



Structural and Biochemical Analysis of Human Schlafen5 Reveals the Molecular Basis of Its Anti-Tumor Function

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Abstract: Background: Schlafen5 (SLFN5) is an IFN-inducible member of the SLFN protein family involved in immune regulation, viral inhibition and tumor control. Recently, SLFN5 was suggested to inhibit the invasion of many kinds of malignant cells, and its expression positively correlates with the overall survival of relevant patients. However, the functional mechanism of SLFN5 remains elusive. Here, we aim to investigate the biochemical properties and anti-tumor mechanism of SLFN5. Methods: We expressed, purified and crystallized the N-terminal domain of human SLFN5 (hSLFN5-N) and solved its structure by molecular replacement. We examined the binding and digestion ability of hSLFN5-N to several kinds of nucleic acid substrates, including DNA, RNA, tRNA and rRNA via electrophoretic mobility shift assay (EMSA) and cleavage assay in vitro. Structure-based mutation screening and cancer-associated hot-spot single-point mutations in hSLFN5 were analyzed to reveal the critical amino acid sites that influence the structure, biochemical and physiological function of the protein. Results: Structurally, hSLFN5-N is characterized by a pseudo-dimeric domain organization with a flexible central valley that is narrower than that of rat (r)SLFN13-N. Unlike rSLFN13-N, hSLFN5-N is incapable of cleaving tRNA and rRNA, but it is able to bind DNA and cleave specific RNA substrates. Site-mutation analysis suggested that positively charged residues surrounding the central valley determine the DNA and RNA affinity of hSLFN5. Structure-based analysis revealed that cancer-associated mutations tend to severely affect the folding stability and function of SLFN5 by disrupting intra-/inter-molecular interactions, incurring unfavorable alterations in local conformation, or altering potential post-translational modifications (PTMs) of the wild-type protein. Besides, SLFN5 had no DNA sequence selectivity in A375 cells, which indicated that its anti-migration activity may mainly depend on its RNA manipulation ability rather than on its DNA affinity as a transcription factor. Conclusions: SLFN5 is a DNA- and RNA-binding protein with endonuclease activity on specific RNA substrates, which determines the role of SLFN5 as a tumor suppressor. Single-point mutations may disrupt the structural stability, RNA manipulation capacity and thus the tumor suppressor function of SLFN5.

Keywords: Schlafen5; crystal structure; DNA binding; RNA cleavage; human cancer

1. Background

SLFN5 belongs to the SLFN protein family with functional implications in various physiological and pathological processes in mammals [1–14], including thymocyte development [15–17], immune regulation [18–21], virus inhibition [7,21–29], and tumor suppression [13,19,30–39]. According to their size and domain composition, SLFN family proteins are categorized into three subgroups [1,25,40,41]. SLFN5 falls into the largest subgroup, subgroup III, characterized by an N-terminal domain (N'-domain), a so-called SWADL domain, and a C-terminal helicase domain that shares homology with the superfamily I of RNA helicases. In contrast, members of subgroup II lack the helicase domain, while those in subgroup I possess only the N'-domain [40,42]. Although the N'-



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domain is conserved across SLFN family members, it exhibits very limited sequence similarity to other proteins, suggesting that it may serve as a distinctive signature of the SLFN family.

The N'-domain of rat SLFN13 (rSLFN13-N) represents the first resolved structure among SLFN family members. It adopts a unique pseudo-dimeric U-pillow-shaped architecture and exhibits endonuclease activity toward tRNAs and rRNAs [23]. Structural modeling suggests that the RNA duplex substrate docks into the valley of rSLFN13-N, where three conserved negatively charged residues within the valley facilitate cleavage of one strand of the duplex [23]. Despite the conservation of the N'-domain, and especially of the three catalytic residues, among the SLFN proteins [23,33,43,44], the RNase activity does not seem to be a common feature of the family: the N'-domains of mouse SLFN1 and human SLFN5 both failed to digest tRNA(Ser) *in vitro* [23]. In addition, SLFN5 has been proposed to function as a transcription factor, suggesting it may directly bind DNA [10,34,45,46]. To elucidate the precise cellular mechanism of SLFN5, further structural and biochemical studies are necessary.

In this study, we determined the crystal structure of the hSLFN5-N. Structural comparison revealed that hSLFN5-N possesses a much narrower valley and a distinct surface electrostatic potential compared to rSLFN13-N, suggesting distinct biochemical properties. Consistently, although hSLFN5-N was unable to digest tRNA or rRNA, it bound both DNA and RNA molecules and exhibited RNase activity toward specific RNA substrates. Single-point mutagenesis of basic residues indicated that hSLFN5 also likely engages nucleic acids within its central valley. Based on the hSLFN5-N structure, we mapped cancer-associated single-point mutations and analyzed their potential impacts on protein stability and function. Our findings suggest that the anti-tumor effect of SLFN5 may be linked to its RNA binding and cleavage capacity rather than its DNA affinity. Collectively, this study reveals key structural distinctions within the SLFN family that underlie their functional diversity, and provides valuable insights for developing SLFN5-based diagnostic and prognostic strategies for related human cancers.

2. Materials and Methods

2.1. Construct Information

The cDNA of *Homo sapiens* (h)SLFN5 was purchased from Source Bioscience. For prokaryotic expression, the constructs and mutants were individually cloned into a modified pET28 vector with an N-terminal His6-tag fusion followed by a PreScission protease (PSP) cleavage site. For eukaryotic expression, hSLFN5 was cloned into the pCAGGS vector with a Flag-tag (pCAGGS-Flag-SLFN5) or the pLVX vector with a tandem S-protein (S)-tag, Flag-tag, and Streptavidin Binding Peptide (SBP)-tag at the N-terminus of the coding sequence for hSLFN5 (pLVX-SFB-SLFN5). Relevant mutants were prepared based on the corresponding recombinant plasmids by site-directed mutagenesis.

2.2. Protein Expression and Purification

hSLFN5 was expressed and purified in a similar way as rSLFN13 [23]. Briefly, recombinant proteins were induced to express in *Escherichia coli* (*E. coli*) Rosetta (DE3). After lysis and centrifugation, the supernatant was filtered and subjected to the first Ni-NTA column (GE Healthcare) purification. Then, the eluted protein was incubated with glutathione S-transferase (GST)-fused PSP to remove the His6-tag and dialyzed overnight. After dialysis, PSP was removed using a GST column and the protein was re-subjected to the second Ni-NTA column purification. The eluted protein was subsequently loaded onto a Superdex200 16/60 column (GE Healthcare) equilibrated with gel filtration buffer containing 20 mM Tris-Cl, pH 8.2, 150 mM NaCl and 1 mM dithiothreitol (DTT). The protein was eluted in a discrete peak corresponding to a molecular mass of approximately 37 kDa. Cell lysis and protein purification were both performed at 4 °C. The yields of SLFN5-N mutants used for biochemical study were comparable with that of the wild-type protein.

2.3. Protein Crystallization

Crystallization trials by the sitting-drop vapor-diffusion method were performed at 4 °C. hSLFN5(12–337) (12 mg mL⁻¹, in a buffer containing 20 mM Tris-Cl, pH 8.2, 150 mM NaCl and 1 mM DTT) was mixed with an equal volume of the reservoir solution containing 100 mM Tris-Cl, pH 7.5, 80 mM MgCl₂, 200 mM glycine, and 1.6 M ammonium sulfate. Crystals appeared after five days and grew to a maximum size in 10 days. For data collection, crystals were flash-frozen in the reservoir solution containing an extra 35% xylitol.

2.4. Structure Determination

The X-ray diffraction data set of hSLFN5(12–337) was collected from a single crystal at beamline BL19U1 of Shanghai Synchrotron Radiation Facility (SSRF). The data set was processed with autoPROC [47]. Phases were

obtained by molecular replacement with Phaser from the CCP4 package [48], and the rSLFN13-N structure (PDB code: 5YD0) was applied as a search model. The models were built with COOT [49] and refined with Refmac5 [50], Phenix [51] and PDB-REDO [52]. Structural validation was carried out using MolProbity [53]. Structural illustrations were prepared using the PyMOL Molecular Graphics System (version 1.7.0.0, Schrödinger LLC (New York, NY, USA); <http://www.pymol.org/> (accessed on 1 February 2019)). X-ray data collection and refinement statistics can be found in Table 1.

Table 1. Crystallographic data collection and refinement.

hSLFN5-N	
Data collection	
Space group	C 1 2 1
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	211.860, 156.442, 131.459
α , β , γ (°)	90, 110.382, 90
Wavelength (Å)	0.97853
Resolution range (Å) *	123.229–3.304 (3.612–3.304)
Resolution in <i>a</i> *, <i>b</i> *, <i>c</i> * (Å)	3.273, 3.882, 3.548
Completeness, spherical (%) *	72.8 (15.6)
Completeness, ellipsoidal (%) *	90.2 (52.9)
<i>R</i> _{merge} *	0.069 (1.101)
<i>R</i> _{meas} *	0.075 (1.193)
<i>R</i> _{pim} *	0.029 (0.454)
<i>R</i> _{sym} *	0.063 (1.007)
CC1/2 (%) *	1.000 (0.731)
<i>I</i> /σ(<i>I</i>) *	13.5 (1.6)
Multiplicity *	6.8 (6.7)
Completeness (%) *	90.2 (52.9)
Redundancy *	3.5 (3.4)
Refinement	
Resolution range (Å) *	41.076–3.295 (3.412–3.295)
No. reflections	43638(220)
No. free test-set reflections *	2162 (13)
<i>R</i> _{work} / <i>R</i> _{free} *	0.2014/0.2472 (0.3703/0.3340)
Average B-factor	164.95
No. modeled atoms	14,075
Protein	14,069
Ligand	6 Zn
Solvent	0
R.m.s. deviations	
Bond lengths (Å)	0.003
Bond angles (°)	0.76
Model validation	
MolProbity score	2.09
Clashscore	12.75
Ramachandran favored/allowed/outliers (%)	92.34/5.88/1.79
Rotamer outliers (%)	0.00

* Values in parentheses are for the highest-resolution shell. The data were processed with autoPROC/STARANISO using an anisotropic cutoff (local mean *I*/σ(*I*) ≥ 1.20). Both conventional spherical completeness and STARANISO ellipsoidal completeness are reported.

2.5. Cell Culture

HEK-293T and A375 cells were individually maintained at 37 °C in high-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 100 U mL^{−1} penicillin and 100 µg mL^{−1} streptomycin. All cell lines are free of mycoplasma and have been authenticated by genotyping.

2.6. Preparation of Nucleic Acid Substrates

For HIV-related RNA, DNA transcription templates were constructed in the pcDNA3.1 vector, and *in vitro* transcription and purification of RNAs were performed as previously described [23]. Briefly, transcription was performed according to the manual of the T7 RiboMAX Express Large Scale RNA Production System (Promega, Madison, WI, USA). The reaction mixture was incubated at 37 °C for 30 min. After treatment with DNase I, the products were resolved by agarose gel and recovered using phenol-chloroform and subsequent ethanol precipitation. The purified RNA was aliquoted and stored at −20 °C if not immediately used. DNA and RNA molecules with different lengths and sequences were purchased from RuiBiotech (Beijing, China). For the native tRNA binding and digestion assay, small RNAs (mainly containing native tRNA) were extracted from cultured 293T cells using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) following the small RNA isolation

protocol. For the rRNA digestion assay, total RNA was extracted from cultured 293T cells using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA).

2.7. Nucleic Acid Cleavage Assay

For the cleavage assay, hSLFN5(12–337) of indicated concentrations was individually incubated with 10 ng μL^{-1} HIV-related RNA, 15 ng μL^{-1} small RNA (mainly composed of native tRNA), 50 ng μL^{-1} total RNA, or 2 μM single-stranded (ss)DNA/RNA, double-stranded (ds)DNA/RNA, random-sequence DNA/RNA, or DNA/RNA hybrid, in the cleavage buffer containing 40 mM Tris-Cl, pH 8.0, 20 mM KCl, 4 mM $\text{MgCl}_2/\text{MnCl}_2$, 2 mM DTT and 5% glycerol for 30 min at 37 °C. Except for the total RNA cleavage products, other cleavage products were analyzed by electrophoresis at 250 V on a 12% denaturing urea-polyacrylamide gel (urea-PAGE) containing 7.5 M urea with 1 $\times$ TBE (89 mM Tris, 89 mM borate and 2 mM EDTA) as the running buffer. The total RNA cleavage products were resolved by agarose gel with 1 $\times$ TBE. After electrophoresis, the gel was stained with SYBR Green II or ethidium bromide (EB) and visualized by UV light.

2.8. EMSA

hSLFN5(12–337) and nucleic acid substrates of different molar ratios were individually incubated in buffer containing 40 mM Tris-Cl, pH 8.5, 20 mM KCl, 2 mM MgCl_2 and 2 mM DTT at room temperature. After incubation, each sample was mixed with a loading buffer containing 3% glycerol and 0.05% bromophenol blue before being loaded onto a 6% native polyacrylamide gel that was pre-run for 45 min at 14.0 V cm^{-1} , 4 °C, in a running buffer containing 40 mM Tris-Cl, pH 8.5. After electrophoresis, the gel was stained with EB and visualized by UV light. The nucleic acid sequences used in biochemical assays are summarized in Table 2.

Table 2. Sequences of nucleic acids used in biochemical assays.

Nucleic acid	Sequence
30 nt ssDNA	5'-GCAGGCTTTCATTGCTACCAAGCAACAAGG-3'
30 bp dsDNA	Sense: 5'-GCAGGCTTTCATTGCTACCAAGCAACAAGG-3' Anti-sense: 5'-CCTTGTGCTTGGTAGCAATGAAAGCCTGC-3'
31 nt ssDNA	5'-GTGAAGGCATTCGCTGCTGTGGTGTCTCGG-3'
31 bp dsDNA	Sense: 5'-GTGAAGGCATTCGCTGCTGTGGTGTCTCGG-3' Anti-sense: 5'-CCGAGAACACCACAGCAGCGAATGCCTTCAC-3'
10 bp DNA (<i>ZEB1</i> promoter)	Sense: 5'-TCCCCTCAAA-3' Anti-sense: 5'-TTTGAGGGGA-3'
7 bp DNA-1	Sense: 5'-AGTAGCA-3' Anti-sense: 5'-TGCTACT-3'
7 bp DNA-2	Sense: 5'-CACAGCC-3' Anti-sense: 5'-GGCTGTG-3'
10 bp DNA-1	Sense: 5'-GCAGTACAGC-3' Anti-sense: 5'-GCTGTACTGC-3'
10 bp DNA-2	Sense: 5'-CAGCAGCCG-3' Anti-sense: 5'-GCGGCTGCTG-3'
18 bp DNA-1	Sense: 5'-ACTCCGCGCGCTCCGGAG-3' Anti-sense: 5'-CTCCGAGCGCGCGGAGT-3'
18 bp DNA-2	Sense: 5'-TCCCCAGACGGCCGCGCC-3' Anti-sense: 5'-GGCGCGCCGCTCTGGGGA-3'
Stem-loop RNA-1	5'-GGCGCCCGAACAGGGACUUGAAAGCGAAAGUAAAGCCAGAGGAGAUCUCUCGACGCAGGAC UCGGCUUGCUGAAGCGCGCACGGCAAGAGGGCGAGGGGCGCGACUGGUGAGUACGCCAAAAA UUUUGACUAGCGGAGGCUAGAAGGAGAGAGAUUGGUGCGAGAGCGUCGG-3'
Stem-loop RNA-2	5'-UGGAAGGGCUAAUUCACUCCCAAAGAAGACAAGAUUCCUUGAUCUGUGGAUCUACCCACAC ACAAGGCUACUUCUCCUGAUUUGGCAGAACUACACACCAGGGCCAGGGGUCAGAUUACCCACUGA CCUUUGGAUGGUGCUACAAGCUAGUACCCAGUUGAGCCAGAUUAGGUAGAAGAGGCCAAUAAA GGAGAGAACCACAGCUUGUACACCCUGUGAGCCUGCAUGGAAUGGAUGACCCUGAGAGAGA AGUGUAGAGUGGAGGUUUGACAGCCGCCUAGCAUUUUAUCACUGGCCCCGAGAGCUGCAUC CGGAGUACUUAAGAACUGCUGACAUCGAGCUUGCUACAAGGACUUCUCCGUGGGGACUUU CCAGGGAGGGCUGGCGUGGGCGGGACUUGGGAGUGGCGAGCCUCAGAUUCUGCAUUAUAGC AGCUGCUUUUUGCCUUCUGGUGUCUCUGGUUAGACCAGAUUCUGAGCCUGGGAGCUCUCU GGCUAACUAGGGAACCCACUGCUUAAAGCCUAAUAAAGCUUGCCUUGAGUGCUUCAA-3'
Stem-loop RNA-3	5'-UGGAAGGGCUAAUUCACUCCCAAAGAAGACAAGAUUCCUUGAUCUGUGGAUCUACCCACAC ACAAGGCUACUUCUCCUGAUUUGGCAGAACUACACACCAGGGCCAGGGGUCAGAUUACCCACUGA CCUUUGGAUGGUGCUACAAGCUAGUACCCAGUUGAGCCAGAUUAGGUAGAAGAGGCCAAUAAA GGAGAGAACCACAGCUUGUACACCCUGUGAGCCUGCAUGGAAUGGAUGACCCUGAGAGAGA AGUGUAGAGUGGAGGUUUGACAGCCGCCUAGCAUUUUAUCACUGGCCCCGAGAGCUGCAUC CGGAGUACUUAAGAACUGCUGACAUCGAGCUUGCUACAAGGACUUCUCCGUGGGGACUUU CCAGGGAGGGCUGGCGUGGGCGGGACUUGGGAGUGGCGAGCCUCAGAUUCUGCAUUAUAGC AGCUGCUUUUUGCCUUCUGGUGUCUCUGGUUAGACCAGAUUCUGAGCCUGGGAGCUCUCU GGCUAACUAGGGAACCCACUGCUUAAAGCCUAAUAAAGCUUGCCUUGAGUGCUUCAAAGUAGU GUGUGCCCGUCUGUUGUGUGACUCUGGUAAACUAGAGAUCCUCAGACCCUUUUGAGCAGUGU GGAAAAUCUCUAGCA-3'
Stem-loop RNA-4	5'-GGUCUCUCUGGUUAGACCAGAUUCUGAGCCUGGGAGCUCUCUGGCUAACUAGGGAACCCACU GCUUAAAGCCUAAUAAAGCUUGCCUUGAGUGCUAAAGUAGUGUGGCCCGUCUGUUGUGUG ACUCUGGUAACUAGAGAUCCUCAGACCCUUUAGUCAGUGGAAAAUCUCUAGCAGUGGC GCCCCAACAGGGACUUGAAAGCGAAAGUAAAGCCAGAGGAGAUCUCUCGACGCAGGACUCGG CUUGCUGAAGCGCGCACGGCAAGAGGGCGAGGGGCGCGACUGGUGAGUACGCCAAAAUUUU GACUAGCGGAGGCUAGAAGGAGAGAGUUGGUGCGAGAGCGUCGG-3'
30 nt ssRNA	5'-GCGGGGAGAGGCGGUUUGCGUAUUGGGCGC-3'

2.9. ChIP-Seq Assay

The SLFN5-overexpressing stable strains of A375 cells were constructed via lentiviral infection using the envelop vector pCMV-VSV-G with pLVX-Flag-SLFN5 or pLVX-SFB-SLFN5, and verified by western blot. The SLFN5- or vector-overexpressing A375 cells were cross-linked with 1% paraformaldehyde, and then harvested for cell lysis. Genomic DNA was sheared by sonication to yield 100–500 bp fragments using a Covaris E220 (Covaris, Woburn, MA, USA), and then the supernatants were subjected to immunoprecipitation (IP) using anti-Flag antibody (Sigma-Aldrich, St. Louis, MO, USA) and subsequent protein A/G bead incubation, or Strep beads. IgG was used as an antibody negative control. After repeated washes, immunoprecipitated DNA was released from protein–DNA complexes by adding proteinase K and NaCl (0.3 M) and heating at 65 °C for 4 h. Then, the DNA was subjected to sequencing library construction using the NEBNext Ultra II DNA Library Prep Kit for Illumina (7645, NEB, Ipswich, MA, USA) according to the manual, and libraries were sequenced using the Illumina NovaSeq 6000 platform (Illumina, CA, USA).

The sequencing data were converted into base sequences using bcl2fastq (version 2.19, Illumina, Inc., San Diego, CA, USA), and the original reads of a specific length were obtained. Next, the DNA sequencing raw reads were preprocessed by filtering out sequencing adapters, short-fragment reads and other low-quality reads. STAR (version 2.5.4b, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA) was then used to map the clean reads to the human hg38 reference genome. Peak detection was performed by MACS2 (Zhang Lab, Harvard University, Cambridge, MA, USA) with a minimum Q-value cut-off of 0.05. The peak recognition, overlap, subtraction, merge and feature-annotation analysis of enriched regions were performed using ChIPseeker (version 1.14.2, Yu Lab, Southern Medical University, Guangzhou, China).

2.10. Western Blot Analysis

The cell pellets after transfection were lysed in ice-cold lysis buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 400 mM NaCl, 10 mM KCl, 1% Nonidet P-40 and 20% glycerol) containing protease inhibitor cocktail and PMSF. After centrifugation at 12,000× g for 15 min, the supernatant was separated by 10% SDS-PAGE and transferred to PVDF membranes (Thermo Scientific, Waltham, MA, USA). After a 1 h incubation with blocking buffer (5% BSA), the membrane was incubated with the corresponding antibodies overnight at 4 °C. The membrane was washed and incubated with HRP-conjugated secondary antibodies. The membranes were washed again, developed with ECL Western Blotting Detection Reagents (Thermo Scientific) and visualized with a ChemiDoc Touch Imaging System (Bio-Rad, Hercules, CA, USA). Antibodies used in this paper were: anti-Flag antibody (Sigma-Aldrich), anti-SLFN5 antibody (Sigma-Aldrich, St. Louis, MO, USA), anti-β-tubulin antibody (ProteinTech, Rosemont, IL, USA), and HRP-conjugated anti-mouse/rabbit secondary antibody (Cell Signaling Technology, Danvers, MA, USA).

2.11. Transwell Assay

Migration experiments *in vitro* were performed using Boyden chambers containing Transwell membrane filter inserts (Corning Costar, Corning, NY, USA). In brief, 5×10^4 cells were seeded into each 24-well Transwell chamber (8 μm pore size) in complete medium with 10% FBS. After 24 h, cells that did not penetrate the filter were wiped off, and cells on the lower surface of the filter were fixed in methanol and then stained with 0.4% crystal violet. The numbers of migrating cells were counted under a light microscope (mean ± SD).

2.12. Data Mining

Information on cancer-related mutations in the human SLFN5 gene was collected from The Cancer Genome Atlas (TCGA), Catalogue of Somatic Mutations in Cancer (COSMIC) and International Cancer Genome Consortium (ICGC) databases. Among hundreds of human cancer-associated mutations in SLFN5, we focused on the single amino acid alterations in hSLFN5-N. The TCGA sequencing data for expression and survival analysis were downloaded from the UCSC Xena platform, and SLFN5 expression data in melanoma were downloaded from the Gene Expression Omnibus (GEO).

3. Results

3.1. Overall Structure of hSLFN5-N

As the full-length SLFN5 protein was not suitable for crystal screening, we tried to express and purify its individual domains. A construct containing residues 12–337 of human SLFN5, which includes the conserved N'-

domain, could be crystallized (Figure 1A). The crystal structure of hSLFN5-N was solved at 3.3 Å and the final model was refined to an R(free) of 0.247 (Table 1). In the structural model, residues 72–74 and 143–168 are missing due to disordered configuration. Similar to rSLFN13-N, hSLFN5-N folds into a U-pillow-shaped architecture with a pseudo-dimeric domain organization, which is composed of an N-terminal lobe (N-lobe), a C-terminal lobe (C-lobe) and a bridging domain (BD). These three domains together form a valley with a width of ~20 Å and a depth of ~20 Å (Figure 1B). The topologically conserved N-lobe and C-lobe are each built of a four-stranded β-sheet and three α-helices. The BD derives from two separate parts of the primary sequence, and consists of two α-helices and two pairs of flanking β-sheets with a two-fold pseudo-symmetry (Figure 1B,C). Despite the topological similarity, α3C, β3C and β4C of the N-lobe are considerably larger than their counterparts α3N, β3N and β4N of the C-lobe. Differences between the N- and C-lobes can also be observed in the spatial arrangement of the α-helices with regard to the four-stranded β-sheet (Figure 1B).

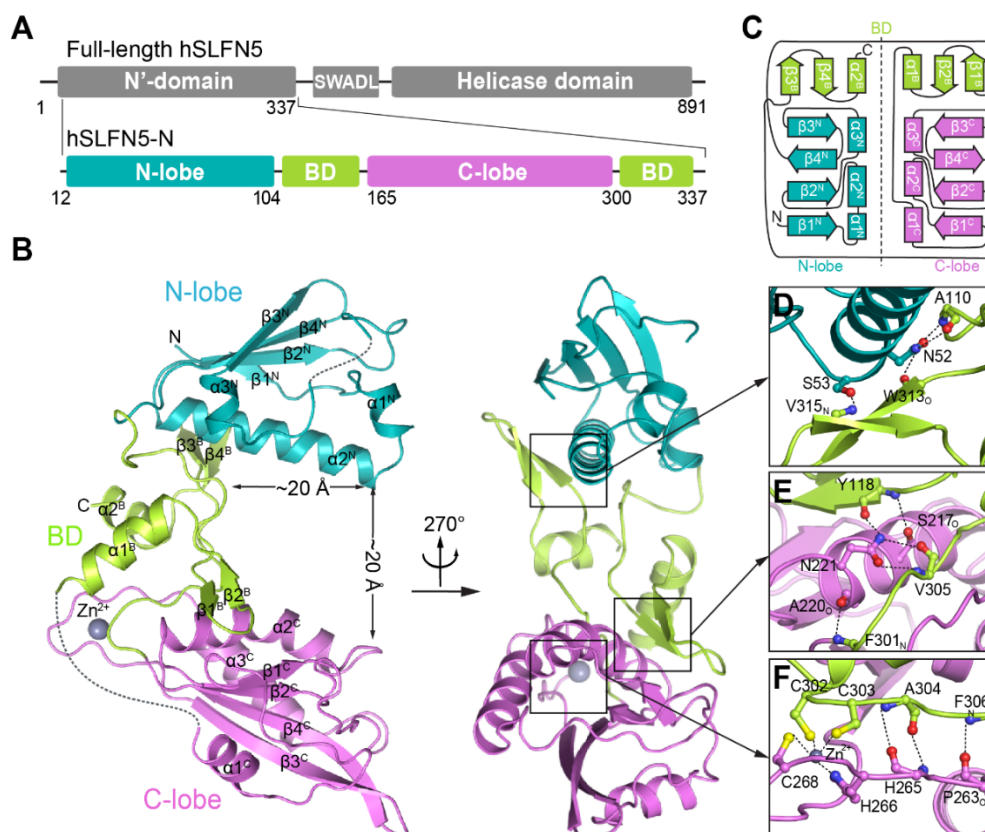


Figure 1. Overall structure of hSLFN5-N. (A) Schematic representation showing the domain organization of crystallized hSLFN5-N based on full-length hSLFN5. (B,D) denotes the bridging domain. Elements for hSLFN5-N are assigned according to the structure. Borders of each element are indicated by residue numbers. (B) Crystal structure of hSLFN5-N. Subdomains of hSLFN5-N are indicated and colored as in (A). Disordered loops are shown as dashed lines. The Zn²⁺ ion is shown as a gray sphere. α-helices and β-strands of each domain are specified. The size of the valley is indicated. (C) The topology diagram of hSLFN5-N. Secondary structural elements (not drawn to scale) are colored as in (B). (D) Interactions between BD and N-lobe. Side chains of involved residues are shown in the same color as the domain they belong to. (E,F) Interactions between BD and C-lobe. Note the CCCH-type zinc finger in (F).

The N-lobe and C-lobe closely interact with the BD at the opposite sides of the central valley. The two β-sheets of the BD each contact the α2N and α2C of the N- and C-lobes. Two conserved asparagines on α2N and α2C play a central role in the inter-domain contact by mediating hydrogen bonds with the corresponding main-chain atoms in the BD (Figure 1D,E). The hydrogen-bond pairs between α2N/α2C and the BD also include S53–V315N, S217O–Y118N and A220O–F301N. In addition, a bulging loop between α2C and β3C of the C-lobe stretches towards the BD and parallels the loop that connects the C-lobe and the BD. These two loops are linked by a line of hydrogen bonds (P263O–P306N, A304O–H265N and H265O–A304N) (Figure 1F). Four residues (H266, C268, C302 and C303) from the two loops form a CCCH-type zinc finger beside the hydrogen-bond line, which is essential for maintaining the stability of the hSLFN5-N molecule (Figure 1F). Structural superimposition of hSLFN5-N with another three SLFN5-N structures yielded root-mean-square deviations (r.m.s.d.) of 1.0 Å, 0.88 Å and 0.92 Å, respectively (Supplementary Figure S1A). The putative RNase catalytic module, formed by

three clustered negatively charged residues (E191, E196 and D233) as inferred from the rSLFN13-N structure, showed high consistency in side-chain orientation [23] (Supplementary Figure S1B). However, subtle differences were observed in the width of the central valley, with our structure possessing the broadest cleft among the compared models for hSLFN5-N. In addition, the four-stranded β -sheets in both the N-lobe and C-lobe showed noticeable variations in length and orientation (Supplementary Figure S1C,D). These structural distinctions point to an inherent flexibility in hSLFN5-N, which may facilitate its specific functional roles, such as substrate recognition and binding. In addition, we performed a structural comparison between our SLFN5-N crystal structure and the reported cryo-electron microscopy (cryo-EM) structure of full-length SLFN5. The two models show consistency in the intramolecular interactions within the N-lobe, C-lobe, and BD (Supplementary Figure S1E–G).

3.2. Structural Comparison between hSLFN5-N and Other SLFNs-N

The N'-domains of the SLFN members (SLFNs-N) share approximately 38% sequence identity and exhibit similarity in overall structural topology, especially between SLFN5-N and SLFN13-N (Figures 2A and S2). As described in the last section, the SLFNs-N share conserved motifs, including the asparagines that mediate the N-/C-lobe–BD interactions, the CCCH-type zinc finger, as well as the putative catalytic module of the acidic amino acid tripod [23] (Figure 2B). Despite these topological similarities, substantial structural differences exist among the SLFNs-N. The most prominent divergence lies in the dimensions of the central valley, which is the putative nucleic acid substrate-binding pocket. The width of this central cleft varies across the SLFN family, ranging from approximately 18 to 23 Å (Figure 2A). This variation is primarily attributable to the conformational flexibility of the α 2N helix at the valley opening, which exhibits significant differences in its tilt angle among family members (Figure 2C). Additional structural variation is observed on the opposite side of the valley, involving the α 2C helix in different SLFN proteins (Figures 2D and S2).

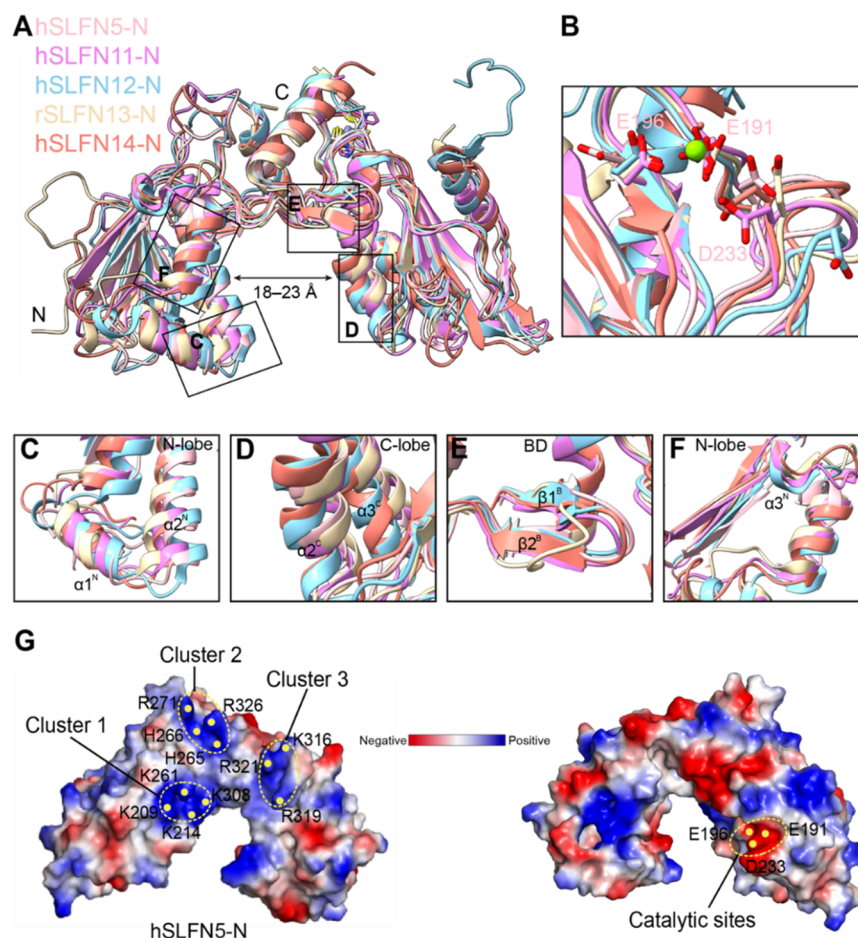


Figure 2. Structural comparison of the N'-domain of hSLFNs. (A) Structural overlay of the N'-domain of hSLFN5, hSLFN11 (7ZEL), hSLFN12 (7LRE), rSLFN13 (5YD0) and hSLFN14 (9JN9). The range of the central-valley widths is indicated. (B) Overlay of the catalytic modules of hSLFN5-N and SLFNs. The zinc ion is shown as a green sphere. (C–F) Local structural comparison between hSLFN5-N and SLFNs. (G) The electrostatic potentials of hSLFN5-N and rSLFN13-N. The residues of positively charged clusters in hSLFN5-N are indicated.

Local conformational differences are also evident in the bridging domain (BD) of hSLFN5-N. Specifically, its two anti-parallel β -strands (β 1B and β 2B), which consist of non-conserved amino acid sequences, are replaced by flexible loops in certain SLFN family members, indicating that this structural motif is not conserved across the family (Figure 2E). Likewise, variations in primary structure lead to another key distinction: the α 3N helix in hSLFN5-N differs in both size and spatial orientation from the corresponding structural element in other SLFNs-N (Figure 2F). Furthermore, analysis of surface electrostatic potential reveals substantial differences in charge distribution among the N'-domains [23,44]. Notably, hSLFN5-N displays more extensive positively charged patches around the central cleft, which are primarily formed by non-conserved residues (Figure 2G).

3.3. The DNA-Binding Activity of hSLFN5-N

Given previous evidence that hSLFN5 localizes to the nucleus and may function as a transcription factor [34], we sought to determine whether its N'-domain directly interacts with DNA *in vitro*. Using electrophoretic mobility shift assays (EMSA), we tested the binding of purified hSLFN5-N to synthesized single-stranded (ssDNA) and double-stranded DNA (dsDNA) molecules. The results showed a concentration-dependent retardation of both ssDNA and dsDNA migration upon addition of hSLFN5-N, indicating direct physical interaction (Figures 3A and S3A). In contrast, rSLFN13-N showed no detectable binding to these DNA molecules under the same conditions (Supplementary Figure S3B). Since hSLFN5 has been reported to transcriptionally activate the *ZEB1* gene by binding a 5'-TCCCCTCA-3' motif in its promoter region [34], we synthesized a 10-bp dsDNA oligonucleotide containing this specific sequence for EMSA analysis. As anticipated, hSLFN5-N efficiently bound this target dsDNA (Figure 3B). Furthermore, we found that alanine substitutions at the putative catalytic residues (E191A, E196A, and D233A) did not significantly impair DNA binding, suggesting that these acidic residues are not essential for the interaction with DNA molecules.

We next investigated the DNA-binding preference of hSLFN5-N. Initial assays using DNA duplexes of varying lengths and sequences revealed that hSLFN5-N bound to all tested substrates with comparable affinity, including two shorter 7-bp dsDNAs (Figure 3C). To further assess sequence dependence, we examined binding to random-sequence DNA oligonucleotides. The results showed that hSLFN5-N effectively retarded the electrophoretic mobility of both 15-nt and 30-nt single-stranded DNA molecules (Figure 3D), indicating a lack of strong sequence specificity.

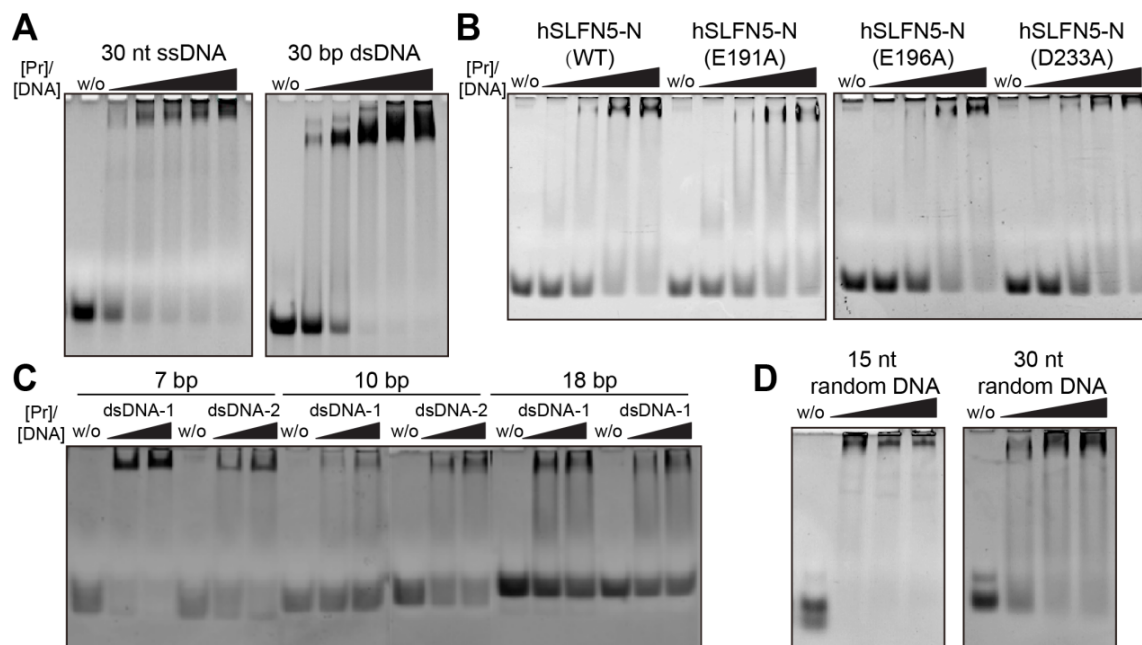


Figure 3. The DNA-binding activity of hSLFN5-N. (A) EMSA result showing the interaction between hSLFN5-N and DNA. hSLFN5-N and DNA of increasing molar ratios (4:1, 8:1, 16:1, 32:1, 64:1) were incubated for 30 min at room temperature. Pr, protein. (B) The DNA-binding activity of hSLFN5-N is independent of the putative catalytic module. Wild-type (WT) hSLFN5-N and the corresponding mutants were individually incubated with a 10-bp dsDNA derived from the *ZEB1* promoter in increasing molar ratios (2:1, 4:1, 8:1, 10:1). (C) hSLFN5-N binds dsDNA of different sequences and lengths. Increasing molar ratios (8:1, 16:1) of hSLFN5-N versus each dsDNA were applied. (D) hSLFN5-N binds ssDNA of different sequences and lengths. Increasing molar ratios (8:1, 16:1, 32:1) of hSLFN5-N versus each random ssDNA were applied.

3.4. Analysis of the Key Amino Acids in hSLFN5 Responsible for Nucleic Acid Affinity

Given the sequence and structural similarity between hSLFN5-N and SLFN13-N, we speculated that hSLFN5 also has the potential to bind nucleic acid substrates in the central valley (Figures 2A and S2). We selected alkaline amino acids surrounding the central valley, including R34, K38, R45, H206, K209, K214, K261, K308, R319, R321 and R326, and individually mutated them to alanine for DNA-binding detection (Figure 4A). DNA molecules with varying sequence and length were introduced into binding assays. Except for mutations H206A and R326A, all other mutations seriously compromised the 7-bp DNA-binding ability of hSLFN5 (Figure 4B). For both random-sequence DNA and a 31-bp DNA substrate, the H206A mutation did not impair binding affinity. In contrast, the R326A mutation exhibited a more pronounced reduction in DNA-binding activity compared with its effect on the shorter 7-bp DNA (Supplementary Figure S4A,B).

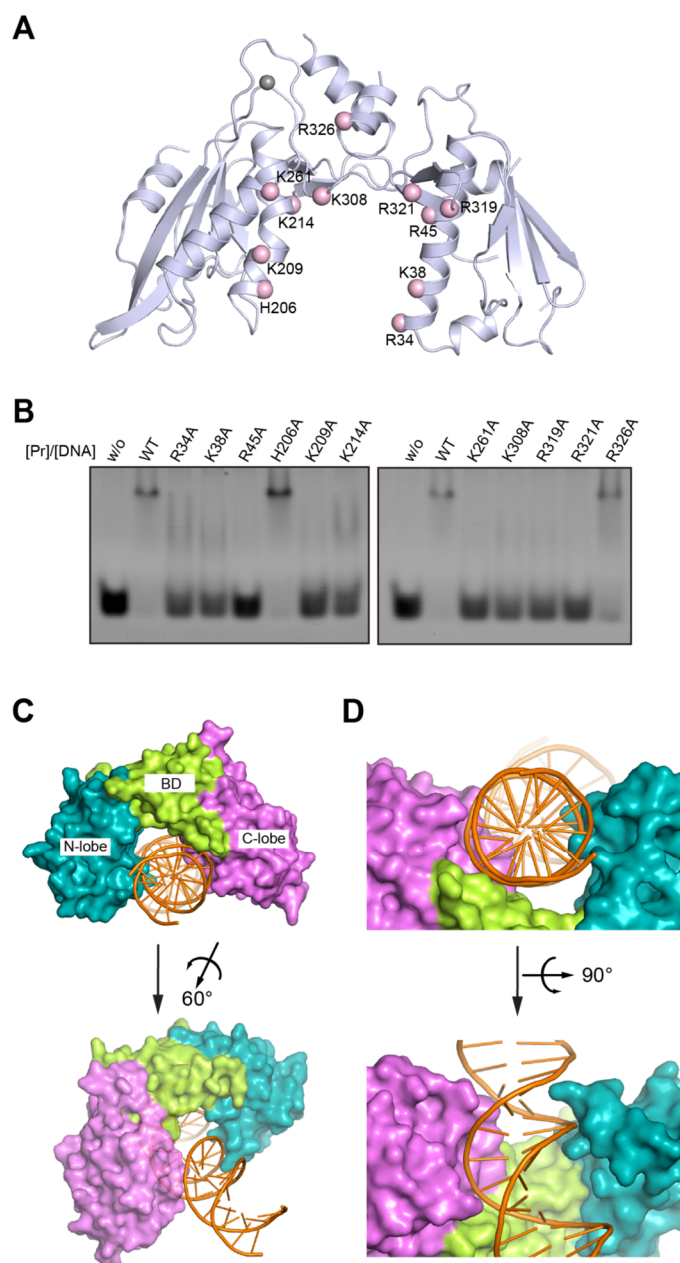


Figure 4. The interaction of hSLFN5-N with nucleic acid substrates. **(A)** Mapping of the positively charged amino acids around the central valley on the structure of SLFN5-N. These sites are shown as spheres. **(B)** The DNA-binding activity of hSLFN5-N is dependent on alkaline amino acids around the central valley. WT hSLFN5-N and the corresponding mutants were individually incubated with 7-bp DNA-1 molecules. The molar ratio of protein to DNA/RNA is 8:1. **(C)** SLFN5-N is shown as a surface representation and the subdomains are color-specified. The coordinates of dsDNA (4LDX, excerpted) are manually docked into the central valley of hSLFN5-N and colored orange. **(D)** Close-up view of hSLFN5-N accommodating the dsDNA. dsRNA fits well in the central valley of hSLFN5-N with no major clash.

Structural analysis provides a potential explanation for these observations. H206 is situated at the entrance of the central valley, and its side chain does not engage in close intermolecular interactions with neighboring residues. Mutation to alanine (H206A) likely preserves proper protein folding and may even slightly enhance DNA affinity by reducing steric hindrance at the substrate-binding interface (Figures 4A,B and S4A,B). In comparison, R326, located in the BD, is relatively distant from the central valley. The R326A mutation disproportionately impaired the binding of longer DNA substrates, suggesting that SLFN5 may employ additional auxiliary residues to engage extended nucleic acids.

Although the magnitude of the effect varied among substrates, most selected mutations in and around the central valley significantly perturbed nucleic acid binding. This strongly implicates the central valley as the primary nucleic acid-binding pocket of SLFN5. Based on these findings, we constructed a structural model of the hSLFN5-N–dsDNA complex, which demonstrates that a dsDNA molecule can be cleanly accommodated within the central valley without major steric clashes (Figure 4C,D).

3.5. Structure-Based Investigations of Cancer-Related Mutations in hSLFN5

According to the TCGA, COSMIC and ICGC databases, mutations of the human *SLFN5* gene were frequently identified from the whole-genome, whole-exome, and transcriptome sequencing data of various human cancers [54–56]. Among these, we identified 225 unique missense mutations, 90 of which are located within the N'-domain. We selected the 43 most frequent missense mutations for in-depth analysis, which correspond to 36 distinct amino acid residues. These mutations were documented in 15 different human cancer types (Table 3). Seven mutations were present in more than one cancer type, with T249M being the most frequently observed, detected across four distinct cancers. We mapped the cancer-associated mutation sites onto the hSLFN5-N structure and observed a broad distribution across the N'-domain: 13 sites localized to the N-lobe, 13 to the C-lobe, and 10 to the BD (Figure 5A and Table 4). Using the available structural information, we subsequently analyzed the potential effects of these mutations on the stability and function of hSLFN5.

In the N-lobe, the R34C and R36W mutations are located at the start of α 2N (Figure 5B). The side chain of the positively charged Arg34 protrudes outward at the entrance of the central valley, and thus may be engaged in binding with nucleic acids. Similar to R34A, the R34C mutation would not cause folding problems, but is likely to directly affect the association between SLFN5 and DNA molecules (Figures 4A,B and S4A,B). R36 is relatively conserved in SLFNs, and forms hydrogen bonds with the main-chain oxygen atoms of R28 and N29 (Figures 5B and S5). Its mutation to the bulky hydrophobic tryptophan disrupts this interaction and would therefore be harmful to the DNA binding of SLFN5. A46 and A49 on α 2N are part of a massive hydrophobic network involving V16, A18, I43, L50, I58, V315 and V320 (Figure 5C). Mutations A46S and A49T are less favored in this environment and may cause folding problems of the protein (Figure 5C). Similarly, A60 of β 2N is surrounded by several hydrophobic residues as well, including A18, V21, I43 and F95, so its mutation to serine or threonine would also adversely affect the structural stability of the protein (Figure 5D). Residues I97 and F98 on β 2N are highly conserved among human and mouse SLFNs (Supplementary Figure S5). The I97N mutation is equivalent to the I135N mutation in mouse (m)SLFN2, which was known as *Elektra* [18,57]. This mutation in mSLFN2 raises the susceptibility of mice to bacterial and viral infection, and causes immunodeficiency by diminishing numbers of T cells and inflammatory monocytes [18]. According to the hSLFN5-N structure, I97 is buried in a hydrophobic cluster involving V47, I58, L86, F95 and V99 (Figure 5E). Its mutation to asparagine would lead to a similar outcome as A60S/A60T. On the other hand, the I97F and F98C mutations do not alter the hydrophobic property of the original residues, and are thus less likely to cause problems in protein folding. Since the side chain of F98 is located on the surface of SLFN5, its mutation to cysteine may incur a new modification so that the physiological function of SLFN5 is disturbed. The highly conserved G54 mediates the turn between α 2N and β 2N (Figure 5F). The G54V mutation deprives the original glycine residue of the configurational flexibility that is commonly found between secondary structural elements, and may therefore incur folding problems for hSLFN5. Finally, mutations I57M, E61Q and N64S emerge on the surface of the molecule, lack intramolecular contact with other residues, and maintain the hydrophobicity or polarity of the original residues. Generally speaking, these mutations would be tolerable with regard to the folding and activity of hSLFN5 (Figure 5G).

Using the same analytical approach, we predicted the potential effects of missense mutations located in the BD and C-lobe, with the results summarized in Table 4. Representative mutations and their local structural environments are illustrated in Supplementary Figures S5 and S6A–J. Consistent with our previous analysis, the R36W mutation—but not R180W—significantly impaired the DNA-binding affinity of SLFN5 (Figures 5B,H,I and S6E). In comparison, the V305M mutation affected both soluble protein yield and DNA-binding activity (Figures 5H,I and S6F). Notably, the most frequently occurring mutation, T249M, did not markedly alter DNA

binding. However, it may abolish potential phosphorylation at this residue, thereby interfering with the physiological function of SLFN5 [58] (Figures 5H,I and S6D).

Collectively, these cancer-associated missense mutations appear to exert diverse effects on SLFN5, including disruptions to protein folding, nucleic acid binding, and post-translational modification. Mutations targeting conserved residues are particularly likely to compromise structural integrity, leading to reduced protein levels in cells.

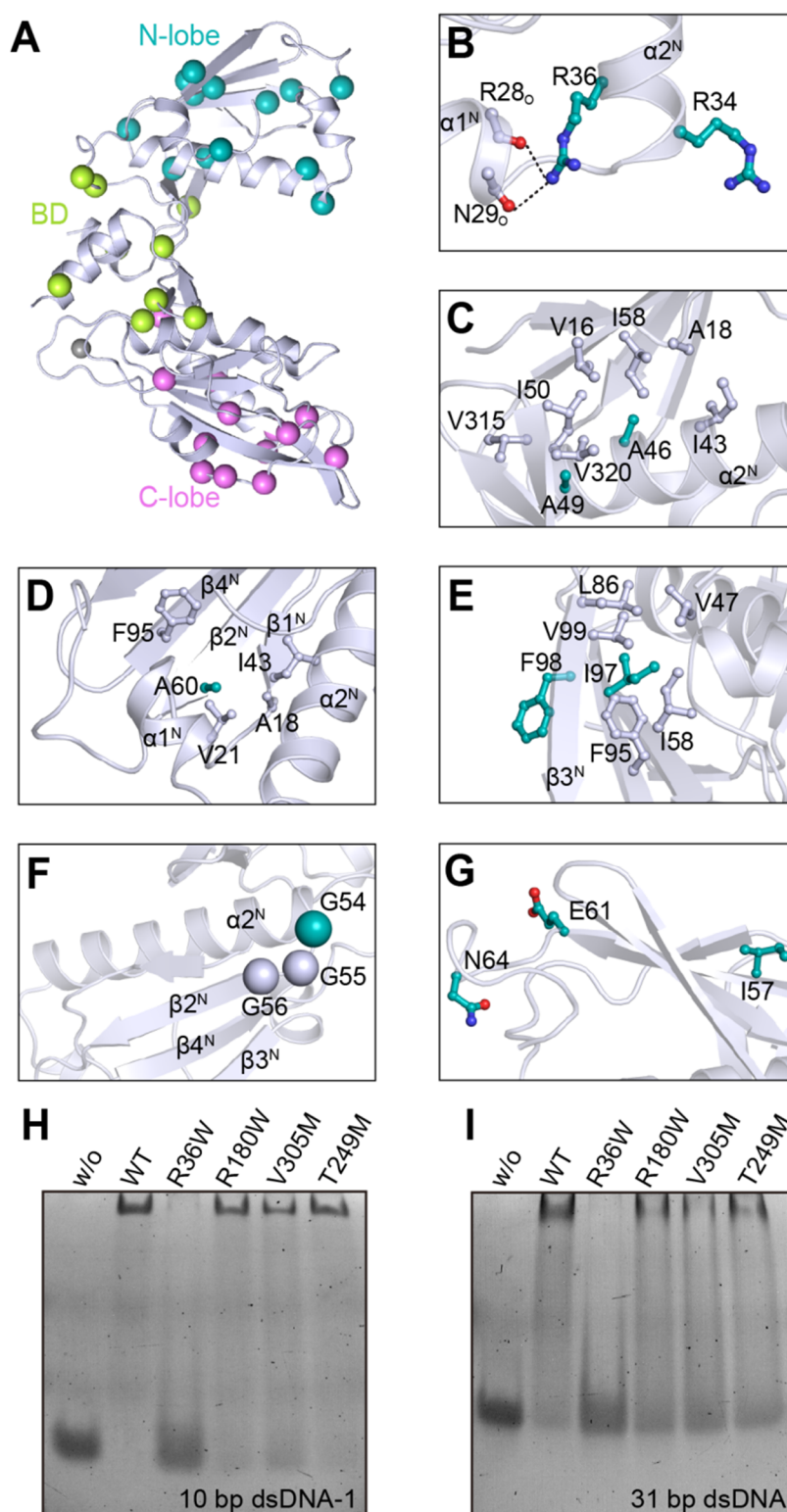


Figure 5. Overview of the distribution of cancer-related mutations. (A) Mapping of the cancer-related SLFN5 mutations on the structure of SLFN5-N. The mutation sites are shown as spheres and colored by the domains. (B–G) Structural details of the mutation sites. Residues subjected to mutations are colored teal, and the surrounding residues are colored gray. (H,I) WT hSLFN5-N and the corresponding mutants were individually incubated with 7-bp DNA-1 (H) or 31-bp DNA (I) molecules. The molar ratio of protein to DNA is 8:1.

Table 3. Summary of reported single-point mutations of hSLFN5-N in human cancers.

Cancer Type	Mutation(s)
Lung cancer	I57M, A60S, D87Y, R178I, E185G, G240R, T249M, S250C
Colorectal cancer	N64S, F98C **, P109S, C139G, R180W ***, R180Q, T249M
Stomach cancer	V78A, I97F, G107D, R180W ***, A295T, Q156P, Q156H
Uterine cancer	R34C, R36W **, A60T, T123A, T249M, V305L
Breast cancer	A46S, G54V, E61Q, R321T
Liver cancer	S115Y **, C139F, A295S, V305M **
Melanoma	F98C **, T249M, P263L, R290C
Blastoma	I97N, S115Y **, V305M **
Cervical cancer	R36W **, E121D
Head and neck	F176C, R180W **
Urinary bladder cancer	D177H, E327Q
Kidney cancer	G257C
Malignant glioma	A49T
Ovarian cancer	G225R **
Germ cell cancer	G225R **

** = two cancer types; *** = three; T249M = four.

Table 4. Summary of the structural analysis of cancer-related SLFN5-N single-point mutations.

Mutation	Domain	Position	Polarity before	Polarity after	Possible Influence
R34C	N-lobe	Exposed	Positively charged	Weak polar	Moderate
R36W	N-lobe	Exposed	Positively charged	Non-polar	Drastic
A46S	N-lobe	Buried	Non-polar	Polar	Moderate
A49T	N-lobe	Buried	Non-polar	Polar	Moderate
G54V	N-lobe	Exposed	Special	Non-polar	Drastic
I57M	N-lobe	Exposed	Non-polar	Non-polar	Very moderate
A60T	N-lobe	Buried	Non-polar	Polar	Moderate
A60S	N-lobe	Buried	Non-polar	Polar	Moderate
E61Q	N-lobe	Exposed	Negatively charged	Polar	Very moderate
N64S	N-lobe	Buried	Polar	Polar	Very moderate
V78A	N-lobe	Buried	Non-polar	Non-polar	Very moderate
D87Y	N-lobe	Exposed	Negatively charged	Polar	Unpredictable
I97N	N-lobe	Buried	Non-polar	Polar	Drastic
I97F	N-lobe	Buried	Non-polar	Non-polar	Moderate
F98C	N-lobe	Exposed	Non-polar	Weak polar	Modification change
G107D	BD	Exposed	Special	Negatively charged	Moderate
P109S	BD	Exposed	Special	Polar	Moderate
S115Y	BD	Buried	Polar	Polar	Unpredictable
E121D	BD	Exposed	Negatively charged	Negatively charged	Unpredictable
T123A	BD	Exposed	Polar	Non-polar	Modification change
C139G	BD	Exposed	Weak polar	Special	Modification change
C139F	BD	Exposed	Weak polar	Non-polar	Modification change
Q156P	BD	Exposed	Polar	Special	Unpredictable
Q156H	BD	Exposed	Polar	Positively charged	Unpredictable
F176C	C-lobe	Buried	Non-polar	Weak polar	Very moderate
D177H	C-lobe	Exposed	Negatively charged	Positively charged	Unpredictable
R178I	C-lobe	Exposed	Positively charged	Non-polar	Moderate
R180Q	C-lobe	Exposed	Positively charged	Polar	Unpredictable
R180W	C-lobe	Exposed	Positively charged	Non-polar	Very moderate
E185G	C-lobe	Exposed	Negatively charged	Special	Moderate
G225R	C-lobe	Buried	Special	Positively charged	Drastic
G240R	C-lobe	Exposed	Special	Positively charged	Moderate
T249M	C-lobe	Exposed	Polar	Non-polar	Modification change
S250C	C-lobe	Exposed	Polar	Weak polar	Unpredictable
G257C	C-lobe	Buried	Special	Weak polar	Very moderate
P263L	C-lobe	Exposed	Special	Non-polar	Drastic
R290C	C-lobe	Exposed	Positively charged	Weak polar	Unpredictable
A295S	C-lobe	Buried	Non-polar	Polar	Unpredictable
A295T	C-lobe	Buried	Non-polar	Polar	Unpredictable
V305M	BD	Buried	Non-polar	Non-polar	Moderate
V305L	BD	Buried	Non-polar	Non-polar	Very moderate
R321T	BD	Exposed	Positively charged	Polar	Modification change
E327Q	BD	Exposed	Negatively charged	Polar	Very moderate

3.6. Critical Sites Affecting the Anti-Tumor Activity of SLFN5

SLFN5 was reported to regulate the anchorage-independent growth, migration and invasion of melanoma cells [30,59]. To investigate the role of SLFN5 in pan-cancer, we analyzed the expression of SLFN5 in multiple tumor tissues and related normal tissues using downloaded sequencing data from the TCGA or GEO database. Compared with normal tissue controls, SLFN5 expression was markedly inhibited in several tumors, including lung squamous cell carcinoma (LUSC), prostatic adenocarcinoma (PRAD), breast cancer susceptibility gene (BRCA) and thyroid cancer (THCA), suggesting the potential of SLFN5 to act as a tumor suppressor (Figure 6A).

We also determined the relationship between SLFN5 expression and patient survival in SKCM and LUSC, and higher SLFN5 expression was positively correlated with progression-free survival (PFS) (Figure 6B). We then verified the knockdown and overexpression of SLFN5 protein in malignant melanoma A375 cells and lung cancer A549 cells, and revealed that their anti-migration rather than anti-proliferation ability was positively correlated with the expression level of SLFN5 as well (Figures 6C–F and S7A,B). Similar results were found in renal clear cell adenocarcinoma 786-O cells and ACHN cells (from ATCC), in which over-expressed wild-type SLFN5 protein both markedly restricted the cellular motility (Supplementary Figure S7C,D). Furthermore, the anti-motility activity of SLFN5-N was only slightly weaker than that of the full-length protein, underscoring the functional importance of the N'-domain in the anti-tumor process (Supplementary Figure S7E,F). A recent study reported that SLFN5 regulates epithelial-mesenchymal transition in breast cancer by suppressing the transcription of *ZEB1*, implying a potential role in transcriptional regulation [34]. However, given that our *in vitro* assays revealed no clear sequence selectivity for DNA binding, we further investigated the DNA-binding preferences of full-length hSLFN5 in A375 cells using chromatin immunoprecipitation sequencing (ChIP-Seq). After controlling for experimental and systematic background, we did not identify any genomic DNA sequences that were specifically enriched by hSLFN5 (Supplementary Figure S8). These results suggest that the anti-melanoma activity of SLFN5 likely depends on functional properties other than specific DNA binding.

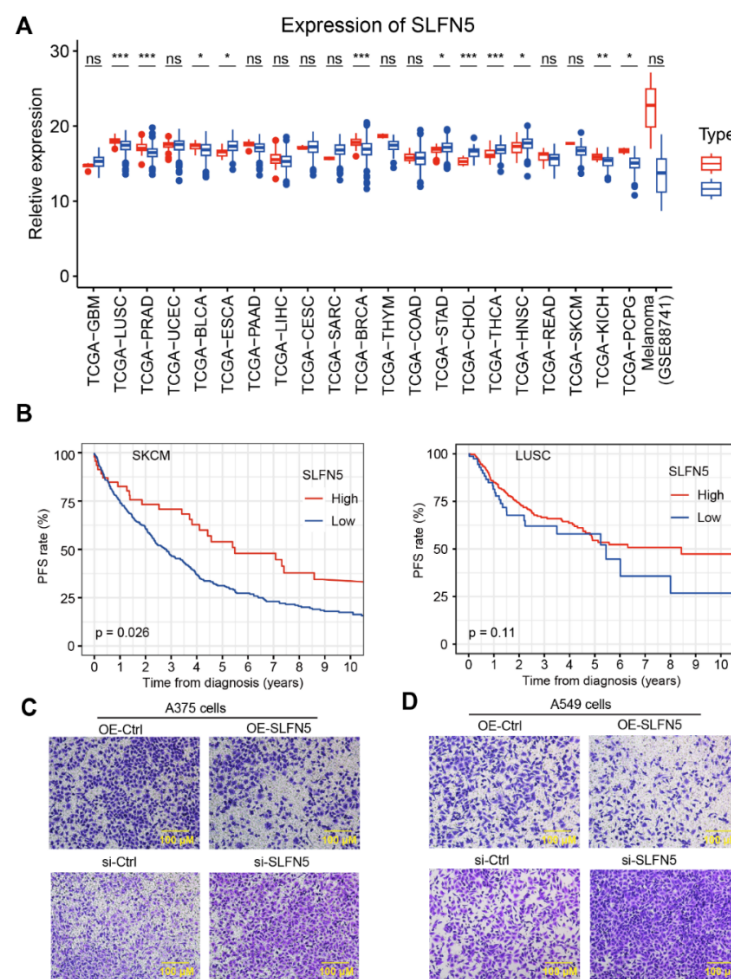


Figure 6. Anti-tumor effect of SLFN5 and related mutations. **(A)** The expression of SLFN5 in multiple tumors and relative normal tissues. The RNA-seq dataset of melanoma data was downloaded from GEO (GSE88741), and other TCGA data were downloaded from the UCSC Xena platform. Differential expression analysis and comparison was performed using Student's *t* test and Benjamini-Hochberg multiple testing correction method. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **(B)** The Kaplan-Meier curves of overall survival for high- and low-expression groups of SLFN5 in SKCM and LUSC. RNA-seq data were downloaded from the UCSC Xena platform. The *P* values from log-rank tests comparing the two Kaplan-Meier curves are shown in each figure. **(C,D)** Migration of A375 cells **(C)** and A549 cells **(D)** transfected with SLFN5-expressing plasmids or anti-SLFN5 siRNA for 24 h. After transfection, 5×10^4 cells were seeded into each chamber and cultured for 20 h prior to detection.

3.7. Anti-Tumor Function of SLFN5 Related to Its RNA Manipulation Ability

Except for hSLFN5, all other human SLFN members have been reported to digest tRNA/rRNA [18,19,35,36]. Our previous study suggested that hSLFN5-N lacked endoribonuclease activity on *in vitro*-transcribed tRNA^{Ser}. Since *in vitro*-transcribed tRNA has no modifications that are essential for amino acid transport, we extracted the native tRNAs from cells and introduced them into cleavage assays. hSLFN5 still had no tRNA cleavage activity regardless of the presence of Mn²⁺ or Mg²⁺ (Figure 7A). Meanwhile, native rRNA resisted cleavage by hSLFN5 as well (Figure 7B). We reasoned that hSLFN5-N either lacks overall nuclease activity, or has a preference for particular types of nucleic acids other than tRNA/rRNA. To further investigate the biochemical properties of SLFN5, we then tested the DNA digestion effect of hSLFN5, since it can accommodate DNA molecules in the central valley. However, ssDNA, dsDNA and random-sequence DNA also resisted digestion by hSLFN5-N (Supplementary Figure S9A,B). After a series of trials, we found that hSLFN5 was able to cleave several stem-loop RNAs transcribed *in vitro* (Figures 7C and S9C). Mutating any of the three residues in the putative catalytic site diminished the nucleolytic activity of hSLFN5; however, the RNA binding ability was not affected (Figure 7C,D). Substitution of positively charged residues surrounding the valley with alanine resulted in reduced RNA binding activity, and thereby reduced nucleolytic activity, indicating a similar pattern to that of DNA binding (Figures 7E,F and S9D–F). The H206A mutation was again an exception and had no effect on RNA binding or cleavage. Besides, hSLFN5, as well as several mutations, made little discrimination in digestion activity among the RNA substrates, suggesting limited substrate bias. Thus, hSLFN5-N is an RNase with a preference for RNA substrates containing a certain sequence or secondary structure, which is distinct from its homologs, underlying the functional diversity of SLFN members.

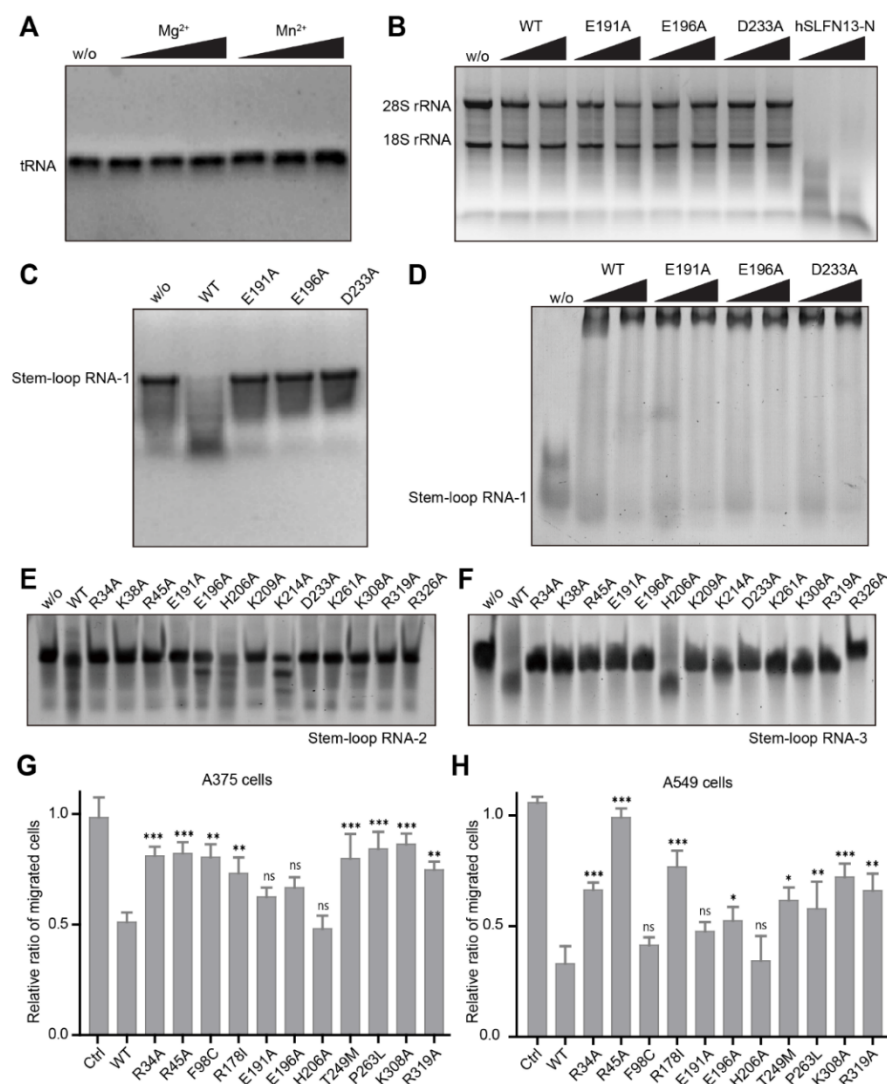


Figure 7. Anti-migration ability of hSLFN5 depends on its RNA processing activity. (A) hSLFN5-N lacks nuclease activity over tRNA. 2 μ M wild-type hSLFN5-N and mutants were individually incubated with small RNAs extracted from HEK-293T cells that are mainly constituted of native tRNAs. (B) hSLFN5-N lacks nuclease activity

over rRNA. hSLFN5-N and mutants of increasing concentrations (2, 4 μ M) were individually incubated with rRNA extracted from HEK-293T cells. hSLFN13-N was used as a positive control. (C) hSLFN5-N cleaves stem-loop RNA-1. 2 μ M wild-type hSLFN5-N and mutants regarding the putative catalytic module were individually incubated with transcribed stem-loop RNA-1 for 30 min at 37 °C. (D) The catalytic module had no effect on the RNA binding ability of hSLFN5-N. Wild-type (WT) hSLFN5-N and the corresponding mutant were individually incubated with stem-loop RNA-1 in increasing molar ratios (4:1, 8:1). (E,F) Stem-loop RNA cleavage by hSLFN5-N and mutants. 1 μ M wild-type hSLFN5-N and mutants were individually incubated with stem-loop RNA-2 (E) and stem-loop RNA-3 (F) for 30 min at 37 °C. (G,H) The effect of wild-type (WT) SLFN5 and several mutants on the migration of A375 cells (G) or A549 cells (H). Error bar indicates standard deviation ($n = 4$ technically independent experiments). One way ANOVA was used for statistical analysis and all mutant groups were compared with WT group. The ns represents not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

On the other hand, the anti-tumor effects of hSLFN5 mutants closely correlated with their performance in RNA manipulation assays. Mutations that impaired RNA cleavage activity—including R34A, R45A, E191A, E196A, K308A, and R319A—consistently reduced the anti-migration capacity of SLFN5 (Figures 7E–H and S9D). In contrast, the H206A mutation, which did not affect RNA cleavage, also had no significant impact on anti-tumor ability, consistent with the functional assay results. Although mutations E191A and E196A nearly abolished RNA digestion, they only partially reduced anti-migration activity. This partial retention of function may be attributed to their preserved RNA-binding affinity (Figure 7C–H). Similarly, cancer-associated mutations—such as F98C, R178I, P263L, and T249M—which are predicted to compromise structural stability or post-translational modification, also diminished the anti-motility function of SLFN5 (Figures 5E, 7G,H and S6D,E,J). Notably, the F98C mutation exhibited a weaker effect on anti-migration in A549 cells than in A375 cells (Figure 7G,H). This observation aligns with sequencing data showing that F98C is present in melanoma and colorectal cancer, but not in lung cancer (Table 3). Taken together, these results suggest that the anti-migration ability of SLFN5 is closely associated with its RNA manipulation activity and potentially with modifications within its N'-domain.

4. Discussion

In this study, we provide a comprehensive structural, biochemical, and physiological characterization of SLFN5, a Schlafen family protein with tumor-suppressive functions. We previously identified SLFN13 as a novel RNase targeting tRNAs and rRNAs [23], and similar RNase activities have been reported for SLFN11, SLFN12, and SLFN14 [1,43,44,60–62]. Based on the structural conservation across SLFN proteins, it has been proposed that they employ a shared clamp-and-cleave mechanism to engage RNA substrates within the central valley [31]. Although SLFN5 shares overall topology and inter-domain architecture with SLFN13, it does not digest tRNA or rRNA. Instead, SLFN5 binds both single-stranded and double-stranded DNA, and cleaves specific stem-loop-structured RNAs, indicating distinct substrate preferences. These functional differences likely arise from its unique structural features. Notably, despite exhibiting some flexibility, the central valley of SLFN5 is significantly narrower than that of SLFN13. While structural modeling suggests that dsRNA fits precisely within the central valley of SLFN13-N, the narrower valley of SLFN5-N appears better suited to accommodate dsDNA [23]. This hypothesis is biophysically supported: a typical B-form dsDNA has a diameter ~ 3 Å smaller than that of dsRNA—a difference that aligns well with the measured width gap between the valleys of SLFN5-N and SLFN13-N [63].

Our results show that the central valley of SLFN5-N, facilitated by adjacent positively charged patches, can bind diverse DNA and RNA structures, though it lacks DNA cleavage activity. Mutational analysis further indicates that RNA recognition by SLFN5 employs an interaction module similar to that used for DNA binding. We propose that SLFN5 may either remodel the valley conformation to engage dsRNA regions, or primarily anchor ssRNA loops within the valley while exploiting other basic surface patches to enhance binding affinity. This is consistent with the observation that mutating basic residues surrounding the valley impairs binding to both dsDNA and ssRNA. We note that the observed structural differences between SLFN5-N and SLFN13-N could reflect either an inherent folding distinction or a context-dependent conformational plasticity of the N-domain. If the latter holds true, it further raises the question of why SLFN5 does not cleave DNA, despite containing a catalytic triad that mediates RNA cleavage in other SLFNs [23,64,65]. Addressing these mechanistic questions will require additional structural data, particularly high-resolution complexes of SLFN5 bound to DNA or RNA substrates. Further structural and biochemical studies on other SLFN family members will also be essential to fully elucidate how structural variation dictates substrate specificity across this protein family.

In the EMSA assays, SLFN5-N exhibited no clear sequence specificity for DNA binding. Consistently, ChIP-Seq analysis in A375 cells did not identify any genomic DNA sequences specifically bound by full-length SLFN5. This result contrasts with earlier ChIP-Seq data from MCF7 cells, which reported preferential binding of SLFN5 to

the 5'-TCCCCTCA-3' motif in the promoter region of *ZEB1* [34], thereby challenging the view of SLFN5 as a sequence-specific transcription factor. At least in A375 cells, the anti-tumor activity of SLFN5 may not depend on specific promoter binding or direct transcriptional regulation. Alternatively, SLFN5 could function through post-transcriptional RNA regulation [66]. Since cellular mRNAs often adopt specific secondary structures to modulate translation, SLFN5 might suppress tumor migration by binding and cleaving mRNAs of key regulators—a model supported by the inverse correlation observed between its RNA-processing activity and cancer cell motility [67,68].

Single-point missense mutations of SLFN5 are frequently found in many kinds of human cancer [54–56,69]. The R36W mutation likely affects the DNA/RNA-binding activity of SLFN5, as Arg36 projects its side chain into the central valley, a region implicated in nucleic acid binding. Substitution with a bulky hydrophobic tryptophan may therefore hinder substrate accessibility. Other identified mutations in SLFN5-N are located outside the central valley and adjacent basic clusters. Many involve the replacement of buried hydrophobic residues with polar or charged amino acids, which could destabilize local or global folding and lead to protein degradation, as some of the mutants failed to be obtained as soluble protein and thus could not be purified in our assay. Similarly, substitutions of glycine residues at secondary-structure turns (e.g., G225R, G240R) may also result in folding defects. In contrast, surface mutations such as F98C, T123A, C139F, T249M, and R321T may not disrupt folding, but instead abolish specific interactions with functional partners or alter key post-translational modifications [58,70]. For example, the T249M mutation, which removes a potential phosphorylation site, significantly compromised the anti-migration activity of SLFN5 in A375 and A549 cells. A complete understanding of these mutational effects will require further studies aimed at identifying SLFN5-regulated downstream factors and characterizing its functionally relevant PTMs.

Supplementary Materials: The following supporting information can be downloaded at: <https://media.sciltp.com/articles/others/2609101452030580/HM-25120013-SM.pdf>, Figure S1. Superimposition of four SLFN5-N structures. (A) Crystal structure of hSLFN5-N (PDB ID: 7CUX) determined by our team were compared with another crystal structure of hSLFN5-N (7Q3Z, 6RRR9) and a hSLFN5-N structure from the full-length SLFN5 structure (7PPJ) determined by electron microscopy. The r.m.s.d between 7CUX with 7Q3Z, 6RR9 and 7PPJ are 1.0 Å, 0.88 Å and 0.92 Å, respectively. (B) Overlay of the catalytic modules. (C,D) Local structural comparison in N-lobe (C) and C-lobe (D). (E–G) Comparison between the cryo-EM structure (7PPJ) and the crystal structure (7CUX) of hSLFN5-N. Residues mediating the interactions between the N-lobe and BD (E), between the C-lobe and BD (F), and within the CCCH-type zinc finger (G) were shown. Figure S2. Sequence alignment between hSLFN5-N and rSLFN13-N. The amino acid sequences of hSLFN5-N (UniProt accession: Q08AF3) and rSLFN13-N (UniProt accession: Q5U311) are aligned using ClustalW2, adapted with permission from Ref. [1]. Residues with a conservation over 80% are in grey shades. α -helices are shown as cylinders and β -strands as arrows for hSLFN5 and rSLFN13. The secondary structure of hSLFN5-N are colored and named as Figure 1B. The secondary structure of rSLFN13-N are colored gray. Figure S3. The DNA binding activity of hSLFN5-N and rSLFN13-N. (A) EMSA result showing the interaction between hSLFN5-N and ssDNA/dsDNA. hSLFN5-N and DNA of increasing molar ratios (1:1, 2:1, 4:1, 8:1) were incubated for 1 h at room temperature. Pr, protein. (B) rSLFN13-N does not interact with dsDNA. rSLFN13-N and dsDNA of increasing molar ratios (4:1, 8:1, 16:1, 24:1, 32:1, 64:1) were incubated for 1 h at room temperature. Figure S4. The key amino acids of hSLFN5-N for DNA affinity. (A) Wild-type (WT) hSLFN5-N and corresponding mutants were individually incubated with 30 nt random DNA in molar ratio 16:1. (B) The DNA binding activity of hSLFN5-N is dependent of the charged amino acids around the central valley. Wild-type (WT) hSLFN5-N and corresponding mutants were individually incubated with 31 bp DNA in molar ratio 8:1 or 16:1. Figure S5. Sequence alignment of the SLFNs-N. Amino acid sequences of human (h)SLFN5 (UniProt accession Q08AF3), hSLFN11 (Q7Z7L1), hSLFN12 (Q8IYM2), hSLFN13 (Q68D06), hSLFN14 (P0C7P3), mouse (m)SLFN1 (Q9Z0I7), mSLFN2 (Q9Z0I6), mSLFN3 (Q9Z0I5), mSLFN4 (Q3UV66), mSLFN5 (Q8CBA2), mSLFN8 (Q7TMF), mSLFN9 (Q7TME9), mSLFN14 (V9GXG1) and rat (r)SLFN13 (Q5U311) are aligned using ClustalW2. Residues with a conservation of 100% are in red shades, greater than 80% in green shades and 60% in grey shades, respectively. The cancer-related mutations of SLFN5 are indicated by dots above the sequence which are colored according to the subdomain they belong to (same color scheme as in Figure 1a). Figure S6. Cancer-related mutations in BD and C-lobe. (A–E) Exposed residues. Thr123 (A), Arg321 (B), C139 (C), Thr249 (D), Phe176, Asp177, Arg178, Arg180 and Glu185 (E). Residues subjected to mutation are colored lemon (from BD) or magenta (from C-lobe), and the surrounding residues are colored gray. Note that Arg178 at the end of $\alpha 1^C$ forms a salt bridge with Glu185. (F) Val305 of C-lobe is surrounded by several hydrophobic residues. (G–J) Glycine and proline mutations. Gly107 and Pro109 (G), Gly225 (H), Gly240 (I), Pro263 (J). Mutations on these residues may alter the flexibility/rigidity of local configuration, and cause folding problems of the protein. Figure S7. Effects of over-expressed SLFN5 on tumor cells migration. (A,B) The expression of SLFN5 in A375 cells (A) and A549 cells (B) was verified by western blot (WB). A375 cells transiently transfected with control siRNA (si-Ctrl), SLFN5 siRNA (si-SLFN5), control vector (OE-Ctrl) or SLFN5 vector (OE-SLFN5) were collected and lysed, total lysates were resolved by SDS-PAGE and immunoblotted with an anti-SLFN5 antibody, anti-Flag antibody or an anti- β -tubulin antibody, as indicated. (C,D) Proliferation of A375 cells

(C) and A549 cells (D). Error bar indicates s.d. (n = 4). (E,F) Migration of 786-o cells (E) and ACHN cells (F) transfected with OE-Ctrl, or OE-SLFN5. 24 h after transfection, the cells were collected and counted. 5×10^4 cells were seeded into chamber to growth for 20 h. (G,H) Both SLFN5 and SLFN5-N inhibited the cell migration in A375 cells (G) and A549 cells (H). SLFN5-N was just slightly weaker than SLFN5 on inhibitory effect. Error bar indicates s.d. (n = 4). Figure S8. ChIP-Seq results of SLFN5 in A375 cells. A375_flag1, results from pLVX-Flag-SLFN5 expressing cells. A375_flag2, results from pLVX-SFB-SLFN5 expressing cells. A375_strep, results from pLVX-SFB-SLFN5 expressing cells. SP1.1 (GSM1661547) and SP1.2 (GSM1661546), validated transcription factors in A375 cells as positive controls. Figure S9. The nucleic acid substrates processing ability of hSLFN5-N. (A,B) hSLFN5-N does not cleave DNAs. hSLFN5-N and mutants of increasing concentrations (2, 4 μ M) were individually incubated with 30-nt ssDNA, 30-nt dsDNA (A) and 30-nt random DNA (B). (C) hSLFN5-N cleaves stem-loop RNAs. 2 μ M hSLFN5-N was individually incubated with transcribed stem-loop RNAs for 30 min at 37 °C in the presence of either Mg^{2+} or Mn^{2+} . hSLFN13-N was used as a positive control. (D) Stem-loop RNA-1 cleavage by hSLFN5-N and mutants. 1 μ M wild-type hSLFN5-N and mutants were individually incubated with stem-loop RNA-1 for 30 min at 37 °C. (E,F) The RNA binding activity of hSLFN5-N and related mutants. Wild-type (WT) hSLFN5-N and corresponding mutants were individually incubated with stem-loop RNA-2, stem-loop RNA-3 (E) or 30-nt ssRNA (F) molecules. The molar ratio of protein and DNA/RNA is 8:1. Reference [71] are cited in supplementary materials.

Author Contributions: S.G.: conceived project. M.L. and J.-Y.Y.: crystallized hSLFN5-N and collected the X-ray diffraction data. J.-Y.Y.: solved the structure. J.-Y.Y., M.L., J.-Y.O. and Z.-W.W.: performed the biochemical experiments. Z.-X.L. and Y.-Q.Z.: analyzed the ChIP-Seq data and RNA-seq data. J.-Y.Y. and S.G. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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References

1. Mavrommatis, E.; Fish, E.N.; Platanius, L.C. The Schlafen Family of Proteins and Their Regulation by Interferons. *J. Interferon Cytokine Res.* **2013**, *33*, 206–210. <https://doi.org/10.1089/jir.2012.0133>.
2. Liu, F.; Zhou, P.; Wang, Q.; Zhang, M.; Li, D. The Schlafen Family: Complex Roles in Different Cell Types and Virus Replication. *Cell Biol. Int.* **2018**, *42*, 2–8. <https://doi.org/10.1002/cbin.10778>.
3. Wu, X.; Schnitzler, G.R.; Gao, G.F.; Diamond, B.; Baker, A.R.; Kaplan, B.; Williamson, K.; Westlake, L.; Lorrey, S.; Lewis, T.A.; et al. Mechanistic Insights into Cancer Cell Killing through Interaction of Phosphodiesterase 3A and Schlafen Family Member 12. *J. Biol. Chem.* **2020**, *295*, 3431–3446. <https://doi.org/10.1074/jbc.ra119.011191>. Correction in *J. Biol. Chem.* **2020**, *295*, 16464–16467. <https://doi.org/10.1074/jbc.AAC120.016548>.
4. Ai, Y.; He, H.; Chen, P.; Yan, B.; Zhang, W.; Ding, Z.; Li, D.; Chen, J.; Ma, Y.; Cao, Y.; et al. An Alkaloid Initiates Phosphodiesterase 3A-Schlafen 12 Dependent Apoptosis without Affecting the Phosphodiesterase Activity. *Nat. Commun.* **2020**, *11*, 3236. <https://doi.org/10.1038/s41467-020-17052-4>.
5. Yue, T.; Zhan, X.; Zhang, D.; Jain, R.; Wang, K.W.; Choi, J.H.; Misawa, T.; Su, L.; Quan, J.; Hildebrand, S.; et al. SLFN2 Protection of tRNAs from Stress-Induced Cleavage Is Essential for T Cell-Mediated Immunity. *Science* **2021**, *372*, eaba4220. <https://doi.org/10.1126/science.aba4220>.
6. Metzner, F.J.; Huber, E.; Hopfner, K.P.; Lammens, K. Structural and Biochemical Characterization of Human Schlafen 5. *Nucleic Acids Res.* **2022**, *50*, 1147–1161. <https://doi.org/10.1093/nar/gkab1278>.

7. Kim, E.T.; Dybas, J.M.; Kulej, K.; Reyes, E.D.; Price, A.M.; Akhtar, L.N.; Orr, A.; Garcia, B.A.; Boutell, C.; Weitzman, M.D. Comparative Proteomics Identifies Schlafen 5 (SLFN5) as a Herpes Simplex Virus Restriction Factor that Suppresses Viral Transcription. *Nat. Microbiol.* **2021**, *6*, 234–245. <https://doi.org/10.1038/s41564-020-00826-3>.
8. Ding, J.; Wang, S.; Wang, Z.; Chen, S.; Zhao, J.; Solomon, M.; Liu, Z.; Guo, F.; Ma, L.; Wen, J.; et al. Schlafen 5 Suppresses Human Immunodeficiency Virus Type 1 Transcription by Commandeering Cellular Epigenetic Machinery. *Nucleic Acids Res.* **2022**, *50*, 6137–6153. <https://doi.org/10.1093/nar/gkac489>.
9. Murai, J.; Tang, S.W.; Leo, E.; Baechler, S.A.; Redon, C.E.; Zhang, H.; Al Abo, M.; Rajapakse, V.N.; Nakamura, E.; Jenkins, L.M.M.; et al. SLFN11 Blocks Stressed Replication Forks Independently of ATR. *Mol. Cell* **2018**, *69*, 371–384.e6. <https://doi.org/10.1016/j.molcel.2018.01.012>.
10. Huang, J.; Wu, C.; Kloeber, J.A.; Gao, H.; Gao, M.; Zhu, Q.; Chang, Y.; Zhao, F.; Guo, G.; Luo, K.; et al. SLFN5-Mediated Chromatin Dynamics Sculpt Higher-Order DNA Repair Topology. *Mol. Cell* **2023**, *83*, 1043–1060.e10. <https://doi.org/10.1016/j.molcel.2023.02.004>.
11. Podvalnaya, N.; Bronkhorst, A.W.; Lichtenberger, R.; Hellmann, S.; Nischwitz, E.; Falk, T.; Karaulanov, E.; Butter, F.; Falk, S.; Ketting, R.F. piRNA Processing by a Trimeric Schlafen-Domain Nuclease. *Nature* **2023**, *622*, 402–409. <https://doi.org/10.1038/s41586-023-06588-2>. Correction in *Nature* **2024**, *632*, E31. <https://doi.org/10.1038/s41586-024-07938-4>.
12. Boon, N.J.; Oliveira, R.A.; Körner, P.R.; Kochavi, A.; Mertens, S.; Malka, Y.; Voogd, R.; van der Horst, S.E.M.; Huisman, M.A.; Smabers, L.P.; et al. DNA Damage Induces p53-Independent Apoptosis through Ribosome Stalling. *Science* **2024**, *384*, 785–792. <https://doi.org/10.1126/science.adh7950>.
13. Ogawa, A.; Izumikawa, K.; Tate, S.; Isoyama, S.; Mori, M.; Fujiwara, K.; Watanabe, S.; Ohga, T.; Jo, U.; Taniyama, D.; et al. SLFN11-Mediated Ribosome Biogenesis Impairment Induces TP53-Independent Apoptosis. *Mol. Cell* **2025**, *85*, 894–912.e10. <https://doi.org/10.1016/j.molcel.2025.01.008>.
14. Ver Donck, F.; Ramaekers, K.; Thys, C.; Van Laer, C.; Peerlinck, K.; Van Geet, C.; Eto, K.; Labarque, V.; Freson, K. Ribosome Dysfunction Underlies SLFN14-Related Thrombocytopenia. *Blood* **2023**, *141*, 2261–2274. <https://doi.org/10.1182/blood.2022017712>.
15. Schwarz, D.A.; Katayama, C.D.; Hedrick, S.M. Schlafen, a New Family of Growth Regulatory Genes that Affect Thymocyte Development. *Immunity* **1998**, *9*, 657–668. [https://doi.org/10.1016/s1074-7613\(00\)80663-9](https://doi.org/10.1016/s1074-7613(00)80663-9).
16. Geserick, P.; Kaiser, F.; Klemm, U.; Kaufmann, S.H.; Zerrahn, J. Modulation of T Cell Development and Activation by Novel Members of the Schlafen (Slfn) Gene Family Harboring an RNA Helicase-like Motif. *Int. Immunol.* **2004**, *16*, 1535–1548.
17. Condamine, T.; Le Luduec, J.B.; Chiffolleau, E.; Beriou, G.; Louvet, C.; Heslan, M.; Tilly, G.; Cuturi, M.C. Characterization of Schlafen-3 Expression in Effector and Regulatory T Cells. *J. Leukoc. Biol.* **2010**, *87*, 451–456. <https://doi.org/10.1189/jlb.0609410>.
18. Berger, M.; Krebs, P.; Crozat, K.; Li, X.; Croker, B.A.; Siggs, O.M.; Popkin, D.; Du, X.; Lawson, B.R.; Theofilopoulos, A.N.; et al. An Slfn2 Mutation Causes Lymphoid and Myeloid Immunodeficiency Due to Loss of Immune Cell Quiescence. *Nat. Immunol.* **2010**, *11*, 335–343. <https://doi.org/10.1038/ni.1847>.
19. Puck, A.; Aigner, R.; Modak, M.; Cejka, P.; Blaas, D.; Stöckl, J. Expression and Regulation of Schlafen (SLFN) Family Members in Primary Human Monocytes, Monocyte-Derived Dendritic Cells and T Cells. *Results Immunol.* **2015**, *5*, 23–32. <https://doi.org/10.1016/j.rinim.2015.10.001>.
20. Vomhof-DeKrey, E.E.; Umthun, J.; Basson, M.D. Loss of Schlafen3 Influences the Expression Levels of Schlafen Family Members in Ileum, Thymus, and Spleen Tissue. *PeerJ* **2020**, *8*, e8461. <https://doi.org/10.7717/peerj.8461>.
21. Zhang, P.; Hu, X.; Li, Z.; Liu, Q.; Liu, L.; Jin, Y.; Liu, S.; Zhao, X.; Wang, J.; Hao, D.; et al. Schlafen 11 Triggers Innate Immune Responses through Its Ribonuclease Activity upon Detection of Single-Stranded DNA. *Sci. Immunol.* **2024**, *9*, eadj5465. <https://doi.org/10.1126/sciimmunol.adj5465>.
22. Li, M.; Kao, E.; Gao, X.; Sandig, H.; Limmer, K.; Pavon-Eternod, M.; Jones, T.E.; Landry, S.; Pan, T.; Weitzman, M.D.; et al. Codon-Usage-Based Inhibition of HIV Protein Synthesis by Human Schlafen 11. *Nature* **2012**, *491*, 125–128. <https://doi.org/10.1038/nature11433>.
23. Yang, J.Y.; Deng, X.Y.; Li, Y.S.; Ma, X.C.; Feng, J.X.; Yu, B.; Chen, Y.; Luo, Y.L.; Wang, X.; Chen, M.L.; et al. Structure of Schlafen13 Reveals a New Class of tRNA/rRNA-Targeting RNase Engaged in Translational Control. *Nat. Commun.* **2018**, *9*, 1165. <https://doi.org/10.1038/s41467-018-03544-x>.
24. Valdez, F.; Salvador, J.; Palermo, P.M.; Mohl, J.E.; Hanley, K.A.; Watts, D.; Llano, M. Schlafen 11 Restricts Flavivirus Replication. *J. Virol.* **2019**, *93*, e00104-19. <https://doi.org/10.1128/jvi.00104-19>.
25. Kim, E.T.; Weitzman, M.D. Schlafens Can Put Viruses to Sleep. *Viruses* **2022**, *14*, 442. <https://doi.org/10.3390/v14020442>.
26. Seong, R.K.; Seo, S.W.; Kim, J.A.; Fletcher, S.J.; Morgan, N.V.; Kumar, M.; Choi, Y.K.; Shin, O.S. Schlafen 14 (SLFN14) Is a Novel Antiviral Factor Involved in the Control of Viral Replication. *Immunobiology* **2017**, *222*, 979–988. <https://doi.org/10.1016/j.imbio.2017.07.002>.

27. Jitobaom, K.; Sirihongthong, T.; Boonarkart, C.; Phakratsakul, S.; Suptawiwat, O.; Auewarakul, P. Human Schlafen 11 Inhibits Influenza A Virus Production. *Virus Res.* **2023**, *334*, 199162. <https://doi.org/10.1016/j.virusres.2023.199162>.
28. Kobayashi-Ishihara, M.; Frazão Smutná, K.; Alonso, F.E.; Argilaguet, J.; Esteve-Codina, A.; Geiger, K.; Genescà, M.; Grau-Expósito, J.; Duran-Castells, C.; Rogenmoser, S. Schlafen 12 Restricts HIV-1 Latency Reversal by a Codon-Usage Dependent Post-Transcriptional Block in CD4⁺ T Cells. *Commun. Biol.* **2023**, *6*, 487.
29. Valenzuela, C.; Saucedo, S.; Llano, M. Schlafen14 Impairs HIV-1 Expression in a Codon Usage-Dependent Manner. *Viruses* **2024**, *16*, 502. <https://doi.org/10.3390/v16040502>.
30. Katsoulidis, E.; Mavrommatis, E.; Woodard, J.; Shields, M.A.; Sassano, A.; Carayol, N.; Sawicki, K.T.; Munshi, H.G.; Plataniias, L.C. Role of Interferon α (IFN α)-Inducible Schlafen-5 in Regulation of Anchorage-Independent Growth and Invasion of Malignant Melanoma Cells. *J. Biol. Chem.* **2010**, *285*, 40333–40341. <https://doi.org/10.1074/jbc.m110.151076>.
31. Sassano, A.; Mavrommatis, E.; Arslan, A.D.; Kroczyńska, B.; Beauchamp, E.M.; Khuon, S.; Chew, T.L.; Green, K.J.; Munshi, H.G.; Verma, A.K.; et al. Human Schlafen 5 (SLFN5) Is a Regulator of Motility and Invasiveness of Renal Cell Carcinoma Cells. *Mol. Cell. Biol.* **2015**, *35*, 2684–2698. <https://doi.org/10.1128/mcb.00019-15>.
32. Zhou, C.; Liu, C.; Liu, W.; Chen, W.; Yin, Y.; Li, C.W.; Hsu, J.L.; Sun, J.; Zhou, Q.; Li, H.; et al. SLFN11 Inhibits Hepatocellular Carcinoma Tumorigenesis and Metastasis by Targeting RPS4X via mTOR Pathway. *Theranostics* **2020**, *10*, 4627–4643. <https://doi.org/10.7150/thno.42869>.
33. Chen, J.; Liu, N.; Huang, Y.; Wang, Y.; Sun, Y.; Wu, Q.; Li, D.; Gao, S.; Wang, H.W.; Huang, N.; et al. Structure of PDE3A-SLFN12 Complex and Structure-Based Design for a Potent Apoptosis Inducer of Tumor Cells. *Nat. Commun.* **2021**, *12*, 6204. <https://doi.org/10.1038/s41467-021-26546-8>.
34. Wan, G.; Zhu, J.; Gu, X.; Yang, Y.; Liu, Y.; Wang, Z.; Zhao, Y.; Wu, H.; Huang, G.; Lu, C. Human Schlafen 5 Regulates Reversible Epithelial and Mesenchymal Transitions in Breast Cancer by Suppression of ZEB1 Transcription. *Br. J. Cancer* **2020**, *123*, 633–643. <https://doi.org/10.1038/s41416-020-0873-z>.
35. Fischietti, M.; Eckerdt, F.; Blyth, G.T.; Arslan, A.D.; Mati, W.M.; Oku, C.V.; Perez, R.E.; Lee-Chang, C.; Kosciuczuk, E.M.; Saleiro, D.; et al. Schlafen 5 as a Novel Therapeutic Target in Pancreatic Ductal Adenocarcinoma. *Oncogene* **2021**, *40*, 3273–3286. <https://doi.org/10.1038/s41388-021-01761-1>.
36. Zhou, C.; Weng, J.; Liu, C.; Liu, S.; Hu, Z.; Xie, X.; Gao, D.; Zhou, Q.; Sun, J.; Xu, R. Disruption of SLFN11 Deficiency-Induced CCL2 Signaling and Macrophage M2 Polarization Potentiates Anti-PD-1 Therapy Efficacy in Hepatocellular Carcinoma. *Gastroenterology* **2023**, *164*, 1261–1278. <https://doi.org/10.1053/j.gastro.2023.02.005>.
37. Akashi, H.; Yachida, N.; Ueda, H.; Yamaguchi, M.; Yamawaki, K.; Tamura, R.; Suda, K.; Ishiguro, T.; Adachi, S.; Nagase, Y.; et al. SLFN11 Is a BRCA-Independent Biomarker for the Response to Platinum-Based Chemotherapy in High-Grade Serous Ovarian Cancer and Clear Cell Ovarian Carcinoma. *Mol. Cancer Ther.* **2024**, *23*, 106–116. <https://doi.org/10.1158/1535-7163.mct-23-0257>.
38. Li, J.; Chen, Z.; Jin, M.; Gu, X.; Wang, Y.; Huang, G.; Zhao, W.; Lu, C. Histone H4K12 Lactylation Promotes Malignancy Progression in Triple-Negative Breast Cancer through SLFN5 Downregulation. *Cell. Signal.* **2024**, *124*, 111468. <https://doi.org/10.1016/j.cellsig.2024.111468>.
39. Wu, Y.J.; Chiao, C.C.; Chuang, P.K.; Hsieh, C.B.; Ko, C.Y.; Ko, C.C.; Chang, C.F.; Chen, T.Y.; Nguyen, N.U.N.; Hsu, C.C.; et al. Comprehensive Analysis of Bulk and Single-Cell RNA Sequencing Data Reveals Schlafen-5 (SLFN5) as a Novel Prognosis and Immunity Biomarker. *Int. J. Med. Sci.* **2024**, *21*, 2348–2364. <https://doi.org/10.7150/ijms.97975>.
40. Neumann, B.; Zhao, L.; Murphy, K.; Gonda, T.J. Subcellular Localization of the Schlafen Protein Family. *Biochem. Biophys. Res. Commun.* **2008**, *370*, 62–66. <https://doi.org/10.1016/j.bbrc.2008.03.032>.
41. Jo, U.; Pommier, Y. Structural, Molecular, and Functional Insights into Schlafen Proteins. *Exp. Mol. Med.* **2022**, *54*, 730–738. <https://doi.org/10.1038/s12276-022-00794-0>.
42. Bustos, O.; Naik, S.; Ayers, G.; Casola, C.; Perez-Lamigueiro, M.A.; Chippindale, P.T.; Pritham, E.J.; de la Casa-Esperón, E. Evolution of the Schlafen Genes, a Gene Family Associated with Embryonic Lethality, Meiotic Drive, Immune Processes and Orthopoxvirus Virulence. *Gene* **2009**, *447*, 1–11. <https://doi.org/10.1016/j.gene.2009.07.006>.
43. Metzner, F.J.; Wenzl, S.J.; Kugler, M.; Krebs, S.; Hopfner, K.P.; Lammens, K. Mechanistic Understanding of Human SLFN11. *Nat. Commun.* **2022**, *13*, 5464. <https://doi.org/10.1038/s41467-022-33123-0>.
44. Luo, M.; Jia, X.; Wang, Z.W.; Yang, J.Y.; Wang, W.; Chen, J.; Ou, J.Y.; Feng, J.X.; Yu, B.; Wang, S.; et al. Structural and Functional Characterization of Human SLFN14. *Nucleic Acids Res.* **2025**, *53*, gkaf484. <https://doi.org/10.1093/nar/gkaf484>.
45. Wan, G.; Liu, Y.; Zhu, J.; Guo, L.; Li, C.; Yang, Y.; Gu, X.; Deng, L.L.; Lu, C. SLFN5 Suppresses Cancer Cell Migration and Invasion by Inhibiting MT1-MMP Expression via AKT/GSK-3 β /Catenin Pathway. *Cell. Signal.* **2019**, *59*, 1–12. <https://doi.org/10.1016/j.cellsig.2019.03.004>.
46. Ding, J.; Wang, S.; Liu, Q.; Duan, Y.; Cheng, T.; Ye, Z.; Cui, Z.; Zhang, A.; Liu, Q.; Zhang, Z.; et al. Schlafen-5 Inhibits LINE-1 Retrotransposition. *iScience* **2023**, *26*, 107968. <https://doi.org/10.1016/j.isci.2023.107968>.

47. Vonnrhein, C.; Flensburg, C.; Keller, P.; Sharff, A.; Smart, O.; Paciorek, W.; Womack, T.; Bricogne, G. Data Processing and Analysis with the *AutoPROC* Toolbox. *Acta Crystallogr. D Biol. Crystallogr.* **2011**, *67*, 293–302. <https://doi.org/10.1107/s0907444911007773>.
48. Collaborative Computational Project, Number 4. The *CCP4* Suite: Programs for Protein Crystallography. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **1994**, *50*, 760–763. <https://doi.org/10.1107/s0907444994003112>.
49. Emsley, P.; Cowtan, K. Coot: Model-Building Tools for Molecular Graphics. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2004**, *60*, 2126–2132. <https://doi.org/10.1107/s0907444904019158>.
50. Winn, M.D.; Murshudov, G.N.; Papiz, M.Z. Macromolecular TLS Refinement in REFMAC at Moderate Resolutions. *Methods Enzymol.* **2003**, *374*, 300–321. [https://doi.org/10.1016/s0076-6879\(03\)74014-2](https://doi.org/10.1016/s0076-6879(03)74014-2).
51. Adams, P.D.; Afonine, P.V.; Bunkóczi, G.; Chen, V.B.; Davis, I.W.; Echols, N.; Headd, J.J.; Hung, L.W.; Kapral, G.J.; Grosse-Kunstleve, R.W.; et al. PHENIX: A Comprehensive Python-Based System for Macromolecular Structure Solution. *Acta Crystallogr. D Biol. Crystallogr.* **2010**, *66*, 213–221. <https://doi.org/10.1107/s0907444909052925>.
52. Joosten, R.P.; Long, F.; Murshudov, G.N.; Perrakis, A. The PDB REDO Server for Macromolecular Structure Model Optimization. *IUCrJ* **2014**, *1*, 213–220. <https://doi.org/10.1107/s2052252514009324>.
53. Chen, V.B.; Arendall, W.B., III; Headd, J.J.; Keedy, D.A.; Immormino, R.M.; Kapral, G.J.; Murray, L.W.; Richardson, J.S.; Richardson, D.C. MolProbity: All-Atom Structure Validation for Macromolecular Crystallography. *Acta Crystallogr. D Biol. Crystallogr.* **2010**, *66*, 12–21. <https://doi.org/10.1107/s0907444909042073>.
54. Cancer Genome Atlas Network. Comprehensive Molecular Characterization of Human Colon and Rectal Cancer. *Nature* **2012**, *487*, 330–337. <https://doi.org/10.1038/nature11252>.
55. Guichard, C.; Amadio, G.; Imbeaud, S.; Ladeiro, Y.; Pelletier, L.; Maad, I.B.; Calderaro, J.; Bioulac-Sage, P.; Letexier, M.; Degos, F.; et al. Integrated Analysis of Somatic Mutations and Focal Copy-Number Changes Identifies Key Genes and Pathways in Hepatocellular Carcinoma. *Nat. Genet.* **2012**, *44*, 694–698. <https://doi.org/10.1038/ng.2256>.
56. Imielinski, M.; Berger, A.H.; Hammerman, P.S.; Hernandez, B.; Pugh, T.J.; Hodis, E.; Cho, J.; Suh, J.; Capelletti, M.; Sivachenko, A.; et al. Mapping the Hallmarks of Lung Adenocarcinoma with Massively Parallel Sequencing. *Cell* **2012**, *150*, 1107–1120. <https://doi.org/10.1016/j.cell.2012.08.029>.
57. Omar, I.; Guterma-Ram, G.; Rahat, D.; Tabach, Y.; Berger, M.; Levaot, N. Schlafen2 Mutation in Mice Causes an Osteopetrotic Phenotype Due to a Decrease in the Number of Osteoclast Progenitors. *Sci. Rep.* **2018**, *8*, 13005.
58. García del Arco, A.; Erhardt, S. Post-Translational Modifications of Centromeric Chromatin. *Prog. Mol. Subcell. Biol.* **2017**, *56*, 213–231. https://doi.org/10.1007/978-3-319-58592-5_9.
59. Mavrommatis, E.; Arslan, A.D.; Sassano, A.; Hua, Y.; Kroczyńska, B.; Platanias, L.C. Expression and Regulatory Effects of Murine Schlafen (Slfn) Genes in Malignant Melanoma and Renal Cell Carcinoma. *J. Biol. Chem.* **2013**, *288*, 33006–33015. <https://doi.org/10.1074/jbc.m113.460741>.
60. Li, M.; Kao, E.; Malone, D.; Gao, X.; Wang, J.Y.J.; David, M. DNA Damage-Induced Cell Death Relies on SLFN11-Dependent Cleavage of Distinct Type II tRNAs. *Nat. Struct. Mol. Biol.* **2018**, *25*, 1047–1058. <https://doi.org/10.1038/s41594-018-0142-5>.
61. Garvie, C.W.; Wu, X.; Papanastasiou, M.; Lee, S.; Fuller, J.; Schnitzler, G.R.; Horner, S.W.; Baker, A.; Zhang, T.; Mullahoo, J.P.; et al. Structure of PDE3A-SLFN12 Complex Reveals Requirements for Activation of SLFN12 RNase. *Nat. Commun.* **2021**, *12*, 4375. <https://doi.org/10.1038/s41467-021-24495-w>.
62. Pisareva, V.P.; Muslimov, I.A.; Tcherepanov, A.; Pisarev, A.V. Characterization of Novel Ribosome-Associated Endoribonuclease SLFN14 from Rabbit Reticulocytes. *Biochemistry* **2015**, *54*, 3286–3301. <https://doi.org/10.1021/acs.biochem.5b00302>.
63. Lipfert, J.; Skinner, G.M.; Keegstra, J.M.; Hensgens, T.; Jager, T.; Dulin, D.; Kober, M.; Yu, Z.; Donkers, S.P.; Chou, F.C.; et al. Double-Stranded RNA under Force and Torque: Similarities to and Striking Differences from Double-Stranded DNA. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 15408–15413. <https://doi.org/10.1073/pnas.1407197111>.
64. Callaghan, A.J.; Aurikko, J.P.; Ilag, L.L.; Grossmann, J.G.; Chandran, V.; Kühnel, K.; Poljak, L.; Carpousis, A.J.; Robinson, C.V.; Symmons, M.F.; et al. Studies of the RNA Degradosome-Organizing Domain of the *Escherichia coli* Ribonuclease RNase E. *J. Mol. Biol.* **2004**, *340*, 965–979. <https://doi.org/10.1016/j.jmb.2004.05.046>.
65. Gan, J.; Tropea, J.E.; Austin, B.P.; Court, D.L.; Waugh, D.S.; Ji, X. Structural Insight into the Mechanism of Double-Stranded RNA Processing by Ribonuclease III. *Cell* **2006**, *124*, 355–366. <https://doi.org/10.1016/j.cell.2005.11.034>.
66. Murchison, E.P.; Hannon, G.J. miRNAs on the Move: miRNA Biogenesis and the RNAi Machinery. *Curr. Opin. Cell Biol.* **2004**, *16*, 223–229. <https://doi.org/10.1016/j.ceb.2004.04.003>.
67. Söderbom, F.; Svärd, S.G.; Kirsebom, L.A. RNase E Cleavage in the 5' Leader of a tRNA Precursor. *J. Mol. Biol.* **2005**, *352*, 22–27. <https://doi.org/10.1016/j.jmb.2005.07.003>.
68. Hirata, A.; Kitajima, T.; Hori, H. Cleavage of Intron from the Standard or Non-Standard Position of the Precursor tRNA by the Splicing Endonuclease of *Aeropyrum pernix*, a Hyper-Thermophilic Crenarchaeon, Involves a Novel RNA Recognition Site in the Crenarchaea Specific Loop. *Nucleic Acids Res.* **2011**, *39*, 9376–9389. <https://doi.org/10.1093/nar/gkr615>.

69. Jiao, Y.; Yonescu, R.; Offerhaus, G.J.A.; Klimstra, D.S.; Maitra, A.; Eshleman, J.R.; Herman, J.G.; Poh, W.; Pelosof, L.; Wolfgang, C.L.; et al. Whole-Exome Sequencing of Pancreatic Neoplasms with Acinar Differentiation. *J. Pathol.* **2014**, *232*, 428–435. <https://doi.org/10.1002/path.4310>.
70. Czuba, L.C.; Hillgren, K.M.; Swaan, P.W. Post-Translational Modifications of Transporters. *Pharmacol. Ther.* **2018**, *192*, 88–99. <https://doi.org/10.1016/j.pharmthera.2018.06.013>.
71. Thompson, J.D.; Higgins, D.G.; Gibson, T.J. CLUSTAL W: Improving the Sensitivity of Progressive Multiple Sequence Alignment through Sequence Weighting, Position-Specific Gap Penalties and Weight Matrix Choice. *Nucleic Acids Res.* **1994**, *22*, 4673–4680.