Electronic Supplementary Information

to the manuscript:

HMGB1 binds to *KRAS* promoter G-quadruplex: a new player in oncogene transcriptional regulation?

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Experimental Section

Materials. The recombinant protein of human high-mobility group box 1 (HMGB1) was purchased from OriGene Technologies (MD, USA). Phosphoramidites and controlled pore glass supports for DNA synthesis were purchased from Link Technologies (UK). Sensor chips, amino coupling reagents and buffers for surface plasmon resonance (SPR) measurements were purchased from GE Healthcare. Dual labeled FAM-5'-AGGGCGGTGTGGGAAGAGGGAAGAGGGGGAGG-3'-TAMRA (F32GT) (FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine) oligonucleotide was obtained from Biomers (Germany). All common chemicals, reagents and solvents were purchased from Sigma Aldrich (Merck group) unless otherwise stated.

Oligonucleotide synthesis and sample preparation. DNA oligonucleotides were chemically synthesized using an ABI 394 DNA/RNA synthesizer (Applied Biosystem, Inc) on controlled pore glass supports at 1 or 5 μmol scale, using standard phosphoramidite solid phase chemistry. In particular, the following oligonucleotides were synthesized: the G-rich KRAS promoter sequence 5'-AGGGCGGTGTGGGAAGAGGGAAGAGGGGGAGG-3' (32G), its complementary C-rich sequence 5'-CCTCCCCCTCTTCCCTCTTCCCACACCGCCCT-3' (32C), and the three labeled sequences FAM-5'-AGGGCGGTGTGGGAAGAGGGAAGAGGGGGAGG-3' (F32G), FAM-5'-CCTCCCCCTCTTCCCTCTTCCCACACCGCCCT-3' (F32C), and 5'-CCTCCCCCTCTTCCCTCTTCCCACACCGCCCT-3'-TAMRA (32CT). After synthesis, the oligomers were detached from the support and deprotected by treatment with concentrated aqueous ammonia at 55 °C for 17 h. The combined filtrates and washings were concentrated under reduced pressure, dissolved in H_2O and purified by high-performance liquid chromatography (HPLC) employing standard protocols. The isolated oligomers were proved to be >98% pure by NMR. The concentration of oligonucleotides was determined by UV adsorption measurements at 90 °C using appropriate molar extinction coefficient values ε (λ = 260 nm) calculated by the nearest-neighbor model. G-quadruplex-forming DNA samples (32G, F32G, and F32GT) and single-stranded C-rich sequences (32C, F32C) were prepared in 50 mM Tris-HCl buffer containing 100 mM KCl at pH 7.4. Duplexes were obtained by mixing equimolar amounts of 32G and 32C (32ds), F32G and 32C (F32ds), or F32G and 32CT (F32dsT), in 50 mM Tris-HCl buffer containing 100 mM LiCl at pH 7.4. All samples were heated at 90 °C for 5 min, gradually cooled to room temperature overnight, and then incubated for 24 h, at 4 °C before data acquisition.

Circular dichroism (CD) experiments. CD experiments were recorded on a Jasco J-815 spectropolarimeter equipped with a Jasco PTC-423S temperature controller. Spectra were recorded in a quartz cuvette with 1 mm path length in the wavelength range of 220–360 nm, and averaged over three scans. The scan rate was set to 100 nm/min, with 1 s response time and 1 nm bandwidth. Buffer baseline was subtracted from each spectrum. For DNA structural characterization, 10 μM of 32G, 32C and 32ds duplex were used. CD spectra of 32C in the pH range 4.0-7.4 were also recorded. CD melting experiments were carried out by collecting data in the range 20-95 °C using a temperature step of 5 °C. The samples were incubated at each temperature for a suitable time to achieve the equilibrium. Reaching equilibrium at each temperature was guaranteed by the achievement of superimposable CD spectra on changing time. The CD melting curves (Fig. S1) were obtained by following changes of CD signal at the wavelength of maximum intensity (263, 270, 276, and 285 nm for 32G, 32ds, 32C at pH 7.4, and 32C at pH 4.5, respectively). Melting temperatures were determined from curve fit using Origin 7.0 software. CD spectra of 32G/HMGB1 mixtures were carried out at 20 °C in a quartz cuvette with 1 cm path length, in the 190–360 nm wavelength range, by using 1 μM G-quadruplex and 1 or 2 μM protein concentration. CD melting experiments of 32G/HMGB1 (1:1) mixture were carried out by following the same procedure reported above for the DNA samples in the absence of protein.

Surface plasmon resonance (SPR) experiments. SPR experiments were performed at 25 °C using a Biacore X100 (GE Healthcare) equipped with a research-grade CM5 sensor chip. HMGB1 protein was immobilized using amine-coupling chemistry and HBS-EP as running buffer (HEPES 10 mM, NaCl 150 mM, EDTA 3 mM, 0.005% Surfactant P20, pH 7.4). The surfaces of flow cells were activated with a 1:1 mixture of 0.1 M NHS (N-hydroxysuccinimide) and 0.1 M EDC (3-(N,N-dimethylamino)propyl-N-ethylcarbodiimide) at a flow rate of 10 μl/ min. The protein at a concentration of 50 μg/mL in 10 mM sodium acetate, pH 4.5, was immobilized at a density of ~3000 RU on the sample flow cell, leaving the reference cell as blank. Unreacted activated groups were blocked by injection of 1.0 M ethanolamine at 10 μL/min over the chip surface. To collect kinetic binding data, DNA molecules were injected at various concentrations (from 0.1 to 10 μM), using 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM KCl as running solution for 32G and 32C, and 50 mM Tris-HCl (pH 7.4) for 32ds. Injections were performed at a flow rate of 30 μL/min. The association time was 120 and 60 s for multi-cycle and single-cycle kinetics, respectively. No regeneration after each sample was required. The data were fit to a simple 1:1

interaction model using the global data analysis option available within the Biacore Evaluation software provided with the device.

Fluorescence titration experiments. Fluorescence titration experiments were performed at 25 °C on a FP-8300 spectrofluorometer (Jasco) equipped with a Peltier temperature controller system (Jasco PCT-818). A sealed quartz cuvette with a path length of 1 cm was used. HMGB1 was excited at 280 nm, and emission spectra were recorded between 285 and 500 nm. Both excitation and emission slit widths were set at 5 nm. Titrations were carried out by stepwise addition (5 µL) of a 32G DNA solution (100 µM) to a cell containing a fixed concentration (3 µM) of protein solution in 50 mM Tris-HCl and 100 mM KCl (pH 7.4). After each addition of 32G, the solution was stirred and allowed to equilibrate for 5 min before spectrum acquisition. The fraction of bound protein (α) at each point of the titration was calculated following the changes of fluorescence intensity at 327 nm, using the following relationship:

$$\alpha = \frac{I_{327} - I_{327}^{free}}{I_{327}^{bound} - I_{327}^{free}}$$

where I_{327} is the fluorescence intensity at 327 nm at the various protein/DNA ratios investigated; I_{327}^{free} and I_{327}^{bound} are the fluorescence intensities of the free and fully bound protein, respectively. Titration curves were obtained by plotting α versus the 32G G-quadruplex concentration. The equilibrium dissociation constant (K_d) and the stoichiometry of interaction were estimated from this plot by fitting the resulting curve by means of nonlinear regression to an independent and equivalent binding site model,[1,2] using the following equation:

$$\alpha = (1/2P_0)\left[(P_0 + nL + 1/K_a) - \sqrt{(P_0 + nL + 1/K_a)^2 - 4P_0nL}\right]$$

where P_0 is the total protein concentration, L the added DNA concentration, n the number of binding sites and K_a the equilibrium association constant. The total protein concentration was corrected for dilution effects resulting from the change in volume incurred by the DNA addition. The experiments were repeated three times, and the obtained results are presented as the mean ± S.D.

Microscale thermophoresis (MST) experiments. MST measurements were performed using the Monolith NT.115 (Nanotemper Technologies, Munich, Germany). The FAM-labeled F32G, F32C, and F32ds were prepared in the appropriate buffers reported above, supplemented with

0.1% Tween. The concentration of the labeled DNA samples was kept constant at 23 nM (F32G) or 20 nM (F32C and F32ds). A serial dilution of the protein (1:2 from 31.3 μM stock solution) in the same buffer used for the oligonucleotides was prepared and mixed with the oligonucleotide solution with a volume ratio of 1:1. Samples were loaded into standard capillaries (NanoTemper Technologies), and MST measurements were performed at 20 °C, using auto-tune LED power and medium MST power. MST analysis was performed in duplicates. Data analysis was performed by employing the MO Affinity Analysis software (v2.3) provided with the instrument. Plots were rendered with GUSSI version 1.2.1 software (http://biophysics.swmed.edu/MBR/software.html).

Förster resonance energy transfer (FRET) experiments. FRET experiments were carried out on a FP-8300 spectrofluorometer (Jasco) equipped with a Peltier temperature controller system (Jasco PCT-818). The experiments were performed by using the dual labeled G-quadruplex-forming sequence F32GT (which has covalently attached the donor FAM and the acceptor TAMRA), and the F32dsT duplex (formed by F32G and 32CT that have covalently attached the donor FAM and the acceptor TAMRA at 5'- and 3'-end, respectively). F32GT and F32dsT were diluted from stock to 1 μM solution in 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM KCl or 100 mM LiCl, respectively. The samples were then annealed by heating to 90 °C for 5 min, followed by cooling to room temperature overnight. Samples at 0.1 μM oligonucleotide concentration (2 mL) were prepared by aliquoting 200 μL of the annealed DNA followed by the addition of 1800 μL of buffer or solutions containing 1 or 2 molar equivalents of HMGB1 protein. Fluorescence emission spectra of labeled DNA molecules in the absence and presence of HMGB1 were recorded at 20 and 100 °C, with excitation at 490 nm and detection in the wavelength range of 500–650 nm. Both excitation and emission slit widths were set at 5 nm. A sealed quartz cuvette with a path length of 1 cm was used. FRET melting experiments were carried out by exciting at 490 nm and recording the fluorescence at 522 nm. Fluorescence readings were taken at intervals of 0.5 °C in the range 20−100 °C, with a constant temperature being maintained for 30 s prior to each reading to ensure a stable value. Final analysis of the data was carried out using Origin 7.0 software.

siRNA transfection. Panc1 cells were seeded at 1×10^5 cells/mL in 12 well plates with the DMEM/F12 (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), one day prior to the transfection. Then, the cells were transfected with 27 nM of human HMGB1 siRNA (Fischer Scientific-Cat No. AM16708) using Lipofectamine 3000 reagent (Invitrogen), according to the manufacturer's instructions. Forty-eight hours after the transfection cells were washed with PBS and then cell pellets were isolated using trypsin

(Invitrogen). Isolated cell pellet was used to extract RNA. Control cells were transfected with Silencer Negative Control No.1 siRNA (Fischer Scientific-Cat No. AM4611). Four biological experiments were performed, and each experiment carried out in duplicates.

RNA extraction and qRT-PCR. Total RNA was isolated with RNeasy Mini kit (Qiagen, Valencia, CA) using the Qiacube automated system. The total RNA measure at 1 µg was used to synthesize cDNA using QuantiTect Reverse Transcription kit (Cat No. 205313-Qiagen). Synthesized cDNA was diluted in 1:3 ratio with nuclease free water. KAPA PROBE FAST qPCR master mix (KAPABIOSYSTEMS- Cat No. KK4703) was used to perform the qRT-PCR. FAM labeled HMGB1 (Hs01923466_g1), FAM labeled KRAS (Hs00364284_g1) and VIC labeled GAPDH (Hs02786624_g1) TaqMan primers were used for qRT-PCR. The real-time PCR detection was performed on the Rotor-Gene Q (Qiagen) system. HMGB1 and KRAS expression levels were calculated using comparative Ct method, relative to GAPDH and data was normalized to the non-transfected control. Statistics were performed with Student's t-test. Values of $P < 0.05$ are considered as significant.

References

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2 L. Cerofolini, J. Amato, V. Borsi, B. Pagano, A. Randazzo and M. Fragai, *J. Mol. Recognit.*, 2015, **28**, 376.

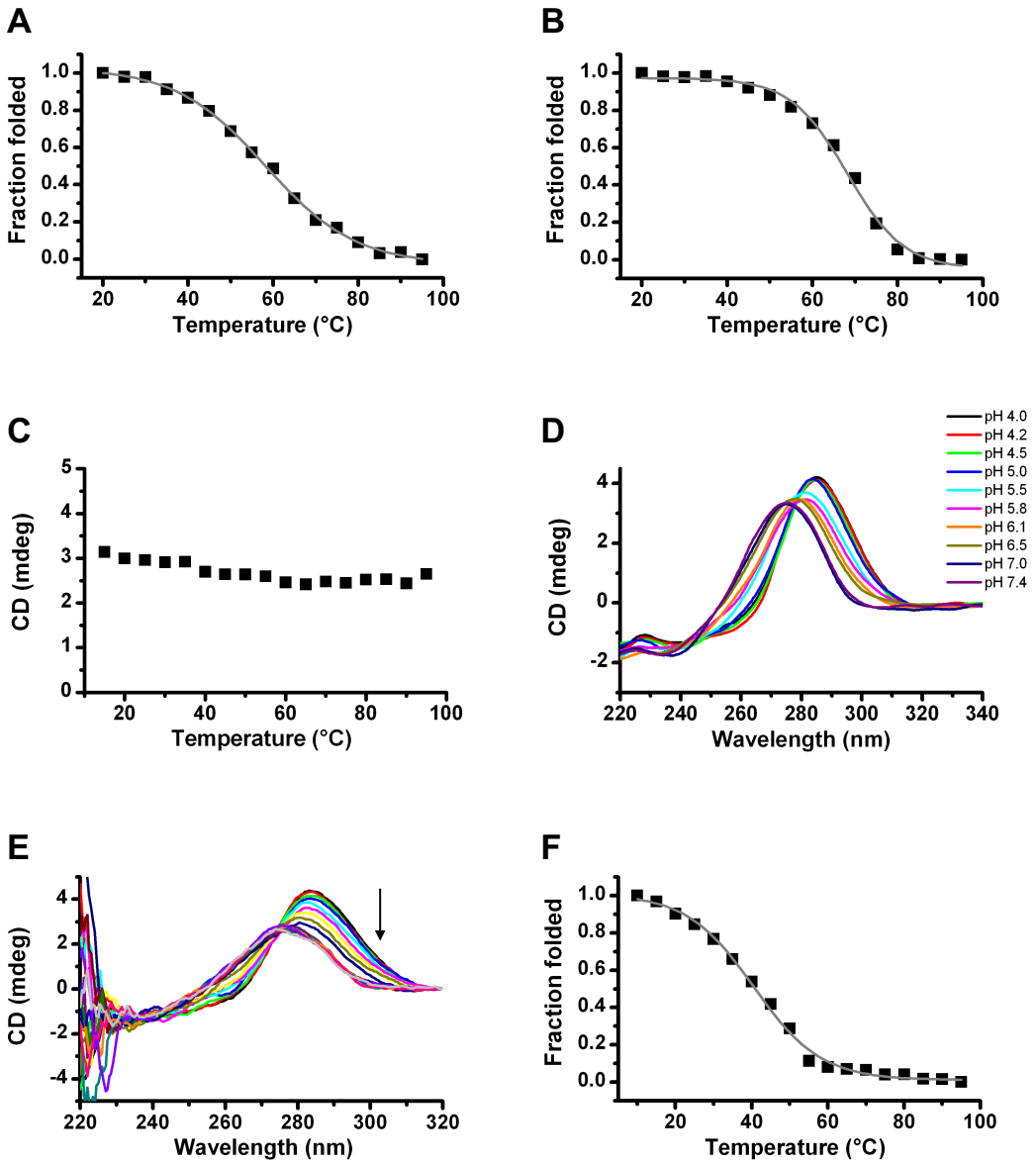

Figure S1. (A, B) Normalized CD melting curves of (A) 32G G-quadruplex-forming oligonucleotide (10 μM) recorded at 263 nm, and (B) 32ds duplex (10 μM) recorded at 270 nm. The T_m values were 58.0 (±0.5) °C and 68.0 (±0.5) °C for 32G and 32ds, respectively. (C) CD melting profile of 32C C-rich oligonucleotide (10 μM) at pH 7.4 recorded at 276 nm. No distinct transition was observed. (D) CD spectra of 32C (10 μM) at different pH values at 10 °C. The spectra suggest that the 32C sequence turned out to be folded into an i-motif-like structure only at pH values lower than 5.0. (E) CD spectra of 32C (10 μM) at pH 4.5 as a function of temperature (from 10 to 95 °C with temperature increase of 5 °C). (F) Normalized CD melting curve of 32C (10 μM) at pH 4.5 recorded at 285 nm. The T_m value was 40.5 (±0.5) °C.

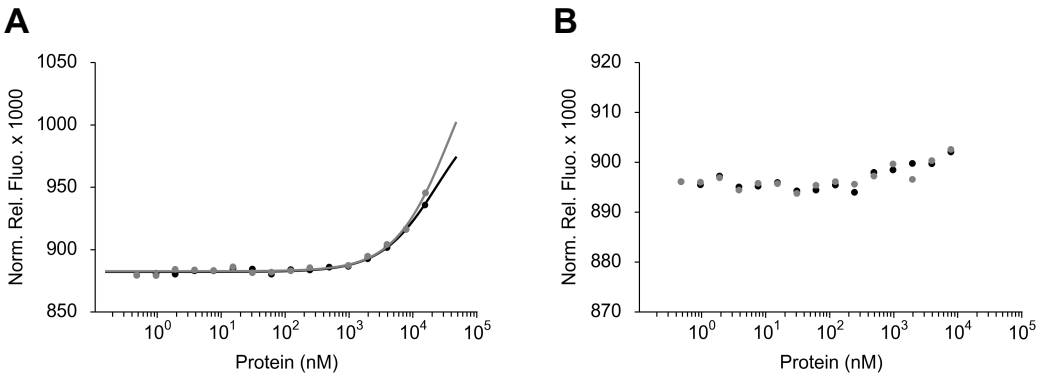

Figure S2. MST measurements of (A) FAM-labeled 32ds duplex (F32ds, prepared by mixing equimolar amounts of F32G and 32C) (20 nM), and (B) 5′FAM-labeled 32C (F32C, 20 nM) at pH 7.4 recorded by adding increasing concentrations of HMGB1 (0.48 nM-15.7 μM). Black and gray data represent two replicates of the same experiment. The estimated K_d value for the protein binding to F32ds was 3.2 (±1.7) × 10^{-5} M. No binding was detected for F32C.

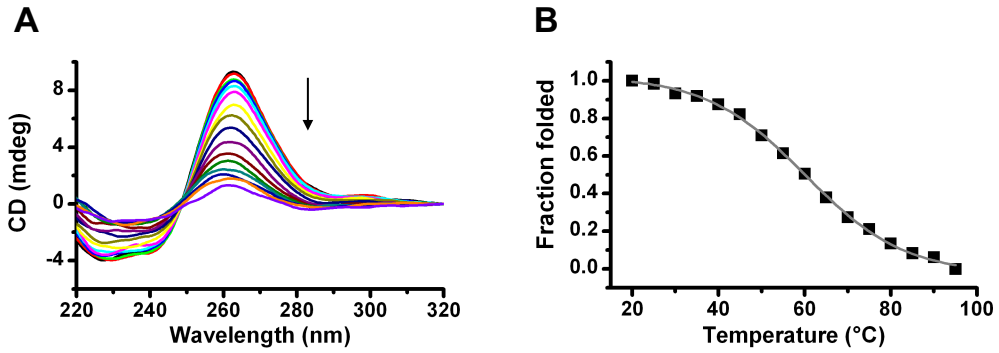

Figure S3. (A) CD spectra of 32G/HMGB1 (1:1) mixtures (1 μM) as a function of temperature (from 20 to 95 °C with temperature increase of 5 °C). (B) Normalized CD melting curve of 32G (1 μM) in the presence of equivalent amounts of HMGB1 recorded at 263 nm. The T_m value was 61.0 (±0.5) °C.

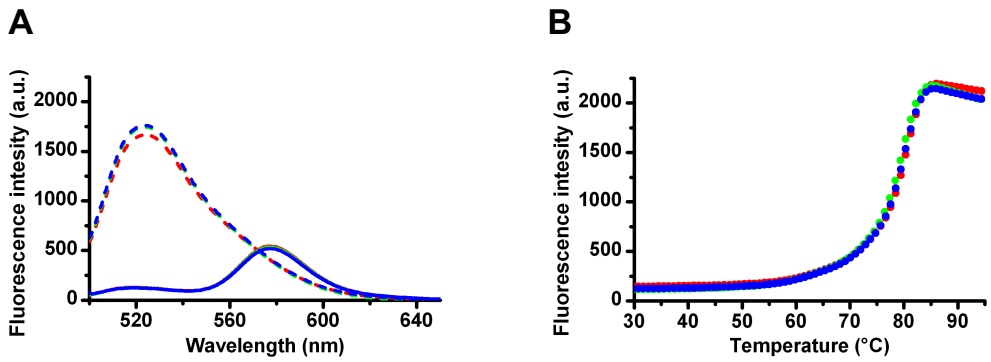

Figure S4. (A) Fluorescence emission spectra of labeled F32dsT duplex (prepared by mixing equimolar amounts of F32G and 32CT) (0.1 μM) obtained exciting FAM at 490 nm in the absence (red) and presence of 1 (green) or 2 (blue) molar equivalents of HMGB1, recorded at 20 °C (solid lines) and 100 °C (dashed lines). (B) FRET-melting curves of F32dsT (0.1 μM) in the absence (red) and presence of 1 (green) or 2 (blue) equivalents of HMGB1.