Faust et al., Supporting Information

Materials and Methods

Protein expression and purification

The pET-HLT-Rev NTD construct was transformed into BL21 Rosetta (Novagen). Cells were plated on ampicillin-selective plates and incubated for 16 h. The next day, colonies were transferred to 3 mL 2xYT medium containing ampicillin (33 mg L^{-1}) and the culture was grown for an additional 16 h in an incubator-shaker at 37 °C. The culture was then transferred (1:100) into 2xYT medium supplemented with antibiotics, and grown at 37 °C until OD at 600 nm reached 0.6. The culture was then transferred to 22 °C and induced with 0.4 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells with Rev NTD were harvested 4 hours post induction by centrifugation and stored at –80 °C.

Bacterial pellets with HLT-Rev NTD were resuspended in buffer: 25 mM NaPi, pH 7.4, 500 mM NaCl, 2 mM βMe, 10% (v/v) glycerol and 10 mM imidazole, supplemented with 10 mM $MgCl_2$, 1 mM PMSF, 0.2 mg mL^{-1} lysozyme and 50 mg L^{-1} DNaseA with addition of 4 M urea, since the protein was found to bind nucleic acids, and lysed by using a microfluidizer (M-110 EHIS; Microfluidics). The solution was centrifuged (20,000 g, 20 min, 4 °C) and the soluble supernatant was separated and filtered. Chromatography was performed by using the AKTA Explorer FPLC system (GE Healthcare Bio-Sciences AB).

The filtered supernatant was loaded on a Ni-Sepharose FF column and refolding was done over 12 hours in a slow decrease in urea concentration down to 0 M. Elution was done using increasing imidazole concentration up to 500 mM. HLT-Rev NTD fractions were pooled according to their molecular weight on SDS-PAGE separation, loaded on a 200 mL gel filtration Sephacryl S100 column (GE Healthcare) pre-equilibrated in the final storage buffer (25 mM NaPi, pH 7.4, NaCl to a final ionic strength of 500 mM, 10% (v/v) glycerol, and 2 mM βMe). Fractions with the pure protein were pooled, concentrated, aliquoted, and stored at –80 °C after fast freezing.

The pET-HLT-Rev CTD construct was transformed into BL21 CodonPlus RIL (Stratagene). Cells were plated on ampicillin-selective plates and incubated for 16 h. The next day, colonies were transferred to 3 mL 2xYT medium containing ampicillin (33 mg L^{-1}) and the culture was grown for an additional 16 h in an incubator-shaker at 37 °C. The culture was then transferred (1:100) into 2xYT medium supplemented with antibiotics, and grown at 37 °C until OD at 600 nm reached 0.6. The culture was then transferred to 17 °C for and induced with 0.1 mM IPTG. Cells with Rev CTD were harvested 16 hours post induction by centrifugation and stored at –80 °C.

The bacterial pellets were resuspended in buffer: 25 mM NaPi, pH 7.4, 500 mM NaCl, 2 mM βMe, 10% (v/v) glycerol and 10 mM imidazole, supplemented with 10 mM $MgCl_2$, 1 mM PMSF, 0.2 mg mL^{-1} lysozyme and 50 mg L^{-1} DNaseA and lysed

by using a microfluidizer (M-110 EHIS; Microfluidics). The solution was centrifuged (20,000 g, 20 min, 4 °C) and the soluble supernatant was separated and filtered. Chromatography was performed by using the AKTA Explorer FPLC system (GE Healthcare Bio-Sciences AB).

The supernatant containing Rev CTD was loaded on a Ni-Sepharose FF column (GE Healthcare) pre-equilibrated with resuspension buffer, and extensively washed. The protein was eluted from the affinity column by gradually increasing the imidazole concentration (up to 500 mM). HLT-Rev CTD fractions were pooled following detection by SDS-PAGE, and loaded on a 200 mL gel filtration Superdex 75 column (GE Healthcare) pre-equilibrated in the final storage buffer (25 mM NaPi, pH 7.4, NaCl to a final ionic strength of 500 mM, 10% (v/v) glycerol, and 2 mM βMe). Fractions with the pure protein were pooled, concentrated, aliquoted, and stored at –80 °C after fast freezing.

MALDI-TOF mass spectrometry confirmed the molecular weights of 19.6 kDa for HLT-Rev NTD and 17.1 kDa for HLT-Rev CTD. Removing the HLT tag from both domains resulted in non-soluble proteins and thus the proteins were left with the tag.

Disorder prediction

The following servers were used to predict the disordered parts of Rev: DisEMBL, GlobPlot, IUPred, RONN, ESpritz, MetaDisorder and PSIPRED[34–41]. The full length sequence of HIV-1 Rev (1-116) was subjected to disorder prediction using the default parameters for all servers.

Circular Dichroism (CD)

CD spectra were recorded by using a J-810 spectropolarimeter (JASCO) equipped with a Peltier thermostat using the supplied Spectra Manager software in a 0.1 cm quartz cuvette in the proteins storage buffer. The samples were heated at a rate of 50 °C per hour, from 25 °C to 70 °C and the CD reading was taken at λ = 222 nm every 0.1 °C. Far-UV CD spectra were collected over 190–260 nm at 4 °C before and after each heating experiment.

The fraction of unfolded protein was calculated by fitting the normalized data using a sigmoidal model, and T_m was defined as the temperature at half height, where t is a constant describing the slope of the melting curve.

$$\chi_{unfolded} = 1 - \frac{1}{1 + \left(\exp\left(\frac{T - Tm}{t}\right)\right)}$$

Analytical size-exclusion chromatography

Analytical size exclusion chromatography was performed on an AKTA Explorer with a Superose 12 micro-analytical column 3.8 × 1 cm (GE Healthcare–Amersham

Pharmacia) equilibrated with buffer (25 mM NaPi, pH 7.4, 500 mM NaCl, 5% (v/v) glycerol and 2 mM βMe). Proteins were eluted with a flow rate of 0.4 mL min^{-1} at 4 °C and the elution profile was monitored by UV absorbance at 220 nm. The column was calibrated with molecular weight standards (GE Healthcare–Amersham Pharmacia). Binding experiments were done on the same column, following incubation of 25 μM of NTD and CTD together for one hour at 4 °C, in a 25 mM NaPi buffer, pH 7.4, ionic strength of 150 mM with NaCl, 5% (v/v) glycerol and 2 mM βMe.

Cross linking

The proteins were diluted to 15 μM in buffer (25 mM NaPi, pH 7.4, ionic strength of 500 mM with NaCl, 5% (v/v) glycerol and 2 mM βMe) and were kept on ice to equilibrate 1 h. The reaction mixtures were treated with 100 μM of the cross-linking agent BS3 (Pierce Thermo Fisher Scientific) and were incubated at 37 °C for 15 min. The reaction was quenched with buffer containing 1.5 M Tris pH 8.8 and the mixtures were left on ice for 1 h. Acetone was added to a final volume of x5 from the initial volume, and samples were incubated at –20 °C for 1 h. Then the samples were spun at 14,000 rpm, 4 °C for 30 minutes, the supernatant was discarded and samples were left to dry before being resuspended in sample buffer and boiled at 95 °C for 5 min. Samples were kept in –20 °C until analysis by SDS-PAGE.

Analytical Ultracentrifugation

Measurements were carried out using an XL-I analytical ultracentrifuge (Beckman-Coulter Inc.), with a UV-visible optics detection system in an An60Ti rotor and 12-mm double sector centerpieces. For sedimentation velocity analysis, 33 μM protein in buffer containing 25 mM NaPi pH 7.4, 500 mM NaCl, 2 mM βMe and 10% (v/v) glycerol was centrifuged at 45,000 rpm at 4 °C. Measurements were automatically recorded every 1 min at 280 nm. The sedimentation coefficient distributions were calculated using the SEDFIT program [https://sedfitsedphat.nibib.nih.gov/software/default.aspx] and displayed with GUSSI [http://biophysics.swmed.edu/MBR/software.html]. The partial specific volume was calculated from the amino acid composition with Sednterp: HLT-Rev: 0.7210 mg mL^{-1}, HLT-Rev NTD: 0.7209 mg mL^{-1}.

Fluorescence

Proteins were diluted to 2 μM in buffer containing 25 mM Hepes, 150 mM NaCl, 2% (v/v) glycerol, 2mM βMe. Since the HLT tag contains an aromatic residue, HLT-Rev was used rather than the cleaved protein and compared to HLT-Rev NTD. Excitation was performed at 280 nm, and emission was monitored between 295 nm and 475 nm using a PerkinElmer Life Sciences LS-50b spectrofluorometer (Perkin Elmer).

Peptide arrays

The CelluSpots™ peptide micro-arrays were synthesized by INTAVIS Bioanalytical Instruments AG. The arrays were first washed with blocking buffer: Phosphate buffered saline (PBS) (10 mM NaPi, 2 mM KPi, 137 mM NaCl and 2.7 mM KCl) with 0.05% (v/v) Tween-20 and 2.5% (w/v) skimmed milk for blocking unspecific binding. HLT-Rev NTD and HLT-Rev CTD were diluted in blocking buffer with 2 mM βMe. The proteins were incubated with the arrays at 4 ºC with gentle shaking overnight. After three washes with PBST the arrays were incubated with anti His-HRP conjugated antibody at room temperature for 1 h and then washed again three times with PBST. Immunodetection was performed using chemiluminescence (ECL reagents, Pierce, Thermo Fisher Scientific).

Mass-Spectrometry

Proteins in their storage buffer were diluted to ~3 µM and set to dry on a plate with Sinapinic acid matrix in a 1:2 ratio (protein : matrix). The samples were then ionized and analysed using a MALDI-TOF instrument (Voyager DE Pro, ABI).

HLT does not induce oligomerization

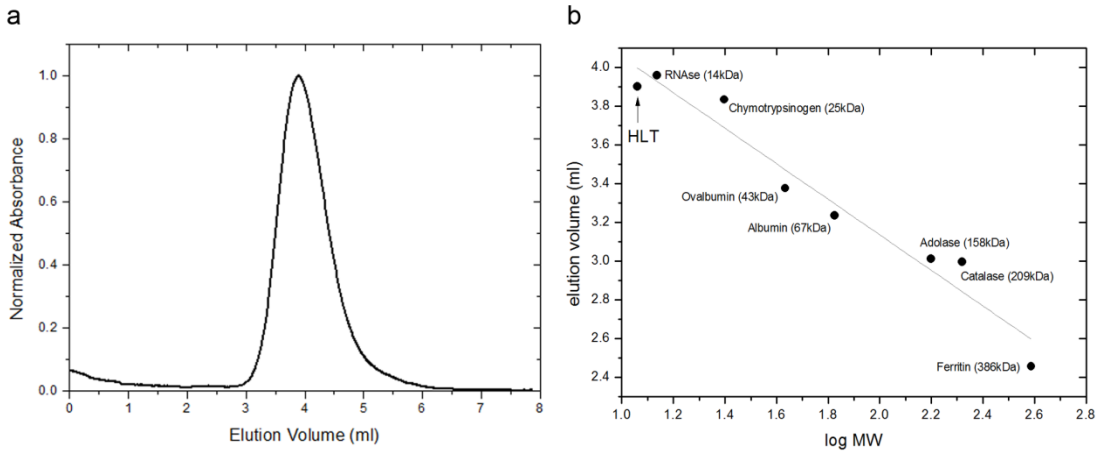

Figure S1. The HLT tag does not oligomerize. A. Analytical SEC of HLT alone. The tag eluted in a single peak. B. The calibration curve of the column. HLT is on the curve, with a correct molecular weight of 11.5 kDa.

The Rev NTD and CTD do not interact with each other

The CD spectrum of a 1:1 mixture of the Rev NTD and CTD was identical to the sum of the different spectra of the two domains and to the spectrum of the full-length Rev, indicating no conformational change when the domains are present together (Fig. S2a). Analytical SEC showed that the NTD and CTD did not form a complex (Fig. S2b). Monitoring the tryptophan fluorescence revealed that the emission spectra of Rev NTD and of full length Rev overlapped, indicating no intramolecular interaction was observed (Fig. S2c). To further test the option of an interaction we designed an array of partly overlapping peptides[27] derived from the full length Rev sequence. Rev NTD bound several peptides on the array, all derived from the NTD itself (especially the known oligomerization domains) but not from the CTD (Table S1 and Fig. S2d). Rev CTD did not bind any Rev-derived peptide on the array (Fig. S2d). Taken together, our results show that no intramolecular interaction between the domains takes place.

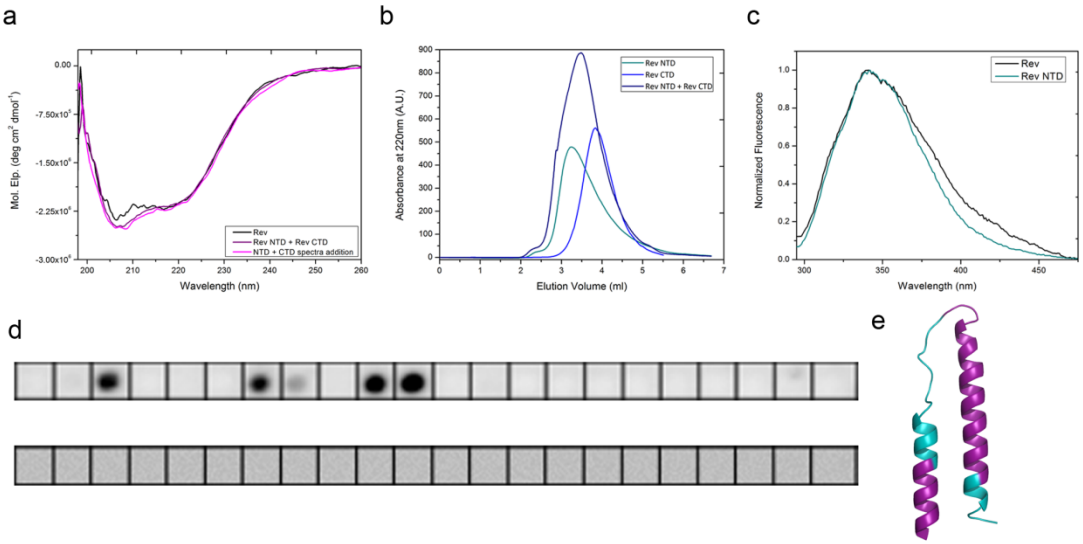

Figure S2. The Rev NTD and CTD do not interact with each other. A. The CD spectrum of the Rev NTD and CTD following co-incubation (purple) overlaps the addition of the two separate spectra of the domains (pink) as well as the spectrum of the full length tagged protein (black). B. Analytical SEC of the NTD and CTD following co-incubation showed that no complex was formed. C. The intrinsic tryptophan fluorescence peak overlaps for Rev NTD and full-length Rev. D. Screening an array of Rev-derived peptides showed that the Rev NTD bound several peptides, all derived from the Rev NTD itself (top). The same array incubated with Rev CTD showed no binding peptides (bottom). E. Rev-NTD binding peptides as revealed in the array screening (purple) shown on the Rev structure (cyan). All peptides are on the N-terminal region.

Table S1. Rev-derived peptides that bound Rev NTD in the array screening*

Spot number	Peptide	Sequence
3	Rev 7-18	DSDEELIRTVRL
7	Rev 30-52	NPEGTRQARRNRRRRWRERQRQI
8	Rev 34-50	TRQARRNRRRRWRERQR
10	Rev 42-58	RRRWRERQRQIHSISER
11	Rev 49-67	QRQIHSISERILGTYLGRS

* All binding peptides are in the N-terminal domain of Rev, and no inter-domain interaction was found.