SUPPLEMENTARY FIGURES

Extended Data Figure 1. Comparison of HCV and CSFV IRES-bound ribosomal complexes.

(**a**) Secondary structures of (left) the HCV IRES and (right) the CSFV IRES. Domain II of each IRES is indicated by a red dashed oval; elements of the pseudoknot and subdomains IIIa - IIIe are color-coded as in Extended Data Fig. 6. (**b**) Cryo EM reconstructions of (Left) the HCV IRES bound to the rabbit 40S subunit at 20Å resolution[8], (Middle) the HCV IRES bound to the 40S subunit of cycloheximide-stalled human 80S ribosomes at 15Å resolution[23] (Accession code EMD-1138) and (right) the CSFV ΔII-IRES bound to rabbit 40S subunits at 8.5Å resolution (this study). In all panels, the IRES-40S subunit is viewed from the solvent side; the 40S subunit is displayed in yellow and the IRES in cyan. The red dashed circles in left and middle panels show a discontinuity in the density of domain II in the HCV IRES bound

to the 40S subunit compared to the HCV IRES bound to 80S ribosomes. The dashed circle in the right hand panel highlights CSFV IRES subdomain $IIId_2$, which has no counterpart in the HCV IRES.

Extended Data Figure 2. Analysis of 40S/ΔII-IRES/eIF3/DHX29 complexes.

40S/ΔII-IRES/eIF3/DHX29 complexes were assembled *in vitro* using CSFV ΔII-IRES mRNA, native eIF2, eIF3 and 40S subunits purified from rabbit reticulocyte lysate and recombinant DHX29, and assayed by toe-printing. Lanes C, T, A and G show the cDNA sequence corresponding to CSFV ΔII-IRES mRNA. The position of the initiation codon is indicated on the left. This analysis revealed (lane 2) that deletion of domain II of the IRES or the presence of DHX29 did not influence IRES's contacts with either 40S subunit (the toe-print stops at $UUU_{387\text{-}9}$, G_{345} and C_{334}) or eIF3 (the toe-print stop at A_{250}) that have been previously observed[2,18]. Moreover, upon addition of the eIF2-TC, 40S/ΔII-IRES/eIF3/DHX29 complexes were quantitatively converted into 48S complexes on the authentic initiation codon AUG_{373} (lane 3). The low-efficiency 48S complex formation on the preceding AUG_{366} was also observed before and was not related to the presence of DHX29[18]. The gel reported in the figure is representative of results obtained from three technical replicates.

Extended Data Figure 3. Unsupervised 3D classification of IRES-bound ribosomal complexes.

Unsupervised 3D classification of IRES-bound ribosomal complexes identified ~423,000 particles inconsistent with the known structure of the 40S subunit (rejects) and six well-populated classes containing complexes of the 40S subunit in a binary complex with the ΔII-IRES (class 1), of the 40S subunit bound to the ΔII-IRES and DHX29 (class 2), of the 40S subunit bound to DHX29 and eIF3 (class 3) and of the 40S subunit bound to the ΔII-IRES, DHX29 and eIF3 in orientation 1 (class 4), in orientation 2 (class 5) and in orientation 3 (class 6), viewed from (left) the back, (center) the intersubunit side and (right) the solvent side.

Extended Data Figure 4. Measured resolution and reference-free 2D classification of IRES-bound ribosomal complexes.

(**a**) Gold Fourier Shell Correlation (FSC) curves of the cryo-EM reconstruction of classes 2 (red line) and 4 (blue line) (also see Extended Data Fig. 3) indicating their estimated resolution. (**b**) Right column on each side, 2D classes obtained by reference-free classification of particles corresponding to 40S/eIF3/DHX29/ΔII-IRES complexes (class 4 in Extended Data Fig. 3). Middle column on each side, projection views of the class 4 cryo-EM map corresponding to the 2D classes. Right column on each side, corresponding views of the segmented 3D map colored as in Figure 1.

Extended Data Figure 5. Correspondence between individual subunits and anthropomorphic features of the eIF3 core complex and 3D variance of the 40S•DHX29•ΔII-IRES•eIF3 map.

(**a**-**b**) Front (upper panels) and back views (lower panels) of cryoEM reconstructions of eIF3 as it appears in class 4 of the CSFV ΔII-IRES•40S•DHX29•eIF3 complex bound to the CSFV ΔII-IRES (**a**) and alone[13] (**b**), labeled to show anthropomorphic terms[12] and the localization of individual subunits in the core complex[11, 24]. (**c**) 3D variance of class 4 Cryo-EM map, filtered to 20Å, and colored according to the computed 3D variance (see Methods), from dark blue for the lowest variance to red for the highest variance. The map is filtered to the resolution at which the 3D variance was estimated (~ 20Å).

Extended Data Figure 6. Comparison between the CSFV and the HCV pseudoknots.

Views of the structures of (**a**) the HCV pseudoknot, from the 3.6Å resolution crystal structure, with an additional crystallization module extending from helix III1[27] (PDB: 3T4B) and (**b**) the CSFV pseudoknot in the context of the 40S-subunit-bound ΔII-IRES (this study) are shown in ribbon representation and colored according to the scheme of the respective secondary structure diagrams (Extended Data Fig. 1a). (**c**, **d**) Closeup views of HCV and CSFV pseudoknots, showing the "main" helix, formed by helix III_1 and pseudoknot (pk) stem 1A (in HCV) or helix III1, pk stem 1a and pk stem 1b (in CSFV), and the "sidecar" helix, which contains subdomain IIIe, pk stem 2 and the two-base-pair helical segment of subdomain IIIf (see Extended Data Fig. 5a and b).

Extended Data Figure 7. Molecular interactions of the CSFV ΔII-IRES with the 40S subunit and interactions of eIF3 with the HCV and CSFV IRESs.

(**a**) Secondary structure diagram of the CSFV ΔII-IRES, with nucleotides shown in different degrees of bold to show qualitatively their flexibility in the cryo-EM map (the more flexible, the bolder). Circled nucleotides interact with the indicated components of the 40S subunit. Ribosomal protein names and residue numbers are indicated according to the *Tetrahymena thermophila* 40S subunit[55]. (**b**-**d**) Secondary structure diagram of the apical region of domain III of (**b**, **c**) the CSFV IRES and (**d**) the HCV IRES. (**b**) contacts of eIF3 with the IRES in the cryoEM map of the 40S/ΔII-IRES/eIF3 complex. (**c** and **d**) sites of strong protection of CSFV and HCV IRESs by native eIF3 from enzymatic cleavage and chemical modification, of protection of the HCV IRES by a 10-subunit form of eIF3 from 1M7 modification, or of interference with binding of eIF3 to the IRES by modification, as indicated in the keys[5,9,11]. Abbreviations: dimethyl sulfate (DMS), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfate (CMCT), diethylpyrocarbonate (DEPC), 1-methyl-7-nitroisatoic anhydride (1M7). The inset panels show (**c**) CSFV and (**d**) HCV IRESs, with helix III4 and subdomains IIIa, IIIb and IIIc in bold.

Extended Data Figure 8. Formation of elongation-competent 80S ribosomes on the HCV IRES depending on the presence of eIF3.

Toe-printing analysis of 48S initiation and 80S pre-termination complexes (pre-TC) assembled on the *wt* and ΔIIIb HCV-(MSTN-STOP) mRNAs with translation components as indicated. The positions of the initiation and stop codons are shown on the left. Lanes C, T, A and G depict the cDNA sequence corresponding to the *wt* HCV-(MSTN-STOP) mRNA. The gel reported in the figure is representative of results obtained from three technical replicates.

Extended Data Figure 9. Unsupervised 3D classification protocol.

Details of the unsupervised 3D classification. The classification included 6 rounds. For each round, the number of the particles included is indicated, as well as their percentages calculated over the full dataset. The classes of rejected particles are crossed out in red and their percentages are indicated, also in red, as calculated over the full dataset. Lines and brackets are drawn in different colors for clarity. Classes generated in rounds 3 to 6 are displayed and colored by radial distance in Chimera UCSF[47] in order to help in the visual discrimination of differences in features among the classes.