Supplementary Material

# Supplementary Figures



**Supplementary Fig 1.** Confirmation of siRNA mediated knockdown on HMGB1.

NHBE cells were treated with DsiRNAs for TYE 563 (transfection control, Cy3) or HMGB1 (R1, R2, R3) and a scrambled siRNA (negative control). 24 h post-transfection, the cells showed successful transfection of TYE 563 into the cells (a-a’’; arrows; Scale bar = 100 μm). HMGB1 knockdown was confirmed by immunoblotting (b, c) and qPCR (d). Significant decrease in HMGB1 protein and mRNA expression was seen on transfection with siRNA.R1 (10 nmol), siRNA.R2 (10 nmol) and siRNA.R3 (10 nmol). Changes in HMGB1 protein expression in NHBE cells with or without siRNA mediated HMGB1 knockdown and treated with ODE (1%) for 24 h was assessed (e). Samples for all assays were derived from the same experiment and were processed in parallel. All the protein bands were normalized over β-actin (37 kD) and percentage intensity relative to control was analyzed. Data was analyzed using one-way ANOVA with Tukey’s multiple comparison test (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001) and represented as mean ± SEM with n = 3/treatment (\* indicates significant difference from control).



**Supplementary Fig 2.** HMGB1 neutralization decreases extracellular secretion of mtDNA.

Mitochondrial DNA (mtDNA) leakage into the cytosol (a) and extracellularly (b) in NHBE cells treated with medium or ODE (1%) followed by medium or HMGB1 neutralization antibody (10 µg/mL) for 8 h per day for 5 days was analyzed via qPCR. mRNA fold change of gene targets of mtDNA release, *ifna1* (c), *ifna4* (d), and *ifnb* (e), were measured by qPCR. For all the assays, samples were derived from the same experiment and were processed in parallel. Data was analyzed using one-way ANOVA with Tukey’s multiple comparison test (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001) and represented as mean ± SEM with n = 3/treatment (\* indicates significant difference from control).



**Supplementary Fig 3.** HMGB1 neutralization promotes mitochondrial biogenesis.

Markers of mitochondrial biogenesis in NHBE cells treated with medium or ODE (1%) followed by medium or HMGB1 neutralization antibody (10 µg/mL) for 8 h per day for 5 days was measured. mRNA expression of *pgc1a* (a), *nrf2* (b) and *tfam* (c) were measured by qPCR. mRNA fold change of gene targets downstream of NRF2, *hmox1* (d), and *nqo1* (e), were measured by qPCR. For all the assays, samples were derived from the same experiment and were processed in parallel. Data was analyzed using one-way ANOVA with Tukey’s multiple comparison test (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001) and represented as mean ± SEM with n = 3/treatment (\* indicates significant difference from control).



**Supplementary Fig 4.** HMGB1 neutralization increases mitochondrial calcium influx.

Intra-mitochondrial calcium levels in mitochondria isolated from NHBE cells treated with medium or ODE (1%) followed by medium or HMGB1 neutralization antibody (10 µg/mL) for 8 h per day for 5 days was visualized (a-a’’’; Scale bar = 100 μm) and quantified by Rhod-2AM staining (b). Using Mito-SOX staining, the levels of superoxide anions (SOX) within the mitochondria was quantified (c). Samples for all assays were derived from the same experiment and were processed in parallel. Data was analyzed using one-way ANOVA with Tukey’s multiple comparison test (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001) and represented as mean ± SEM with n = 6/treatment (\* indicates significant difference from control).

# Supplementary Tables

**Supplementary Table 1.** Control and HMGB1 DsiRNA sequences

|  |  |  |  |
| --- | --- | --- | --- |
| Gene | Duplex name | Sequence (5’→3’) | |
| HMGB1 | Ri.HMGB1.13.1 (R1) | Sense | CUGAUAAAAGGUUUUGUCAAACATT |
| Antisense | CCGACUAUUUUCCAAAACAGUUUGUAA |
| Ri.HMGB1.13.2 (R2) | Sense | GCAAAAUGUCAUCAUAUGCAUUUTT |
| Antisense | UCCGUUUUACAGUAGUAUACGUAAAAA |
| Ri.HMGB1.13.3 (R3) | Sense | CCAUUGGUGAUGUUGCGAAGAAACT |
| Antisense | CAGGUAACCACUACAACGCUUCUUUGA |
| Negative  Control | DsiRNA (NC) | Sense | CGUUAAUCGCGUAUAAUACGCGUAT |
| Antisense | AUACGCGUAUUAUACGCGAUUAACGAC |

**Supplementary Table 2.** Primer sequences

|  |  |  |
| --- | --- | --- |
| Gene symbol | Primer Sequence (5’→3’) | |
| *mtdna* | Forward | TGAGGCCAAATATCATTCTGAGGGGC |
| Reverse | TTTCATCATGCGGAGATGTTGGATGG |
| *mtnd1* | Forward | GGCTATATACAACTACGCAAAGGC |
| Reverse | GGTAGATGTGGCGGGTTTTAGG |
| *16s* | Forward | CCGCAAGGGAAAGATGAAAGAC |
| Reverse | TCGTTTGGTTTCGGGGTTTC |
| *hmgb1* | Forward | GCGAAGAAACTGGGAGAGATGTG |
| Reverse | GCATCAGGCTTTCCTTTAGCTCG |
| *nos2* | Forward | GCTCTACACCTCCAATGTGACC |
| Reverse | CTGCCGAGATTTGAGCCTCATG |
| *ifna* | Forward | AGAAGGCTCCAGCCATCTCTGT |
| Reverse | TGCTGGTAGAGTTCGGTGCAGA |
| *ifna4* | Forward | GTTCCAGAAGGCTCAAGCCATC |
| Reverse | TAGGAGGCTCTGTTCCCAAGCA |
| *ifnb* | Forward | CTTGGATTCCTACAAAGAAGCAGC |
| Reverse | TCCTCCTTCTGGAACTGCTGCA |
| *tfam* | Forward | GTGGTTTTCATCTGTCTTGGCAAG |
| Reverse | TTCCCTCCAACGCTGGGCAATT |
| *pgc1a* | Forward | CCAAAGGATGCGCTCTCGTTCA |
| Reverse | CGGTGTCTGTAGTGGCTTGACT |
| *nrf2* | Forward | CACATCCAGTCAGAAACCAGTGG |
| Reverse | GGAATGTCTGCGCCAAAAGCTG |
| *gstp1* | Forward | TGGAAGGAGGAGGTGGTTACCA |
| Reverse | GGTAAAGGGTGAGGTCTCCATC |
| *hmox1* | Forward | CCAGGCAGAGAATGCTGAGTTC |
| Reverse | AAGACTGGGCTCTCCTTGTTGC |
| *nqo1* | Forward | CCTGCCATTCTGAAAGGCTGGT |
| Reverse | GTGGTGATGGAAAGCACTGCCT |
| *hif1a* | Forward | TATGAGCCAGAAGAACTTTTAGGC |
| Reverse | CACCTCTTTTGGCAAGCATCCTG |
| *vhl* | Forward | GACACACGATGGGCTTCTGGTT |
| Reverse | ACAACCTGGAGGCATCGCTCTT |
| *slc2a6* | Forward | TCACCAAGTCCTTCCTGCCAGT |
| Reverse | CACAGCAGCCTGTGAACACCAG |
| *eno1* | Forward | AGTCAACCAGATTGGCTCCGTG |
| Reverse | CACAACCAGGTCAGCGATGAAG |
| *foxj1* | Forward | ACTCGTATGCCACGCTCATCTG |
| Reverse | GAGACAGGTTGTGGCGGATTGA |
| *cfap157* | Forward | CAGCAGGAACTGGCTAATGAGC |
| Reverse | ACGTCACTGTCCTCTTCATCGC |
| *cld2* | Forward | GTGACAGCAGTTGGCTTCTCCA |
| Reverse | GGAGATTGCACTGGATGTCACC |
| *ocld* | Forward | ATGGCAAAGTGAATGACAAGCGG |
| Reverse | CTGTAACGAGGCTGCCTGAAGT |

**Supplementary Table 3.** Array gene list

|  |  |  |
| --- | --- | --- |
| **Assay ID** | **Gene Symbol(s)** | **Gene Name(s)** |
| Hs99999901\_s1 | 18s rRNA | - |
| Hs01060284\_m1 | ATP12A | ATPase H+/K+ transporting non-gastric alpha2 subunit |
| Hs00167575\_m1 | ATP4A | ATPase H+/K+ transporting alpha subunit |
| Hs01026288\_m1 | ATP4B | ATPase H+/K+ transporting beta subunit |
| Hs00900735\_m1 | ATP5A1 | ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle |
| Hs00969569\_m1 | ATP5B | ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide |
| Hs01101219\_g1 | ATP5C1 | ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1 |
| Hs01076982\_g1 | ATP5F1 | ATP synthase, H+ transporting, mitochondrial Fo complex subunit B1 |
| Hs00829069\_s1 | ATP5G1 | ATP synthase, H+ transporting, mitochondrial Fo complex subunit C1 (subunit 9) |
| Hs01086654\_g1 | ATP5G2 | ATP synthase, H+ transporting, mitochondrial Fo complex subunit C2 (subunit 9) |
| Hs00266085\_m1 | ATP5G3 | ATP synthase, H+ transporting, mitochondrial Fo complex subunit C3 (subunit 9) |
| Hs01046892\_gH | ATP5H | ATP synthase, H+ transporting, mitochondrial Fo complex subunit D |
| Hs00273015\_m1 | ATP5I | ATP synthase, H+ transporting, mitochondrial Fo complex subunit E |
| Hs01081389\_g1 | ATP5J | ATP synthase, H+ transporting, mitochondrial Fo complex subunit F6 |
| Hs04194830\_s1 | ATP5J2 | ATP synthase, H+ transporting, mitochondrial Fo complex subunit F2 |
| Hs00538946\_g1 | ATP5L | ATP synthase, H+ transporting, mitochondrial Fo complex subunit G |
| Hs00426889\_m1 | ATP5O | ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit |
| Hs00429389\_m1 | ATP6V0A2 | ATPase H+ transporting V0 subunit a2 |
| Hs01084784\_m1 | ATP6V0D2 | ATPase H+ transporting V0 subunit d2 |
| Hs00375969\_m1 | ATP6V1C2 | ATPase H+ transporting V1 subunit C2 |
| Hs00369807\_m1 | ATP6V1E2 | ATPase H+ transporting V1 subunit E2 |
| Hs00373169\_m1 | ATP6V1G3 | ATPase H+ transporting V1 subunit G3 |
| Hs01018008\_g1 | BCS1L | BCS1 homolog, ubiquinol-cytochrome c reductase complex chaperone |
| Hs00971639\_m1 | COX4I1 | cytochrome c oxidase subunit 4I1 |
| Hs00261747\_m1 | COX4I2 | cytochrome c oxidase subunit 4I2 |
| Hs00362067\_m1 | COX5A | cytochrome c oxidase subunit 5A |
| Hs00426948\_m1 | COX5B | cytochrome c oxidase subunit 5B |
| Hs01924685\_g1 | COX6A1 | cytochrome c oxidase subunit 6A1 |
| Hs00193226\_g1 | COX6A2 | cytochrome c oxidase subunit 6A2 |
| Hs00266375\_m1 | COX6B1 | cytochrome c oxidase subunit 6B1 |
| Hs00376070\_m1 | COX6B2 | cytochrome c oxidase subunit 6B2 |
| Hs00269977\_m1 | COX6C | cytochrome c oxidase subunit 6C |
| Hs01652418\_m1 | COX7A2 | cytochrome c oxidase subunit 7A2 |
| Hs00190880\_m1 | COX7A2L | cytochrome c oxidase subunit 7A2 like |
| Hs00371307\_m1 | COX7B | cytochrome c oxidase subunit 7B |
| Hs00187909\_m1 | COX8A | cytochrome c oxidase subunit 8A |
| Hs00418377\_m1 | COX8C | cytochrome c oxidase subunit 8C |
| Hs00357717\_m1 | CYC1 | cytochrome c1 |
| Hs00383379\_m1 | LHPP | phospholysine phosphohistidine inorganic pyrophosphate phosphatase |
| Hs00244980\_m1 | NDUFA1 | NADH:ubiquinone oxidoreductase subunit A1 |
| Hs00190004\_m1 | NDUFA10 | NADH:ubiquinone oxidoreductase subunit A10 |
| Hs00418300\_m1 | NDUFA11 | NADH:ubiquinone oxidoreductase subunit A11 |
| Hs04187282\_g1 | NDUFA2 | NADH:ubiquinone oxidoreductase subunit A2 |
| Hs01547166\_g1 | NDUFA3 | NADH:ubiquinone oxidoreductase subunit A3 |
| Hs00800172\_s1 | NDUFA4 | NDUFA4, mitochondrial complex associated |
| Hs01634019\_g1 | NDUFA5 | NADH:ubiquinone oxidoreductase subunit A5 |
| Hs00899690\_m1 | NDUFA6 | NADH:ubiquinone oxidoreductase subunit A6 |
| Hs01561430\_m1 | NDUFA7 | NADH:ubiquinone oxidoreductase subunit A7 |
| Hs00204417\_m1 | NDUFA8 | NADH:ubiquinone oxidoreductase subunit A8 |
| Hs00192290\_m1 | NDUFAB1 | NADH:ubiquinone oxidoreductase subunit AB1 |
| Hs00605903\_m1 | NDUFB10 | NADH:ubiquinone oxidoreductase subunit B10 |
| Hs00190006\_m1 | NDUFB2 | NADH:ubiquinone oxidoreductase subunit B2 |
| Hs00427185\_m1 | NDUFB3 | NADH:ubiquinone oxidoreductase subunit B3 |
| Hs00853558\_g1 | NDUFB4 | NADH:ubiquinone oxidoreductase subunit B4 |
| Hs00159582\_m1 | NDUFB5 | NADH:ubiquinone oxidoreductase subunit B5 |
| Hs00159583\_m1 | NDUFB6 | NADH:ubiquinone oxidoreductase subunit B6 |
| Hs00188142\_m1 | NDUFB7 | NADH:ubiquinone oxidoreductase subunit B7 |
| Hs00922355\_g1 | NDUFB8 | NADH:ubiquinone oxidoreductase subunit B8 |
| Hs00601381\_mH | NDUFB9 | NADH:ubiquinone oxidoreductase subunit B9 |
| Hs00159587\_m1 | NDUFC1 | NADH:ubiquinone oxidoreductase subunit C1 |
| Hs01072843\_m1 | NDUFC2 | NADH:ubiquinone oxidoreductase subunit C2 |
| Hs00192297\_m1 | NDUFS1 | NADH:ubiquinone oxidoreductase core subunit S1 |
| Hs00190020\_m1 | NDUFS2 | NADH:ubiquinone oxidoreductase core subunit S2 |
| Hs01549083\_m1 | NDUFS3 | NADH:ubiquinone oxidoreductase core subunit S3 |
| Hs00942568\_m1 | NDUFS4 | NADH:ubiquinone oxidoreductase subunit S4 |
| Hs02578754\_g1 | NDUFS5 | NADH:ubiquinone oxidoreductase subunit S5 |
| Hs00190035\_m1 | NDUFS6 | NADH:ubiquinone oxidoreductase subunit S6 |
| Hs00257018\_m1 | NDUFS7 | NADH:ubiquinone oxidoreductase core subunit S7 |
| Hs00159597\_m1 | NDUFS8 | NADH:ubiquinone oxidoreductase core subunit S8 |
| Hs00957930\_m1 | NDUFV1 | NADH:ubiquinone oxidoreductase core subunit V1 |
| Hs00221478\_m1 | NDUFV2 | NADH:ubiquinone oxidoreductase core subunit V2 |
| Hs00221479\_m1 | NDUFV3 | NADH:ubiquinone oxidoreductase subunit V3 |
| Hs00192329\_m1 | OXA1L | OXA1L, mitochondrial inner membrane protein |
| Hs00535680\_g1 | PPA1 | pyrophosphatase (inorganic) 1 |
| Hs00602575\_m1 | PPA2 | pyrophosphatase (inorganic) 2 |
| Hs00417200\_m1 | SDHA | succinate dehydrogenase complex flavoprotein subunit A |
| Hs01042482\_m1 | SDHB | succinate dehydrogenase complex iron sulfur subunit B |
| Hs01698067\_s1 | SDHC | succinate dehydrogenase complex subunit C |
| Hs01098144\_g1 | SDHD | succinate dehydrogenase complex subunit D |
| Hs00199138\_m1 | UQCR11 | ubiquinol-cytochrome c reductase, complex III subunit XI |
| Hs00163415\_m1 | UQCRC1 | ubiquinol-cytochrome c reductase core protein I |
| Hs00996395\_m1 | UQCRC2 | ubiquinol-cytochrome c reductase core protein II |
| Hs04194251\_g1 | UQCRFS1 | ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1 |
| Hs03045181\_g1 | UQCRH | ubiquinol-cytochrome c reductase hinge protein |
| Hs00416927\_g1 | UQCRQ | ubiquinol-cytochrome c reductase complex III subunit VII |
| Hs99999905\_m1 | GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| Hs99999909\_m1 | HPRT1 | hypoxanthine phosphoribosyltransferase 1 |
| Hs99999908\_m1 | GUSB | glucuronidase beta |
| Hs99999903\_m1 | ACTB | actin beta |
| Hs99999907\_m1 | B2M | beta-2-microglobulin |
| Hs00609297\_m1 | HMBS | hydroxymethylbilane synthase |
| Hs00183533\_m1 | IPO8 | importin 8 |
| Hs99999906\_m1 | PGK1 | phosphoglycerate kinase 1 |
| Hs99999902\_m1 | RPLP0 | ribosomal protein lateral stalk subunit P0 |
| Hs99999910\_m1 | TBP | TATA-box binding protein |
| Hs99999911\_m1 | TFRC | transferrin receptor |
| Hs99999901\_s1 | 18S | Eukaryotic 18S rRNA |
| Hs00824723\_m1 | UBC | ubiquitin C |

**Supplementary Table 4.** Characteristics of primary antibodies

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Epitope** | **Species/Clone** | **Dilution** | **Catalog #** | **Supplier** |
| β-Actin | Mouse Monoclonal | 1:10,000 | ab6276 | AbCama |
| LaminB1 | Rabbit Polyclonal | 1:1000 | ab16048 | AbCama |
| GAPDH | Rabbit Polyclonal | 1:2500 | ab9485 | AbCama |
| HMGB1 | Rabbit Polyclonal | 1:5000 | ab79823 | AbCama |
| MFN1 | Mouse Monoclonal | 1:1000 | sc-166644 | Santa Cruzb |
| MFN2 | Mouse Monoclonal | 1:1000 | sc-100560 | Santa Cruzb |
| OPA1 | Mouse Monoclonal | 1:1000 | sc-393296 | Santa Cruzb |
| DRP1 | Mouse Monoclonal | 1:1000 | sc-271583 | Santa Cruzb |
| Parkin | Mouse Monoclonal | 1:1000 | sc-32282 | Santa Cruzb |
| PINK1 | Mouse Monoclonal | 1:1000 | sc-517353 | Santa Cruzb |
| BNIP3 | Mouse Monoclonal | 1:1000 | sc-56167 | Santa Cruzb |
| Cytochrome C | Mouse Monoclonal | 1:1000 | sc-13156 | Santa Cruzb |
| PGC1α | Mouse Monoclonal | 1:1000 | sc-518025 | Santa Cruzb |
| NRF2 | Mouse Monoclonal | 1:1000 | sc-365949 | Santa Cruzb |
| TFAM | Mouse Monoclonal | 1:1000 | sc-376672 | Santa Cruzb |
| HIF1α | Mouse Monoclonal | 1:1000 | sc-13515 | Santa Cruzb |
| TLR9 | Mouse Monoclonal | 1:1000 | sc-47723 | Santa Cruzb |
| cGAS | Mouse Monoclonal | 1:1000 | sc-515777 | Santa Cruzb |
| IFI16 | Mouse Monoclonal | 1:1000 | sc-8023 | Santa Cruzb |
| IRF3 | Mouse Monoclonal | 1:1000 | sc-33641 | Santa Cruzb |

aCambridge, United Kingdom

bDallas, Texas, USA

**Supplementary Table 5.** Characteristics of secondary antibodies

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Expression system** | **Conjugate** | **Species/Clone** | **Dilution** | **Catalog #** | **Supplier** |
| Donkey/IgG | Alexa Fluor® 680 | Rabbit Polyclonal | 1:10,000 | A10043 | Invitrogenc |
| Rabbit/IgG | Alexa Fluor® 680 | Mouse Polyclonal | 1:10,000 | A27031 | Invitrogenc |
| Donkey/IgG | IRDye® 800CW | Rabbit Polyclonal | 1:10,000 | 926-32213 | LI-CORd |

cThermofisher Scientific, USA

dNebraska, USA