Mechanistic understanding of human SLFN11

Felix J. Metzner1,2, Simon J. Wenzl1,2, Michael Kugler3,2, Stefan Krebs1, Karl-Peter Hopfner1 & Katja Lammens1

Schlafen 11 (SLFN11) is an interferon-inducible antiviral restriction factor with tRNA endoribonuclease and DNA binding functions. It is recruited to stalled replication forks in response to replication stress and inhibits replication of certain viruses such as the human immunodeficiency virus 1 (HIV-1) by modulating the tRNA pool. SLFN11 has been identified as a predictive biomarker in cancer, as its expression correlates with a beneficial response to DNA damage inducing anticancer drugs. However, the mechanism and interdependence of these two functions are largely unknown. Here, we present cryo-electron microscopy (cryo-EM) structures of human SLFN11 in its dimeric apoenzyme state, bound to tRNA and in complex with single-strand DNA. Full-length SLFN11 neither hydrolyses nor binds ATP and the helicase domain appears in an autoinhibited state. Together with biochemical and structure guided mutagenesis studies, our data give detailed insights into the mechanism of endoribonuclease activity as well as suggestions on how SLFN11 may block stressed replication forks.
verify by biochemical analysis. Together, the data give detailed insights into substrate recognition and processing by SLFN11 as well as its regulation by phosphorylation. The mode of ssDNA binding by dimeric SLFN11 suggests a mechanism how the protein might block stalled replication forks. Taken together, our manuscript describes a new avenue to a mechanistic understanding of Schlafen proteins and specifically how SLFN11 acts as a double-edged sword with two functions.

**Results and discussion**

**Overall structure of dimeric SLFN11 apoenzyme**

SLFN11 consists of an N-terminal endonuclease domain (residues 1–353), termed Slfn core domain, followed by a linker domain (residues 354–576) and a C-terminal domain with homology to superfamily I (SF I) DNA/RNA helicases (residues 577–901) (Fig. 1a). To provide a structural basis for the nuclease and ATPase functions, we used cryo-EM to solve the structure of full-length human SLFN11 (Fig. 1b). We recorded

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**Fig. 1 | Structure of full-length human SLFN11.**  
**a** Domain architecture of SLFN11 with indicated key functional features. **b** Ribbon representation of the full-length human SLFN11 dimer with highlighted structural features in dark red. **c** Detailed view of the dimer interface I between helicase domains. **d** Detailed view of the dimer interface II between Slfn core domains. **e** Detailed view of the linker-helicase interface. SWAVDL motif and corresponding residues are coloured in dark red. **f** Structural differences of SLFN11 and SLPN5 (PDB code 7PPJ) in the conformation of the helicase domains. The SLFN11 ID-helix is depicted in green. The position of the missing C-lobe of the helicase domain of SLPN5 is depicted as a transparent cartoon model based on the AlphaFold model42.

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datasets of SLFN11 and SLFN11E209A and 2D classifications yielded classes of monomeric as well as C2 symmetric dimeric SLFN11. 3D reconstruction of the SLFN11 dimer resulted in a map with a global resolution of 2.86 Å, allowing model building of residues 7–899 (Fig. 1b). Furthermore, monomeric and dimeric SLFN11E209A were reconstructed from two different datasets, resulting in maps with global resolutions of 4.0 Å and 3.25 Å, respectively (Supplementary Fig. 1a). Regarding a single protomer, the Slfn core domain forms a horseshoe-like shape as previously described for other Schlafen family members (Fig. 1a). A zinc ion is coordinated by residues H1285, C287, C321 and C322 forming a zinc finger motif (Supplementary Fig. 1b).

The SLFN11 dimer exhibits a ring-like structure with the individual protomers interacting via two interfaces (Fig. 1b–d). Interface I is located between the N-lobes of the helicase domains and is stabilized by several salt bridges (R590 to E726 and K591 to E725) (Fig. 1c). Interface II is located between the Slfn core domains that interact in a head-to-tail orientation and is formed by mostly polar and charged residues (E78, Q79, R82, S88, R134, T138, S139, R141, E147) (Fig. 1d). A related dimer interface was reported for SLFN12 which is stabilized in its dimeric form by small molecule-induced PDE3A binding (Supplementary Fig. 1c).

In solution, two peaks corresponding to the molecular weights of monomeric (104 kDa) and dimeric (208 kDa) SLFN11 were detected by mass photometry analysis (Supplementary Fig. 1d). In line with the polar nature of the dimer interfaces, the equilibrium between monomer and dimer is highly salt-sensitive (Supplementary Fig. 1d).

Mechanism of the SLFN11 endoribonuclease activity

SLFN11 has been reported to cleave type II tRNAs, leading to translational inhibition in a codon usage-dependent manner. By modulation of the tRNA pool, SLFN11 can inhibit viral protein translation during e.g. HIV infections or translation of human ATM and ATR in response to stalled replication forks. In the dimer structure, the lined-up Slfn core domains form a central channel. The endonuclease active sites are located in this central groove in close proximity to interface II (Fig. 2). To identify the binding mode to tRNA, we structurally analysed SLFN11 in the presence of a mixture of different yeast tRNAs. 3D coordinates of the nuclease active site with active site residues (E209 and E214) (Fig. 1d). A related dimer interface was reported for SLFN12 which is stabilized in its dimeric form by small molecule-induced PDE3A binding (Supplementary Fig. 1c).

In solution, two peaks corresponding to the molecular weights of monomeric (104 kDa) and dimeric (208 kDa) SLFN11 were detected by mass photometry analysis (Supplementary Fig. 1d). In line with the polar nature of the dimer interfaces, the equilibrium between monomer and dimer is highly salt-sensitive (Supplementary Fig. 1d).

The linker domain, harbouring the conserved SWAVDL motif, interacts with the helicase C-lobe (Fig. 1e) and connects to the helicase domain via a long α-helix that we will refer to as the inter-domain (ID)-helix (Fig. 1f). Notably, the structure of human SLFN5 revealed a strikingly different conformation of this region, despite a high sequence conservation (Fig. 1f and Supplementary Fig. 2). Due to the different folds, the helicase N-lobes of SLFN5 and SLFN11 adopt relative conformations which are rotated by approximately 180° to each other with regard to the respective linker domains. While monomeric SLFN11 exhibits the same overall conformation as the protomers in the dimer, the Slfn core C-lobe is not visible in the map of monomeric SLFN11 (Supplementary Fig. 1a). This suggests conformational flexibility and indicates that dimerization is needed to stabilize the C-lobes of the Slfn core domains in a defined conformation.

**Fig. 2** | SLFN11 tRNA recognition and nuclease activity. a Cryo-EM density map for SLFN11 bound to tRNA with proposed structural model of docked yeast tRNA\textsuperscript{\textit{p}} (PDB code 5AXM). b Bottom view of Slfn core domains with highlighted nuclease active sites. Cryo-EM density map for tRNA bound between N- and C-lobes of Slfn core domains. c Structural models of type I (tRNA\textsuperscript{\textit{p}}} (PDB code 2FMT) and type II (tRNA\textsuperscript{\textit{p}}}, model created by RNAComposer\textsuperscript{33,34}). Variable loop of type II tRNA is coloured in orange and SLFN11 cleavage site in pink (nt 75). SLFN11 endonuclease activity towards respective tRNAs demonstrated in nuclease assays. d Close-up view of the nuclease active site with active site residues (E209 and E214) coordinating a magnesium ion. e Metal ion dependency of SLFN11 endonuclease activity examined by nuclease assay. Effect of mutations of nuclease active site residues on cleavage of tRNA\textsuperscript{\textit{p}} monitored by nuclease assay. f Multiple sequence alignment of nuclease active site residues of human Schlafen family members with mutated residues highlighted in red. b Characterization of dimerization-induced nuclease activity of SLFN11 monitored by nuclease assay. Experiments in c, e, f, h were performed in duplicates. One representative replicate is shown. Source data for c, e, f, h are provided as a Source Data file.
reconstruction showed additional density in the groove between the Slfn core domain N- and C-lobes, fitting one tRNA molecule (Fig. 2a). This shows that a single tRNA molecule is bound and cleaved by the SLFN11 dimer. Two conserved positively charged patches, previously described to contribute to efficient tRNA cleavage by rSLFN13, are in close proximity to the tRNA" (Supplementary Fig. 3a). Although the resolution of the map did not allow for detailed tRNA model building, the density indicates that substrate recognition occurs in the Slfn core N- and C-lobes of both protomers of the dimer (Fig. 2b).

To precisely map the SLFN11 endonucleolytic cutting site, we sequenced tRNA^Ser cleavage products (Supplementary Fig. 3e). SLFN11 cleaves tRNA^Ser mainly at a single cutting site, positioned 10 nucleotides from the 3′ end, between nucleotides 75 and 76. This is in line with the cleavage pattern observed for rSLFN13. In the cryo-EM map, SLFN11 interacts with the acceptor stem and T-loop of the tRNA. It is positioned in close proximity to both endonuclease active sites, approximately 10 and 20 nucleotides from the 3′ end of the tRNA, respectively. However, based on the endonuclease activity assays and the sequencing results, only the active site that is closer to the 3′ end is cleavage proficient. The variable loop which is not clearly visible in our density, could be involved in binding, since it would be positioned in proximity to the Slfn core domain N- and C-lobes of both protomers of the dimer (Fig. 2b).

To biochemically verify whether SLFN11 dimerization is required for endonuclease activity, we performed an in trans complementation assay where the nuclease inactive SLFN11E214A was titrated to a constant and limiting concentration of SLFN11*. The nuclease activity was increased with the addition of SLFN11E214A, while SLFN11E214A alone was inactive (Fig. 2b). This confirms that dimeric SLFN11 is the nuclease active species and that a single active site is sufficient for tRNA cleavage. A double mutant of dimer interface I located in the helicase domains (K591D, Y722A) did not influence tRNA cleavage while the introduction of a third mutation in dimer interface II (R82D) rendered the nuclease inactive (Supplementary Fig. 3g). In summary, dimerization of SLFN11 is necessary for substrate specificity and cleavage site orientation in the active site. In line with our results, it has been hypothesized that the ribonuclease activity of subgroup II Schlafen family member SLFN12 might be stimulated by dimerization."
Although there is no direct interaction between S753 and ssDNA in the cryo-EM structure, the S753D mutant is deficient in DNA binding as monitored by EMSA (Supplementary Fig. 6b, c). This hints towards a functional connection between ssDNA binding to the helicase domain and the nuclelease activity of the Slfn core domain.

**SLFN11 exhibits an autoinhibited ATPase conformation**

The helicase domain of SLFN11 harbours the essential Walker A and B motifs of SF I DNA/RNA helicases which usually couple ATP hydrolysis to translocation along or unwinding of DNA or RNA (Supplementary Fig. 2). Mutation of the Walker B motif has been shown to cause defects in chromatin opening activity of SLFN11 in response to replication stress. Thus, we analysed nucleotide binding and hydrolysis by SLFN11. Addition of different nucleotides (ATP, ADP and ATPγS) causes only minor changes in the inflection temperature of SLFN11, indicating no interaction with the tested nucleotides (Fig. 4a). In contrast, SLFN5 shows a significant increase in unfolding temperature in presence of ATP and ATPγS (Fig. 4a).

We structurally overlaid the helicase domains of SLFN11 and DNA2, another SF I DNA/RNA helicase in its ADP-bound state (Fig. 4b). This illustrates that in SLFN11, ATP binding is sterically blocked by the ID-helix. The conserved glutamine residue of the predicted Q-motif is highlighted in bold. e Fluorescence anisotropy assay documenting the effect of K652D mutation on the binding of SLFN11 towards 50 nt ssDNA. The data were fit to a cooperative binding equation. Data are represented as mean values ± SEM from four independent experiments. Source data for a, b, e are provided as a Source Data file.

The monomeric cryo-EM structure adopts a similar helicase domain arrangement and ID-helix fold as the SLFN11 dimer (Supplementary Fig. 1a), indicating a second necessary step to trigger an active ATPase conformation. However, the characteristic strand separating Pin motif that is found in most SF I helicases with double strand unwinding activity is missing in SLFN11 (Supplementary Fig. 7b). Together with the absence of dsDNA binding elements (SF1A helicase domains 1B and 2B) in SLFN11, this demonstrates that the protein alone is not a strand opening helicase.

In summary, human SLFN11 is a manganese-dependent type II tRNA endoribonuclease. One tRNA molecule is bound in the positively charged groove of the SLFN11 core domain dimer and both protomers are involved in substrate recognition. Although the tRNA is in close proximity to both nuclease active sites, only one cleavage reaction occurs 10 nucleotides from the 3’ end. The active site residues E209, E214 and K216 are essential for catalysis. These residues are conserved in human SLFN11, 12, 13 and 14 that were shown to be nuclease proficient. Previous studies implied that helicase or ATPase activity is needed for replication fork blockage and/or chromatin opening. SLFN11 on its own is not proficient in ATP hydrolysis nor ATP binding in vitro. Hence, an additional factor e.g. binding partner, modification or signal is needed to activate ATPase activity. Despite the high sequence conservation of the Schlafen family members, the subtle differences in the endonuclease active site, DNA binding groove and helicase domain lead to divergent functions within this protein family. The presented data shed light on the structural and functional attributes of human SLFN11 and how it might bind to replication forks. The structural and mutational data explain the regulatory and inhibitory effects resulting from the phosphorylation of SLFN11. The inability of the phospho-mimetic S753D mutant to cleave tRNA and to bind
ssDNA might be a hint at a functional connection between the nuclease and helicase domains. We conclude that SLFN11 may act as a double-edged sword with two functions within one protein which might be regulatory connected. Further in cellulo studies are needed to uncover the whole regulatory picture of SLFN11 activation and to clarify the possible coupling or separation of these two functions. Our biochemical and structural investigation of full-length SLFN11 presents an important basis to facilitate these studies.

**Methods**

**Protein expression and purification**

A construct encoding for full-length SLFN11 with an N-terminal double FLAG-tag and a HRV 3C cleavage site was purchased from GenScript. SLFN11 was cloned into the pFASTBac1 expression vector using Gibson assembly for expression in insect cells\(^3\). *Spodoptera frugiperda* SF21 insect cells (Thermo Fisher) were used for virus generation. Expression was carried out in *Trichoplusia ni* High Five cells (Invitrogen) at 27 °C and 95 rpm for 72 h. After 72 h, the cells were centrifuged at 30,000 g at 4 °C for 60 min. The supernatant was incubated for 45 min. Beads were applied onto a 5 ml column (Bio-Rad) and washed with lysis buffer. RPA was eluted by adding 5 CV imidazole (buffer B (20 mM HEPES pH 7.8, 1 M NaCl, 2 mM MgCl\(_2\), 5 mM KCl, 0.1 mM EDTA, 4 mM imidazole) supplemented with protease inhibitors. Cells were lysed by sonication and the lysate was centrifuged at 30,000 g at 4 °C for 45 min and the supernatant was incubated with pre-equilibrated Ni\(^2+\)-NTA agarose beads (Qiagen), and washed with lysis buffer. RPA was eluted by applying a linear NaCl gradient (10 CV) in elution buffer (20 mM HEPES pH 7.8, 300 mM NaCl, 2 mM MgCl\(_2\), 5 mM KCl, 0.1 mM EDTA, 350 mM imidazole). The eluate was pooled and dialyzed directly concentrated using a centrifugal filter unit (Amicon, MWCO 30 kDa). Concentrated SLFN11 was applied onto either Superdex 200 5/150 or Superose 6 increase 5/150 column (GE Healthcare) equilibrated in buffer A. Peak fractions were used for cryo-EM grid preparation.

SLFN11 mutants were prepared by site-directed mutagenesis PCR and expressed and purified as the wild-type protein. SLFN5 was expressed and purified following a similar protocol, with the difference, that the pH of the buffers was adjusted to pH 7.1\(^\text{\textcircled{1}}\).

RPA was cloned into the pBBGia expression vector using the biGbac system\(^\text{\textcircled{2}}\). *Spodoptera frugiperda* SF21 insect cells (Thermo Fisher) were used for virus generation. RPA was expressed in *Trichoplusia ni* High Five cells (Invitrogen) similarly to SLFN11. Cell pellets were resuspended in lysis buffer (20 mM HEPES pH 7.8, 300 mM NaCl, 2 mM MgCl\(_2\), 5 mM KCl, 0.1 mM EDTA, 4 mM imidazole) supplemented with protease inhibitors. Cells were lysed by sonication and the lysate was centrifuged at 30,000 g at 4 °C for 60 min. The supernatant was applied onto pre-equilibrated Ni\(^2+\)-NTA agrose beads (Qiagen), and incubated for 45 min. Beads were applied onto a 5 ml column (Bio-Rad) and washed with lysis buffer. RPA was eluted by adding 5 CV imidazole buffer (20 mM HEPES pH 7.8, 300 mM NaCl, 2 mM MgCl\(_2\), 5 mM KCl, 0.1 mM EDTA, 350 mM imidazole). The eluate was pooled and dialyzed overnight into buffer A (20 mM HEPES pH 7.8, 100 mM NaCl, 2 mM MgCl\(_2\), 5 mM KCl, 0.1 mM EDTA). The clarified protein solution was applied onto a pre-equilibrated HiTrap Heparin HP column (GE Healthcare). RPA was eluted by applying a linear NaCl gradient (10 CV) using buffer B (20 mM HEPES pH 7.8, 1 M NaCl, 2 mM MgCl\(_2\), 5 mM KCl, 0.1 mM EDTA). Peak fractions were pooled and flash frozen in liquid nitrogen.

**Sample preparation and cryo-EM data acquisition**

Purified SLFN11\(^\text{\textcircled{1}}\) was dialyzed into dialysis buffer (20 mM HEPES pH 7.5, 60 mM NaCl, 2 mM MgCl\(_2\), 1 mM DTT) and pre-incubated for 30 min with breyer’s yeast tRNAs mixture (Sigma-Aldrich). The sample
was diluted in cryo-EM buffer (20 mM HEPES pH 7.5, 2 mM MgCl₂, 1 mM DTT) to a final concentration of 3.5 μM SLFN11 and 10 μM tRNA mixture. 4.5 μl was applied on a glow discharged QUANTIFOIL® R2/1 + 2 nm carbon Cu200 grid. The sample was vitrified in liquid ethane using an EM GP plunge freezer (Leica, 10 °C and 90% humidity).

Freshly purified SLFN11D209A was diluted in cryo-EM buffer (50 mM Glycine pH 9, 200 mM NaCl, 2 mM MgCl₂, 1 mM DTT) to a final concentration of 5 μM. 4.5 μl was applied on a glow discharged QUANTIFOIL® R2/1 Cu200 grid. The sample was vitrified in liquid ethane using an EM GP plunge freezer (Leica, 10 °C and 90% humidity).

Freshly purified SLFN11wt was pre-incubated for 30 min with 60 nt ssDNA and 1 mM ADP. The sample was diluted in cryo-EM buffer (100 mM Glycine pH 9, 2 mM MgCl₂, 1 mM DTT) to a final concentration of 100 nM or 300 nM. Movies were recorded for 60 s and analyzed by 3D ctf re

cryoelectron microscope (300 kV) equipped with a GIF quantum energy filter (slit width 20 eV) and a Gatan K2 Summit direct electron detector software (ESB, 2012.1.27REL, TEM User Interface Titan ISS-4, Digital Micrograph 3.2.2.1461.0).

For the structure of SLFN11wt and SLFN11wt bound to tRNA 8,569 movies were collected with a total electron dose of 44.03 e⁻/Å², fractionated into 40 movie frames over 8 s. For the structure of SLFN11E209A monomer 3,212 movies were collected with a total electron dose of 43.58 e⁻/Å², fractionated into 40 movie frames over 8 s. For the structure determination of SLFN11E209A dimer 7,078 movies were collected (of which 4,420 movies at a tilt angle of 25°) with a total electron dose of 44.03 e⁻/Å², fractionated into 40 movie frames over 8 s. For the structure of SLFN11wt dimer 223,491 particles. The model of SLFN11wt bound tRNA was generated by docking of the corresponding density map using UCSF ChimeraX 1.247. All structures were prepared with UCSF ChimeraX.

Cryo-EM data collection

Cryo-EM data were collected using an FEI Titan Krios G3 transmission electron microscope equipped with a GIF quantum energy filter (slit width 20 eV) and a Gatan K2 Summit direct electron detector software (ESB, 2012.1.27REL, TEM User Interface Titan ISS-4, Digital Micrograph 3.2.2.1461.0). For the structure of SLFN11wt and SLFN11wt bound to tRNA 8,569 movies were collected with a total electron dose of 49.65 e⁻/Å², fractionated into 40 movie frames over 8 s. For the structure determination of SLFN11wt dimer 7,078 movies were collected (of which 4,420 movies at a tilt angle of 25°) with a total electron dose of 44.03 e⁻/Å², fractionated into 40 movie frames over 8 s. For the structure of SLFN11E209A monomer 3,212 movies were collected with a total electron dose of 43.58 e⁻/Å², fractionated into 40 movie frames over 8 s. For the structure determination of SLFN11E209A bound to ssDNA 6,419 movies were collected (of which 4,088 movies at a tilt angle of 25°) with a total electron dose of 43.33 e⁻/Å², fractionated into 40 movie frames over 8 s. All datasets were collected with defocus values ranging from -1.1 to -2.9 μm and a pixel size of 1.046 Å.

Cryo-EM image processing

Movie frames were motion corrected using MotionCor2 1.4.5. All subsequent cryo-EM data processing steps were carried out using cryoSPARC 3.3.1 and the resolutions reported here are calculated based on the gold-standard Fourier shell correlation (FSC = 0.143). In total, five datasets were processed. The CTF parameters of the datasets were determined using patch CTF estimation (multi).

Model building and refinement

Atomic models were built by rigid body docking of models predicted by AlphaFold242 into the cryo-EM density. For building of ssDNA, ssDNA bound to DNA2 (PDB code 5AXM) was used as a starting model. As the sequence of the bound ssDNA cannot be determined from the map, the five nucleotides closest to the 5' end of the 60 nt sequence were modelled into the C2 symmetric ssDNA bound SLFN11 map. The models were partially rebuilt in Coot 0.9-pre41. Missing parts were built de-novo. Atomic models were improved by ISOLDE 1.2.244 and real space refinement in PHENIX 1.1745,46 using the maps with highest resolution, respectively. The model of SLFN11wt bound tRNA was generated by docking of RNA100 (PDB code 5AXM) to the corresponding density map using UCSF ChimeraX 1.247. All structures were prepared with UCSF ChimeraX.
Nuclease assay
Nuclease activity of SLFN11 was examined by a gel-based nuclease assay. The nuclease reaction was performed in nuclease buffer (25 mM Tris pH 7.5, 120 mM NaCl, 2 mM MgCl₂, 1 mM DTT) with 50 nM SLFN11 and 50 nM 6-FAM labelled nucleic acid substrate, unless otherwise indicated. 2 mM MnCl₂ was added if not stated otherwise. Reactions were started by adding the substrate and incubated at 37 °C for 45 min. Samples were mixed with loading dye (15% Ficoll, 20 mM Tris pH 7.6, 40 mM NaCl) and applied to a self-cast 15% denaturing polyacrylamide gel (Rotiphores™ DNA sequencing system). Gels were run in 0.5 TBE at 270 V (Bio-Rad) for 50 min. Gels were imaged by a Typhoon™ FLA 7000 (GE Healthcare) and analysed using GIMP 2.10.2 and ImageJ 1.8.0. The effect of dimerization on the nuclease activity of SLFN11 was examined using a gel-based nuclease assay with settings described above. 30, 150, and 300 nM SLFN11 were titrated to 30 nM SLFN11. Reactions were started by adding 50 nM 6-FAM labelled tRNA. The effect of ssDNA on the nuclease activity of SLFN11 was examined using a gel-based nuclease assay as described above. 50 nM SLFN11 or SLFN11K652D were incubated with or without 100 nM 50 nt ssDNA. Reactions were started by adding 50 nM 6-FAM labelled tRNA. Uncropped gels are provided in the Source Data file.

Electrophoretic mobility shift assay (EMSA)
Binding of SLFN11 to nucleic acid substrates was monitored by electrophoretic mobility shift assay (EMSA). Either 37.5–300 nM SLFN11 (for DNA substrates) or 62.5–500 nM SLFN11 (for tRNA substrates) was incubated with 40 nM 6-FAM labelled substrates at 4 °C for 30 min in EMSA buffer (25 mM HEPES pH 7.5, 60 mM KCl, 8% glycerol, 2 mM MgCl₂, 1 mM DTT). Samples were mixed with loading dye (15% Ficoll, 20 mM Tris pH 7.6, 40 mM NaCl) and applied to a NativePAGE 3–12% Bis-Tris gel (Thermo Fisher). The electrophoresis was performed in 1× NativePAGE running buffer (Thermo Fisher) at 100 V for 120 min at 4 °C. The gels were imaged using a Typhoon™ FLA 7000 (GE Healthcare) and analysed using GIMP. Uncropped gels are provided in the Source Data file.

Affinity measurement by fluorescence anisotropy
Initial protein dilutions (0, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 nM) of SLFN11 were prepared in assay buffer (25 mM Tris pH 7.5, 120 mM NaCl, 2 mM MgCl₂, 1 mM DTT). Protein dilutions were then mixed with 6-FAM labelled DNA (Supplementary Table 2) in assay buffer without NaCl (final DNA concentration of 10 nM) in a 1:1 (v/v) ratio (final volume: 20 µl, Greiner Flat Bottom Black 384 well plate). The reaction was incubated at 25 °C for 30 min and the fluorescence anisotropy was subsequently measured at an excitation wavelength of 470 nm and an emission wavelength of 520 nm using an Infinite M1000 microplate photometer (Tecan). Protein dilutions were analysed with Prism 6.07 (GraphPad Software). The datasets were extracted from each value of a dilution series and the datasets were fitted to a Hill model to determine the apparent dissociation constants.

Nano differential scanning fluorimetry (nanoDSF)
Binding of SLFN11 to various substrates was examined by nanoDSF (Tycho NT.6, NanoTemper Technologies). 300 nM SLFN11 was incubated with 300 nM of 50 nt ssDNA or 50 bp dsDNA in nanoDSF buffer (25 mM Tris pH 7.5, 60 mM NaCl, 2 mM MgCl₂, 1 mM DTT) for 30 min on ice, respectively. For testing of dependence on salt (NaCl) and ions (Mg²⁺, Mn²⁺, Ca²⁺, and Zn²⁺), nanoDSF buffer was adjusted accordingly. Interaction with nucleotides was performed similarly, where SLFN11 or SLFN5 (300 nM) were incubated with or without corresponding nucleotides (1 mM) in nanoDSF buffer. The samples were loaded into glass capillaries and the internal fluorescence at 330 nm and 350 nm was measured while a thermal gradient was applied. Data were analysed using the internal Tycho NT.6 software 1.3.2.878 and plotted with Prism (GraphPad Software).

ATP Hydrolysis assay
A fluorescence-based ATPase assay was conducted to determine the ATPase rate of SLFN11. SLFN11 (250 nM) was incubated with 150 nM of different DNA or RNA substrates in ATPase buffer (25 mM Tris pH 7.5, 50 mM NaCl, 1 mM DTT, 2 mM MgCl₂, 0.1 mg ml⁻¹ BSA) at 4 °C for 30 min. RPA was used at a concentration of 250 nM. SLFN11:substrate complexes were combined with 0.1 mM NADH in reaction buffer (25 mM Tris pH 7.5, 50 mM NaCl, 1 mM DTT, 2 mM MgCl₂, 0.1 mg ml⁻¹ BSA, 0.5 mM PEP (phosphoenolpyruvate), 1 mM ATP, 25 µM lactate dehydrogenase/ pyruvate kinase (Sigma-Aldrich)) in a 384 well plate (Greiner). Hexokinase from Saccharomyces cerevisiae (1.5 nM, Sigma-Aldrich) supplemented with 300 µM glucose served as the positive control. The fluorescence of NADH was measured at 37 °C using an Infinite M1000 microplate photometer (Tecan). The reaction was monitored for 45 min (20 s intervals) using an excitation wavelength of 340 nm and an emission wavelength of 460 nm. Data were analysed using Prism (GraphPad Software).

tRNA sequencing
For the identification of the SLFN11 cleavage position in tRNA, a nuclease reaction was performed as described above. Briefly, 1 µM tRNA substrate in nuclease buffer (25 mM Tris pH 7.5, 120 mM NaCl, 2 mM MgCl₂, 1 mM DTT) was incubated with 11 nM SLFN11 at 37 °C for 45 min. 15 µl of the reaction mixture were purified on a Sephadex G-25 column (Roche Quick Spin columns for radiolabeled RNA). The eluate (20 ng µl⁻¹) was converted to an Illumina sequencing library with the SMARTer smRNA-Seq kit (Takara) following the manufacturer’s instruction. The RNA was enzymatically polyadenylated, then reversely transcribed with an oligo dT primer and a template-switching primer, obtaining a cDNA that was extended with primer-templated sequences on both ends of the original RNA. The cDNA was amplified with barcoded primers, converting it into a sequencing-ready Illumina-compatible library. The cDNA library was sequenced on a NextSeq1000 (Thermo Fischer) in 60 bp paired-end mode. The obtained sequencing reads were demultiplexed and poly-A tails at the end as well as three nucleotides at the beginning, that were introduced by the template-switching mechanism, were removed. The reads were mapped to the sequence of tRNA using Burrows-Wheeler Aligner 0.17.7. The start positions of the mapped reads were visualized in a histogram using Prism (GraphPad Software).

Mass spectrometry
2 µl of purified SLFN11 (3.2 µM, purified from Trichoplusia ni High Five cells) were diluted with 5 µl 100 mM NH₄HCO₃ and 3 µl water. Proteins were reduced by addition of 1 µl DTE (50 mM in 50 mM NH₄HCO₃) and incubated for 30 min at 37 °C. Carbamidomethylation of cysteines was performed by adding 2 µl of iodoacetamide (100 mM in 50 mM NH₄HCO₃) and 30 min incubation at RT. For protein digestion, 20 ng of Trypsin (Promega) was added to the sample and incubated at 37 °C overnight. After digestion, the sample was acidified with 2 µl 15% formic acid. Liquid chromatography-mass spectrometry analysis was performed on an Ultimate 3000 nano-LC system (Thermo Fisher) coupled with a Q Exactive HF-X mass spectrometer (Thermo Fisher). Peptides were separated with an EasySpray reversed-phase column (PepMap 1) was converted to an Illumina sequencing library with the SMARTer smRNA-Seq kit (Takara) following the manufacturer’s instruction. The RNA was enzymatically polyadenylated, then reversely transcribed with an oligo dT primer and a template-switching primer, obtaining a cDNA that was extended with primer-templated sequences on both ends of the original RNA. The cDNA was amplified with barcoded primers, converting it into a sequencing-ready Illumina-compatible library. The cDNA library was sequenced on a NextSeq1000 (Thermo Fischer) in 60 bp paired-end mode. The obtained sequencing reads were demultiplexed and poly-A tails at the end as well as three nucleotides at the beginning, that were introduced by the template-switching mechanism, were removed. The reads were mapped to the sequence of tRNA using Burrows-Wheeler Aligner 0.17.7. The start positions of the mapped reads were visualized in a histogram using Prism (GraphPad Software).
Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data that support this study are available from the corresponding author upon request. The coordinates of the SLFN11α, SLFN11β and dimer, and SLFN11α ssDNA-bound structure have been deposited in the Protein Data Bank (PDB) under the accession code 7ZEL, 7ZEP, and 7ZES, respectively. The SLFN11α dimer cryo-EM reconstruction is available at the Electron Microscopy Data Bank (EMDB) under the accession code EMD-14690. The SLFN11 dimer reconstruction of SLFN11α bound to tRNA is available at the EMDB under the accession code EMD-14695. The SLFN11 monomer and dimer reconstruction of SLFN11α bound at the EMDB under the EMDB accession code EMD-14693 and EMD-14691, respectively. The SLFN11 dimer reconstruction of SLFN11α bound to ssDNA is available at the EMDB under the accession code EMD-14692. MS spectra were searched using the human subset of the Swiss-Prot database [https://www.uniprot.org/]. Source data are provided with this paper.

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Author contributions
F.J.M., S.J.W. and K.L. conceived the project. F.J.M., S.J.W., M.K. and K.L. designed all structural and biochemical experiments. F.J.M., S.J.W. and M.K. conducted all structural and biochemical experiments. F.J.M., S.J.W., M.K. and K.L. carried out the cryo-EM data collection and analysis. S.K. performed tRNA sequencing analysis. K.-P.H. helped with the analysis and interpretation of the results. F.J.M., S.J.W., M.K. and K.L. wrote the manuscript. K.L. and K.-P.H. provided funding. All authors discussed and commented on the results and the manuscript.

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Correspondence and requests for materials should be addressed to Katja Lammens.

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