Proteasome activator PA28γ stimulates degradation of GSK3-phosphorylated insulin transcription activator MAFA

Kenichi Kanai, Shinsaku Aramata, Sayo Katakami, Kunio Yasuda and Kohsuke Kataoka

Laboratory of Molecular and Developmental Biology, Graduate School of Biological Science, Nara Institute of Science and Technology, 8916-5 Takayama-cho, Ikoma 630-0192, Japan

(Correspondence should be addressed to K Kataoka; Email: kkataoka@bs.naist.jp)

(K Kanai is now at Department of Tissue Morphogenesis, Max-Planck Institute for Molecular Biomedicine, Roentgenstrasse 20, 48149 Muenster, Germany)

Abstract

MAFA is a member of the MAF family of basic leucine zipper transcription factors and is a critical regulator of insulin gene expression and islet β-cell function. To be degraded by the proteasome, MAFA must be phosphorylated by GSK3 and MAP kinases at multiple serine and threonine residues (Ser49, Thr53, Thr57, Ser61, and Ser65) within its amino-terminal domain. In this study, we report that MAFA degradation is stimulated by PA28γ (REGγ and PSME3), a member of a family of proteasome activators that bind and activate the 20S proteasome. To date, only a few PA28γ-proteasome pathway substrates have been identified, including steroid receptor coactivator 3 (SRC3) and the cell cycle inhibitor p21. The PA28γ-mediated degradation of MAFA is regulated by phosphorylation at multiple sites within its amino-terminal region. We also found that a PA28γ mutant (N151Y) that did not stimulate p21 degradation enhanced MAFA degradation, and another mutant (K188D) that promoted greater p21 degradation did not enhance MAFA degradation. These results suggest that PA28γ stimulates MAFA degradation through a novel molecular mechanism that is distinct from that for the degradation of p21.

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Introduction

MAFA is a member of the MAF family of basic leucine zipper transcription factors. In avians, MAFA/L-MAF has been identified as a neuroretina-specific transcript (Benkhelifa et al. 1998) or a transcription factor that binds to the lens-specific enhancer in crystallin genes (Ogino & Yasuda 1998). Gain- and loss-of-function experiments in the developing chicken embryo revealed that MAFA/L-MAF is a key regulator of lens development (Reza et al. 2002).

In mammals, MAFA has been identified as a transactivator of the insulin gene that binds to a critical cis-regulatory element within the promoter termed C1/RIPE3b (Kataoka et al. 2002, Olbrot et al. 2002, Kajihara et al. 2003, Matsuoka et al. 2003). MAFA is exclusively expressed in β-cells in the pancreas (Kataoka et al. 2004, Zhang et al. 2005, Nishimura et al. 2006) and plays a critical role in establishing β-cell-specific expression of the insulin gene together with the other β-cell-enriched transcription factors, PDX1 and BET2/NEUROD (Aramata et al. 2005, Docherty et al. 2005, Kaneto et al. 2005). MAFA also regulates a set of β-cell-specific genes, including glucose transporter 2 (glu2) and granuphilin (Zhang et al. 2005, Kato et al. 2006, Wang et al. 2007). In Mafa knockout mice, islets are normal at birth, but β-cells exhibit a deficiency in glucose-stimulated insulin secretion over time and show progressive degeneration by 8–12 weeks of age (Zhang et al. 2005, Artner et al. 2007, Nishimura et al. 2008). These observations indicate that MAFA regulates the maturation, functional maintenance, and survival of β-cells. Recently, it was also shown that MAFA regulates intra-thymic expression of insulin and affects susceptibility to type I diabetes (Nosó et al. 2010).

Previous studies have demonstrated that phosphorylation plays critical roles in the regulation of MAFA activity. The amino-terminal domain of MAFA is first phosphorylated by an unidentified priming kinase at Ser65 and then sequentially by GSK3 at Ser61, Thr57, Thr53, and Ser49 (Han et al. 2007, Rocques et al. 2007). Thr57 and Ser65 can also be phosphorylated by ERK and/or p38 MAP kinase (Benkhelifa et al. 2001, Ochi et al. 2003, Sii-Felice et al. 2005). Phosphorylation at these sites is required for proteasomal degradation of MAFA protein (Ochi et al. 2003, Han et al. 2007, Rocques et al. 2007), and mutations at these phosphorylation sites influence the transforming and differentiation-inducing activities of MAFA (Benkhelifa et al. 2001, Nishizawa et al. 2003, Ochi et al. 2003, Pouponnot et al. 2006, Rocques et al. 2007). However, the molecular mechanism by which phosphorylated MAFA is degraded is unknown.
PA28γ (REGγ and PSME3) is a member of a family of proteasome activators that bind the 20S subunit of the proteasome (Dubiel et al. 1992, Ma et al. 1992). PA28γ is predominantly localized within the nucleus and forms homoheptamers, whereas PA28α and PA28β, other members of this family, form heteroheptamers in the cytoplasm (Realini et al. 1997, Wojcik et al. 1998). Biochemical and crystal structural analyses of PA28 family members have suggested that PA28 heptamers associate with both ends of the 20S proteasome like ‘caps’ through interactions between the proteasome and the carboxy-terminal tail of each monomer. On these interactions, the ‘activation loop’ of PA28 induces a conformational change of the 20S proteasome subunit and stimulates the protease activity (Whitby et al. 2000, Forster et al. 2005). Experiments using peptide substrates revealed that PA28α and PA28β stimulate three types of 20S proteasome catalytic activities: trypsin-like, chymotrypsin-like, and post-glutamyl peptidyl hydrolyzing activities. In contrast, PA28γ stimulates only trypsin-like activity (Realini et al. 1997).

To date, only a few protein substrates of the PA28γ-proteasome pathway have been identified. These substrates include steroid receptor coactivator 3 (SRC3), hepatitis C virus core protein, the tumor suppressor p53, and the cell cycle inhibitors p21 (CIP1/WAF1), p16 (INK4A), and p19 (ARF) (Morishita et al. 2003, Li et al. 2006, 2007, Chen et al. 2007, Zhang & Zhang 2008). PA28γ directly binds to SRC3 and p21 and promotes their proteasomal degradation independent of poly-ubiquitination and the 19S subunit. In contrast, PA28γ stimulates the degradation of p53 by enhancing its ubiquitination by MDM2. These observations suggest that PA28γ is a multi-functional protein involved in the degradation of a subset of nuclear proteins.

In this study, we demonstrate that degradation of MAFA is stimulated by PA28γ. PA28γ bound to MAFA and induced its proteasomal degradation in a phosphorylation-dependent manner. In addition, PA28γ attenuated MAFA-driven transcriptional activation of the insulin promoter. Using well-characterized PA28γ mutants (N151Y and K188D) (Zhang et al. 1998, Li et al. 2001), we found that PA28γ stimulated degradation of MAFA and p21 through distinct molecular mechanisms.

Materials and methods

Plasmids

The mammalian expression vector for hemagglutinin (HA)-tagged mouse Mafa (pHygEF2/HA-m-Mafα) and its derivatives (IRES-EGFP fusions and amino acid substitution mutants) were described previously (Han et al. 2007).

To construct the expression vectors for the PA28α, PA28β, and PA28γ FLAG epitope fusion proteins (pHygEF2/FLAG-PA28α, FLAG-PA28β, and FLAG-PA28γ), cDNA fragments containing the entire open frame of mouse PA28α, PA28β, and PA28γ were amplified from total RNA isolated from MIN6 mouse insulinoma cells by reverse transcriptase (RT)-PCR using the following primers: Pa28α, 5’-AGAACCTTAGTATGGCCACACTGAGGTCATCC-3’ and 5’-AGACCGGCGCTCAATAGATCATTCCCTGGTTT-3’; Pa28β, 5’-AGAACCTAGTATGGCCACACTGAGGTCATCC-3’ and 5’-AGACCGGCGCTCAATAGATCATTCCCTGGTTT-3’. The amplified fragments were cloned into the pHygEF2/FLAG-SUMO-2 plasmid (Kanai et al. 2010) by replacing the SpeI-NotI fragment.

To construct the FLAG-tagged GSK3β expression vector (pHygEF2/FLAG-GSK3β), the mouse GSK3β open reading frame was amplified by RT-PCR from mouse liver total RNA using the following primers: 5’-ACTAGTACCATGGATTACAAGGATGACGACGATA-3’ and 5’-GGGAAGGCCTCATGTCGGGGCCAGAGAAACC-3’. The amplified fragment was inserted into the pCRII-TOPO plasmid (Invitrogen) by TA cloning. The SpeI-NotI fragment was excised from the resulting plasmid and inserted into the pHygEF2 vector following digestion with XbaI and NotI.

All of the PA28γ (N151Y and K188D) and GSK3β (S9A and K85R) point mutants were generated by site-directed overlap extension PCR mutagenesis (Ho et al. 1989).

Transfection, immunoprecipitation, and immunoblotting

NIH3T3 or 293T cells grown in 24-well plates were transfected with a total of 0.8 μg plasmid using 2 μl lipofectamine 2000 reagent (Invitrogen). In1024 cells were transfected with a total of 1.6 μg plasmid using 4 μl lipofectamine 2000 (Invitrogen). After 24 h of transfection, whole cell extracts were prepared by the direct addition of 4X SDS sample buffer (200 mM Tris–HCl (pH 6.8), 8% SDS, 400 mM dithiothreitol, 0-2% bromophenol blue, and 40% glycerol) and then subjected to immunoblot analysis as described previously (Kataoka et al. 2002).

For immunoprecipitation, HeLa cells grown in 6-well plates were transfected with a total of 3-2 μg plasmid using 8 μl lipofectamine 2000 (Invitrogen). Whole cell extracts were prepared by cell lysis in 800 μl NETN buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris–HCl, pH 7.5, and 0-1%
NP-40) containing a protease inhibitor cocktail (Nacarai tesque, Kyoto, Japan), sonicated for 20 s, and then centrifuged. Aliquots of the extracts were subjected to immunoprecipitation using anti-HA-agarose (Roche) and then examined by immunoblot analysis. The antibodies used were as follows: anti-HA (MBL, Nagoya, Japan), anti-FLAG (M2, Sigma), anti-GFP (Clontech, Mountain View, CA, USA), anti-TF-IID (TAPI-binding protein (TBP) SI-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-c-Maf (M-153, Santa Cruz), and anti-PA28γ (PW8190; Enzo Life Sciences, Plymouth Meeting, PA, USA). MG132, epoxomicin, lactacystin (Merck), SB216763 (Tocris Bioscience, Ellsville, MO, USA), and cycloheximide (CHX, Sigma) were obtained commercially.

RNAi

NIT-1 cells grown in 24-well plates were transfected with 15 pmol siRNA using PrimaPort (Credia Japan, Kyoto, Japan) and were subjected to a second round of transfection 24 h later. A synthetic siRNA targeting mouse Pa28γ was purchased from Invitrogen (Psme3-MSS208070).

The pEF3-GFPbs-U6 plasmid to express short-hairpin RNA (shRNA) was designed to clone a double synthetic oligonucleotide into the U6 small nuclear RNA promoter using BseRI-BglII restriction sites. The oligonucleotides used for silencing human PA28γ were as follows: 5'-GAACCAAGGTGGTTTGTGAttcaagagaTACAAACACCTTGGTTCCttttt-3' and 5'-GATCaaaaaGGAACCAAGGTGGTTTGTGAtctcttgaaTCACAAACACCTTGGTTTca-3'.

Luciferase assay

The luciferase reporter plasmid (pGLA/h-ins-p) was constructed by inserting a KpnI-HindIII fragment of the pGL2-based h-ins-p-luc plasmid (Kataoka et al. 2002) into pGL4.10 (Promega). pEF-Rluc were described previously (Kataoka et al. 2001). NIH3T3 cells grown in 24-well plates were transfected with a total of 0.8 μg plasmid DNA (0.05 μg pGL4/h-ins-p, 0.05 μg pEF-Rluc, and 0.7 μg of the expression plasmids) using lipofectamine 2000 (Invitrogen). Cells were harvested 24 h after transfection, and the firefly and Renilla luciferase activities were measured using the Dual Luciferase

**Figure 1** PA28γ decreased the amount of MAFA protein. (A) Schematic structure of the pHygEF2/HA-m-MafA-IRES-EGFP expression plasmid. EF1α, elongation factor 1α; IRES, internal ribosome entry site; EGFP, enhanced green fluorescent protein; bZip, basic leucine zipper. (B) NIH3T3 cells were transfected with a constant amount (0.2 μg) of pHygEF2/HA-m-MafA-IRES-EGFP and an increasing amount (0.2, 0.4, and 0.6 μg) of the FLAG-tagged PA28γ (F-PA28γ) expression plasmid. Total cell extracts were analyzed by immunoblot using anti-HA, anti-FLAG, and anti-EGFP antibodies. TATA-binding protein (TBP) was used as a loading control. (C) Knockdown of PA28γ. 293T cells were transfected with expression vectors for HA-MAFA (0.4 μg) and Pa28γ-targeted shRNA (0.4 μg). Cell extracts were analyzed for HA-MAFA and endogenous PA28γ. (D) The insulinoma-derived cell line NIT-1 was transfected with Pa28γ-targeted siRNA. Endogenous MAFA and PA28γ proteins were analyzed by immunoblot. An asterisk indicates non-specific (NS) cross-reacting material serving as a loading control. (E) NIH3T3 cells were transfected with 0.2 μg HA-m-MafA-IRES-EGFP expression plasmid together with 0.6 μg expression plasmids for F-PA28γ, F-PA28α, F-PA28β, or F-PA28α plus F-PA28β as indicated. Total cell extracts were immunoblotted with the indicated antibodies.
Cell extracts were prepared, and the expression levels of the transgenes were analyzed by immunoblotting with anti-HA and anti-FLAG antibodies. Expression of EGFP was also analyzed to normalize the transfection efficiency, and TBP was monitored as a loading control. The amount of HA-MAFA was reduced by F-PA28γ co-expression in a dose-dependent manner (Fig. 1B).

To examine the role of endogenous PA28γ in the regulation of MAFA protein levels, we used 293T cells because HA-MAFA protein is relatively unstable in this cell line (see Fig. 3D). 293T cells were transfected with an expression plasmid for HA-MAFA together with the shRNA expression vector designed to target PA28γ. Immunoblot analysis showed that the amount of MAFA was increased by the knockdown of PA28γ (Fig. 1C).

The effect of PA28γ knockdown on the amount of endogenous MAFA protein was examined. An siRNA designed to target PA28γ was transfected into a β-cell-derived cell line NIT-1 in which endogenous MAFA protein was expressed. Immunoblot analysis showed that endogenous PA28γ was reduced in cells transfected with Pa28γ-siRNA and that endogenous MAFA protein was increased in these cells (Fig. 1D). These results indicated that PA28γ might negatively regulate the amount of MAFA protein by inducing degradation.

Then, the effect of PA28α and PA28β, other PA28 family members, on MAFA reduction was determined. PA28γ predominantly localizes in the nuclei and forms a homohexameter, whereas PA28α and PA28β localize in the cytoplasm and form heteromeric hexamers (Realini et al. 1997, Wojcik et al. 1998). As shown in Fig. 1E, PA28α or PA28β alone or in combination did not significantly influence the amount of HA-MAFA.

**Phosphorylation of MAFA is required for PA28γ-mediated reduction**

Previous studies have shown that MAFA degradation is dependent on phosphorylation at multiple serine and threonine residues (Ser49, Thr53, Thr57, Ser61, and Ser65) in the amino-terminal domain (Han et al. 2007, Rocques et al. 2007). To investigate the role of these phosphorylation sites in MAFA downregulation by PA28γ, a MAFA substitution mutant (5A) in which these serine and threonine residues were replaced by alanine residues was examined (Fig. 2A). As we previously observed (Han et al. 2007), the electrophoretic mobility of the 5A mutant protein was higher than that of wild-type (WT) MAFA due to the absence of phosphorylation at the mutated sites (Fig. 2B). In contrast to WT MAFA, the 5A mutant was resistant to PA28γ-mediated reduction. These results suggested that phosphorylation at these amino acid residues is required for the PA28γ-mediated reduction of the amount of MAFA.

To determine whether PA28γ affects the transcriptional activity of MAFA, a luciferase reporter gene driven

**Results**

**PA28γ reduces the amount of MAFA protein**

To test the possible involvement of PA28γ in the regulation of the amounts of MAFA protein, an expression plasmid for HA-tagged full-length MAFA (HA-MAFA) fused to IRES-EGFP under the control of the constitutively active EF1α promoter was used (Fig. 1A). We transfected NIH3T3 cells with a constant amount of this plasmid together with an increasing amount of an expression plasmid for FLAG-tagged PA28γ (F-PA28γ). NIH3T3 was used as a recipient cell for this assay because HA-MAFA protein is relatively stable in this cell line (Han et al. 2007, see also Fig. 3C).
by the human insulin promoter was co-transfected into NIH3T3 cells together with expression plasmids for HA-MAFA and F-PA28γ alone or in combination (Fig. 2C). As previously demonstrated, both WT MAFA and the 5A mutant stimulated reporter activity to a similar degree (Han et al. 2007). Co-expression of F-PA28γ reduced transcriptional activation by WT MAFA, but not by the 5A mutant. These results suggested that PA28γ suppressed the transcriptional activity of MAFA by reducing the amount of protein.

**PA28γ enhances proteasomal degradation of MAFA**

To determine whether PA28γ reduces the amount of MAFA by enhancing its degradation, NIH3T3 cells were treated with the proteasome inhibitors MG132, epoxomicin, or lactacystin. These drugs blocked the effect of PA28γ on MAFA (Fig. 3A). In 293T cells, MG132 treatment increased the amount of MAFA protein (Fig. 3B). Knockdown of PA28γ resulted in an accumulation of MAFA, and MG132 did not induce further increase in MAFA in these cells. These results indicated that PA28γ promoted the degradation of MAFA by way of the proteasome.

The degradation rates of MAFA in the absence or presence of F-PA28γ were measured. Nascent protein synthesis was blocked in transfected cells using CHX treatment and the amount of HA-MAFA was measured by immunoblotting. WT HA-MAFA disappeared more rapidly in the presence of F-PA28γ than in its absence (Fig. 3C). The phosphorylation-deficient MAFA mutant (5A) was stable, and its degradation rate was

![Image of Figure 3](https://www.endocrinology-journals.org/pa28gamma-dependent-degradation-of-mafa)
not significantly affected by the presence of F-PA28γ. The reduction of endogenous PA28γ by shRNA resulted in a significant increase in the half-life of WT HA-MAFA protein (Fig. 3D). These results demonstrated that PA28γ promoted proteasomal degradation of MAFA that depended on its phosphorylation status.

PA28γ associates with MAFA depending on phosphorylation

The interaction between MAFA and PA28γ was examined by the co-expression of WT or 5A HA-MAFA and F-PA28γ in HeLa cells. Cell extracts were subjected to immunoprecipitation with an anti-HA antibody and immunoblot analysis with anti-HA and anti-FLAG antibodies (Fig. 4). In the context of WT HA-MAFA co-expression, F-PA28γ was detected in the anti-HA immunoprecipitate. However, the MAFA 5A mutant did not co-immunoprecipitate F-PA28γ. Thus, MAFA interacted with PA28γ in a phosphorylation-dependent manner.

GSK3β cooperates with PA28γ to induce MAFA degradation

We previously demonstrated that MAFA is first phosphorylated at Ser65 by an unidentified kinase and is then sequentially phosphorylated at Ser61, Thr57, Thr53, and Ser49 by GSK3 (Han et al. 2007). We thus investigated the role of GSK3 in PA28γ-mediated degradation of MAFA. Treatment of the transfected cells with SB216763, a specific inhibitor of GSK3, induced the accumulation of hypo-phosphorylated forms of HA-MAFA and blunted the PA28γ-mediated degradation of MAFA (Fig. 5A). This result indicated that the phosphorylation of MAFA by GSK3 is required for PA28γ-mediated protein degradation.

Then, NIH3T3 cells were transfected with a constant amount of the HA-MAFA expression plasmid and an increasing amount of the F-PA28γ expression vector. Expression plasmids for WT, constitutively active (CA), or kinase-deficient (KD) forms of GSK3β were also included. Cell extracts were analyzed for protein expression by immunoblotting (Fig. 5B). In this experimental setting, the amount of the PA28γ expression vector was kept low so that PA28γ alone promote only marginal degradation of WT MAFA. However, co-expression of GSK3β, particularly the CA form, enhanced MAFA degradation by PA28γ. In contrast, the KD form of GSK3β had little effect, indicating that the stimulation of PA28γ-mediated MAFA degradation by GSK3β depends on its kinase activity. The amount of the 5A form of MAFA was not largely affected by co-expression of F-PA28γ or either form of GSK3β.
These results indicate that GSK3β cooperates with PA28γ to induce the degradation of MAFA through the phosphorylation of its target serine and threonine residues.

**PA28γ-mediated degradation of MAFA and p21 occurs through distinct molecular mechanisms**

To investigate the molecular mechanism by which PA28γ stimulates MAFA protein degradation, we utilized two well-defined PA28γ mutants. It has been proposed that the PA28γ heptamer binds to the 20S proteasome and stimulates its trypsin-like activity (Realini et al. 1997). The N151Y mutant is capable of binding to the 20S proteasome but is unable to enhance its catalytic activity (Zhang et al. 1998). In contrast, the K188D mutant is hyperactive, activating not only the 20S proteasome trypsin-like activity but also its chymotrypsin-like and post-glutamyl peptidyl hydrolyzing activities (Li et al. 2001).

To confirm the characteristics of these mutants, we tested their ability to stimulate degradation of the p21/CIP1, a previously identified PA28γ target (Chen et al. 2007, Li et al. 2007). As expected, p21 degradation was enhanced by WT PA28γ but not by the N151Y mutant (Fig. 6A), whereas the K188D mutant more potently induced p21 degradation. We then tested the effect of these mutants on MAFA stability (Fig. 6B). The defective N151Y and the WT PA28γ induced MAFA degradation to a similar extent, whereas the hyperactive mutant K188D did not induce MAFA degradation. These results indicated that PA28γ stimulates the degradation of p21 and MAFA by distinct molecular mechanisms.

**Discussion**

Previous studies have shown that the phosphorylation of MAFA at multiple sites (Ser49, Thr53, Thr57, Ser61, and Ser65) by a priming kinase and GSK3 are critical for its proteasomal degradation (Han et al. 2007, Rocques et al. 2007), but the molecular mechanism underlying this degradation was unknown. In this study, several lines of evidence suggest that PA28γ is involved in the degradation of MAFA. Overexpression of PA28γ stimulated the degradation of MAFA depending on the phosphorylation and attenuated transactivation of the insulin promoter. Furthermore, GSK3 cooperated with PA28γ to enhance MAFA degradation. Conversely, the knockdown of PA28γ using siRNA or shRNA increased endogenous and exogenous MAFA protein, while pharmacological inhibition of GSK3 eliminated the PA28γ-mediated degradation of MAFA. Co-transfection and co-immunoprecipitation experiments revealed that PA28γ binding to MAFA was dependent on phosphorylation.

Previous reports have demonstrated that PA28γ directly binds to p21 and SRC3 and stimulates their degradation via the 20S proteasome by an unknown mechanism that is not dependent on poly-ubiquitination and the 19S regulatory subunit (Chen et al. 2007, Li et al. 2007). Using two well-characterized PA28γ mutants, N151Y and K188D, we showed that PA28γ stimulates the degradation of MAFA and p21 by distinct molecular mechanisms. The N151Y mutant can bind to the 20S proteasome but is unable to enhance its protease activity. Here, the N151Y mutant did not stimulate p21 degradation but it enhanced MAFA degradation. In contrast, the ‘hyperactive’ K188D mutant stimulated p21 degradation more than WT PA28γ but it did not enhance MAFA degradation. Additional studies are required to elucidate the exact molecular mechanism to explain how PA28γ enhances MAFA degradation.

PA28γ knockout mice exhibit growth retardation, and embryonic fibroblasts derived from them have defects in mitosis and apoptosis (Murata et al. 1999). Thus, it has been proposed that p21 and p53 are potential targets of PA28γ in the regulation of cell cycle progression and apoptosis (Chen et al. 2007, Li et al. 2007, Zhang & Zhang 2008), but the exact role of PA28γ in the cell cycle and other biological processes is largely unknown. In this study, MAFA was identified as a new target of PA28γ. Examination of the development and function of pancreatic β-cells in PA28γ-knockout mice is warranted to determine whether PA28γ is involved in β-cell morphology and function. As MAFA regulates cell differentiation as well as oncogenic transformation (Nishizawa et al. 2003, Pouponnot et al. 2006), PA28γ may also be involved in these processes. Further studies of PA28γ and its
target proteins will help define its role in the degradation of a subset of nuclear proteins important in a variety of biological processes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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