Supplementary Information

The Ubiquitin E3 ligase FBXO22 degrades PD-L1 and sensitizes cancer cells to DNA damage

Sarmishtha De [a1], Elise G Holvey-Bates [a], Kala Mahena[a], Belinda Willard[b], George R Stark[a1]

[a] Department of Cancer Biology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195

[b] Proteomics Core, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195

[1]Corresponding authors: Sarmishtha De, Department of Cancer Biology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195, Mobile: (216)5480170, Email: des2@ccf.org; George R Stark, Department of Cancer Biology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195. Tel: (216)4446062, Email: starkg@ccf.org.

SI Materials and Methods

Cells and Reagents. A549 and Calu1 cells were maintained in DMEM or DMEM-F12 respectively, supplemented with 5% (vol/vol) heat-inactivated FBS, 50 units/mL penicillin, and 50 μg/mL streptomycin. PC9, H1299, MDAMB231, MCF7, and HCT116 cells were maintained in RPMI medium supplemented with 10% (vol/vol) FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. H196 cells were grown in RPMI medium supplemented with 10% (vol/vol) FBS, 1% Hepes, 1% sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin. hTERT-HME cells were grown in Medium 171 with mammary epithelial growth supplement (Invitrogen). All cultures were kept in 5% CO2 at 37 °C. For Western analyses, the antibody against FBXO22 was from Santa Cruz Biotechnology (clone FF-7, cat. # sc-100736) and that against CDK5 was from Cell Signaling (cat. # 2506). The antibody against ubiquitin (clone P4D1-A11, cat. # 05-944) was purchased from Sigma-Millipore, and that against PD-L1 was from Thermo Fisher Scientific (PA5-28115). GAPDH-HRP (clone 0411, cat. # sc-47724) conjugated antibody was from Santa Cruz Biotechnology. For immunoprecipitation of PD-L1, the antibody was from Proteintech Group (cat. # 17952-1-AP). Roscovitine (Seliciclib, CYC202) was purchased from Selleck Chemicals (cat. # S1153). MG132 (cat. # 474790) and N-ethylmaleimide (NEM, cat. # 04259) were from Sigma-Aldrich. Transfection reagents Lipofectamine (cat. # 18324012) and Lipofectamine plus (cat. # 11514015) were purchased from Invitrogen.

Constructs, Virus Production, and Infection. Validation-based insertional mutagenesis (VBIM) vector constructs and their use were described in detail previously (1-3). Human full-length WT FBXO22 and mutant FBXO22 constructs were generated in the lentiviral

vector pLCMVpuro at the EcoR1 and Sal1 sites. Total RNA was isolated from cells by using Qiagen RNeasy Mini kit according to the protocol provided by the manufacturer. Reverse transcription was performed by using oligo DT20 primers with the SuperScript III First-Strand Synthesis System kit and protocol (Invitrogen). Standard PCR reactions were performed by using FBXO22-specific forward and reverse primers with a stop codon in the reverse primer. The PCR product was cloned into lentiviral vector pLCMV-puro at EcoR1 and Sal1. shRNAs against human CDK5 (TRCN0000194974) and PD-L1 (TRCN0000056914) were obtained from Sigma-Aldrich. To produce infectious virus, 293T cells were transfected transiently with pCMVDR8.2 and pVSV-G helper plasmids as well as the plasmid of interest by using Lipofectamine (30 ul) and Lipofectamine Plus (20 ul) in 6 ml of optiMEM. The virus produced was collected 24 and 48 h after infection, 4 μg/mL of polybrene was added, and the preparation was used to infect cells.

Identifying overexpressed RNAs by high throughput sequencing. EBC-1 cells were infected with VBIM-LVs and exposed cumulatively to 3 rounds of ionizing radiation, followed by expansion of the population after each round. To characterize the VBIM insertions enriched in the IR resistant population, VBIM-targeted RNAs were assessed by high throughput next generation RNA sequencing (HT-NGS). RNA-Seq generated ~2000 reads containing VBIM vector sequence fused to a cellular RNA, out of 143 million total reads. All VBIM-tagged sequences of more than 15 nucleotides were aligned to the human Ref-seq database.

Knocking out FBXO22 genes using CRISPR-Cas9. The CRISPOR software program (4) was used to select sgRNA sequences against human FBXO22 (NC_000015 REGION: 75903878.. 75942511). The selected sgRNA sequence

(TCAGCGTGTGCCGCTTATGGAGG, targeted to Exon 2) was cloned into pLentiCRISPRv2 according to the protocol described earlier (5, 6). To produce pseudo-lentiviruses, 4×10^6 HEK 293T cells were plated on 10 cm plates and were transfected the next day with 3 μg pLentiCRISPRv2 (with sgRNA), 1.6 μg psPAX2, and 1 μg pMD2.G using Lipofectamine and Lipofectamine Plus. The supernatant suspensions were harvested 24 and 48 h after transfection, combined, and stored at −80 °C. To construct knockout cells, 20×10^4 A549 cells were plated into one well of a 6-well plate and transduced with diluted pseudo-lentivirus (0.5ml virus plus 0.5ml culture media). Twenty-four hours after transduction, the cells were cultured in medium containing 1 μg/ml puromycin for 3 days. Puromycin-resistant cells were cloned by limiting dilution and single-cell clones were selected for further amplification. The protein expression of FBXO22 in each clone was examined by the Western method. Vector control cells were generated using an identical infection and cloning strategy with pLenti-CRISPRv2 empty vector pseudo lentiviruses.

Cell Survival Assay. Five hundred cells were plated in each well of 24-well plates, allowed to attach overnight, and exposed to IR. After 7 days, the cells were lysed with 1 M NaOH and diluted 50-fold before the A_{260} was measured, as an indication of the total amount of nucleic acid. The fraction of surviving cells was calculated relative to untreated controls (2). To determine the effect of Roscovitine, alone or in combination with cisplatin, the cells were plated at 3,000-5,000 in each well of 24-well plates and allowed to attach overnight. The cells were treated with either Roscovitne or cisplatin as single agents or in combination. After 72 h, the cells were lysed with 1M NaOH and diluted 50-fold before the A_{260} was measured. The fraction of surviving cells was calculated relative to vehicle controls.

Immunoblotting. Immunoblotting was performed as described (3). In brief, protein extracts from the cells were separated by 10% SDS-PAGE, transferred to PVDF membrane, blocked by 5% nonfat milk and sequentially incubated with the indicated primary antibodies and horseradish peroxidase (HRP)-linked secondary antibody (Cell Signaling, Beverly, MA). SuperSignal West Pico plus Chemiluminescent Substrate (ThermoFisher Scientific) was used for detection. Microsoft PowerPoint and Photoshop CS5 were used to crop images from unprocessed images.

In vivo ubiquitination assay. To detect PD-L1 ubiquitination, Calu1 cells were treated with 30 μM of the proteasome inhibitor MG132 for 6 h, washed with PBS, pelleted, and lysed. For A549 cells, the cells were stimulated with interferon-γ (1ng/ml) for 16 h, and further incubated with MG132 (30 μM) for another 6 h before lysis. The cells were lysed in buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet p-40, 0.1% SDS, 10 mM of deubiquitinase inhibitor, NEM, and a mixture of protease and phosphatase inhibitors (Roche Applied Science). After pre-clearing with protein A/G agarose plus (Santa Cruz Biotechnology) beads for an hour, the cell lysates were incubated with anti-PD-L1 overnight at 4 °C. Then protein A/G agarose plus beads were added for incubation for 2 more hours. The beads were extensively washed with the buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet p-40, 0.1% SDS) to remove PD-L1-associated proteins. PD-L1 was eluted by boiling the beads with SDS loading buffer for 10 min, followed by Western analysis to visualize polyubiquitylated protein bands.

Detection of cell membrane PD-L1 level. Flow cytometry was performed to determine the cell surface levels of PD-L1. The cells were labeled with PE-conjugated mAb against PD-L1 (clone MIH2, cat. # 393607) was obtained from BioLegend. Dead cells were

eliminated by DAPI staining (1 μg/mL, added immediately prior to sorting). PE-positive cells were sorted using a FACS Aria II flow cytometer. Isotype control IgG was used to gate PD-L1-positive cells.

Identification of novel phosphorylated residues in FBXO22 by Mass-Spectrophotometry. The samples were subjected to protease digestion and LC-MS/MS analysis was performed using a Fusion Lumos LC-MS instrument. The observed triply charged peptide has an observed m/z of 1008.816 Da and is within -2.1 ppm of the expected mass. This spectra is dominated by several doubly charged C-terminal y ions. The masses of the y_7 and y_{19} ions is consistent with phosphorylation on either S160 or S162. In order to determine the degree of phosphorylation on this peptide following IR treatment, chromatograms for the unmodified and phosphorylated forms of the GIVTPGIVVTPMoGSGSNRPQEIEIGESGF peptide were plotted and these chromatograms were used for the calculation of the peak area ratios PA (phospho)/PA(unmod) (8).

Statistical analysis. Values were expressed as means ± SD. Data were analyzed using the Student *t* test or by two-tailed ANOVA, using GraphPad Prism software. *P* values of <0.05 are considered statistically significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

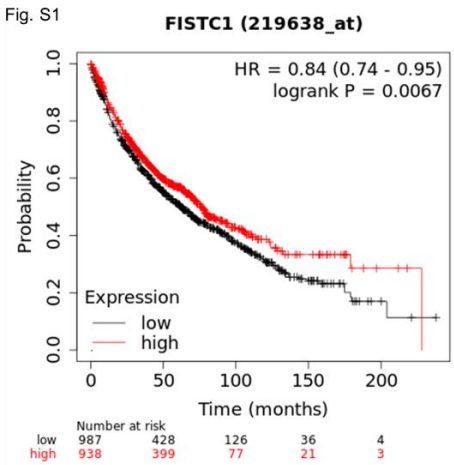

Fig. S1. Kaplan–Meier estimates of overall survival of lung cancer patients having high and low FBXO22 expression.

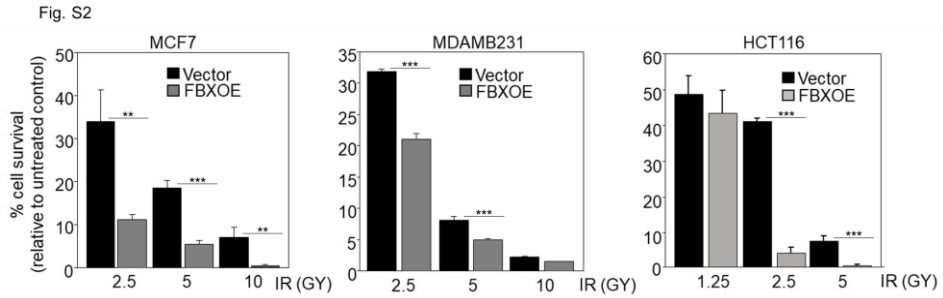

Fig. S2. FBXO22 over expression increases the sensitivity to IR in breast and colon cancer cells. Cells over expressing (FBXOE) were exposed to IR or left untreated. Seven days after exposure, the cells were lysed with NaOH and the A_{260} was measured as an indication of the total amount of nucleic acid. The fraction of surviving cells was calculated relative to untreated control.

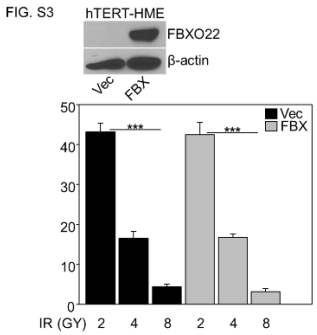

Fig. S3. FBXO22 over expression did not increase sensitivity to IR. FBXO22 was over expressed in hTERT HME cells **(upper panel).** Cell survival was measured after treatment with IR (lower panel).

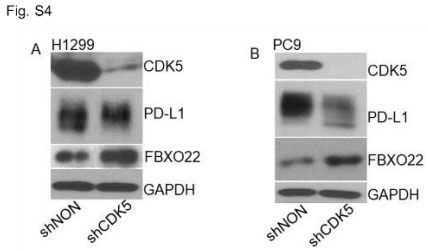

Fig. S4. Down regulation of CDK5 decreases PD-L1 expression and increases FBXO22 levels in NSCLC cells. *A*, *B*. The expression levels of CDK5, PDL1, and FBXO22 were assayed by the Western method in H1299 (*A*) and PC9 (*B*) cells after knock-down of CDK5.

Fig. S5

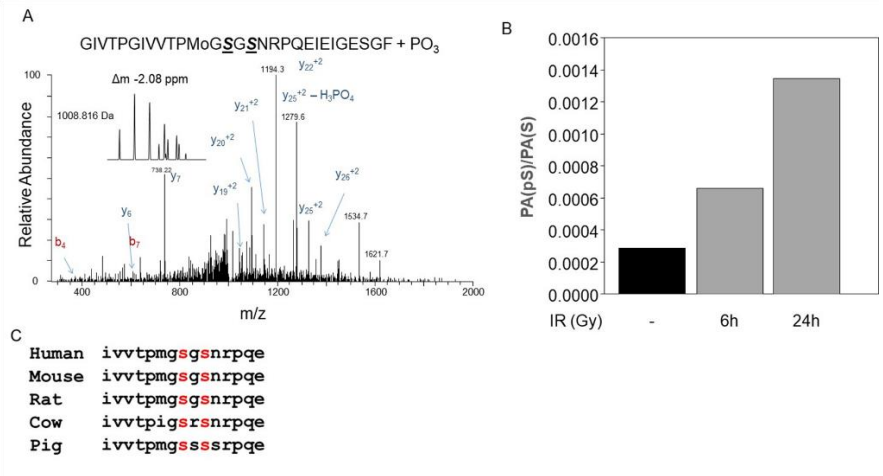

Fig. S5. Identification of residues phosphorylated in FBXO22 in response to IR. *A.* Tandem mass spectrometry **(**MS/MS) analysis of peptides corresponding to amino acids 157-175 of FBXO22. A549 cells over expressing FBXO22 were treated with IR (10 Gy) or left untreated. The samples were collected 6 or 24 h after exposure to IR. *B.* Abundance of FBXO22 phosphorylated on S160 or S162, 6 or 24 h after treatment (10 Gy). *C.* Conservation of the sequences surrounding S160 or S162 in mammals.

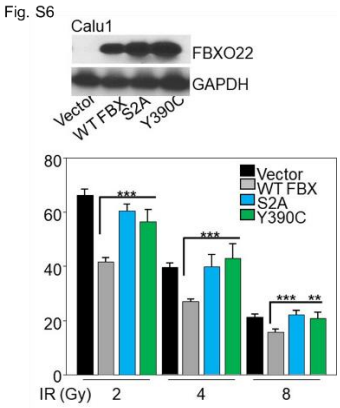

Fig. S6. Phosphorylation of FBXO22 is required to increase sensitivity to IR. Calu1 cells over expressing WT or mutants of FBXO22 were exposed to IR, and the cell viability was determined 7 days later. Data shown represent means ± SD of two experiments, in which each measurement was performed in triplicate.

SI References

1. Lu T, *et al.* (2009) Validation-based insertional mutagenesis identifies lysine demethylase FBXL11 as a negative regulator of NFkappaB. *Proc Natl Acad Sci U S A* 106(38):16339-16344.

2. De S, Cipriano R, Jackson MW, & Stark GR (2009) Overexpression of kinesins mediates docetaxel resistance in breast cancer cells. *Cancer Res* 69(20):8035-8042.

3. De S, Dermawan JK, & Stark GR (2014) EGF receptor uses SOS1 to drive constitutive activation of NFkappaB in cancer cells. *Proc Natl Acad Sci U S A* 111(32):11721-11726.

4. Concordet JP & Haeussler M (2018) CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. *Nucleic Acids Res* 46(W1):W242-W245.

5. Sanjana NE, Shalem O, & Zhang F (2014) Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods* 11(8):783-784.

6. Shalem O, *et al.* (2014) Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* 343(6166):84-87.

7. De S, *et al.* (2015) Erlotinib protects against LPS-induced endotoxicity because TLR4 needs EGFR to signal. *Proc Natl Acad Sci U S A* 112(31):9680-9685.

8. Willard BB, Ruse CI, Keightley JA, Bond M, & Kinter M (2003) Site-specific quantitation of protein nitration using liquid chromatography/tandem mass spectrometry. *Anal Chem* 75(10):2370-2376.