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DOI:

[10.1074/jbc.RA118.004469](https://doi.org/10.1074/jbc.RA118.004469)

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*Document Version*

Publisher's PDF, also known as Version of record

*Citation for published version (Harvard):*

Spiliotopoulos, A, Blokpoel Ferreras, L, Densham, RM, Caulton, SG, Maddison, BC, Morris, JR, Dixon, JE, Gough, KC & Dreveny, I 2018, 'Discovery of peptide ligands targeting a specific ubiquitin-like domain-binding site in the deubiquitinase USP11', *Journal of Biological Chemistry*. <https://doi.org/10.1074/jbc.RA118.004469>

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<http://www.jbc.org/cgi/doi/10.1074/jbc.RA118.004469>

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## Discovery of peptide ligands targeting a specific ubiquitin-like domain-binding site in the deubiquitinase USP11

Anastasios Spiliotopoulos<sup>1,2#</sup>, Lia Blokpoel Ferreras<sup>1</sup>, Ruth M. Densham<sup>3</sup>, Simon G. Caulton<sup>1</sup>, Ben C. Maddison<sup>4</sup>, Joanna R. Morris<sup>3</sup>, James E. Dixon<sup>1</sup>, Kevin C. Gough<sup>2\*</sup> and Ingrid Dreveny<sup>1\*</sup>

<sup>1</sup> Centre for Biomolecular Sciences, School of Pharmacy, University of Nottingham, Nottingham NG7 2RD, United Kingdom; <sup>2</sup> School of Veterinary Medicine and Science, Sutton Bonington Campus, College Road, Sutton Bonington, Leicestershire, LE12 5RD, UK; <sup>3</sup> Birmingham Centre for Genome Biology and Institute of Cancer and Genomic Sciences, Medical and Dental Schools, University of Birmingham, Birmingham B15 2TT, UK; <sup>4</sup> ADAS, School of Veterinary Medicine and Science, Bonington Campus, College Road, Sutton Bonington, Leicestershire, LE12 5RD, UK.

Running title: *USP11 selective peptide ligands*

<sup>#</sup>Present address: UCB Pharma, 208 Bath Rd, Slough SL1 3WE, UK.

<sup>\*</sup>To whom correspondence should be addressed: Dr Kevin Gough, School of Veterinary Medicine and Science, Sutton Bonington Campus, College Road, Sutton Bonington, Leicestershire, LE12 5RD. UK. [kevin.gough@nottingham.ac.uk](mailto:kevin.gough@nottingham.ac.uk), Dr Ingrid Dreveny, Centre for Biomolecular Sciences, School of Pharmacy, University of Nottingham, Nottingham NG7 2RD United Kingdom. [ingrid.dreveny@nottingham.ac.uk](mailto:ingrid.dreveny@nottingham.ac.uk)

**Keywords:** peptide interaction, peptides, protease, crystal structure, deubiquitylation (deubiquitination), ubiquitin, ubiquitin-dependent protease, phage display, cell-penetrating peptide (CPP), DNA damage response, molecular recognition, USP11

### ABSTRACT

Ubiquitin specific proteases (USPs) reverse ubiquitination and regulate virtually all cellular processes. Defined non-catalytic domains in USP4 and USP15 are known to interact with E3 ligases and substrate recruitment factors. No such interactions have been reported for these domains in the paralog USP11, a key regulator of DNA double-strand break repair by homologous recombination (HR). We hypothesized that USP11 domains adjacent to its protease domain harbour unique peptide-binding sites. Here, using a next-generation phage display (NGPD) strategy, combining phage display library screening with next generation sequencing, we discovered unique USP11 interacting peptide motifs. Isothermal titration calorimetry disclosed that the highest affinity peptides ( $K_D$  of  $\sim 10 \mu\text{M}$ ) exhibit exclusive selectivity for USP11 over USP4 and USP15 *in vitro*. Furthermore, a crystal structure of a USP11-peptide complex revealed a previously unknown binding site in USP11's non-catalytic ubiquitin-like

(UBL) region. This site interacted with a helical motif and is absent in USP4 and USP15. Reporter assays using USP11-WT versus a binding pocket-deficient double mutant disclosed that this binding site modulates USP11's function in HR-mediated DNA repair. The highest affinity USP11 peptide binder fused to a cellular delivery sequence induced significant nuclear localization and cell cycle arrest in S phase, affecting the viability of different mammalian cell lines. The USP11 peptide ligands and the paralog-specific functional site in USP11 identified here provide a framework for the development of new biochemical tools and therapeutic agents. We propose that an NGPD-based strategy for identifying interacting peptides may be applied also to other cellular targets.

There are  $\sim 54$  ubiquitin specific proteases (USPs) encoded in the human genome, most of which harbour a canonical cysteine protease catalytic triad. USPs specifically reverse

ubiquitination of a range of substrates to regulate virtually all cellular processes including protein degradation, signalling pathways, DNA damage repair, transcription and receptor endocytosis (1). USPs harbour a catalytic domain that is flanked or interspersed by additional domains for versatile functionality and specificity (2,3). For most USPs, interactions involving these domains are poorly understood, and only few selective agents to probe a particular USP's function are available (4-10). Human ubiquitin specific protease 11 (USP11) is best known for its role in DNA damage repair by homologous recombination (HR) (11,12). Knockdown of USP11 results in spontaneous activation of the DNA damage response and sensitivity to genotoxic stress agents (13) and ionizing radiation (14). DNA damage repair by HR mostly occurs in S and G2 phases of the cell cycle and is suppressed in G1. As part of the mechanism controlling this process, USP11 regulates the ubiquitination status of the BRCA2-PALB2 complex and USP11 is upregulated in S phase (12).

USP11 silencing also inhibits TGF $\beta$  pathway signalling (15) and USP11 is involved in NF- $\kappa$ B signalling pathways. The stability of several other important proteins relevant to health and disease is regulated by USP11, including p21 (16), RAE1 (17) and cIAP2 (18). Consistent with an important regulatory role, USP11 is dysregulated in a number of cancers including pancreatic cancer (19), glioma, ovarian and breast cancers, as well as hematological malignancies (12).

USP11 harbours a core protease domain as well as non-catalytic regions containing an N-terminal domain present in USPs (DUSP) and internal ubiquitin-like (UBL) domains (20) (Fig. 1A). USP11 shares this domain architecture with paralogs USP15 and USP4, which are known as the DUSP-UBL (DU) family of USPs (20-23).

We previously determined the crystal structure of the USP11 N-terminal DU domains and showed that USP11's non-catalytic domains do not directly auto-activate or inhibit the activity of USP11 (20). In contrast, the USP4 N-terminal DU domains are required for efficient turnover of the enzyme by promoting ubiquitin release (24). The USP4 and USP15 DU domains interact with E3 ligases (25) and the substrate recruitment factor SART3 (26,27), but no interactions have yet been reported for these domains in USP11. We hypothesized that USP domains adjacent to the protease domain

would be good targets for the identification of unique binding sequences as they significantly differ between USPs. Here, a next generation phage display (NGPD) approach was applied to screen for peptide ligands against the USP11 N-terminal domains. NGPD combines the diversity of phage display libraries with the screening capability of next generation sequencing platforms. This allows the replacement of immunoassay screening of several hundred randomly picked phage clones after conventional phage panning with the more comprehensive analysis of the sequence of potentially millions of ligand genes to determine enrichment of particular sequences which can then be selected for screening in biochemical assays. This method has been applied previously to the identification of peptides with specific binding traits (28-30). We discovered USP11 specific peptides harbouring consensus motifs that do not interact with either paralog USP4 or USP15 and identified a novel binding site in the USP11 N-terminal ubiquitin-like domain. Together, the data show that the NGPD strategy can be used to uncover highly selective ligands and novel binding sites for USPs as a basis for the development of new probes or anti-proliferative agents.

## Results

### *A next generation phage display strategy for the isolation of USP11 peptide ligands*

A NGPD approach was applied to the identification of USP11 ligands as schematically depicted in Fig. 1B. A randomized linear peptide library fused to the gpVIII protein was used in order to isolate peptides that interact with the USP11 N-terminal DU domains (DUSP and UBL domains in tandem; USP11<sub>DU</sub>; Fig. 1A). After three biopanning rounds, phage ssDNA was extracted, purified and peptide sequences amplified and deep sequenced. In order to rank the identified sequences, a two proportion Z test based method (31,32) was applied to round 3 output phage and analysis of the 50 sequences with the highest Z scores after three rounds of panning (Fig. S1) revealed the presence of two sequence motifs: [YNHC]-[+-]-L-[+-]- $\phi$ -R (motif 1, based on 18 peptides) and a L-x-L- $\phi$ -x-x-S-[RP] (motif 2, based on 29 peptides) whereby x stands for any residue, [+-] for charged residues and  $\phi$  for non-polar residues. A graphical representation of the residue frequency within the motifs as computed by the

Multiple EM for Motif Elicitation (MEME) algorithm (33) is shown in Fig. 1C. The thermodynamic binding parameters of synthetic peptides representative of both motifs including the N-terminal phage protein sequence (AEGEF), AEGEFYKLIKIRTPQ (referred to as FYLIR peptide) and AEGEFLELLKASRW (referred to as LxLL peptide), were determined by isothermal titration calorimetry (ITC). Peptides harbouring either motif bound USP11<sub>DU</sub> with dissociation constants of about 10  $\mu\text{M}$  ( $8.86 \pm 1.13 \mu\text{M}$  for AEGEFYKLIKIRTPQ and  $6.92 \pm 2.25 \mu\text{M}$  for AEGEFLELLKASRW; Fig. 1D). We subsequently focused on the FYLIR peptide and showed that full-length USP11 and its N-terminal domains alone display comparable dissociation constants indicating that the presence of the protease domain has no influence on the interaction (Fig. S2).

#### ***A helical motif in the peptide ligand binds to a novel USP11 UBL domain binding site***

At present no binding sites or interaction partners have been identified for the USP11 N-terminal domains. We therefore focused on exploring the molecular basis of the interactions between USP11 and the peptide ligand. Co-crystallization trials of USP11<sub>DU</sub> in the presence of FYLIR peptide yielded monoclinic crystals of space group  $P2_1$  that diffracted to 1.3  $\text{\AA}$  resolution after optimization. The structure was determined by molecular replacement and refined to an  $R_{\text{work}}/R_{\text{free}}$  of 15.7/17.8%. Data collection and refinement statistics are summarized in Table 1. The DUSP and UBL domains in the peptide complex structure are arranged in tandem (Fig. 2A), analogous to the structures of the rat USP11 orthologue (20) and homologues USP4 (SGC) and USP15 (21,22), but unlike the previous structure of human USP11<sub>DU</sub> (20). Unexpectedly, the structure revealed that the peptide occupied an elongated cleft in the USP11 UBL domain (Fig. 2), with clear electron density for peptide residues Gly3-Arg14 observed in both copies of the asymmetric unit (Fig. S3A). The peptide's main interacting core comprises nine residues (residues 5-13; FYKLIKIRTP) (Fig. 2C, D), with a buried interaction interface area of  $\sim 750 \text{\AA}^2$ . Interestingly, peptide residues Glu2-Lys7 adopt an  $\alpha$ -helical conformation when in complex with USP11, which is stabilized by T-shaped  $\pi$ - $\pi$  stacking interactions of residues Phe5 and Tyr6. Phe5, which can be described as the first tooth of

the bidentate-like interaction, is nearly completely buried by occupying a hydrophobic pocket (referred to as major pocket). Tyr6 is partially solvent exposed and engages in additional contacts with the USP11 UBL domain.

The pocket is formed predominantly by side chains of USP11 residues His161, Trp200, Leu208, Ile230, Glu232, Pro241, Ser242 and Leu245. Hydrogen bonding interactions between main chain carbonyls and amine groups of peptide residues Lys9, Arg11 and USP11 Leu208 are also key features of the interaction (Fig. 2C, D). The peptide's Ile10 occupies a second hydrophobic pocket (USP11 Leu245, Trp200). Ile10 "anchors" the peptide together with the guanidinium group of FYLIR peptide Arg11 that forms a salt bridge with USP11 Asp209 in what could be described as the 'second tooth' of the bidentate-like interaction. The peptide's Arg11 is also in close proximity to another acidic residue, USP11 Glu205 (Fig. 2C). Compared to the unliganded USP11 UBL domain (PDB code 4MEL (20)), minor conformational changes in the structure are observed. The side chain of His161 partially masks the pocket in the unliganded structure. The loop between strands S3 and S4 in the UBL domain (S3S4 loop, residues N203-S207) becomes less flexible in the peptide bound structure due to interactions of USP11 Glu205 with the peptide's Arg11. Moreover, a flexible-to-order transition of Leu245 due to interactions with the peptide's Phe5 is also observed between the unliganded and liganded USP11<sub>DU</sub> structures (Fig. S3B).

#### ***The 'FYLIR' peptide ligand is highly specific for USP11 in vitro***

USP11 shares its domain structure with USP4 and USP15 (20-24). The USP11 UBL domain shares 32% sequence identity with the corresponding UBL domains in USP4 and USP15, whereas these domains in USP4 and USP15 share 72% sequence identity (Fig. 3). We therefore tested whether the peptide ligand is specific for USP11. Interestingly, an interaction between FYLIR peptide and the DU domains of either USP4 or USP15 by ITC was not observed, suggesting that this binding cleft for the peptide is specific to USP11 (Fig. 4C). A superposition of the three structures revealed differences in the main USP11 binding pocket ('major pocket') compared to USP15 and USP4 (Fig. 4A, B). In particular,



USP11 Leu208 is substituted by Phe188 and Tyr192 in USP15 and USP4, respectively. At the position of USP11 Ser242, USP15 and USP4 harbour arginine residues Arg222 and Arg226, respectively (Fig. 3, 4A). The arginine residues are engaged in  $\pi$ -stacking and ion- $\pi$  interactions with the aromatic side chains of USP15 Phe188 and USP4 Tyr192 and consequently occlude the pocket. Therefore, the smaller side chains of Leu208 and Ser242 may be the key residues that allow USP11 to engage with the peptide ligand.

To further verify the key role of this pocket for ligand binding in solution, we generated a USP11\_DU Leu208Phe and Ser242Arg double mutant (USP11\_DU<sup>L208F, S242R</sup>) to render this region USP15-like. USP11\_DU and USP11\_DU<sup>L208F, S242R</sup> behaved similarly on gel filtration. No aggregation and an equivalent elution volume was observed for the mutant disclosing that the mutations are compatible with folding resulting in a similar hydrodynamic radius to USP11\_DU. Furthermore, FL-USP11<sup>L208F, S242R</sup> was active (Fig. S4). ITC experiments confirmed that ligand binding to USP11\_DU<sup>L208F, S242R</sup> was abolished (Fig. 5A). We also investigated whether this binding pocket is involved in interactions with motif 2 (LxLL peptide). ITC experiments with the USP11 pocket-deficient double mutant USP11\_DU<sup>L208F, S242R</sup> and this peptide resulted in no detectable binding, showing that both peptide binding motifs require the ‘major pocket’ for interaction with USP11 (Fig. 5B). Whether in addition to altering the pocket region, these mutations affect the conformation of FL-USP11 is not known.

#### ***The UBL binding pocket of USP11 is required for its function in homologous recombination***

The ITC data showed that peptides harbouring either motif interacted with the major pocket in the UBL domain. USP11 is most well-known for its role in DNA damage repair by homologous recombination (HR) (11,12) and depletion of USP11 results in HR repair defects (13,14). In order to explore whether the identified binding site is required for USP11 function, HR GFP-reporter assays were carried out in U20S-DR3 reporter cells (34). These cells contain an integrated non-functional GFP gene, which has been interrupted by inclusion of an I-Sce1 restriction site. Also contained within the integrated region is another incomplete GFP sequence that can be used

as a template for HR repair across the I-Sce1 site. A double strand break within the reporter can be induced by expression of the nuclease I-Sce1 and reconstitution of the GFP gene and subsequent expression of functional GFP protein can be used as a marker of successful HR-mediated repair. To this end, the formation of GFP products was measured from the integrated substrate in USP11 depleted cells transfected with *I-Sce1* (34), and complemented with USP11wt or the UBL binding site mutant USP11<sup>L208F, S242R</sup> (Fig. 5C). The percentage of GFP positive cells normalised to the RFP transfection efficiency was significantly lower in the case of the USP11 binding site mutant compared to USP11wt and similar to the RFP control. This shows that the USP11<sup>L208F, S242R</sup> mutant cannot complement USP11wt for HR indicating the importance of this binding site for USP11’s function in HR, either directly or due to its contribution to the conformation of the enzyme.

#### ***Transduction of a ‘FYLR’ peptide agent results in differential effects on cell viability***

It was hypothesized that peptide binding may affect the catalytic turnover of substrates by steric hindrance as the peptide binding site is located in proximity to the catalytic triad in the protease domain. When the impact of the FYLR peptide on the USP11 catalytic activity was assessed using the model substrates 7-amino-4-methylcoumarin modified ubiquitin (Ub-AMC) and K63-linked FRET di-ubiquitin carboxytetramethylrhodamine (TAMRA), the observed slight reduction in catalytic activity was not statistically significant compared to controls (unpublished observation).

We next evaluated the effect of the FYLR peptide ligand on cells in culture. The novel GET system, developed for highly efficient nucleic acid, protein and peptide delivery (35), was used to deliver the FYLR peptide. GET is based on the use of cell-penetrating peptides modified with heparin-binding sequences, which enhance cellular uptake by endocytosis. The synthetic peptide, GET-FYLR, designed for these studies harboured an N-terminal TAMRA fluorescent tag, for easy visualization, fused to the GET (P21 and 8R (35)) and FYLR sequences. GET-FYLR was efficiently delivered to all cell lines tested (including human glioblastoma KNS-42 and U87, cervical carcinoma HeLa, mammary carcinoma MCF7, pancreatic carcinoma Panc1, and fibroblast NIH3t3 and BJ6

cell lines). Confocal imaging of HeLa cells, treated for 24 hours with a low dose of GET-FYLIR (10  $\mu$ M) showed localization of the GET-FYLIR peptide in both the cell cytoplasm and the nucleus; with the highest accumulation in nucleoli (Fig. 6A). This showed that the GET-FYLIR peptide is efficiently delivered to subcellular compartments where endogenous USP11 protein is localized (36) and that the GET-FYLIR peptide efficiently escapes endosomal vesicles (as endosomal escape can be a limitation for delivered peptides). Having established efficient delivery, the peptide's effect was investigated by assessing cell viability.

Interestingly, a significant cell-type specific decrease in cell viability upon delivery of the GET-FYLIR peptide (up to 50  $\mu$ M) was observed (Fig. 6B). Panc1 cell lines were particularly sensitive to GET-FYLIR dose, whereas other cell lines, especially BJ6 human dermal fibroblasts, were relatively insensitive. After 24 h incubation with 10  $\mu$ M of GET-FYLIR, Panc1 cells showed a loss of viability (~50%); an almost complete loss of viability (to ~12%) was observed at a dose of 20  $\mu$ M. Viability of BJ6, U87 and NIH3t3 cell lines was retained at 10  $\mu$ M dose with a minimal effect at a 20  $\mu$ M dose. In order to confirm that the differential effect on cell viability was not due to peptide uptake efficiency, GET-FYLIR initial uptake (1 h) and long-term uptake (24 h) with low dosages in NIH3t3 cells (a relatively insensitive cell line) were compared to uptake in Panc1 cells (a sensitive cell line to GET-FYLIR dose) (Fig. S5). The lowest dose tested (1  $\mu$ M; Fig. S5A) showed little difference in cell uptake in either cell-type for short (1 h) or long (24 h) exposures. Importantly, uptake increased with longer exposures. However, at a higher dose (5  $\mu$ M; Fig. S5B), cell viability effects in Panc1 but not NIH3t3 cells were observed, with increased uptake in Panc1 cells for both 1 h and 24 h incubations. Interestingly, the uptake was similar at 1 h and 24 h incubations for Panc1 cells at this higher dose. This possibly suggests that cell membrane permeability may have been affected even after 1 h incubation in Panc1 cells. Uptake by NIH3t3 cells was significantly lower than Panc1 cells at this dose for both incubation times. These data suggests that the GET-FYLIR peptide is similarly taken up in the different cell lines, but that uptake is significantly altered once the concentration begins to compromise cell viability. In order to confirm that GET-FYLIR was

specifically producing this change in cell viability and uptake, a scrambled control peptide where the FYLIR sequence was replaced by the sequence APKFEIRRGTYKLE (GET-Scrambled) was also used. The scrambled peptide was efficiently delivered and there was no difference in the uptake profile between NIH3t3 and Panc1 cells. Furthermore the GET-Scrambled peptide had no significant effect on Panc1 cells (up to 10  $\mu$ M) (Fig. S6). This suggests therefore that cell-type specific changes in cell uptake, at dosages in which the cell viability was compromised, were attributable to the activity of the FYLIR peptide.

In order to further characterize the effect of the GET-FYLIR peptide, cell cycle analysis was conducted on cell lines that were GET-FYLIR sensitive (Panc1) and relatively resistant (NIH3t3). Using genome copy number analyses by flow cytometry, we demonstrated that Panc1 cells treated with GET-FYLIR (20  $\mu$ M) showed a significantly increased fraction of cells in S phase (36.9% to 52.8%;  $p < 0.0001$ ;  $n = 7$ ), as well as significant alterations in all other fractions (Fig. 6C). In NIH3t3 cells, GET-FYLIR had smaller effects on cell cycle distribution (24.8% to 37.5%,  $p < 0.0001$ ,  $n = 5$ ). Furthermore, in Panc1 cells, the subG0 fraction (cells containing less than one diploid genome) increased ~9-fold (0.9% to 8%;  $p < 0.0001$ ;  $n = 7$ ), whereas this effect was not significant in NIH3t3 cells (0.2% to 0.8%;  $p = 0.9992$ ;  $n = 5$ ), suggesting DNA fragmentation and cell death is triggered in Panc1 cells by GET-FYLIR. These results suggest that, when efficiently delivered to cells, FYLIR has a cell-type specific effect on cell viability, associated with cell cycle arrest and S phase progression.

## Discussion

The present study describes the discovery for the first time of unique peptide ligands for a ubiquitin specific protease by the means of next generation phage display. The highest affinity peptide with a 'FYLIR' motif interacts with USP11 with a dissociation constant of ~10  $\mu$ M. This ligand is highly specific for USP11 by occupying an unexpected unique site in the N-terminal UBL domain that is neither present in the paralogs, USP4 and USP15, or any of the other USPs encoded in the human genome. UBL domains are often involved in interactions and occur frequently in USPs (37). In the most well studied member of the

USP family USP7, UBL domains recognize binding motifs in the epigenetic regulators UHRF1 (38) and DNMT1 (39) amongst other binding partner proteins (40), but this is achieved through different recognition surfaces. The USP11 crystal structure showed that both the S3-S4 loop that is often involved in engaging binding partners in UBL domains (41) as well as a conserved unique C-terminal sequence extension to the UBL core (sequence WPSAQL) are part of the peptide binding site.

A single to double-digit micromolar starting affinity, as reported here, is often observed for peptide identification from phage display. For example, peptides isolated to Japanese encephalitis virus, insulin-degrading enzyme or IL17A had affinities in the 2.5-12  $\mu$ M range (42-44). Subsequent affinity maturation has been successfully employed to achieve affinities in the nanomolar range (42). Additional peptide phage libraries can also be constructed by randomizing the residues which are adjacent to the ones critical for the interaction (soft randomization) based on structural information and thus improve the affinity of the parental FYLIR peptide. This will be the focus of future investigations.

For the majority of USPs the roles of non-catalytic regions, such as the N-terminal USP11<sub>DU</sub> domains, are poorly understood. However, USP7 accessory domains have been shown to have unique specificities, where [PA]-x-x-S motifs in p53, Hdm2 and Epstein-Barr nuclear antigen 1 are recognized by a central binding cleft in the N-terminal TRAF-like domain (45) and the USP8 rhodanese domain harbours a ligase recognition site (46). Knowledge of binding sites and the availability of agents targeting the non-catalytic domains can be beneficial for drug discovery programmes due to the potential for increased selectivity compared to active site inhibitors. This is the first report of specific binding sequence motifs for USP11. A high resolution crystal structure revealed a binding mode with a helical segment that bears hallmarks of typical peptide-protein interactions such as an amphiphilic nature and the presence of a leucine, tyrosine, isoleucine and phenylalanine at the interface (47). Interestingly, both peptide motifs identified in this unbiased NGPD approach use the major pocket for interactions. In addition, a role of this binding site in homologous recombination assays was

confirmed by comparing USP11<sup>wt</sup> and the binding pocket-deficient mutant USP11<sup>L208F, S242R</sup>. This may be due to a direct contribution of the particular mutated residues to interactions, but could in principle also be due to a change in enzyme conformation.

Furthermore, a fluorescently labelled peptide agent was designed and showed efficient transduction of several cell lines. Interestingly, the FYLIR peptide based agent blocked cell proliferation and affected viability by arresting the cell cycle predominantly in S phase. USP11's role in DNA damage repair by homologous recombination, which is important in S phase and is highly suppressed in G1 (12), is consistent with the peptide's effects being, at least in part, attributed to interference with USP11 function. Indeed, mutation of the FYLIR peptide's USP11 binding site affected homologous recombination in U2OS cells. A significant fraction of USP11 localizes to chromatin in cells before and upon induction of DNA damage (13). The GET-FYLIR peptide localizes with the USP11 subcellular distribution and is enriched in nucleoli.

Several reports link USP11 to cell cycle progression and proliferation although the detailed mechanisms are only partially understood. For example, (i) cell growth assays in MCF-7 and MDA-MB-231 cells show an increase in cell numbers upon USP11 overexpression, while USP11 knockdown leads to growth inhibition (48); (ii) USP11 itself is under cell cycle control and turns over rapidly in G1 cells, especially upon DNA damage induction, whereas expression of USP11 in S phase is high and insensitive to DNA damage (12). Knockdown of USP11 results in the activation of DNA damage response pathways, leading to hypersensitivity of cells to genotoxic stress (13); (iii) in primary human fibroblasts knock-down of USP11 results in characteristics of senescence, including proliferative arrest and enlarged nucleoli (49) and (iv) USP11 interacts with p21 to regulate cell cycle progression and DNA damage responses (16). Interestingly, we found that in Panc1 cells that have previously been shown to have high amounts of USP11 mRNA and undergo dose dependent cell death upon treatment with mitoxantrone that inhibits USP11 (19), cell viability is highly affected by FYLIR peptide exposure. USP11 has been recognized as a novel anti-cancer target by preventing repair of double strand breaks in cancer

cells via synthetic lethality (11). Nevertheless, further studies will be required to decipher the detailed mechanism of action of the GET-FYLIR peptide agent.

Taken together, we identified USP11 interaction motifs, a novel binding site in the USP11 UBL domain and demonstrate the ability of a peptide agent containing a USP11 interaction motif to differentially affect cell lines. Therefore, as well as providing insight into USP11 recognition, the findings may also pave the way for the development of novel peptidomimetic agents. To our knowledge, outside of epitope mapping, this is the first example of using NGPD to define protein-binding motifs. The strategy will be applicable to other members of the USP family for the identification of unique ligands and binding sites and the development of molecular probes and/or therapeutic agents.

## Experimental procedures

### Constructs

DNA constructs for this study were generated by standard molecular biological techniques. Constructs of human USP11 spanning the DUSP-UBL domains (USP11\_DU), residues 1-244 or 24-244, USP11 (1-920) as well as USP15\_DU and USP4\_DU in pET26b and FL-USP11 in pColdI have previously been described (20,21). The double mutants USP11\_DU<sup>L208F, S242R</sup> and FL-USP11<sup>L208F, S242R</sup> were generated by site directed mutagenesis using the QuickChange method. For HR assays, FL-USP11 was cloned into the cDNA3.1(+)-mRFP vector using *KpnI* and *XhoI* restriction sites and a corresponding construct harbouring L208F and S242R mutations created by the QuickChange method.

### Protein production

All proteins were expressed and purified according to protocols described previously (20). Protein expression was induced by adding 0.5 mM IPTG to BL21-CodonPlus cell cultures grown at 37 °C in 2xYT medium to mid-log phase. Cells harboring the USP11\_DU (residues 1-244 or 24-244), the double mutant USP11\_DU<sup>L208F, S242R</sup>, USP15\_DU, and USP4\_DU plasmids were harvested after 4 h. For FL-USP11 constructs, cells were grown for 72 h at 10 °C. For all DU constructs cells were lysed by sonication in 20 or 50 mM Tris-Cl pH 7.5, 150 mM NaCl, 20 mM imidazole. Cells

containing FL-USP11 were lysed in 50 mM Tris-Cl, 300 mM NaCl, 5% (v/v) glycerol, 20 mM imidazole. Samples were purified using HiTrap chelating columns (GE Healthcare) precharged with nickel sulfate. Size exclusion chromatography was performed using a Superdex 75 16/60 column (GE Healthcare) pre-equilibrated with 50 mM Tris-Cl, pH 7.5, and 150 mM NaCl for the DU constructs or a Superdex 200 16/60 column (GE Healthcare), pre-equilibrated with 50 mM Tris-Cl, pH 7.5, 300 mM NaCl, and 1% glycerol for the FL-USP11.

### Phage display and biopanning

The peptide phage display library used for biopanning experiments (kindly provided by Professor Franco Felici, University of Molise, Italy; designated PC89VIII) is based on the PC89 phagemid vector (50) with a nonapeptide inserted at the N-terminus of the phage major coat protein pVIII and a reported diversity of approximately 10<sup>7</sup>. The library was transformed into *E. coli* TG1 supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5(rK-mK) [F' traD36 proAB lacIqZΔM15] which was the strain used for all manipulations and phage rescue. Libraries were grown to mid-log phase (OD<sub>600</sub>~0.6), superinfected with M13KO7 helper phage (Pharmacia LKB) and incubated overnight at 30 °C (250 rpm) to produce phage particles. Phage was harvested and PEG precipitated only for the first panning round. For all subsequent selection rounds (3 in total) supernatant phage was used directly. Biopanning was carried out once as previously described (51) and proteins were immobilized on cobalt beads (Dynabeads) (solution phase panning). A total of 100 μg of USP11\_DU was immobilized for each round. In parallel, the phage isolated at each round against USP11\_DU were also bound against an unrelated His-tagged control protein under the same conditions. This control protein was a fragment of flagellin, a virulence factor from *E. coli* O157:H7, amino acid residues 164 to 495, the protein was used as it is unrelated to the target proteins and carried a C-terminal His-tag. Bound phage were washed 10 to 20x with PBS (10 mM phosphate buffer pH 7.2-7.4 with 150 mM NaCl) plus 0.1% (v/v) Tween (PBST) and then PBS. Bound phage were eluted from the beads with 100 mM triethylamine and neutralized in 1 M Tris pH 7.4. Half of the eluted phage was used for infection of TG1 cells, which were plated and used to make glycerol stocks.



**Deep sequencing**

The ssDNA of eluted phage for round 3 of selection against the USP11\_DU target and the control protein was extracted and purified using the QIAprep Spin M13 Kit. Subsequently, phage ssDNA was precipitated with ethanol and stored as a dry pellet to be used as a PCR template. For the amplification of the unknown peptide sequences, a two-step PCR strategy was followed as previously described (32). The strategy produced an amplicon of approximately 330 bp and each sample contained a unique barcoded sequence. Each sample's DNA concentration was determined by the Agilent 2100 Bioanalyser using the DNA 1000 kit. Based on the concentration readings for the peak of interest samples were pooled in equal amounts, purified by the Agencourt AMPure XP kit and run on a 2% (w/v) agarose gel. The band of ~330 bp was excised from the gel and purified using the NucleoSpin Gel and PCR Clean-up kit. Finally, the concentration and size of the pooled DNA was measured on a Bioanalyser. NGPD was carried out once for each round of panning.

**Ion torrent data file processing and sorting of sequences**

Deep sequencing of all samples was performed using an Ion Torrent PGM technology commercial service by the Department of Biochemistry at the University of Cambridge on a 318 chip. Approximately 500 Mbase of readings were obtained per chip, which translates to  $\sim 10^5$  reads on average per barcode and a mean length of 100 bp. The number of meaningful reads per panning round for each sample was found to be approximately  $4 \times 10^4$ . Subsequently, processing of all sequences in quality FASTQ file format was conducted. The first processing step consisted of the demultiplexing of the FASTQ file in order to sort the sequences according to their barcodes and generate individual FASTQ files, each representing the repertoire of peptides eluted from one of the panning rounds binding to either the target protein or the control protein. Each FASTQ file was then converted to FASTA format, translated in all three reading frames and concatenated into one file containing all frames. Using Perl scripts, only sequences of interest, flanked between conserved PC89 gpVIII protein sequences (AEGEF and DPAKAA motifs were used), were kept in a single file. The peptide sequence repertoires of eluted

polyclonal phage binding to either the protein of interest or the control protein were then compared. Ultimately, all sequences were exported to excel files with frequencies: the number of copies of the sequence isolated against the target protein and the number of copies of the same sequence isolated against the control protein. A two proportion Z test was then used to compare the sequence populations and sort them by Z scores which reflects the ratio as well as the absolute frequencies of each peptide sequence and allows their ranking by relative statistical importance (31). For peptide motif generation from the obtained sequences the MEME algorithm was used (33).

**Isothermal titration calorimetry (ITC)**

ITC data were measured on a MicroCal VP-ITC or a PEAQ ITC instrument to obtain the binding parameters for peptide ligand-USP11\_DU interactions. The sample cell contained 30  $\mu$ M protein in PBS buffer. Each peptide (at a concentration of 300 or 600  $\mu$ M) was titrated into the sample cell in 8  $\mu$ l (VP-ITC) or 2  $\mu$ l (PEAQ ITC) injections at a temperature of 25 °C. Spacing was typically 180 sec and a stirring speed of 300 rpm was used. The data were analysed using NITPIC (52), SEDPHAT (53) and GUSI software. ITC experiments were repeated at least twice independently apart from the USP15\_DU, USP4\_DU and the FL-USP11 titrations with FYLIR peptide, which were conducted once.

**Protein crystallization, data collection and structure determination**

Samples of the USP11 DUSP-UBL domains (residues 24-244) at concentrations of 4 and 8 mg/ml in 100 mM NaCl, 50 mM Tris-Cl, pH 7.5, 1% glycerol were mixed with the FYLIR peptide (AEGEFYKLIKIRTPR) at a ratio 1:4 and crystallization trays were set up by the sitting drop vapour diffusion method at 10 °C. Crystals grew within 3 days and the initial conditions were further optimized in separate trays varying the pH and the precipitant concentration. In 0.01 M tri-sodium citrate and 16% PEG6000 rectangular single crystals grew within a day. Crystals were flash cooled after soaking in a cryoprotectant solution of 0.01 M tri-sodium citrate, 16% PEG6000, 20% glycerol and 10% ethanediol. Crystals of the USP11\_DU-FYLIR complex diffracted to 1.3 Å resolution and a dataset was collected at beamline

I02 at Diamond Light Source, UK at a wavelength of 0.97949 Å and a temperature of 100 K. Data were processed using XDS (54) and AIMLESS (55) and the structure was solved by molecular replacement using individual DUSP and UBL domain coordinates from the human USP11\_DU structure at 3.0 Å (PDB code 4MEL (20)) as search model with PHASER (56). Data collection statistics are summarized in Table 1.

### **Model building, refinement and validation**

Model building and adjustments of the two molecules of the asymmetric unit, each binding to a peptide at the same location, were conducted using COOT (57). Structure refinement was carried out in PHENIX (58); data refinement statistics are shown in Table 1. The quality of the model was assessed by MOLPROBITY (59). The final model consists of two USP11\_DU molecules in the asymmetric unit that associate via an interface area of 812 Å<sup>2</sup>, but no tight packing at the interface occurs. Either 12 or all 14 residues of the FYLIR peptide were observed in each copy of the asymmetric unit. LigPlot<sup>+</sup> (60) was used to analyze the interactions of peptide residues with USP11\_DU. In the final model there are no Ramachandran outliers with 98.7% of residues located in favored regions. Refinement statistics are summarized in Table 1. All structure figures were generated with Pymol ([www.pymol.org](http://www.pymol.org)).

### **Cell Lines**

Cell lines (human glioblastoma KNS-42 and U87, cervical carcinoma HeLa, mammary carcinoma MCF7, pancreatic carcinoma Panc1, and fibroblast NIH3t3 and BJ6 cell lines) except KNS-42 were cultured at 37 °C in 5% CO<sub>2</sub> in Dulbecco's modified Eagle's media (DMEM; Sigma), supplemented with 10% (v/v) Fetal Calf Serum (FCS, Sigma), 4.5 g/L D-Glucose, 2 mM L-glutamine and 100 units/ml penicillin and 100 units/ml streptomycin (Invitrogen). KNS-42 cells were cultured in DMEM/F-12 (1:1). Cell passage was carried out using 0.05% (w/v) trypsin-EDTA (Invitrogen). U20S-DR3-GFP reporter cell lines for homologous recombination were a kind gift from Jeremy Stark (City of Hope, Duarte, CA).

### **DNA repair reporter assays**

U20S reporter cell lines were simultaneously co-transfected with siRNA using Dharmafect1

(Dharmacon) and DNA (RFP-USP11 or RFP-USP11<sup>L208F, S242R</sup> and ISceI endonuclease expression constructs) using FuGene6 (Promega). The media was replaced after 16 hours and cells were grown for a further 48 hours before harvesting with trypsin and fixation in 2% paraformaldehyde (Sigma) in PBS. Using a CyAn flow cytometer, a minimum of 10,000 cells were analysed for each sample and RFP and GFP double positive cells were scored and normalised to RFP-transfection efficiency. Each individual experiment contained three technical repeats. Graphs shown are combined data from six independent experiments and error bars show SEM.

### **GET-FYLIR peptides**

Synthetic peptides were obtained from Biomatik or PeptideSynthetics. These harboured an N-terminal red-fluorescent 5-Carboxytetramethylrhodamine (TAMRA) modified GET sequence composed of P21 (KRKKKGKGLGKKRDPCLRKYK) and 8R (RRRRRRRR) fused to the FYLIR peptide (AEGEFYKLIKIRTPR), to create GET-FYLIR (TAMRA-KRKKKGKGLGKKRDPCLRKYKRRRRRRRR AEGEFYKLIKIRTPR-NH<sub>2</sub>). In the equivalent scrambled GET control peptide the FYLIR sequence was replaced by the scrambled sequence **APKFEIRRGTYKLE** (GET-Scrambled).

### **Cell uptake**

Cells treated with GET-FYLIR or GET-Scrambled were analysed on an Astrios Cell sorter using a 488 nm green laser (40,000 cells; gated on live cells by forward/side scatter). Gmean was used as the average cell intensity. All data sets were combined for the statistical analysis. A Sidak's multiple comparisons test was applied. N refers to the number of biological replicates.

### **Confocal microscopy**

HeLa cells were plated onto 10 mm diameter glass coverslips (1x10<sup>5</sup> cells/coverslip). Cells treated with GET-FYLIR were fixed for 10 min in 3.7% (w/v) paraformaldehyde (Sigma), stained with Hoechst 33342 1 µg/ml (Molecular Probes) for a further 10 min, washed and mounted. Cells were imaged using a LSM880C confocal microscope (Zeiss, Germany). A 63x objective lens was used with a 488 nm laser used for Hoechst 33342 and a

561 nm DPSS laser for GET-FYLIR transduced cells. Images were captured and processed using ZEN software (Zeiss, Germany).

#### ***Cell viability***

Cells were plated in 96-well plates ( $2.5 \times 10^4$  cells/well) and treated with GET-FYLIR (0-50  $\mu$ M) for 24 hours. Cells were also treated with GET-Scrambled as a control. After treatment, cells were washed with PBS and then incubated for 15 min at 37 °C with a 100  $\mu$ l Presto Blue solution (10% Presto Blue, Invitrogen in HBSS, Sigma). Change in the fluorescence was measured using a plate reader with the excitation/emission wavelengths set at 560/590 nm (Infinite® 200 PRO, TECAN), with untreated cells used as a control. All data sets were combined for the statistical analysis. A Sidak's multiple comparisons test was applied. N refers to the number of biological replicates.

#### ***Cell cycle analysis***

Cells were plated in 24 well plates ( $1 \times 10^5$  cells/well) and treated with GET-FYLIR (20  $\mu$ M)

for 24 hours. The cells were treated with trypsin, detached from the plate and centrifuged at 200 g for 5 min. The supernatant was aspirated and the cells were resuspended in PBS. The cells were fixed in 70% ethanol for 1 hour at 4 °C. The cells were washed with PBS and incubated in propidium iodide staining solution (0.1% (v/v) Triton X-100, 10  $\mu$ g/ml PI (Sigma) and 100  $\mu$ g/ml DNase-free RNase A in PBS) for 30 min at room temperature. Cells were analyzed using an FC500 flow cytometer (Beckman Coulter) equipped with a 488 nm laser, data from at least 10000 cells were acquired for each sample. Cells were gated based on forward and side scatter and doublets excluded by height/area analysis. Cell cycle percentages were calculated by curved fit using Weasel V3.0 (Walter and Eliza Hall Institute of Medical Research). Data shown are representative. All data sets were combined for the statistical analysis. A Sidak's multiple comparisons test was applied. N refers to the number of biological replicates. All curves and statistical analyses were produced using Prism 7 (GraphPad Software).

**Acknowledgements:** Crystallographic data were obtained with the support of Diamond Light Source, UK and the European Synchrotron Radiation Facility (ESRF), Grenoble, France. We are grateful to colleagues at ESRF for providing assistance in using beamline ID23-2 and at Diamond in using beamline I02 and colleagues at the Advanced Data Analysis Centre, University of Nottingham for advice on bioinformatics data analysis. We also thank the University of Birmingham Tech Hub Facility for FACS support. Coordinates and structure factor files of the USP11\_DU – FYLIR peptide complex have been deposited in the Protein Data Bank under accession code 5OK6.

**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article.



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**FOOTNOTES**

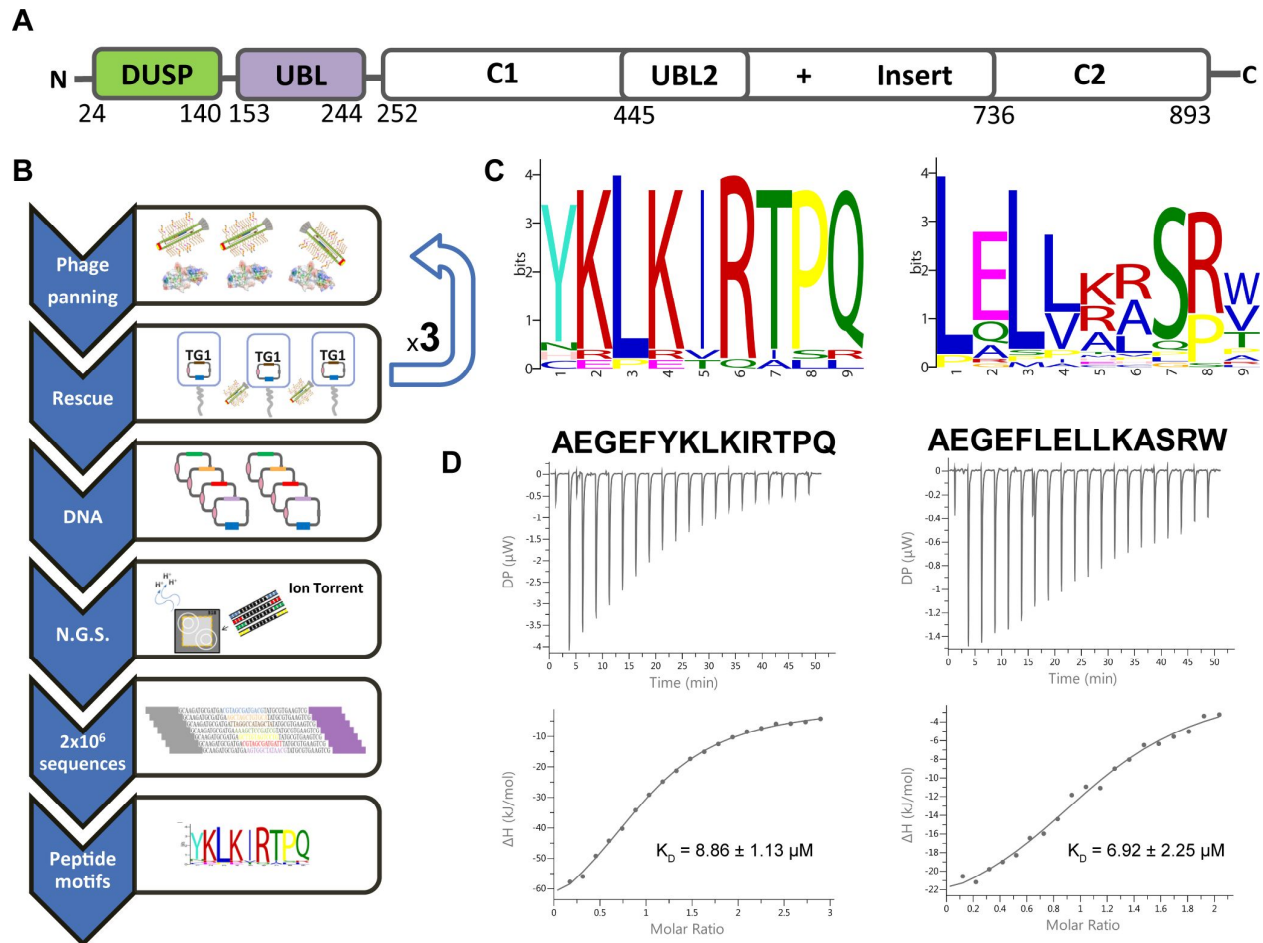
This work was supported by the Biotechnology and Biological Sciences Research Council [grant number BB/H012656/1], to ID. JRM is HEFCE funded. AS was supported by a PhD Studentship funded by the University of Nottingham. RMD was funded by Cancer Research UK [grant number C8820/A19062].

The abbreviations used are: USP, ubiquitin specific protease; NGPD, next generation phage display; ITC, isothermal titration calorimetry; UBL, ubiquitin-like; DUSP, domain present in USPs

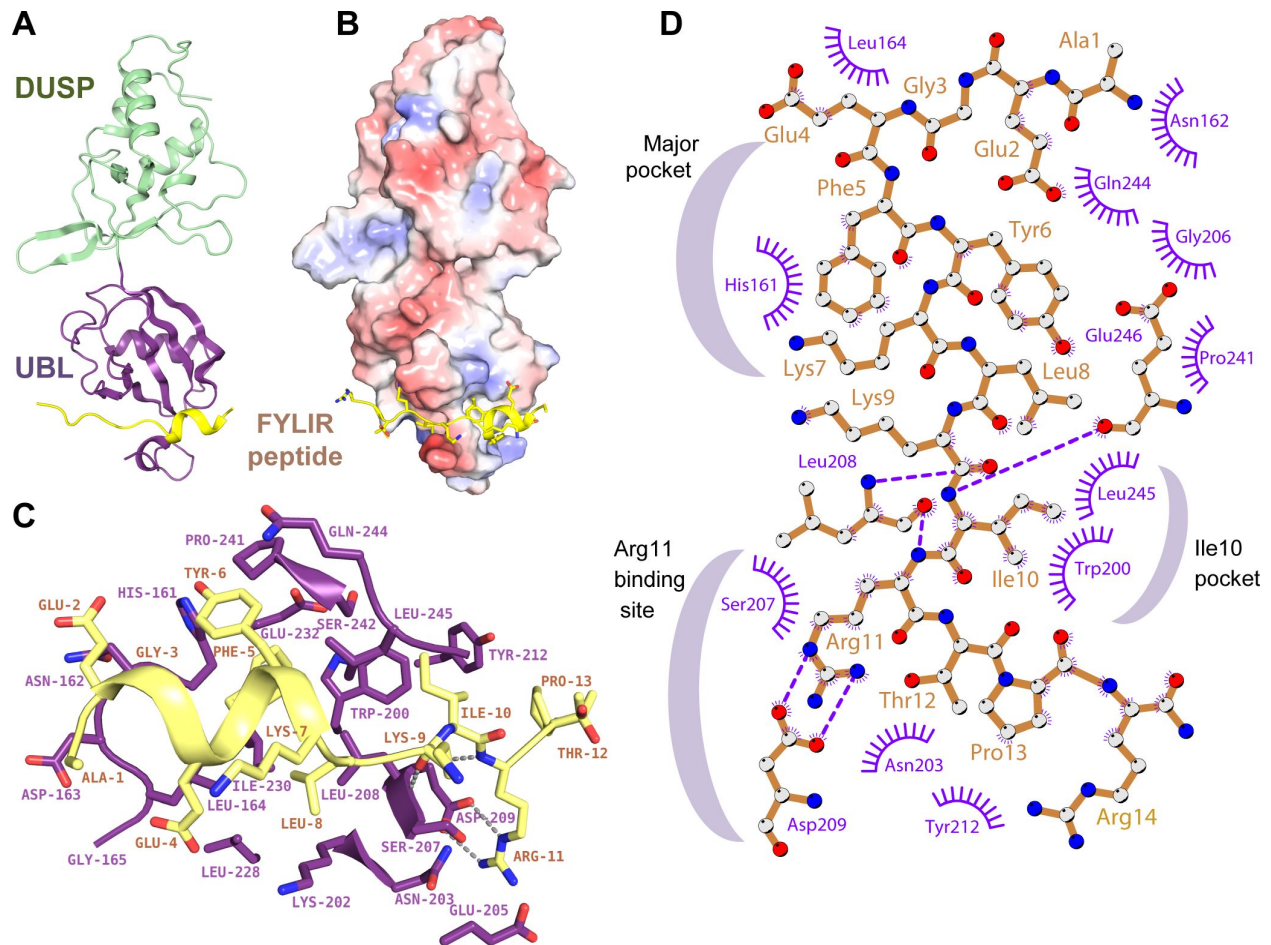
Table 1 Crystallographic data collection and refinement statistics

	USP11_DU-FYLIR (AEGEFYKLLKIRTPR)
<b>Data collection</b>	
Space group	P2 <sub>1</sub>
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	65.77, 45.51, 100.61
β (°)	102.68
Resolution (Å)	1.30
<i>R</i> <sub>merge</sub>	0.054 (0.678)*
<i>R</i> <sub><i>pin</i></sub>	0.027 (0.409)
<i>I</i> / σ <i>I</i>	15.1 (2.1)
CC1/2	0.999 (0.799)
Completeness (%)	98.3 (89.6)
Redundancy	4.1 (3.4)
Wilson B-factor (Å <sup>2</sup> )	13.8
<b>Refinement</b>	
Resolution range (Å)	49-1.35
No. of reflections	140596 (13740)
<i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub>	0.157/0.178
No. atoms	
Protein	3986
Non-peptide ligand	38
Water	649
<i>B</i> -factors (Å <sup>2</sup> )	
Protein	19.66
Peptide	28.07
Water	32.36
R.m.s. deviations	
Bond lengths (Å)	0.015
Bond angles (°)	1.35

\*Values in parentheses are for highest-resolution shell.



**Figure 1. Discovery of USP11 binding sequences.** (A) Schematic representation of the human USP11 domain structure. The N-terminal DUSP-UBL domains used as bait in the next generation phage display (NGPD) experiments (USP11\_DU) are labelled and depicted in green and purple, respectively. (B) Flow chart of the NGPD approach. Three iterative rounds of phage selection (panning) against USP11\_DU were carried out and the eluted phage were bound to the target (USP11\_DU) and an unrelated control protein in parallel. The phagemid vectors from the output phage isolated against the target and control proteins were isolated and the DNA region encoding the peptides was amplified and deep sequenced. Peptide sequences seen to be enriched against the target protein compared to the control were listed and motifs identified. (C) Amino acid sequence motifs identified by the MEME algorithm after the third round of biopanning. (D) ITC data of USP11\_DU with FYLR (AEGEFYKLRTPQ) and LxLL (AEGEFLELLKASRW) peptides. Thermograms (top) and binding isotherms (bottom) fitted using a one binding site model with associated  $K_D$  values are shown. DP, differential power.

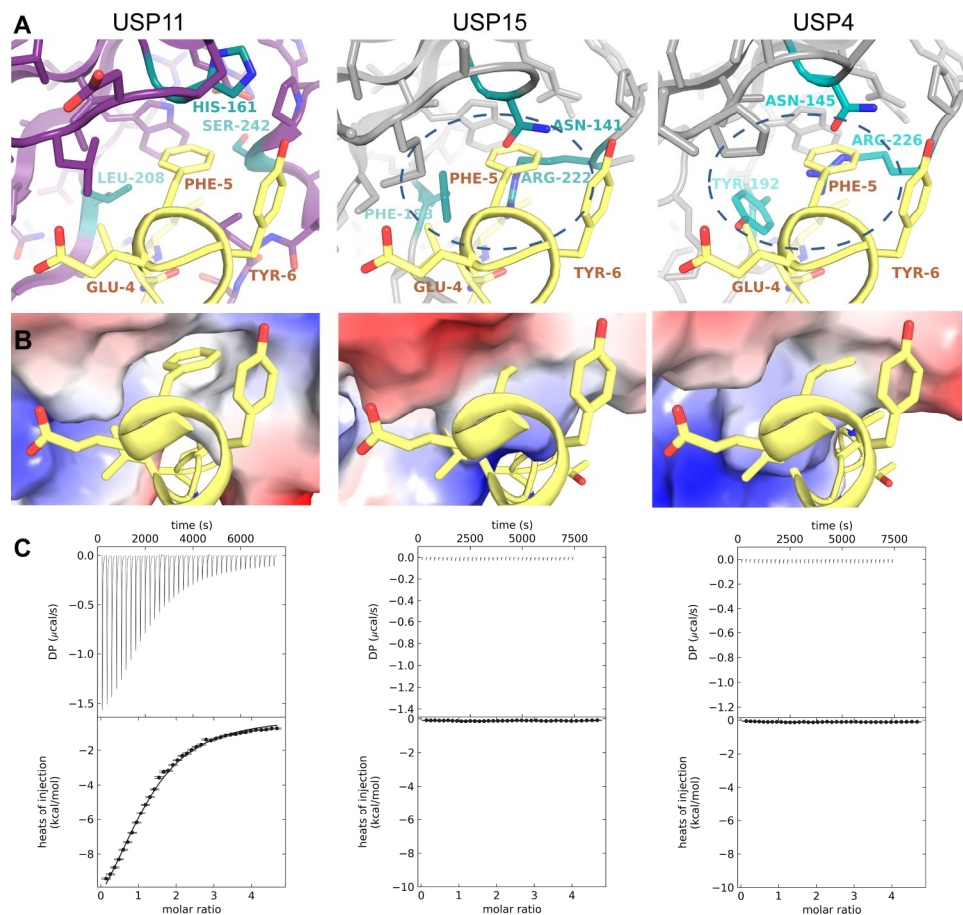


**Figure 2. Molecular basis of USP11-FYLIR peptide interaction** (A) Cartoon representation of the USP11\_DU-FYLIR peptide complex crystal structure. The USP11 DUSP and UBL domains are depicted in green and purple, respectively with the FYLIR peptide (AEGEFYKLRTPR) shown in yellow (B) Electrostatic potential surface representation of USP11\_DU in complex with the FYLIR peptide in yellow cartoon representation. Side chains are shown as sticks in the same orientation as in A. (C) Close-up view of the molecular basis of the interactions. The peptide is bound to the USP11 UBL domain, with residues 5-12 predominantly contributing to the interaction. Key residues involved in the interaction are labelled and shown as sticks. Colour code is the same as in A. (D) Schematic representation of FYLIR peptide-USP11 interactions generated using Ligplot+ (60). Peptide residues are labelled in brown, whereas USP11 residues are labelled in purple. Hydrogen bonding interactions are indicated as purple, dashed lines and USP11 residues engaging in hydrophobic interactions with the peptide are depicted in purple. Crescent shapes indicate USP11 binding pockets involved in FYLIR peptide binding.

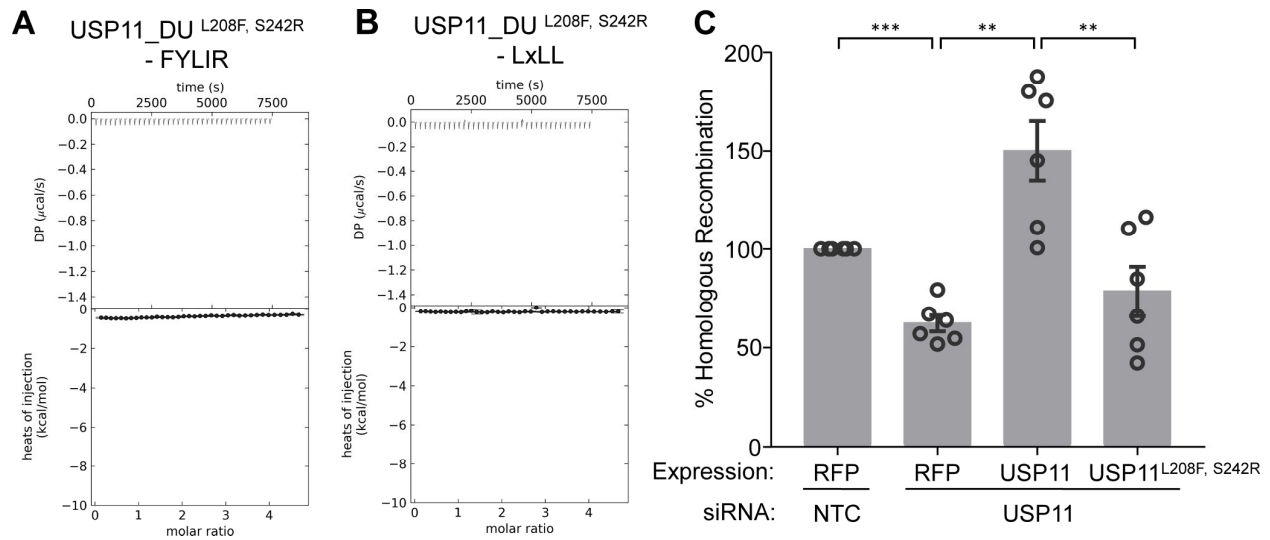




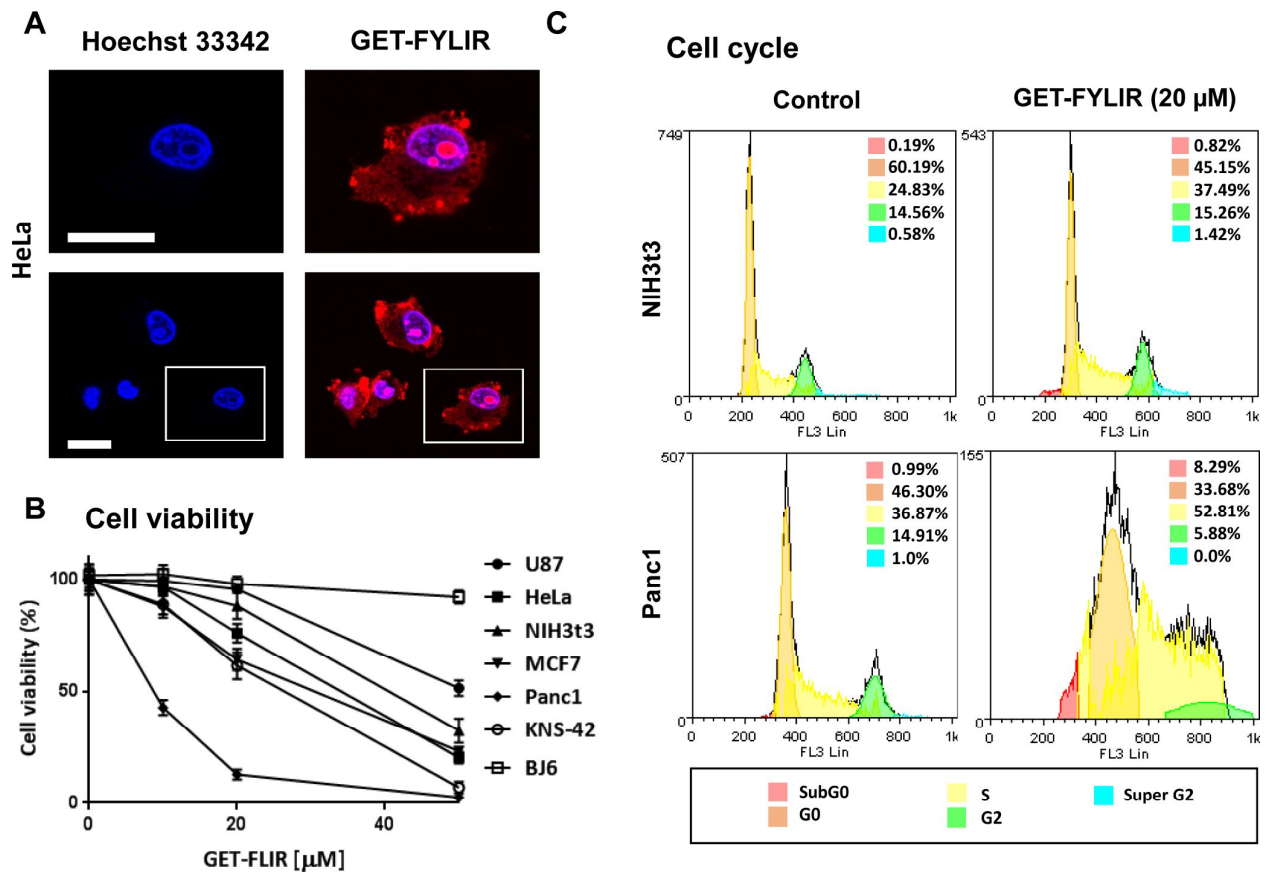
**Figure 3. Sequence alignment of USP11, USP4 and USP15 DU domains.** Structure-based sequence alignment using PROMALS3D (61) of human USP11 (PDB code 4MEL (20)), USP15 (PDB code: 3T9L (21)) and USP4 (PDB code: 3JYU, SGC, unpublished) with secondary structure elements above the sequences indicated (e=strand; h=helix). The DUSP domain is shaded green, whereas the UBL domain is shaded in purple. Sequence conservation is depicted as per PROMALS3D default representation (bold and uppercase letters (such as G); aliphatic residues (I, V, L): l; aromatic residues (Y, H, W, F): @; hydrophobic residues (W, F, Y, M, L, I, V, A, C, T, H): h; alcohol residues (S, T): o; polar residues (D, E, H, K, N, Q, R, S, T): p; tiny residues (A, G, C, S): t; small residues (A, G, C, S, V, N, D, T, P): s; bulky residues (E, F, I, K, L, M, Q, R, W, Y): b; positively charged residues (K, R, H): +; negatively charged residues (D, E): -; charged (D, E, K, R, H) with the exception that identical residues are indicated using an asterisk symbol. USP11 UBL domain residues located at the interface upon peptide binding are highlighted in red.



**Figure 4. Molecular basis of FYLIR peptide USP11 specificity** (A) Close-up views of FYLIR peptide binding to the ‘major pocket’ in the USP11 UBL domain (left panel) compared to USP15 (centre panel) and USP4 (right panel), where steric clashes occur when the peptide is modelled into the same position (highlighted by a dashed ellipse). Key residues involved in the interaction (USP11) or preventing peptide binding (USP15, USP4) are depicted in cyan and labelled. (B) Close-up views of electrostatic surface representations of FYLIR peptide binding to the ‘major pocket’ in the USP11 UBL domain (left panel) compared to USP15 (centre panel) and USP4 (right panel), where the binding pocket is occluded. (C) ITC data of FYLIR peptide with USP11\_DU, USP4\_DU and USP15\_DU, showing that peptide-ligand binding is highly specific for USP11.



**Figure 5. Effects of a UBL binding site mutant on peptide recognition and homologous recombination** (A) ITC data of the USP11\_DU<sup>L208F, S242R</sup> double mutant that mimics USP15 showing that binding of the FYLIR peptide ligand is completely abolished upon mutating residues in the UBL pocket (referred to as major pocket). (B) ITC data of titrations of peptide AEGEFLRLLNFTKP harbouring motif 2 (LxLL) with USP11\_DU<sup>L208F, S242R</sup>. The interaction of this peptide ligand with USP11 is also abolished upon mutating residues in the UBL pocket. (C) Homologous Recombination GFP-reporter assays with USP11 siRNA and RFP-USP11 WT or mutant USP11<sup>L208F, S242R</sup>. Each individual experiment contains three technical repeats. Data presented is the overall mean calculated from the means of each individual experiment (n=6). % GFP and RFP double positive cells were normalised to RFP transfection efficiency; error bars = SEM; p-values were computed using the Welch's T-test and are shown as \*\*\* p<0.0005, \*\* p<0.005 and \* p<0.05 (NTC v siUSP11: p=0.0003; siUSP11 v siUSP11+WT: p=0.0017; siUSP11 v siUSP11+USP11<sup>L208F, S242R</sup>: p=0.0049).



**Figure 6. Cellular effects of FYLIR peptide agent.** (A) GET-FYLIR has cytoplasmic, nuclear and nucleolar localization. HeLa cells were treated with GET-FYLIR (10  $\mu$ M) for 24 h and assessed by confocal microscopy (nuclei were counterstained with Hoechst 33342). The white scale bar is 20  $\mu$ m. (B) GET-FYLIR decreases cell viability cell-type specifically and dose dependently. Cell viability (Presto Blue assay) was assessed after 24 h incubation with GET-FYLIR (0, 10, 20 and 50  $\mu$ M) in KNS-42, U87, BJ6, HeLa, NIH3t3, MCF7 and Panc1 cells. Cell viability was expressed as percentage of cell viability  $\pm$  s.d. (n=3 biological repeats) (C) GET-FYLIR induces S phase arrest cell-type specifically. Cell cycle analysis was conducted using NIH3t3 cells (n=5 biological repeats) and Panc1 (n=7 biological repeats) cells untreated or incubated with GET-FYLIR (20  $\mu$ M) for 24 h. Cells were fixed, stained with propidium iodide staining solution and analysed for DNA content. The distribution and percentage of cells in SubG0, G0, S, G2 and super G2 phase of the cell cycle are indicated.

**Discovery of peptide ligands targeting a specific ubiquitin-like domain-binding site in the deubiquitinase USP11**

Anastasios Spiliotopoulos, Lia Blokpoel Ferreras, Ruth M Densham, Simon G Caulton, Ben C Maddison, Joanna R Morris, James E Dixon, Kevin C Gough and Ingrid Dreveny

*J. Biol. Chem.* published online October 29, 2018

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Access the most updated version of this article at doi: [10.1074/jbc.RA118.004469](https://doi.org/10.1074/jbc.RA118.004469)

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