**Article:**
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## Supplemental Methods

### Generation of fluorescently labelled RSSs for RAG cleavage assays

PCR reactions contained either 1 ng of annealed 12-RSS (DAR39/40) or 23-RSS (DG61/62) oligonucleotides as template, 25 pmol of each primer, Alexa Fluor 488-labelled RSS-F and unlabelled RSS-R (20+21; Table S1), 250 µM dNTPs and Herculase II Fusion DNA polymerase. PCR comprised 35 cycles of 10 seconds at 95℃, 15 seconds at 60℃ and 15 seconds 72℃. The reactions were gel purified and the purified RSS probe was stored in 10 mM Tris-HCl pH 7.5 at 4℃.

### In vitro RAG cleavage and binding assays

RAG cleavage assays were performed using fluorescently labelled RSS probes (above). Reactions (10 µl) contained 25 mM MOPS pH 7.0, 50 mM potassium acetate, 1 mM DTT, 100 µg/ml BSA, 1 mM $MgCl_2$ (1 mM $MnCl_2$ for Figure 4C), 8 nM fluorescently labelled oligonucleotide substrate, 20 nM unlabelled partner oligonucleotide (except Fig 4C), 100 ng (400 nM) HMGB1 and 150 ng of purified RAG proteins. Where indicated, HMGB1 was omitted. Initial reactions (Figure 2A) were for 10 minutes in the presence of DMSO which enhances RAG cleavage. Subsequent cleavage reactions were in the absence of DMSO for 60 minutes to accurately determine the effects of HMGB1[21].

Reactions were incubated at 37℃ before stop buffer was added to give final concentrations: 50 mM Tris-HCl pH 8.0, 0.1% SDS, 5 mM EDTA, 0.175 mg/ml proteinase K, 1 x DNA loading buffer, followed by incubation at 37℃ for 30 minutes. The reaction was loaded onto a 15% polyacrylamide gel in 1 x TAE and separated for 2 hours at 170V. Gels were visualised using a FLA-5100 imager (Fujifilm, Tokyo, Japan).

RAG-RSS binding assays were performed using radioactively labelled oligonucleotide substrates (Table S1) to improve sensitivity and the same conditions as above, except the divalent cation was $Ca^{2+}$. For the binding reactions shown, 20% DMSO was included, but equivalent patterns were observed in the absence of DMSO. For the 6xHis-HMGB1 supershift assay,

100 ng of 6xHis-HMGB1 and 0.5 μl of mouse anti-6x-His monoclonal antibody (STJ96906; St John's Laboratory, London, United Kingdom) were used. Following incubation at 37℃ for 10 minutes, reactions were chilled on ice for 5 minutes and loaded onto a 4% (19:1) polyacrylamide gel in 0.5 x TBE, pre-chilled to 4℃. The gels were run at 170V for 2 hours at 4℃, dried a nd exposed to a phosphorimager and visualised using an FLA-5100 imager (Fujifilm). Oligonucleotide sequences are given in Table S1.

Cloning of lentiviral vectors for HMGB1 knockdown

The lentivirus expressing dCas9-KRAB was generated by firstly deleting VP64 from lenti-dCAS-VP64_Blast (Addgene Cambridge, MA, USA; 61425, a gift from Feng Zhang) via PCR, using primers 22+23 (Table S1) and Q5 DNA polymerase to generate the lenti-dCas9-T2A-BLAST vector. The cDNA sequence encoding the KRAB domain from human ZNF10 (aa 2-97) was amplified from HEK293T cell cDNA using Q5 DNA polymerase and primers 24+25 (Table S1); this was then ligated into the lenti-dCas9-T2A-BLAST vector between the dCas9 and T2A-BLAST sequences to generate lenti-dCas9-KRAB-T2A-BLAST, which was verified by sequencing. To target dCas9-KRAB to the HMGB1 TSS, a guide RNA sequence complementary to the HMGB1 TSS was cloned into lenti sgRNA(MS2)_zeo (Addgene 61427, a gift from Feng Zhang) using primers 26+27 (Table S1).

Generation of lentiviral particles

Lentiviral particles were generated by seeding HEK293T cells at a density of 3.5 x $10^6$ cells in 10 cm dishes, 24 hours prior to transfection. Cells were co-transfected with either 4 μg lenti-dCas9-KRAB-T2A-BLAST or 4 μg lenti sgRNA(MS2)-Zeo containing the HMGB1 TSS guide RNA sequence, together with 4 μg of LV packaging vector pCMVR8.74, a gift from Didier Trono (Addgene plasmid #22036), 2 μg of the vector that expresses the coat protein, pMD2.G, a gift from Didier Trono (Addgene plasmid #12259), and 30 μg PEI per dish. The media was changed 16 hours post transfection and the supernatant containing lentiviral particles was harvested at 48 hours and used immediately for transduction.

Cloning of lentiviral vectors and transduction to over-express HMGB1/2

To generate the HMGB1ΔC-P2A-Puro lentiviral vector, firstly, a fragment containing the U6 promoter and gRNA scaffold was deleted from lentiCRISPR v2 (Addgene 52961, a gift from Feng Zhang) by digestion with KpnI (R3142S, New England Biolabs) and EcoRI (R3101S, New England Biolabs) and the overhangs removed using T4 DNA polymerase (M0203S, New England Biolabs). Next, Cas9 was removed via PCR using primers 28+29 (Table S1) and Q5 polymerase. Rat HMGB1 cDNA was amplified from pETM11-HMGB1 also using Q5 DNA polymerase, and primers 30+31 (Table S1); this was ligated into the lenti-P2A-Puro vector and its sequence verified.

The HMGB2ΔC-P2A-Puro and HMGB2-P2A-Puro lentiviral vectors were generated in a similar way. First, the mouse HMGB2 cDNA sequence was amplified from NIH3T3 cDNA using Q5 polymerase and primers 32+33 (HMGB2ΔC) and 32+34 (HMGB2; Table S1); these were also ligated into the lenti-P2A-Puro vector and their sequences verified.

Lentiviral particles were produced as described above, using 4 μg of lentiviral transfer vector, 4 μg pCMVR8.74, 2 μg pMD2.G and 30 μg PEI per 10 cm dish. HMGB1 knock-down cells were transduced with either the HMGB1ΔC-P2A-Puro, HMGB2-P2A-Puro or HMGB2ΔC-P2A-Puro lentivirus as described previously followed by antibiotic selection with 5 μg/ml puromycin dihydrochloride (sc-108071B, Santa Cruz Biotechnology). The presence of HMGB1ΔC-P2A, HMGB2-P2A-Puro and HMGB2ΔC-P2A was confirmed by western blotting using either a mouse monoclonal anti-HMGB1 antibody (sc-56698, Santa Cruz Biotechnology) or a rabbit monoclonal anti-HMGB2 antibody (14163T, CST, Danvers, MA, USA).

**Table S1 - Primers used in this study**

| # | Name | Sequence | Reference |
|---|---|---|---|
| 1 | DR55 | AGAGGGACTGGATTCCAAAGTTCTC | 33 |
| 2 | 1233 | CTTTCATTGCCATACG | 34 |

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|---|---|---|---|
| 3 | SJ-F | CTGCTTGCTGTTCTTGAATGG | |
| 4 | SJ-R | TACAGCCAGACAGTGGAGTA | |
| 5 | CAT-F | CGTATGGCAATGAAAGACGGTGAGC | 35 |
| 6 | CAT-R | CGAAGAAGTTGTCCATATTGGCCAC G | 35 |
| 7 | JH299-SJ Probe | /56-FAM/CCAGTCTGT/ZEN/AGCACTGTG CACAGT/3IABkFQ/ | |
| 8 | R401W_F | GTCACTGACGTGGAGGGCGCAGAAA C | |
| 9 | R401W_R | AGGAGATGCTGGCGAGGC | |
| 10 | R504Q_F | GCATGCTCTTCAGAATGCCGAGA | |
| 11 | R504Q_R | AAAGGTTGAAAAATCTGCCTC | |
| 12 | R401L_F | GTCACTGACGCTAAGGGCGCAGAAA C | |
| 13 | R401L_R | AGGAGATGCTGGCGAGGC | |
| 14 | R401K_F | TCACTGACGAAAAGGGCGCAG | |
| 15 | R401K_R | CAGGAGATGCTGGCGAGGC | |
| 16 | DAR39 (12RSS top) | GATCTGGCCTGTCTTACACAGTGCTA CAGACTGGAACAAAAACCCTGCAG | 7 |
| 17 | DAR40 (12RSS bottom) | CTGCAGGGTTTTTGTTCCAGTCTGTA GCACTGTGTAAGACAGGCCAGATC | 7 |
| 18 | DG61 (23RSS top) | GATCTGGCCTGTCTTACACAGTGGT AGTACTCCACTGTCTGGCTGTACAAA AACCCTGCAG | 7 |
| 19 | DG62 (23RSS bottom) | CTGCAGGGTTTTTGTACAGCCAGAC AGTGGAGTACTACCACTGTGTAAGA CAGGCCAGATC | 7 |
| 20 | 488-RSS-F | /Alexa-Fluor-488/TGATCTGGCCTGTCTTA | |
| 21 | RSS-R | GATCTGCAGGGTTTTTGT | |
| 22 | VP64_Del F | GAGGGCAGAGGAAGTCTGC | |
| 23 | VP64_Del R | GTCGCCTCCCAGCTGAGA | |
| 24 | hZNF10_KRAB_F | GATGCTAAGTCACTAACTGC | |

| | | | |
|---|---|---|---|
| 25 | hZNF10_KRAB_R | AACTGATGATTTGATTTCAAATG | |
| 26 | HMGB1 TSS Guide Top BsmBI | CACCGGTTACAGAGCGGAGAGAGTG | |
| 27 | HMGB1 TSS Guide Bottom BsmBI | AAACCACTCTCTCCGCTCTGTAACC | |
| 28 | P2A F | GCAACAAACTTCTCTCTGC | |
| 29 | EF1a R | CTGTGTTCTGGCGGCAAAC | |
| 30 | rHMGB1F | GCCGCCACCATGGGCAAAGGAGATCCTAA | |
| 31 | rHMGB1ΔCR | TTTCGCTGCATCAGGTTT | |
| 32 | mHMGB2F | GCCGCCACCATGGGCAAGGGTGACCC | |
| 33 | mHMGB2ΔCR | CTTTCCTGCTTCACTTTTG | |
| 34 | mHMGB2R | TTCTTCATCCTCCTCTTCTTCCTCG | |