Supplementary information

Supplementary methods

Electrophoretic mobility shift assay (EMSA)

Rev peptide 33-55 and full length Rev (Rev FL) WT or W45A were expressed in E. *coli* tagged with ZZ (protein A) and His and purified over the His6-tag using Ni-NTA agarose (Quiagen). Unlabelled RRE SLIIB (36 pmol / 81 pmol) was mixed with indicated molar fold excess of Rev peptide / Rev FL in PBS buffer, incubated for 15 minutes at RT and loaded on a 8% acrylamide gel in TBE. The gels were stained with toluedene blue to visualize RNA. Toluedene blue is a basic thiazine metachromatic dye with high affinity for acidic molecules, thus useful for nucleic acid staining (1). It can also stain protein at higher concentration, as it can be observed at the bottom of the gel in Supplementary Figure 1B (free Rev displays a different colour than Rev-RNA complexes seen above).

GST pulldown

GST-tagged UHMs (42 µg) were mixed with 42 µg of untagged ULMs in 100 µl PBS buffer supplemented with 2 mM β-mercaptoethanol and 0.1% (v/v) IGEPAL at 4 ºC and mixed vigorously for 1 h. For GST precipitation, 3 µg of glutathione-Sepharose (GSH 4B, Amersham) was added and mixed vigorously for 15 min. The glutathione-Sepharose was sedimented by centrifugation and washed twice with the buffer described above. Results were analyzed on Coomassie-stained SDS-PAGE gels.

Fluorescence microscopy

HeLa cells (5,000 cells/well) were seeded in 96-well tissue cultured treated PerkinElmer View plates 24 hours before transfection. Cells were transfected with GFP, Rev-GFP or Rev(W45A)-GFP using FuGENE HD Transfection Reagent (Roche Diagnostic). 48 hours after transfection, cells were fixed with 2% formaldehyde for 10 minutes, washed with PBS and each well was imaged using an Operetta High-Content Screening device (PerkinElmer). Nucleus of a cell was counterstained with Hoechst33342. In each well, cells with nuclear Rev expression were quantified afterwards with the PerkinElmer Harmony software. The nuclei of individual cells were identified based on Hoechst staining of DNA. The cytoplasm of each cell was then identified based on the detection of Rev-GFP staining surrounding individual nuclei. Several hundred of cells were analyzed and following formula was set up to calculate the ratio cytoplasm/nucleus: number of cells with cytoplasmic Rev signal/ number of cells with nuclear Rev signal.

Supplementary figures

Figure S1

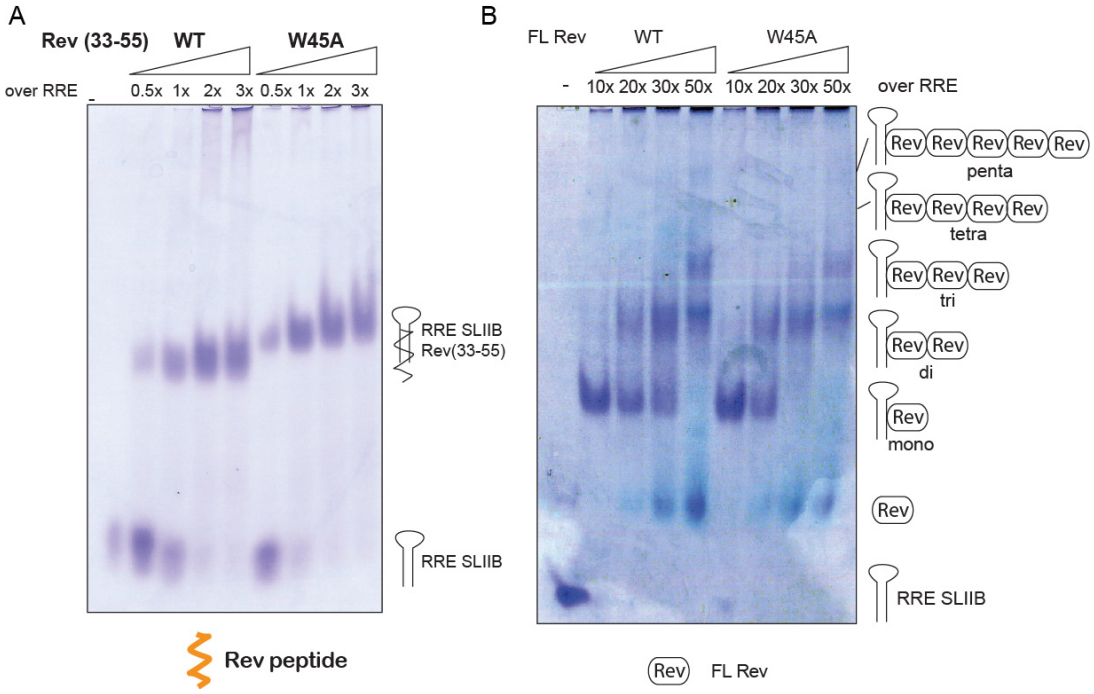

Figure S1. Both Rev WT and W45A bind RRE SLIIB.

A-B. EMSA experiments analyzing Rev binding to RRE SLIIB. RNA is detected on native acrylamide gel using toluedene blue **A.** RRE SLIIB was mixed with indicated increasing amounts of Rev 33-55 WT or W45A in PBS buffer, incubated for 15 minutes at room temperature and resolved on the gel. **B.** RRE SLIIB was mixed with indicated increasing amounts of full-length Rev WT or W45A in PBS buffer, incubated for 15 minutes at room temperature and resolved on the gel. With increasing Rev concentration more Rev molecules accumulate on SLIIB, as illustrated on the right side of the gel.

Figure S2

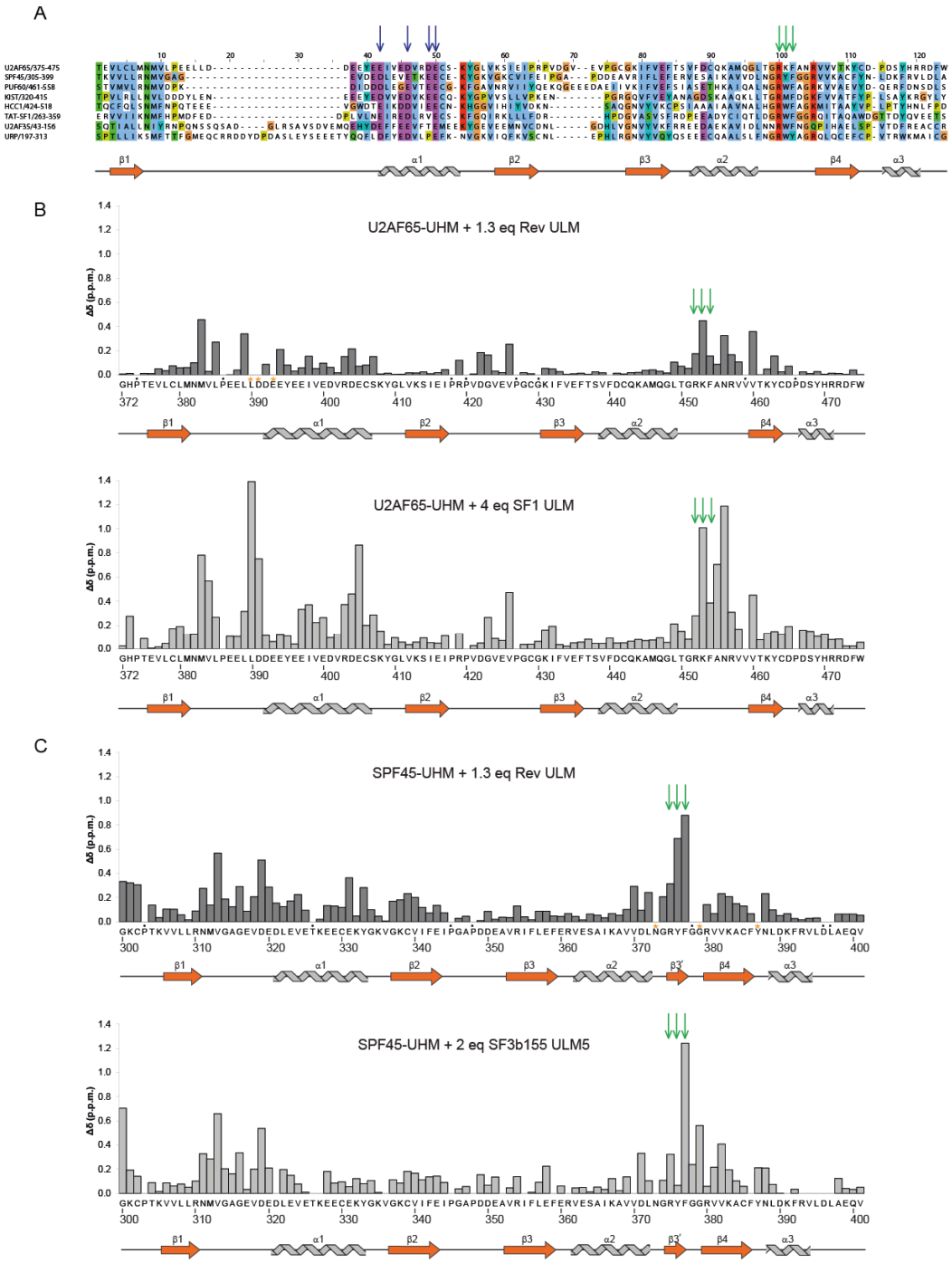

Figure S2. The binding interface of U2AF65 and SPF45 UHMs with the Rev ULM is similar to the interaction with SF3b155 and SF1 ULMs.

A. Alignment of human UHM domains. Residues that bind ULMs are marked by arrows; purple arrows: negatively charged residues from helix α1, green arrows: *RXF* motif. **B and C.** Chemical shift perturbations were calculated upon binding of excess ULMs as: (Δδ(1H)2 + (0.2 x Δδ(15N))2)1/2. Black dots indicate unassigned residues, orange asterisks indicate residues that could not be tracked in the HSQC spectra due to slow/intermediate exchange regime. Secondary structurel elements of U2AF65 and SPF45 UHMs are depicted below the graphs. Binding of SF3b155 and SF1 ULMs to UHMs are plotted based on published data: (2,3).

Figure S3

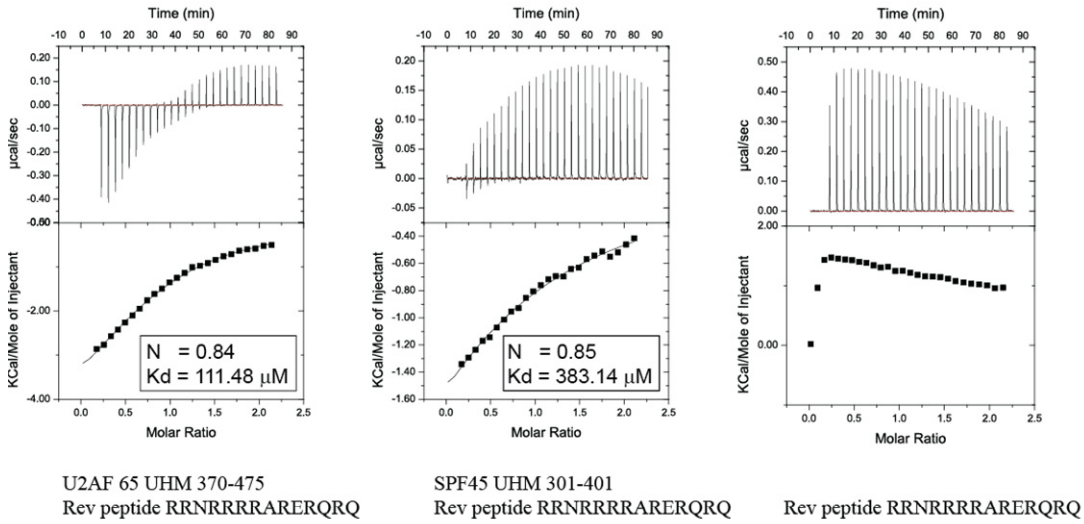

Figure S3. Rev W45A has a reduced affinity to U2AF65 and SPF45 UHMs.
Isothermal titration calorimetry of U2AF65/SPF45 UHMs and Rev ULM W45A (38-51). 2000 µM Rev peptide was titrated to 200 µM UHMs. The graphs are representative of 2-3 independent ITC measurements. Control titration of Rev peptide to the buffer (last graph) was subtracted from experimental runs before the K_D calculation.

Figure S4

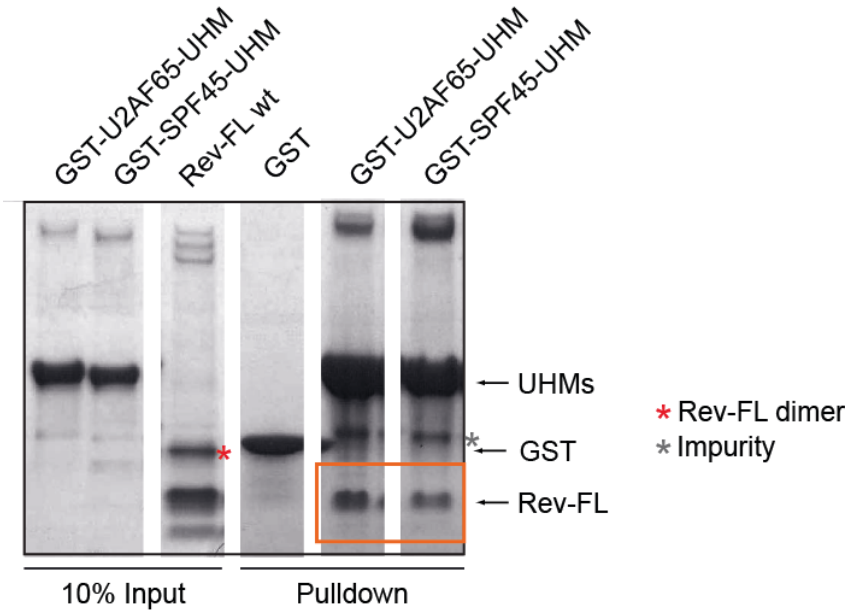

Figure S4. Rev protein binds U2AF65 and SPF45 UHMs.
Coomassie-stained SDS-PAGE analysis of GST pulldown experiments. Indicated GST-tagged UHMs and GST alone were used to precipitate untagged full-length Rev protein.

Figure S5

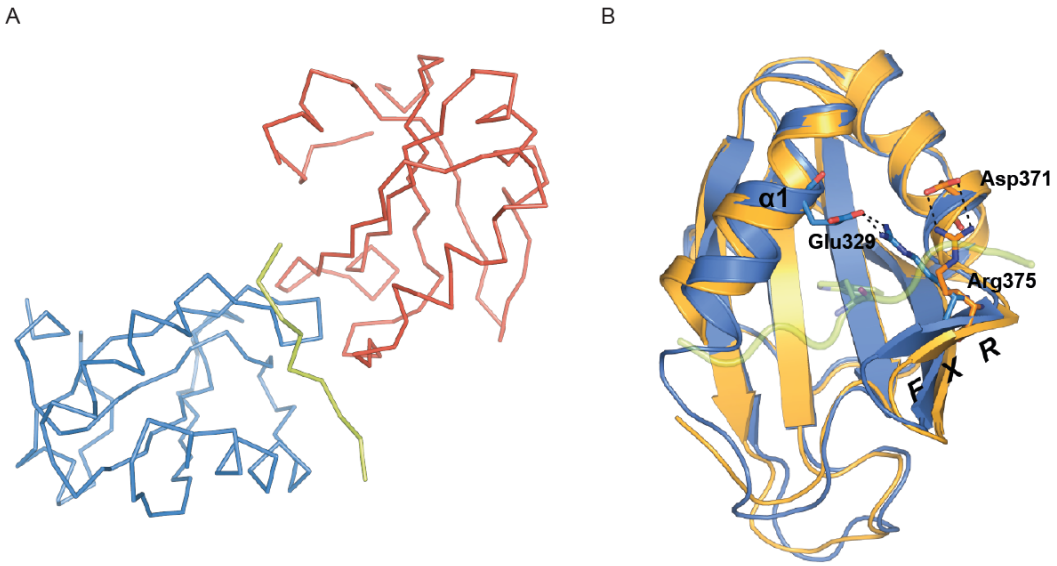

Figure S5. Asymmetric unit content of SPF45-Rev crystals and overlay of free and Rev-bound SPF45.
A. Ribbon representation of SPF45 UHM and Rev peptide molecules in the asymmetric unit. Bound SPF45 copy is shown in blue, Rev ULM ligand in green, and unbound SPF45 copy in red. **B.** Overlay of cartoon representations of free SPF45 (2PE8, gold) and SPF45 bound to Rev peptide (blue). Rev peptide is shown in transparent green. The helix α1 and sheet β3' (containing the RXF motif) are the areas of main conformational changes upon Rev binding. Their closing up can be appreciated in the figure. Arg375 forms a salt bridge with Asp371 in the free form and with Glu329 in the bound form, thus contributing to the stabilization of the "closed" conformation (all three residues involved in salt bridge formation are shown as sticks).

Figure S6

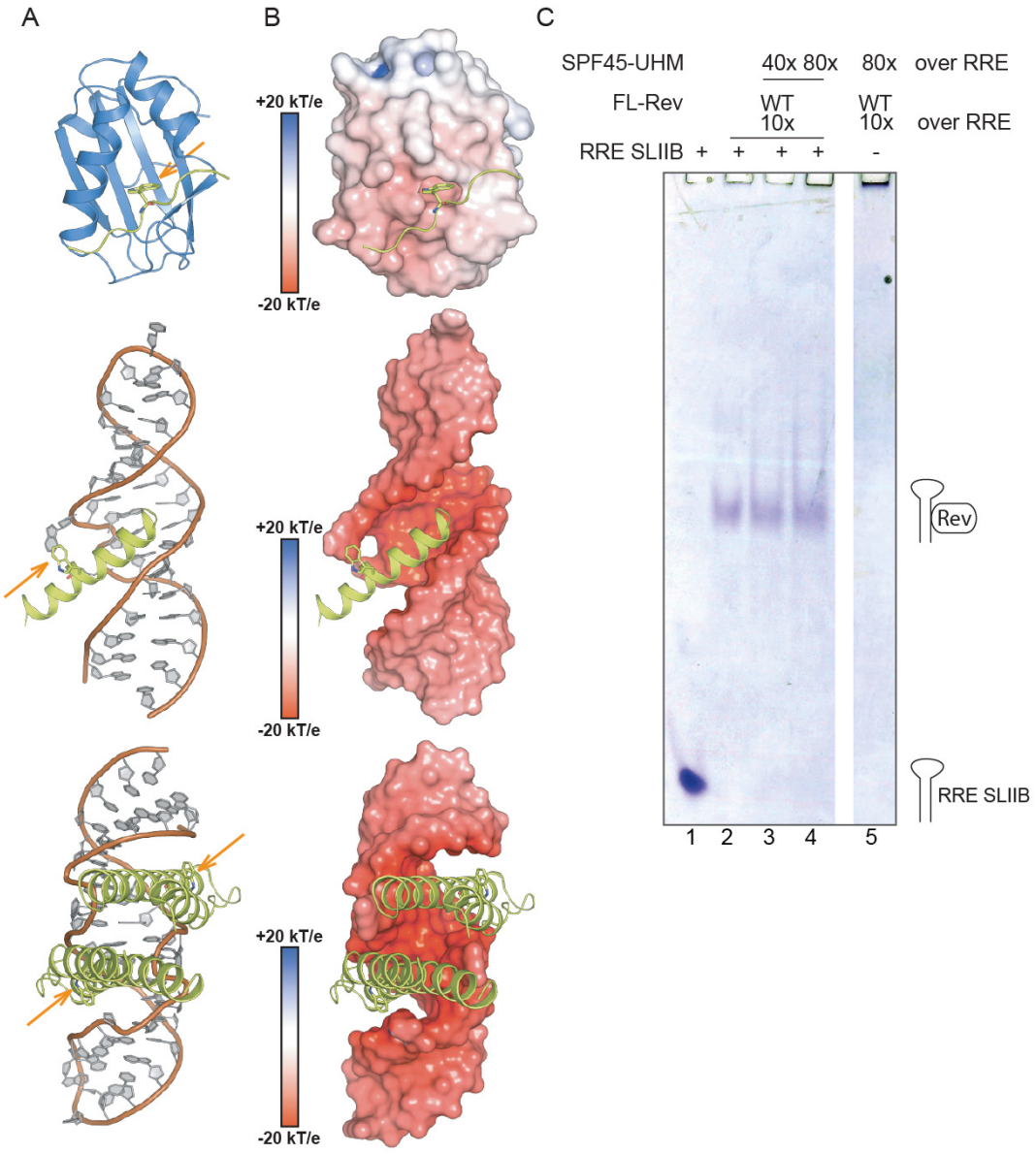

Figure S6. Rev binding to RRE RNA and UHM is mutually exclusive.
A-B. Comparison of Rev binding (green cartoon) to SPF45 UHM (top), SLIIB RNA (middle), and to RRE RNA (bottom) (PDB: present, 1ETG and 4PMI) (4,5). Top: Rev 41-49, SPF45 301-401; middle: Rev 33-55, RRE RNA 41-79; bottom: Rev 1-70, RRE RNA 36-82 **A.** Ribbon and stick representations of the complexes. The location of Trp 45 is indicated with an orange arrow. **B.** Heat maps showing the electrostatic surface potential of SPF45 UHM, SLIIB and RRE RNAs when bound to Rev protein. **C.** EMSA experiment. RRE SLIIB was incubated with 10x molar excess of full-length Rev protein (lane 2-4). Increasing amounts (40- and 80-fold over RRE) of SPF45 UHM were added (lane 3 and 4). The complexes were resolved on an acrylamide gel and stained with toluedene blue. A shift is observed in lanes 2-4 indicating Rev-bound RRE. No supershift can be seen upon addition of SPF45 UHM to the Rev:RRE complex.

Figure S7

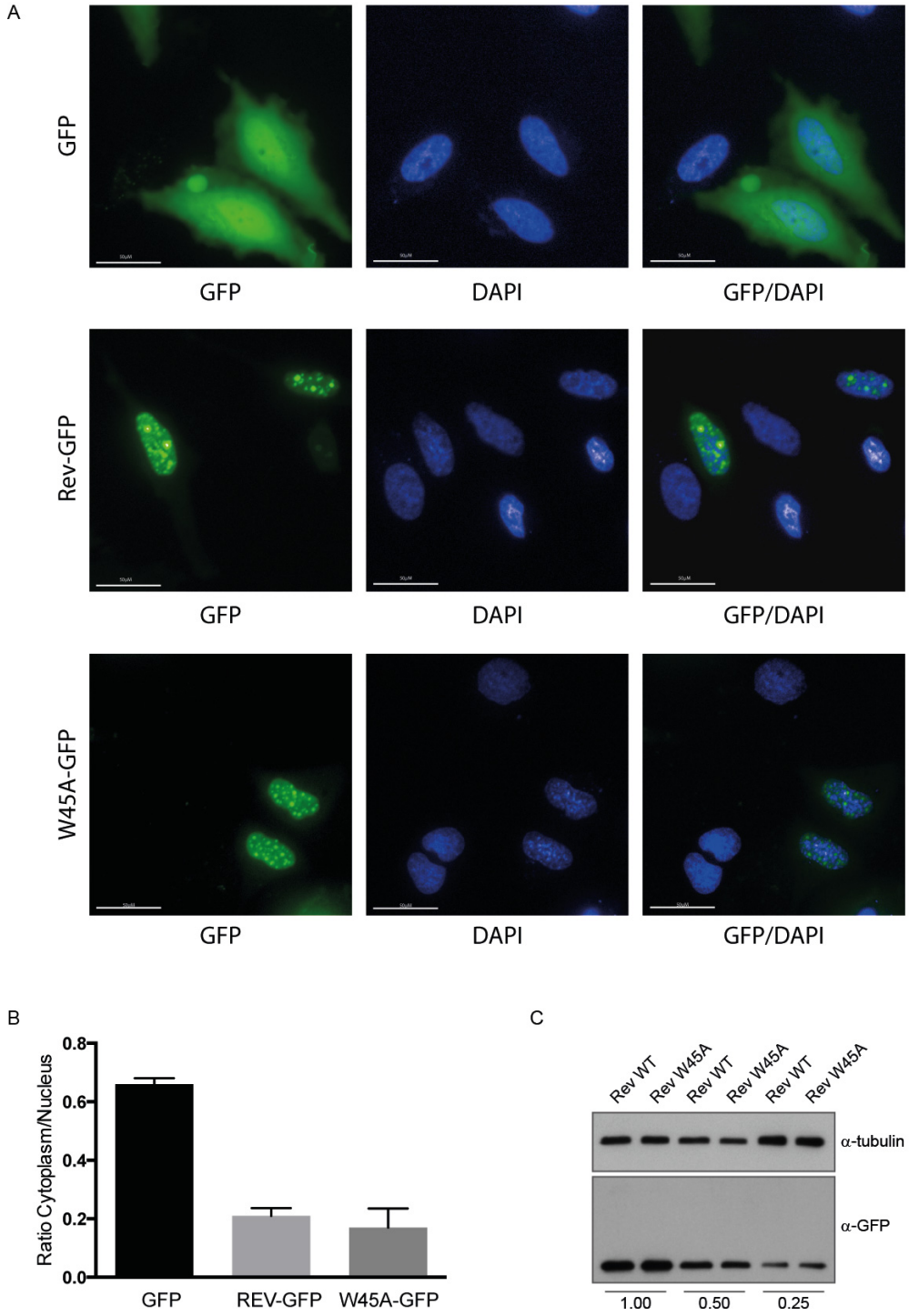

Figure S7. Expression levels and cellular distribution of Rev WT and W45A are equivalent. HeLa cells were transiently transfected with GFP-tagged Rev and assayed 48 hours after transfection. **A.** The cells were fixed, incubated with Hoechst33342 in order to visualize the nuclei and imaged. Scale bar, 50 µm. **B**. The levels of cytoplasmic and nuclear GFP signal was quantified in several hundred individual cells; the average values are presented in the graph. **C.** Assessment of Rev protein levels through Western blotting. The values 1.00, 0.50 and 0.25 inform about decreasing amounts of pCsRevsg143 plasmid used for transfecting HeLa cells.

Figure S8

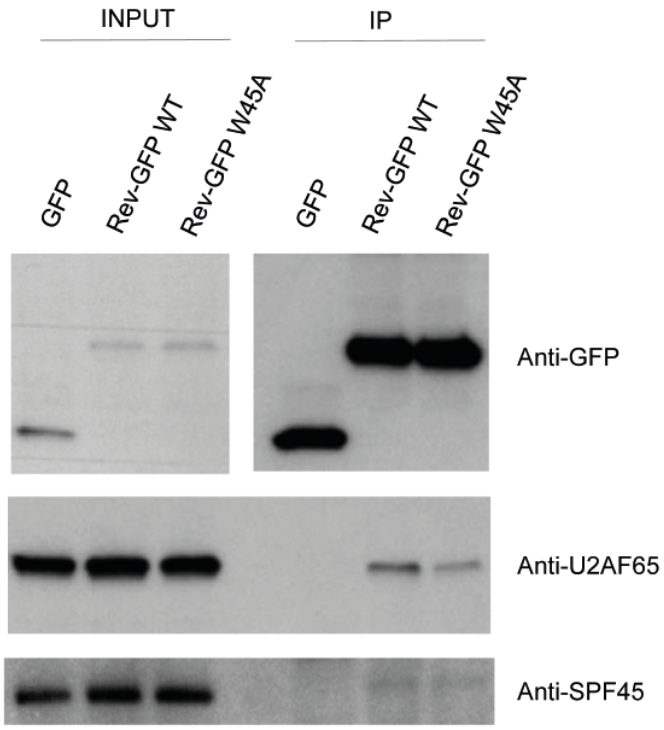

Figure S8. Rev copurifies with U2AF65 but not with SPF45 in HeLa cells.
HeLa cells were transiently transfected with GFP-tagged Rev WT or W45A and assayed 48 hours after transfection. Extracts were subjected to immunoprecipitation with α-GFP antibody. 1% of input and 50% of the immunoprecipitate were analyzed by Western blot, using α-GFP, α-SPF45 and α-U2AF65 antibodies. While U2AF65 can be detected in the Rev-coprecipitate, no unambiguous signal can be seen for SPF45.

References

1. Sridharan, G. and Shankar, A.A. (2012) Toluidine blue: A review of its chemistry and clinical utility. *Journal of oral and maxillofacial pathology : JOMFP*, **16**, 251-255.
2. Corsini, L., Bonnal, S., Basquin, J., Hothorn, M., Scheffzek, K., Valcarcel, J. and Sattler, M. (2007) U2AF-homology motif interactions are required for alternative splicing regulation by SPF45. *Nature structural & molecular biology*, **14**, 620-629.
3. Selenko, P., Gregorovic, G., Sprangers, R., Stier, G., Rhani, Z., Krämer, A. and Sattler, M. (2003) Structural basis for the molecular recognition between human splicing factors U2AF65 and SF1/mBBP. *Molecular cell*, **11**, 965-976.
4. Battiste, J.L., Mao, H., Rao, N.S., Tan, R., Muhandiram, D.R., Kay, L.E., Frankel, A.D. and Williamson, J.R. (1996) Alpha helix-RNA major groove recognition in an HIV-1 rev peptide-RRE RNA complex. *Science*, **273**, 1547-1551.
5. Jayaraman, B., Crosby, D.C., Homer, C., Ribeiro, I., Mavor, D. and Frankel, A.D. (2014) RNA-directed remodeling of the HIV-1 protein Rev orchestrates assembly of the Rev-Rev response element complex. *eLife*, **3**, e04120.