# Supplementary Information

### Additional cell line information

B16F10 cells were kindly provided by Dr R Kalaria from ACTREC, Mumbai. An immortalized astrocytic cell line, SVG and PHA (primary human astrocytes of fetal origin were kindly provided by Dr Pankaj Seth from National Brain Research Institute, Manesar, India.

### Histopathology and Immunohistochemistry

Histological sections of normal brain and tumor tissues were examined by light microscopy using H&E preparation. Tumor sections of diffusely infiltrating astrocytomas and oligodendrogliomas were graded using the WHO grading scheme by the neuropathologist (VS). Paraffin sections (4 μm) from the tumor tissue and control samples were collected on silane coated slides and IHC was performed. The antigen retrieval was performed by heat treatment of the deparaffinized sections in a microwave oven at 600 W for 30 minutes for IMP3, IGF2, Akt-2 and pAkt in citrate buffer (10 mM, pH 6.0). All sections were further treated with methanol and 5% hydrogen peroxide to block the endogenous peroxidases followed by washes with PBS buffer (pH 7.4 – 7.6). Skimmed milk powder (5%) was used to block background staining for 45 minutes. After the initial processing steps, sections were incubated overnight with the primary antibody at $4^0$ C (anti-IMP3, DAKO, Denmark, Clone 69.1, 1:80) (Anti-Human IGF-2 clone – Recombinant, R&D systems, USA, dilution 1:40) (Anti-Human Akt-2 Clone-302501; Santa Cruz diluion 1:20) (Anti-p-Akt1/2/3 Clone - Thr 308-R; Santa Cruz, dilution 1:50). This was followed by incubation with supersensitive non-biotin HRP detection system (QD440-XAK, Biogenex, USA). 3, 3'-Diaminobenzidine (Sigma-Aldrich, St. Louis, USA) was used as the chromogenic substrate. Glioblastoma tumors that showed markedly increased mRNA levels of IMP3 served as positive controls for IMP3 staining. A negative control slide in which the primary antibody is excluded was incorporated with each batch of staining. Cytoplasmic staining was noted for IMP3, IGF2, nuclear for Akt2 and nuclear/cytoplasmic for pAkt. The nuclear and cytoplasmic immunohistochemical staining were scored semi-quantitatively on a three-point scale of 0-2, where 0 = no staining, 1+ = mild staining, and 2+ = strong staining. Only 2+ nuclear and cytoplasmic positivity was considered for analysis. The immunopositivity was assessed in more than 1000 cells from each tumor specimen. The labeling index (LI) was expressed as percentage of cells that showed 2+ staining among the total number of cells counted**.** A nuclear and/or cytoplasmic LI of ≥10% was considered to label a tumor positive for each of the markers. The IHCs on the mouse brain sections were done with the VECTOR M.O.M immunodetection kit (VECTOR laboratories, California, USA) as per the manufacturer's protocol. For immunohistochemical analysis of B16F10 xenografts, the protocol for embedding, sectioning and immunohistochemical staining was followed as previously described (1).

### RNA isolation and RT-qPCR

RNA isolation and RT-qPCR were carried out as has been described before (2). Total RNA was extracted from the frozen tissue by using the TRI reagent (Sigma). The RNA samples were quantified by measuring the absorbance using a spectrophotometer and visualized on a MOPS formaldehyde gel for quality assurance. The relative quantification of the expression levels of selected genes was carried out using a two-step strategy: In the first step, cDNA was generated from RNA derived from different tissue samples using a cDNA archive kit (ABI PRISM); subsequently, real-time quantitative PCR was carried out in an ABI PRISM 7900 (Applied Biosystems) sequence detection system with the cDNA as template using gene-specific primer sets and a Dynamo kit containing SYBR green dye (Finnzymes). All measurements were made in triplicates. The genes GARS (glycyl-tRNA synthetase), AGPAT1 (1-acylglycerol-3-phosphate O-acyltransferase 1), ATP5G1 [ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C1 (subunit 9)], and RPL35A (ribosomal protein L35a) were used as internal controls because their expression levels were

found to be unaltered in microarray experiments. Delta-delta CT method was used for the calculation of ratios. Statistical significance was tested by ANOVA using GraphPad PRISM software. Sequences of reverse transcription-PCR primers and conditions used will be provided on request. Each dot in figure 1 A and Supplementary figure SF 6 represents the median transcript levels from one sample after normalization with the internal reference genes.

For the Real Time RT-PCR experiments in Figure 1A, 1C, 2A, 4A, 5B, 5C, 5D and 5E, and supplementary Figure SF1A-L, SF2A, SF4A, SF4B, SF5A, SF5F and SF8 – again all measurements were made in triplicates and multiple internal reference genes were used for normalization in each case. The delta-delta CT method was used for calculation of ratios wherein the average Ct value of the internal controls was used to normalize for the expression levels of each gene studied. The Delta Ct values of the test sample were then subtracted from the Delta Ct value of the experimental samples to arrive at the delta-delta Ct value. The fold change or the log 2 transformed expression values were then arrived at for the various experiments.

In supplementary figure SF7A, the transcript levels of mouse IMP3 were determined after normalization with the levels of cyclophilin and the fold change in transcript levels has been calculated and depicted.

**Transfection, Adenovirus preparation and western blotting**

To raise stable clones over expressing IMP3, cells were transfected with the pCEP4-IMP3 construct using escort III (Sigma) as per the manufacturer's protocol. 24 hours after transfection, the transfectants were selected on hygromycin (200-400 µg/ml) for 3-4 weeks after which individual clones were further amplified. Stable clones were then confirmed by real time qPCR and western blotting before further characterization. For H1299, the clone pools were further used for characterization after validation. Adenoviruses for over expression of IGF2BP3/IMP3 were prepared as has been previously described (3) (4).

Protein lysates from the tissue samples and from cell lines were prepared in RIPA buffer and used for immunoblotting after quantitation by Bradford's reagent. The following antibodies have been used in these studies : Anti-IMP3 antibody (Santa Cruz), Anti-IMP3 antibody (Sigma), Anti-Actin HRP (Sigma), Anti-PCNA (Oncogene), Anti-IGF2 (Abcam), Anti-IGF2 antibody (Santa Cruz), Anti-Cyclophilin B (Santa Cruz), Thr37/46 Phospho 4EBP1 (Cell Signaling), 4EBP1 (Cell Signaling), Ser 2448 phospho mTOR (Cell Signaling), mTOR (Cell Signaling) Ser 473 phospho Akt (Cell Signaling), Akt (Cell Signaling), phospho MEK1/2 (Cell Signaling), MEK1/2 (Cell Signaling), Phospho ERK1/2 (Cell Signaling), ERK1/2 (Cell Signaling).

**siRNA**

The human IMP3 and cyclophilin siRNA duplexes were designed and chemically synthesized by Dharmacon Reasearch (Lafayette, CO). The SMARTpool siRNA is a mixture of four different siRNA duplexes targeting distinct coding region sequences of IMP3 (Genbank™ accession number NM_006547). The siRNA duplexes were dissolved in the 1X universal RNA oligo buffer (20mM KCl, 6mM HEPES-KOH (pH 7.5), 0.2 mM $MgCl_2$). SiRNA transfections (200 nM) were carried out using Dharmafect (Dharmacon Research) as per the manufacturer's instructions. The cells were plated for the various experiments 96 hours after siRNA transfection to ensure efficient knockdown.

**Cell Proliferation and chemosensitivity assays**

For cell proliferation, cells were plated in a 96 well plate and at appropriate time points MTT (20 µl of 5 mg/mL) was added to the cells. Three hours after MTT addition, the formazan crystals were dissolved in DMSO (200 µl) and measured as absorbance at 550 nm. For chemosensitivity assays, 24 hours after plating, the cells were treated with the various cytotoxic drugs and incubated at 37 ºC, 5% $CO_2$ for 45 hours. At this point, MTT was added to the cells and 3 hours later the assay was terminated and absorbance was measured as above. The absorbance by the control cells was

considered to be 100% and all samples were normalized to the control cells. All assays were carried out in triplicates and the mean value was used to generate the graph.

**Soft agar colony formation assay and Invasion assay**

Stable clones or transfected cells were harvested by trypsinization and 2.5 X $10^4$ cells were resuspended in 0.3 % noble agar and overlaid on 0.6% agar in a 35 mm dish. The number of colonies that appeared after 3-4 weeks were counted microscopically (10X bright field), over 10 representative fields. The matrigel based invasion assay was carried out using the BD matrigel inserts as per the manufacturer's instructions (Becton Dickinson). The invasion index was calculated as indicated by the manufacturer's protocol after counting five fields from both the control and the matrigel inserts for each cell type. These assays were carried out in duplicates.

**Subcutaneous tumor growth and lung metastasis assays**

All procedures were performed according to the local animal ethics commission protocols. In the first set of experiments nude mice were divided into two groups (7 animals per group), and 100 µL of a single cell suspension of $5X10^6$ cells / mL was injected subcutaneously. Using a caliper, the tumor diameters were measured once every three days, and tumor volumes were determined using the following formula: $V = D \times d \times 0.52$ ($V$, tumor volume; $D$, the biggest dimension; $d$, the smallest dimension). The death of the mice was recorded and used for Kaplan- Meier survival analysis.

In the second set of experiments to measure lung metastasis, C57BL/6 mice (8 animals per group) were injected intravenously with 100 µL of a single cell suspension of $3X10^6$ B16F10 cells per animal through the lateral tail vein. The animals were sacrificed 30 days after injection and the lungs were isolated. The lungs were subsequently fixed and the number of macro-metastatic nodules in each case was recorded. The counting of metastatic nodules was done by two researchers in a blinded manner.

**RNA Immuno-precipitation**

RNA immuno-precipitation assays were carried out as has been detailed previously (5, 6). Briefly, cells with low levels of endogenous IMP3 were infected with Ad IMP3 and Ad GFP at 20 MOI respectively. 48 hours post infection, the cells treated with 1% formaldehyde to crosslink the cellular RNAs and proteins and the cells were subsequently harvested in Polysome Lysis Buffer (100mM KCl, 5mM MgCl2, 10mM HEPES (pH 7.0), 0.5% NP40, 1 mM DTT, 100 units/ml Rnase Out (RNase inhibitor, 100 units/ml Invitrogen, Cat no # 10777-019), 400 µM Vanadyl ribonucleoside complexes (NEB, Cat no S1402S) and protease inhibitor cocktail) and frozen at -80ºC to maximize the lysis efficiency. Subsequently, the lysates was cleared by centrifugation at 14000 rpm and the amount of protein in the supernatant was quantified by Bradford's reagent (Biorad). Equilibriated protein A agarose beads were then incubated with equal amounts of the IMP3 specific antibody (Anti-IMP3, N19, sc-47893, Santa Cruz) and the conspecific control antibody (Anti-Cyclophilin B antibody, sc-20361, Santa Cruz) for 16 hours. Equal amount of the total cell lysates from the control virus (Ad-GFP) and Ad-IMP3 infected samples was then used for pre clearing. The pre-cleared lysates were then split into two aliquots one of which was used for immuno-precipitation with the control antibody and the other with the IMP3 specific antibody. The bound RNAs were then extracted with TRI reagent and then quantified by nanodrop. Equal amounts of RNA were then used for cDNA synthesis with the archive cDNA synthesis kit (ABI Prism) from which equal amounts of cDNA were used for Real Time PCR based quantitation of the transcript levels.

**Polysome fractionation**

U138 glioma cells transfected with cyclophilin or IMP3 siRNA for four days and subjected to polysome analysis as described below. Briefly, after siRNA treatment, cycloheximide (100 µg/mL) was added 15 mins before harvesting. Polysome lysates were prepared in polysome extraction buffer ( (5mM Tris-HCl pH 7.5, 1.5mM KCl, 5mM MgCl2, 100 µg/mL Cycloheximide, 1mM DTT, 200

units/mL RNase Out, 200 µg/mL tRNA, 0.5% Triton X 100, 0.5% Sodium deoxycholate and protease inhibitor cocktail). After fifteen minutes incubation on ice, KCl concentration in the lysates was raised to 150 mM and the lysates were cleared by centrifugation at 3000g. These lysates were subsequently loaded on a 10-50% sucrose gradient and centrifuged for 2 hours at 36000 rpm. After centrifugation, gradients were collected into 0.5 mL fractions while the absorbance at 254 nm ($A_{254}$) was continuously monitored using an automatic fractionator. The fractions corresponding to polysome (# 10 to 14) and non-polysome (# 1 to 9) were pooled, RNA was isolated and used for RT –PCR analysis. The relative quantification of the IGF-2 transcripts was carried out using a two-step strategy: In the first step, cDNA was generated from RNA derived from different tissue samples using a cDNA archive kit (ABI PRISM); subsequently, real-time quantitative PCR was carried out in an ABI PRISM 7900 (Applied Biosystems) sequence detection system with the cDNA as template using gene-specific primer sets and a Dynamo kit containing SYBR green dye (Finnzymes). All measurements were made in duplicates. The Ct values of IGF-2 transcript between cyclophilin and IMP3 siRNA transfected cells within polysome or non-polysome fractions were used to calculate the relative fold change.

**Cycloheximide treatment of cells**:

Cycloheximide (50 µg/ ml) was added to H1299 vector and IMP3 stable cells 24 h after plating the cells at a seeding density of 2.5 x $10^6$ cells/ 90 mm dish. Cells were harvested at different time points as indicated and subjected to western blotting to detect IGF-2 and (Anti-IGF-2, Abcam) and actin (Anti-actin HRP, Sigma).

**Intracranial Tumor growth and Bioluminiscent Imaging**

Parental U87MG cells were obtained from the American Tissue Type Collection. Stable U87-MG-Luciferase cell line was prepared by transfection of a Luciferase containing plasmid followed by selection on G418 to obtain stable clones. These U87-MG-Luciferase stable cells were subsequently transfected with the human IMP3 or cyclophilin siRNA duplexes from Dharmacon Reasearch (Lafayette, CO) as described above. The SMARTpool siRNA against IMP3 is a mixture of four different siRNA duplexes targeting distinct coding region sequences of IMP3 (Genbank™ accession number NM_006547). SiRNA transfections (200 nM) were carried out using Dharmafect (Dharmacon Research) as per the manufacturer's instructions. The siRNA transfected cells were subsequently used for an intracranial injection to monitor the effect of IMP3 knockdown on glioma tumor growth. All procedures were performed according to the local animal ethics commission protocols. In these experiments nude mice were divided into two groups (5 animals per group), and 5 µL of a single cell suspension of 0.5X$10^6$ cells in PBS was injected into the cortex of NIH nude/nude mice (Coordinates – 1mm posterior, 2 mm lateral and 1 mm depth) over a period of 5 minutes using a Kopf stereotaxic manual microinjector.

From one week after tumor implantation, the mice were imaged with the Xenogen IVIS system (Xenogen Corporation, Alameda, CA) to record the bioluminescent signal emitted from the implanted tumor cells. The IVIS 100 cooled CCD camera system was used for emitted light acquisition and Living Image software (Xenogen Corp.) was used for data analysis. Animals received an intraperitoneal injection of 2mg D-Luciferin (Gold Biotechnology) with isofluorane anesthesia. Bioluminiscence imaging and recording were carried out at the peak photon emission for all the animals. Data were analyzed based on total photon flux emission (photons/s) in the region of interest over the intracranial space. The average photon flux for each group has been plotted in the figure along with the corresponding standard deviations.

**References:**


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**Supplementary Figure Legends**

**Figure SF1 – Transcript levels of the various IGF family members across the different grades of astrocytoma.**

**Panels A – L Scatter plots for IGF-1, IGF-2, IGF-1R, IGF-2R, IGFL1, IGFL2, IGFL3, IGFL4, IGF2BP1, IGF2BP2, IGF2BP3/IMP3, IGF2AS transcript levels across the different grades of astrocytoma**

Log 2-transformed gene expression ratios obtained from RT-qPCR analysis of indicated samples are plotted for the indicated genes. Each dot represents data derived from one sample. For each sample, fold change in gene expression is calculated over its mean expression in normal brain samples after normalization with six different internal reference genes - AGPAT, GARS, ATP5G1, GAPDH, ACTB and RPL35A. After analysis, no significant change in the transcript levels of the various IGF family members was found except for IGF2BP3/IMP3.

**Figure SF2 – Stable over-expression of IMP3 in H1299, non-glioma cells leads to increased proliferation, invasion and anchorage-independent growth.**

**A)** Fold change in the IMP3 transcript levels obtained from RT-qPCR analysis of the H1299 vector stable clone and the IMP3 stable clones are plotted. For each sample, fold change in gene expression is calculated over its expression in the vector stable H1299 cells. The insert shows the western blotting analysis for the vector and IMP3 stable clones with the indicated antibodies.

**B)** Viability was measured by MTT assay at indicated time points for H1299 vector and IMP3 stable clones and plotted. The assays were carried out in triplicates and the mean value for each cell type at each time point was used to generate the graph.

**C)** H1299 - vector and IMP3 stable clones were subjected to soft agar colony formation assay and the photographs of representative fields are shown.

**D)** The number of colonies in the soft agar assay as described in **C** are counted and shown. A paired t test for difference in their colony formation abilities is significant ($p=0.0012$).

**E)** The invasion index of the vector and IMP3 stable H1299 cells was determined based on the BD$^{TM}$ matrigel assay as per the manufacturer's instructions 24 hours after seeding. A paired t test for difference in their invasion index is significant ($p=0.0357$).

**Figure SF3 – Adenoviral over-expression of IMP3 also leads to increased proliferation.**

**A, B and C)** Viability was measured by MTT assay at indicated time points for HaCaT (**A**), SVG (**B**) and H1299 (**C**) cells infected with GFP or IMP3 adenoviruses and plotted. The assays were carried out in triplicates and the mean value for each cell type at each time point was used to generate the graph.

**Figure SF4 – siRNA mediated IMP3 knockdown in U138 glioma cells increases the chemosensitivity of cells.**

**A)** Fold change in the cyclophilin transcript levels obtained from RT-qPCR analysis of mock, cyclophilin and IMP3 siRNA treated U138 cells at the indicated time points are plotted.

**B)** Fold change in the IMP3 transcript levels obtained from RT-qPCR analysis of mock, cyclophilin and IMP3 siRNA treated U138 cells at the indicated time points are plotted.

**C)** 14 days after plating, the mock, cyclophilin or IMP3 siRNA transfected U138 cells were fixed, stained with crystal violet and photographed.

**D, E and F)** U138 cells were either cyclophilin (black) or IMP3 (grey) siRNA transfected as per the manufacturer's instructions. The cells were treated with the indicated concentrations of Taxol (I), Temozolomide (J) or Adriamycin (K) 24 hours after plating, and 48 hrs after drug addition, the proportion of live cells was quantified by MTT assay. The percentage viability was calculated after normalization to the OD values of untreated cells. The assays were carried out in triplicates and the mean value for each cell type at each time point was used to generate the graph. A t test for difference in their difference in the chemosensitivity of the cyclophilin and IMP3 siRNA treated cells was found to be significant – for, Taxol 1, 2, 3 and 4 μg/mL $p=0.0579$, 0.0208, 0.0007, 0.0566 respectively. For Temozolomide 50, 200, 400 and 600 μM, $p=0.2783$, 0.0128, 0.0473, 0.0377 respectively. For Adriamycin 0.4, 0.8, 2 and 5 μg/mL, $p=0.0339$, 0.0736, 0.0223, 0.0002 respectively. Generally a p value of $p\leq0.05$ was considered significant. A $p\leq0.05$ is represented as *, while $p\leq0.01$ is represented as **, while *** is used to represent $p\leq0.0001$

**Figure SF5 – Effect of IMP3 over expression in murine melanoma derived B16F10 cells.**

**A)** Fold change in the IMP3 transcript levels over its expression in the parental B16F10 cells obtained from RT-qPCR analysis of the B16F10 vector stable clone and the IMP3 stable clone are plotted. The insert shows the western blotting analysis for the vector and IMP3 stable clones with the indicated antibodies

**B)** Viability was measured by MTT assay at indicated time points for B16F10 vector stable clone and the IMP3 stable clone and plotted.

**C)** B16F10 vector stable clone and the IMP3 stable clone were subjected to soft agar colony formation assay and the photographs of representative fields are shown.

**D**) The number of colonies in the soft agar assay as described in **C** are counted and shown. ANOVA for difference in their colony formation abilities is significant ($p=0.0058$).

**E)** Sections from B16F10 xenograft derived from vector stable (***a*** and ***b***) and IMP3 stable (***c*** and ***d***) were stained for IMP3 protein by immunohistochemistry. While the vector clone derived tumors show little or no positivity for IMP3, the tumors derived from the IMP3 stable clone show extensive staining for IMP3.

**F)** Fold change in the cyclophilin and IMP3 transcript levels obtained from RT-qPCR analysis of mock, cyclophilin and IMP3 siRNA treated U87-MG-Luc cells at 96 hours after transfection are plotted after normalization with the mock.

**Figure SF6 – IMP3 positive cells are seen to be more invasive in human patient samples and increased IMP3 expression correlates with poor prognosis.**

**A).** IMP3 positive cells are seen to be present in the infiltrating front ***(a),*** the subpial zone ***(b)*** and peri-vascular region ***(c*** and ***d)*** of GBM tumors**.** The yellow arrows indicate IMP3 positive cells.

**B)** Kaplan-Meier survival estimates for 83 GBM samples are calculated based on IMP3 staining. Survival curves for the groups positive and negative for IMP3 in univariate analysis were generated. The cases that were positive for IMP3 (black line) had a shorter survival than the cases that were negative (red line), and this difference was statistically significant (Gehan-Breslow $p=0.0441$).

**Figure SF7 – Over-expression of IMP3 in a mouse model of glioma.**

**A)** Fold change in the murine IMP3 transcript levels obtained from RT-qPCR analysis of the multiple glioma tumor samples (mouse model) and normal mouse brain are plotted.

**B)** Equal amounts of total protein lysates from multiple glioma tumor samples (mouse model) and normal mouse brain were subjected to western blotting to detect levels of murine IMP3 and tubulin proteins.

**C)** Mouse brain with glioma (mouse model) stained for IMP3. The tumor region of the mouse brain **(Box A)** and subpial region **(Box B**) stained positive for IMP3. The yellow arrows indicate IMP3 positive cells.

**Figure SF8 – IGF-2 transcript levels across the different grades of astrocytoma.**

Log2-transformed gene expression ratios obtained from RT-qPCR analysis of indicated samples are plotted for IGF-2. Each dot represents data derived from one sample. For each sample, fold change in gene expression is calculated over its mean expression in normal brain samples after normalization with the internal reference genes. After analysis, no significant change in the median log2ratio of IGF2 transcript levels was found across the different grades of glioma (Normals = -0.10, DO II = -0.65, DA II = 0.005, AO III = -0.26, AA III = -0.22 and GBMs = 0.38). ANOVA for difference in the IGF2 transcript levels across the grades is not significant (p=0.1301).

**Figure SF9**

U138 glioma cells transfected with cyclophilin or IMP3 siRNA for four days and subjected to polysome analysis as described in experimental procedures. The polysome profiles of both conditions

are shown.