Supplementary Information: Methods

Cell Lines

HeLa cervical carcinoma and B16.F10 murine melanoma were grown in RPMI supplemented with 2mM (final) glutamine and 10% bovine calf serum. BJ human fibroblasts were cultured in DMEM supplemented with 2mM glutamine (final) and 10% bovine calf serum.

Antibodies

Phosphorylated p70 S6 Kinase (p-S6K), Thr389 (1A5, #9206), mTOR (7C10, #2983), RAPTOR (24C12, #2280), p-mTOR2448 (#2971), RICTOR (53A2, #2114), p-mTOR2481 (#2974) were all obtained from Cell Signaling Technologies (Danvers, MA). Rabbit anti-sera recognizing PA200 has been previously described (35). Antibodies used as loading controls include mouse anti-HSP70 (SPA-810, Stressgen, Victoria BC), anti-ERp57 (35), or anti-alpha tubulin (Sigma, clone DM 1A).

Transfection

To ensure ample nutrients prior to transfection, all transfection experiments were initiated by plating HeLa cells three days prior to transfection at 1.5×10^6 per 15 cm diameter dish. After 48 hours, cells were expanded into two dishes with fresh media. After 24 hours, cells were transfected using Amaxa (Lonza) nucleofection solution V and program X-005. Greater than 98% transfection efficiency was routinely obtained as

measured using fluorescent RNA oligonucleotides. Knockdown (>95%) was verified in each experiment by Western blot.

Radiation Exposure

For untransfected cells (Figure 1A) cells were plated in complete growth media and allowed to rest for 2-3 hours. Cells were exposed to 5 Gy irradiation using Philips Mark 1 Cesium irradiator at a dose rate of 0.472Gy/min. For transfected cells, cells were allowed to recover for forty-eight hours, then harvested and replated in complete media for 2-3 hours prior to irradiation.

Extracellular glutamine measurements

Untransfected HeLa cells were plated at 1.5 million cells per dish (10cm diameter, $2x10^4/cm^2$) with 15ml growth media ($2x10^4/cm^2$). After adherence overnight, nutrients were replenished prior to exposure to 5 Gy IR. Forty-eight hours after IR exposure (2.073 Gy/min), supernatants were harvested and analyzed for glutamine levels using a Bioprofile Flex instrument (Nova Biomedical, Waltham MA). Glutamine consumed was calculated as (initial glutamine level – remaining glutamine level)/million cells.

Clonogenic survival

HeLa cells were transfected with PA200 specific siRNA or non-specific siRNA (Ctrl). Forty-eight hours after transfection and plating in RPMI+10% bovine calf serum supplemented with 2mM (total) glutamine, cells were harvested and replated in 6 well dishes (50 or 100 cells / well) with fresh growth media. Two to 3 hours later, cells were

exposed to 2 or 5 Gy IR at room temperature at a dose rate of 2.073 Gy/min. After irradiation, cells were incubated at 37°C, 5%CO_2 for 13 days or 7 days or a group of cells was supplemented on day 7 with 2mM glutamine and colony formation assessed on day 13 post-IR exposure.

Proteasome Activity Assay

To assess proteasome activity, cells were lysed by three rounds of freeze-thaw in 25mM Tris-HCl, 5mM EDTA, 5mM EGTA at 25 million cells per ml. Post nuclear supernatants were incubated in the presence of the LLE-MCA (100 micromolar) or LLVY-MCA (100 micromolar) in the presence or absence of the inhibitors MG132 (5 micromolar final, for LLE-MCA) or Velcade (20 nM final, for LLVY-MCA). Released fluorescence was measured by excitation at 360nm (40nm band pass filter) and emission 460nm (40 nm band pass filter) on a BioTek Synergy 2 plate reader (Winooski, VT).

Nutrient depletion

Immediately after transfection, cells were plated at the indicated densities in 6 well dishes ($1.3x10^{6}$ cells/well = $14.4x10^{4}$ cells/cm^{2}) in 3 mls of media. For nutrient replete conditions, media was replaced daily. Seventy two hours after transfection, cells were lysed directly in the dish with 1%Tx100, 0.5mM PMSF, 5mM NEM, 1mM NaF, 5mM beta-glycerophosphate, in Tris buffered saline.

Growth Assays

After transfection with PA200 specific siRNA (black bars) or non-specific siRNA (Ctrl si, white bars), HeLa cells were cultured in 2mM glutamine for 48 hours. Cells were then harvested, washed and placed in RPMI media + 10% bovine calf serum supplemented with 0.05mM or 0.2mM L-glutamine for 72 hours ($0.33x10^4/cm^2$). Cell growth was assessed using Promega cell proliferation kit (MTS assay).

Glutamine limitation experiments

After transfection, cells were plated at $1.5x10^6$ cells per 15 cm diameter dish to recover for 48 hours. Cells were harvested and re-plated at 10000 cells per well 96 well dish in 200 microliters of media with indicated concentration of glutamine. 72 hours after replating metabolic activity was measured using Promega proliferation kit (MTS assay) according to manufacturers recommendations.

Amino acid analysis

Cells were treated as indicated in the figure legend. Cells were harvested and treated with 5% trichloroacetic acid for 30 minutes on ice. Precipitated proteins were pelleted and the supernatants were frozen in dry ice-methanol and stored frozen at -80°C until analysis at the Advanced Protein Technology Centre Hospital for Sick Children, Toronto Ontario Canada.