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## Supplemental Information

# The Mitochondrial-Encoded Peptide MOTS-c Translocates to the Nucleus to Regulate Nuclear Gene Expression in Response to Metabolic Stress

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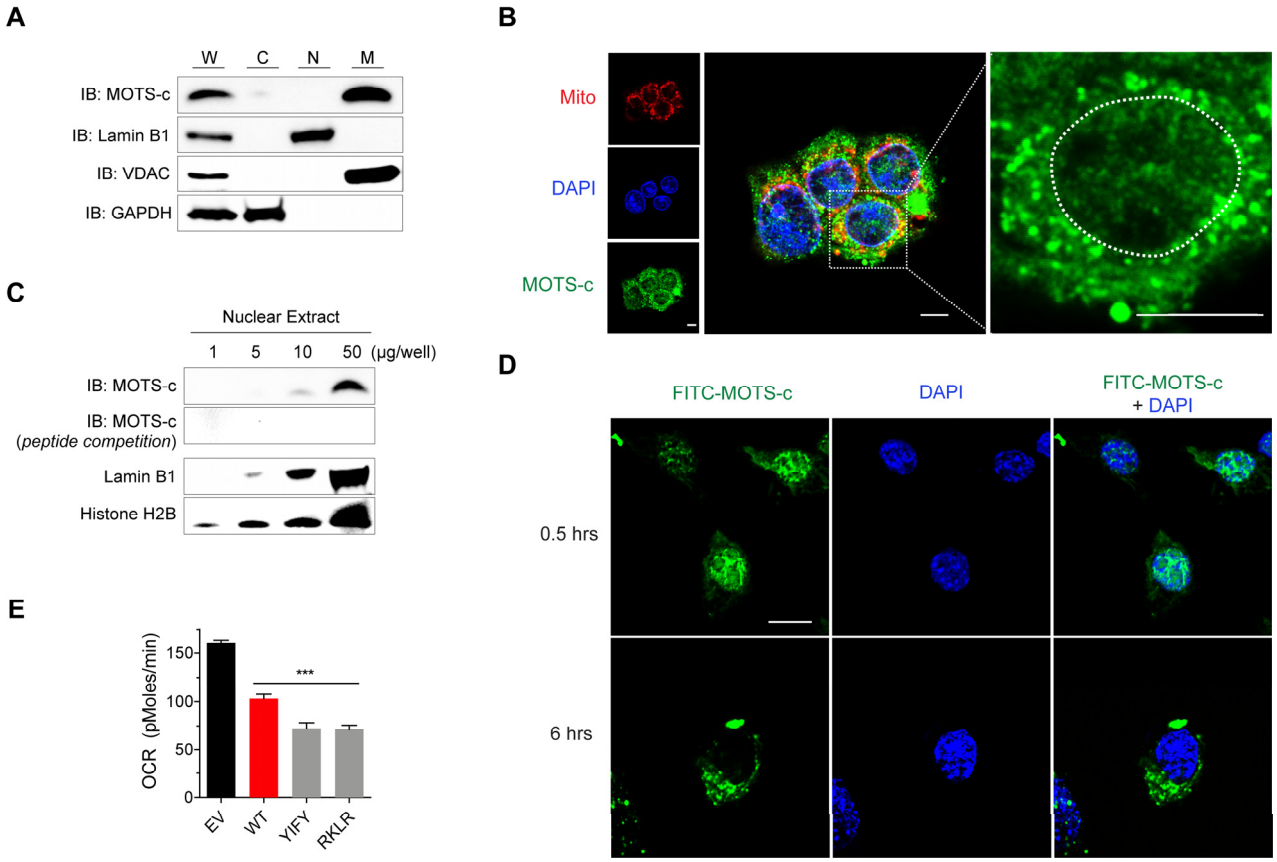


**Figure S1, Related to Figure 1. MOTS-c is a nuclear peptide encoded in the mitochondrial genome**. (**A**) Subcellular distribution pattern of MOTS-c by immunoblotting (equal amounts of total protein loaded for C, N, M) in resting HepG2 cells. (**B**) Immunofluorescence staining for endogenous MOTS-c in resting HepG2 cells. Nucleus is outlined by white dashed lines. Representative images shown (n=3). Cells were co-stained with DAPI (nucleus) and mitotracker red (mitochondria). W: whole cell lysate, C: cytosol, N: nucleus, M: mitochondria. (**C**) Immunoblot of endogenous MOTS-c from nuclear extracts of resting HepG2 cells. Antibody specificity to MOTS-c was confirmed by neutralizing peptide competition. Lamin B1 and histone H2B were used as nuclear loading controls. (**D**) Localization of exogenously treated FITC-MOTS-c peptide (1 μM) in C2C12 myoblasts by confocal microscopy. (**E**) Oxygen consumption rate (OCR), normalized to relative protein concentration, of HEK293 cells that were stably transfected with empty vector (EV) or MOTS-c vectors (WT, YIFY mutant, RKLR mutant) (n=15). WT: wild-type MOTS-c, YIFY: $_{8}$YIFY$_{11}$>$_{8}$AAAA$_{11}$ mutant MOTS-c, and RKLR: $_{13}$RKLR$_{16}$>$_{13}$AAAA$_{16}$ mutant MOTS-c. Scale bar, 10 μm. Error bars represent mean ± s.e.m. ***$p$<0.001 by Student's *t*-test.

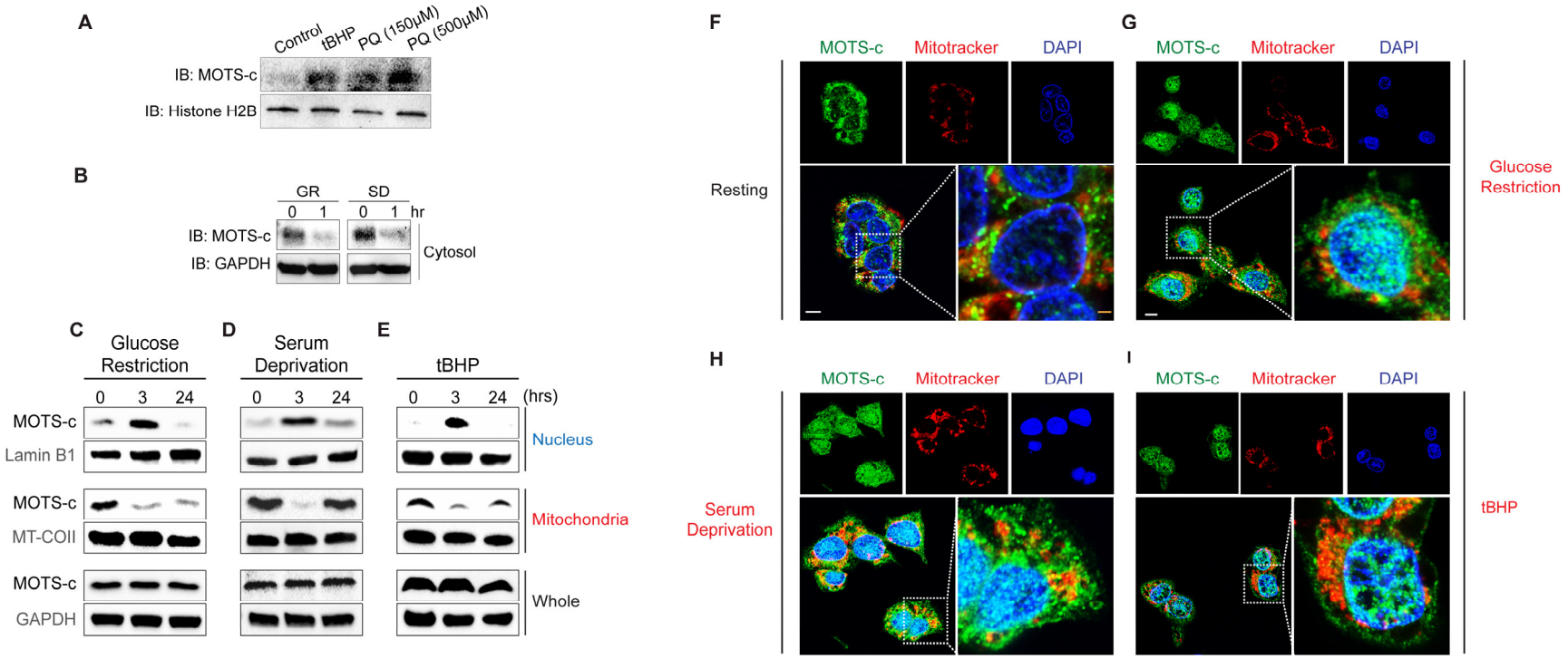


**Figure S2, Related to Figure 2. MOTS-c translocates to the nucleus in response to metabolic stress**. (**A**) MOTS-c levels in the nucleus with and without tBHP (150 μM) or paraquat (PQ) at the indicated concentrations. (**B**) Cytosolic MOTS-c levels detected by immunoblotting with and without GR or SD (1 hr). 50 ug of protein was loaded and the membrane was blotted with 2 ng/uL of MOTS-c antibody (final concentration) and exposed for 4 minutes to detect cytosolic MOTS-c. (**C-I**) Spatial and temporal assessment of MOTS-c localization in HepG2 cells after glucose restriction (GR; 0.5 g/L), serum deprivation (1% FBS), and *tert*-butyl hydrogen peroxide (tBHP; 100 μM) by (**C-E**) subcellular fraction immunoblots and (**F-I**) immunofluorescence microscopy. Subcellular fractions were purified at 0, 3, and 24 hours post-stress, and confocal microscopy images were acquired 3 hours post-stress. Representative images shown (n=3). Cells were co-stained with DAPI (nucleus) and mitotracker red (mitochondria). Scale bar, white: 10 μm, yellow: 2 μm.

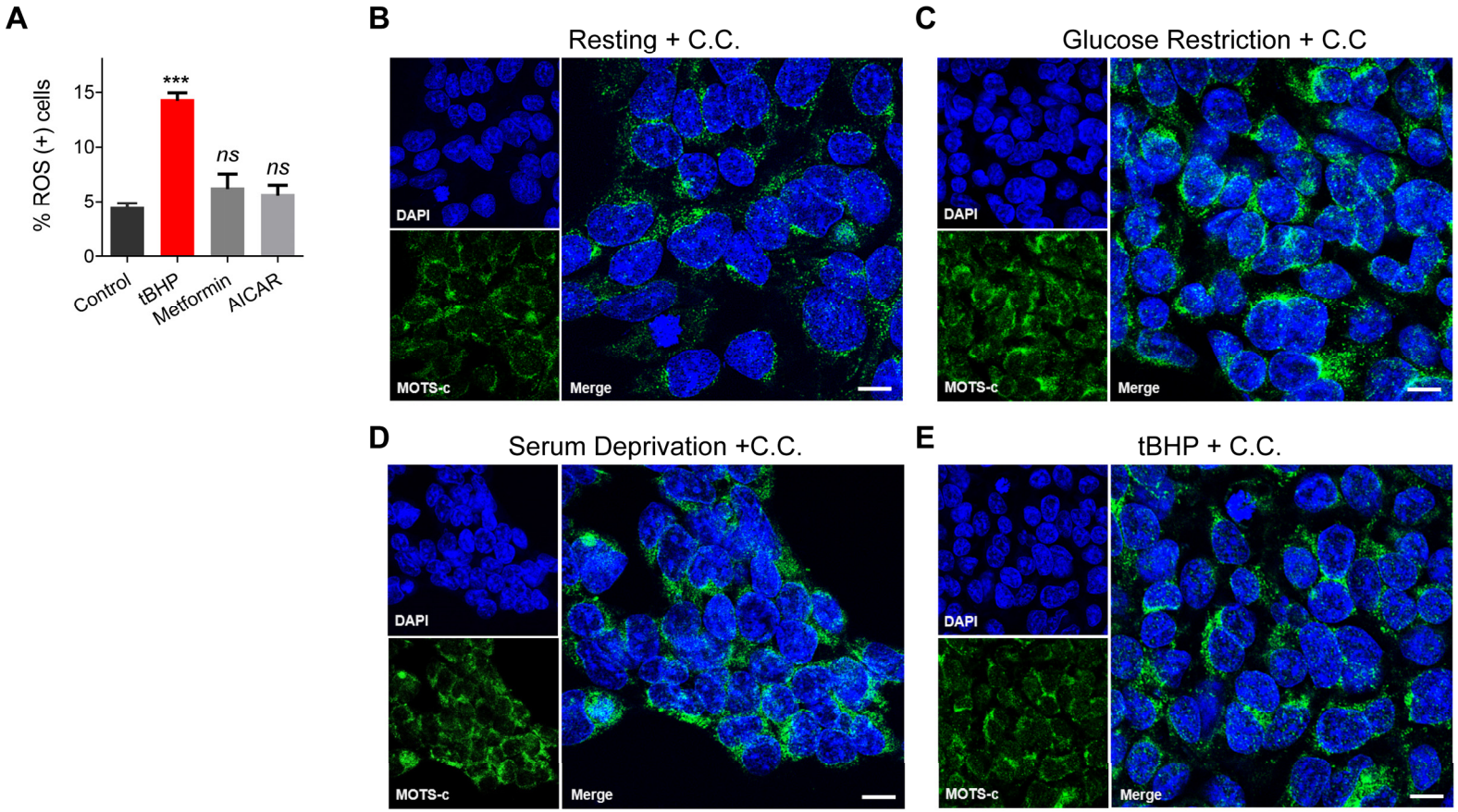


**Figure S3, Related to Figure 3. AMPK inhibition prevents stress-induced nuclear translocation of MOTS-c.** (**A**) Flow cytometry based on DHE to assess time-dependent reactive oxygen species (ROS) production in response to a 1-hour treatment with tBHP (100 μM), metformin (5mM), and AICAR (2mM) in HEK293 cells (n=4). (**B-E)** The level of nuclear MOTS-c by immunofluorescence microscopy in (**B**) resting HEK293 cells and after one hour of (**C**) GR, (**D**) SD, (**E**) tBHP (100 μM) co-treated with compound C (10 μM), an AMPK inhibitor, for 30 minutes prior to stress. Representative images shown (n=3). Cells were co-stained with DAPI (nucleus). Error bars represent mean ± s.e.m. ***$p$<0.001 by Student's $t$-test. Scale bar, 10 μm.

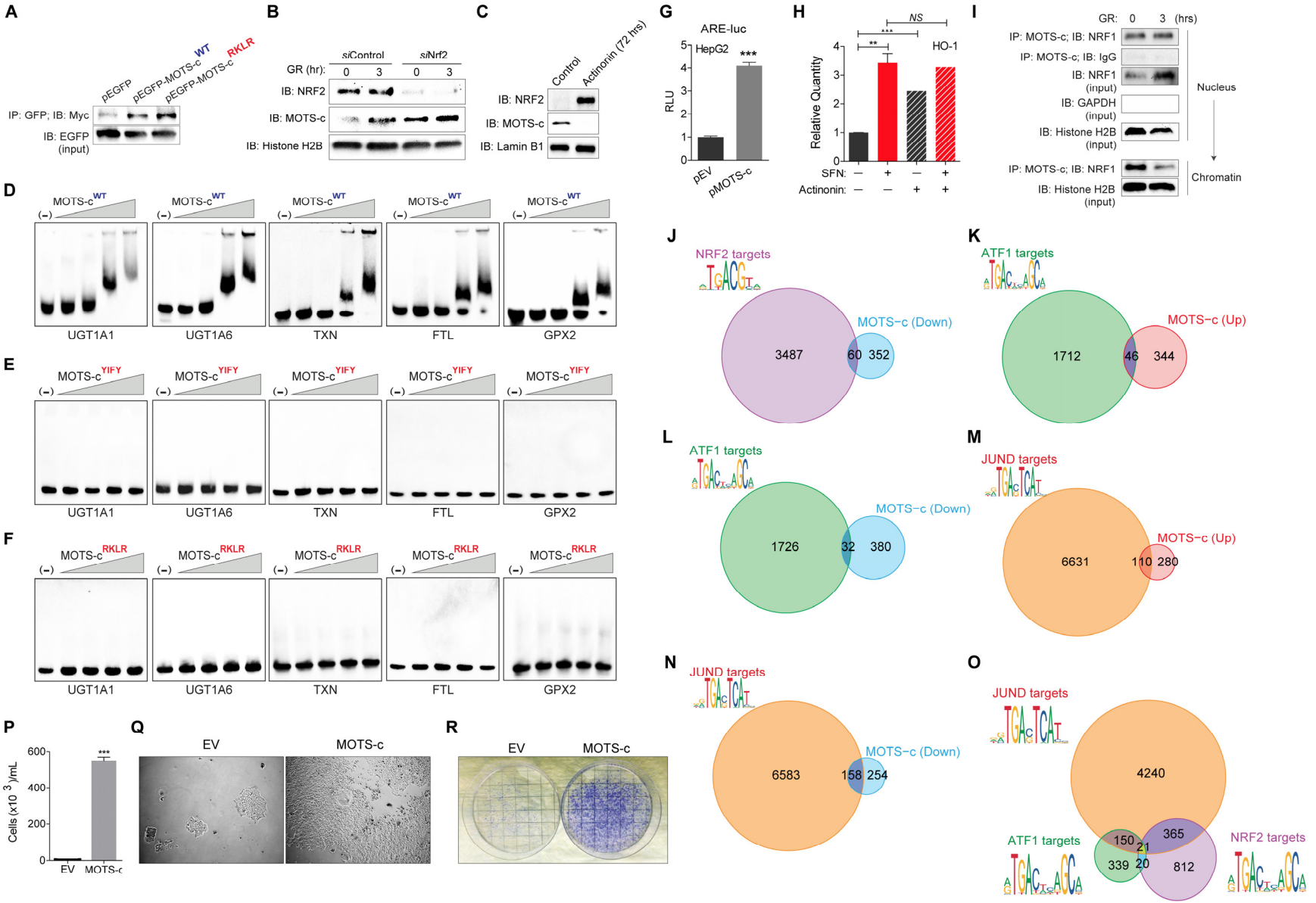
A
pEGFP
pEGFP-MOTS-c WT
pEGFP-MOTS-c RKLR
IP: GFP; IB: Myc
IB: EGFP (input)
B
siControl
siNrf2
GR (hr): 0 3 0 3
IB: NRF2
IB: MOTS-c
IB: Histone H2B
C
Control
Actinonin (72 hrs)
IB: NRF2
IB: MOTS-c
IB: Lamin B1
D
MOTS-c WT
UGT1A1
UGT1A6
TXN
FTL
GPX2
E
MOTS-c YIFY
F
MOTS-c RKLR
G
ARE-luc
HepG2
***
RLU
pEV
pMOTS-c
H
NS
**
***
HO-1
Relative Quantity
SFN:
Actinonin:
I
GR: 0 3 (hrs)
IP: MOTS-c; IB: NRF1
IP: MOTS-c; IB: IgG
IB: NRF1 (input)
IB: GAPDH (input)
IB: Histone H2B (input)
Nucleus
Chromatin
J
NRF2 targets
MOTS-c (Down)
3487
60
352
K
ATF1 targets
MOTS-c (Up)
1712
46
344
L
ATF1 targets
MOTS-c (Down)
1726
32
380
M
JUND targets
MOTS-c (Up)
6631
110
280
N
JUND targets
MOTS-c (Down)
6583
158
254
O
JUND targets
4240
ATF1 targets
NRF2 targets
150
21
365
339
20
812
P
Cells (x10 3 )/mL
600
400
200
0
***
EV
MOTS-c
Q
EV
MOTS-c
R
EV
MOTS-c

**Figure S4, Related to Figure 4. MOTS-c interacts with NRF2 and binds to DNA fragments containing consensus ARE sequences**. (**A**) Co-immunoprecipitation of EGFP-MOTS-c (WT and RKLR mutant) and Myc-tagged Nrf2 (Myc-Nrf2) in HEK293 cells determined by immunoblotting. (**B**) MOTS-c levels in nuclear extracts in response to GR (3 hrs) with and without *NRF2* knockdown using siRNA. (**C**) NRF2 levels in nuclear extracts with and without actinonin treatment (100 μM; 72 hours), which depletes MOTS-c (Lee et al., 2015). (**D-F**) EMSA analysis of ARE motifs in several Nrf2 target genes by incubation with (**D**) WT, (**E**) YIFY mutant MOTS-c, or (**F**) RKLR mutant MOTS-c peptide at 0, 0.5, 1.5, 3, and 6 μg. WT: wild-type MOTS-c, YIFY: $_{8}$YIFY$_{11}$>$_{8}$AAAA$_{11}$ mutant MOTS-c, and RKLR: $_{13}$RKLR$_{16}$>$_{13}$AAAA$_{16}$ mutant MOTS-c. Representative images are shown (n=3). (**G**) ARE luciferase reporter activity on cells transfected with pEV or pMOTS-c in HepG2 cells. Firefly luciferase activity was normalized to *Renilla* luciferase activity (n=4). (**H**) qRT-PCR analysis of HO-1 expression in response to the sulforaphane (SFN; 10 μM) and actinonin (150 μM) in HEK293 cells (n=3). (**I**) Co-immunoprecipitation of MOTS-c and NRF1 from nuclear extracts and chromatin extracts derived thereof of HEK293 cells after GR (3 hrs) determined by immunoblotting. (**J-O**) RNA-seq-derived Venn diagrams of genes upregulated by MOTS-c and *bona fide* target genes as indicated (n=6). (**P-R**) HEK293 cells stably transfected with MOTS-c plasmid (or EV) underwent GR+SD for 4 days and allowed to recover for 7 days in complete media. Survival was assessed by (**P**) live cell counts using trypan blue exclusion, (**Q**) phase contrast images, and (**R**) crystal violet staining of cells (n=6). Error bars represent mean ± s.e.m. $^{**}p<0.01$, $^{***}p<0.001$ by Student's *t*-test.

**Real-time PCR primer sequences**

| Gene Name | Forward primer | Reverse primer | Reference |
|---|---|---|---|
| HO-1 | CTCAAACCTCCAAAAGCC | TCAAAAACCACCCCAACCC | [(Reichard et al., 2007)] |
| NQO-1 | TGCAGCGGCTTTGAAGAAGAAAGG | TCGGCAGGATACTGAAAGTTCGCA | [(Seng et al., 2009)] |
| RPL27 | CATTGATGATGGCACCTCAG | TTGTGGGCATTAGGTGATTG | This study |

**ChIP-qPCR primer sequences**

| Gene Name | Forward primer | Reverse primer | Reference |
|---|---|---|---|
| HO-1 | GGCTCCCAGAGAACAGTTAGAAAAG | GGACTTGGCCAGGCTATGC | [(Reichard et al., 2007)] |
| NQO-1 | CCCTTTTAGCCTTGGCACGAAA | TGCACCCAGGGAAGTGTGTTGTAT | [(Sahdeo et al., 2014)] |

**EMSA oligonucleotide sequences**

| Gene Name | Sequence | Reference |
|---|---|---|
| HO-1 | CCTCCCAAACATGACGCAGCAGAAATGC | [(Rushworth et al., 2005)] |
| NQO-1 | CAGTCACAGTGACTCAGCAGAATCT | [(Wang et al., 2007)] |
| UGT1A1 | GGACTTGGCACTTGGTAAGCACGCAATGAACAGTCA | [(Yueh and Tukey, 2007)] |
| UGT1A6 | CAGAAGCTCAGGTGAAAGCTGACACGGCCATAGT | [(Munzel et al., 2003)] |
| TXN | GGTCACCGTTACTCAGCACTTTGTGGGGTTCAC | [(Kim et al., 2001)] |
| FTL | GAGCTCAGCATGACTCAGCAGTC | [(Hintze and Theil, 2005)] |
| GPX2 | AATTGTACAGTGAGAGGGCAGG | [(Banning et al., 2005)] |

**Table. S1, Related to Figure 4. List of primers and oligonucleotides used for real-time PCR, ChIP-qPCR, and EMSA.**

**Table. S2, Related to Figure 4. Gene ontology (GO) analysis on RNA-seq performed on HEK293 cells that overexpressed MOTS-c (*vs* EV) under GR (3 hrs).**