Supplementary figures

Figure S1: RFP-substrates and RFP-substrates-Ub chimeras. The different substrates were cloned with an N terminus His-RFP. A TEV recognition site was inserted between the RFP and the substrate. A second set of substrates also contained a C-terminus fusion of wild type ubiquitin.

Figure S2: The linker length between the RFP and the ubiquitin domains in RFP-Ub does not affect the ubiquitination efficiency. RFP-Ub variants with different linker length were ubiquitinated by E2B, E2D2, E2D3, E2D4 and E2T as described in Figure 2. Reactions with the indicated E2s were conducted on RFP-20AA-Ub, RFP-40AA-Ub and RFP-80AA-Ub and analyzed by Western blot **(A)** and coomassie staining **(B)**.

Table S1: The distance between the E2 recruitment site and the substrate does not alter the ubiquitination properties. Mass Spectrometry analysis of ubiquitination reactions performed with RFP-Ub variants (RFP-Ub, RFP-20AA-Ub, RFP-40AA-Ub and RFP-80AA-Ub) are presented in Figure 2. Ubiquitination occurred on both the RFP and Ub portions in all variants by all examined E2s.

Figure S3: Ribbon cartoon for the RFP-Ub chimeras. The crystal structures of RFP and Ubiquitin were fused *in-silico* to generate a schematic representation of the RFP-Ub chimeras. A TEV recognition site was inserted between the RFP and the ubiquitin portions. The ubiquitin domain was linked directly to the TEV recognition sequence (no linker) or through a stretch of 20, 40 and 80 amino acids extracted from a region predicted to be unstructured in b-casein. The lysine residues found to be ubiquitinated (Table S1) in the chimeras are depicted.

Figure S4: The 80AA casein linker does not contain a cryptic site that recruit the E2 directly. RFP-80AA lacking the C-terminus domain was generated as a control to eliminate the possibility that the linker acts as a cryptic site in recruiting the E2. Ubiquitination was performed on RFP-80AA and RFP-80AA-Ub in the presence of the indicated E2s. Reactions were analyzed by Western blot with anti ubiquitin.

Figure S5: Double titration experiment of RFP-HERPc ubiquitination by E2G2 and gp78c. (A) Initially, ubiquitination was conducted on RFP-HERPc in the absence **(left panel)** and presence **(right panel)** of increasing gp78c concentration. After the optimal gp78c concentration was determined (indicated with an arrow), a titration of RFP-HERPc ubiquitination with a growing concentration of E2G2 was conducted. The optimal concentration of E2G2 with gp78c is indicated with an arrow. All of the reaction mixtures were boiled in sample buffer, separated on SDS-PAGE and western blotted with anti ubiquitin antibody.

Figure S6: MS data for the BARD/BRCA ubiquitinations used to generate Table1. In support of data shown in Table 1, MASCOT derived protein views and MS/MS spectra for representative samples are presented according to the numbering given in the supplementary table. All peptide species of the substrate protein identified in the MASCOT search are listed, followed by a representative MS/MS spectrum for each detected ubiquitinated peptide species (GlyGly modification on the indicated lysine). The identified b and y fragment ions matched to the MS/MS data are indicated in the MS/MS spectrum and also as bold red numbers in the table. The following fragment ions are indicated: b: N-terminal fragment; b^{++}: b doubly charged; b*: b-NH_3; b^{*++}: b* doubly charged; b^0: b-H_2O; b^{0++}: b^0 doubly charged; y: C-terminal fragment; y^{++}: y doubly charged; y*: y-NH_3; y^{*++}: y* doubly charged; y^0: y-H_2O; y^{0++}: y^0 doubly charged. The second table shows a list of alternative sequence interpretations per MS/MS spectrum with corresponding peptide scores. **(B)** Results of orbitrap analyses. All

detected peptide species are shown for samples listed in the preceding table. Ubiquitinated peptide species are marked with a pounds symbol (#).

Figure S7: MS data for the p53/MDM2/MDMX ubiquitinations used to generate in Table 2. (A) Ion Trap MS data. MASCOT derived protein views and MS/MS spectra for representative samples RFP-p53/E2D2/MDM2, MDMX (#7), RFP-p53-Ub/E2D3/MDM2 (#14) and RFP-p53-Ub/E2N/E2V1/MDM2 (#15) are presented. All peptide species of the substrate protein identified in the MASCOT search are listed, followed by a representative MS/MS spectrum for each detected ubiquitinated peptide species (GlyGly modification on the indicated lysine). The identified b and y fragment ions matched to the MS/MS data are indicated in the MS/MS spectrum and also as bold red numbers in the table. The following fragment ions are indicated: b: N-terminal fragment; b^{++}: b doubly charged; b*: b-NH_3; b^{*++}: b* doubly charged; b^0: b-H_2O; b^{0++}: b^0 doubly charged; y: C-terminal fragment; y^{++}: y doubly charged; y*: y-NH_3; y^{*++}: y* doubly charged; y^0: y-H_2O; y^{0++}: y^0 doubly charged. The second table shows a list of alternative sequence interpretations per MS/MS spectrum with corresponding peptide scores. **(B)** Results of orbitrap analyses. All detected peptide species are shown for samples listed in the preceding table. Ubiquitinated peptide species are marked with a pounds symbol (#).

Figure S8: Comparison of the MS peaks corresponding to the unique peptides generated upon trypsinization of K48 and K11 ubiquitin conjugates. The peaks magnitude is an indicative of relative increase or decreased in the amounts of K11 and K48 conjugates and is attributed, in the current example, to the E2 enzyme used, as well as the presence or absence of MDMX.

Figure S9: RFP-p53 ubiquitination in the presence of MDM2 and MDMX. RFP-p53 was subjected to ubiquitination with the indicated E2s in the presence of MDM2 alone **(left panel)** or a mixture of MDM2 and MDMX **(right panel)**. Reaction mixtures were boiled in sample buffer, separated on SDS-PAGE and western blotted with anti ubiquitin. Similar up-scaled reactions were analyzed by mass spectrometry and are presented in Table 2.

Figure S10: Raw MS data for the RFP-Ub 20/40/80AA linker ubiquitinations. MS analysis was performed using an Orbitrap and all the detected peptides are presented. Ubiquitinated peptides are marked with a pound symbol (#).

Figure S11: Ion trap MS data for the HERP/gp78 ubiquitinations. All detected peptides for the regarding substrate protein are listed in the MASCOT derived protein view for samples RFP-HerpC/E2G2/gp78 (#11) and RFP-HerpC-Ub/E2G2/gp78 (#14). MS was performed using a high capacity ion trap.

Figure S12: Raw MS data for the p53/N/V1/MDM2/MDMX ubiquitination. Since non canonical (K11) ubiquitinations have been detected in this setting, the MS chromatography as well as MS/MS spectra are presented.

Figure S13: Sequence of fusion proteins RFP-p53-Ub and RFP-BARD-BRCA-Ub. The positions of all detected modified lysine residues in the fusion proteins are indicated, followed by their position in the human sequence of the regarding protein as defined in the SwissProt database (in brackets).