

**Fig. S1. Nsp13-K288R mutant protein has no detectable ATPase or helicase activity.** (A) Purified recombinant Nsp13-K288R protein (5,000 ng) after final dialysis (see *Experimental Procedures*), resolved by SDS-PAGE and detected by Coomassie-staining. (B) Representative native polyacrylamide gel analysis of products from Nsp13-K288R reaction mixtures with a 19-bp RNA partial substrate conducted in the presence of 2 mM ATP and 1 mM Mg<sup>2+</sup> for 15 min at 37 °C. Filled triangle represents heat-denatured RNA substrate control. Products from reaction mixture containing Nsp13-WT is shown. (C) Representative thin layer chromatography of a kinetic analysis of Nsp13-K288R (0.05 and 0.5 nM) ATPase activity in the presence of a 60 nt ssRNA effector. The two chromatography sheets containing reactions from one experiment were spliced together and no lanes were omitted.



**Fig. S2.** dT<sub>200</sub> titration to determine polynucleotide concentration suitable for helicase trap in singleturnover kinetic experiments. The indicated concentrations of dT<sub>200</sub> were pre-incubated with Nsp13 (0.1625 nM) for 5 min at RT. Subsequently, ATP and radiolabeled 30-bp RNA partial duplex substrate (0.25 nM) was added to the reaction mixture and allowed to incubate at 37 °C for 60 min. Reactions were quenched and analyzed by native polyacrylamide gel electrophoresis and ImageQuant as described under *Experimental Procedures*.



**Fig. S3. Strand-specific inhibition of Nsp13 RNA helicase activity by sugar-phosphate backbone discontinuity.** (A) Depiction of the partial duplex RNA substrates with a polyglycol linker (PGL) in the top or bottom strand. (B) Quantitative assessment of Nsp13 helicase activity from a 15-min incubation at 37 °C as a function of protein concentration in the presence of 2 mM ATP and 5 mM Mg<sup>2+</sup> on the PGL backbone-modified substrates (0.25 nM) shown in (A). Data represent the average of at least three independent experiments with SD indicated by error bars.



**Fig. S4. Chemical identity of nucleic acid strands dictates Nsp13 helicase unwinding efficiency.** (A) Depiction of the partial duplex substrates with DNA or RNA in the top or bottom strand. (B) Quantitative assessment of Nsp13 helicase activity from a 15-min incubation at 37 °C as a function of protein concentration in the presence of 2 mM ATP and 5 mM Mg<sup>2+</sup> on the nucleic acid substrates (0.25 nM) shown in (A). Data represent the average of at least three independent experiments with SD indicated by error bars.



**Fig. S5. Nsp13 disrupts high affinity interaction of streptavidin bound to 3' biotinylated ssDNA or ssRNA.** Quantitative assessment of Nsp13-catalyzed streptavidin displacement kinetic reactions incubated at various time points (1 - 16 min) at 37 °C in the presence of 1 mM MgCl<sub>2</sub> and 2 mM ATP using 39-mer single-stranded RNA and DNA substrates (0.25 nM) with a biotin moiety at the 3' end. Biotinylated oligonucleotide was preincubated with 0.75 nM streptavidin monomer for 10 min and reactions were initiated by the addition of 1 μM biotin immediately followed by Nsp13 (1.6 nM). Data represent the average of at least three independent experiments with SD indicated by error bars.



**Fig S6. Effect of Divalent Salts on Nsp13 ATPase Activity.** (A) 0.4 nM Nsp13 was incubated with 1 mM ATP in Nsp13 reaction salts with either MgCl<sub>2</sub>, MnCl<sub>2</sub>, CaCl<sub>2</sub> or CoCl<sub>2</sub> and 1.6 µM 60 nt RNA effector. Reactions were quenched at 0 and 10 minutes. (B) 0.4 nM Nsp13 was incubated with 1 mM ATP in Nsp13 reaction salts with 1.6 µM 60 nt RNA effector and mixtures of divalent salts either in a 1:1 or a 1:4 ratio resulting in a final concentration of either 2 or 5 mM divalent salt in each reaction. (C) 4 nM Nsp13 was incubated with 1 mM ATP in Nsp13 reaction salts with increasing amounts of MgCl<sub>2</sub> in the absence of effector RNA. Reactions from these ATPase assays were quenched at 0 and either 10 (A,B) or 30 (C) minutes and run on PEI-cellulose TLC sheets for 20 minutes in 1M Formic Acid and 0.8 M LiCl. % ATP hydrolyzed was calculated by dividing the released phosphate by the total of ATP and phosphate in each lane. Background from the t=0 was subtracted out from each measurement. Data represent the average of at least three independent experiments with SD indicated by error bars.



**Fig S7. Effect of MgCl<sub>2</sub> on Nsp13's binding to the DNA substrate.** Nsp13 (2.5 nM) was incubated with the 16-bp DNA forked duplex substrate (0.25 nM) for 15 min at RT. (A) Binding mixtures were assessed by EMSA, as described in *Experimental Procedures*. (B) Quantitative analysis is shown. Data represent average of at least three independent experiments with SD indicated by error bars.

Start	6xHis	TEV Protease Site 🔸 🛛 🛛	Isp13 Begin
ATGTCGTACTACCATCA	CCATCACCATCACGATTACGATATCCCAACGACCGA	AAACCTGTATTTTCAGGGCGCCATGGGATCC	GCTGTCGGCGCTTGCGTGCTCTGCAACAGCCAGACCTCTCTGCGTTGCGGTGCT
мзүүнн	нннн рур гртте	. N L Y F Q G A M G S	A V G A C V L C N S Q T S L R C G A
TGTATCCGCCGTCCTTT	CCTCTGTTGCAAGTGCTGTTACGACCACGTGATCTC	CACTAGCCACAAACTGGTGCTGAGCGTGAAT	CCTTACGTGTGCAATGCCCCCGGTTGCGATGTCACCGACGTGACTCAGCTGTAC
CIRRFF	LCCKCCIDHVIS	, I S H K L V L S V N	PIVCNAPGCDVIDVIQLI
L G G M S Y	V C K S H K D D T S F D	CCTCTGCGCTAACGGCCAAGTGTTCGGCCTCT	TACAAGAACACTTGCGTGGGTAGCGACAACGTGACCGACTTTAACGCCATCGCC
100101	1085888881517	TOMNOVIOT	1 K W 1 C V C D D W V I D I W X I X
100000000000000000000000000000000000000			
T C D W T N	A G D Y T L A N T C T F	ACGUUTCAAAUTGITTGUUGUUGAAAUTUTGA	A A BECTACOGA A GAGACOTTCA A GOTOTCCTACOGTA COGTACOGTO GAGA
CTCCTCACCCACCCTCA	ACTCCATCTCA CCTCCCA ACTCCCTA A ACCCCCTCC	CONTRATA CORTA CALCARCE	TACCECCTCACCAACAACAECAACCTCCAAATCECCCAATATACCTTCCACAAC
V L S D R E	L H L S W E V G K P R P	P L N R N Y V F T G	Y R V T K N S K V Q I G E Y T F E K
GGCGACTATGGCGATGC	TGTCGTGTACCGTGGCACTACTACCTACAAGCTCAA	CGTCGGCGACTATTTCGTGCTCACTAGCCAC	
G D Y G D A	V V Y R G T T T Y K L N	V G D Y F V L T S H	T V M P L S A P T L V P Q E H Y V R
ATCACCGGTCTGTACCC	CACTCTGAACATCTCCGACGAGTTCAGCTCCAACGT	GGCCAACTACCAAAAGGTGGGTATGCAGAAG	TACTCCACTCTGCAAGGTCCTCCCGGTACCGGTAAGTCCCACTTTGCTATCGGT
ITGLYP	TLNISDEFSSNV	'ANYQKVGMQK	YSTLQGPPGTGKSHFAIG
CTGGCTCTGTATTACCC	CTCCGCCCGTATCGTGTACACTGCTTGCTCCCACGC	TGCTGTCGACGCTCTCTGCGAGAAGGCTCTG	AAGTACCTCCCCATCGACAAGTGTAGCCGTATTATCCCCGCTCGTGCTCGCGTC
LALYYP	SARIVYTACSHA	AVDALCEKAL	KYLPIDKCSRIIPARARV
GAGTGCTTCGACAAGTT	CAAGGTGAACTCCACTCTGGAGCAGTACGTCTTCTG	TACCGTGAATGCTCTGCCCGAAACCACCGCCC	GATATCGTCGTCTTCGACGAGATCAGCATGGCTACCAACTACGATCTGTCCGTC
ECFDKF	K V N S T L E Q Y V F C	TVNALPETTA:	DIVVFDEISMATNYDLSV
GTCAATGCTCGTCTGCG	CGCTAAGCATTACGTGTACATCGGCGATCCCGCTCA	ACTGCCCGCTCCTCGTACTCTGCTGACCAAGO	GCACTCTGGAGCCCGAGTACTTCAACAGCGTCTGCCGTCTGATGAAGACCATC
VNARLR	AKHYVYIGDPAQ	2 L P A P R T L L T K	GTLEPEYFNSVCRLMKTI
GGCCCCGACATGTTTCT	GGGTACTTGTCGCCGTTGTCCCGCTGAGATTGTCGA	CACTGTGTCCGCTCTGGTCTACGACAACAAG	CTCAAGGCTCACAAGGACAAGAGCGCTCAGTGCTTCAAAATGTTCTACAAGGGT
GFDHFL	GICKKCFALIVD	JIVSALVIDNK	L K M N D K S M Q C F K M F I K G
V T T H D V	S S A T N R P O T G V V	GCGTGAGTITCTGACCCGCATCCCGCTIGGC	R K A V F T S P Y N S O N A V A S K
<b>3 TTOTOOTOTOTOT</b>		~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	
I L G L P T	O T V D S S O G S E Y D	) Y V I F T O T T E T	A H S C N V N R F N V A I T R A K V
			Nsn13 End DYKDDDDK Tag Stop
GGCATTCTGTGCATCATGTCCGATCGCGACCTCTATGACAAGCTCCAATTCACCTCTCGGAGATCCCCCGTCGCAACGTGCTTACTTGCAAGGACGACGACGACGACGATAGTAA			
GILCIM	S D R D L Y D K L Q F T	SLEIPRRNVA	TLQDYKDDDDK*

**Fig. S8. Nsp13 coding sequence optimized for insect cell expression.** Sequence of the insert coding for Nsp13 which was codon-optimized for insect cell expression. The insert was inserted into the pFastBac HT B vector using BamHI and XhoI restriction sites as noted in *Methods*. Lines above the DNA sequence indicate regions of interest and amino acids are below the sequence.



**Fig. S9. Recombinant SARS-CoV-2 Nsp13 purification.** (A) Scheme for purification of recombinant SARS-CoV-2 Nsp13 protein. ① Hi5 insect cells previously infected with baculovirus containing recombinant Nsp13 were lysed and cell debris pelleted after a centrifugation step. ② The supernatant was filtered through a 0.45 micron PVDF filter. ③ The filtered lysate was injected into a 1 ml HisTrap HP column followed by imidazole washes and elution with a high concentration of imidazole. ④ The eluted protein was dialyzed to remove imidazole and high salt using a centrifugal MWCO filter device. ⑤ The 6xHis tag was cleaved off of the Nsp13 using TEV protease overnight at 4°C. ⑥ The cleaved protein was dialyzed to remove β-mercaptoethanol. ⑦ Nsp13 was bound to anti-FLAG beads followed by washes and elution with excess 3XFLAG peptide. ⑧ The elution was poured into a column and eluted Nsp13 was collected. ⑨ The eluted Nsp13 was dialyzed to exchange into the storage buffer and remove any 3XFLAG peptide. (B) Coomassie-stained SDS polyacrylamide gel showing Nsp13 fraction from affinity purification. Lysate (Lys), Supernatant (Sup), Filtered (Fil), 20 mM Imidazole wash (20-I), 40 mM imidazole wash (40-I), Elution (Elu), Dialysis flow-through (DFT), TEV protease cleaved protein (TEV), molecular weight markers (MW), Unbound (UB), washes (Wash). The final dialyzed recombinant Nsp13 protein is shown in **Fig. 1.**