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## ABLATION OF B7-H3 BUT NOT B7-H4 RESULTS IN HIGHLY INCREASED TUMOR BURDEN IN A MURINE MODEL OF SPONTANEOUS PROSTATE CANCER

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### Abstract

The costimulatory molecules B7-H3 and B7-H4 are overexpressed in a variety of human tumors and have been hypothesized as possible biomarkers and immunotherapeutic targets. Despite this potential, the predominating uncertainty about their functional implication in tumor-host interaction hampers its evaluation as a target for cancer therapy. By means of a highly physiologic, spontaneous tumor model in mice, we establish a causal link between B7-H3 and host tumor control and found B7-H4 to be redundant.

### Introduction

The immune system can recognize spontaneous malignancies and mediate their elimination [1;2], which is greatly influenced by the tumor microenvironment. Cancer immunotherapy

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Dr. Allison is a paid consultant for Jounce Therapeutics. The authors have no additional financial interests.

#### Author contributions

K.K. and S.H. designed, performed and analyzed the experiments; R.M., J.W., R.W. and G.G. performed experiments; K.K. and S.H. prepared the manuscript; P.S. gave methodical advice; all authors contributed to the scientific discussion and manuscript editing.

aims at promoting the activation of immune effector mechanisms or blocking inhibitory pathways to elicit and sustain an endogenous antitumor response. In this regard, co-signaling molecules as accessory modulators of T-cell responses are of particular interest as they are determining factors between tolerance and immunity [3]. Their targeting for cancer immunotherapy has gained conceptual validation in recent years, as several efforts have shown success in Phase II and Phase III clinical trials or received FDA approval as standard-of-care therapies for the treatment of cancer patients [4]. A prerequisite for improving clinical benefit from combination therapies and for the development of new strategies is a clear understanding of the factors critically involved in shaping the antitumor immune response. In addition, the identification of biomarkers is vital for the rational choice of combinatorial treatments and the advancement of individualized immunotherapy.

With these objectives, we focused on the co-signaling molecules B7-H3 and B7-H4 in a stringent genetic study. Both molecules have been related to immune-modulatory processes in the tumor microenvironment as they are overexpressed on a variety of human tumors. While B7-H4 expression has been correlated with poor clinical outcome, studies investigating associations of B7-H3 expression with survival have shown conflicting results [5]. Furthermore, the receptor(s) for B7-H3 or B7-H4 have not been identified conclusively, and the physiologic role of B7-H3 particularly is elusive, as both immune-stimulatory and immune-inhibitory capacities have been demonstrated *in vitro* and *in vivo* [6;7].

To causally determine the function and relevance of B7-H3 and B7-H4 in cancer progression, we employed the TRAMP (transgenic adenocarcinoma of the mouse prostate) model, in which male mice develop spontaneous prostate cancer with a history similar to the human disease [8] and in which we were able to genetically ablate B7-H3 or B7-H4.

## Materials and Methods

### Mice

TRAMP<sup>+</sup> mice were a gift from N. Greenberg, maintained in the hemizygous state and crossed with B7-H3<sup>-/-</sup> or -B7-H4<sup>-/-</sup> mice. Male TRAMP<sup>+</sup> and TRAMP<sup>-</sup> littermate control mice of wt, B7-H4<sup>-/-</sup> and B7-H4<sup>-/-</sup> breedings were used for analysis at indicated time points. B7-H3<sup>-/-</sup> mice were a gift from T. Mak. The generation of B7-H4<sup>-/-</sup> was previously described [9]. All breedings were maintained on the C57BL/6 background and the full C57BL/6 background of all strains confirmed by analysis of 1449 SNPs. C57BL/6 mice were purchased from The Jackson Laboratory and CD45.1<sup>+</sup> B6.SJL mice were from Taconic. All mice were bred and maintained under specific pathogen-free (SPF) conditions and animal experimentation was conducted in accordance with institutional guidelines.

### RNA preparation and real-time PCR

RNA was isolated from homogenized tissue using the RNeasy kit (Qiagen). 1-5µg RNA was reverse-transcribed using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare). Quantitative PCR was performed on a real-time PCR system (Applied Biosystems/7500) using TaqMan primer/probe gene expression assays for each gene analyzed (Applied Biosystems). GAPDH was used as the endogenous control. Relative changes in gene

expression were calculated using the Ct method. Focused qPCR arrays (PAMM-135Z, PAMM-074Z) were purchased from Qiagen.

### Tumor evaluation and Histopathology

Mice were weighed prior to euthanization by CO<sub>2</sub> inhalation at indicated time points (between 6 and 36 weeks of age). The whole urogenital tract, as well as the anterior, ventral and dorsolateral prostate lobes were dissected and individually weighed. All weights were normalized to whole body weight. Dorsolateral prostate lobes were fixed in 10% buffered neutral formalin and embedded in paraffin. 5µm-thick tissue sections (8 spaced cuts per mouse) were routinely stained with hematoxylin and eosin and microscopically examined.

### Isolation of cells from mouse prostate and lymph nodes

The dorsolateral prostate lobes were dissected, mechanically disrupted and digested using DNase (Sigma-Aldrich) and liberase (Roche) for 30 minutes at 37°C in complete RPMI media and filtered through a 70 µm cell strainer (BD Biosciences). Lymphocytes were enriched using centrifugation over Histopaque-1119 (Sigma). Tumor cells for *in vitro* culture were depleted of CD45<sup>+</sup>-infiltrating cells by FACS, 10<sup>4</sup> cells were cultured in 200 µl of a 4mg collagen matrix (Matrigel, BD Biosciences) for 5 days, released by incubation in Dispase (BD Biosciences) for 2h at 37°C and counted. Draining (periaortic) and non-draining (brachial) lymph nodes were dissected, mechanically disrupted and filtered through a 70 µm cell strainer (BD Biosciences).

### Flow cytometry

Fluorescently labeled antibodies were purchased from eBioscience or BD Biosciences. Dead cells were stained using the fixable aqua dead cell stain kit (Molecular Probes), Surface markers were stained in the presence of 2.4G2 mAb (MSKCC Monoclonal Antibody Core Facility) to block FcγR binding. The intracellular staining kit from eBioscience was used after stimulation with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma), 1 µM ionomycin (Sigma) and GolgiStop (BD Biosciences) in complete RPMI at 37°C for 4h. Anti B7-H3-PE and anti B7-H4-PE fluorescence intensity was amplified using Faser Kit (Miltenyi Biotec). Samples were acquired using a LSR II flow cytometer (Becton Dickinson) and analyzed using FlowJo software (Tree Star). The absolute number of cells per tumor sample was quantified using fluorescent microbeads (Life Technologies) and normalized to a known sample volume, which was then normalized to tumor wet weight.

### Immunohistochemistry

Tissue was fixed in 10% buffered neutral formalin and embedded in paraffin. 5µm-thick tissue sections were stained with anti Ki67 (MSKCC Pathology Core Facility) and the Ki-67 index (percentage of cells showing nuclear expression of Ki-67) was assessed using a light microscope.

### Bone marrow chimeras

Bone marrow chimeras were generated using bone marrow from the femurs of CD45.1<sup>+</sup>B6.SJL or CD45.2<sup>+</sup>B7-H3<sup>-/-</sup> mice. 6–7 weeks old recipient CD45.2<sup>+</sup>TRAMP<sup>+</sup> B7-

H3<sup>-/-</sup>CD45.2<sup>+</sup>TRAMP<sup>+</sup> or TRAMP<sup>-</sup> controls were lethally irradiated exposing them to two, 24h spaced doses of 600 Gy and reconstituted with 5x10<sup>6</sup> bone marrow cells. 12 weeks later, mice were bled to confirm chimerism. Lymph nodes (LN), urogenital tracts and individual prostate lobes were harvested and analyzed when mice were 30 weeks old.

### Proliferation assays

T cells were purified from LN cell suspensions by magnetic cell sorting with CD90 Microbeads (Miltenyi Biotec). Cells were cultured for 48 h in round-bottom plates coated over night at 4°C with 0.5 µg/ml anti CD3 and either B7-H3 Ig or control Ig at indicated concentrations. Proliferation was monitored by the addition of <sup>3</sup>H-methyl-thymidine (1 µCi/well) for the last 24h of culture. Cells were harvested onto glass-fiber filters using a Tomtec harvester, and filters were counted using a MicroBeta scintillation counter (Perkin-Elmer).

### Cytotoxicity assays

RMA and RMA-S cells were obtained from Dr. Ljunggren (Karolinska Institute). All cell lines were tested and validated to be mycoplasma free; no genomic authentication was performed. RMA and RMA-S cells were transfected with B7-H3, irrelevant protein (Thy1.1) or empty vector using the MSCV retroviral expression system. Cells expressing high levels of the transfected proteins were sorted by FACS. For measurement of *in vivo* NK cell-mediated cytotoxicity, RMA and RMA-S cells were labelled with different concentrations of cfse or eflour670 (eBioscience) and injected i.v. into naïve C57BL/6 mice or mice that had received 100µg of depleting anti-NK1.1 antibody (PK-136, BioXCell) 48h earlier. Labelled cells were recovered from lungs 4h post injection. Percent cytotoxicity was determined as 100-(100\*(%RMA-S/%RMA)/(%RMA-s/%RMA in NK cell-depleted mice)). For *in vitro* CD8<sup>+</sup> T cell-mediated cytotoxicity, SPASTc mice were used, which express a TCR specific for the TRAMP-C2 tumor antigen SPAS-1 derived H8 peptide [10]. SPASTc splenocytes were activated for 6 days with H8 peptide and 100 U/ml of recombinant human IL2 (PeproTech), subsequently used as effectors and H8-pulsed RMA cells as targets in a LDH release Cytotox-One Assay (Promega) following manufacturer's instructions. Percent cytotoxicity was determined as 100×((Experimental - Effectors alone)/(Maximum LDH Release - Effectors alone)).

### Statistical analysis

Analysis was performed using Graph Prism software (GraphPad). Where data sets were normally distributed, t-tests were performed.

## Results and Discussion

We analyzed the expression of B7-H3 and B7-H4 mRNA and protein in primary TRAMP<sup>+</sup> tumors *ex vivo* by qPCR and flow cytometry, respectively. Reflecting the situation in human prostate cancer [11], expression of both molecules was markedly elevated in tumors and the expression increased with cancer progression (Fig. 1a,b and Supplementary Fig. 1). To determine the impact of B7-H3 and B7-H4 on tumor growth, we analyzed TRAMP<sup>+</sup> tumor progression in mice deficient in either molecule at different stages of tumor development. Tumor burden was assessed by weight of the entire urogenital tract (UG) and the dissected

dorsolateral (DL) prostate lobes, as well as by histologic analysis. Strikingly, mice lacking B7-H3 showed dramatically increased tumor sizes (Fig 1 c-g). Tumor development in TRAMP mice starts with puberty at week five to six. The aggravated tumor burden observed in TRAMP<sup>+</sup>B7H3<sup>-/-</sup> mice was detectable as early as 12 weeks of age and increased over time. Histopathologic analysis revealed a higher abundance of neoplastic epithelium and increased atypia in TRAMP<sup>+</sup>B7H3<sup>-/-</sup> mice compared to TRAMP<sup>+</sup>wt mice (Supplementary Fig. 2). In contrast to the drastic impact of B7-H3 expression on tumor development, tumor progression was found to be independent of B7-H4 (Fig. 1e-g). TRAMP tumors develop due to androgen-driven expression of the complete SV40 T-antigen under the control of the probasin promoter [8;12]. To address potential interference by differences in levels of testosterone or androgen receptor expression that could account for the phenotype, we analyzed their levels in wt and B7-H3<sup>-/-</sup> mice and found them to be comparable (Supplementary Fig. 3). Recently, a non-immunologic, tumor cell-autonomous function of B7-H3 has been described using siRNA-mediated downregulation of B7-H3 in cancer cell lines [13]. To test whether B7-H3 influences proliferation of primary TRAMP<sup>+</sup> tumor cells in a cell-intrinsic manner, we first evaluated their expression of the cell cycle marker Ki67. We found it to be expressed at comparable levels in tumor cells extracted from early as well as late-stage TRAMP<sup>+</sup>B7-H3<sup>-/-</sup> tumors and corresponding wt controls (Supplementary Fig. 4). Secondly, we isolated primary tumor cells from TRAMP<sup>+</sup>wt and TRAMP<sup>+</sup>B7H3<sup>-/-</sup> mice, expanded them in a three-dimensional collagen matrix *ex vivo* and confirmed equal rates of proliferation (Supplementary Fig. 4). Results from both assays suggest a cell-extrinsic mode of action for B7-H3 during tumor development. To test whether this effect is immune-mediated, we characterized the tumor microenvironment by assessing mRNA expression of genes implicated in immune responses within the DL prostates of 12-, 18- and 30-week old TRAMP<sup>+</sup> wt and TRAMP<sup>+</sup>B7-H3<sup>-/-</sup> mice. Consistently, IL10 and TGFβ transcripts were markedly elevated in the tumors of 30-week old TRAMP<sup>+</sup>B7-H3<sup>-/-</sup> mice, while IFNγ transcripts were less abundant in B7-H3<sup>-/-</sup> tumors (Supplementary Fig. 5). These data adumbrate an altered and potentially more suppressive immune environment in the absence of B7-H3. Correspondingly, we found a slight trend towards a decrease in the relevant effector cytokine expression by B7-H3<sup>-/-</sup> tumor-infiltrating CD8<sup>+</sup> T cells as well as NK cells (Supplementary Fig. 5). Previous studies have reported B7-H3 to either promote or inhibit T-cell proliferation and effector responses in various assays [14]. In our *in vivo* setting, B7-H3 did not have a prominent direct effect on effector CD4<sup>+</sup> or CD8<sup>+</sup> T-cell proliferation or CD8<sup>+</sup> T or NK cell-mediated cytotoxicity as tested by <sup>3</sup>H-thymidine incorporation in the presence of plate-bound B7-H3Ig or specific lysis of B7-H3-transfected target cells, respectively (Supplementary Fig. 6). Conclusively, searching for the primary difference on the cellular effector level, we observed a significant accumulation of FoxP3<sup>+</sup> T cells (Treg) in B7-H3<sup>-/-</sup> tumors (Fig. 2a-c), while at the same time percentages of Tregs in non-draining and tumor-draining lymph nodes were the same in TRAMP<sup>+</sup>wt and TRAMP<sup>+</sup>B7-H3<sup>-/-</sup> mice (Supplementary Fig. 7). An overview of other tumor-infiltrating cell populations is given in Supplementary Fig. 8. Lack of B7-H3 led to an increased proliferative activity of Tregs (Fig. 2d). Taken together, our results demonstrate an effective immune-regulation due to the expression of B7-H3, but not the expression of B7-H4, on the local tumor-microenvironment.

B7-H3 is predominantly expressed in non-hematopoietic tissue [6] but its expression has also been detected in activated lymphocytes *in vitro* [15], activated macrophages [16] as well as on endothelial cells of the tumor-associated vasculature [17]. We confirm the notion of a hematopoietic expression of B7-H3 by *ex vivo* cytometric analysis (Supplementary Fig. 9), raising the question of whether it is B7-H3 expression on tumor tissue or on tumor-infiltrating lymphocytes (TIL) that accounts for the phenotype of the altered tumor burden. To address this, we generated bone marrow chimeric TRAMP<sup>+</sup> mice in which B7-H3 was absent from the mostly radio-sensitive hematopoietic compartment (B7-H3<sup>-/-</sup>→TRAMP<sup>+</sup>wt), or the radio-resistant tissues only (wt→TRAMP<sup>+</sup>B7-H3<sup>-/-</sup>) and subsequently assessed tumor progression. While chimeric B7-H3<sup>-/-</sup>→TRAMP<sup>+</sup>wt tumors were comparable to solely wt (wt→TRAMP<sup>+</sup>wt) controls, chimeric wt→TRAMP<sup>+</sup>B7-H3<sup>-/-</sup> tumors were significantly larger and comparable to B7-H3<sup>-/-</sup> (B7-H3<sup>-/-</sup>→TRAMP<sup>+</sup>B7-H3<sup>-/-</sup>) tumors (Fig. 2e,f). Notably, the percentages of FoxP3<sup>+</sup> cells were significantly increased only in wt→TRAMP<sup>+</sup>B7-H3<sup>-/-</sup> but not in B7-H3<sup>-/-</sup>→TRAMP<sup>+</sup>wt tumors (Fig. 2g) reflecting the phenotype in non-chimeric B7-H3<sup>-/-</sup> and wt tumors, respectively. Therefore, the absence of B7-H3 in radio-resistant tissue, most likely in the tumor, and not cells of hematopoietic origin accounts for a local increase in Treg numbers and the elevated tumor burden.

In summary, this genetic study affirms the potential of both B7-H3 and B7-H4 to serve as biomarkers in prostate cancer but clearly shows a hereof-detached function of immune-regulation. The vast impact specifically of B7-H3 on solid tumor growth in the applied model of tumorigenesis highlights its potential in shaping the tumor microenvironment and fosters the notion of B7-H3 as a valuable immunomodulatory target in cancer immunotherapy. There is a growing body of evidence describing a direct association of an increased accumulation of FoxP3<sup>+</sup> cells in the microenvironment of human solid tumors and an adverse clinical outcome [18]. Immune modulation by interfering local Treg activity also significantly contributes to the success of therapeutic interventions targeting co-signaling molecules in tumor immunotherapy [19].

There are burning questions arising from this substantive evidence of exacerbated tumor burden in the absence of B7-H3 as well as from other recent studies about the differing functions attributed to B7-H3, particularly in view of the ongoing clinical evaluation of an anti-B7-H3 antibody targeting tumor cells (MGA271, MacroGenics). Although the full mechanistic underpinnings of the impact of B7-H3 on tumor development remains to be established, it is tempting to conjecture on the basis of our findings that B7-H3 acts, directly or indirectly, inhibitory upon Tregs which could result in an impaired antitumor immune response in its absence. In order to verify this hypothesis, the foremost goal is to find all types of receptors for B7-H3, their expression profile and to study the immediate downstream responses at the cellular and molecular level.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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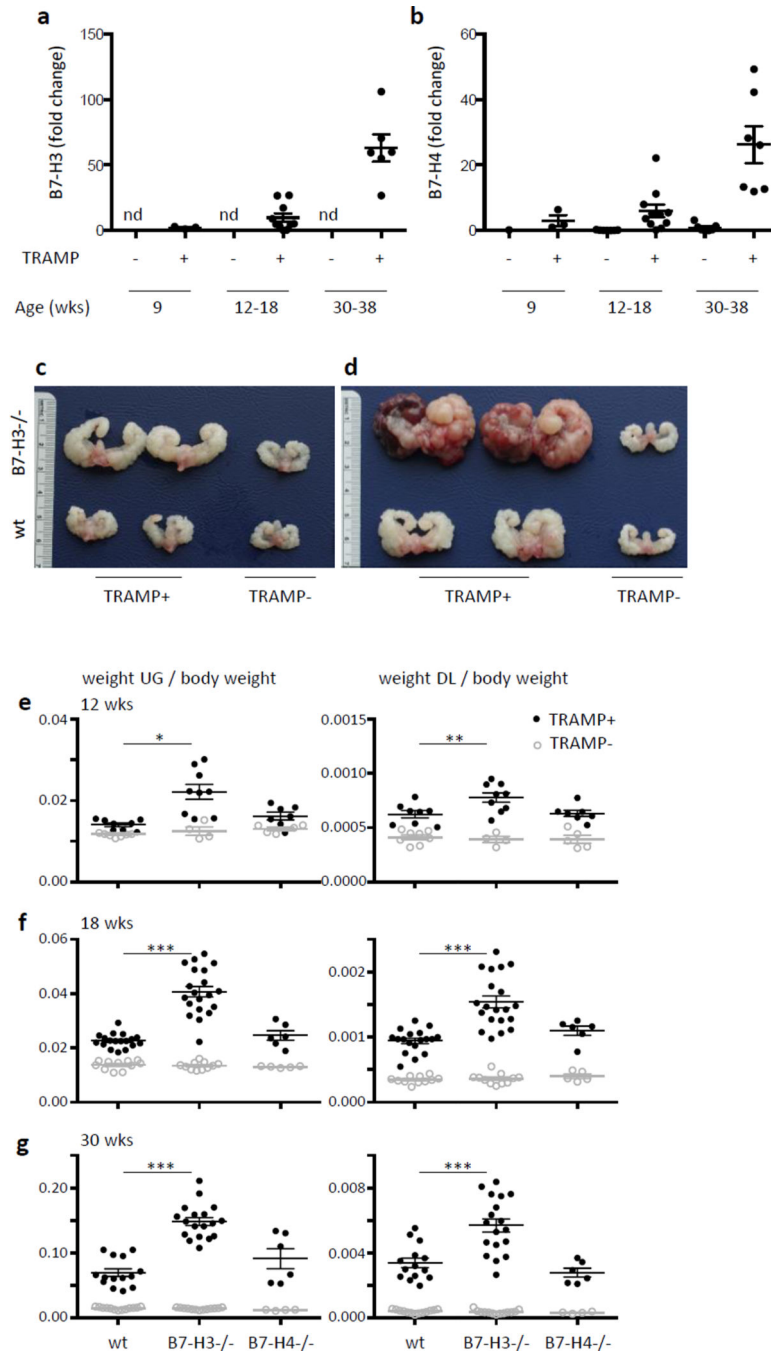
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**Figure 1. Greatly increased growth of murine prostate tumors in the absence of B7-H3**  
 (a) B7-H3 and (b) B7-H4 mRNA expression in the DL prostate lobes of male TRAMP<sup>+</sup> or age-matched TRAMP<sup>-</sup> littermate control mice at indicated time points measured by qPCR and calculated by  $2^{-(C_t)} \pm \text{SEM}$ . (n = 3 per group) (c) Representative images of UG of 18-week and (d) 30-week old TRAMP<sup>+</sup>wt, TRAMP<sup>+</sup>B7-H3<sup>-/-</sup> and age-matched TRAMP<sup>-</sup>wt or TRAMP<sup>-</sup>B7-H3<sup>-/-</sup> littermate control mice, respectively. (e) Masses of UG and DL prostates of TRAMP<sup>+</sup>wt, TRAMP<sup>+</sup>B7-H3<sup>-/-</sup>, TRAMP<sup>+</sup>B7-H4<sup>-/-</sup> and corresponding TRAMP<sup>-</sup>wt, TRAMP<sup>-</sup>B7-H3<sup>-/-</sup> or TRAMP<sup>-</sup>B7-H4<sup>-/-</sup> littermate control mice, respectively, evaluated at

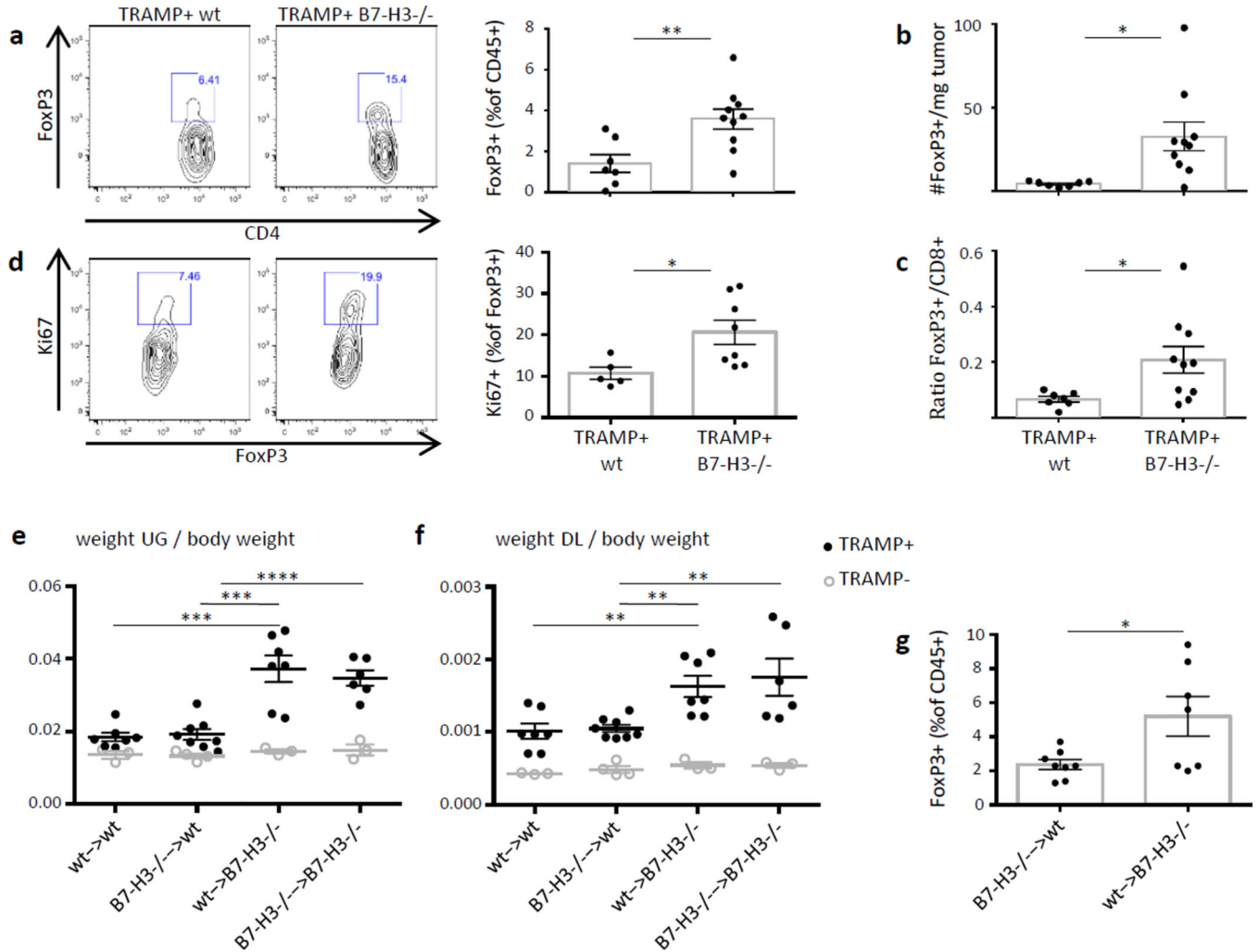
12 weeks, (f) 18 weeks and (g) 30 weeks of age and normalized to whole body weight  $\pm$ SEM. (TRAMP<sup>+</sup>wt: n=39, TRAMP<sup>+</sup>B7-H3<sup>-/-</sup>: n=48, TRAMP<sup>+</sup>B7-H4<sup>-/-</sup>: n=21, TRAMP<sup>-</sup>wt: n=33, TRAMP<sup>-</sup>B7-H3<sup>-/-</sup>: n=28, TRAMP<sup>-</sup>B7-H4<sup>-/-</sup>: n=14) Unpaired t test. \* p< 0.05, \*\* p< 0.01, \*\*\* p< 0.001.

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**Figure 2. Lack of B7-H3 expression on radio-resistant tissue accounts for elevated tumor growth and accumulation of regulatory FoxP3<sup>+</sup> T cells**  
 Percentages of tumor-infiltrating (a) FoxP3<sup>+</sup> cells and (b) Ki67<sup>+</sup>FoxP3<sup>+</sup> cells, (c) total FoxP3<sup>+</sup> cell-counts per mg of tumor, and (d) ratio of FoxP3<sup>+</sup> to CD8<sup>+</sup> T cells isolated from TRAMP<sup>+</sup>wt and TRAMP<sup>+</sup>B7-H3<sup>-/-</sup> tumors at 18 weeks of age and analyzed by flow cytometry ± SEM (n = 5). Masses of (e) UG and (f) DL prostates of indicated bone marrow chimeric TRAMP<sup>+</sup>wt, TRAMP<sup>+</sup>B7-H3<sup>-/-</sup> and corresponding TRAMP<sup>-</sup> control mice evaluated at 30 weeks of age and normalized to whole body weight ±SEM (TRAMP<sup>+</sup>: n = 6). Percentages of tumor-infiltrating (g) FoxP3<sup>+</sup> cells and (f) Ki67<sup>+</sup>FoxP3<sup>+</sup> cells isolated from indicated bone marrow chimeric TRAMP<sup>+</sup> mice at 30 weeks of age. Unpaired t test. \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001