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Research paper

# Acetylation of translationally controlled tumor protein promotes its degradation through chaperone-mediated autophagy

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ABSTRACT

Translationally controlled tumor protein (*Tpt1*/TCTP) is a multi-functional cytosolic protein whose cellular levels are finely tuned. TCTP regulates protein behavior by favoring stabilization of protein partners or on the contrary by promoting degradation of others. TCTP has been shown to be transcriptionally and translationally regulated, but much less is known about its degradation process. In this study, we present evidence that chaperone-mediated autophagy (CMA) contributes to TCTP regulation. CMA allows lysosomal degradation of specific cytosolic proteins on a molecule-by-molecule basis. It contributes to cellular homeostasis especially by acting as a quality control for cytosolic proteins in response to stress and as a way of regulating the level of specific proteins. Using a variety of approaches, we show that CMA degradation of TCTP is Hsc70 and LAMP-2A dependent. Our data indicate that (i) TCTP directly interacts with Hsc70; (ii) silencing LAMP-2A in MEFs using siRNA leads to inhibition of TCTP downregulation; (iii) TCTP is relocalized from a diffuse cytosolic pattern to a punctate lysosomal pattern when CMA is upregulated; (iv) TCTP is degraded *in vitro* by purified lysosomes. Importantly, using lysine-mutated forms of TCTP, we show that acetylation of Lysine 19 generates a KFERQ-like motif and promotes binding to Hsc70, lysosome targeting and TCTP degradation by CMA. Altogether these results indicate that TCTP is degraded by chaperone-mediated autophagy in an acetylation dependent manner.

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## 1. Introduction

Translationally controlled tumor protein (TCTP) is a cytosolic protein that is highly conserved through phylogeny and ubiquitously expressed in eukaryotic organisms (Hinojosa-Moya et al., 2008). *Tpt1*/TCTP is regulated at transcriptional and post-transcriptional levels. Rapid translation of TCTP is adjusted through the storage of untranslated *tpt1* mRNA and their mobilization upon mitotic signaling (Bommer et al., 2002; Bommer et al., 2010). Although the extracellular role of TCTP has been shown to be associated to histamine release (MacDonald et al., 1995), the intracellular functions of TCTP have been partially elucidated (Amson et al., 2013). TCTP is involved in the cell cycle (Burgess et al., 2008; Cucchi et al., 2010; Gachet et al., 1999), proliferation and growth (Chen et al., 2007b), DNA repair (Hong and Choi, 2013; Zhang et al., 2012) and apoptosis (Liu et al., 2005; Susini et al., 2008; Yang et al., 2005). From a functional standpoint, TCTP often regulates protein behavior by favoring stabilization of protein partners. TCTP can undergo various post-translational modifications (Baylot et al., 2012; Chen et al., 2012; Munirathinam and Ramaswamy, 2012; Yarm, 2002), and notably phosphorylation by polo-like kinase decreases its microtubule-stabilizing activity (Yarm, 2002). Studies on tumor reversion revealed that TCTP is a key factor regulating this process (Amson et al., 2013; Telerman and Amson, 2009; Tuynder et al., 2004; Tuynder et al., 2002). By analyzing the gene expression profile between tumor cells and revertant counterparts that have a suppressed malignant phenotype, significant downregulation of TCTP in the revertants was observed (Tuynder et al., 2002). Accordingly, high TCTP levels are correlated with breast cancer aggressiveness for which it is an independent prognostic factor (Amson et al., 2012).

TCTP cellular expression is known to be regulated both at the transcriptional and translational levels. Recently, the mechanism of growth-dependent up-regulation of TCTP synthesis has been characterized (Bommer et al., 2015). It was found that TCTP mRNA

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translation is controlled through mTORC1 signaling by means of the presence of a 5′-terminal oligopyrimidine tract (5′-TOP) on the TCTP mRNA. However, homeostasis of a specific protein is a function of its production and removal rates. We have previously reported extracellular release of TCTP in association with exosomes through p53 activation (Lespagnol et al., 2008). In the present study, we focused on the molecular mechanisms by which TCTP can be degraded. Several pathways have been described for degradation of cytosolic proteins. Until recently, macroautophagy and ubiquitin-proteasome degradation were the only reported pathways. However, a process called chaperone-mediated autophagy (CMA) was shown to apply to various cytosolic proteins in mammalian cells (Arias and Cuervo, 2011; Kaushik and Cuervo, 2012). In contrast to regular macroautophagy, CMA allows lysosomal degradation of specific cytosolic proteins on a molecule-by-molecule basis. The selectivity of this pathway is conferred through recognition by the cytosolic chaperone Hsc70 (70 kDa heat shock cognate) of a pentapeptide biochemically related to KFERQ in the sequence of CMA substrate. The presence of Q at one end of the pentapeptide is critical but can be replaced by a N (Cuervo, 2010). The substrate-chaperone complex is targeted to the lysosome and interacts with the cytosolic tail of lysosome-associated membrane protein type 2A (LAMP-2A). After unfolding, the substrate, assisted by a lysosomal form of Hsc70, translocates into the lysosomal lumen through a multimeric complex formed by LAMP-2A assembly, and is rapidly degraded by lysosomal hydrolases. Basal levels of CMA activity are detectable in almost all mammalian cells and contribute to the maintenance of cellular homeostasis as well as to specialized functions depending on the cell type and degraded substrate. CMA is upregulated upon various conditions ranging from prolonged starvation to different cellular stresses.

In the present study, using both *in cellulo* and *in vitro* approaches, we demonstrate that CMA participates in TCTP regulation at a post-translational level by lysosomal degradation. Importantly, our data show that degradation through CMA preferentially occurs for a specific pool of TCTP, requiring acetylation of the protein.

## 2. Materials and methods

### 2.1. Ethics statement

All experiments were carried out in accordance with the European Community Council Directive of November 24, 1986 (86/609/ECC). This study was approved by the local branch of the 'Comité National de Réflexion Ethique sur l'Expérimentation Animale' (CNREEA n°36) under the reference CEEA-LR-12127. All efforts were made to minimize animal suffering and to reduce the number of rats used.

### 2.2. Materials

Nycodenz (Proteogenix), sulfo-SBED (Pierce), his-tagged Hsc70 (StressMarq Biosciences Inc.) and doxycycline (Fisher BioReagents) were used. Sodium cyanoborohydride, *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide, 6-aminonicotinamide (6-AN), nicotinamide (NAM), MG-132, spautin-1, 3-methyladenine, bafilomycin-A1, wortmannin, proteinase K, phosphocreatine, creatine phosphokinase, apyrase and chymostatin were from Sigma; leupeptin, pepstatin A and RNase A were from Euromedex. Trichostatin (TSA) was obtained from Invivogen. Lactalbumin (Sigma) was reduced and carboxymethylated (RCMLA) according to Ewbank and Creighton (Ewbank and Creighton, 1993). Prestained and unstained molecular markers were from Thermo Scientific. Immobilon-P transfer membranes were from Millipore and [$^{14}$C]formaldehyde was obtained from American Radiochemical Chemicals. Dynabeads Protein G were obtained from Life Technologies (10003D), and streptavidin-HRP was from Amersham (RPN1231V). Glutathione sepharose 4B (17-0756-01) and PreScission protease (27-0843-01) were from GE Healthcare. Oxyfluor was from Oxyrase, Inc. Protease inhibitor cocktail (11697498001) was from Roche. GST-Trap® _M was obtained from chromotek.

### 2.3. Antibodies

The following primary antibodies were used in Western blotting experiments: rat polyclonal anti-hTCTP (home-made against recombinant human TCTP); rat monoclonal anti-Hsc70 (1B5) from Stressgen; mouse monoclonal anti-β-actin (AC-15), rabbit polyclonal anti-GAPDH (G9545), mouse monoclonal anti-ubiquitin (6C1) and mouse monoclonal anti-acetylated tubulin (6-11B-1) antibodies from Sigma; rabbit polyclonal anti-LAMP-2A (AMC2) from Invitrogen; rabbit polyclonal anti-VDAC2 (ab126120), rabbit monoclonal anti-ATG5 (ab108327) and anti-ATG7 (ab133528) antibodies from Abcam; rabbit anti-LC3 (L7543) from Sigma; rabbit anti-p62 (PM045) from MBL and rabbit monoclonal anti-acetylated lysine (9814) from Cell Signalling Technology. HRP-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

The following primary antibodies were used for fluorescence microscopy: rat monoclonal anti-mouse LAMP-2 (PAN-reactive) antibody (GL2A7) purified from hybridoma supernatant (DSHB, Iowa city), rabbit polyclonal anti-LAMP-2A antibody (AMC2). Fluorescently labeled secondary antibodies: donkey anti-rabbit IgG conjugated with Alexa Fluor 488 (A21206) and donkey anti-rat IgG conjugated with Alexa Fluor 594 (A21209) were purchased from Invitrogen.

Rabbit monoclonal anti-TCTP antibody (ab133568, Abcam) was used together with Dynabeads protein G (10003D, Life technologies) for TCTP immunoprecipitation from HeLa cells.

### 2.4. Cells

Breast adenocarcinoma MCF7 cells, HeLa cells, mouse embryo fibroblasts (MEFs), m5-7 cells (Hosokawa et al., 2006) and ATG7$^{-/-}$ MEFs (Komatsu et al., 2005) were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% heat inactivated foetal bovine serum, 2 mM GlutaMax-I™, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 UI/ml penicillin and 100 μg/ml streptomycin. When needed, m5-7 cells were grown in the presence of doxycycline (100 ng/ml) for 4 days (Hosokawa et al., 2006) to achieve ATG5 extinction. For starvation treatment, cells were washed three times and incubated in DMEM. For Western blot experiments, cells were washed once in PBS before trypsinization. Cell pellets were washed twice in ice-cold PBS and lysed in RIPA (150 mM NaCl, 20 mM Tris, pH 7.4, 1 mM EDTA, 1% DOC, 1% NP40, 2 mM DTT and antiprotease cocktail) for 20 min on ice. After centrifugation (20 min; 20,000*g*, 4 °C), the protein concentration in supernatants was determined using Coomassie protein assay reagent (1856209, Thermo Scientific) and BSA as standard.

### 2.5. DNA constructs

Generation of pGEX-6P-1-hTCTP was described elsewhere (Amzallag et al., 2004). The PAmCherry1-N1 plasmid was obtained from Dr Vladislav Verkhusha (Subach et al., 2009b) *via* Addgene (Addgene plasmid 31928). As EcoRI and XhoI restriction sites are inverted in these two plasmids, hTCTP cDNA was amplified by PCR using primers containing these sites compatible with the destination vector, then ligated into pPAmCherry1-N1 after EcoRI-XhoI digestion and gel purification of both insert and plasmid. Single or multiple point mutations were generated by site directed mutage-

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