Cellular prion protein mediates early apoptotic proteome alternation and phospho-modification in human neuroblastoma cells

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Anti-apoptotic properties of physiological and elevated levels of the cellular prion protein (PrPc) under stress conditions are well documented. Yet, detrimental effects of elevated PrPc levels under stress conditions, such as exposure to staurosporine (STS) have also been described. In the present study, we focused on discerning early apoptotic STS-induced proteome and phospho-proteome changes in SH-SY5Y human neuroblastoma cells stably transfected either with an empty or PRNP-containing vector, expressing physiological or supraphysiological levels of PrPc, respectively. PrPc-overexpression per se appears to stress the cells under STS-free conditions as indicated by diminished cell viability of PrPc-overexpressing versus control cells. However, PrPc-overexpression becomes advantageous following exposure to STS. Thus, only a short exposure (2 h) to 1 μM STS results in lower survival rates and significantly higher caspase-3 activity in control versus PrPc-overexpressing cells. Hence, by exposing both experimental groups to the same apoptotic conditions we were able to induce apoptosis in control, but not in PrPc-overexpressing cells (as assessed by caspase-3 activity), which allowed for filtering out proteins possibly contributing to protection against STS-induced apoptosis in PrPc-overexpressing cells. Among other proteins regulated by different PrPc levels following exposure to STS, those involved in maintenance of cytoskeleton integrity caught our attention. In particular, the finding that elevated PrPc levels significantly reduce profilin-1 (PFN-1) expression. PFN-1 is known to facilitate STS-induced apoptosis. Silencing of PFN-1 expression by siRNA significantly increased viability of PrPc-overexpressing versus control cells, under STS treatment. In addition, PrPc-overexpressing cells depleted of PFN-1 exhibited increased viability versus PrPc-overexpressing cells with preserved PFN-1 expression, both subjected to STS. Concomitant increase in caspase-3 activity was observed in control versus PrPc-overexpressing cells after treatment with siRNA-PFN-1 and STS. We suggest that reduction of PFN-1 expression by elevated levels of PrPc may contribute to protective effects PrPc-overexpressing SH-SY5Y cells confer against STS-induced apoptosis.

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Apoptosis is essential for maintenance of cellular homeostasis as part of normal development of the nervous system. At the same time apoptosis is also a characteristic of many neurodegenerative disorders. Furthermore, reduced apoptotic cell death or its obstruction is one of the critical cellular changes during malignant transformation.

Considering that cellular prion protein (PrPc) is necessary for propagation of prion diseases and that apoptosis has been described in the brains of patients affected by these diseases, a more complete understanding of PrPc impact on apoptotic cell death is required. Moreover, PrPc appears to be involved in the pathogenesis of Alzheimer disease and in promoting invasiveness of different cancer cell types, both of which are accompanied by dysregulated apoptosis.

Although expression of PrPc at physiological levels is known to exert protective, anti-apoptotic effects in vitro as well as in vivo, other evidence strongly suggest that PrPc overexpression in different cell lines sensitises cells to apoptotic stimuli. It appears that an augmented susceptibility to apoptotic stimuli, such as staurosporine (STS), is governed by a p53-dependent pathway. Moreover, in vivo findings demonstrated that PrPc overexpression can induce spontaneous neurodegeneration, and that local PrPc overexpression in muscles leads to primary myopathy, most likely via a p53 pathway. Earlier, we reported disturbed cellular homeostasis following PrPc overexpression in human neuroblastoma SH-SY5Y cells, but were unable to show that a sole overexpression of PrPc can alter p53 levels.

Yet, another study employing mouse neuroblastoma N2a cell line suggested that physiological levels of PrPc have a decisive protective role against STS-mediated cell death.

Keeping in mind that elevated PrPc levels per se may provoke neurodegeneration, that neurodegenerative diseases, including prion diseases are characterized by neuronal
apoptosis,19,20 and that rise in PrPc expression promotes invasiveness and survival of cancer cells,5,7 the aforementioned conflicting findings on PrPc expression levels and its associated pro- and/or anti-apoptotic properties should be further elucidated.

This study aimed at revealing largely unknown proteome and phospho-proteome changes of early apoptotic events following treatment of human neuroblastoma SH-SY5Y control cells, stably overexpressing an empty vector, with apoptotic agent STS versus SH-SY5Y cells stably over-expressing PrPc exposed to the same apoptotic agent.

STS is a non-selective protein kinase inhibitor that has been extensively used as one of the most potent pro-apoptotic stimuli in a variety of cells.21–23 Although molecular mechanisms of STS-induced apoptosis are still not completely clear an involvement of caspase activation is certain.

By identifying early changes in protein expression patterns between physiological and PrPc overexpressing levels, on ‘the edge of apoptosis’ (already present in control, but not in PrPc-overexpressing cells, as assessed by caspase-3 activation) we aimed at filtering out proteins contributing to previously observed expression level-mediated pro- and/or anti-apoptotic PrPc properties. Identification of these candidate proteins might improve our understanding of PrPc function both in health and disease.

Results

To identify early apoptotic changes following 2-h exposure to 1µM STS a quantitative proteome and phospho-proteome analysis was performed using human neuroblastoma SH-SY5Y cells stably transfected either with PRNP or an empty vector, respectively. An introduction of pCineoPRNP plasmid into SH-SY5Y cells treated with either DMSO or STS resulted in an average 5- (P = 3.7 × 10⁻⁵) to 7-fold (P = 6.5 × 10⁻⁵) higher expression of PrPc in PrP-loverexpressing (designated PrP) versus control SH-SY5Y cells (designated ctrl), as quantified by ELISA measurements (Figure 1). Remarkably, PrP cells demonstrated diminished viability in MTS assay as compared with control cells, both under treatment-free conditions (P = 1 × 10⁻⁷) and following DMSO treatment (P = 6.5 × 10⁻⁵). After STS treatment an increased viability rates of PrP versus control cells were observed (P = 0.019) (Figure 2a). Concomitantly, caspase-3 activity, a marker of programmed cell death, was significantly (P = 0.017) increased in control as compared with PrP cells, both subjected to STS treatment (Figure 2b).

Densitometric analysis of silver-stained 2-DE gels revealed altogether 14 (Table 1), whereas phospho-protein staining revealed 10 (Table 2) differentially expressed proteins (Figures 3 and 4) across all compared experimental groups. To be able to differentiate between protein expression changes induced by either STS or the solvent (DMSO) six different experimental groups were compared (Tables 1 and 2).

The threshold for identification of up-/down-regulated proteins was set to 1.5- (proteome) and 1.4-fold (phospho-proteome) changes, respectively. Based on this criterion, we detected 11 up- and 10 down-regulated proteins (five distinct proteins were repeatedly found to be differentially regulated across different transfection/treatment groups) following silver staining of 2-DE gels throughout all experimental groups.

Exemplarily, actin-interacting protein 1 was detected as differentially regulated in PrP⁺STS/ctrl+STS and PrP⁺STS/ctrl+DMSO group. Seven proteins exhibited a 2-fold or higher regulation after silver staining of 2-DE (Table 1). Two experimental groups: PrP⁺DMSO/ctrl+STS and PrP⁺STS/ctrl+DMSO did not demonstrate any differentially regulated proteins following silver staining.

According to above mentioned criterion, we detected 4 up- and 7 down-regulated phosphorylated proteins throughout all experimental groups. Altogether, a majority of differentially expressed proteins (45.5%) appertains to a protein metabolism/folding group of proteins, 22.7% to energy metabolism, 18.2% of proteins belong to cytoskeleton group of proteins and finally 13.6% to stress response group (Figure 5).

Owing to the emerging role of PrPc in maintenance of cytoskeleton organization,25 we decided to analyse expression level of two differentially regulated cytoskeletal proteins, PFN-1 and transgelin-2 using western blot analysis (Figure 6). Profilin-1 protein expression was 1.64-fold (P = 0.042) lower in PrP⁺STS versus ctrl+STS group with a score of 627, whereas transgelin-2 expression was 3.65-fold (P = 0.008) higher in PrP⁺STS as compared with ctrl+STS group with a score of 393 (Table 1). Densitometric analysis of western blot protein bands revealed ~1.5-fold lower expression of Pfn1 in PrP⁺STS/ctrl+STS and PrP⁺STS/ctrl+DMSO as compared with ctrl+STS group (P = 0.021). Nearly the same ratio of differential expression (1.5-fold) was retained between all the other groups compared, with the following levels of significance: ctrl+DMSO versus ctrl+STS (P = 0.001); PrP⁺DMSO versus PrP⁺STS (P = 0.046) and ctrl+DMSO versus PrP⁺DMSO (P = 0.0002). A significant elevation of transgelin-1 expression is observed in PrP⁺STS group as compared with all three other groups (P = 0.0003). Intriguingly, p53 expression pattern is analogous to that of Pfn1 (Figure 6a). Although not detected as regulated in densitometric analysis of 2-DE gels, we decided to check p53 expression levels between different experimental groups because of previously reported involvement of this protein in the exertion of PrPc function when
siRNA-PFN-1 versus siRNA-PFN-1, no protein bands neither in ctrl nor in PrP cells

mediated expressional regulation of PFN-1 as well as its Figures 8a and 6a). We also verified that level of PrP c-previously for siRNA-untreated cells (comparison between after transfection with non-targeting mock control as obtained

demonstrated lower survival rates (Figure 8b). 

Discussion

Former reports on PrPc overexpression in different experimental models remain controversial. Wild-type PrPc-overexpression can either exert protective effects as shown in BAX and TNF-α-mediated cell death in vitro28,29 or can induce neurodegeneration and primary myopathy in vivo14,16 Several earlier studies investigating pro- and anti-apoptotic features of PrPc using STS as an apoptotic agent, found that PrPc-overexpression renders cells more susceptible to apoptotic cell death, whereas cells devoid of PrPc expression demonstrate reduced susceptibility to STS-induced apoptosis. The mechanism of STS-induced PrPc-regulated cell death appears to be p53-dependent and to involve caspase-3 activation.12,13,26 However, the possibility that mechanism of action vary dependent on different cell types and STS concentrations used still persists.30 Hence, findings on mouse neuroblastoma N2a cell line showed that downregulation of PrPc sensitizes cells to STS-induced cytotoxicity and apoptosis, whereas an overexpression of PrPc has little or no effect.18 Interestingly, in the present study cell viability of PrPc-overexpressing cells under both control (cell medium) and DMSO conditions was significantly diminished as compared with SH-SY5Y cells expressing endogenous levels of PrPc. However, after exposure to stress conditions (STS treatment) PrPc-overexpression seemed to become advantageous and cell survival rates were even higher than in the control group.

The study target was to display early apoptotic proteome and phospho-proteome changes. The functional roles of identified proteins indicate that most STS-provoked apoptotic changes are linked to protein metabolism/folding, energy metabolism, stress response and cytoskeleton organization.

challenged with STS.26 Indeed, expression of p53 was 1.4-fold higher in ctrl+STS versus PrP+STS group (P = 0.0003). Ctrl+STS group showed 2.3-fold increase in p53 expression as compared with ctrl+DMSO group (P = 0.0005) and 3.9-fold increase in PrP+STS versus PrP+DMSO group (P = 7.2 × 10−5). Finally, a 2.3-fold decrease in p53 expression was observed in PrP+DMSO as compared with ctrl+DMSO group (P = 0.010).

Owing to the fact that PFFN-1 promotes STS-induced apoptosis,27 and elevated levels of PrPc downregulate its expression in SH-SY5Y cells, we decided to examine the link between PrPc and PFN-1 further.

Immunofluorescence analysis additionally verified PrPc-mediated expressional regulation of PFFN-1 as well as its altered co-localization pattern in ctrl+STS versus PrP+STS group (Figure 7).

To test if farther downregulation of PFN-1 has an effect on cell viability and caspase-3 activity of PrPc versus ctrl cells treated with STS, we performed PFN-1 gene expression silencing using siRNA approach. Western blot analysis demonstrated comparable PFN-1 protein expression pattern after transfection with non-targeting mock control as obtained previously for siRNA-untreated cells (comparison between Figures 8a and 6a). We also verified that level of PrPc overexpression following treatment with siRNA remained unchanged (data not shown). In cells transfected with siRNA-PFN-1, no protein bands neither in ctrl nor in PrP cells were visible (Figure 8a). Cell viability of PrPc cells treated with siRNA-PFN-1 versus ctrl cells treated with siRNA-PFN-1 both exposed to STS was significantly increased (P = 0.0006), whereas caspase-3 activity was decreased (P = 0.0216). In addition, PrP cells treated with siRNA-PFN-1 exhibited significantly higher survival rates (P = 0.0139) and slightly decreased caspase-3 activity (P = 0.0311) versus PrP cells treated with mock control, both subjected to STS (Figure 8b and c). Remarkably, ctrl cells treated with siRNA-PFN-1 demonstrated lower survival rates (P = 0.0022) as compared with ctrl cells treated with mock control, both subjected to STS (Figure 8b).
### Table 1: List of proteins identified from 2-DE gels of control and PrP\(^c\)-overexpressing SH-SY5Y cells treated either with DMSO or STS

| Groups compared | Spot no. | Fold change | Peptides matched | Score | State change | P-value | Protein ID | Accession no. |
|-----------------|----------|-------------|------------------|-------|--------------|---------|------------|---------------|
| PrP\(^{+STS}\)/ctrl\(^{+DMSO}\) | 375      | 1.95        | 10               | 166   | ↑ PrP\(^{+STS}\) | 0.003   | Succinate dehydrogenase (ubiquinone) flavoprotein subunit, mitochondrial | P31040 |
|                  | 384      | 1.60        | 9                | 127   | ↑ PrP\(^{+STS}\) | 0.048   | Actin-interacting protein 1 | O75083 |
|                  | 607      | 5.06        | 15               | 523   | ↑ PrP\(^{+STS}\) | 0.021   | Alpha-enolase | P06733 |
|                  | 702      | 1.52        | 3                | 81    | ↑ PrP\(^{+STS}\) | 0.049   | Stomatin-like protein 2 | Q9UJZ1 |
|                  | 1006     | 1.95        | 11               | 205   | ↑ PrP\(^{+STS}\) | 0.011   | Proteasome subunit alpha type-3 | P25788 |
|                  | 1049     | 3.19        | 3                | 100   | ↑ PrP\(^{+STS}\) | 0.020   | Alpha N-terminal protein methyltransferase 1 A | Q9BV86 |
|                  | 1042     | 10.16       | 6                | 92    | ↑ PrP\(^{+STS}\) | 0.0008  | Peroxiredoxin-4 | Q13162 |
|                  | 1016     | 1.60        | 14               | 395   | ↑ PrP\(^{+STS}\) | 0.018   | Ubiquitin-conjugating enzyme E2 O | Q9C0C9 |
|                  | 1105     | 1.76        | 5                | 54    | ↑ PrP\(^{+STS}\) | 0.048   | Adenine phosphoribosyl transferase | P07741 |
|                  | 1161     | 1.54        | 8                | 124   | ↑ PrP\(^{+STS}\) | 0.008   | Transgelin-2 | P37802 |
|                  | 1157     | 3.65        | 22               | 393   | ↑ PrP\(^{+STS}\) | 0.032   | 40 S ribosomal protein S12 | P25398 |
|                  | 1293     | 2.07        | 7                | 136   | ↑ PrP\(^{+DMSO}\) | 0.042   | Profilin-1 | P07737 |
|                  | 1294     | 2.45        | 3                | 86    | ↑ PrP\(^{+DMSO}\) | 0.019   | Ubiquitin-conjugating enzyme E2 K | Q9Y2D3 |
|                  | 1161     | 1.84        | 8                | 124   | ↑ PrP\(^{+DMSO}\) | 0.042   | Profilin-1 | P07737 |
|                  | 375      | 1.59        | 10               | 166   | ↓ ctrl\(^{+STS}\) | 0.020   | Succinate dehydrogenase (ubiquinone) flavoprotein subunit, mitochondrial | P31040 |
| PrP\(^{+DMSO}\)/ctrl\(^{+DMSO}\) | 1108     | 1.90        | 3                | 86    | ↓ ctrl\(^{+STS}\) | 0.028   | Ubiquitin-conjugating enzyme E2 K | Q9Y2D3 |
|                  | 1161     | 1.58        | 8                | 124   | ↓ ctrl\(^{+STS}\) | 0.009   | Adenine phosphoribosyl transferase | P07741 |
|                  | 1294     | 2.07        | 6                | 263   | ↓ ctrl\(^{+STS}\) | 0.013   | Profilin-1 | P07737 |
|                  | 384      | 1.90        | 9                | 127   | ↓ PrP\(^{+DMSO}\) | 0.024   | Actin-interacting protein 1 | O75083 |

Fourteen different proteins were identified from 2-DE gels of SH-SY5Y cells stably transfected either with pCneoPRNP or an empty vector. The number of spots corresponds to their location on the gel (Figure 3). The comparison of protein regulation between distinct transfection groups, fold change, number of peptides matched, ion score, state change, significance (unpaired Student's t-test), protein identification and Swiss-Prot accession numbers are given for each spot. The depiction of transfection groups is as follows: ctrl\(^{+DMSO}\) = empty vector transfected cells treated with DMSO; ctrl\(^{+STS}\) = empty vector transfected cells treated with STS; PrP\(^{+DMSO}\) = PrP\(^c\)-overexpressing cells treated with DMSO; PrP\(^{+STS}\) = PrP\(^c\)-overexpressing cells treated with STS.
Ten different proteins were identified from phospho-stained 2-DE gels of SH-SY5Y cells stably transfected either with pCIneo PRNP or an empty vector (protein spot no. 394 remained unidentified). The number of spots corresponds to their location on the gel (Figure 4). The comparison of protein regulation between distinct transfection groups, fold change, number of peptides matched, ion score, state change, significance (unpaired Student’s t-test), protein identification and Swiss-Prot accession numbers are given for each spot. The depiction of transfection groups is as given in Table 1.

A cytoskeleton protein that showed significant changes between control and PrP<sup>S</sup>-overexpressing cells after STS treatment was profilin-1 (PFN-1). This protein is ubiquitously expressed actin-binding protein, which when overexpressed facilitates STS-induced apoptosis. It is well-established that actin-binding proteins are of utmost importance for the adhesion of cells to extracellular matrices (ECM) and for cell survival because of their role in linkage of integrins to actin cytoskeleton. The fact that apoptotic cells display an early detachment from ECM and the rearrangements of actin cytoskeleton, an elevated expression of PFN-1 in control as compared with PrP<sup>S</sup>-overexpressing SH-SY5Y cells, both subjected to STS, might indicate an increased susceptibility of the former group to STS-mediated apoptotic cell death. In addition, control versus PrP<sup>S</sup>-overexpressing SH-SY5Y cells subjected to DMSO showed likewise significantly higher expression of PFN-1. It appears that STS treatment itself is sufficient to up-regulate PFN-1 expression, whereas PrP<sup>S</sup> overexpression seems to be able to decrease it. Of interest, N-terminal sequence of PrP<sup>S</sup> possesses extended poly-(L proline) II (PPII) helix and profilin family is known to bind to polyproline ligands advancing actin polymerization and cell motility, which becomes important during tumor progression. Hence, downregulation of PFN-1 was reported in different adenocarcinoma (i.e., breast, pancreatic). Loss of PFN-1 expression led to increased motility and invasiveness of MDA-MB-231 breast cancer cells, whereas PFN-1 overexpression suppressed micro-metastasis of MDA-MB-231 cells in nude mice. Conversely, an upregulation of PrP<sup>S</sup> expression was detected in metastatic gastric cancer, and its expression in pancreatic ductal adenocarcinoma was correlated with a marked decrease in patients’ survival. Interestingly, we showed a significant re-distribution of PFN-1 in PrP<sup>S</sup>-overexpressing versus control cells both exposed to STS. Furthermore, silencing of PFN-1 by siRNA resulted in a significant increase in viability and decrease in caspase-3 activity in PrP<sup>S</sup>-overexpressing versus control cells under STS conditions. Even more important, a significant raise in viability and concomitant diminishment of caspase-3 activity was observed in PrP<sup>S</sup>-overexpressing cells in which PFN-1 was...
silenced versus PrP<sup>c</sup>-overexpressing cells treated with mock control, both exposed to STS. These findings suggest that downregulation of PFN-1 may have a role in PrP<sup>c</sup>-mediated protection against apoptotic cell death induced by STS. The established link between PFN-1 and PrP<sup>c</sup> definitely deserves further attention.

Remarkably, p53 another tumor suppressor protein crucial for maintaining genetic stability and cellular redox status<sup>44,45</sup> exhibited in the present study nearly identical expression pattern as the one observed for PFN-1. We detected significantly higher levels of p53 expression in control as compared with PrP<sup>c</sup>-overexpressing cells under both experimental conditions. In resting cells p53 levels are low, whereas its levels increase because of rapid stabilization following exposure to different DNA-damaging agents and other stress stimuli.<sup>46</sup> In earlier study, we observed no differences in p53 expression after stable PrP<sup>c</sup>-overexpression in SH-SY5Y cells.<sup>17</sup> However, it must be emphasized that in the latter study SH-SY5Y control cells were not stably transfected with an empty pCIneo vector. Nevertheless, p53-dependent

Figure 3 Silver-stained 2-DE gels of empty vector- and PrP<sup>c</sup>-overexpressing SH-SY5Y in the presence of DMSO/STS. Linear 17 cm IPG strips (pH 3-10) were used and loaded with 130 µg of proteins. Labelling on the gel represents the location of 14 protein spots detected as differentially regulated between distinct transfection/treatment groups. Upper panels represent 2-DE gel pattern of SH-SY5Y cells stably transfected with an empty vector (designated ctrl), whereas lower panels represent the 2-DE pattern of SH-SY5Y cells stably overexpressing PrP<sup>c</sup> (designated PrP). The panels on the left side depict 2-DE pattern following DMSO treatment, whereas panels on the right side depict 2-DE pattern following STS treatment. The protein identity of spots is listed in Table 1.
activation of caspase-3 was repeatedly verified following STS treatment of both PrPc-overexpressing and cells with endogenous PrPc levels.13,26 We suggest that higher expression of PFN-1 and p53 in control as compared with PrPc-overexpressing SH-SY5Y cells subjected to STS might contribute to apoptosis (caspase-3 activation) in the former versus the latter group.

Transgelin is another protein affecting dynamics of the actin cytoskeleton.47 In the present study, western blot analysis exhibited approximately 45-fold higher expression of transgelin-2 in PrPc-overexpressing SH-SY5Y cells subjected to STS as compared with all other experimental conditions. Meanwhile, transgelin was identified as a repressor of metallo-matrix proteinase 9 (MMP-9) expression involved in ECM remodeling.48,49 Likewise, PrPc was shown to decrease MMP-9 transcript levels in neuronal cells.50 Moreover, a surplus of MMP-9 pro-apoptotic

Figure 4 2-DE phospho-proteome of empty vector- and PrPc-overexpressing SH-SY5Y in the presence of DMSO/STS. Linear 17 cm IPG strips (pH 5-10) were used and loaded with 130 μg of proteins. Labelling on the gel represents the location of 11 protein spots detected as differentially regulated between distinct transfection/treatment groups (spot no. 394 remained unidentified). Upper panels show representative gels of control cells, expressing endogenous levels of PrPc, whereas lower panels show representative gels of PrPc-overexpressing SH-SY5Y cells. The panels on the left side: DMSO (vehicle control); the panels on the right side: STS treatment. The protein identity of spots is listed in Table 2.

Figure 5 Distribution of all differentially regulated total and phospho-proteins in 2-DE in the form of functional categories.
versus anti-apoptotic properties, especially during neuronal apoptosis, let us assume that PrP<sub>c</sub>-mediated upregulation of transgelin-2 following STS treatment presumably led to down-regulation of MMP-9 and, thus to a reduction of its pro-apoptotic properties.

Peroxiredoxin-4 (Prx-4), which belongs to a stress response functional group of proteins is an ER-localized enzyme that exhibits antioxidant properties and promotes cell survival. Peroxiredoxin-4 appears to provide a cytoprotective effect against environmental factors that raise levels of hydrogen peroxide in the lumen of ER. We observed 10-fold higher expression of Prx-4 in PrP<sup>c</sup>-overexpressing versus control cells, both subjected to STS. We presume that PrP<sup>c</sup>-overexpressing SH-SY5Y cells subjected to STS undergo less ER stress than the control cells because of their high Prx-4 expression. In line with this connotation, Cusinato et al. showed that when cellular ER stress is present STS effects are more potent.

The fact that protein phosphorylation is the most common post-translational modification in eukaryotic cells involved in all basic cellular processes prompted us to investigate phospho-proteome of PrP<sup>c</sup>-overexpressing cells following STS treatment. Inositol monophosphatase 1 (IMPA1) phosphorylation status was decreased in PrP<sup>c</sup>-overexpressing cells subjected to STS as compared with control cells subjected to the same conditions. Inhibition of IMPA1 is characterized by depletion of free inositol and a subsequent decrease of inositol-1,4,5-triphosphate (IP<sub>3</sub>) levels resulting in induction of autophagy, which is generally known to block the induction of apoptosis. Another protein’s phosphorylation level was exclusively increased by PrP<sup>c</sup>-overexpression treated with STS: calcium-binding mitochondrial carrier protein ScAMC-1. This protein is beneficial for countering Ca<sup>2+</sup> overload-induced cell death, and STS is known to provoke cytosolic and mitochondrial Ca<sup>2+</sup> overload, a critical event for initiation of cell death.

Figure 6 Profilin-1, transgelin-2 and p53 are differentially regulated between empty vector- and PrP<sup>c</sup>-overexpressing SH-SY5Y cells following DMSO/STS treatment. Western blot analysis shows a marked upregulation of profilin-1 in both empty vector (designated ctrl) and PrP<sup>c</sup>-overexpressing (designated PrP) SH-SY5Y cells subjected to STS as compared with DMSO treatment. An overexpression of PrP<sup>c</sup> significantly decreases profilin-1 expression, regardless of DMSO/STS treatment. Transgelin-2 expression was detected only in cells overexpressing PrP<sup>c</sup> subjected to STS treatment, whereas no signal was detected in PrP<sup>c</sup>-overexpressing cells subjected to DMSO. Likewise, no signal was obtained in control cells subjected either to DMSO or STS. p53 displays a similar expression pattern as profilin-1 with significantly higher expression in control as compared with PrP<sup>c</sup>-overexpressing cells under both experimental conditions. β-actin expression below is given as a control for an equal protein load. The displayed Western blots are representatives of three independent experiments (a). Expression level of each protein displayed by western blot was quantified by densitometric analysis and is shown as a diagram (b-d). Data were normalized against β-actin and are given as a ratio of each protein/β-actin ± S.D., *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.
Provanal et al. identified two proteins modulated after prion infection, which revealed changed phosphorylation status in the present study: stomatin-like protein 2, involved in mitochondrial biogenesis, and T-complex protein 1, a molecular chaperone. Not only that stomatin-like protein 2 phosphorylation status was decreased in PrPc-overexpressing cells, but also its expression level was down-regulated following PrPc-overexpression in SH-SY5Y cells treated with STS. On the contrary, T-complex protein 1 showed increased phosphorylation on PrPc overexpression.

Cystathionine-beta synthase (CBS), an enzyme primarily localized in the brain, important for cellular H2S production, exhibited diminished phosphorylation in PrPc-overexpressing cells subjected to both DMSO and STS. H2S appears to have oxygen sensor properties and to be involved in regulation of energy metabolism. CBS phosphorylation at Ser 227 has been shown to modulate its catalytic activity and thereby H2S production. The possible role of PrPc in regulating phosphorylation status of CBS remains to be elucidated.

Altogether, PrPc-overexpression in SH-SY5Y cells per se appears to stress the cells as observed by decreased cell viability under control conditions. However, after exposure to STS, higher levels of PrPc become beneficial in terms of cell survival and apoptosis as compared with endogenous PrPc levels. Proteome and phospho-proteome indicate major changes in protein metabolism/folding, energy metabolism, cytoskeleton and stress response group between both experimental groups. Although not identified during proteome analysis, we confirm differential regulation of tumor suppressor p53 by different PrPc levels under non-apoptotic and apoptotic conditions.

In our opinion the established link between PrPc and PFN-1 deserves a special attention given PFN-1 role in tumor suppression on one side and PrPc role as a promoter of cell death and disease.

### Figure 7
Co-localization of profilin-1 and PrPc in SH-SY5Y cells following DMSO/STS treatment. Co-immunostaining of profilin-1 and PrPc shows a marked alteration of profilin-1 in both empty vector (designated ctrl) and PrPc-overexpressing (designated PrP) SH-SY5Y cells subjected to STS as compared with DMSO treatment (a). Quantification of images taken in different regions of SH-SY5Y cells fixed after STS treatment showed a significant re-distribution of profilin-1 in PrP-positive cells. Pearson’s co-localization correlation coefficient rp (−1 ≤ rp ≤ 1) and graph was generated by ImageJ (Bethesda, MD, USA) (WICF plugin) software. Densitometric analysis was performed from four independent experiments and the level of significance was calculated using one-way ANOVA Friedman test: *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001 (b-d)
induced apoptosis, but also in the light of emerging roles of these two proteins in cancer.

Materials and Methods
Cell culture, stable transfection and staurosporine treatment. SH-SYSY human neuroblastoma cells constitutively and stably expressing full-length human PrP<sup>c</sup> were established and maintained as previously described. In addition, control transfected SH-SYSY cells stably expressing pCIneo plasmid (Promega, Mannheim, Germany) without a human prion protein gene (PRNP) insert were generated in the same manner. Both cell lines were grown in Dulbecco's modified Eagle medium (DMEM; Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS; Biochrom), 1% penicillin/streptomycin (P/S; Biochrom) and 1% L-glutamine (Biochrom) at 37 °C, 5% CO2 supply and 95% humidity. Six weeks after addition of 400 μg/ml Genetecin (Gibco/Invitrogen, Karlsruhe, Germany) both stably transfected cell lines were selected and further maintained in the same medium with a lower Genetecin concentration (200 μg/ml).

Before induction of apoptotic cell death by STS in empty vector- and PRNP-transfected SH-SYSY cells, cell medium containing Genetecin was aspirated and a fresh medium without Genetecin was added. Apoptosis was induced by exposure of cells to 1μM STS (Sigma-Aldrich, Taufkirchen, Germany) for 2 h. Staurosporine was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). Control cells received vehicle, 0.1% DMSO. After 2 h of exposure to either STS or DMSO cells were washed twice with phosphate-buffered saline (PBS) and collected for further analyses.

siRNA and transfection. For silencing of profilin-1 gene expression 200 000 cells per well in a 6-well plate were seeded in complete DMEM medium (Biochrom) before transient transfection with siRNA duplexes, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 24 h in complete DMEM, cells were re-suspended in Opti-MEM I reduced serum medium (ThermoFischer Scientific, Darmstadt, Germany) and transfected either with 200 pmol/l profilin-1-specific siRNAs (catalog number sc-36316-Santa Cruz Biotechnology, Dallas, TX, USA) containing three different sequences: GUGUCCUGGUGGUUGAAGA, CACGGUGGUUUGAUCAACA, and CCCCAUACCUUAUUGCU64 or with equimolar non-targeting siRNA duplex (control siRNA negative control: Eurogentec, Cologne, Germany) as a negative control. Six hours post-transfection Opti-MEM was replaced with complete DMEM and cells were incubated for another 18 h. Subsequently, 1μM STS or DMSO were added for another 2 h before processing cells for MTS proliferation and caspase-3 activity assay (please see below).

Silver staining and densitometric analysis. The 17cm gels were silver stained as previously described. Briefly, gels were fixed in 50% methanol, 12% acetic acid for 1 h at room temperature (RT) on an orbital shaker, washed for 40 min in 50% ethanol and sensitized with 0.8 mM sodium thiosulfate for 60 s. The gels were then incubated in freshly prepared silver nitrate solution (0.2% silver nitrate and 0.026% formaldehyde) for 20 min at RT followed by three washing steps of 20 s each, in distilled water. Finally, gels were placed in a developing solution (6% sodium carbonate, 0.0185% formaldehyde and 0.05% sodium thiosulfate) until standard markers were stained completely and adequate spots visualized. Gels were scanned with a Gel CanoScan 8400 F (Canon, Tokyo, Japan). Densitometric analyses were performed using Delta 2D software version 3.6 (Decodon GmbH, Greifswald, Germany). Furthermore, the intensities of the spots were normalized by dividing the intensity of each spot by the sum of all spot intensities on the corresponding gels. Fold change, standard deviation and Student's t-test significance level were calculated using Microsoft excel software. Spots having at least 1.5-fold expression changes (P<0.05) were considered statistically significant. The spots of interest were excised from the silver stained gels, in-gel protein digestion and extracted peptides were subjected to the mass spectrometric based MS/MS protein identification analysis (please see Supplementary Table 1) as described earlier. Acceptance criteria was set to at least two sequenced peptides per protein spot. Four independent 2-DE experiments were performed.

Phospho-specific staining of 2-DE gels. Gels were fixed twice in a solution containing 50% methanol and 10% acetic acid for 45 min and washed three times in double distilled water for 15 min each. Gels were incubated in Pro-Q Diamond phospho-stain (Invitrogen, Paisley, UK) overnight in the dark at RT, distilled in 20% acetonitrile and 50 mM sodium acetate three times for 30 min,
followed by three washing steps in double distilled water for 5 min each. Gels were scanned using an imaging instrument (FLA-5100 Fuji photo film, Düsseldorf, Germany) at a wavelength of 532 nm. Fold change, standard deviation and Student's t test level of significance were calculated using Microsoft excel software. Spots having at least 1.4-fold expression changes (P<0.05) were considered statistically significant. In-gel protein digestion and MS/MS analysis (please see Supplementary Table 2) were performed as described in the section 'Silver staining and densitometric analysis'. Acceptance criteria was set to at least two sequenced peptides per protein spot. Four independent 2-DE experiments were performed.

Antibodies and immunoblot analysis. Primary antibodies used for immunoblot analysis were monoclonal mouse anti-transgelin-2 (1:1000) (Abcam, Cambridge, UK), monoclonal mouse anti-p53 (1:1000) (Abcam), monoclonal mouse anti-beta-actin (1:5000 Abcam) and monoclonal rabbit anti-profilin-1 (1:1000) (Abcam). Secondary antibodies used were horseradish peroxidase (HRP)- conjugated rabbit anti-mouse (IBA, Goettingen, Germany) and goat anti-rabbit (Jackson Immunoresearch Laboratories, Palo Alto, CA, USA).

Cell lysis extraction and immunoblotting were performed as described previously. Briefly, cells were lysed (50 mM Tris–HCl, pH = 8, 1% Triton X 100, 0.5% CHAPS, 1 mM DTT), and lysates were cleared of cell debris (1 min at 1000× g, 4 °C). Cell lysates were supplemented with protease and phosphatase inhibitor cocktail (Roche, Mannheim, Germany) and were separated on 12.5% SDS-PAGE. Immunoblotting was performed using above mentioned primary antibodies overnight at 4 °C. Membranes were then rinsed three times in 1 x Tris-buffered saline with Tween-20 (TBS-T) and incubated with the corresponding HRP-conjugated secondary antibody (diluted 1:2000; 1:5000) for 1 h at RT. Immunoreactivity was detected after immersion of the total cell lysate (50 μg) was then incubated with 200 μl caspase-3 specific substrate DEVD-pNA for 4–5 h at 37 °C. Caspase-3-mediated release of p-nitroaniline (pNA) was measured by absorbance at 405 nm. Background absorbance from the control (untreated cells) was subtracted from the samples after the final absorbance was obtained. All caspase assays were performed in quadruplicates.

### Statistical analysis

Densitometric values of 2-DE gels and western blot bands as well as the values obtained from cell viability and caspase-3 activity assays were statistically assessed using unpaired two-sided Student's t test. Means and S.D. were calculated from three to four independent set of experiments. Differences with P<0.05 value were considered statistically significant.

### Conflict of Interest

The authors declare no conflict of interest.

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