Supporting Information

Metal Assisted Protein quantitation (MAPq): Multiplex analysis of protein expression using lanthanide-modified antibodies with detection by inductively coupled plasma mass spectrometry

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Table of Contents

Supporting methods

Characterization of CRISPR-CAS9 XPC knockout cells

HEK293T cells were transfected with a CRISPR-Cas9 construct targeting *XPC* (gRNA 5'-CTGCGCAGTTCGCGTCCCCG-3'). TA subcloning of the target region followed by sequencing was used to identify insertions/deletions created by gene editing. Compared to HEK293T WT reference sequence (Req#38940), the chosen XPC- clone contained a 1 bp deletion in the codon for Arg12 (denoted in red in gRNA sequence). Reduction of *XPC* mRNA in 293T XPC- cells was confirmed by qRT-PCR using SYBR® PowerUp mastermix (ThermoFisher) and the *XPC* primers Fwd 5'-agaaaatgtgcagcgatggt-3' and Rev 5'-agaacacctctagccactggtc-3'. *XPC* expression was normalized to the housekeeping gene tubulin (*TUBB;* Fwd 5'-ttcaatctccctccaagctc-3' and Rev 5'-gggaaggattccacttgaca-3'). Total protein was extracted from 293T WT and XPC- cells using urea lysis buffer (8% sucrose, 2M urea, 4% SDS). Protein (30 µg) was electrophoresed on a NuPAGE™ 4-12% Bis-Tris protein gel (ThermoFisher), transferred to PVDF membrane, blocked (5%), and probed with anti-XPC antibody (Bethyl A301-122A) and anti-β-actin antibody (CST 4970S). Signal was detected by anti-rabbit-HRP secondary antibody and chemiluminescence on a digital imager (BioRad). Additionally, XPA, XPC, and XPG protein were quantified by MAPq as described to confirm the specificity of the XPC CRISPR targeting. For phenotypic analysis, UV sensitivity was measured by exposing 293T WT and XPC- cells to increasing doses of UV-C radiation (0-50 J/m^2; germicidal lamp). 3 days post-irradiation, cells were stained with crystal violet to determine % survival in irradiated wells compared to non-irradiated wells.

Determination of lanthanide labeling efficiency and antibody levels

To do this, we measured the concentration of each antibody by measuring protein concentration at 280 nm absorbance. We then converted this to number of molecules of each antibody.

Lanthanide concentrations on each antibody were measured using ICP-MS and this number also converted to molecules. This allowed us to determine the number of lanthanide molecules on each antibody (Table S1). Lanthanide concentrations were then measured in each sample. Because we calculated the number of lanthanides/antibody, we were then able to determine the number of antibodies in each samples.

Preparation of cell extracts for western blotting

SW480 cells were cultured in DMEM (high glucose, 25mM), 10% FBS, and 1% penicillin-streptomycin. Cells were seeded in 100 mm plates and allowed to adhere for 24 hr prior to treatment with vehicle (0.1% DMSO) or 10μM ICG-001 for an additional 48 hr. Cells were harvested by washing each plate with 5 mL of ice-cold PBS twice and scraped with 1 mL ice-cold PBS into 1.5 mL Eppendorf tubes. Cells were pelleted by centrifugation at 425 *g*, 4 °C, for 5 min. Cell pellets were then stored at -80 °C until processing. Cells were separated into nuclear and cytoplasmic fractions using the NE-PER kit (Thermo Scientific) supplemented with protease inhibitor cocktail and 2 mM DTT.

HEK293T cells were cultured in DMEM with either low (5 mM) or high (25 mM) glucose supplemented with 10% FBS. Cells were conditioned in elevated glucose for a minimum of 3 weeks. Cells were seeded on 150 mm plates and allowed to adhere for 24 hr, after which plates were washed with PBS. Each dish was treated with 1 mL lysis buffer (2 M urea, 8% sucrose, 4% SDS and Roche cOmplete, EDTA-free protease inhibitor cocktail and placed on a shaker for 5 min at 4 °C. Cells were then scraped and applied to QIAshredder (Qiagen) to shear the DNA. Samples were heated to 95 °C and stored at -80 °C. Cells isolated from liver tissue (described above) were processed similarly with addition of lysis buffer directly to cell pellets. Protein concentrations were determined using the Biorad Bradford Assay kit. Cytosolic (40 μg), or nuclear extracts (20 μg) prepared from SW480 cells were loaded onto a 4-20% gradient Tris-Glycine protein gel (Lonza) in 2X Laemmli buffer and electrophoresed at 150 volts for 1 hr. Gels were then transferred onto PVDF membrane overnight (25 volts at 4 °C). Membranes were blocked in 5%

nonfat milk in a wash buffer (200 mM NaCl and 0.1% Tween-20 in TBS) for 1 hr at room temperature. Primary antibodies were diluted in high salt TBST (25 mM Tris pH 8, 300 mM NaCl, and 0.1% Tween (v/v)) and added to the membranes. Membranes were then incubated at 4 °C with gentle rocking on a Heidolph rotator overnight after which membranes were washed 4x at room temperature for 30 min with high salt TBST. Secondary antibodies conjugated to HRP were added at a dilution of 1:20,000 in high salt TBST and incubated for 1 hr at room temperature. After 30 min of washing in wash buffer, membranes were subjected to ECL substrate (GE Healthcare) and visualized using a Biorad Gel Documentation System. HEK293T and liver cell extracts (30 µg) were loaded onto a 4-12% gradient Bis-Tris protein gel (NuPAGE) in SDS-PAGE loading buffer (62.5 mM Tris, pH 8.0, 1.5% SDS, 8% glycerol, 0.005% bromophenyl blue) and electrophoresed at 150 volts for 90 min. Gels were then transferred onto PVDF membranes overnight (20 volts at 4 °C). Membranes were incubated with blocking buffer (5% blocking powder (Bio-Rad) in PBS + 0.05% Tween-20) for 1 hr and then incubated with primary antibodies diluted (1:1000) in blocking buffer overnight at 4 °C. Blots were then washed 3x 5 min with PBS + 0.05% Tween-20 and secondary antibodies (IRDye® 680RD goat anti-rabbit or IRDye® 800RD goat anti-mouse) were diluted (1:10,000) in blocking buffer added for 1 hr. Blots when then washed again and visualized using the Odyssey CLx Infrared Imaging System (LI-COR Biosciences).

Animal care and tissue collection

Lepr$^{wt/db}$ mice derived from C57BL/6J stock from the Jackson Laboratories were bred for 5 generations with C57BL/6 mice obtained from the City of Hope Animal Resource Center as previously described to obtain Lepr$^{db/db}$ mice.[23] DNA was isolated from 1 to 2 mm tail snips and genotyped as previously described.[23] At 26 weeks of age, *Lepr$^{wt/wt}$* and *Lepr$^{db/db}$* mice were euthanized and perfused with PBS (pH 8) for isolation of liver tissue. Samples were immediately flash frozen in liquid nitrogen and stored at -80 °C. Livers were collected from 5 Lepr$^{wt/wt}$ and 5 Lepr$^{db/db}$ mice. All procedures were approved under City of Hope IACUC Protocol #02016.

Cell isolation from liver tissue

The Miltenyi gentleMACs liver dissociation kit (Miltenyi 130-105-807) was used to dissociate cells from 100 mg of liver tissue according to the manufacturer's instructions. After enzyme addition, tissue was placed into gentleMACS C tubes (Miltenyi 130-093-237) and cells isolated using the Miltenyi gentleMACS Octo Dissociator with heating (Miltenyi 130-096-427) using program 37C_m_LIDK_1. Cells were counted using a hemocytometer, suspended in 10% DMSO, and frozen at -80 °C. Isolated cells were fixed, permeabilized, and probed with antibodies as described above. An average of $5x10^7$ cells were isolated per 100 mg of liver tissue.

Image pixilation

Western blot images, saved as tiff files, were analyzed using the UN-SCAN-IT version 5.1 software from Silk Scientific Inc. Images were loaded into the software and individual protein bands were highlighted and assigned pixel values based on intensity. Pixel values for each band were then analyzed using Excel and plotted using GraphPad Prism software. Data represents two independent replicates.

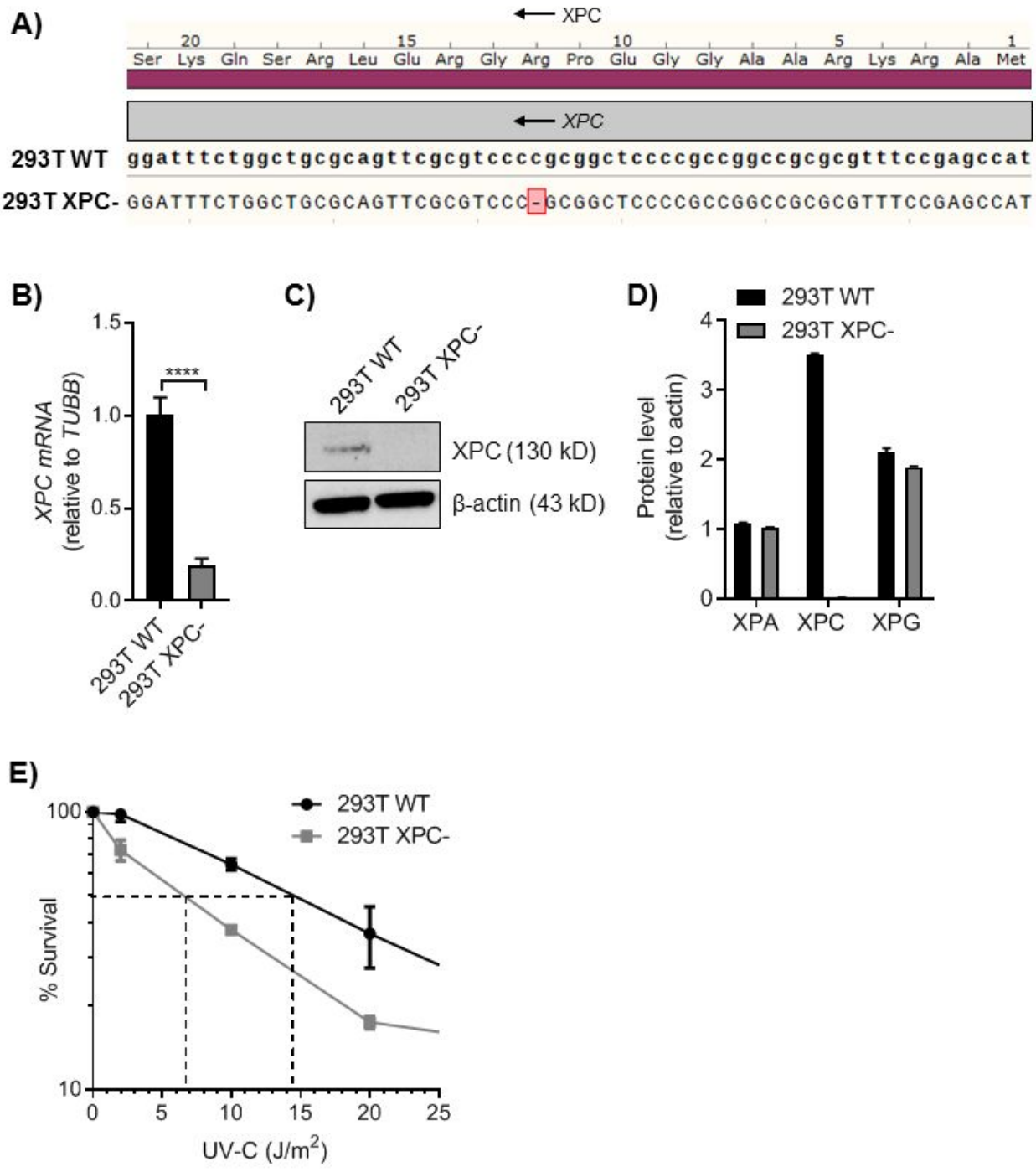
A)
XPC
20 15 10 5 1
Ser Lys Gln Ser Arg Leu Glu Arg Gly Arg Pro Glu Gly Gly Ala Ala Arg Lys Arg Ala Met
XPC
293T WT ggatttctggctgcgcagttcgcgtccccgcggctccccgccggccgcgcgtttccgagccat
293T XPC- GGATTTCTGGCTGCGCAGTTCGCGTCCC-GCGGCTCCCCGCCGGCCGCGCGTTTCCGAGCCAT
B)
XPC mRNA (relative to TUBB)
1.5
1.0
0.5
0.0
****
293T WT
293T XPC-
C)
293T WT
293T XPC-
XPC (130 kD)
β-actin (43 kD)
D)
293T WT
293T XPC-
Protein level (relative to actin)
4
3
2
1
0
XPA XPC XPG
E)
293T WT
293T XPC-
100
10
% Survival
0 5 10 15 20 25
UV-C (J/m2)

Figure S1. Analysis of antibody specificity in XPC CRISPR-CAS9 knockdown cells. HEK293T WT cells were transfected with a CRISPR-Cas9 construct targeting XPC. A) T/A subcloning revealed a 1bp deletion in Arg12 in 293T XPC- cells (Req#38940). Knock out was confirmed by B) qRT-PCR (n=6; paired t test), C) Western blot, and D) MAPq protein analysis. The non-targeted XP proteins XPA and XPG were also measured by MAPq to confirm that they were not impacted by gene editing. E) For phenotypic analysis, UV sensitivity was measured by exposing 293T WT and XPC- cells to increasing doses of UV-C radiation. Surviving cells were assessed by crystal violet staining; dashed lines indicate 50% survival.

Antibody	Lanthanide label	Total amount of labeled antibody (molecules)	Total amount of lanthanide (molecules)	Lanthanides/ Antibody
Actin	Yb^{171}	2.33e13±1.18e11	1.241e15±0.98e13	53.12±1.22
AKT	Nd^{146}	4.19e13±2.78e11	2.82e15±1.77e13	67.32±0.96
p-AKT	Tb^{159}	3.29e13±2.61e11	2.51e15±1.06e13	76.31±0.50
Alpha tubulin	Eu^{151}	8.55e13±9.77e11	4.46e15±7.93e13	52.44±1.08
β-Catenin	Sm^{147}	1.19e12±2.88e10	6.67e13±3.44e11	56.19±2.74
CBP	Gd^{156}	5.62e12±2.02e11	2.35e14±6.06e12	43.61±1.70
CSB	Er^{167}	7.40e13±6.51e11	4.20e15±1.81e13	56.88±0.72
EGLN3	Nd^{148}	7.84e13±3.35e11	5.23e15±1.60e13	66.67±0.24
EphB2	Dy^{164}	4.12e12±8.89e10	1.11e14±4.16e12	26.86±0.55
Ephrin B2	Gd^{160}	4.16e12±8.39e10	2.76e14±6.32e12	66.22±0.23
ERK	Yb^{174}	5.18e13±3.72e11	3.38e15±4.04e13	65.22±0.48
p-ERK	Ho^{165}	9.11e13±5.95e11	8.98e15±3.48e13	98.59±0.30
esRAGE	Tm^{169}	5.23e13±3.75e11	2.08e15±3.55e13	39.65±0.40
GAPDH	Nd^{150}	9.85e13±3.19e11	4.81e15±1.28e13	48.78±0.11
Glo1	Eu^{153}	4.19e13±5.57e10	1.95e15±3.51e13	46.62±0.89
HIF1-α	Dy^{161}	6.70e13±1.45e12	1.85e15±1.22e14	27.65±1.41
NFκB	Pr^{141}	6.13e13±5.18e11	7.01e15±6.15e13	114.44±1.87
P300	Dy^{162}	5.57e12±5.67e10	1.64e14±3.28e12	28.83±0.84
p38	Sm^{154}	3.36e13±2.47e11	2.27e15±8.72e12	67.38±0.58
p-p38	Nd^{144}	7.63e13±1.16e12	3.64e15±1.27e13	47.72±1.66
PDGFA	$E^{168}r$	9.11e13±9.17e11	6.25e15±5.64e13	68.61±1.30

RAGE	Lu^{175}	9.57e13±4.85e11	7.24e15±5.02e13	75.66±0.90
S100A4	Er^{166}	4.07e12±5.22e10	8.76e13±7.57e11	21.52±0.17
Survivin	Er^{170}	3.96e12±6.01e10	5.66e14±6.95e12	143.21±2.78
XPA	Yb^{172}	3.16e13±4.72e11	2.62e15±9.06e13	82.83±3.90
XPC	Dy^{163}	5.12e13±9.51e11	9.78e14±2.27e13	19.13±0.59
XPC	Nd^{142}	1.20e13±5.31e11	1.14e15±5.96	95.59±3.35
XPG	Gd^{155}	9.57e13±2.52e10	6.76e15±3.12e13	70.62±0.33
VEGF	Gd^{157}	6.67e13±4.47e11	4.42e15±9.69e13	66.29±1.03

Table S1. Lanthanide-antibody labeling efficiency. Lanthanide-conjugated antibodies were analyzed using ICP-MS and total amount of lanthanide in each sample determined. This number was then normalized to the total amount of antibody in each sample to calculate the number of lanthanides/antibody. Three independent experiments revealed a coefficient of variation ≤10%.

Antibody	Vehicle (ng/mL)	ICG-001 (ng/mL)	Vehicle Protein/Actin ratio	ICG-001 Protein/Actin ratio
β-Catenin Sm^{147}	0.020 ± 0.009	0.022 ± 0.005	0.147 ± 0.023	0.121 ± 0.052
CBP Gd^{156}	0.034 ± 0.006	0.021 ± 0.009	0.183 ± 0.040	0.157 ± 0.018
Ephrin B2 Gd^{160}	0.013 ± 0.004	0.012 ± 0.004	0.065 ± 0.009	0.097 ± 0.020
P300 Dy^{162}	0.025 ± 0.0054	0.013 ± 0.004	0.136 ± 0.027	0.104 ± 0.005
EphB2 Dy^{164}	0.021 ± 0.007	0.023 ± 0.001	0.173 ± 0.052	0.132 ± 0.56
S100A4 Er^{166}	0.018 ± 0.003	0.016 ± 0.005	0.147 ± 0.027	0.098 ± 0.049
Survivin Er^{170}	0.012 ± 0.003	0.008 ± 0.005	0.096 ± 0.018	0.045 ± 0.021

Table S2. Average quantitative protein measurements from ICP-MS in vehicle (0.1% DMSO) and ICG-001 treated SW480 cells.

Antibody	5 mM Glucose (ng/mL)	25 mM Glucose (ng/mL)	5 mM protein/actin ratio	25 mM protein/actin ratio
XPA Yb^{172}	1.390 ± 0.012	0.448 ± 0.009	0.110 ± 0.001	0.086 ± 0.003
XPC Dy^{163}	2.289 ± 0.003	0.593 ± 0.010	0.181 ± 0.001	0.114 ± 0.003
XPG Gd^{155}	2.189 ± 0.151	0.574 ± 0.023	0.173 ± 0.012	0.110 ± 0.005
HIF-1α Dy^{161}	2.686 ± 0.142	0.845 ± 0.012	0.213 ± 0.011	0.162 ± 0.004

Table S3. Average quantitative protein measurements from ICP-MS in HEK293T cells in 5 mM and 25 mM glycemic conditions.

Antibody	Lepr$^{wt/wt}$	Lepr$^{db/db}$	Lepr$^{wt/wt}$ protein/actin ratio	Lepr$^{db/db}$ protein/actin ratio
XPA Yb^{172}	0.035 ± 0.018	0.029 ± 0.005	0.558 ± 0.189	0.357 ± 0.036
XPC Dy^{163}	0.015 ± 0.01	0.012 ± 0.003	0.220 ± 0.033	0.149 ± 0.018
XPG Gd^{155}	0.043 ± 0.026	0.038 ± 0.010	0.649 ± 0.136	0.462 ± 0.057
HIF-1α Dy^{161}	0.038 ± 0.024	0.028 ± 0.009	0.586 ± 0.159	0.336 ± 0.064

Table S4. Average quantitative protein measurements from ICP-MS in Lepr$^{wt/wt}$ and Lepr$^{db/db}$ mice.