

Targeting ITGB2-Driven Tumor Cell-Intrinsic Signaling Inhibits Melanoma Progression

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Abstract

ITGB2 serves as a key integrin that drives immune cell activation and migration. It has long been regarded as expressed solely in hematopoietic cells. As a result, the potential role of ITGB2 expressed within cancer cells themselves in the advancement and treatment of solid tumors has received limited attention. We employed single-cell and bulk RNA sequencing, real-time quantitative PCR, multiplex immunofluorescence, flow cytometry, immunoblotting, and ICAM-1-dependent adhesion and proliferation assays. These approaches helped reveal functional ITGB2 expression intrinsic to melanoma cells, its links to clinical tumor progression, its activation status, its role in protumorigenic signaling, and its roles in adhesion and proliferation. Analyses were conducted using patient melanoma samples and established human and murine melanoma cell lines. In vivo tumorigenicity experiments were carried out in immunocompromised NSG mice, immunocompetent wild-type C57BL/6 mice, and Icam1 knockout C57BL/6 mice to examine the downstream effects of the melanoma-ITGB2 pathway on tumor growth and metastasis. The cancer cell-intrinsic ITGB2 pathway was inhibited through CRISPR/Cas9-generated *Itgb2* knockout, ITGB2-blocking antibodies, ITGB2 activation via CD44 crosslinking, and pharmacologic blockade of ITGB2-linked Wnt signaling using LGK974, zamaporvint, and the FDA-approved agent pyrvinium pamoate repurposed for oncology. The findings establish that ITGB2 is expressed outside the hematopoietic compartment and exerts protumorigenic effects directly within melanoma cells. Melanoma cell ITGB2 facilitated binding to ICAM-1, enhanced cancer progression in preclinical models, showed higher levels in clinical metastatic melanomas compared with primary tumors or benign nevi, and served as a predictor of sentinel lymph node metastasis in patients with primary melanoma. In line with this, blocking melanoma cell-intrinsic ITGB2 with antibodies or *Itgb2* gene knockout strongly reduced ICAM-1-mediated adhesion, tumor growth, and metastatic spread. The interaction between melanoma cell ITGB2 and ICAM-1 triggered downstream Wnt signaling; pharmacologic suppression of this pathway inhibited ITGB2-driven tumorigenesis in melanoma. This study challenges the established view that ITGB2 expression is confined to leukocytes by identifying a tumor cell-intrinsic ITGB2:ICAM-1:Wnt signaling axis that promotes tumor growth and represents a genuine therapeutic target in melanoma.

Keywords: ITGB2, Integrin $\beta 2$, CD18, Melanoma, Metastasis, ICAM-1, Wnt, CD44

Introduction

The integrin family of adhesion receptors plays a central role in regulating various immune cell activities,

including activation, expansion, and tissue homing [1]. Among the 24 known integrin $\alpha\beta$ heterodimers [2], some have been linked to cancer development, including melanoma [3]. However, therapeutic inhibition of most of these integrins has shown limited success against tumor growth due to overlapping functions and broad cellular distribution [2]. In contrast, ITGB2 ($\beta 2$) integrins are distinctive because they function as non-redundant receptors specialized for hematopoietic cell proliferation and directed migration to inflammatory sites, including tumors [1]. ITGB2-dependent homing primarily occurs

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through binding to intercellular adhesion molecule (ICAM)-1 [2]. Targeting ITGB2 family members on immune cells alone is enough to completely halt their activation and trafficking [1, 4], highlighting the dominant importance of the ITGB2:ICAM-1 interaction over other adhesion systems in immune cell regulation. This is clearly illustrated in leukocyte adhesion deficiency-1 (LAD-1), a disorder caused by ITGB2 loss-of-function mutations that prevent immune cell activation and migration, leading to severe, recurrent infections [1].

The ITGB2 common β chain associates with four different α subunits—ITGAD (α D, CD11d), ITGAL (α L, CD11a, LFA-1), ITGAM (α M, CD11b, Mac-1), or ITGAX (α X, CD11c)—with ITGAL being the primary functional partner in T cell-mediated responses [2]. Until recently, all four ITGB2 heterodimers were considered to be expressed exclusively on leukocytes and absent from other cell types [5]. This assumption has been questioned by emerging evidence of ITGB2 expression in tumor cells in some epithelial cancers, though its precise role in tumor development remains incompletely understood [6-8]. In this study, we document ITGB2 gene and protein expression in non-hematopoietic melanoma cells. Crosslinking of the CD44 hyaluronan receptor, which is known to control ITGAL/ITGB2 activation on leukocytes [9], similarly upregulated and conformationally activated ITGAL/ITGB2 on melanoma cells. Tumor cell-intrinsic ITGB2 enhanced in vitro adhesion to ICAM-1, supported in vivo tumor formation in both human and mouse melanoma models, was more abundant in clinical metastatic lesions than in primary melanomas or benign nevi, and was associated with sentinel lymph node (SLN) metastasis among patients with primary tumors.

Pharmacologic or genetic inhibition of melanoma cell-intrinsic ITGB2—via blocking antibodies or CRISPR/Cas9-mediated *Itgb2* knockout—eliminated ICAM-1-dependent adhesion by melanoma cells and markedly reduced tumor growth in immunocompromised NOD/SCID/IL2R γ -null (NSG) mice. In immunocompetent C57BL/6 hosts, ITGB2 blockade or genetic deficiency of host *Icam1* substantially decreased spontaneous metastasis of B16-F10 and YUMM5.2 tumors. Melanoma-specific *Itgb2* knockout also impaired primary tumor development in wild-type mice but not in *Icam1*^{-/-} hosts. Unbiased RNA sequencing identified the Wnt pathway as a key downstream effector of the melanoma cell ITGB2:ICAM-1 interaction.

Consistent with this, pharmacologic inhibition of Wnt signaling effectively countered ITGB2-mediated adhesion and tumor progression. Overall, these results reveal a tumor cell-intrinsic ITGB2:ICAM-1:Wnt axis that accelerates melanoma growth and identify it as a valid therapeutic target. The work overturns the conventional understanding of ITGB2 as leukocyte-restricted and uncovers a “leukocyte mimicry” strategy [10] that solid tumor cells exploit to drive malignancy.

Materials and Methods

Cell culture

Authenticated, mycoplasma-free human A2058 (Cat# CRL-3601, RRID: CVCL_1059), A375 (Cat# CRL-1619, RRID: CVCL_0132), and MDA-MB-435S (Cat# HTB-129, RRID: CVCL_0622) as well as murine B16-F10 (Cat# CRL-6475, RRID: CVCL_0159), YUMM1.7 (Cat# CRL-3362, RRID: CVCL_JK16), YUMM3.3 (Cat# CRL-3365, RRID: CVCL_JK36), YUMM4.1 (Cat# CRL-3366, RRID: CVCL_JK38), and YUMM5.2 (Cat# CRL-3367, RRID: CVCL_JK43) melanoma cell lines were obtained from the American Type Culture Collection (ATCC, Gaithersburg, MD). Human melanoma LOX-IMVI cells (RRID: CVCL_1381) were from MilliporeSigma (Burlington, MA), and C8161 cells (RRID: CVCL_6813) were from Dr. Mary Hendrix (Children’s Memorial Research Center, Chicago, IL). FEMX cells (RRID: CVCL_A011) were from Dr. Udo Schumacher (University Hospital Hamburg-Eppendorf, Hamburg, Germany). The human HSB-2 (Cat# CCL-120.1, RRID: CVCL_1859) and murine EL-4 (Cat# TIB-39, RRID: CVCL_0255) T lymphoblastic lines as well as human umbilical vein (HUVEC, Cat# CRL-1730, RRID: CVCL_2959) and murine C166 (Cat# CRL-2581, RRID: CVCL_6581) endothelial cells were obtained from ATCC. All cell lines were used at low passage, < 70% confluency, and were cultured in DMEM or RPMI-1640 medium (Life Technologies, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, MilliporeSigma) and 1% (v/v) penicillin/streptomycin (Life Technologies) as described [11]. HUVECs were cultured in fibronectin-coated (MilliporeSigma, 20 μ g/ml) flasks and maintained in EGM-2 BulletKit Endothelial Cell Growth Medium (Lonza, Basel, Switzerland).

Clinical specimens

All research using human tissue samples received approval from the Institutional Review Boards at Mass General Brigham (protocols 2022P002062, 2022P000827, and 2013P001014), the University of Zurich in Switzerland (biobank protocol PB.2018_00194), and the University of Bonn in Germany (protocol 187/16). Written informed consent was obtained from each participant. The investigations adhered fully to the principles outlined in the Declaration of Helsinki. Single-cell suspensions from human melanoma tissues were prepared by enzymatic digestion with collagenase, as previously reported [12].

Antibodies and biologic reagents

The following abs and reagents were used for flow cytometric (FC) analysis: PE-conjugated anti-human ITGB2 ab (clone MEM-48, Thermo Fisher Scientific, Waltham, MA, Cat# A15766, RRID: AB_2534546) or PE-conjugated mouse IgG1 isotype control ab (clone MOPC-21, Thermo Fisher Scientific, Cat# MA1-10415, RRID: AB_2536783), APC-conjugated anti-human ITGB2 ab (clone MEM-48, Thermo Fisher Scientific, Cat# MA1-19456, RRID: AB_1071389) or APC-conjugated mouse IgG1 isotype control ab (clone MOPC-21, Thermo Fisher Scientific, Cat# MA5-18093, RRID: AB_2539476), PE-conjugated anti-human ITGB2 ab (clone MEM-148, Novus Biologicals, Centennial, CO, Cat# NB500-480PE, RRID: AB_3191646) or PE-conjugated mouse IgG1 isotype ab (clone MG1, Novus Biologicals, Cat# NBP1-97005PE, RRID: AB_3242285), PE-conjugated anti-human ITGB2 ab (clone KIM-127, Leinco Technologies, St. Louis, MO, Cat# C552) or PE-conjugated mouse IgG1 isotype ab (clone HKSP, Leinco Technologies, Cat# I-103), APC-conjugated anti-human ITGAL ab (clone HI111, BioLegend, San Diego, CA, Cat# 301212, RRID: AB_314150) or APC-conjugated mouse IgG1 isotype ab (clone MOPC-21, BioLegend, Cat# 400120, RRID: AB_2888687), Pacific Blue-conjugated anti-human ICAM-1 ab (clone HCD54, BioLegend, Cat# 322716, RRID: AB_893384) or Pacific Blue-conjugated mouse IgG1 isotype ab (clone MOPC-21, BioLegend, Cat# 400131, RRID: AB_2923473), BV570-conjugated anti-human CD3 ab (clone UCHT1, BioLegend, Cat# 300436, RRID: AB_2562124) or BV570-conjugated mouse IgG1 isotype ab (clone MOPC-21, BioLegend, Cat# 400160, RRID: AB_10900440), AF700-conjugated anti-human CD19 ab (clone SJ25C1, BioLegend, Cat# 363034, RRID: AB_2616936) or AF700-conjugated mouse IgG1

isotype ab (clone MOPC-21, BioLegend, Cat# 400144, RRID: AB_2923250), PerCP-conjugated anti-human/mouse ITGAM ab (clone M1/70, BioLegend, Cat# 101230, RRID: AB_2129375) or PerCP-conjugated rat IgG2b isotype ab (clone RTK4530, BioLegend, Cat# 400630, RRID: AB_893676), SB645-conjugated anti-human CD31 ab (clone WM-59, Thermo Fisher Scientific, Cat# 64-0319-42, RRID: AB_2717093) or SB645-conjugated mouse IgG1 isotype ab (clone P3.6.2.8.1, Thermo Fisher Scientific, Cat# 64-4714-82, RRID: AB_470111), PE-conjugated anti-human CD140a ab (clone 16A1, BioLegend, Cat# 323506, RRID: AB_2268113) or PE-conjugated mouse IgG1 isotype ab (clone MOPC-21, BioLegend, Cat# 400113, RRID: AB_326435), BV711-conjugated anti-human CD56 ab (clone 5.1H11, BioLegend, Cat# 362542, RRID: AB_2565920) or BV711-conjugated mouse IgG1 isotype ab (clone MOPC-21, BioLegend, Cat# 400168), PE/Dazzle594-conjugated anti-human CD45 ab (clone HI30, BioLegend, Cat# 304052, RRID: AB_2563567) or PE/Dazzle594-conjugated mouse IgG1 isotype ab (clone MOPC-21, BioLegend, Cat# 400176, RRID: AB_2923261), BV785-conjugated anti-human ITGAX ab (clone 3.9, BioLegend, Cat# 301644, RRID: AB_2565778) or BV785-conjugated mouse IgG1 isotype ab (clone MOPC-21, BioLegend, Cat# 400170, RRID: AB_11219601), FITC-conjugated anti-mouse ITGB2 ab (clone M18/2, BioLegend, Cat# 101405, RRID: AB_312814) or FITC-conjugated rat IgG2a isotype ab (clone RTK2758, BioLegend, Cat# 400506, RRID: AB_2736919), AF647-conjugated anti-mouse ITGB2 ab (clone M18/2, BioLegend, Cat# 101414, RRID: AB_2265032) or AF647-conjugated rat IgG2a isotype ab (clone RTK2758, BioLegend, Cat# 400526, RRID: AB_2864284), PE/Cy7-conjugated anti-mouse ITGAL ab (clone M17/4, BioLegend, Cat# 101122, RRID: AB_2562781) or PE/Cy7-conjugated rat IgG2a isotype ab (clone RTK2758, BioLegend, Cat# 400522, RRID: AB_326542), PE-conjugated anti-mouse ICAM1 ab (clone YN1/1.7.4, BioLegend, Cat# 116108, RRID: AB_313699) or PE-conjugated rat IgG2b isotype ab (clone RTK4530, BioLegend, Cat# 400608, RRID: AB_326552), anti-human/mouse CD44 ab (clone 5F12, Thermo Fisher Scientific, Cat# MA5-12394, RRID: AB_10984604) or mouse IgG1 isotype ab (clone PPV-06, Thermo Fisher Scientific, Cat# MA1-10405, RRID: AB_2536773) and goat anti-mouse IgG Fc ab (Thermo Fisher Scientific, Cat# 31170, RRID: AB_228290), anti-human/mouse CD44 ab (clone IM7, Thermo Fisher

Scientific, Cat# 14-0441-82, RRID: AB_467246) or rat IgG2b isotype control ab (clone eB149/10H5, Thermo Fisher Scientific, Cat# 14-4031-82, RRID: AB_470099) and goat anti-Rat IgG Fc ab (Thermo Fisher Scientific, Cat# 31226, RRID: AB_228348).

The following abs were used for immunoblotting: unconjugated rabbit anti-human ITGB2 ab (clone D4N5Z, Cell Signaling Technology, Danvers, MA, Cat# 73663, RRID: AB_2799842), unconjugated rabbit anti-mouse ITGB2 ab (clone E9O7W, Cell Signaling Technology, Cat# 72607, RRID: AB_3083057), unconjugated rabbit anti-mouse non-phospho (non-p, active) β -catenin (Ser33/37/Thr41) ab (clone D13A1, Cell Signaling Technology, Cat# 8814, RRID: AB_11127203), unconjugated rabbit anti-mouse LEF-1 ab (clone C12A5, Cell Signaling Technology, Cat# 2230, RRID: AB_823558), unconjugated rabbit anti-mouse p-VANGL2 (Thr78, Ser79, Ser82) ab (clone 7H7C2, Thermo Fisher Scientific, Cat# MA5-38241, RRID: AB_2898157), unconjugated rabbit anti-mouse VANGL2 ab (Thermo Fisher Scientific, Cat# 21492-1-AP, RRID: AB_11182263), horseradish peroxidase (HRP)-linked anti-rabbit IgG (Cell Signaling Technology, Cat# 7074, RRID: AB_2099233), HRP-linked anti- β -actin ab (clone D6A8, Cell Signaling Technology, Cat# 12620, RRID: AB_2797972).

The following abs and reagents were used for cell adhesion, proliferation, or in vivo tumorigenicity studies: recombinant human ICAM-1 (Abcam, Waltham, MA, Cat# ab82125), recombinant mouse ICAM-1 (Abcam, Cat# ab277758), Ultra-LEAF anti-human ITGB2 ab (clone TS1/18, BioLegend, Cat# 302116, RRID: AB_2561483) or Ultra-LEAF mouse IgG1 isotype ab (clone MOPC-21, BioLegend, Cat# 400166, RRID: AB_2927801), Ultra-LEAF anti-mouse ITGB2 ab (clone M18/2, BioLegend, Cat# 101421, RRID: AB_312810) or Ultra-LEAF rat IgG2a isotype ab (clone RTK2758, BioLegend, Cat# 400573, RRID: AB_11147167), anti-mouse ITGB2 ab (clone GAME-46, BD Biosciences, Franklin Lakes, NJ, Cat# BDB557440, RRID: AB_396703) or mouse IgG1 κ (clone R3-34, BD Biosciences, Cat# BDB553922, RRID: AB_479672), pyruvinium pamoate (MedChemExpress, Monmouth Junction, NJ, Cat# HY-A0293), LGK974 (Selleck Chemicals, Houston, TX, Cat# S7143), zamaporvint (Selleck Chemicals, Cat# E1486), and vemurafenib (Selleck Chemicals, Cat# S1267).

The following abs and reagents were used for immunohistochemistry and immunofluorescence:

unconjugated mouse anti-human ITGB2 ab (clone MEM-48, Novus Biologicals, Cat# NB500-379, RRID: AB_10000712), Dako REAL Detection System, Alkaline Phosphatase/RED (Agilent Dako, Santa Clara, CA, Cat# K5005), Biotin-conjugated goat anti-mouse IgG (Thermo Fisher Scientific, Cat# 31800, RRID: AB_228305), AF546-conjugated goat anti-mouse IgG1 (Thermo Fisher Scientific, Cat# A-21123, RRID: AB_2535765), and AF488-conjugated goat anti-mouse IgG1 (Thermo Fisher Scientific, Cat# A-21121, RRID: AB_2535764), unconjugated rabbit anti-human SOX10 (clone EPR4007, Abcam, Cat# ab155279, RRID: AB_2650603), unconjugated rabbit anti-human CD3 (clone SP162, Abcam, Cat# ab135372, RRID: AB_2884903), unconjugated rabbit anti-human CD31 (clone EPR3094, Abcam, Cat# ab76533, RRID: AB_1523298), unconjugated rabbit anti-human ICAM-1 (MilliporeSigma, Cat# SAB5700809, RRID: AB_3669069) and Cy3-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific, Cat# A10520, RRID: AB_10563288) or AF488-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific, Cat# A-11008, RRID: AB_143165), unconjugated mouse anti-human SOX10 (clone 1D2C8, Proteintech, Rosemont, IL, Cat#66786-1-Ig, RRID: AB_2882131) and AF647-conjugated goat anti-mouse IgG2a (Thermo Fisher Scientific, Cat# A-21241, RRID: AB_2535810), unconjugated mouse anti-human PU.1 (clone G148-74, BD Biosciences, Cat# 554268, RRID: AB_395335) and AF488-conjugated goat anti-mouse IgG2a (Thermo Fisher Scientific, Cat# A-21131, RRID: AB_2535771).

Immunofluorescence and immunohistochemistry staining protocols

Multicolor immunofluorescence was performed to detect ITGB2, along with SOX-10, CD3, CD31, PU.1, and/or ICAM-1, with DAPI nuclear counterstain, on FFPE tumor tissues collected from individuals with melanoma, following established methods [11-13]. In brief, the paraffin-embedded sections were heated at 56 °C for 30 min, deparaffinized in xylene for 10 min, and then progressively rehydrated in a graded ethanol series (100%, 95%, 75%, and 50% v/v) with 2-min incubations at each step. Antigen unmasking was achieved by immersing the slides in pH 6.0 Target Antigen Retrieval solution (Agilent Dako) and subjecting them to pressure cooking at 110 °C for 45 min inside a decloaking chamber (Biocare Medical). Nonspecific binding was prevented by applying a blocking mixture containing

10% goat serum plus avidin and biotin blockers (Vector Laboratories). The slides were next incubated overnight at 4 °C with mouse anti-human ITGB2 antibody (diluted 1:100 or 1:200; Novus Biologicals). After three 5-min rinses in 0.1% Tween 20 in TBS, biotinylated goat anti-mouse IgG secondary antibody (1:200; Thermo Fisher Scientific) was added for 1 h at room temperature, followed by additional washes and 1-h incubation with streptavidin-Cy5 (1:50; Thermo Fisher Scientific) or AF546-conjugated goat anti-mouse IgG1 (1:500; Thermo Fisher Scientific).

After re-blocking with 10% goat serum, the tissues were incubated overnight at 4 °C with one of the following primary antibodies: rabbit anti-human SOX-10 (1:500; Abcam), rabbit anti-human CD3 (1:100; Abcam), rabbit anti-human CD31 (1:100; Abcam), mouse anti-human PU.1 (1:100; BD Biosciences), or rabbit anti-human ICAM-1 (1:200; MilliporeSigma). Following a blocking step with 0.1% Sudan Black B (MilliporeSigma) and TBS-T washes, appropriate fluorophore-labeled secondary antibodies were applied for 1 h at room temperature, including Cy3-conjugated goat anti-rabbit IgG (1:2000; Thermo Fisher Scientific), AF647-conjugated goat anti-mouse IgG2a (1:2000; Thermo Fisher Scientific), AF488-conjugated goat anti-mouse IgG2a (1:1000; Thermo Fisher Scientific), or AF488-conjugated goat anti-rabbit IgG (1:1000; Thermo Fisher Scientific). Finally, sections were coverslipped with ProLong Glass Antifade mounting medium containing NucBlue or DAPI (Thermo Fisher Scientific). Images were acquired using an EVOS FL Auto 2 fluorescence imaging system (Thermo Fisher Scientific).

Separate immunohistochemistry for ITGB2 followed the identical preparatory steps. Still, it utilized the Dako REAL Detection System with Alkaline Phosphatase/RED substrate (Agilent Dako) as recommended by the manufacturer, after overnight exposure to the anti-ITGB2 mouse antibody at 1:100 (Novus Biologicals).

To investigate links between ITGB2 levels and melanoma advancement, multicolor immunofluorescence was applied to a tissue microarray. ITGB2 expression in primary melanoma samples from 107 patients (University of Bonn collection) was correlated with documented sentinel lymph node metastasis status (assessed by J.L.). The TMA [14] included duplicate 2-mm cores sourced from benign common nevi (n = 7), primary melanomas (n = 24), and metastatic melanomas (n = 13). Nevi were obtained from

2013 diagnoses, while melanoma cases dated from 1997–2007; all were preserved as FFPE blocks at BWI. Histological review of hematoxylin and eosin-stained slides by two independent dermatopathologists (G.F.M. and C.G.L.) confirmed all clinical diagnoses before the study.

Staining was performed as described previously [11–14] using mouse anti-human ITGB2 (1:200; Novus Biologicals), mouse anti-human SOX10 (1:500; Proteintech), and DAPI. Secondary labeling employed AF488-conjugated goat anti-mouse IgG1 (1:1000; Thermo Fisher Scientific) and AF647-conjugated goat anti-mouse IgG2a (1:2000; Thermo Fisher Scientific). Isotype-matched controls and appropriate tissue controls were included throughout. Two images per intact core or primary tumor specimen were taken at 20X objective magnification. Blinded manual quantification in ImageJ software (NIH) determined the percentage of ITGB2+SOX10+ double-positive cells among all nuclear SOX10-positive melanoma cells across replicate cores.

RNA isolation and quantitative reverse transcription PCR (RT-qPCR)

Total RNA was isolated from cultured cell lines and tumor xenografts with the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany, Cat# 74134), or from mouse lung samples using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Cat# 74704), strictly following the supplier's guidelines, as previously outlined [11, 12, 15]. The purified RNA served as template for cDNA synthesis employing the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific, Cat# 11754050). Subsequent RT-qPCR reactions were run in triplicate for in vitro cell samples and in duplicate for murine tissue specimens. Amplification was performed with Fast SYBR Green Master Mix (Thermo Fisher Scientific) or TaqMan assays (Thermo Fisher Scientific) using primer/probe sets. Reactions took place on QuantStudio 3 or QuantStudio 5 Real-Time PCR instruments (Applied Biosystems, Waltham, MA). Standard thermal cycling conditions consisted of an initial 95 °C hold for 20 s, followed by 40 cycles of 95 °C for 1 s, with melt curve analysis included where relevant. For human 18S rRNA detection, a distinct program was used: 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 s, 60 °C for 20 s, and 68 °C for 1 min, ending with a melt curve confirmation, consistent with earlier reports [12]. TaqMan-based reactions were performed according to the vendor's recommended cycling parameters.

Transcript abundance was normalized to human 18S rRNA or to human/mouse β -actin (ACTB/Actb) as endogenous controls. The $2^{-\Delta\Delta Ct}$ determined fold-change values (delta-delta Ct) approach [11, 12, 15, 16]. Any samples displaying Ct values above 35, exceeding one melt peak, matching the water-only negative control, or showing housekeeping gene Ct >25 in TaqMan runs were classified as not detected (nd).

Genomic DNA isolation and qPCR

Genomic DNA was purified from samples using the DNeasy Blood and Tissue Kit (Qiagen, Cat# 69504) per the manufacturer's directions, as noted in prior work. For quantification of melanoma lung metastases, 0.5 μ g of genomic DNA was combined with TaqMan qPCR primers targeting EGFP and run on a QuantStudio 5 Real-Time PCR system (Applied Biosystems). Normalization was performed relative to murine Actb levels, with cycling parameters set according to the manufacturer's standard protocol.

Flow cytometry analysis

Surface expression of the ITGB2 heterodimer and ICAM-1 protein was assessed on human and mouse melanoma cell lines, human HSB-2 and mouse EL-4 T-lymphoblastic cells, HUVEC and mouse C166 endothelial cells, as well as single-cell suspensions prepared from melanoma patient tumors. Multi-parameter flow cytometry was performed according to established procedures [11, 12, 15, 16]. Dead cells were excluded by incubating for 10 min at room temperature in the dark with Zombie NIR Fixable Viability Dye (1:1000; BioLegend, Cat# 423106) according to the product instructions. Fc receptor-mediated nonspecific binding was prevented using Human TruStain FcX (BioLegend, Cat# 422302) for 10 min at room temperature for human cells or TruStain FcX PLUS anti-mouse CD16/32 antibody (BioLegend, Cat# 156604) for 10 min at 4 °C for murine samples, as detailed previously [13, 15]. Staining was performed for 30 min at 4 °C with fluorochrome-labeled antibodies (5–10 μ g/mL) in flow cytometry buffer (PBS supplemented with 2% v/v FBS). Cells were washed afterward, and all experiments included isotype-matched control antibodies along with gating to remove dead cells and doublets [11-16]. Data acquisition occurred on either a FACSCanto (BD Biosciences) or an Aurora spectral flow cytometer (Cytek, Fremont, CA), and results were processed with FlowJo software version 10.8.1 (TreeStar, Ashland, OR).

CD44 crosslinking experiments

The influence of CD44 crosslinking on ITGB2 surface expression and activation was examined by flow cytometry using the protocol described above. In brief, both human and murine melanoma cells were pretreated for 1 h at 37 °C with anti-CD44 antibody (10 μ g/mL; clones 5F12 or IM7, Thermo Fisher Scientific) or matched isotype control antibodies in flow cytometry buffer. Following a wash step, crosslinking was induced by adding goat anti-mouse or goat anti-rat IgG Fc secondary antibodies (10 μ g/mL; Thermo Fisher Scientific) for 45 min at 37 °C in the same buffer. Cells were washed twice and then stained for ITGB2 and its partnering α subunits on the cell surface as outlined in the standard flow cytometry procedure.

Immunoblotting

Wild-type, Itgb2-deficient (KO), or Cas9-control melanoma cells were harvested and disrupted on ice using cold RIPA buffer (Thermo Fisher Scientific) that had been supplemented with a Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology, Cat# 5872). After vortexing the mixtures for 30 min at 4 °C [11, 12], protein levels in the cleared lysates were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) according to the provided instructions. The extracted proteins were then separated through SDS-PAGE and transferred onto Immobilon-P PVDF membranes (MilliporeSigma). To reduce background, the membranes were blocked for at least 1 hour at room temperature in TBS-T buffer containing 5% (w/v) non-fat dry milk (Thermo Fisher Scientific). Overnight incubation at 4 °C was followed by either anti-mouse ITGB2 (clone E9O7W, 1:1000; Cell Signaling Technology) or anti-human ITGB2 (clone D4N5Z, 1:1000; Cell Signaling Technology). Three washes in TBS-T were followed by a 1-hour room-temperature exposure to HRP-conjugated secondary antibody (1:2000; Cell Signaling Technology). Signal development employed Lumi-Light Western Blotting Substrate (MilliporeSigma), with bands captured on HyBlot CL Autoradiography Films (Thomas Scientific, Swedesboro, NJ) using a Kodak Min-R mammography processor (Kodak, Rochester, NY) [11-13, 16]. Where required, ReBlot Plus Strong Antibody Stripping Solution (MilliporeSigma) was applied according to manufacturer directions to remove prior antibodies. The stripped membranes were re-blocked and reprobed overnight at 4 °C with HRP-conjugated anti- β -actin

(clone D6A8, 1:1000; Cell Signaling Technology) in TBS-T containing 5% non-fat dry milk, then visualized as described.

For Wnt pathway modulation studies, YUMM5.2 cells were grown in 60-mm dishes with complete RPMI-1640 medium (10% heat-inactivated FBS and 1% penicillin/streptomycin). Following overnight serum starvation in serum-free RPMI-1640 plus 1% penicillin/streptomycin at 37 °C and 5% CO₂, treatment was carried out using pyrvinium pamoate (6.5 µM), LGK974 (20 µM), zamaporvint (300 nM), or matched vehicle for time periods between 20 min and 12 h. Lysates from inhibitor-treated wild-type YUMM5.2 cells, vehicle controls, Itgb2 KO cells, and Cas9 controls were prepared and run on SDS-PAGE as above. Transfer to membranes was performed using the Trans-Blot Turbo system (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol. Blocking occurred for ≥1 h at room temperature in 5% milk/TBS-T. Primary antibodies incubated overnight at 4 °C included rabbit anti-mouse active (non-phosphorylated) β-catenin (Ser33/37/Thr41) (clone D13A1, 1:1000; Cell Signaling Technology), rabbit anti-mouse LEF-1 (clone C12A5, 1:1000; Cell Signaling Technology), and rabbit anti-mouse p-VANGL2 (Thr78, Ser79, Ser82) (clone 7H7C2, 1:500; Thermo Fisher Scientific). After TBS-T washes, HRP-secondary antibody (1:1000; Cell Signaling Technology) was added for 1 h at room temperature. Visualization was performed using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) on a Fusion FX Imaging System (Vilber Lourmat, Collégien, France). Some blots were stripped with Restore PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific) when suitable, or processed on separate membranes. Loading control probing was performed overnight at 4 °C with HRP-conjugated anti-β-actin (clone D6A8, 1:1000) or unconjugated rabbit anti-mouse VANGL2 (1:500), the latter followed by HRP-secondary (1:2000), all in 5% milk/TBS-T, and developed as above.

Establishment of melanoma lines stably expressing nuclear GFP

Nuclear GFP-expressing stable variants of B16-F10 and YUMM5.2 melanoma cells were created by inserting a nuclear localization sequence into the GFP coding region of the MSCV-N-Flag-HA-GFP plasmid (Addgene #41034, RRID: Addgene_41034). Retroviruses were generated by transfecting HEK-293EBNA cells (ATCC

CRL-10852, RRID: CVCL_6974) with this construct together with packaging plasmids GagPolDS and pN8e-VSV-G using Lipofectamine 2000 (Thermo Fisher Scientific) [11]. Viral particles were collected from the medium 48–72 h later and filtered [12]. Melanoma cells were infected and placed under puromycin selection, then subjected to repeated FACS sorting on either a BD FACSAria II or Beckman Coulter MoFlo Astrios EQ at the Beth Israel Deaconess Medical Center Flow Cytometry Core (Boston, MA) until GFP positivity exceeded 95%.

Production of stable Itgb2 knockout lines in B16-F10 and YUMM5.2 melanoma cells

Stable Itgb2 knockout (KO) versions of B16-F10 and YUMM5.2 melanoma cells were established via CRISPR/Cas9-mediated gene editing, following previously reported approaches [15]. Initially, stable Cas9-expressing monoclonal lines were generated in both cell lines through lentiviral delivery. Lenti-X 293T packaging cells (Takara Bio, San Jose, CA, Cat# 632180, RRID: CVCL_4401) were co-transfected with the lentiCas9-EGFP expression construct (Addgene plasmid #63592, RRID: Addgene_63592), along with the packaging plasmids psPAX2 (Addgene #12260, RRID: Addgene_12260) and pMD2.G (Addgene #12259, RRID: Addgene_12259), using Lipofectamine 3000 (Thermo Fisher Scientific) as directed by the manufacturer. Supernatants containing lentiviral particles were collected 48–72 hours after transfection, concentrated by centrifugation at 20,000 rpm for 6 hours, and passed through 0.45 µm filters. The virus was then used to infect B16-F10 and YUMM5.2 cells in the presence of 10 µg/ml polybrene (MilliporeSigma). EGFP-positive cells were subsequently isolated by single-cell sorting into 96-well flat-bottom plates (Thermo Fisher Scientific) on a MoFlo Astrios EQ cell sorter (Beckman Coulter) at the Beth Israel Deaconess Medical Center Flow Cytometry Core (Boston, MA). Several individual clones were propagated, and those maintaining >95% EGFP expression were verified using an Aurora Spectral Analyzer (Cytek).

To ensure the Cas9-EGFP clones retained growth properties comparable to the parental wild-type lines, selected Cas9-expressing clones and unmodified control cells were implanted subcutaneously into the flanks of C57BL/6 wild-type mice, and tumor development was monitored. A Cas9-EGFP clone that displayed *in vivo* growth kinetics matching the wild-type cells was chosen

for further editing. This clone was then transduced with a plasmid carrying two guide RNAs (TGCCCCCGTTGGTCGAACTC and GTGACTTTCCGGCGGGCCAA) directed against murine *Itgb2*, along with a transient blue fluorescent protein (BFP) marker for selection (VectorBuilder, Chicago, IL). 48 hours post-transduction, double-positive (BFP+ EGFP+) cells were single-cell-sorted into 96-well plates as described above. Emerging colonies were expanded and evaluated for *Itgb2* gene deletion and loss of ITGB2 protein by quantitative RT-PCR, flow cytometry, and Western blotting [15].

Confirmation of genomic disruption at the *Itgb2* locus involved extracting DNA from promising clones using the DNeasy Blood & Tissue Kit (Qiagen), followed by PCR amplification of the targeted region with Platinum Taq DNA Polymerase (Thermo Fisher Scientific) and primers flanking the gRNA sites [15]. The reaction conditions included 1.5 mM MgCl₂, an initial denaturation at 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 62 °C for 30 s, and 68 °C for 20 s, plus a final extension at 68 °C for 10 min. Primer sequences were: forward 5'-CCTGACCTCAGATCCCTCCT-3' and reverse 5'-GGTAATTGCCATGCGGGTTC-3'. The resulting 328 bp amplicons representing individual alleles were purified from gels, cloned into the pCR2.1 vector using the TA Cloning Kit (Thermo Fisher Scientific), and introduced into One Shot TOP10 chemically competent *E. coli* (Thermo Fisher Scientific) [15]. Plasmids isolated via the ZymoPURE Plasmid Miniprep Kit (Zymo Research, Irvine, CA) were submitted for Sanger sequencing (GENEWIZ/Azenta Life Sciences, Burlington, MA) [15], confirming homozygous knockout of *Itgb2*.

ICAM-1-mediated cell adhesion assays

Cell adhesion to ICAM-1 was evaluated in melanoma cells using 96-well plates with white walls and clear bottoms (Thermo Fisher Scientific), following an established protocol [17] with several adjustments. In brief, replicate wells (quadruplicate) were coated with 25 µg/ml recombinant human ICAM-1 (Abcam, Cat# ab82125) or 50 µg/ml recombinant mouse ICAM-1 (Abcam, Cat# ab277758) diluted in PBS (Life Technologies). Plates were centrifuged at 2,000 rpm for 5 minutes and then incubated for 2 hours at 37 °C. After coating, wells were blocked for 30 minutes at 37 °C with either 1% (w/v) BSA or 1% (v/v) FBS in PBS. Wild-type, *Itgb2* KO, or Cas9 control cells were resuspended in

adhesion buffer consisting of RPMI 1640 supplemented with 0.2% BSA.

For antibody blockade experiments, wild-type melanoma cells were pretreated on ice (4 °C) for 30 minutes with 20 µg/ml function-blocking anti-human ITGB2 antibody (clone TS1/18, BioLegend) or anti-mouse ITGB2 antibody (clones M18/2, BioLegend or GAME-46, BD Biosciences), compared against isotype-matched controls. In selected experiments, cells were also incubated in parallel with 50 mM EDTA (Thermo Fisher Scientific) in Ca²⁺/Mg²⁺-free PBS. For Wnt pathway inhibitor studies, wild-type mouse cells were first treated with ITGB2-blocking or control antibodies in adhesion buffer, then exposed to pyrvinium pamoate (6.5 µM), LGK974 (20 µM), zamaportin (10 µM), or vehicle for an additional 30 minutes on ice at 4 °C. *Itgb2* KO and Cas9 control cells were similarly preincubated with the Wnt inhibitors or vehicle.

Cell suspensions (100 µl per well) containing 4–5 × 10⁴ melanoma cells or 1.5 × 10⁵ T lymphoblastic cells were added to the prepared plates and allowed to adhere for 45 minutes at 37 °C. Non-adherent cells were removed by washing the wells three times with Ca²⁺/Mg²⁺-containing PBS (Thermo Fisher Scientific) using a gentle squirt bottle. The number of adherent cells was measured using the Promega CellTiter-Glo 2.0 Assay (Promega, Madison, WI, Cat# G9242) on a GloMAX 96 Microplate Luminometer (Promega) according to the manufacturer's instructions [16]. Results are expressed as the percentage of adherent cells relative to the total input cell number.

3D tumor sphere proliferation assays

The impact of *Itgb2* deletion on melanoma cell growth in three-dimensional tumor spheres was assessed using the Promega CellTiter-Glo 2.0 Cell Viability Assay (Promega) on a GloMAX 96 Microplate Luminometer, as outlined above. Ultra-low attachment 96-well plates were first treated with 60 µl per well of Anti-Adherence Rinsing Solution (Stemcell Technologies, Cambridge, MA), centrifuged at 2,000 rpm for 5 minutes, aspirated, and rinsed with RPMI-1640 medium. Cells were resuspended at 8 × 10³ cells per 100 µl in culture medium composed of RPMI 1640 containing 1% (w/v) methylcellulose, 1% (v/v) FBS, and 1% (v/v) penicillin/streptomycin. The suspension was filtered through 0.35 µm Cell Strainer Snap Caps (Corning, Corning, NY) before seeding 100 µl per well [11, 12]. In certain experiments, cells were exposed to vemurafenib

(20 μ M) or vehicle control. Cultures were maintained for 48 hours at 37 °C in a 5% CO₂ incubator.

Mouse models and in vivo tumor formation experiments

Wild-type C57BL/6 mice (RRID: IMSR_JAX:000664), Icam1tm1Jcgr knockout C57BL/6 mice [18] (RRID: IMSR_JAX:002867), and immunodeficient NOD/SCID IL-2R $\gamma^{-/-}$ (NSG) mice (RRID: IMSR_JAX:005557) were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in the BWH animal facility according to prior descriptions [11, 12, 16]. Both male and female animals (minimum 6 weeks old) were used, with groups balanced for sex and age. All procedures complied with National Institutes of Health guidelines for animal care and were conducted under the approved BWH IACUC protocol 2016N000112.

Human melanoma lines C8161 and MDA-MB-435S, or mouse lines B16-F10 and YUMM5.2, were injected subcutaneously into the flanks of recipient mice. Dosing was 1×10^6 cells per inoculum for C8161 or MDA-MB-435S into NSG mice, 1×10^5 cells for B16-F10 into wild-type C57BL/6, Icam1 KO C57BL/6, or NSG mice, and 1×10^6 cells for YUMM5.2 into the same strains [11, 12, 16].

In antibody treatment studies, mice received intraperitoneal injections of 200 μ g of Ultra-LEAF blocking anti-human ITGB2 (clone TS1/18, BioLegend) or anti-mouse ITGB2 (clone M18/2, BioLegend), or isotype-matched controls, every 3 days, beginning on the day of tumor cell injection and continuing throughout the study. For Wnt inhibition experiments, animals were administered pyrvinium pamoate (0.3 mg/kg [19]) or vehicle by intraperitoneal injection daily. In other cohorts, submaximal doses of LGK974 (3 mg/kg body weight/day [20, 21]) or zamaporvint, or vehicle, were provided continuously by incorporation into custom rodent chow (Research Diets, Inc., New Brunswick, NJ). Tumor dimensions were recorded at least weekly, and volume was calculated using the standard formula $TV \text{ (mm}^3\text{)} = (\pi/6) \times 0.5 \times \text{length} \times (\text{width})^2$ [12, 16]. Monitoring continued until the experimental endpoint, signs of disease, or when tumor burden necessitated euthanasia per protocol. The maximum allowable tumor size per BWH IACUC guidelines is 2 cm in any dimension; this limit has never been exceeded. Additional euthanasia criteria included tumor ulceration or moribund status, which were also respected in all experiments [12, 16].

Bulk RNA sequencing (total mRNA-seq)

For transcriptome profiling of melanoma tissues via total mRNA sequencing, freshly collected tumor specimens were immediately snap-frozen in liquid nitrogen, stored temporarily at -80 °C, and shipped to GENEWIZ/Azenta for downstream handling. GENEWIZ/Azenta performed RNA isolation, library preparation, and sequencing. Total RNA was purified from the frozen samples using the RNeasy Plus Mini Kit (Qiagen, Cat# 74134) according to the manufacturer's protocol. RNA concentration was determined with a Qubit 2.0 Fluorometer (Thermo Fisher Scientific), while RNA quality and integrity were assessed using RNA ScreenTape on an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA). Sequencing libraries were generated using the NEBNext Ultra II RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA) according to the manufacturer's recommendations. Poly(A) mRNA was first captured using Oligo(dT) beads, then fragmented for 15 minutes at 94 °C. Second-strand cDNA synthesis was performed, after which the cDNA ends were repaired and 3' adenylated. Universal adapters were ligated, indexes were added, and PCR-amplified libraries were run with a limited number of cycles. Library quality was verified on the Agilent TapeStation, and quantification was done using the Qubit 2.0 Fluorometer and KAPA Library Quantification qPCR assay (KAPA Biosystems, Wilmington, MA). Multiplexed libraries were clustered across three lanes of a flow cell and sequenced on an Illumina HiSeq 4000 platform (Illumina, San Diego, CA) with 2×150 bp paired-end reads, following the manufacturer's guidelines.

Bioinformatics processing and differential expression analysis of RNA-seq data

Raw sequencing reads were converted to gene-level counts using the bcbio-nextgen RNA-seq pipeline (version 1.2.9-8eb78b7; <https://bcbio-nextgen.readthedocs.org>). Initial quality assessment of the raw data was conducted with FastQC v0.11.9 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Reads were aligned to the *Mus musculus* mm10 (UCSC 2011/12) genome assembly employing STAR v2.6.1d [22]. Alignment quality was evaluated for metrics such as coverage uniformity, ribosomal RNA contamination, distribution across genomic features (exons, introns), library complexity, and other parameters using FastQC v0.11.9, Qualimap v2.2.2d

[23], MultiQC v1.11 (<https://github.com/ewels/MultiQC>), and additional custom scripts. Transcript abundances in Transcripts Per Million (TPM) were estimated at the isoform level using Salmon v1.6.0, as isoform-level quantification improves accuracy when aggregating to gene-level estimates. Gene-level counts were summarized from Salmon TPMs using tximport, and differential expression analysis was performed with DESeq2 v1.38.3 [24] in R (version 4.2.2; <https://www.R-project.org/>).

Analyses were conducted independently for each mouse strain (wild-type C57BL/6, *Icam1* knockout C57BL/6, and NSG) to compare Cas9 control versus *Itgb2* KO tumors. Log2 fold changes were shrunk using the apleglm method [25] to minimize noise. Differentially expressed genes were defined as those with a shrunk log2 fold change greater than 0.5 and an adjusted p-value < 0.05 (false discovery rate-corrected). Genes showing differential regulation in both NSG and wild-type C57BL/6 backgrounds, but not in *Icam1* knockout C57BL/6 mice, were selected for downstream protein-protein interaction network analysis using STRING (<https://string-db.org>). A full-network analysis was run with a minimum interaction confidence score of 0.371. Heatmaps and volcano plots were produced from connected nodes in the main network using the pheatmap (<https://CRAN.R-project.org/package=pheatmap>) and EnhancedVolcano R packages (<https://github.com/kevinblighe/EnhancedVolcano>). The displayed STRING network highlights major clusters identified at a higher confidence threshold (interaction score 0.54).

Evaluation of publicly available single-cell RNA sequencing datasets

The Seurat object from Pozniak *et al.* [26] was obtained and analyzed using the Seurat dotplot function to display both the percentage of cells expressing ITGB2 and ICAM1 and their average expression levels. Expression was further compared between ABCB5⁺ and ABCB5[−] cells and between NGFR⁺ and NGFR[−] populations. Since ABCB5⁺ and NGFR⁺ cells generally exhibited higher sequencing depth, data were normalized before visualization. Sequencing depth was approximated by the number of detected genes per cell; quartiles were defined based on ABCB5[−] and NGFR[−] cells. ABCB5⁺ and NGFR⁺ subsets were then randomly downsampled to match the quartile distribution of the negative

populations before generating dot plots for ITGB2 and ICAM1.

Data from Goyal *et al.* [27] were downloaded, and pathway activity scores were computed by summing expression of all genes belonging to the canonical Wnt signaling pathway GO term and the non-canonical Wnt signaling pathway GO term. These scores, together with ITGB2 and ICAM1 expression, were visualized using Seurat dotplots. Additional examination of ITGB2, ICAM1, and CD44 expression across patient-derived melanoma cells, T cells, and endothelial cells was conducted on the Tirosh *et al.* [28] dataset.

Statistical evaluation

Group comparisons were performed using two-tailed Student's t-test, Mann-Whitney U test, Wilcoxon signed-rank test, or Fisher's exact test for two-group analyses; one-way ANOVA followed by Dunnett's post-hoc test or Kruskal-Wallis test with Dunn's post-hoc test for three or more groups; and repeated-measures two-way ANOVA or mixed-effects models with Šidák's multiple comparisons test for time-course data such as tumor growth curves. Statistical significance was defined as $p < 0.05$. All analyses were performed using GraphPad Prism version 10.1.1 (GraphPad, San Diego, CA).

Results and Discussion

Melanoma cells produce ITGB2, whose expression and functional activation are controlled by CD44

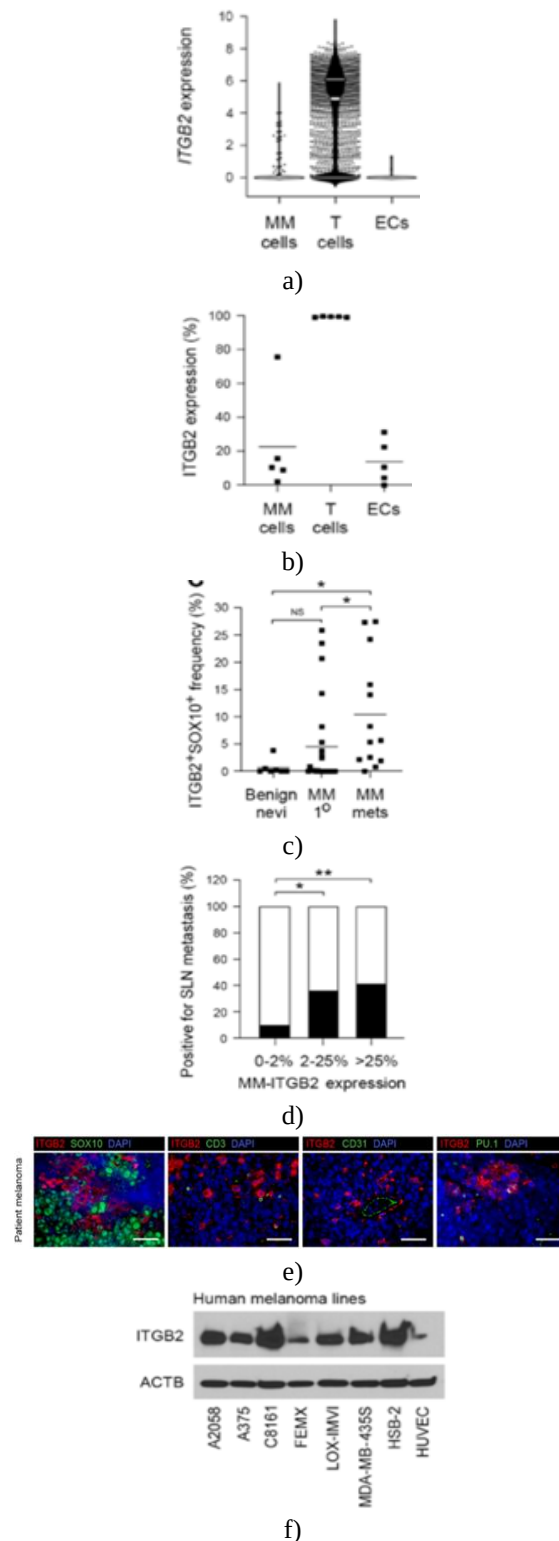
Examination of a publicly available single-cell RNA-seq dataset [28] revealed that the human ITGB2 gene is expressed in melanoma cells across 13 of 14 patient tumor samples analyzed. The expression range overlapped with that seen in T cells (positive control) and was higher than in endothelial cells (ECs); (**Figure 1a**). Surface protein detection by flow cytometry confirmed ITGB2 on $22.5 \pm 13.5\%$ (mean $\pm$ SEM) of melanoma cells across five of five patients (**Figure 1b**), in comparison with $99.4 \pm 0.1\%$ on tumor-infiltrating T cells and $13.7 \pm 5.8\%$ on ECs (**Figure 1b**).

Immunofluorescence analysis of a clinical tissue microarray (TMA [14]) showed that the average proportion of ITGB2⁺SOX10⁺ melanoma cells was markedly elevated in metastatic lesions ($10.4 \pm 2.9\%$, $n = 13$ patients) relative to primary melanomas ($4.5 \pm 1.6\%$, $n = 24$ patients) and benign nevi ($0.7 \pm 0.5\%$, $n = 7$ patients); (**Figure 1c**). In an expanded set of primary melanoma specimens from 105 patients, ITGB2

positivity on cancer cells was < 2% in 40 cases, 2–25% in 36 cases, and >25% in 29 cases. The two groups with detectable to high ITGB2 expression exhibited a higher rate of sentinel lymph node (SLN) metastases than the low/absent ITGB2 group (**Figure 1d**). Multiplex immunofluorescence staining distinguished ITGB2 expression on nuclear SOX10+ melanoma cells from that on CD3+ T cells, CD31+ endothelial cells, or PU.1+ macrophages within patient tumor samples (**Figure 1e**). Single-cell RNA-seq profiling of the four known ITGB2 heterodimer α -subunit partners [2] indicated that ITGAL transcripts were prominently expressed in melanoma cells. In contrast, ITGAD, ITGAM, and ITGAX showed minimal or absent expression. Flow cytometry corroborated this pattern, revealing substantial surface ITGAL ($6.6 \pm 3.6\%$) but very low ITGAM ($2.4 \pm 1.4\%$) and ITGAX ($1.1 \pm 1.0\%$) on patient-derived melanoma cells (n = 5). RT-qPCR verified ITGB2 as well as ITGAD, ITGAL, ITGAM, and ITGAX transcripts in multiple human melanoma cell lines (A2058, A375, C8161, FEMX, LOX-IMVI, MDA-MB-435S), HSB-2 T-lymphoblastic control cells, and HUVEC. Comparable murine transcripts for *Itgb2*, *Itgad*, *Itgal*, *Itgam*, and *Itgax* were detected in B16-F10, YUMM1.7, YUMM3.3, YUMM4.1, and YUMM5.2 melanoma lines, as well as EL-4 T-cell lymphoma and C166 endothelial controls. Western blotting confirmed ITGB2 protein in the listed human melanoma lines and HSB-2 cells, with only trace amounts in HUVEC (**Figure 1f**). Parallel analysis showed ITGB2 protein in murine melanoma lines and EL-4 cells, but not in C166 cells (**Figure 1g**).

Crosslinking the hyaluronan receptor CD44 with specific antibodies on melanoma cells led to strong upregulation of baseline surface ITGB2 protein in every human (**Figure 1h**) and murine line tested (**Figure 1i**), with increases exceeding 70-fold in some cases. CD44 crosslinking also boosted the main melanoma-expressed pairing subunit, ITGAL, without affecting ITGAM or ITGAX in both human and murine melanoma cells. Furthermore, CD44 engagement promoted a conformational shift in ITGB2 toward an open, activated state detectable by the activation-sensitive antibodies KIM-127 (**Figure 1j**) and MEM-148 (**Figure 1k**). Patient melanoma cells displayed higher CD44 surface levels than those on tumor-infiltrating T cells and ECs, consistent with a regulatory role for CD44 in controlling ITGAL/ITGB2 on melanoma cells. Collectively, these findings demonstrate that melanoma cells express the

ITGB2 heterodimer and that CD44 induces its expression and activation.



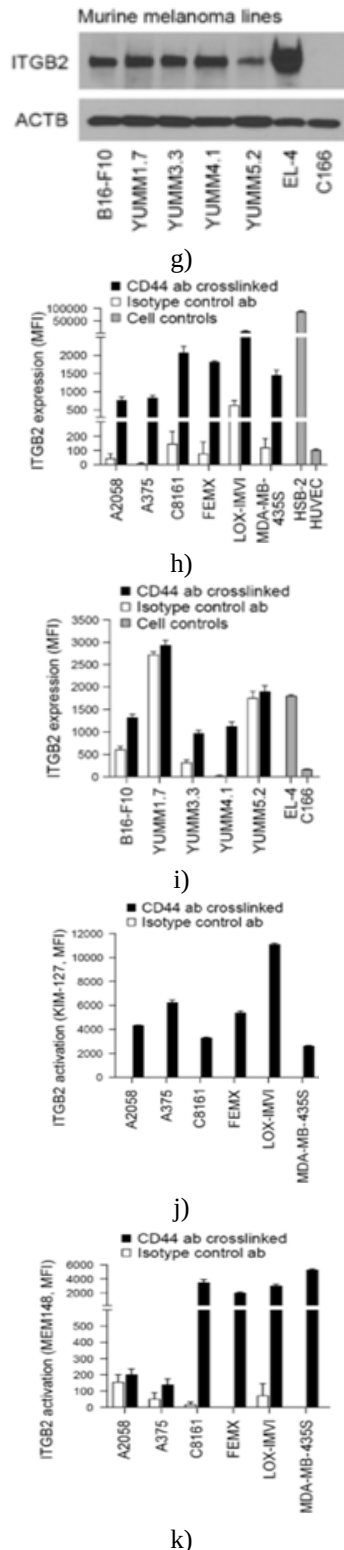


Figure 1. Melanoma cell-autonomous ITGB2 expression and its activation through CD44.

(a) Single-cell (sc) RNA sequencing was used to compare human ITGB2 transcript expression in

melanoma (MM) cells with that in tumor-infiltrating T lymphocytes and endothelial cells (ECs). Data are displayed as violin plots with overlaid individual cells, where the bold white line denotes the median and the thin white lines represent the upper and lower quartiles. (b) Flow cytometric analysis quantified the proportion (mean) of cells expressing surface ITGB2 protein among patient-derived melanoma cells, T cells, and ECs ($n = 5$ patients). (c) Multicolor immunofluorescence staining of a melanocytic tissue microarray (TMA) was performed to determine the mean percentage of ITGB2⁺SOX10⁺ cells in benign nevi ($n = 7$), primary melanoma specimens ($n = 24$), and metastatic melanoma samples ($n = 13$). Statistical significance was evaluated using the Kruskal–Wallis multiple-comparisons test. (d) Immunostaining of primary melanoma specimens ($n = 105$) was used to classify tumors according to the proportion of ITGB2-positive melanoma cells (0–2%, $n = 40$; 2–25%, $n = 36$; >25%, $n = 29$). The incidence of sentinel lymph node (SLN) metastasis was then compared across these groups. Black bars indicate ITGB2-positive melanoma cells, whereas white bars indicate ITGB2-negative cells. Statistical analysis was conducted using Fisher's exact test. (e) Representative multiplex immunofluorescence images of primary melanoma biopsies demonstrating co-localization of ITGB2 (red) with the melanocyte nuclear marker SOX-10 (green, first panel), the pan-T-cell marker CD3 (green, second panel), the endothelial marker CD31 (green, third panel), or the macrophage marker PU.1 (green, fourth panel). Nuclei were visualized with DAPI (blue). Scale bars represent 50 μ m. (f, g) Representative immunoblot analyses showing ITGB2 protein expression in (f) human melanoma cell lines (A2058, A375, C8161, FEMX, LOX-IMVI, and MDA-MB-435S) together with HSB-2 T-lymphoblastic leukemia cells and HUVEC endothelial cells as controls, and (g) murine melanoma cell lines (B16-F10, YUMM1.7, YUMM3.3, YUMM4.1, and YUMM5.2) alongside EL-4 T-cell lymphoma cells and C166 endothelial cells. (H, I) Flow cytometry was used to assess the impact of CD44 antibody-mediated crosslinking (black bars) compared with isotype control antibody treatment (white bars) on ITGB2 surface abundance, expressed as mean fluorescence intensity (MFI $\pm$ SEM), in (h) human melanoma cell lines and (i) murine melanoma cell lines. Corresponding control cells are indicated by gray bars. (j, k) Following CD44 antibody crosslinking, the activation status of melanoma-associated ITGB2 was

evaluated by flow cytometry using the activation-specific ITGB2 antibody clones j (KIM-127) and k (MEM-148). Data are presented as MFI $\pm$ SEM. Results shown are representative of at least three independent experiments ($n \geq 3$). * $P < 0.05$; ** $P < 0.01$; NS, not significant.

Antibody-mediated inhibition of melanoma cell-intrinsic ITGB2 suppresses ICAM-1-dependent adhesion, tumor growth, and metastatic dissemination

To investigate the functional contribution of tumor cell-derived ITGB2 to melanoma progression, we first examined its role in adhesion to ICAM-1, one of its principal ligands [2]. Human metastatic melanoma cell lines C8161 and MDA-MB-435S, together with ITGB2-positive HSB-2 control cells, exhibited robust attachment to immobilized human ICAM-1, with adhesion levels significantly exceeding those observed on negative control substrates (**Figure 2a**). Blocking ITGB2 with a neutralizing antibody or disrupting integrin activity using ethylenediaminetetraacetic acid (EDTA) markedly reduced ICAM-1-mediated adhesion (**Figure 2a**), consistent with the established inhibitory effects of EDTA on integrin function [29].

Comparable findings were obtained using murine models. B16-F10 and YUMM5.2 melanoma cells, as well as EL-4 control cells, adhered efficiently to murine ICAM-1, whereas treatment with either an ITGB2-blocking antibody or EDTA significantly impaired this interaction (**Figure 2b**).

To determine whether ITGB2 contributes to tumor growth in vivo, NSG mice bearing xenografts of human C8161 or MDA-MB-435S melanoma cells received a human-specific ITGB2-neutralizing antibody. Antibody treatment significantly inhibited tumor expansion compared with controls (**Figure 2c**). Likewise, administration of an anti-mouse ITGB2-blocking antibody substantially reduced the growth of murine B16-F10 and YUMM5.2 tumors implanted in NSG mice (**Figure 2d**).

Supporting these observations, analysis of tumor tissues demonstrated that expression of melanoma-intrinsic ITGB2/*Itgb2* and ICAM1/*Icam1* transcripts was approximately 4- to more than 1,000-fold elevated in vivo relative to matched in vitro cultures, consistent with enhanced ITGB2 signaling within the tumor microenvironment.

In contrast, treatment of immunocompetent C57BL/6 mice with an ITGB2-neutralizing antibody produced

different outcomes. Antibody administration accelerated the growth of B16-F10 tumors. It did not significantly alter the growth of YUMM5.2 tumors compared with isotype-treated controls (**Figure 3a**). These effects coincided with a pronounced reduction in T-cell infiltration into both tumor models following ITGB2 blockade (**Figure 3b**), suggesting that inhibition of host immune-cell ITGB2 counterbalanced the direct antitumor effects observed in T-cell-deficient NSG mice (**Figure 2d**).

Despite this limitation, ITGB2 inhibition produced a striking suppression of metastatic spread. In mice bearing subcutaneous GFP-labeled tumors of comparable size, pulmonary metastases were reduced by approximately eightfold in the B16-F10 model and by more than 270-fold in the YUMM5.2 model, as quantified by genomic GFP copy number in lung tissue using qPCR (**Figure 3c**).

To determine whether melanoma-derived ITGB2 requires host ICAM-1, B16-F10 and YUMM5.2 cells were implanted into *Icam1*^{-/-} C57BL/6 mice, which subsequently received either ITGB2-neutralizing antibodies or isotype controls (**Figure 3d**). As anticipated, loss of host *Icam1* decreased the abundance of tumor-infiltrating T cells (**Figure 3e**), resembling the immune-deficient NSG setting in which melanoma-targeted ITGB2 inhibition effectively restrained tumor growth (**Figure 2d**).

However, neutralization of ITGB2 produced little additional effect on tumor growth in *Icam1*^{-/-} mice (**Figure 3d**), indicating that melanoma cell-derived ITGB2 relies on host ICAM-1 to support tumor development. Notably, the absence of host *Icam1* itself resulted in larger B16-F10 and YUMM5.2 tumors (**Figure 3d**) than those observed in wild-type mice expressing ICAM-1 (**Figure 3a**), a finding consistent with the greater accumulation of intratumoral T cells in the wild-type setting (**Figure 3e**).

Remarkably, although primary tumors were larger in *Icam1*^{-/-} hosts, metastatic dissemination to the lungs was markedly diminished compared with wild-type animals, with the reduction being especially pronounced for YUMM5.2 tumors (**Figure 3f**). These findings further support the concept that interactions between melanoma cell-intrinsic ITGB2 and host ICAM-1 play a critical role in promoting metastatic progression (**Figures 3c and 3f**).

Collectively, these results demonstrate that a melanoma cell-intrinsic ITGB2–ICAM-1 signaling axis drives both primary tumor growth and metastatic dissemination.

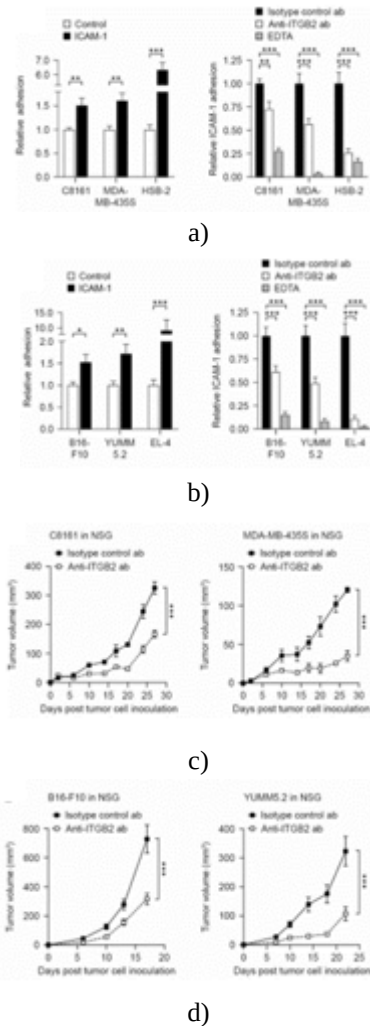
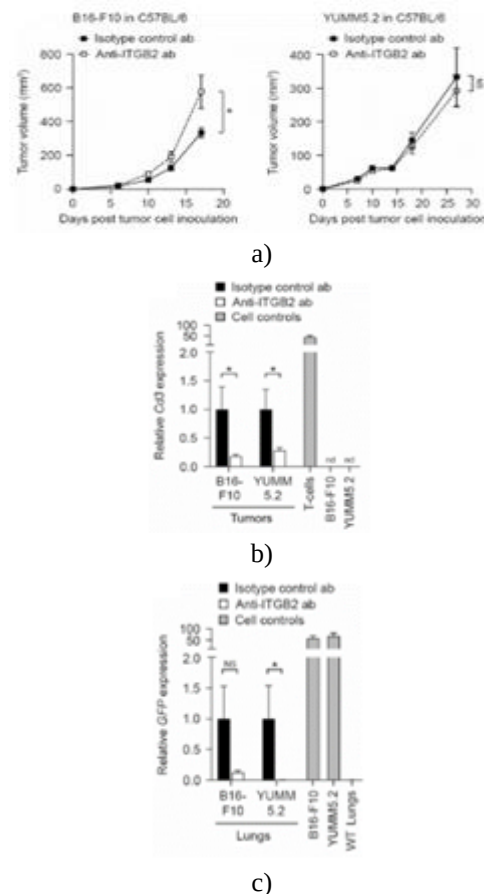


Figure 2. Antibody-mediated inhibition of melanoma cell-autonomous ITGB2 suppresses ICAM-1-dependent adhesion and tumor growth.

(a, b) Relative in vitro adhesion (mean $\pm$ SEM) of (a) human melanoma cell lines C8161 and MDA-MB-435S, together with the positive control HSB-2 cells, and (b) murine melanoma cell lines B16-F10 and YUMM5.2, along with EL-4 positive control cells, to immobilized ICAM-1 compared with a negative coating control. Cells were analyzed either without treatment (left panels) or following exposure to an ITGB2-blocking antibody, the pan-integrin inhibitor EDTA, or an isotype-matched control antibody (right panels). (c, d) In vivo tumor growth curves (mean $\pm$ SEM) showing the effects of ITGB2 neutralization. (c) NSG mice bearing xenografts

of human C8161 or MDA-MB-435S melanoma cells were treated with a human-specific ITGB2-blocking antibody or its isotype control. (d) NSG mice implanted with murine B16-F10 or YUMM5.2 melanoma cells received either an anti-mouse ITGB2-blocking antibody or an isotype control antibody, and tumor progression was monitored over time. Data presented in (a) and (b) are representative of, or combined from, at least three independent experiments ($n \geq 3$). Comparisons between two groups were performed using the unpaired Student's t-test. In contrast, analyses involving three groups were conducted using one-way ANOVA followed by Dunnett's multiple-comparisons post hoc test. For (c) and (d), each treatment cohort included 5–20 mice. Tumor growth differences were evaluated using repeated-measures two-way ANOVA or a mixed-effects model, followed by Šidák's multiple-comparisons correction. Statistical significance is indicated as $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***). See also Figures 3–5.



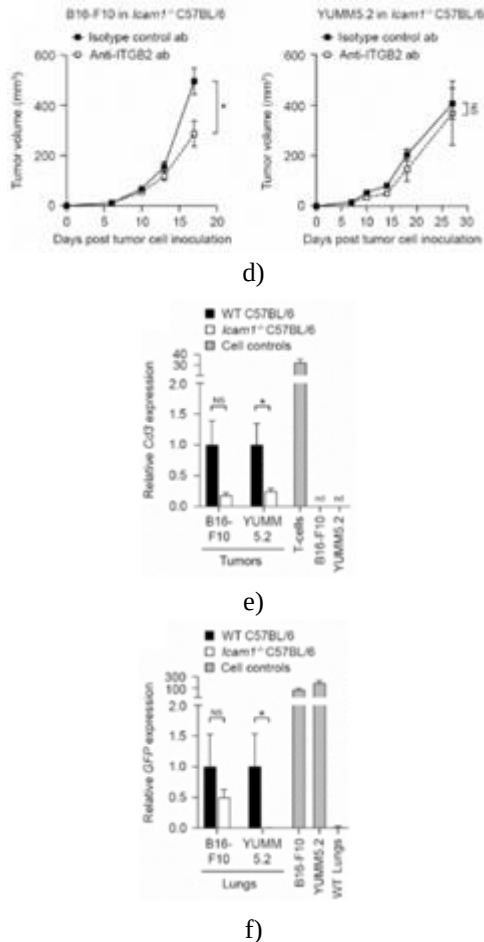


Figure 3. Antibody-mediated blockade of ITGB2 or genetic deficiency of host Icam1 suppresses melanoma metastasis.

(a–c) Impact of treatment with an anti-mouse ITGB2 blocking antibody compared to isotype control on tumor formation by B16-F10 and YUMM5.2 cells implanted into wild-type (WT) C57BL/6 mice. (a) Tumor volume progression over time (mean $\pm$ SEM), (b) relative levels of intratumoral T cells, and (c) relative lung metastatic burden of GFP-tagged melanoma cells, quantified via qPCR detection of genomic Cd3 or GFP sequences in tumor and lung samples, respectively. Primer specificity for Cd3 was confirmed with murine T cells (positive control) and B16-F10/YUMM5.2 cells (negative control). GFP primer specificity was verified using GFP-expressing B16-F10 and YUMM5.2 cells (positive) and tumor-free WT mouse lungs (negative). (d–f) Corresponding effects of anti-ITGB2 blocking antibody versus isotype control on B16-F10 and YUMM5.2 tumorigenesis in Icam1^{-/-} C57BL/6 mice. (d) Tumor growth curves (mean $\pm$ SEM), (e) intratumoral T-cell

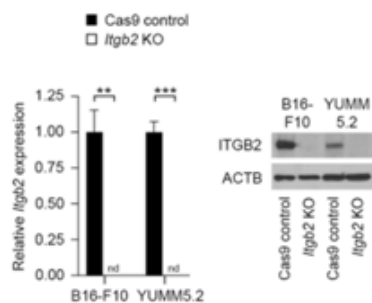
infiltration, and (f) lung metastasis were assessed by qPCR with the same positive and negative controls described above. Panels (a) and (d) included $n = 16$ – 20 mice per treatment arm. Data in (b, c, e, f) represent results from at least three independent experiments (pooled or representative). Control groups shown in (b/e) and (c/f) are shared. Tumor growth differences were evaluated by repeated-measures two-way ANOVA or mixed-effects model with Šidák's post-hoc correction. Unpaired Student's t-test analyzed data in (b, c, e, f). *, $P < 0.05$; NS, not significant; nd, not detected. See also **Figures 4 and 6**.

CRISPR/Cas9-mediated Itgb2 knockout specifically in melanoma cells impairs ICAM-1 adhesion and restricts tumor progression.

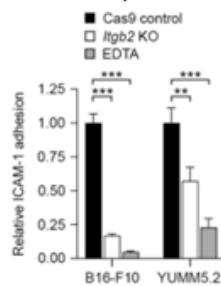
To directly examine the cancer cell-autonomous role of ITGB2, stable Itgb2 knockout lines of B16-F10 and YUMM5.2 melanoma cells were generated via CRISPR/Cas9. Complete loss of Itgb2 at the genomic and protein levels was confirmed by sequencing (data not shown), RT-qPCR, and Western blotting (**Figure 4a**). Melanoma-intrinsic deletion of Itgb2 markedly reduced adhesion to surface-bound ICAM-1 (**Figure 4b**) and diminished three-dimensional spheroid growth in vitro (**Figure 4c**). Consistent with earlier observations of ITGB2 blockade in NSG mice (**Figures 2c and 2d**), tumor cell-specific Itgb2 knockout strongly suppressed B16-F10 and YUMM5.2 tumor development in both immunodeficient NSG hosts (**Figure 4d**) and immunocompetent C57BL/6 mice (**Figure 4e**).

In Icam1^{-/-} C57BL/6 recipients, YUMM5.2 Itgb2-knockout tumors showed no significant growth difference relative to controls (**Figure 4f**), highlighting the dependence of this protumorigenic ITGB2 activity on host ICAM-1. By contrast, B16-F10 Itgb2-knockout cells still exhibited significantly impaired tumor growth even in Icam1^{-/-} hosts (**Figure 4f**), pointing to an additional ICAM-1-independent contribution of tumor-intrinsic ITGB2 in this model. Melanoma cells themselves expressed ICAM-1 in culture, with further upregulation observed in vivo. Notably, tumor cell-associated Icam1 levels were substantially higher in B16-F10 grafts compared with YUMM5.2 grafts when grown in Icam1^{-/-} versus WT hosts (**Figure 4g**). This pattern suggests a greater reliance on cancer cell-derived ICAM-1 (versus host-derived) for B16-F10 progression, which may underlie its reduced dependence on host ICAM-1 for ITGB2-driven growth (**Figure 4f**).

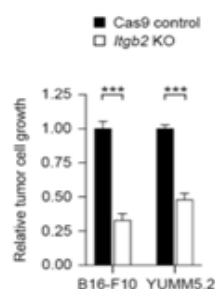
Supporting this differential dependency, human melanoma cell lines displayed broad heterogeneity in ICAM1 mRNA and ICAM-1 protein abundance, spanning from nearly undetectable to levels surpassing those in T cells and control endothelial cells. Similar variability in ICAM1 transcript (**Figure 4h**) and ICAM-1 protein (**Figure 4i**) expression was evident across patient-derived melanoma specimens. Multicolor immunofluorescence staining of clinical melanoma samples indicated that ITGB2-expressing tumor cells frequently localized near ICAM-1-positive cells (**Figure 4j**). Flow-cytometric analysis further revealed elevated ICAM-1 expression on ITGB2⁺ melanoma cells compared with ITGB2⁻ melanoma cells from patients. Collectively, these data establish a key cancer cell-intrinsic function for ITGB2 in driving ICAM-1-dependent melanoma tumorigenesis and support the therapeutic potential of disrupting melanoma ITGB2:ICAM-1 interactions.



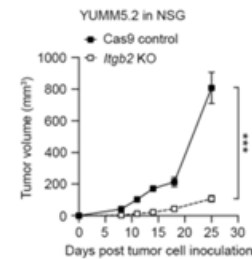
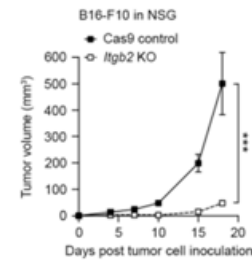
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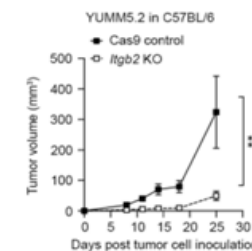
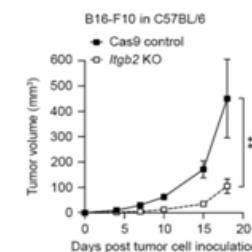
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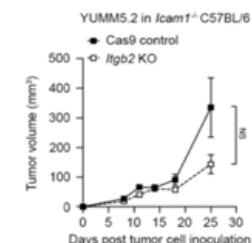
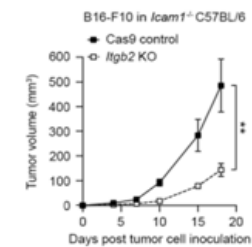
c)



d)



e)



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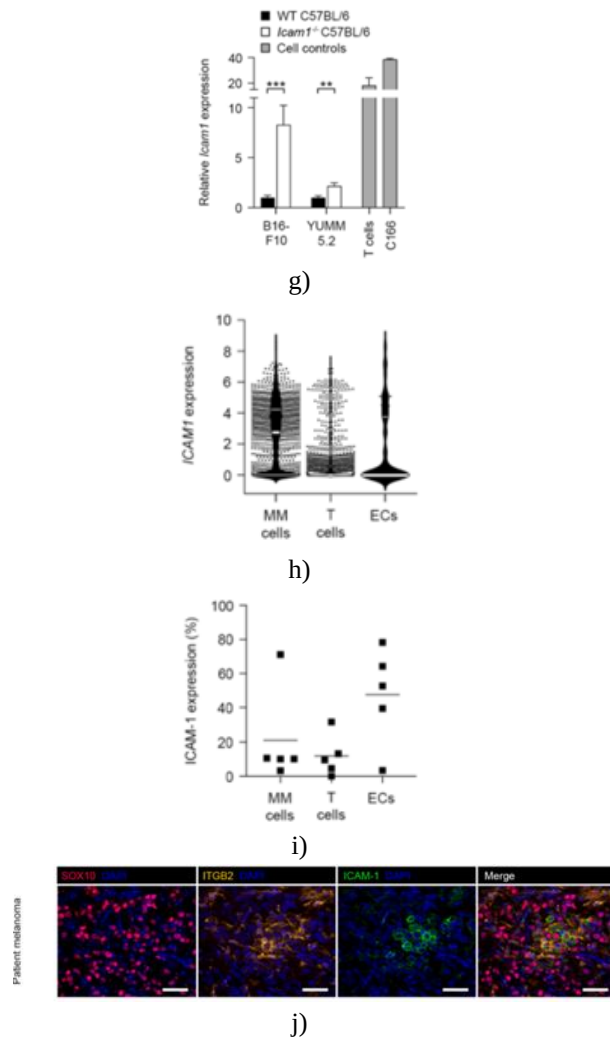


Figure 4. CRISPR/Cas9-mediated melanoma cell-specific deletion of *Itgb2* reduces binding to ICAM-1 and impedes subsequent tumor expansion.

(a) Confirmation of stable CRISPR/Cas9-generated *Itgb2* knockout at both mRNA (RT-qPCR, left) and protein (immunoblot, right) levels in B16-F10 and YUMM5.2 melanoma cells. (b–f) Comparison of *Itgb2* KO cells versus Cas9 control counterparts for (b) in vitro adhesion to immobilized ICAM-1 (mean $\pm$ SEM), with or without EDTA as negative control, (c) in vitro proliferation measured by CellTiter-Glo luminescence (mean $\pm$ SEM), and (d–f) in vivo tumor growth kinetics (mean $\pm$ SEM) in (d) NSG, (e) C57BL/6, and (f) *Icam1*^{−/−} C57BL/6 mice. (g) *Icam1* mRNA levels in B16-F10 and YUMM5.2 tumors harvested from WT C57BL/6 (black bars) versus *Icam1*^{−/−} C57BL/6 hosts (white bars), with murine T cells and C166 endothelial cells included as positive controls (gray bars). (h) Violin

plots from single-cell RNA-seq showing ICAM1 expression in patient melanoma (MM) cells, tumor-infiltrating T cells, and endothelial cells (ECs); median indicated by thick white line, quartiles by thin white lines, with individual cells as dots. (i) Mean percentages of surface ICAM-1 protein expression on patient MM cells, T cells, and ECs ($n = 5$ patients) assessed by flow cytometry (FC). (j) Representative multiplex immunofluorescence images ($n = 4$ patients) of clinical melanoma tissue stained for the melanocytic marker nuclear SOX-10 (red), ITGB2 (yellow), and ICAM-1 (green), with merged view shown; nuclei counterstained with DAPI (blue). Scale bars = 50 μ m. Data in panels (a, b, c, g) are representative of or pooled from at least three independent experiments. Statistical comparisons used unpaired Student's t-test for two groups and one-way ANOVA with Dunnett's post-test for three or more groups. Panels (d–f) included $n = 10$ mice per melanoma variant. Tumor growth was analyzed by repeated-measures two-way ANOVA. **, $P < 0.01$; ***, $P < 0.001$; NS, not significant; nd, not detected. See also **Figures 2 and 4**.

Melanoma cell ITGB2:ICAM-1 engagement triggers downstream Wnt pathway activation; pharmacologic blockade of this axis inhibits ITGB2-driven tumor progression.

To comprehensively map the downstream signaling pathways engaged by melanoma cell ITGB2 in response to ICAM-1 during tumorigenesis, we conducted unbiased RNA-seq profiling of *Itgb2* KO versus control YUMM5.2 tumors propagated in NSG, WT, or *Icam1*^{−/−} C57BL/6 mice. This approach identified 151 consistently altered differentially expressed genes (DEGs) in *Itgb2* KO tumors, shared between NSG and WT C57BL/6 hosts but absent in *Icam1*^{−/−} animals (**Figure 5a**); (top 51 DEGs shown). STRING network analysis [30] highlighted three primary interconnected gene modules linked to melanoma ITGB2 activity: Wnt pathway components, integrin/ECM-related molecules, and growth factor-associated networks. Among these, Wnt-associated genes, such as *Wnt5a*, *Wnt5b*, *Notum*, and *Dkk2*, showed the highest connectivity scores (**Figure 5b**), consistent with prior links between Wnt signaling and ICAM-1 regulation in cancer [31].

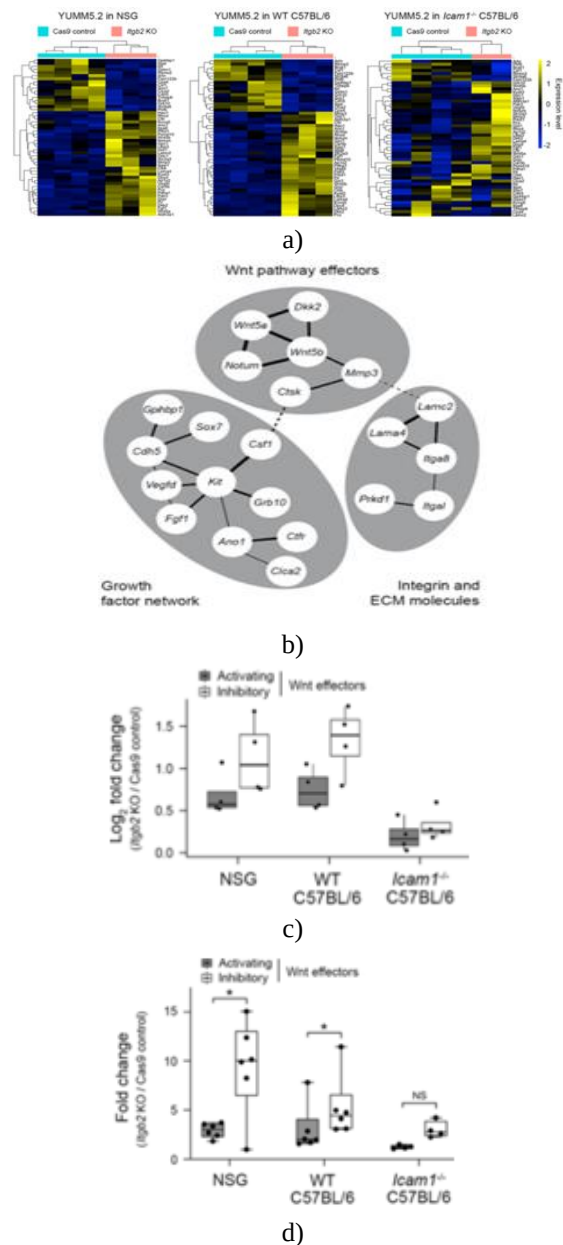
Given the known roles of Wnt in melanoma cell-state switching [32] and BRAF inhibitor resistance [33, 34], we evaluated associations between ITGB2 and ICAM1 and between these genes and proliferative versus

invasive/neural crest-like phenotypes and therapy resistance. Examination of published scRNA-seq datasets [26, 27] showed comparable ITGB2 and ICAM1 levels across phenotypes, with ITGB2 relatively enriched in proliferative (mitotic) over invasive (neural crest-like) cells and higher in ABCB5+ pro-proliferative [35] versus NGFR+ pro-invasive [26] populations. Expression of both Wnt pathway genes and ITGB2:ICAM1 was elevated in BRAF-resistant compared to treatment-naïve melanoma cells, and *Itgb2* deletion increased cellular sensitivity to the BRAF inhibitor vemurafenib.

Targeted inspection of *Icam1*-dependent DEGs from **Figure 5a** annotated in the Gene Ontology Biological Process (GOBP) database as canonical or non-canonical Wnt components identified upregulation of both positive (*Frat2*, *Kpna1*, *Wnt5a*, *Wnt5b*) and negative (*Dkk2*, *Igfbp4*, *Kank1*, *Notum*) regulators [36] upon *Itgb2* KO in NSG and WT C57BL/6 hosts, but not *Icam1*^{-/-} hosts. Wnt antagonists were more prominently induced than agonists (**Figure 5c**), indicating net suppression of Wnt signaling in *Itgb2*-deficient tumors. Independent RT-qPCR validation in a separate in vivo cohort confirmed these Wnt gene changes and the dominance of inhibitory over activating effectors (**Figure 5d**). Immunoblotting corroborated reduced levels of canonical Wnt mediators active (non-phosphorylated) β -catenin and LEF-1, along with milder decreases in non-canonical p-VANGL2, in *Itgb2* KO versus control YUMM5.2 cells (**Figure 5e**).

To functionally validate Wnt pathway participation in ITGB2:ICAM-1 signaling, we tested three distinct Wnt antagonists: the FDA-approved agent pyrvinium pamoate (repurposed for oncology [19, 37]), LGK974, and zamaporvint [20, 21]. All three compounds inhibited both canonical and (to a lesser degree) non-canonical Wnt activity (**Figure 5f**) and blocked ITGB2:ICAM-1-mediated melanoma cell adhesion (**Figure 5g**). Like ITGB2 antibody blockade, these inhibitors also reduced ICAM-1 adhesion in isotype control-treated YUMM5.2 cells (**Figure 5h**). Co-treatment with pyrvinium or zamaporvint, plus an ITGB2-neutralizing antibody, produced synergistic inhibition of adhesion (**Figure 5h**). In vivo, *Itgb2* KO or systemic administration of pyrvinium, LGK974, or zamaporvint significantly restrained YUMM5.2 tumor progression in both NSG and C57BL/6 mice relative to vehicle controls (**Figure 6a**). Wnt inhibitors also curbed growth of isotype-treated wild-type tumors and synergized with ITGB2 antibody in NSG hosts. Still, this cooperation was not observed in

immunocompetent C57BL/6 mice (**Figure 6b**). Importantly, the antitumor effects of all three Wnt antagonists — mirroring upstream *Itgb2* genetic deletion or antibody blockade — were completely lost in *Icam1*^{-/-} C57BL/6 recipients (**Figures 6a and 6b**). These results reveal a tumor cell-autonomous ITGB2:ICAM-1:Wnt signaling axis that promotes melanoma growth and underscore its promise as a target for direct therapeutic intervention against ITGB2-dependent malignancy.



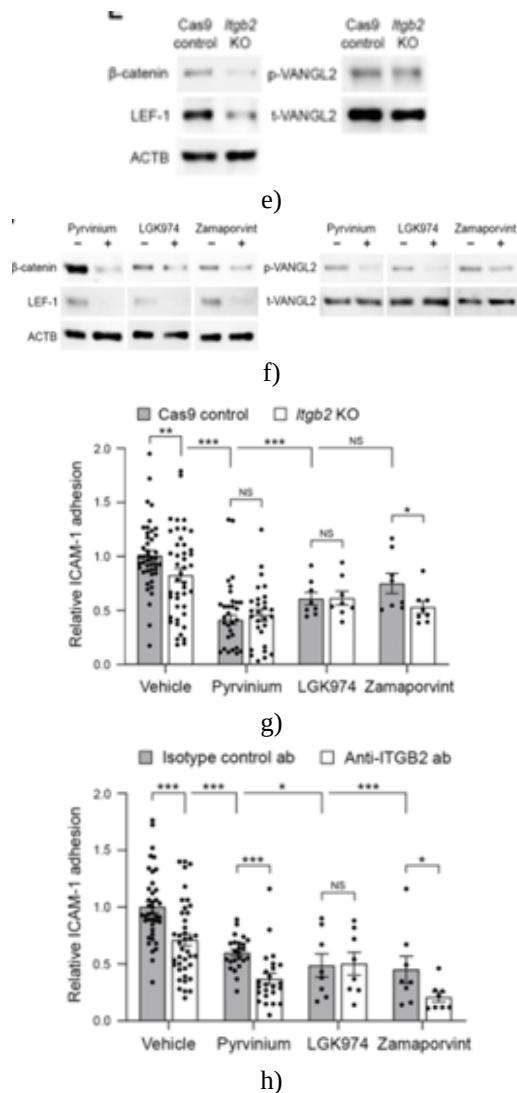


Figure 5. Melanoma cell ITGB2 engagement with ICAM-1 promotes Wnt pathway activation, and blockade of this pathway, in turn, impairs tumor cell attachment to ICAM-1.

(a) Heatmap illustrations of the 51 most interconnected differentially expressed genes (DEGs) identified by RNA-seq in *Itgb2*-deficient versus control YUMM5.2 melanomas. These transcripts displayed matching directional changes in tumors from NSG hosts (left) and immunocompetent wild-type (WT) C57BL/6 animals (middle), yet this pattern was absent in *Icam1*^{-/-} C57BL/6 recipients (right). (b) STRING-based protein

association map highlighting 22 DEGs from (a) that exhibited the most robust connectivity. Gray ovals outline major network modules. Direct protein linkages are depicted with line widths reflecting interaction confidence (thicker = stronger), while indirect connections appear as dashed lines. Unclustered proteins were not included. Significance was evaluated using the paired Wilcoxon test. (c) Box-and-whisker plots summarizing log₂ fold changes for Wnt-associated DEGs in *Itgb2* KO relative to Cas9 control tumors (corresponding to panel a), classified according to Gene Ontology Biological Process (GOBP) annotations. Genes were separated into activator (Frat2, Kpna1, Wnt5a, Wnt5b) and inhibitor (Dkk2, Igfbp4, Kank1, Notum) groups, with medians shown as horizontal lines. (d) RT-qPCR confirmation (expressed as fold change) of the Wnt effector genes listed in (c) from an independent set of *Itgb2* KO versus Cas9 control YUMM5.2 tumors isolated from NSG, WT C57BL/6, or *Icam1*^{-/-} C57BL/6 mice. Medians are marked by horizontal bars within box-and-whisker plots. (e) Western blot examples depicting levels of canonical Wnt effectors — active (non-phospho) β -catenin and LEF-1 — along with ACTB as loading control (left), and the non-canonical marker p-VANGL2 with its total counterpart (right) in *Itgb2* KO compared to Cas9 control YUMM5.2 cells. (f) Representative immunoblots (as in e) of Wnt pathway proteins in YUMM5.2 cells following exposure to the Wnt antagonists pyrvinium pamoate, LGK974, or zamaporvint versus vehicle treatment. (g and h) Quantitative assessment (mean $\pm$ SEM) of melanoma cell adhesion to coated ICAM-1 via CellTiter-Glo readout for (g) *Itgb2* KO versus Cas9 control YUMM5.2 lines and (h) wild-type YUMM5.2 cells incubated with anti-ITGB2 blocking antibody or isotype control, either alone or combined with pyrvinium pamoate, LGK974, zamaporvint, or vehicle. Paired Student's t-test was applied for statistical evaluation. Panels (a-d) derive from $n = 2-6$ tumors per condition across the respective mouse strains. Findings in (e-h) come from at least $n = 2-7$ separate experiments (representative or pooled). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant. See also Figures 2, 3 and 6.

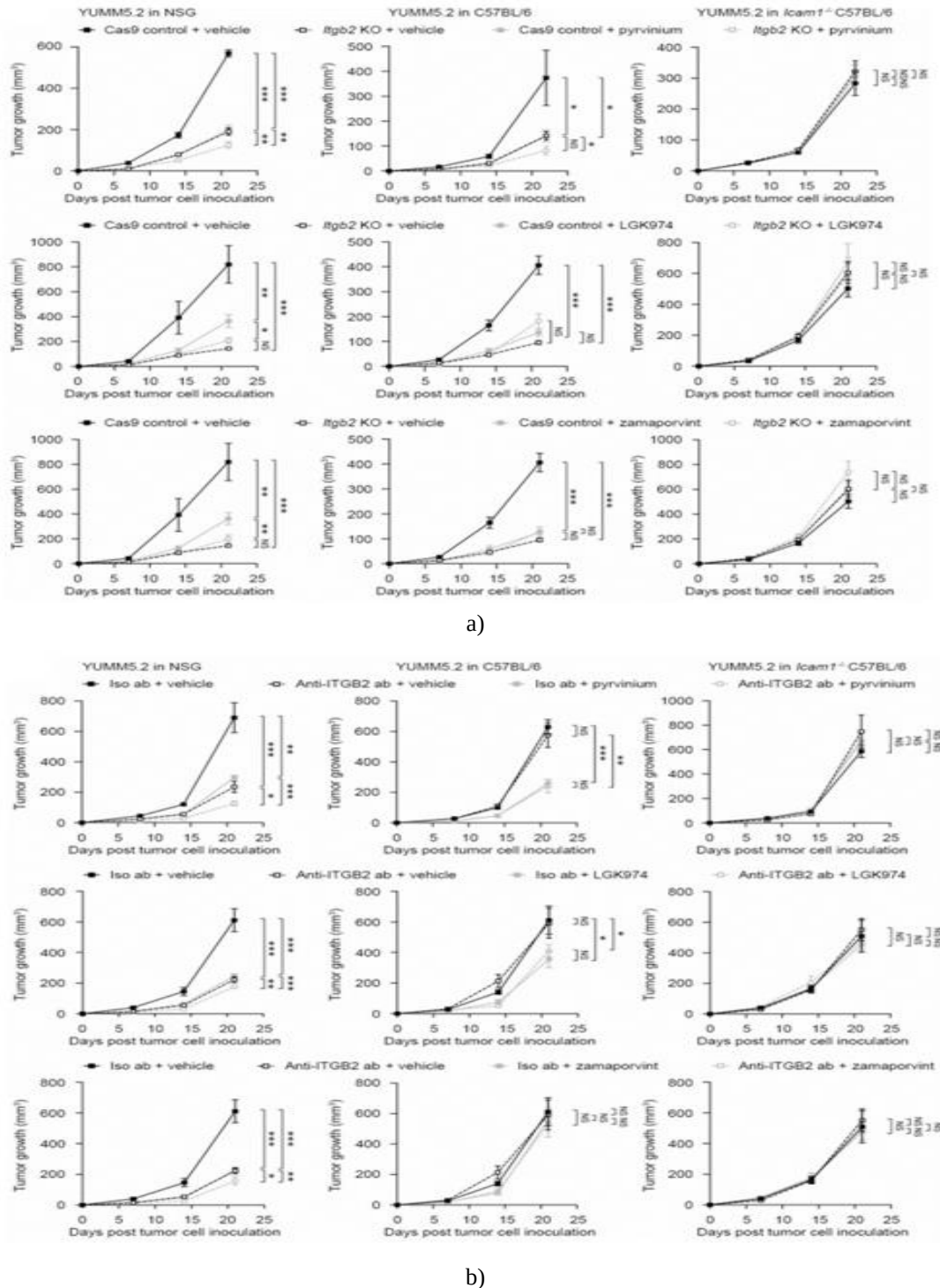


Figure 6. Wnt pathway inhibition restricts melanoma progression driven by the ITGB2:ICAM-1 interaction in living animals.

(a and b) Tumor volume curves (mean ± SEM) for (a) *Itgb2* KO versus Cas9 control YUMM5.2 cells or (b) wild-type YUMM5.2 cells receiving anti-mouse ITGB2 blocking antibody versus isotype control, either alone or

combined with the Wnt antagonists pyrvinium pamoate, LGK974, zamaporvint, or vehicle. Experiments were performed in NSG (left), wild-type (WT) C57BL/6 (middle), or *Itcam1*^{-/-} C57BL/6 mice (right). Since

LGK974 and zamaporvint studies were run in parallel, their shared vehicle control groups are identical. Panels (a and b) included $n = 6$ –10 mice per treatment arm. Tumor growth differences were analyzed by repeated-measures two-way ANOVA or mixed model with Šídák's multiple comparisons correction. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant. See also **Figure 5**.

ITGB2 family adhesion receptors play essential roles in leukocyte activation, expansion, and migration [38]. Following their initial identification, ITGB2-containing integrin complexes were considered restricted to hematopoietic cells, encompassing both adaptive and innate immune populations as well as blood cancers [5, 39, 40]. Only a limited number of more recent studies have reported ITGB2 expression in certain epithelial tumors [6-8], though its precise role in cancer development remains unclear. Using multiple complementary approaches, the present work demonstrates that melanoma cells in patient samples and in established human and mouse melanoma lines functionally express ITGB2. The proportion of ITGB2+SOX10+ tumor cells increased progressively from benign nevi to primary melanomas and metastatic lesions in clinical tissues. Additionally, higher frequencies of ITGB2-positive cancer cells in primary patient melanomas were associated with sentinel lymph node (SLN) involvement, supporting a direct role for tumor-cell-intrinsic ITGB2 in disease progression.

Ligation of the hyaluronan receptor CD44 [41] on melanoma cells triggered both increased expression and functional activation of ITGAL/ITGB2, detected by conformation-specific antibodies, thereby recapitulating CD44-mediated control of this integrin in immune cells [9]. Alternative alpha subunits (ITGAD, ITGAM, ITGAX) showed minimal expression in patient-derived melanoma cells and were not upregulated by CD44 stimulation. Therefore, regulation of melanoma ITGB2 heterodimer assembly, abundance, and activation state likely depends on further unidentified modulators and regulatory mechanisms operating beyond CD44 alone. These factors may differ under culture conditions or in the *in vivo* microenvironment, thereby influencing or selecting for the prevalence of ITGB2-expressing melanoma subpopulations. Analogous to its behavior in leukocytes [2], melanoma cell ITGB2 interacted with ICAM-1, and this binding was disrupted by antibody-mediated ITGB2 neutralization, EDTA treatment, or genetic Itgb2 deletion. Collectively, these observations

identify a novel form of “hematopoietic mimicry” [10] in which melanoma cells repurpose leukocyte-associated ITGB2 mechanisms to support their own malignant progression.

Consistent with this model, both antibody-based ITGB2 blockade and CRISPR/Cas9-driven tumor cell-specific Itgb2 knockout substantially impaired the expansion of human and murine melanomas in immunodeficient NSG mice, echoing the established growth-inhibitory impact of ITGB2 disruption in T lymphocytes [42]. In immunocompetent C57BL/6 hosts, Itgb2 knockout likewise curtailed tumor formation. However, systemic administration of ITGB2 antibodies failed to slow primary tumor growth in this setting, likely due to concurrent depletion of tumor-infiltrating T cells. Nevertheless, the same antibody treatment markedly decreased spontaneous lung metastasis relative to isotype control when primary tumors were size-matched, revealing separate contributions of melanoma cell ITGB2 to proliferation and metastatic spread. In T cell-competent animals, the detrimental effect of blocking T cell ITGB2 on antitumor immunity offset the direct growth-suppressive action on cancer cells, but did not interfere with metastasis reduction. This highlights the need for cancer cell-selective ITGB2 inhibitors that spare T-cell function. The robust tumor inhibition achieved through melanoma-specific Itgb2 knockout provides strong proof of concept for the viability of such targeted strategies. Supporting this idea, a recent study employed chimeric antigen receptor (CAR) T cells directed against a leukemia-specific ITGB2 conformation, successfully eliminating malignant cells without harming normal hematopoietic populations [40]. Analogous conformation-specific epitopes could be harnessed to selectively neutralize the active form of ITGB2 expressed by melanoma cells. Earlier broad-spectrum ITGB2 inhibitors tested clinically for inflammatory and autoimmune conditions [2, 5], such as erlizumab (rhuMAB) and rovelizumab (LeukArrest) [43], were ultimately discontinued due to safety concerns. Although these agents potently inhibited ITGB2 activity in immune cells, a subset of patients developed progressive multifocal leukoencephalopathy due to impaired clearance of the *JC polyomavirus* [43]. These experiences nevertheless confirm ITGB2 as a druggable target and emphasize the importance of creating cancer cell-restricted inhibitors.

Additional support for pursuing tumor-intrinsic ITGB2 comes from statins, which are widely used FDA-

approved drugs recently shown to possess disruptive activity against ITGB2:ICAM-1 [44] and to demonstrate antitumor effects in various cancers [45]. Although initially designed to lower cholesterol synthesis [45], statins can directly associate with ITGB2 and prevent its binding to ICAM-1 [44]. In view of the current results, the anticancer benefits previously attributed to statins [44, 45] may partly stem from direct interference with melanoma cell ITGB2:ICAM-1 signaling, meriting dedicated follow-up studies in the oncology setting.

Our observations of lower numbers of tumor-infiltrating T cells in syngeneic *Icam1*^{-/-} compared with wild-type C57BL/6 mice align with the well-documented function of endothelial ICAM-1 in facilitating ITGB2-dependent T cell recruitment [38]. Host *Icam1* deficiency, much like ITGB2 blockade, also led to a strong reduction in spontaneous lung metastasis when primary B16-F10 and YUMM5.2 tumors were of similar size. These data therefore position ICAM-1 as a key regulator of both T-cell and cancer-cell trafficking. Additionally, deletion of host *Icam1* abolished the tumor growth restraint caused by melanoma-specific *Itgb2* knockout in YUMM5.2 models, reinforcing the contribution of host-derived ICAM-1 to ITGB2-driven tumor progression. In contrast, *Itgb2*-deficient B16-F10 tumors grew at comparable rates in *Icam1*-null and wild-type hosts, implying that tumor cell-produced ICAM-1 can sometimes substitute for the missing host protein. Supporting this notion, melanoma cells, like leukocytes, constitutively express ICAM-1 at varying levels, as shown here and elsewhere [46]. Indeed, *Icam1* transcript levels in tumors were markedly elevated in B16-F10 relative to YUMM5.2 grafts recovered from *Icam1*-deficient animals, indicating that both host and cancer cell sources of ICAM-1 contribute to ITGB2-mediated growth. While other ICAM family proteins can also engage ITGB2 [38], their potential influence on tumorigenesis remains to be determined. Overall, however, the present results highlight cancer cell ITGB2:ICAM-1 binding as a central driver of melanoma advancement.

The ITGB2:ICAM-1 interaction regulated downstream Wnt signaling [47-49], as revealed through unbiased RNA-seq, quantitative RT-PCR, and protein blotting. Loss of melanoma *Itgb2* triggered upregulation of several Wnt-related genes within tumor cells in NSG and wild-type but not *Icam1*^{-/-} C57BL/6 hosts. Notably, inhibitory Wnt regulators were induced more strongly than activators, consistent with net attenuation of the

pathway upon disruption of the *Itgb2*:*Icam1* axis. Western blot analyses confirmed decreased abundance of canonical Wnt effector proteins in *Itgb2* KO cells compared with controls, with minimal impact on non-canonical signaling. These molecular changes match prior reports linking elevated tumoral ICAM-1 to enhanced canonical Wnt activity in patients [31]. They also correspond with our detection of heightened Wnt, ITGB2, and ICAM1 expression in BRAF inhibitor-resistant versus sensitive patient melanoma cells [27], together delineating a tumor cell-autonomous ITGB2:ICAM-1:Wnt circuit. Beyond Wnt, our RNA-seq and STRING network analyses identified additional gene clusters downstream of melanoma *Itgb2*:*Icam1*, such as growth factor pathways, suggesting that ITGB2 may control other actionable targets worthy of future exploration. Nevertheless, pharmacologic inhibition of Wnt signaling with clinically relevant agents — LGK974 [20], zamaporvint [21], and the FDA-approved compound pyrvinium pamoate [19, 37] — effectively blocked ITGB2:ICAM-1-dependent adhesion and substantially impaired melanoma outgrowth in NSG and wild-type C57BL/6 mice, while these effects were lost in *Icam1*^{-/-} hosts. Taken together, these data establish Wnt as a major effector pathway lying downstream of melanoma ITGB2:ICAM-1 and emphasize the translational value of attacking multiple nodes within this tumor-intrinsic signaling network.

Conclusion

This study identifies a previously unrecognized melanoma cell-intrinsic ITGB2:ICAM-1:Wnt signaling axis that promotes tumor development—disrupting either melanoma ITGB2:ICAM-1 interactions or the downstream Wnt cascade markedly inhibited tumor formation in multiple preclinical models, establishing the tumoral ITGB2 pathway as a legitimate therapeutic vulnerability in melanoma and potentially in other ITGB2-expressing solid cancers [6-8]. Our results also provide a strong rationale for advancing next-generation [40, 50] cancer cell-selective ITGB2 inhibitors that spare leukocyte ITGB2. Such tumor-specific agents could effectively restrain cancer progression while circumventing the detrimental immunological side effects associated with non-selective ITGB2 blockade [5, 43].

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Conflict of Interest: None

Financial Support: None

Ethics Statement: None

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