B7H6 is a functional ligand for NKp30 in rat and cattle and determines NKp30 reactivity towards human cancer cell lines

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Abbreviations used in this article: BAG-6: BCL2-associated athanogene 6; EGFP: enhanced GFP; EST: expressed sequence tag; HA: hemagglutinin; ILC: innate lymphoid cells; MFI: mean fluorescence intensity; siRNA: short interfering RNA; SH2/3: Src homology domain 2/3; TM: transmembrane; UTR: untranslated region

Abstract

NK cells kill cancer cells and infected cells upon activation by cell surface receptors. Human NKp30 is an activating receptor expressed by all mature NK cells. The B7 family member B7H6 has been identified as one ligand for NKp30. Several alternative ligands have also been reported, and the field remains unsettled. To this end, we have identified full-length functional B7H6 orthologs in rat and cattle, demonstrated by phylogenetic analysis and transfection experiments. In cell-cell contact-dependent assays, chimeric NKp30 reporter cells responded strongly to B7H6 in rat and cattle. Likewise, rat NKp30 expressing target cells induced strong activation of B7H6 reporter cells. Together, these observations demonstrate that B7H6 is conserved as a functional ligand for NKp30 in mammalian species separated by more than 100 million years of evolution. B7H6 and NKp30 are pseudogenes in laboratory mice. The rat thus represents an attractive experimental animal model to study the NKp30-B7H6 interaction *in vivo*. B7H6 was widely expressed among human cancer cell lines, and the expression level correlated strongly with the activation of human NKp30 reporter cells. Furthermore, siRNA knockdown of B7H6 abolished NKp30 reporter responses, suggesting that B7H6 is the major functionally relevant expressed ligand for NKp30 on these cancer cell lines.

Introduction

Natural killer (NK) cells are large granular lymphocytes with the ability to recognize and kill cancer cells and infected cells [1-4]. The molecular basis for NK cell recognition of target cells is increasingly well understood. NK cell effector functions such as cytotoxicity or cytokine release are regulated by signals from different cell surface receptors, and thus by a balance of simultaneous inhibitory and activating signaling events [5]. Whereas many NK cell receptors belong to receptor families with both activating and inhibitory members, the natural cytotoxicity receptors NKp30, NKp44 and NKp46 are single-member family receptors with activating functions [6]. NKp30, like NKp46, is expressed by all mature human NK cells [6] as well as subsets of innate lymphoid cells (ILC) [7, 8]. Surface expression of NKp30 has also been reported to be inducible on endometrial epithelium following progesterone stimulation, and on cord blood T cells and $V\delta1^+$ T cells after cytokine stimulation [6]. NKp30 is a type 1 transmembrane protein belonging to the immunoglobulin superfamily consisting of one V-set Ig domain, a short stalk, a transmembrane region and a short cytoplasmic tail [9]. The NKp30 transmembrane region contains an arginine residue that forms an ionic bond to a dimeric transmembrane adaptor protein (CD3ζ-CD3ζ or FcRεIγ-CD3ζ) that activates NK cells by recruiting the tyrosine kinase Syk. Three alternative NKp30 splice variants encode different cytoplasmic regions, with seemingly different modulatory effects on NKp30 function [6, 10].

Several alternative ligands have been reported for NKp30 in the human. These include several pathogen-encoded protein ligands (in chronological order): soluble pp65 from CMV [11], HA from poxviruses including *Vaccinia* virus [12, 13] virus, Duffy binding-like domain 1α from *Plasmodium falciparum* [14], and recently also β-1,3-glucans from the pathogenic fungi *Cryptococcus neoformans* and *Candida albicans* [15]. With regards to cell-encoded ligands, NKp30 has been reported to bind heparan sulphates [16], Galectin-3 [17], the intracellular protein BCL2-associated athanogene 6 (BAG6) (also called HLA-B associated transcript 3)

[18] and the transmembrane cell surface protein B7H6 [19]. Several lines of evidence support B7H6 as a functional ligand for human NKp30, including X-ray crystallography studies [20, 21].

B7H6 consists of two extracellular Ig domains, a transmembrane region and a long cytoplasmic tail. It is not clear whether B7H6 has an integral signaling function. Intriguingly, B7H6 is widely expressed on cancer cell lines and is also expressed by tumor cells *in situ* [22]. In contrast, B7H6 does not seem to be expressed to any great extent by normal human tissues under steady state, although it can be upregulated on myeloid cells under inflammatory conditions [23]. B7H6 thus appears to a large extent to represent a comparably specific cancer marker, and chimeric antigen receptor-based therapy strategies towards B7H6 are in development [24, 25].

A role for NKp30 in NK cell killing of cancer cells *in vivo* has not been clearly established, but is under active investigation by several laboratories. The field however remains unsettled as to whether the different proposed ligands for NKp30 are relevant and functional *in vivo* in cell-cell interaction. NKp30 is expressed by NK cells in the rat [26] but is only a pseudogene in the mouse [27], and experimental animal models to study the interaction between NKp30 and B7H6 are currently lacking.

In this report, we have investigated whether functional orthologs of B7H6 exist in two non-primate species; rat and cattle; and to what extent B7H6 is a functional ligand for NKp30 in these species. Using cell-cell contact based reporter cell assays, we have also investigated the correlation between NKp30 binding and B7H6 expression by a panel of human cancer cell lines. siRNA knockdown of B7H6 expression abolished NKp30 reactivity towards cancer cells, suggesting that B7H6 is most functionally relevant cancer cell-encoded ligand for NKp30.

Results

Molecular cloning of functional bovine and rat B7H6 cDNAs

In order to investigate functional homologues of human B7H6, public sequence databases were searched for rodent and bovine genes with homology to human B7H6. A bovine mRNA sequence obtained searching GenBank was used to design primers, and a full-length bovine B7H6 cDNA was cloned from spleen RNA by RT-PCR. Searching rat databases did not yield a full-length B7H6 sequence, but a short sequence with homology to the N-terminal Ig domain exon of human B7H6 was retrieved from an EST database. The 5´-UTR, leader and Ig V-set domain sequence of a rat cDNA was then obtained by rapid amplification of cDNA ends (RACE) cloning using cDNA from the rat myeloid cell line RMW. A 3´-UTR primer was then generated based on sequence analysis of a bacterial artificial chromosome clone and the Ig C-set, transmembrane, cytoplasmic and 3´-UTR regions were cloned and sequenced. Comparing the whole protein, identity to human B7H6 was 37% for cattle and 26% for rat (Fig. 1A). When comparing the ligand-binding Ig domains, bovine and rat B7H6 were more similar to the human ortholog (54% and 49% identity, respectively). Rat B7H6 contained an unusually long N-terminal leader peptide of 79 residues (Fig. 1A). Despite the unusual length of the rat B7H6 leader it contains a hydrophobic stretch near the C-terminal end and a putative signal peptidase cleavage site (as predicted by Signal P). Transfection experiments with different constructs encoding the native polypeptide with an internal (ectodomain) HA tag or a C-terminal YFP tag induced surface expression of rat B7H6, demonstrating that the leader peptide is a fully functional ER sorting signal (Supporting information Fig. 1). Rat B7H6 contains a stalk region not observed in human or cattle. Apart from the lacking stalk region, the gene structures of rat and bovine *B7h6* (also termed *Ncr3lg1*) were similar (Supporting information Fig. 2). An alternative splice variant excluding this stalk exon was

also observed (data not shown). The transmembrane and cytoplasmic regions were remarkably different between the three species. Although functional data are lacking, it has been reported that human B7H6 contains an intracytoplasmic domain homologous to GAG polyprotein, as well as an immunoreceptor tyrosine-based inhibition (ITIM)-like motif, and SH2 and SH3-binding domains [19]. Those features were not conserved in cattle and rat (Fig. 1A). With regards to NKp30, the extracellular region of bovine NKp30 shared 78% amino acid identity compared to human, whereas rat NKp30 was 65% identical (Fig. 1B). In concordance with previous reports by others, searching available laboratory mouse (*Mus musculus*) sequence databases did not retrieve intact genes capable of encoding full-length NKp30, whereas Ryukyu mice (*Mus caroli)* have a seemingly functional *Nkp30* gene sharing 77.1% amino acid identity with the rat ortholog. We also searched available mouse sequence databases for a B7H6 ortholog. A short genomic sequence highly similar to the leader peptide of rat B7H6 was found on chromosome 3. Moreover, a sequence homologous to the N-terminal Ig domain was detected on chromosome 7, but contained a frameshift mutation. Mouse sequences with high similarity to the C-terminal Ig domain were not retrieved. Thus, in all investigated mouse species, a gene encoding a functional B7H6 molecule is lacking (data not shown).

Phylogenetic analysis of several immunoglobulin superfamily proteins showed that rat, cattle and human B7H6 cluster together (Fig. 1C). Searching human databases with rat or bovine B7H6 retrieved human B7H6 as the single best hit. Moreover, forward and reverse similarity searches between rat, cattle B7H6 and several other mammalian species also invariably yielded single hits, suggesting that B7H6 is conserved as a single ortholog among mammalian species (data not shown). In the phylogenetic analysis, B7H6 clustered together with the other members of the B7 family (Fig 1C, dark grey area). Rat and cattle NKp30 grouped together with members of the CD28 family (Fig 1C, light grey area). Of note, CTLA-4 was more

similar to NKp30 than to CD28, somewhat surprising given that CTLA-4 and CD28 bind the same ligands.

B7H6 is a physiological ligand for NKp30 in rat and cattle

Due to controversy in the field with regards to ligand specificity of human NKp30, we wanted to determine whether B7H6 is a physiological ligand for NKp30 also in other species. To this end, we generated EGFP-producing reporter cell lines stably expressing chimeric receptors consisting of the ectodomain of bovine or rat NKp30 fused to the cytoplasmic region of mouse CD3ζ. Upon overnight co-incubation, rat NKp30 reporter cells responded strongly towards 293T cells transiently transfected with rat B7H6 (Fig. 2A). In control experiments, the NKp30 reporters did not respond to 293T targets transfected with empty vector, and untransfected BWN3G cells did not respond to B7H6-transfected 293T targets. Additionally, we generated reporter cells expressing the ectodomain of rat B7H6 fused to the cytoplasmic region of mouse CD3ζ. The rat B7H6 reporter cells responded strongly towards CHO cells stably transfected with rat NKp30, corroborating the data from the inverse experiment, demonstrating that B7H6 is a physiological ligand for NKp30 in the rat as well as in the human (Fig. 2B). We also generated reporter cells expressing the ectodomain from cattle B7H6. The bovine B7H6 reporters responded strongly towards 293T cells transfected with bovine NKp30, whereas the control experiments were negative (Fig. 2C). This indicated that B7H6 is a physiological ligand for NKp30 also in cattle. Using the rat, cattle and human reporter lines, no cross-species binding between NKp30 and B7H6 could be demonstrated (data not shown). Together, our data indicate that NKp30 and B7H6 are conserved as a receptor-ligand pair between primates, rodents and ruminants, separated by more than 100 million years of evolution.

B7H6 transcription and surface expression in the rat

In the human, B7H6 expression has been reported to be restricted to cancer cell lines, certain tumors *in situ,* and monocytes and neutrophils in inflammatory conditions. We wanted to investigate whether the transcription profile is similar in the rat. qPCR on cDNA derived from rat cell lines showed expression in myeloid lineage cells in the rat, but not on NK cell lines or embryonic fibroblasts (Fig. 3A). RT-PCR on a large panel of tissues from two inbred rat strains (DA and PVG) also showed transcription of B7H6 in muscle, testis, and spleen (data not shown). In the absence of a mAb towards rat B7H6, this could not be investigated at the protein level. To investigate if the B7H6 mRNA detectable by RT-PCR led to surface expression, we performed reporter cell assays. The B7H6 $mRNA^+$ cell lines RMW (myeloid), R2 (macrophage) and RBL-2H3 (basophilic leukemia) all activated rat NKp30 reporter cells (Fig. 3B), indicating that B7H6 is expressed at the cell surface in these cell lines. RMW cells reproducibly induced stronger responses than R2 and RBL-2H3 targets. This was not reflected in their relative mRNA levels, suggesting intracellular retention or rapid endocytosis and degradation of B7H6 protein in RBL-2H3 cells. Alternative explanations include different expression of adhesion molecules necessary for synapse formation.

B7H6 expression on human cancer cell lines is highly correlated with NKp30 reporter cell activation

Others have previously demonstrated surface expression of B7H6 on several human cancer cell lines. To re-investigate this, we analyzed 20 human carcinoma cell lines and the embryonal kidney cell-derived line 293T for surface expression of B7H6 by flow cytometry. A continuum of B7H6 expression was observed, from $B7H6^-$ cells to cells expressing B7H6 at intermediate and high levels (Fig. 4A). To investigate to what extent these cancer cells

were recognized by human NKp30, we generated a human NKp30 reporter cell line and performed overnight co-incubation assays with each cancer cell line. In these assays, cancer targets that express B7H6 at a high level also strongly activated the NKp30 reporters, whereas $B7H6^-$ cancer targets did not induce NKp30 reporter responses (Fig. 4A). Linear regression analysis of B7H6 expression level (MFI) by cancer cell lines and degree of NKp30 reporter activation (% $EGFP^+$ cells) showed high correlation (R^2=0.8) (Fig. 4B). This suggested that recognition of the cancer cells by NKp30 was mostly (or solely) dependent on B7H6 expression.

Others have reported that the ectodomain of B7H6 can be shed from tumor cells in soluble form. The human NKp30 reporter cells did not respond to plastic wells precoated with culture supernatant (complete medium or PBS supernatant from 4 h culture) from the cancer cell lines HCT15, FO-1, CaCo-2, LoVo, KYSE-70, MCF-7, OVCAR-3, PC3, SK-BR-3, T47D, WM239 and HT29 (data not shown), indicating that B7H6 ectodomain shedding is not a universal feature of cancer cells.

siRNA knockdown of B7H6 on cancer cells abrogates reactivity with NKp30

To further investigate if the NKp30 reactivity towards tumor cells was dependent on B7H6 we performed siRNA-mediated knock-down of B7H6 in selected cell lines. Cancer cell lines were treated with B7H6 siRNA or control siRNA 72 hours before overnight incubation with NKp30 reporter cells. Knockdown efficiency varied between cell lines, however a clear reduction in reporter activation was always observed (Fig. 5A). High correlation between B7H6 expression (MFI) and reporter activation (% $EGFP^+$ reporter cells) was maintained as shown by linear regression analysis (R^2=0.75) (Fig. 5B). These data indicate that B7H6 was the only ligand expressed by these cancer cell lines that was recognized by NKp30 reporters.

CTLA4 is not an alternative receptor for B7H6

In terms of tumor evolution, it might be seen as surprising that a high fraction of cancers have not lost B7H6 expression as a result of selection pressure imposed by NK cells. One possible explanation for this could be that B7H6 might interact with alternative receptors with inhibitory functions, either on NK cells or other immune cells. Our phylogenetic analysis showed that NKp30 is more closely related to CTLA4 than to other CD28 family members (Fig. 1C). Although it would seem unlikely that CTLA4 could also bind to B7H6 and negatively regulate antitumor immune responses, we generated reporter cells expressing the ectodomain of human CTLA4 to investigate this possibility. Using $B7H6^{+}$ targets, no reporter responses were induced in overnight reporter assays. As a positive control, the reporters responded strongly to crosslinking with antibody. These data indicate that CTLA4 is not a receptor for B7H6 (Fig. 6).

Discussion

Several alternative ligands have been reported for NKp30 in the human [6]. In addition to the pathogen-encoded protein ligands pp65 (CMV) [11], HA (poxviruses) [12, 13], Duffy binding-like domain 1α (plasmodium) [14] and fungal wall β-1,3-glucans [15], several cellular encoded ligands have been reported, including heparan sulphates [16], BAG6 [18] and B7H6 [19]. The field remains somewhat unsettled as to the functional role of the different candidate NKp30 ligands.

In an attempt to clarify this, we set out to investigate if B7H6 is a functional ligand for NKp30 in other mammalian species. In this paper, we have cloned and identified full-length orthologs of human B7H6 in rat and in cattle. RT-PCR analysis demonstrated that B7H6 can be expressed at the mRNA level as full-length open reading frame transcripts, and transfection experiments induced protein expression at the cell surface. In reporter cell assays, target cells expressing rat B7H6 strongly triggered rat NKp30 reporter responses. Vice versa, rat NKp30-expressing target cells triggered rat B7H6 reporters, demonstrating that B7H6 is a functional ligand for NKp30 in the rat, with sufficient affinity to activate cellular responses in physiological cell-cell contact-based experiments. Similar results were obtained in cattle, where the NKp30-B7H6 interaction induced strong reporter cell responses. Thus, NKp30 and B7H6 is a functional receptor/ligand pair in mammals outside primates, suggesting this could also apply for other species outside ruminants and rodents. Interestingly, forward and reverse sequence similarity searches between a number of other mammalian species invariably returned one single B7H6 homologue in most species (data not shown), supporting the possibility that this receptor-ligand interaction is widely conserved among mammals. Putative orthologs of NKp30 and B7H6 have been identified in amphibians (*Xenopus*) and in cartilaginous fish (shark), but have not been found in bony fish, chicken or opossum,

suggesting that B7H6 may have been a ligand for NKp30 at the beginning of vertebrate evolution [28]. It is not clear why these molecules have later been selectively lost in some species.

Others have reported that *Nkp30* is a pseudogene in *Mus musculus*, but encodes a seemingly intact open reading frame in *Mus caroli* [27]. We found that B7H6 only exists as a fragmented pseudogene in the mouse, including *Mus caroli*. Accordingly, the rat represents the most accessible experimental animal to study the NKp30-B7H6 interaction *in vivo,* including infection models and experimental tumor development. With availability of monoclonal antibodies towards rat B7H6, the rat will also provide an experimental model for the study of how surface expression of B7H6 is regulated in different cells and tissues under varying physiological and pathological conditions.

Our observations of NKp30 reporter cell reactivity with rat myeloid cell lines combined with RT-PCR analysis suggest that B7H6 may also be expressed by subsets of primary myeloid cells in the rat. Whereas an early report did not detect B7H6 expression in resting, healthy tissues [19], earlier functional data have indicated that NKp30 is involved in the killing of dendritic cell subsets by NK cells [29] and B7H6 expression on $CD16^{+}CD14^{+}$ monocytes and granulocytes was found to be inducible by proinflammatory cytokines or ligands of Toll-like receptors [23]. B7H6 also appears to be expressed in atopic dermatitis, inducible by proinflammatory cytokines [8]. Besides myeloid cells under conditions of inflammation, B7H6 is widely expressed by human cancer cell lines [19, 22, 30-33] and chimeric antigen receptor-based cancer therapies directed against B7H6-expressing tumors are in development [25].

Our finding that B7H6 was not a ligand for CTLA4 (in our hands) suggests that there may be other mechanisms that allow $B7H6^{+}$ cancers to develop while avoiding elimination by the

immune system. Some members of the B7 family can dimerize to form homodimers or heterodimers with other B7 family members[34]. Reporter cells expressing rat or bovine B7H6 did not show self-reactivity, indicating that B7H6 homodimerization in *trans* does not occur. We cannot exclude the possibility of *cis* homodimerization, but if this were the case it did not seem to affect binding to NKp30, based on the strong reactivity of B7H6 reporters with $NKp30^{+}$ targets. A recent report has suggested a role for cancer cell-expressed B7H6 in inducing immunosuppressive mechanisms via NKp30-expressing ILC2 [7], suggesting a mechanism whereby B7H6 expression by cancer cells can support tumor survival and providing a possible explanation of how the negative selective pressure imposed by NK cells could be balanced out.

Corroborating previous reports, we found high B7H6 surface expression on nine out of 19 cancer cell lines, weak expression on two and very weak or no expression on eight of these lines. Supporting that B7H6 is a functional ligand for human NKp30, we observed a strong correlation between the level of B7H6 surface expression and the ability to activate human NKp30 reporters. Here, we did not investigate surface expression of alternative previously reported cellular ligands for NKp30, such as BAG6 and galectin-3. siRNA knockdown of B7H6 on the same cell lines correlated strongly with reduced reporter responses. Although this should not be taken as proof, our observations should inspire concern that other putative ligands for NKp30, despite some level of affinity, might be irrelevant or nonfunctional in cell-cell contact situations, and suggests that NK cell killing of cancer cells through NKp30 relies on B7H6 surface expression.

In this paper, we have not investigated the capability of NKp30 to respond to proposed pathogen-encoded ligands. Recent reports point towards a role for B7H6 as an infection-induced ligand [35-37]. Proteomic analysis of CMV-infected cells found that B7H6 surface expression is induced by deletion variants of CMV but not by the wild-type virus, and that the

US18 and US20 viral genes act to suppress B7H6 surface expression [37]. Although the factors that regulate B7H6 expression are not yet understood, this ligand has an important role in identifying targets for NK cells as a result of malignant transformation and possibly also intracellular infection.

We have here found that B7H6 is conserved as a functional ligand for NKp30 between primates, rodents and ruminants, indicating that B7H6 represents an ancient mechanism to flag targets for NK cells, dating back at least 100 million years in mammalian evolution. Our observation that B7H6 is a functional ligand for NKp30 in the rat provides a novel opportunity to investigate the functional role of this receptor/ligand pair in experimental animal models of cancer, infection and autoimmune disease.

Materials and Methods

Molecular cloning of bovine and rat B7H6

Bovine B7H6 cDNA clones were obtained by RT-PCR from spleen RNA with primers based on a predicted transcript sequence (GenBank ID: NM_001206792.1) (forward: 5'-GCTATTGCAATGGCGAAGA-3'; reverse: 5'-GATTTGCTGATGCGTTGAG-3') using *Pfu Turbo* polymerase (Agilent Technologies). The bovine B7H6 cDNA sequence has been submitted to GenBank (accession no. MH237865).We searched rat databases for sequences with homology to human B7H6 and identified an EST (GenBank accession no. CV105261.1) which could correspond to the first extracellular Ig domain of a rat B7H6 ortholog. Based on this, we generated a gene-specific reverse primer (5'-CGACCTTGCATTGGTATTCTCCTGCTTC-3') and the 5'-UTR (163 bp), leader and Ig-V domains were obtained using RACE cloning (GeneRacer, Invitrogen) and RNA isolated from the RMW cell line. RT-PCR products were cloned into pCR 2.1-Topo vector (Invitrogen, San Diego, CA) and sequenced by Sanger sequencing (BigDye Terminator v3.1 kit, Thermo Fisher Scientific). The obtained 5´ part of the sequence allowed us to identify a bacterial artificial chromosome sequence (GenBank accession no. AC120807.4) that appeared to contain the entire gene, from which we designed a putative 3'-UTR primer that was used to clone a full open reading frame rat B7H6 cDNA by RT-PCR ((forward: 5'-TGACCCACCGTGCTCTAAGACGA-3'; reverse 5'-CCACGAATACTGTGTCCTTGACCTG-3')) (GenBank accession no. MH237864)

Sequence analysis

Genomic or EST sequence information was obtained using BLAST and related search algorithms browser applications at the NCBI (www.ncbi.nlm.nih.gov) and Ensembl (www.ensembl.org) web sites. Sequence analysis, alignments and phylogenetic analysis was performed with the DNASTAR Lasergene 9 program package, Clustal X [38] and NJplot. Transmembrane regions and signal peptides were predicted using TMPred and SignalP [39], respectively.

Transcription analysis

Real-time qunatitative or conventional semi-quantitative RT-PCR of rat B7H6 expression in tissues and cell lines was performed using gene-specific primers from neighboring exons. Total RNA from cell lines or primary cells was isolated using TRIzol reagent according to the manufacturer's instructions (Life Technologies). First-strand cDNA synthesis was carried out using M-MLV RNase H^- reverse transcriptase (Promega) using 1 µg total RNA in a 20 µl reaction as previously detailed [40]. qPCR was performed in triplicates with a standard TaqMan protocol with specific primers and FAM-TAMRA probes for rat B7H6 and HPRT, respectively, spanning a splice junction site (Platinum Quantitative PCR Supermix-UDG with ROX (Invitrogen); 7900HT thermal cycler (Applied Biosystems); ΔΔCt method). Statistical analysis was performed with non-paired Students t-test. Semi-quantitative RT-PCR was performed using Dynazyme II DNA Polymerase (Thermo Fisher Scientific), with hot-start and five initial cycles of touchdown PCR followed by 30-35 cycles at optimal annealing temperatures.

Primary cells and cell lines

The following rat cell lines were used: RMW (a myeloid cell line derived from *in vitro* culture of splenocytes) [41], RBL-2H3 (basophilic leukemia cell line) [42]; R2 (macrophage cell line) [43] and rat embryonic fibroblasts. The following human cell lines were used: Breast cancer: SK-BR-3, MCF7, T47D, MDA.MB.231; melanoma: WM9, WM35, WM239, FO-1; colon cancer: LoVo, CaCo-2, HCT15, HCT116, HT29; esophageal squamous cell carcinoma: KYSE-70; prostate cancer: PC3, DU145; ovarian carcinoma: OVCAR-3; glioblastomas: U87, SF126; esophageal squamous epithelium: HET1A; embryonic kidney: 293T. The BWN3G cell line (BW5147 mouse thymoma cells stably transfected with EGFP under control of a 3xNFAT response element promoter) has been described previously [44]. All cell lines used were routinely screened for mycoplasma infection and maintained in complete medium (RPMI 1640 supplemented with 1 mM sodium pyruvate, 1% antibiotic/antimycotic solution and 10% FBS (all from Invitrogen).

Expression constructs and transient transfections

The full open reading frame of rat NKp30 was amplified from BN rat spleen cDNA by PCR using *PfuTurbo* polymerase (Agilent Technologies), cloned (pCR2.1-TOPO vector, Invitrogen) and sequenced. An expression construct encoding the open reading frame of rat NKp30 was generated in the BSRα vector. An expression construct encoding bovine B7H6 with an N-terminal FLAG tag was generated in the pFLAG-CMV3 vector (SigmaAldrich). A full-length rat B7H6 expression construct with a C-terminal YFP tag was generated in pEYFP-N1 (Clontech). All constructs were verified by sequencing. A plasmid encoding bovine NKp30 in the pExpress-1 vector (IMAGE ID 8053487) was purchased from Source Bioscience**.** For transient transfections of 293T or CHO-K1 cell lines, 6.5 µg of plasmid DNA

resuspended in PBS was mixed with 32 µg of polyethyleneimine (Polysciences) resuspended in water, incubated 25 min, then added to a 25 cm^2 flask containing 6 mL complete medium and cells growing at 60-80% confluence. After 24 hours, the cells were washed twice with PBS and kept in complete medium until they were harvested for flow cytometric analysis and reporter assays 48 hours after transfection start.

Antibodies and flow cytometry

The following mAbs were used: M2 (anti-FLAG, Sigma-Aldrich), HA.11 (anti-HA, Covance Research Products), 875001 (anti-hB7H6, R&D Systems), P30-15 (anti-hNKp30-Alexa Fluor 647, Biolegend) and W6/32-Alexa Fluor 647 (anti-human MHC class I). A polyclonal Alexa Fluor 647-conjugated goat-anti-mouse IgG was used as secondary antibody (Thermo Fisher Scientific). Samples were analyzed with FACSCalibur or FACSCanto II flow cytometers using CellQuest Pro, FACSDiva (both BD Biosciences) and FlowJo software. Flow cytometry procedures were in accordance with standard methodological guidelines[45].

Imaging flow cytometry

CHO-K1 cells were stably transfected with a construct encoding full length rat B7H6 with a C-terminal EYFP tag were analyzed for surface versus intracellular staining with a 5-laser 12-channel ImageStreamX imaging flow cytometer (Amnis) using a 40x lens. Cells were washed in PBS and fixed with 2% paraformaldehyde (Thermo Fisher Scientific) in PBS for 10 minutes at room temperature before acquisition. Bright-field area was set to a lower limit of 50 µm to eliminate debris, and single cells were identified based on area and aspect ratio gating. Data was analyzed using the IDEAS 4.0 software (Amnis).

Generation of reporter cell lines

A chimeric receptor expression construct was made in the pBSRα-EN vector, encoding the leader and extracellular domains of rat B7H6 followed by a membrane-proximal section containing HA (YPYDVPDYA) and FLAG (DYKDDDK) epitope tags, coupled to the transmembrane region of human CD8 and the cytoplasmic domain of mouse CD3ζ. Human, bovine and rat NKp30 and bovine B7H6 constructs were also generated in pBSRα-EN, but encoding an N-terminal FLAG tag followed by respective extracellular domains, coupled to the transmembrane region of human CD8 and the cytoplasmic domain of mouse CD3ζ. All constructs were verified by sequencing. To obtain stably transfected receptor reporter cells, 3×10^6 BWN3G cells were mixed with 20 µg linearized plasmid at 4°C in complete medium and electroporated at 120 V, 960 µF (GenePulser, Bio-Rad Laboratories) in a 2-mm cuvette. After 24 hours, cells were seeded at 1.000 to 10.000 cells/well in 96-well plates and selected in complete medium supplemented with 1.6 mg/ml Geneticin (G-418 disulphate; ThermoScientific) and 1 mg/ml Hygromycin B (Invitrogen). Stable clones with bright surface expression identified by flow cytometry (anti-FLAG mAb M2 and/or anti-HA mAb HA.11), were further tested for EGFP expression after receptor crosslinking: 96-well plates were coated with 10µg/ml polyclonal goat anti-mouse IgG (Jackson ImmunoResearch) in 50mM sodium carbonate buffer (pH 9.3) at 4°C overnight, blocked with 10mg/ml BSA in PBS for 30 minutes at room temperature, and coated with anti-FLAG or anti-HA mAb (10 µg/ml) for 1-2 hours at 37°C. Plates were washed and 5×10^4 reporter cells were added for overnight incubation. EGFP production was measured by flow cytometry, gating to exclude human target cells (HLA class I^+) (Supporting information fig. 3). Despite the fact that bovine NKp30 was readily expressed as a full length construct, we were not able to generate bovine NKp30 reporter cells with sufficient surface expression for reporter cell activation.

Reporter assays

5×10^4 target cells were mixed with 5×10^4 reporter cells in flat-bottom 96-well plates and incubated in 200 µl complete medium at 37°C overnight (20-24 hours). EGFP production by reporter cells was measured by flow cytometry. Target cells were distinguished from the mouse reporter cells in flow cytometric analysis by staining with an anti-human MHC class I antibody (mAb W6/32).

siRNA-mediated knock-down

To target B7H6 expression in tumor cell lines, a mix of four siRNAs complementary to human B7H6 (ON-TARGETplus SMARTpool, Dharmacon, ThermoScientific) was used. In a 24-well plate, 3.6×10^5 cells were plated in each well. After 24 hours, 7.2 pmol siRNA (B7H6 or control) was mixed with 1.2 µL RNAiMAX (ThermoScientific) (both dissolved in OptiMEM), incubated at room temperature for 20 minutes and added to the cells in 500 µl of complete medium. 60-66 hours after transfection start, cells were washed twice in OptiMEM, and complete medium was added. Cells were harvested for reporter assays 72 hours after transfection start. Surface expression of B7H6 was analyzed by flow cytometry at 72 and 96 hours after transfection start.

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Conflict of interest

The authors declare no financial or commercial conflicts of interest.

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587 # Figure legends

588 **Figure 1. Molecular cloning of rat and bovine B7H6.** Peptide sequence alignments of
589 human, rat and bovine B7H6 (A) and NKp30 (B) are shown. Identical residues and gaps are
590 indicated by dashes and dots, respectively. Exons encoding signal sequence, Ig superfamily
591 domains (Ig V- and C-set), stalk, transmembrane (TM) and cytoplasmic regions are indicated.
592 Putative TM regions are underlined. The conserved B-F strand disulfide bond cysteine
593 residues are shaded in gray. GenBank accession numbers: rB7H6: MH237864; btB7H6:
594 MH237865; hB7H6: NP_001189368.1; rNKp30: AAP13457.1; btNKp30: AAI09615.1;
595 hNKp30 (isoform a): AAH52582.1. (C) Phylogram displaying amino acid sequence similarity
596 between B7H6, NKp30 and a selection of Ig superfamily receptors including members of the
597 CD28 and B7 families. The phylogram is based on alignment of exons encoding the
598 extracellular Ig domains. Human CD3γ was selected as outgroup. The B7 family ligands
599 (dark grey background) and CD28 family receptors (light grey background) clustered together.
600 Values at nodes represent percent frequencies of branch association based on 1000 bootstrap
601 repetitions. Branch length of 0.05 corresponds to 5% sequence dissimilarity. h, *Homo sapiens*;
602 bt, *Bos taurus;* r, *Rattus norvegicus*.

603

604 **Figure 2. NKp30 and B7H6 is a functional receptor ligand pair in rat and cattle.** EGFP
605 production by reporter cells after overnight co-incubation with the indicated target cells was
606 assessed by flow cytometry. (A) Reporter cells expressing a chimeric receptor consisting of
607 the extracellular domain of rat NKp30 coupled to the intracellular region of mouse CD3ζ were
608 incubated with 293T target cells transfected with rat B7H6 (left) or empty vector (293T.EV,
609 middle). Untransfected BWN3G cells (BW.-) incubated with B7H6 target cells were used as
610 an additional negative control (right). (B) Similarly; reporter cells expressing a rat

611 B7H6/mouse CD3ζ chimeric receptor were incubated with CHO-K1 cells stably transfected to
612 express rat NKp30 (left) or untransfected (middle). Untransfected BWN3G cells (BW.-)
613 incubated with CHO.rNKp30 served as an additional control (right). (C) Bovine B7H6
614 reporter cells were incubated with 293T target cells transfected with a bovine NKp30
615 construct (left) or empty vector (middle). Untransfected BWN3G against 293T.NKp30 target
616 cells is also shown (right). The percentage of $EGFP^+$ reporter cells is indicated in the upper
617 right corner. For each cell line, one representative experiment of at least three experiments is
618 shown.

619

620 **Figure 3. B7H6 expression by rat cells.** (A) qPCR analysis of B7H6 transcription in the
621 indicated rat cell lines. Expression of B7H6 relative to the endogenous control HPRT is
622 shown, normalized to RMW samples. (B) EGFP production by rat NKp30 reporter cells after
623 overnight incubation with the indicated target cells; RBL-2H3, R2, RMW or embryonic
624 fibroblasts (R.E.F). Percentage of $EGFP^+$ cells is indicated in the upper right corner. The
625 results shown are representative of at least three individual experiments.

626

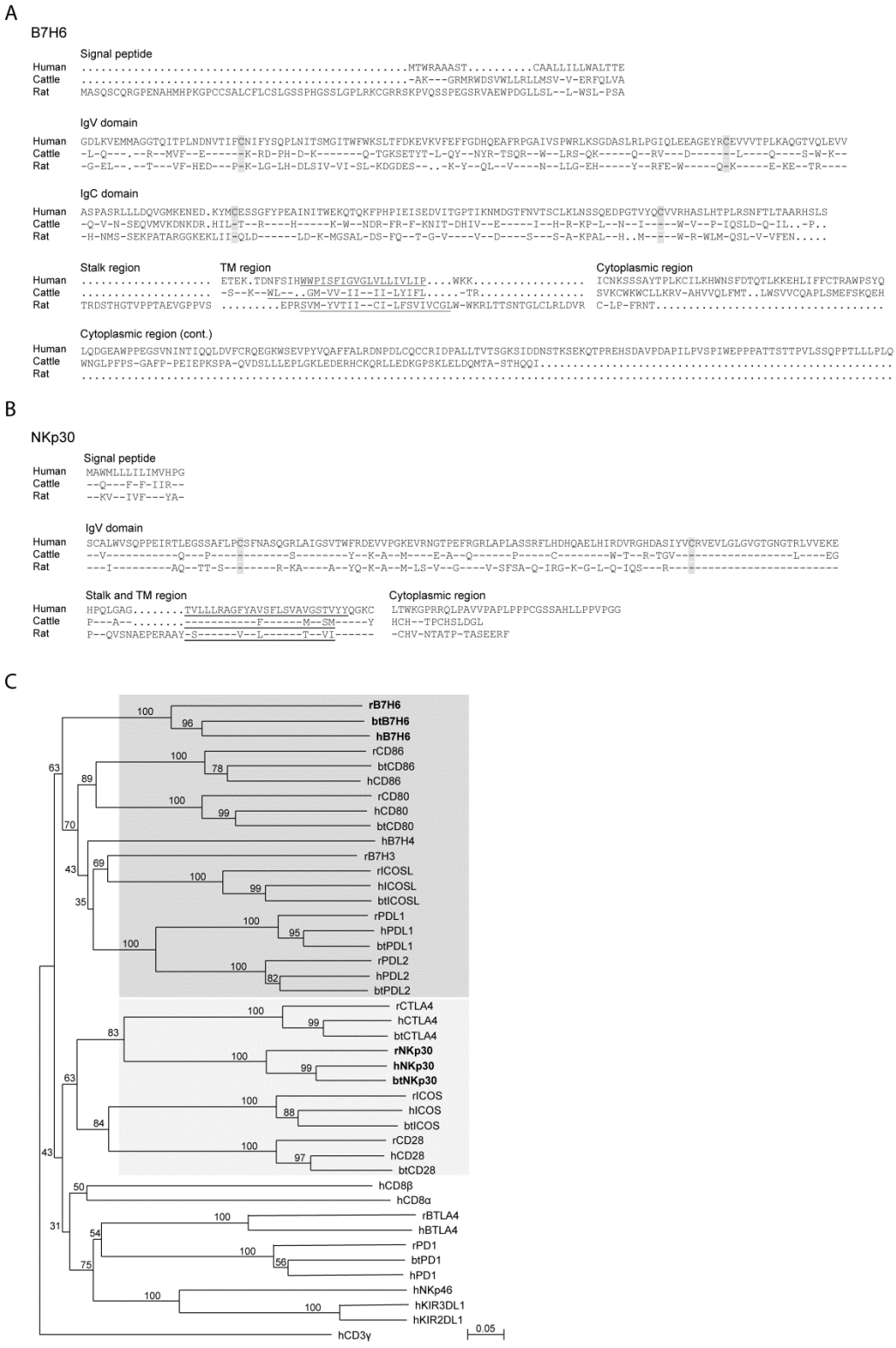
627 **Figure 4. B7H6 is widely expressed by human cancer cell lines and activates NKp30**
628 **reporter cells.** (A) Histograms (right) show B7H6 surface expression (solid line) on the
629 indicated 21 different human cancer cell lines and 293T cells as assessed by flow cytometry
630 using an anti-hB7H6 mAb. The shaded area represents isotype control. Dot plots (left) show
631 EGFP production by reporter cells expressing the extracellular domain of human after
632 overnight incubation with target cell lines. The percentage of $EGFP^+$ reporter cells is
633 indicated in the upper right corner. The plots shown are representative of at least four
634 individual experiments. (B) Linear regression analysis correlating surface expression (MFI) of

B7H6 by cancer cell lines with degree of NKp30 reporter cell activation (percentage of $EGFP^+$ reporter cells).

Figure 5. siRNA-mediated knockdown of B7H6 expression on cancer cells abolished NKp30 reporter cell responses. (A) Histograms (upper rows) of the indicated cell lines show B7H6 surface expression after transfection with control siRNA (solid line) or B7H6 siRNA (dashed line). Shaded area represents isotype control. Dot plots (lower rows) show EGFP production by NKp30 reporter cells after overnight incubation with target cells treated with indicated siRNA (control or B7H6). Results shown are representative of at least four individual experiments. (B) Linear regression analysis correlating surface expression (MFI) of B7H6 on cancer cell lines with degree of activation of NKp30 reporter cells. Filled diamonds: B7H6 siRNA; open circles: control siRNA.

Figure 6. B7H6 is not a ligand for the CD28 family member CTLA4. Reporter cells expressing human CTLA4 with an HA epitope tag (A) were incubated with $B7H6^{bright}$ 293T cells as targets overnight, and analyzed for EGFP expression by flow cytometry (B). As controls, reporter cells were incubated in plastic wells precoated with an anti-HA mAb or isotype control. The results shown are representative of at least three independent experiments.

654 **Figure 1**



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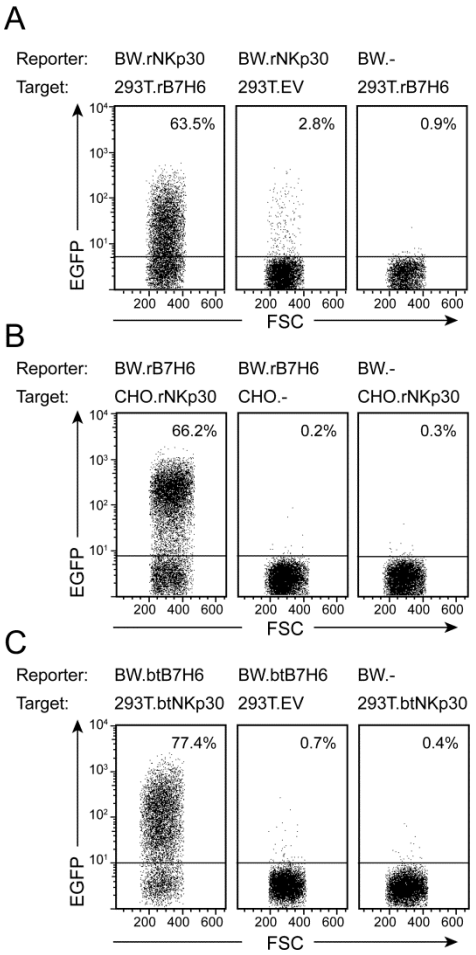
657 **Figure 2**



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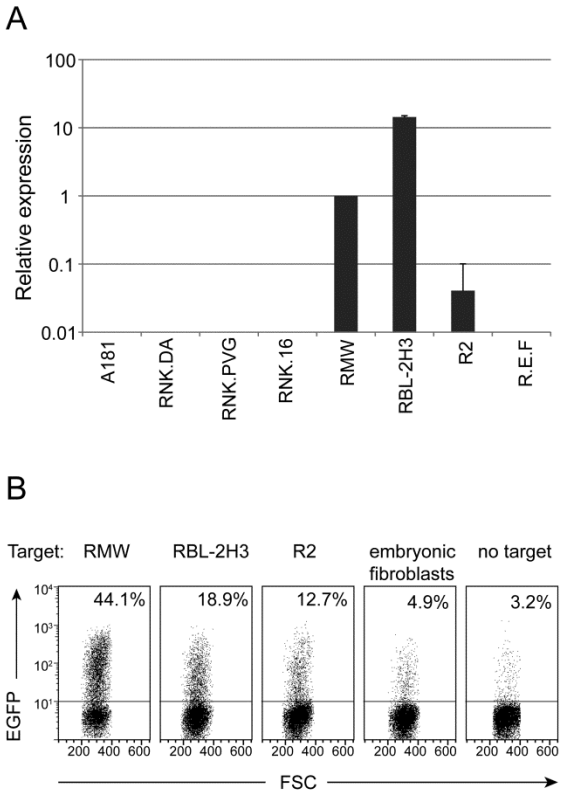

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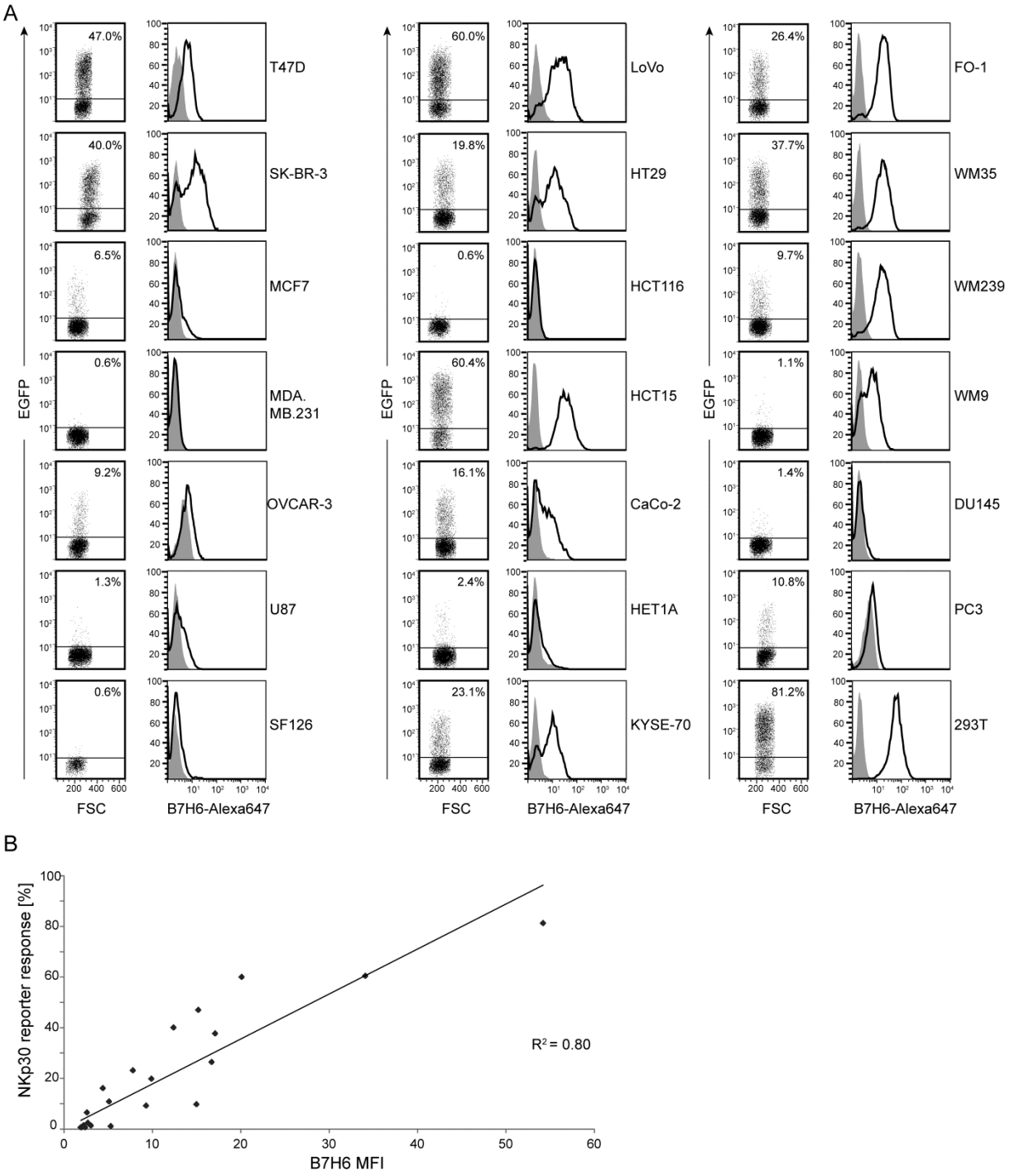
Figure 4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EGFP
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T47D
40.0%
SK-BR-3
6.5%
MCF7
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MDA.
MB.231
9.2%
OVCAR-3
1.3%
U87
0.6%
SF126
60.0%
LoVo
19.8%
HT29
0.6%
HCT116
60.4%
HCT15
16.1%
CaCo-2
2.4%
HET1A
23.1%
KYSE-70
26.4%
FO-1
37.7%
WM35
9.7%
WM239
1.1%
WM9
1.4%
DU145
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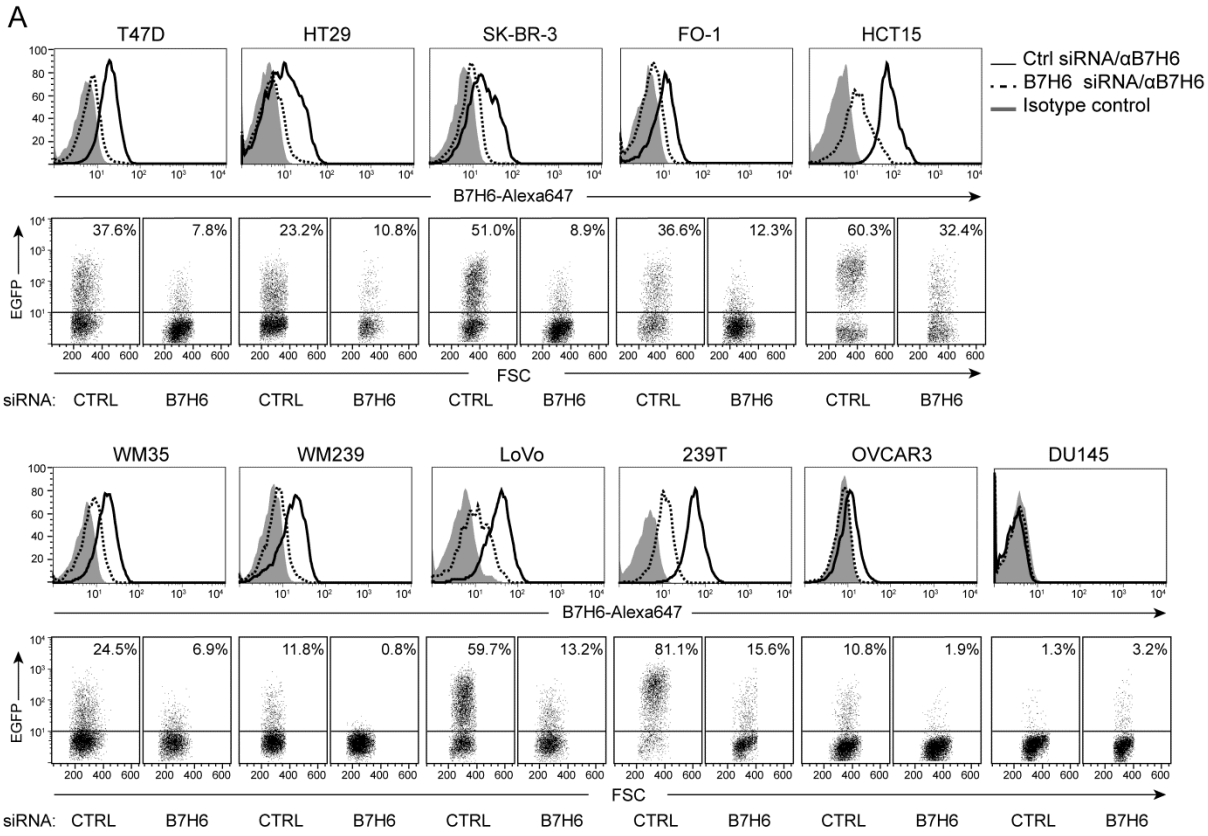
PC3
81.2%
293T
B
NKp30 reporter response [%]
B7H6 MFI
R² = 0.80

667 **Figure 5**



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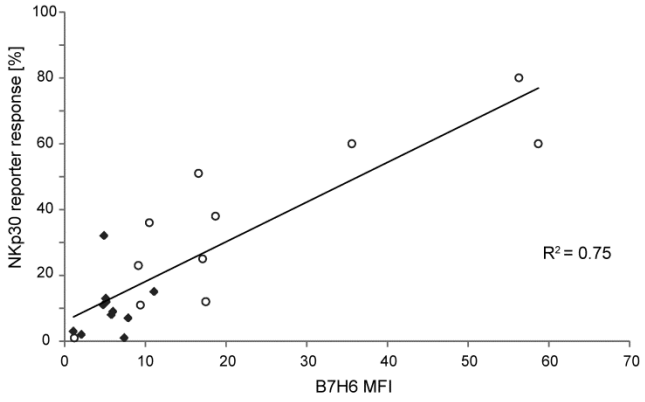

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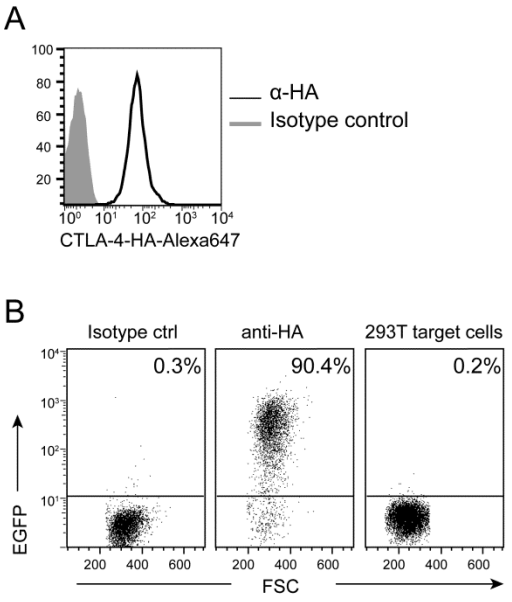
671 **Figure 6**



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