A fluorescent bimolecular complementation screen reveals MAF1, RNF7 and SETD3 as PCNA-associated proteins in human cells

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The proliferating cell nuclear antigen (PCNA) is a conserved component of DNA replication factories, and interactions with PCNA mediate the recruitment of many essential DNA replication enzymes to these sites of DNA synthesis. A complete description of the structure and composition of these factories remains elusive, and a better knowledge of them will improve our understanding of how the maintenance of genome and epigenetic stability is achieved. To fully characterize the set of proteins that interact with PCNA we developed a bimolecular fluorescence complementation (BiFC) screen for PCNA-interactors in human cells. This 2-hybrid type screen for interactors from a human cDNA library is rapid and efficient. The fluorescent read-out for protein interaction enables facile selection of interacting clones, and we combined this with next generation sequencing to identify the cDNAs encoding the interacting proteins. This method was able to reproducibly identify previously characterized PCNA-interactors but importantly also identified RNF7, Maf1 and SetD3 as PCNA-interacting proteins. We validated these interactions by co-immunoprecipitation from human cell extracts and by interaction analyses using recombinant proteins. These results show that the BiFC screen is a valuable method for the identification of protein-protein interactions in living mammalian cells. This approach has potentially wide application as it is high throughput and readily automated. We suggest that, given this interaction with PCNA, Maf1, RNF7, and SetD3 are potentially involved in DNA replication, DNA repair, or associated processes.

Introduction

The process of chromosomal replication is a complex one. Not only must the DNA sequence be completely copied, and any mistakes produced during the copying process accurately rectified, but the associated chromatin structures must also be properly reproduced to generate 2 daughter cells containing the same information as the parent cell. Failures in these copying processes result in the inheritance of altered genetic and epigenetic information, which can lead to cell death, or the development of cancer. While the enzymatic requirements for the accurate copying of genetic information are well understood, the mechanisms that exist to control and coordinate the other events of chromosomal replication are less well defined.1

Proliferating cell nuclear antigen (PCNA) is a conserved DNA sliding clamp protein essential for DNA replication in eukaryotic cells. This small (29kDa monomer size in humans) protein forms a homotrimeric toroidal structure which encircles the DNA at the replication fork. Its capacity to translocate over the newly synthesized duplex means that it can act as a sliding recruitment protein; proteins which bind to PCNA are brought into the vicinity of the active replication fork and the nascent DNA strands. Thus, although it possesses no enzymatic activity, PCNA is used to concentrate the enzymes of DNA replication at their sites of action and modulate their activity.2-4

Many proteins have been convincingly shown to interact with PCNA. These include the DNA polymerases epsilon and delta (PolE and PolD), required for DNA synthesis;5-8 flap endonuclease 1 (Fen1) and ligase I (Lig1) for Okazaki fragment processing9,10; Mut S homologs 3 (MSH3) and 6 (MSH6) for mismatch repair11,12; chromatin assembly factor 1 (Caf-1)13; and DNA methyltransferase 1 (DNMT1) for epigenetic inheritance,14 among many others. Thus PCNA is central to many of the processes that must occur in a coordinated way as chromosomal replication proceeds.15 PCNA also interacts with additional partners involved in more specialized pathways. As examples, PCNA also regulates translesion DNA synthesis via interaction with polymerase eta (PolH)16-18; cell cycle arrest via p2119-21; S-phase specific protein degradation via the Cullin 4 (Cul4)/DNA damage binding protein 1 (DDB1) ubiquitin

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ligase,22–24 nucleotide excision repair via xeroderma pigmento-
sum proteins A and G (XPA/XPG),25,26 and base excision repair
via apurinic/apyrimidinic endonuclease 1 (Ape1),27 uracil DNA
glycosylase (UNG2)28 and 3-methyladenine-DNA glycosylase
(MPG)29; and this list is by no means exhaustive.

For the most part, interactions between PCNA and its protein
partners have been identified on a case-by-case basis. Many inter-
actions were initially identified by immunoprecipitation or in
vitro binding of recombinant proteins, and it was subsequently
noticed that PCNA-interacting proteins often contain the PCNA
interacting protein (PIP) motif.30,31 This is a small peptide motif
with consensus sequence Q-x-x-[LIM]-x-x-[FY]-[FY], derivatives
of which are found in PolD3, Fen1, Lig1, MSH3, MSH6, Caf-1,
DNMT1, PolH, p21, XPG, Ape1, UNG2 and MPG. Crystal
structures of PCNA with interacting proteins or peptides have
demonstrated that this motif is a direct binding surface, interact-
ing with the inter-domain connecting loop of PCNA in a mainly
hydrophobic manner.32 Proteins which contain such a motif on
a solvent-exposed surface are therefore good candidates for
PCNA interactors. However, there are some characterized
PCNA-interacting proteins that do not contain such a motif. As
examples, the catalytic subunit of PolD likely binds PCNA via a
“KA-box”,33 and the NER protein XPA uses a so-called APIM
(AlkB homolog 2 PCNA interacting motif) for its PCNA interac-
tion.34 All these motifs are degenerate and short, thus a bioinfor-
matics-based search of the human proteome is unlikely to
identify specifically all the true PCNA-interactors. Given the
importance of PCNA in regulating the processes that ensure
genome and epigenome stability through replication, it has been
previously noted that a full characterization of the PCNA-inter-
actome would be desirable.4,35

We developed an in-cell screening approach to identify PCNA
interaction partners. The format of our screen will allow it to
report on interactions that happen in the context of active repli-
cation sites, even those that are DNA-dependent or transient,
interactions that could be missed in a purification-based strategy.
We based our screen on bimolecular fluorescence complementa-
tion (BiFC), the process whereby 2 fragments of a fluorescent
protein, individually non-fluorescent, can combine to give a fluo-
rescent species when brought into close proximity by the interac-
tion of “bait” and “prey” proteins (Fig. 1A).36,37 A similar
system has previously been used in the identification of proteins
that interact with the protein kinase PKB/Akt.38 Here, we com-
bined this strategy with fluorescent activated cell sorting (FACS)
and next generation sequencing to develop a novel format for
screening for protein interactions in real time in living mamma-
lian cells.

Results

To identify novel PCNA interacting proteins in human cells
we used a bimolecular complementation (BiFC) approach with a
PCNA “bait” (Fig. 1A). This comprises the full length PCNA
open reading frame with the C-terminal portion of Venus fluo-
rescent protein39 (CTV: amino acids 159–238) fused to its C-
terminus. The split point of Venus was selected at between amino
acids 158 and 159 after Remy et al.38 The construct also contains
a linker sequence to minimise potential perturbations to PCNA
folding,40 a nuclear localization signal (NLS) to ensure appropri-
cate cellular location and a FLAG epitope for detection. Using
indirect immunofluorescence after transient transfection in
MRC5 cells we showed that the PCNA_CTV is recruited to focal
sites of DNA replication (so-called replication factories41,42)
where it co-localized with EdU incorporation in a triton-resistant
manner (Fig. 1B). This suggests that the bait construct is loaded
onto chromatin in a manner reminiscent of endogenous PCNA
demonstrating that this tagged version of PCNA can recapitulate
the essential cellular activity of PCNA.

We generated a cell line derived from HEK293 cells that sta-
bles expresses this construct from the CMV promoter. Western
blotting of total cell extracts from control and bait cells using a
polyclonal antibody against PCNA showed that the construct is
expressed at levels well below that of endogenous PCNA
(Fig. 1C - inputs), and immunoprecipitation of the
PCNA_CTV from cell extracts using the anti-FLAG monoclonal
antibody co-precipitated endogenous PCNA
(Fig. 1C - antis). Thus, the tagged bait is able to associate normally with endogenous
PCNA to form mixed trimers, implying that its function is
unlikely to be dramatically impaired.

To validate and optimise the BiFC screen parameters we con-
structed positive and negative control “prey” constructs based on
pCEP4, an episomally maintained plasmid. They express the tar-
get prey with the N-terminal part of Venus (NTV: amino acids
1–158) fused to the N-terminus (Fig. 1A). The constructs also
contain an NLS, linker sequence and the HA epitope. Expression
is driven by the CMV promoter and selection is by hygromycin
resistance. As a positive control we fused the N-terminal part of
Venus to the Fen1 open reading frame. The interaction between
Fen1 and PCNA is well documented, occurs at replication facto-
ries and is mediated by the PIP box motif of Fen1.39,43 Mutation
of the Fen1 PIP-box abolishes its interaction with PCNA44,45 so
we used a mutated negative control version (L340A, F343A, F344A:
PIP*) was also generated. We transiently transfected
PCNA_CTV (bait) expressing cells with these NTV_Fen1 con-
structs. The wild type (WT) NTV_Fen1 transfection generated
yellow fluorescent signal as a result of bimolecular fluorescence
complementation (BiFC) mediated by the interaction between
PCNA_CTV and NTV_Fen1 (Fig. 2A). Importantly, far less
yellow fluorescence was detected in cells transfected with either
the empty prey vector, or the mutated NTV_Fen1 PIP* con-
struct that is unable to interact with PCNA (Fig. 2A). This shows
that the BiFC signal is a sensitive and accurate reporter of biolog-
ically relevant protein associations.

To verify that we could isolate cells by flow sorting on the
basis of these differences in BiFC signal we transfected bait
PCNA_CTV cells with the empty vector or one of the 2
NTV_Fen1 prey plasmids, and analyzed the cells on a MoFlo
fluorescence cytometer after 24 hours (Fig. 2B). Untransfected
cells show only endogenous levels of yellow auto-fluorescence
(none are brighter than a threshold set at 108 units). Transfection
with empty vector or NTV_Fen1 PIP* constructs do result in
low amounts of yellow fluorescence: 3.4% and 4.7% cells, respectively. This represents the cumulative, spontaneous refolding of Venus in the absence of targeted association between fusion partners. However, transfection with NTV_Fen1 WT, which is able to interact with PCNA and mediate specific BiFC resulted in significantly increased yellow fluorescence, with 9.9% cells demonstrating bright yellow fluorescence. Thus the BiFC methodology can be combined with cell sorting to enrich cells with specific BiFC signal.

In order to use this method to characterize the PCNA interactome we converted a human cDNA library into a prey library using Gateway™ mediated recombination. This library was transfected into $5 \times 10^6$ bait PCNA_CTV cells, and cells were analyzed on a MoFlo cytometer for yellow fluorescence after 24 hours. At this time cells with an intensity of greater than 108 were taken as positive for interaction, these comprised 3.8% of the total NTV_Fen1 transfected cells and 0.6% of the cells transfected with the library (Fig. 3A). In total 13000 BiFC positive cells were isolated. These were returned to culture, 65000 unsorted but library transfected cells were also returned to culture to act as a control. After 24 hours hygromycin was added to select for cells that had received prey plasmids. The cells were grown under selection for 4 weeks. After this time FACS analysis confirmed that a substantial proportion of the sorted cells were still exhibiting BiFC yellow fluorescence: 38.2% in the control NTV_Fen1-transfected and 10.1% in the cDNA library-transfected population (Fig. 3A). After further expansion, DNA was extracted from the library-transfected, sorted population and the unsorted control. These DNA preparations were used as templates for PCR-mediated amplification of the library sequences present using plasmid-specific probes. 500ng of these PCR products were used to generate a 454 sequencing library, which was analyzed in multiplex format on the FLX junior 454 platform. A total of 66694 and 32135 reads was obtained for sorted...
and control libraries respectively. After trimming of vector sequences and removal of genomic contaminants these reads aligned to 501 and 504 unique cDNAs in the sorted and control screens.

This depth of sequencing enabled identification of the most abundant cDNAs in the starting library, which are most likely to contribute false positive results in the screen. To take this bias into account when analyzing the data we looked specifically at those cDNAs which were rare or not present in the unsorted control dataset, but which were abundant in the sorted screen. To do this in an unbiased way we ranked the cDNAs identified in each data set in order of their abundance. We then calculated the change in rank for each between the library control and the screen enriched populations. A positive change in rank demonstrates a relative enrichment by the screen procedure, which is likely to be the result of true positive interaction. Indeed, using this approach the known PCNA interactors PolD2 and Fen1, were identified by the screen. All cDNAs with a change in rank of greater than 50 places are provided in supplementary Table 1.

We performed a gene ontology (GO) based analysis using the DAVID algorithm46 to determine whether any particular biological pathways or functions were over-represented in the screen. We found however that the gene groups represented in the screen hits corresponded to ribosomal proteins, followed by proteins of oxidative metabolism, and actin and myosin related groups. These pathways were also over-represented in the library control dataset. We therefore concluded that GO analysis was of limited use in this case due to the biased nature of the input material. To validate the screen, we selected 4 cDNAs that were enriched in this PCNA interaction screen, but not previously reported to interact with PCNA, for further study: those encoding LMO4, Maf1, RNF7 and SetD3. These candidates were manually selected because database searches suggested that they are predicted to be nuclear proteins with potentially interesting roles not limited to skeletal muscle and additionally, their size should permit expression in <i>E. coli</i>, facilitating initial study.

To confirm that the presence of these cDNAs in the sorted library is a result of a PCNA interaction we cloned the full length cDNAs of LMO4, MAF1, RNF7 and SETD3 into the BiFC prey vector and transfected them independently into bait cells. These were analyzed for yellow fluorescence on a confocal microscope (Fig. 3B). MAF1, RNF7 and SETD3 constructs all generated yellow fluorescence resulting from BiFC, showing that these proteins can associate with PCNA. On the other hand, expression of a full-length LMO4 prey construct did not generate detectable BiFC, suggesting that LMO4 is a likely false positive.

To independently validate the interaction between these proteins and PCNA we performed immunoprecipitations and <i>in vitro</i> interaction analyses. A V5-tagged version of SetD3 was transiently transfected into MRC5 cells, from which extracts were made. PCNA was immunoprecipitated from these cell extracts, and precipitates were probed for the presence of...
co-precipitated V5-SetD3 (Fig. 4A). SetD3 specifically co-precipitated with PCNA, indicating that the identified interaction is physiological. Similarly, we expressed V5-tagged Maf1 in human cells by transient transfection. Immunoprecipitation of PCNA using a polyclonal antibody, specifically co-precipitated Maf1 (Fig. 4B). This immunoprecipitation only co-purified a small amount of the Maf1 protein, so to further verify the interaction with PCNA we additionally tested it in vitro. Recombinant Maf1 protein was expressed in E. coli. Soluble E. coli extracts containing HA-tagged Maf1 were mixed with E. coli extracts containing S-tagged recombinant PCNA, or controls, and proteins were isolated on S-resin. Resin-associated proteins were analyzed by immunoblotting (Fig. 4C). This demonstrated that recombinant Maf1 can directly associate with PCNA, validating our immunoprecipitation and BiFC findings. The RNF7 gene is expressed in several isoforms and the sequencing reads from the screen did not enable us to distinguish whether isoform 1 or isoform 4 was able to interact with PCNA. We thus cloned the open reading frames for both these proteins and expressed them in E. coli. In vitro pull down experiments, as described above for Maf1, show that both RNF7v1 and RNF7v4 specifically associate with PCNA (Fig. 4D). Overexpression of either isoform of V5-strep-RNF7 reduced cell viability in MRC5 cells (data not shown) precluding investigation of this interaction by co-immunoprecipitation. Collectively, these results validate the BiFC screening protocol and highlight 3 novel PCNA interacting proteins that may have important roles in DNA replication, repair, or other cellular pathways involving PCNA. During S phase of the cell cycle PCNA becomes concentrated in replication factories in the nucleus, which can be visualized as focal nuclear substructures using fluorescence microscopy. We assessed the sub-nuclear localization of Maf1, SetD3 and RNF7 by transfecting MRC5 cells with constructs expressing GFP-tagged versions targeted to the nucleus with a nuclear localization signal (NLS). Co-transfection of RFP-tagged PCNA was used to indicate the positions of replication factories in S phase cells. We could not detect localized enrichment of Maf1, SetD3 or RNF7 at replication factories (Fig. S1). Furthermore, while PCNA is retained at replication foci after extraction of nuclear soluble proteins with detergent prior to fixation, most signal from Maf1, SetD3 and RNF7 is removed by even very limited detergent treatment (Fig. S1). This suggests that if these proteins are involved in DNA replication, this role does not require enrichment or tight retention of their activity at replication sites. PCNA also has a role during the DNA synthesis phase of nucleotide excision repair, and it is specifically recruited to damaged DNA sites. We used a localized UV-irradiation procedure to assess whether Maf1, SetD3 or RNF7 are enriched at sites of UV induced damage. Immunofluorescence using an anti-XPA antibody was used to mark the
position of ongoing DNA repair. No increased concentration of Maf1, SetD3 or RNF7 proteins was detected at repair sites (Fig. S2). While detergent resistant recruitment of PCNA was readily detected at sites of localized irradiation, extraction with triton prior to fixation removed all the Maf1, SetD3 and RNF7 signal (Fig. S2). This suggests that, as for replication, if these proteins are involved in nucleotide excision repair, this role does not require enrichment or tight retention of their activity at repair sites. It is certainly possible that these proteins might have active roles at replication or repair sites that are not detected by these approaches, or alternatively that the PCNA interactions are used during other cellular processes that we have not yet analyzed.

**Discussion**

As a key player regulating the events of chromosomal replication, PCNA is fundamental to genome and epigenome stability. Recently, the first pathogenic mutation in PCNA in humans was reported, and was shown to cause disease by its inability to properly interact with a subset of protein partners.\textsuperscript{51,52} This additionally raises the possibility that the loss or mutation of PCNA-interacting proteins could similarly contribute to human disease. Thus, understanding the extent, mechanism and regulation of protein traffic on PCNA is of great importance. Although PCNA was identified as critical for DNA replication and repair almost 20 years ago,\textsuperscript{53} there still has not been a systematic investigation.
of the PCNA-interactome, although the need for such a study has been highlighted.\textsuperscript{4,5} Instead there have been several independent studies using a variety of methodologies generating as many as 238 reported PCNA interaction partners to date (http://thebiogrid.org/111142). Here, we sought to design a strategy capable of detecting all potential PCNA interactions in human cells. To do this we utilised the BiFC methodology. We reasoned that this was eminently suitable for our question because: a) It places the interactions in a human cell system that should recapitulate any post-translational modification events that may be necessary for interaction. b) Our system targets the expressed reporters to the nucleus, thus enabling discovery of interactions that occur within a relevant intracellular environment. c) The refolding of the BiFC split-Venus construct has been reported to stabilize interactions between bait and prey constructs.\textsuperscript{54} Although this may result in higher false positive rates (as we did observe) this phenomenon should enhance the detection of weak or transient interaction events that might be missed by other methods. In addition this screen provides real-time readout of interaction and is high throughput in format. These characteristics means that this format can be readily developed in the future to study dynamic changes in the PCNA interactome such as might occur upon drug treatment or other cellular stresses. Here we utilised HEK293 cells as a readily transfecetable human cell line, but the method should be applicable to other cell types.

The PCNA interaction partners that we identified here have not previously been reported to have a role in DNA replication. SetD3 is a conserved histone H3-methyltransferase.\textsuperscript{55,56} It is abundantly expressed in muscle and promotes muscle-differentiation by regulating the transcription of muscle-related genes. In our experiments the BiFC signal from the PCNA-SetD3 interaction is found both in cytoplasm and nucleus. Consistent with this, the endogenous protein has been reported previously to localize to both compartments in human cells.\textsuperscript{57} The exogenous overexpression of \textit{Danio rerio} SetD3 in either mouse or human cell lines led to decreased cell viability and increased apoptosis.\textsuperscript{56} In contrast, we have generated cells expressing human SetD3 with either V5- or HA_NTV- epitope tags without observed perturbations to cell proliferation or viability (data not shown). The \textit{D. rerio} and human SetD3 proteins share 78\% identity, differing mostly in their C-termini, which contain a putative substrate binding domain. It therefore seems possible that species-specific binding events at the C-terminus of SetD3 mediate this toxicity, and as such this domain warrants further investigation.

The \textit{MAF1} gene is conserved from yeast to humans.\textsuperscript{58} In \textit{S. cerevisiae} it is a negative regulator of RNA polymerase III in response to lack of nutrients and growth factors, and replication stress,\textsuperscript{59,60} and in humans it can regulate all 3 RNA polymerases.\textsuperscript{61} Maf1 has been suggested to have a tumor suppressor function: it represses oncogenesis, reducing anchorage-independent growth and tumor formation in mice\textsuperscript{62} and human cells,\textsuperscript{61} and in \textit{Drosophila melanogaster}, dMaf1 depletion leads to an increase in growth rate and body size.\textsuperscript{63} Although both our \textit{in vitro} and \textit{in cell} experiments have shown a weak interaction between human Maf1 and PCNA, we do not yet know the biological function of this association.

The \textit{RNF7} gene (alternatively known as \textit{RBX2}) is also highly conserved during evolution (it is the homolog of \textit{S. cerevisiae} Roc2) and the protein is present in both the cytoplasm and the nucleus in human cells.\textsuperscript{64} It was first identified as a redox-inducible and apoptosis-protective antioxidant protein that decreases the production of ROS.\textsuperscript{65} It is overexpressed in various cancers, including lung, colon, stomach and liver cancer.\textsuperscript{66,67} More recently it was shown to be a component of specific Cullin-RING E3 ubiquitin ligases, in which it binds Cullin-1 or Cullin-5 to promote the ubiquitination and subsequent proteasomal degradation of substrates.\textsuperscript{68,69} It is of note that one of PCNA's cellular roles is to target PIP degron-containing substrates to the Cul4/DDB1 ubiquitin ligase.\textsuperscript{22-24} This complex contains RNF75 (RBX1), which has significant homology to RNF7. Although no direct interaction has been reported between PCNA and RNF75, given our results it will be of interest to ascertain whether there is a direct physical binding of PCNA to RNF75 that could be important for this degradation function. Alternatively PCNA might contribute, via the RNF7-interaction, to the proteasomal degradation of other proteins that are not targets of the Cul4/DDB1 ligase. In \textit{S. cerevisiae}, a yeast 2-hybrid assay shows no interaction between Roc2 and PCNA,\textsuperscript{70} suggesting that the role of this interaction may be specific to higher eukaryotes.

We note that none of the interaction partners here identified contain canonical PCNA interaction motifs. This suggests there are additional modes of PCNA interaction, not previously detected by the predominantly candidate-based approaches for identification of PCNA interactors that have been utilised to date. The three novel interaction partners reported here also do not appear to be enriched at DNA replication factories nor nucleotide excision repair sites in human cells. There are many reasons why this might be the case. It is now clear that PCNA interactions can be utilised to initiate protein degradation, thus Maf1, SetD3 or RNF7 might be destroyed following PCNA binding. It is also possible that the interactions with these factors are not utilised at the DNA replication forks, but during other cellular functions in which PCNA plays important roles. The screen reported here used a cDNA library derived from skeletal muscle. As a non-proliferating, non UV exposed tissue it is possible that the screen will actually be biased against detection of proteins involved in DNA replication and nucleotide excision repair. PCNA is crucial for other processes of DNA repair: base excision repair and probably homologous recombination,\textsuperscript{71,72} and it is also possibly involved in transcription and signaling,\textsuperscript{4} thus the novel interactions identified here may well be required for other important biological pathways in which PCNA is implicated. It is clear that even subtle perturbation to PCNA’s partner profile can cause unexpected cellular phenotypes and have dramatic consequences for human health.\textsuperscript{52} The identification of these novel PCNA partners provides a new avenue for understanding the multifaceted and complex roles of this fascinating protein.
Materials and Methods

Cell lines and transfection
MRC5 (SV40-transformed) and HEK293 and derived lines were grown in DMEM glutamax (Invitrogen) with 10% FBS, supplemented with penicillin and streptomycin at 37°C; 5% CO₂. Cells were transfected with PEI or FugeneHD. Selection was with 750 µg/ml G418 or 100 µg/ml hygromycin where used.

Plasmids
For BiFC experiments, human cDNA encoding PCNA was cloned into the bait vector, which was derived from pcDNA3.1 (Invitrogen) engineered to produce the C-terminal portion of Venus fluorescent protein (amino acids 159–230), a nuclear localization signal (PKKKRK), the FLAG epitope (DYKDDDDK) and a flexible hydrophilic linker sequence (GEGQGQGQGPGRGYAYRS). cDNAs encoding human Fen1, RNF7v1 and v4, Maf1 and SetD3 were cloned into an engineered pDEST prey expression vector derived from pCEP4 (Invitrogen) containing the HA epitope (YPYDVPDYA) the N-terminal portion of Venus (amino acids 1–158) and an NLS and linker as above and attR sites for recombination; using the Gateway system (Invitrogen). 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The cDNA library used was derived from skeletal muscle (male, 24 years) with an average insert size of 1.6 kb in the pCMV•SPORT6 vector (Invitrogen catalog number 11327–012). The library cDNAs were exchanged into the pDONRzreo vector (Invitrogen) using a BP reaction according to the manufacturers protocols (Invitrogen). An LR reaction was then used to transfer the cDNA library into the engineered prey pDEST vector on pCEP4 as above.

Fluorescence activated cell sorting
Cells were analyzed and sorted on a MoFlo cytometer (Beckman Coulter) in FACS sort buffer: HBSS supplemented with 25 mM HEPES and 5 mM EDTA. A 100µm nozzle was used at 30psi. Venus fluorescence was detected with a 100mW, 514 laser line with a 546 nm filter. After sorting cells were returned to conditioned medium to aid recovery.

DNA preparation and PCR
DNA was prepared from sorted cells and unsorted controls using the GeneJet DNA purification kit (Thermo Scientific) according to the manufacturer’s instructions. This was used as a template to amplify library cDNA inserts by PCR using Phusion DNA polymerase (New England Biolabs) and pCEP4-specific primers (CATTTATGCCAGTACATGACCTT and GCAA-TAGCATCACAATTTCCACA) according to manufacturer’s protocols.

454 sequencing
500 ng PCR product from the above reactions was used submitted for 454 Alicon sequencing using the Roche FLX Junior sequencer in the Department of Biochemistry, University of Cambridge. Raw read data was trimmed using Flexbar to remove vector and adapter sequences and then blastn was used to query the human nucleotide genomic and transcript databases for sequence matches. Genomic contaminants carried over from the PCR reactions were removed manually, as were mitochondrial genes.

Immunofluorescence and EdU localization
Cells growing on glass coverslips were either fixed directly in 2% formaldehyde in PBS for 20 minutes at room temperature, or treated with 0.1% or 0.2% Triton X-100 in CSK (10 mM Pipes, pH 7.0; 300 mM sucrose; 100 mM NaCl; 3 mM MgCl₂; 1× complete protease inhibitor cocktail (Roche)) for removal of soluble nuclear proteins prior to fixation. Where used, 12µM EdU was added to culture medium for 10 minutes prior to fixation. EdU was visualised using the Click-iT® Edu Alexa Fluor® 647 Imaging Kit (Invitrogen) according to the manufacturer’s instructions. Immunofluorescent detection of bait and prey proteins was using anti-FLAG M2 antibody (Sigma) or anti-HA monoclonal antibody 12CA5 (Abcam). XPA was detected using anti-XPA monoclonal antibody (abcam). After permeabilisation of cells (0.5% Triton X-100 in PBS for 5 minutes), blocking was for in 3% BSA (Sigma) solution in phosphate buffered saline (Gibco) containing 0.1% Tween-20 (Sigma). Primary antibodies were incubated on cells at a 1/1000 dilution in blocking buffer for 2 hours at room temperature. After washing 3 times in blocking buffer secondary antibody goat-anti-mouse-Alexa Fluor 488 or 633 (Molecular Probes) was used at a dilution of 1/1000 in blocking buffer for 1 hour. After three further washes in blocking buffer coverslips were mounted in Aqua-Poly mount with DAPI. Immunofluorescent signal was visualised on a Leica TCS confocal using a 63× objective with oil immersion. The following excitation (Ex.) and emission (Em.) settings were used: DAPI: Ex. 405 nm; Em. 415–518 nm. GFP/Alexa Fluor 488: Ex. 488 nm; Em. 500–600 nm. Venus (BiFC): Ex. 514 nm; Em. 524–618 nm. RFP/Alexa Fluor 555: Ex. 543nm; Em. 561–615 nm. Alexa Fluor 633/647: Ex. 633 nm; Em. 645–769 nm.

Recombinant protein production
His-S or His-HA tagged fusion proteins were produced in BL21-CodonPlus (DE3)-RIPL competent cells (Agilent) at 30°C for 4h after addition of 1mM IPTG. Cells were collected by centrifugation and lysed in cold PBS with 1mM PMSF and 0.1%
Triton X-100, using a Diagenode Bioruptor (8 cycles of 30s on High). Soluble fractions were kept at ~80°C.

For PCNA in vitro interaction, lysates were mixed and then incubated with S-agarose beads (Novagen) at 4°C overnight with agitation. Beads were washed in cold PBS with 0.1% Triton X-100 and then resuspended in Laemmli SDS-PAGE buffer. Negative controls were performed using lysates from cells expressing only His-S or His-HA-GST.

**Cell extracts**

HEK or MRC5 cells were washed in PBS with 1mM iodoacetamide then incubated 30min on ice in extract buffer (10% glycerol, 0.01% Igepal, 40 mM NaCl, 50 mM Tris pH7.5, 2 mM MgCl2, 1U/ml Benzonase (Novagen) and then adjusted to equalise by the addition of extract buffer.

**Cell lysates** were centrifuged at 17000 g, for 15 minutes at 4°C. Cell lysates were centrifuged at 17000 x g, for 15 minutes at 4°C. Protein concentration was determined by Bradford assay (Sigma) and then adjusted to equalse by the addition of extract buffer.

**Immunoprecipitation**

For analysis of PCNA_CTV bait binding to endogenous PCNA Flag pull-downs were performed by incubating extracts from HEK 293 PCNA_CTV stable cell lines, or controls, with PCNA Flag pull-downs were performed by incubating extracts from HEK 293 PCNA_CTV stable cell lines, or controls, with PCNA Flag pull-downs were performed by incubating extracts from HEK or MRC5 cells transfected with V5-tagged prey vectors were made as above. Before immunoprecipitation, a buffer exchange was performed using Zeba spin desalting column (Pierce) into PCNA-binding buffer (10% glycerol, 0.01% Igepal, 25 mM NaCl, 25mM Tris pH7.5, 1 × complete protease inhibitors (Roche)). The NaCl concentration was adjusted to 150mM and samples were incubated a further 10 minutes on ice. Cell lysates were centrifuged at 17000 x g, for 15 minutes at 4°C. Protein concentration was determined by Bradford assay (Sigma) and then adjusted to equalse by the addition of extract buffer.

**Western-blot analysis**

Proteins were resolved by SDS-PAGE, transferred to a PVDF membrane (Whatman), and probed with the following antibodies: mouse anti-PCNA (ab29), rabbit anti-V5 (ab9116) and mouse anti-HA (ab16918) from Abcam. Membranes were then incubated with the appropriate HRP-conjugated secondary antibodies and detected by chemiluminescence using a ChemiDoc MP System (Bio-Rad) and Clarity or DURA detection reagents (Bio-Rad/Pierce). For RNF7 experiments, an additional 2mM DTT were added to the sample buffer in order to reduce samples and obtain monomers.

**Localized UV irradiation**

MRC5 were transfected at least 24h prior to irradiation. Irradiation was performed as previously described. Briefly, cells grown on coverslips to around 80% confluency were washed in PBS. Excess PBS was removed and isopore membrane filters with 5 μm pores (Millipore) were placed on top of cells before irradiation with UVC (254 nm) at 100J/m². After filter removal, cells were put back in medium and allowed to recover for 30 minutes in the incubator prior to further treatment or fixation.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Supplemental Material**

Supplemental data for this article can be accessed on the publisher’s website.
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