Targeted mass spectrometry enables robust quantification of FANCD2 mono-ubiquitination in response to DNA damage

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Abstract

The Fanconi anemia pathway is an important coordinator of DNA repair pathways and is particularly relevant to repair of DNA inter-strand crosslinks. Central to the pathway is monoubiquitination of FANCD2, requiring the function of multiple proteins in an upstream Fanconi core complex. We present development and analytical characterization of a novel assay for quantification of unmodified and monoubiquitinated FANCD2 proteoforms, based on peptide immunoaffinity enrichment and targeted multiple reaction monitoring mass spectrometry (immuno-MRM). The immuno-MRM assay is analytically characterized using fit-for-purpose method validation. The assay linear range is >3 orders of magnitude with total repeatability <16% CV. In proof-of-principle experiments, we demonstrate application of the multiplex assay by quantifying the FANCD2 proteoforms following mitomycin-c treatment in an isogenic pair of *FancA*-corrected and uncorrected cell lines, as well as primary peripheral blood mononuclear cells from Fanconi Anemia patients. Additionally, we demonstrate detection of endogenous FANCD2 monoubiquitination in human breast cancer tissue. The immuno-MRM assay provides a potential functional diagnostic for patients with Fanconi Anemia with defects in the upstream FA complex or FANCD2, and a potential test for predicting sensitivity to DNA cross-linking agents in human cancers.
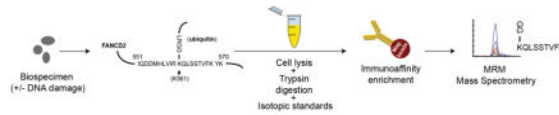
Graphical Abstract

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Conflict of Interest statement

A.G.P. is the founder of Precision Assays.

Keywords

Fanconi anemia; multiple reaction monitoring; DNA damage response; immuno-MRM

1. Introduction

Fanconi anemia (FA) is an inherited disorder characterized by progressive bone marrow failure and an increased risk of cancers, especially during early adulthood. FA patients harbor mutations in a set of genes encoding the FA pathway, a network of 21 proteins that is specialized for repairing DNA inter-strand cross-links [1]. Activation of the FA core complex results in the monoubiquitination of FANCD2 and FANCI, which interact with several downstream components (e.g. BRCA1, Rad51, BRCA2) for DNA repair [2]. Cells with mutations in any of the core complex proteins lack the ability to monoubiquitinate FANCD2, making FANCD2 ubiquitination a convergence point in the pathway, with an estimation of >90% FA patients defective in this step [1,3]. FA patients experience excessive toxicities with chemotherapy or radiation, so early diagnosis is essential to inform therapeutic decisions [4]. Additionally, somatic mutations In FA genes render tumor cells sensitive to DNA crosslinking agents, so identification of FA pathway defects provides an opportunity for therapeutic targeting [5–7].

Diagnosis of FA based on presenting symptoms alone is difficult and unreliable due to the broad phenotypic spectrum of FA and overlapping features with other genetic (and nongenetic) diseases. A significant subset of FA patients lack characteristic physical findings [8]. Currently, the standard for FA diagnostic testing is manual enumeration of chromosomal breakage following exposure to clastogens [9,10]. In this test, T lymphocytes in peripheral blood are stimulated with phytohaemagglutinin (PHA) to proliferate in the presence or absence of DNA cross-linking agents, typically mitomycin C (MMC) or diepoxybutane (DEB), followed by counting chromosomal breaks and fusions in metaphase spreads. Chromosomal breakage may also be assessed in skin fibroblasts, since somatic mosaicism may result in a falsely negative blood test. The approach is laborious and difficult to standardize, partly because results are dependent on several variables (e.g. culture time, exposure time, concentration, threshold for positive/negative). Molecular subtyping by DNA sequencing or complementation analysis is increasingly utilized clinically, though interpretation is complicated by variants of unknown significance [11], ascertainment of biallelic mutation distribution, and distinguishing constitutional versus somatic mutations [12,13].

Since pathogenic mutations in FA genes functioning upstream of the FANCD2 complex impair FANCD2 monoubiquitination, an assay to directly quantify the level of monoubiquitinated FANCD2, and thus determine defects in the pathway, would provide a complementary functional readout to existing assays. However, testing of FANCD2

monoubiquitination with traditional immunoassays or intracellular localization assays are low throughput and semi-quantitative [3,14], establishing a need for a more effective quantitative assay.

Targeted, multiple reaction monitoring mass spectrometry (MRM) is a quantitative approach capable of quantifying analyte peptides with high sensitivity and specificity [15,16]. MRM has been used in clinical laboratories for decades to measure small molecules [17], and is increasingly applied in protein and peptide measurement [18–21]. The *targeted* MRM mode of proteomic analysis is different from untargeted ("shotgun") discovery proteomics, where methods are designed to profile as many peptides as possible by matching MS/MS spectra to protein databases. In contrast, for MRM, protein lysates from biospecimens are proteolyzed (typically with trypsin), and proteotypic peptide sequences *unique to the protein* of interest ("proteotypic peptides") are *targeted* for detection by MRM mass spectrometry (Figure 1A). Instrument and method parameters are optimized to enable the most sensitive and specific measurement of the targeted peptide as a surrogate for the protein of interest. Furthermore, a synthetic, HPLC-purified, stable isotope-labeled version of the analyte peptide is spiked into the biospecimen at a known concentration as an internal standard. The internal standard peptide serves several functions, including: i) to control for much of the preanalytical and all of the analytical variation in the assay, ii) to enable precise, relative quantification of the endogenous analyte, and iii) to help confirm specificity of the assay. The high specificity of MRM-based assays is based on detection of multiple MRM transitions (i.e. combinations of precursor/fragment ions), the chromatographic co-elution of analyte and internal standard, and the consistency of the relative transition ratios between the endogenous and standard peptides. In addition to being highly specific, MRM is standardizable and transferrable across laboratories [22–25], is capable of highly multiplexed measurements [25,26], and is capable of quantifying cell signaling events through chromatographic enrichment of low abundance analytes (e.g., ubiquitinated or phosphorylated peptides) [27–30]. A public repository of highly characterized targeted MS assays has been created [31], and the assay platform is beginning to gain acceptance in clinical laboratories [32,33]. For example, an MRM-based assay targeting a single proteotypic peptide sequence unique to the human thyroglobulin protein is used by clinical laboratories as a surrogate to quantify thyroglobulin (2768 amino acids, ~300kDa) in patients that make autoantibodies that interfere with conventional immunoassays [34,35]. Other examples include similar assays developed for apolipoproteins [36] and troponin [37].

MRM-based assays have several advantages over conventional protein measurement technologies (e.g., Western blotting, immunohistochemistry, ELISA), such as direct detection of the analyte by a mass spectrometer (contributing to high specificity), the use of internal standards (enabling reproducibility and inter-laboratory standardization), the capability to readily multiplex analysis of many target peptides, a large linear range (typically 10^3), and relatively less time and cost required for assay development [38]. In MRM, cross reactivity of antibodies usually does not affect specificity, because the mass spectrometer directly detects the target of interest, and interferences are detected based on analytical criteria [39]. In contrast, Western blots (WB) can feature multiple nonspecific bands and uncertainties about which band represents the analyte of interest. While WB is semi-quantitative at best and has not been standardized across laboratories, MRM uses

HPLC-purified, synthetic, internal stable isotope-labeled standard peptides (spiked into the biospecimen at a known concentration) for standardization across laboratories [22–24].

We recently demonstrated the utility of peptide immuno-MRM (coupling immuno-affinity enrichment of peptides to MRM) to quantify the pharmacodynamics of post-translationally modified (e.g. phosphorylated and ubiquitinated) peptides in response to DNA damage [28,30]. Here, we present a multiplex immuno-MRM assay for quantifying the unmodified and monoubiquitinated proteoforms of a proteotypic peptide unique to FANCD2 (K561). The assay is analytically characterized using fit-for-purpose method validation, including linearity, repeatability, and stability. Proof-of-principle application of the assay is demonstrated using human immortalized cell lines (congenic FancA+/−) and Fanconi Anemia patient primary human T cells treated with the DNA cross-linking agent MMC. We also demonstrate detection of monoubiquitinated FANCD2 in breast tumor tissue. The assay can be multiplexed with other immuno-MRM assays [28,30] to profile more comprehensively the DNA damage response in a variety of sample types and conditions, making it useful for a range of biomedical research studies.

2. Materials and Methods

See supplementary material for full materials and methods.

3. Results and discussion

3.1 Method development and parameter optimization

An overview of the workflow for the immuno-MRM assay is shown in Figure 1A. Development of the assay requires identification of a proteotypic peptide sequence (i.e. unique to the protein of interest) for measurement, generation of affinity reagents for enrichment of the targeted peptide, and optimization of analytical parameters for detection of the endogenous and synthetic peptide standards. To develop an assay, we first identified the target peptide analyte as the tryptic fragment surrounding the K561 site of ubiquitination on FANCD2. A blast search confirmed that this sequence is unique to the FANCD2 protein. Ubiquitination covalently attaches the ubiquityl moiety to the lysine at position 561, blocking the site from enzymatic digestion. Additionally, there is a trypsin cleavage site at the lysine residing on the ubiquityl moiety. Thus, following trypsin digestion, the modified protein produces a peptide encompassing amino acids 561–569 containing a –GG remnant on K561, and the unmodified protein produces a fully tryptic peptide of amino acids 562–569 (see Figure 1B). We sought to develop an immuno-MRM assay capable of measuring both forms of the peptide (monoubiquitinated and unmodified).

We followed established protocols to generate affinity reagents for use in peptide immunoaffinity enrichment [38,40]. The modified peptide containing the ubiquitin (i.e. –GG) remnant was used as the immunogen sequence in generating an anti-peptide rabbit monoclonal antibody. Using established protocols [40], monoclonal supernatants were screened against both forms of the peptide (modified and unmodified) to obtain a clone that was capable of immuno-precipitating both forms. Purified antibodies were subjected to quality control by BCA assay for protein concentration, SDS gel to confirm intact antibody,

and recovery efficiency in immuno-MRM enrichments to confirm antibody activity (data not shown).

Synthetic analyte peptides (i.e., HPLC-purified, heavy stable isotope-labeled versions of the modified and unmodified tryptic peptides) were used to optimize the mass spectrometer parameters [41]. Tandem mass spectrometry was used to analyze the HPLC-purified synthetic peptides to confirm the peptide identity and select the best combinations of precursor and fragment ions (i.e. transitions) to be used for identification and quantification of the peptide during MRM. Following best practices [16,42], the top 4–6 transitions were identified for testing in preliminary response curves in a background matrix of pooled lysates prepared from LCLs +/− 10 Gy ionizing radiation. For the final assay, we selected the 3 best transitions that were associated with the highest intensity and free from background noise and interferences. Collision energy was optimized for each transition to produce the most complete fragmentation (i.e. the highest intensities) [41]. Optimized transition ions and collision energies are reported in Supplementary Table 1. Consistent with best practices [39,43], the specificity for detection of the analyte was confirmed in all runs by detection of multiple transitions, co-elution of the analyte peptide and internal standard, and identical relative abundances of their transition ions.

3.2 Fit-for-purpose characterization: Response curves

The immuno-MRM assay targeting the FANCD2 analyte peptides was characterized using fit-for-purpose method validation to determine the assay performance and analytical figures of merit [21,44,45]. Response curves were generated in a background matrix consisting of an equal mixture of four components: LCL GM07057 + 10 Gy ionizing radiation (IR) (1 hr), LCL GM07057 + mock IR (1 hr), LCL GM01526 + 10 Gy IR (1 hr), LCL GM01526 + mock IR (1 hr) The curves were used to characterize the linear range, lower limits of quantification (LLOQ), and upper limits of quantification (ULOQ). All points were analyzed in triplicate with nine replicates for the blank samples. The linear range was > 3 orders of magnitude for both peptides (Figure 1C), with LLOQs of 1.4 and 0.7 fmol/mg for the ubiquitinated and unmodified peptides, respectively. Curves were linear at the highest concentration point measured, thus ULOQs were a minimum estimate of > 4000 fmol/mg, as were the linear ranges. Assay performance in the response curves is reported in Supplementary Table 2, and data are provided in Supplementary Table 3.

3.3 Fit-for-purpose characterization: Repeatability

Repeatability experiments were used to determine the expected intra-assay (within day) and inter-assay (between day) repeatability. Heavy peptides were spiked into 500 μg aliquots of pooled protein lysates from LCLs (GM07057/GM01526) +/− ionizing radiation (same pool of lysates used in response curves) at three concentrations (1.5, 150, 1500 fmol/mg) with constant concentration of light peptide (200 fmol/mg). Complete process triplicates (including tryptic digestion) were prepared and analyzed on five independent days. For the ubiquitinated peptide assay, the mean intra-assay variability was 6%, 7%, and 4% CV, and the mean inter-assay variability was 17%, 7%, and 10% for the low, medium, and high concentration samples, respectively. For the unmodified peptide assay, the mean intra-assay variability was 8%, 8%, and 7% CV, and the mean inter-assay variability was 8%, 9%, and

6% for the low, medium, and high concentration samples, respectively. Repeatability performance is reported in Supplementary Table 2 and data are provided in Supplementary Table 3.

3.4 Fit-for-purpose characterization: Peptide stability

Peptide stability in the processed samples was evaluated to determine safe handling conditions of the samples. Heavy peptides were spiked into 500 μg aliquots of pooled protein lysates from LCLs (GM07057/GM01526) +/− IR (same pool of lysates used in response curves) at 150 fmol/mg, and light peptides were added at 200 fmol/mg. Aliquots of the sample were enriched in duplicate. Control samples were analyzed immediately, and test samples were stored at 4°C in the autosampler for approximately 6 hours and 24 hours, with additional aliquots subjected to 1 freeze-thaw, 2 freeze-thaws, and storage at −80°C for approximately five months. Variation and percent difference relative to the fresh sample were evaluated (Supplementary Table 2 and data in Supplementary Table 3). All points fell within the expected range except for the ubiquitinated peptide in the sample with two freeze-thaws (35% CV and percent difference 24%). These results demonstrate that the samples are sufficiently preserved under all conditions up to a single freeze-thaw.

3.5 Repeatability of endogenous analyte detection

The final assay was validated by measuring the amount of endogenous analyte expression in aliquots of a common sample prepared and analyzed over multiple days. Individual aliquots of 500 μg of protein lysate from pooled LCLs (GM07057/GM01526) +/− IR were analyzed over five days. For the ubiquitinated assay, the mean intra-assay and inter-assay variability was 13% and 10% CV. For the unmodified peptide, the mean intra-assay and inter-assay variability was 8% and 11% CV. The total assay variability was calculated by taking the square root of the sum of squares for the intra- and inter-assay variability. Total assay variability was 16% and 13% CV for detection of the endogenous ubiquitinated and unmodified analyte peptides, respectively. Supplementary Table 2 shows the repeatability for measurement of endogenous analytes and data are provided in Supplementary Table 3.

3.6 Quantification of FANCD2 proteoforms in immortalized human cell lines

To demonstrate the utility of the assay in determining defects in the FA pathway, we measured the ubiquitination of FANCD2 in response to DNA damage in human cells. As described previously [46], an isogenic uncorrected/corrected pair of lymphoblast cell lines was prepared from the *FANCA*-deficient HSC72 cell line, by introducing a wild type FANCA cDNA. We compared the ability of HSC72 and *FANCA*-corrected HSC72 cells to ubiquitinate FANCD2 in response to the DNA crosslinking agent mitomycin-C (MMC). An additional wildtype lymphoblast cell line, GM07057, was included as a positive control. Cells were harvested following 2 hours and 24 hours of incubation in MMC, and mock-treated negative controls were harvested at 2 hours. Protein lysates were generated, and 500 μg aliquots were analyzed using the multiplexed immuno-MRM assay targeting monoubiquitinated and unmodified FANCD2 peptides. Experiments were performed in biological triplicate and analyzed in a blinded fashion (data are provided in Supplementary Table 4).

As shown in Figure 2A (upper panel), all three cell lines express FANCD2 (using results from the non-modified QSLSSTVFK peptide). The GM07057 (wildtype) and *FANCA*-corrected HSC72 cells express FANCD2 ubK561 at baseline and show an increase in the amount of FANCD2 ubK561 at the 2 and 24 hour time-points compared to the mock-treated cells. However, no FANCD2 ubK561 is detected in the *FANCA*-deficient HSC72 cells (Figure 2A, lower panel), due to disruption of the Fanconi Core complex [3,47]. These data demonstrate the utility of the assay to detect defects in the Fanconi Core complex in immortalized cells.

3.6 Quantifiation of ubiquitinated FANCD2 in primary human cells

To determine if the assay could quantify defects in the DNA damage response in primary human cells, we isolated and expanded peripheral blood mononuclear cells (PBMCs) from three FA patients and a healthy control. PBMCs were exposed to MMC for a period of 2 hours and 24 hours. Negative controls were mock-treated. Protein lysates were proteolyzed with trypsin, and 500 μg aliquots were analyzed by the multiplex immuno-MRM assay.

As shown in Figure 2B (upper panel), FANCD2 protein is expressed at much higher levels in the control and FA-A patients compared to the FA-D2 patient, as has been described [3]. Additionally, the assay confirms induction of FANCD2 ubK561 in the control PBMC, while no FANCD2 ubK561 signal above the limits of detection was observed in the FA patient cells (Figure 2B, lower panel). Genomic analysis confirms that patient 1 harbored biallelic mutations in *FANCA*, while patient 3 had mutations in the *FANCD2* gene. Patient 2 was diagnosed with FA by chromosomal breakage, but had an undetermined subtype (as mentioned above, molecular subtyping by DNA sequencing is complicated by variants of unknown significance [11], ascertainment of biallelic mutation distribution, and distinguishing constitutional versus somatic mutations [12,13]). Patient 2 is an example where the immuno-MRM assay provides a complementary method of assessing a molecular pathway to inform diagnosis.

Finally, we tested the feasibility of quantifying the monoubiquitinated and unmodified FANCD2 peptides in human tumor tissue (Figure 2C). An aliquot of 500 μg of protein lysate from a frozen human breast cancer biospecimen was analyzed using the immuno-MRM assay. The endogenous expression of FANCD2 and the monoubiquitinated FANCD2 were detected in the cancer sample, showing the utility for application of the assay in tissue biopsies. Given the signal-to-noise ratio in the specimen tested, we estimate the amount of input material could be decreased as much as five-fold and still maintain detection of the modification, compatible with analysis of core needle biopsies.

4. Conclusion

We describe a novel, multiplexed quantitative assay for unmodified and monoubiquitinated proteoforms of FANCD2. The assay is highly reproducible, provides good sensitivity, and can be run at moderate throughput. The specificity of the assay is confirmed based on an abundance of evidence, including nine criteria that exceed widely recognized best practices for targeted peptide measurements [39,44]: (i) The sequence (QLSSTVFK) is unique to the FANCD2 protein, based on a blast search of the uniprot human protein database; (ii) The

proteotypic peptide analyte QLSSTVFK (±monoubiquitination) and its synthetic, stable isotope-labeled internal standards co-elute (i.e. have the same retention time and peak shape) during the liquid chromatography separation; (iii) Three independent transitions are detected for each peptide, consistent with best practices [39,43]; (iv) The transitions from the analyte peptides and internal standards have the same relative abundances, again consistent with best practices [39,43]; (v) The linear response of the assay follows expected behavior using spiked analytes and standards; (vi) The unmodified FANCD2 analyte peptide *is not detected* in the biological samples (i.e., Fanconi patient PBMCs) known to lack functional FANCD2 protein (Figure 2B); (vii) The monoubiquitinated FANCD2 analyte peptide *is not detected* in the biological samples (lymphoblast cell lines, patient PBMCs) known to lack expression of the FANCD2 or FANCA proteins (Figure 2A–B); (viii) MRM-based detection of the monoubiquitinated FANCD2 analyte peptide *is restored* in the FANCA-deficient cell line when the wild type FANCA gene is added back to the cells (Figure 2A); (ix) The quantity of the monoubiquitinated proteoform detected by the assay is increased following treatment of cells with a DNA cross-linking agent (MMC) (Figure 2A–B), consistent with known biology. The assay is currently configured to perform precise, relative quantification of a surrogate peptide for the FANCD2 protein (in both its unmodified and ubiquitinated forms). Because several factors can affect peptide recovery during sample processing (e.g. trypsin digestion efficiency and peptide solubility), the accuracy of the assay for quantifying the full length protein could be improved by incorporating protein standards and potentially incorporating multiple representative peptides for the unmodified protein.

The assay can be further multiplexed with other immuno-MRM assays to build upon a panel of analytes capable of measuring the activation of the DNA damage response [28,30]. Thus, the immuno-MRM assay provides a potential functional diagnostic for patients with Fanconi Anemia with defects in the upstream FA complex or FANCD2, and a potential test for predicting sensitivity to DNA cross-linking agents in human cancers. This test provides a useful functional analysis of variants of unknown clinical significance in FA genes upstream of and including FANCD2 by quantitatively assessing FANCD2 monoubiquitination and FANCD2 levels. Clinical diagnosis is critical to inform clinical management, choice of therapy, and family counseling. Additionally, this assay identifies somatic acquired defects in the Fanconi anemia pathway, a functional vulnerability that could inform therapeutic targeting using DNA cross-linking agents (e.g. platinum compounds) in oncology. Since this is a functional assay, mechanisms that would be missed by genetic sequencing, such as epigenetic silencing of FA genes in cancer cells, would be flagged by this method [5].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

FA	Fanconi anemia
MRM	multiple reaction monitoring
PTMs	post-translational modifications
MS	mass spectrometry
DDR	DNA damage response
MMC	mitomycin-C
LCL	lymphoblast cell line
WB	Western blot
VUS	variants of undetermined significance
IR	ionizing radiation

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Highlights

- Ubiquitination of FANCD2 is central to the Fanconi Anemia DNA repair pathway.

- We developed and analytically validated a novel multiplex immuno-MRM assay to quantify unmodified and monoubiquitinated FANCD2 proteoforms.

- We demonstrate detection and quantification of signaling deficiencies in Fanconi Anemia cell lines and primary cells, as well as quantification in human breast tumor tissue.
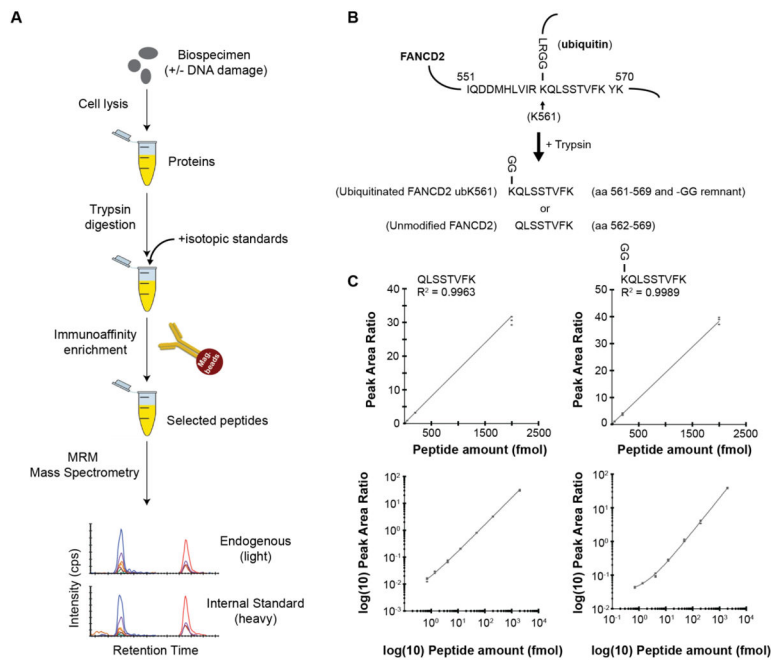
Fig. 1. Development and characterization of the multiplex assay

(**A**) Description of the assay workflow: A variety of sample types can be analyzed by MRM, with previous applications demonstrated in plasma, serum, cell line lysates, and tissue specimens. Proteins are extracted from the biological sample using standard procedures amenable to downstream mass spectrometry. The protein lysates are enzymatically digested, typically using trypsin, and an HPLC-purified, stable isotope-labeled standard (SIS) peptide corresponding to each analyte is spiked-in at a known concentration to act as an internal standard. Although each pair of spiked-in SIS peptide and its endogenous analyte peptide share the same amino acid sequence (and hence the same chemical and physical properties), they are separately detected by the mass spectrometer due to the m/z difference introduced by the stable isotope label. The analyte peptides, along with their stable-isotope analogs, are enriched from the complex sample using anti-peptide antibodies. Measurement of the enriched analytes and standards is performed using quantitative mass spectrometry in a technique called multiple reaction monitoring (MRM). MRM focuses the analytical capability of the instrument on the specific analyte of interest by using a combination of mass filters in a 'transition' (i.e. a specific combination of precursor and fragment ions). Transitions can be cycled through sequentially to allow multiplexing. Specificity is confirmed through orthogonal biophysical properties of the analyte peptides: detection of multiple m/z transitions per peptide, chromatographic co-elution (i.e. identical retention time) of the analyte and internal standard, and identical relative abundance of transitions in the analyte and internal standard. The peak area ratio (light endogenous analyte peptide to heavy SIS peptide) is used for precise, relative quantification. Synthetic peptide standards allow the assays to be highly standardized across laboratories. (**B**) Design of the assay for the unmodified and monoubiquitinated FANCD2 proteoforms. Ubiquitination of FANCD2 at lysine 561 (depicted in the uppermost figure) blocks the lysine from proteolysis during trypsin digestion. This results in formation of the peptide sequence from amino acids 561–

569 containing a –GG remnant on the modified lysine. Peptides containing the modified remnant and the unmodified tryptic peptide were selected for assay development. (**C**) Characterization of the immuno-MRM assay by response curves. Curves are plotted on linear and log(base 10) scales. Error bars are the standard deviation of technical triplicates.
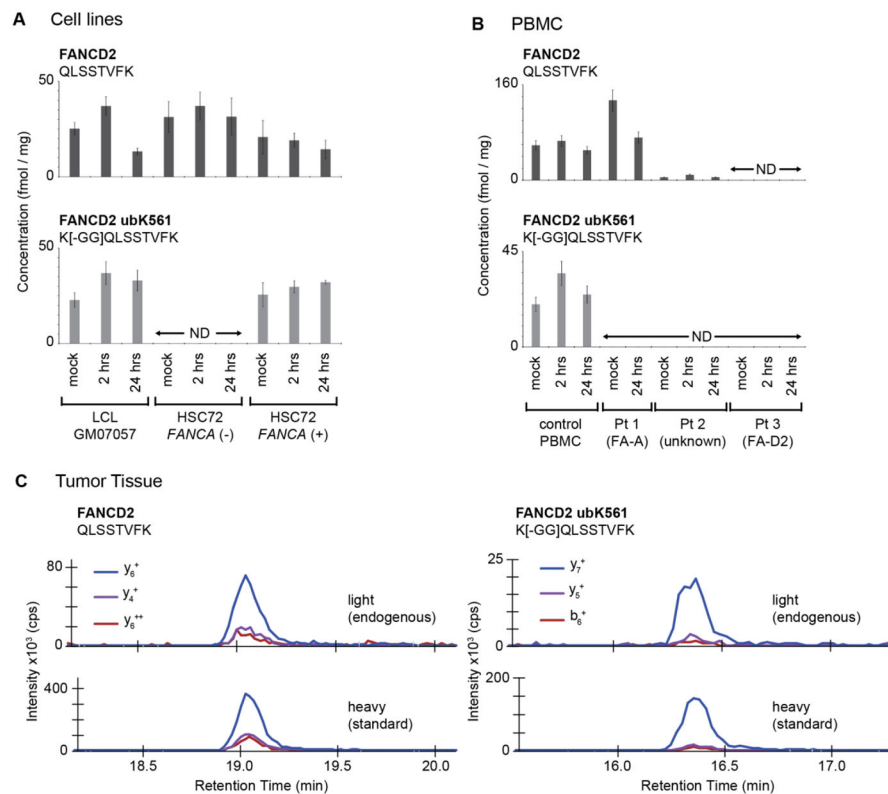
Fig. 2.
Quantification of total and monoubiquitinated FANCD2 in human-derived cell lines and primary human cells and tissue. (A) Concentration of monoubiquitinated FANCD2 peptide and unmodified FANCD2 peptide in human LCLs treated with MMC. Error bars are the standard deviation of biological triplicates or the expected error based on the characterized repeatability of the assay. 'ND' is not detected (i.e. below the lower limit of quantification). The FA patient complementation group assignment, where known, is indicated in parentheses. (B) Concentration of monoubiquitinated FANCD2 peptide and unmodified FANCD2 peptide in primary human PBMCs treated with MMC. Error bars are the expected error based on the characterized total CV of the assays. 'ND' is not detected (i.e. below the lower limit of quantification). (C) MRM transition ion chromatograms for light (endogenous) and heavy (internal standard) peptides detected in human breast tumor tissue. Specificity is confirmed by detection of multiple transitions from each of the endogenous and heavy-labeled standard peptides, as well as equivalent retention times and relative intensities of transitions for light and heavy peptides.