability assays, differential effects between the two cells type for a same treatment condition were assessed by unpaired Student’s t-test. Paired and unpaired Student’s t-tests were used for MCA and immunofluorescence experiments, respectively. For comet assays, unpaired Student’s t-test was applied to compare a time point to the non-treated (NT) condition for a unique cell line, while paired Student’s t-test was used to compare values for a same point between two cell lines. P-values < 0.05 were considered significant (indicated by asterisks): *p < 0.05; **p < 0.01, ***p < 0.001. For multiple comparisons of siRNA-treated cells, different responses of treatments were analyzed by one-way ANOVA. P-values < 0.05 were considered significant (indicated by dollars): $p < 0.05, $$p < 0.01, $$$p < 0.001. Comparison among data were done using Tukey’s HSD Post-hoc test.

In these experiments, when different between the non-treated (NT) and a treated condition was assessed, the results are indicated above the error bar of the treated condition except for clonogenic assays in which the difference between two cell lines for a same condition is indicated below the error bar. When the difference between two treated conditions was assessed, the results are indicated above a horizontal line. When multiple comparisons classify the different conditions in groups, these groups are indicated by lowercase letter at the bottom of the bars in the histograms. Statistical analyses and plots were generated using GraphPad Prism 6.0 software or Excel.

**References**

1. Jinadasa, R. N., Bloom, S. E., Weiss, R. S. & Duhamel, G. E. Cytolethal distending toxin : a conserved bacterial genotoxin that blocks cell cycle progression, leading to apoptosis of a broad range of mammalian cell lineages. *Microbiology* 157, 1851–1875 (2011).
2. Nesić, D., Hsu, Y. & Stebbins, C. E. Assembly and function of a bacterial genotoxin. *Nature* 429, 429–433 (2004).
3. Song, J., Gao, X. & Galán, J. E. Structure and function of the Salmonella Typhi chimaeric A(2)B(5) typhoid toxin. *Nature* 499, 350–354 (2013).
4. Hu, X. & Stebbins, C. E. Dynamics and Assembly of the Cytolethal Distending Toxin. *Proteins Struct. Funct. Bioinforma.* 65, 843–855 (2006).
5. Ge, Z. et al. Bacterial cytolethal distending toxin promotes the development of dysplasia in a model of microbiially induced hepatocarcinogenesis. *Cell. Microbiol.* 9, 2070–2080 (2007).
6. DiRenzo, J. M. Breaking the Gingival Epithelial Barrier: Role of the Aggregatibacter actinomycetemcomitans Cytolethal Distending Toxin in Oral Infectious Disease. *Cells* 3, 476–499 (2014).
7. Elwell, C. A. & Dreyfus, L. A. DNase I homologous residues in CdtB are critical for cytolethal distending toxin-mediated cell cycle arrest. *Mol. Microbiol.* 37, 952–963 (2000).
8. Lara-Tejero, M. & Galán, J. E. A Bacterial Toxin That Controls Cell Cycle Progression as a Deyoxyribonuclease I-Like Protein. *Science* 290, 354–357 (2000).
9. Li, L. et al. The Haemophilus ducreyi cytolethal distending toxin activates sensors of DNA damage and repair complexes in proliferating and non-proliferating cells. *Cell. Microbiol.* 4, 87–99 (2002).
10. Hassane, D. C., Lee, R. B. & Pickett, C. L. Campylobacter jejuni Cytolethal Distending Toxin Promotes DNA Repair Responses in Normal Human Cells. *Infect. Immun.* 71, 541–543 (2003).
11. Guillera, L. et al. Myc is Required for Activation of the ATM-Dependent Checkpoint Responses in Response to DNA Damage. *PLoS One* 5, 1–9 (2010).
12. Fedor, Y. et al. From single-strand breaks to double-strand breaks during S-phase: a new mode of action of the *Escherichia coli* Cytolethal Distending Toxin. *Cell. Microbiol.* 15, 1–15 (2013).
13. Cortes-Bratti, X., Karlsson, C., Lagergård, T., Thelestam, M. & Frissan, T. The Haemophilus ducreyi Cytolethal Distending Toxin Induces Cell Cycle Arrest and Apoptosis via the DNA Damage Checkpoint Pathways. *J. Biol. Chem.* 276, 5296–5302 (2001).
14. Aaloui-El-Azhier, M. et al. Role of the ATM-checkpoint kinase 2 pathway in CDT-mediated apoptosis of gingival epithelial cells. *PLoS One* 5, 1–10 (2010).
15. Elwell, C., Chao, K., Patel, K. & Dreyfus, L. *Escherichia coli* CdtB Mediates Cytolethal Distending Toxin Cell Cycle Arrest. *Infect. Immun.* 69, 3418–3422 (2001).
16. Iyama, T. & Wilson, D. M. 3rd DNA repair mechanisms in dividing and non-dividing cells. *DNA Repair (Amst).* 12, 620–636 (2013).
17. Caldecott, K. W. Single-strand break repair and genetic disease. *Nat. Rev. Genet.* 9, 619–631 (2008).
18. Aparicio, T., Baer, R. & Gautier, J. DNA double-strand break repair pathway choice and cancer. *DNA Repair (Amst).* 19, 169–175 (2014).
19. Petermann, E., Orta, M. L., Issacva, N., Schultz, N. & Helleday, T. Hydroxyurea-Stalled Replication Forks Become Progressively Inactivated and Require Two Different RAD51-Mediated Pathways for Restart and Repair. *Mol. Cell* 37, 492–502 (2010).
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58. Gaillard, H., García-Muse, T. & Andrés, A. Replication stress and cancer. Nat. Rev. Cancer 15, 983–1000 (2015).

59. Shechter, D., Dormann, H. L., Allis, C. D. & Hake, S. B. Extraction, purification and analysis of histones. Nat. Protoc. 5, 1445–1457 (2010).

60. Lebaillly, P. et al. DNA damage in B and T lymphocytes of farmers during one pesticide spraying season. Int. Arch. Occup. Environ. Health 88, 963–972 (2015).

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Author Contributions
E.B. designed and performed experiments, analyzed data and wrote the manuscript. Y.M. and A.L. obtained and screened the HCT116 DNA repair deficient cell lines. M.C. and E.B.-R. designed and performed the comet assays. B.S. discussed the data and revised the manuscript. G.M., supervised this study, obtained funding and wrote the manuscript. J.V. supervised this project, designed and performed experiments, obtained funding and wrote the manuscript.

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