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Supplementary Materials for

Dysfunctional MnSOD leads to redox dysregulation and activation of prosurvival AKT signaling in uterine leiomyomas

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This PDF file includes:

Supplementary Materials

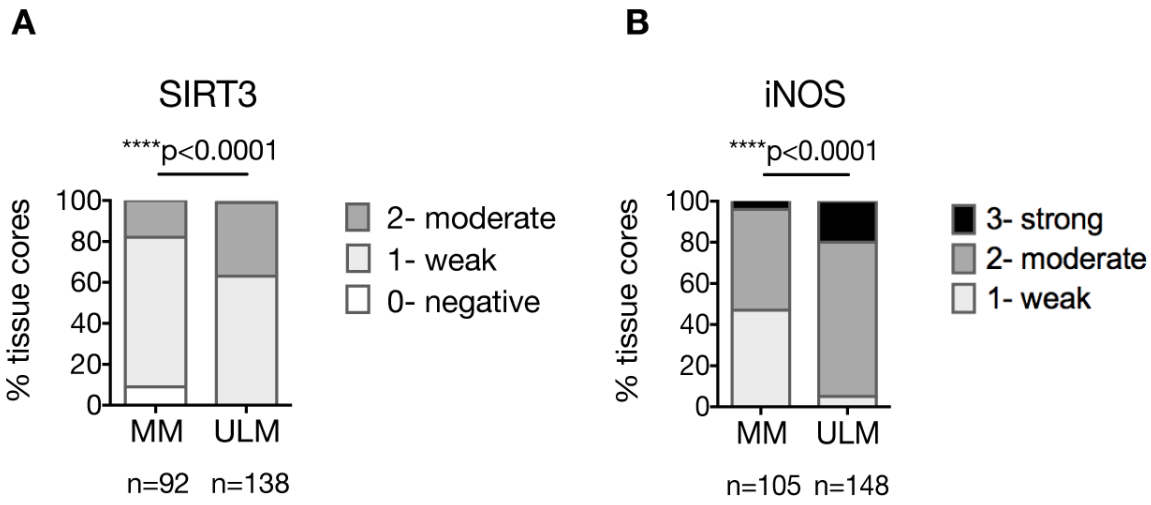


fig. S1. SIRT3 and iNOS protein levels in ULM. (**A** and **B**) The TMA containing 60 matched MM and ULM specimens was immunostained with SIRT3 and iNOS antibody as described in Materials and Methods. The staining intensity of well-preserved matched MM/ULM tissue cores was scored numerically as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong). Statistically significant differences between normal MM and ULM were evaluated using a Chi-square test (****p<0.0001).

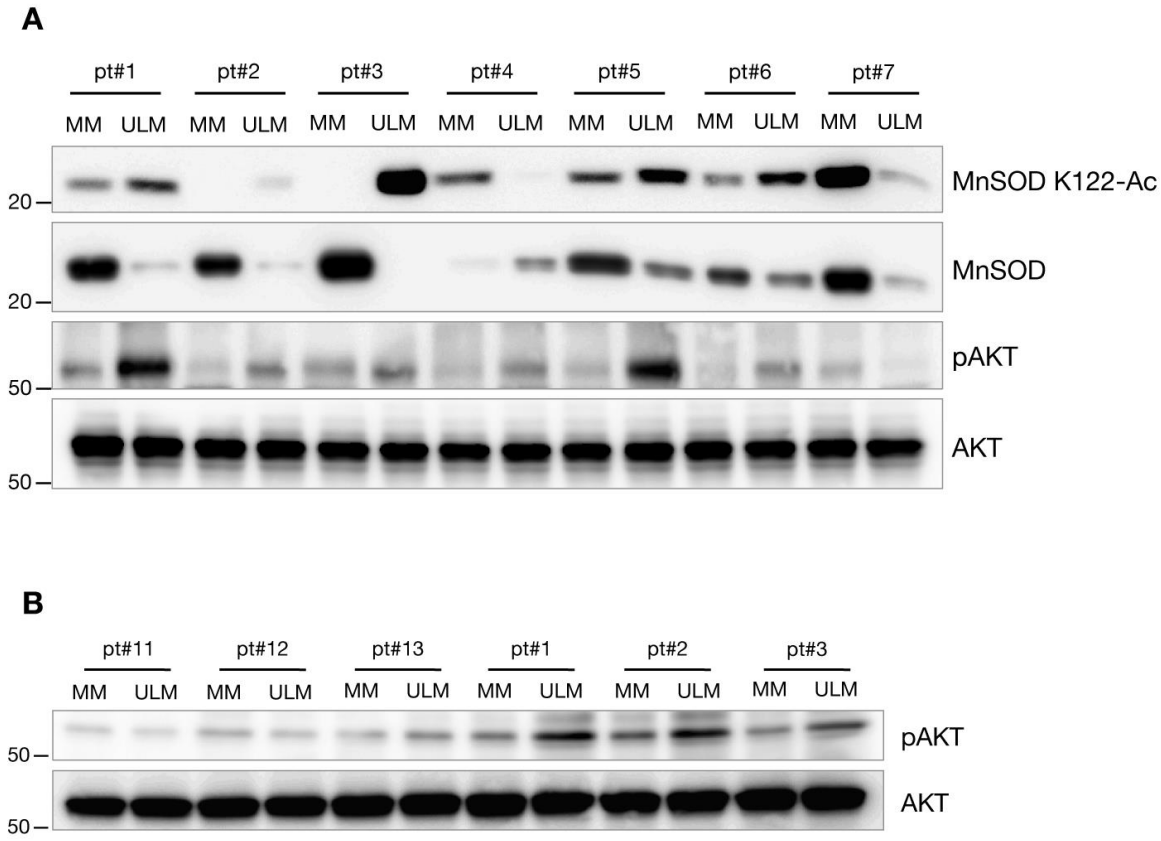


fig. S2. Differential expression of MnSOD K122-Ac, MnSOD, and pAKT in MM and ULM cells. (**A**). Western blot showing the expression profile of MnSOD K122-Ac, MnSOD and pAKT(S473) (pAKT) proteins across 7 different untreated patient-derived MM and ULM cells. panAKT (AKT) was used as loading control. (**B**). Immunoblotting of pAKT levels in MM and ULM cells from patients #11-13 (Fig. 1B) that did not shown differences in MnSOD activity. Patients #1-3 were used as controls.

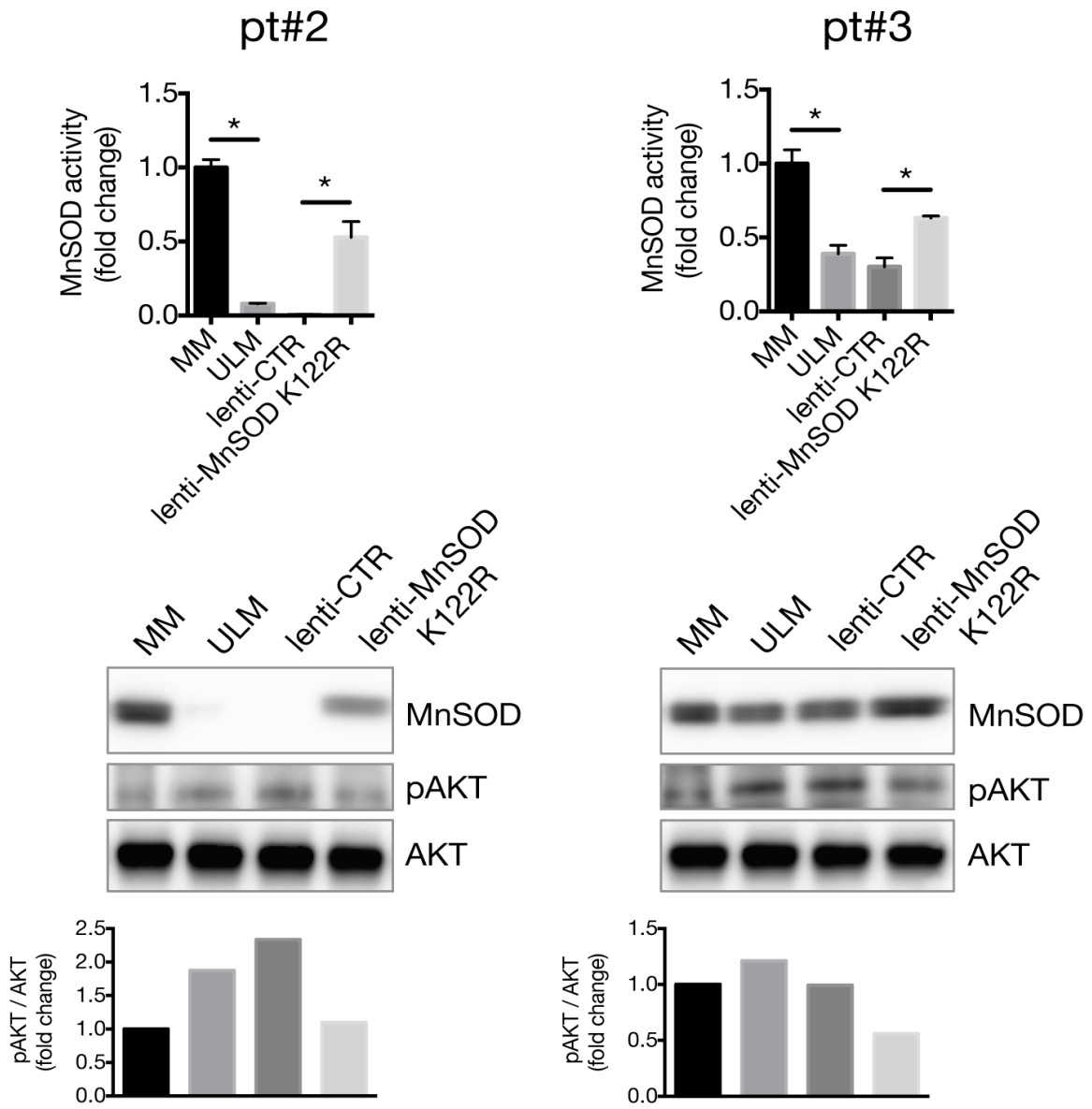
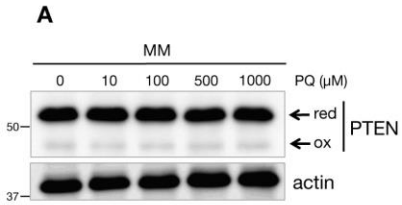
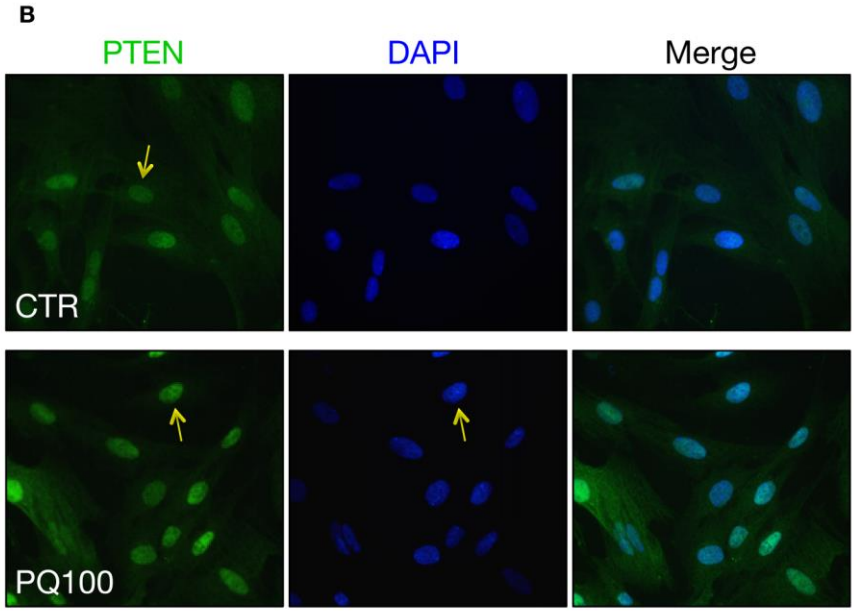
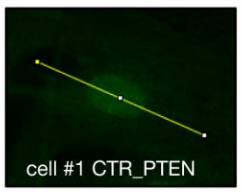
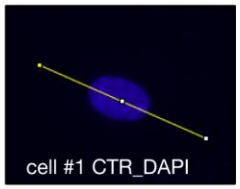


fig. S3. Overexpression of MnSOD reduces pAKT levels in ULM cells from multiple patients. ULM cells were transiently infected with a control lentivirus (lenti-CTR) or mutant MnSOD K122-R (lenti-MnSOD K122-R) as described in Materials and Methods. Cells were subsequently lysed and MnSOD activity as well as MnSOD and pAKT protein levels assessed. Data presented here are from two independent experiment/patients aside from those shown in Fig. 2D. MnSOD activity was measured as previously described in ULM cells infected with lenti-CTR and lenti-MnSOD K122-R (*p=0.0286; unpaired t-test) as well as in uninfected MM and ULM cells, used as controls. MnSOD, pAKT and total AKT levels were also analyzed by western blotting and corresponding densitometric analysis presented below the blot.


A
MM
0
10
100
500
1000
PQ (μM)
50
red
ox
PTEN
37
actin


B
PTEN
DAPI
Merge
CTR
PQ100


C
cell #1 CTR_PTEN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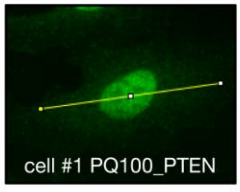
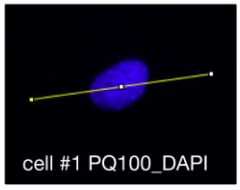
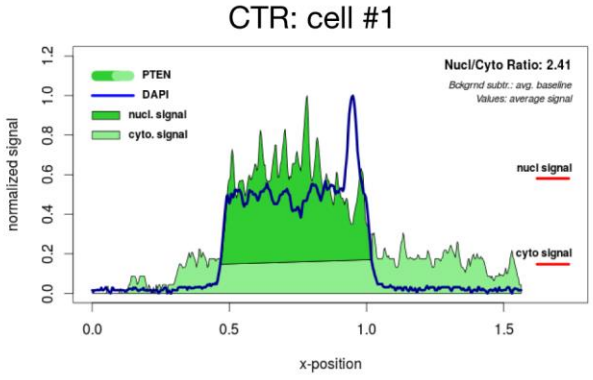
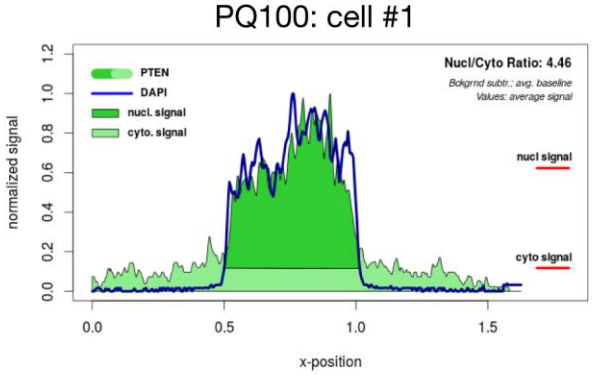

cell #1 PQ100_PTEN


cell #1 PQ100_DAPI


D
CTR: cell #1
PTEN
DAPI
nucl. signal
cyto. signal
Nucl/Cyto Ratio: 2.41
Bckgrnd subtr.: avg. baseline
Values: average signal
nucl signal
cyto signal
normalized signal
x-position


PQ100: cell #1
PTEN
DAPI
nucl. signal
cyto. signal
Nucl/Cyto Ratio: 4.46
Bckgrnd subtr.: avg. baseline
Values: average signal
nucl signal
cyto signal
normalized signal
x-position

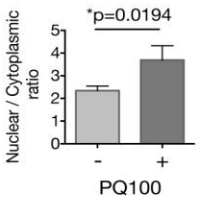

E
*p=0.0194
Nuclear / Cytoplasmic ratio
PQ100

fig. S4. PQ causes PTEN nuclear translocation in ULM cells. (**A**). MM cells were exposed to the indicated concentrations of paraquat (PQ) for 6 hours in serum-free medium and cellular protein extracts were analyzed under non-reducing conditions and immunoblotted with anti-PTEN and anti-actin antibodies. One representative western blotting of three independent experiments is shown (n=3). (**B**) ULM cells were treated with 100 µM PQ for 6 hours in serum-free medium and then immunofluorescence staining was performed for subcellular distribution of PTEN. Representative pictures of at least three independent experiments are shown. For each experimental condition, the nuclear/cytoplasmic ratio of PTEN was determined in at least 10 cells as follows. Stacked blue (DAPI) and green (PTEN) images were analyzed in ImageJ. A line selection was drawn across the nucleus and cytoplasm of each cell as shown in (**C**) using the line tool. The signal profile in each channel was retrieved (**D**). The signal in the DAPI channel was used as a mask to discriminate between nuclear and extra-nuclear signal in the green channel via an R script. The yellow arrow in (B) indicates a representative cell used for the analysis shown in the above figure. The R code used for this analysis was packaged and made available at *http://www.github.com/dami82/CellSignalingTools*. The bar plot in (**E**) shows the nuclear/cytoplasmic distribution of PTEN as mean values ± SD of at least 10 cells per condition from three independent experiments. (*p=0.0194, Unpaired t-test, n=3).

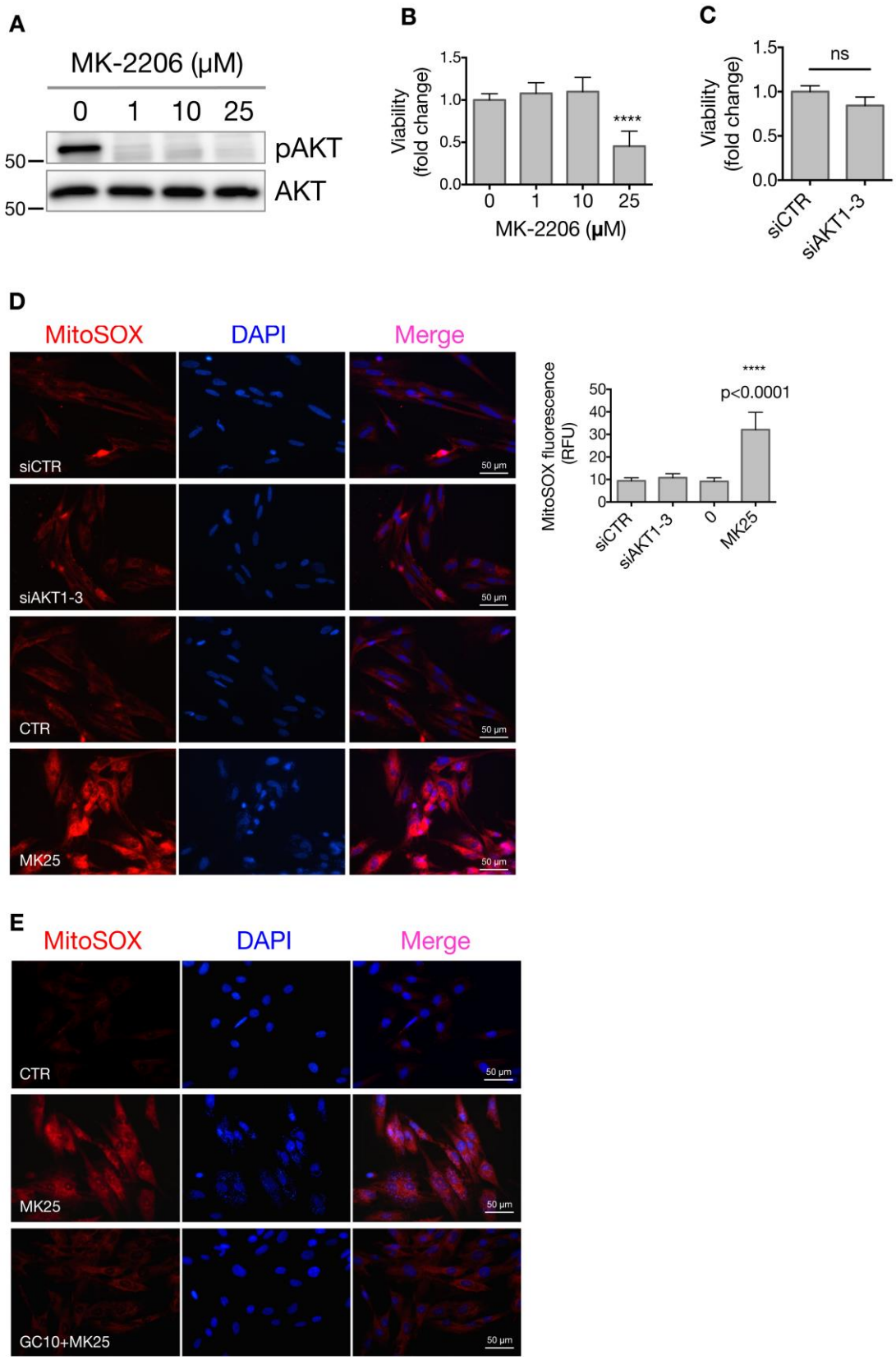

A
MK-2206 (μM)
0 1 10 25
50
pAKT
50
AKT
B
Viability (fold change)
1.5
1.0
0.5
0.0
****
0 1 10 25
MK-2206 (μM)
C
Viability (fold change)
1.5
1.0
0.5
0.0
ns
siCTR
siAKT1-3
D
MitoSOX
DAPI
Merge
siCTR
siAKT1-3
CTR
MK25
50 μm
MitoSOX fluorescence (RFU)
50
40
30
20
10
0
****
p<0.0001
siCTR
siAKT1-3
0
MK25
E
MitoSOX
DAPI
Merge
CTR
MK25
GC10+MK25
50 μm

fig. S5. Different effects of MK-2206 and AKT silencing on ULM cell viability and superoxide generation. (**A**) and (**B**). ULM cells were treated at the indicated concentrations of MK-2206 or vehicle (DMSO, corresponding to 0 µM) for 24 hours. (**A**). Protein extracts were subjected to SDS-PAGE followed by immunoblotting with pAKT(S473) (pAKT) and AKT antibodies. A representative western blot from three independent experiments is shown. (**B**). ULM cell viability was determined using WST-1. Results are expressed as mean±SD from three independent experiments (****$p<0.0001$, One-way ANOVA). (**C**). AKT knockdown has a different impact on superoxide generation and viability than MK-2206 in ULM cells. ULM cells were reverse transfected with siAKT1, siAKT2, siAKT3 and a non-targeting siRNA (siCTR) using Lipofectamine RNAiMAX (fig. S5A). After 72 hours, viability of ULM cells was analyzed using WST-1 (Unpaired t-test, One-way ANOVA, n=3). (**D**). Following AKT knockdown, ULM cells were stained with MitoSOX for superoxide detection. Representative pictures from three independent experiments are shown. Fluorescence intensity of more than 10 cells per condition was quantified as described in Materials and Methods. MK-2206 (25 µM, 6 hours treatment) was used as positive control. Data are shown as mean ± SD from three independent experiments (****$p<0.0001$, One-way ANOVA, n=3). (**E**). MM cells were stained with MitoSOX for superoxide detection and treated for 6 hours with MK-2206 (25 µM) alone or in combination with GC4419 (10 µM). Representative pictures from three independent experiments are shown.

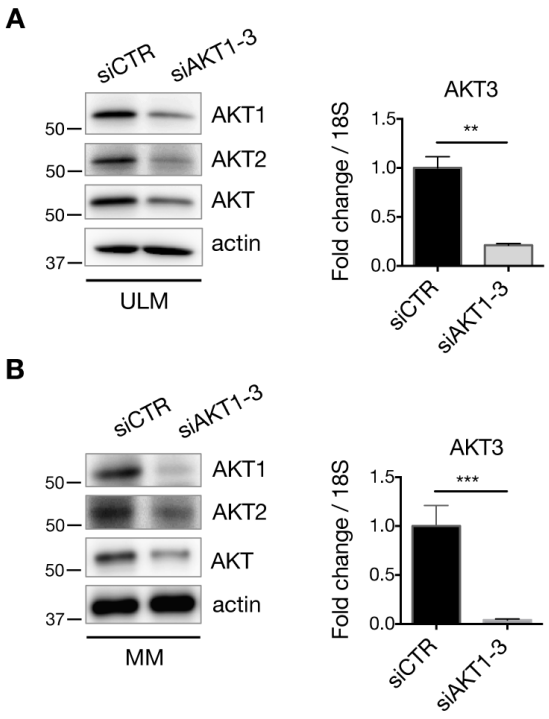


fig. S6. AKT silencing in ULM and MM cells. (**A** and **B**). ULM and MM cells were reverse transfected with siAKT1, siAKT2, siAKT3 and a non-targeting siRNA (siCTR) using Lipofectamine RNAiMAX. AKT1, AKT2 and panAKT (AKT) knockdown was verified by western blotting using anti-AKT1, anti-AKT2 and anti-panAKT antibodies, while AKT3 knockdown was determined by RT-PCR using 18S as reference gene (**p=0.0061, ***p=0.001, Unpaired t-test).