Expanded View Figures

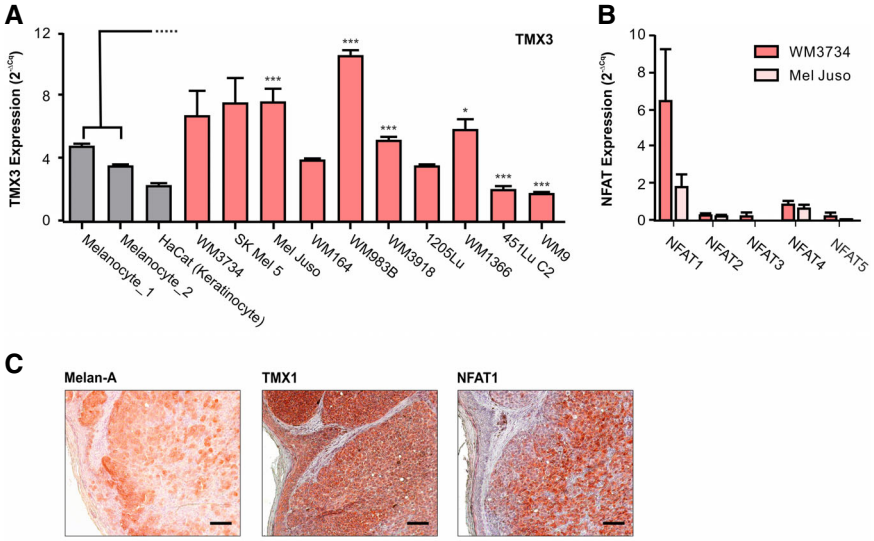

Figure EV1. TMX and NFAT expression in melanoma cells and human melanoma samples.

A mRNA expression of TMX3 was quantified by RT–qPCR in melanocytes from two donors, in keratinocytes, and in a panel of genetically distinct melanoma cell lines.

B mRNA expression of NFAT isoforms in melanoma.

C Melan-A, TMX1, and NFAT1 staining (IHC) in nodular melanoma *patient II* (out of four).

D TMX1 and NFAT1 staining (IHC) of paraffin-embedded samples of healthy human tissue (donors D1–D4) and progressing stages of melanoma (patient numbers P1–P18).

Data information: In (A), data are normalized to the expression of the control protein TBP and are presented as mean ± SEM ($n \geq 3$). In (B), data are normalized to the expression of the control protein TBP and are presented as mean ± SEM ($n \geq 6$). In (C, D), scale bar: 50 μm. Statistical significance was addressed using unpaired, two-tailed Student's *t*-test, $^{*}P < 0.05$; $^{***}P < 0.005$.

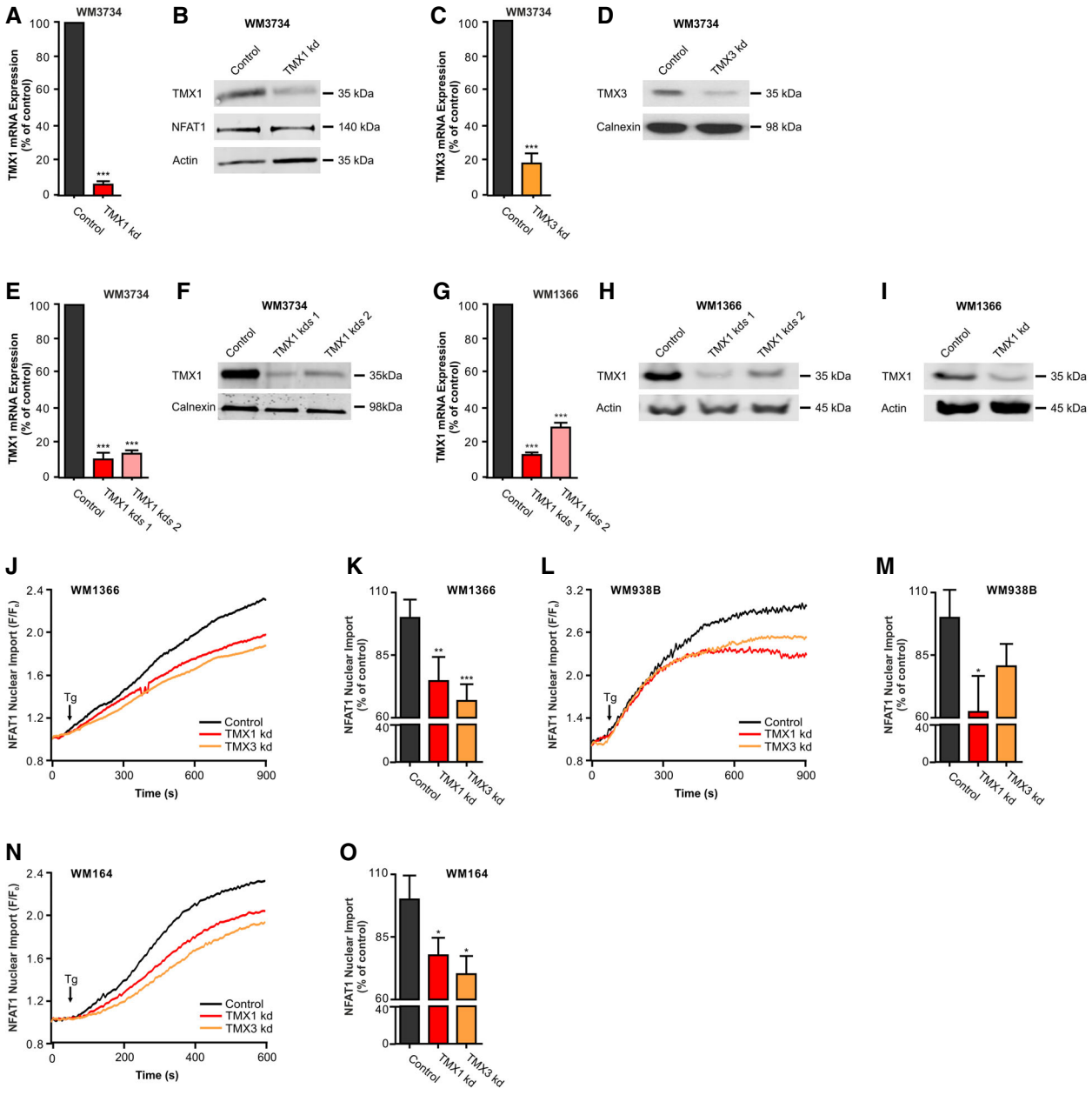

Figure EV2. Transient and stable downregulation of TMX1 and TMX3, and NFAT translocation.

A mRNA expression of TMX1-silenced WM3734 melanoma cells, quantified by RT–qPCR.
B Immunoblot of WM3734 melanoma cells and TMX1-silenced cells probed for TMX1, NFAT1, and actin as a loading control.
C mRNA expression of TMX3-silenced WM3734 melanoma cells, quantified by RT–qPCR.
D Immunoblot of WM3734 melanoma cells and TMX3-silenced cells probed for TMX3 and calnexin.
E mRNA expression of WM3734 melanoma cells with stable knockdown of TMX1 (two clones), quantified by RT–qPCR.
F Immunoblot of WM3734 melanoma cells with stable knockdown of TMX1 (two clones) probed for TMX1 and calnexin.
G mRNA expression of WM1366 melanoma cells with stable knockdown of TMX1 (two clones), quantified by RT–qPCR.
H Immunoblot of WM1366 melanoma cells with stable knockdown of TMX1 (two clones) probed for TMX1 and actin.
I Immunoblot of WM1366 melanoma cells with transient knockdown of TMX1 probed for TMX1 and actin.
J–O Nuclear translocation of NFAT1-GFP in TMX1- or TMX3-silenced (siRNA) melanoma cells after stimulation with thapsigargin in Ringer's buffer with 1 mM Ca^{2+}. Time-dependent nuclear import of NFAT1 in WM1366 (J), WM938B (L), and WM164 (N); corresponding normalized endpoint quantification (K, M, and O).

Data information: In (A, C, E, and G), data are normalized to the expression of TBP and are presented as mean ± SEM (*n* values: A control = 5, TMX1 kd = 7; C = 3; E = 5; G = 4). In (J–O), data are presented as mean ± SEM (*n* values: WM1366, control = 53, TMX1 kd = 49, TMX3 kd = 63; WM938B, control = 16, TMX1 kd = 12, TMX3 kd = 27; WM164, control = 46, TMX1 kd = 56, TMX3 kd = 44). Statistical significance was addressed using unpaired, two-tailed Student's *t*-test, $*P < 0.05$; $**P < 0.01$; $***P < 0.005$.

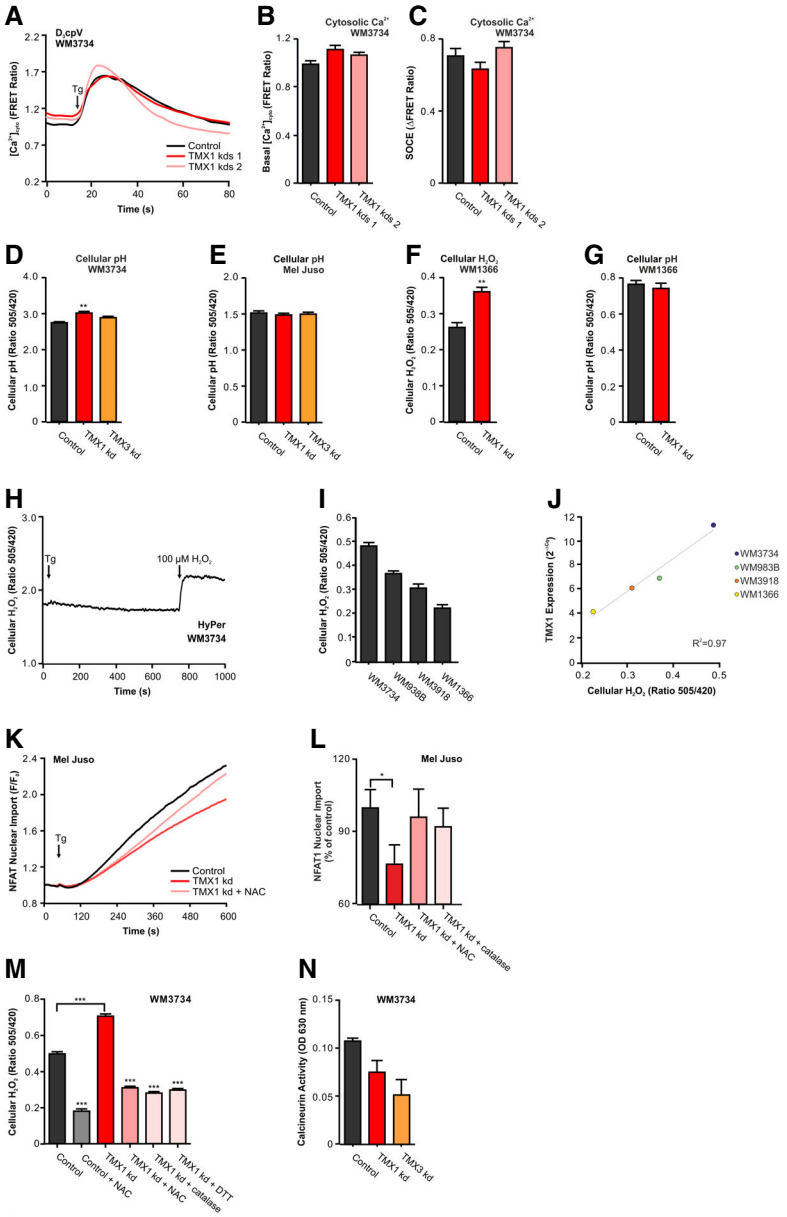

Figure EV3. Calcium, H_2O_2 and pH measurements, H_2O_2 and TMX1 expression correlation, NFAT1 translocation, antioxidants, and calcineurin activity.

A–C (A) Cytosolic calcium after stimulation with thapsigargin in Ringer's buffer with 0.25 mM Ca^{2+} measured with the FRET sensor D_3cpV. (B) Quantification of basal cytosolic calcium levels and (C) SOCE quantification (plateau–basal) for WM3734 after stable silencing of TMX1 (two clones).

D, E Cellular pH was measured using the H_2O_2-insensitive but pH-sensitive SypHer 48 h after silencing of TMX1 or TMX3 in WM3734 (D) and Mel Juso cells (E).

F, G Cellular H_2O_2 (F) and cellular pH (G) in WM1366 after transient knockdown of TMX1.

H Cellular H_2O_2 was measured with HyPer after stimulation with thapsigargin (1 µM); additionally, 100 µM H_2O_2 was added for maximal oxidation.

I Cellular ROS were measured in melanoma cell lines using HyPer.

J Correlation between TMX1 expression and cellular H_2O_2 measured with HyPer. Expression data are from Fig 1A.

K, L NFAT1-GFP nuclear import was induced by thapsigargin (Tg; 1 µM) in TMX1-silenced Mel Juso cells and after pre-incubation with 100 µM N-acetyl-L-cysteine (NAC) for 48 h (K). Quantification of the data from (K) and upon treatment with PEG-catalase (50 U/ml) (L).

M Cellular H_2O_2 was measured with HyPer 25 min after treatment with antioxidants (NAC: 100 µM; catalase: 50 U/ml; DTT 2 mM).

N Calcineurin activity in WM3734 melanoma cells was measured with an enzymatic assay, 48 h after siRNA transfection.

Data information: In (A–C), data are presented as mean ± SEM (*n* values: control = 75, TMX1 kds 1 = 68, TMX1 kds 2 = 78). In (D, E), data are presented as mean ± SEM (*n* values: WM3734: control = 142, TMX1 kd = 153, TMX3 kd = 164; Mel Juso: control = 72, TMX1 kd = 95, TMX3 kd = 101). In (F, G), data are presented as mean ± SEM (*n* values: HyPer: control = 144, TMX1 kd = 170; SypHer: control = 134, TMX1 kd = 136). In (H), data are presented as mean (*n* = 8). In (I), data are presented as mean ± SEM (*n* values: WM3734 = 26, WM938B = 26, WM3918 = 18, WM1366 = 33). In (K, L), data are presented as mean ± SEM (*n* values: control = 63, TMX1 kd = 47, TMX1 kd + NAC = 39, TMX1 kd + catalase = 99). In (M), data are presented as mean ± SEM (*n* values: control = 115, control + NAC = 94, TMX1 kd = 175, TMX1 kd + NAC = 26, TMX1 kd + catalase = 42, TMX1 kd + DTT = 42). In (N), data are presented as mean ± SEM (*n* = 2). Statistical significance was addressed using unpaired, two-tailed Student's *t*-test, $*P < 0.05$; $**P < 0.01$; $***P < 0.005$.

Figure EV4. ER H_2O_2, ER stress, mitochondrial Ca^{2+} uptake, MCU, NCLX, and EM of mitochondria–PM contacts.

A ER-HyPer measurements before and after reduction with 2 mM DTT.
B ER-HyPer measurements before and after oxidation with 2 mM H_2O_2.
C ROS levels were measured with ER-targeted HyPer in WM3734 cells with transient knockdown of TMX1 or TMX3.
D mRNA expression of XBP1s in melanoma cells, 48 h after TMX1 or TMX3 knockdown. The positive control was treated for 4 h with thapsigargin (1 μM) to induce ER stress.
E Immunoblot of melanoma cells stained for protein disulfide isomerase (PDI) and the ER stress marker immunoglobulin heavy chain-binding protein (BiP); GAPDH was used as loading control.
F, G Mitochondrial pH was measured using the H_2O_2-insensitive but pH-sensitive mito-SypHer, 48 h after silencing of TMX1 or TMX3 in WM3734 (F) and Mel Juso cells (G).
H–J Mitochondrial Ca^{2+} uptake in WM3734 melanoma cells with stable knockdown of TMX1 measured with the protein sensor 4mt-TNXL after addition of Ringer's buffer containing 1 mM Ca^{2+} and stimulation with thapsigargin (Tg, 1 μM) (H). Quantification of basal mitochondrial Ca^{2+} levels (I) and mitochondrial Ca^{2+} uptake (plateau–basal) (J).
K Basal mitochondrial H_2O_2 levels measured with mito-HyPer 8 h after treatment with BAPTA-AM (10 μM).
L Mitochondrial Ca^{2+} uptake (plateau–basal) of WM3734 cells with stable knockdown of TMX1 8 h after treatment with NAC (100 μM) measured with 4mt-D3cpV in Ringer's buffer containing 0.25 mM Ca^{2+}.
M Normalized mRNA expression of MCUa, MCUb (mitochondrial calcium uniporter), and NCLX (mitochondrial Na^+-Ca^{2+} exchanger) in melanoma cells with stable knockdown of TMX1 (two clones).
N Immunoblot of melanoma cells with transient (kd) or stable (kds) knockdown of TMX1 stained for MCU, NCLX, and actin (60 μg protein).
O, P Representative electron micrographs of control and TMX1 knockdown HeLa cells (O); 1 defines exemplary marked mitochondria. Corresponding quantification of the distance coefficient (see Materials and Methods) (P).
Q Cellular pH was measured in TMX1-silenced WM3734 with additional knockdown of NOX4 or after inhibiting NOX4 with GKT137831 (140 nM).

Data information: In (A, B), data are presented as mean (*n* values: A = 5; B = 3). In (C), data are presented as mean ± SEM (*n* values: control = 169, TMX1 kd = 179, TMX3 kd = 206). In (D), data are normalized to TBP and are presented as mean of duplicates from one experiment. In (E), the immunoblots are representatives of two experiments. In (F, G), data are presented as mean ± SEM (*n* value: Mel Juso: control = 74, TMX1 kd = 73, TMX3 kd = 83; WM3734: control = 87, TMX1 kd = 85, TMX3 kd = 105). In (H–J), data are presented as mean ± SEM (*n* value: control = 94, TMX1 kds = 83). In (K), data are presented as mean ± SEM (*n* value: control + BAPTA = 248, TMX1 kd = 347, TMX1 kd + BAPTA = 264). In (L), data are presented as mean ± SEM (*n* value: control = 36, TMX1 kds = 23, TMX1 kds + NAC = 41). In (M), data are normalized to TBP and are presented as mean ± SEM (*n* value: MCUa = 7, MCUb = 7, NCLX = 3). In (P), data are presented as mean ± SEM (*n* values: control = 73, TMX1 kd = 46). In (Q), data are presented as mean ± SEM (*n* values: control = 265, TMX1 kd = 233, TMX1 kd + NOX4 kd = 187, TMX1 kd + GKT = 194). Statistical significance was addressed using unpaired, two-tailed Student's *t*-test, $*P < 0.05$; $**P < 0.01$; $***P < 0.005$.

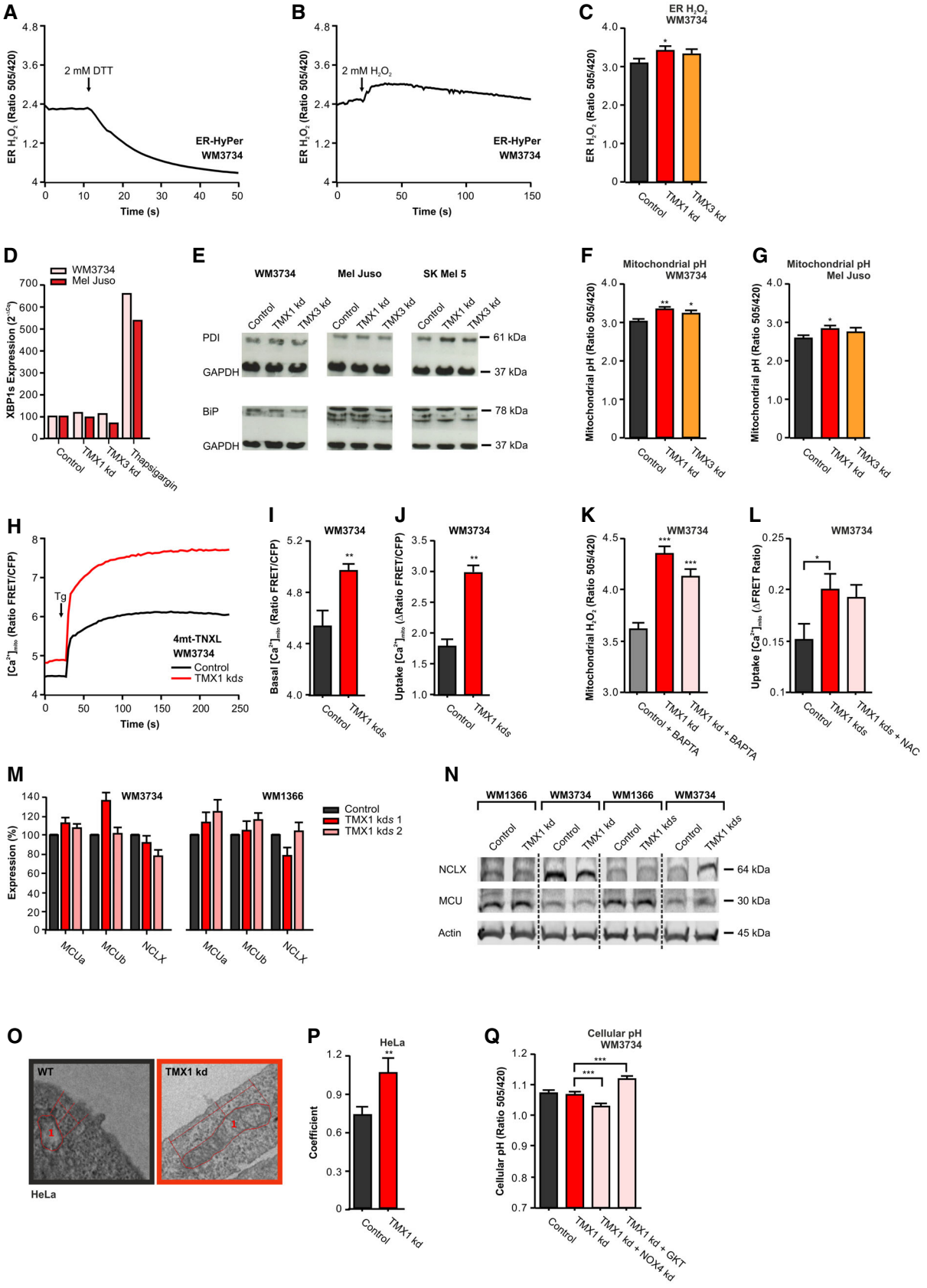
A
2 mM DTT
ER-HyPer
WM3734
ER H2O2 (Ratio 505/420)
Time (s)
B
2 mM H2O2
ER-HyPer
WM3734
C
ER H2O2
WM3734
Control
TMX1 kd
TMX3 kd
D
WM3734
Mel Juso
XBP1s Expression (2-ΔCt)
Thapsigargin
E
WM3734
Mel Juso
SK Mel 5
PDI
GAPDH
BiP
61 kDa
37 kDa
78 kDa
F
Mitochondrial pH
WM3734
Mitochondrial pH (Ratio 505/420)
G
Mitochondrial pH
Mel Juso
H
Tg
4mt-TNXL
WM3734
Control
TMX1 kds
[Ca2+]mito (Ratio FRET/CFP)
Time (s)
I
WM3734
Basal [Ca2+]mito (Ratio FRET/CFP)
TMX1 kds
J
WM3734
Uptake [Ca2+]mito (ΔRatio FRET/CFP)
K
WM3734
Mitochondrial H2O2 (Ratio 505/420)
Control + BAPTA
TMX1 kd + BAPTA
L
WM3734
Uptake [Ca2+]mito (ΔFRET Ratio)
TMX1 kds + NAC
M
WM3734
WM1366
Control
TMX1 kds 1
TMX1 kds 2
Expression (%)
MCUa
MCUb
NCLX
N
WM1366
WM3734
WM1366
WM3734
NCLX
MCU
Actin
64 kDa
30 kDa
45 kDa
O
WT
TMX1 kd
HeLa
P
HeLa
Coefficient
Q
Cellular pH
WM3734
Cellular pH (Ratio 505/420)
TMX1 kd + GKT
TMX1 kd + NOX4 kd

Figure EV4.

Figure EV5. TMX1, TMX3, BRAF, and NFAT1 in melanoma behavior, adaptation, mitochondrial respiration, melanoma patient survival, and NFAT1 translocation.

A Proliferation (72 h) of melanoma cells after treatment with different concentrations of the drug dipyridamole, which prevents NFAT–calcineurin interaction.
B Proliferation (48 h) of WM1366 after transient knockdown of TMX1 and TMX3.
C Transwell migration (48 h) of WM1366 after transient knockdown of TMX1 and TMX3.
D Immunoblot probing for TMX1 and actin in lysates isolated from tumor samples collected 45 days after mouse injection (corresponding to the data shown in Fig 6I–K). Samples labeled with "A" represent the control group, while "B" and "C" represent groups with the two different clones of TMX1 shRNA knockdown.
E Representative immunoblots of tumor-extracted lysates probed for phosphorylated AKT (S473) and total AKT.
F Representative immunoblots for TMX1, phosphorylated AKT (S473), and total AKT expression in lysates extracted from TMX1- and TMX3-silenced WM3734 melanoma cells grown in standard culture conditions. HSP90 was used as a loading control.
G–I The oxygen consumption rate (OCR) was determined in control and stable TMX1 knockdown WM3734 (two clones) seeded in glucose-containing medium following standard treatment with oligomycin, FCCP, and rotenone + antimycin A (G). The basal respiration (H) and maximal respiration (I) were quantified.
J–L Kaplan–Meier survival plots and log rank for the correlation between mRNA expression levels and survival probability of melanoma patients, separated in groups with high or low expression of TMX1 (J), TMX3 (K), and NFAT1 (L); shaded areas depict the 95% confidence intervals of the survival probabilities, and significance was assessed using log-rank tests.
M, N Schoenfeld's test for Cox analysis. The *P*-values for individual Schoenfeld's test are indicated in the figure (global Schoenfeld's test $P = 0.8694$).
O, P NFAT1-GFP nuclear import was induced by thapsigargin (Tg; 1 μM) in Ringer's buffer containing 0.25 mM Ca^{2+} in TMX1-silenced WM3734 cells with and without additional knockdown of BRAF (O); endpoint quantification (P).
Q, S The role of BRAF mutation status on NFAT1, TMX1, and TMX3 expression in melanoma cells. mRNA levels of TMX1 (Q), NFAT1 (R), and TMX3 (S) in a panel of melanoma cell lines characterized by their BRAF mutation status.

Data information: In (A–C), data are presented as mean ± SEM ($n = 3$). In (G–I), data are presented as mean ± SEM (*n* values: control = 7, TMX1 kds 1 = 6, TMX1 kds 2 = 6). In (O, P), data are presented as mean ± SEM (*n* values: control = 100, TMX1 kd = 90, TMX1 kd + BRAF kd = 121). In (Q–S), data are normalized to the expression of TBP (expression data are extracted from Figs 1A and B, and EV1A; BRAF mutations are shown in Appendix Table S1). Statistical significance was addressed using unpaired, two-tailed Student's *t*-test, $*P < 0.05$; $**P < 0.01$; $***P < 0.005$.

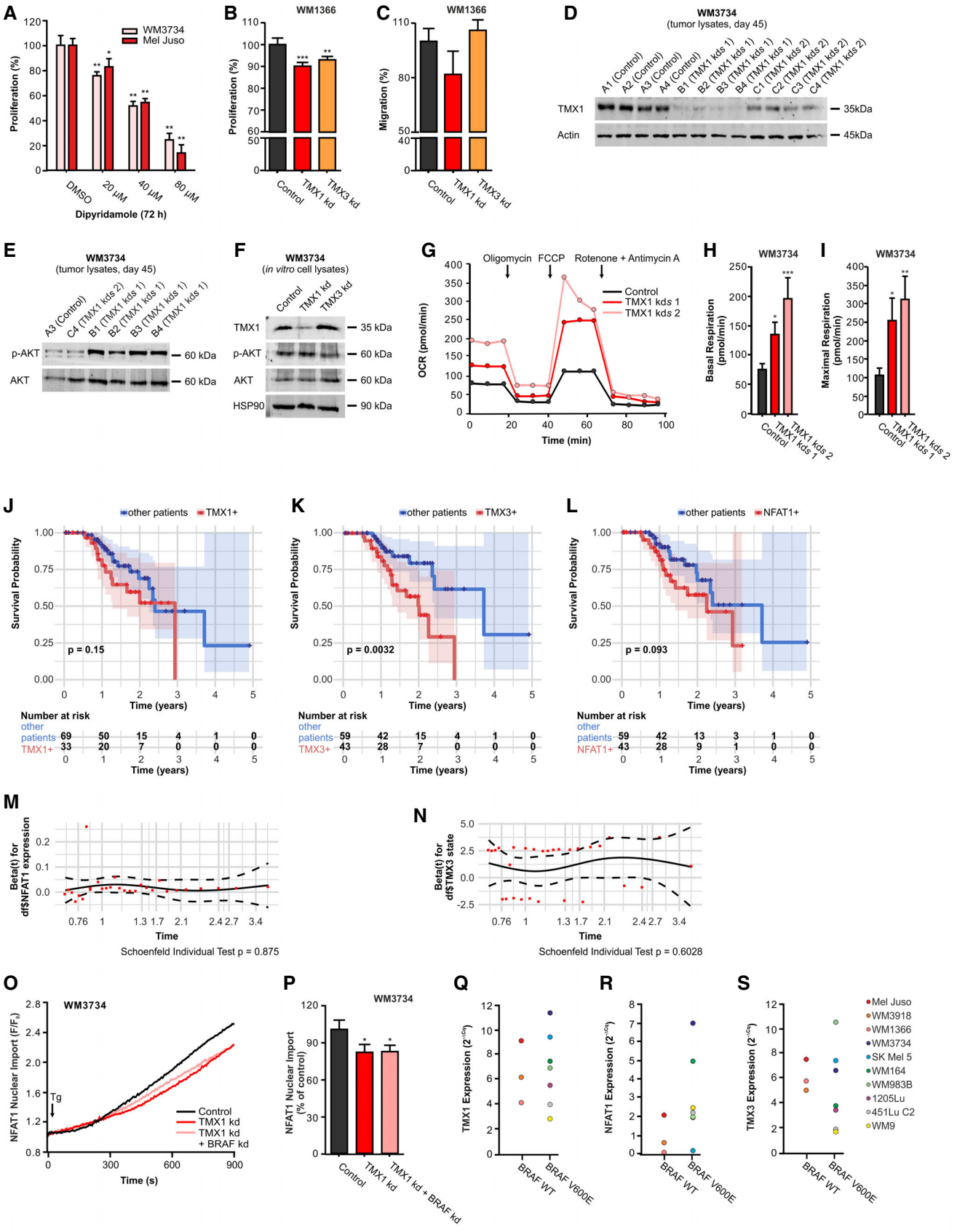
A
WM3734
Mel Juso
Proliferation (%)
DMSO
20 μM
40 μM
80 μM
Dipyridamole (72 h)
B
WM1366
Proliferation (%)
Control
TMX1 kd
TMX3 kd
C
WM1366
Migration (%)
Control
TMX1 kd
TMX3 kd
D
WM3734
(tumor lysates, day 45)
A1 (Control)
A2 (Control)
A3 (Control)
A4 (Control)
B1 (TMX1 kds 1)
B2 (TMX1 kds 1)
B3 (TMX1 kds 1)
B4 (TMX1 kds 1)
C1 (TMX1 kds 2)
C2 (TMX1 kds 2)
C3 (TMX1 kds 2)
C4 (TMX1 kds 2)
TMX1
Actin
35kDa
45kDa
E
WM3734
(tumor lysates, day 45)
A3 (Control)
C4 (TMX1 kds 2)
B1 (TMX1 kds 1)
B2 (TMX1 kds 1)
B3 (TMX1 kds 1)
B4 (TMX1 kds 1)
p-AKT
AKT
60 kDa
60 kDa
F
WM3734
(in vitro cell lysates)
Control
TMX1 kd
TMX3 kd
TMX1
p-AKT
AKT
HSP90
35 kDa
60 kDa
60 kDa
90 kDa
G
Oligomycin
FCCP
Rotenone + Antimycin A
Control
TMX1 kds 1
TMX1 kds 2
OCR (pmol/min)
Time (min)
H
WM3734
Basal Respiration (pmol/min)
Control
TMX1 kds 1
TMX1 kds 2
I
WM3734
Maximal Respiration (pmol/min)
Control
TMX1 kds 1
TMX1 kds 2
J
other patients
TMX1+
Survival Probability
p = 0.15
Time (years)
Number at risk
other patients 69 50 15 4 1 0
TMX1+ 33 20 7 0 0 0
0 1 2 3 4 5
Time (years)
K
other patients
TMX3+
Survival Probability
p = 0.0032
Time (years)
Number at risk
other patients 59 42 15 4 1 0
TMX3+ 43 28 7 0 0 0
0 1 2 3 4 5
Time (years)
L
other patients
NFAT1+
Survival Probability
p = 0.093
Time (years)
Number at risk
other patients 59 42 13 3 1 0
NFAT1+ 43 28 9 1 0 0
0 1 2 3 4 5
Time (years)
M
Beta(t) for df$NFAT1 expression
Time
Schoenfeld Individual Test p = 0.875
N
Beta(t) for df$TMX3 state
Time
Schoenfeld Individual Test p = 0.6028
O
WM3734
NFAT1 Nuclear Import (F/F0)
Tg
Control
TMX1 kd
TMX1 kd + BRAF kd
Time (s)
P
WM3734
NFAT1 Nuclear Import (% of control)
Control
TMX1 kd
TMX1 kd + BRAF kd
Q
TMX1 Expression (2-dCt)
BRAF WT
BRAF V600E
R
NFAT1 Expression (2-dCt)
BRAF WT
BRAF V600E
S
TMX3 Expression (2-dCt)
BRAF WT
BRAF V600E
Mel Juso
WM3918
WM1366
WM3734
SK Mel 5
WM164
WM983B
1205Lu
451Lu C2
WM9

Figure EV5.