Ubx5-Cdc48 assists the protease Wss1 at DNA-protein crosslink sites in yeast

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Abstract

DNA-protein crosslinks (DPCs) pose a serious threat to genome stability. The yeast proteases Wss1, 26S proteasome, and Ddi1 are safeguards of genome integrity by acting on a plethora of DNA-bound proteins in different cellular contexts. The AAA ATPase Cdc48/p97 is known to assist Wss1/SPRTN in clearing DNA-bound complexes; however, its contribution to DPC proteolysis remains unclear. Here, we show that the Cdc48 adaptor Ubx5 is detrimental in yeast mutants defective in DPC processing. Using an inducible site-specific crosslink, we show that Ubx5 accumulates at persistent DPC lesions in the absence of Wss1, which prevents their efficient removal from the DNA. Abolishing Cdc48 binding or complete loss of Ubx5 suppresses sensitivity of wss1Δ cells to DPC-inducing agents by favoring alternate repair pathways. We provide evidence for cooperation of Ubx5-Cdc48 and Wss1 in the genotoxin-induced degradation of RNA polymerase II (RNAPII), a described candidate substrate of Wss1. We propose that Ubx5-Cdc48 assists Wss1 for proteolysis of a subset of DNA-bound proteins. Together, our findings reveal a central role for Ubx5 in DPC clearance and repair.

Keywords: Cdc48/p97; DNA-protein crosslink; protease; UBX protein; yeast

Subject Categories: DNA Replication, Recombination & Repair; Post-translational Modifications & Proteolysis

Introduction

DNA-protein crosslinks (DPCs), also known as protein-DNA adducts, are formed by the covalent association of a protein with DNA. If left unresolved, these highly mutagenic and cytotoxic lesions can interfere with essential DNA transactions, cause a severe block to the progression of replication and transcription machinery, and therefore jeopardize the fidelity of genome integrity (Stingele et al., 2017). DPCs are classified into two groups, non-enzymatic and enzymatic, depending on the nature of the crosslinked protein and the triggering mechanism that leads to DPC formation. Non-enzymatic DPCs arise from non-specific crosslinking after exposure to metabolic products such as formaldehyde or acetaldehyde or by the action of exogenous agents (UV-light, IR, etc.), while enzymatic DPCs are the result of abortive enzymatic reactions that require the establishment of a covalent DNA-enzyme reaction intermediate (Stingele et al., 2015; Stingele & Jentsch, 2015; Vaz et al., 2017; Zhang et al., 2020).

DNA Topoisomerase 1 (Top1) is prone to enzymatic DPC formation, as it forms a covalent link with DNA to relax torsional stress that emerges during DNA replication and transcription (Pommier et al., 2016). To do so, Top1 nicks DNA and covalently binds one strand to assemble in a transient entity known as a Top1 cleavage complex (Top1cc; Pommier, 2006). Top1-DNA crosslinks can appear when the enzymatic cleavage occurs close to certain DNA lesions (abasic sites, DNA breaks, base mismatch, etc.) or be induced by camptothecin (CPT), a Top1-poison that inserts into the catalytic pocket of Top1 and stabilizes Top1ccs (Staker et al., 2002). Using a similar catalytic mechanism, Flp recombinase introduces a nick at a specific FRT target site. A Flp mutant carrying the point mutation H305L will drive the formation of a stable covalent crosslink upon FRT cleavage. The genetic requirements for cells to repair this DPC-like lesion on DNA are the same as for cells treated with the Top1 poison CPT (Nielsen et al., 2009; Serbyn et al., 2020, 2021).

Along with the canonical DSB repair pathways such as nucleotide excision repair (NER) and homologous recombination (HR; Ide et al., 2011), numerous other pathways have been implicated in the processing of trapped Top1ccs (Pommier et al., 2014; Vaz et al., 2017; Fielden et al., 2018; Ide et al., 2018). In yeast, the tyrosyl-DNA phosphodiesterase Tdp1, which directly hydrolyzes the bond between Top1 and the DNA, was among the first DNA repair enzymes described to have relevance for the elimination of Top1-DNA crosslinks (Yang et al., 1996; Pouliot et al., 1999, 2001). Tdp1 is not able to process intact Top1ccs and prior proteolytic digestion or denaturation is essential to enable hydrolysis by Tdp1 (Yang et al., 1996; Deblmhote et al., 2002; Pommier et al., 2014). Recently, the yeast protease Wss1 (SPRTN or DVC1 in mammals), Ddi1, and 26S proteasome were shown to participate in the proteolysis of the protein moiety of a DPC (Stingele et al., 2014; Serbyn et al., 2020). It is proposed that remodeling or initial processing of the adducts is
required before proteolysis, which may involve the AAA ATPase Cdc48 (known as p97/VCP; Nie et al, 2012; Stingele et al, 2014; Balakirev et al, 2015; Fiedlen et al, 2020).

Cdc48/p97 is an abundant essential chaperone involved in a wide variety of cellular processes, including but not limited to cell cycle regulation, membrane fusion, transcriptional control, and ubiquitin-dependent protein degradation (Woodman, 2003; Ye, 2006; White & Lauring, 2007; Stach & Freemont, 2017). Several studies investigated its role in DNA replication (Yamada et al, 2000; Mouisset et al, 2008; Deichsel et al, 2009; Ramadan et al, 2017) and DNA repair (Zhang et al, 2000; Vaz et al, 2013; Torrecilla et al, 2017), including DPC repair. In most of the processes, Cdc48/p97 processing is initiated by unfolding of ubiquitin conjugated to its targets (Ye, 2006; Twomey et al, 2019). The substrate specificity is achieved through interaction with numerous regulatory adaptors (Hanzelmann & Schindelin, 2017), of which UBX proteins constitute the largest known group (Schuberth & Buchberger, 2008). Supporting the idea of a role for Cdc48/p97 in DNA-bound protein clearance, Cdc48/p97 mediates chromatin extraction and disassembly of some protein complexes (Jentsch & Rumpf, 2007; Schuberb & Haines, 2007; Maric et al, 2014; Frattini et al, 2017; Ramadan et al, 2017). It was linked to processes involved in SPRTN/Wss1 proteolysis (Lin et al, 2008; Davis et al, 2012; Ghosal et al, 2012; Mosbech et al, 2012; Nie et al, 2012; Stingele et al, 2014; Balakirev et al, 2015; Maskey et al, 2017; Fiedlen et al, 2020; Kroning et al, 2022) and is also required for proteasome-mediated degradation of stalled RNA Polymerase II (RNAPII) following UV-induced DNA damage (Verma et al, 2011; Lafon et al, 2015; He et al, 2017). Particularly, a specialized complex containing mammalian p97 and its cofactor TEX264 is required for clearance of TOP1-DNA adducts by SPRTN (Fiedlen et al, 2020), raising the question of cofactor specificity regarding DPC substrates. The ability of Cdc48/p97 to counteract DPCs appears crucial; however, the mechanistic basis of how it is achieved is still not fully defined.

In this work, we investigate the involvement in DPC repair of Ubx5, a Cdc48 co-factor of the UBX protein family that has previously been implicated in UV-induced degradation of the largest subgroup of RNAPII, Rpb1 (Verma et al, 2011). Based on our findings, Ubx5 has a negative effect in Saccharomyces cerevisiae mutant strains exhibiting DPC persistence related to the unavailability of the DPC-protease Wss1. By using an in vivo inducible DPC system, we observed that Ubx5 and Cdc48 accumulation at sites of unrepaired DPC lesions delays their clearance. We further demonstrate that loss of interaction between Ubx5 and Cdc48 is sufficient to suppress the growth phenotype and drug sensitivity of yeast mutant strains accumulating DPCs. Finally, loss of Ubx5 restores the impaired genotoxin-induced degradation of Rpb1 in the absence of the protease Wss1, in a Ddi1-dependent manner. We propose a new mechanism in which Ubx5 mediates Cdc48 recruitment to adducts on chromatin, such as DNA-protein crosslinks or stalled RNA Polymerase II.

Results

Multiple Cdc48 co-factors suppress effects of mutations affecting Top1-DNA crosslinks

Aiming to unravel Cdc48 adaptors that define substrate-specificity in DPC repair, we intersected the list of known CDC48 interactors with the genetic network of the tdpl-degron + auxin wss1Δ (tdpl-degron) mutant defective in Top1cc repair. A list of physical and genetic CDC48 interactors was extracted from the yeast Saccharomyces Genome Database (SGD; Cherry et al, 1997; Fig EV1A). For these selected genes, we then analyzed the relative number of transposition events in auxin-treated tdpl-degron wss1Δ mutant (Serbyn et al, 2020; Fig 1A). We noted increased transpositions in the UBX5 gene body (Fig 1B) and confirmed that ubx5Δ substantially rescues the growth phenotype of freshly dissected yeast spores (Figs 1C and EV1B), as well as the CPT sensitivity (Fig 1D) of the tdplwwss1 double mutant defective in Top1ccs repair (Stingele et al, 2014; Balakirev et al, 2015). It is worth pointing out that Ufd1 and Npl4, both known to be core adaptors of the Cdc48-Ufd1-Npl4 complex recruited to double-strand breaks (DSBs; Meerang et al, 2011) and DNA-bound proteins (Balakirev et al, 2015), are suppressors of tdplwwss1 as well (Fig 1A). Moreover, Ubx5 protein levels are decreased in the tdpl-degron wss1Δ mutant, emphasizing the negative effect of Ubx5 in that cellular context (Fig1E and F; tdpl-degron wss1Δ + auxin). Loss of Wss1 alone already causes reduction of steady-state Ubx5 levels (Fig 1E and F; tdpl-degron wss1Δ no auxin), suggesting that the ubx5Δ suppression may represent the adaptive response to Wss1 deficiency. From these data, we presumed that Ubx5 may have an inhibitory effect in the absence of the repair enzymes Wss1 and Tdp1.

Ubx5-Cdc48 interaction interferes with the resistance to DPCs in the absence of the Wss1 protease

Wss1 protease is required for cellular resistance against formaldehyde (FA) and hydroxurea (HU; Fig 2A, O’Neill et al, 2004; Maddi et al, 2020; Serbyn et al, 2020). While it is well documented that HU induces replication stress, several studies reported that HU creates free nitrooxide radicals (Yarbro, 1992; King, 2003), which are prone to create DPCs (Dizdaroglu et al, 1989; Nakano et al, 2003), raising the possibility that DPCs may increase in response to HU. As levels of Ubx5 are downregulated in the absence of Wss1 (Fig 1E and F), we hypothesized that Ubx5 loss could restore wss1Δ tolerance to these DPC-inducing agents. Indeed, ubx5Δ reverts FA and HU hypersensitivity of the wss1Δ mutant to WT-like levels (Fig 2A). In contrast, ubx5Δ did not suppress hypersensitivity to Top1ccs in the absence of Tdp1 (Fig EV2A). From these data, we inferred that Ubx5 is genetically linked to Wss1 but not to the Top1-specific repair factor Tdp1. Given its genetic interactions, Ubx5 protein might target a broad range of DPCs for repair, similar to Wss1.

To gain further insights into Ubx5 function in DPC repair, we addressed the role of Ubx5 domains. Yeast Ubx5 consists of four known domains: an amino (N)-terminal UBA, central UAS, UIM, and a carboxyl (C)-terminal UBX (Fig2B). To assess their role, we complemented ubx5Δ wss1Δ with HA-tagged Ubx5 constructs overexpressed from a galactose-inducible promoter (Fig 2C and D). Complementation by ubx5Δubx5Δ, ubx5Δubx5Δ, or ubx5Δubx5Δ domain mutants was detrimental to cell growth and compromised resistance to several drugs, similar to complementation by the full-length protein (Fig 2C). However, the ubx5Δubx5Δ construct was not well expressed (Fig 2D), not allowing us to conclude about the role of the UAS domain. WT-like phenotypes of ubx5Δubx5Δ and ubx5Δubx5Δ variants suggest that these domains are not involved in Wss1-related functions of Ubx5 in DNA repair. On one hand, these data argue that Wss1-linked function of Ubx5 neither...
requires ubiquitin binding by the UBA domain (Husnjak & Dikic, 2012), nor interaction with ubiquitin via the UIM motif that is important for RNAPII turnover after UV treatment (den Besten et al., 2012). On the other hand, the residual ubiquitin binding of ubx5ubaΔ and ubx5uimΔ variants may be sufficient to cause drug sensitivity in wss1Δ. In the same line, the non-physiological overexpression of these constructs can potentially mask a partial loss of function.

The UBX domain of Ubx5 mediates the interaction with Cdc48 (Decottignies et al., 2004; Hartmann-Petersen et al., 2004; Schuberth et al., 2004; Alexandru et al., 2008; Sasagawa et al., 2010), and we additionally tested whether this domain is critical for DPC resistance. Interestingly, the ubx5ubxΔ mutant was expressed at WT-like levels (Fig 2D) and showed partial rescue of ubx5Δ wss1Δ, even in the presence of DPC-inducing drugs (Fig 2C), suggesting the important role of Ubx5-Cdc48 interaction.

On top of that, when the genomic copy of UBX5 was replaced by ubx5ubxΔ, the mutant suppressed the growth defects of tdp1Δ wss1Δ spores (Fig EV2B), restored HU and FA resistance of wss1Δ (Fig EV2C and D), and restored CPT resistance of tdp1Δ wss1Δ (Fig EV2D), similarly to ubx5Δ.
Altogether, these observations reinforce the idea that the Ubx5-Cdc48 interaction, mediated by the UBX domain, is severely deleterious in the absence of Wss1 upon DPC induction.

UBX proteins Ubx4 and Ubx5 are detrimental to DPC resistance

Ubx4 is another UBX-containing protein (Schuberth & Buchberger, 2008) identified as a mild suppressor of tdp1wss1 (Fig 1A) that presents a highly transposed UBX domain in the screen (Fig EV3A). The ubx4ΔUBX mutant partially rescued HU sensitivity of wss1Δ cells (Fig EV3B), while the full UBX4 deletion did not (Fig EV3C). Similarly, ubx4ΔUBX modestly rescued the growth defect of tdp1wss1 (Fig EV3B), suggesting that Ubx4 could function as an alternative Cdc48 adaptor needed for DPC repair but is still required for other cellular functions. These data involve several members of the UBX proteins family in DPC repair.

We speculated that Ubx5 and Ubx4 target Cdc48 to assist Wss1 in DPC removal, each to a different extent. To explore this idea, we stressed a double ubx4Δ ubx5Δ mutant with several DPC inducers, as this should lead to similar sensitivities as a WSS1 deletion (Fig EV3C and D). However and surprisingly, neither the double mutant nor the single mutants showed sensitivities to the drugs tested, also in combination with tdp1Δ (Fig EV3D). Despite the nosyous effects of UBX proteins in the absence of Wss1, UBX proteins are not participating in all Wss1 cellular functions.

Taken together, these findings strongly suggest that the UBX proteins Ubx5 and Ubx4, both cofactors of Cdc48, are detrimental upon DPC accumulation in wss1Δ due to their interaction with the segregase Cdc48 via their UBX domain.

Loss of the Cdc48 adaptor Ubx5 improves repair efficiency of the crosslinked Flp

Ubx5 mutants restore a WT-like phenotype in the absence of the repair enzymes Wss1 and Tdp1. Accumulation of Top1 crosslinked to DNA in tdp1Δ wss1Δ was previously demonstrated (Stingele et al, 2014). We reasoned that loss of UBX5 would lead to a decreased number of crosslinked proteins detected on DNA. To test this hypothesis, we used the previously described Flp-nick system (Fig 3A; Nielsen et al, 2009), which creates a Top1-like crosslink at a single FRT locus artificially introduced into chromosome VI of the yeast (S. cerevisiae) genome. This system uses a galactose-inducible mutant Flp recombinase flp-H305L, which triggers a covalent crosslink “Flp-cc” at the FRT site, thus allowing to precisely follow the molecular events linked to the appearance, detection, and repair of the Flp-cc. Similar to the genetic interactions observed in the W303 strain background (Fig 1D), ubx5Δ also suppressed cell growth defects resulting from flp-H305L expression on 2% galactose in the Flp-nick system (Fig 3B). Of note, as the Flp-nick system mimics a Top1cc, endogenous Top1 was removed in the following experiments.

Figure 2. Loss of interaction between Cdc48 and Ubx5 restores growth of cells accumulating chromatin lesions.

A Ubx5 loss rescues wss1Δ sensitivity to formaldehyde (FA) and hydroxyurea (HU). Cells were grown in YEPD and either treated with 40 mM FA for 15 min or spotted directly on 100 mM HU. Plates were incubated for 2 days at 30°C.
B Schematic representation of Ubx5 domains. UBA domain targets Ubx5 to ubiquitinated substrates. UBX domain is a Cdc48 binding module. UIM is implicated in Rpb1 degradation after UV treatment. UAS domain has an unknown function.
C HA-UBX5 (pGAL o/e): vector, UBX5, UBAΔ, UASΔ, UIMΔ, UBXΔ, UBAΔUBXΔ. No UBX5 expr. UBX5 expressed
D Protein levels of overexpressed HA-Ubx5 mutants shown in (C) were analyzed by immunoblotting (as in Fig 1E).
To monitor changes in \textit{flp-H305L} retention at the FRT site, cells were first synchronized in the G1-phase of the cell cycle by the addition of α-factor prior to and during the 2 h galactose induction of the \textit{flp-H305L} mutant (Fig 3D, upper panel). \textit{flp-H305L} induction was then stopped with glucose and cells were released from α-factor G1 arrest and allowed to enter the cell cycle (Fig 3D, glu 60 min and 120 min; Fig 3C for cell-cycle analysis). Consistently, and as we formerly reported (Serbyn et al., 2020, 2021), \textit{flp-H305L} was more persistent at the FRT site at 120 min in \textit{tdp1Δ wss1Δ top1Δ} compared to a WT strain, which showed rapid removal of the Flp-cc from the FRT site (Fig 3D, compare strains 1 and 2). Since UBX5 deletion suppresses the \textit{tdp1Δ wss1Δ top1Δ} phenotype, we hypothesized that Ubxs5 removal may lead to faster clearance of the protein crosslink. Indeed, Flp-cc repair dynamics in \textit{ubx5Δ tdp1Δ wss1Δ top1Δ} was very similar to WT (Fig 3D, strain 3). These results suggest that the rescue observed in \textit{ubx5Δ} is probably due to a reduced amount of DPCs on the DNA.

We then reasoned that the possible negative effect of Ubxs5 could be due to its presence at the damage site. We therefore tested Ubxs5 recruitment at the FRT site using the Flp-nick system. We observed that while Ubxs5 was not recruited at the Flp-cc site in a WT-like strain, there was a significant enrichment of Ubxs5 upon removal of the Wss1 and Tdp1 repair enzymes that were not detected in the absence of FRT (Fig 3E). Notably, this great accumulation of Ubxs5 at a DPC locus became significantly detectable during the S- and G2-phases, underlining the role Ubxs5 may have only at certain stages of the cell cycle. A straightforward interpretation of these findings is that Ubxs5 prevents the elimination of the Flp-cc (and probably Top1cc, and other DPCs) from the DNA by hyper-accumulating at the damage location when Wss1 and Tdp1 are unavailable.

\textbf{Ubxs5 participates in Cdc48 targeting at the crosslinked Flp}

The collected data led us to wonder whether Ubxs5 was involved in targeting Cdc48 to DPC sites. Indeed, we observed that Ubxs5 was greatly enriched at the FRT site and that Ubxs5 was detrimental due to its connection with Cdc48. We therefore attempted to detect Cdc48 at the crosslinked Flp. Although no significant enrichment was observed in a WT strain following DPC induction with galactose (Fig 4A, strain 1), Cdc48 was excessively present at the induced DPC site in the absence of Wss1 and Tdp1 (Fig 4A, strain 2). Remarkably, in line with the above-mentioned hypothesis, the Cdc48 enrichment at the FRT was alleviated upon loss of Ubxs5 (Fig 4A, strain 3).

Regulation and targeting of Cdc48 to its multiple cellular substrates is predominantly ubiquitin-dependent (Ye, 2006). Using again the Flp-nick system, we tested whether ubiquitin occupancy at the FRT was changing in the different mutants examined (Fig 4B). In conjunction with what we observed before, increased ubiquitin signal was detected upon DPC induction especially in the absence of Wss1, and Tdp1 (Serbyn et al., 2021; Fig 4B). This is not surprising given that the Flp-cc remains bound at the FRT site in this strain background (Fig 3D, strain 2). Curiously, in the absence of Ubxs5, we still detected significant accumulation of ubiquitin in the vicinity of the Flp-bound locus upon Flp-cc induction, with the highest peak after 30 min of glucose repression (Fig 4B). Later timepoints revealed that the ubiquitin kept accumulating in \textit{tdp1Δ wss1Δ top1Δ}, while it was not the case for \textit{ubx5Δ tdp1Δ wss1Δ top1Δ}, respectively, correlating with the kinetics of Flp-cc remaining bound or being removed (Fig 3D). These data suggest that Cdc48 accumulates on unrepairated Flp-cc, probably targeted by Ubxs5. The remaining ubiquitin species in \textit{ubx5Δ tdp1Δ wss1Δ} likely facilitate DPC repair.

\textbf{Other repair pathways are active in \textit{ubx5Δ tdp1Δ wss1Δ}}

We postulated that UBX5 deletion could lead the cells toward an alternative repair pathway. The aspartic protease Ddi1 has recently been characterized as a novel DPC repair player acting in parallel to Wss1 (Svoboda et al., 2019; Serbyn et al., 2020), and it was recently shown to be a ubiquitin-dependent protease (Vip et al., 2020). We thus speculated that Ddi1 could play a role in improving the growth of \textit{ubx5Δ tdp1Δ wss1Δ} or \textit{ubx5Δ wss1Δ}. By performing genetic analyses, we observed that Ddi1 was indeed essential for \textit{ubx5Δ tdp1Δ wss1Δ} survival, as spores additionally lacking Ddi1 were unable to proliferate (Figs 5A and EV4A). Likewise, Ddi1 was crucial for the viability of the \textit{ubx5Δ wss1Δ} mutant under FA or HU stress (Fig 5B). Indeed, \textit{wss1Δ ddi1Δ} is very sensitive to DPCs and \textit{ubx5Δ} is no longer proficient in suppressing \textit{wss1Δ} in the absence of Ddi1 (Fig 5B; Serbyn et al., 2020). However, the \textit{ubx5Δ ddi1Δ} double mutant did not reveal a strong phenotype on the tested drugs (Fig 5B).

We further tested the requirements of other known Top1ccs and DPC repair factors (Liu et al., 2002; Deng et al., 2005; Pommier, 2006; Alvaro et al., 2007; Stinglee et al., 2014; Sun et al., 2020b; Figs EV4B–G and EV5). Among the pathways tested, deletion of RAD52 (homologous recombination, Fig EV4B), RAD9 (checkpoint activation, Fig EV4C), SGS1 (RecQ helicase, Fig EV4D), SRS2 (anti-recombinase, Fig EV4E), RAD27 and MRE11 (structural nucleases, Fig EV4F and G), strongly affected growth of \textit{ubx5Δ tdp1Δ wss1Δ} spores, emphasizing the essentiality of these enzymes and their respective repair pathways to counteract DPC appearance in yeast mutants lacking Wss1 and Tdp1. Surprisingly, abrogation of the non-homologous end-joining (NHEJ) pathway (yku70Δ, Fig EV5A), the translesion synthesis pathway (rev3Δ, Fig EV5B) as well as deletion of the nucleotide excision repair (NER) pathway (rad4Δ, Fig EV5C) had no effect on \textit{ubx5Δ tdp1Δ wss1Δ} spore survival. Taken together, these genetic analyses (Fig EV5D) show that checkpoint pathways, recombination, multiple nucleases, and Ddi1 promote the growth of \textit{ubx5Δ tdp1Δ wss1Δ}.

\textbf{Ddi1 provides resistance toward DPCs to cells lacking Ubxs5 and Wss1}

Because the Ddi1 and Wss1 proteases work in parallel pathways, Ddi1 becomes more important when Wss1 is absent. Thus, Ddi1 expression is elevated in cells lacking Wss1 (Fig 5C and D). Interestingly, Ddi1 protein levels remained overexpressed following deletion of UBX5 on top of the \textit{wss1Δ} mutation, indicating that it still plays a relevant role to help both the \textit{ubx5Δ wss1Δ} and the \textit{ubx5Δ tdp1Δ wss1Δ} mutants in dealing with different sources of stress (Figs 1D, 2A, and 5B). Accordingly, ChIP-qPCR analyses at the FRT locus revealed that Ddi1 is recruited in \textit{tdp1Δ wss1Δ top1Δ} (Fig 5E), as previously observed (Serbyn et al., 2021). Even though the \textit{ubx5Δ tdp1Δ wss1Δ top1Δ} mutant demonstrated more efficient Flp-cc elimination from the FRT (Fig 3D), Ddi1 was recruited to the same extent. This enrichment of Ddi1 suggests that it is a key contributor to its role in repairing DPCs directly.
Figure 3. Loss of Ubx5 has a suppressing effect on crosslinked Flp.

A Schematic representation of the "Flp-nick" system initially described in (Nielsen et al., 2009). The mutant flp-H305L recombinase is expressed from a galactose-inducible promoter and targeted to the Flp Recognition Target (FRT) site introduced in the yeast genome next to ARS607, on chromosome VI. FRT consists of three DNA elements (orange) recognized by the Flp recombinase. The location of qPCR primers used for all subsequent ChIP-qPCR analyses is indicated in black, at +200 bp, +500 bp, and +1,000 bp from the FRT.

B The ubx5Δ mutation rescues growth defects caused by Flp-nick galactose-induction in the tdp1Δ wss1Δ top1Δ mutant. Indicated strains were grown in YEP-2% raffinose prior to plating on YEP-2% glucose or YEP-2% galactose plates. Plates were incubated for 3 days at 30°C.

C Cell cycle progression of Flp-nick strains and time points used in (D), monitored by fluorescence-activated cell sorting (FACS). ns, non-synchronized; α-f, alpha-factor; gal, galactose; raf, raffinose.

D flp-H305L-3HA dynamics at the FRT site in different mutants. Cells were grown in 2% raffinose (raf), synchronized in G1 with alpha-factor (α-f) during induction of flp-H305L expression with 3% galactose (gal). Induction was stopped by addition of glucose (glu), and cells were released into the cell cycle. Samples were collected at the indicated time points. Levels of flp-H305L-3HA are indicated in black. Data are presented as the percentage of input showing the means ± SDs for n = 5 independent biological replicates. Significance was determined by ordinary one-way ANOVA using Tukey’s multiple comparison test (n.s., non-significant; * P < 0.05; ** P < 0.01; *** P < 0.001). No FRT binding site and no Flp-H305L-3HA expression (raf) were used as negative controls. See (C) for cell cycle analyses by FACS.

E Ubx5 occupancy at the Flp-bound FRT site. Levels of Ubx5-TAP were monitored by ChIP and qPCR following formaldehyde crosslinking in cells grown as described in (D). The graph shows enrichment of qPCR signals over the unrelated intergenic region. Data are presented as means ± SDs for n = 5 independent biological replicates. P-values for +200 bp were defined by 2-way ANOVA using Tukey’s multiple comparisons test (n.s., non-significant; * P < 0.05; ** P < 0.01; *** P < 0.001). In addition to the indicated mutations, all strains are bar1Δ Ubx5-TAP. No FRT strain and raffinose (raf) samples were used as negative controls.
in ubx5Δ-mediated suppression of tdp1Δ wss1Δ and wss1Δ hyper-
sensitivity to DPC-inducing drugs, probably by mediating proteolytic
digestion of the DPC. Indeed, this last point is demonstrated by the
inability of the Ddi1 catalytic mutant (D220A; Fig 5F) to restore
digestion of the DPC. Indeed, this last point is demonstrated by the
ubx5Δ hyper-
Δ wss1Δ and wss1Δ hyper-
sensitivity to DPC-inducing drugs, probably by mediating proteolytic
digestion of the DPC. Indeed, this last point is demonstrated by the
ubx5Δ and Wss1.

Overall, these results indicate that Ddi1 provides resistance to DPC-inducing drugs in cells lacking
ubx5Δ and Wss1.

Figure 4. Cdc48 recruitment at crosslinked Flp depends on Ubx5.

A Cdc48 occupancy at the Flp-bound FRT site. Levels of Cdc48-TAP were mon-
tered by ChIP and qPCR following formaldehyde crosslinking in asynchro-
nous cells before and after Flp-cc induction with galactose. The graph
shows enrichment of qPCR signal over the unrelated intergenic region. Data
are presented as means ± SDs of three to four independent biological rep-
licates. P-values for +200 bp were defined by 2-way ANOVA using Tukey’s
multiple comparisons test (n.s., non-significant; ****P < 0.0001). In addition
to the indicated mutations, all strains are Cdc48-TAP. No FRT strain
was used as a negative control.

B Ubiquitination of the FRT locus. Asynchronous cultures were grown in 2%
raffinose (raf) or additionally supplemented with 3% galactose (gal) to
induce flp-H305L expression. Cells were also collected during glucose
repression of flp-H305L expression at time points 30 min (glu 30’) and 120
min (glu 120’). Ubiquitin antibody was used for ChIP-qPCR analysis follow-
ing formaldehyde crosslinking. Data are shown as the mean ± SDs of three
to six independent biological replicates. P-values for +200 bp were defined
by 2-way ANOVA using Tukey’s multiple comparisons test (n.s., non-
significant, *P < 0.05, **P < 0.001, ***P < 0.0001). In addition to all indi-
cated mutations, all strains are barΔ Ddi1-TAP.

Ubx5 prevents efficient turnover of stalled RNAPII in the absence of Wss1

Several studies have revealed that the largest subunit of RNAPII, Rpb1, is prone to stalling and degradation after exposure to geno-
toxic agents (Beaudenon et al., 1999; Malik et al., 2008; Verma
et al., 2011; Wilson et al., 2013). The UV-induced degradation of Rpb1 depends on Cdc48, as well as its cofactor proteins Ubx4 and
UbX5 (Verma et al., 2011). Albeit DNA-bound Rpb1 is currently not
shown to be a DPC, its degradation after UV or HU exposure still
engages the DPC-proteases Ddi1 and Wss1 (Serbyn et al., 2020). It is
still unclear how Ddi1 and Wss1 may assist Ubx4-Ubx5-Cdc48 in
the extraction of stalled Rpb1, but it is likely that stalled Rpb1 is a
substrate of these two proteases.

In light of the suppressing effect of ubx5Δ on wss1Δ cells and the
requirement of Ddi1 in this context, we aimed to assess how Rpb1
stability was altered in these conditions. We evaluated Rpb1 protein
levels in total yeast cell extracts following HU treatment. As for-
merly reported, Rpb1 was rapidly degraded in response to HU expo-
sure in WT cells, while loss of Ubx5 or Wss1 hindered this process
(Fig 6A and B). Surprisingly, the double ubx5Δ wss1Δ mutant was
proficient for Rpb1 turnover, although both proteins alone are
strictly required for degradation. Additionally, Rpb1 turnover in
ubx5Δ wss1Δ was dependent on Ddi1 (Fig 6A and B). These obser-
vations reveal that the function of Wss1 in Rpb1 degradation can be
bypassed if UBX5 is deleted, strongly favoring the Ddi1-dependent
pathway.

With respect to prior work that described Rpb1 accumulation on
chromatin in wss1Δ (Serbyn et al., 2020), we also examined DNA-
bound Rpb1 in the mutants generated in this study. Consistently,
Rpb1 accumulation can easily be observed in wss1Δ (Fig 6C, lane 2;
Fig 6D), and this stabilization is counteracted in ubx5Δ wss1Δ (Fig 6C,
lane 4). Finally, Rpb1 was highly abundant on chromatin in the
triple mutant ubx5Δ wss1Δ ddi1Δ (Fig 6C, lane 5; Fig 6D),
emphasizing the importance of Ddi1 in the suppressing effect of
ubx5Δ. Taken together, these data suggest that Ubx5 contribution to
the clearance of stalled DNA-bound Rpb1 is relevant only in the
presence of Wss1. This argues that Wss1 and Ubx5 may cooperate
for genotoxin-induced Rpb1 turnover. When Wss1 and Ubx5 are lost, the pathway for degradation of stalled RNAPII is compromised and strongly relies on Ddi1.

Discussion

With the progression of studies on DNA-protein crosslinks, several repair routes have been discovered but the contribution of each pathway still needs to be elucidated. This study aimed to gain a deeper understanding of the involvement of the AAA ATPase Cdc48 in the cellular response to DPCs. Here, we show that the UBX protein Ubx5 is the major Cdc48 adaptor capable of suppressing the phenotype associated with the defective Wss1-dependent route of proteolytic DPC processing. Interruption of Ubx5-Cdc48 interaction was sufficient to abrogate phenotypes linked to loss of Wss1 or deletion of both critical Top1cc repair genes WSS1 and TDP1. Our data indicate that Ubx5 is targeted to DPC sites and prevents them from proteolysis if Wss1 enzymatic activity is unavailable. Moreover, Wss1 cannot efficiently perform its proteolytic activity without prior action of Ubx5-Cdc48, revealing an unexpected role of Ubx5 in coordinating the cooperation between Cdc48 and Wss1 in the removal of DNA-bound proteins. We exploit these observations to propose a model for Ubx5-Cdc48 and Wss1 co-action in DPC elimination (Fig 7), in which Ubx5 targets Cdc48-Ufd1-Npl4 to ubiquitinated DPCs for initial processing of the protein adduct, followed by Wss1 recruitment to complete proteolysis.

Ubx5 targets Cdc48 and assists Wss1 at DNA-bound proteins

An unexpected finding from our work is that cells deprived of Ubx5-Wss1 are resistant to DPCs (Fig 2A) and present a normal Rpb1 turnover following exposure to HU (Fig 6A and B), although both Ubx5 and Wss1 activities are important to mediate extraction of Rpb1 from chromatin (Fig 6C and D; Verma et al, 2011; Serbyn et al, 2020). Given the established connection between SPRTN/Wss1 and p97/Cdc48 in several reports (Davis et al, 2012; Ghosal et al, 2012; Mosbeck et al, 2012; Stengele et al, 2014; Balakirev et al, 2015; Fielden et al, 2020; Kroning et al, 2022), we suggest that the protease Wss1 is assisted by the unfolding activity of Ubx5-Cdc48 to remove chromatin-bound proteins. It remains to be elucidated which comes first, Wss1 or Cdc48? On one hand, Cdc48 might extract by-products generated by Wss1 enzymatic activity. On the other hand, the activity of Cdc48 may be required to prepare the DPC and facilitate its proteolysis by Wss1.

For several reasons, we envision that Ubx5-Cdc48 is targeted before Wss1. In support of this hypothesis, we were able to detect a substantial accumulation of both Ubx5 and Cdc48 at a Top1cc-like locus, especially in the absence of Wss1 (Figs 3E and 4A). Experiments in human cells showed that p97/Cdc48 enabled proteolysis of TOP1cc by SPRTN/Wss1, probably by unfolding the adduct to increase accessibility for SPRTN cleavage (Fielden et al, 2020). Overall, the data support a model in which Ubx5-Cdc48 engaged on a crosslinked protein is unable to complete substrate degradation without Wss1. Consequently, in the absence of Wss1, Ubx5-Cdc48 is targeting and masking the DNA-bound protein (Figs 3E and 4A), which will prevent easy access to parallel repair factors, such as Ddi1 (Fig 5). In this view, we propose that if Ubx5-Cdc48 is unavailable, the function of Wss1 would be partially compromised as the initial processing and unfolding of the adduct would not occur (Fig 7). Despite our efforts, we could not detect Wss1 at DPCs, and our view of the recruitment of Wss1 to DPCs strongly relies on genetic data.

Is Ubx5-Cdc48 strictly required for Wss1 enzymatic activity on DPCs?

Our data showing that ubx5A mutant cells are not sensitive to DPCs induced by FA, HU (Fig 2A), or CPT (Figs EV2A and EV3C) argue against the above-mentioned model. However, it can also suggest that, although Ubx5 is crucial for turnover of some DNA-bound proteins following exposure to genotoxins (Fig 6A and B), it may be dispensable for removal of other types of adducts potentially targeted by Wss1. This statement is supported by some recent in vitro experiments. Using biochemical reconstitution of purified proteins, the authors revealed that SPRTN/Wss1 is sufficient for proteolysis of loosely folded DPCs, but p97/Cdc48 is required for proteolytic cleavage of tightly folded DPCs (Kroning et al, 2022). One could therefore propose that the reliance on Ubx5-Cdc48 unfolding activity depends on the structural characteristics of the DPC encountered. Thus, if Ubx5-Cdc48 is engaged on tightly folded DPCs, it might absolutely require Wss1’s activity to finalize the extraction, which is demonstrated in our experiments by a great accumulation of both Ubx5 and Cdc48 at DPCs in the absence of Wss1 (Figs 3E and 4A). As shown by ubx5A which alleviates wss1Δ phenotypes (Fig 2A), Wss1 could be assisted by Ubx5-Cdc48 in a wide variety of DPCs, whereas the protease may absolutely rely on Ubx5-Cdc48 unfolding activity only for a specific subset of tightly folded DPCs. This model also partially explains why wss1Δ mutant cells complemented with a Wss1 allele deficient in Cdc48 binding are resistant to HU exposure (Maddi et al, 2020), but not to Top1-crosslink accumulation (Stengele et al, 2014). In the same line, the mammalian SPRTN-SHP mutant allele deficient in p97 binding is not processive in the repair of TOP1ccs (Fielden et al, 2020).

Alternatively, in the absence of Ubx5-Cdc48, Wss1 may eventually still be able to process DPCs, but in a longer time frame. A recent study highlighted that p97-UFD1-NPL4 activity has a high ubiquitin threshold (Deegan et al, 2020; Fujisawa et al, 2022) and that mammalian UBXD7 (Ubx5 homolog) reduces it (Fujisawa et al, 2022). It is proposed that UBXD7 promotes the formation of productive p97-UFD1-NPL4 complexes on chains of only five ubiquitins. Yet, absence of UBXD7 does not hinder the efficiency of p97-UFD1-NPL4 on longer ubiquitin chains. It is conceivable to attribute a similar function to yeast Ubx5-Cdc48 in DPC processing, by promoting Wss1 recruitment or activity toward substrates with short ubiquitinated substrates. This could explain why both Ubx5 and Cdc48 are not detected in WT-like condition on a DPC, as they may promote efficient removal (Figs 3E and 4A). In this model, the absence of Ubx5-Cdc48 complex does not necessarily prevent Wss1 targeting and activity. However, once Ubx5-Cdc48 is engaged, it relies on the protease for efficient repair. Additional investigation is required to reveal the extent to which Wss1 relies on Ubx5-Cdc48 complex.

Ddi1 as an alternative pathway for deficient Wss1

Although Wss1 may be the primary response to DPCs, an excess of DPCs could saturate this pathway and cells may require alternative
repair pathways, such as Ddi1. However, if Ubx5-Cdc48 is targeted first, it requires Wss1 to finalize adduct proteolysis, as proposed in the previous paragraph. Consequently, this may result in a slow dissociation of Ubx5-Cdc48 from the DNA-bound adduct in wss1Δ, which would compete with other repair factors for the access to the lesion and therefore be lethal. Supporting our model, a previous study performed in mammalian cells suggested that UBXD7 (Ubx5 homolog) was shielding substrates to protect them from proteasomal factors, leaving them for p97-dependent processing (Alexandru et al., 2008). Consequently, the use of p97-inhibitor induces...
trapping of UBXD7 on substrates, while absence of UBXD7 leaves the substrate accessible for engaging proteasome factors.

In agreement with this idea, we observed a substantial accumulation of both Ubx5 and Cdc48 at the Flp-cc in the absence of Wss1 and Tdp1 (Figs 3E and 4A). In addition, ubiquitin and Ddi1 levels detected at the Flp-cc remained high upon Ubx5 deletion, although DPCs were processed (Figs 3D, 4B, and 5C–E). Indeed, the amount of DPC is lower in $\text{ubx5}^{\Delta} \text{tdp1}^{\Delta} \text{wss1}^{\Delta}$ compared to $\text{tdp1}^{\Delta} \text{wss1}^{\Delta}$.

These data suggest that the activity of Ddi1, or its access to Flp-cc may be inhibited by a prolonged association of Ubx5-Cdc48 on the lesion. More to that, Ddi1 requires very long ubiquitin chains (Yip et al., 2020). It is therefore tempting to speculate that accumulation of Ubx5-Cdc48 may block the growth of ubiquitin chains. Consequently, lack of Ubx5 may allow faster growth of long ubiquitin chains and, accordingly, efficient recruitment and proteolysis by Ddi1. In line with this idea, the catalytic activity of Ddi1 remains essential for cellular resistance of the $\text{ubx5}^{\Delta} \text{wss1}^{\Delta}$ mutant towards DPCs and DNA-bound proteins (Figs 5F and G, and 6).

Figure 6. Ubx5 loss promotes Rpb1 degradation in the absence of the DPC protease Wss1.

A Ubx5 loss restores HU-induced Rpb1 degradation in wss1Δ and requires Ddi1. Rpb1 levels were defined in cells growing in the presence of 200 mM HU and 100 μg/ml cycloheximide (CHX) at the indicated time points. Rpb1 and Pgk1 levels in total cell extracts were probed by immunoblotting and quantified using fluorescent secondary antibodies. See (B) for quantifications. Images show immunoblotting using chemiluminescence.

B Rpb1 turnover representative of pictures shown in (A). Relative Rpb1 to Pgk1 levels were set to 1 in the respective non-treated samples. Graphs show values of means ± SDs of three to six independent biological replicates.

C, D Rpb1 chromatin enrichment following HU treatment. (C) Chromatin fractions were isolated from cells treated for 2 h with 200 mM HU and 100 μg/ml CHX. Pgk1 and H3 were used as controls to monitor the fractionation. (D) Quantifications of Rpb1 and H3 levels in chromatin fractions. Relative Rpb1 to H3 levels were set to 1 in the respective WT samples. Quantifications of eight biological replicates are presented as means ± SDs. Significance was defined by ordinary one-way ANOVA using Dunnett’s multiple comparison test and WT as a control (n.s., non-significant; **P < 0.01).
While we propose that ubiquitination is a platform for the recruitment of Ubx5-Cdc48-Wss1 and the protease Ddi1 to DPCs, it should not be neglected that other cellular mechanisms rely on ubiquitination as well. For example, previous studies shed light on the importance of the proteasome in ubiquitin-dependent DPC degradation (Borgermann et al., 2019; Larsen et al., 2019; Sparks et al., 2019; Sun et al., 2020a). Although we detected the ubiquitin signal in close vicinity to the Flp-cc (Fig4B, + 200 bp), it remains unclear whether the modification is placed on the DPC itself, or involves other proteins.

Homologous recombination and nucleotide excision repair in DPC repair

In previously published findings, a synthetic lethality interaction was reported between yeast Wss1 and Rad52-dependent HR pathway (Stingele et al., 2014). A similar interplay was found in mammalian cells depleted of BRCA2-dependent HR and SPRTN (Ruggiano et al., 2021). In support of these findings, our results show that HR is critical for yeast cell survival in the absence of Ubx5, Tdp1, and Wss1 (Fig EV4B), suggesting that HR-dependent processing of DPCs acts in parallel to Wss1. Loss of Ubx5 may allow the action of other activities, such as HR, to compensate for the absence of Wss1 and Tdp1.

We extensively used the Flp-nick system and showed that Ubx5 was consistently enriched at the FRT site in the absence of Wss1 (Fig 3E). Interestingly, microscopy experiments revealed the presence of the HR component Rad52 at the FRT site (Nielsen et al., 2009). Therefore, Ubx5 may not only prevent the access of repair factors such as Ddi1 to Flp-cc, but also restrict the accessibility of HR factors.

Previous studies have investigated the contribution of the NER pathway in the elimination of DPCs (Reardon & Sancar, 2006; Baker et al., 2007; de Graaf et al., 2009; Nakano et al., 2007, 2009; Stingele et al., 2014), and a synthetic lethality interaction was identified between yeast Wss1 and NER (Stingele et al., 2014). However, our genetic analyses challenge these observations. Our data indicate that the Ubx5-mediated suppression of DPC repair-deficient strains does...
not rely on NER (Fig EV5C). Yeast cells depleted of Wss1, Tdp1, and NER are viable in the absence of Ubx5 (Fig EV5C). In the context of Ubx5-suppression, NER appears to play a minor role compared to other pathways, such as HR or proteolytic digestion by Ddi1.

Our study assessed the relationship between Ubx5, Wss1, and other repair mechanisms only in unstressed conditions (Figs EV4 and EV5), whereas the synthetic lethality phenotype of Wss1 and NER-deficient strains was observed after treatment with FA (Stingele et al, 2014). Therefore, a viable ubx5Δ tdp1Δ wss1Δ mutant depleted of NER may exhibit acute sensitivity to FA-induced DPCs. Although our observations are contradictory to some published reports, they are consistent with other studies that attributed a minor role to NER in repairing DPCs (Quievryn & Zhitikovich, 2000; de Graaf et al, 2009; Nakano et al., 2007, 2009; Zecevic et al, 2010). Understanding the exact contribution of these pathways in yeast will require additional work.

**Cdc48 uses different adaptors to target different DPC types**

The large variety of Cdc48 adaptors raises the question of how many can act in DPC repair pathways and whether they are redundant. Indeed, Cdc48/p97 can associate with its adaptors in different ways to form various assemblies (Alexandru et al, 2008; Schuberth & Buchberger, 2008; Buchberger et al, 2015); Cdc48/p97 can also bind cofactors in a hierarchical manner to provide additional substrate specificity (Hanzelmann et al, 2011). For instance, the mammalian UBXD7 protein only binds to p97 in complex with UFD1-NPL4 and not to p97 alone (Hanzelmann et al, 2011). It is therefore not surprising to find both Ufd1 and Npl4 as suppressors of the tdp1Δ ubx5Δ wss1Δ mutant along with Ubx5, despite the essentiality of the UFD1 gene (Fig 1A), supporting a model in which Cdc48 recruitment to DPCs is toxic in this cellular context.

Similarly, SPRTN/Wss1 interacts specifically with p97/Cdc48 bound to UFD1-NPL4 and to p97 alone (Hanzelmann et al, 2011). It is therefore not surprising to find both Ufd1 and Npl4 as suppressors of the tdp1Δ ubx5Δ wss1Δ mutant along with Ubx5, despite the essentiality of the UFD1 gene (Fig 1A), supporting a model in which Cdc48 recruitment to DPCs is toxic in this cellular context.

Among the UBX protein family, Ubx4 also turned out to be an interesting suppressor of tdp1Δ ubx5Δ wss1Δ (Figs 1A and EV3A and B). Given that Ubx4 and Ubx5 are redundant and in charge of a similar function in RNAPII turnover following UV damage (Vermel et al, 2011), one could hypothesize a redundant role for Ubx4 and Ubx5 in DPC repair as well. This could provide another explanation for why ubx5Δ shows no sensitivity toward DPC-inducing agents (Figs 2A, EV2A, and EV3C), as Ubx4 could take over its role. However, a double ubx4Δ ubx5Δ mutant is not sensitive to DPCs induced by several drugs (Fig EV3C and D), arguing against this proposal. One could speculate that in the case of Top1cc, Tdp1 may still be taking care of the repair and this could explain the absence of a phenotype for the ubx4Δ ubx5Δ mutant. For instance, Top1cc-sensitivity of yeast cells can be revealed only by simultaneous depletion of Wss1 and Tdp1 (Stingele et al, 2014). However, additional deletion of TDP1 in ubx4Δ ubx5Δ did not change CPT resistance (Fig EV3D). As proposed above, the activity of Wss1 may not strictly depend on UBX proteins interacting with Cdc48, providing a partial answer to this lack of additivity.

In contrast, ubx5Δ completely abolished Cdc48 accumulation at a DPC site in tdp1Δ wss1Δ (Fig 4A), suggesting that the segregation is not targeted by other cofactors in the context of Flp-cc. Yet, our data do not exclude the possibility of distinct substrate specificities for Ubx4 and Ubx5 in DPC repair. Ubx5 is perhaps the major substrate targeting adaptor for Cdc48 regarding Top1ccs, but we do not exclude the role other Cdc48 cofactors can have in DPC repair. In favor of this explanation, it was previously reported that Ubx5 is one of the UBX proteins that binds the most strongly to Cdc48, while Ubx4 has a weak binding (Schuberth et al, 2004). Consequently, other Cdc48 cofactors may be involved in DPC repair along with Wss1, that we might not be able to reveal with our genetic approach identifying Top1cc repair factors (Fig 1A).

Cdc48 is able to be directed to mixed SUMO-ubiquitin chains, which could trigger the recruitment of the protease Wss1 to these chains (Nie et al, 2012). However, the SUMO-binding capacity of Wss1 remains essential for proteolytic cleavage in vivo (Mullen et al, 2010; Stingele et al, 2014; Balakirev et al, 2015). Consistently and given the number of Cdc48 cofactors, Ubx5 could perhaps assist Wss1 in the cleavage of mainly ubiquitinated DPCs, while other cofactors may be required for other types of signals. In mammals, the activity of SPRTN on protein adducts is also dependent on both sumoylation and ubiquitin signals (Ruggiano et al, 2021).

To our surprise, the Cdc48 cofactor Doa1 did not appear as a suppressor of tdp1Δ ubx5Δ wss1Δ in the transposon screen (Serbyn et al, 2020; Fig 1A). Contrasting this observation, Doa1 was previously shown to work in DPC repair along with Cdc48 and Wss1 in a ternary complex, capable of targeting SUMO substrates (Balakirev et al, 2015), although Doa1 was thought to be ubiquitin specific (Mullally et al., 2006). These conclusions were drawn from precipitation experiments performed without any stress conditions, and the function of Doa1 in DPC repair was not addressed directly. Doa1 may also be involved in the repair of a specific DPC subtype not tested in our study.

Altogether, our observations support the view that Cdc48/p97 assists Wss1/SPRTN in the repair of a wide range of DPCs, depending on the cofactor it is associated to. It remains to be understood how many Cdc48/p97 cofactors have a relevant function in DPC repair biology and in which specific context they are required. Future studies will reveal how Ubx4 and Ubx5, and probably other Cdc48 cofactors, are orchestrated in DPC repair. This work may provide new insights into the resistance associated with TOP1 inhibitors and help in predicting responses to topoisomerases-targeted therapy.

**Limitations of the study: the removal of DPCs and stalled RNAPII**

We employed *in vivo* Flp-cc and stalled Rpb1 as two different targets of DPC repair and Cdc48 activities. While Flp-cc involves the creation of a covalent linkage between *flp-H305L* and the DNA, it is not the case for stalled Rpb1. However, it is possible that a prolonged stalling of Rpb1 may lead to a DPC-like lesion. For instance, this has been observed for inhibitor-induced trapping of Poly(ADP-ribose) polymerase −1 and −2 (PARP-1 and PARP-2; Murai et al, 2012), which are so tightly bound to DNA that it results in a DPC-like
lesion, or a “pseudo”-DPC (Stingele et al., 2017). Consistently, the repair of DPCs and the degradation of stalled Rpb1 may share similar mechanisms, including Ubx5, Cdc48, and Wss1. However, some responses may differ. One important and interesting question is whether the degradation of Rpb1 fully follows the same mechanisms used to degrade protein adducts, such as Flp-cc.

Materials and Methods

Reagents and Tools table

| Reagent/Resource | Reference or Source | Identifier or Catalog Number |
|------------------|---------------------|-----------------------------|
| **Experimental Models** | | |
| *S. cerevisiae* strains – See Appendix Table S1 for the list of strains | This Study | N/A |
| *E. coli* DH5alpha | N/A | N/A |
| **Recombinant DNA** | See Appendix Table S2 for the list of plasmids | This Study | N/A |
| ** Antibodies** | | |
| Mouse anti-HA (monoclonal, clone 16B12); for WB 1:2;000; for ChIP 1 µg/1 mg protein | Biolegend | 901502; RRID: AB_2565006 |
| Mouse anti-Ubiquitin (monoclonal, clone FK2); for ChIP 1 µg/1 mg of protein | Calbiochem | ST200-100UG; RRID: AB_2043482 |
| Mouse anti-Rpb1 (monoclonal, clone 8WG16); for WB 1:5,000 | Biolegend | 664912; RRID: AB_2650945 |
| Mouse anti-Pgk1 (monoclonal, clone 22C5D8); for WB 1:3,000 | Abcam | ab113687; RRID: AB_10861977 |
| Rabbit anti-Histone H3 (polyclonal); for WB 1:2;000 | Invitrogen | P15-16183; RRID: AB_10985434 |
| Fluorescent secondary Goat IRDye 800CW anti-mouse; for WB 1:4;000 | LI-COR | 926-32210; RRID: AB_621842 |
| Secondary Goat anti-Mouse-HRP; 1:5,000 | DAKO | P0447; RRID: AB_2617137 |
| Secondary Goat anti-Rabbit-HRP; 1:5,000 | DAKO | P0448; RRID: AB_2617138 |
| **Oligonucleotides and sequence-based reagents** | | |
| OFS4363_FRT +200bp_forward; 500 nM AAGTTCGACATGGGCTTCAG | Serbyn et al (2020) | N/A |
| OFS4364_FRT +200bp_reverse; 500 nM TCGTTTGGAGGACCTTTGAG | Serbyn et al (2020) | N/A |
| OFS4365_FRT +500bp_forward; 300 nM CGGGCAGTAGCTCATCAAGT | Serbyn et al (2020) | N/A |
| OFS4366_FRT +500bp_reverse; 300 nM CATGAAGAGGGTGAGGAGGA | Serbyn et al (2020) | N/A |
| OFS4367_FRT +1kb_forward; 600 nM CGGCCTGATCATTCATTCCA | Serbyn et al (2020) | N/A |
| OFS4368_FRT +1kb_reverse; 600 nM CGGACATCACAATCTTGCAC | Serbyn et al (2020) | N/A |
| OFS2788_intergenic_forward; 500 nM TGTTCTTTAAAGGGTATGGTCA | Gali et al (2017) | N/A |
| OFS2789_intergenic_reverse; 500 nM TGGCGCAGTAGCTTGTGAAACC | Gali et al (2017) | N/A |
| See Appendix Table S3 for full list of oligonucleotides | This Study | N/A |
| **Chemicals, enzymes and other reagents** | | |
| Zymolyase-20T | Amsbio | 120491-1 |
| Phusion High-Fidelity DNA Polymerase | Thermo Scientific | F530L |
| Auxin (3-indoleacetic acid) | Sigma-aldrich | I2886 |
| Camptothecin | Lucerna-Chem | Cat. no 0215973225 |
| Etoposide | Sigma-aldrich | E1383 |
| Hydroxyurea | Bio Basic | HB0528 |
| Formaldehyde | Sigma-aldrich | 1.04003 |
| MG132 | Enzo Life Sciences | BML-P1102 |
### Reagents and Tools table (continued)

| Reagent/Resource                                    | Reference or Source | Identifier or Catalog Number |
|-----------------------------------------------------|---------------------|------------------------------|
| Cycloheximide                                        | Sigma-Aldrich       | C7698                        |
| WesternBright ECL HRP substrate                     | Advansta            | K-12045                      |
| WesternBright sirius HRP substrate                  | Advansta            | K-12043                      |
| cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail| Roche               | 469159001                    |
| NEM (N-Ethylmaleimide)                              | Thermo Scientific   | 40526                        |
| Dynabeads Protein G for immunoprecipitation         | Invitrogen          | 100090                       |
| Dynabeads Pan Mouse IgG                             | Invitrogen          | 11041                        |
| Alpha Factor                                         | PRIMM               | 201307-00007                 |
| Glusulase                                           | Perkin Elmer        | NEE154001Ea; lot: 2607947    |
| Propidium Iodide                                     | Sigma               | P4170                        |
| Proteinase K                                        | Carl Roth           | 7S28.3                       |
| 4X Bolt LDS Sample Buffer                            | Life Technologies   | B0007                        |
| RNase A, PureLink                                    | Invitrogen          | 12091-021; lot: 1772940; 2364671 |
| The Bio-Rad protein assay                            | Bio-Rad             | 500-0006                     |
| Phire Green Hot Start II PCR Master Mix              | Thermo Scientific   | F126L                        |
| NEBuilder HiFi DNA Assembly Cloning Kit              | NEB                 | E5520                        |
| NucleoSpin Gel and PCR Clean-Up                     | Macherey-Nagel     | Cat. No 740609.250           |
| SYBR Select Master Mix for CFX                      | Applied biosystems  | 4472942                      |
| MinElute PCR Purification Kit                        | Qiagen              | 28006                        |

**Deposited data**

Unprocessed imaging (immunoblots, spot assays, tetrades) data

This Study

Mendeley data: https://doi.org/10.17632/hzwvpgs7n8.1

**Software**

- **Prism 8.0.1**
  - GraphPad Software, Inc. RRID: SCR_002798
- **Kaluza**
  - Beckman Coulter RRID: SCR_016182
- **SnapGene 3.2.1**
  - GSL Biotech LLC RRID: SCR_015052

**Other**

- **MagNA Lyser Instrument**
  - Roche 3358976001
- **Bioruptor Twin**
  - Diagenode UCD-400
- **Galios 8 colors 2 Lasers Flo Cytometer**
  - Beckman Coulter B43619
- **Real-Time PCR Detection System**
  - Bio-Rad CFX connect and CFX96
- **0.5 mm Glass Beads**
  - BioSpecProducts Cat. No 11079105

**Methods and Protocols**

**Yeast strains and growth conditions**

All *S. cerevisiae* yeast strains used in this study (listed in Appendix Table S1) were derived from W303 or S288C genetic backgrounds. Genomic mutations were introduced by transformation and verified by colony PCR, phenotypic analyses, and/or sequencing. Epitope insertions were checked by immunoblotting. Other mutants were obtained by standard techniques and tetrad dissection. TAP-tagged strains were obtained from the yeast fusion library (Ghaemmaghami et al., 2003). Oligonucleotides, template plasmid, or DNA used for PCR and transformation are listed in Appendix Tables S2 and S3. Additional details on strain construction are available upon request.

Yeast cells were grown at 30°C in YEP- (1% yeast extract, 2% peptone) or SC- (1.7 g/l yeast nitrogen base; 5 g/l ammonium sulfate; 0.87 g/l dropout mix) liquid media, or grown on plates supplemented with 20 g/l agar. As a source of sugar, 2% glucose, 2% raffinose, or 2–3% galactose was added. Selection for dominant markers was performed on YEPD-based medium (YEP-2% glucose) supplemented with 200 µg/ml G418, 200 µg/ml cloNAT, or 50 µg/ml Hygromycin B.

**E. coli strains and growth conditions**

DH5α *E. coli* bacterial strains (listed in Appendix Table S2) were grown at 37°C in LB medium or on LB-2% agar plates supplemented with 50 µg/ml of ampicillin for plasmid selection.
**Yeast transformation**

Five milliliters of exponentially growing cells were resuspended in 80 μl LiTE buffer (100 mM LiAc; 10 mM Tris pH 7.5; 1 mM EDTA). Thirty eight microliters of cells were mixed with 100 μg/ml salmon sperm ssDNA, 37.28% PEG4000, and 200 ng DNA to transform (plasmid DNA or purified PCR product). Cells were incubated for 1 h at 30°C and then supplemented with 6% DMSO, and a heat shock was performed for 10 min at 42°C. Cells were plated on selective media and grown for 2–3 days before isolation of single colonies.

**Genetic crosses and tetrad dissection**

Haploid strains of opposite mating types were mixed and spotted overnight on a YEPD plate at 30°C, to allow mating and diploid construction. Diploids were selected by streaking the mating mixture overnight onto a plate selecting for the diploid genotype. Spore formation was induced by transferring the diploid cells to KAc sporulation medium (20 g/l potassium acetate; 2.2 g/l yeast extract; 0.5 g/l glucose; 0.87 g/l dropout mix; 20 g/l agar; pH 7) for 4–5 days at 30°C. Before tetrad dissection, the ascus cell wall was digested with 0.5 mg/ml Zymolyase (Amsbio, 120491-1) treatment for 5 min at room temperature. Haploid spores from single tetrads were separated with a micromanipulator and grown on a YEPD plate for 3 days at 30°C. The individual phenotype of the dissected spores was determined by replica plating on agar plates containing selective media (various drop-out and drug media).

**Construction of recombinant DNA**

Recombinant plasmid DNA was constructed using NEB Builder HiFi DNA Assembly Cloning Kit (NEB, E5520). PCR templates and oligonucleotides used to generate products are provided in Appendix Tables S2 and S3, respectively. Additional details on construction are available upon request.

**Colony PCR**

Genomic mutations were checked by direct resuspension of yeast strains in Phire Green Hot Start II PCR Master Mix (Thermo Scientific, F126L) supplemented with oligonucleotides at 0.5 μM.

**Spot assays**

Yeast mutant growth and sensitivities were assessed by spot assay. Cells were grown exponentially in the appropriate medium at 30°C under continuous rotation and diluted to OD₆₀₀ = 1–1.5. 10-fold serial dilutions were spotted on agar plates containing the indicated concentrations of auxin, camptothecin (CPT), and hydroxyurea (HU). To treat cells with formaldehyde (FA), 1 ml of yeast culture was incubated with indicated concentrations of FA for 15 min under vacuum. Pellets were resuspended in 100 μl of Urea Buffer (50 mM Tris–HCl pH 7.5; 5 mM EDTA; 6 M Urea; 1% SDS), mixed with 200 μl of glass beads, and subjected to bead-beating in a MagNa Lyzer instrument (Roche) 5 times for 45 s at 4°C, 1 min of pause in between. Final solubilization was performed at 65°C for 10 min and centrifugation 10 min at 16,000 g. Addition of 100 μl sample buffer (3% SDS; 15% Glycerol; 0.1 M Tris pH 6.8; 0.0133% bromophenol blue; 0.95 M 2-mercaptoethanol) and boiled for 10 min. Protein samples were separated by SDS–PAGE and transferred onto nitrocellulose membrane. After transfer, membranes were blocked for 30 min with 5% milk dissolved in TBS-T (150 mM NaCl, 20 mM Tris–HCl, 0.05% Tween, pH 7.4) and incubated with appropriate antibodies indicated in each figure. See the Reagents and Tools table for a list of antibodies used in this study. Signals were revealed with Western Bright ECL HRP (Advanta, K-12045) or Sirius HRP (Advanta, K-12043) substrates.

**Flp-nick induction, chromatin immunoprecipitation (ChIP), and quantitative real-time PCR (qPCR)**

Induction of the flp-H30SL-3HA expression was performed as described in (Nielsen et al, 2009). Briefly, yeast cells were grown to log phase in YEP- 2% raffinose (no transcription of the locus). Where indicated, cells were synchronized in G1 with 200 ng/ml α-factor (PRIMM, 201307-00007) for 1.5 h. Induction was performed for 2 h by addition of 3% galactose. Cells were then washed twice with 20 ml cold YEP (no sugar) medium and released into warm YEP-2% glucose (YPD) medium. One milliliter of cells were collected to monitor cell-cycle progression at desired time points.

Cells were harvested at the different time points by centrifugation at 1,650 g for 3 min, washed twice with cold 1× Phosphate Buffer Saline (PBS) and frozen in 2 ml screw-cap tubes. For Ddi1-TAP, Ubx5-TAP, Cdc48-TAP, and Ubiquitin ChIP, cells were fixed before pelleting by addition of 1% formaldehyde (FA) for 15 min at room temperature, then quenched with 250 mM glycine for 5 min, and kept on ice for at least 10 min. All subsequent steps were performed at 4°C. The frozen cells were resuspended in 1 ml of cold FA lysis buffer (50 mM HEPES-KOH, pH 7.5; 140 mM NaCl; 1 mM EDTA; 1% Triton X-100; 0.1% sodium deoxycholate; protease inhibitor cocktail (Roche)) and lysed by bead-beating with 500 μl of 0.5 mm glass beads with five cycles of 30 s at 6,000 rpm with 1 min pause in between each bead beating cycle, on a MagNA Lyser Instrument (Roche). lysates were then recovered in a new tube by centrifugation and further spun at 16,000 g for 30 min. Pellets were resuspended in 1 ml of FA lysis buffer and subjected to DNA sonication for 20 cycles of 30 s in a Bioruptor Twin (Diagenode). Following pelleting by 15 min centrifugation at 16,000 g, soluble fractions were transferred to a new tube and protein concentration was measured by Bio-Rad protein assay (500-0006). For each ChIP, 1 μg of protein (1/10 of input transferred in a new tube) was incubated together with 1 μl of anti-HA antibodies (BioLegend, 901502 anti-HA.11 clone 16B12, for flp-H30SL-3HA ChIP), or 1 μl of anti-Ubiquitin antibodies (Calbiochem, ST1200-100UG clone FK2), or 20 μl of Dynabeads Pan Mouse IgG (Invitrogen, 11041; for Ddi1-TAP, Ubx5-TAP and Cdc48-TAP ChIP) overnight at 4°C with rotation. Prewashed Protein G Dynabeads (Invitrogen, 100009D) were then added for 3 h to recover antibodies. Beads were collected on magnetic stands and washed once with 500 μl of FA lysis buffer, twice with 500 μl of FA-500 buffer (50 mM HEPES-KOH, pH 7.5; 500 mM NaCl; 1 mM EDTA; 1% Triton X-100; 0.1% sodium deoxycholate), twice with 500 μl of Buffer III (10 mM Tris–HCl pH 8; 1 mM EDTA; 250 mM...
LiCl; 1% IGEPAL; 1% sodium deoxycholate) and finally once with 500 μl of TE buffer (50 mM Tris–HCl pH 7.5; 10 mM EDTA). Proteins were eluted twice with 100 μl of elution buffer B (50 mM Tris–HCl pH 7.5; 1% SDS; 10 mM EDTA) for 8 min at 65°C. Eluted and input samples were incubated for 2 h at 42°C with 0.75 mg/ml Proteinase K (Carl Roth, 7528.3) and decrosslinked at 65°C for at least 12 h. DNA was purified with the MinElute PCR purification kit (Qiagen, 28006) and eluted from the columns twice with 30 μl of elution buffer from the kit. Real-time qPCRs were then performed using SYBR Select Master Mix (Applied Biosystems, 4472942) with oligonucleotide pairs listed in the Reagents and Tools Table (FRT +200 bp; +500 bp; +1,000 bp or intergenic) and a Real-Time PCR machine (Bio-Rad). Results are presented as percent of input or normalized to the intergenic region.

**Cell cycle analysis by flow cytometry analysis (FACS)**

For flow cytometry analysis, 1 ml of yeast cells at OD_{600} = 0.5 were harvested by centrifugation and resuspended in 70% ethanol allowing storage at 4°C for up to 1–2 weeks. Next, cells were pelleted at 3,500 g for 2 min and washed with 300 μl of 50 mM sodium citrate (NaCl) pH 7.2. A second centrifugation at 3,500 g for 10 min was performed, and cells were resuspended in 250 μl NaCl +5 μl RNase A (InVitrogen, 12091-021) and incubated for 1 h at 37°C. Staining with 25 μg/ml Propidium Iodide (PI, Sigma, P4170) was performed at 37°C for 1 h. Cells were then sonicated in a Bioruptor Twin (Diagenode) during five cycles of 5 s, before flow cytometry analysis on a Gallios flow cytometer (Beckman Coulter).

**Analysis of Rpb1 levels following hydroxyurea treatment**

Overnight cultures were diluted to OD_{600} = 0.2. When cells reached OD_{600} = 0.8, hydroxyurea (HU; Bio Basic, HB0528) was added to a final concentration of 0.2 M along with 100 μg/ml cycloheximide (Sigma-Aldrich, C7698) to prevent protein synthesis. Cultures collected at different time points were subjected to TCA protein extraction and immunoblotted with antibodies against Rpb1 (BioLegend, 664912 clone 8WG16) or anti-Pgk1 (Abcam, ab113687 clone 22C5D8) antibodies. Fluorescent secondary antibodies (LI-COR, 926-32210) were used for quantification analyses.

**Isolation of chromatin**

The protocol was adapted from Kubota et al. (2012). Fifty OD of yeast cells were harvested by spinning down cultures for 3 min at 1,650 g and washed once with 1× cold Phosphate Buffer Saline (PBS) and frozen. Pelleted cells were resuspended in 1 ml of pre-spheroblasting buffer (100 mM PIPES/KOH, pH 9.4; 10 mM DTT; 0.1% sodium azide) and incubated for 10 min at room temperature and centrifuged for 3 min at 1,800 g. To induce spheroblasts formation, cells were resuspended in 1 ml spheroblasting buffer (50 mM KH_{2}PO_{4}/K_{2}HPO_{4}, pH 7.4; 0.6 M Sorbitol; 0.1 mM MgCl_{2}; 1 mM DTT; 20 mM β-glycerophosphate; 1 mM PMSF; 0.01% IGEPAL; protease inhibitor (Roche)) and tubes were centrifuged for 5 min at 5,000 g. The supernatant was subjected to a second spin at 5,000 g for 5 min, and a final spin at 16,100 g for 20 min to obtain the nuclei in the pellet. Nuclei were resuspended in 200 μl of EB-X buffer (50 mM HEPES/KOH, pH 7.5; 100 mM KCl; 2.5 mM MgCl_{2}; 0.1 mM ZnSO_{4}; 2 mM sodium fluoride; 0.5 mM Spermidine; 0.25% Triton X-100; 1 mM DTT; 20 mM β-glycerophosphate; 1 mM PMSF; protease inhibitor cocktail) and lysed for at least 10 min on ice. The entire preparation was then transferred on 500 μl of EBX-Buffer (EB-X buffer; 30% sucrose) and spun at 16,000 g for 10 min. Chromatin pellet was gently resuspended in 1 ml EB-X buffer and finally centrifuged at 10,000 g for 2 min. The chromatin was resuspended in 30 μl of 1.5 × Bolt LDS Sample Buffer (Life Technologies, B0007) and analyzed by immunoblotting with antibodies indicated on each figure. The quality of the fractionation was controlled by immunoblotting against PGK1 (cytoplasm contamination; Abcam, ab113687 clone 22C5D8) and H3 (chromatin extraction; Invitrogen, Pa5-16183).

**Quantification and statistical analysis**

Volcano plot from Fig1A was generated using Prism 8. Gene bodies in Figs 1B and EV3A are defined as the ORF. Statistical details can be found in figure legends. Prism 8 was used to quantify P-values and generate graphs. The mean and standard deviation (SD) are reported on the graphs.

**Data availability**

This paper does not report original code.

Unprocessed imaging data were deposited at Mendeley Data: https://doi.org/10.17632/hzvwpgs7n8.1.

Further information and requests for resources and reagent should be direct to the lead contact, Françoise Stutz (francoise.stutz@unige.ch).

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**Author contributions**

Audrey Noireterre: Conceptualization; data curation; formal analysis; validation; investigation; visualization; methodology; writing – original draft; writing – review and editing. Natalia Serbyn: Data curation; validation; investigation; methodology; writing – review and editing. Ivona Bagdiul: Data curation; validation; investigation; methodology. Françoise Stutz: Conceptualization; supervision; funding acquisition; project administration; writing – review and editing.
Disclosure and competing interests statement
The authors declare that they have no conflict of interest.

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**Expanded View Figures**

### A

**CDC48 known interactors**

| Gene | Gene | Gene |
|------|------|------|
| CTF4 | PHO23 | UBX2 |
| CUZ1 | POC4 | UBX3 |
| DFM1 | RAD23 | UBX4 |
| DOA1 | RFU1 | UBX5 |
| DSK2 | RPN10 | UBX6 |
| FZO1 | RPN4 | UBX7 |
| HMG2 | SGS1 | UFD1 |
| HRD1 | SHP1 | UFD2 |
| HSL1 | STT3 | UFE1 |
| LGE1 | THI7 | VMS1 |
| MRE11 | UBI4 | VPS27 |
| NPL4 | UBP2 | |
| OTU1 | UBP3 | |

### B

![Image of tetrads]

**Figure EV1.** (related to Fig 1). Additional Cdc48 interactors.

A. List of known CDC48 physical and genetic interactors extracted from the yeast Saccharomyces Genome Database. The genes in orange correspond to tdp1-degron wss1Δ + auxin highest suppressors plotted and labeled in Fig 1A.

B. Complete genotype characterization of tetrads shown in Fig 1C.
Figure EV2. (related to Fig 2). The UBX domain of Ubx5 is detrimental in the absence of DPC repair factors.

A ubx5Δ is not suppressing defects linked to tdp1Δ. The 12geneΔOHSR mutant (Chinen et al., 2011) was used to reveal sensitivity to camptothecin (CPT). Cells were grown in YEPD and plated on 1 μg/ml CPT. Plates were incubated for 3 days at 30°C.

B Loss of UBX domain of Ubx5 is sufficient to suppress growth defects of tdp1Δ wss1Δ. Tetrads were analyzed after dissection of the diploid (TOP1/tdp1Δ; WSS1/wss1Δ; UBX5/ubx5ubxΔ; TOP1/top1Δ).

C The UBXΔ mutant restores drug resistance of wss1Δ. Cells were grown in YEPD and either treated with 40 mM FA for 15 min or spotted directly on 100 mM HU. Plates were incubated for 2 days at 30°C.

D The UBXΔ mutant restores the growth and increases drug resistance of tdp1Δwss1Δ. Cells were grown in YEPD. Serial dilutions were spotted on a medium supplemented with 1 mM auxin and 5 μg/ml Camptothecin (CPT) or 100 mM hydroxyurea (HU). Plates were incubated for 2 days at 30°C.
Figure EV3. (related to Fig 2). Genetic interactions of Ubx4 and Ubx5 in DPC repair.

A. The C-ter UBX domain of Ubx4 is highly transposed in tdp1wss1Δ. Snapshot depicting transposon coverage of UBX4 gene body in the tdp1-degron wss1Δ + auxin and one of the WT libraries. The height of the bars represents the number of reads for each transposon.

B. Loss of the C-ter UBX domain of Ubx4 suppresses wss1Δ and mildly suppresses tdp1wss1Δ. Cells were grown in YEPD and spotted on a medium supplemented with 1 mM auxin, 5 µg/ml CPT, or indicated concentrations of HU. Plates were incubated for 2 days at 30°C.

C. Analysis of sensitivities of ubx4Δ ubx5Δ mutant in combination with wss1Δ. Cells were grown in YEPD and either treated with 40 mM FA for 15 min or spotted directly on 100 mM HU. Plates were incubated for 2 days at 30°C.

D. Analysis of sensitivities of ubx4Δ ubx5Δ mutant in combination with tdp1Δ. Cells were grown in YEPD and either treated with 40 mM FA for 15 min or spotted directly on 100 mM HU. Plates were incubated for 2 days at 30°C.
Figure EV4. (related to Fig 5). Genetic analyses reveal essential repair pathways for DPC processing in the absence of Ubx5.

A Complete genotype characterization of tetrads shown in Fig 5A.

B-G Genetic interactions of rad52A (B), rad9A (C), sgs1A (D), srs2A (E), rad27A (F), and mre11A (G) with tdp1A, wss1A and ubx5A. Tetrads were analyzed after dissection of the diploid (TDP1/tdp1A; WSS1/wss1A; UBX5/ubx5A) in combination with one of the following: (RAD52/rad52A); (RAD9/rad9A); (SGS1/sgs1A); (SRS2/srs2A); (RAD27/rad27A); (MRE11/mre11A).
Figure E5. (related to Fig 5). Non-essential repair pathways for DPC processing in the absence of Ubx5.

A–C Genetic interactions of yku70Δ (A), rev3Δ (B) and rad4Δ (C) with tdp1Δ, wss1Δ and ubx5Δ. Tetrads were analyzed after dissection of the diploid (TDP1/tdp1Δ; WSS1/wss1Δ; UBX5/ubx5Δ) in combination with one of the following: (YKU70/yku70Δ); (REV3/rev3Δ); (RAD4/rad4Δ).

D Summary table of all tetrad analyses. Red: negative genetic interactions.
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Appendix References (for Appendix Table S1)
| Internal Database Number | Genotypic background | Genotype | Source |
|--------------------------|----------------------|----------|--------|
| **BASIC STRAINS**        |                      |          |        |
| FSY 4976                | W303                 | W303-1A {MATa; his3-11_15; leu2-3_112; ura3-1; trp1D2; ade2-1; can1-100} | Euroscarf |
| FSY 4977                | W303                 | W303-1B {MATalpha; his3-11_15; leu2-3_112; ura3-1; trp1D2; ade2-1; can1-100} | Euroscarf |
| FSY 7311                | BY4741              | MATa; his3Δ1; leu2Δ0; lys20; met15Δ0; ura3Δ0 | This study |
| FSY 8374                | 12geneD0HSR (BY4741) | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pdr3Δ0 pdr6Δ0 pdr1Δ0 yrf1Δ0 snq2Δ0 pdr5Δ0 pdr10Δ0 yor1Δ0 pdr15Δ0 pdr11Δ0 pdr12Δ0 aus1Δ0 RME1 (ins308A) | Chinen et al., 2011 |
| FSY 7561                | Flp-nick Flp-HA (W303) | Cir0; leu2-3_112::pGAL10-flp-H305L-3HA-HIS3; FRT-tetO-URA3; TetR-mRFP MATa | Serbyn et al., 2020 |
| FSY 7400                | Flp-nick Flp micr. (W303) | Cir0; leu2-3::pGAL-flp(H305L)-LEU2; TetR-mRFP(iYGL119W); tetOx224(iYFR020W)-URA3-FRT; Nup49-GFP | Serbyn et al., 2020 |
| FSY 7625                | (diploid)            | W303     | This study |
| FSY 7718                | (diploid)            | W303     | This study |
| FSY 8781                | Flp-nick Flp-HA (W303) | Tdp1-AID*-6HA-HPH; ura3-1::pADH-Os.TIR1-URA3.MATa | This study |
| FSY 8779                | (diploid)            | W303     | This study |
| FSY 9477                | BY4741              | Ubx5-TAP-HIS3; MATa | Ghaemmagha mi et al., 2003 |

**Appendix Table S1 – Yeast strains used in this study**
| ID     | Strain       | Genotype                                                      | Reference         |
|--------|--------------|---------------------------------------------------------------|-------------------|
| FSY 8259 | W303        | HA-Ddi1 (no marker); MATa                                    | Serbyn et al., 2020 |
| FSY 7989 | W303        | ubx5D::KAN; wss1D::HIS3; ddi1D::TRP1; MATalpha                | This study        |
| FSY 9445 | W303        | ubx5D::KAN; ddi1D::TRP1; MATa                                 | This study        |
| FSY 8012 | W303        | wss1D::HIS3; ddi1D::TRP1; MATa                                | Serbyn et al., 2020 |
| FSY 9438 | W303        | ubx5D::KAN; wss1D::HIS3; ddi1D::TRP1; MATa                    | This study        |
| FSY 9447 | W303        | HA-Ddi1 (no marker); ubx5D::KAN; MATa                       | This study        |
| FSY 8268 | W303        | HA-Ddi1 (no marker); wss1D::HIS3; MATa                       | Serbyn et al., 2020 |
| FSY 9441 | W303        | HA-Ddi1 (no marker); ubx5D::KAN; MATa                        | This study        |
| FSY 8269 | W303        | HA-ddi1-d220a (no marker); ubx5D::KAN; MATa                 | Serbyn et al., 2020 |
| FSY 7988 | W303        | ubx5D::KAN; ddi1D::TRP1; MATalpha                            | This study        |
| FSY 9443 | W303        | HA-ddi1-d220a (no marker); ubx5D::KAN; wss1D::HIS3; MATa     | This study        |
| FSY 9438 | W303        | ubx5D::KAN; wss1D::HIS3; ddi1D::TRP1; MATa                    | This study        |
| FSY 7718 | W303        | tdp1D::URA3/TDP1; wss1D::HIS3/WSS1; ubx5D::KAN/UBX5; MATa/alpha | This study        |
| FSY 9296 |             | 12geneD0HSR ubx5D::KAN; MATa                               | This study        |
| FSY 8655 |             | 12geneD0HSR tdp1D::HPH; MATa                                | Serbyn et al., 2021 |
| FSY 9311 |             | 12geneD0HSR ubx5D::KAN; tdp1D::HPH; MATa                    | This study        |
| FSY 9428 | W303        | ubx5-ubxD::KAN; MATa                                        | This study        |
| FSY 6014 | W303        | wss1D::HIS3; tdp1D::URA3; tcp1D::MATa                        | This study        |
| FSY 4978 | W303        | MATa/alpha                                                  | This study        |
| FSY 9436 | W303        | ubx5-ubxD::KAN; wss1D::HIS3; Tdp1-AID*-6HA::HPH; ura3-1::pADH-Os.TIR1-URA3; MATa | This study        |
| FSY 7626 | W303        | ubx4-ubxD::KAN (diploid)                                    | This study        |
| FSY 8234 | W303        | ubx4D::TRP1; ubx5D::KAN; MATalpha                           | This study        |
| FSY 5856 | W303        | tdp1D::URA3; MATa                                          | Serbyn et al., 2020 |
| FSY 9251 | W303        | rad52D::NAT; MATalpha                                       | This study        |
| FSY 7373 | W303        | rad9D::HPH; MATalpha                                        | This study        |
| FSY 5863 | W303        | sgs1D::HPH; MATalpha                                        | This study        |
| FSY 7381 | W303        | srs2D::TRP1; MATalpha                                       | This study        |
| FSY 5821 | W303        | rad27D::LEU2 MATalpha                                       | Serbyn et al., 2021 |
| FSY 7124 | W303        | mre11D::HPH; MATa                                          | Serbyn et al., 2021 |
| FSY 9244 | W303        | yku70D::HPH MATa                                            | This study        |
| FSY 9245 | W303        | rev3D::TRP1 MATalpha                                         | This study        |
| FSY 7718 | W303        | rad4D::TRP1/RAD4; tcp1D::URA3/TDP1; wss1D::HIS3/WSS1; ubx5D::KAN/UBX5; MATa/alpha | This study        |
| FSY 7835 | W303        | ubx5D::KAN; wss1D::HIS3; tcp1D::URA3; MATa                   | This study        |
## Appendix Table S2 – Plasmids used in this study

| Internal Database number | Source | Comments |
|--------------------------|--------|----------|
| **Plasmids used for transformation** | | |
| pFS 1336 | pRS315 (empty) | Stutz lab stock | N/A |
| pFS 4218 | pRS315-HA-UBX5 | This study | Constructed by gibson assembly - checked by sequencing |
| pFS 4277 | pJG4-5-pGAL-HA-UBX5 | This study | Constructed by gibson assembly - checked by sequencing |
| pFS 4278 | pJG4-5-pGAL-HA-uimD | This study | Constructed by gibson assembly - checked by sequencing |
| pFS 4279 | pJG4-5-pGAL-HA-ubxD | This study | Constructed by gibson assembly - checked by sequencing |
| pFS 4334 | pJG4-5-pGAL-HA-ubaD | This study | Constructed by gibson assembly - checked by sequencing |
| pFS 4335 | pJG4-5-pGAL-HA-uasD | This study | Constructed by gibson assembly - checked by sequencing |
| pFS 4336 | pJG4-5-pGAL-HA-ubaDubxD | This study | Constructed by gibson assembly - checked by sequencing |
| pFS 668 | pJG4-5-pGAL (empty) | Stutz lab stock | N/A |
| **Plasmids used as a template for deletion, tag or gene insertion** | | |
| pFS 2161 | pFA6a-KANMX | Stutz lab stock | N/A |
| pFS 2162 | pFA6a-TRP1 | Stutz lab stock | N/A |
| pFS 2484 | pFA6a-TAP-HIS3 | Stutz lab stock | N/A |
| pFS 3073 | pUG73-LEU2 | Stutz lab stock | N/A |
| pFS 4314 | pFA6a-TAP-1ADH1-LoxP-LEU2-LoxP | This study | for genomic TAP-LEU2 tagging Generated by gibson assembly of 2 PCR products with OFS_4766 + 4902 on pFS 2484 and OFS_4903 + 4904 on pFS 3073 |
## Appendix Table S3 – Oligonucleotides used in this study

| Internal Database number | Sequence Used for | Source | Used for: |
|--------------------------|-------------------|--------|-----------|
| OFS_5061                 | GAAGTCGACCAGTCCTCATT | This study | PCR amplification of genomic Cdc48-TAP (Figure 6) |
| OFS_5010                 | CATTAGCGACAAGTTTCTCCG | This study | PCR amplification of genomic Cdc48-TAP (Figure 4) |
| OFS_4403                 | TGTTGTCGTGCAGACCTTCT | This study | PCR amplification of genomic Ubx5-TAP (Figure 3) or genomic ubx5D::KAN |
| OFS_4404                 | TTCCTTGATGACGCCCAC | This study | PCR amplification of genomic Ubx5-TAP (Figure 3) or genomic ubx5D::KAN |
| OFS_5073                 | ACGACAAGGCAGACATAAGCACACTTTTTCGGCTAAAAGGATCTCGTA | This study | to generate rad4D::TRP1 (pFS 2162) used in Figure EV5 |
| OFS_5074                 | AACATACTTTCTTAATTTGGAACAGTGCCTATTGgaattcgagctcgtttaaac | This study | cloning to generate pFS 4216_pRS315-HA-Ubx5 (Figure 1) insert Ubx5 in pRS315 |
| OFS_4635                 | CATAGACTACGACGTGTGGCAATAATAACATACCTTTTCGCTAA | This study | to generate ubx5D::TRP1 (pFS 2162) used in Figure 1 |
| OFS_4343                 | TGGAATAATTTGGGAATTTACTCTGTG | Stutz lab | cloning to generate pFS 4216_pRS315-HA-Ubx5 (Figure 1) insert Ubx5 in pRS315 |
| OFS_4342                 | TCATTCCGTAACTCTTCTACCTTCT | Stutz lab | cloning to generate pFS 4216_pRS315-HA-Ubx5 (Figure 1) insert Ubx5 in pRS315 |
| OFS_4780                 | TTCGGGAGAGGGGATCGATGGGAAAAGTA | This study | cloning Ubx5 under pGAL promoter (Figure 2) |
| OFS_4781                 | TTCGCGCCACTCGAGAAG | This study | cloning Ubx5 under pGAL promoter (Figure 2) |
| OFS_4782                 | TATGCCTCTTCCCGAATCGGGGAAGGAAAAGTGAAGA | This study | cloning Ubx5 variants under pGAL promoter (Figure 2) |
| OFS_4783                 | CTCGAGTCGCCGAATATTTTCTATCTTACCAGGTCAAGCTTC | This study | cloning Ubx5 variants under pGAL promoter (Figure 2) |
| OFS_4784                 | CTCGAGTCGCCGAATATTTTCTATCTTACCAGGTCAAGCTTC | This study | cloning Ubx5 variants under pGAL promoter (Figure 2) |
| OFS_5097                 | CAGATATTGCCTCTCCCCAGGAGGGGCTTACCTGCAAGC | This study | cloning Ubx5D variant under pGAL promoter (Figure 2) |
| OFS_4550                 | AACCATTCTCAGGAGGAGGGTTTTGGGCCTTTTCTCTTCAGTGTTCTAATCTTGATGGCCTTTTCTCTACTA | Stutz lab stock | cloning Ubx5D variant under pGAL promoter (Figure 2) |
| OFS_4551                 | CTGCTTCCTTGAATTTGTTTTGAGGCTTTTCTCTACTA | Stutz lab stock | cloning Ubx5D variant under pGAL promoter (Figure 2) |
| OFS_4552                 | CTAAGGGTGATCGGCTCACTTCAATACACAAATCGGAGAAGAATGCC | This study | cloning Ubx5 uasD variant under pGAL promoter (Figure 2) |
| OFS_4553                 | ATGTCATCTTGGATCCGAGGAGGTTTTGGAGGCTTACCTCAGCTG | This study | cloning Ubx5 uasD variant under pGAL promoter (Figure 2) |
| OFS_4554                 | TCGAAAGAATAGCGAACAGCAGCTAATAACAGCAGAATACACGGAATACTGGGTTTGAAAGTGAATTTAAAGCAGGGAAGGAAAATGGAGA | This study | cloning Ubx5 uasD variant under pGAL promoter (Figure 2) |
| OFS_4555                 | TTGATAAAAGTAAAAGGAGGAGACATGGTATTAGGTTATGATAGGCTTTTGTATGAATTGAGCTGTAAACG | This study | PCR amplification of 4262 to amplify URA3-FRT and generate FSY 7599 (Figure 3) |
| OFS_4556                 | CGAAGCGATTAGCTTTGGAAGGAA | This study | PCR amplification of 4262 to amplify URA3-FRT and generate FSY 7599 (Figure 3) |
| OFS_1653                 | CGAAGCAGGTATTGGAAGGAA | Stutz lab stock | generate FSY 7599 (addition of bar1D (no marker) amplified from pDNA) (Figure 3) |
| OFS_1654                 | CGAAGCAGGTATTGGAAGGAA | Stutz lab stock | generate FSY 7599 (addition of bar1D (no marker) amplified from pDNA) (Figure 3) |
| OFS_4720                 | TGCGATGAGAATGGAGGAA | This study | PCR amplification of 4262 to amplify URA3-FRT and generate FSY 7599 (Figure 3) |
This study to generate Ddi1-TAP-LEU2 from pFS 4314 (Figure 5)

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to generate Ddi1-TAP-LEU2 from pFS 4314 (Figure 5)

This study to construct pFS 4314_pFA6a-TAP-LEU2

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