Figure EV1. Identification and characterization of a recombinant helicase core of *Saccharomyces cerevisiae* Sen1 suitable for structural studies.

A SDS–PAGE analysis of Sen1<sub>1976-1880</sub> tagged with C-terminal CPD-His<sub>8</sub>. Lane E shows the elution fraction after Ni<sup>2+</sup>-affinity purification step, and lane InsP<sub>6</sub> shows the tag cleavage after the protein was incubated with 400 µM inositol hexakisphosphate (InsP<sub>6</sub>) for 20 min at 4°C. The protein before and after tag cleavage is smaller than expected: Theoretical molecular weights of Sen1<sub>1976-1880</sub>-CPD-His<sub>8</sub> and Sen1<sub>1976-1880</sub> are ~126 kDa and ~102 kDa, respectively. Left lane M shows a molecular weight marker.

B Time course analysis of the ATP-dependent 3'-5' duplex unwinding activity of Sen1 proteins. Reactions were performed in the presence of 5 nM of Sen1 and 2 nM of substrate. An RNA:DNA duplex composed of a 44-mer RNA annealed to a 19-mer DNA molecule to provide a 3'-end 25-nt single-strand overhang was used as the substrate (see Appendix Table S1 for sequence details). The asterisk (*) denotes the presence of a FAM at the 5' end of the DNA.

C Snapshots of the electron density maps at important regions of the structure described in the text. The 2Fo–Fc maps are contoured at 1.7σ.
Figure EV2. Biochemical and structural properties of Sen1, and comparison with Upf1.

A  Zoom-in view of the nucleotide binding site in Sen1 (left) and Upf1 (right) (PDB: 2XZO, Chakrabarti et al, 2011). The adenine ring is sandwiched between an apolar surface of RecA1 and an aromatic residue protruding from the short linker that connects RecA1 to RecA2 (Tyr1655, corresponding to Tyr638_{Upf1} and Tyr442_{MGMBP}). In addition, the conserved side chain of Gln1339 (corresponding to Gln413_{Upf1} and Gln196_{MGMBP}) forms a bidentate hydrogen-bond interaction with the N6 and N7 moieties of the adenine ring.

B  Comparison of the structures of yeast Sen1_{Hel}-ADP, human UPF1_{Hel}-AMPPNP (PDB: 2GJK, Cheng et al, 2007), UPF1_{Hel}-ADP-AlF_{4^-}-RNA (PDB: 2XZO, Chakrabarti et al, 2011), and yeast Upf1_{Hel-CH}-ADP-AlF_{4^-}-RNA (PDB: 2XZL, Chakrabarti et al, 2011). The molecules in a side-view orientation (90° clockwise rotation around a vertical axis with respect to the front-view in Fig 4A).

C  Comparison of the RNA-binding sites of Sen1 (left) and Upf1 (right) (PDB: 2XZO, Chakrabarti et al, 2011).

D–F  Functional analysis of the Sen1_{Hel} T1289A, R1293A mutant harboring substitutions at conserved positions at the predicted RNA-binding surface. (D) Fluorescence anisotropy assays. Curves represent three independent measurements. (E) ATP hydrolysis assays. Values correspond to the average and SD of three independent experiments. (F) IVTT assays performed in the same conditions as in Fig 1C. The images correspond to different gels migrated and processed in parallel. The values of nascent RNA released correspond to one out of two independent experiments.
Figure EV2.
Figure EV3. Analysis of the impact of the “prong” mutations on the affinity of Sen1 hel for the RNA.
Electrophoretic mobility shift assay (EMSA) using a 5'-end fluorescently labeled 44-mer RNA as the substrate (DL3316, see Appendix Table S1) at 2 nM and Sen1 variants at 10, 20, 40, 80, and 160 nM at the final concentrations. Gels were migrated and processed in parallel. The values correspond to the mean of two independent experiments. At high protein concentrations, Sen1 forms high-order complexes with the RNA that are retained in the wells of the gel.
Figure EV4. Analysis of the phenotype of the ΔLP mutant in vivo.

A A Sen1 variant harboring the ΔLP cannot support cell viability. A Δsen1 strain (YDL2767) covered by an URA3-containing plasmid (pFL38) expressing wild-type (wt) Sen1 was transformed with a TRP1-plasmid (pFL39) carrying either the wt or a ΔLP version of SEN1. After over-night growth in non-selective medium, cells initially harboring both plasmids were plated on minimal medium (CSM) containing 5-fluoroorotic acid (5-FOA) to select for cells that have lost the URA3 plasmid (and can therefore survive thanks to the TRP1 plasmid-borne SEN1 copy). The absence of cells growing in 5-FOA and containing the TRP1 plasmid expressing Sen1ΔLP indicates that the ΔLP deletion is lethal.

B The Sen1ΔLP mutant is strongly defective in transcription termination in vivo. Northern blot analyses of two well-characterized NNS-targets, snR47 and snR13, in a Sen1-AID (auxin-induced degron, Nishimura et al, 2009) strain carrying a plasmid (pFL39) expressing either the wt or a ΔLP version of SEN1. A strain harboring an empty vector was included as a positive control for termination defects. To detect the primary products of NNS-dependent termination that are processed/degraded by the exosome, the strain was also deleted in the exonuclease RRP6. Sen1-AID was depleted for 1 h by the addition of 100 μM indole-3-acetic acid (a natural auxin) to monitor the capacity of the plasmid-borne versions of SEN1 to induce transcription termination. The strong accumulation of longer RNA species in the sen1ΔLP mutant compared to the wt is indicative of major termination defects. Under non-depletion conditions, the strain harboring the mutant protein exhibits a dominant-negative phenotype (partial termination defects), indicating that Sen1ΔLP has similar expression levels compared to the endogenous Sen1. The ACT1 transcript is used as a loading control.
Figure EV5. Multiple sequence alignment of the helicase domain of Upf1-like helicases. The multiple alignment was done using Clustal Omega, and the conservation was calculated using BLOSUM62 and is shown in purple.