Tumor Necrosis Factor-induced Microtubule Stabilization Mediated by Hyperphosphorylated Oncoprotein 18 Promotes Cell Death

Katia Vancompernolle†, Tom Boonefaes, Matthias Mann‡, Walter Fiers, and Johan Grooten
From the Department of Molecular Biology, Ghent University and Flanders Interuniversity Institute for Biotechnology, K. L. Ledeganckstraat 35, B-9000 Ghent, Belgium and the §Protein and Peptide Group, EMBL, Meyerhofstrasse 1, 69117 Heidelberg, Germany

Tumor necrosis factor (TNF)-induced cell death in the fibrosarcoma cell line L929 occurs independently of caspase activation and cytochrome c release. However, it is dependent on mitochondria and is characterized by increased production of reactive oxygen intermediates that are essential to the death process. To identify signaling molecules involved in this TNF-induced, reactive oxygen intermediate-dependent cell death pathway, we performed a comparative study by two-dimensional gel electrophoresis of phosphoproteins from a mitochondria-enriched fraction derived from TNF-treated and control cells. TNF induced rapid and persistent phosphorylation of the phosphorylation-responsive regulator of the microtubule (MT) dynamics, oncoprotein 18 (Op18). By using induced overexpression of wild type Op18 and phosphorylation site-deficient mutants S25A/S38A and S16A/S63A in L929 cells, we show that TNF-induced phosphorylation on each of the four Ser residues of Op18 promotes cell death and that Ser16 and Ser63 are the primary sites. This hyperphosphorylation of Op18 is known to completely turn off its MT-destabilizing activity. As a result, TNF treatment of L929 cells induced elongated and extremely tangled microtubules. These TNF-induced changes to the MT network were also observed in cells overexpressing wild type Op18 and, to a lesser extent, in cells overexpressing the S25A/S38A mutant. No changes in the MT network were observed upon TNF treatment of cells overexpressing the S16A/S63A mutant, and these cells were desensitized to TNF-induced cell death. These findings indicate that TNF-induced MT stabilization is mediated by hyperphosphorylation of Op18 and that this promotes cell death. The data suggest that Op18 and the MT network play a functional role in transduction of the cell death signal to the mitochondria.

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gene-inductive activities of TNF.

To identify molecules that are involved in the cytokotoxic process downstream of the receptor-proximal events, we studied lysesates from cells that had been stimulated with TNF for 1.5 h.

To date, three proteins with reproducible and large increases in phosphorylation upon TNF treatment have been detected; in this study we examined one of those proteins, oncprotein 18 (Op18, stathmin). Op18 is a conserved cytoplasmic phosphoprotein that is highly expressed in a wide variety of cancers, including a subset of leukemias and breast carcinomas (14–16). Its high abundance seems to be necessary for the maintenance of the transformed phenotype (17). Op18 is a phosphorylation-responsive regulator of microtubule (MT) dynamics that increases the catastrophe rate of MTs (depolymerization or shrinkage phase of individual MTs) in a dose-dependent manner (18, 19).

Op18 can be phosphorylated in vivo on four Ser residues (Ser16, Ser25, Ser38, and Ser63) (20) and is a target for both cell cycle and cell surface receptor-coupled kinase systems (for a review, see Ref. 21). Phosphorylation on all four Ser residues completely turns off its MT-destabilizing activity, leading to cell cycle and cell surface receptor-coupled kinase systems (for a role for Op18 may be to regulate the MT system in response to progression through the cell cycle. However, the actual role of Op18 in mitosis could be rather passive, and the only thing that may be required during mitosis is switching off its microtubule-destabilizing activity.

The high stoichiometry of Op18 phosphorylation by several receptor-regulated kinase systems suggests that the primary role for Op18 may be to regulate the MT system in response to external signals in interphase cells. In this paper, we describe for the first time a functional role for Op18 in TNF-induced cell death.

MATERIALS AND METHODS

Cell Lines and Cultures

All L929 cells were cultured in Dulbecco's modified Eagle's medium supplemented with heat-inactivated fetal calf serum (5%, v/v), heat-inactivated newborn calf serum (5%, v/v), penicillin (100 units/ml), streptomycin (0.1 mg/ml), and l-glutamine (2 mM), at 37 °C in a humidified incubator under a 5% CO₂ atmosphere.

Reagents

Murine TNF was produced in Escherichia coli and purified to at least 99% homogeneity in our laboratory. It had a specific activity of 1.9 × 10⁹ IU/mg of protein (National Institute for Biological Standards and Control, Potters Bar, UK), contained 4 ng of endotoxin/mg of protein, and was used at 1000 IU/ml. Recombinant human interferon-α, which is also active on murine cells, was a generous gift from Dr. C. Weissmann (University of Zürich); it had a specific antiviral activity of 7.9 × 10³ units/mg, as determined on murine cells in a L929 cell/vesicular stomatitis virus assay and was used at 1000 units/ml. Propidium iodide (PI) and cycloheximide (CHX) (all from Sigma) were used at concentrations of 30 μM and 50 μg/ml, respectively. The polyclonal anti-Op18 anti-serum was a generous gift from Dr. M. Gullberg (University of Umeå, Umeå, Sweden).

Plasmids

The cDNAs encoding WT Op18 and phosphorylation site-deficient mutants were generous gifts from Dr. M. Gullberg (University of Umeå). Plasmid pSP64Mx, containing the murine Mx promoter, was donated by Dr. C. Weissmann (25). Plasmid pSV2neo contains the bacterial neo genes used as a selection marker in transfection experiments (26).

Construction of Expression Plasmids

Inserts encoding the mutants and WT Op18 were isolated as Xhol/Smal fragments from the Bluescript pBS plasmid and cloned as blunted fragments into the blunted BamHI site of the pSP64Mx expression vector.

Measurement of TNF-induced Cell Death by Flow Cytometry

Cell death in L929 and L929-derived cell lines was induced by the addition of TNF (1000 IU/ml) to the cell suspension. Cell death was measured by quantifying PI-positive cells by FACS (FACSCalibur, Beckton Dickinson, San Jose, CA). The PI dye was excited with an argon-ion laser at 488 nm; PI fluorescence was measured above 590 nm using a long pass filter. Routinely, 3,000 cells were analyzed. Cell death is expressed as the percentage of PI-positive cells in the total cell population.

Radiolabeling of Cells and Preparation of the Subcellular Protein Fractions

L929 cells were plated 48 h prior to the experiment. ³²P labeling was carried out as described in Ref. 13. TNF treatments (1000 IU/ml, 1.5 h) were done in the presence of CHX, to synchronize cell death. To simplify the two-dimensional phosphoprotein pattern and subsequent computer analysis, we prepared two subcellular fractions: the cytosolic protein fraction, containing soluble cytoplasmic molecules and molecules derived from single-membrane organelles, was obtained as the supernatant from digitonin (0.03%)-permeabilized cells. After rinsing once with excess phosphate-buffered saline buffer, the remaining cell fraction was lysed in a CHAPS (2%)-containing buffer as described in Ref. 13. This lystate was then centrifuged (20,000 × g), and the supernatant was used as the organelle fraction; it is enriched for mitochondrial and cytoskeleton-derived proteins. The three protein spots detected with increased phosphorylation upon TNF treatment were present in both subcellular fractions.

Two-dimensional Gel Electrophoresis

Isoelectric Focusing—Isoelectric focusing was carried out on 18-cm IPG strips, pH 4–7 (Amersham Pharmacia Biotech), according to the manufacturer’s instructions. Protein samples were precipitated with ethanol and redissolved in lysis buffer.

SDS-Polyacrylamide Gel Electrophoresis—The second dimension (SDS-polyacrylamide gel electrophoresis) was run on large horizontal Excell gradient gels, 12–14% acrylamide (Amersham Pharmacia Biotech), according to the manufacturer’s instructions.

Western Blotting—Proteins were separated by SDS-polyacrylamide gel electrophoresis (14%) and transferred to a polyvinylidene difluoride membrane (Hybond-P, Amersham Pharmacia Biotech). The blots were incubated with the desired antibody, followed by ECL-based detection (Amersham Pharmacia Biotech).

Amino Acid Sequence Analysis by Nanoelectrospray Mass Spectrometry

Amino acid sequence analysis of peptides derived from an in situ digest of a Coomassie Blue-stained two-dimensional gel protein spot was performed as described previously (27).

Immunofluorescence Microscopy

L929 cells were grown on glass coverslips for 48 h. Following TNF treatment, cells were fixed with methanol at −20 °C. MTs were stained with a 1:1 mixture of 1:200 anti-tubulin (Harlan Sera-Lab, Crawley Down, UK) and detected with a fluorescein isothiocyanate-conjugated goat anti-rat IgG antibody (Harlan Sera-Lab). Coverslips were mounted in VectaShield and analyzed using a Zeiss Axioskop microscope.

RESULTS

TNF Induces Increased Phosphorylation of Op18—Fig. 1A shows the autoradiogram of the two-dimensional gels from TNF-treated and control samples. The protein spot with increased phosphorylation identified as Op18 is indicated by the arrow. It was identified by nanoelectrospray mass spectrometry analysis of tryptic peptides derived from an in situ digest of the excised protein spot (data not shown). The identity of the spot was further confirmed by Western blotting using an anti-polyclonal antibody against Op18 (Fig. 1B). As shown, TNF treatment increased the amount of the mono- and diphostorylated forms of Op18, which correspond with the γ₁, α₂, and α₁ forms, according to the pattern described in Ref. 20. The γ₁ form is phosphorylated on either Ser¹⁶, Ser²⁵, Ser³⁸, or Ser⁶³; the α₂ form is phosphorylated on either Ser²⁵/Ser³⁸, Ser¹⁶/Ser²⁵, Ser⁶³/Ser³⁸, or Ser²⁵/Ser⁶³, the α₁ form is phosphorylated on either Ser¹⁶/Ser²⁵, Ser³⁸, or Ser²⁵/Ser⁶³.
TNF-induced Hyperphosphorylation of Op18 Promotes Cell Death—To investigate the role of Op18 and its phosphorylation in TNF-induced cell death, we overexpressed WT Op18 and the phosphorylation site-deficient mutants Op18 S25A/S38A and Op18 S16A/S63A in L929 cells. We chose an inductive expression vector for two reasons. First, it has been shown that overexpression of phosphorylation site-deficient mutants of Op18 causes a block during mitosis (18). A second and more important reason is the large clonal variability in sensitivity to TNF-induced cell killing, ranging in L929 subclones from almost resistant to complete cell killing in 3 h.²Because of the latter, it is unwise to compare TNF sensitivity among different clones. However, use of an inductive expression vector circumvents this clonal variability, because TNF sensitivity can be compared in the same clone upon induced expression of the protein.

Murine fibrosarcoma L929 cells were transfected with pSP64MxOp18 WT and phosphorylation site-deficient mutants using the calcium phosphate procedure. In this expression vector, Op18 is under the control of the murine Mx promoter that is inducible by type I interferon (IFN). Although the murine Mx promoter has been reported to result in tightly controlled heterologous gene expression in VERO cells (28), we found quite leaky expression in our system.

After a preliminary screen of G418-resistant clones, clones were further selected according to the following criteria: low leak expression, strong induced expression, and little or no cell toxicity upon induced expression of the protein. As a result, we obtained five clones expressing WT Op18 and only two clones per mutant with a low level of leak expression that was well tolerated by the cell and strongly induced expression of the proteins.

For induction, cells were incubated with 1000 IU/ml human interferon-α for 16 h prior to TNF treatment. Levels of the ectopically expressed proteins in the Op18 clones in the noninduced and induced states and the endogenous levels of Op18 in the control clones (Neo) are shown in Fig. 2, A and B. Essentially, all Op18 clones had detectable leak expression but also a strong induced expression.

TNF-induced cell death in the different clones was measured as a function of time by flow cytometry, using PI uptake as the parameter for the number of dead cells (5). For practical reasons, the experiments were performed in two sets. In the first set, we measured the effect of induced expression of WT Op18 on TNF-induced cell death. In a second set of experiments, we measured the effect of induced expression of the phosphorylation site-deficient mutants on TNF-induced cell death in comparison with clones overexpressing WT Op18 and control clones. The experiments were repeated several times, and similar results were obtained each time. Representative experiments are shown in Fig. 2. C and D show the percentage of cell death after 5 h of TNF treatment of the different clones in noninduced and induced conditions. The same results are presented in E and F, respectively, as the percentage increase in cell death in induced over noninduced conditions. As Fig. 2 clearly indicates, induced overexpression of WT Op18 caused a significant increase (average of 40–50%) in TNF-induced cell death compared with the same clones in the noninduced state. A slight increase (average of 6%) in cell death was also observed in control clones (Neo), due to human interferon-α treatment of the cells. An increase (average of 34%) in cell death was also observed in the clones overexpressing the Op18 S25A/ S38A mutant. On the contrary, no increase, or even a decrease (average of 2%), in cell death was observed in the clones

² K. Vancompernolle, unpublished observations.
overexpressing the Op18 S16A/S63A mutant. This decrease would be even more pronounced if the slight increase in cell death in the human interferon-α-treated control clones were taken into consideration.

To verify whether there was a correlation between the phosphorylation status of the ectopically expressed proteins and the effect on TNF-induced cell death, we examined the two-dimensional gel pattern of the phosphoisosforms of WT Op18 and mutants in TNF-treated and control cells (Fig. 3). Based on the two-dimensional pattern of the phosphoisosforms described in Ref. 20, TNF induces phosphorylation on all four Ser residues (Ser16, Ser25, Ser38, and Ser63) of WT Op18, with a marked increase in the α2 and α3 phosphoforms. In Fig. 3, the triple (α12) and quadruple (α22) phosphorylated forms are evident. The triple and quadruple phosphorylated forms were not detected in parental cells (Fig. 1B), probably because they were below the limit of detection. This hyperphosphorylation on all four Ser residues leads to maximum inactivation of the MT destabilizing activity of Op18 (23, 22, 29). The β0 isoform was also apparent upon TNF treatment; this isoform is derived from the same mRNA as the α0 form but has an as yet unknown post-translational modification (30). In the clone overexpressing the Ser25/Ser38 mutant, TNF still increased the amount of monophosphorylated and diphosphorylated forms and thus represents phosphoryla-

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**Fig. 2.** Effect of induced overexpression of WT Op18 and phosphorylation site-deficient mutants on TNF-induced cell death in L929 cells. (A) and (B), expression levels (exogenous plus endogenous) of WT Op18 and mutants in the selected clones in the noninduced (ni) and induced (i) condition. Note that basically all Op18 clones have some leak expression. Endogenous levels of Op18 are hardly detectable in the control (Neo) clones (the same amount of total protein was loaded as for the Op18 clones). (C) and (D), percentage of cell death after 5 h of TNF treatment of the different clones in the noninduced (white bars) and induced condition (black bars). (E) and (F), percentage increase in cell death of the induced condition over the noninduced condition for the same experiment as represented in (C) and (D), respectively.
phosphorylation of Ser25 and Ser38 is required for phosphorylation of the MT-destabilizing activity of Op18 by phosphorylation on Ser16 and Ser63 (23). However, this might be different in interphase cells. Strikingly, upon TNF treatment, no change in the phosphorylation pattern was observed in the clone overexpressing the S16A/S63A mutant.

Taken together, these results clearly indicate that TNF-induced hyperphosphorylation of Op18 promotes cell death, and phosphorylation on Ser16 and Ser63 seems to be a prerequisite for this. These data correlate well with the down-regulation of the MT-stabilizing activity of Op18 by phosphorylation described in the literature. Specifically, phosphorylation of either Ser16 or Ser63 in combination with Ser25 and Ser38 inhibits the MT-stabilizing activity of Op18 (23), while phosphorylation of both Ser 16 and 63 is sufficient to inhibit the MT destabilizing activity of Op18 in interphase and mitotic cells (24).

TNF-induced Microtubule Stabilization Is Mediated by Hyperphosphorylated Op18—Given the function of Op18 as a regulator of MT dynamics, we tested whether TNF actually induced changes in the organization and polymerization status of the MTs in L929 cells. As shown by immunofluorescence microscopy in Fig. 4, parental L929 cells have a fine and not very dense network of MT filaments distributed throughout the cell, excluding only the nucleus. After only 10 min of TNF treatment, at which point increased phosphorylation of Op18 was already detectable, the MT network had changed. Specifically, there was an overall increase in the immunofluorescence signal, showing a denser microtubule network. Even more pronounced changes in the organization of the MTs were evident after 1.5–3 h of TNF treatment (Fig. 4). A strong increase in the immunofluorescence signal was observed and a very dense and extremely tangled network of MTs was present. Additionally, the MTs were considerably more elongated and extended toward the rim of the cell. In addition, TNF-treated cells had a more spread phenotype (before they started to die), in comparison with control cells.

To investigate whether these TNF-induced changes in the MT network were mediated by phosphorylation of Op18, we examined the organization of the MT network in stable L929 clones that inducibly overexpressed WT Op18 and the phosphorylation site-deficient mutants Op18 S25A/S38A and Op18 S16A/S63A. Overexpression of WT Op18 and phosphorylation site-deficient mutants resulted in depolymerization of MTs, in agreement with previous reports (18, 22). In Fig. 5 (left panels), it can be seen that there are few microtubules present in the untreated cells (control) and that they are not homogeneous in thickness. Furthermore, the overall fluorescence signal was weak, indicating low amounts of MT polymer. TNF treatment of cells overexpressing WT Op18 resulted in the appearance of a dense network of elongated MTs. Additionally, the MTs appeared thicker than those in TNF-treated parental cells (Fig. 4). A TNF-induced increase in MTs was also observed in cells overexpressing the Op18 S25A/S38A mutant, although to a lesser extent than in cells overexpressing the WT protein (Fig. 5). However, the MTs were not as thick as in the TNF-treated cells overexpressing WT Op18. On the contrary, no change in the MT network could be detected upon TNF treatment of cells overexpressing the Op18 S16A/S63A mutant. It is noteworthy that the degree of tangling of MTs observed after TNF treatment is less pronounced in cells overexpressing WT Op18 than in parental cells. This may be because untreated cells overexpressing WT Op18 have lower levels of MT polymer than parental cells. In this case, higher levels of phosphorylated Op18 would be required to cause the same morphological change in the MT network observed after TNF treatment of parental cells. It is also possible that the TNF-induced changes in the MT network may be caused not only by phosphorylated Op18 but also by other regulators of MT dynamics.

Taking the data together, we draw the conclusion that TNF induces the stabilization of MTs, mediated by hyperphosphorylation of Op18. This MT stabilization is responsible for promotion of TNF-induced cell death.

DISCUSSION

In this study, we have identified Op18 as a protein that becomes rapidly and persistently phosphorylated upon TNF treatment of the fibrosarcoma cell line L929. TNF-induced cell death in these cells is independent of caspase activation and cytochrome c release but is dependent on mitochondria (7, 8) and is accompanied by increased production of reactive oxygen species (ROS).

Fig. 3. Phosphorylation status of the ectopically expressed WT Op18 and mutants in TNF-treated (1.5 h) and control cells. The in vitro phosphorylation status of Op18 and mutants in TNF-treated and control cells was analyzed by two-dimensional gel electrophoresis followed by Western blotting with a rabbit polyclonal anti-Op18 antibody. Equal amounts of total protein lysate were loaded on all gels. TNF induced phosphorylation of all four Ser residues (Ser16, Ser25, Ser38, and Ser63) in clones overexpressing WT Op18. Note the prominent increase of the double (α2, α1) and triple phosphorylated forms (α3, αβ). TNF also induced an increase of the mono- and diphosphorylated forms in cells overexpressing the Op18 S25A/S38A mutant. However, no change in phosphorylation status could be detected upon TNF treatment of cells overexpressing the Op18 S16A/S63A mutant.
It has been shown that phosphorylation of either Ser16 or Ser63 in combination with Ser25 and Ser38 inhibits the activity of Op18 (23) and that dual phosphorylation on Ser16 and Ser63 by protein kinase A is necessary and sufficient to switch off the MT-destabilizing activity of Op18 in intact cells (24). Phosphorylation on Ser25 and Ser38 are of only minor importance in regulating the activity of Op18 (23); this may explain why overexpression of the Op18 S25A/S38A mutant still has a cell death-promoting role, since its MT-destabilizing activity still can be turned off.

On the contrary, overexpression of the Op18 S16A/S63A mutant does not promote and may even decrease TNF-induced cell death, because its MT-destabilizing activity cannot be turned off. There are several possible explanations as to why TNF-induced cell death is not completely blocked by the latter. First, endogenous WT Op18 is still present, and thus the mutant protein has to compete with the WT protein. Second, TNF can also modulate the activity of other MT regulatory proteins that may all contribute to cell death. Furthermore, the MT system is not the only event involved in TNF-induced cell death in L929 cells. This suggests that TNF-induced cell death is the result of a concerted series of events.

In summary, we conclude that TNF-induced hyperphosphorylation of Op18 promotes cell death and that phosphorylation on Ser16 and Ser63 is necessary for this. Furthermore, the TNF-induced changes in the MT network (MT stabilization) can be attributed to the TNF-induced phosphorylation of Op18, and MT stabilization is in fact responsible for the TNF-induced cell death-promoting activity of phosphorylated Op18.

Analysis of the phosphorylation status of the ectopically expressed proteins after TNF treatment also reveals that the hierarchical order of Op18 phosphorylation is different from that in mitosis. During mitosis, dual phosphorylation on Ser25 and Ser38 is required for phosphorylation on Ser16 and Ser63 (23). However, in TNF-treated cells, no change in phosphorylation of the Op18 S16A/S63A mutant could be detected, while there was an increase in the mono- and diphosphorylated forms of the Op18 S25A/S38A mutant. Thus, during TNF-induced cell death, phosphorylation on Ser16 and/or Ser63 is required before phosphorylation on Ser25 and/or Ser38 can occur. Therefore, the hierarchical order of Op18 phosphorylation might determine a differential way of regulating MT-stabilizing activity of Op18 during cell proliferation and cell death.

The TNF-induced multisite phosphorylation of Op18 also indicates that several kinases become activated. It is tempting to speculate that protein kinase A is one of the likely candidates, since it has been shown that protein kinase A is activated by TNF (31), and that induced expression of the catalytic subunit of protein kinase A can switch off the activity of Op18 in vivo by phosphorylation on Ser16 and Ser63 (24). However, several other kinases can also phosphorylate Op18 (21, 32), and the kinases involved in TNF-triggered phosphorylation of Op18 remain to be determined.

Op18 and the MT network seem not only to play a role in TNF-induced cell death, but it has been reported that activation of p53 in cells also appears to target MT regulatory proteins. Notably, Op18 and MAP4 are down-regulated following p53 induction in, among others, MCF-7 cells (33). Furthermore, it has been shown that restoration of p53 function in these cells sensitizes cells that are TNF-resistant (because of the presence of mutated p53) to the cytotoxic action of TNF (34). Downregulation of Op18 would also result in MT stabilization, since it has been shown that microinjection of neutralizing anti-Op18 antibodies into newt long cells results in an increase of MT polymer and an associated decrease in catastrophe frequency.
TNF-induced Phosphorylation of Op18

(35). This may mean that Op18 is not only targeted by TNF via the kinase pathway but also transcriptionally, via p53.

The question of whether Op18 phosphorylation and MT stabilization directly or indirectly contribute to the TNF-induced mitochondria- and reactive oxygen intermediate-dependent cell death pathway remains unresolved. Currently, more and more data are emerging that indicate that microtubules play a role in signal transduction (reviewed in Ref. 36). MTs, together with MAPs and/or motor proteins, could contribute to the transmission of signals from the cell membrane to downstream targets in the cytoplasm. Three models have been proposed for this: 1) MT sequestering and delivery, 2) MT delivery, in which motor proteins are involved, and 3) MT scaffolding of signaling molecules (36). Thus, it could be that the role of Op18 in TNF signaling to cell death is indirect and that only the function of phosphorylated Op18 is to stabilize the MTs. This would mean that a primary signaling cascade is initiated starting from the TNF receptor and leading to MT stabilization that in turn may contribute to and promote the transmission of the death signal to the mitochondria. This primary signaling cascade presumably involves not only Op18; TNF might also modulate the activity of other MT regulatory molecules. The exact mechanism of signal transfer from the MTs to the mitochondria remains to be determined.

However, a more direct role of Op18 in the signal transfer to the mitochondria is not excluded. It is interesting to note that Op18 is differentially expressed in the brain of mitochondrial Mn-superoxide dismutase-deficient mice (37). This Mn-superoxide dismutase deficiency causes oxidative stress and mitochondrial dysfunction, two phenomena also occurring in TNF-induced cell death in L929 cells. As a result, it could be that Op18, in addition to regulating MT dynamics, has an additional, as yet unidentified cellular function.

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