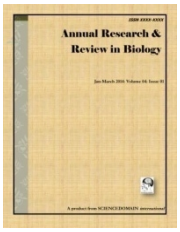

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TRAP1 is a Novel Interaction Partner of PML, Localized with PML in Nuclear Bodies and Relocating with PML to the Cytoplasm Following Stress

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Authors' contributions

Author EWS designed the study and performed the experiment and analyses. Authors EWS and OM wrote the manuscript. Both authors EWS and OM read and approved the final manuscript.

Short Research Article

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ABSTRACT

Aims: The aim of the study was to identify a novel interaction partner of PML and to study the localization of the novel protein complex in cells.
Study Design: Isolation of PML and interacting protein complexes by co-immunoprecipitation using a PML antibody and subsequent mass spectrometry analyses.
Place and Duration of Study: Department of Medical and Molecular Genetics, Guy's Hospital, London between 2002 and 2005 and Cancer Stem Cell Innovation Centre and Department of Tumor Biology, Norwegian Radium Hospital, Oslo between 2011-2014.
Methodology: Interacting proteins of PML were isolated from K562 cell lysates by large scale co-immunoprecipitation and mass spectrometry. The complex formation was confirmed by standard co-immunoprecipitations and confocal microscopy. The cellular localization of the protein complex was further studied by confocal microscopy in different cell types and following exposure to stress.
Results: We have identified tumor necrosis factor receptor protein 1 (TRAP1) as an

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interacting partner of PML. A fraction of TRAP1 is localized to PML nuclear bodies (NBs) in human cancerous cells and normal mouse embryonic fibroblasts, indicating conservation across species. PML and TRAP1 are both implicated in regulation of cell death and survival, with PML acting as an inducer of apoptosis and TRAP1 as protector from cell death. Exposure to stress, results in relocation of TRAP1 - PML from NBs to cytoplasmic punctate structures, indicating a role for this complex in stress-response.
Conclusion: TRAP1 and PML interact in normal and cancerous cells. The complex may play a role in stress response.

Keywords: TRAP-1; PML; NBs; nuclear bodies; mass spectrometry.

ABBREVIATIONS

ER: Endoplasmic Reticulum; MEFs: Mouse Embryonic Fibroblasts; TGF: Transforming Growth Factor; TNF: Tumour Necrosis Factor; TRAP: TNF Receptor Associated Protein.

1. INTRODUCTION

The PML protein has been linked to a number of cellular processes, including apoptosis, growth regulation, senescence and response to viral pathogens [1,2,3]. The pro-apoptotic functions of PML have been clearly demonstrated *in vivo* and in cells derived from Pml "knock-out" mice. For instance, mouse cells which lack Pml exhibit decreased sensitivity to apoptosis induced by tumor necrosis factor (TNF)-α, Fas ligand or transforming growth factor (TGF)-β and are defective in apoptosis induced by ceramide, interferon or irradiation [4,5]. Within normal tissues and cultured cells, the majority of PML is associated with multi-protein structures, known as PML nuclear bodies (NBs), while a smaller fraction of PML is found free in the nucleoplasm or cytoplasm [6]. The importance of PML NB integrity became clear through studies of acute promyelocytic leukaemia cells harboring the t(15:17) translocation, where PML NBs disruption due to the expression of the PML-RARα fusion protein correlated with disease [7]. Since then, researchers have mainly focused on understanding the role of PML NBs. However, during the last decade, specific roles have also been demonstrated for the smaller fraction of cytoplasmic PML, for instance in TGF-β signaling [4]. Furthermore, cytoplasmic PML localizes to mitochondria-associated membranes, where the protein is implicated in regulating calcium influx from the endoplasmic reticulum (ER) to the mitochondria [8]. These findings highlight that PML not only has a function within PML NBs, where the majority of PML protein is located, but that cytoplasmic PML also plays key roles. Here we describe the successful isolation and identification of a novel interacting partner of PML, namely TNF receptor associated protein (TRAP)1, and we further show that the TRAP1 – PML complex is relocated to the cytoplasm following cellular stress and apoptotic stimuli.

2. MATERIALS AND METHODS

2.1 Cell Lines and Culturing

K562 cells (ATCC) or early passage (<p6) primary mouse embryonic fibroblasts (MEFs) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), glutamax and 100U/ml penicillin and 100µg/ml streptomycin. MEFs were generated from mouse embryos 12-14 days post conception. All experimental

procedures performed on mice and derivation of MEF primary cells were approved by the King's College London Ethical Review Process Committee and carried out under the UK Home Office License 70/5761.

2.2 Co-immunoprecipitation, SDS-PAGE and Mass Spectrometry Analysis

K562 cells were lysed in NP40 lysis buffer (0.5% NP40, 50mM Tris-HCl pH 8.0, 150mM NaCl, 1mM EDTA, 1mM DTT, protease inhibitor cocktail (Roche Diagnostics). Large scale co-immunoprecipitation was performed using mouse anti-PML (PGM3) antibody (Santa Cruz Biotechnology) as bait, to pull out endogenous protein complexes harboring PML. Protein A/G agarose beads slurry (Santa Cruz Biotechnology) was added and the mixture incubated with rotation for 2 hours. The isolated proteins were resolved by SDS-PAGE, and the gel was stained in Colloidal coomassie blue G-250 (Sigma). Proteins were excised from the gel and digested with trypsin in-gel. The tryptic digests were analyzed by liquid chromatography (LC) performed on a 100- by 0.18-mm C18 column and peptides were eluted with a gradient of aqueous acetonitrile (ACN) (5 to 65% ACN over 30 min starting at 5 minutes post-injection) containing 0.1% formic acid at a flow rate of 2 µl/min and subsequent tandem mass spectrometry (MS). The obtained peptide sequences were matched with the NCBI database following analyses of the MS-MS spectra using a ProteomeX system (Thermo Electron, Hemel Hempstead, Hertfordshire, UK).

2.3 Co-Immunoprecipitation and Western Blotting

K562 cells were lysed in NP40 lysis buffer (0.5% NP40, 50mM Tris-HCl pH 8.0, 150mM NaCl, 1mM EDTA, 1mM DTT, protease inhibitor cocktail (Roche Diagnostics). Cell extracts were incubated overnight at 4 degrees, with primary antibodies mouse anti-PML (PGM3) antibody (Santa Cruz Biotechnology) or mouse anti-TRAP1 antibody (Abcam). MEF cell extracts were incubated with rabbit polyclonal antibody raised against full-length human PML [9]. Immunoprecipitation using normal mouse serum IgG (Santa Cruz Biotechnology) was also performed as a negative control, to ensure the protein of interest was not precipitated un-specifically. Protein A/G agarose bead slurry (Santa Cruz Biotechnology) was added and the mixture incubated with rotation for 2 hours. After washing, immunoprecipitated proteins were resolved by SDS-PAGE. For Western blotting, the following primary antibodies were used: mouse anti-PML antibody (PGM3) (1:500 dilution) or mouse anti-TRAP1 antibody (1:10000 dilution).

2.4 Confocal Microscopy

Cells were fixed in 4% paraformaldehyde, permeabilized in 0.2% triton-X and incubated overnight in 10% FCS in PBS. Subsequently, cells were incubated with primary antibodies: rabbit polyclonal antibody raised against full-length human PML [9] (1:500 dilution) or mouse anti-TRAP1 antibody (Abcam) (1:200 dilution) and appropriate secondary conjugated antibodies (Dako) (1:2000). Confocal microscopy was performed using LSM 510 Meta Axioplane Imaging microscope (Zeiss).

2.5 Cellular Stress Induction

MEFs were untreated, subjected to 42ºC heat shock for 1 hour and subsequent 5 hour recovery, or exposed to a combination of 1µg/ml cyclohexamide and 50ng/ml murine tumour necrosis factor (Tnf), which induces apoptosis in MEFs [10].

2.6 Apoptotic Assay

Detection of DNA fragmentation, associated with apoptosis, was performed using the Apoalert DNA fragmentation assay kit (Clontech, Hampshire, UK). All cells were stained with propidium iodide (red). Cells undergoing apoptosis incorporated fluorescein-dUTP at the 3'-hydroxyl ends of fragmented DNA (green). The overlay image of an apoptotic cell appeared yellow. Slides were viewed using LSM 510 Meta Axioplane Imaging microscope (Zeiss). Minimum 100 randomly chosen cells/sample were evaluated and the number of apoptotic cells scored. On some occasions, cells were subject to confocal microscopy following apoptosis induction.

3. RESULTS

3.1 TRAP1 is a Binding Partner of PML

We have identified TRAP1 as a novel interaction partner of endogenous PML in human chronic myeloid leukaemia cells (K562) through a large-scale co-immunoprecipitation using an antibody against PML as bait, followed by mass spectrometry analyses of the isolated proteins. TRAP1 corresponded to a strong band of approximately 75 kDa following SDS-PAGE separation Fig. 1A, and its identity was confirmed following MS analysis of peptides generated by tryptic digestion of the band Fig. 1B. As many as 29 peptides matched the TRAP1 protein sequence in the human NCBI database Fig. 1C.

We subsequently performed standard co-immunoprecipitation analyses in K562 cell lysates and confirmed that TRAP1 was indeed detectable in the PML-bound fractions. To ensure specificity of the association, we also confirmed that TRAP-1 was not detectable in the non-specific IgG bound control fractions Fig. 2A. As further confirmation, PML was also detectable in co-immunoprecipitates of TRAP1 using a TRAP1 antibody as bait Fig. 2A. We subsequently investigated whether the TRAP1-PML complex could also be detected in normal mouse cells and found that Trap1 was detectable in co-immunoprecipitates of Pml from MEF lysates Fig. 2B. These findings confirm that TRAP1 is indeed in complex formation with PML both in cancer cells and normal cells and also indicate that the association is conserved across species.

3.2 Sub-fractions of TRAP1 and PML Co-localize in PML NBs

Since proteins which would not normally form complex formations within the live cell can associate in cell lysates due to rupturing of cell membranes and organelles, we investigated the cellular distribution of endogenous PML and TRAP1 in fixed cells. Confocal microscopy analyses of fixed MEFs showed that Pml displayed the typical punctate nuclear staining characteristics of Pml NBs in virtually all cells Fig. 2C. Trap1 also displayed a punctate nuclear localization pattern, which co-localized in part with Pml. These findings provide further indication that Trap1 associates with Pml and is a constituent in a subset of Pml NBs.

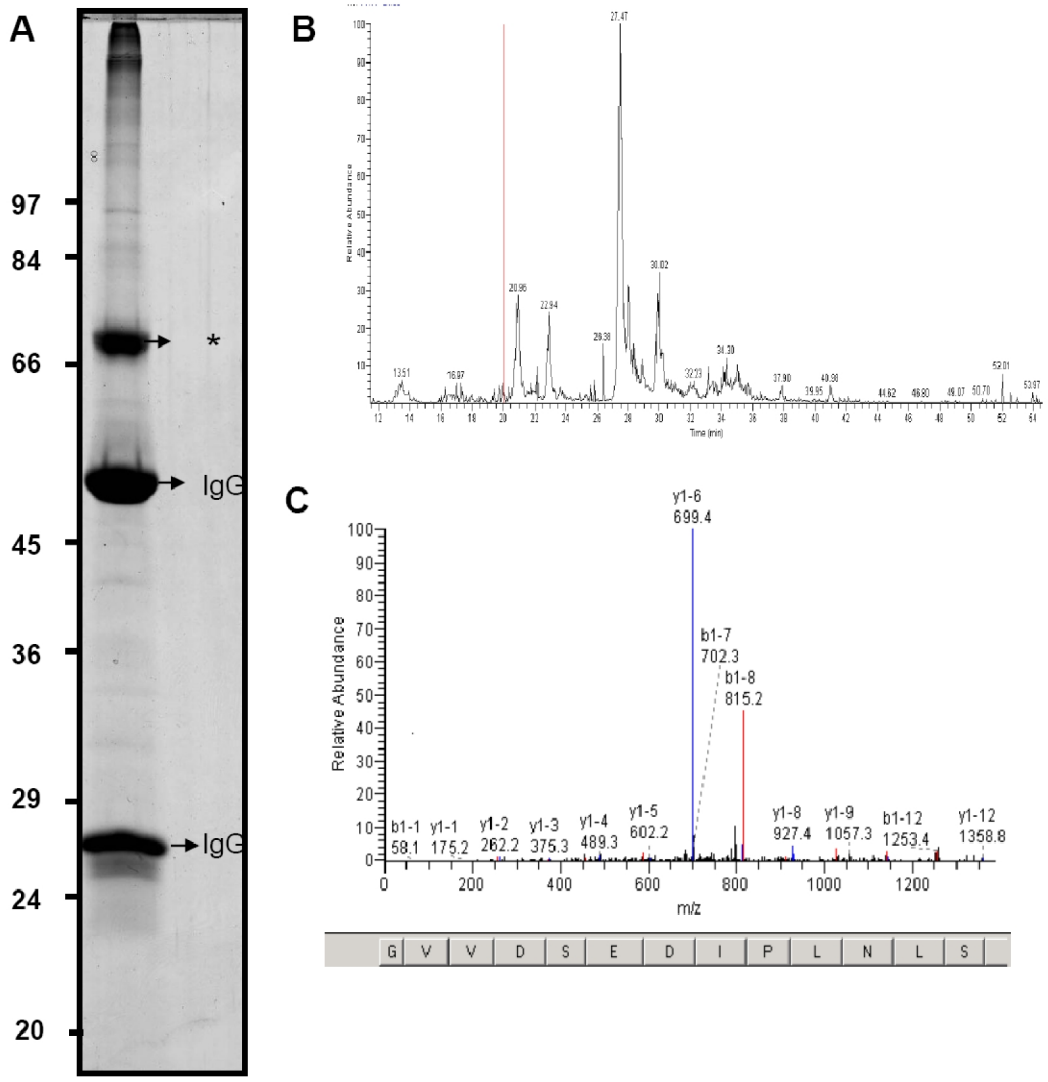

Fig. 1. TRAP1 is a complex binding partner of PML, identified by LC-MS/MS

*(A) K562 cell extracts were subject to large scale co-immunoprecipitation utilising PML antibody (PGM3). Bound proteins were separated by SDS-PAGE and stained by coomassie blue. (B) Reverse phase separation of tryptic peptides resulting from in-gel digestion of band marked * and subsequent (C) MS/MS spectrum of a tryptic peptide derived from the gel band marked *. The sequence obtained by performing TurboSEQUEST analyses is shown boxed beneath the spectrum*

3.3 Trap1 and Pml are Relocated from Pml NBs during Heat Shock and Reform after Recovery

Since PML NBs are known to dissociate following heat shock [11,12], we investigated the cellular location of Trap1 and Pml in MEFs after 1 hour of 42ºC heat shock. As expected, we found that Pml NBs were dispersed and that Pml staining appeared diffuse both in the nucleus and the cytoplasm. Interestingly, Trap1 previously localized to NBs now also appeared dispersed Fig. 3A. Following 5 hours recovery from heat shock, Pml NBs reformed

in the majority of the cells and again Trap1 started to co-localize with Pml in NBs of a subset of the cells Fig. 3B.

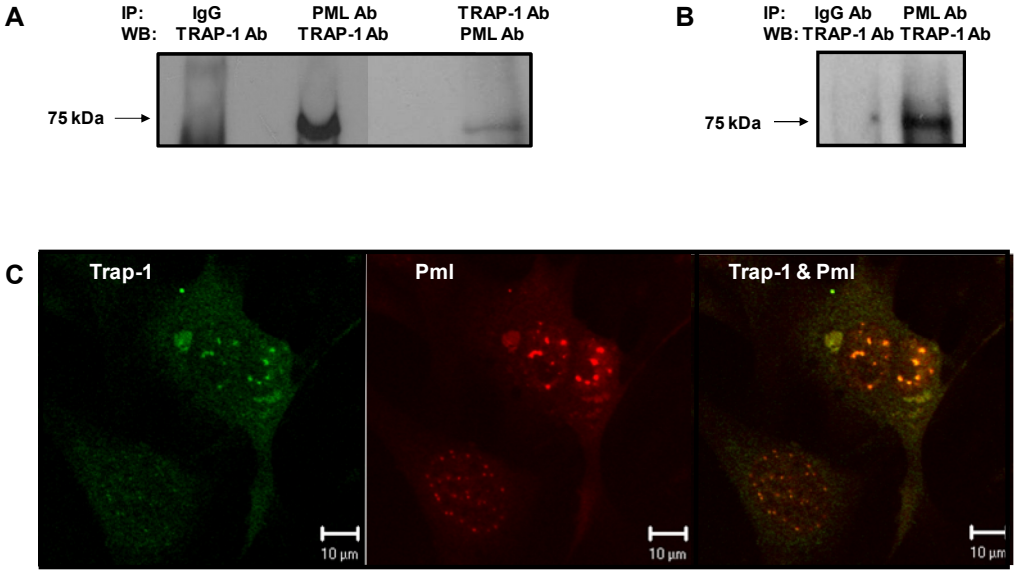

Fig. 2. The TRAP1-PML complex is confirmed across species

(A) K562 cell lysates were immunoprecipitated (IP) with either mouse serum IgG (control) or mouse anti-PML antibody and bound TRAP1 was revealed by western blotting (WB) with TRAP1 antibody. K562 cell lysates were also IP with mouse anti-TRAP1 antibody and bound PML was revealed by WB with PML antibody. (B) MEF cell lysates were IP with either rabbit serum IgG (control) or rabbit anti-PML antibody. Bound TRAP1 was revealed by WB using mouse anti-TRAP1 antibody. (C) The cellular distribution of TRAP1 (green) and PML (red) was evaluated by Confocal microscopy in fixed MEFs

3.4 Trap1 and Pml Co-localize in Punctate Structures in the Cytoplasm Following Induction of Apoptosis with Cycloheximide and TNF

The pro-apoptotic functions of PML have been demonstrated in numerous *in vitro* studies as well as in mice, using the Pml knock out mouse model [13]. TRAP1, on the other hand, has been associated with pro-survival functions [14,15,16,17,18]. Since PML and TRAP1 both have been implicated in mechanisms regulating survival and cell death, we evaluated the distribution of Pml and Trap1 in MEFs, following exposure to apoptosis-inducing stimuli. When MEFs were exposed to cycloheximide combined with Tnf [10], Pml displayed marked punctate Pml-positive staining within the cytoplasm Fig. 3C, in addition to the punctate nuclear staining pattern typical of Pml NBs. Interestingly, in contrast to untreated cells, Trap1 staining was restricted to the cytoplasm in virtually every cell, co-localising with the cytoplasmic fraction of Pml in the vast majority of cells Fig. 3C. Nearly all MEFs stimulated with cycloheximide in combination with Tnf underwent apoptosis (indicated by positive “Apo-alert” staining), while induction of apoptosis was not detected in untreated MEFs Fig. 3D.

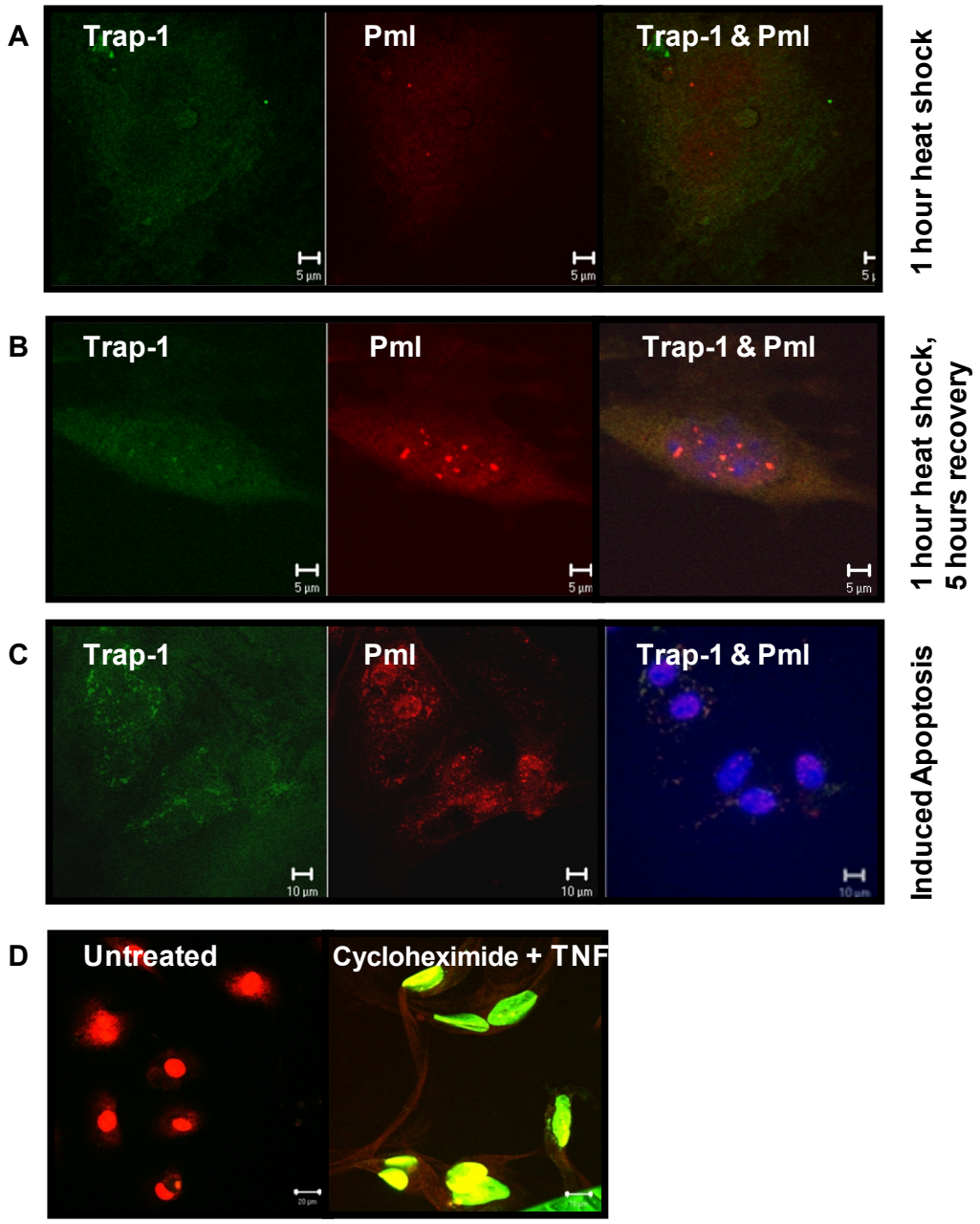

Fig. 3. TRAP1 and Pml co-localize in a subset of Pml NBs and relocate to the cytoplasm following cellular stress

*The cellular distribution of TRAP1 and PML was evaluated by confocal microscopy in **(A)** MEFs exposed to 1 hour heat shock, **(B)** MEFs exposed to 1 hour heat shock and 5 hours recovery and **(C)** MEFs exposed to cycloheximide combined with Tnf. The endogenous proteins were immune-stained with PML antibody (red) and TRAP1 antibody (green). The overlay is shown in yellow. On some occasions nuclear DNA was stained with Hoechst, visualizing the nucleus. **(D)** MEFs treated as in (C) were subjected to apoptosis assay confirming that cycloheximide combined with TNF indeed induced apoptosis in virtually all cells (yellow), whilst untreated cells appeared red*

4. DISCUSSION

In this study we have identified TRAP1 as a novel association partner of PML. The fact that TRAP1 was identified by 29 peptides matching the NCBI database following immunoprecipitation and LC/MS-MS analyses, and the subsequent confirmation by co-immunoprecipitations using either PML antibody or TRAP1 antibody to pull out the respective partner strongly indicates that the interaction is specific. However, during cell lysis, proteins which would not normally co-localize in the cell could interact *in vitro*. We therefore showed by confocal microscopy that a sub-fraction of TRAP1 and PML co-localized in the PML NBs, indicating that the proteins indeed are associated also in fixed cells. Furthermore, the association was observed both in cancerous and normal cells and across species. However, further analyses would have to be performed to determine whether the interaction is direct or indirect.

TRAP1 was first identified as an interacting partner of TNF receptor 1 (TNFR1) [19] and later found to also interact with the Retinoblastoma protein (Rb) [20]. The fact that neither TNFR1 nor Rb localize to the mitochondria, where TRAP1 is predominantly localized in many cell types, indicated that TRAP1 had functions outside the mitochondria [21]. In agreement with this, Chechetto and Gupta demonstrated that the sub-cellular localization of TRAP1 varied in different cell types and tissues, and that the TRAP1 protein was indeed observed outside mitochondria [22]. More recently, it was shown that TRAP1 localizes to the ER, where TRAP1 interacts with the proteasome regulatory particle TBP7/Rpt3 [23].

In this study we found that a fraction of TRAP1 co-localized with a subset of PML NBs and we determined the sub-cellular localization of both TRAP1 and PML following PML NB disruption. Following heat shock of MEFs we found that Pml NBs were dispersed, as expected [11,12]. Interestingly, Trap1 was also dispersed. Following recovery from heat shock and re-formation of Pml NBs, a sub-fraction of Trap1 was again localized to the Pml NBs. These observations indicate that PML is required for the recruitment of TRAP1 to NBs, as is the case for the majority of PML NB constituents, including BLM, CBP and DAXX [24,25].

TRAP1 is 50% similar to HSP90 [21], a well-characterized chaperone with pro-survival activity [26]. TRAP1, like HSP90, possesses ATPase activity, but there is no evidence of TRAP1 functioning as a classical chaperone [27]. However, it was recently suggested that TRAP1 refolds damaged proteins in the ER [23]. Although the function of TRAP1 has not been nearly as well studied as the function of PML, TRAP1 has an anti-apoptotic function [14,15,16,17,18,28], and silencing of TRAP1 results in induction of apoptosis [16]. More specifically, TRAP1 protects cells from apoptosis by antagonizing reactive oxygen species [15], and inhibition of TRAP1 activity increases sensitivity to chemotherapy [14]. In comparison, the role of PML in apoptosis has been relatively well studied [13]. The majority of studies have focused on the role of nuclear PML, but in the last decade it has become clear that the smaller fraction of cytoplasmic PML plays a distinct role in apoptosis [29].

Since both PML and TRAP1 have been implicated in apoptosis, we exposed MEFs to apoptosis-inducing agents and subsequently investigated the cellular localization of the two proteins. We found that PML NBs dispersed, resulting in a nuclear diffuse as well as cytoplasmic staining pattern for PML and relocation of TRAP1 from the nucleus to the cytoplasm. Interestingly, the two proteins also in part co-localized in cytoplasmic punctate structures. Cytoplasmic PML has been found to play a role in induction of apoptosis by regulating calcium-release from the ER [8] and a more specific function for cytoplasmic PML

has also been demonstrated in TGF-β-induced apoptosis [4]. On the contrary, following herpes simplex virus-1 infection, cytoplasmic PML promotes cellular resistance to viral infection and is thus part of a defense mechanism [30].

5. CONCLUSION

In this study, we have identified TRAP1 as a novel binding partner of PML using co-immunoprecipitations and LC-MS/MS. Importantly the findings have been validated by additional techniques and in other cell types, showing conservation across species. In response to cellular stress, the complex re-locates from the NBs to the cytoplasm, indicating a role in stress response. However, the potential functional relevance of the interaction remains to be investigated, with the key question being whether PML performs a pro-survival or pro-apoptosis function in conjunction with TRAP1.

ETHICAL APPROVAL

All experimental procedures performed on mice and derivation of MEF primary cells were approved by the King's College London Ethical Review Process Committee and carried out under the UK Home Office License 70/5761

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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