**SUPPLEMENTARY DATA**

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

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**I. Supplementary figures and legends (Figures S1 to S9)**

**II. Supplementary table**

Table S1 includes all primer sequences used in the study.

**III. Supplementary materials and methods**

**IV. Supplementary references**

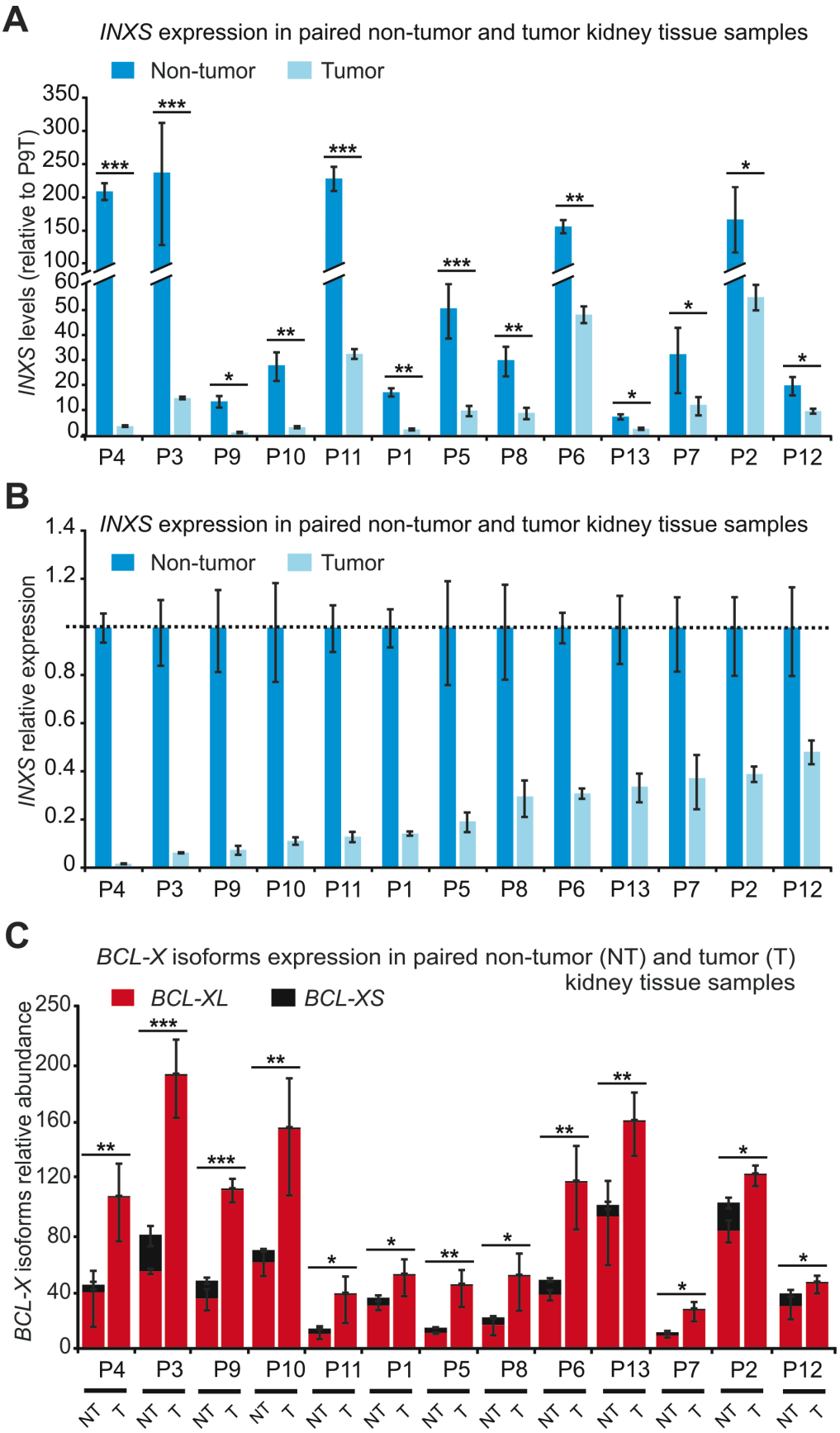


Figure S1. Levels of *INXS* lncRNA and of *BCL-X* mRNA isoforms in non-tumor and matched tumor samples from kidney cancer patients. (A) *INXS* expression in non-tumor (dark blue) and

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.

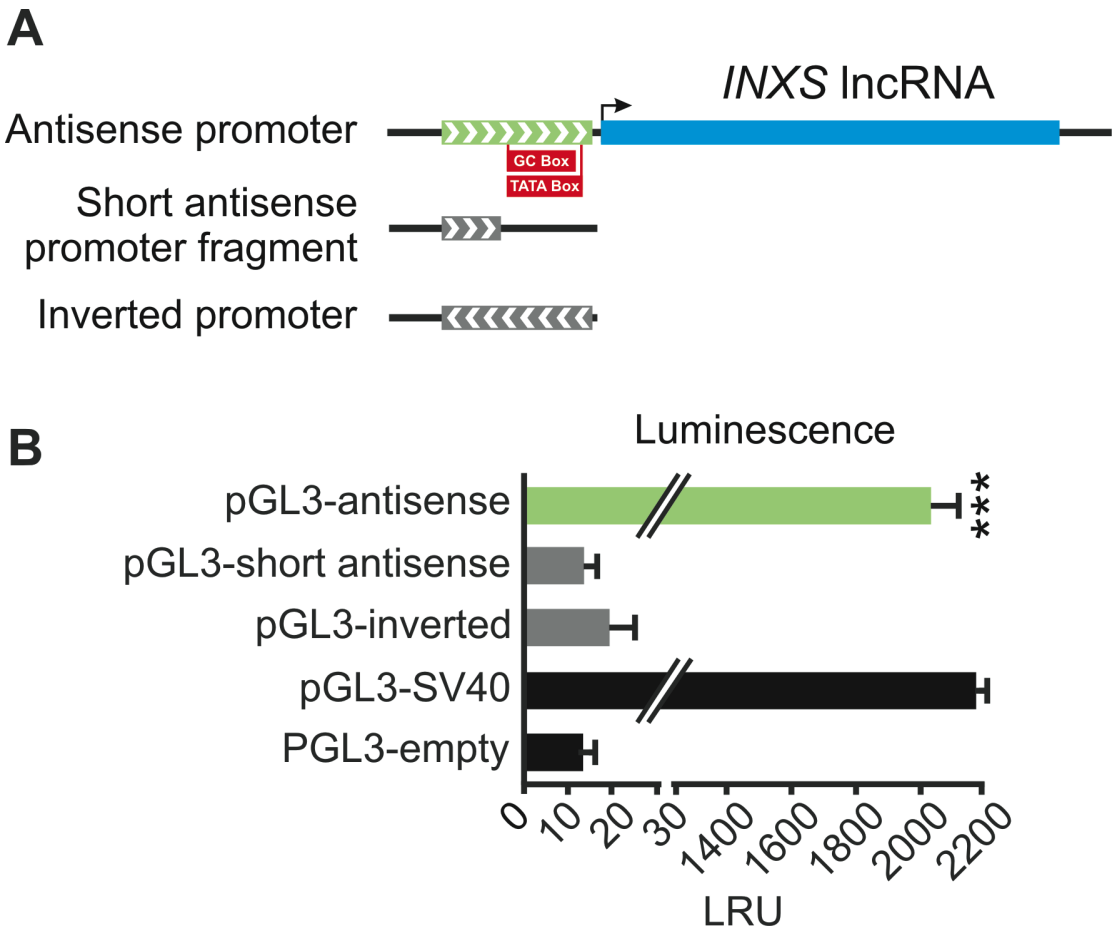


Figure S2. *INXS* promoter assay. (A) Genomic localization of the three different DNA segments (green and gray arrow-headed bars) used in the construction of pGL3-modified plasmid vectors. Arrowheads inside green and gray bars indicate sequence orientation in the plasmid vector construct. (B) Luciferase activity measured in cells transfected with pGL3-antisense, pGL3-short antisense and pGL3-inverted constructs. As a control, cells transfected with a vector containing the SV40 promoter (pGL3-SV40) or a vector without a promoter insert (pGL3-empty vector) were tested. LRU, Luminescence Relative Units; the data are the mean ± SD of three independent experiments.

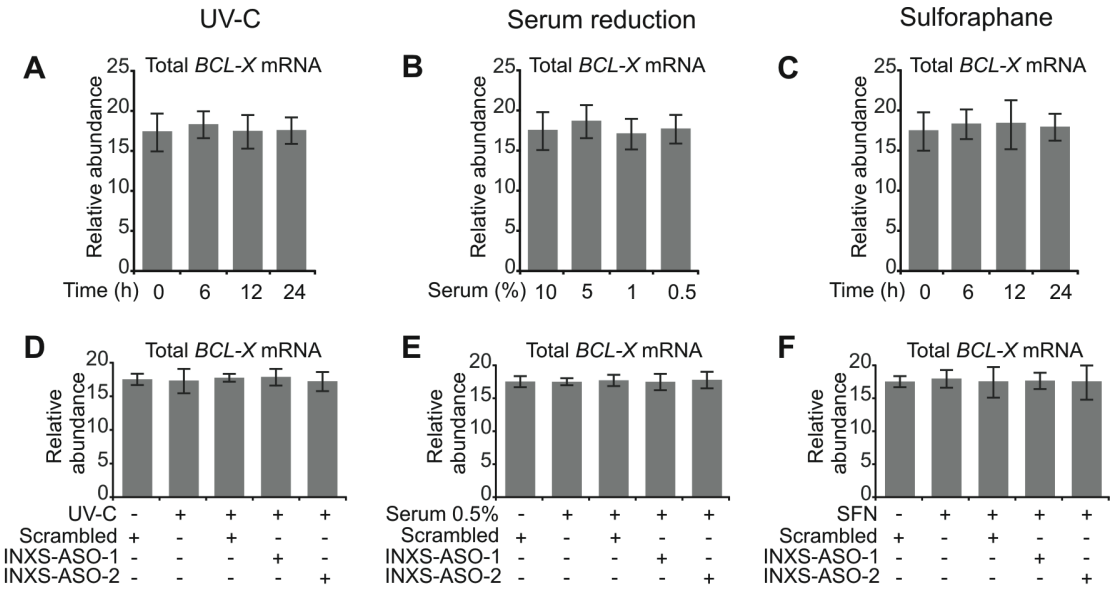


Figure S3. Total *BCL-X* mRNA levels in 786-O cells exposed to apoptosis inducing agents in the absence or the presence of *INXS* knockdown. (A, B, C) In 786-O cells exposed to (A) 40 J/m2 UV-C light, (B) serum reduction or (C) sulforaphane the levels of total *BCL-X* mRNA have not changed. The data are the mean ± SD of three independent experiments. (D, E, F) In 786-O cells treated with two different *INXS*-knockdown antisense oligonucleotides (INXS-ASO-1 or -2) or with a control scrambled antisense oligonucleotide, exposure to (D) 40 J/m2 UV-C light, (E) serum reduction or (F) sulforaphane has not changed the levels of total *BCL-X* mRNA. The data are the mean ± SD of three independent experiments.

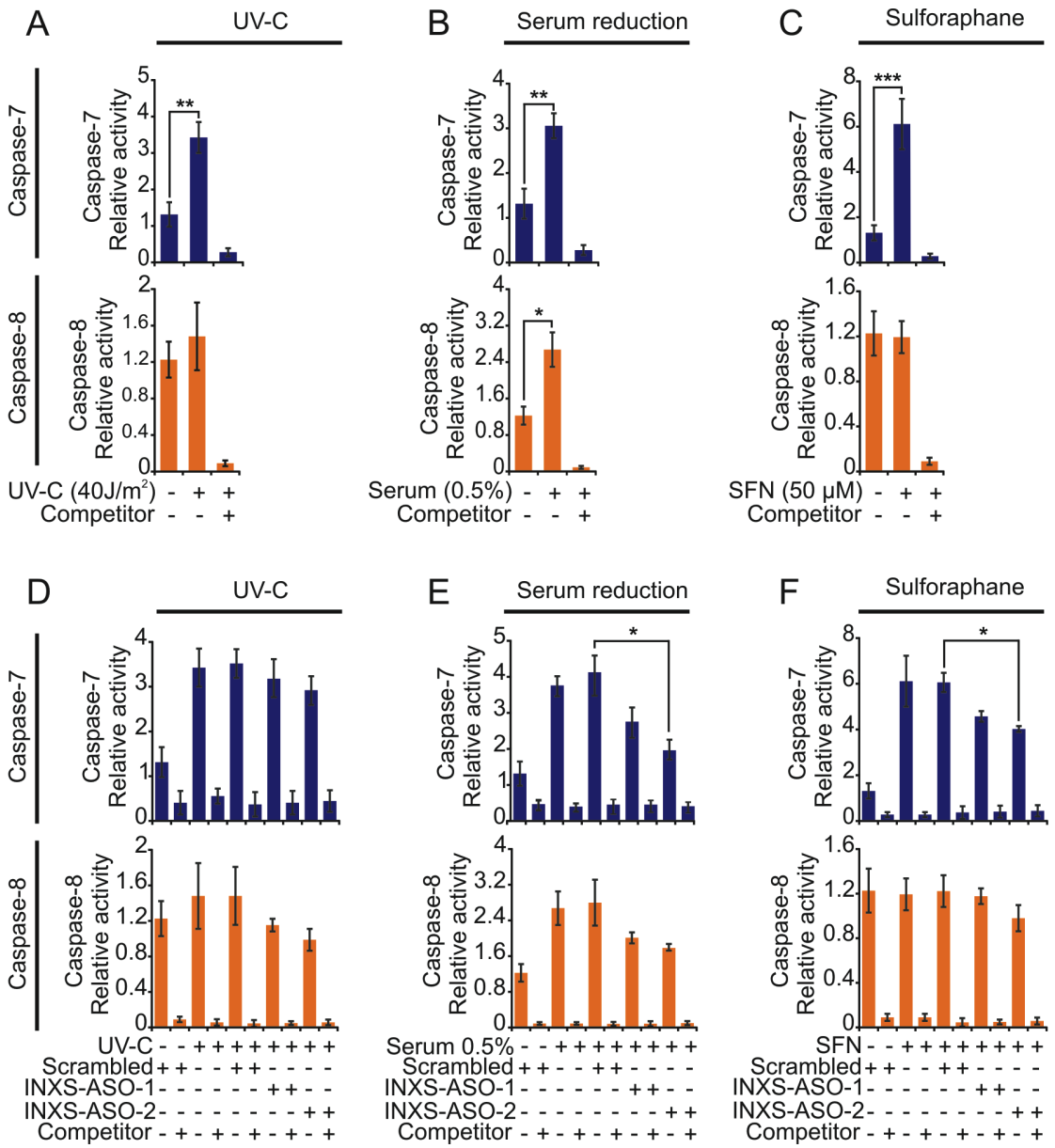


Figure S4. Caspase activation assay in the 786-O cell line following exposure to apoptosis inducing agents in the absence or presence of INXS knockdown. (A) Caspase 7 (blue bars) is activated 24 h after exposure to 40 J/m2 UV-C, while caspase 8 (orange bars) is not affected. The data are the mean ± SD of three independent experiments. (B) Caspase 7 (blue bars) and caspase 8 (orange bars) are activated 24 h after serum reduction (0.5%). The data represent the mean ± SD of three independent experiments. (C) Caspase 7 (blue bars) is activated 24 h after treatment with SFN (50 μM), while caspase 8 (orange bars) is not affected. The data are the mean ± SD of three independent experiments. (D) Exposure to 40 J/m2 UV-C light in the presence of two distinct INXS-knockdown antisense oligonucleotides (INXS-ASO-1 or -2) does not affect caspase 7 (blue bars) and caspase 8 (orange bars) activation. The data are compared with scrambled oligonucleotides, and are the mean ± SD of three independent experiments. (E) Serum reduction (to 0.5%) 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 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.

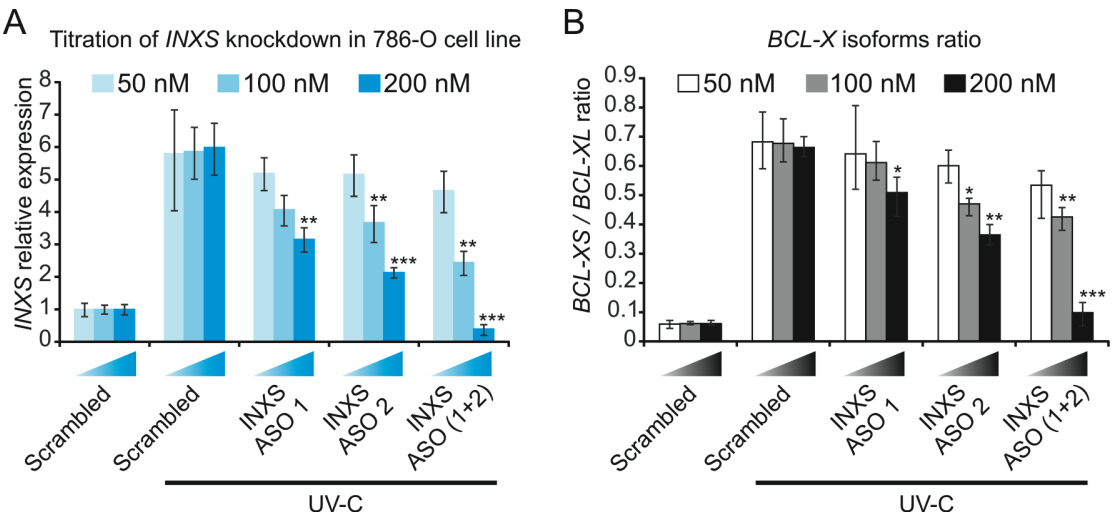


Figure S5. Titration of *INXS* knockdown in the 786-O cell line exposed to UV-C light, using modified antisense oligonucleotides INXS-ASO-1 and -2. (A) The levels of *INXS* lncRNA were measured in the 786-O cell line exposed to UV-C light upon cell transfection with each one of the two modified antisense 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.

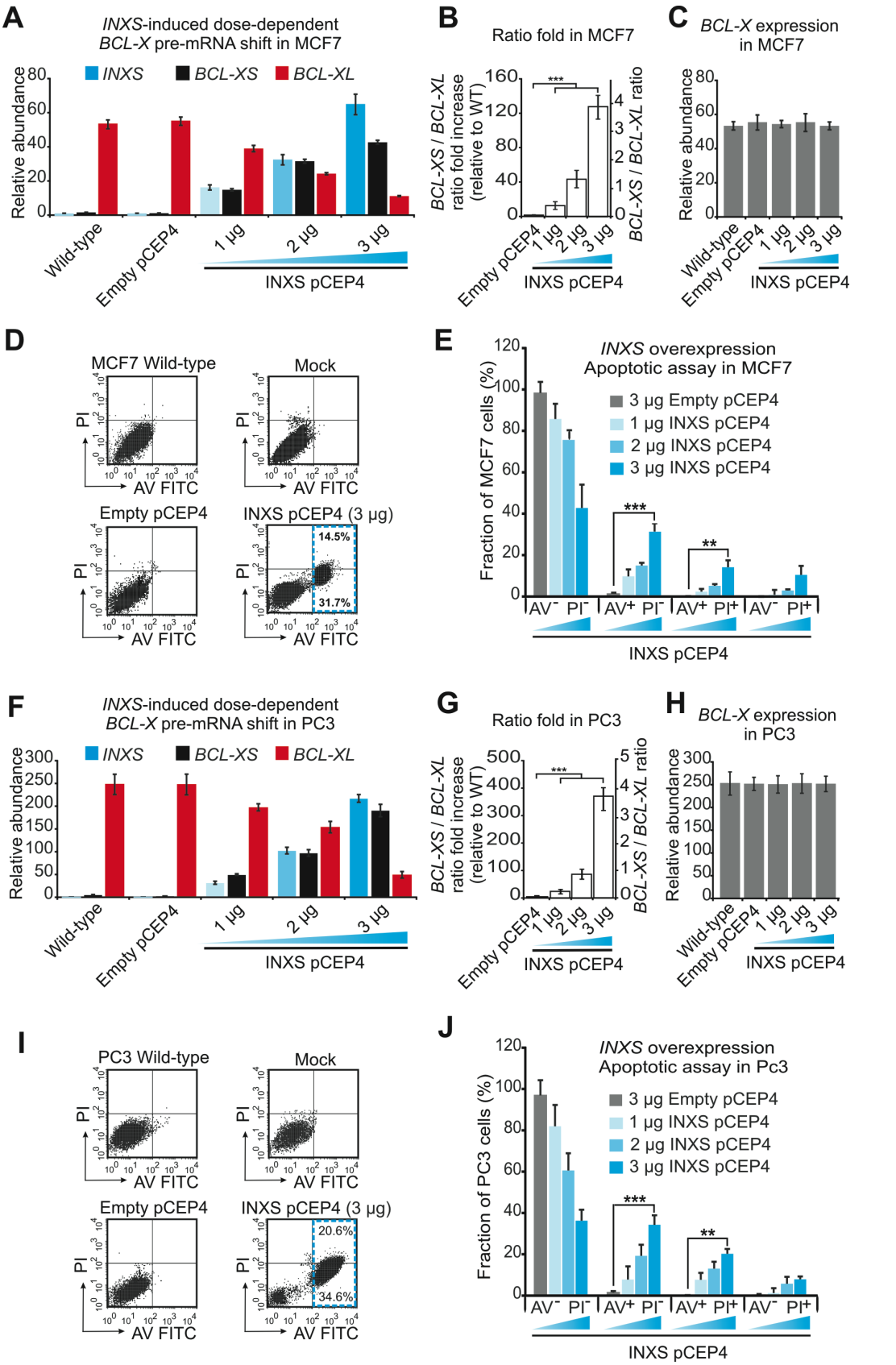


Figure S6. Overexpression of *INXS* induces a shift from the long to the short *BCL-X* isoform and promotes apoptosis in MCF7 and PC3 tumor cell lines. (A, F) The levels of INXS lncRNA

(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.

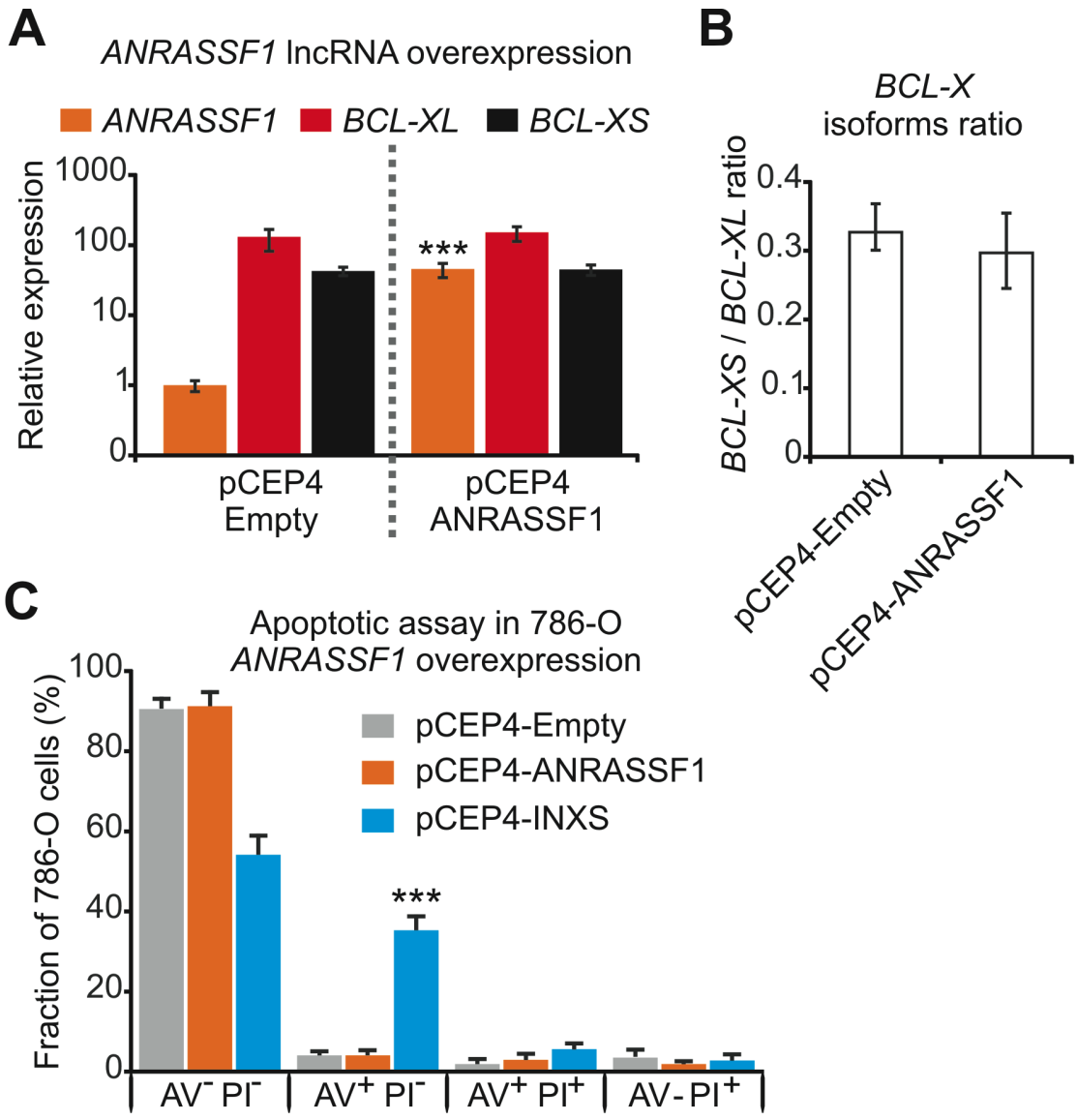


Figure S7. Overexpression of the unspliced antisense lncRNA *ANRASSF1* does not induce a change in *BCL-X* isoforms abundance or apoptosis. (A) The levels of *BCL-XS* (black) and *BCL-XL* (red) mRNA isoforms were measured by RT-qPCR in 786-O kidney tumor cells 24h after transient transfection with the pCEP4-ANRASSF1 plasmid (*ANRASSF1* lncRNA 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.

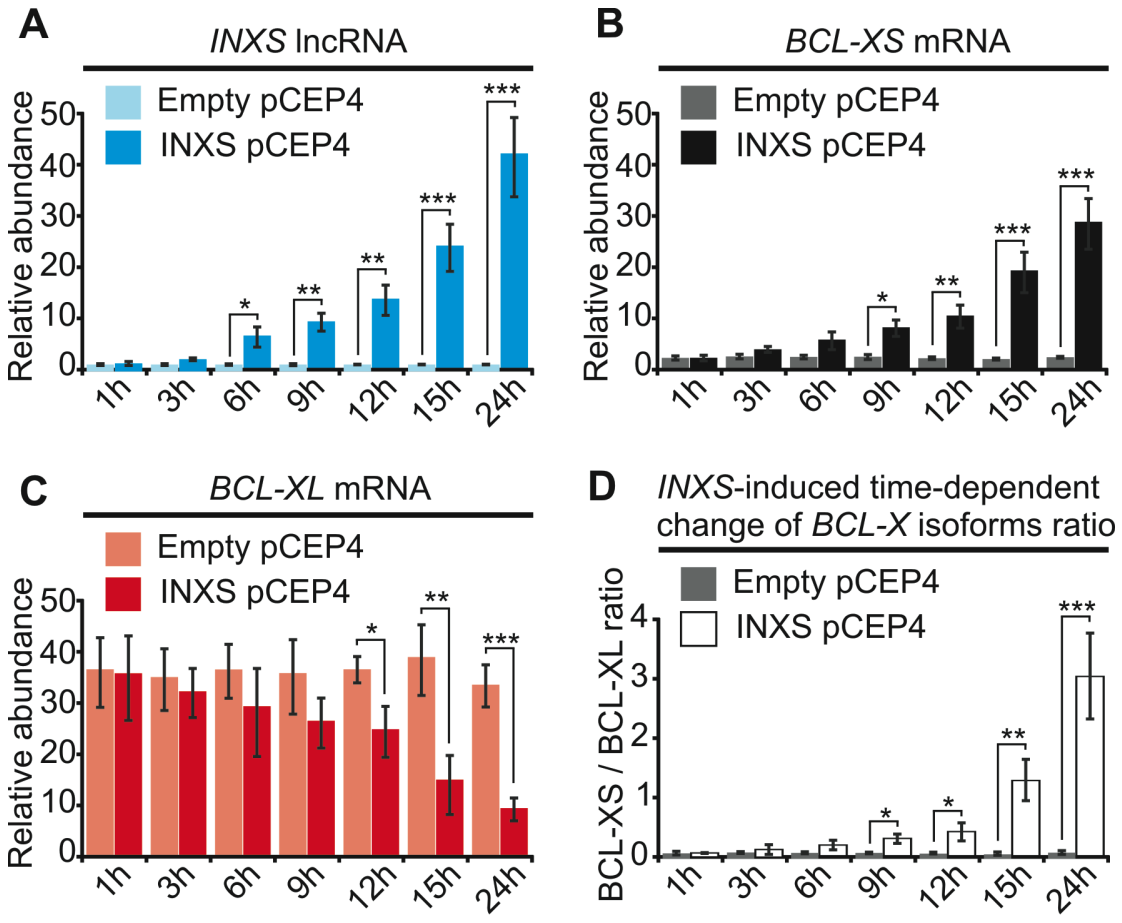


Figure S8. Time course of changes in *INXS* lncRNA and in *BCL-X* mRNA isoforms expression following transfection with pCEP4-INXS plasmid. (A) *INXS* lncRNA levels in 786-O cells transfected with pCEP4-empty (light blue) or with pCEP4-INXS plasmid (dark blue), measured at the indicated times after transient transfections. (B) *BCL-XS* mRNA levels measured in the same cells as in A. (C) *BCL-XL* mRNA levels measured in the same cells as in A. (D) *BCL-XS*/*BCL-XL* ratio in the same cells as in A. In all panels, the data are the mean ± SD of three independent experiments.

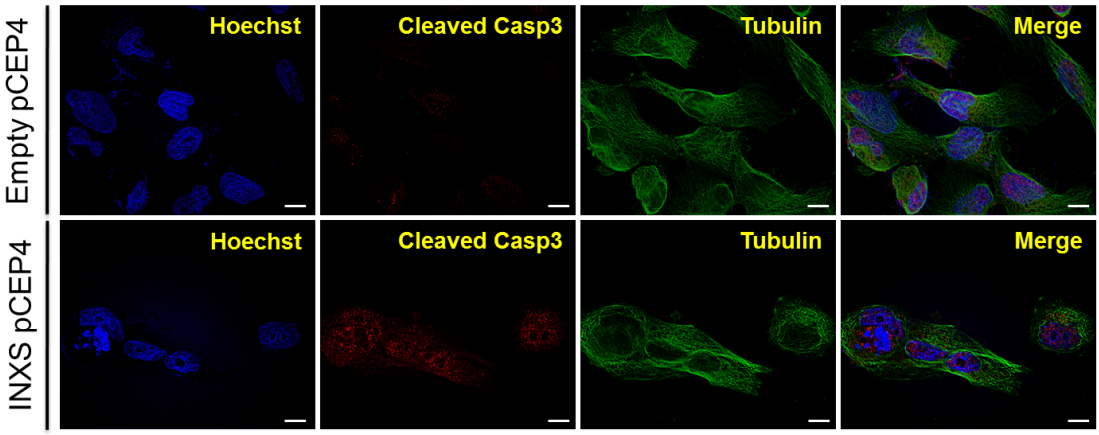


Figure S9. Immunofluorescence detection of active caspase 3 in 786-O kidney tumor cells. Upper panels shows control cells at 24 h after transfection with pCEP4-empty plasmid, and lower panels show cells at 24 h after transfection with pCEP4-INXS plasmid. Cells were stained with Hoechst dye (left), with 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 | CTCCTCTCCCGACCTGTGATACAAAAG |
| | 5'RACE-Inner | GAGATTCAGAGTCCACTGGTGCTTTCG |
| *INXS* | 3'RACE-Outer | CCTAAAGGGACTTCTCAATGGGGTTC |
| | 3'RACE-Inner | CTTCTCGAGCTCACTAGGCCGGGTA |

**Cloning**

| Target | Primer | Sequence |
|---|---|---|
| *INXS* | Forward[§] | AGTAGCTAGCGTGGTGAAATGAGGCCAGTC |
| | Reverse[§] | TTATCTCGAGCAGGAAAACGTGGTCTCAGC |

**Strand-specific RT-qPCR**

| Target | Primer | Sequence |
|---|---|---|
| *INXS* | Sense/Forward | CCCCCTCCAGGTACCAGAAC |
| | Antisense/Reverse | CCACTGGTGCTTTCGATTTGA |

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

| Target | Primer | Sequence |
|---|---|---|
| Total *BCL-X* | Forward | CACAGCAGCAGTTTGGATGC |
| | Reverse | ATATGCTGTCCCTGGGGTGA |
| *BCL-XL* | Forward | TAAACTGGGGTCGCATTGTG |
| | Reverse | AGGTAAGTGGCCATCCAAGC |
| *BCL-XS* | Forward | GCAGTAAAGCAAGCGCTGAG |
| | Reverse | GTTCCACAAAAGTATCCTGTTCAAAG |
| *GAPDH* | Forward | TGCACCACCAACTGCTTAGC |
| | Reverse | GGCATGGACTGTGGTCATGAG |
| *pre-tRNA$^{Tyr}$* | Forward | AAAAAACCGCACTTGTCTCCTTCG |
| | Reverse | CCTTCGATAGCTCAGCTGGTAGAG |
| *5S rRNA* | Forward | AGGCGCCTCCTTCAGCGTCT |
| | Reverse | CAGGCGGTCTCCCATCCAAG |
| *18S rRNA* | Forward | GCAGGCGCGGGTAACC |
| | Reverse | AAGCTTATGACCCGCACTTACTG |
| *7SK* | Forward | GACATCTGTCACCCCATTGA |
| | Reverse | GCGCAGCTACTCGTATACCC |
| *ACTB* | Forward | CGAGGCCCCCCTGAAC |
| | Reverse | GCCAGAGGCGTACAGGGATA |
| *MYC* | Forward | TCAAGAGGTGCCACGTCTCC |
| | Reverse | TCTTGGCAGCAGGATAGTCCTT |
| *TUBA1C* | Forward | TCAACACCTTCTTCAGTGAAACG |
| | Reverse | AGTGCCAGTGCCAACTTCATC |
| *SNORD15A* | Forward | GAAGAGATGATGACGAGTCTGACT |
| | Reverse | GAAATTACTTCAACCAGGGCTCTTT |
| *HPRT1* | Forward | TGAGGATTTGGAAAGGGTGT |
| | Reverse | GAGCACACAGAGGGCTACAA |
| *CCDN1-v1* | Forward | CCAGAGTGATCAAGTGTGAC |
| | Reverse | GGGACATCACCCTCACTTAC |

| | | |
|---|---|---|
| *SRSF1-v1* | Forward | TGCTACGGCTTCTGCTACGA |
| | Reverse | GATCTCATGAGGGAGAAACTGCC |
| *SRSF1-v2* | Forward | CCCGGATGTAGGCAGTTTCT |
| | Reverse | TTCAGGCAAGGTTGTCCAAG |
| *ANRASSF1* | Forward | GGCAATTAGAACGCTCCTTG |
| | Reverse | CTGTGCTAGGCGATAGAGATCC |

**Promoter assay**

| Target | Primer | Sequence |
|---|---|---|
| Antisense promoter | Forward[§] | AGTA**AAGCTT**TGCCAGCAAACAGTGCTT |
| | Reverse[§] | TTAT**GCTAGC**AAGAGGTTAACTCCTTGGAAATAC |
| Inverted promoter | Forward[§] | TTAT**GCTAGC**TGCCAGCAAACAGTGCTT |
| | Reverse[§] | AGTA**AAGCTT**AAGAGGTTAACTCCTTGGAAATAC |
| Short antisense | Forward[§] | AGTA**AAGCTT**TGCCAGCAAACAGTGCTT |
| | Reverse[§] | TTAT**GCTAGC**TGAGAATTGTCAGATTTTGGG |

**Knockdown**

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

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

| Target | Primer | Sequence |
|---|---|---|
| *BCL-X* | Forward | ATGTCTCAGAGCAACCGGGAGCTG |
| | Reverse | TCATTTCCGACTAAGGTGAGCC |
| *BCL-2* | Forward | GGTGGAGCTCTTCAGG |
| | Reverse | ACAGTTCCAAGGCATCC |

(*) = Phosphorothioate;

(m) = 2'-*O*-Methyl;

[§] 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 (GTTCCACAAAAGTATC/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-X 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 *Nhe*I and *Xho*I 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 $10^6$ 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 $10^6$ 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) fluorigenic 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$^{TM}$ 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$^{TM}$ DNase (Ambion) at 37$^{o}$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 $10^6$ 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 $CO_2$ 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-$\alpha$-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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