The International Journal of Biochemistry & Cell Biology 44 (2012) 132–138

Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Differential regulation of PML–RARα stability by the ubiquitin ligases SIAH1/SIAH2 and TRIAD1

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ARTICLE INFO

Article history:
Received 29 June 2011
Received in revised form 5 October 2011
Accepted 16 October 2011
Available online 22 October 2011

Keywords:
Ubiquitinylating enzymes
TRIAD1
SIAH
UBCH8
Proteasomal degradation
PML–RARα

ABSTRACT

The ubiquitin proteasome system plays an important role in normal and malignant hematopoiesis and relies on the concerted action of three enzyme families. The E2 ubiquitin conjugase UBCH8 (ubiquitin conjugating enzyme [human] 8) cooperates with the E3 ubiquitin ligases SIAH1 and SIAH2 (seven in absentia homolog 1/2) to mediate the proteasomal degradation of oncoproteins. One such protein is the leukemia fusion protein PML–RARα (promyelocytic leukemia–retinoic acid receptorα) that is associated with acute promyelocytic leukemia. A limited number of UBCH8 interaction partners that participate in the UBCH8-dependent depletion of cancer-relevant proteins are known. We report here that TRIAD1 (two RING fingers and DRIL [double RING finger linked] 1), an E3 ubiquitin ligase relevant for the clonogenic growth of myloid progenitors, binds UBCH8 as well as PML–RARα. Moreover, there is concurrent induction of TRIAD1 and UBCH8 upon combinatorial treatment of acute promyelocytic leukemia cells with the pro-apoptotic epigenetic modulator valproic acid and the differentiation inducing agent all-*trans* retinoic acid. However, in sharp contrast to SIAH1/SIAH2 and UBCH8, TRIAD1 binding to PML–RARα has no effect on its turnover. In summary, our data exclude TRIAD1 as crucial regulator of the leukemic determinant PML–RARα, but highlight the prominence of the UBCH8/SIAH axis in PML–RARα degradation.

1. Introduction

In light of the crucial role of the ubiquitin proteasome system (UPS) for health and disease, detailed characterization of this system and its regulators is highly warranted (Kirkin and Dikic, 2011). Tagging of proteins with lysine K48-linked polyubiquitin chains allows their rapid elimination via the proteasome, an intracellular multi-protease complex (Hochstrasser, 2009). Ubiquitinylation is catalyzed by an E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzymes, and E3 ubiquitin ligases. The E1 activates ubiquitin ATP-dependently and transfers it to an E2 enzyme. RING domain E3s mediate substrate binding and recruit E2s, thereby allowing the transfer of the E2 ubiquitin cargo to a substrate. Dedicated substrate/E3 pairing permits precise ubiquitinylation. Specificity of ubiquitin ligases is additionally guaranteed by their ability to associate with different ubiquitin conjugases. Furthermore, ubiquitin ligases themselves can be targeted for proteasomal degradation by their cognate ubiquitin conjugase(s). In line with these findings we could show in a previous study that the E3 ubiquitin ligase SIAH1 (seven in absentia homolog 1) and the E2 ubiquitin conjugase UBCH8 (ubiquitin conjugating enzyme [human] 8) cooperatively promote the turnover of the UBCH8 interacting E3 ligase RLIM (RING finger LIM domain-binding protein) (Krämer et al., 2008).

Various studies indicate that the UPS regulates proliferation, differentiation, and apoptosis during hematopoiesis (Crawford et al., 2008; Heuzé et al., 2008; Marteijn et al., 2006). An example for successful therapeutic activation of the UPS is acute promyelocytic leukemia (APL), a subtype of acute myeloid leukemias characterized by blasts blocked at the promyelocytic stage (Okuno et al., 2004). In 95% of all cases, APL cells express PML–RARα (promyelocytic leukemia–retinoic acid receptor α) encoded by

Abbreviations: APL, acute promyelocytic leukemia; ATRA, all-*trans* retinoic acid; HDACi, histone deacetylase inhibitor; PML–RARα, promyelocytic leukemia–retinoic acid receptor α; SIAH, seven in absentia homolog; TRIAD1, two RING fingers and DRIL (double RING finger linked) 1; UBCH, ubiquitin conjugating enzyme (human); VPA, valproic acid.

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doi:10.1016/j.biocel.2011.10.008

the translocation t(15;17) (Perissi et al., 2010). In contrast to the RARα transcription factor, PML–RARα is not activated upon physiological concentrations of retinoic acid. Subsequently, differentiation genes positively controlled by RARα become repressed by PML–RARα under physiological conditions (Boukarabila et al., 2009; Epping et al., 2007; Mercurio et al., 2010; Perissi et al., 2010). Pharmacological doses of all-*trans* retinoic acid (ATRA) evoke proteasomal degradation of PML–RARα and a co-repressor/co-activator exchange turning PML–RARα into a potent transcriptional activator. Consequently, promyelocytic leukemia cells become mature white blood cells (Brown et al., 2009; Duprez et al., 2003; Mercurio et al., 2010; Perissi et al., 2010).

HDACI (histone deacetylase inhibitors) alter gene expression and cellular signaling by inducing histone and non-histone protein acetylation (Buchwald et al., 2009; Spange et al., 2009). Such agents are able to correct dysregulated repressive transcription patterns. Moreover, by augmenting the expression of the ubiquitin conjugase UBCH8, these drugs trigger the proteasomal degradation of proteins associated with tumorigenesis. One example is PML–RARα, as it is targeted for degradation by the HDACi-modulated UBCH8–SIAH1–proteasome axis (Buchwald et al., 2009; Krämer et al., 2008). Contrary to retinoids, HDACi mainly evoke caspase-dependent apoptosis of PML–RARα-positive NB4 cells. A deeper insight of regulatory mechanisms controlling the abundance of this oncoprotein may provide new avenues for therapy of patients suffering from APL (Buchwald et al., 2010; Krämer et al., 2008; Mercurio et al., 2010; Müller and Krämer, 2010; Perissi et al., 2010).

In this study we aimed for the identification of so far unknown UBCH8 interacting proteins that might participate in the UBCH8-dependent degradation of cancer-relevant oncoproteins. By systematic yeast two-hybrid (Y2H) screens we identified the ubiquitin ligase TRIAD1 (Two RING fingers and DRIL [double RING finger linked]-1) as a novel UBCH8 interaction partner. *In vitro* binding and cellular co-localization studies confirmed the association of TRIAD1 with UBCH8. Co-expression studies revealed that TRIAD1 itself is not targeted for degradation by UBCH8 and its cooperating SIAH ubiquitin ligases. We furthermore disclose the interaction of TRIAD1 with the UBCH8 substrate PML–RARα. Moreover, we show that ATRA and the HDACi valproic acid (VPA) concurrently induce UBCH8 and TRIAD1 expression in cells derived from the bone marrow of a patient with APL in relapse. Our work though also demonstrates that the interaction of TRIAD1 with PML–RARα and UBCH8 neither leads to PML–RARα protein destabilization nor does it interfere with the very potent UBCH8/SIAH1- or UBCH8/SIAH2-mediated degradation of PML–RARα.

2. Materials and methods

2.1. Drugs and chemicals

The proteasome inhibitor Z-Leu-Leu-Leu-al (MG132) was purchased from Axxora; ATRA and VPA were from Sigma–Aldrich.

2.2. Cell lines

Cell lines were cultured as stated (Buchwald et al., 2010; Krämer et al., 2008).

2.3. Plasmids

The following plasmids have been described previously: UBCH8-V5, GST-UBCH8, FLAG-PML–RARα, Myc-PML–RARα-GFP, pEGFP, SIAH1^{C72S}; SIAH1, SIAH2 (Krämer et al., 2008); HA-SIAH1 (Crone et al., 2011); Myc-SIAH2 (Germani et al., 1999); TRIAD1-Myc (Marteijn et al., 2005). The plasmid encoding GST-UBCH7 was created using the primers 5′-CGGGATCCCGATGGCGGCCAGCAGGAGGC-3′ and 5′-GGAATTCCTTAGTCCACAGGTCGCTTTTCCCCTA-3′. The PCR product was ligated into BamHI/EcoRI sites of pGEX-5X-1 (GE Healthcare). The UBCH7-V5 encoding plasmid was constructed by PCR amplification using 5′-GCCACCATGGCGGCCA-GCAGGAGGC-3′ and 5′-GTCCACAGGTCGCTTTTCCCCATATT-3′ and ligated into the pcDNA3.1/V5-His-TOPO vector as stated by the manufacturer (Invitrogen). All constructs were verified by sequencing.

2.4. Yeast two-hybrid screen

Automated Y2H screens were performed as described in references (Albers et al., 2005; Albert et al., 2003; Lamesch et al., 2007 human genes). Human cDNA libraries from testis and brain (Clonetech) as well as a library of individually cloned full-length open reading frames (from cDNAs of 10.070 different genes) were screened for full-length UBCH8 as the bait.

2.5. Quantitative real-time PCR

Cellular mRNA was isolated and cDNA was synthesized as explained in (Krämer et al., 2008). Data obtained were analyzed with the delta-Cq quantification model (Hellemans et al., 2007) using three reference genes (*HMBS*, *GAPDH*, and *RPL13A*). These were verified with the geNorm program (Vandesompele et al., 2002). Primer sequences for quantitative real-time PCR (qPCR) were: TRIAD1 fwd 5′-CGGGTACAGGAGCCTAGAGCTCGCCG-3′ and rev 5′-GGATTGTGGCACAGTCTGTGGGTGCG-3′; UBCH8 fwd 5′-TGGCGAG-CATGCGAGTGGTGAAGG-3′ and rev 5′-CTGGACAGGTTCCGCAG-GTATGGG-3′; GAPDH fwd 5′-TGCACCACCAACTGCTTAGC-3′ and rev 5′-GGCATGGACTGTGGTCATGAG-3′; RPL13A fwd 5′-CCT-GGAGGAGAAGAGGAAAGAGA-3′ and rev 5′-TTGAGGACCTCTGTG-TATTTGTCAA-3′; HMBS fwd 5′-GGCAATGCGGCTGCAA-3 and rev 5′-GGGTACCCACGCGAATCAC-3′.

2.6. Transfection assays

Transient protein expression in HEK293T cells was achieved with PEI (Sigma–Aldrich) or Lipofectamine2000 (Invitrogen) (Krämer et al., 2009). Cells were harvested after 24 h. Empty vectors pcDNA3.1 or pSG5, as applicable regarding the coding plasmids, were used to obtain equal amounts of total DNA transfected (2 μg/10^6 cells). We noted that it is important to use the same plasmid backbone for this as each appeared to have a different impact on general transfection efficiency. A plasmid encoding green fluorescent protein (pEGFP, 0.05 μg) was co-transfected to monitor transfection efficiency where applicable.

2.7. Cell lysis, immunoblot, immunofluorescence, and microscopy

Lysate preparation and immunoblot techniques are summarized in (Buchwald et al., 2010; Krämer et al., 2008). Microscopy analyses of HeLa cells were performed as described (Knauer et al., 2007). NB4 suspension cells were fixed on frosted glass slides by cytospin centrifugation for 10 min at 100 g before staining.

2.8. GST pull-down

This method was carried out as described in references (Buchwald et al., 2010; Krämer et al., 2008).