Global crotonylome reveals CDYL-regulated RPA1 crotonylation in homologous recombination–mediated DNA repair

Huajing Yu1*, Chen Bu2*, Yuncheng Liu1, Tianyu Gong1, Xiaoping Liu1, Shumeng Liu3, Xiaojun Peng2, Wenting Zhang1, Yani Peng1, Jianguo Yang1, Lin He1, Yu Zhang1, Xia Yi1, Xiaohan Yang1, Luyang Sun1, Yongfeng Shang1,3, Zhongyi Cheng2†, Jing Liang1†

Previously, we reported that chromodomain Y–like (CDYL) acts as a crotonyl–coenzyme A hydratase and negatively regulates histone crotonylation (Kcr). However, the global CDYL-regulated crotonylome remains unclear. Here, we report a large-scale proteomics analysis for protein Kcr. We identify 14,311 Kcr sites across 3734 proteins in HeLa cells, providing by far the largest crotonylome dataset. We show that depletion of CDYL alters crotonylome landscape affecting diverse cellular pathways. Specifically, CDYL negatively regulated Kcr of RPA1, and mutation of the Kcr sites of RPA1 impaired its interaction with single-stranded DNA and/or with components of resection machinery, supporting a key role of RPA1 Kcr in homologous recombination DNA repair. Together, our study indicates that protein crotonylation has important implication in various pathophysiologic processes.

INTRODUCTION

In eukaryotic cells, protein posttranslational modifications (PTMs) trigger rapid functional adaptations to various extracellular signals by modulating enzymatic activity, protein stability, interacting platform, and so on. Dysregulation of protein PTMs can lead to various pathological conditions such as developmental defects and malignant transformation. With the advancement of modern mass spectrometry (MS) technology, a group of short-chain lysine acylations including propionylation, butyrylation, 2-hydroxyisobutyrylation, succinylation, malonylation, glutarylation, crotonylation, and β-hydroxybutyrylation has been identified (1, 2). These acylations share structural similarity with the well-studied lysine acetylation. However, functional elucidation of these newly identified acylations is still in the infancy stage (2).

Histone lysine crotonylation (Kcr) was originally identified as a histone PTM that is associated with actively transcribed genes in several types of eukaryotic genomes (1). The precursor of Kcr is crotonyl–coenzyme A (CoA), a metabolic intermediate containing a four-carbon acyl chain with one double bond. The regulatory enzymes of histone Kcr have been actively investigated. It has been reported that classic histone acetyltransferases p300, pCAF, and MOF also have histone crotonyltransferase activity (3, 4), while classic histone deacetylases HDAC1/2/3 and SIRT1/2/3 are able to remove Kcr under different conditions (5, 6). Previously, we reported that chromodomain Y–like protein CDYL, a transcriptional corepressor and a reader for H3K9me2/3 and H3K27me2/3 (7–9), acts as crotonyl-CoA hydratase to negatively regulate histone Kcr (10), and we showed that this activity is intrinsically linked to its transcription repression activity and is implicated in mammalian spermatogenesis (10). Recently, several proteomics studies have expanded Kcr substrates to nonhistone proteins (11–14). However, the functional impact of protein Kcr remains to be explored, and whether CDYL regulates Kcr on nonhistone proteins is not known.

Replication protein A (RPA) is a single-stranded DNA (ssDNA)–binding protein in eukaryotic cells. Binding of RPA prevents ssDNA from winding back on itself or forming secondary structures; thus, RPA plays a key role during DNA metabolic processes such as replication, repair, and homologous recombination (HR) in meiosis. Human RPA is a heterotrimer containing RPA1, RPA2, and RPA3, in which RPA1 is responsible for ssDNA end resection during HR, and RPA1 collaborates with RAD51, BRCA2, and RAD52 to stimulate strand exchange (18, 19). Moreover, RPA1 interacts with both WRN and DNA2L, thereby stimulating WRN-mediated double-stranded DNA end unwinding and DNA2L-mediated ssDNA degradation (20). Corresponding to its multifaceted engagement in various aspects of DNA metabolism, RPA1 has been shown to be regulated by various PTMs such as acetylation, phosphorylation, ubiquitination, and SUMOylation (21–24). However, whether and how Kcr regulates the function of RPA1 are unclear.

In the current study, we used a quantitative proteomics approach to gain a global view of the crotonylome alterations in response to CDYL knockout (KO). We identified 14,311 Kcr sites across 3734 proteins in HeLa cells and generated by far the largest crotonylome dataset. We specifically characterized the functional significance of CDYL-regulated Kcr of RPA1 and demonstrated an important role of this regulation in mediating repair of camptothecin (CPT)–induced DNA damage by promoting the interaction of RPA1 with ssDNA and/or other HR factors.

†Corresponding author. Email: liang_jing@hsc.pku.edu.cn (J.L.); zhongyi_cheng@ptm-biolab.com (Z.C.)

*These authors contributed equally to this work.
†Coresponding author. Email: liang_jing@hsc.pku.edu.cn (J.L.); zhongyi_cheng@ptm-biolab.com (Z.C.)
RESULTS

Global landscape of CDYL-regulated crotonylome in mammalian cells

We previously reported that CDYL acts as a crotonyl-CoA hydratase to negatively regulate histone Kcr (10). To gain a global view of CDYL-regulated crotonylome, especially Kcr of nonhistone substrates, we used an integrated approach involving SILAC (stable isotope labeling by amino acids in cell culture) labeling, HPLC (high-performance liquid chromatography) fractionation, immunoaffinity enrichment, and high-resolution LC-MS/MS (liquid chromatography–tandem MS) to investigate Kcr substrates upon depletion of CDYL in HeLa cells (Fig. 1A). In brief, wild-type (WT) or CDYL KO HeLa cells were metabolically labeled with either the "heavy" form $^{13}$C$_6$$^{15}$N$_4$-arginine (Arg) and $^{13}$C$_6$$^{15}$N$_2$-lysine (Lys) or "light" form $^{12}$C$_6$$^{14}$N$_4$-arginine (Arg) and $^{12}$C$_6$$^{14}$N$_2$-lysine (Lys). A total of 5 x $10^8$ cells for each labeling were harvested and lysed, and the resulting protein lysates were mixed in a 1:1 ratio. After trypsin digestion, peptides were separated into 12 fractions by high-pH reversed-phase HPLC, and Kcr-containing peptides were enriched with immobilized anti-Kcr and analyzed by LC-MS/MS. A total of 14,311 Kcr sites across 3734 proteins were identified, with 14,008 Kcr sites from 3677 proteins quantified (table S1). Among these Kcr proteins, 1381 (36.9%) had a single Kcr site and 695 (18.6%) had more than six Kcr sites (Fig. 1B). Compared with earlier studies (14), our system detected significantly more Kcr sites, which were fairly overlapped with the previously reported crotonylome dataset (Fig. 1C).

To investigate whether the proteomics distribution of Kcr overlaps with other lysine acylations to any degree, Kcr sites were compared with previously determined lysine acetylation (Kac) and succinylation (Ksucc) sites in HeLa cells (25). The results showed only 254 overlapped sites among Kcr, Kac, and Ksucc, 898 overlapped sites between Kcr and Ksucc, and 716 overlapped sites between Kcr and Kac (Fig. 1D), suggesting that these three acylations have distinct functional implications. We further examined the subcellular distribution of crotonylome. Roughly equal amounts of Kcr were found in the cytoplasm and the nucleus, while about 18% of Kcr were found in the mitochondria (Fig. 1E). The distribution of Kcr is different from that of Ksucc, which is largely localized to the mitochondria (26), highlighting distinct functionalities for these two modifications.

We next examined the amino acids flanking the identified Kcr sites against all human background sequences using iceLogo (27). Evident enrichment of negatively charged glutamic acid was found at −1 and +1 positions of Kcr sites (Fig. 1, F and G). Consistently, analysis using Motif-X algorithms identified EKxxxxxE, KEKxxxK, and KxxxEK as significantly overrepresented hotspots for Kcr sites (table S2) (28). These results are in agreement with a recent crotonylome study (13). Further comparisons of the enriched motifs for Kcr to that for Kac or Ksucc indicate that these three modifications have different sequence alignments (Fig. 1, F to G, and fig. S1, A and B) (25). Structural analysis of Kcr proteins using NetSurfP showed that approximately 30% of Kcr sites were found in $\alpha$ helices, 6% were located in $\beta$ strands, and the remaining 64% were seen in disordered coils (Fig. 1H). The distribution pattern of Kcr exhibited no significant difference from that of total protein lysine residues, suggesting that there was no structural preference for Kcr, at least in HeLa cells. The average surface accessibility of protein Kcr was significantly lower ($P < 0.001$) than that of total protein lysine residues (Fig. 1H), implying that Kcr is preferably located within protein structures. Analysis of potential Kcr-regulated intracellular pathways by Gene Ontology (GO) revealed that Kcr proteins are involved in diverse biological processes including translational initiation, RNA splicing, and amino acid metabolism (Fig. 1I), and comparing these pathways to Kac- or Ksucc-regulated intracellular pathways indicates overlapped yet different biological processes (fig. S2).

Quantitative analysis of Kcr proteome in CDYL-deficient cells

We next quantified the changes of protein Kcr in response to CDYL KO relative to total protein abundance in HeLa cells. The cutoff ratio for significant Kcr changes between CDYL KO and WT cells was set to above 1.5 or below 0.67. The results showed that 1141 Kcr sites in 759 proteins were up-regulated, and 933 Kcr sites in 528 proteins were down-regulated in CDYL KO cells (Fig. 2, A and B, and table S1). KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis revealed that up-regulated Kcr proteins are mainly involved in RNA splicing, metabolism, RNA transport, and DNA replication, whereas down-regulated Kcr proteins are enriched in pathways related to endocytosis, adherens junction, and tight junction. Given that CDYL mainly acts as a crotonyl-CoA hydratase to negatively regulate Kcr, as we previously reported (10), we reasoned that up-regulated Kcr proteins are more likely to be the direct targets of CDYL (Fig. 2C and table S1).

Our previous work demonstrated that CDYL is mainly a chromatin-bound protein in the nucleus (10). We thus analyzed the chromatin-associated interaction network in CDYL-regulated Kcr proteome based on the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database. Using MCODE (Minimal Common Oncology Data Elements), a number of highly associated subnetworks of Kcr proteins were identified from CDYL-regulated Kcr proteome. Consistent with the role of CDYL in negative regulation of Kcr, Kcr levels on most of these chromatin-associated proteins, such as RPA1, POLD1, APEX1, XRCC5, and KDM1A, were up-regulated in CDYL-deficient HeLa cells (Fig. 2D). In particular, RPA1 represents one of the central nodes in the subnetworks of these Kcr proteins (Fig. 2D). Because RPA1 plays a key role in DNA metabolism processes and previous studies suggest that the function of RPA1 could be regulated by PTMs under various conditions, as stated earlier, we thus focused on RPA1 to investigate the biological consequence of CDYL-regulated Kcr.

CDYL negatively regulates Kcr of RPA1 on K88, K379, and K595

Next, RPA1, APEX1, and HP1 were selected to validate the crotonylome data. To this end, total cellular extracts from WT or CDYL KO cells were immunoprecipitated with anti-PanKcr followed by immunoblotting with antibodies against the respective proteins. The results showed that these proteins were crotonylated in HeLa cells and that their Kcr levels significantly increased upon CDYLKO, with comparable levels of total proteins (Fig. 3, A and B). Reciprocal immunoprecipitation with anti-RPA1 followed by immunoblotting with the antibody against PanKcr confirmed increased level of RPA1 Kcr upon CDYL depletion, supporting the notion that CDYL negatively regulates Kcr of RPA1 (Fig. 3C). In comparison, CDYL KO did not affect Kac of RPA1 (Fig. 3C), reinforcing the notion that CDYL specifically regulates Kcr of RPA1 but not other RPA1 modifications and consistent with the enzymatic activity of CDYL as a crotonyl-CoA hydratase. Examination of the site specificity of CDYL-regulated Kcr of RPA1 revealed a total of eight Kcr sites on RPA1.
Fig. 1. Profiling Kcr proteome in HeLa cells. (A) Schematic representation of experimental workflow for SILAC quantification of Kcr in WT and CDYL KO HeLa cells. (B) Pie chart showing the distribution of the number of identified Kcr sites per protein. (C) Venn diagram comparing the total numbers of Kcr sites in previous study and our study. (D) Venn diagram showing overlap between quantifiable Kcr, Kac, and Ksucc sites in HeLa cells. (E) Venn diagram showing cellular compartment distribution of Kcr proteins. (F) Motif analysis of all identified Kcr proteins. (G) Icelogo representation showing flanking sequence preferences for all Kcr sites. (H) Distribution of all lysines and crotonylated lysines in structured regions of proteins. (I) Bar graphs showing representative ontology annotations enriched with Kcr proteome.
Fig. 2. Quantification analysis of crotonylome in response to CDYL KO. (A) Histogram showing the ratio distribution of quantifiable Kcr sites between CDYL KO and WT HeLa cells. (B) Scatterplot showing the quantification of Kcr sites in relation to peptide intensities. (C) Bar graphs showing KEGG pathway associated with all identified and CDYL-regulated Kcr proteins. (D) Protein-protein interaction network of the chromatin-associated Kcr proteins based on the STRING database.
Fig. 3. Characterization of CDYL-regulated Kcr of RPA1. (A) The expression of the indicated proteins was measured by Western blotting in HeLa cells. (B) Immunoprecipitations in WT and CDYL KO HeLa cells with anti-PanKcr or anti–immunoglobulin G (IgG) followed by immunoblotting with antibodies against the indicated proteins. (C) Immunoprecipitations in WT and CDYL KO HeLa cells with anti-RPA1 or anti-IgG followed by immunoblotting with anti-PanKcr or anti-PanKac. (D) Schematic diagram of RPA1. (E) K88, K379, and K595 are major Kcr sites of RPA1 in vivo. Immunoprecipitation assays were performed in HeLa cells overexpressing indicated FLAG-tagged RPA1 constructs with anti-FLAG followed by immunoblotting (IB) with anti-FLAG or anti-PanKcr. (F) The specificity of antibodies against RPA1 K88cr, RPA1 K379cr, and RPA1 K595cr was verified by dot blot assays. The nitrocellulose membrane was spotted with the indicated amounts of uncrotonylated or crotonylated RPA1 peptides and immunoblotted with the indicated antibodies. (G) Verification of the specificity of anti–RPA1-K88cr, anti–RPA1-K379cr, or anti–RPA1-K595cr by Western blotting. Immunoprecipitation assays were performed in HeLa cells expressing the indicated FLAG-tagged RPA1 constructs with anti-FLAG followed by immunoblotting with the indicated antibodies, respectively. (H) The Kcr level of RPA1 is increased in CDYL KO cells. WT and CDYL KO HeLa cells were transfected with FLAG-RPA1, followed by immunoblotting with the indicated antibodies. (I) Top: Coimmunoprecipitation assays with lysates from HeLa cells expressing FLAG-CDYL using anti-FLAG followed by immunoblotting with antibodies against the indicated proteins. Bottom: Coimmunoprecipitation assays with lysates from HeLa cells expressing anti-CDYL followed by immunoblotting with antibodies against the indicated proteins. (J) Top: GST pull-down assays with GST-fused CDYL and in vitro transcribed/translated RPA1. Bottom: Coomassie brilliant blue staining of the purified GST and GST-CDYL. (K) HeLa cells were treated with UV (20 J/m²), IR (10 Gy), HU (1 mM), CPT (1 μM), VP16 (40 nM), or dimethyl sulfoxide (DMSO) for 8 hours. Western blotting was performed with the indicated antibodies. Each scale bar represents the mean ± SD for triplicate experiments. Mean data are normalized to RPA1. *P < 0.05 versus lane 1 (two-tailed unpaired Student’s t test). (L) In the presence or absence of CPT treatment, cellular extracts from WT and CDYL KO HeLa cells were immunoblotted with the indicated antibodies.
from our LC-MS/MS results. Upon CDYL KO, the levels of K88cr, K379cr, and K595cr increased 1.476-, 1.394-, and 1.582-fold, respectively, whereas the levels on other RPA1 Kcr sites did not significantly change (Fig. 3D and fig. S3). Replacing K88, K379, or K595 with arginine (R) by site-specific mutagenesis followed by immunoprecipitation assays in HeLa cells transfected with FLAG-tagged WT or point mutant of RPA1 detected decreased Kcr of RPA1 with K88R, K379R, and K595R (Fig. 3E).

To further confirm the LC-MS/MS results, we generated polyclonal antibodies specifically recognizing RPA1 K88cr, RPA1 K379cr, or RPA1 K595cr, respectively. The specificity of these antibodies was verified by dot blotting assays using corresponding peptides with or without Kcr modification (Fig. 3F). Western blotting of total cell lysates showed that both crotonylated RPA1 and unmodified RPA1 run at approximately 70 kDa (fig. S4). In addition, immunoprecipitation assays in HeLa cells transfected with FLAG-tagged WT RPA1 or point-mutant RPA1-K88R, RPA1-K379R, or RPA1-K595R confirmed that K to R mutagenesis abolished the recognition by respective RPA1 Kcr antibodies (Fig. 3G). Moreover, Western blotting revealed that the levels of all three Kcr sites on RPA1 significantly increased upon CDYL KO (Fig. 3H). Together, these observations support the notion that CDYL negatively regulates Kcr of RPA1 by targeting K88, K379, and K595.

Kcr of RPA1 is up-regulated upon DNA-damaging insults
To further characterize the biological impact of CDYL-regulated Kcr of RPA1, we first performed coimmunoprecipitation experiments to examine whether RPA1 is physically associated with CDYL. Both overexpressed FLAG-CDYL and endogenous CDYL were readily detected in HeLa cell lysates immunoprecipitated with an RPA1 antibody (Fig. 3I), supporting the physical interaction between CDYL and RPA1 in vivo. In addition, glutathione S-transferase (GST) pull-down experiments with bacterially expressed GST-CDYL and in vitro transcribed/translated RPA1 further demonstrated a direct interaction between CDYL and RPA1 (Fig. 3J).

As stated earlier, RPA1 is an ssDNA-binding protein important in DNA metabolic events such as replication and repair. To investigate whether Kcr of RPA1 plays a role in these processes, we first examined whether the levels of RPA1 Kcr could be influenced by replication stress and/or DNA damage. To this end, HeLa cells were treated with hydroxyurea (HU) to induce replication stress or treated with ultraviolet (UV), ionizing radiation (IR), cytotoxic anticancer drug etoposide (VP16), or CPT to induce DNA lesions (Fig. 3K). Replacement of RPA1 with K88R, K379R, or K595R with arginine (R) by site-specific mutagenesis followed by immunoprecipitation assays in HeLa cells transfected with FLAG-tagged WT or point mutant of RPA1 detected decreased Kcr of RPA1 with K88R, K379R, and K595R (Fig. 3L), RPA1-K595cr (Fig. 3K). Comparison of the effect of CPT on WT and CDYL KO cells revealed that while depletion of CDYL resulted in increases in Kcr levels with anti–RPA1-K88cr and anti–RPA1-K379cr, or anti–RPA1-K595cr, as expected, treatment of CPT did not induce further increase of Kcr on these three sites in CDYL KO cells (Fig. 3L), suggesting that depletion of CDYL abolished CPT-induced increase of RPA1 Kcr and supporting the importance of CDYL in regulating RPA1 Kcr dynamics in response to CPT insult.

Kcr of RPA1 enhances the interaction of RPA1 with ssDNA and/or HR factors
CPT abolishes the religation activity of topoisomerase I and generates double-strand breaks (DSBs), making it a well-established agent to induce replication fork collapse, S-phase arrest, and HR in DSB repair in tumor cells (29). To investigate the mechanism underlying the function of RPA1 Kcr in CPT-induced DSB repair, we first examined whether Kcr of RPA1 affected its ability to bind ssDNA. We generated another RPA1 mutant in which all K88, K379, and K595 were simultaneously mutated to arginine (RPA1-3KR) to mimic decrotonylated RPA1. FLAG-tagged WT or RPA1-K88R, RPA1-K379R, RPA1-K595R, or RPA1-3KR was then transfected into HeLa cells. DNA-protein binding assay using biotinylated ssDNA and lysates from these cells revealed that the ssDNA-binding affinity of RPA1 was compromised by all RPA1 mutants, with the more evident effect seen with RPA1-K379R and RPA1-K595R (Fig. 4A). We additionally used electrophoretic mobility shift assays (EMSAs) to examine whether Kcr of RPA1 affected its ssDNA-binding ability in vitro. To this end, FLAG-tagged RPA1 proteins were purified using anti-FLAG M2 affinity gel from human embryonic kidney (HEK) 293T cells transfected with WT RPA1 or individual RPA1 mutant (Fig. 4B), and the proteins were incubated with DNA substrates labeled with cyanine 3 (Cy3) dye. The results confirmed that all RPA1 mutants had a compromised binding to ssDNA, supporting a notion that Kcr of RPA1 promotes its ssDNA-binding activity (Fig. 4C). Notably, RPA1-3KR exhibited the most evident defect in ssDNA binding in EMSA experiments, while RPA1-K379R and RPA1-K595R had more evident defects in ssDNA binding in the assay using cell extracts (Fig. 4A). This discrepancy is likely due to the reaction difference in which additional RPA1-associated factors from cell lysates could influence the affinity of RPA1 toward ssDNA.

We next examined whether Kcr of RPA1 could affect its recruitment to the sites of CPT-induced DNA damage. To this end, HeLa cells were transfected with FLAG-tagged WT RPA1 or FLAG-tagged RPA1-K88R, RPA1-K379R, RPA1-K595R, or RPA1-3KR followed by treatment with CPT, fixed, and subsequently subjected to immunofluorescence staining. The results showed that all transfectants were recruited to the loci of DSB with roughly equal efficiency (Fig. 4D), arguing against a possibility that Kcr affects the recruitment of RPA1 to DNA damage sites.

We thus examined whether Kcr of RPA1 could affect its interaction with other HR factors. To this end, HeLa cells were transfected with FLAG-tagged WT RPA1 or FLAG-tagged RPA1-K88R, RPA1-K379R, RPA1-K595R, or RPA1-3KR. Lysates were collected from the transfected cells, and coimmunoprecipitation experiments were performed with anti-FLAG followed by immunoblotting with antibodies against known RPA1-associated HR factors. Compared to WT RPA1, RPA1-K88R exhibited a weakened interaction with BLM, DNA2L, MRE11, NBS1, and RAD51, with the interaction with RAD51 mostly affected, while RPA1-K379R and RPA1-K595R displayed compromised interactions with WRN, MRE11, and RAD51. The interaction of RPA1-3KR with BLM, DNA2L, WRN, MRE11, NBS1, and RAD51 was all compromised (Fig. 4E). Collectively, these results indicate that Kcr is important to the interaction of RPA1 with HR factors.
**Fig. 4.** Kcr of RPA1 affects its ability to bind ssDNA and/or HR factors. (A) Cellular extracts from HeLa cells overexpressing the indicated FLAG-tagged constructs were incubated with biotinylated ssDNA. Bound proteins were pulled down using streptavidin-coated beads and subjected to Western blot analysis with the indicated antibodies. (B) FLAG-tagged RPA1-WT, RPA1-K88R, RPA1-K379R, RPA1-K595R, or RPA1-3KR was expressed and purified with anti-FLAG M2 affinity gel from HEK293T cells and stained with Coomassie brilliant blue. (C) FLAG-tagged RPA1-WT, RPA1-K88R, RPA1-K379R, RPA1-K595R, or RPA1-3KR (0.2, 0.4, and 0.8 μM) was used for EMSA experiments using Cy3-labeled ssDNA substrates. Free ssDNA substrates and protein ssDNA were detected with a Typhoon FLA 9500 imager. (D) HeLa cells were treated with control or RPA1 siRNA (which targets 3′ untranslated region). Twenty-four hours later, cells were transfected with the indicated FLAG-tagged constructs. After 47 hours, cells were treated with 4 μM CPT or DMSO for 1 hour, fixed, and stained with anti-BLM and anti-FLAG. More than 60 cells per sample were analyzed at each independent experiment, and representative images are shown. (E) Coimmunoprecipitation assays with lysates from HeLa cells overexpressing the indicated FLAG-tagged constructs using anti-FLAG followed by immunoblotting with antibodies against the indicated proteins. Each scale bar represents the mean ± SD for triplicate experiments. Mean data are normalized to indicated proteins. *P < 0.05 versus WT (two-tailed unpaired Student’s t test).
To further explore the biological impact of Kcr-dependent interaction of RPA1 with HR factors, we first examined whether Kcr of RPA1 could affect RAD51 foci formation upon CPT insult. For this purpose, endogenous RPA1 was depleted in HeLa cells by specific small interfering RNAs (siRNAs), and RPA1 expression was reconstituted in these cells with WT or RPA1 mutants. Immunofluorescence staining showed that CPT-induced RAD51 foci formation was all reduced in cells expressing the RPA1 mutants, consistent with their weakened interaction with RAD51 (Fig. 5A).

We then determined whether Kcr of RPA1 could affect CPT-induced DSB resection, as mutation of the Kcr sites of RPA1 compromised the ability of the protein to interact with several components of the resection machinery including the MRN complex, BLM, and DNA2L. To this end, WT or RPA1 mutants were reconstituted in HeLa cells in which endogenous RPA1 was knocked down by siRNA. Using a bromodeoxyuridine (BrdU) antibody staining technique that only detects DNA in single-stranded form (20, 31), we found that CPT-triggered ssDNA generation was significantly compromised in cells reconstituted with RPA1 mutants especially with RPA1-3KR (Fig. 5B), supporting a role for RPA1 Kcr in proper function of the resection machinery upon CPT stress.

To further investigate the role of RPA1 Kcr in regulating HR repair of DSB, we performed green fluorescent protein (GFP)-based chromosomal reporter assays with DR-GFP-U2OS, a stable cell line well established to measure HR (29). RPA1 expression was reconstituted with WT RPA1, RPA1-K88R, RPA1-K379R, RPA1-K595R, or RPA1-3KR in DR-GFP-U2OS cells, in which endogenous RPA1 was knocked down by siRNA. The knockdown efficiency of endogenous RPA1 and equal expression of the transfected constructs were validated and assessed by Western blotting (Fig. 5C). Evidently, knockdown of RPA1 was associated with a significant decrease in the percentage of GFP$^+$ DR-U2OS cells, an effect that could be rescued by overexpression of siRNA-resistant WT RPA1 but not RPA1-K88R, RPA1-K379R, RPA1-K595R, or RPA1-3KR (Fig. 5C), supporting the importance of RPA1 Kcr in HR repair.

Kcr of RPA1 is important to cell survival during DNA damage

To investigate whether the involvement of RPA1 Kcr in HR repair could extend to a pathophysiologically relevant cellular response, we further examined the effect of RPA1 Kcr on cell survival and apoptosis by colony formation and flow cytometry assays, respectively. Without CPT treatment, knockdown of RPA1 in HeLa cells led to a decrease in the colony numbers, an effect that could be largely rescued by overexpression of WT RPA1, RPA1-K88R, RPA1-K379R, RPA1-K595R, or RPA1-3KR to a similar extent. In contrast, upon treatment with CPT, while WT RPA1 could still rescue the reduced colony numbers in RPA1-deficient cells, all the RPA1 mutants failed to do so (Fig. 6A). We overexpressed CDYL in HeLa cells to examine whether it could phenocopy the effect of RPA1 mutants and found that these cells also exhibited significantly increased sensitivity to CPT treatment (Fig. 6A), consistent with negative regulation of RPA1 Kcr by CDYL. We also treated cells with HU and VP16 and examined whether reconstitution of RPA1 with RPA1-K88R, RPA1-K379R, RPA1-K595R, or RPA1-3KR could rescue the viability of RPA1-depleted HeLa cells under these conditions. While cells reconstituted with RPA1-K595R and RPA1-3KR were more sensitive to HU treatment, all RPA1 mutants could sufficiently rescue cell viability upon VP16 treatment (Fig. 6A), suggesting that Kcr of RPA1 might play differential roles under different DNA-damaging conditions. Flow cytometric analysis for the effect of RPA1 Kcr on cell apoptosis with or without the treatment of CPT revealed that knockdown of RPA1 in HeLa cells caused a significant increase in CPT-induced cell apoptosis, an effect that could be rescued by overexpression of WT RPA1, but not RPA1-K88R, RPA1-K379R, RPA1-K595R, or RPA1-3KR (Fig. 6B). We noticed that cells in which endogenous RPA1 was depleted exhibited less CPT-induced apoptosis compared with cells reconstituted with the mutants, possibly due to an additive effect of compromised RPA1 function or the toxicity of defective mutant overexpression. Nevertheless, these results support the idea that Kcr of RPA1 is important to cell survival and antiapoptotic response under DNA-damaging conditions.

DISCUSSION

In the current study, we used quantitative proteomics approach to characterize global crotonylome under CDYL KO. We identified 14,311 Kcr sites in 3734 proteins, generating by far the largest crotonylome dataset (11–14). Of a total of 14,311 Kcr sites, 8430 sites (58.90%) have not been reported in mammalian cells. Therefore, our study expands the scope of Kcr proteome and provides a comprehensive cellular landscape of Kcr. We report that 1141 Kcr sites from 759 proteins increased by more than 1.5-fold and 933 Kcr sites from 528 proteins decreased by more than 0.67-fold upon CDYL KO. Considering that CDYL functions as a crotonyl-CoA hydratase to negatively regulate protein Kcr, we reasoned that the up-regulated Kcr proteins are more likely to be the direct targets of CDYL. On the basis of its hydration capacity, it is conceivable that CDYL regulates Kcr on proteins directly associated with CDYL or localized in the vicinity of this protein. In addition to RPA1, we found that several previously identified CDYL-binding partners, such as HP1, HDAC1, and MCM3 (11), are modified by Kcr and exhibit increased Kcr upon CDYL KO, although how Kcr influences the functional interplay between CDYL and these proteins is currently unknown.

Among CDYL-regulated Kcr proteins, RPA1 is an essential regulator of eukaryotic DNA metabolism. We found that CDYL negatively regulates K88cr, K379cr, and K595cr of RPA1, and we showed that Kcr levels of RPA1 elevate in responding to CPT-induced DSB. Our results suggest that K379cr and K595cr of RPA1 are more important for the ssDNA-binding ability of RPA1, possibly due to the fact that both K379 and K595 reside in the DNA-binding domain of RPA1 (Fig. 3D). Moreover, mutations of the Kcr sites of RPA1 impede the interaction of RPA1 with HR factors including BLM, DNA2L, WRN, MRE11, RAD51, and NBS1 and compromise RAD51 foci formation, ssDNA generation, and efficient HR repair during CPT-induced DSB, indicating that Kcr of RPA1 is an important regulatory means under CPT insult. Using colony formation assays, we demonstrated that overexpression of CDYL could phenocopy the effects of RPA1 mutants on the sensitivity to CPT treatment, consistent with negative regulation of RPA1 Kcr by CDYL. However, we are aware that the role of CDYL during CPT-induced DSB is probably more complex. We demonstrated previously that CDYL is required for HR-mediated DNA repair by coordinating with histone chaperone CAF-1 and DNA helicase minichromosome maintenance (MCM) for proper chromatin structure at damage sites (29). Dr. Ayoub’s group has also demonstrated that CDYL promotes homology-directed repair of DSBs using ‘traffic-light reporter’ system (30). In addition, overexpression of CDYL in cells
Fig. 5. Kcr of RPA1 enhances the function of the resection machinery in HR repair. (A) HeLa cells were treated with RPA1 siRNA for 24 hours before cells were transfected with the indicated FLAG-tagged constructs. After 47 hours, cells were treated with 4 μM CPT or DMSO for 1 hour, fixed, and stained with anti-RAD51 and anti-FLAG. Only nuclei showing more than five colocalized foci were considered as positive. More than 60 cells per sample were analyzed at each independent experiment. Error bars indicate ±SD for the results of triplicate assays. ***P < 0.001 (two-tailed unpaired Student's t test). (B) HeLa cells were transfected and treated with DMSO or CPT as in (A). Cells were then fixed and stained with anti-BrdU and anti-FLAG. BrdU (20 μM) was added to the medium 15 min before CPT treatment. Error bars indicate ±SD for the results of triplicate assays. **P < 0.01 and ***P < 0.001 (two-tailed unpaired Student’s t test). ns, not significant. (C) DR-GFP-U2OS cells were transfected with control or RPA1 siRNA as indicated. Twenty-four hours later, cells were transfected with HA-I–Sce I expression plasmids together with pcDNA3.1-3×FLAG-C vector or FLAG-tagged RPA1 constructs for 48 hours before they were collected and analyzed by fluorescence-activated cell sorting. Data represent the mean ± SD for triplicate experiments. ***P < 0.001 (two-tailed unpaired Student’s t test).
could affect Kcr of a panel of proteins including Kcr of histones. Nevertheless, it is increasingly clear that CDYL plays an important role in HR DNA repair.

On the basis of distinct dynamics of Kcr of RPA1 (Fig. 3K) and different ability of RPA1 mutants to rescue cell viability under different DNA-damaging insults (Fig. 6A), we propose that Kcr of RPA1 plays differential roles in different repair pathways. Given our observations that all RPA1 mutants exhibit a compromised ssDNA-binding activity and affect the interaction of RPA1 with other DNA repair regulators, it is possible that the functional defects
of these mutants are moderate and/or are compensated by other factors under normal conditions or upon VP16 treatment, whereas Kcr of RPA1 is more critical for cells in response to CPT and HU. Further investigations are warranted to delineate the role of RPA1 Kcr under different cellular environments.

As stated earlier, it has been reported that RPA1 is regulated by various PTMs including phosphorylation, SUMOylation, ubiquitination, and acetylation (21–24). For example, RPA1 is ubiquitinated at multiple sites outside its DNA-binding channel, and ubiquitination of RPA1 plays a signaling role upon replication fork stalling rather than targets the protein for proteasomal degradation (24). RPA1 has also been reported to undergo SUMOylation at K449 and K577, thereby facilitating the recruitment of RAD51 to DNA damage foci to initiate DNA repair through HR (21). In addition, recent studies indicate that RPA1 is acetylated by PCAF/GCN5 at K163, and this modification functions to stabilize the RPA1/XPA complex during nucleotide excision repair (22, 23). We found that K88cr, K379cr, and K595cr of RPA1 are involved in HR repair during CPT-induced DSBs. Although our study indicates that CDYL influences Kcr of RPA1 on these sites, we do not exclude the possibility that RPA1 Kcr could be regulated by other factors such as crotonyl transferases and/or deoctonases, which could also contribute to the dynamics of RPA1 Kcr upon CPT treatment. Intriguingly, we detected moderately reduced levels of CDYL upon CPT treatment (Fig. 3K), which could be one of the reasons for the increased RPA1 Kcr under this condition. However, no detectable reduction of CDYL was found upon other treatments, underpinning the complexity of the regulation of RPA1 Kcr under DNA-damaging insults. K88, K379, and K595 of RPA1 have also been previously reported to be ubiquitinated (K595 is also SUMOylated) by proteomics studies (24, 32–35), although the functional impact of these ubiquitinations has not been characterized. Because our observations regarding the function of Kcr on these sites are mainly derived from K to R mutagenesis, we thus do not exclude the possibility that other modifications of these sites also play a role during CPT-induced HR repair. Characterization of the crotonyltransferase that targets RPA1 in response to DNA damage and deciphering its mechanistic action, such as mapping the interaction domain with RPA1 and creating RPA1 mutants that cannot interact with its crotonyltransferase, would help to better clarify the function of Kcr of RPA1 on these sites. How multiple PTMs coordinately regulate the function of RPA1 in different DNA metabolic events remains to be further investigated.

The precursor of Kcr is crotonyl-CoA, a metabolic intermediate often generated from fatty acid oxidation. Undoubtedly, a close functional connection between cell metabolism and regulation of protein Kcr in various biological contexts can be envisioned. In line with this, a recent study reported that short-chain fatty acid metabolites and their corresponding CoAs generated by gut microbes promote histone Kcr and affect gene transcription in host colons (6). Kcr proteins identified in our current study have been implicated in multiple biological processes such as RNA splicing, metabolic pathways, and RNA transport. In particular, we found that multiple factors that are involved in fatty acid β-oxidation and ketogenesis, such as ACOX1 and ACAT1, are subjected to CDYL regulation, suggesting potential feedback loops between cellular pools of acetyl-CoA/short-chain acyl-CoA and protein Kcr in metabolic pathways. It is important to clarify the detailed regulatory mechanisms in this aspect in future investigations.

In summary, our study is the first systematic analysis of Kcr substrates upon CDYL KO and provides by far the largest proteo-nylome dataset in mammalian cells. Further investigations of the functions of protein Kcr in diverse cellular pathways will deepen our understanding of the complex protein PTM code and provide clues for future drug development to combat diseases including cancer.

**MATERIALS AND METHODS**

**Antibodies and reagents**

Commercial antibodies used were as follows: anti-FLAG (Sigma), anti-CDYL (Sigma, HPA035578; for Western blotting), anti-CDYL (Abcam, ab5188), anti-BrdU (Abcam, ab6326), anti-RPA1 (Abclonal, A0990), anti-RAD51 (Abclonal, A6268), anti-WRN (Abclonal, A6855), anti-DNA2L (Abcam, ab2179), anti-BLM (Abcam, ab96488), anti-MRE11 (Cell Signaling Technology, 4847), anti-NBS1 (Cell Signaling Technology, 14956), anti-RAD50 (Cell Signaling Technology, 3427), anti-Myc, anti-hemagglutinin (HA), anti-β-actin (MBL), anti-PanKac (PTM, 101), anti-PanKcr (PTM, 502; for Western blotting), and anti-PanKcr–conjugated agarose beads (PTM, 503; for immunoprecipitation). A CDYL antibody that we generated previously (peptide antigen: KQKESTLRTNRTSPNN; B&M) was used for immunoprecipitation. The polyclonal antibodies against RPA1 K88cr, RPA1 K379cr, and RPA1 K595cr were generated by immunizing rabbits with two synthetic crotonyl peptides corresponding to residues surrounding K88, K379, and K595 of RPA1, respectively. HU, CPT, and VP16 were from Sigma. Streptavidin agarose resins were from Thermo Fisher Scientific. Protein A/G and Sepharose CL-4B beads were from GE Healthcare Biosciences, and protease inhibitor mixture cocktail was from Roche Applied Science.

**Plasmids and siRNAs**

The complementary DNA (cDNA) for WT RPA1 was amplified by polymerase chain reaction and ligated into pcDNA3.1(−) plasmid containing 3xFLAG tag. RPA1 mutants including K88R, K379R, K595R, and 3KR were generated using the QuikChange Lightning Site-Directed Mutagenesis Kit. The siRNAs were purchased from the GenePharma Company of Shanghai. The target sequences were as follows: RPA1 siRNA, 5′-UUAUCAUCAAGCAGGAAUUAU-3′; CDYL siRNA, 5′-CAGAGAAUAACUCACUAATT-3′.

**Cell culture and transfection**

HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (HyClone) supplemented with 10% fetal bovine serum (FBS). All transfections were carried out using Lipofectamine 2000 (Invitrogen) with the final concentration at 25 nM.

**SilAC labeling**

SilAC labeling and quantitative proteomics analysis were performed as previously described (36). Briefly, WT or CDYL KO HeLa cells were grown in DMEM supplemented with 10% FBS and either heavy form [13C6] 15N4-arginine (Arg10) and [13C6]14N2-lysine (Lys5) or light form [13C6] 15N4-arginine (Arg10) and [12C6]14N2-lysine (Lys5), respectively. The cells were grown for more than 10 generations before being harvested to achieve more than 97% labeling efficiency. After that, the cells were further expanded in SILAC medium to desired cell number (5 × 107) in 15 mm–by–150 mm plates.
Nuclear extraction
Cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS) before they were lysed in NETN buffer (100 mM NaCl, 50 mM tris-HCl, 1 mM EDTA, 0.5% NP-40) supplemented with protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Calbiochem) and histone deacetylase (HDAC) inhibitor [50 mM nicotinamide (NAM) and 3 μM trichostatin A (TSA)] on ice for 30 min followed by centrifugation at 1000g for 10 min at 4°C. The supernatant was discarded, and the pellet was collected as nuclear fraction samples.

Protein extraction and trypsin digestion
Samples were sonicated three times on ice using a high-intensity ultrasonic processor (Scientz) in lysis buffer (8 M urea, 1% protease inhibitor cocktail, 3 μM TSA, and 50 mM NAM). The remaining debris was removed by centrifugation at 12,000g at 4°C for 10 min. The supernatant was then collected, and protein concentration was determined with a BCA kit according to the manufacturer’s instructions. For digestion, the protein solution was reduced with 5 mM dithiothreitol (DTT) for 30 min at 56°C and alkylated with 11 mM iodoacetamide for 15 min at room temperature in darkness. The protein sample was then diluted by adding 100 mM triethylammonium bicarbonate (TEAB) to urea concentration of less than 2 M. Last, trypsin was added at 1:50 trypsin-to-protein mass ratio for the first overnight digestion and 1:100 trypsin-to-protein mass ratio for the second 4-hour digestion.

HPLC fractionation
The tryptic peptides were fractionated into fractions by high-pH reversed-phase HPLC using a Thermo Betasil C18 column (5-μm particles, 10 mm ID, 250 mm length). Briefly, peptides were first separated with a gradient of 8 to 32% acetonitrile (pH 9.0) over 60 min into 60 fractions. Then, the peptides were combined into 10 fractions and dried by vacuum centrifugation.

Kcr peptide enrichment
Tryptic peptides were dissolved in NETN buffer [100 mM NaCl, 1 mM EDTA, 50 mM tris-HCl, 0.5% NP-40 (pH 8.0)] and then incubated with antibody beads (PTM Bio Inc., Hangzhou) at a ratio of 15-μl beads per milligram of protein at 4°C overnight. The antibody beads were washed four times with NETN buffer and twice with double-distilled H2O. The Kcr peptides were then eluted by adding elution buffer with 0.1% trifluoroacetic acid. The eluted peptides were cleaned with C18 ZipTips (Millipore) according to the manufacturer’s instructions before being subjected to LC-MS/MS analysis.

LC-MS/MS analysis
The tryptic peptides were dissolved in 0.1% formic acid (solvent A) and directly loaded onto a homemade reversed-phase analytical column (15 cm length, 75 μm ID). The gradient was composed of an increase from 6 to 23% solvent B (0.1% formic acid in 98% acetonitrile) over 26 min, 23 to 35% in 8 min, and climbing to 80% in 3 min and then holding at 80% for the last 3 min, all at a constant flow rate of 400 nl/min on an EASY-nLC 1000 ultra performance liquid chromatography (UPLC) system. The peptides were subjected to nanospray ionization (NSI) source followed by MS/MS in Q Exactive Plus (Thermo Fisher Scientific) coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. The m/z (mass/charge ratio) scan range was 350 to 1800 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS using normalized collision energy (NCE) setting as 28, and the fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure alternated between one MS scan followed by 20 MS/MS scans with 15.0-s dynamic exclusion. Automatic gain control was set at 5 × 10^4. Fixed first mass was set to 100 m/z.

Database searching and protein quantification
The resulting MS/MS data were processed using MaxQuant with integrated Andromeda search engine (v.1.5.2.8). Tandem mass spectra were searched against human Swiss-Prot database (20,203 sequences) concatenated with reverse decoy database. Trypsin/P was specified as cleavage enzyme allowing up to four missing cleavages. The mass tolerance for precursor ions was set to 20 parts per million in first search and 5 parts per million in main search, and the mass tolerance for fragment ions was set to 0.02 Da. Carbamidomethyl on Cys was specified as fixed modification, and Kcr modification and oxidation on Met were specified as variable modifications. False discovery rate (FDR) was adjusted to <1%, and minimum score for modified peptides was set to >40.

Bioinformatics analysis
Motif-X software (http://motif-x.med.harvard.edu/) was used to analyze the model of sequences constituted with amino acids in specific positions of Kcr-21-mers (10 amino acids upstream and downstream of the site) in all protein sequences (28), the minimal number of peptide occurring in one motif “occurrences” was set to 20, and the motif analysis statistics test significance threshold value was set to 0.0000001. The amino acid sequence heat map is a two-dimensional data matrix where every row is an amino acid and every column is a position. One cell in the heat map matrix is colored according to the log10 P value for that position and amino acid, which was calculated using the Fisher’s exact test method. The iceLogo (https://iomics.ugent.be/icelogoserver/) was used to examine the properties of amino acids surrounding the modification sites using t test with P < 0.05, and the “choosing scoring system” was set as “percentage difference.” Secondary structures were predicted using NetSurfP. GO term and KEGG pathway enrichment were performed using DAVID 6.8 (37, 38). STRING database (http://string-db.org/) was used for protein-protein interaction (PPI) analysis. Cytoscape (version 3.0) software was used to display the network (39).

Immunoprecipitation and Western blotting
Briefly, cellular lysates were prepared by incubating the cells in lysis buffer [50 mM tris-HCl, 150 mM NaCl, 0.5% NP-40, and 2 mM EDTA (pH 7.5)] or BCI100 buffer [20 mM tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 0.5% Triton] containing protease inhibitor cocktail for 20 min at 4°C, followed by centrifugation at 14,000g for 15 min at 4°C. Protein concentration of the lysates was determined with a BCA kit according to the manufacturer’s protocol. Overall, 5% (1:20) cellular extracts were used for input. For immunoprecipitation, 500 μg of protein lysates was incubated with 2 μg of specific antibodies for 12 hours at 4°C with constant rotation; 60 μl of 50% protein A or G agarose beads was then added, and the incubation was continued for an additional 2 hours. Beads were then washed five times using the lysis buffer. Between washes, the beads were collected by centrifugation at 500g for 5 min at
4°C. The precipitated proteins were eluted from the beads by resuspending the beads in 2× SDS–polyacrylamide gel electrophoresis (PAGE) loading buffer and boiling for 10 min. The resultant materials from immunoprecipitation or cell lysates were resolved using 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. For Western blotting, membranes were incubated with appropriate antibodies for 1 hour at room temperature or overnight at 4°C followed by incubation with a secondary antibody. Immunoreactive bands were visualized using a Western blotting Luminol reagent (Santa Cruz Biotechnology) according to the manufacturer’s recommendation (40).

**Purification of recombinant proteins**

GST fusion constructs were expressed in BL21 cells, and crude bacterial lysates were prepared by sonication in TEDGN [50 mM tris- HCl (pH 7.4), 1.5 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, and 0.4 M NaCl] in the presence of the protease inhibitor cocktail. Recombinant GST-fused proteins used in pull-down assays were affinity-purified with glutathione Sepharose 4B according to the manufacturer’s instructions (GE Healthcare, New England Biolabs). For EMSA, HEK293T cells expressing full-length FLAG-tagged RPA1-WT, RPA1-K88R, RPA1-K379R, RPA1-K595R, and RPA1-3KR were collected and lysed in lysis buffer [50 mM tris-HCl (pH 7.4), 500 mM NaCl, 1% NP-40, 1 mM EDTA, 10% (v/v) glycerol, and 1 mM DTT] supplemented with protease inhibitor cocktail. The resulting lysate was incubated with anti-FLAG M2 affinity gel for 2 hours, and the beads were washed five times with lysis buffer. The immobilized proteins were eluted with 3×FLAG peptide and used in EMSAs as described below or resolved on SDS-PAGE followed by Coomassie brilliant blue staining.

**GST pull-down assay**

CDYL was fused to GST, expressed in bacteria, and purified using standard protocols. In vitro transcription and translation experiments were done with rabbit reticulocyte lysate (TNT Systems, Promega) according to the manufacturer’s recommendation. The glutathione beads were incubated with the GST-CDYL and in vitro transcribed/translated RPA1 in the buffer containing 10 mM NaHepes (pH 7.5), 150 mM NaCl, 0.005% Tween 20, and 2 mM DTT and then were washed in the buffers containing 10 mM NaHepes (pH 7.5), 150 to 300 mM KCl, 0.5 to 1.0% Tween 20, and 2 mM DTT. Unless stated elsewhere, 20% of the total protein was used as input.

**Detection of ssDNA**

BrdU (20 μM) was added to the medium for 15 min and was removed by a brief wash before CPT treatment (4 μM, 1 hour), preextraction, and fixation. BrdU in ssDNA patches was detected as described below without a DNA denaturation step (31).

**Immunofluorescence staining and confocal microscopy**

HeLa cells growing on six-well chamber slides were washed once with PBS, extracted with CSK buffer [10 mM Pipes (pH 7.0), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, and 0.5% Triton X-100] for 2 min, washed again with cold PBS, and fixed in 4% (w/v) paraformaldehyde for 10 min. Cells were permeabilized with 0.2% Triton X-100 in PBS, blocked with 0.8% bovine serum albumin (BSA), and incubated with appropriate primary antibodies coupled to Alexa Fluor 488 (Jackson ImmunoResearch; mouse, 115-585-003, 1:100), Alexa Fluor 488 (Jackson ImmunoResearch; mouse, 115-545-003, 1:100), or Alexa Fluor 555 (Life Technologies; rat, A21434, 1:100, for BrdU staining). Cells were then washed for four times, and a final concentration of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (0.1 μg/ml; Sigma) was included in the final wash to stain nuclei. Confocal laser scanning microscopy images were obtained using a Zeiss LSM 510 microscope equipped with an argon laser (365 nm, 20-Hz pulse). The images were captured and analyzed using Adobe Photoshop CS4.

**DNA-protein binding assay**

The ssDNA [30 nucleotides (nt), *5′-ACGCTGCCAATTCTACTACGTGCCTTGCTA] was labeled at the 5′ end with biotin. HeLa cells transiently transfected with plasmids encoding FLAG-tagged WT RPA1 or RPA1 mutants were lysed in binding buffer [10 mM tris-HCl (pH 7.5), 100 mM NaCl, BSA (10 μg/ml), 10% glycerol, 0.5% NP-40]. Biotinylated ssDNA was incubated with the lysates for 30 min at room temperature. Streptavidin beads were then added followed by further incubation with the lysates for 1 hour at room temperature. The samples were subjected to SDS-PAGE and analyzed by Western blotting.

**Electrophoretic mobility shift assay**

The DNA substrates (ssDNA-1, 30 nt, *5′-ACGCTGCCAATTCTACTACGTGCCTTGCTA; ssDNA-2, 30 nt, *5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT[DNA]) were labeled at the 5′ end with Cy3. The reaction was performed in a binding buffer [25 mM tris-HCl (pH 7.5), 100 mM NaCl, 5 mM MgCl2, 1 mM DTT, BSA (100 μg/ml), 5% glycerol, 0.05% Triton X-100] with the respective ssDNA substrate (5 nM) for 15 min at room temperature. Loading dye (50% glycerol) was added, and the reaction mixtures were separated by 1% agarose gel in TAE buffer [40 mM tris-HCl (pH 7.5) and 1 mM EDTA] at 90 V for 70 min. The electrophoresis was carried out in a gel tank surrounded by ice. Cy3-labeled DNA was visualized using Typhoon FLA 9500 (41).

**Colony formation assay**

HeLa cells were treated with control siRNA or specific siRNA targeting RPA1 3′ untranslated region 24 hours before cells were transfected with the indicated FLAG-tagged expression constructs. Forty hours after transfection, cells were treated with or without 1 μM CPT for 8 hours. Same numbers of cells for each group were seeded into six-well plate, and the above treatment was repeated twice (every 2 days). Cells were allowed to grow into colonies for a total of 14 days, fixed with 4% (w/v) paraformaldehyde for 10 min, and stained with 0.5% crystal violet for 20 min. The number of colonies per well was counted, and the planting efficiency and surviving fraction for given treatments were calculated on the basis of the survival rates of control cells. Each experiment was performed in triplicate and repeated at least three times.

**Flow cytometry**

For measurement of repair efficiency, DR-GFP-U2OS cells were trypsinized, washed with PBS, collected with FACSCalibur, and analyzed by FlowJo. For apoptosis assays, HeLa cells were processed using Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) according to the manufacturer’s instructions and analyzed using a FACSCalibur flow cytometer.
Statistical analysis
Group data were analyzed by two-tailed unpaired Student’s t test (GraphPad Prism software, version 5.01) and expressed as means ± SD unless otherwise indicated. P < 0.05 was considered to be statistically significant.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/11/eaay4697/DC1

Fig. S1. Sequence logo analysis of Kac and Ksucc peptides.
Fig. S2. Pathway analysis of Kac and Ksucc proteins.
Fig. S3. Full MS and MS/MS spectra for K379cr, K88cr, and K595cr of RPA1.
Fig. S4. Full Western blots of HeLa cell lysates using RPA1-K88cr, RPA1-K379cr, RPA1-K595cr, or RPA1 antibodies.
Fig. S5. Change of RPA1 Kcr under different DNA-damaging insults.
Table S1. Complete list of Kcr sites identified in WT and CDYL KO HeLa cells.
Table S2. Motif analysis of Kcr, Kac, and Ksucc peptides.

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**Competing interests:** Z.C. is a co-founder and the chief executive officer of PTM Bio Inc. The other authors declare that they have no competing interests. **Data and materials availability:** The MS proteomics data including the annotated mass spectra have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org/cgi/GetDataset) via the PRIDE partner repository with the dataset identifier PXD013616 and will be available publicly upon acceptance. All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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