The presence of C/EBPα and its degradation are both required for TRIB2 mediated leukaemia

Caitríona O'Connor[1], Fíona Lohan[1], Joana Campos[1], Ewa Ohlsson[3,4,5], Mara Salomè[1], Ciarán Forde[2], Raik Artschwager[7], Robert M. Liskamp[7], Mary R. Cahill[8], Patrick A. Kiely[6], Bo Porse[3,4,5], Karen Keeshan[1]

[1]Paul O'Gorman Leukaemia Research Centre, Institute of Cancer Sciences, University of Glasgow, Scotland

[2]University College Cork, Ireland

[3]The Finsen Laboratory, Rigshospitalet, Faculty of Health Sciences, University of Copenhagen, Denmark

[4]Biotech Research and Innovation Center (BRIC), University of Copenhagen, Copenhagen, Denmark

[5]Danish Stem Cell Centre (DanStem) Faculty of Health Sciences, University of Copenhagen, Denmark

[6]Department of Life Sciences, Materials and Surface Science Institute and Stokes Institute, University of Limerick, Ireland

[7]School of Chemistry, University of Glasgow, Glasgow, G12 8QQ, UK

[8]Department of Haematology, Cork University Hospital, Ireland

Corresponding author: Karen Keeshan. Paul O'Gorman Leukaemia Research Centre, Institute of Cancer Sciences, University of Glasgow, Scotland

Tel: 0044 141 301 7895

Email: Karen.keeshan@glasgow.ac.uk

Running Title: Deciphering the C/EBPα and TRIB2 relationship

Abstract

C/EBPα (p42 and p30 isoforms) is commonly dysregulated in cancer via the action of oncogenes, and specifically in acute myeloid leukaemia (AML) by mutation. Elevated TRIB2 leads to the degradation of C/EBPα p42, leaving p30 intact in AML. Whether this relationship is a cooperative event in AML transformation is not known and the molecular mechanism involved remains elusive. Using mouse genetics our data reveal that in the complete absence of C/EBPα TRIB2 was unable to induce AML. Only in the presence of C/EBPα p42 and p30 were TRIB2 and p30 able to cooperate to decrease the latency of disease. We demonstrate the molecular mechanism involved in degradation of C/EBPα p42 requires site-specific direct interaction between TRIB2 and C/EBPα p42 for the K48-specific ubiquitin-dependent proteasomal degradation of C/EBPα p42. This interaction and ubiquitination is dependent on a critical C-terminal lysine residue on C/EBPα. We show effective targeting of this pathway pharmacologically using proteasome inhibitors in TRIB2 positive AML cells. Together, our data show that excess p30 cooperated with TRIB2 only in the presence of p42 to accelerate AML and the direct interaction and degradation of C/EBPα p42 is required for TRIB2-mediated AML.

Keywords: C/EBPα p42, p30, TRIB2, proteasome

Introduction

The C/EBPα (CCAAT/enhancer binding protein α) transcription factor is commonly mutated in acute myeloid leukaemia (AML) and dysregulated in a number of cancers (i.e. liver, prostate, lung, squamous cell carcinoma). Mutations in CEBPA occur exclusively in haematological diseases and result in perturbed C/EBPα protein expression and function. C/EBPα promoter methylation perturbs C/EBPα expression in AML(1), CML(2), and head and neck squamous cell carcinoma(3). In AML, oncogene-mediated dysregulation of C/EBPα mRNA expression and/or protein activity occurs in cytogenetically abnormal subtypes of AML, including AMLs with t(8;21)[AML1-ETO], inv(16)[CBFb-MYH11], t(15;17)[PML-RARA], and t(3;21)(AML-1-MDS-1-Evi-1 fusion protein) translocations, and in cytogenetically normal (CN)-AMLs with FLT3-ITD mutation, or with elevated TRIB2 expression(4). C/EBPα protein is also dysregulated by a number of posttranslational modifications including phosphorylation, sumoylation and ubiquitination(5).

CEBPA is an intronless gene with N-terminal transcriptional activation domains and a C-terminal basic region leucine zipper (LZ) domain. CEBPA mutations, which occur across the entire coding region, are present in 5% and 10% of childhood and adult AML respectively(6). Somatic and inherited mutations in CEBPA co-occur, are often bi-allelic, and are found with higher frequency in cytogenetically normal (CN) AML(7,8). CEBPA is a favourable prognosis factor in AML, specifically for cases that present with double mutations in CEBPA, typically an N-terminal and a C-terminal basic region LZ gene mutation. C/EBPα protein exists as two isoforms, a full length C/EBPα p42 isoform classified as a tumour suppressor, and an N-terminally truncated p30 isoform classified as an oncogene. N-terminal mutations typically are frame-shift mutations that lead to a premature stop in translation of p42, while retaining p30 expression. In contrast, C-terminal mutations typically are in-frame insertions/deletions in the LZ domain that disrupt DNA binding or dimerization. C/EBPα functions by forming stable homo-and heterodimers with itself and other C/EBP family members, which act to stabilize their protein expression(9).

TRIB2 is a potent oncogene capable of inducing fully penetrant AML in murine models, and can cooperate with other AML oncogenes to disrupt signaling pathways and transcription factors in AML

disease(10-13). TRIB2 expression was shown to be elevated in a cohort of AML patients with a mixed myeloid/lymphoid phenotype and a dysregulated CEBPA gene expression signature. In fact, TRIB2 leads to the degradation of C/EBPα p42 via E3 ligase COP1 binding whilst sparing p30 from degradation, resulting in disturbed granulopoiesis. This modulation of C/EBPα was found to be critical for the induction of AML *in vivo*(10,13). E2F1 cooperates with C/EBPα p30 to activate the Trib2 promoter in preleukaemic cells resulting in elevated TRIB2 expression. This was shown to be an important mechanism regulating TRIB2 expression and survival of AML cells(12).

In normal cells, C/EBPα is a key transcription factor in the transition from the pre-granulocyte-macrophage (pre-GM) to the granulocyte-macrophage progenitor (GMP) stage of differentiation, and in the transcriptional and epigenetic control of haematopoietic stem cell (HSC) self-renewal(14-16). In GMP progenitor cells, C/EBPα p42 is found bound to the Trib2 promoter and inhibits E2F1-mediated activation of Trib2. At the HSC stage, C/EBPα acts to prime HSCs for differentiation along the myeloid lineage via DNA binding to regulatory regions of genes induced during differentiation(16). Although conditional *Cebpa* knockout mice do not develop disease, the loss of *Cebpa* in adult HSCs leads to an increased number of functional and proliferative HSCs(15,17) and loss of HSC pool maintenance in serial transplantations(16). *Cebpa* expression is required for AML disease initiation by the oncogene MLL-ENL but not for disease maintenance(18). In contrast, *Cebpa* is required for the maintenance of Hoxa9/Meis1 AML disease(19). Loss of *Cebpa* did not abrogate BCR/ABL induced leukaemia but it did alter the resultant disease phenotype(20). Thus, it appears that a certain threshold of C/EBPα expression is important for the development of myeloid leukaemic disease. This is supported by the observation that leukaemia-derived CEBPA mutations are virtually never null mutations(21,22). The combination of N- and C-terminal mutations have distinct features that result in leukaemia development(23,24). Murine knockin studies investigating p30 oncogenicity in the absence of C/EBPα p42 showed that p30 induced fully penetrant transplantable AML(25). p30 is sufficient for haematopoietic commitment to the myeloid lineage, it enables transition from the common myeloid progenitor (CMP) to the GMP stage. However it does not retain the ability to control proliferation and myeloid progenitors have greatly increased self renewal capacities(25).

While TRIB2 disrupts the ratio of C/EBPα p42:p30 and functions to degrade C/EBPα, it is unclear whether the loss of C/EBPα p42, or the expression of C/EBPα p30 is critical for TRIB2 mediated AML disease. Here we show using mouse genetics that the presence of C/EBPα is paradoxically required for TRIB2 induced AML, and only in the presence of C/EBPα p42 is there a cooperative effect seen with TRIB2 and C/EBPα p30. We dissect the molecular details of the degradative relationship between TRIB2 and C/EBPα p42, and show that the direct interaction between TRIB2 and C/EBPα p42 is required for TRIB2-mediated ubiquitin-dependent proteasomal degradation of C/EBPα p42. We identify lysine 313 of C/EBPα as a site of TRIB2-mediated ubiquitination. We show that we can target TRIB2 AML cells with proteasome inhibition. Together, these data show that C/EBPα presence is required for the initiation of TRIB2 induced AML and subsequent C/EBPα p42/p30 disruption is critical for TRIB2 AML disease.

Results

C/EBPα p42 accelerates AML in the presence of TRIB2 and excess p30

In order to establish if C/EBPα is essential for TRIB2-induced AML, we performed a bone marrow transplant using a previously described conditional *Cebpa*$^{fl/fl}$*; Mx1Cre* mouse model and used polyinosinic-polycytidylic acid (pIpC) to facilitate ablation of C/EBPα in the haematopoietic compartment. Two weeks post deletion, c-Kit^{+} haematopoietic stem and progenitor cells (HSPCs) from the BM of pIpC treated control *Cebpa*$^{fl/fl}$ and *Cebpa*$^{\Delta/\Delta}$ animals were transduced with either empty MigR1 or MigR1-TRIB2 retrovirus. Transduced cells were transplanted into irradiated recipients, and monitored for disease progression. As expected, all mice reconstituted with *Cebpa*$^{fl/fl}$ cells expressing TRIB2 developed lethal AML with a median latency of 33 weeks, accompanied by accumulation of GFP^{+} cells (mean fraction of *Cebpa*$^{fl/fl}$ GFP-expressing cells 93% +/- 5.2%) in the bone marrow and splenomegaly (data not shown). In striking contrast, TRIB2 transduced *Cebpa*$^{\Delta/\Delta}$ HSPCs did not give rise to AML (Fig. 1A and supplemental fig. 1B). Notably, 14 months after transplantation CD45.2^{+}GFP^{+} cells were detected suggesting that the TRIB2 expressing *Cebpa*$^{\Delta/\Delta}$ cells are able to properly home to the bone marrow but do not give rise to leukaemia. Therefore, while TRIB2 functions to degrade C/EBPα p42 leading to AML(10), the complete absence of C/EBPα abrogates TRIB2 oncogenicity. Thus, it appears that a certain threshold of C/EBPα (p42 or p30) expression is necessary for the initiation of TRIB2 AML.

Having established the necessity of C/EBPα expression for TRIB2 AML, we next sought to elucidate whether p42 degradation or p30 accumulation is the key driver of this pathway, and whether p30 can cooperate with TRIB2 to accelerate disease onset. In order to investigate this we designed a transplant model which utilised donor cells from an established *Cebpa* conditional knockout model that only expressed p30 isoform (referred to here as *Cebpa*$^{\Delta/p30}$), or were heterozygous for this allele still expressing p42 on one allele (referred to as *Cebpa*$^{fl/p30}$ cells) (See methods and Fig. 1B). CD45.2^{+} donor cells from pIpC treated 8-14 week old *Cebpa*$^{fl/+}$*, Cebpa*$^{fl/p30}$ *and Cebpa*$^{fl/p30}$*; Mx1Cre* mice were harvested two weeks post deletion, transduced with MigR1 or MigR1-TRIB2 retrovirus and transplanted into CD45.1^{+} recipients. CD45.2 and GFP expression were monitored at 7 weeks post-

transplant in peripheral blood (supplementary fig. 1C-D) and every 2 weeks thereafter, showing recipient reconstitution with donor cells. The cohort of mice transplanted with TRIB2 transduced *Cebpa*$^{fl/+}$ cells developed AML disease as previously published(10) with a median latency of 49 weeks. However, mice transplanted with TRIB2 transduced *Cebpa*$^{fl/p30}$ cells developed a much more aggressive AML with an accelerated latency of 29 weeks (Fig. 1C) as evidenced by accumulation of GFP^{+} cells in the bone marrow, elevated white blood cell counts and splenomegaly (supplemental fig. 1E-J). Mice transplanted with MigR1 transduced *Cebpa*$^{\Delta/p30}$ cells developed AML with a median latency of 40 weeks, as expected and previously published for *Cebpa*$^{L/L}$ mice(25)("L" refers to p30), and no significant acceleration of the disease was seen in mice transplanted with TRIB2 transduced *Cebpa*$^{\Delta/p30}$ cells (median latency of 35 weeks) (Fig. 1D). Comparing TRIB2-AML in *Cebpa*$^{fl/p30}$ and *Cebpa*$^{\Delta/p30}$ cohorts, p42 accelerated AML in the presence of TRIB2 and excess p30 (Fig. 1E). Control cohorts of mice transplanted with *Cebpa*$^{fl/p30}$ and *Cebpa*$^{fl/+}$ cells transduced with MigR1 as expected did not develop any disease. Flow cytometry analysis showed that mice in both of these groups had normal haematopoietic compartments (supplemental fig. 1E-I). Flow cytometry analysis revealed that the leukaemic profile of GFP^{+} *Cebpa*$^{fl/+}$-TRIB2, *Cebpa*$^{fl/p30}$-TRIB2, *Cebpa*$^{\Delta/p30}$-TRIB2 and *Cebpa*$^{\Delta/p30}$-MigR1 AML cells was predominantly c-Kit^{+} (Fig. 1F). Protein analysis of the leukaemic bone marrow cells between the GFP^{+} *Cebpa*$^{fl/+}$-TRIB2, *Cebpa*$^{fl/p30}$-TRIB2, *Cebpa*$^{\Delta/p30}$-TRIB2 and *Cebpa*$^{\Delta/p30}$-MigR1 AML cells groups verified expected p42 and p30 expression levels in mice (leukaemic ratio p42:p30 in *Cebpa*$^{fl/+}$-TRIB2 cohort, p42 and increased p30 in *Cebpa*$^{fl/p30}$-TRIB2 and *Cebpa*$^{fl/p30}$-MigR1 cohort, comparable p30 in the absence of p42 in *Cebpa*$^{\Delta/p30}$-MigR1 and *Cebpa*$^{\Delta/p30}$-TRIB2 cohorts (Fig. 1G). Taken together these data show that despite TRIB2 functioning to degrade p42, C/EBPα is essential for the initiation of TRIB2 mediated AML and only in the presence of C/EBPα p42 is there a cooperative effect seen with p30.

Identification of specific amino acids involved in the TRIB2 and C/EBPα interaction

Having shown the requirement for C/EBPα p42 in the initiation of TRIB2-induced AML we next focused on the interaction between C/EBPα and TRIB2. We first determined using subcellular fractionation and confocal microscopy that the co-localisation of TRIB2-C/EBPα was nuclear

(supplementary fig. 2A and B). Using GST pull-down experiments we show that the C-terminal leucine zipper (LZ) domain of C/EBPα is required for the interaction with TRIB2 as a GZ mutant expressing an unrelated LZ could no longer bind TRIB2. However, TRIB2 can still bind a C/EBPα LZ mutant (L12V) that can no longer dimerise, indicating TRIB2 binding to C/EBPα is independent of amino acids involved in C/EBPα dimerization (supplementary Fig. 2C, D and E). To more precisely identify specific regions of interaction with TRIB2, we SPOT-synthesized a peptide array containing a series of 18-amino acid overlapping peptides identical to the C-terminus LZ domain of C/EBPα. GST-TRIB2 bound to eight peptides within the C-terminus LZ of C/EBPα (Fig. 2A). To identify the specific amino acids required for the interaction, we used a Specific Alanine Scanning Substitution Array (SASSA), where consecutive amino acids of the parent peptides were substituted to an Alanine. This identified residues R333, R339 and R343 of C/EBPα as potential mediators of interaction with TRIB2 (Fig. 2B). Using site-directed mutagenesis (SDM) we show that mutation of R339A abolished the interaction with GST-TRIB2 in a pull-down assay (Fig. 2C). This was confirmed by co-immunoprecipitation in mammalian cells (supplementary fig. 2F) indicating that R339 of C/EBPα is required for interaction with TRIB2.

Reciprocally, we used TRIB2 peptide arrays that covered the entire TRIB2 protein to identify specific amino acids in TRIB2 responsible for the interaction with C/EBPα. Recombinant C/EBPα protein bound to peptides that spanned from amino acids 25-318 indicating extensive interaction of C/EBPα with TRIB2 (Fig. 3A). SASSA of amino acids in peptide 2 (DHVFRAVLHSHGEELVCK) identified R77, which is homologous to R107 in TRIB1 that exists as a gain of function TRIB1 mutation in AML(26). SASSA of amino acids in peptide 4 (PEILNTSGSYSGKAADVW) identified a putative GSK3 phosphorylation site (which has the consensus GSK3 phosphorylation site motif S/TXXXS/T). SASSA of peptide 5 (HPWFSTDFSVSNSGFGAK) identified K322 (Fig. 3B). Three mutants R77A, S227A/S229A/S231A/K233A (4-AA) and K322A were generated (Fig. 3C). Co-immunoprecipitation showed that mutation of the sequence S227A/S229A/S231A/K233A (4-AA) abolished the interaction with C/EBPα completely, and K322A and R77A while reduced could still bind C/EBPα (Fig. 3D and supplementary Fig. 2G).

Ubiquitin-mediated mechanism of TRIB2-induced p42 degradation

We next assessed the ability of TRIB2 to induce ubiquitin specific degradation of C/EBPα. A ubiquitination assay was performed in 293T cells transfected with C/EBPα, TRIB2 and Ubiquitin (Ub). Increased C/EBPα ubiquitination was observed in TRIB2 expressing cells compared to controls and this was exclusively K48 mediated ubiquitination (proteasome degradation K48 specific polychains as opposed to non-degradative ubiquitination mediated by K63 polychains) as detected by K48 and K63 specific antibodies (Fig. 4A). This was confirmed using ubiquitin mutants which do not express the K48 ubiquitin polychain (UB/K48R-HA) and the K63 ubiquitin polychain (UB/K63R-HA) in a ubiquitination assay (Fig. 4B compare lanes 7, 8 and 9). We show using the R339A C/EBPα binding mutant that the direct binding between TRIB2 and C/EBPα was required for TRIB2-mediated ubiquitination of C/EBPα compared to the control mutant R343A that retains the TRIB2 interaction (Fig. 4C). Reciprocally, using the TRIB2 binding mutant S227A/S229A/S231A/K233A (4-AA) revealed a loss in C/EBPα ubiquitination as compared to the mutants R77A and K322A that retain higher C/EBPα binding affinity or WT TRIB2 (Fig. 4D). Together these data show that the direct binding interaction between TRIB2 and C/EBPα was required for TRIB2-mediated ubiquitination of C/EBPα.

To investigate the site on C/EBPα susceptible to TRIB2 mediated ubiquitination, Ubpred online software analysis of C/EBPα was performed and identified a number of lysine residues as potential ubiquitination sites with low to high confidence scores. K313 was predicted with medium confidence as a potential ubiquitination site (Fig. 5A and B) which has been identified as a recurrent C/EBPα mutation (K313dup) that results in an extra lysine adjacent to K313. This duplication occurs in 10% of C/EBPα mutated AMLs and identified to have a shorter protein half-life than wildtype C/EBPα as determined by cycloheximide assay(27). Mutation of K313 to K313R resulted in the loss of TRIB2-induced C/EBPα ubiquitination as compared to WT C/EBPα (Fig. 5C). As a control we show that this mutation retains functional C/EBPα transcriptional activity as both WT and mutant C/EBPα stimulated the G-CSFR luciferase reporter construct, a known C/EBPα target gene (Fig. 5D). These

data show that K313 serves as the site of ubiquitin conjugation on C/EBPα in the presence of TRIB2 and mutation of K313 abrogates TRIB2-mediated ubiquitin-dependent proteasomal degradation of C/EBPα. This site is also present in p30 yet we do not observe TRIB2-mediated p30 ubiquitination (Fig. 5E), suggesting that perhaps the N terminus of C/EBPα p42 functions in the degradative complex.

Proteasome Inhibition selectively targets AML with high TRIB2 expression

Our data suggest that inhibition of the proteasome would effectively inhibit the function of TRIB2 by abrogating C/EBPα protein degradation and would be an effective pharmacological targeting strategy in TRIB2 positive AMLs. To test this U937 cells (express detectable levels of endogenous TRIB2 and C/EBPα) transduced with retroviral MigR1-GFP control and MigR1-TRIB2-GFP were treated with 10 nM bortezomib (a reversible inhibitor of the proteasome chymotryptic activity resulting in the accumulation of K48 ubiquitin linked proteins). An increase in cytotoxicity in the TRIB2 overexpressing cells compared to MigR1 control transduced cells was observed and cells expressing TRIB2-VPM that cannot degrade C/EBPα behaved similarly to control cells (Fig. 6A panel 1 and 2). Overexpression of MigR1-C/EBPα was able to rescue bortezomib induced cell death in U937 cells (Fig. 6A panel 3). In C/EBPα negative leukaemia cells, K562 and Kasumi 1 bortezomib toxicity was not increased following TRIB2 overexpression supporting the specificity of bortezomib on the TRIB2-C/EBPα axis (Fig. 6A panel 4 and 5). Second generation irreversible proteasome inhibitors(28,29) also showed selective killing of high TRIB2 (lentiviral Phr-GFP) expressing AML cells as assessed by cell viability (Fig. 6B). Bortezomib treatment rescued the degradation of C/EBPα p42 seen with TRIB2 overexpression in U937 cells (Fig. 6C). In fact, p30 expression remains upon TRIB2 overexpression, confirming our ubiquitination data in figure 5. We next established a human AML orthotopic xenograft model in which U937 cells were propagated in NSG xenografts and mice were either treated with bortezomib *in vivo* (Fig. 6D and described in methods), or TRIB2 was knocked down using shRNA lentivirus (Fig. 6E(12)). *In vivo* bortezomib treatment significantly impaired the engraftment of U937 cells as determined by lower % of engrafting GFP^+ cells positive for human antibody CD45 expression (Fig. 6F). Additionally, engraftment of U937 cells expressing

shTRIB2 lentivirus in NSG xenografts showed that in comparison to control (shctrl), knockdown of TRIB2 impaired the progression of U937 orthotopic xenografts (Fig. 6G). Together our data show that TRIB2 expressing AML cells can be pharmacologically targeted with proteasome inhibition due, in part, to inhibition of the TRIB2 proteolytic function on C/EBPα.

Discussion

Although C/EBPα dysfunction is a common occurrence in different cancers, details on the mechanisms involved in bringing about the loss of C/EBPα protein expression in *Cebpa* wild type and mutated AML are yet to be fully elucidated. In this study we identify the molecular mechanism involved in the dysregulation of C/EBPα expression via TRIB2 in AML. We and others(10,13,30) have previously demonstrated that TRIB2 overexpression induces AML and that it degrades C/EBPα p42. Here we provide novel insights on this process and show that the presence of C/EBPα p42 is required not only to initiate TRIB2 AML, but also for TRIB2 to cooperate with C/EBPα (p42 loss and increased p30) in driving AML disease. Our data support the emerging notion that C/EBPα functions in the initial transformation, as supported by the data showing that C/EBPα regulated a transcriptional program essential for initiation yet dispensable for the maintenance of MLL-ENL disease(18). Our data clearly show loss of C/EBPα p42 via TRIB2 induced ubiquitin-mediated degradation and excess p30 expression cooperates to accelerate AML.

Using bone marrow transduction and transplantation approaches we show that C/EBPα p42 isoform expression from one *Cebpa* allele is necessary and sufficient for TRIB2 to cooperate with p30 isoform in AML. The addition of TRIB2 does not further accelerate AML induced by excess p30 itself, but does accelerate AML when p42 is present. This suggests that p30 is necessary for TRIB2-mediated AML induction. The possibility remains that TRIB2 may convert p42 to p30 to form additional p30 protein but we have not any evidence for this here. Under normal physiological conditions the protein levels of C/EBPα p42 are greater than p30 protein levels, and when this balances changes toward more p30 levels it is considered “leukaemic”, as observed in leukaemic cell lines and AML patients(22). We showed K313 in the C terminus of C/EBPα p42 was critical for TRIB2-mediated ubiquitination and proteasomal degradation. This lysine residue is a common mutation in AML patients (~10% of all C/EBPα mutations) which results in the duplication of the single lysine to KK leading to reduced protein stability(27). Using mouse genetics it was shown that this duplication of K313 to K313KK (K313dup) resulted in an increased proliferation of long-term HSCs (LT-HSCs) leading to an expansion of premaligant HSCs not seen with the N-terminal

mutations(23). Our data show that the absence of this lysine by mutation to an arginine (K313R) abrogates the susceptibility of C/EBPα to degradation whilst retaining its DNA binding and transcriptional activation function. This suggests that TRIB2 degrades C/EBPα p42 via ubiquitination of K313 and duplication of this residue as occurs in AML patients samples would lead to an increase in C/EBPα p42 degradation in the presence of TRIB2. Patients with K313dup had TCR rearrangements and CD7 expression(27), lymphoid features associated with unfavourable outcome and which we have previously linked with TRIB2 and C/EBPα perturbation(31). Previous investigation of the combination of a C-terminal mutation (K313 duplication) with C/EBPα p30 expression revealed co-operation and provides a possible explanation for the high prevalence of one N-term mutation and one C-term mutation in ~90% of biallelic C/EBPα mutant AMLs(23). We propose that the K313dup mutation in patients contributes to leukaemic transformation by increasing the susceptibility of C/EBPα to ubiquitination resulting in increased degradation of C/EBPα p42. A thorough investigation of TRIB2 function in K313 mutant patient cells would further validate this.

C/EBPα p42 R339 and K313 amino acids are both present in p30 yet TRIB2 does not degrade p30. One explanation may be that the protein complexes containing TRIB2 and C/EBPα p42 or TRIB2 and p30 are different. Our previous work showed that TRIB2 binds with COP1 E3 ligase and this interaction is necessary for C/EBPα p42 degradation(13). While our peptide mapping clearly shows specific sites in both TRIB2 and C/EBPα responsible for the direct interaction, in the absence of crystal structure information we cannot predict further based on our data the reason why p30 is not targeted by TRIB2 for degradation. Our data does not discount a role for p30 in TRIB2 mediated AML; p30 presence may, for example, activate a unique set of genes, inhibit other C/EBPs or bind to a unique set of proteins distinct from C/EBPα p42 as previously reported(32,33). Indeed, U937 cells express endogenous C/EBPα but are less sensitive to bortezomib induced killing compared to TRIB2 overexpressing cells. This suggests that TRIB2 must be affecting something in addition to C/EBPα p42 degradation to provide synergy in AML. Our data support further investigations into the proteomic profiling of AML, with a focus on the involvement of C/EBPα ubiquitination and the need

for an in-depth understanding of the C/EBPα ubiquitin regulatory pathway in normal versus leukaemic (CEBPA WT and mutant) settings.

Whilst TRIB2 itself is an oncogene capable of driving AML, the molecular interaction identified here between TRIB2 and C/EBPα leading to dysregulated C/EBPα may have implications in other models of AML and indeed other cancers. It is clear that the loss of C/EBPα function is a common occurrence in a number of different cancer types. What is not clear at this point is the extent of the involvement of TRIB2 function across different haematological malignancies and other cancers. To date, cooperation between TRIB proteins and other oncogenes in AML has been demonstrated, most often with Hoxa9 and Meis1, suggesting that TRIB2 protein function downstream of commonly occurring oncogenic events may impact on C/EBPα(34,35). TRIB2 is found elevated in lung cancer and associated with the E3 ligase TRIM21(30). In liver cancer cells, TRIB2 associated with other E3 ligases including β-TrCP, COP1 and Smurf1(36). Thus, there is a strong link between TRIB2 and the ubiquitin-proteasome system (UPS) in cancer.

As shown here, high TRIB2 expressing cells are sensitive to bortezomib induced cellular toxicity. However, inhibition of the proteasome also targets other proteins, E3 ligases and their substrates. We currently have limited knowledge of TRIB2 degradation substrates in general, and indeed knowledge of TRIB2 as a substrate itself of the UPS in cancer. This study highlights the important molecular interplay between TRIB2 and C/EBPα in driving AML, and provides a framework for further investigations into targeting the UPS as an emerging cancer therapy.

Materials and Methods

Murine Bone Marrow Transduction and Transplantation

Cebpa$^{fl/fl}$*; Mx1cre* were crossed to *Cebpa*$^{+/p30}$ ((25)p30 referred to as "L" allele previously) to yield *Cebpa*$^{fl/+}$*; Mx1Cre, Cebpa*$^{fl/p30}$*; Mx1cre, Cebpa*$^{fl/+}$ and *Cebpa*$^{fl/p30}$. Genotyping and evaluation of *Cebpa* excision methods provided in SI. 8-14 week old *Cebpa*$^{fl/fl}$*, Cebpa*$^{fl/fl}$*; Mx1Cre, Cebpa*$^{fl/+}$*, Cebpa*$^{fl/p30}$ *and Cebpa*$^{fl/p30}$*; Mx1Cre* mice were polyinosinic-polycytidylic acid (pIpC) treated (3 injections every second day with 300 ug pIpC) to excise the *Cebpa* allele. Resultant genotypes are *Cebpa*$^{fl/fl}$ (expressing solely p42 alleles which can give rise to p30), *Cebpa*$^{\Delta/\Delta}$ (null for p42 and p30), *Cebpa*$^{fl/+}$ (expressing solely p42 alleles which can give rise to p30), *Cebpa*$^{fl/p30}$ (expressing one p42 and one solely p30 allele), and *Cebpa*$^{fl/p30}$*; Mx1Cre* (referred to as *Cebpa*$^{\Delta/p30}$, solely expressing one p30 allele) (Fig. 1B). Two weeks post deletion, CD45.2^{+} BM cells were collected and either c-Kit purified by magnetic-activated cell sorting (Fig. 1A), or total bone marrow (Fig. 1C-D) was transduced with 40% titre matched retrovirus in the presence of IL3, IL6 and SCF. Freshly transduced unsorted cells (1x10^{6} Fig. 1A, 3x10^{6} Fig. 1C) were injected by tail vein into lethally irradiated CD45.1^{+} recipients generating *Cebpa*$^{fl/fl}$-TRIB2 (n=12), *Cebpa*$^{\Delta/\Delta}$-TRIB2 (n=10), *Cebpa*$^{fl/+}$-MigR1 (n=9), *Cebpa*$^{fl/+}$-TRIB2 (n=4), *Cebpa*$^{fl/p30}$-MigR1 (n=6), *Cebpa*$^{fl/p30}$-TRIB2 (n=8), *Cebpa*$^{\Delta/p30}$-MigR1 (n=6), *Cebpa*$^{\Delta/p30}$-TRIB2 (n=8). Mice were monitored for 14 months and euthanized when they developed physical signs of disease (behavioural changes, laboured breathing, hunched posture) or high white blood cell counts. For NOD-SCID IL2Rγnull (NSG) mice Xenograft transplantation, 1x10^{6} U937 cells transduced with shTRIB2-GFP (n=5) or shCtrl-GFP (N=8) were transplanted into non-irradiated NSG mice via tail vein injection. To assess bortezomib *in vivo* efficacy, GFP ctrl transduced U937 cells were injected and 5 days post injection mice were randomized and treated with either bortezomib (0.5mg/kg, n=3) or PBS (i.p. administration, n=3) at days 5, 7 and 11 post-transplant which was well tolerated. Mice were sacrificed on day 16. Animals were maintained at the University of Copenhagen and the University of Glasgow, housed in accordance with their respective institutional guidelines. All work was carried out under the approval of the Danish Ethical Committee and the Home Office of the United Kingdom.

Flow Cytometry

Cell suspensions were stained on ice in FACS Buffer (PBS ph7.0, 10mM Hepes, 0.02% sodium azide, 0.2% BSA). Analytical flow cytometry was performed on a FACS Canto (Becton Dickinson), sorting was performed on a FACS Aria II (Becton Dickinson) and data was analyzed using FlowJo software (Treestar, Version 10). Dead cells were excluded using DAPI and doublets excluded based on FSC-H and FSC-A. For apoptosis assays, Annexin V-PE (BD Biosciences 556422) or Annexin V-APC (BD Biosciences 550475) was used.

Details of vectors, antibodies, cell culture/transfections and real-time PCR provided in supplementary information (SI).

Ubiquitination Assay, Co-Immunoprecipitation and Western Blotting.

293T cells transfected with described vectors were treated with 10μM MG-132 five hours prior to lysis where indicated. For ubiquitination assay, cells were lysed in 1% SDS, sonicated and supernatants precleared with Protein G Sepharose 4 fast flow beads (GE healthcare) then incubated with C/EBPα antibody overnight. For Co-Immunoprecipitation (CO-IP), cells were lysed in Tris buffer (50mM Tris pH 7.4, 150 mM NaCl, 1mM EDTA, 0.5% NP-40, 5% glycerol, with protease and phosphatase inhibitors) and precleared lysates were incubated with C/EBPα antibody overnight. Whole cell proteins were isolated by RIPA lysis buffer (50mM Tris, pH 8.0, containing 0.5% NP-40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EDTA, 20mM N-Ethylmaleimide with protease and phosphatase inhibitors) or by direct lysis (2X SDS sample buffer). Details of antibodies provided in SI. For GST-IP, 3mg of indicated cell lysate was added to 50μL of GSH slurry with pull down performed using 25ug of GST or GST-TRIB2 (Details of GST protein production provided in SI). Densitometric analysis of bands was carried out with ImageJ Software. $n \geq 3$ for all CO-IP and ubiquitination assays.

Spot synthesis of peptides and overlay analysis.

Peptide arrays were performed as previously described(37,38) (n=2) and details provided in SI. Briefly, peptide arrays encompassing the C-terminus of C/EBPα and the entire TRIB2 protein were generated and incubated with GST-TRIB2 or purified C-teminal C/EBPα (Genway). Arrays were

incubated with secondary antibodies and bound protein detected using ECL or the Odyssey® Infrared Imaging System (LI-COR Biosciences).

Statistical analysis

Statisitcal analysis and graphing was performed on GraphPad Prism (version 5.03). When comparing 2 groups an unpaired, two-tailed Student's t-test was used. The Log-rank test was used to compare groups on a survival curves. Statistical significance was attained when the *P* value ≤ 0.05 and was indicated in the related figure legends and graphs.

The authors declare no conflict of interest

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Authorship Contributions

Contribution: KK designed the study. FL, CO'C, CF, EO, MS and JC performed the research. BP provided transgenic mouse models. BP, PK, RL, RA and MC provided essential reagents and expertise. KK, FL, CO'C, MS, EO and JC analysed the data. CO'C and FL made the figures. KK wrote the paper. All authors edited the paper.

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Figure Legends

Figure 1: C/EBPα p42 accelerates AML in the presence of TRIB2 and excess p30 (A) Kaplan-Meier survival curve of mice reconstituted with either *Cebpa*$^{fl/fl}$ or *Cebpa*$^{\Delta/\Delta}$ bone marrow cells transduced with TRIB2 expressing retrovirus. The median survival of mice reconstituted with *Cebpa*$^{fl/fl}$ -TRIB2 cells was 33 weeks (n=12), none of the mice reconstituted with *Cebpa*$^{\Delta/\Delta}$-TRIB2 cells developed leukaemia within 60 weeks (n=10). ***P<0.0001 by Log-rank test. (B) Schematic representation of *Cebpa* alleles (C) Kaplan-Meier survival curve of mice reconstituted with either *Cebpa*$^{fl/+}$ or *Cebpa*$^{fl/p30}$ bone marrow cells transduced with MigR1 or TRIB2 expressing retrovirus. None of the mice reconstituted with *Cebpa*$^{fl/+}$-MigR1 (n=9) or *Cebpa*$^{fl/p30}$-MigR1 (n=6) developed leukaemia within 60 weeks. The median survival of mice reconstituted with *Cebpa*$^{fl/+}$-TRIB2 cells was 49 weeks (n=4), while the median survival of mice reconstituted with *Cebpa*$^{fl/p30}$-TRIB2 was 29 weeks (n=8). ***P<0.0001 by Log-rank test. (D) Kaplan-Meier survival curve of mice reconstituted with *Cebpa*$^{\Delta/p30}$ bone marrow cells transduced with MigR1 or TRIB2 expressing retrovirus. The median survival of mice reconstituted with *Cebpa*$^{\Delta/p30}$-MigR1 cells was 40 weeks (n=6), while the median survival of mice reconstituted with *Cebpa*$^{\Delta/p30}$-TRIB2 cells was 35 weeks (n=8). P=0.8111 by Log-Rank test. (E) Kaplan-Meier survival curve comparing mice reconstituted with *Cebpa*$^{fl/p30}$-TRIB2 or *Cebpa*$^{\Delta/p30}$-TRIB2 bone marrow cells. *P≤0.05 by Log-rank test (F) Average percentage of c-Kit expression in CD45.2+ BM of each group +/- SD (G) Protein analysis of C/EBPαp42 and p30 expression in bone marrow of mice. ns=non-specific band.

Figure 2: Identification of R339 as crucial C/EBPα amino acid in TRIB2 binding (A) Peptide arrays encompassing C-terminus of C/EBPα were generated as described in Materials and Methods. Eight binding peptides unique to C/EBPα were identified. The table displays the amino acid identity of these peptides. This array is representative of two independent SPOT-synthesised arrays. Dark spots are indicative of peptide binding. (B) SASSAs on a region spanning the C-terminus of C/EBPα were probed with GST-TRIB2. The binding of GST-TRIB2 to each alanine substituted peptide was quantified by densitometry and presented as a percentage of the control unmutated WT sequence, with an underlay identifying the amino acid which was substituted with an alanine. >50% reduction in

binding intensity indicative of involvement in the interaction. (C) Western blot analysis of 293T cell lysates expressing WT C/EBPα and C/EBPα mutants (Left panel). GST pull-down, performed in the presence of MG132, using GST-TRIB2 followed by Western blot analysis for GST (top and bottom panels) and C/EBPα (middle panel).

Figure 3: Identification of the C/EBPα binding site on TRIB2 (A) Peptide arrays encompassing TRIB2 were generated as described in Materials and Methods. Five binding peptides spanning amino acids 25-318 of C/EBPα protein were identified. The table displays the amino acid identity of these. This array is representative of two independent SPOT-synthesised arrays. Dark spots are indicative of peptide binding. (B) SASSAs on regions spanning TRIB2 were probed with C/EBPα protein. The binding of C/EBPα protein to each alanine substituted peptide was quantified by densitometry and presented as relative intensity of the control unmutated WT sequence, with an underlay identifying the amino acid which was substituted with an alanine. >50% reduction in binding intensity indicative of involvement in the interaction. (C) 293T lysates expressing WT TRIB2 and mutants R77A, 4-AA (S227A/S229A/S231A/K233A) immunoblotted with anti-TRIB2 and actin serving as a loading control. (D) TRIB2 binding mutants 4-AA (S227A/S229A/S231A/K233A), R77A and K322A and WT C/EBPα were expressed in 293T cells and C/EBPα was immunoprecipitated and lysates were immunoblotted for TRIB2 and C/EBPα (top panels). Western blot of total input lysates shows levels of protein expression (bottom panels). Densitometric analysis was performed and the ratio of IP:Input, normalised to Actin are presented (underneath bottom panels).

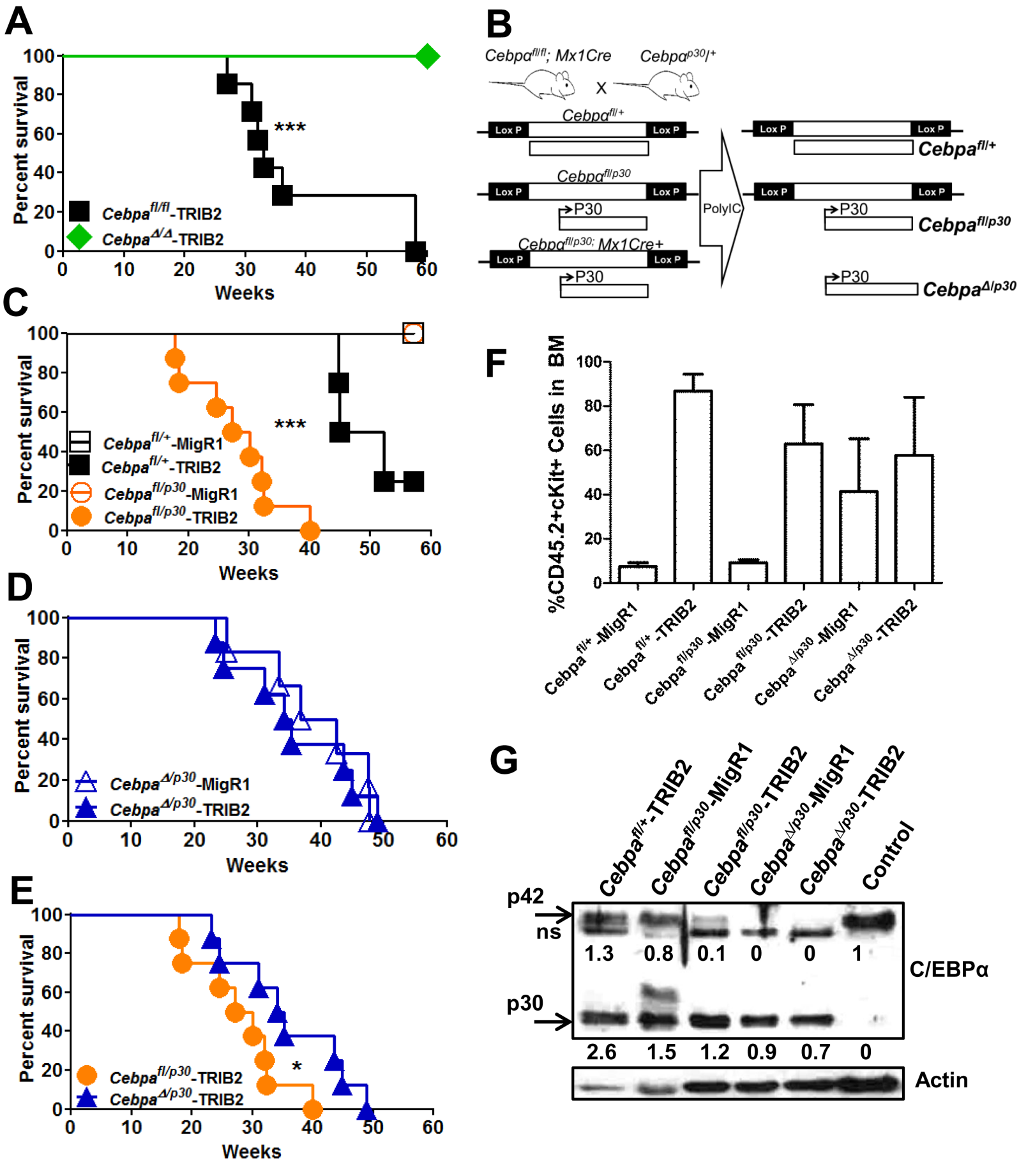
Figure 4: TRIB2 induces binding dependent K48 specific ubiquitin-dependent proteasomal degradation of C/EBPα on Lysine K313 (A) 293T cells were transfected with ubiquitin-HA, TRIB2, C/EBPα and PHMA empty vector control. Ubiquitination assay was performed. Immunoblotting for HA detects ubiquitination (top IP panel), K63 species of polyubiquitination (second IP panel), K48 species of polyubiquination (third IP panel) and C/EBPα detects IP protein (bottom IP panel). Immunoblotting for TRIB2 and C/EBPα detects input levels and actin serves as a loading control (input panels). (B) Ubiquitination assay as outlined in (A) including ubiquitin mutants that do not express the K48 (K48R) ubiquitin polychain and the K63 (K63R) ubiquitin polychain. (C)

Ubiquitination assay as outlined in (A) including C/EBPα mutants; R339A and R343A. (D) Ubiquitination assay as outlined in (A) including TRIB2 binding mutants R77A, 4-AA (S227A/S229A/S231/K233A) and K322A. Indicated experiments (B-D) were treated with 10μM MG-132 for 5hrs before ubiquitination assay was performed.

Figure 5: TRIB2 ubiquitinates C/EBPα on K313 (A) Ubpred analysis of C/EBPα Ensembl gene ID (ENSRNOG00000010918) showing the probability of each Lysine acting as ubiquitination sites. (B) Schematic diagram displaying the localisation of K313 in C/EBPα. (C) Ubiquitination assay performed with C/EBPα-K313R and TRIB2 in 293T cells. (D) 293T cells were co-transfected with the G-CSFR promoter firefly luciferase constructs containing either the C/EBPα WT (WT GCSFR) or mutant binding sites (Mut GCSFR), and either an empty PHMA vector or vector containing C/EBPα or K313R, along with a pRL-TK Renilla luciferase internal control plasmid. Luciferase activity was measured 24 hours post-transfection. Bar chart represents reporter luciferase activity for each sample normalised for renilla values, and graphed relative to the control sample. Results analysed using two-tailed unpaired t-test and representative of 3 independent experiments. (E) Ubiquitination assay performed with C/EBPα p30 and TRIB2 in 293T cells.

Figure 6: **Modulation of TRIB2 levels sensitizes AML cells to cell death induced by proteasome inhibition** (A) Sorted GFP^+ cells transduced with MigR1 control, MigR1-TRIB2, MigR1-VPM or MigR1-C/EBPα retrovirus were treated +/- 10nM bortezomib for 16-24hrs and analysed by flow cytometry for cell death by DAPI. Graph of fold change in cell death as determined by DAPI positive cells. Data displayed is average of 3 technical replicates, and is representative of 2 independent experiments. (ns denotes not significant, *P<0.05,**P<0.005 by Student´s unpaired *t* test). (B) Sorted GFP^+ U937 cells transduced with either Phr or Phr-TRIB2 were treated with 10nM bortezomib, 500nM sulfonyl fluoride (SF), 10nM carfilzomib (Cfz) or DMSO only for 16hrs. Cytotoxicity was assessed by Dapi staining. Representative FACS plots of 3 independent experiments with 2 technical replicates each are shown. (C) Sorted GFP^+ U937 cells transduced with either Phr or Phr-TRIB2 were treated for 6 and 8h with 10nM bortezomib or DMSO only (NDC, no drug control) and cell lysates were analysed for C/EBPα expression by Western blotting. TRIB2 overexpression was confirmed and

tubulin was used as loading control. Representative of 2 independent experiments. (D) Schematic representation of the AML xenograft study. NOD-SCID IL2Rγ^{null} mice (NSG) were transplanted with GFP^{+}*hCD45*$^{+}$ U937 cells (n=6). Each group was randomized into 2 groups for treatment with 0.5mg/kg bortezomib or PBS, i.p., on a regime schedule of 3 injections for 16 days. (E) TRIB2 gene knockdown was confirmed at the mRNA level in shTRIB2-U937 cells selected with 2μg/ml puromycin for 48h. Values represent gene expression relative to shctrl-U937 cells and normalized to the reference gene ABL (***P<0.001 by Student´s *t* test). (F) GFP and hCD45 expression in BM were quantified by flow cytometry as a measure of disease burden in control PBS (n=3) and bortezomib (n=3) treated animals and data are shown by representative FACS contour plots (lower panel) and bar graphs (upper panel) with means ± SD (*P<0.05 by Student´s *t* test). (G) GFP and hCD45 expression in BM were quantified by flow cytometry as a measure of disease burden in shctrl (n=5) and shTRIB2 U937 (n=8) mice and data are shown by representative FACS contour plots (lower panel) and bar graphs (upper panel) with means ± SD (***P<0.001 by Student´s *t* test).


A
Percent survival
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***
Cebpa fl/fl -TRIB2
Cebpa Δ/Δ -TRIB2
B
Cebpa fl/fl; Mx1Cre
Cebpa p30/+
X
Cebpa fl/+
Cebpa fl/p30
Cebpa fl/p30; Mx1Cre+
Lox P
P30
PolyIC
Cebpa fl/+
Cebpa fl/p30
Cebpa Δ/p30
C
Percent survival
Weeks
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Cebpa fl/+ -MigR1
Cebpa fl/+ -TRIB2
Cebpa fl/p30 -MigR1
Cebpa fl/p30 -TRIB2
D
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Cebpa Δ/p30 -TRIB2
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Cebpa fl/p30 -TRIB2
Cebpa Δ/p30 -MigR1
Cebpa Δ/p30 -TRIB2
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Cebpa fl/+ -TRIB2
Cebpa fl/p30 -MigR1
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Cebpa Δ/p30 -MigR1
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Control
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1.3
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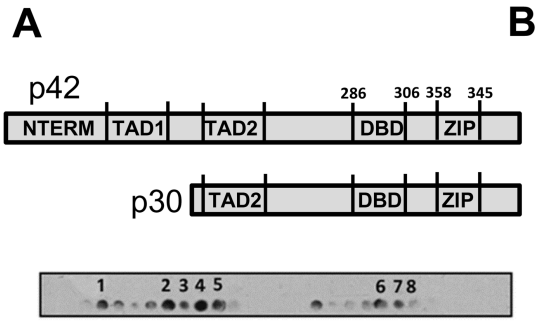
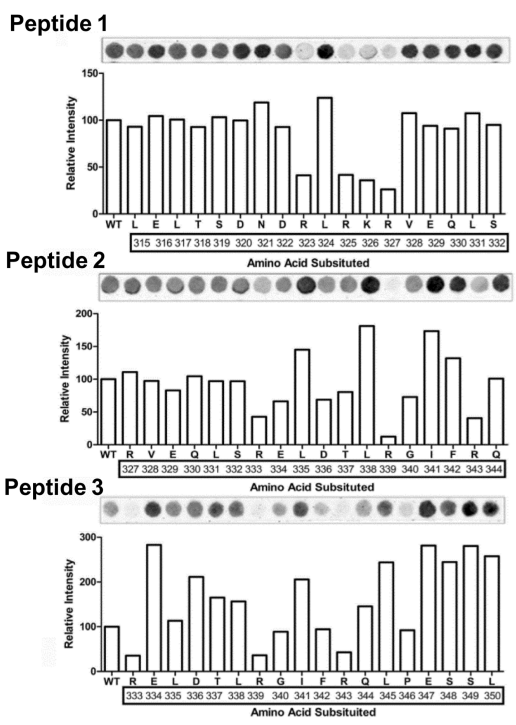
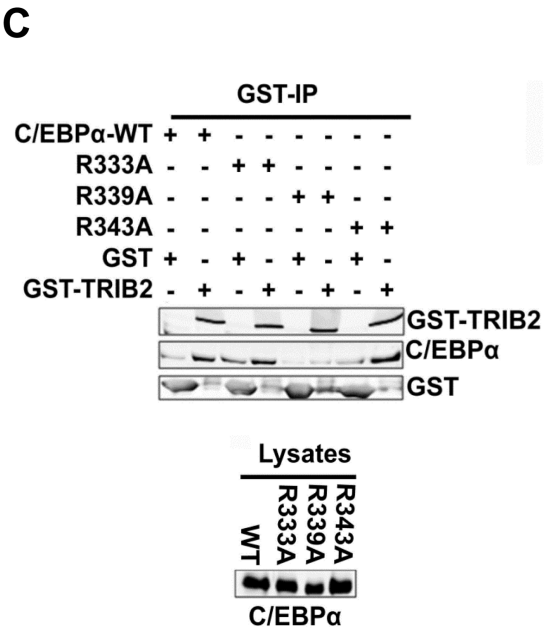
C/EBPα
Actin

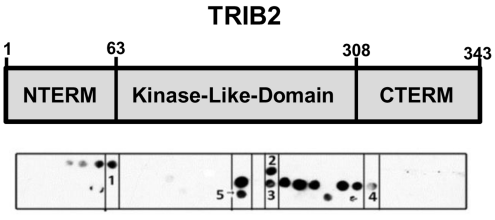


Peptide	Residue	Amino Acid Sequence
1	312-331	L-E-L-T-S-D-N-D-R-L-R-K-T-V-E-Q-L-S
2	326-343	R-V-E-Q-L-S-R-E-L-D-T-L-R-G-I-F-R-Q
3	319-346	Q-L-S-R-E-L-D-T-L-R-G-I-F-R-Q-L-P-E
4	322-349	R-E-L-D-T-L-R-G-I-F-R-Q-L-P-E-S-S-L
5	334-362	D-T-L-R-G-I-F-R-Q-L-P-E-S-S-L-V-K-A
6	325-342	K-R-V-E-Q-L-S-R-E-L-D-T-L-R-G-I-F-P
7	329-345	E-Q-L-S-R-E-L-D-T-L-R-G-I-F-R-Q-L-P
8	331-348	S-R-E-L-D-T-L-R-G-I-F-R-Q-L-P-E-S-S



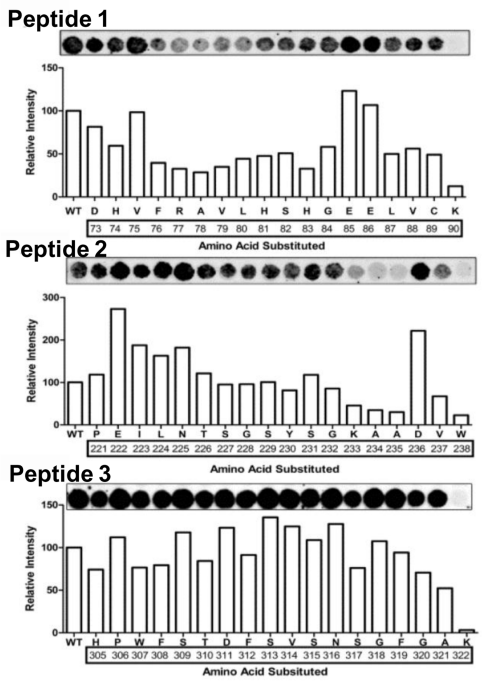


A

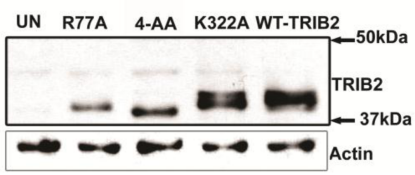


Peptide	Residue	Amino Acid Sequence
1	25-43	E-E-L-S-S-I-R-S-A-E-P-S-Q-S-F-S-P-N
2	69-87	P-L-E-G-D-H-V-F-R-A-V-H-L-H-S-G-E-E
3	189-207	T-R-V-K-L-E-S-L-E-D-A-Y-I-L-R-G-D-D
4	217-234	A-Y-V-S-P-E-I-L-N-T-S-G-S-Y-S-G-K-A
5	300-318	E-I-L-D-H-P-W-F-S-T-D-F-S-V-S-N-S-G

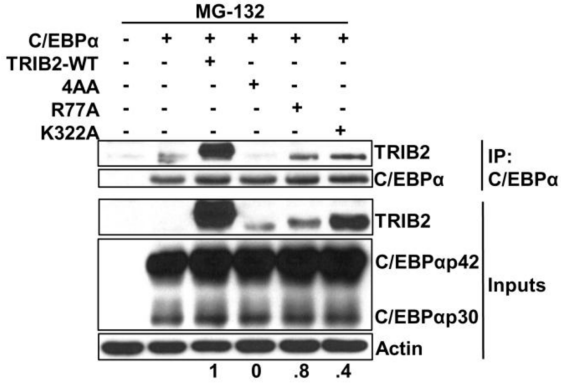
B

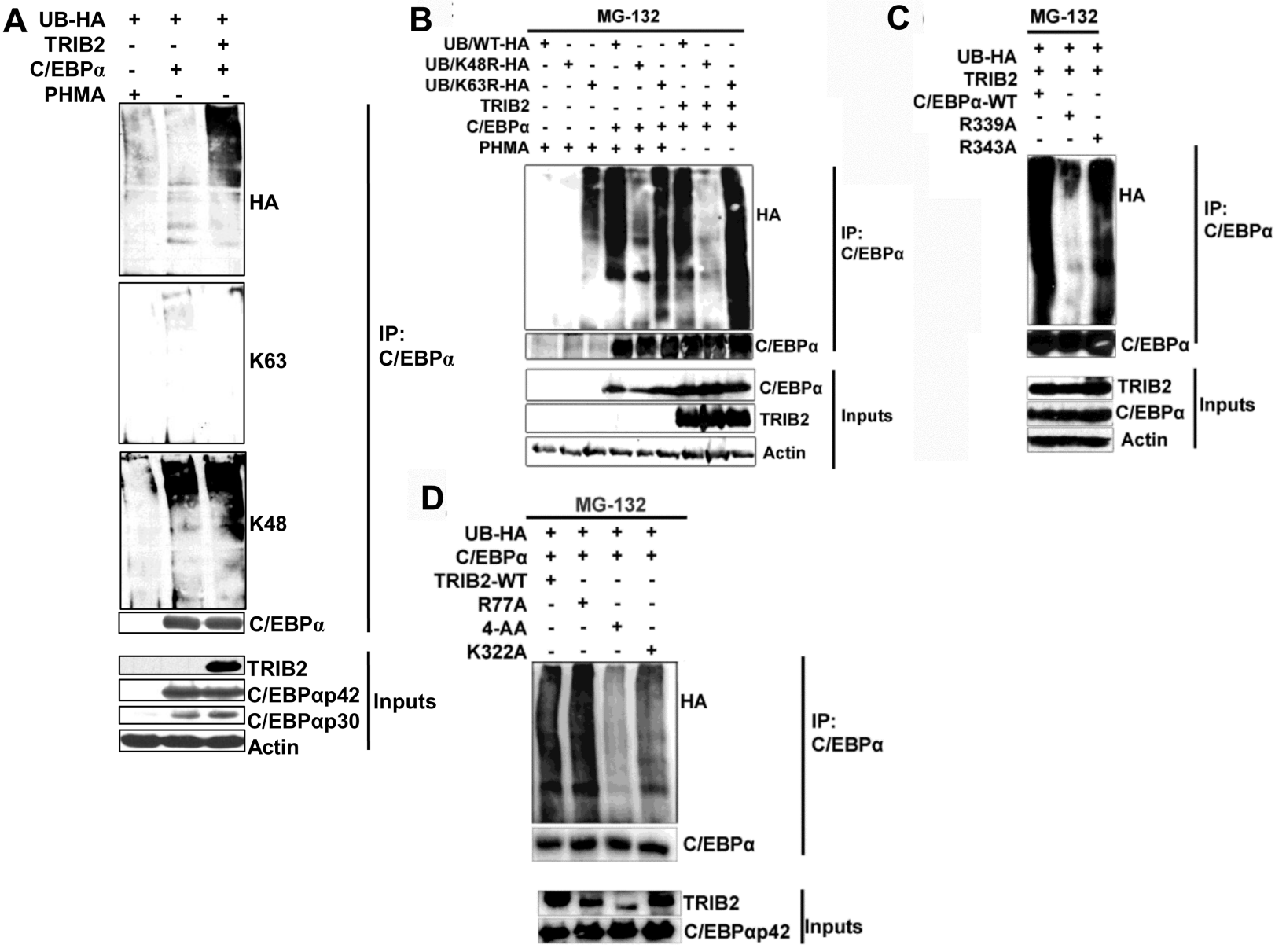


C



D




A
UB-HA + + +
TRIB2 - - +
C/EBPα - + +
PHMA + - -
HA
K63
K48
C/EBPα
IP: C/EBPα
TRIB2
C/EBPαp42
C/EBPαp30
Actin
Inputs
B
MG-132
UB/WT-HA + - - + - - + - -
UB/K48R-HA - + - - + - - + -
UB/K63R-HA - - + - - + - - +
TRIB2 - - - - - - + + +
C/EBPα - - - + + + + + +
PHMA + + + + + + - - -
HA
C/EBPα
IP: C/EBPα
C/EBPα
TRIB2
Actin
Inputs
C
MG-132
UB-HA + + +
TRIB2 + + +
C/EBPα-WT + - -
R339A - + -
R343A - - +
HA
C/EBPα
IP: C/EBPα
TRIB2
C/EBPα
Actin
Inputs
D
MG-132
UB-HA + + + +
C/EBPα + + + +
TRIB2-WT + - - -
R77A - + - -
4-AA - - + -
K322A - - - +
HA
C/EBPα
IP: C/EBPα
TRIB2
C/EBPαp42
Inputs

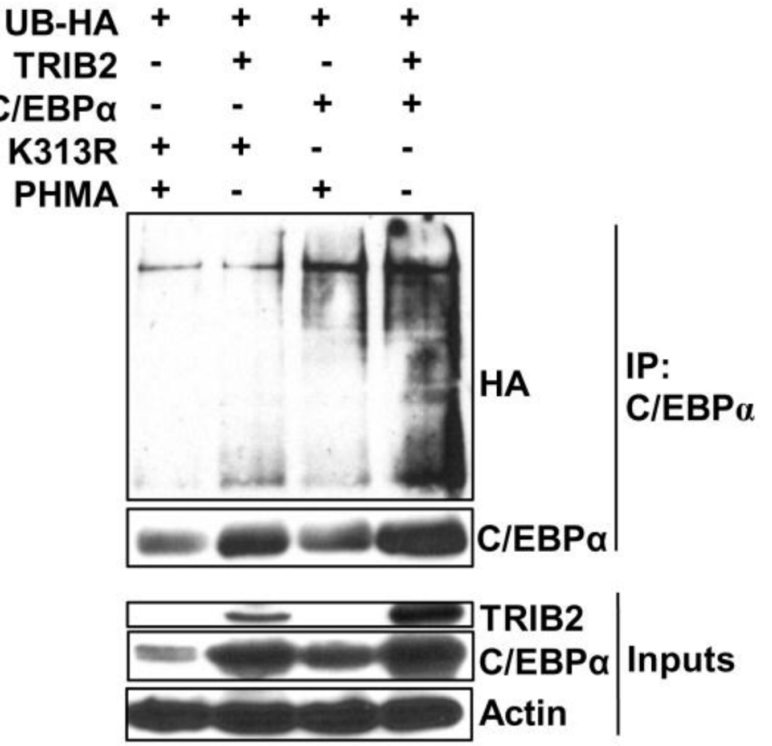
A

Low 0.62 ≤ s ≤ 0.69
Med 0.69 ≤ s ≤ 0.84
High 0.84 ≤ s ≤ 1.00

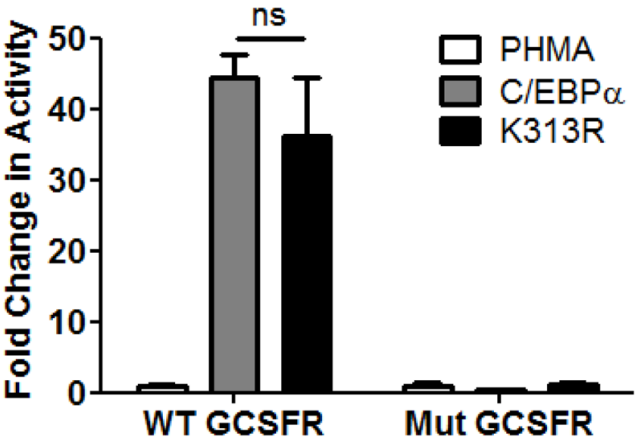
Residue	Score	Ubiquitinated
90	0.76	-Med
92	0.64	-Low
159	0.85	-High
169	0.90	-High
250	0.71	-Med
313	0.77	-Med

B

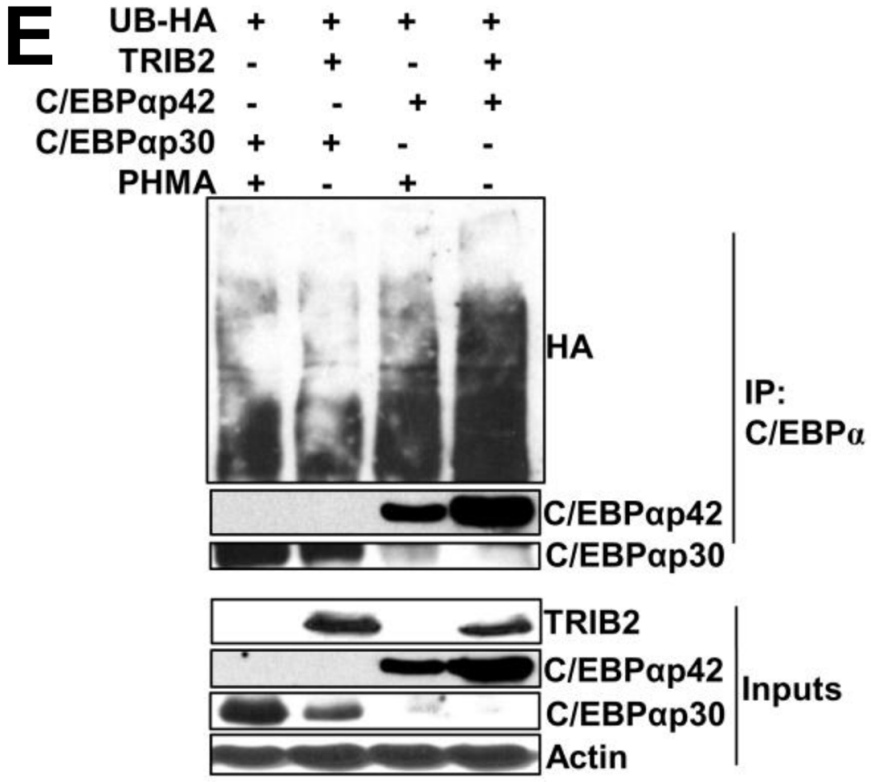
C

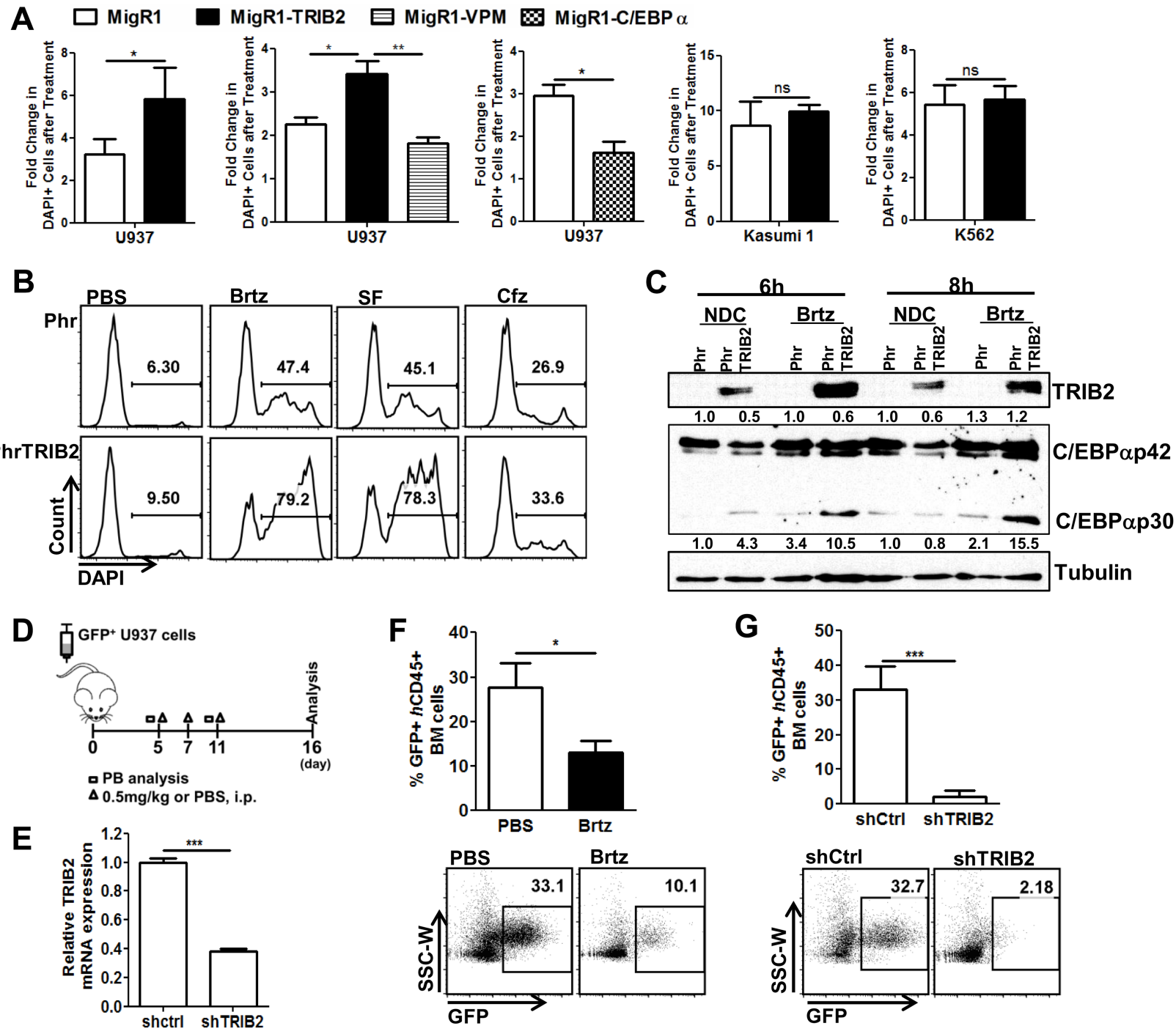


D



E




A
MigR1
MigR1-TRIB2
MigR1-VPM
MigR1-C/EBP α
Fold Change in DAPI+ Cells after Treatment
U937
Kasumi 1
K562
ns
B
PBS
Brtz
SF
Cfz
Phr
PhrTRIB2
6.30
47.4
45.1
26.9
9.50
79.2
78.3
33.6
Count
DAPI
C
6h
8h
NDC
Brtz
Phr
PhrTRIB2
TRIB2
1.0 0.5 1.0 0.6 1.0 0.6 1.3 1.2
C/EBPαp42
C/EBPαp30
1.0 4.3 3.4 10.5 1.0 0.8 2.1 15.5
Tubulin
D
GFP+ U937 cells
Analysis
0 5 7 11 16 (day)
PB analysis
0.5mg/kg or PBS, i.p.
E
Relative TRIB2 mRNA expression
shctrl
shTRIB2
F
% GFP+ hCD45+ BM cells
PBS
Brtz
33.1
10.1
SSC-W
GFP
G
shCtrl
shTRIB2
32.7
2.18