The E3 ubiquitin-protein ligase Triad1 inhibits clonogenic growth of primary myeloid progenitor cells

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Author contribution
CAJE-V performed the subtractive PCR and Northern blot analysis, BAVDR cloned Triad1 and performed database analyses, JAFM, GN and AM performed CFU-GM assays, JAFM and BAVDR cloned and sequenced Triad1 constructs, BAVDR, JAFM and LVE generated the Triad1 antibody and performed Triad1 immunofluorescence and real-time PCR, JAFM performed yeast-two-hybrid studies, Western blot analyses, in vitro ubiquitination, proteasome activity and liquid culture studies, JAFM, TDW, BL, JHJ and BAVDR designed the studies and BAVDR wrote the paper.

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Data deposition
The in this manuscript reported human Triad1 sequences have been deposited in Genbank (accession numbers AF099149-AF099153).
Abstract

Protein ubiquitination plays important roles in a variety of basic cellular processes. Proteins are ubiquitinated by E2-E3 ubiquitin ligase complexes. Depending on the type of ubiquitin chain conjugated, proteins are either targeted for degradation by the proteasome or their activity is specifically altered. We describe a novel conserved nuclear protein, Triad1, which is strongly induced during myeloid differentiation. Triad1 contains a TRIAD motif that harbours 2 RING finger structures. Triad1 binds the E2 ubiquitin conjugating enzyme UbcH7 as well as ubiquitinated proteins and supports the formation of ubiquitin chains that are recognized by the proteasome. The biological function of Triad1 in myelopoiesis was studied by performing CFU-GM assays using retrovirally transduced primary murine bone marrow cells. Triad1 severely inhibited myeloid colony formation. In contrast, two Triad1 RING finger point mutants that failed to bind UbcH7 did not affect colony formation. Moreover, proteasome inhibition counteracted the inhibition of colony formation exerted by wild type Triad1. In liquid cultures, Triad1 did not influence differentiation but strongly inhibited proliferation resulting in a G0/G1 accumulation. We conclude that proteasomal degradation of proteins that are ubiquitinated by Triad1 affects the clonogenic growth of primary myeloid progenitor cells.
Introduction

Granulocytes and monocytes develop from common myeloid progenitor cells through a complex network of cell growth, differentiation and apoptosis regulating factors. Alterations in these processes may cause acute myeloid leukemia, which is characterized by uncontrolled proliferation of immature myeloid cells that fail to differentiate towards mature functional cells\textsuperscript{1,2}. In leukemia mutations occur in genes encoding tyrosine kinases like Flt3, c-kit, N-ras and transcription factors like AML1, CBFB, GATA1, PU.1, C/EBP\textgreek{a} and RAR\textgreek{a}\textsuperscript{1,2}. While the transcription factors predominantly play a role in lineage commitment, activation of tyrosine kinases is thought to result in proliferative and/or survival signals\textsuperscript{1,2}. Recent studies have shown that several proteins involved in myelopoiesis (including proteins mutated in leukemia) are inactivated through ubiquitin (Ub)-proteasomal degradation pathways\textsuperscript{3-7}. This form of targeted protein degradation is accomplished by the covalent conjugation of Ub to substrate proteins, usually in the form of a multi-Ub chain, which marks these proteins for progressive degradation by the 26S proteasome\textsuperscript{8,9}. Protein ubiquitination is catalyzed through a cascade of reactions. Ub is first activated by the ATP-dependent Ub-activating E1 enzyme and subsequently transferred to one of a set of E2 Ub-conjugating enzymes. The E2 enzymes act in conjunction with accessory E3 Ub protein ligases. In the E2-E3 complex, the E3 component binds to protein substrates, allowing the E2 to form a multi-Ub chain linked to a lysine of the substrate protein\textsuperscript{8,9}. Thus, the E3 Ub ligases play a crucial role in this process as these proteins recognize the cellular proteins destined for ubiquitination\textsuperscript{10}. To date, several types of E3 Ub ligases have been described. These include HECT, RING finger, U-box, SOCS-box, F-box and cullin ligases\textsuperscript{11-20}. While the first three types of proteins are actively involved in substrate ubiquitination the last three do not ubiquitinate substrate proteins themselves but are part of larger protein complexes that exhibit Ub ligase activity\textsuperscript{15-19}. Despite the fact that several proteins that play an important role in myelopoiesis are known to be targeted by the Ub-proteasome pathway, only a few E3 Ub ligases involved in myelopoiesis have been described. These include the cullin Ub ligase Cul-4A implicated in myelopoiesis\textsuperscript{4,21}. Cul-4a heterozygous mice have diminished multipotent progenitors and enforced Cul-4A expression inhibits granulocytic differentiation of myeloid PLB-985 cells\textsuperscript{21}. An important target of Cul-4A in myelopoiesis may be the transcription factor HoxA9 that is targeted by Cul-4A for ubiquitination resulting in its proteasomal degradation\textsuperscript{4}. Another group of Ub ligases involved in myelopoiesis are the SOCS proteins. All of these proteins contain a suppressor of cytokine signaling
(SOCS) box that provides in the scaffold for interaction with Ub ligase complexes\textsuperscript{15,16}. One of the main functions of the SOCS box proteins is to recruit substrate proteins to ligase complexes resulting in their ubiquitination. SOCS3 is induced upon G-CSF treatment, binds to the activated G-CSF receptor and negatively regulates G-CSF receptor-mediated STAT activation\textsuperscript{22}. In addition, mice with loss of SOCS3 function in the hematopoietic compartment develop neutrophilia\textsuperscript{23}. Although it is not exactly clear how SOCS proteins inhibit cytokine signaling, it has been suggested that the ubiquitination and subsequent degradation of signaling molecules may be involved in this process\textsuperscript{24}. Finally, another important E3 Ub ligase involved in myelopoiesis is the RING finger protein Cbl. Cbl catalyzes the ubiquitination of several activated receptor tyrosine kinases including the colony-stimulating factor 1 (CSF-1) receptor\textsuperscript{25}. The ligand binding stimulated ubiquitination of these receptors results in their internalization and targeting to lysosomes where they are degraded. As this inhibits these receptors to recycle through early endosomes to the cell surface, Cbl negatively regulates receptor signaling\textsuperscript{25}. Here, we identify a novel E3 Ub ligase, Triad1, to be strongly induced during granulocytic and monocytic differentiation and show that the Ub ligase activity of Triad1 inhibits the proliferation of committed myeloid progenitor cells.
Methods

Subtractive PCR, 5’ and 3’ RACE and classical and real-time RT-PCR

Subtractive PCR was performed using polyA+ mRNA isolated from 0 and 18 hours ATRA treated NB4 cells (PCR-select cDNA subtraction kit, Clontech). Subtracted radiolabeled PCR fragments were isolated from polyacrylamide gel, re-amplified, cloned in pCR2.1 and sequenced. Methods for cDNA synthesis and classical and real-time RT-PCR were as described\textsuperscript{26}. 5’RACE was performed using the Marathon cDNA amplification kit (Clontech) on 1.0 \(\mu\)g of human spleen mRNA. Relevant primers are indicated in supplementary Table 1.

Plasmid constructs

Triad1 coding sequences were PCR amplified using cDNA derived from NB4 cells and cloned in pCR2.1. A 10 aa human \textit{myc} epitope was introduced at the 3’ end of the coding sequence of \textit{Triad1} by PCR and cloned into pSG5 and non-tagged Triad1 was cloned into pcDNA3.1. Triad1 coding sequences were generated by PCR and cloned in pEGFP-C1 to generate GFP-Triad1, pFastBacHT to generate His-Triad1 in Hi5 cells (Baculovirus expression system, Invitrogen) and in pGEX-2TK to generate GST-Triad1. For retroviral transduction Triad1 coding sequences were inserted into the retroviral pLRZS vector. For yeast-two-hybrid assays sequences coding for Triad1 aa 1-119 and 109-493 were cloned in pGAD-C. All primers used for cloning purposes and for generating the Triad1 point mutants H158A and C161A by site-directed mutagenesis are indicated in supplementary Table 1. The integrity of all Triad1 sequences was checked by sequence analysis.

Immunofluorescence, Northern blotting, GST- and His-captures and immunoprecipitations

In these studies antibodies against CD34, CD3, CD14, CD15, CD45 (Beckman Coulter), Mac1 (BD Biosciences), GFP (7.1/13.1 Roche), \textit{myc} (9E10, Santa Cruz), UbcH7 and UbcH9 (Transduction laboratories), Ub (6C1,Sigma), Flag (M2, Sigma), annexinV (AlexaFluor 647, Molecular Probes) and Triad1 were used. Triad1 antibodies were generated by immunizing rabbits with a KLH-coupled peptide corresponding to Triad1 aa 473-486, followed by affinity purification using peptides immobilized on Affigel 10 columns (Biorad). Human hematopoietic subsets were isolated by FACS with appropriate antibodies.
Granulocytes were selected based on CD45 positivity and scatter plot. Cell purity was confirmed to be more than 95% by flow cytometric analysis. For immunofluorescence experiments cells were stained with indicated primary antibodies followed by staining with a secondary antibody. For detection of endogenous Triad1 by multicolor flow cytometry (Cytomics FC500, Becton Dickinson), cells were stained with a Triad1 antibody using fix-and-perm approaches as described followed by blocking of cells with rabbit serum and subsequent staining for relevant surface markers. For Triad1 Northern blot hybridization 10 µg total RNA isolated from NB4 cells treated with ATRA (10^-6 M) for various time points or 1.0 µg mRNA from hematopoietic cells was hybrized using Triad1 clone 14-5 as a probe. For GST-captures, 10 µg of immobilized GST or GST-Triad1 was incubated with cell lysates from 10^7 NB4 cells. For capture of Histagged proteins, lysates from transfected 293T cells were incubated with His-select beads (Sigma) under denaturing conditions (6M guanidiumchloride). For immunoprecipitations, lysates from transfected COS cells were incubated with indicated antibodies. Bound proteins were washed, separated by SDS-PAGE, immunoblotted and stained with relevant antibodies.

Yeast-two-hybrid analysis

For yeast-two-hybrid analyses UbcH bait and Triad1 prey constructs were transfected to the yeast strain AH109 (Clontech). This strain contains two selectable markers (histidine and adenine) and a lacZ reporter. A positive interaction in yeast was defined by growth of blue colonies 4 days after plating cells on leucine, tryptophane, adenine and histidine deficient SD medium containing X-α-gal.

Proteasome activity measurements and in vitro ubiquitination assays

Proteasome activity was determined in primary murine bone marrow cells after treatment with 10^-8 M MG132. For measurements, 67.5 µg protein lysates (concentration determined using Bradford assay) were incubated with the proteasome substrate Suc-Leu-Leu-Val-Tyr-AMC (Boston Biochem) for 2 hrs at 37°C as described. AMC liberated from the substrate by proteasome activity was determined by measuring fluorescence (Fluorstar, BMG, Isogen Life Sciences). For in vitro ubiquitination assays His-Triad1 was isolated from Hi5 cells (Baculovirus Bac-to-Bac system, Invitrogen) using His-select spin columns followed by elution using 250 nM imidazole. Imidazole was removed from His-Triad1 protein
eluates using a Microcon YM-10 filter (Amicon) and protein concentrations were determined with the Bradford assay. Ubiquitination assays were performed using 1.6 μg reticulocyte fraction IIA (Boston Biochem), bovine Ub (Sigma) or recombinant UbK48-only (Boston Biochem) with freshly prepared His-Triad1 for 60 min at 37°C followed by staining of ubiquitinated proteins in Western blot analysis using a Ub specific antibody (C61, Sigma) as described30.

**Clonogenic assays (CFU-GM) and myeloid cell proliferation**

Retroviral vectors (pLZRS) were transfected to retroviral producer phoenix cells (kindly provided by Dr. G. Nolan). Primary murine bone marrow cells isolated from femurs from 6 weeks old female CBA/CA mice were prestimulated for two days with SCF, IL-12, IL-3, Flt-3, TPO and α-TGFβ and subsequently infected with retroviruses on retronectin coated dishes. After 24 hrs GFP positive cells were sorted by FACS and seeded in semi-solid medium (5,000 cells/well) containing pokeweed mitogen stimulated mouse spleen conditioned medium, horse serum and SCF. Colonies were counted 5 days later yielding between 83 to 125 colonies after CFU-GM of empty vector transduced cells and morphologically analyzed after May-Grünwald-Giemsa staining. To determine proliferation in liquid assays, 5,000 cells were seeded in 96-well plates using the above-mentioned medium without agar. Relative cell numbers were determined by FACS using fluorescent beads. Apoptosis and DNA histograms were determined by flowcytometer using AnnexinV and propidium iodide staining, respectively. Percentages of cells in various phases of cell cycle were determined using ModFit software (Verity).
Results

Identification of genes induced during granulocytic differentiation

Acute promyelocytic leukemia cells can be forced to differentiate along the granulocytic lineage by the vitamine A derivative all-trans retinoic acid (ATRA). To identify genes involved in granulocytic differentiation we performed a subtractive PCR screen using RNA isolated from promyelocytic leukemia (NB4) cells before and 18 hours after ATRA treatment. Northern blotting using one of the subtracted clones (clone 14-5) showed the induction of a 4 Kb mRNA in NB4 cells 24 hours after ATRA treatment to reach maximum levels at 48 hours (Figure 1a). The subtracted clone appeared identical to human randomly cloned expressed cDNA sequences identified in database searches (not shown). By using these sequences in database walks a 3.9 Kb cDNA contig was constructed. This contig was confirmed by amplification of overlapping RT-PCR fragments and 5'- and 3'-RACE (Figures 1b and c). Sequence analysis of the 3.9 Kb transcript identified a poly-adenylation signal (ATAAA) 24 bp upstream of the 3’ end of the transcript and a translational start codon at cDNA position 145. This start codon is accompanied by a consensus Kozak sequence and is followed by a 1479 bp open reading frame (ORF, supplementary Figure 1). The coding sequences were confirmed by sequence analysis of 12 independent clones (Genbank accession number AF099149). In line with multiple bands observed in RT-PCR (see Figure 1b), three Triad1 clones contained different insertions of extra sequences present at cDNA position 399 possibly representing splice variants (Genbank accession numbers AF099150, AF099151, and AF099152).

Identification of Triad1

The 1479 bp ORF in the 3.9 Kb transcript predicts the formation of a 493 aa protein (Figure 1d). We have called this protein Triad1 referring to its cysteine-histidine rich TRIAD motif. The TRIAD (two RING fingers and DRIL) motif consists of two RING fingers that flank the DRIL (double RING finger linked) domain. The DRIL (a.k.a. IBR) domain resembles other cysteine-rich motifs as the LAP/PHD, LIM and RING finger domains. Recent data indicate that the C-terminal RING finger (RING2) of the TRIAD motif has a distinct topology from the classical RING finger structure and that it binds only one zinc atom instead of two. Further Triad1 protein analysis identified an acidic N-terminal region, two C-terminal coiled coil regions and two putative nuclear localization signals. To determine whether Triad1 is conserved during
evolution we performed database searches and identified full length murine, *D. melanogaster*, and *C. elegans* orthologs (supplementary Fig. 1). The conservation in the last 231 aa C-terminal part of Triad1 between *D. melanogaster* and *C. elegans* compared to human Triad1 is very high being 80% and 64% identical, respectively. Comparison of complete murine and human Triad1 shows that the proteins are 98% identical. The strong evolutionary conservation of Triad1 suggests that it exerts important biological functions.

**Triad1 is induced during normal granulocytic/monocytic differentiation**

Triad1 gene expression is induced during forced granulocytic differentiation of promyelocytic leukemia (NB4) cells. To determine the relevance of this observation we analyzed *Triad1* expression in normal human hematopoiesis. *Triad1* Northern blot hybridization using RNA isolated from total bone marrow, mature granulocytes and T-cells revealed strongest *Triad1* expression in granulocytes (Figure 2a). In agreement, *Triad1* quantitative RT-PCR detected high expression in mature granulocytes and low expression in T-cells and immature CD34+ cells, indicating that *Triad1* is induced during maturation of immature CD34+ cells towards mature granulocytes (Figure 2b). Further expression studies showed detectable *Triad1* expression by real-time PCR in a large set of human tissues tested indicating that the function of Triad1 is probably not limited to hematopoiesis (Figure 2c).

To determine the subcellular localization of Triad1 a C-terminally myc epitope-tagged Triad1 (Triad1-myc) expression vector was transfected to L88/5 stromal cells. Indirect immunofluorescence (IF) using the myc epitope recognizing antibody 9E10 revealed a clear nuclear diffuse staining with exclusion of the nucleoli (Figure 3a). In cells with highest IF levels, some cytoplasmic staining was also observed. To determine the endogenous Triad1 expression in primary hematopoietic cells an affinity purified Triad1 antibody was used in indirect IF. Analysis of immature CD34+ and T-cells revealed a weak nuclear staining (Figure 3b). In contrast, a strong nuclear localization was observed in both granulocytes and monocytes. While the localization in monocytes was nuclear diffuse, a ring-shaped nuclear organization was observed in granulocytes (Figure 3b). To determine at what stage Triad1 was induced during myelopoiesis we stained fresh bone marrow and leukopheresis samples with a Triad1 antibody and analyzed the cells by flowcytometry. Co-staining with CD34 (immature progenitors), CD3 (T-cells), CD14 (monocytes) or CD15
granulocytes) showed that Triad1 is lowly expressed in immature CD34+ and T-cells, and that the expression gradually increases upon maturation towards committed myelo-monocytic progenitors to reach highest levels in mature monocytes and granulocytes (Figure 3c and supplementary Figure 2). Together, these data indicate that Triad1 is induced at the mRNA and protein level during differentiation of immature blood cells towards both monocytes and granulocytes and that it localizes predominantly to the nucleus in primary human hematopoietic cells.

**Triad1 is an E3 Ub ligase**

The RING finger structure provides in an interface for interaction with E2 Ubcs and all RING finger proteins that bind Ubcs exhibit Ub ligase activity\(^20\). Since Triad1 harbors two RING fingers we tested whether it binds to Ubcs using yeast-two-hybrid assays. For these experiments two Triad1 prey-fragments were used, one coding for amino acids 1-119, Triad1\(^{[1-119]}\), lacking the TRIAD structure and one for amino acids 109-493, Triad1\(^{[109-493]}\), including the TRIAD structure. No interaction was observed between Triad1\(^{[1-119]}\) and a panel of Ubcs\(^{28}\) tested including cdc34, UbcH5, -6, -7, -8, -9, -10 and 13 (not shown). In contrast, a clear interaction between Triad1\(^{[109-493]}\) and UbcH7 was observed as indicated by growth of yeast cells on medium lacking both histidine and adenine (Figure 4a). When less stringent yeast-two-hybrid conditions were used (histidine selection only) an interaction between Triad1\(^{[109-493]}\) and UbcH6 and UbcH13 (a.k.a. Bendless) was also found (data not shown). Since endogenous UbcH7 is readily detectable in NB4 cells we incubated cell lysates from these cells with GST-Triad1 to confirm the UbcH7 interaction as observed in yeast-two-hybrid assays. Bound proteins were stained with an UbcH7 antibody in Western blot analysis, showing that Triad1 efficiently binds endogenous UbcH7 from NB4 cells (Figure 4b). Staining for UbcH9 in these experiments was negative while UbcH9 is also easily detectable in NB4 cells by Western blotting (not shown). To test whether the N-terminal RING finger was involved in UbcH7 binding we constructed two independent Triad1 RING finger point mutants in which one of the predicted zinc coordinating amino acids were changed into alanine (H158A, C161A). Mutations were introduced in the Triad1 prey-construct (Triad1\(^{[109-493]}\)) and used in yeast-two-hybrid assays. While normal Triad1\(^{[109-493]}\) interacts with UbcH7 in yeast-two-hybrid assays, both point mutants did not interact with UbcH7 indicating that the N-terminal RING finger of Triad1 is essential for UbcH7 binding (Figure 4a). To further test whether Triad1 might function as
Ub ligase we determined whether Triad1 binds ubiquitinated proteins. To this end, Triad1-myc and Flag-tagged Ub (Flag-Ub) were co-expressed in the presence or absence of proteasome inhibitors followed by myc immunoprecipitation (IP). Triad1 staining of myc-immunoprecipitated proteins in Western blot analysis revealed that proteasome inhibition resulted in stabilization of Triad1. Flag staining of proteins bound to Triad1 detected a smear of ubiquitinated proteins that appeared more intense when transfected cells were treated with a proteasome inhibitor, suggesting that Triad1 binds ubiquitinated proteins (Figure 4c). As the observed smear might represent exclusively ubiquitinated Triad1 species instead of ubiquitinated proteins bound to Triad1, we repeated the experiment using GFP-Triad1 and Flag-Ub. Subsequent GFP IP followed by Triad1 staining in Western blot analysis revealed the presence of unmodified Triad1 while Flag staining revealed a smear of ubiquitinated proteins, including proteins with sizes smaller compared to Triad1. As the smaller proteins do not represent Triad1 we concluded that Triad1 binds ubiquitinated proteins (Figure 4d).

To further test whether Triad1 functions as E3 Ub ligase, we performed an in vitro ubiquitination assay using reticulocyte fraction IIA (enriched in E1 and E2 activity and devoid of Ub and proteasome activity), with or without Ub and His-Triad1. Staining of reaction mixtures with a Ub specific antibody in Western blot analysis revealed the presence of Triad1-dependent ubiquitinated proteins (Figure 4e). Triad1 binds to UbcH7. In concert with E3 Ub ligases, UbcH7 supports the formation of Ub chains linked through lysine 48 (K48) that are recognized by the proteasome. To test whether Triad1 can catalyze the formation of Ub chains linked through K48, we also used recombinant Ub carrying only one lysine residue at position 48 (UbK48-only), showing that Triad1 catalyzes the formation of Ub chains linked through K48. The interaction between Triad1 and UbcH7 as well as ubiquitinated proteins and the in vitro ubiquitination assay strongly suggest that Triad1 functions as E3 Ub ligase that may target proteins for proteasomal degradation.

Triad1 is targeted by the Ub -proteasome pathway

In addition to their substrates, many Ub ligases are ubiquitinated themselves. Ubiquitination of Triad1 was studied by co-expressing Triad1 and His-tagged Ub (His-Ub) followed by selection of His-tagged proteins under denaturing conditions. Staining with a Triad1 specific antibody detected discrete bands with higher molecular weight compared to unmodified Triad1 indicating that Triad1 is ubiquitinated (Figure 5a). Proteasome inhibition resulted in an increase in ubiquitinated Triad1 forms, indicating that
ubiquitinated Triad1 is subject to proteasomal degradation. Similar results were obtained when Triad1 and Flag-Ub were co-expressed followed by Flag IP under non-denaturing conditions (Figure 5b). The discrete Triad1 Ub-modified forms observed in these IPs combined with the smear of ubiquitinated proteins detected upon Triad1 IP when cotransfected with Ub as shown in Figures 4c and d further indicate that, apart from being ubiquitinated itself, Triad1 also binds ubiquitinated proteins.

*The Ub ligase activity of Triad1 regulates myelopoiesis*

To determine the biological relevance of the induction of Triad1 during myeloid differentiation, we tested whether Triad1 expression affected the clonogenic growth of primary immature murine bone marrow cells in CFU-GM assays. Primary murine bone marrow cells were transduced with pLZRS-derived viruses containing a GFP-IRES-Triad1 sequence allowing for separate GFP and Triad1 expression. Fractions with low, intermediate and high GFP expression were sorted. GFP expression correlated with Triad1 expression as shown by Western blot analysis (Figure 6a and supplementary Figure 3a). Compared to empty vector controls (GFP alone), Triad1 expression resulted in a concentration-dependent inhibition of clonogenic growth. While low Triad1 expression did not inhibit clonogenic growth, intermediate Triad1 expression resulted in an inhibition of over 60%, whereas highest Triad1 expression resulted in a strong inhibition of clonogenic growth of over 80% (Figure 6a). In addition, colonies derived from high and intermediate Triad1 expressing cells were significantly smaller compared to empty vector controls (supplementary Figure 3b). To determine whether Triad1 affects myeloid differentiation, colonies were recovered from semi-solid medium and morphologically analyzed. This revealed a modest Triad1-dependent increase in the percentage of granulocytes and decrease in erythroblasts compared to empty vector transduced cells (Figure 6b).

The observed inhibition of clonogenic growth suggests a role for Triad1 in the development of immature committed murine bone marrow cells towards mature myeloid cells. In many Ub ligases the RING finger is essential for Ubc binding as well as Ub ligase activity. Mutations in RING fingers abrogating the interaction with Ubecs result in impaired ubiquitination. To test whether the Ub ligase activity of Triad1 was involved in the clonogenic inhibition of myeloid progenitor cells, we introduced the H158A and C161A RING finger mutations that abrogate UbcH7 binding in full-length Triad1 coding sequences in the retroviral
vectors and used these constructs in CFU-GM colony assays. Strikingly, in contrast to normal Triad1, expression of the Triad1 point mutants did not affect the clonogenic growth of primary immature cells (Figure 6c). Because the mutant proteins were expressed at levels comparable to wild type Triad1 as shown by Western blot analysis this indicates that the N-terminal RING finger of Triad1 plays an important role in the regulation of proliferation and or differentiation of myeloid progenitor cells. In E3 Ub ligases, the RING finger structure has been shown to be essential for Ub ligase activity. As UbcH7 can catalyze the formation of Ub chains resulting in protein degradation and the Triad1 RING finger mutations abrogate the inhibitory effect on clonogenic growth, our data suggest that the ubiquitination and subsequent proteasomal degradation of Triad1 target proteins results in clonogenic inhibition. To test this, we determined whether the inhibition of clonogenic growth exerted by Triad1 could be relieved by proteasome inhibition. For these experiments $10^{-8}$ M of the proteasome inhibitor MG132 was used because at this concentration limited toxicity on empty vector transduced cells was observed in liquid assays (Figure 6d). At this concentration, the bone marrow cells showed an increase in ubiquitinated species and decreased proteasomal activity (Figures 6e and f, respectively). Interestingly, proteasome inhibition partially reversed the Triad1-induced suppression of colony formation (Figure 6g). When corrected for the suppressive effects of MG132 on empty vector transduced cells, an over two-fold increase in Triad1 positive colonies was observed when the proteasome was inhibited (Figure 6h). Moreover, the colonies derived from MG132 treated Triad1 transduced cells were significantly larger as compared to non-treated cells (supplementary Figure 3b). In addition to the proteasome, MG132 inhibits other proteases. To rule out the possibility that the increase in colony numbers of Triad1 transduced cells after treatment with MG132 depended on other mechanisms rather then inhibition of the 26S proteasome, we repeated the experiments using other more specific proteasome inhibitors including PS-341, epoxomycin, and lactacystin. This yielded similar results as observed for MG132 (Figure 6h). Thus the growth inhibitory effect exerted by Triad1 relies on the N-terminal RING finger and can be counteracted by proteasome inhibition indicating that degradation of Triad1 targets are implicated in myelopoiesis.

To further investigate how Triad1 affected CFU-GM colony formation, we cultured Triad1 retrovirally transduced bone marrow cells in liquid medium and measured proliferation, cell cycle progression (DNA histograms), differentiation and apoptosis. Compared to empty vector control, Triad1 expression resulted in
a dramatic inhibition of proliferation (Figure 7a). This was accompanied by a marked increase in the percentage of cells in G0/G1 phase, and an increase in annexin V positivity (Figures 7b and c). In these experiments no significant changes in myeloid differentiation as indicated by Mac1 positivity was observed (Figure 7d). Thus, the inhibition of clonogenic growth by Triad1 is probably caused by a cell cycle arrest associated with increased apoptosis. Based on these findings we conclude that the ubiquitination and degradation of Triad1 target proteins contributes to the development of early committed myeloid progenitors towards mature monocytes and granulocytes.
Discussion

The modification of proteins with ubiquitin plays an important role in a variety of basic cellular processes, including cell division, differentiation and apoptosis. Protein poly-ubiquitination linked through lysine 48 of Ub results in degradation of tagged proteins by the 26S proteasome. Proteins are ubiquitinated by E2-E3 Ub ligase complexes. The E3 Ub ligases play a key-role in this process as these enzymes determine which cellular proteins are ubiquitinated. We report here that the E3 Ub ligase Triad1 is induced during both granulocytic and monocytic differentiation (Figures 1-3). Triad1 contains two RING finger structures. By providing an interface for interaction with E2 Ub conjugating enzymes, the RING finger constitutes an essential domain in many E3 Ub ligases. In line with this, we found that Triad1 physically interacts with the E2 Ub conjugating enzyme UbcH7 through its N-terminal RING finger and to ubiquitinated proteins (Figure 4a-d). Moreover, in vitro ubiquitination experiments showed that Triad1 supports ubiquitination of both wild type and UbK48-only (Figure 4e). Ub chains linked through lysine 48 are efficiently recognized by the proteasome resulting in substrate degradation. This indicates that Triad1 may mark proteins for proteasomal degradation.

Triad1 exhibits a predominantly nuclear staining in all cell types tested (Figure 3). In stromal cells (L88/5) and in primary monocytes the nuclear staining is diffuse, while a ring-shaped localization is observed within the nuclear lobes of primary granulocytes (Figure 3). Although these data may suggest that Triad1 substrates are primarily nuclear proteins a protein-interaction map of the Drosophila proteome revealed that Drosophila Triad1 (CG5709) binds in addition to Ubc7 (CG12799) to both cytoplasmic and nuclear proteins. These include cytoplasmic orthologs of signaling molecule Traf3 involved in TNF-receptor mediated signal transduction and E3 ub ligase Hakai involved in ubiquitination of E-cadherin (respectively, CG4394 and CG10263), but also nuclear proteins including the ortholog of the transcription factor HOX11L2 (CG7937) implicated in acute lymphoblastic leukemia development and the ortholog of the helix-loop-helix transcription factors NHLH1 and NHLH2 (CG3052) implicated in neurogenesis.

Forced Triad1 expression in primary murine bone marrow cells inhibited colony formation in CFU-GM assays. Two N-terminal RING finger point mutants that are unable to bind the E2 UbcH7 did not inhibit clonogenic growth indicating that the N-terminal RING finger of Triad1 is involved in the inhibition of clonogenic growth (Figures 6a and c). As the RING finger is involved in the catalytic activity (i.e. Ub
conjugation)\(^{13,20}\) in virtually all E3 RING Ub ligases, this suggested that the ubiquitination of Triad1 target proteins caused the inhibition of clonogenic growth. In conjunction with E3 Ub ligases, UbcH7 is known to catalyze the formation of Ub chains that result in proteasomal degradation of tagged proteins. To further study the clonogenic inhibition of Triad1 in CFU-GM assays we repeated the experiments in the presence of proteasome inhibitors and found that this partially reversed the effects of Triad1 (Figures 6g and h). We conclude that proteasomal degradation of Triad1 targets plays an important role in myelopoiesis. In liquid cultures of transduced bone marrow cells, Triad1 expression resulted in a strong inhibition of proliferation while differentiation remained unaltered. The inhibition of proliferation was associated with a G0/G1 accumulation and increased apoptosis (Figure 7).

Triad1 is a member of the class of TRIAD (or RING-IBR-RING) proteins\(^ {31,32}\). Since the description of this class of proteins several TRIAD proteins have been reported to function as E3 Ub ligase including Triad3, Parkin, Parc and p53RFP\(^ {39-42}\). These Ub ligases are involved in various biological processes including immunity, neuronal development, and p53 functioning. Triad3 targets Toll-like receptors 4 and 9 for ubiquitination\(^ {40}\). By sensing antigens, Toll-like receptors play a role in both innate and adapted immunity. Ligand binding of these receptors result in NF-\(\kappa B\) activation and Triad3 regulates the ubiquitination and concomitant turnover of these receptors thereby controlling the intensity and duration of Toll-like receptor function\(^ {40}\). Another well-studied TRIAD protein is Parkin which is, like Triad1, highly expressed in fetal brain and involved in neuronal development\(^ {43}\). Congenital mutations in Parkin cause juvenile parkinsonism and acquired loss-of-heterozygosity of Parkin has been described in various human malignancies\(^ {44-48}\). The TRIAD proteins Parc and p53RFP are involved in p53 function\(^ {41,42}\). Parc functions as E3 Ub ligase that binds to p53. Although Parc does not ubiquitinate p53, it sequesters this protein in the cytoplasm. The other TRIAD protein, p53RFP, is a p53 target gene. p53RFP binds to the cyclin-dependent kinase inhibitor p21waf1. As p53RFP overexpression results in reduced p21waf1 levels, p53RFP might function as Ub ligase for p21waf1\(^ {41}\). The majority of the reported TRIAD proteins have been shown to interact with UbcH7, which is in line with the observation that Triad1 also interacts with UbcH7. By using less stringent conditions in yeast-two-hybrid assays, we also observed an interaction between Triad1 and UbcH13 (not shown). Interestingly, UbcH13 catalyzes the formation of Ub chains (linked through lysine 63) that are not recognized by the proteasome but that alter the activity of proteins\(^ {49-51}\). Two recent studies have shown that
the TRIAD protein Parkin also binds to UbcH13 in addition to UbcH7 and that it can catalyze the formation of both lysine 48- and 63-linked Ub chains\textsuperscript{30,62}. As Triad1 may bind UbcH13 it will be important to test whether it also exhibits activity that supports ubiquitination linked through lysine 63 of Ub. Nevertheless, the inhibition of clonogenic growth exerted by Triad1 described here depends at least for a part on targeting proteins for proteasomal degradation because proteasome inhibition partially reversed the effects of Triad1. To further understand how the Ub-proteasome pathway contributes to myelopoiesis, it will be of great interest to identify which cellular proteins are targeted for ubiquitination by Triad1.
Acknowledgments

We thank Dr. Martin Scheffner for valuable discussions and help in setting up ubiquitination assays and for providing us with pcDNA3.1-His-Ub, Herman Swarts for generating Triad1 Baculovirus, Dr. Philip James for two hybrid vectors, Dr. Yukio Okano for Ubc yeast-two-hybrid bait constructs, Dr. Toshiaki Suzuki for pcDNA3.1-Flag-Ub, Dr. G. Nolan for pLZRS and the phoenix cell line ϕ-NX-A.
References


Figure legends

Figure 1. Identification of Triad1. A. Northern blot hybridization using subtracted clone 14-5 detecting the ATRA-mediated induction of a 4 Kb transcript in NB4 cells (time of ATRA incubation is given at the top in hours). Rehybridization with a GAPDH probe indicates equal loading. B. Triad1 5' and 3' RACE and RT-PCR fragments as depicted in Figure 1C (lane numbers correspond to fragment numbers in Figure 1C). Alternative splicing results in the detection of two RT-PCR fragments (lane 2). RACE and PCR fragments 1-5 were amplified using primer combinations indicated in supplementary Table 1. C. Schematic representation of the 3.9 Kb ATRA induced Triad1 transcript (see supplementary Fig. 1 for sequences). Indicated are the 1479 ORF (open box) and overlapping cDNA and 5' and 3' RACE fragments as well as the localization of fragment 14-5 representing the clone identified in the subtractive PCR screen. D. Schematic representation of Triad1 protein domains. A = acidic domain; R1 and R2 are RING finger structures; D = DRIL domain; CC = coiled coil domain.

Figure 2. Triad1 is highly expressed in granulocytes. A. Northern blot hybridization using Triad1 clone 14-5 as a probe detects high Triad1 expression in mature granulocytes compared to complete bone marrow and T-cells. B. Triad1 quantitative RT-PCR detects high expression in granulocytes and low expression in immature CD34+ and T-cells. Data are from two samples from unrelated healthy volunteers. Expression in CD34+ cells was set at 1.0 and values were normalized for PBGD expression as described. C. Triad1 real-time PCR detects high expression in peripheral blood compared to bone marrow (expression in total bone marrow was set at 1.0). Amongst non-hematopoietic tissues highest expression was observed in fetal brain.

Figure 3. Triad1 is a predominantly nuclear protein and is highly expressed in mature granulocytes and monocytes. A. IF of Triad1-myc reveals a nuclear diffuse localization with exclusion of nucleoli in L88/5 cells. In cells with strong nuclear staining some cytoplasmic staining is also observed. Phase contrast image (phase contrast) indicating cellular structures are given at the right. Immunofluorescence of empty vector transduced cells did not reveal significant staining (not shown). B. A Triad1 specific affinity
purified antibody detects low endogenous Triad1 expression in CD34+ and T-cells and high nuclear expression in mature granulocytes and monocytes. DNA was stained with DAPI and merged pictures are indicated. Representative results from one of three unrelated healthy volunteers are indicated. Triad1 IF detects in granulocytes a ring-shaped nuclear sublocalization. Strongest Triad1 expression is observed in the DAPI dull region (arrows) within the lobes of the nuclei. As negative control, cells were stained with pre-immune serum taken prior to peptide vaccination, revealing no significant signals (not shown). C. Intracellular Triad1 was stained on fresh bone marrow (n=1) or leukopheresis material (n=1) and analyzed by flowcytometer yielding similar results. The figure shows the mean fluorescence of indicated compartments of the leukopheresis sample. Lowest expression was observed in CD3+ and CD34+ cells. Within the CD14+ fraction lowest expression was observed in the more immature fraction (Mo1) compared to mature monocytes (Mo2). Likewise, in immature myelocytic fractions (Gr1 including promyelocytes and Gr2 including myelocytes/metamyelocytes) lower expression was observed compared to mature granulocytes (Gr3). Cells were co-stained for Triad1 and CD45 in combination with CD34, CD3, CD14 or CD15. Within the CD14+ and CD15+ populations immature and mature compartments were defined based on CD45 staining and side scatter as described (see also supplementary Figure 2)54. Staining with pre-immune serum resulted in 30-100-fold lower signals (not shown).

**Figure 4. Triad1 is an Ub ligase. A.** Human Triad1 interacts with UbcH7 in yeast-two-hybrid assays as shown by growth on double histidine and adenine selection (left plate). The Triad1 mutants H158A and C161A fail to bind to UbcH7 (right plate). Used bait- (left plate) and prey-constructs (right plate) are indicated. EV = empty vector control. B. GST-Triad1 efficiently captures UbcH7 from human NB4 cell lysates while GST alone does not. C. Co-transfection of Triad1-myc and Flag-Ub followed by myc IP and Flag staining detects a smear of ubiquitinated proteins [X-(ub)n] bound to Triad1. The amount of ubiquitinated proteins bound to Triad1 is more intense when cells were treated with a proteasome inhibitor (12.5 µM MG132). D. Cotransfection of GFP-Triad1 and Flag-Ub followed by GFP IP. Immunoprecipitates were split in two parts and run on same gel as indicated and independently stained for Triad1 and Flag respectively revealing GFP-Triad1 (arrowhead) and a smear of ubiquitinated proteins ([X-(ub)n] including proteins with sizes smaller compared to Triad1. Asterisk represents IgH. E. *In vitro* Ub ligase assay.
Reticulocyte fraction IIA, Triad1 (0.03 ug) or 10*Triad1 (0.3 ug) without Ub (-), with wild type Ub (WT) or UbK48-only (K48) were incubated followed by staining of ubiquitinated proteins. Note the marked increase of both wild type and K48-only ubiquitinated species after addition of Triad1. Increased dose of Triad1 correlates with increase in ubiquitinated proteins. Equal protein transfer between the tested conditions is indicated by the Ub band (lower panels).

**Figure 5. Triad1 is ubiquitinated and targeted for degradation by the proteasome.** A. Cotransfection of Triad1 and His-Ub followed by selection of ubiquitinated proteins using His-select beads under denaturing conditions followed by Triad1 staining detects multiple discrete ubiquitinated Triad1 forms [Triad1-(ub)_n]. Proteasome inhibition (12.5 µM MG132) results in an increase in Triad1 ubiquitination. Left lane contains positive control containing lysate from Triad1 (arrowhead) transduced 293T cells. Asterisk indicates an a-specific band. B. Cotransfection of Flag-Ub and Triad1 followed by Flag IP and staining for Triad1 detects multiple ubiquitinated Triad1 species (Triad1-(ub)_n) that were more intense when cells were treated with a proteasome inhibitor prior to cell lysis. Note that unmodified Triad1 (arrow) co-immunoprecipitates with ubiquitinated Triad1.

**Figure 6. The Ub ligase activity of Triad1 inhibits clonogenic growth of primary murine bone marrow cells.** A. Empty vector (EV) or Triad1 retrovirally transduced murine bone marrow cells with increasing expression (Triad1 I, II and III, respectively) were used in CFU-GM revealing a Triad1 concentration dependent inhibition of clonogenic growth (n=3). Triad1 staining using lysates from 100,000 transduced bone marrow cells revealed no detectable levels in EV transduced cells and increasing expression in cells with increasing GFP positivity (fractions II and III, respectively). NC = negative control. B. Morphological analysis of cells grown in CFU-GM shows a modest increase in the percentage of granulocytes and decrease in erythroblasts upon Triad1 expression. C. Triad1 RING mutants H158A and C161A do not inhibit clonogenic growth showing that the N-terminal RING finger is essential for the clonogenic inhibition exerted by wild type Triad1 (n=2). Comparable expression of wild type Triad1 and mutants was shown by Western blotting using lysates from 100,000 transduced bone marrow cell (equal loading was checked by Ponceau staining, not shown). D. Treatment of murine bone marrow cells grown in liquid medium with
MG132 indicates maximal tolerated dose with limited toxicity of $10^{-8}$ M. 5,000 cells were seeded in 96 wells and cultured for 3 days. Relative cell numbers were determined by flowcytometer using fluorescent beads (Beckman Coulter). **E.** Treatment of primary murine bone marrow cells with $10^{-8}$ M MG132 for two days resulted in an increase in ubiquitinated proteins ($x$-$(ub)_n$). **F.** Proteasome activity measurements (indicated as fluorescence units (FU)) on lysates taken from primary murine bone marrow cells that were treated with $10^{-8}$ M MG132 for one or two days showed clear proteasomal inhibition. **G.** Proteasome inhibition (MG132) relieves the suppressive effect of Triad1 in CFU-GM ($n=3$). MG132 was added to cells prior to cell seeding. For all tested conditions observed numbers of colonies were indicated relative to number of empty vector (EV) obtained colonies (set at 100). **H.** Treatment of Triad1 transduced cells with independent proteasome inhibitors counteracted the suppressive effects of Triad1 on colony formation. The number of obtained colonies of mock treated Triad1 transduced cells was set at 100 and relative colony numbers obtained for cells treated with indicated proteasome inhibitors are indicated ($10^{-8}$ M MG132 ($n=3$), $10^{-9}$ M PS341 ($n=2$), $5\times10^{-10}$ M epoxomycin = epox ($n=1$) and $10^{-8}$ M lactacystin = lacta ($n=1$)).

**Figure 7.** Triad1 inhibits proliferation of committed myeloid progenitors in liquid cultures. **A.** Proliferation of transduced bone marrow cells in liquid medium indicating dramatic growth inhibition of Triad1 transduced cells compared to empty vector transduced cells. Cell number at start of culture was set at 100 and relative numbers during culture time (days) were plotted. Open diamonds and closed boxes represent respectively empty vector and Triad1 transduced cells. **B.** Reduced growth rate of Triad1 transduced cells is accompanied by an increase in the percentage of cells in G0/G1 phase (DNA histograms were determined by flowcytometer using propidium iodide staining, see supplementary Figure 3c). **C.** Triad1 transduced cells grown in liquid medium show enhanced annexin V positivity compared to empty vector transduced cells. **D.** Triad1 expression does not result in alterations in the percentage of Mac1 positive cells compared to empty vector transduced cells.
Table 1. Sequences of Triad1 oligonucleotides

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For amplification of overlapping Triad1 RT-PCR fragments 2, 3, 4 (Figure 1) primer combinations T1V-T1AB, T1Z-T1P and T1K-T1O were used, respectively. For 3’ RACE primers AO was used for cDNA synthesis followed by PCR using primers AP1 and T1D, followed by hemi-nested PCR using primers AP1 and T1B. For 5’ RACE primers AP2 and T1X were used in PCR followed by hemi-nested PCR using primers AP2 and T1Y. T1M4 and T1M5 were used to for the construction of a C-terminally myc tagged Triad1 PCR construct. The 30 bp myc-tag encoding sequence is underlined. Primers H158AF, H158AR, C161AF en C161AR were used to construct Triad1 H158A and Triad1 C161, respectively. For generation of GST-Triad1 and GFP-Triad1 primer pairs T1exp1 and T1exp2 were used. For cloning full length Triad1 in pCDNA3.1 and retroviral vectors primers T1M4 and T1exp2 were used. For cloning Triad1-119 and Triad1 109-493 in pGAD vector primer combinations T1exp1-T1G1 and T1G2-T1exp2 were used, respectively. For Triad1 qPCR primers T1qF and T1qR and probe (TET-label) T1qP were used. BamHI and EcoRI sites introduced by PCR for cloning purposes are indicated (bold italics).
1. A. ATRA (10^-6) M
   0 24 48 96 hrs
   Triad1
   GAPDH

   B. M  1  2  3  4  5  M
   Triad1 protein
   TRAM domain
   1  R1  D  R2  CC  CC
   14 93

   C. Triad1 transcript
   5'  1  2  3  14 15  4  5  3' 3' 3'
   1  14 1000 1623 2000 3000 3392

   D. Triad1 transcript

2. A. Triad1
   BM granulocytes T-cells
   GADPH
   B. CD34+ granulocytes T-cells
   Relative Triad1 expression
   0 5 10 15 20 25

   C. Relative Triad1 expression
   blood bone marrow fetal brain lung heart liver
   spleen thymus prostate
   spinal cord uterus placenta
   skeletal muscle colon liver kidney
   Relative Triad1 expression
3.

A. Triad1-myc, α-myc 9E10

Phase contrast

B. α-Triad1

DAPI merged

CD34+ T-cells monocytes granulocytes

C. Mean fluorescence intensity

CD34+ CD3+ Mo1 Mo2 Gr1 Gr2 Gr3

CD14+ CD15+

Immature mature Immature mature
6. A. 

![Graph showing cell numbers vs. time]

B. 

![Bar graph showing percentage of cells]

C. 

![Graph showing proteasome activity]

D. 

![Bar graph showing cell numbers vs. concentration of MG132]

E. 

![Image showing IB: α-Ub and IB: α-actin]

F. 

![Graph showing proteasome activity over time]

G. 

![Graph showing cell numbers vs. treatment]

H. 

![Graph showing cell numbers vs. treatment]

7. A. 

![Graph showing cell numbers vs. days in culture]

B. 

![Graph showing % cells in G0/G1 vs. days in culture]

C. 

![Graph showing % Annexin V+ vs. days in culture]

D. 

![Graph showing % MAC1+ vs. days in culture]
The E3 ubiquitin-protein ligase Triad1 inhibits clonogenic growth of primary myeloid progenitor cells

Jurgen A Marteijn, Liesbeth van Emst, Claudia A Erpelinck-Verschueren, Gorica Nikoloski, Aswin Menke, Theo de Witte, Bob Lowenberg, Joop H Jansen and Bert A van der Reijden