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Supplemental Material to:

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The deubiquitylase USP15 stabilizes newly synthesized REST and rescues its expression at mitotic exit

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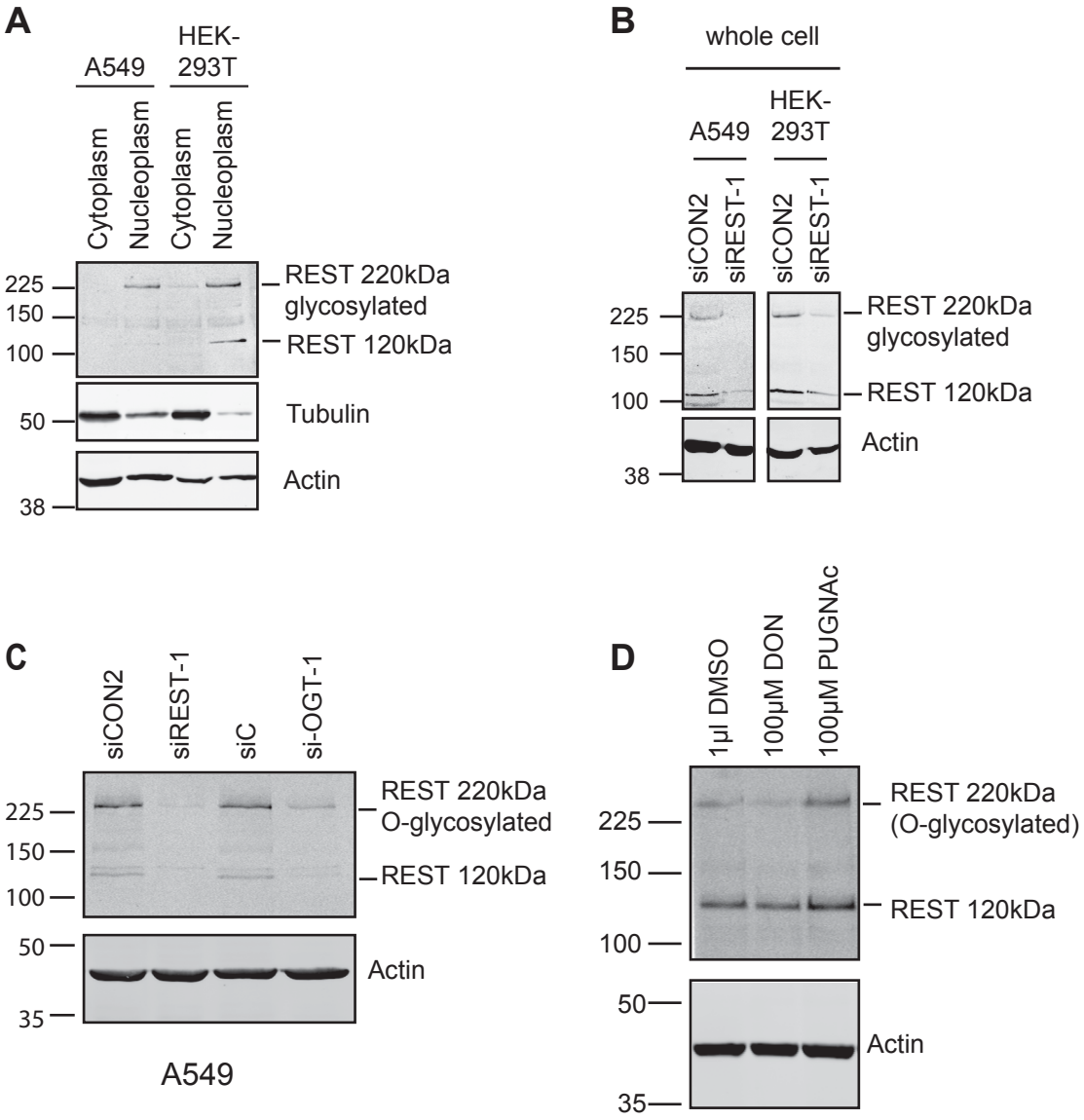
A
A549
HEK-293T
Cytoplasm
Nucleoplasm
Cytoplasm
Nucleoplasm
225
150
100
50
38
REST 220kDa glycosylated
REST 120kDa
Tubulin
Actin
B
whole cell
A549
HEK-293T
siCON2
siREST-1
siCON2
siREST-1
225
150
100
38
REST 220kDa glycosylated
REST 120kDa
Actin
C
siCON2
siREST-1
siC
si-OGT-1
225
150
100
50
35
REST 220kDa O-glycosylated
REST 120kDa
Actin
A549
D
1µl DMSO
100µM DON
100µM PUGNAc
225
150
100
50
35
REST 220kDa (O-glycosylated)
REST 120kDa
Actin

Fig. S1

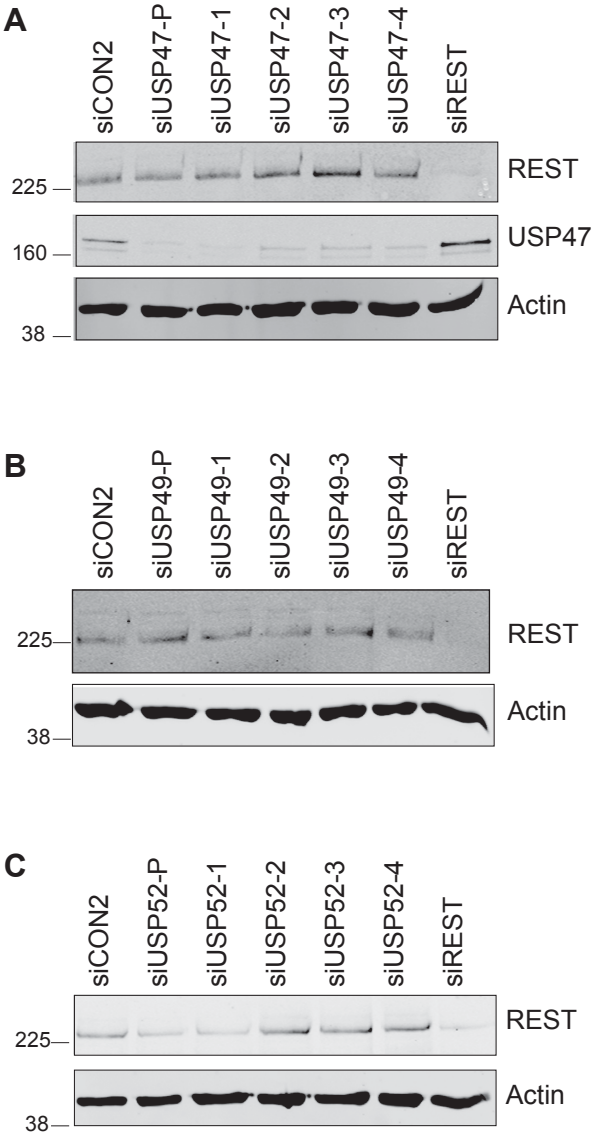
A
siCON2
siUSP47-P
siUSP47-1
siUSP47-2
siUSP47-3
siUSP47-4
siREST
225
160
38
REST
USP47
Actin
B
siCON2
siUSP49-P
siUSP49-1
siUSP49-2
siUSP49-3
siUSP49-4
siREST
225
38
REST
Actin
C
siCON2
siUSP52-P
siUSP52-1
siUSP52-2
siUSP52-3
siUSP52-4
siREST
225
38
REST
Actin

Fig. S2

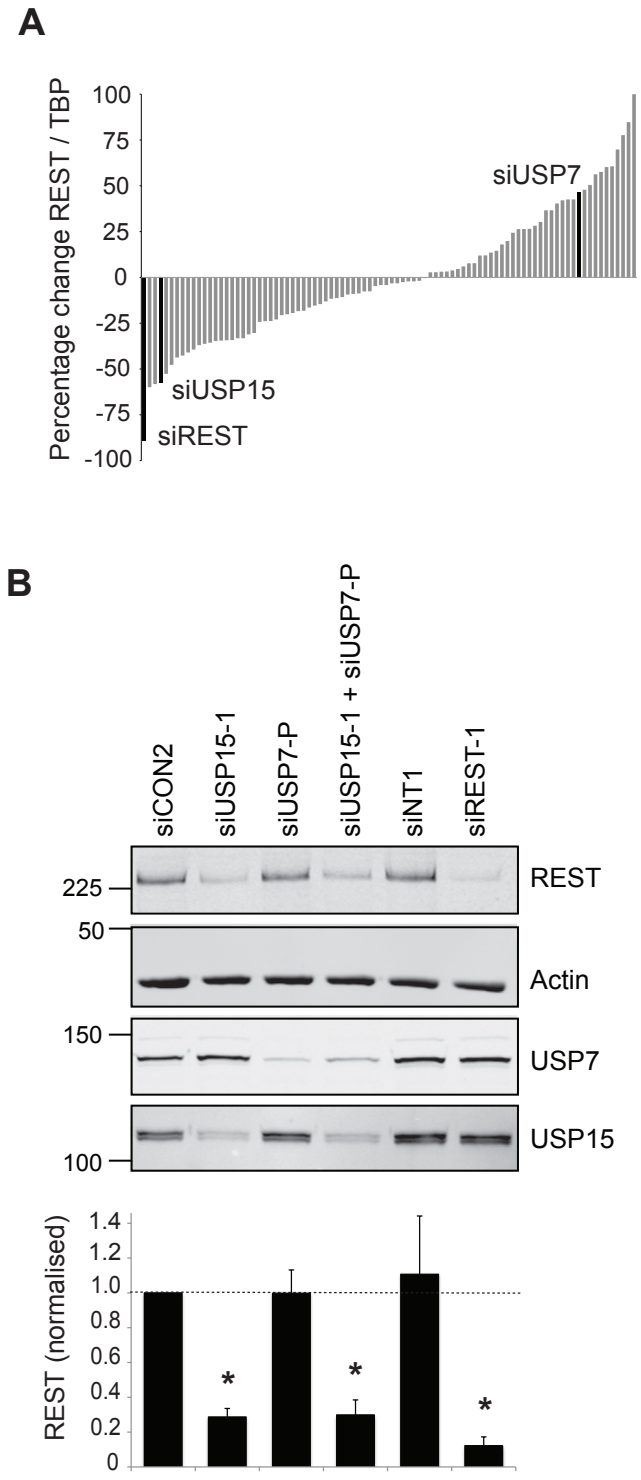
A
Percentage change REST / TBP
100
75
50
25
0
-25
-50
-75
-100
siUSP7
siUSP15
siREST
B
siCON2
siUSP15-1
siUSP7-P
siUSP15-1 + siUSP7-P
siNT1
siREST-1
225
50
150
100
REST
Actin
USP7
USP15
REST (normalised)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
*

Fig. S3

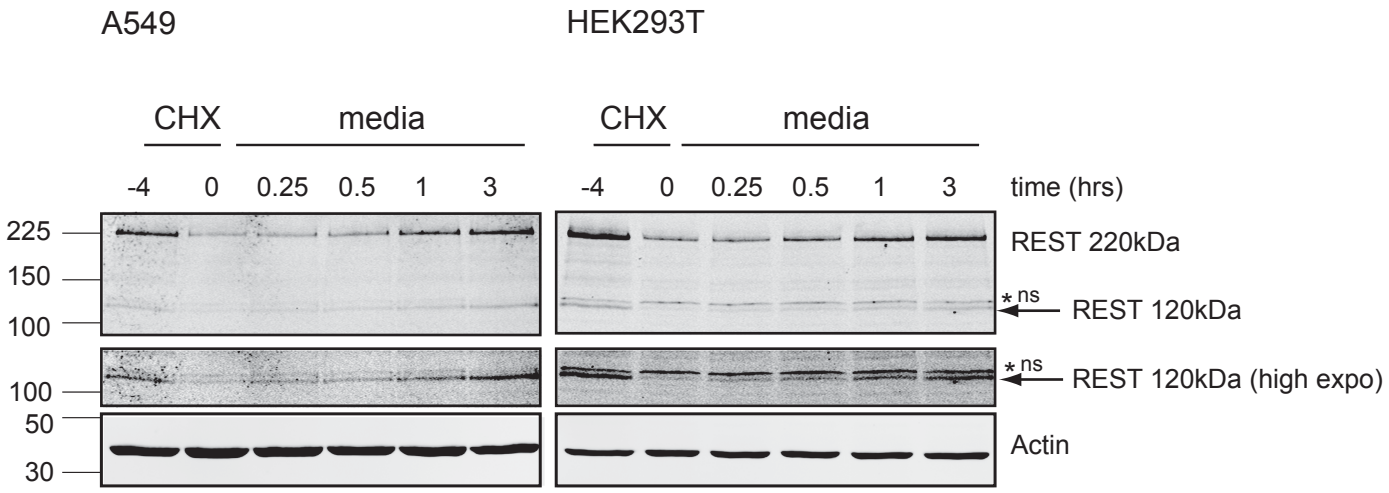
A549
HEK293T
CHX
media
CHX
media
-4
0
0.25
0.5
1
3
-4
0
0.25
0.5
1
3
time (hrs)
225
150
100
100
50
30
REST 220kDa
* ns
REST 120kDa
* ns
REST 120kDa (high expo)
Actin

Fig. S4

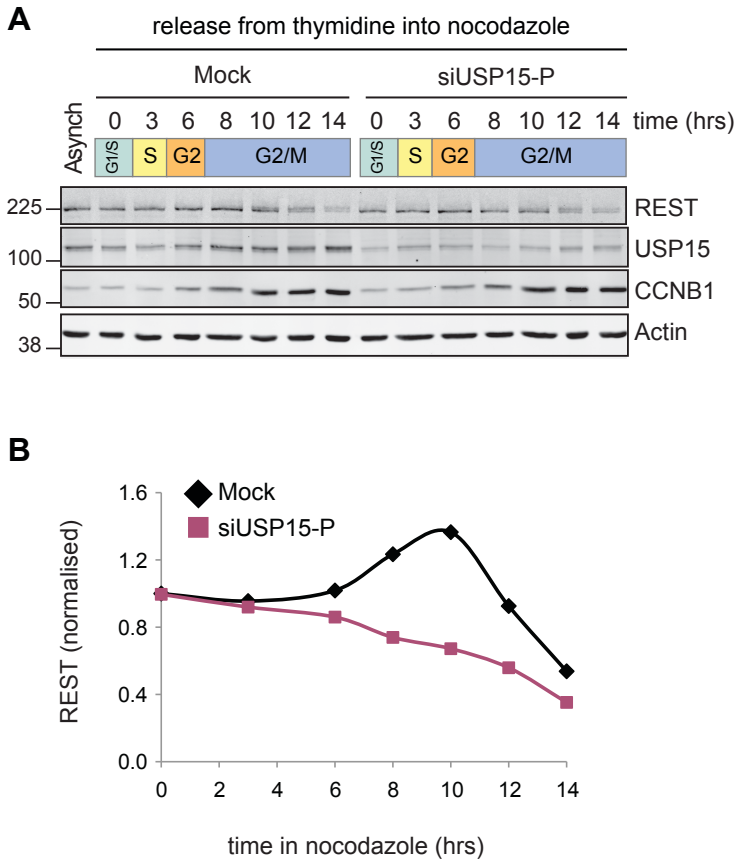
A
release from thymidine into nocodazole
Mock
siUSP15-P
Asynch
0 3 6 8 10 12 14
0 3 6 8 10 12 14
time (hrs)
G1/S
S
G2
G2/M
G1/S
S
G2
G2/M
225
100
50
38
REST
USP15
CCNB1
Actin
B
Mock
siUSP15-P
REST (normalised)
1.6
1.2
0.8
0.4
0.0
0 2 4 6 8 10 12 14
time in nocodazole (hrs)

Fig. S5

Supplementary Figure Legends

Fig. S1. Characterization of REST expression in model cell lines.

A. Fractionated protein extracts were prepared from A549 or HEK-293T cells and immunoblotted for REST using a rabbit polyclonal anti-REST antibody (07-579, Millipore). REST expression is in most part restricted to the nucleoplasm. 220kDa glycosylated REST is the predominant form in A549 NSCLC cells, but an equivalent amount of nascent 120kDa REST is present in HEK-293T cells. **B.** A549 or HEK-293T cells were transfected with a control siRNA or siRNA targeting REST, whole cell extracts were prepared 72hrs later in Laemmli buffer and probed for REST. 120kDa REST is more apparent in these extracts and again is more prevalent in HEK-293T cells. The REST targeting siRNA reduces both the 120kDa and 220kDa immunoreactive bands. **C**. Prolonged inhibition of O-glycosylation by depletion of OGT leads to a loss of both 220kDa and 120kDa REST, suggesting REST is unstable when not glycosylated. A549 Cells were transfected with siRNAs as indicated for 72 hrs prior to immunoblotting. **D.** Transient pharmacological intervention in *O*-glycosylation by addition of an inhibitor (DON) lead to loss of 220kDa REST with only a mild decrease in 120kDa REST. Addition of the OGT agonist (PUGNAc) increased 220kDa REST. Drugs were added to HEK-293T cells at the indicated concentrations for 15 hours, prior to lysis in hot SDS extraction buffer.

Fig. S2. Deconvolution of other candidate DUBs.

A549 cells were transfected as indicated for 72 hours with either siRNA pools used in the DUB library screen, or with the four individual siRNAs that composed these pools. REST abundance was assessed after depletion of: **A**, USP47, **B**, USP49, or **C**, USP52. None of these candidates were validated as potential REST DUBs.

Fig. S3. USP7 depletion does not affect REST protein levels in asynchronous A549 cells.

A. USP7 was not identified as a candidate DUB for REST in A549 cells by siRNA screening. The dataset from Figure 1A is shown highlighting the position of the USP7 siRNA within the ranking. **B.** USP7 depletion did not diminish REST or augment the effect of USP15. USP7 (using an independent siRNA pool) and USP15 were depleted from A549 cells for 72hrs, either in parallel or in concert as indicated. Knockdown efficiency was 89% for USP7 and 85% for USP15; siCON2 and siNT1 are non-targeting control siRNAs. REST was estimated by immunoblotting and the quantification relative to actin for three independent experiments is shown below (*$P<0.05$, error bars show std dev).

Fig. S4. Newly synthesized REST appears in both 120kDa unmodified and 220kDa O-glycosylated forms within 30 min of release from a translational block.

A549 or HEK293T cells were treated with cycloheximide for 4 hours, before washing out the inhibitor and releasing into fresh medium (0hrs) to allow new protein synthesis. Whole cell protein extracts were sampled from 15 min to 3hrs and analysed by immunoblotting. A non-specific band is indicated by an asterisk (*ns).

Fig. S5. USP15 does not antagonise degradation of phosphorylated REST during prometaphase arrest.

In USP15 depleted cells, REST fails to accumulate as cells enter G2/M, but still degrades on prolonged G2/M arrest. A549 cells were transfected with siRNA prior to thymidine synchronisation, then released from G1/S into media containing nocodazole. **A.** Protein expression was monitored by immunoblotting over 14hrs and representative blots are shown. **B.** Total REST and USP15 were quantified from duplicate gels, normalised to actin and plotted relative to their level in control cells.