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MARCH Proteins Mediate Responses to Anti-tumor Antibodies

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Abstract

CD98, which is required for the rapid proliferation of both normal and cancer cells, and MET, the hepatocyte growth factor receptor, are potential targets for therapeutic anti-tumor antibodies. Here we report that the anti-proliferative activity of a prototype anti-CD98 antibody, UM7F8, is due to antibody-induced Membrane-Associated Ring CH (MARCH) E3 ubiquitin ligase-mediated ubiquitination and downregulation of cell surface CD98. MARCH1-mediated ubiquitination of CD98 is required for UM7F8's capacity to reduce CD98 surface expression and its capacity to inhibit the proliferation of murine T cells. Similarly, CD98 ubiquitination is required for UM7F8's capacity to block the colony forming ability of murine leukemia initiating cells. To test the potential generality of the paradigm that MARCH E3 ligases can mediate the anti-proliferative response to anti-tumor antibodies, we examined the potential effects of MARCH proteins on responses to Emibetuzumab, an anti-MET antibody currently in clinical trials for various cancers. We report that MET surface expression is reduced by MARCH1, 4, or 8-mediated ubiquitination and that Emibetuzumab-induced MET ubiquitination contributes to its capacity to downregulate MET and inhibit human tumor cell proliferation. Thus, MARCH E3 ligases can act as co-factors for anti-tumor antibodies that target cell surface proteins, suggesting that the MARCH protein repertoire of cells is a determinant of their response to such antibodies.

Keywords

MARCH; Ubiquitin; CD98; MET; therapeutic antibodies

Introduction

Cell surface proteins are the targets of many anti-tumor antibodies. For example, the type II transmembrane protein CD98 (CD98hc, SLC3A2) enables rapid proliferation and clonal expansion of cellular populations by supporting integrin signaling and amino acid transport (1–3), the latter through di-sulfide linkage to a number of amino acid transporters(4). CD98 expression correlates with poor prognosis in many cancers and modest changes in CD98

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expression can impact rapid proliferation(4). CD98 is also important in tumorigenesis. For example, Ras-driven skin tumors depend on CD98 expression because CD98 regulates integrin mechanotransduction(5), in part by altering sphingolipid metabolism(6). Indeed, antibodies targeting human CD98 can inhibit a variety of tumors(7, 8) and Acute Myelogenous Leukemia (AML) colony forming cells(9). Thus, CD98 represents a potential target for tumor suppressing antibodies.

MET/Hepatocyte growth factor receptor/C-MET is also a potential target for antibody-based therapeutics, although toxicities have limited some such antibodies(10). MET promotes cancer growth in many cancer types and upregulation of MET surface expression can lead to ligand-independent signaling and resulting proliferation(11, 12). Emibetuzumab, a humanized monoclonal anti-MET antibody, which is currently in PhaseII/PhaseIII clinical trials, inhibits MET by inducing lysosomal accumulation of MET. Emibetuzumab must be bivalent to neutralize MET function and works in a kinase independent manner(13). Whereas ligand-induced downregulation of MET surface expression is well appreciated, little is known about how MET surface expression is regulated by Emibetuzumab in the absence of ligand. In particular, there is limited insight into the molecular mechanisms whereby Emibetuzumab promotes internalization and lysosomal targeting of MET without eliciting agonist activity.

The Membrane Associated RING-CH (MARCH) family proteins are a novel class of transmembrane E3 ubiquitin ligases that regulate stability of transmembrane protein substrates. MARCH family members ubiquitinate membrane proximal lysine residues resulting in lysosomal degradation(14–17). In general, transmembrane receptors are substrates of a subset of MARCH family members; for example, CD98 is a substrate of MARCH1 and MARCH8(14, 16, 18). In addition to biosynthetic regulation of CD98 expression, we previously reported that MARCH1-mediated ubiquitination of CD98 limits expression of endogenous CD98 and clonal expansion of T cells(16). The growing list of MARCH substrates indicates that MARCH proteins can influence a wide range of biological processes through ubiquitination of transmembrane receptors including CD98, CD44, MHC class I and II, CD86, CD25, ICAM, TRAILR1/2 and the Insulin receptor (14, 19–25). MARCH8 can also restrict HIV replication by directed ubiquitination of the ENV protein suggesting MARCH8 also plays a role in innate immunity(25). Thus, MARCH family members can regulate many cellular processes through targeted ubiquitination and lysosomal degradation of transmembrane receptors.

A subset of antibodies targeting CD98 limit cell proliferation, and this effect is enhanced by crosslinking the CD98 antibody (7, 9). MARCH1/8 over-expression lead to ubiquitination of CD98 membrane-proximal cytoplasmic lysines leading to CD98 degradation (16). We hypothesized that MARCH-mediated ubiquitination and downregulation of CD98 was linked to the anti-proliferative effects of anti-CD98. Here we report an anti-human CD98, clone UM7F8 (26)., that blocks cell proliferation induces ubiquitination and downregulation of CD98 thereby limiting cell proliferation. Blocking ubiquitination inhibits the capacity of UM7F8 to suppress antigen-driven proliferation of T cells and of the colony-forming ability of AML initiating cells,. We tested the paradigm that MARCH-mediated ubiquitination could contribute to the downregulation of other cancer-

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relevant targets by studying Emibetuzumab-induced downregulation of MET and inhibition of tumor cell proliferation. We report that MET surface expression is regulated by MARCH1/4/8-mediated ubiquitination of conserved membrane proximal lysine residues and that Emibetuzumab induces ubiquitination of these lysine residues resulting in MET downregulation and inhibition of tumor cell proliferation. Thus, MARCH proteins are co-factors that can dictate responses to therapeutic antibodies against transmembrane receptors.

Materials and Methods

Animals, Cell Lines and Reagents

Slc3a2 $^{fl/fl}$, *Rosa26* $^{CRE-ERT2}$ mice were described previously(9). *Slc3a2* $^{f/f}$, *CD4 CRE*+, *OT1*+, mice were described previously(2). *March1* $^{-/-}$ mice were provided by Satoshi Ishido (27). *March1* $^{-/-}$ mice were bred onto the *OT1* background using animals obtained from Jackson labs. *March1* $^{-/wt}$ (heterozygous) mice on the *OT1* background were crossed to generate *March1* $^{-/-}$, *OT1* donor mice and *March1* $^{wt/wt}$, *OT1* control donors. All animals were housed at the University of California, San Diego animal facility and all experiments were approved by the Institutional Animal Care and Use Committee. RAMOS, HeLa-CCL2 and Jurkat cells were obtained from ATCC. HeLa Flp-In-TREX cells were a generous gift from Anne-Claude Gingras(28). 293T-LX cells were purchased from Clontech. MKN45 cells were a gift from Sheila Crowe and authenticated as MKN45 by IDEXX. MKN45, RAMOS, Jurkat and primary T cells were maintained in complete RPMI media (Gibco) + 10% FBS (Sigma), 1x L-glutamine (Gibco), 1x Penicillin/Streptomycin (Gibco), 1x Beta-mercapto ethanol (Thermo). 293T-LX, HeLa and HeLa Flp-In Trex cells were maintained in 1xDMEM (Corning), 10% FBS (Sigma), 1x L-glutamine (Gibco), 1x Penicillin/Streptomycin (Gibco). Upon revival cell lines were verified as negative for *Mycoplasma species* using the mycoplasma detection kit (BioTool). All cell lines were maintained continuously for a maximum of six weeks. Ammonium chloride and doxycycline were obtained from Sigma, ProteinG sepharose was purchased from GE. CFSE was purchased from Biolegend. Methocult media was purchased from StemCell Technologies.

Antibodies

Anti-CD98 clone UM7F8, mIgG1 control antibody clone MOPC21, anti-mouse cKit clone ACK45 and anti-human NGFR clone C40–1457 were purchased from BD. Anti-Mouse CD90.1 Clone OX-7, anti-CD98 clone MEM108, and anti-mouse CD98 Clone RL388, were purchased from Biolegend. Anti-CD98 H300 was purchased from Santa Cruz Biotechnology. Anti-Ubiquitin clone P4D1 and anti-Met clone L41G3 were purchased from CST. Goat Anti-human IgG F(ab')2, Goat Anti-mouse IgG F(ab')2, and Goat anti-mouse IgG F(ab') were purchased from Jackson. Humanized anti-human MET (LY2875358/Emibetuzumab) and non-cross competing MET antibody (LSN2148068) have been described previously and were provided by Eli Lilly(13). LSN2148068 was conjugated to DyLight650 using a DyLight microscale conjugation kit (Life Technologies) according to the manufacturer's directions. Secondary reagents for western blot (Goat anti-mouse IgG-800, Goat anti-mouse IgG-680, Goat anti-rabbit IgG-800 and goat anti-rabbit IgG-680 were obtained from Licor.

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Plasmids and DNA Manipulations.

R777-E131 Hs.MET was a gift from Dominic Esposito (Addgene plasmid # 70415). pSpCas9(BB)-2A-Puro (PX459) was a gift from Feng Zhang (Addgene plasmid # 48139). pMSCV-MLL_AF9-IRES-htNGFR was kindly provided by Scott Armstrong. pMSCV-NRAS^{G12V}-IRES-YFP was kindly provided by Scott Lowe(29). All plasmids were constructed by PCR amplification of insert sequences and cloning into target vectors using InFusion cloning (Clontech). Retroviral and lentiviral plasmids were maintained in STBL3 *E. Coli* (Life Technologies), other expression vectors were maintained in Stellar *E. Coli* (Clontech). All plasmids were verified by direct sequencing. The following vectors were engineered for this manuscript: pcDNA5FRT-MET, pcDNA5FRT-MET_4KR, pcDNA5FRT-MET-KD, pcDNA5FRT-MET_4KR_KD were generated by site directed mutagenesis using R777-E131 Hs.MET as template. MET with c-terminal HA tags were amplified by PCR using the vectors described above and cloned into pcDNA3.1 hygro. pcDNA3.1-Puro-MET_EGFP and pcDNA3.1-Puro-MET_4KR-EGFP were cloned by amplification of tagless MET and MET_4KR and fused to EGFP by infusion cloning. All other DNA constructs have been described previously(16). Plasmid maps and primer sequences are available upon request.

Antibody Treatments

Ramos and HeLa cells were treated for 24 hours with the indicated cocktails of antibody. When treated in combination with crosslinking, antibodies and cross linker were premixed before adding to cells. 24 hours post-treatment, cells were enumerated by flow cytometry as described previously(16). Ubiquitination assays for CD98 and MET were performed as described previously(16). Plasmid vectors expressing kinase dead (Y1234A/Y1235A) MET and kinase dead MET_4KR were used to transiently transfect HeLa cells for MET ubiquitination assays.

Transduction and Manipulation of Mouse T cells

Retrogenic bone marrow Chimeras were performed as described previously(16). For CD98 downregulation assays in T cells, splenocytes from *March1*$^{-/-}$, *OT1* or *March1*$^{wt/wt}$, *OT1* littermate control mice were stimulated with 1nM cognate *OT1* peptide (SIINFEKL) and 10U/ml of recombinant human IL-2 (NCI Frederick) for 48 hours. CD8 T cells were purified using the Untouched mouse T cell kit (Thermo), washed and re-plated with fresh IL-2. T cells were infected with the indicated retroviral vectors encoding human CD98 by spinocculation for 90min at 1800rpm. 24 hours post-infection cells were treated with anti-human CD98 or control IgG and crosslinked with anti-mouse IgG F(ab’)2. 24 hours post treatment (48 hours post infection) surface expression of CD98 was determined by flow cytometry with a non-cross competing CD98 antibody (clone MEM108).

Generation of Human CD98 Expressing MLL

MLL_AF9/NRAS mosaic MLL was generated as described previously using *Slc3a2* $^{fl/fl}$, *Rosa26*$^{CRE-ERT2}$ donor mice(9) with the following modifications: Established MLL was infected with MSCV-IRES-GFP retroviral vectors encoding either 8KR CD98, CD98 or empty vector control. At this point cells were also treated overnight with 4-hydroxy

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tamoxifen (Sigma). GFP+/YFP+/c-Kit+ AML cells were sorted and used for colony formation assays in the presence of anti-CD98 UM7F8 as described previously(9).

MARCH-Mediated Downregulation of MET

HeLa cells were transfected as described previously(16) and surface expression of MET was determined using an anti-MET antibody LSN2148068 by flow cytometry.

MET KO cell lines

MET was targeted in HeLa cells or HeLa Flp-In TREX cells by transfection with pSpCAS9–2A-Puro encoding a guide RNA (5’- GGTGTTTCCGCGGTGAAGTT –3’) targeting residue 335 to 354 of NM000245.3. 24 hours post-transfection cells were selected transiently on 2μg/ml of puromycin. After 24 hours in puromycin, the drug was washed away and cells were monitored for a population of MET negative cells. After five days in culture, MET KO cells were purified by depleting MET expressing cells with MET antibody (LY2875358). MET KO HeLa and HeLa FlpIn TREX cell pools were validated by flow cytometry and western blot. MET KO HeLa-FlpIn TREX cells were rescued with either wt MET or 4KR-MET by transfection with a rescue vector (pcDNA5FRT-MET, pcDNA5FRT-MET_4KR, pcDNA5FRT-MET-KD or pcDNA5FRT-MET_4KR_KD) and pOG44 in a 10:1 ratio. Isogenic cells with a single integrated copy of the rescue vector were selected on 200μg/ml of Hygromycin for two weeks. For characterization see Supplementary Figure S1.

RNAi

siRNAs targeting human MARCH8 (Smartpool) or non-targeting pools of up to five siRNAs were purchased from Dharmacon. MKN45 cells were transfected with Dharmafect4 according to the manufacturer’s directions. Knockdown efficiency was determined by qPCR of MARCH8 transcripts relative to GAPDH.

In vitro proliferation Assay

MKN45 cells were transiently transfected with either pcDNA3.1-Puro-MET_EGFP or pcDNA3.1-Puro-MET_4KR_EGFP using lipofectamine3000 according to the manufacturer’s directions at a ratio of 1μg of DNA to 3μl of lipofectamine3000. At 24 hours post-transfection cells were selected in 2μg/ml of puromycin for 24 hours. At 48 hours post-transfection, cells were split and sorted for GFP+ (transfected) cells. Recovered cells were plated at a density of 500 cells/well in a 96 well plate. Cells were treated with Emibetuzumab or control IgG with or without crosslinking as described above. At 96 hours post-treatment, viable cell number was determined by Celltiter96 Aqueous assay (Promgea). Relative cell number for each condition was determined relative to untreated control.

Depmap Analysis

MET dependency (CERES score, x-axis) versus MARCH8 expression (TPMlog2, y-axis) for 650 cancer cell lines was accessed from the depmap database (https://depmap.org/portal/), current as of July 22nd 2019. The search parameters used were as follows: 1) X-axis: Gene: MET(RCCP, HGFR, DFNB97), dataset: Combined RNAi (Broad, Novartis Marcotte). 2) Y-axis: Gene: MARCH8 (CMIR, MIR, c-MIR, MARCH-VIII,

RNF178), dataset: Expression Public 19Q2). MKN45, EBC1, SNU5 and U87MG cells were highlighted using the cell line highlighter function.

Results

Anti-CD98 Limits Lymphoma cell proliferation and downregulates cell surface CD98.

Treatment with an anti-CD98, UM7F8, limits proliferation of Ramos human Burkitt Lymphoma cells and, as we previously reported (7), F(ab')2-induced crosslinking of UM7F8 further reduced cell number (Figure1A). Because CD98 expression is required for proliferation of lymphocytes and other cells (1–3), we examined the effect of UM7F8 on surface expression of CD98. Treatment with UM7F8 slightly reduced expression of cell surface CD98 and crosslinking of UM7F8 with anti-IgG F(ab')2 greatly increased this downregulation (Figure1B, 1C). At a saturating dose, treatment with UM7F8 reduced surface expression of CD98 by more than 60% (Figure1C).

Anti-CD98 (UM7F8) acts by inducing MARCH1-Mediated ubiquitination of CD98.

MARCH1 and MARCH8 ubiquitinate CD98 on membrane proximal lysine residues leading to subsequent lysosomal degradation(16, 30). Treatment of Jurkat or Ramos cells with UM7F8 followed by crosslinking induced robust ubiquitination of endogenous CD98 (Figure1D). The smeared band representing ubiquitinated CD98 on SDS gels is consistent with combinations of mono-ubiquitin modifications of the 8 cytoplasmic lysines previously reported by us and others(16, 30). A mutant of CD98 in which eight membrane proximal lysine residues are substituted for arginine (8KR) is resistant to ubiquitination by MARCH1 or MARCH8, thus preventing subsequent downregulation(16). We used this mutant of CD98 to ask whether UM7F8-induced downregulation of CD98 was mediated by ubiquitination at these sites. Crosslinked UM7F8 failed to induce ubiquitination of 8KR-CD98 in Jurkat cells, whereas ubiquitination of wild type CD98 was readily detectable (Figure1E). Accordingly, in transduced mouse OT1 T cells expressing human CD98, crosslinked UM7F8 failed to reduce expression of 8KR-CD98 in sharp contrast to wild type CD98 whose expression was reduced by >30%(Figure1F). Therefore, UM7F8 induces downregulation of CD98 through ubiquitination of cytoplasmic lysine residues.

CD98 expression is regulated by MARCH1-mediated ubiquitination in mouse T cells(16). To assess whether MARCH1 is an E3 ubiquitin ligase responsible for UM7F8-induced downregulation of CD98, we transduced *March1*$^{-/-}$ or *March1*$^{+/+}$ littermate mouse T cells with human CD98. Crosslinked UM7F8 did not significantly change expression of human CD98 in *March1* $^{-/-}$ T cells, whereas human CD98 was significantly reduced by ~25% in *March1*$^{+/+}$ T cells (Figure1G). Taken together, these data show that crosslinked UM7F8 induces MARCH1-mediated ubiquitination of CD98 resulting in reduced CD98 expression.

Anti-CD98 (UM7F8) induced CD98 ubiquitination limits T cell proliferation.

CD98 enables the clonal expansion essential for adaptive immunity(4) yet is dispensable for homeostatic proliferation(2). UM7F8 reduced expression of CD98 (Figure 1B,C) and inhibited T cell proliferation(26); thus, we hypothesized that UM7F8-induced downregulation of CD98 was responsible for its capacity to inhibit T cell proliferation.

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Because UM7F8 is specific for human CD98, we used retrogenic bone marrow reconstitution to generate naïve mouse T cells dependent upon human CD98 by virtue of deletion of the endogenous mouse CD98 alleles and ectopic expression of human CD98(2, 16). We used this retrogenic approach to generate OT1 T cells dependent upon either 8KR or wild type human CD98 for clonal expansion(Figure2A). Naïve, mouse-CD98 null, human-CD98+, OT1 T cells were purified from the spleens of transplanted recipient mice (Figure2B), stimulated with cognate peptide antigen (SIINFEKL) and then treated with UM7F8 or IgG control. UM7F8 reduced proliferation of human CD98-bearing T cells to a level similar to that seen in CD98 null T cells. In sharp contrast, the proliferation of human 8KR CD98-bearing T cells was unaffected by UM7F8(Figure2C). Quantification of these effects revealed that UM7F8 treatment had no detectable effect on the proliferation index of 8KR CD98-bearing T cells, yet the proliferation index of human CD98-bearing T cells was profoundly reduced (Figure2D). UM7F8 did not affect wild type OT1 T cells, which lack human CD98 (Figure2C). Therefore, UM7F8 inhibition of T cell proliferation depends on its capacity to induce ubiquitination of CD98 on cytoplasmic lysine residues and subsequent downregulation.

CD98 also enables proliferation of a wide variety of cancer cells(3, 5, 9) such as in a murine model of Acute Myelogenous Leukemia (AML)(9). We employed this model to ask if UM7F8 could limit clonal expansion in the context of cancer initiating cells. We replaced the endogenous mouse CD98 with retrovirally expressed human CD98. Hematopoietic stem cells from inducible CD98 knockout mice (*Slc3a2*$^{f/f}$, *Rosa26*$^{CRE\text{-}ERT2}$) were transformed by retroviral transduction with an MLL-AF9 fusion and activated NRAS to generate AML initiating cells(29). These cells were amplified by passage through congenic recipient mice. When the congenic recipient animals showed signs of AML, the secondary leukemic cells were isolated from the spleen. Sorted *Slc3a2*$^{f/f}$, *Rosa26*$^{CRE\text{-}ERT2}$ AML initiating cells (NGFR+, YFP+) were transduced with retroviruses encoding a human CD98 resistant to MARCH1/8-mediated ubiquitination (8KR), human CD98 control, or empty vector and treated with 4 hydroxy-tamoxifen to inactivate the mouse CD98 alleles. Sorted GFP+ AML initiating cells were tested for clonal expansion *in vitro* upon anti-CD98 or IgG treatment (Figure3A). Treatment of 8KR AML initiating cells with UM7F8 had no detectable effect on clonal expansion compared to IgG control (Figure3B). In sharp contrast, UM7F8 treatment reduced colony formation of human CD98 AML initiating cells by more than 50% (Figure3B). To verify that the resistance to UM7F8 was not due to inefficient inactivation of the endogenous mouse CD98, cells were removed from methyl cellulose after counting and CD98 expression was assessed by flow cytometry. Both human 8KR CD98 and wild type human CD98-bearing AML initiating cells were negative for endogenous mouse CD98 (Figure3C). Therefore, anti-CD98 (UM7F8) limits proliferation of normal and cancer cells by inhibiting CD98 function through MARCH-mediated ubiquitination and downregulation.

MET is a MARCH1/4/8 substrate and regulated via ubiquitination of membrane proximal lysine residues.

The foregoing studies showed that the capacity of this anti-CD98 to inhibit proliferation of T cells and AML colony formation was dependent on antibody-induced MARCH-mediated ubiquitination and resulting downregulation of the antibody's target. The effect of other

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anti-tumor antibodies could be influenced by downregulation of their target antigens. To test this idea, we examined the effect of antibody-induced ubiquitination of MET on its downregulation by Emibetuzumab, an anti-MET antibody that promotes MET lysosomal degradation (13). To determine if MET was a MARCH substrate, we first screened for MARCH family members that induce downregulation of endogenous MET by transient over expression in HeLa cells. MET was downregulated by MARCH1, MARCH4 and MARCH8 (Figure4A). We did not detect downregulation of endogenous MET by ectopic expression of MARCH2,3,5,6,7, and 10 (data not shown). Catalytically inactive MARCH8 was unable to downregulate MET, indicating MET downregulation requires MARCH8-mediated ubiquitination (Figure4A). Analysis of MET downregulation as a function of MARCH1/4/8 expression revealed that these three MARCH family members downregulate MET with equal efficiency (Figure4B). MET and CD98 are regulated by distinct MARCH family members (Figure4C, D). MARCH4 downregulated MET but had no effect on CD98. In contrast MARCH1 and MARCH8 downregulated both CD98 and MET (Figure4C, D).

MARCH family members ubiquitinate membrane proximal lysine residues, targeting transmembrane proteins for lysosomal degradation(14, 16). Alignment of the transmembrane and membrane proximal cytoplasmic residues of mammalian MET proteins revealed four conserved membrane proximal lysine residues in human MET, (K976, K977, K979 and K982) (Figure4E). We generated a MET mutant (4KR-MET) wherein these four membrane proximal lysine residues were mutated to arginine. To test the role of these residues in MET regulation we generated MET-knockout HeLa Flp-In-TREX cells by CRISPR mediated genome editing (FigureS1). MET knockout cells were rescued by a single integration of Tet inducible, epitope-tagged 4KR-MET or MET. Induction of 4KR-MET and MET in isogenic HeLa lines was verified by western blot (FigureS1). 4KR-MET was resistant to MARCH4/8 mediated downregulation, suggesting that ubiquitination of these membrane proximal lysine residues targets MET for lysosomal degradation (Figure 4F). To further confirm that endogenous MET is regulated by MARCH family members, we used siRNAs to silence MARCH8 in MKN45 human gastric cancer cells, which lacked detectable MARCH1 and MARCH4 mRNA, and observed a 50% increase in MET surface expression (Figure4G,H,I). Taken together these data demonstrate that MET is a MARCH1/4/8 substrate and surface expression of MET is regulated by MARCH-mediated ubiquitination of membrane proximal lysine residues.

Emibetuzumab causes MARCH-mediated ubiquitination of MET.

Having established MET as a bona fide MARCH-substrate, we asked whether the effects of Emibetuzumab could be mediated by MARCH-dependent ubiquitination. Emibetuzumab efficiently downregulated endogenous MET from the surface of HeLa cells and this effect was increased by crosslinking (Figure5A). Emibetuzumab alone reduced MET surface expression by almost 60% and crosslinking increased downregulation to greater than 80% (Figure5B). To determine if the downregulation of MET by Emibetuzumab is mediated by MARCH family members, we utilized a mutant of MET resistant to MARCH1/4/8 induced downregulation, 4KR-MET. 4KR-MET was resistant to Emibetuzumab induced downregulation (Figure5C). 4KR-MET was only partially resistant to crosslinked Emibetuzumab induced downregulation (Figure5C). We hypothesized that

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4KR-MET, although resistant to MARCH1/4/8 mediated ubiquitination, could still be regulated by ligand induced autophosphorylation and degradation through c-cbl. Thus, we prepared a 4KR-kinase dead (4KR-KD) mutant of MET and tested it for downregulation with Emibetuzumab(31). 4KR-KD-MET was resistant to downregulation when cells were treated with Emibetuzumab under crosslinking conditions (Figure5D). We next confirmed Emibetuzumab induced ubiquitination of MET, whereas 4KR-MET was resistant to Emibetuzumab induced ubiquitination (Figure5E). We observed a significant ~15% reduction in Emibetuzumab-induced MET downregulation in MARCH8 silenced MKN45 cells (Figure5F). Finally, we asked if blocking ubiquitination of MET would render MKN45 cells resistant to Emibetuzumab's anti-proliferative activity. Emibetuzumab did not block the proliferation of MKN45 cells expressing 4KR-MET, whereas Emibetuzumab reduced proliferation of MKN45 cells expressing wild type MET (Figure5G). Thus, MARCH-mediated ubiquitination and degradation of MET contributes to the mechanism of action of Emibetuzumab.

Discussion

The work reported here provides new insight into the mechanism of action of antibodies against CD98 and MET, two membrane proteins that are potential therapeutic targets in a variety of cancers. CD98 enables rapid proliferation of cancer cells by supporting adhesive signaling and amino acid transport(1, 2, 5, 9, 32). Here we report that an antibody targeting human CD98 (UM7F8), previously shown to block proliferation, induces ubiquitination and downregulation of cell surface CD98, thereby inhibiting the proliferation of both AML initiating cells and T cells. The role of MARCH proteins in responses to anti-tumor antibodies is not limited to CD98 as established with our analysis of Emibetuzumab, an anti-MET antibody. We report that MET is a substrate for MARCH1/4/8 mediated ubiquitination and downregulation and that Emibetuzumab, which is currently in PhaseII/III clinical trials against non-small cell lung cancer and hepatocellular carcinoma, limits proliferation of tumor cells by promoting MARCH mediated-ubiquitination and degradation of MET. Although we cannot preclude the possibility that CD98 and/or MET were internalized and not degraded, removal from the cell surface is sufficient limit both CD98 and MET function(13, 16). Thus, MARCH proteins are cellular co-factors that can influence responses to antibodies against tumor cell transmembrane proteins.

MARCH proteins enforce anti-tumor antibody-mediated downregulation of membrane proteins. We found that the effects of both an anti-CD98 and Emibetuzumab can be mitigated by blocking MARCH-mediated ubiquitination. For example, mutation of target ubiquitin acceptor sites blocked the anti-proliferative effects of both antibodies. Importantly, mutation of membrane proximal lysines, the known targets of MARCH proteins was sufficient to inhibit ubiquitination, downregulation, and blockade of proliferation (14). The importance of the membrane proximal lysines points to the role of MARCH proteins rather than other, non-membrane-tethered, E3 ubiquitin ligases that could also lead to ubiquitination of more distal lysines. The role of the MARCH1 in UM7F8-mediated downregulation of CD98 was confirmed by the preservation of cell surface CD98 in UM7F8-treated *March1* $^{-/-}$ T cells. Similarly, silencing MARCH8 reduced the ability of Emibetuzumab to decrease the expression of MET in MKN45 Cells. Thus, MET is a novel

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MARCH substrate and MARCH proteins can therefore regulate both MET expression and the effect of a therapeutic anti-MET antibody. MARCH proteins can ubiquitinate other transmembrane protein targets, such as CD44 and MHCI and thus antibodies against these proteins may also lead to MARCH-mediated downregulation. As shown here, loss of MARCH protein-ubiquitation can reduce the effects of certain anti-tumor antibodies. In addition to MARCH1,4, and 8, other E3 ligases, such as C-Cbl, can ubiquitinate and downregulate transmembrane proteins. Indeed, as shown here, when Emibetuzamab is crosslinked, it induces kinase-dependent downregulation of c-MET, presumably via c-Cbl-induced ubiquitationation. Thus, the principle that anti-body-induced MARCH-dependent ubiqutination can lead to downregulation of the targets of anti-tumor antibodies may extend to other ubiquitin ligases..

Target oligomerization plays a role in antibody-induced MARCH-dependent downregulation of transmembrane receptors. We confirmed our previous work(7) that secondary crosslinking markedly potentiated anti-CD98 downregulation and bivalency is important in the capacity of Emibetuzumab to induce MET downregulation and growth inhibition(13). We note that F(ab')2-mediated crosslinking of Emibetuzumab resulted in a marked increase in downregulation even with the MET-4KR mutant. Because intense clustering of MET could induce autophosphorylation resulting in c-Cbl-mediated ubiquitination of membrane distal lysines leading to proteosomal degradation(33), we speculated that this could cause downregulation of MET-4KR. In support of this idea, a kinase-dead MET-4KR was completely resistant to crosslinked Emibetuzumab. Internalization and lysosomal degradation of MHCI, another MARCH1/4/8 substrate, is also enhanced by antibody crosslinking(34). Antibody crosslinking may trigger surface protein quality control mechanisms through conformational changes and recruitment to microdomains that enforce interactions with MARCH family members(34, 35). In stark contrast, monovalent antibodies targeting CD98 can bypass the lysosomal degradation pathway enabling efficient cycling from luminal to apical surfaces of brain microvasculature endothelial cells, effectively crossing the blood-brain barrier(36). These data support the notion that antibody-induced target oligomerization leads to MARCH dependent degradation and other means of inducing receptor clustering may have a similar effect.

Our studies provide new insights into the mechanism of action of two anti-tumor antibodies. Anti-CD98 antibodies have long been known to inhibit cell proliferation(8, 26, 37); however their mechanism of action has not been established. Indeed, the capacity of CD98 to support proliferation is dependent on two functions: 1) Its ability to support integrin signaling(2, 3) and 2) its ability to form a disulfide-linked heterodimer with light chains that enable support of amino acid transport(38). Antibodies to the extracellular domain of CD98 would not have access to the transmembrane and cytoplasmic domains that are necessary and sufficient for support of integrin signaling(39), nor would they have access to the endoplasmic reticulum where the disulfide-bonded heterodimers form(40). The present work shows that CD98 antibodies can inhibit proliferation by inducing downregulation of CD98. Similarly, many MET antibodies have agonist function that results in autophosphorylation and Cbl-mediated downregulation(10). In contrast, Emibetuzumab can induce degradation of MET without inducing autophosphorylation(13). We show that Emibetuzumab initiates an additional downregulation pathway, MARCH-mediated ubiquitination of MET, that

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limits MET expression and therefore proliferation of tumor cells. Thus, MARCH-mediated ubiquitination is a novel mechanism whereby anti-tumor antibodies can cause degradation of their membrane protein targets.

The MARCH protein repertoire can dictate responses to anti-tumor antibodies. Consistent with other MARCH substrates, both MET and CD98 are regulated by a specific subset of MARCH family members (15, 16, 23, 41–44). Here we report that MARCH-mediated ubiquitination of membrane proximal lysines is required for the anti-proliferative effects of these antibodies. MKN45, SNU5, U87MG, and EBC-1 tumor cells are MET-dependent and express MARCH8 as reported in Depmap, as of August 2019 (45). Treatment with Emibetuzumab reduces MET expression by ~50% and limits xenograft tumors derived from these cell lines(13). These Emibetuzumab sensitive cell lines were dependent on MET in an RNAi selection screen also reported in Depmap indicating that MARCH8 expression and MET dependency together may mark a tumor as a candidate for sensitivity to Emibetuzumab. e This paradigm of MARCH-mediated down regulation may apply to other tumor cell transmembrane antigens that serve as antibody targets for inhibition of cell proliferation or induction of cell death. Conversely, antibody binding to tumor cells can also serve to mark those cells for antibody-dependent cytotoxicity or complement–mediated lysis; in this instance MARCH dependent downregulation of the antibody and its target could oppose the therapeutic response. Because membrane proteins differ in susceptibility to each MARCH protein, the repertoire of MARCH proteins expressed by a particular cancer can be a determinant of the therapeutic response to certain anti-tumor antibodies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key Points:

- MARCH E3 ubiquitin ligases regulate responses to anti-tumor antibodies
- The tumor's MARCH protein repertoire may determine sensitivity to those antibodies.

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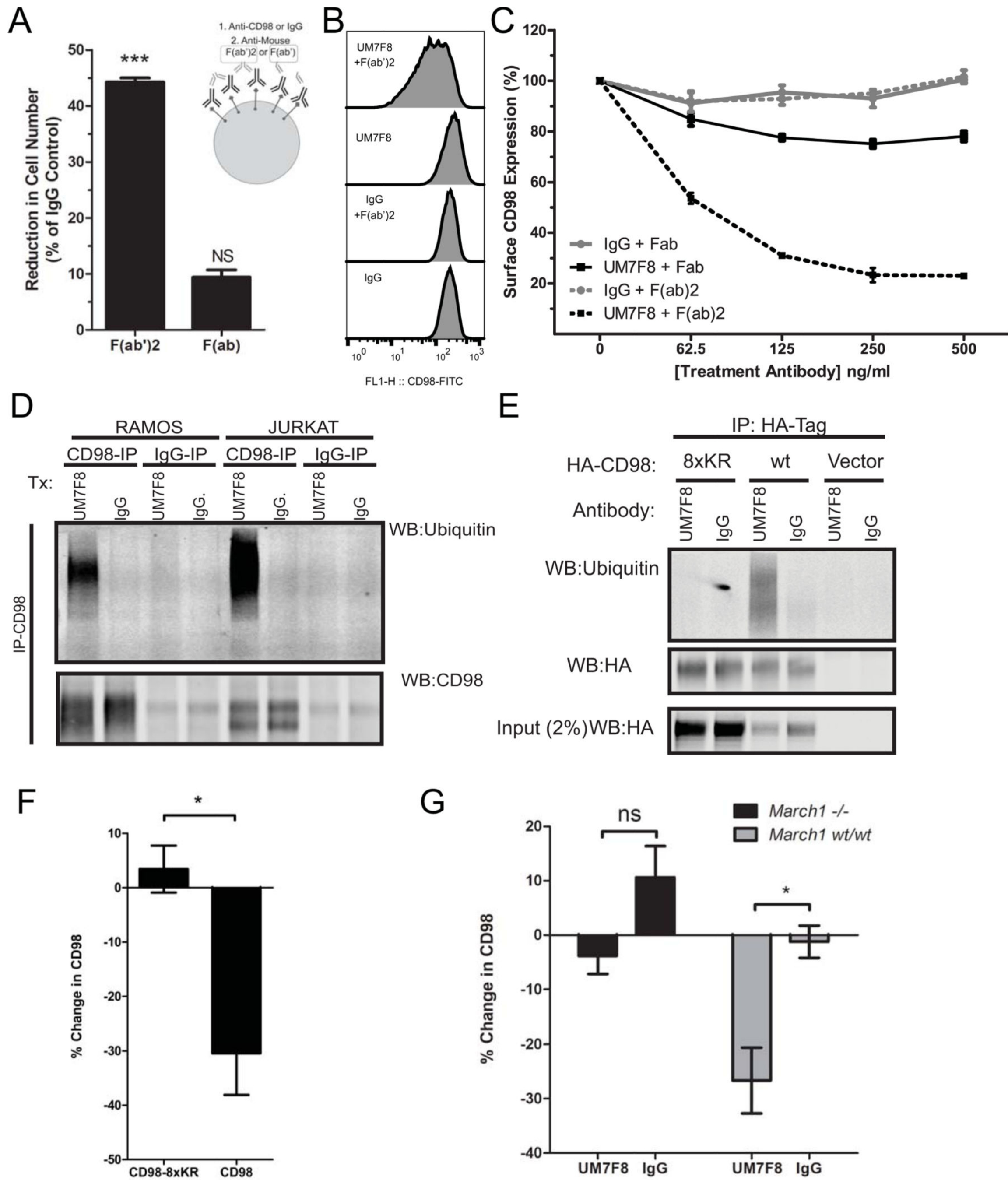

Figure 1: Anti-CD98 limits proliferation by MARCH1 mediated ubiquitination and downregulation of CD98.

(A) Inset: Schematic of antibody treatment regimen. Cells are treated 1) with either anti-CD98 (Clone UM7F8) or isotype control (IgG) and 2) with crosslinking anti-mouse IgG F(ab')2 or non-crosslinking anti-mouse IgG F(ab'). The mean change in cell number relative to IgG control is depicted ± SEM from two experiments performed in triplicate of RAMOS cells treated as outlined above. (B) Histograms depicting surface expression of CD98 on RAMOS cells treated as in (A). (C) Dose response of UM7F8 downregulation of surface CD98. RAMOS cells were treated with either clone UM7F8 or Control IgG in combination

with F(ab')2 or F(ab'). Surface expression of CD98 is depicted ± SEM from three independent experiments. (D) Endogenous CD98 immunoprecipitated from RAMOS and Jurkat cells treated with either UM7F8 or IgG control and in the presence of F(ab')2 was assayed for recovered CD98 or covalent modification with ubiquitin by immunoblotting. (E) Jurkat cells stably expressing HA-tagged 8KR-CD98, a mutant of CD98 resistant to ubiquitination on cytoplasmic K residues (8KR), or its wild type counterpart (wt) were treated as in (D) and HA-tagged CD98 8KR or *wt* was immunoprecipitated and probed for recovered HA-tagged CD98 or covalent modification with ubiquitin by immunoblotting. (F) OT1 T cells were stimulated with cognate peptide (SIINFEKL) antigen and were then transduced with retroviruses encoding human CD98 and a Thy1.1 marker. Transduced cells were treated with UM7F8 or IgG as in (D) and surface expression of human CD98 was detected by flow cytometry. The mean percent change in surface expression of ectopically expressed human CD98 relative to IgG control from three independent experiments (n=3 donor mice) is depicted ± SEM. (G) Primary T cells from *March1* $^{-/-}$ mice or *March1* $^{+/+}$ littermate controls were stimulated, transduced with human CD98 and treated with anti-CD98 or control as in (F). The mean percent change in human CD98 expression relative to untreated cells ± SEM from three independent experiments (N=3 littermate pairs) is depicted. *** denotes $P < 0.001$ by one way ANOVA with Bonferroni's posttest, NS denotes not significant. * denotes $P < 0.05$ by Student's T test, NS denotes not significant. Raw data appear in Supplemental Figure S2.

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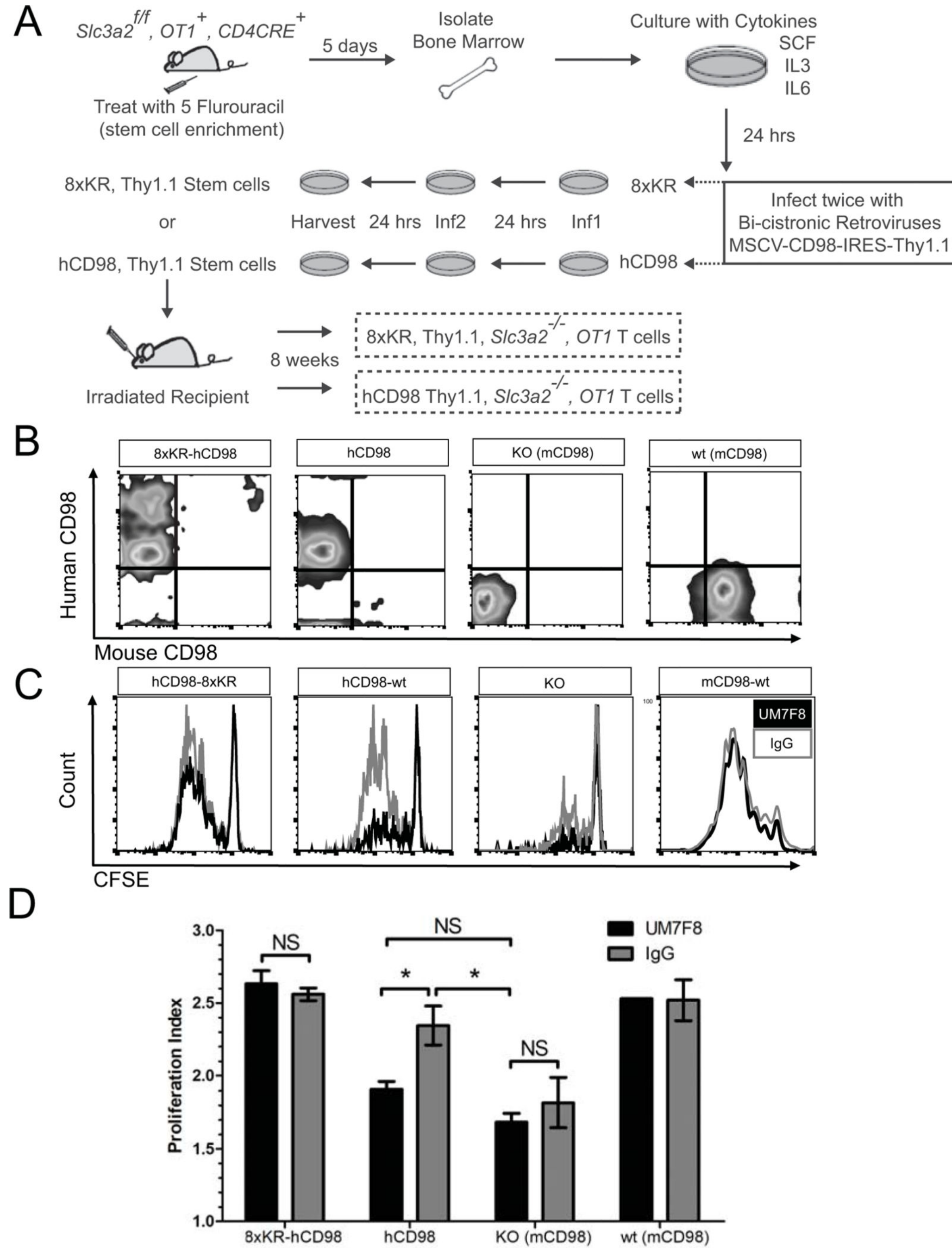

Figure 2: Anti-CD98 blocks CD98-dependent proliferation of T cells by promoting CD98 ubiquitination.

(A) Schematic of retrogenic derivation of OT1 T cells with customized human CD98 genotypes. Briefly, donor OT1 mice with conditional knockout of endogenous mouse CD98 in all T cells (*Slc3a2 f/f*, *CD4 CRE+*, *OT1+*) were treated with 5-Flurouracil to deplete cycling cells enriching hematopoietic stem cells. Five days after 5FU treatment bone marrow was isolated and cultured in the presence of cytokines to support stem cell cycling (SCF, IL-3, IL6). After 24 hours in culture, cells were transduced with retroviruses expressing either 8KR or wt human CD98 with a Thy1.1 surrogate marker. Transduced cells were

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transplanted into lethally irradiated recipient mice. Approximately 8 weeks after transplant CD8+ T cells derived from donor bone marrow were detectable in circulation. (B) CD98 phenotype of purified naïve OT1 T cells from the spleens of retrogenic mice used for experiments. (C) Purified OT1 T cells of the indicated phenotypes confirmed in (B) were labeled with CFSE and stimulated with SIINFEKL peptide. Representative histograms depicting CFSE dilution of the indicated stimulated human CD98 positive, mouse CD98 negative, $CD8^+$ OT1 T cells stimulated with SIINFEKL peptide, in the presence of UM7F8 (black trace) or IgG (grey trace) and anti-mouse F(ab')2. (D) The mean proliferation index ± SEM from three independent experiments (n=3 donor mice) is depicted. * denotes $P < 0.01$, NS denotes not significant by one way ANOVA and Bonferroni post-test.

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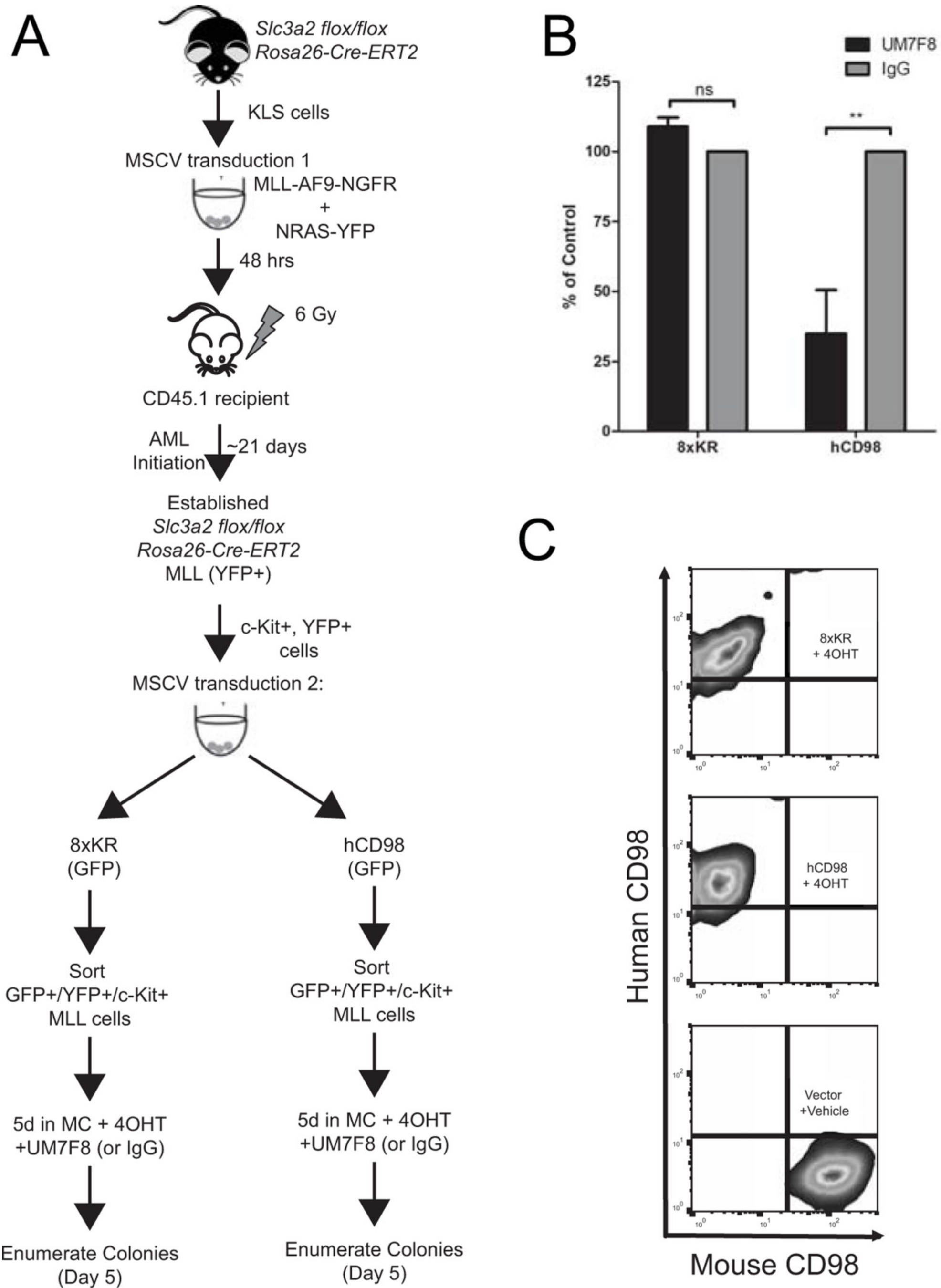

Figure 3: Anti-CD98 induced ubiquitination blocks CD98 dependent colony formation of cancer initiating AML cells.

(A) Overview of CD98 customized MLL-AF9/NRAS AML. Briefly, hematopoietic stem cells (KLS) from donor mice with inducible global knockout of endogenous mouse CD98 were transduced with retroviruses encoding oncogenes (MLL-AF9 fusion and NRAS) sufficient to initiate AML. Retroviruses included a surrogate marker to select transduced cells (human NGFR and YFP for MLL-AF9 and NRAS, respectively). Cancer initiating cells were transplanted into recipient mice to initiate AML. After approximately 21 days established AML cells were recovered and transduced with retroviruses encoding either the

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8KR mutant of human CD98 (8KR), wt human CD98 (hCD98) or empty EGFP vector. Viable, EGFP+ AML initiating cells were plated in methyl cellulose media containing 4 hydroxy tamoxifen (4OHT) or ethanol (Vehicle), and treated with UM7F8 or IgG and Anti-mouse F(ab')2. (B) Colony formation assays. After 5 days in culture colonies were enumerated. Colony formation in the presence of UM7F8 expressed as a percent of IgG treatment from two independent experiments performed in triplicate is shown ± SEM. ** denotes $P < 0.01$, NS denotes not significant by one-way ANOVA and Bonferroni post test. (C) Verification of human and mouse CD98 phenotypes in established AML (EGFP+) after treatment with anti-CD98 and subsequent clonal expansion. Raw colony counts appear in Supplemental Figure S2 panel F.

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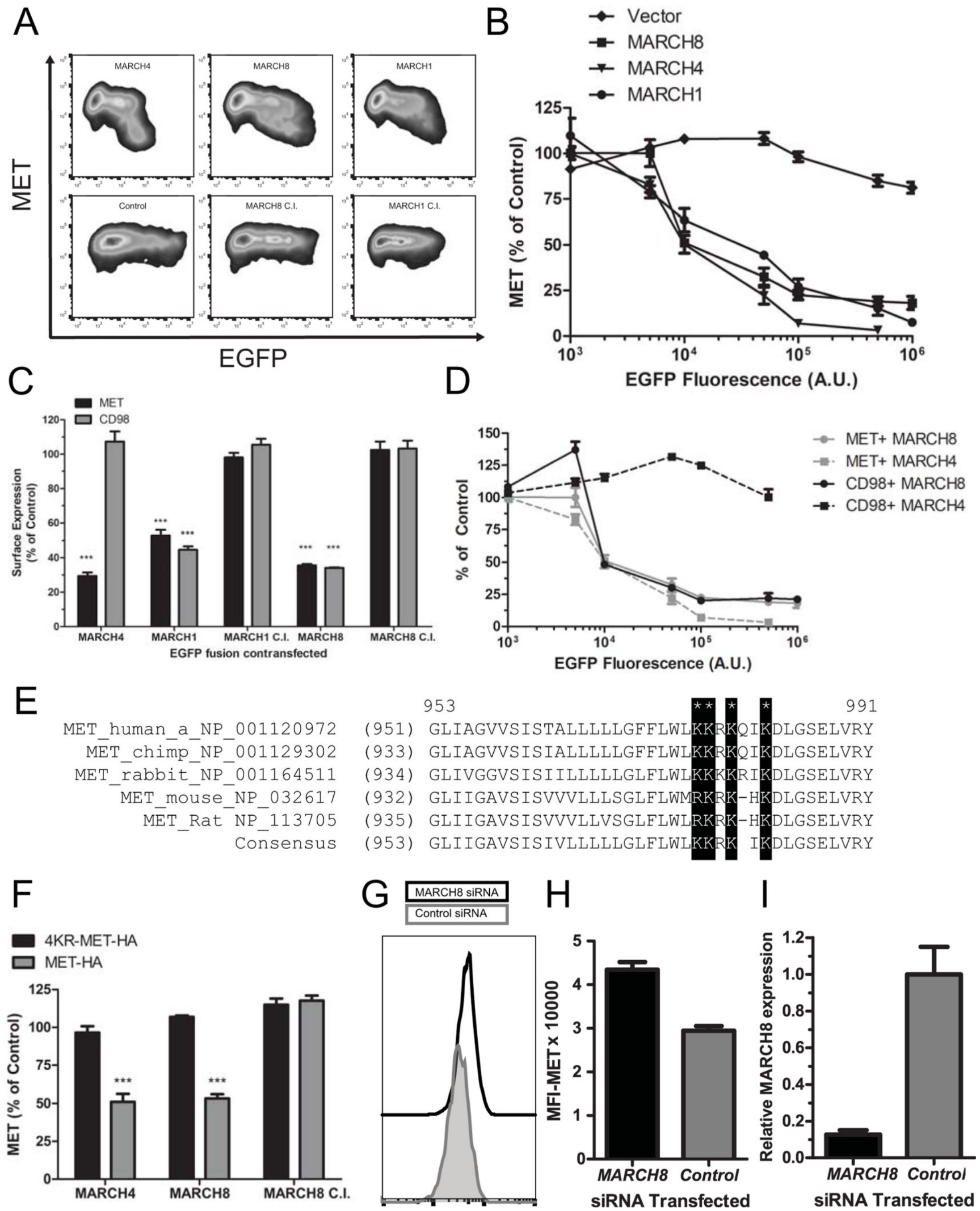

Figure 4: MET is regulated by MARCH1/4/8 through ubiquitination of membrane proximal cytoplasmic lysine residues.

(A) Surface expression of endogenous MET in HeLa cells transfected with either EGFP, MARCH4-EGFP, MARCH1-EGFP, MARCH8-EGFP or catalytically inactive mutants of MARCH1 or 8-EGFP. Dot plots are representative of three independent experiments. (B) Surface expression of endogenous MET in HeLa cells transfected with MARCH4-EGFP, MARCH1-EGFP, MARCH8-EGFP or a catalytically inactive mutant of MARCH8-EGFP (C.I.) relative to EGFP control as a function of MARCH expression (EGFP fluorescence) mean±SEM from three independent experiments is shown. (C) Differential MARCH

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sensitivity of CD98 and MET in HeLa cells. Cells transfected with cDNA encoding either MARCH4-EGFP or MARCH8-EGFP as in A were assayed for cell surface CD98 or MET Mean% of control±SEM from three independent experiments for MET and two independent experiments is shown for CD98. (D) as in (B) depicting CD98 or MET expression as a function of MARCH4 or MARCH8 expression. Note that the CD98 is resistant to MARCH4. (E) Amino acid alignment of the membrane proximal region of mammalian MET proteins. Conserved lysines are shaded. All four conserved membrane proximal lysines of human MET were mutated to arginine to generate 4KR-MET (marked by asterisks). (F) Surface expression of MET as a percentage of EGFP control from MET-knock out HeLa-FlpIn TREX cells, rescued with a single inducible integration of either HA-tagged 4KR MET or HA-tagged MET ± SEM from three independent experiments is depicted. (G) Surface expression of endogenous MET from MKN45 cells 72 hours post-transfection with either MARCH8 siRNA or control (non-targeting) siRNA. (H) quantification of MET surface expression from 3 independent experiments performed as in (G). (I) Validation of MARCH8 knockdown in MKN45 cells treated with MARCH8 or control siRNAs. Mean relative MARCH8 expression 72 hours post transfection from three independent experiments is depicted. *** denotes significantly different from EGFP control, $P < 0.01$, by one way ANOVA and Dunnet's post test. Raw data appear in Supplemental Figure S3.

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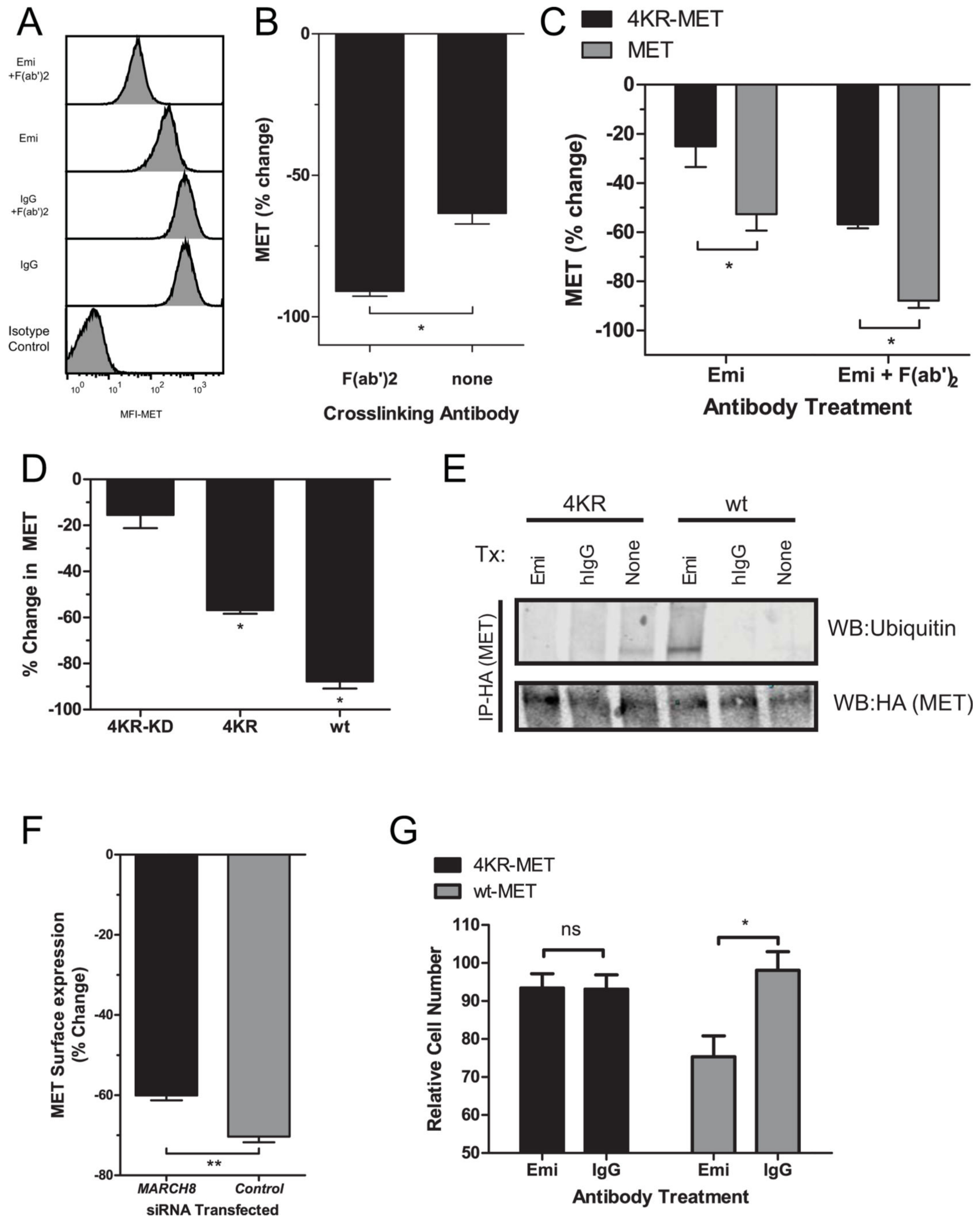

Figure 5: Emibetuzumab induces MARCH8-dependent ubiquitination and downregulation.

(A) Downregulation of endogenous MET with Emibetuzumab in HeLa cells. Histograms depicting MET surface expression 24 hours post-treatment with Emibetuzumab (Emi) or human IgG4 isotype control (IgG), with and without anti-human F(ab')2. (B) Quantification of Emibetuzumab induced downregulation of MET in HeLa cells. Cells were treated and surface MET expression determined as in (A). 24 hours post-treatment the percent change in MET relative to hIgG4 isotype control with or without anti-human F(ab')2 was determined. Mean percent change in MET surface expression ± SEM from three

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independent experiments is depicted. (C) 4KR-MET is partially resistant to Emibetuzumab induced downregulation. 24 hours post-treatment surface expression of MET or 4KR-MET was determined as in (B). Mean percent change in surface expression of MET wt or 4KR-MET± SEM from three independent experiments is depicted. (D) A Kinase Dead+ 4KR mutant of MET is resistant to Emibetuzumab induced downregulation. as in (C) with the indicated MET mutant or wt. Mean percent change in MET surface expression± SEM from three independent experiments is depicted. (E) Immunoblot of Immunoprecipitated HA-tagged MET for covalent modification with ubiquitin from HeLa cells stably expressing HA-tagged 4KR-MET (4KR) or its wt counterpart 24 hours post-treatment with 100nM Emibetuzumab or hIgG4 isotype control. (F) Emibetuzumab induced downregulation is impaired by MARCH8 knockdown in MKN45 cells. As in (B) comparing the mean percent change in endogenous MET surface expression of MKN45 cells 72 hours post-transfection with the indicated siRNA from 3 independent experiments is depicted. (G) 4KR-MET renders MKN45 cells resistant to the anti-proliferative effects of Emibetuzumab. The Emibetuzumab sensitive cell line, MKN45, was transfected with 4KR-MET-EGFP or MET-EGFP. 24 hours post-transfection viable, EGFP positive cells were sorted, plated then treated with either Emibetuzumab (Emi), hIgG4 Isotype control (IgG). 96 hours post treatment viable cell number was determined by MTS assay. Mean relative cell number compared to untreated cells ± SEM from four independent cultures is depicted. * denotes $P < 0.01$ by one way ANNOVA and Bonferroni post-test. Raw data appear in Supplemental Figure S4.