Supporting Information

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SI Materials and Methods

Reagents, Cell Culture, and Antibodies. Human melanoma cell lines were described previously (1). Human adult melanocyte lines (HEMa-LP, HEMa-DP, and HEMn-LP) were purchased from Banksia Scientific and cultured as previously described (1). Human fresh melanoma isolates containing more than 95% tumor cells were prepared according to our published methods (1). Studies using human tissues were approved by the human research ethics committees of University of Newcastle and Royal Prince Alfred Hospital, Australia. Antibodies against GAPDH, GRP78, GSK3α, Mcl-1,β-catenin, and p53 were purchased from Santa Cruz Biotechnology. Antibodies against PARP, phospho-Mcl-1 (Ser159/Thr163), and si-GSK3α were purchased from Cell Signaling. Mature hsa-miR-149* mimics (double-stranded oligonucleotides designed to mimic the function of endogenous microRNA) and anti–has-miR-149* (antisense RNA oligonucleotides designed to inhibit the function of endogenous microRNA) were purchased from Thermo Fisher Scientific. Oligonucleotides were used at concentrations of 100 nM.

Xenograft Model. Mel-RM cells (1×10^7) transduced with the vector alone or anti-miR-149* were injected s.c. into each flank of male athymic nude mice (Shanghai SLAC Laboratory; n = 6 mice per group). Tumor growth was monitored by measurements of tumor diameters with a sliding caliper three times a week. The tumor volume was recorded as length × width × height. Mice were killed and tumors were weighed at 36 d after transplantation. Studies on animals were conducted with approval from the animal research ethics committee of the University of Science and Technology of China.

Transcriptional Reporter Assays and Transfection. PcDNA3.0-p53 plasmid (200 ng) or empty vector was individually cotransfected into Mel-RM cells, together with appropriate GPC1 promoter reporter plasmids (p53 BR, 100 ng) by using Lipofectamine 2000 (Invitrogen). In each transfection, cells were also cotransfected with Renilla luciferase reporter plasmid. Firefly and Renilla luciferase activity were assayed using a Dual-Luciferase Reporter Assay System (Promega). Fold activation values were measured relative to the levels of Firefly luciferase activity in cells transfected with empty vectors and normalized by Renilla luciferase activities. GSK3α-3′UTR and GSK3α-3′UTR-mut were constructed into pSI-CHECK2-report plasmid (Promega). Plasmids and miR-149* mimics or inhibitor were cotransfected into Mel-RM cells (2.5×10^5) by using DharmaFECT Duo Transfection Reagent (Thermo Fisher Scientific). Fold activation values were measured relative to the levels of Renilla luciferase activity in cells transfected with negative control oligo and normalized by Firefly luciferase activities.

1. Nguyen T, Zhang XD, Hersey P (2001) Relative resistance of fresh isolates of melanoma to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. *Clin Cancer Res* 7(3, suppl):966s–973s.

RNA Isolation and mRNA Profiling. Total RNA from Mel-RM cell lines was isolated with TRIzol reagent (Invitrogen). To identify different expression of microRNAs, we performed microRNA microarray studies (Exiqon). The filtered results were subjected to cluster software (Cluster 3.0) and TreeView analysis (Stanford University) for unsupervised hierarchical clustering by genes and arrays. Metric (distance) and linkage were set as Euclidean distance and average, respectively.

qRT-PCR Analysis. Total RNA was reverse-transcribed from 50 ng total RNA with MMLV Reverse Transcriptase using the following specific primers: RT-miR-149, 5′>GTCGTATCCAGTGCGTGTCGTGGAG TCGGCAATTGCACTGGATACGACGGGAGTG < 3′; RT-miR-149*, 5′>GTCGTATCCAG TGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGCACAGC < 3′; and RT-U6, 5′>C GCTTCACGAATTTGCGTGTCAT < 3′. Real-time RT-PCR for microRNAs was performed using a Stratagene Mx3000P (Agilent Technologies) with SYBR Green Master Mix. As a control, the small housekeeping U6 was amplified and quantified. The specific primers used in this reaction were as follows: miR-149, forward, 5′>GTTTCTGGCTCCGTGT<3′; miR-149, reverse, 5′>CAGTGCGTGTCGTGGAGT<3′; miR-149*, forward, 5′>GTTAGGGAGGGAC GGG<3′; miR-149*, reverse, 5′>CAGTGCGTGTCGTGGA GT<3′; U6, forward, 5′>GCTTCGGCAGCACATAT ACTAAAAT <3′; and U6, reverse, 5′>CGCTTCACGAATTTGCGTGTCAT<3′.

Quantification of ISH and IHC Results. Staining of the proteins and miR-149* were evaluated semiquantitatively by three independent observers. The intensity of staining was scored on a scale of 0 to 4. The percentage of positive cells was estimated from 0% to 100%. An immunoreactive score was developed by multiplying the percentage of positive cells by the intensity score and dividing it by 10.

Statistic Analysis. Statistical analysis was carried out using Microsoft Excel 2003 software. Two-tailed Student *t* test was used to assess differences in values between experimental groups. A *P* value lower than 0.05 was considered to indicate statistical significance.

Knockdown of p53 and miR-149*. Sigma MISSION Lentiviral Transduction Particles for shRNA-mediated knockdown of p53 were used for knockdown of p53 according to the manufacturer's instruction. The sequence used to inhibit hsa-miR-149* was 5′>GCACA GCCCCCG TCCCTCCC T <3′. LentiLox 3.7 vector was used to express short, single-stranded anti–miR-149*.

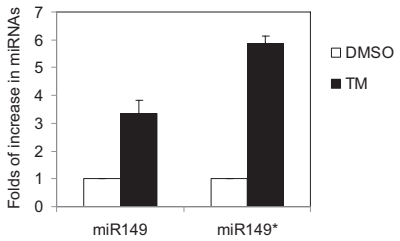

Fig. S1. miR-149 is up-regulated by ER along with miR-149* in melanoma cells. Mel-RM cells were treated with TM (3 μM) for 12 h. Total RNA was extracted by using TRIzol and subjected to qRT-PCR analysis (values are presented as mean ± SD; $n = 3$).

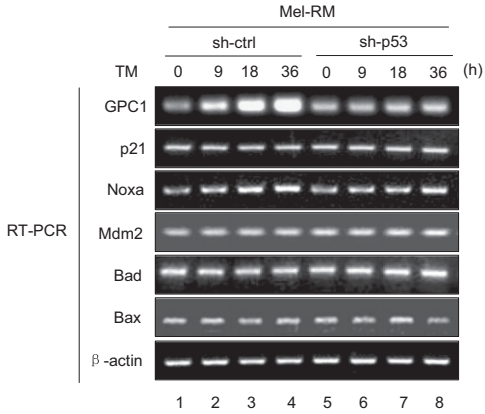

Fig. S2. Induction of ER stress leads to p53-dependent up-regulation of the GPC1 and Noxa mRNAs. Mel-RM cells transduced with the control shRNA or shRNA for p53 were treated with TM for indicated periods. Total RNA was extracted and subjected to semi–RT-PCR analysis of the expression of GPC1, p21, Noxa, Mdm2, Bad, Bax, and β-actin (as a loading control) at the mRNA level.

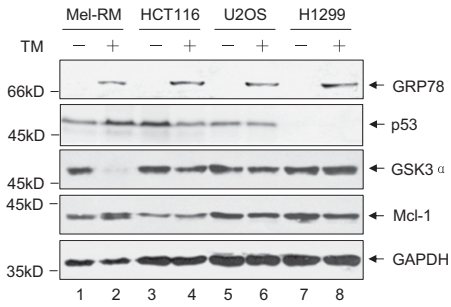

Fig. S3. Induction of ER stress by TM up-regulates endogenous p53 and Mcl-1 and down-regulates GSK3a in Mel-RM melanoma cells, but not in other nonmelanocyte-origin cancer cells. Melanoma (Mel-RM), colon cancer (HCT116), osteosarcoma (U2OS), and lung cancer (H1299) cells were treated with TM for 12 h. The expression of GRP78, p53, GSK3a, and Mcl-1 was analyzed by using Western blotting. GAPDH was used as loading control. Induction of GRP78 is a commonly used marker of activation of the ER stress response.

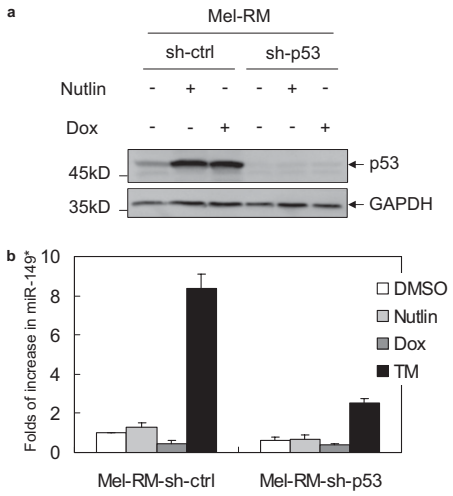

Fig. S4. Induction of p53 by nutlin and doxorubicin is not associated with up-regulation of miR-149*. (*A*) Whole-cell lysates from Mel-RM cells treated with nutlin (10 μM, 24 h) or doxorubicin (1 μg/mL,12 h) were subjected to Western blot analysis. (*B*) Total RNA from Mel-RM cells treated with nutlin (10 μM, 24 h), doxorubicin (1 μg/mL,12 h) was subjected to qRT-PCR analysis of miR-149* expression. Total RNA from Mel-RM cells treated with TM (3 μM, 24 h) was included as a control.

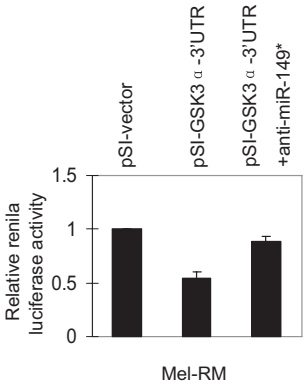

Fig. S5. Inhibition of miR-149* enhanced the activity of the 3′UTR of GSK3a. Mel-RM cells were introduced with indicated plasmids and oligonucleotides for 24 h. Reporter activity was measured by luciferase assays. The results showed that pSI-luciferase activity was suppressed in Mel-RM cells in the presence of the 3′UTR of GSK3a, but the suppression is reversed when miR-149* was inhibited. Dada shown are the mean ± SD of three individual experiments.

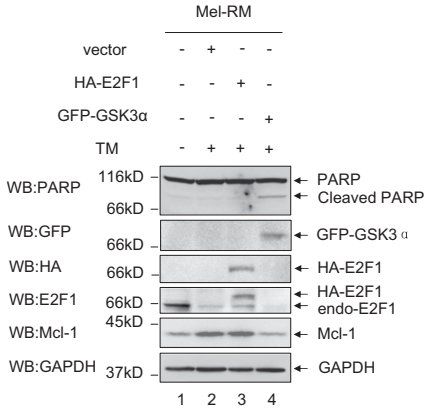

Fig. S6. Overexpression of GSK3α, but not overexpression of E2F1, inhibits induction of Mcl-1 and renders melanoma cells sensitive to ER stress-induced apoptosis. Mel-RM cells were transduced with empty vector, pcDNA3.0-HA-E2F1, and pEGFP-C1-GSK3α. Twenty-four hours later, cells were treated with TM (10 μM) for another 36 h. Whole-cell lysates were subjected to Western blotting analysis of PARP, GSK3α, E2F1, Mcl-1, and GAPDH (as a loading control).

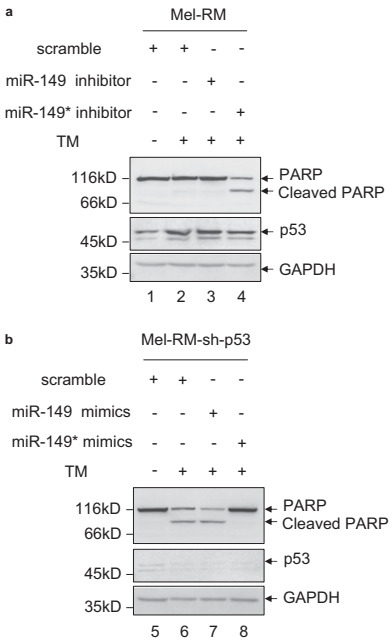

Fig. S7. miR-149 does not play a role in regulation sensitivity of melanoma cells to ER stress-induced apoptosis. Mel-RM cells (*A*) and Mel-RM cells deficient in p53 (cells from Mel-RM sublines established by shRNA knockdown; *B*) were transfected with the indicated oligonucleotide (100 nM). Twenty-four hours later, cells were treated with TM for another 36 h. Whole-cell lysates were then subjected to Western blotting analysis of PARP, p53, and GAPDH (as al loading control.)

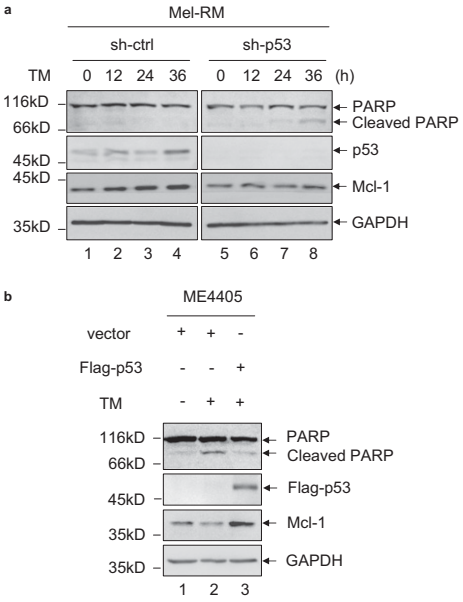

Fig. S8. (*A*) Mel-RM cells with or without p53 were treated with TM (10 μM) at indicated periods, and cell lysates were subjected to Western blotting analysis of PARP, p53, Mcl-1, and GAPDH (as a loading control). (*B*) Ectopic expression of p53 up-regulates Mcl-1 and protects against ER stress-induced apoptosis in ME4405 cells. ME4405 cells (p53-null) were introduced with empty vector and pcDNA3.0-flag-p53. Twenty-four hours later, cells were treated with TM (10 μM) for another 36 h. Whole-cell lysates were subjected to Western blotting analysis of PARP, p53, Mcl-1, and GAPDH (as a loading control).

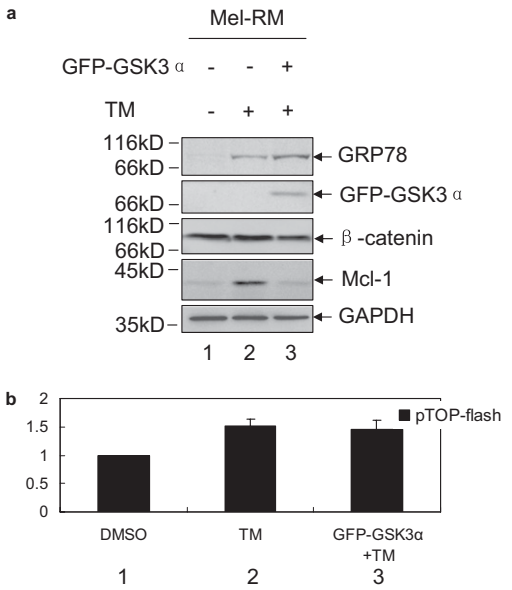

Fig. S9. GSK3a does not impinge on the expression of β-catenin, nor does it regulate the activity of the Wnt/β-catenin pathway. (*A*) Mel-RM cells were transfected with or without pEGFP-C1-GSK3α. Twenty-four hours later, cells were treated with TM for another 24 h. Whole-cell lysates were subjected to Western blotting analysis of GRP78, GSK3a, β-catenin, Mcl-1, and GAPDH (as a loading control). (*B*) Mel-RM cells were transfected with the pTOP-flash luciferase report construct with or without cotransfection with pEGFP-C1-GSK3α. Twenty-four hours later, cells were treated with TM (3 μM) for another 24 h. The report activity was measured by using luciferase assays.

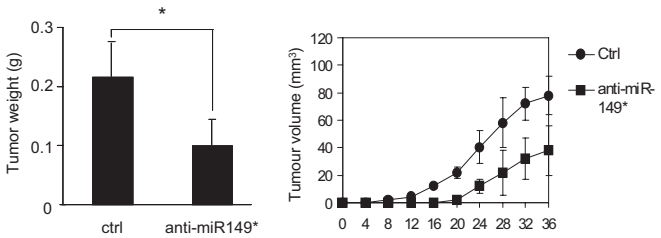

Fig. S10. Inhibition of miR-149* retards melanoma xenograft growth in nude mice. Mel-RM cells with or without miR-149* stably inhibited by anti–miR-149* were transplanted into flanks of nude mice. *Left*: Weights of the xenografts harvested at 36 d after transplantation. *Right*: growth curves of melanoma xenografts in nude mice. Values are presented as mean ± SD (n = 6). Two-tailed Student t test: $^{*}P < 0.05$, $^{**}P < 0.001$.

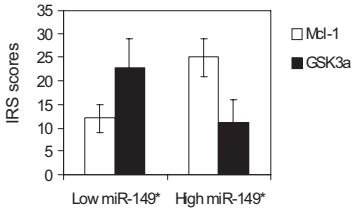

Fig. S11. Quantitative analysis of miR-149* expression on melanoma tissue sections as shown in Fig. 4*F* in relation to the expression levels of Mcl-1 and GSK3a.

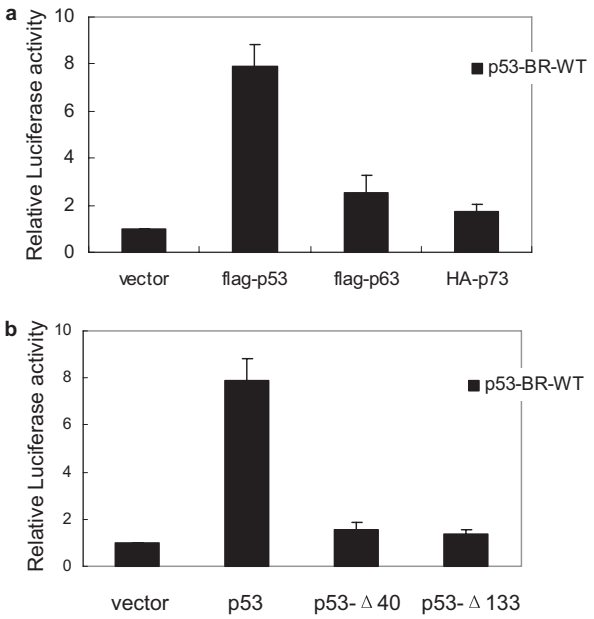

Fig. S12. (*A* and *B*) p53-BR-WT luciferase activity was increased by overexpression of p53, but not p63, p73, p53-Δ40, and p53-Δ133. Mel-RM cells were transfected with the indicated plasmids for 24 h. Reporter activity was measured by luciferase assays. Data shown are the mean ± SD of three individual experiments.

Table S1. TM up-regulates miR-149* but does not notably alter the expression of other miRNAs that have been shown to be p53 targets

miRNAs	3 h/0 h	6 h/0 h	12 h/0 h	24 h/0 h	36 h/0 h
hsa-miR-149*	1.15	3.52	4.57	7.73	6.90
hsa-miR-149	1.02	0.99	1.13	1.55	1.92
hsa-miR-34a†	1.20	1.00	1.06	1.00	1.15
hsa-miR-34b†	1.26	1.11	1.37	1.54	1.81
hsa-miR-34c-3p†	0.81	0.89	0.68	1.19	0.94
hsa-miR-107†	0.88	1.01	1.06	1.06	0.88
hsa-miR-192†	0.89	0.97	0.94	1.20	0.76
hsa-miR-215†	0.78	1.31	1.27	0.87	0.78
hsa-miR-765	1.30	1.45	1.91	2.32	3.42
hsa-let-7b	0.58	1.03	0.82	0.88	0.76
hsa-let-7a	1.06	0.79	0.78	0.73	0.79

Numbers represent fold change in levels of expression.
†miRNAs reported to be regulated by p53.