Extended Data Fig. 1. *SINEUP-DJ-1* enhances DJ-1 mRNA translation.

(a-b) *SINEUP-DJ-1* activity. RNA levels. Canonical *SINEUP-DJ-1* was tested along with mutated forms. Total RNAs were extracted at 48 h after HEK293T cell transfection, reverse-transcribed and analyzed through real time PCR. **a)** DJ-1 mRNA relative expression was quantified and reported on the graph using empty pCS2+ as negative control (Ctrl=1, dotted line). *GAPDH* mRNA was used to normalize. No significant variation was observed (ordinary one-way ANOVA, Holm-Sidak's multiple comparisons test) **b)** Expression of mutated constructs was also assessed through Real-time PCR. SINEUP mutant RNA expression was quantified compared to *SINEUP-DJ-1* RNA levels. All plots indicate mean ± SD, and representation of n = 3 independent biological replicates. **(c-e) SINEUP RNA pull-down. c)** *DJ-1* mRNA expression for RPD. **d)** *SINEP-DJ-1* expression for RPD. **e)** *DJ-1* mRNA expression for ribosome fractionation. **(f-g) Ribosome fractionation. f)** *SINEP-DJ-1* expression for ribosome fractionation. DJ-1 mRNA and *SINEP-DJ-1* RNA relative expression was quantified and reported on the graph using empty pCS2+ as negative control (Ctrl=1, dotted line). *GAPDH* mRNA was used to normalize. **g)** Lysates from control and treated cells were fractionated by sucrose gradients and separated through a UV detector continuously measuring the solution absorbance (260 nm). The relative distributions (in %) of *GAPDH* mRNA were analyzed by RT-qPCR in each of the 13 gradient fractions. RNA distribution was reported as the mean ± SD of 3 replicates.

Extended Data Fig. 2. The invSINEB2 element of *AS Uchl1* acts as an IRES in *cis*.

a) IRES activity assay in U2OS cells. IRES activity. SINEB2 element, in direct (dirSINEB2) and in inverted (invSINEB2) orientations, and *c-myc* IRES were separately cloned between Rluc and Fluc sequences in pRUF-dual luciferase reporter vectors. Empty pRUF-MCS and pRUF-*c-myc* IRES were respectively used as negative and positive controls. Luciferase intensities were measured at 48 h after U2OS cell transfection of each pRUF dual luciferase reporter plasmid (reported in Figure1). Results are shown as the detected intensity ratio of Fluc and Rluc (Fluc/Rluc) normalized against the empty pRUF-MCS background signal. IRES activity was calculated compared to the negative control (Ctrl=1, dotted line). Reported graph shows that invSINEB2 induced Fluc protein translation *in cis* at higher levels than the positive control. Plot indicates mean ± SD of 4 biological replicates performed in duplicate; $^{*}p < 0.05$, $^{**}p < 0.01$ were considered significant. Ordinary one-way ANOVA with Holm-Sidak's multiple comparisons test was used to calculate significances.

(b-h) IRES activity (promoter-less plasmids). Empty pRUF-MCS and pRUF-*c-myc* IRES, both SV40 promoter-less were used as negative controls. **b)** Rluc expression was quantified and showed on the graph as mean luminescence intensity (RFU) of n = 3 independent replicates. **c)** Rluc mRNA

relative expressions were quantified and reported on the graph using non-transfected cells (only LIPO) as control = 1. **d)** Fluc activity was plotted on the graph as mean luminescence intensity (RFU) of n = 3 independent replicates. Expression of Fluc protein was calculated compared to the LIPO control. **e)** Rluc mRNA levels were quantified and reported on the graph using, as control, non-transfected cells. **f)** Fluc mRNA levels relative to Rluc expression (Fluc/Rluc) were reported on the graph. **g and h)** qRT-PCR assessed the expression of the inserted invSINEB2, dirSINEB2 and *c-myc*-IRES sequences. RNA levels were normalized on *GAPDH* mRNA expression. All plots indicate mean ± SD, and represent n=4 independent replicates. **(i-n) IRES activity (bicistronic plasmids).** SINEB2 element, in direct (dirSINEB2) and in inverted (invSINEB2) orientations, and *c-myc* IRES were separately cloned between Rluc and Fluc sequences in pRUF-dual luciferase reporter vectors. Empty pRUF-MCS and pRUF-*c-myc* IRES were used as negative and positive controls respectively. **i)** Rluc activity was quantified and reported on the graph as mean luminescence intensity (RFU) of n = 3 independent replicates. **(j-k)** Total RNAs were extracted at 48 h after HEK293T cell transfection, reverse-transcribed, and analyzed with real-time PCR. **j)** Rluc mRNA relative expressions were quantified and reported on the graph using empty pRUF as control (Ctrl=1, dotted line). **k)** Relative expressions of Fluc mRNAs were calculated compared to the empty control. **l)** Fluc mRNA levels relative to Rluc expression (Fluc/Rluc) were reported on the graph assessing the absence of cryptic promoters and thus of shorter transcripts containing only Fluc or Rluc sequences. No significant variation was observed. **m)** qRT-PCR confirmed the expression of the inserted *c-myc*-IRES, invSINEB2 and dirSINEB2 sequences. RNA levels were normalized on *GAPDH* mRNA expression. All plots indicate mean ± SD, and represent n=4 independent replicates. **n)** Specific region of cDNAs, derived from extracted RNAs, were amplified by performing two different PCRs (PCR1 and PCR2). For PCR1, a pSV40 forward primer (annealing downstream the TSS and upstream Rluc CDS) was used in combination with a reverse primer targeting Rluc CDS. PCR1-derived amplicons were loaded on an agarose gel and images captured. Double bands represent long and short amplicons made from unprocessed and processed transcripts respectively as a consequence of the presence of an intronic sequence downstream of the pSV40 promoter of the pRUF vector backbone. PCR2 was performed using the same pSV40 forward primer used in PCR1 in combination with a reverse primer annealing to Fluc CDS. Derived amplicons were visualized on agarose gel. All derived amplicons showed the expected size confirming the transcription of a unique "bicistronic" RNA for each construct. **(o-t) IRES activity (IVT mRNA).** SINEB2 element, in inverted (invSINEB2) orientation, and *c-myc* IRES were separately cloned between Rluc and Fluc sequences in pRUF-dual luciferase reporter vectors. Vectors were linearized and derived mRNAs *in vitro* transcribed. Bicistronic mRNA with no IRES sequences and with *c-myc* IRES were used as negative and positive controls respectively. Total

RNA was extracted at 6 h following HEK293T cell transfection, reverse-transcribed, and analyzed with real-time PCR. **o)** Rluc mRNA relative expression was reported on the graph using non-transfected cells as control (Ctrl=1). **p)** Relative expression of Fluc mRNA was analyzed as previously described for Rluc mRNA. **q)** Fluc mRNA levels relative to Rluc expression (Fluc/Rluc) were reported on the graph to assess the same mRNA expression levels for the two CDSs. **r and s)** qRT-PCR confirmed the expression of the inserted invSINEB2 and *c-myc*-IRES elements. RNA levels were normalized on *GAPDH* mRNA expression. All plots indicate mean ± SD, and represent n=4 independent replicates. **t)** Rluc-Fluc whole bicistronic mRNA levels were reported on the graph. *GAPDH* was used to normalize.**(u-v) circRNA expression (circularizing vector).** GFP-circularizing vectors were transfected in HEK293T cells. 48 h after transfection, cells were harvested and RNA extracted. Non-transfected cells were used as control (Ctrl=1). Circular GFP with IRES in antisense orientation was the negative control while circGFP with an embedded *c-myc* IRES was the positive control. RNAs were extracted, reverse transcribed and analyzed with real time PCR. *GAPDH* was used to normalize. **u)** Expression of circular GFP was confirmed in all constructs. **v)** Linear *GFP* mRNA relative expressions were quantified and reported on the graph. As expected, linear counterpart resulted much lower compared to the circRNA over-expression. All graphs indicate mean ± SD, and represent n=3 independent replicates.

Extended Data Fig. 3. Viral and cellular IRES sequences act as ED in SINEUP RNAs.
a-b) *DJ-1* mRNA and SINEUP RNA levels. *SINEUP(IRES)-DJ-1* comprises a BD, targeting *DJ-1* mRNA (-40/+4) in an antisense orientation, and different IRES sequences (viral or cellular) acting as potential EDs. SINEUP(IRES) constructs were transiently expressed in HEK 293T cells. Total RNAs were extracted at 48 h after transfection, reverse transcribed and analyzed with real time PCR. RNA levels were normalized on *GAPDH* mRNA. Empty pCS2+ was used as control (Ctrl=1, dotted line). **a)** *DJ-1* mRNA relative expressions were quantified and reported on the graph. No significant variations were observed. **b)** qRT-PCR confirmed the expression of all transfected *SINEUP(IRES)-DJ-1* constructs. All plots indicate mean ± SD, and represent n=4 independent replicas. (**c-e) *SINEUP(IRES)* activity in HepG2 cells. c)** Reassuming plot reporting *SINEUP(IRES)-DJ-1* activity. SINEUP constructs were transiently expressed in HepG2 cells. SINEUP activity was measured 48 h after transfection with western blot. Western blot analysis was carried out using anti-DJ-1 Ab to detect and quantify DJ-1 proteins. β-actin was used to normalize. Empty pCS2+ was used as control (Ctrl=1, dotted line). All SINEUP(IRES) RNAs increased DJ-1 protein levels in a canonical SINEUP-like mechanism. Plot reports DJ-1 fold induction mean ± SD; it represents at least 4 independent biological replicates; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$ were considered significant

(ordinary one-way ANOVA, Holm-Sidak's multiple comparisons test). **d)** RNA levels. Total RNAs were extracted, reverse-transcribed, and analyzed with real time PCR. RNA levels were normalized on *GAPDH* mRNA. Empty pCS2+ was used as control (Ctrl=1, dotted line). *DJ-1* mRNA relative expressions were quantified and reported on the graph. No significant variations were observed. **e)** Expression of all transfected *SINEUP(IRES)-DJ-1* constructs were also assessed with real time PCR. All plots indicate mean ± SD and represent n=4 independent replicates. **f) Endogenous *c-myc* mRNA polysome distribution.** The relative distribution (in %) of *c-myc* mRNA, in non-transfected cells, was analyzed through Real-Time PCR using two different oligo pairs: one pairing at the level of IRES element and one at the 3'UTR of *c-myc* mRNA. RNA distributions were reported as the mean ± SD of 3 replicates. **(g-j) *miniSINEUP(IRES)* activity in HEK293T cells. g)** Schematic overview of *miniSINEUP-DJ-1* and *miniSINEUP(IRES)-DJ-1*. Mini synthetic SINEUP targeting *DJ-1* mRNA (yellow) comprising exclusively a BD (yellow) and an invSINEB2 as ED (black). *miniSINEUP(IRES)-DJ-1* comprises a BD, targeting *DJ-1* mRNA (-40/+4) in an antisense orientation, and different IRES sequences (viral or cellular) acting as potential EDs (light grey). **h)** Representative western blot images and reassuming plot reporting *miniSINEUP(IRES)-DJ-1 activity*. miniSINEUP constructs were transiently expressed in HEK 293T cells and their activity measured 48 h after transfection by western blot. Western blot analysis was carried out using anti-DJ-1 Ab to detect and quantify DJ-1 proteins. β-actin was used to normalize. Empty pCS2+ was used as control (Ctrl=1, dotted line). As expected, all miniSINEUP(IRES) RNAs increased DJ-1 protein levels. Plot reports DJ-1 fold induction mean ± SD; it represents at least 4 independent biological replicates performed in duplicate; $^{****}p < 0.0001$ was analyzed with ordinary one-way ANOVA, Holm-Sidak's multiple comparisons test. **i)** RNA levels. Total RNAs were extracted, reverse transcribed and analyzed with real time PCR. RNA levels were normalized on *GAPDH* mRNA. Empty pCS2+ was used as control (Ctrl=1, dotted line). *DJ-1* mRNA relative expressions were quantified and reported on the graph. No significant variations were observed. **j)** Expression of all transfected miniSINEUP(IRES) constructs was assessed with real time PCR. All plots indicate mean ± SD, and represent n=4 independent replicates.

Extended Data Fig. 4. Synthetic circular SINEUP RNAs.

Circularized SINEUP RNA maintains its activity in *trans*. *circSINEUP(c-myc IRES)-GFP* activity. Experiments were performed as described in methods ("Cell Lines and Transfection Conditions"). HEK293T cells were co-transfected with circRNA-expressing vector and pEGFP (to express GFP protein) in a 6:1 ratio. 48 h after transfection, cells were collected and RNA extracted. Empty pCDNA3.1(-) was used as negative control (Ctrl=1, dotted line) and linear *miniSINEUP-GFP*

and *miniSINEUP(c-myc IRES)-GFP* were the positive controls. RNAs were extracted, reverse transcribed and analyzed with real time PCR. RNA expressions. *GAPDH* was used to normalize. **a)** *GFP* mRNA levels. Empty pCDNA3.1(-) was the negative control (Ctrl=1, dotted line). *GFP* mRNA relative expressions were quantified and reported on the graph. No significant variations were observed. **b)** SINEUP and circSINEUP RNA. Expression of all transfected constructs were confirmed with real time PCR. All graphs indicate mean ± SD, and represent n=3 independent replicates.

Extended Data Fig. 5. CircMyc is a circSINEUP.

(a-f) circMyc increases target protein levels without altering their mRNA levels, validation results. HEK293T were transfected with pcDNA3.1(+) ZKSCAN1 MCS Exon circMyc (n=6). Control cells were transfected with empty pcDNA3.1(+) ZKSCAN1 MCS Exon Vector (as described in methods "Cell Lines and Transfection Conditions"). 48 h after transfection, cells were harvested; cell pellets were divided into two tubes in order to extract both proteins and RNA from the same sample. **a)** RNA-seq volcano plot. log_2FC is reported on nthe x-axis while $-log_{10}$ of the FDR-corrected p-values are plotted on the y-axis. Dots associated to negative values of log_2FC represent downregulated genes, dots on the right represent upregulated genes. Red dots represent genes codifying for proteins whose fold inductions (FC>1.5) were validated with western blot. Expression levels of the genes of interest were validated with real time PCR (Figure S6c). Blue dot represents over-expressed circMyc. $p < 0.05$ was considered significant. CircMyc levels were validated with real time PCR (Figure S6c). **b)** Mass spectrometry results validation. Total protein extract was analyzed with western blot. β-actin and GAPDH were used to normalize; fold-inductions were calculated relative to the empty pcDNA3.1(+) ZKSCAN1 MCS Exon Vector. PXK, MFN, CHK1, GUCY1B1 Abs were used to detect levels of proteins of interest. circMyc overexpression led to upregulation of all proteins of interest. Plots report protein fold induction mean ± SD; they represent 6 independent biological replicates; $^{*}p < 0.05$ and $^{**}p < 0.01$ were considered significant (one-sample t-test). **c)** circMyc target mRNA levels. Extracted RNAs were reverse-transcribed and analyzed with real time PCR. *GAPDH* was used to normalize. Empty pcDNA3.1(+) ZKSCAN1 MCS Exon Vector was the negative control (Ctrl=1, dotted line). Target mRNA relative expressions were quantified and reported on the graph. No significant variations were observed. **d)** circMyc RNA expression (from pcDNA3.1(+)ZKSCAN1 MCS Exon Vector). Overexpression of circMyc was observed with qRT-PCR using specific divergent primers, normalized on *GAPDH* mRNA levels and compared to the empty control. The graph indicates mean ± standard deviation (n=6). **e)** *Pxk* mRNA expression. As discussed in Figure 6c, HEK293T cells were transfected with pcDNA3.1(+)-Laccase2-circMyc (n=9). Empty pcDNA3.1(+)-Laccase2 vector was the negative control. 48 h after transfection, cells

were harvested and RNA extracted. Total RNA was reverse transcribed and analyzed with real time PCR. *GAPDH* was used to normalize. Empty pcDNA3.1(+)-Laccase 2 MCS Exon Vector was the negative control (Ctrl=1, dotted line). *Pxk* mRNA relative expressions were quantified and reported on the graph. No significant variations were observed. **f)** circMyc, from pcDNA3.1(+)-Laccase 2 vector, RNA expression. Over-expression of circMyc was observed with qRT-PCR using specific divergent primers, normalized on *GAPDH* mRNA levels and compared to empty pcDNA3.1(+)-Laccase 2 MCS Exon Vector. The graph indicates mean ± standard deviation (n=6). **(g and h) circMyc increases PXK protein levels in both mESC WT and mESC *Dicer*$^{-/-}$ cells.** *Pxk-flag* cDNA was co-transfected with circMyc(1:12) in wild type mouse embryonic stem cells (mESC) or *Dicer* knock-out mESCs. After 48 h, cells were harvested and proteins and RNA were extracted. Empty pcDNA3.1(+)-Laccase2 vector, cotransfected with *Pxk-flag*, was used as negative controls; *Pxk-flag* WT + circMyc was the positive control. Western blot analysis was performed, anti-PXK Ab was used to detect PXK protein. PXK fold induction was calculated compared to the empty control (Ctrl=1, dotted line). β-actin was used to normalize. circMyc induced PXK expression in both WT and *Dicer*$^{-/-}$ mESC . circMyc activity was plotted as mean fold-induction values ± SD. $^{*}p < 0.05$ was considered significant. Ordinary one-way ANOVA, with Holm-Sidak's multiple comparisons test, was used to calculate significance. **(i)** *Pxk* mRNA relative expression. *GAPDH* was used to normalize. Empty pcDNA3.1(+)-Laccase 2 cotransfected with *Pxk-flag* was the negative control (Ctrl=1, dotted line). *Pxk* mRNA relative expressions were quantified and reported on the graph. No significant variations were observed. **(j)** Over-expression of circRNAs was observed with qRT-PCR using specific divergent primers, normalized on *GAPDH* mRNA levels and compared to empty pcDNA3.1(+)-Laccase 2 MCS Exon Vector. Graph indicates mean ± standard deviation (n=3). **(k-n) circMyc: a circSINEUP able to interact with *Pxk* mRNA inducing its protein translation.** HEK293T cells were transfected with pcDNA3.1(+)-Laccase2-circMyc (n=4). Empty pcDNA3.1(+)-Laccase2 vector was the negative control. (**k** and **l**) Following CHX treatment, 1/10 of the sample was used to perform total RNA extraction to assess both *Pxk* mRNA and circMyc expression levels. *GAPDH* was used to normalize. Empty pcDNA3.1(+)-Laccase was the negative control (Ctrl=1, dotted line). **m)** As described in Figures 1d and 5d, lysates from control and treated cells were fractionated by sucrose gradients and separated through an UV detector reading the RNA amount (254 nm) in each fraction. The relative distributions (in %) of *GAPDH* mRNA were analyzed by RT-qPCR in each of the 13 gradient fractions for both control and treated samples. RNA distribution was reported as the mean ± SD of 3 replicates. CircMyc RNA did not affect *GAPDH* mRNA distribution. **n)** circMyc RNA Pull Down (RPD). CircMyc was purified using biotinylated DNA probes whose length ranges around 20 nts. As negative control, RPD was performed in the absence of targeting

probes. qRT-PCRs show the enrichment of circMyc and *Pxk* mRNA upon circMyc RPD. Data are shown as means of both circMyc and *Pxk* mRNA enrichment versus input relative to the negative control ± SD of 6 replicates. **(o-q) *CircMyc* mutants.** circMyc mutants were generated deleting both BD1 and 2 (circMyc ΔBD1-2) or inverting their sequence orientations (circMyc invBD1-2). HEK293T cells were transfected with circMyc mutants (n=3). Empty pcDNA3.1(+)-Laccase2 vector was the negative control while pcDNA3.1(+)-Laccase2-circMyc was the positive control. 48 h after transfection, cells were harvested and proteins and RNAs were extracted. **o)** Western blot analysis was performed, anti-PXK Ab was used to detect PXK protein. PXK fold induction was calculated compared to the empty control (Ctrl=1, dotted line). β-actin was used to normalize. Neither circMyc ΔBD1-2 nor circMyc invBD1-2 induced PXK expression. circMyc mutant activities were plotted as mean fold-induction values ± SD. $^{*}p < 0.05$ was considered significant. Ordinary one-way ANOVA, with Holm-Sidak's multiple comparisons test, was used to calculate significance. **p)** *Pxk* mRNA expression. Total RNA was reverse-transcribed and analyzed with real time PCR. *GAPDH* was used to normalize. Empty pcDNA3.1(+)-Laccase 2 MCS Exon Vector was the negative control (Ctrl=1, dotted line). *Pxk* mRNA relative expressions were quantified and reported on the graph. No significant variations were observed. **q)** circRNA expression. Overexpression of circRNAs were observed with qRT-PCR using specific divergent primers, normalized on *GAPDH* mRNA levels and compared to empty pcDNA3.1(+)-Laccase 2 MCS Exon Vector. Both circMyc mutants were expressed at lower levels than WT circMyc. The graph indicates mean ± standard deviation (n=3).

Extended Data Fig. 6. Pairing rules of circMyc RNA/Pxk mRNA.

***Pxk-flag* mutants RNA.** Each *Pxk* mutant construct was cotransfected with circMyc in HEK293T cells (1:12). After 48 h, cells were harvested and proteins and RNA were extracted. Empty pcDNA3.1(+)-Laccase2 vector cotransfected with different *Pxk*-mutants were used as negative controls; *Pxk-flag* WT + circMyc was the positive control. **a)** ΔTSs *Pxk* mutant mRNA. RNA was extracted, reverse-transcribed and analyzed with qRT-PCR. *GAPDH* was used to normalize. Empty pcDNA3.1(+)-Laccase 2 cotransfected with each *Pxk* mutant was the negative control (Ctrl=1, dotted line). *Pxk* mRNA relative expressions were quantified and reported on the graph. No significant variations were observed. **(b and d)** circRNA expression. Overexpression of circRNAs was observed with qRT-PCR using specific divergent primers, normalized on *GAPDH* mRNA levels and compared to empty pcDNA3.1(+)-Laccase 2 MCS Exon Vector. Graph indicates mean ± standard deviation (n=3). **c)** TSs *Pxk* mutant mRNA. Total RNA was extracted at 48 h after cotransfection, reverse-transcribed and analyzed with qRT-PCR. *GAPDH* was used to normalize. Empty pcDNA3.1(+)-Laccase 2 co-transfected with each *Pxk* mutant was the negative control (Ctrl=1, dotted line). *Pxk*

mRNA relative expressions were quantified and reported on the graph. No significant variations were observed.