Supplementary information

Supplementary Methods

Supplementary Figure Legends

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Cell culture, reagents and clinical samples

Human melanoma cell lines A375, A2058 and WM35 were grown in Dulbecco's modified Eagle's medium (Gibco, Grand Island, USA). B16F10 melanoma cells (TCM2, Chinese Academy of Sciences, Shanghai, China) and PBMCs derived from C57BL/6 mouse (Jackson Lab, Bar Harbor, USA) were grown in RPMI 1640 Medium (Gibco). All the cultured cells were supplemented with 10% fetal bovine serum (Gibco) and 1% Pen Strep Glutamine (Gibco). Cells were grown at 37 ℃ in 5% CO_2 and lifted with 0.05% Trypsin-EDTA (Gibco). In co-culture system, B16F10 melanoma cells and PBMCs derived from C57BL/6 mouse were incubated in 12-well plates, with melanoma cells on the bottom layer and PBMCs suspended in the medium for 24 hours. All these melanoma cell lines were authenticated by short-tandem repeat (STR) fingerprinting in center of DNA typing in Fourth Military Medical University and tested for mycoplasma contamination. The phosphorylated JAK2 inhibitors WP1066 (S2796, Selleck, Houston, USA) was dissolved in DMSO and used at the concentration of 2.5 μM for 24 hours. Protein synthesis inhibitor Cycloheximide (HY-12320, MCE, South Brunswick, USA) was dissolved in DMSO and used at the concentration of 5 nM for 0, 4 hours and 8 hours, respectively. Proteasome inhibitor MG132 (474790, Calbiochem, Darmstadt, Germany) was dissolved in DMSO and used at the concentration of 5 μM for 24 hours.

11 melanoma patients treated with anti-PD-1 antibody were included in this study. Five patients were treated with toripalimab and the other six were treated with pembrolizumab. Patients were categorized into 6 responders (CR, PR, or SD of greater than 6 months with no progression) and 5 non-responders (PD or SD for less than or equal to 6 months before disease progression) as

evaluated by standard Response Evaluation Criteria In Solid Tumors (RECIST v1.1) guidelines.[1] The tumor tissues and peripheral blood were obtained before the initiation of the indicated treatment from melanoma patients who were diagnosed as melanomas at AJCC stage III or stage IV. Informed consent was obtained from all the enrolled patients and the study was approved by the ethics committee of Xijing Hospital, Fourth Military Medical University. Clinical information of each patient is displayed in Figure 1A and Supplementary Table S1. Moreover, peripheral blood was extracted after 6 weeks of the treatment with anti-PD-1 antibodies and PBMCs were separated by lymphosep (DKW) according to the manufacturer's recommended procedures. Flow cytometry was used for analyzing the characteristics of these cells with indicated interventions.

ChIP assays

ChIP assays were performed as previously described. [2] Briefly, 3-5 × 10^7 cells were crosslinked with 1% paraformaldehyde at room temperature for 15 minutes and sonicated to generate chromatin fragments of 200-600 bp. Fragmented chromatin was then immunoprecipitated overnight with STAT3 antibody (ab119352, abcam), followed by washes and elution. ChIP DNA from A375 and A2058 cells transfected with A20 siRNA or control siRNA was quantified by qRT-PCR. The *PD-L1* primers used were: Forward: 5'-GAGGAAGTCACAGAATCCACGA-3', Reverse: 5'-AAAGTCAGCAGCAGACCCAT-3'.

Mass spectrum analysis

As previously described,[3] the protein bands of interest were excised from Coomassie blue-stained gel. Each gel slice was diced into small pieces (1 mm ×1 mm) and placed into a 1.5-ml tube. A gel

piece that was removed from a protein-free region of the gel was used as a parallel control. Sample preparation used for Q-Exactive mass spectrometry was performed according to the standard protocol as described previously. [4] Gel slices were de-stained and digested in 20 μl of sequencing grade trypsin at 37 °C overnight. The protein digests were later desalted for MS and MS/MS analysis, which were performed with the use of the QExactive system (Thermo Fisher Scientific, USA). Afterward, Proteome Discoverer software (version 1.4; Thermo Fisher Scientific, USA) was applied for protein identification and quantitation.

Plasmid vectors and siRNA transfection

Small interfering RNA (GenePhama, Shanghai, China) was used to knockdown A20 and PHB expression. Lipofectamine 3000 transfection Reagent kit (L3000-015, Invitrogen, Carlsbad, USA) was used for transfection according to the manufacturer's recommended procedures. Mouse CRISPR/Cas9 plasmid (sc-423436, Santa Cruz, Dallas, USA) was used to obtain the knockout of A20 in B16F10 melanoma cells with the use of Lipofectamine 3000 transfection Reagent kit. After 72 hours of transfection, puromycin (ab141453, abcam, Cambridge, UK) was used to remove the cells with no transfection efficiency. Then the screened cells were separated in 96 wells plate as 1 cell/well to incubate. Gene sequencing was used to obtain the clone with A20 knockout. The PD-L1 overexpression plasmid (CD274-pEX-4 plasmid), A20 overexpression plasmid (A20-pHB plasmid), wild type PHB (PHB WT-pEX-3) and mutant PHB plasmids (PHB mut186-pEX-3 and PHB mut202-pEX-3) as well as empty plasmid vectors were designed and made by GenePharma, and transferred into melanoma cells with Lipofectamine 3000 transfection Reagent kit according to the manufacturer's recommended procedures. Neomycin (S2568, Selleck) and puromycin (abcam) was used to remove the non-transfected cells. The sequences of used siRNAs against A20 and PHB were

as follows: siRNA A20 seed sequence-1: 5‘-CAUGCACCGAUACACACUUTT-3’; siRNA A20 seed sequence-2: 5‘-CUGGAAGAAAUACACAUAUTT-3’; siRNA PHB seed sequence-1: 5‘-AGCCAGCUUCCUCGCAUCUdTdT-3; siRNA PHB seed sequence-2: 5‘-CCCAGAAAUC ACUGUGAAAdTdT-3’.

Immunoprecipitation

For immunoprecipitation (IP) analysis, cultured cells were solubilized and protein extracts (500 μg) were used for immunoprecipitation with antibodies against A20, PHB or STAT3. Cells with indicated treatment were collected and put into immunoprecipitation assay using Pierce Co-IP kit (Thermo Scientific) according to manufacturer’s instructions. In brief, 20 μg rabbit polyclonal anti-A20 antibody (ab93868, Abcam), rabbit monoclonal anti-PHB antibody (ab75766, Abcam) or mouse monoclonal anti-STAT3 antibody (9139, Cell Signaling Technology) was added directly to the resin in the spin column. The column was capped and incubated at room temperature for 90-120 mins using a rotating body or mixer. After the antibody was immobilized, the protein extracts (500 μg) was added to the resin, followed by being evenly turned over at 4 °C overnight. Immune complexes were washed extensively 4 times with Tris-buffered saline containing 0.05% Tween-20 detergent and subjected to western blot.

Western blot

Western blot was performed as described previously.[5] In brief, samples were separated with SDS-PAGE, transferred to polyvinylidene difluoride membrane and probed with the corresponding antibodies. The dilution of antibodies were as follows: anti-human A20 monoclonal rabbit (1:1000, ab92324, abcam), anti-mouse A20 monoclonal mouse (1:1000, ab13597, abcam),

anti-phospho-STAT3 (Y705) monoclonal rabbit (1:1000, ab76315, abcam), anti-STAT3 monoclonal mouse (1:1000, 9139, Cell Signaling Technology, Cambridge, UK), anti-phosphor- STAT3 (S727) polyclonal rabbit (1:1000, 9134, Cell Signaling Technology), anti-JAK2 rabbit polyclonal rabbit (1:1000,ab39636,abcam), anti-PHB monoclonal rabbit (1:1000, ab75766, abcam), anti-human PD-L1 monoclonal rabbit (1:1000,ab205921, abcam), anti-mouse PD-L1 monoclonal rabbit (1:1000,ab213480, abcam), anti-Ubiquitin (linkage-specific K48) monoclonal rabbit (1:1000,ab140601, abcam),anti-c-myc monoclonal rabbit (1:1000,ab32072, abcam), anti-mcl-1 monoclonal rabbit (1:1000,ab32087, abcam), anti-mouse and anti-rabbit secondary antibodies (1:3000, 115-035-003, Jackson ImmunoResearch, West Grove,USA). Signals were detected using Western ECL Substrate (Thermo Scientific).

Real-Time PCR

Total RNA was extracted by RNAiso Plus (Takara, Kyoto, Japan). Isolated RNA was reversely transcripted into cDNA using the First Strand cDNA Synthesis Kit (Takara) according to the manufacturer's instruction. Subsequent gene expression was then analyzed using SYBR Select Master Mix (Takara). We designed and used the following gene-specific primers: 5'-GGTGCCGACTACAAGCGAAT-3' (Forward) and 5'- AGCCCTCAGCCTGACATGTC-3' (Reverse) for PD-L1, 5'-GCAGATAGCCAAGGGTATGAGTTACC-3' (Forward) and 5'-TTTTGCCAGCCCA AAATCTGT-3' (Reverse) for PHB, and designed forward primers span exon-exon junctions where possible.

Tumor tissue microarray

Melanoma tissue microarray (M1004e) was purchased from US Biomax Inc. (Rockville,USA). Detailed information can be accessed via http://www.biomax.us/tissue-arrays/Melanoma/ME1004e.

Mouse xenograft tumor model

All mice were maintained under pathogen-free conditions at the Fourth Military Medical University, Xi'an, China, and were at the age of 4-6 weeks at the time of cell implantation. Mice were kept at 12 hours/12 hours light cycle and received standard food and water. All animal studies and experimental procedures were approved by the Animal Care and Use Committee of the animal facility at the Fourth Military Medical University. The experimental design and number of mice assigned to each treatment were based on prior experience with similar models and provided sufficient statistical power to discern significant differences.

In C57BL/6 mice, the xenograft mice model was established with the subcutaneous implantation with wild type-B16F10 melanoma cells or B16F10 melanoma cells transfected with either CRISPR/Cas9 A20 KO plasmid (sc-423436, Santa) or CD274-pEX-4 plasmid (GenePhama) which encodes PD-L1 protein. 5×10^5 cells were injected subcutaneously per mouse. In the case of anti-PD-1 antibody treatment, the mice were randomly divided into 4 groups and, once the tumor volume reached around 100mm,^{3} the mice were intraperitoneally injected with anti-PD-1 antibodies (100μg/mouse) (Clone J43, Bioxcell, West Lebanon, USA) every second day. For the depletion of immune cells, mice were injected i.p. with 200μg monoclonal antibodies against $CD8^+$T cells with anti-CD8 antibody (clone 2.43, Bioxcell) on the first day of treatment and followed by every 2 days throughout the experiment. For mouse blood samples, up to 2ml blood per mouse was extracted and PBMCs were separated according to the manufacturer's recommended procedures of lymphosep (DKW). Lymphocytes were calculated and incubated in co-cultured system with B16F10 cells as

human cells co-culture system mentioned above. Flow cytometry was used for analyzing the characteristics of these cells with indicated interventions.

Flow cytometry analysis

For the analysis of the characteristics of infiltrating $CD8^+$T cells in tumor, flow cytometry assay was immediately performed at the time of specimen collection on freshly obtained melanoma patients or mouse tumor specimens after tumor dissociation. Tumors were collected, cut and minced in HBSS containing collagenase IV (sigma), and then incubated in 37℃ incubator for 45mins with shake. PBS with trypsin and DNase (sigma) were used for another 15 mins' incubation in shaking-37℃ incubator. The digested tissues were washed with PBS twice and then filtered with 80μM filter. Then single cell suspensions were incubated with ACK lysis buffer (Invitrogen) for 5 mins in room temperature to remove red blood cells as previously described.[6] Cells were then re-suspended and analyzed on a Beckman Coulters Gallios flow cytometer. Antibodies were used as follows: anti-human CD3 (317308, biolegend, San Diego, USA), anti-human PD-L1 (329708, biolegend), anti-mouse PD-L1 (329718, biolegend), anti-human CD8 (344704, biolegend), anti-mouse CD8a (100706, biolegend), anti-human Ki67 (350526, biolegend), anti-mouse Ki67 (652406, biolegend), anti-human PD-1 (329908, biolegend), anti-mouse PD-1 (135216, biolegend), anti-human/mouse Granzyme B (515403, biolegend), anti-mouse Granzyme B (372220, biolegend), anti-human Perforin (308105, biolegend), anti-mouse Perforin (154306, biolegend). Samples were analyzed with Cytexpert software.

For the detection of cell death in melanoma with or without the knockout of A20, melanoma cells with indicated treatment were harvested by trypsinization, washed twice with 4 °C PBS and re-suspended in binding buffer. Annexin V-PE and 7AAD solution (4A Biotech, Beijing, China)

were then added to stain the cells before analysis by flow cytometry (Beckman Coulter). The apoptotic rate was assessed and calculated after at least three independent experiments, with at least three biological replicates each time.

Gene set enrichment analysis

To perform the gene set enrichment analysis, GSEA software was used to derive the absolute enrichment scores using C2 curated subset (experimentally validated gene sets) of the Molecular Signature Database version 6.0, [7,8] based on KEGG pathways. [9]

Cell co-culture system

For the construction of the *in vitro* co-culture system, PBMCs were isolated from peripheral whole blood of C57BL/6 mouse that were burdened with B16F10 tumor using Ficoll (DKW, China). The PBMCs were seeded at 1×10^6 cells/well and cultured with B16F10 melanoma cells (2.5×10^5 cells/well) in the presence of 100 ng/ml anti-CD3ε mAb-coated co-culture system. The PBMCs and B16F10 cells were cultured in RPMI 1640 supplemented with 10% FBS, L-glutamine, antibiotic and IL-2 (100 U/mL) at 37℃ in a 5%-CO_2 incubator. After 72h, cells were harvested and go through FACS analysis. For the detection of apoptotic melanoma cells, group of $CD45^-$ cells from the co-cultured system were gated, and PI and Annexin V staining were used for the detection of cell apoptosis. For the analysis of $CD8^+$T cells, group of $CD45^+CD3^+CD8^+$cells were gated, and the percentage of Ki-67^+ and Granzyme B^+ cells were analyzed with the use of the corresponding antibodies described in **Flow cytometry analysis** part.

Histology and immunohistochemical staining analysis

Melanoma tissues were fixed in 10% neutral buffered formalin and paraffin-embedded (FFPE). 2μm FFPE consecutive tumor sections were stained with Hematoxylin (Dako, Copenhagen, Denmark) and Eosin G (Dako). For immunohistochemical staining analysis, paraffin-embedded melanoma tissues of tumor tissue microarray (TMA) or implanted tumor in C57BL/6 mice were de-paraffinized and rehydrated with graded ethanol dilutions. After antigen retrieval in Tris-EDTA Buffer (10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0), goat serum was added to block nonspecific binding for 30 mins. Tissue sections were then incubated with anti-mouse/human A20 (1:50, ab92324, abcam), anti-human PHB (1:100, ab 75766, abcam), anti-human PD-L1 (1:250, ab213524, abcam) or anti-mouse PD-L1 (1:100, ab238697, abcam) at 4°C overnight, followed by anti-rabbit alkaline phosphatase secondary antibody. The section was then incubated in Fast Red solution, and subsequently counterstained with hematoxylin and mounted with glycerol. The staining intensity was calculated as integrated optical density (IOD) using Image-Pro® Plus software (version 5.1; Media Cybernetics, Inc., Rockville, MD, USA) as previously described.[10]

Immunofluorescence staining analysis

Melanoma cells for immunofluorescence staining analysis were first washed with PBS, fixed with 4% paraformaldehyde for 10–20 mins and permeabilized with 0.5% Triton for 15 mins. After washing the slides with PBS, cells were blocked with normal goat sera at room temperature for 1 hour. Then, cells were incubated with primary antibody diluted in the blocking solution at 4°C overnight. Paraffin-embedded tissue sections were de-paraffinized and rehydrated with graded ethanol dilutions. After antigen retrieval in Tris-EDTA Buffer (10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0), goat serum was added to block nonspecific binding for 30 mins,

followed by the incubation with primary antibody. The primary antibodies were as follows: anti-human/mouse A20, 1:200, ab13597, abcam; anti-Melan-A, 1:200, ab51061, abcam; anti-phospho-STAT3 Y705, 1:100, 9145, Cell Signaling Technology; anti-STAT3, 1:1500, #9139, Cell Signaling Technology; anti-PHB, 1:100, ab75766, abcam; anti-CD8, 1:100, ab237709, abcam; anti-CD45, 1:100, 60287-1-Ig, Proteintech. Cells or tissue sections were then washed with PBS and probed with the secondary antibodies conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, USA) and Alexa Fluor 568 (Invitrogen) for 1 hour and were stained with DAPI (Roche, Basel, Switzerland). Images were obtained using an inverted confocal laser scanning microscope (Zeiss). The staining intensity was calculated as integrated optical density (IOD) using Image-Pro® Plus software (version 5.1; Media Cybernetics, Inc., Rockville, MD, USA) as previously described [68].

ELISA assay

ELISA analysis on culture medium of melanoma cells with/without A20 knockdown was performed using the Human IL-6 ELISA Kit (Neobioscience) according to the manufacturer's instructions. The absorbance (A450) was measured with a plate reader (Bio-Rad).

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Supplementary Figure Legends

Figure S1. (A) Immunofluorescence staining analysis of the subcellular distribution of A20 in melanoma tissues (n=5). Scale bar = 50μm. **(B)** Immunoblotting analysis shows expression of A20 in B16F10 melanoma cells with or without the knockout of A20 expression. **(C)** Apoptotic tumor cells were analyzed by flow cytometry analysis in B16F10 cells with or without the knockout of A20. **(D)** CD8 expression in peripheral blood lymphocytes of C57BL/6 mice intraperitoneally injected with anti-CD8 antibody was determined by flow cytometry at day 10 after the first injection. **(E)** Analysis in TCGA SKCM database shows the association between *CD274* level and *TNFAIP3* level. *P* value was calculated by linear regression analysis. **(F)** The histogram illustrates relative mRNA expression of PD-L1 in A375 and A2058 cells with or without the knockdown of A20. *P* values were calculated by paired Student's *t*-test.

Figure S2. (A) The apoptosis of melanoma cells with indicated interventions in the co-culture system. *P* values were calculated by paired Student's *t*-test. **(B)** Western blots shows expression of JAK2 in melanoma cells with indicated interventions of A20. **(C)** ELISA assay revealed changes of IL-6 level in supernatant of cultured melanoma cells. **(D)** Western blot analysis shows expression of SOCS3 in melanoma cells with indicated interventions of A20. **(E)** Western blot analysis shows the expression of PHB after the indicated interventions of A20. **(F)** Relative mRNA expression of PHB in A375 and A2058 cells after the indicated interventions of A20. **(G)** IHC staining of the PHB proteins in TMAs consisting of 18 nevus, 62 primary melanomas and 20 metastatic melanomas. Scale bar = 100μm. **(H)** The correlation between *CD274* and *PHB* mRNA expressions in TCGA SKCM database. r value was calculated by Spearman correlation, and *P* value was calculated by two-tailed Student's *t*-test. **(I)** The correlation between *TNFAIP*3 and *PHB* mRNA expressions in

TCGA SKCM database. r value was calculated by Spearman correlation, and *P* value was calculated by two-tailed Student's *t*-test.