Supporting Information

for

Enhanced Binding Affinity for an i-Motif DNA Substrate Exhibited by a Protein Containing Nucleobase Amino Acids

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Figure S1. RRM1 of human hnRNP LL. (A) 3D structure (C-score -0.78, TM score 0.61±0.14, RMSD 5.9 ± 3.7 Å) generated by I-TASSER software using the structure of *Mus musculus* RRM domain of BAB28521 protein (pdb 1WEX) as a template. Residues most probably involved in binding to the *BCL2* i-motif DNA are shown in violet, residues mutated in the present study are ilustrated in the structure; (B) Prediction of amino acid residues of RRM1 most probably involved in binding to *BCL2* i-motif DNA, as determined by I-TASSER (dark blue square), DP-bind (red square) and NCBI-CDD (green square). Residues conserved between human hnRNP LL and hnRNP L proteins are marked by yellow squares, mutations shown experimentally to reduce RNA binding of hnRNP L are marked by light blue squares.

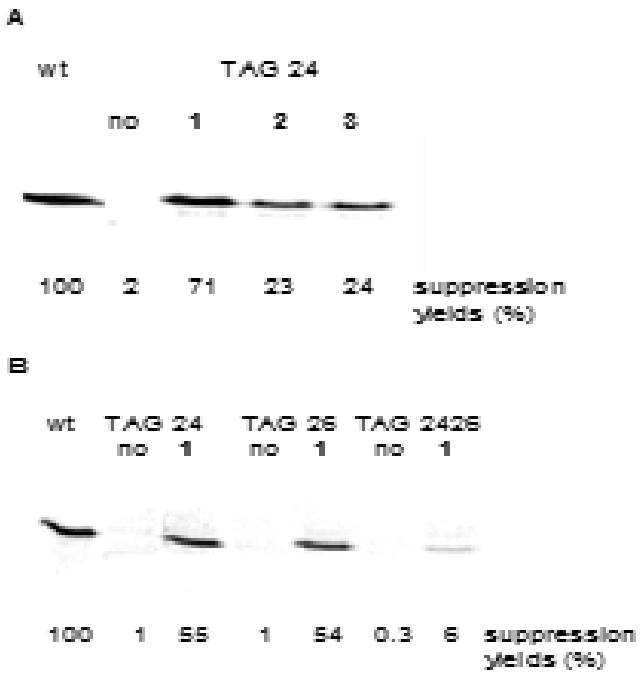


Figure S2. SDS-polyacrylamide gel showing (A) incorporation of nucleobase amino acids **1**, **2** and **3** into position 24 of RRM1, (B) incorporation of amino acid **1** into position 24, position 26 or both positions of RRM1. Suppression yields are shown. Absence of activated suppressor tRNA is indicated by "no".

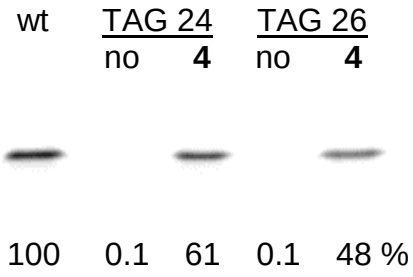


Figure S3. SDS-PAGE analysis of the incorporation of nucleobase amino acid **4** into positions 24 and 26 of RRM1. The ^{35}S-methionine labeled protein product was quantified by the use of a phosphorimager.

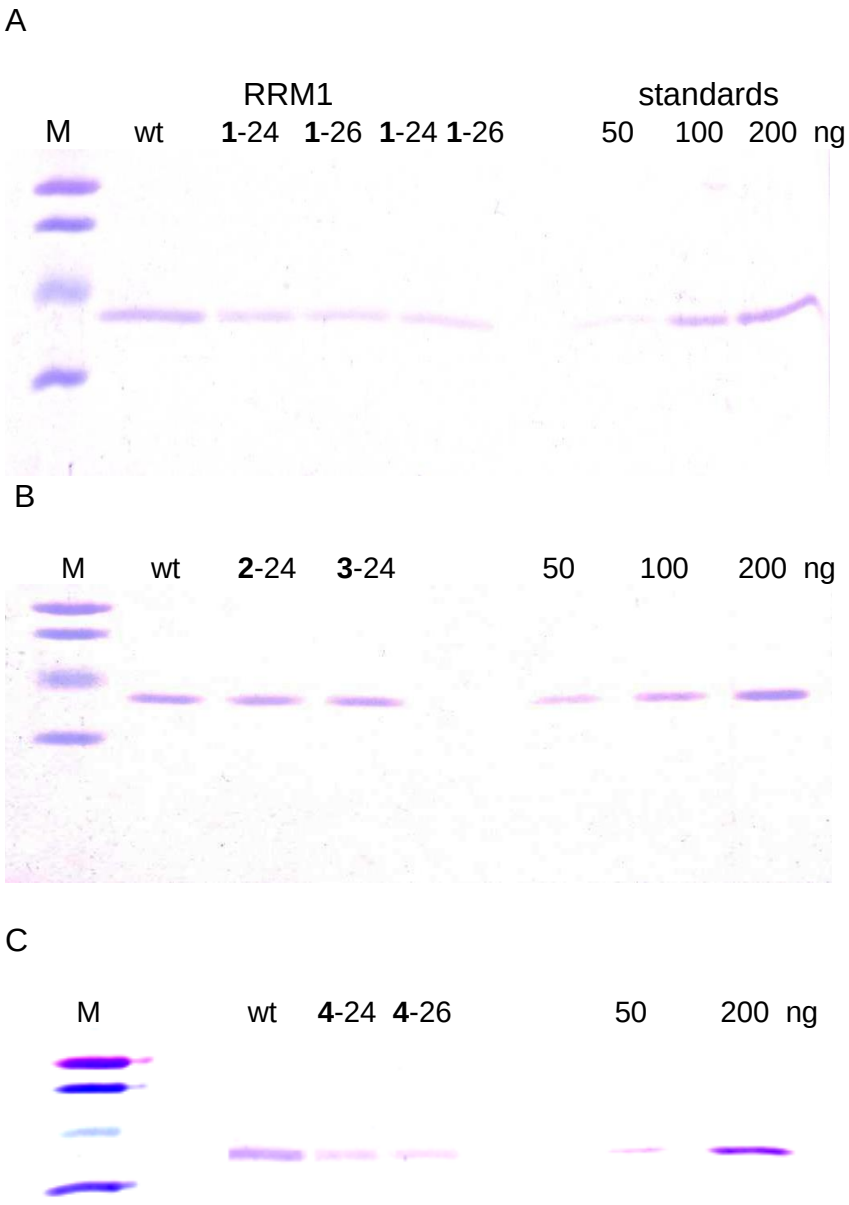


Figure S4. Characterization of wild-type (wt) and modified RRM1 samples after concentration and analysis by denaturing SDS-polyacrylamide gel electrophoresis and Coomassie R-250 staining. M - molecular weight standards: 10, 15, 20 and 25 kDa.

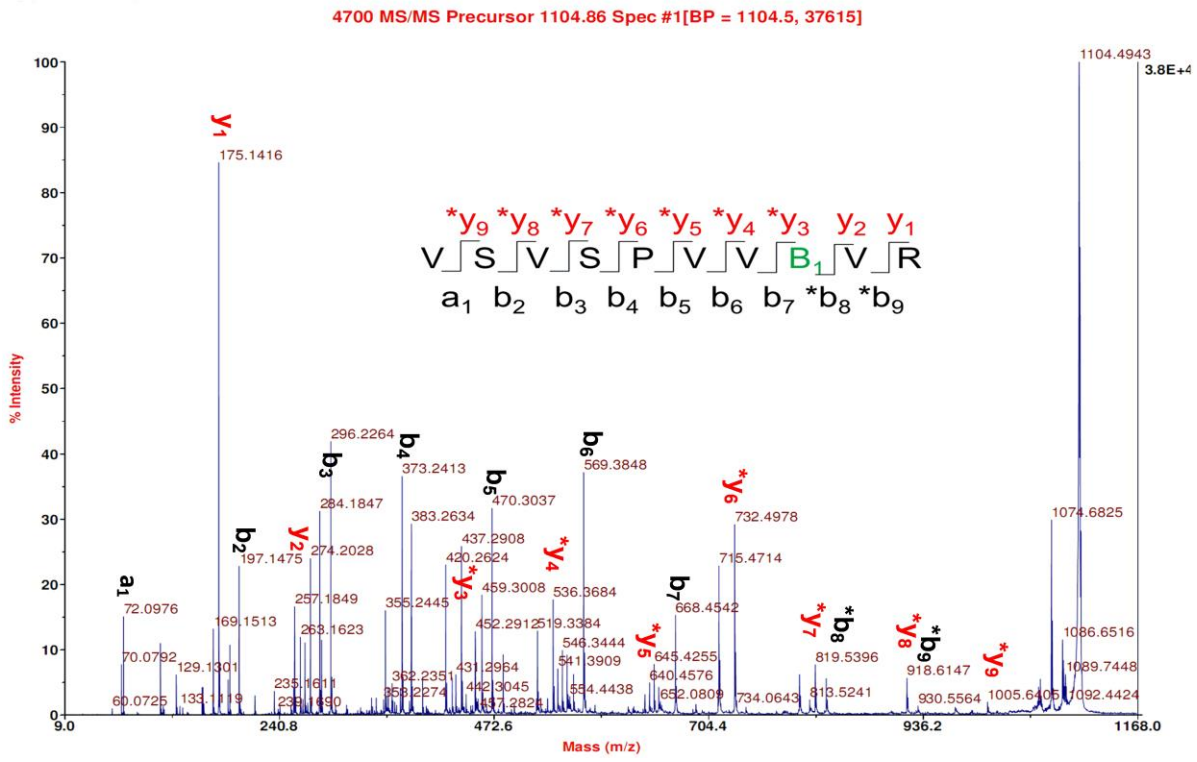


Figure S5. MS/MS analysis of RRM1 having nucleobase amino acid 1 at position 24, obtained by the use of an AB SCIEX TOF/TOF 4800 PLUS SYSTEM.

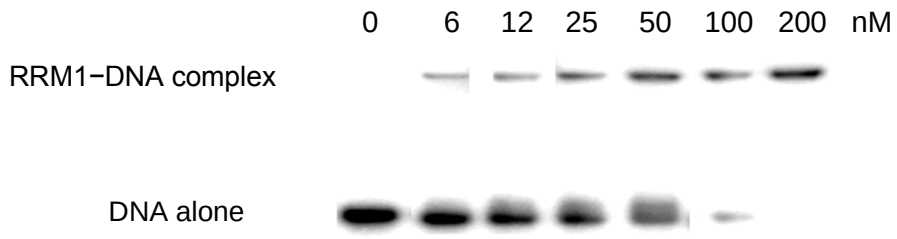


Figure S6. EMSA analysis of the binding of the ^{32}P-radiolabeled *BCL2* i-motif DNA (10 nM) to wild-type RRM1 (*in vivo* sample). Polyacrylamide gel electrophoresis of two-fold dilutions of the RRM1 sample with *BCl2* i-motif DNA.

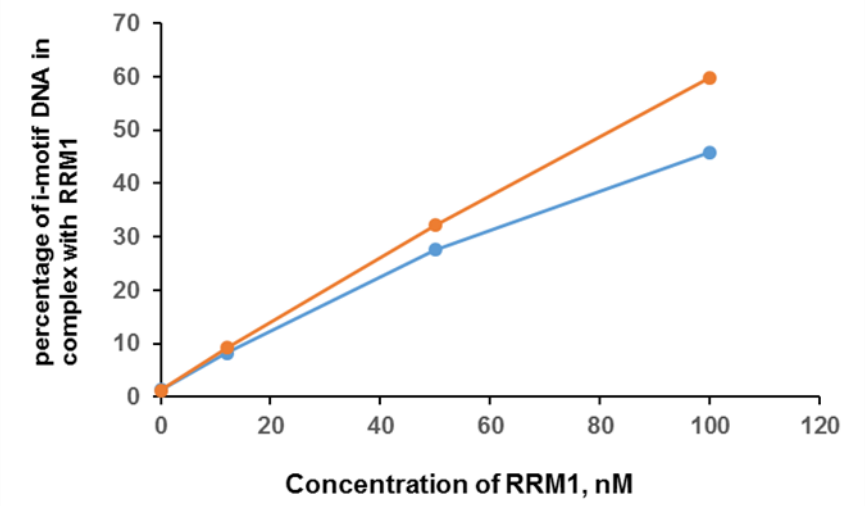


Figure S7. Comparison of *BCL2* i-motif DNA binding ability of wild-type RRM1 samples prepared *in vitro* (blue) and *in vivo* (orange).

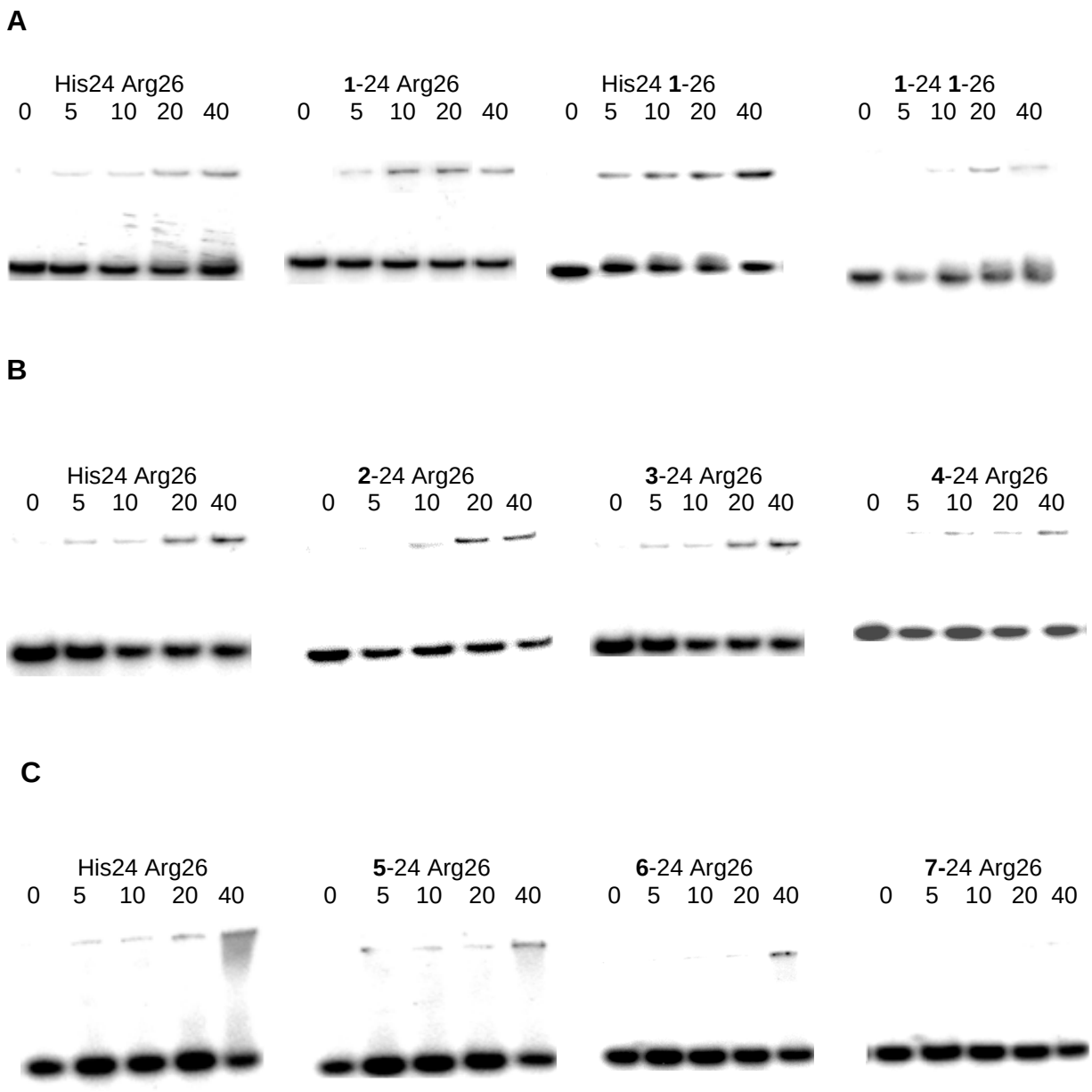


Figure S8. EMSA study of the *BCL2* i-motif DNA (10 nM) binding properties of different RRM1 constructs. Examples of polyacrylamide gel electrophoretic analysis of samples of RRM1s (5 − 40 nM concentrations) with (A) nucleobase amino acid **1** in different positions, (B) nucleobase amino acids **2**, **3** and **4** at position 24 in complex with the *BCL2* i-motif DNA and (C) amino acids **5**, **6** and **7** at position 24.

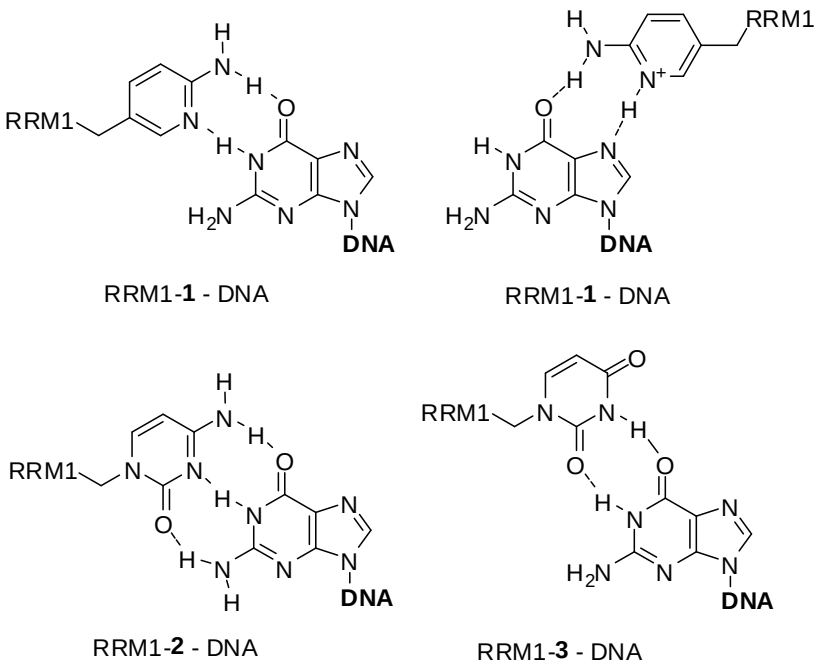


Figure S9. Possible modes of binding of RRM1s containing nucleobase amino acids **1**, **2** and **3** to G14 in the i-motif DNA.

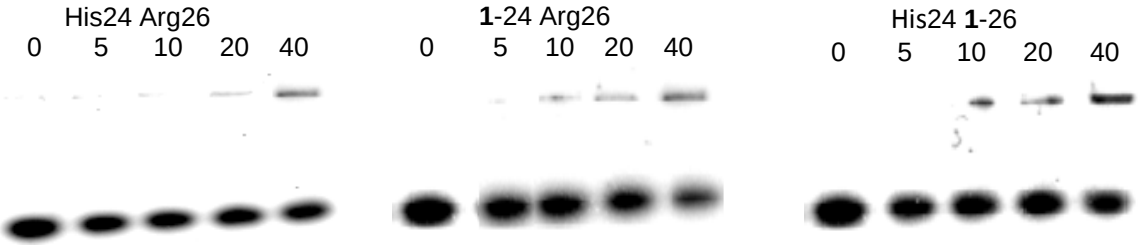
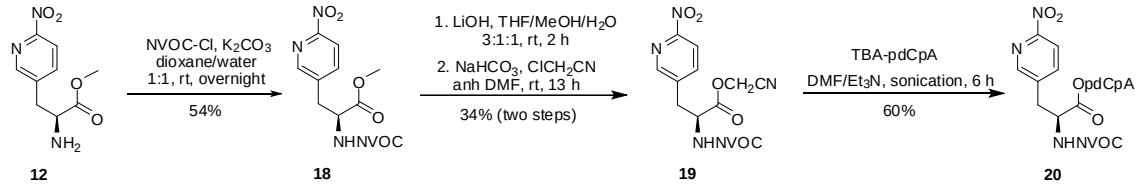


Figure S10. EMSA study of the modified *BCL2* i-motif DNA (A14; 10 nM) binding properties with modified RRM1s containing nucleobase amino acid **1** in RRM1 positions 24 or 26. Examples of polyacrylamide gel analyses of the three RRM1s (5 − 40 nM concentrations) are presented.

Scheme S1. Synthesis of the NVOC-Protected pdCpA Ester of Nucleobase Amino Acid 4



Chemical Syntheses

General Experimental Procedures. All experiments requiring anhydrous conditions were conducted in flame-dried glassware fitted with rubber septa under a positive pressure of dry nitrogen or argon. Reactions were performed at room temperature unless otherwise indicated. Analytical thin layer chromatography (TLC) was performed using glass plates precoated with silica gel (0.25 mm, 60 Å pore size, 230-400 mesh, Silicycle) impregnated with a fluorescent indicator (254 nm). TLC plates were visualized by exposure to ultraviolet light (UV). Flash column chromatography was performed employing silica gel (60 Å pore size, 40-63 μm, standard grade, Silicycle). An acetone cooling bath was cooled to the appropriate temperature by the addition of small portions of dry ice.

Chemicals were purchased from Aldrich Chemical Co., Sigma Chemical Co. or Combi Blocks. THF was distilled under argon from sodium-benzophenone ketyl and CH_2Cl_2 was distilled under argon from calcium hydride.

^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian INOVA 400 (400 MHz) spectrometer at 25 °C. Proton chemical shifts are expressed in parts per million (ppm, δ scale) and are referenced to residual protium in the NMR solvent ($CDCl_3$: ^{1}H, δ 7.26 ppm, ^{13}C, δ 77.16 ppm; CD_3OD: ^{1}H, δ 3.31 ppm, ^{13}C, δ 49.00 ppm). Splitting patterns are designated as follows: s, singlet; br s, broad singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. High resolution mass spectra were obtained at the Arizona State University CLAS High Resolution Mass Spectrometry Facility or the Michigan State University Mass Spectrometry Facility. HPLC purification was performed with a Waters 600 pump coupled with a Varian ProStar 340 detector and a Grace Econosil C_{18} column (250 × 10 mm, 5 μm). The tetranbutylammonium (TBA) salt of pdCpA was prepared using Dowex 50W×8, 200-400 mesh activated in its TBA form.

5-Methyl-2-nitropyridine (9).[1] A sample of 6.49 g (60.0 mmol) of 2-amino-2-methylpyridine (**8**) was dissolved in 150 mL of conc H_2SO_4 and cooled to 0 °C. To the mixture was added dropwise 75 mL of 30% H_2O_2. The reaction mixture was allowed to warm to room temperature and was then stirred overnight. The reaction mixture was neutralized with a saturated aqueous solution of Na_2CO_3 and extracted with three 300-mL portions of CH_2Cl_2. The combined organic layer was dried over Na_2SO_4. After concentration under diminished pressure **9** was obtained as a light yellow solid: yield 5.00 g (60%); silica gel TLC R_f 0.40 (10:3:1 hexanes–CH_2Cl_2–ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 2.39 (s, 3H), 7.75 (ddd, 1H, J = 8.3, 2.2 and 0.7 Hz), 8.04 (d, 1H, J = 8.3 Hz) and 8.31 (d, 1H, J = 2.2 Hz); ^{13}C NMR ($CDCl_3$) δ 18.3, 117.5, 139.9, 140.5, 148.9 and 154.8. This material was used directly in the next step.

Warning: *Mixtures of concentrated H_2SO_4 and H_2O_2 (also known as piranha solutions) are highly reactive and must be handled with great caution, and with appropriate protective clothing/equipment. Admixture of H_2O_2 to conc H_2SO_4 is exothermic, must be done slowly and requires cooling. The reverse order of addition must be avoided, and can potentially result in explosions, as can contact of the mixture with organic solvents. The ratio of H_2SO_4 to H_2O_2 is also important.*[1b,c]

5-(Bromomethyl)-2-nitropyridine (10).[2] To a stirred solution containing 250 mg (1.81 mmol) of 5-methyl-2-nitropyridine (**9**) in CCl_4 was added 37.0 mg (0.20 mmol) of 2, 2'-azobisisobutyronitrile and 320 mg (1.80 mmol) of *N*-bromosuccinimide. The reaction mixture

was heated at 80 °C for 4 h under argon. The cooled reaction mixture was concentrated under diminished pressure and the residue was purified by chromatography on a silica gel column (10 × 4 cm). Elution with 4:1 hexanes–ethyl acetate gave the desired product **10** as a colorless oil: yield 210 mg (54%); silica gel TLC R_f 0.35 (4:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 4.55 (s, 2H), 8.08 (d, 1H, J = 8.0 Hz), 8.24 (d, 1H, J = 8.5 Hz) and 8.64 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 27.2, 118.3, 140.1, 140.6, 149.0 and 156.2; mass spectrum (APCI), *m*/*z* 216.9623 $(M+H)^+$ ($C_6H_6BrN_2O_2$ requires *m*/*z* 216.9613).

(2*R*,5*S*)-3,6-Dimethoxy-2-isopropyl-5-((6-nitropyridin-3-yl)methyl)-2,5-dihydropyrazine (11). To a stirred solution containing 1.00 g (5.43 mmol) of Schöllkopf's reagent in 30 mL of anhydrous THF at –78 °C was added dropwise 2.38 mL (5.94 mmol) of 2.5 M BuLi in THF. The reaction mixture was stirred at –78 °C for 2 h under argon and then a solution containing 1.07 g (4.95 mmol) of **10** in 10 mL of anhydrous THF was added. The reaction mixture was allowed to warm to room temperature and stirred for an additional 16 h under argon, then diluted with satd aq K_2CO_3 and extracted with three 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 4 cm). Elution with 5:1 hexanes–ethyl acetate gave **11** as a light yellow oil: yield 500 mg (32%); silica gel TLC R_f 0.30 (4:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.59 (d, 3H, J = 6.8 Hz), 0.91 (d, 3H, J = 6.8 Hz), 2.10-2.14 (m, 1H), 3.18-3.26 (m, 2H), 3.56-3.57 (m, 1H), 3.62 (s, 3H), 3.68 (s, 3H), 4.26-4.30 (m, 1H), 7.78 (dd, 1H, J = 8.4 and 2.4 Hz), 8.11 (d, 1H, J = 8.4 Hz) and 8.40 (d, 1H, J = 2.0 Hz); ^{13}C NMR ($CDCl_3$) δ 16.7, 18.9, 31.9, 36.8, 52.57, 52.63, 55.6, 60.9, 117.3, 140.5, 140.8, 150.2, 155.5, 161.5 and 164.8; mass spectrum (APCI), *m*/*z* 321.1563 $(M+H)^+$ ($C_{15}H_{21}N_4O_4$ requires *m*/*z* 321.1563).

Methyl (*S*)-2-Amino-3-(6-nitropyridin-3-yl)propionate (12). To a stirred solution containing 66.0 mg (0.21 mmol) of **11** in 4 mL of THF at 0° C was added 3 mL of 2 N aq HCl. The reaction mixture was stirred at room temperature for 1.5 h. The reaction mixture was then slowly poured into 50 mL of satd aq $NaHCO_3$ and then extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 3:1 ethyl acetate–methanol gave **12** as a yellow oil: yield 39.0 mg (84%); silica gel TLC R_f 0.60 (3:1 ethyl acetate–methanol); ^{1}H NMR ($CDCl_3$) δ 3.02-3.21 (m, 2H), 3.68 (s, 3H), 3.80 (t, 1H, J = 6.8 Hz), 4.80 (br s, 2H), 8.04 (dd, 1H, J = 8.4 and 2.0 Hz), 8.23 (d, 1H, J = 8.4 Hz) and 8.45 (d, 1H, J = 2.4 Hz); ^{13}C NMR ($CDCl_3$) δ 38.2, 52.7, 56.2, 118.9, 142.1, 142.3, 142.4, 150.6 and 175.7; mass spectrum (APCI), *m*/*z* 226.0833 $(M+H)^+$ ($C_9H_{12}N_3O_4$ requires *m*/*z* 226.0828).

Methyl 3-(6-Aminopyridin-3-yl)-(*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-propionate (13). To a solution of 39.0 mg (0.17 mmol) of **12** in 5 mL of MeOH was added catalytic amount of 10% Pd/C and the reaction was placed under 1 atm of H_2 (g) overnight. The catalyst was removed by filtration through a Celite pad and the filtrate was concentrated under diminished pressure. The residue was dissolved in 1 mL of 1:1 dioxane–water and 62.0 mg (0.45 mmol) of K_2CO_3 was added followed by 55.0 mg (0.20 mmol) of NVOC-Cl. The reaction mixture was stirred at room temperature overnight, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:3 methanol–ethyl acetate gave **13** as a yellow solid:

yield 18.1 mg (23% for two steps); silica gel TLC R_f 0.45 (1:1 methanol–ethyl acetate); ^{1}H NMR (CD_3OD) δ 2.78-3.05 (m, 2H), 3.71 (s, 3H), 3.89 (s, 3H), 3.91 (s, 3H), 4.36-4.39 (m, 1H), 5.36-5.47 (m, 2H), 6.54 (d, 1H, J = 8.4 Hz), 7.09 (s, 1H), 7.37 (d, 1H, J = 8.4 Hz) and 7.72-7.74 (m, 2H); ^{13}C NMR (CD_3OD) δ 34.9, 37.1, 52.8, 56.8, 57.0, 64.6, 109.3, 110.4, 110.7, 122.6, 129.7, 140.4, 140.7, 147.8, 149.5, 155.4, 158.1, 159.7 and 173.8; mass spectrum (APCI), *m/z* 435.1512 $(M + H)^+$ ($C_{19}H_{23}N_4O_8$ requires *m/z* 435.1516).

Cyanomethyl 3-(6-Aminopyridin-3-yl)-(*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)propionate (14). To a stirred solution containing 21.8 mg (0.05 mmol) of **13** in 1 mL of 1:3:1 water–THF–methanol was added 150 µL (3.59 mg, 0.15 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 2 h, and then concentrated under diminished pressure. The residue was dissolved in 1 mL of anhydrous DMF and 25.0 mg (0.30 mmol) of $NaHCO_3$ was added followed by 10.0 µL (11.3 mg, 0.15 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C overnight under argon, then diluted with 20 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:3 methanol–ethyl acetate gave the desired product **14** as a light yellow solid: yield 7.37 mg (32% for two steps); silica gel TLC R_f 0.51 (1:3 methanol–ethyl acetate). ^{1}H NMR (CD_3OD) δ 2.83-3.06 (m, 2H), 3.90 (s, 3H), 3.92 (s, 3H), 4.46-4.50 (m, 1H), 4.92 (s, 2H), 5.36-5.48 (m, 2H), 6.57 (d, 1H, J = 8.4 Hz), 7.08 (s, 1H), 7.43 (dd, 1H, J = 8.0 and 2.4 Hz), 7.72 (s, 1H), 7.77 (s, 1H) and 7.98 (s, 1H); mass spectrum (APCI), *m/z* 460.1472 $(M + H)^+$ ($C_{19}H_{23}N_4O_8$ requires *m/z* 460.1468).

3-(6-Aminopyridin-3-yl)-(*S*)-2-((4,5-dimethoxy-2-nitrobenzyloxy)carbonylamino)-propionic Acid pdCpA Ester (15). To a stirred solution containing 5.20 mg (4.00 µmol) of pdCpA tetrabutylammonium salt in 100 µL of 9:1 anhydrous DMF–triethylamine was added 9.64 mg (21.0 µmol) of **14**. The reaction mixture was sonicated for 8 h. The reaction mixture was purified by HPLC on a C_{18} reversed phase column (250 × 10 mm) using a linear gradient of 99:1 → 1:99 50 mM aq ammonium acetate, pH 4.5–acetonitrile. The retention time of the desired product was 20.2 min. The fractions containing the product were lyophilized to afford **15** as a colorless solid: yield 2.21 mg (54%); mass spectrum (ESI), *m/z* 1037.2203 $(M-H)^-$ ($C_{37}H_{43}N_{12}O_{20}P_2$ requires *m/z* 1037.2192).

Methyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(6-nitropyridin-3-yl)propionate (18). To a stirred solution containing 30.0 mg (0.13 mmol) of **12** in 1 mL of 1:1 dioxane–water was added 55.2 mg (0.39 mmol) of K_2CO_3 followed by 41.3 mg (0.20 mmol) of NVOC-Cl. The reaction mixture was stirred at room temperature for 12 h under argon, then diluted with 50 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with ethyl acetate gave **18** as a yellow solid: yield 33.4 mg (54% for two steps); silica gel TLC R_f 0.53 (1:10 methanol–ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 3.21-3.43 (m, 2H), 3.78 (s, 3H), 3.96 (s, 3H), 3.98 (s, 3H), 4.69-4.73 (m, 1H), 5.48 (s, 2H), 6.95 (s, 1H), 7.70 (s, 1H), 7.85 (d, 1H, J = 8.4 Hz), 8.20-8.22 (m, 1H) and 8.40 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 34.5, 49.5, 54.0, 56.7, 56.9, 63.8, 110.2, 113.6, 117.3, 126.6, 136.9, 140.8, 148.6, 149.0, 153.7, 154.1, 154.3, 156.5 and 169.3; mass spectrum (APCI), *m/z* 465.1261 $(M + H)^+$ ($C_{19}H_{21}N_4O_{10}$ requires *m/z* 465.1258).

Cyanomethyl (*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(6-nitropyridin-3-yl)propionate (19). To a stirred solution containing 46.4 mg (0.10 mmol) of **18** in 1 mL of 1:3:1 water–THF–methanol was added 300 µL (7.18 mg, 0.30 mmol) of 1 N LiOH. The reaction mixture was stirred at room temperature for 2 h, and then concentrated under diminished pressure. The residue was dissolved in 1 mL of anhydrous DMF under argon and 50 mg (0.60 mmol) of $NaHCO_3$ was added followed by 20.0 µL (25.0 mg, 0.30 mmol) of chloroacetonitrile. The reaction mixture was stirred at 23 °C overnight under argon, then diluted with 20 mL of brine and extracted with two 50-mL portions of EtOAc. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 1 cm). Elution with 1:10 methanol–ethyl acetate gave **19** as a light yellow solid: yield 29.0 mg (34% for two steps); silica gel TLC R_f 0.50 (1:10 methanol–ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 3.27-3.43 (m, 2H), 3.96 (s, 3H), 3.98 (s, 3H), 4.75-4.89 (m, 3H), 5.46-5.50 (m, 3H), 6.93 (s, 1H), 7.69 (s, 1H), 7.90 (d, 1H, J = 8.4 Hz), 8.23 (d, 1H, J = 8.4 Hz) and 8.45 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 34.9, 49.6, 54.2, 56.6, 56.7, 64.8, 108.5, 111.2, 113.5, 118.3, 126.5, 137.9, 140.8, 148.7, 149.5, 153.6, 154.4, 155.3, 156.2 and 169.5; mass spectrum (APCI), *m/z* 490.1206 $(M + H)^+$ ($C_{20}H_{20}N_5O_{10}$ requires *m/z* 490.1210).

(*S*)-2-((4,5-Dimethoxy-2-nitrobenzyloxy)carbonylamino)-3-(6-nitropyridin-3-yl)propionic Acid pdCpA Ester (20). To a stirred solution containing 5.2 mg (4.0 µmol) of pdCpA tetrabutylammonium salt in 100 µL of 9:1 anh DMF–anhydrous triethylamine was added 10.3 mg (21 µmol) of **19**. The reaction mixture was sonicated for 6 h. The reaction mixture was purified by HPLC on a C_{18} reversed phase column (250 × 10 mm) using a linear gradient of 99:1 → 1:99 50 mM aq ammonium acetate, pH 4.5 – acetonitrile. The retention time of the desired product was 21.1 min. The fractions containing the product were lyophilized to afford **20** as a colorless solid: yield 2.45 mg (60%); mass spectrum (ESI), *m/z* 1067.1948 $(M-H)^-$ ($C_{37}H_{41}N_{12}O_{22}P_2$ requires *m/z* 1067.1934).

Biochemical Experiments

Site-directed Mutagenesis of the RRM1 Gene. Mutagenesis of RRM1 gene at the positions corresponding to His24 and Arg26 in the expressed protein was carried out by the use of a modified Quik-ChangeTN site-directed mutagenesis protocol.[6] pETRRM1wt plasmid, having the wild-type RRM1 gene, and three different primers, based on the desired mutations, 5′ CTGTTTCTCCGGTTGTTTAGGTTCGTGGTCTGTGCG 3′ (RRM1-24), 5′ CTCCGGTTGTTCACGTTTAGGGTCTGTGCGAATCTG 3′ (RRM1-26), and 5′ CTGTTTCTCCGGTTGTTTAGGTTTAGGGTCTGTGCGAATCTG 3′ (RRM1-2426), were used.

Oligonucleotide primer phosphorylation was performed in reaction mixtures (20 µL total volume) containing 100 pmol of primer, 1 mM ATP, 70 mM Tris buffer, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT and 1 unit of T4 polynucleotide kinase that were incubated at 37 ºC for 1 h and then chilled on ice. Then 40 µL of deionized water, 240 µL of 5 M NH_4OAc and 750 µL of cold ethanol were added. The combined solution was mixed and incubated at –20 ºC for 20 min and centrifuged at 12,000 × g for 20 min. The pellet was washed with 70% ethanol, air-dried, and dissolved in 50 µL of RNase-free water.

The polymerase chain reaction (PCR) was carried out in 50 μL (total volume) of 35 mM Tris-HCl, pH 8.0, containing 300 ng of template, 14 pmol of primer, 10 nmol of dNTPs, 12 mM KOAc, 5 mM DTT, 0.05 % Triton X-100, 0.05 mM EDTA, 2.5 U of Pfu polymerase and 20 U of Taq DNA ligase. The thermal cycler was programmed as follows: pre-incubation at 95 °C for 2 min, 18 cycles at 95 °C for 1 min, 45 °C for 1 min and 65 °C for 8 min, then final extension for 7 min at 72 °C and cooling to room temperature. One μL of restriction endonuclease *Dpn*I was added and the reaction mixture was incubated at 37 °C for 1 h. Then the samples were subjected to denaturation at 95 °C for 1 min, followed by 2 cycles at 95 °C for 1 min, 50 °C for 1 min and 70 °C for 8 min. The samples were then precipitated by the successive additions of NaOAc, pH 5.2, to a concentration of 0.1 M and 3 vol of cold ethanol. After incubation for 20 min at –20 ˚C, the samples were centrifuged, and the pellets were washed with 70% ethanol, air-dried and dissolved in 10 μL of purified water. DH5α high efficiency competent cells (> 10^7cfu/μg) were transformed using 5 μL of the PCR products per 50 μL of cell suspension and the transformants were selected on LB agar with 30 μg/mL kanamycin and incubated at 37 ºC for 18-24 h.

Preparation of Aminoacyl-tRNA$_{CUA}$s. The activation of suppressor tRNA$_{CUA}$s was carried out as described previously.[3,4] Briefly, the reaction was carried out in 100 μL reaction mixture (total volume) of 100 mM Na HEPES buffer, pH 7.5, containing 1.0 mM ATP, 15 mM $MgCl_2$, 100 μg of suppressor tRNA$_{CUA}$-C$_{OH}$, 0.5 A_{260} unit of *N*-NVOC-protected aminoacyl-pdCpA, 15% DMSO, and 100 units of T4 RNA ligase. The reaction mixture was incubated at 37 °C for 1.5 h and quenched by the addition of 0.1 vol of 3 M NaOAc, pH 5.2. The N-protected aminoacylated tRNA was precipitated with 3 vol of cold absolute ethanol and the pellet was redissolved in RNase-free water. The efficiency of ligation was estimated by 8% polyacrylamide–7 M urea gel electrophoresis (pH 5.0).[5] The *N*-NVOC-protected aminoacyl-tRNA$_{CUA}$s were deprotected by irradiation with a 500 W mercury-xenon lamp for 3 min at room temperature. The solution was centrifuged, and the supernatant was adjusted to 0.3 M NaOAc and treated with 3 vol of cold ethanol to precipitate the aminoacylated tRNA. The tRNA pellet was collected by centrifugation, washed with 70% aq ethanol, air dried and dissolved in 20 μL of RNase free water.

***In Vitro* Protein Translation.** Protein translation reactions were carried out in 20-2000 μL of incubation mixture containing 0.2-0.4 μL/μL of S-30 system, 100 ng/μL of plasmid, 35 mM Tris acetate, pH 7.4, 190 mM potassium glutamate, 30 mM ammonium acetate, 2 mM DTT, 0.2 mg/mL total *E. coli* tRNA, 3.5% PEG 6000, 20 μg/mL folinic acid, 20 mM ATP and GTP, 5 mM CTP and UTP, 100 μM amino acids mixture, 0.5 μCi/μL of ^{35}S-methionine and 1 μg/mL rifampicin. In the case of plasmids having a gene with a TAG codon, a suppressor tRNA (activated with nucleobase amino acid **1**, **2**, **3** or **4**) was added to a concentration of 0.3-0.6 μg/μL. Reactions were carried out at 37 °C for 1 h and terminated by chilling on ice. Aliquots from *in vitro* translation mixtures were analyzed by SDS-PAGE followed by quantification of the radioactive bands by phosphorimager analysis.

Purification of RRM1 Samples. Samples of RRM1, prepared by *in vitro* translation, were diluted about three times with 100 mM Tris-HCl, pH 8.3 (wash buffer) and loaded on a Strep-tactin Sepharose column (volume 100 μL) (IBA GmBH), that had been equilibrated with the same buffer. The column was washed with 2 mL of the same buffer and bound proteins were eluted with six 0.05-mL portions of the same buffer, supplemented with 2.5 mM desthiobiotin (elution buffer). Fractions were analyzed by SDS-PAGE (phosphorimager detection) and the

fractions containing RRM1 were combined and concentrated/desalted using 10 kDa filtration devices (Amicon Ultra, Millipore Corp, Billerica, MA). To estimate amount of protein in each sample of RRM1 (wild type and mutants), a known concentration of wild-type RRM1, prepared by *in vivo* translation (40 μM) was used as a standard for Coomassie staining (Figure S4). To verify the concentration of RRM1 in the *in vitro* wild-type sample, an EMSA was run using the sample prepared *in vivo* as a standard (Figure S6). The concentration of protein in the *in vitro* sample was normalized based on EMSA data during the parallel testing of both samples. The two samples of wild-type RRM1 (prepared *in vivo* and *in vitro*) were diluted to the same concentration (250 nM) and EMSA was run again to complete the sample comparison (Figure S7). Additionally, to normalize the amount of each *in vitro* sample of RRM1 (wild-type and mutant), thus enabling the derivation of more exact EMSA data, the radioactivity in the bands after electrophoretic analysis of the final samples were also determined (phosphoimager detection).

Electrophoretic Mobility Shift Assay (EMSA). Oligonucleotides (39 nt), 5′ CAGCCCCGCTCCC**G**CCCCCTTCCTCCCGCGCCCGCCCCT 3′ and 5′ CAGCCCCGCTCCC**A**CCCCCTTCCTCCCGCGCCCGCCCCT 3′, corresponding to the wild-type and mutant *BCL2* i-motif DNAs, respectively, were phosphorylated using T4 polynucleotide kinase. The reaction mixture (total volume 80 μL), contained 32 pmol of oligonucleotide and 39 pmol ^{32}P-ATP (80 μCi) (Amersham) in 70 mM Tris buffer, pH 7.6, with 10 mM $MgCl_2$, 5 mM DTT and 2 U of T4 polynucleotide kinase, and was incubated at 37 ºC for 2 h and then chilled in ice. Separation of the oligonucleotide from unreacted ^{32}P-ATP was accomplished by the use of a MicroSpin G-25 column (GE Healthcare). The volume of solution containing the purified radiolabeled oligonucleotide was 96 μL; this was diluted 20-fold into 83 mM Tris-HCl, pH 6.5.

Samples of RRM1 were used for 2-fold serial dilutions with 10 mM Tris-HCl, pH 6.5, and 2 μL of each dilution was mixed with 3 μL of the oligonucleotide solution containing 83 mM Tris-HCl, pH 6.5, and 16.6 fmol/μL of ^{32}P-labeled oligonucleotide. The reaction mixtures (5 μL total volume) were incubated for 20 min at room temperature. Four μL of loading solution (30% glycerol, 0.005% bromophenol blue and xylene cyanol FF) was added to each reaction and samples were analyzed on a native 12% polyacrylamide gel, prepared with10 mM Tris acetate, pH 7.8. Before the samples were loaded, the gel was run for 30 min at 10 V per 1 cm of gel in 10 mM Tris acetate, pH 7.8. Samples (4 μL) were loaded and the electrophoresis was run for 2 h at 80 V and 4 °C. For the purpose of comparison, the binding capacity of all analyzed samples was expressed in units of half-binding concentration (BC_{50}), which was estimated based on a linear function (R^2 >0.95) in the 5−40 nM concentration region (Figure S7), which was determined for both wild-type samples (*in vivo* and *in vitro* preparations) and was within the concentration range for all prepared RRM1 mutants.

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