SUPPLEMENTARY DATA

Long noncoding RNA INXS is a critical mediator of BCL-XS induced apoptosis

Carlos DeOcesano-Pereira, Murilo S. Amaral, Kleber S. Parreira,
Ana C. Ayupe, Jacqueline F. Jacysyn, Gustavo P. Amarante-Mendes,
Eduardo M. Reis, Sergio Verjovski-Almeida

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Figure S1. Levels of INXS lncRNA and of BCL-X mRNA isoforms in non-tumor and matched tumor samples from kidney cancer patients. (A) INXS expression in non-tumor (dark blue) and...
paired tumor (light blue) tissues across a panel of 13 clear-cell renal cell carcinoma patient samples (P1 to P13). Expression for each patient is shown relative to P9 tumor sample. The data are the mean ± SD of three technical replicates. (B) INXS expression in the non-tumor (dark blue) sample of each patient is set to 1 and that of the paired tumor (light blue) sample is calculated relative to the non-tumor. Patients are sorted in the ascending order of INXS relative level in the tumor. (C) BCL-XS (black) and BCL-XL (red) mRNA isoform levels in non-tumor (NT) and paired tumor (T) tissues across the panel of 13 clear-cell renal cell carcinoma patient samples. Expression levels are relative to the expression of the BCL-XS isoform in the tumor tissue of each patient. The data are the mean ± SD of three technical replicates.
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only with INXS-ASO-2. Caspase 8 is not affected. The data are compared with scrambled oligonucleotide, and are the mean ± SD of three independent experiments. (F) Treatment with SFN (50 µM) in the presence of two distinct INXS-knockdown antisense oligonucleotides (INXS-ASO-1 or -2) significantly abrogates caspase 7 activation only with INXS-ASO-2. Caspase 8 (orange bars) is not affected. The data are compared with scrambled oligonucleotides, and are the mean ± SD of three independent experiments.
Figure S5. Titration of INXS knockdown in the 786-O cell line exposed to UV-C light, using modified antisense oligonucleotides INXS-ASO-1 and -2. (A) The levels of INXS IncRNA were measured in the 786-O cell line exposed to UV-C light upon cell transfection with each one of the two modified antisense oligonucleotides INXS-ASO-1 and -2 for 24 h, using 50nM (light blue), 100nM (blue) and 200nM (dark blue) oligonucleotide, either separately or together. (B) ASO concentrations are indicated by the different color bars. For each of the transfections, the levels of BCL-XS and BCL-XL mRNA isoforms were measured by RT-qPCR and the BCL-XS/BCL-XL mRNA ratio is shown. The data are the mean ± SD of three independent experiments.
Figure S6. Overexpression of INXS induces a shift from the long to the short BCL-X isoform and promotes apoptosis in MCF7 and PC3 tumor cell lines. (A, F) The levels of INXS lncRNA

(A) INXS-induced dose-dependent BCL-X pre-mRNA shift in MCF7

(B) Ratio fold in MCF7

(C) BCL-X expression in MCF7

(D) MCF7 Wild-type vs. Mock vs. Empty pCEP4

(E) INXS overexpression

(F) INXS-induced dose-dependent BCL-X pre-mRNA shift in PC3

(G) Ratio fold in PC3

(H) BCL-X expression in PC3

(I) PC3 Wild-type vs. Mock vs. Empty pCEP4

(J) INXS overexpression

(A, F) The levels of INXS lncRNA
(blue bars), BCL-XS mRNA (black bars) and BCL-XL mRNA (red bars) were measured by RT-qPCR in (A) MCF7 breast cancer cells, and (F) PC3 prostate cancer cells after 24 h of transient transfection with increasing amounts of pCEP4-INXS plasmid. In parallel, these RNAs were measured in control cells, either wild-type cells or cells transfected with empty plasmid. All expression levels are shown as relative abundance with respect to the endogenous INXS in wild-type cells, and are the mean ± SD of three independent experiments. (B, G) In the two cell lines, the increase in BCL-XS/BCL-XL ratio depends on the extent of INXS overexpression. In all cases, the data represent the mean ± SD of three independent experiments. (C, H) In the two cell lines, the total BCL-X mRNA does not change upon INXS overexpression. In all cases, the data represent the mean ± SD of three independent experiments. (D, I) Apoptosis was detected in the (D) MCF7 and (I) PC3 cell lines transfected with 3 µg pCEP4-INXS plasmid. These results were obtained by flow cytometry using double labeling with Annexin V FITC (AV FITC, x-axis) and propidium iodide (PI, y-axis). The percentage of cells that were labeled with AV FITC is shown in the quadrants marked with broken blue lines. (E, J) The results from each quadrant in D and I are shown as the fraction of labeled cells relative to the total. The data are the mean ± SD of three independent experiments.
Figure S7. Overexpression of the unspliced antisense IncRNA ANRASSF1 does not induce a change in BCL-X isoforms abundance or apoptosis. (A) The levels of BCL-XS (black) and BCL-XL (red) mRNA isoforms were measured by RT-qPCR in 786-O kidney tumor cells 24h after transient transfection with the pCEP4-ANRASSF1 plasmid (ANRASSF1 IncRNA levels = orange bars). (B) BCL-XS/BCL-XL mRNA ratio in these cells was not reduced upon ANRASSF1 overexpression. (C) Upon transfection of 786-O cells with pCEP4-ANRASSF1 plasmid (orange bars) or with pCEP4-Empty (grey bars), apoptosis was measured by flow cytometry using double labeling with Annexin V FITC (AV) and propidium iodide (PI). The plot shows the fraction of labeled cells relative to the total. Cells transfected with pCEP4-INXS (blue bars) were used as a positive control. The data are the mean ± SD of three independent experiments.
Figure S8. Time course of changes in \textit{INXS} lncRNA and in \textit{BCL-X} mRNA isoforms expression following transfection with pCEP4-INXS plasmid. (A) \textit{INXS} lncRNA levels in 786-O cells transfected with pCEP4-empty (light blue) or with pCEP4-INXS plasmid (dark blue), measured at the indicated times after transient transfections. (B) \textit{BCL-XS} mRNA levels measured in the same cells as in A. (C) \textit{BCL-XL} mRNA levels measured in the same cells as in A. (D) \textit{BCL-XS}/\textit{BCL-XL} ratio in the same cells as in A. In all panels, the data are the mean ± SD of three independent experiments.
Figure S9. Immunofluorescence detection of active caspase 3 in 786-O kidney tumor cells. Upper panels shows control cells at 24 h after transfection with pCEP4-empty plasmid, and lower panels show cells at 24 h after transfection with pCEP4-INXS plasmid. Cells were stained with Hoechst dye (left), with anti-cleaved active caspase 3 (center left) and with anti-tubulin (center right). On the far right panel, the images were merged. Scale bars, 10 μm.
## II. Supplementary table

### Table S1. List of primers used in the present study

#### 5' and 3' RACE

| Target | Primer | Sequence                  |
|--------|--------|---------------------------|
| INXS   | 5'RACE-Outer | CTCTCTCCCCCACCTGTGATACAAAAG |
| INXS   | 5'RACE-Inner  | GAGATCCAGAGTCACCTGTTCTTTCG |
| INXS   | 3'RACE-Outer  | CTAAGGGACTTCTCAATGGGGTTC   |
| INXS   | 3'RACE-Inner  | CCTCTGAGCTCAGTGGCCGGTA    |

#### Cloning

| Target | Primer | Sequence                  |
|--------|--------|---------------------------|
| INXS   | Forward  | AGTAGCTAGCAGTGGAATGAGGCCAGTC |
| INXS   | Reverse  | TTATCTCGAGCAGAAACGGCTGAG  |

#### Strand-specific RT-qPCR

| Target | Primer | Sequence                  |
|--------|--------|---------------------------|
| INXS   | Sense/Forward  | CCCCCCTCCAGGTACCAACACAGAC |
| INXS   | Antisense/Reverse | CCACTGCGTCTTTGATATAG  |

#### RT-qPCR of total BCL-X, of BCL-X isoforms and of controls and normalizers

| Target   | Primer | Sequence                  |
|----------|--------|---------------------------|
| Total BCL-X | Forward  | CACAGCAGCAGTTGGATGC       |
| BCL-XL   | Reverse | ATATGCTGTCCTGCGGGTGA     |
| BCL-XS   | Forward | TAAACTGGGGTGCACTTTGC      |
| GAPDH    | Reverse | GAGTAGCTAGCGGCTGAGCAGAAG |
| pre-IrRNA^Tyr | Forward | AAAAAACGCAGCTTCCGCTTCCG |
| 5S rRNA  | Reverse | AGGCCGCTCCTCCAGGGCTCT    |
| 18S rRNA | Forward | GCAGGCGCCGGTACAGCGAGCC   |
| 7SK      | Forward | GATTATATGGTACCCTCTTCTT   |
| ACTB     | Reverse | GCCGAGCTAAGCAGTGCAGCG    |
| MYC      | Forward | TCAAGAGGCTCCAGCTACAGC    |
| TUBA1C   | Reverse | AGTGCCAGTGCCAGCTACTACT   |
| SNORD15A | Forward | GAAGAGAGATGAGCGAGTCTGACT |
| HPRT1    | Reverse | GAAATTACTTCAACACAGGGGCTT |
| CCN1-v1  | Forward | TGGAGGTGGGAAAGGGGTG       |
|          | Reverse | GAGCACACAGAGGCTACAA      |
|          |        | GGGACATCACCTCAGTTTAC   |
| SRSF1-v1  | Forward | TGCTACGGCTTCTGCTACGA |
|-----------|---------|----------------------|
|           | Reverse | GATCTCATGAGGGAGAAACTGCC |
| SRSF1-v2  | Forward | CCCGGATGTAGGCAGTTTCT |
|           | Reverse | TTCAGGCAAGGTGTCAGAG |
| ANRASSF1  | Forward | GGCAATTAGAAGCTCCTTG |
|           | Reverse | CTGTGCTAGGGCATAGAGATCC |

**Promoter assay**

| Target                  | Primer      | Sequence                                      |
|-------------------------|-------------|-----------------------------------------------|
| Antisense promoter      | Forward ²   | AGTAAGCTTTGCGAGCAACAGTGTCT                    |
|                         | Reverse ²   | TTATGCTAGCAAGGTTAATCGCTGGAAATAC             |
| Inverted promoter       | Forward ²   | TTATGCTAGCTGCCAGCAACAGTGTCT                  |
|                         | Reverse ²   | AGTAAGCTTTAGAGGTAACTCGTTGAAATAC             |
| Short antisense         | Forward ²   | AGTAAGCTTTGCGAGCAACAGTGTCT                  |
|                         | Reverse ²   | TTATGCTAGTTGCGAGCAACAGTGTCT                |

**Knockdown**

| Target     | Oligo    | Sequence                                      |
|------------|----------|-----------------------------------------------|
| INXS       | INXS ASO-1 | mG*mC*mC*mU*mC*A*C*C*T*C*A*C*C*mA*mG*mU*mC*mU |
| INXS       | INXS ASO-2 | mC*mA*mC*mC*mU*C*C*T*C*C*C*G*A*mC*mC*mU*mG*mU |
| Control    | ASO-Scrambled | mA*mC*mU*mA*mC*G*A*T*C*A*C*A*C*mU*mC*mU|mU*mU |

**RNA-binding protein immunoprecipitation assay (RIP)**

| Target | Primer      | Sequence                                      |
|--------|-------------|-----------------------------------------------|
| BCL-X  | Forward     | ATGTCTCAGAGCAACCGGAGCTG                       |
|        | Reverse     | TCATTTCCGACTAGTGAGGCC                        |
| BCL-2  | Forward     | GGTGGAGCTTCTCGAG                           |
|        | Reverse     | ACAGTTCAAGGCATCC                           |

(*) = Phosphorothioate;
(m) = 2'-O-Methyl;
² Sequence of primers containing restriction sites used for cloning are labeled in bold and underlined.
III. Supplementary materials and methods

Cell culture

Cell lines 786-O, HK-2, HepG2, 769-P, CaKi-1, MCF7, HEK293 and HeLa from ATCC, plus cell line RC-142, were maintained in DMEM (Cultilab) containing 10% fetal bovine serum (v/v) (Cultilab). PC3 (ATCC) cell line was maintained in DMEM/F12 (Cultilab). THLE-3 (ATCC) cell line was maintained in BEGM Bullet Kit without Gentamycin/Amphotericin (GA) and with Epinephrine 5 ng/ml, EGF 70 ng/ml, Phosphoethanolamine containing 10% fetal bovine serum (v/v) (Cultilab). MCF10A (ATCC) cell line was maintained in HAMF12 (Cultilab) supplemented with EGF (Sigma), choleric toxin (Sigma), insulin (Sigma) e hydrocortisone (Sigma), 5% horse serum (v/v) (Cultilab). RWPE-1 (ATCC) cell line was maintained in Keratinocyte Serum Free Medium (K-SFM, Gibco), supplemented with BPE (Gibco) and EGF (Gibco). LNCaP (ATCC) cell line was maintained in RPMI (Gibco) containing 10% fetal bovine serum (v/v) (Cultilab).

Patient samples

Samples of renal cell carcinoma tissue and of adjacent non-tumor tissue from the freshly-frozen tissue bank of Hospital de Cancer, Instituto Nacional de Cancer, Rio de Janeiro, were obtained with informed consent from patients submitted to radical nephrectomy and were snap-frozen in liquid nitrogen within 10 min from resection. Samples were analyzed anonymously.

Animals

Four-week-old and six-week-old athymic female BALB/c nude mice, weighing 20 – 24 g, were obtained from the Animal Production Area of the Institute of Chemistry, Universidade de São Paulo. The animals were monitored daily and maintained under specific-pathogen-free conditions. All experiments were performed in compliance with national guidelines and regulations, in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA), and have been approved by the Internal Animal Care and Use Committee of Institute of Chemistry, Universidade de São Paulo.

RT-qPCR

For the absolute quantification of INXS lncRNA across the different cell lines in Figure 1C, we measured INXS in the cDNA samples by qPCR with Power SYBR Green (Applied Biosystems) and compared with the measurements by qPCR of a dilution curve of INXS PCR product that had been purified and independently quantified by UV absorbance (1). For this absolute quantification we used cDNA generated by strand-specific reverse transcription (RT) using 3 µg of total RNA in 20 µl of RT reaction with SuperScript III (Invitrogen) and the INXS-Antisense/Reverse primer, followed by qPCR with the same primer (Antisense/Reverse) plus the Sense/Forward primer (primer sequences are described in Table S1) using 5 µl of the 8-
fold diluted RT reaction in 20 µl of qPCR. For the sense control transcript, RT was performed with the INXS-Sense/Forward primer, followed by qPCR with the pair of primers. Two controls for the RT step, one without primer (- primer) and the other without reverse transcriptase (-RT) were performed, followed by qPCR with the pair of primers, in order to confirm the absence of RNA self-priming and of genomic DNA contamination in the RT, respectively (2).

For the experiments of Figure 1B and Figure 7A we used strand-specific RT as described above, in order to measure the antisense INXS and to preclude the detection of pre-mRNA eventually present in the sample, and PCR with 40 and 35 cycles was used, respectively.

For measuring INXS in all other experiments except those in Figures 1B, 1C and 7A, as well as for measuring protein-coding mRNAs, oligo-dT primed reverse transcription was performed using 1 µg of total RNA in 20 µl of RT reaction with SuperScript III (Invitrogen), followed by qPCR using 5 µl of the 8-fold diluted RT reaction in 20 µl of qPCR. For cell lines assays, transcript levels were normalized to GAPDH, and represented as relative abundance using the delta Ct method (3). For kidney tissue samples, HPRT1 was used as normalizer.

For measuring BCL-XL isoform by qPCR, a pair of primers was designed that annealed to the unique segment present on exon 2 of the long isoform. For measuring BCL-XS, the reverse primer was designed in a manner that it spanned the exon 2/exon 3 junction; the 5’-portion of the primer is complementary to the 5’-end of exon 3 of BCL-XS and the 3’-portion of the primer is complementary to the 3’-end of exon 2. The slash in the sequence (GTTCCACAAAGTATC/CTGTTCAAAG) marks the exons boundary. For measuring total BCL-X mRNA, independent of the isoforms, a pair of primers was designed to target a sequence on exon 2 that is common to both isoforms. The primer sequences are listed in Table S1.

For estimating the copy numbers of INXS and BCL isoforms in the 786-O tumor cell line, we assumed 20 pg total RNA per cell; the absolute level of INXS (236 copies per ng total RNA, from Figure 1C) corresponds to approximately 5 copies of INXS per cell. Using the relative abundances (Ct values) of INXS, BCL-XS and BCL-XL that were measured under the same conditions in 786-O (oligo-dT primed reverse transcription and the same amount of input cDNA in the qPCR) we estimated approx. twice as much BCL-XS in the cell (12 copies per cell) and 35-fold higher BCL-XL compared to INXS (167 copies per cell).

Biogenesis by RNA polymerase II, 5’-cap structure and stability assays
To test for the presence of a methylguanosine 5’-cap modification in INXS, total RNA (10 µg) from HeLa cells was treated with 1 unit Terminator 5’-phosphate-dependent exonuclease (5-Exo, Epicentre) for 2 h at 30°C, with or without prior treatment with 10 units tobacco acid pyrophosphatase (TAP) for 1 h at 37°C, according to the manufacturer’s protocol. After exonuclease treatment, qPCR for INXS was performed. TUBA1C tubulin gene was assayed as a control. The results were normalized using the level of SNORD15A snoRNA, a non-capped RNA (4). The primers used are listed in Table S1.
For RNA Polymerase II inhibition and RNA decay assays, 5x10^5 cells were cultured for 24 h. Subsequently, for the RNAP II inhibition assays, the medium was replaced by fresh medium with 50 µg/ml α-amanitin (Sigma) or vehicle (water) and maintained for 9h. For the RNA decay assay, the culture medium was replaced by medium with 10 µg/ml actinomycin D (Invitrogen) or vehicle (DMSO) during 0, 1, 3, 6 and 8 h. After treatment, the cells were harvested and total RNA was extracted. For the RNAPII inhibition assay, qPCR was performed for INXS and for ACTB, MYC, pre-tRNA^Tyr, 7SK, which were assayed as positive and negative controls. The results were normalized using the level of 5S RNA. For the decay assay, at each time point the INXS and MYC levels were measured using qRT-PCR and normalized to that of an untreated sample. The MYC gene was assayed as a control for half-life estimation. The primers used are listed in Table S1.

**Cell fractionation**

Nuclear and cytoplasmic fractionation and RNA isolation were performed as previously described (5). The RNA was used for reverse transcription and qPCR as described above. The results were normalized relative to the level of GAPDH detected in the same fraction, either cytoplasmic or nuclear (6). Adequate cell fractionation was controlled by separating the total RNA extract of the fractions by electrophoresis in the BioAnalyzer (Agilent) and detecting the presence of a 32S rRNA band exclusively in the nuclear fraction (7). Additionally, enrichment of MALAT1 lncRNA (1) and of 45S rRNA in the nuclear fraction was measured by RT-qPCR as a nuclear fraction control, as well as the enrichment of 18S rRNA was measured by RT-qPCR as a cytoplasmic fraction control. Also, a western blot of the protein extracts from the fractions was performed, and has detected GAPDH (Sigma, G8795 anti-GAPDH Ab) only in the cytoplasmic fraction and histone H3 (Millipore, 06-599B anti-acetyl-histone H3 Ab) only in the nuclear fraction.

**Induction of endogenous INXS expression**

Cells (786-O) on 100-mm plates were exposed to UV-C for 40 s at 1 J.m^{-2}.s^{-1} immediately following the replacement of the culture medium by PBS, and subsequently grown in culture medium with 10% serum for up to 24 h. For INXS knockdown followed by UV-C induction, 60-mm plates were used, and knockdown (see below) was carried out prior to UV-C exposure. For the serum reduction assay, cells were grown for 24 h with different serum concentrations, as indicated in the figures. For SFN treatment, 50µM DL-sulforaphane (Sigma) was added to cells in DMEM medium with 10% serum and cultured for up to 24 h.

**Promoter assay**

Putative promoter sequences were amplified from genomic DNA (primers are listed in Table S1) and cloned into pGL3-basic vector in the direct (pGL3-antisense) or inverted (pGL3-inverted) orientation. A Dual-Luciferase Reporter Assay System (Promega) was used with HEK293 cells according to the manufacturer’s protocol.
Transient plasmid transfection

The INXS full-length cDNA was cloned into the NheI and XhoI sites of pCEP4 expression vector (Invitrogen), and it was sequence-verified. The cells were transfected with pCEP4-INXS or with empty vector, using 1 µg of plasmid per 10⁶ cells and FuGENE HD (Promega), and grown for 24 h prior to qPCR or apoptosis assays. For the time-course assay, cells were transfected with 1 µg of plasmid per 10⁶ cells and FuGENE HD (Promega), and grown for the indicated periods of time prior to qPCR. For the plasmid titration assay, the cells were transfected with the different amounts of plasmid that are indicated in the figures, and the assay was carried out after 12 h.

Oligonucleotide transfection

For silencing of INXS lncRNA, the cells were plated for 24 h in 60 mm-diameter plates in OPTI-MEM (Invitrogen) medium. Subsequently, two distinct modified 20-mer oligos (500 nM, final concentration, IDT) targeting INXS or a modified scrambled oligo (IDT) were separately transfected using lipofectamine RNAimax (Invitrogen); total RNA was extracted 24 h after transfection. Oligonucleotide sequences are listed in Table S1.

We have titrated the effectiveness of the two ASOs in reducing INXS levels in the 786-O kidney tumor cell line after induction of INXS by UV-C exposure (Figure S5). Using the two distinct ASOs in the range 50, 100 and 200 nM, each one separately and the two in combination, we found a dose-dependent progressively more marked reduction of INXS with increasing concentrations of the ASOs, and the most effective reduction of INXS was obtained with the combination of ASO-1 plus ASO-2 at 200 nM (Figure S5A). In parallel, we observed a progressive change in the BCL-X isoforms, and a progressive reduction in the BCL-XS/BCL-XL ratio with increasing ASOs (Figure S5B). Again, the most effective reduction was obtained with the combination of ASO-1 plus ASO-2 at 200 nM; a similar extent of reduction was obtained for each of the two ASOs separately, at 500 nM each (see Figure 5 in the main text).

Note that in spite of obtaining a pronounced INXS knockdown effect with the combination of ASO-1 plus ASO-2 at 200 nM, we have favored the ability to test the two distinct ASOs separately, in order to be able to collect independent evidence of phenotypic effects elicited for each of the two distinct ASOs, which adds to the evidence of a specific effect related to INXS knockdown. Therefore, for further tests with the three apoptosis-inducing agents (UV-C, serum starvation and sulforaphane, in Figure 5) we used each ASO separately at 500 nM concentration (8), rather than testing the two ASOs in combination.

Fluorimetric determination of caspase activity

Four million cells were washed twice with PBS, and lysed in 50 µl of ice-cold lysis buffer (150 mM NaCl, 20 mM Tris pH 7.5, 1% Triton X-100, 10 µg/ml leupeptin, 5 µg/ml aprotinin and 100 µM PMSF). The cell lysates were incubated on dry ice for 1 min, followed by 10 min
incubation at 4°C, and supernatants were obtained by centrifugation at 15,000 g for 15 min at 4°C. Fifty µl of lysate supernatants were then transferred to a 96-well flat bottom microplate containing 100 µl of caspase 3 (Ac-DEVD-AFC), caspase 7 (Ac-VDVAD-AFC), caspase 8 (Ac-IETD-AFC) or caspase 9 (Ac-LEHD-AFC) fluorogenic substrates, in the presence or absence of the respective caspase inhibitor (Kamiya Biomedical Company) at 20 µM final concentration. The samples were read on a spectrofluorometer (SpectroMax, GEMINI XS, Molecular Device) at 400 nm excitation and 505 nm emitted light.

RNA-binding protein immunoprecipitation assay (RIP)
Native RIP was performed using the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer’s instructions, with 5 µg of each of the following antibodies: Rabbit IgG Purified (Millipore, PP64B) or anti-Sam68 (Santa Cruz Biotechnology, sc-333). The bead-antibody complexes were washed six times, and resuspended in RIP Wash Buffer. Before purification of RNAs, 10% of each of the resuspended bead complexes was saved, and used for western blot with anti-Sam68. The RNA was extracted from the remaining sample, treated with TURBO™ DNase (Ambion) at 37°C for 30 min, purified using the RNeasy Micro Kit (Qiagen), and the RNA was quantified using RiboGreen (Invitrogen). Reverse transcription and qPCR were performed with these samples, as described in the main text.

Western blotting analysis
In parallel with the RIP assay, the immunoprecipitated proteins attached to the beads were analyzed by western blotting with anti-Sam68 antibody (Santa Cruz Biotechnology) using ECL detection reagents (Amersham, GE Healthcare). For this purpose, in the RIP procedures after the beads were washed and prior to the RNA extraction steps, 10% of the final volume containing the bead-antibody complexes from the RIP assay was saved to perform western blot and detect Sam68. The beads were suspended in Laemmli Sample Buffer and incubated at 95 °C for 5 min, then briefly spun down, and the supernatant was loaded on SDS-PAGE and run at 90V for 2 h. One percent of the cell lysate was separated before immunoprecipitation, and also loaded in parallel, as a control input. The proteins were transferred to a nitrocellulose membrane (Amersham, GE Healthcare), and were developed with anti-Sam68 antibody (Santa Cruz Biotechnology) at 1:200 dilution, using ECL western blotting detection reagents (Amersham, GE Healthcare).

For BCL-XS and BCL-XL proteins detection, we incubated cell lysates from 10⁶ cells in 100 µl RIPA buffer with 30 µl of protein A-sepharose 4B conjugate suspension (101042, Invitrogen) for 1 h, discarded the resin, and incubated the supernatant overnight with 1 µl anti-BCL-X antibody (556361, BD Pharmingen) and 50 µl of protein A-sepharose 4B conjugate suspension (101042, Invitrogen). Subsequently the resin was washed with 20 mM Tris-HCl pH 7.0 buffer, suspended in 25 µl of gel loading buffer, boiled for 10 min, and 20 µl of the supernatant was loaded on 15% SDS-PAGE and run at 90V for 2 h. The proteins transferred
to nitrocellulose membranes were detected with the same anti-BCL-X antibody (556361, BD Pharmingen) at 1:750 dilution, followed by anti-rabbit secondary antibody (Alexa Fluor 750 Goat Anti-Rabbit IgG; Molecular Probes) at 1:10000 dilution, and the membrane was scanned with the Odyssey near-infrared scanner (LI-COR, Biosciences). The relative intensities of the BCL-XS and BCL-XL bands were quantified with the Odyssey software.

Nude mouse xenograft assays
Viable 786-O cells (10^7), as determined by trypan blue staining, were suspended in 200 µL DMEM (Cultilab) without fetal bovine serum and were injected subcutaneously into one flank of nude mice. Palpable tumors formed after 15 - 20 days. When each tumor reached an average volume of 250 mm^3, the mouse was randomly assigned to one of two groups, and the injections of either pCEP4-empty (n = 6) or pCEP4-INXS (n = 6) plasmid were started (day zero). The test consisted of an intra-tumor injection of 10 µg plasmid dissolved in 50 µl (final volume) of TurboFect in vivo Transfection solution (Fermentas), according to the manufacturer's protocol. The injection was applied on day 0 and every third day thereafter, over a period of 15 days of injection. The tumor size was measured in two perpendicular diameters, with a caliper, before each injection. The tumor volume was calculated using the formula 0.5 X (greatest longitudinal diameter) X (greatest transverse diameter)^2 [9]. The animals were euthanized 8 –11 weeks after tumor implant, using CO₂ inhalation, when the tumor volume exceeded 600 mm^3 in the animals treated with pCEP4-empty (control). Autopsies were performed, and the tumors were weighed.

Fluorescence microscopy
For immunofluorescence experiments, cells transfected with pCEP4-INXS or pCEP4-empty were collected 24 h after transfection and fixed with 2% p-formaldehyde for 15min. The cells were plated on coverslips, incubated for 5 minutes with TBS-0.1% Triton X-100 for permeabilization and then blocked with TBS-0.1% Triton X-100 1% BSA for 1h at room temperature. Subsequently, the coverslips were incubated with anti-cleaved caspase 3 (Asp175) (5A1E) Rabbit mAb (9664, Cell Signaling) and anti-α-tubulin (sc-53646, Santa Cruz Biotechnology) antibodies. The visualization of cleaved caspase 3 was performed with specific anti-IgG conjugated with Alexa Fluor-555 (A-21422, Molecular Probes) and the α-tubulin with Alexa Fluor-488 (A-11001, Molecular Probes). The nuclear DNA was stained with 20µg/ml Hoechst 33342 (H1399, Invitrogen). The images were obtained on Nikon Eclipse E600 microscope and deconvoluted using the Huygens Essential software image processing.

In vivo optical imaging system
Tumors were monitored by near-infrared optical imaging using the Odyssey scanner (LI-COR, Biosciences). Mice were injected intravenously via tail vein with 1 nmol of IRDye 800CW EGF Optical Probe (LI-COR, Biosciences) dissolved in PBS per animal, and 12 h after injection the
animals were scanned for in vivo fluorescence imaging according to the manufacturer's protocol.

**Statistical tests**

Data are represented as the mean ± SD. Statistical significance was calculated using a two-way ANOVA followed by the Bonferroni test as a post-test or using a two-tailed paired Student t-test. P<0.05 was considered significant, and statistical significance is denoted in the figures with one asterisk (P<0.05), two asterisks (P<0.01) or three asterisks (P<0.001).

**IV. Supplementary references**

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