SUPPLEMENTARY INFORMATION

Phosphorylation of Mcl-1 by CDK1-cyclin B1 initiates its Cdc20-dependent destruction during mitotic arrest

Margaret E. Harley, Lindsey A. Allan, Helen S. Sanderson and Paul R. Clarke

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Antibodies and reagents

The following antibodies were used for immunoblotting according to standard protocols: total caspase-3 (Santa Cruz Biotechnology); Bcl-x_L, Bim (Cell Signalling); Noxa (AbCam). Histone H3 phospho-Ser10 (Millipore) was used for flow cytometry. The caspase inhibitor, Z-VAD-FMK (Biomol), was used at 10 μM for 2 h prior to further treatment. Staurosporine (Calbiochem) was used at 3 μM for 5 h.

Determination of mitotic index by flow cytometry

Ser10 phospho-histone H3 analysis by flow cytometry was carried out as described previously (Allan & Clarke, 2007).

FIGURE LEGENDS

Supplementary Figure 1. Mcl-1 depletion sensitizes U2OS cells to nocodazole-induced apoptosis. Mcl-1 was depleted from U2OS cells using siRNA. After 48 h, cells were treated with 250 ng/ml nocodazole for a further 48 h or left untreated. Sub-G1 DNA content (<2*N*) of asynchronous (As) and nocodazole-treated cells (Noc) was analysed by flow cytometry.

The percentage of apoptotic cells (boxed) was greater in cells depleted of Mcl-1 compared to control siRNA (Luc), nocodazole-treated cells. The efficiency of Mcl-1 depletion at 48 h was determined by Western blotting (lower panel).

Supplementary Figure 2. Mitotic arrest is maintained in synchronised U2OS cells. U2OS cells were treated with 100 ng/ml nocodazole for 2 h. Mitotic cells were isolated and re-plated for a further 2 or 4 h so that cells were arrested in mitosis for a total of 2, 4 or 6 h. Then floating cells were collected together with any adherent cells before the mitotic index (%, boxed) was determined by flow cytometry, using Ser10 phospho-histone H3 as a marker of mitosis. The majority of cells collected after 2 h nocodazole treatment are mitotic (75%) and this is maintained at each subsequent time point, demonstrating that cells synchronized in mitotic arrest by this protocol do not slip out of mitosis but remain arrested in mitosis.

Supplementary Figure 3. Bcl-2 and Bcl-x_L are not degraded during mitotic arrest. U2OS cells were synchronised in mitosis by the 2 h nocodazole treatment and re-plating protocol. Cell lysates were immunoblotted for Bcl-2 and Bcl-x_L.

Supplementary Figure 4. Mitotic degradation of endogenous Mcl-1 is not caspase dependent. U2OS cells were treated with 10 μM caspase inhibitor Z-VAD-FMK for 2 h, prior to mitotic arrest with 100 ng/ml nocodazole (Noc) (upper panels). As a positive control to confirm the efficacy of Z-VAD-FMK, a broad spectrum protein kinase inhibitor, staurosporine (STS), was used to induce apoptosis. In this case, U2OS cells were treated with 10 μM Z-VAD-FMK for 2 h, before addition of 3 μM STS for 5 h (lower panels). Samples were analysed by immunoblotting. Z-VAD-FMK failed to inhibit the destruction of Mcl-1 in

nocodazole-arrested cells, but did inhibit the cleavage of caspase-3 in response to staurosporine.

Supplementary Figure 5. Specificity of antibodies generated against Mcl-1 phosphorylation sites. Antibodies were tested against BSA-conjugated phosphopeptides. Dot blots show that pT92 Mcl-1 and pS64 Mcl-1 are specific to their phosphorylated peptides and do not recognise the corresponding non-phosphorylated peptides or BSA.

Supplementary Figure 6. Phosphorylation of Mcl-1 at Thr92 and Ser64 during the cell cycle. U2-Mcl-1-WT cells were synchronised at the G1/S boundary by double thymidine block then released into the cell cycle. Samples were analysed by SDS-PAGE and immunoblotting at the times indicated after release from the block. Asynchronous (As) and nocodazole-arrested (NM) samples are shown for comparison. Phosphorylation of both Thr92 and Ser64 was detected in samples enriched with mitotic cells (10-16 h), as indicated by phosphorylated Ser10 of histone H3.

Supplementary Figure 7. Mcl-1 is phosphorylated at Thr92 in mitosis and during mitotic arrest. Endogenous Mcl-1 was immunoprecipitated from U2OS cells growing in asynchronous culture (As), mitotic cells harvested by wash off (M) or from cells following treatment with nocodazole (100 ng/ml) for 2 h prior to separation of mitotic (NM) and adherent (NA) cells. Immunoprecipitates were immunoblotted for Mcl-1 phosphorylated at Thr92 or total Mcl-1. Total cell lysates used for the precipitations are shown (Input). Phosphorylation of Mcl-1 at Thr92 was detected only in mitotic cells: both during normal mitosis and during mitotic arrest following nocodazole treatment.

Supplementary Figure 8. Flow cytometry profiles of data from one experiment contained in graph in Figure 8A. U2-Mcl-1-WT, U2-Mcl-1-T92A, U2-Mcl-1-S64A and U2-eV cells were treated with 250 ng/ml nocodazole for 45 h. Flow cytometry was used to measure sub-G1 (<2*N*) DNA content (%, boxed) of asynchronous and nocodazole-treated cells. Cells expressing the T92A mutant of Mcl-1 are resistant to nocodazole-induced apoptosis.

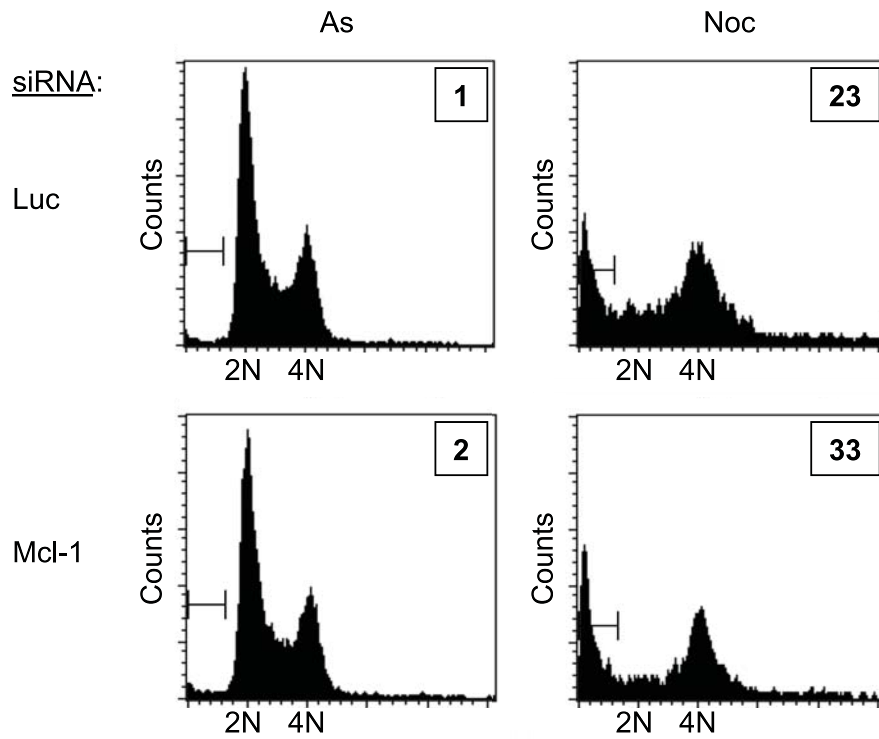
Supplementary Figure 9. Cells expressing the D-box mutant of Mcl-1 are resistant to nocodazole-induced apoptosis. U2-Mcl-1-WT, U2-Mcl-1-D-box and U2-Mcl-1-ΔIR cells were treated with 250 ng/ml nocodazole for 45 h. Flow cytometry was used to measure sub-G1 (<2*N*) DNA content (%, boxed) of asynchronous and nocodazole-treated cells.

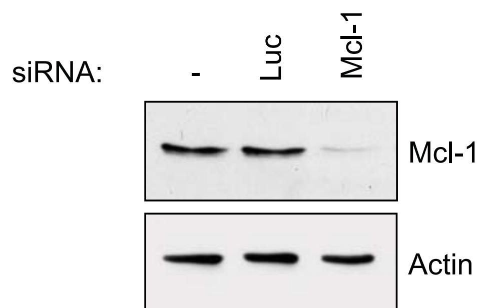
Supplementary Figure 10. U2OS cells stably transfected with Mcl-1 (WT, T92A or D-box mutant) were treated with nocodazole (250 ng/ml) for 45 h. Cells were analysed for sub-G1 DNA content by flow cytometry (+/- SEM, n=3). Statistical analyses were performed using a two-tailed unpaired Student's *t*-test. The percentage of cells with sub-G1 DNA content in T92A or D-box mutant cells was significantly different from WT ($p<0.002$). The expression level of Mcl-1 WT, T92A or D-box mutant proteins was determined by Western blotting (lower panel).

Supplementary Figure 11. Phosphorylation of Mcl-1 at Ser64 and Thr92 does not affect binding to Noxa or Bim. The co-precipitation of proteins with Flag-Mcl-1 was examined in U2-Mcl-1-WT, U2-Mcl-1-T92A, U2-Mcl-1-S64A and U2-eV cells in asynchronous culture (left panels) or treated with 10 μM MG132 for 2 h, prior to addition of 100 ng/ml nocodazole (Noc) for 4 h (right panels). The cell lysates (Input) and Flag-tagged Mcl-1 immunoprecipitates (Flag IP) were analysed by SDS-PAGE and immunoblotting with the

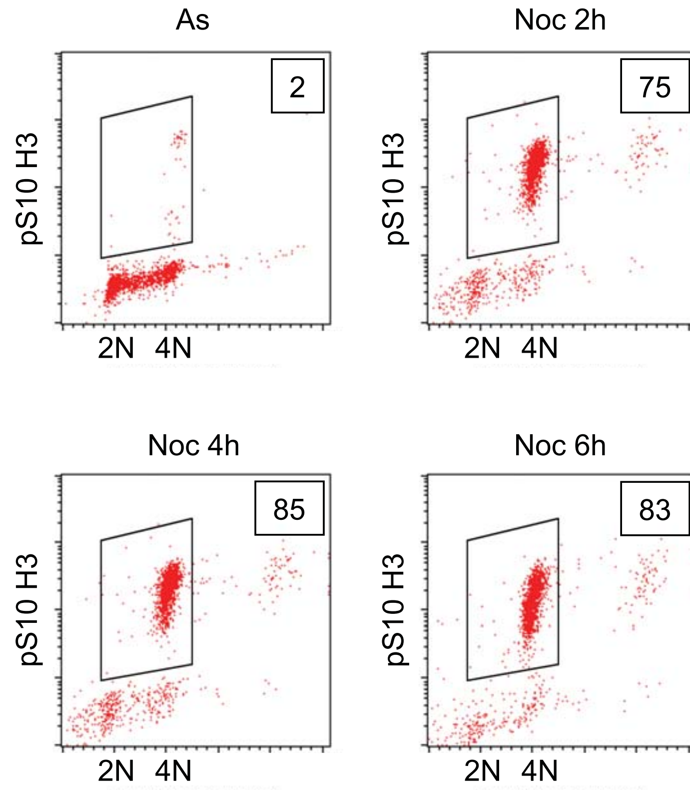
specified antibodies. Association of the pro-apoptotic proteins Noxa and Bim with Mcl-1 was not inhibited by the T92A or S64A phosphorylation site mutants of Mcl-1, even when phosphorylated Mcl-1 was stabilized in cells arrested in mitosis.

SUPPLEMENTARY FIGURES

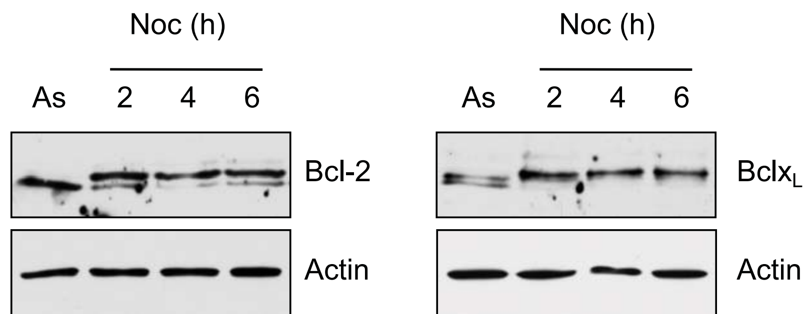




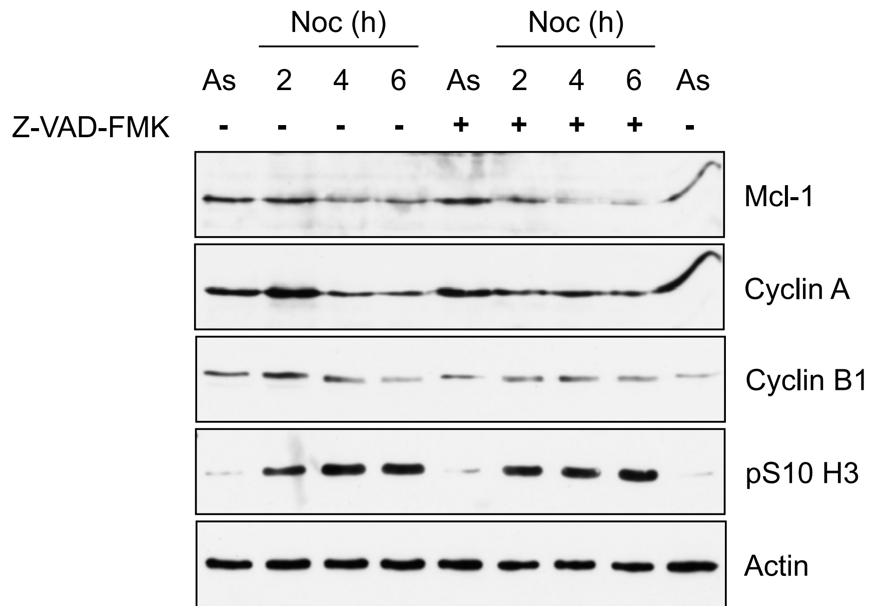
Supplementary Figure 1

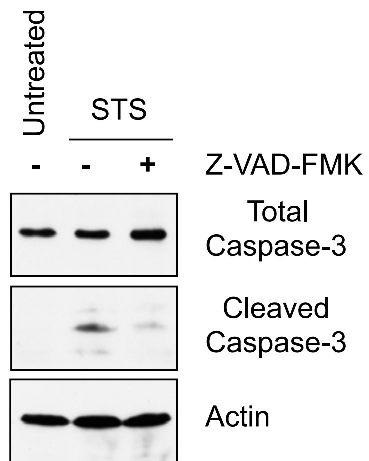


Supplementary Figure 2

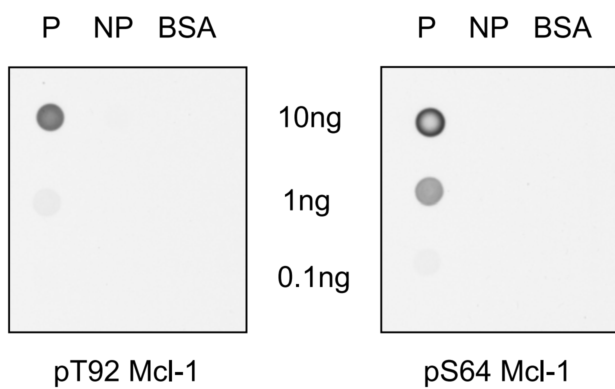


Supplementary Figure 3

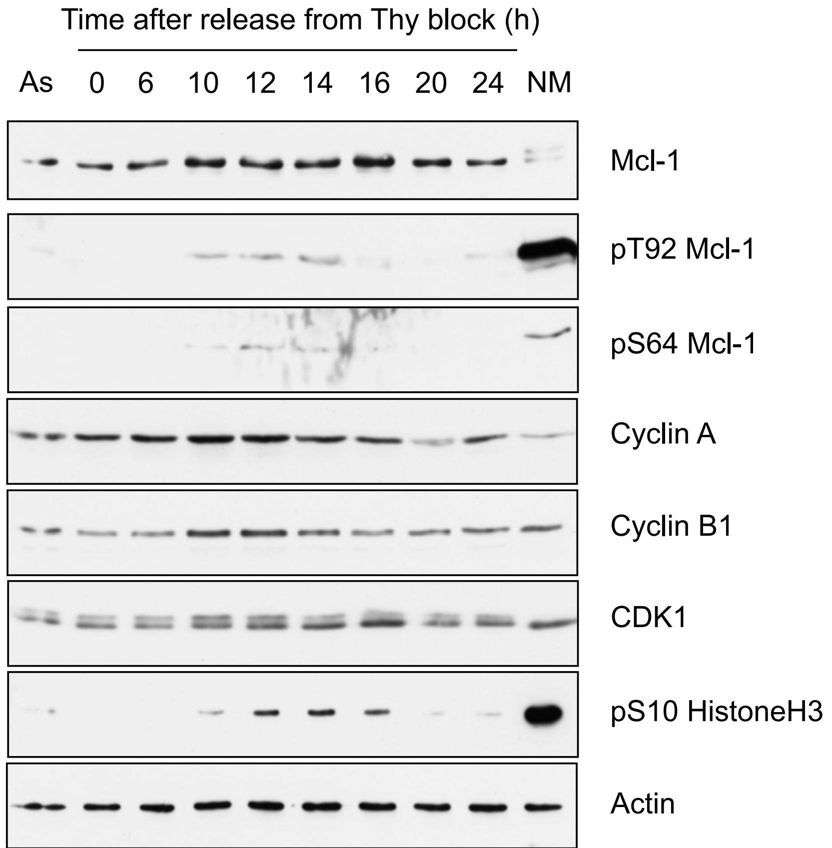




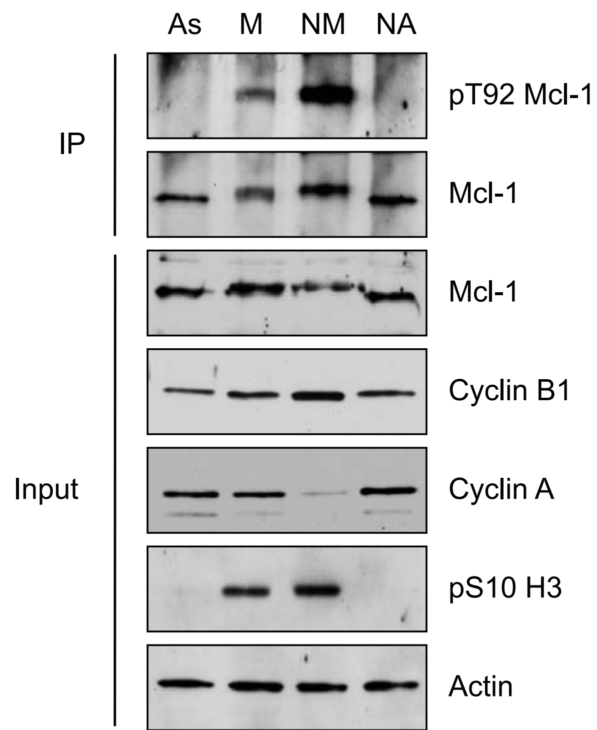
Supplementary Figure 4



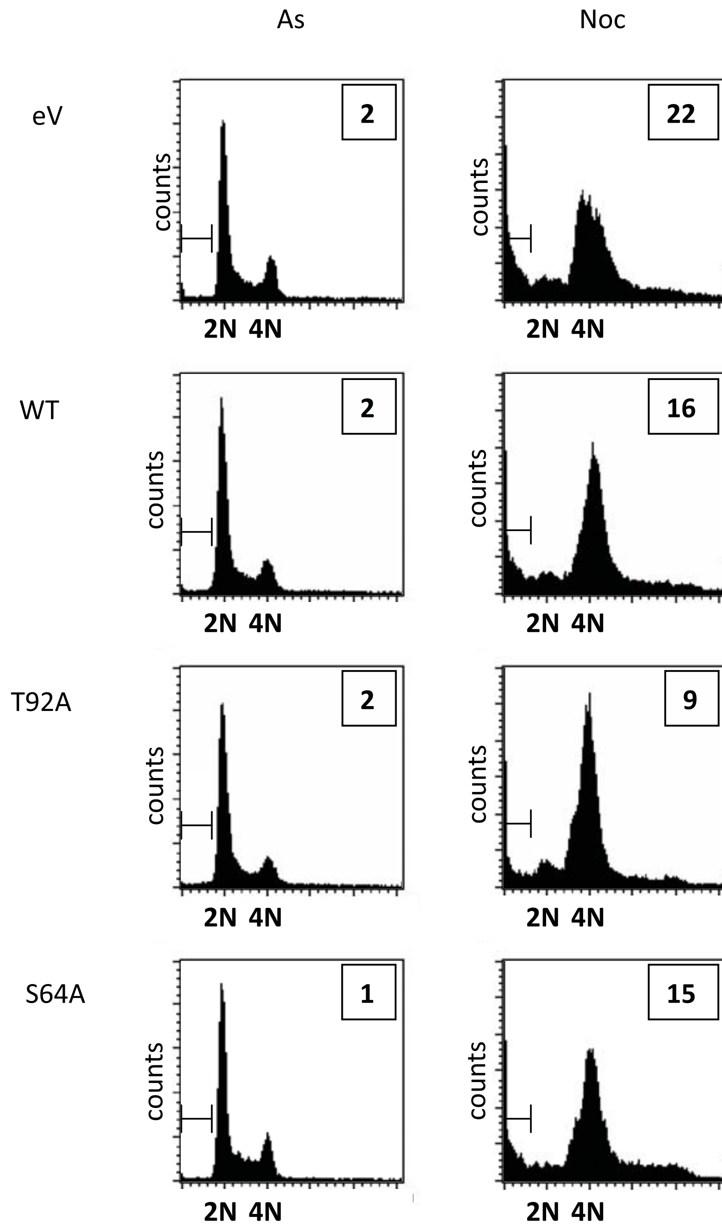
Supplementary Figure 5



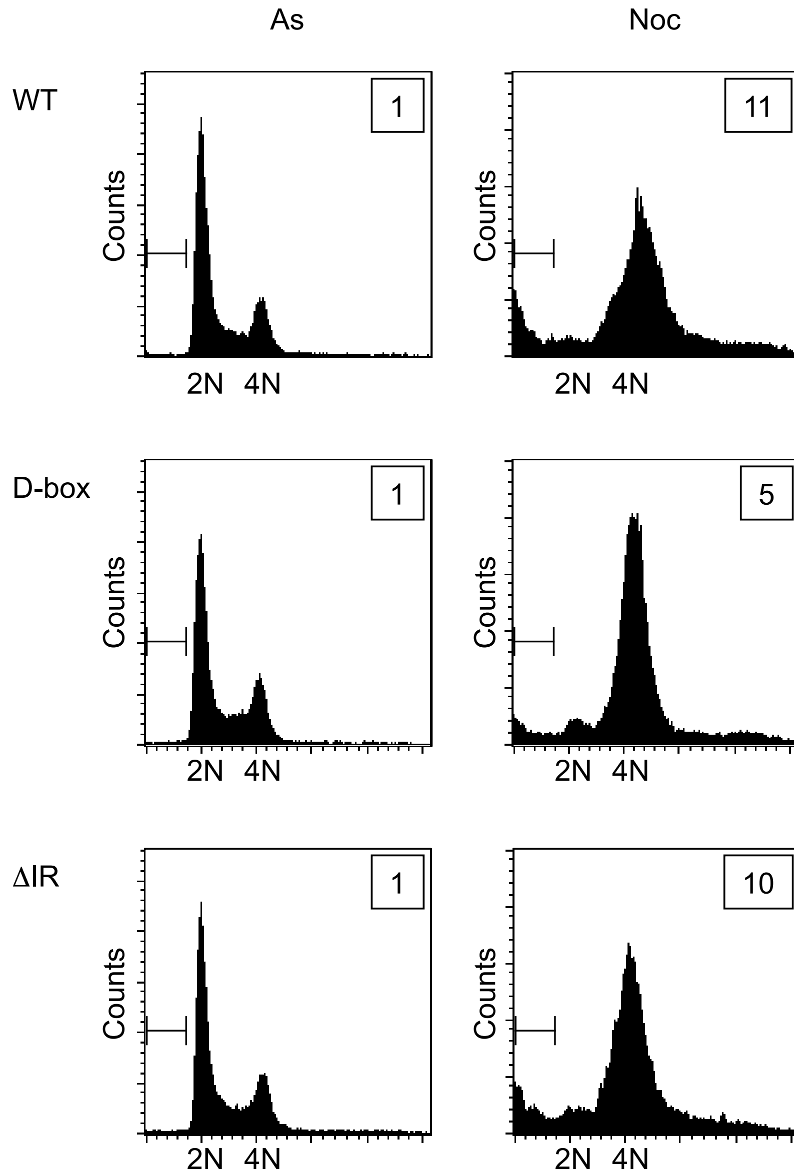
Supplementary Figure 6



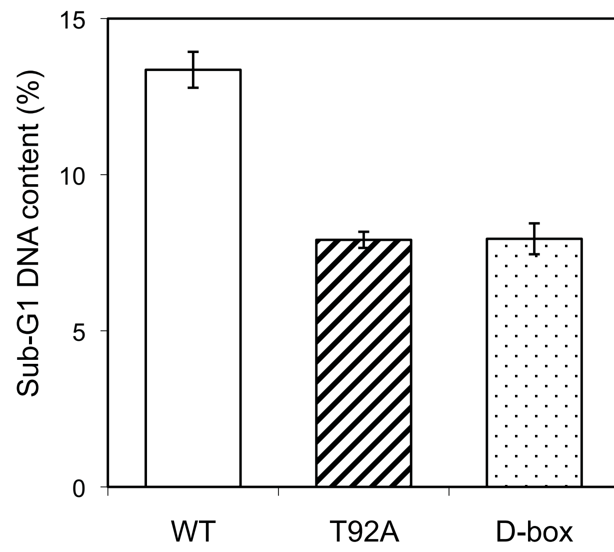
Supplementary Figure 7

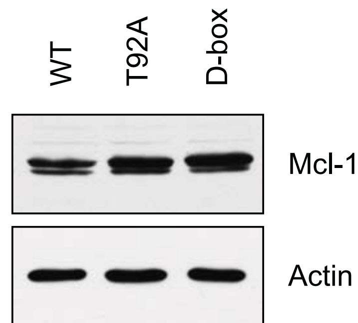


Supplementary Figure 8

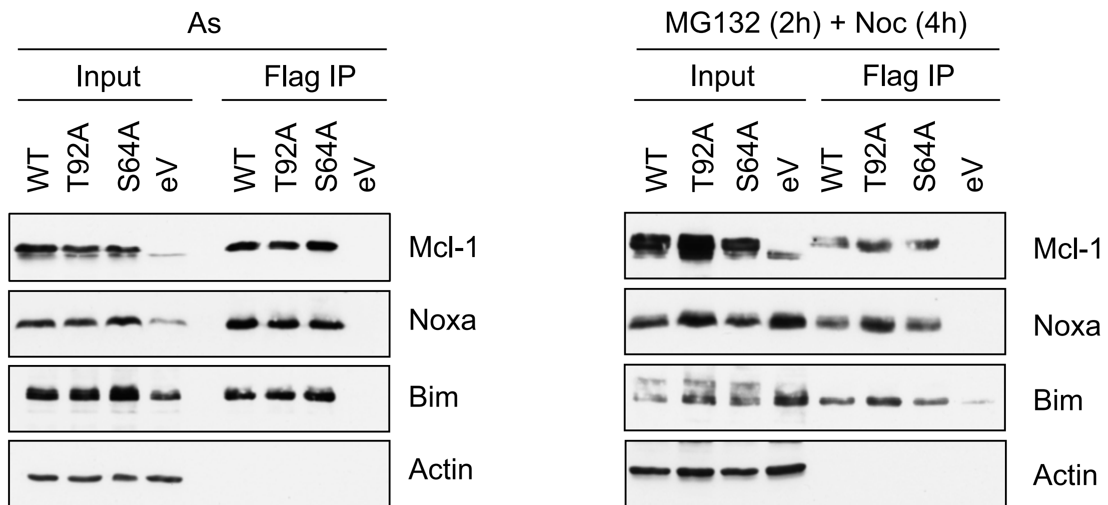


Supplementary Figure 9





Supplementary Figure 10



Supplementary Figure 11