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## The fatal attraction between pro-prion and filamin A: prion as a marker in human cancers

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### Abstract

Pancreatic cancer is the fourth leading cancer causing deaths in the USA, with more than 30,000 deaths per year. The overall median survival for all pancreatic cancer is 6 months and the 5-year survival rate is less than 10%. This dismal outcome reflects the inefficacy of the chemotherapeutic agents, as well as the lack of an early diagnostic marker. A protein known as prion (PrP) is expressed in human pancreatic cancer cell lines. However, in these cell lines, the PrP is incompletely processed and exists as pro-PrP. The pro-PrP binds to a molecule inside the cell, filamin A (FLNa), which is an integrator of cell signaling and mechanics. The binding of pro-PrP to FLNa disrupts the normal functions of FLNa, altering the cell's cytoskeleton and signal transduction machineries. As a result, the tumor cells grow more aggressively. Approximately 40% of patients with pancreatic cancer express PrP in their cancer. These patients have significantly shorter survival compared with patients whose pancreatic cancers lack PrP. Therefore, expression of pro-PrP and its binding to FLNa provide a growth advantage to pancreatic cancers. In this article, we discuss the following points: the biology of PrP, the consequences of binding of pro-PrP to FLNa in pancreatic cancer, the detection of pro-PrP in other cancers, the potential of using pro-PrP as a diagnostic marker, and prevention of the binding between pro-PrP and FLNa as a target for therapeutic intervention in cancers.

### Keywords

Creutzfeldt-Jakob disease; cytoskeleton; filamin A; glycosylphosphatidylinositol anchor; pancreatic ductal cell adenocarcinoma; peptide signal sequence; pre-pro-prion; prion; pro-prion; signal transduction; transmissible spongiform encephalopathy

### From scrapie to prion

Transmissible spongiform encephalopathy (TSE) is a group of subacute, fatal and infectious neurodegenerative diseases in animals and humans [1]. Scrapie, a form of TSE in sheep and goats, has been an endemic in the UK since the 1750s, and has been known to be transmissible since the early 1900s [2]. For decades, owing to its atypical features, the etiologic agent of TSE remained baffling. In 1967, Griffith proposed that the TSE agent

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might be a self-replicating protein [3]. In 1982, Prusiner and his associates isolated and characterized the pathogen, which they named proteinaceous infectious particle or scrapie prion (PrP<sup>Sc</sup>) (Table 1) [4–6]. Subsequently, it was found that PrP<sup>Sc</sup> is an aberrant, rogue isoform of a normal cellular prion protein (PrP) [7]. Prusiner proposed that all prion diseases share a common pathogenic mechanism, which is based on the conformational conversion of the normal PrP into the infectious and pathogenic, PrP<sup>Sc</sup> [8]. It is thought that the accumulation of PrP<sup>Sc</sup> in the brain then causes neuropathology. Since then, prion diseases have been used synonymously with TSE. Prion disease, such as Creutzfeldt–Jakob disease (CJD) in humans is rare, with an incidence of approximately one case/million people/year [1]. Owing to its rarity, over the years, human prion disease has not received much attention. However, in 1996, a new human prion disease known as variant CJD (v-CJD) gained widespread attention and caused grave public health concerns. It was thought that v-CJD was caused by the transmission of bovine spongiform encephalopathy (mad cow disease) from cattle to humans, owing to the consumption of meats from affected cattle [9]. Since peaking in 2000, the cases of v-CJD have dropped significantly over the years. Therefore, it appears that the original hysteria of a v-CJD epidemic has not materialized.

### Normal PrP

The human prion gene, *PRNP*, is located on chromosome 20, at 20p13, with a three-exon structure. The third exon contains the entire open reading frame of the protein. At a protein level, the PrP is a glycosylphosphatidylinositol (GPI)-anchored, highly conserved and ubiquitously expressed glycoprotein [10,11].

Human PrP is first synthesized as a pre-pro-PrP polypeptide of 253 amino acids (Figure 1). The first 22 amino acids at the N-terminus contain the leader peptide sequence. The last 22 amino acids at the C-terminus contain the GPI anchor peptide signal sequence (GPI-PSS). Both of these sequences are removed in the endoplasmic reticulum and, thus, are absent from the mature PrP.

Mature, human PrP has 209 amino acids from residue 23–231, and can arbitrarily be divided into three major domains. The N-terminal domain encompasses the first 90 amino acids, and has a conserved motif of five repeating octapeptides. NMR studies have revealed that the N-terminal domain of PrP is flexible, and lacks noticeable secondary structure. The central domain is located between amino acid 110 and 130. On the other hand, the C-terminal region contains a well-defined, globular domain that has two potential N-linked glycosylation sites and a disulfide bridge [12,13]. The protein backbone of a mature PrP has a molecular mass of approximately 23 kDa. The addition of two N-linked glycans, and a GPI anchor completes the maturation of GPI-anchored PrP, which has a molecular mass of approximately 34–39 kDa. Despite the fact that PrP is a relatively small protein, the synthesis, processing and transit of PrP are complex, cell-context dependent and not completely understood [14].

Since the cloning of *PRNP*, more than 30 PrP-binding partners have been identified. However, the normal physiologic function of PrP remains elusive [15–17]. Cell-surface PrP has been reported to function as an adhesion molecule. This hypothesis is supported by findings that demonstrate that cell surface PrP binds glycosaminoglycan [18,19], laminin receptor [20], selectin [21] and neural cell-adhesion molecule (NCAM) [22]. These molecules are important in cell–cell as well as cell–matrix interactions. This interpretation is further strengthened by a recent finding demonstrating that PrP is involved in the proliferation as well as the distribution of desmosomal-associated proteins, such as desmoglein in human and mouse enterocytes [23].

The octapeptide-repeat region of PrP binds divalent cations, such as  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  [24,25]. Binding of  $\text{Cu}^{2+}$  enables PrP to function as an antioxidant in a cell context-dependent manner [24]. Thus, PrP functions as a metal transporter and plays a role in regulating oxidative stress [24,25]. In addition, PrP has also been reported to interact with stress-inducible protein [26], plasminogen [27], heat shock proteins [28], dystroglycan [29], glypican 1 [30], Grb2 [31] and tubulin [32], as well as lipids [33] and nucleic acids [34]. However, the biological significance of these interactions is not clear.

Accumulated evidence suggests that cell surface PrP also participates in signal transduction [35]. PrP is present on the cell surface in lipid rafts or caveolae, which are microdomains on the cell membrane that are important in organizing signal-transducing platforms [36]. Supporting this interpretation is evidence demonstrating that PrP colocalizes and interacts with caveolin 1 – a major component of the caveolae [37–39]. Caveolin 1 can positively or negatively impact multiple signal-transducing pathways, such as G protein-coupled receptors, MAPK/ERK, PKC, as well as Src family kinases, such as Fyn and Src [37,40]. It appears that PrP can participate in numerous signal-transducing pathways either by binding directly to the signaling molecule or by interacting with caveolin 1.

Prion protein plays a role in apoptosis in a cell context-dependent manner. PrP regulates the activity of p53 and sensitizes neurons to apoptotic stimuli [41]. On the other hand, PrP has also been reported to have antiapoptotic function. For example, neuronal cell lines from *Prnp*<sup>-/-</sup> mice are more susceptible to apoptosis than similar cell lines from wild type, *Prnp*<sup>+/+</sup> mice [42]. In a cAMP/PKA-dependent manner, PrP provides protective signals to neurons [43]. PrP inhibits the functions of Bax and, thus, protects cells against Bax-mediated apoptosis [44]. PrP also protects neurons against metal-induced toxicity (e.g., manganese) and cell death by interfering with its transport [45]. In a human breast cancer cell lines, PrP protects the cells from TNF-induced cell death [46]. To unravel these seemingly contradictory findings, it has been recently suggested that PrP functionality is pathway-dependent; PrP is proapoptotic during endoplasmic reticulum stress and antiapoptotic during oxidative stress-induced cell death [47]. PrP also binds amyloid- $\beta$  oligomers, which then cause synaptic dysfunction and contributes to neurodegeneration in Alzheimer's disease [48]. Another study suggests that whether PrP is neuroprotective also depends on the underlying pathogenic mechanism [49].

While most of the PrP-interacting partners bind to the N-terminus or the C-terminus globular domain of PrP, the central region of PrP, from residues 113–128, also contain a stretch of highly conserved, small hydrophobic amino acids. This region can function as a transmembrane domain in cell lines [50]. Furthermore, expression and accumulation of this aberrant PrP isoform in transgenic mice causes neurodegeneration [51]. However, whether this region is important in the normal biology of PrP is not clear.

Adding to the complexity is the recent realization that cell-surface PrP is proteolytically cleaved by multiple proteases, such as ADAM10 and TACE [52]. Some of the truncated PrP species are present on the cell surface, and may be important in prion functionality as well as the pathogenesis of prion disease. In addition to the cell surface, a GPI-anchored PrP is detected in the nucleus of proliferation cells [23]. However, the function of the GPI-anchored PrP in the nucleus is not clear. PrP is also detected in body fluids, such as blood [53], milk [54] and urine [55]. The PrP species detected in blood and urine have been reported to be infectious, indicating the presence of PrP<sup>Sc</sup> [53,55]. Most of the normal PrP species detected in the circulation are truncated PrP species lacking a portion of the C-terminal globular domain.

At present, it is difficult to conceive how a relatively small protein can interact with so many partners that are present in different cellular compartments. It should be noted that despite all the important functions that have been attributed to PrP, a *Prnp*<sup>-/-</sup> mouse is apparently normal without any overt aberrant phenotype [56]. Therefore, the normal physiologic function of PrP remains an enigma.

## The GPI anchor

Prion protein is a GPI-anchored protein [8]. It is not clear why some proteins are GPI anchored while others are not. It has been estimated that approximately 0.5–1% of the mammalian genome encodes for GPI-anchored proteins [57–59]. The biosynthesis of the GPI anchors and their attachment to proteins are complex, and protein- and cell-context dependent [57–59]. At least 24 genes have been identified to be important in this process. The common core structure of the GPI anchor is synthesized in the endoplasmic reticulum in a stepwise mechanism. The first step is the transfer of *N*-acetyl-glucosamine (GlcNAc) from UDP-GlcNAc to phosphatidylinositol (PI) to yield GlcNAc-PI. This reaction is catalyzed by a  $\alpha$ 1–6 GlcNAc transferase complex, which is composed of seven gene products: PIG-A, PIG-C, PIG-H, GPI-1, PIG-Y, PIG-P and DPM2. The second step is the de-*N*-acetylation by PIG-L to generate GlcN-PI. Subsequently, three mannose residues are sequentially added. The last step in GPI anchor processing is attachment of the already assembled GPI structure, *en bloc*, to the newly synthesized pro-protein in a transamidase reaction. This attachment occurs concurrently with the cleavage of the C-terminal GPI-PSS, at a site known as the  $\omega$  site [57–59]. The  $\omega$  residue for mammalian proteins is confined to amino acids glycine, serine, cysteine, alanine, aspartic acid and asparagine. There is no other obvious motif in the GPI-PSS that signals the transamidase reaction.

In general, the GPI-PSS contains between 15 and 25, small hydrophobic amino acids, similar to a typical transmembrane domain. Substitution of a single amino acid at the  $\omega$  site of Qa2, a normally GPI-anchored protein, prevents its GPI anchor modification [60]. Nonetheless, Qa2 is still present on the cell surface, as an integral membrane protein; in this case, the Qa2 uses the GPI-PSS as a surrogate transmembrane domain. Using an *in vitro* GPI anchor assembly assay, Chen *et al.* reported that the GPI-PSS of PrP is intrinsically inefficient (14%) compared with nine other GPI-PSS (ranging from 24 to 60%) in accepting the GPI anchor [61]. However, the significance of this finding is not known.

Interestingly, it has been reported that exchanging the GPI-PSS of NCAM for the GPI-PSS of carcinoembryonic antigen generates a mature protein that has a NCAM external domain, but carcinoembryonic antigen-like biological properties [62–64]. Based on these findings, it is postulated that the GPI-PSS possesses cryptic biological information that specifies the addition of a particular functional GPI anchor that, ultimately, determines the function of the mature protein. It is interesting to note that while the coding region of the human *PRNP* and mouse *Prnp* is approximately 85% conserved, their GPI-PSSs are identical. On the other hand, their N-terminal peptide sequences, which are also deleted prior to maturation, share only 60% identity [65]. The significance of this conservation is not known.

Accumulated evidence suggests that aberrations in the GPI-anchor assembly pathway may contribute to oncogenesis. mRNAs for some genes in the GPI-anchor pathway, such as *PIG-T*, *PIG-U* and *GPAAI*, are upregulated in bladder, breast, and head and neck cancers [66,67]. Overexpression of *PIG-T* and *GPAAI* in 3T3 cells enhances their invasiveness *in vitro* and growth *in vivo*. Conversely, downregulation of *PIG-T* and *GPAAI* in breast cancer cell lines inhibits their growth [68]. Increased *GPAAI* expression is associated with hepatocarcinoma's poor cellular differentiation and poor prognosis [69]. The underlying mechanisms causing these effects are not known. The oncogene, *KRAS*, is frequently

mutated in human cancers [70,71]. In yeast, Ras regulates the function of a complex, GPI-*N*-acetylglucosaminyltransferase, which catalyzes the first step in GPI anchor biosynthesis, transferring a GLcNAc from UDP-GlcNAc to an acceptor PI in the endoplasmic reticulum. Furthermore, the GTP-bound Ras is associated with the GPI-*N*-acetylglucosaminyltransferase complex and inhibited its activity *in vivo* [72]. It is worth noting that some of the genes essential for GPI anchor modification are located in chromosomal regions, which are amplified, deleted or mutated in human cancers. For example, in human pancreatic cancer 8q24.3 (*GPAA-1*), 9p13.3 (*PIG-O*) and 14q11–24 (*PIG-H*) were amplified, while 16p13.3 (*PIG-Q*), 17p13.1–12 (*MPDU-1*), 1p36 (*PIG-V*) and 21q22.2 (*PIG-P*) were deleted [73–75]. Some of these chromosomal abnormalities may have impacted the integrity of the GPI anchor pathway genes.

Since PrP has been associated with cellular survival, proliferation and differentiation, aberrant PrP function may contribute to tumorigenesis. It is reported that PrP is overexpressed in human gastric cancers and contributes to their resistance to chemotherapeutic agents [76]. An expression microarray study found that *PRNP* is also overexpressed in human colorectal cancers [77]. *PRNP* is one of the 25 genes that are overexpressed in pancreatic cancer cell lines [78]. However, the mechanism by which overexpression of PrP promotes tumorigenesis is not clear. In 2007, we initiated a study to screen for PrP expression in a panel of human tumor cell lines, starting with pancreatic cancers.

### PrP in pancreatic ductal cell adenocarcinoma

The majority of the human pancreatic cancers are ductal cell adenocarcinoma (PDAC), which is the fourth leading cause of cancer deaths in the USA [79,80]. Patients with PDAC have dismal prognosis as the overall median survival is 6 months and the 5-year survival rate is less than 10%. Progression of human PDAC correlates with a series of histological changes from a flat, columnar epithelium to a papillary, mucinous epithelium, with increasing loss of cellular architecture and with nuclear atypia [81–83]. These lesions are referred to as pancreatic intraepithelial neoplasia (PanIN). PanIN is divided into three stages: PanIN-1, -2 and -3. These morphological changes are accompanied by increasing numbers of genetic alterations.

Genome-wide aberrations have impeded pinpointing the culprit genes in the etiology of human PDAC. Nevertheless, there is significant progress in identifying the molecular mechanisms underlying PDAC carcinogenesis. The most common genetic lesions found in human PDAC are mutations in *KRAS*, *TP53*, *DPC4* and *CDNK2A* [84–86]. It is now generally accepted that the *KRAS* mutation is one of the earliest, and most important genetic lesion in the development of PDAC; the majority of PDAC cases have a mutation in codon 12 of *KRAS*, substituting a glycine with aspartate, valine or arginine.

Our research has demonstrated that PrP is expressed in a panel of human PDAC cell lines ( $n = 7$ ) [87]. However, in contrast to normal PrP, which is glycosylated and GPI anchored, in the PDAC cell lines, PrP is neither glycosylated nor GPI anchored; it exists as pro-PrP retaining its GPI-PSS (Figure 1). This deficiency is not caused by a global failing of the GPI anchor machinery in the PDAC cell lines; two normally GPI-anchored proteins, CD55 and flotillin 1 remain GPI anchored in the PDAC cell lines. Despite lacking a GPI anchor, the pro-PrP is present on the PDAC cell surface, using the GPI-PSS as a transmembrane domain. Our immunoblotting results with multiple anti-PrP monoclonal antibodies (mAbs) suggest that pro-PrP is the only PrP species detected in the PDAC cell lines. However, some other PrP species, such as glycosylated or GPI-anchored PrP, may be present in these cells but at levels that are under the detection limit of immunoblots.

To further study the biological functions of pro-PrP, we attempted to identify proteins in the PDAC cell lines that physically interact with pro-PrP and, thus, could be copurified with PrP. While many proteins did copurify with anti-PrP mAbs as well as control mAbs, one protein, with a relative molecular mass of 280 kDa, consistently copurified only with anti-PrP mAbs but not with control mAbs. Mass spectrometry analysis revealed that this protein is filamin A (FLNa). FLNa is an actin-binding cytolinker, which connects cell surface molecules to the actin filaments of the cytoskeleton and, thus, integrates signaling events with cellular mechanical forces [88–90]. Upon binding F-actin filaments, FLNa promotes high-angle branching of actin filaments to maintain a 3D orthogonal network, which is important in cell-shape maintenance as well as cell migration. *FLNA* is mapped to chromosome Xq28 [89,90]. Two transcript variants, encoding different isoforms, have been found for *FLNA*. In males, FLNa deficiency caused by a null mutation is embryonic lethal. In females, depending on the nature and local of the mutation, it causes several developmental syndromes involving neuronal, skeletal and connective tissues [89,90]. The *Flna*<sup>-/-</sup> mouse is nonviable owing to widespread developmental defects in the heart and vascular structures [91,92]. Whether abnormality in FLNA contributes to tumorigenesis is not known; patients with *FLNA* mutations are not known to have a higher incident of cancers.

Native FLNa is a homodimer; each subunit contains a F-actin-binding domain at the N-terminus followed by 24  $\beta$ -sheet Ig-like domains at the C-terminus [89–91] (Figure 2). More than 40 proteins have been reported to interact with FLNa. These molecules include signal-transducing molecules, other scaffolding proteins and adhesion molecules, as well as growth factor receptors [89–91]. All FLNa-interacting partners bind to the C-terminal Ig-like domains from domain 10 to domain 24 of FLNa. Domain 24 is the dimerization domain and is essential for optimal FLNa function [88–90].

The atomic structure of the interface between the FLNa Ig-like domain and some of the common FLNa binding partners has been resolved [93–95]. These FLNa-binding partners share a conserved hydrophobic amino-acid motif. Unexpectedly, Clustal<sup>W</sup> alignment suggests that the GPI-PSS of pro-PrP contains a FLNa-binding motif. Binding of FLNa to pro-PrP was first demonstrated by *in vitro* pull-down assays using recombinant FLNa and recombinant pro-PrP. FLNa only binds pro-PrP but not mature PrP, which lacks the GPI-PSS. Second, in the PDAC cell lines, pro-PrP and FLNa are also copurified by immunoprecipitation in both directions. Third, PrP and FLNa colocalize in the PDAC cell lines, as revealed by immunofluorescent staining and observed in a confocal microscope [87].

The presence of an FLNa-binding motif in the GPI-PSS appears to be specific for PrP. We examined 14 GPI-PSS from other normally GPI-anchored proteins and we found that only the GPI-PSS of PrP has the FLNa-binding motif. Therefore, even if some other normally GPI-anchored proteins also exist as proproteins, retaining their GPI-PSS, in the PDAC cell lines, they are not expected to bind FLNa. The reason that a FLNa-binding motif is present in a normally discarded region of PrP is not known.

More recent studies reveal that pro-PrP has multiple binding sites at the C-terminal Ig-like domains of FLNa, including domains 10, 16, 17, 18, 20, 21 and 23 [Li CY, Yu S, Nakamura F *et al.*, Pro-prion promotes melanoma cells spreading and migration by binding to FLNa and modulates its interaction with integrin  $\beta$ 1. Submitted]. This finding is not unexpected because the Ig-like domains are highly conserved. However, whether all these binding sites are available for pro-PrP binding in native, dimeric, FLNa is not known. On the other hand, we found that the last five amino acids at the C-terminal end of the PrP GPI-PSS are critical for FLNa binding. Removal of these five amino acids completely eliminates its FLNa-

binding capacity. These results suggest that the GPI-PSS of PrP is able to transverse the membrane bilayer, and some of the amino acids are exposed in the cytosolic side of the membrane, enabling it to bind FLNa (Figure 3).

## Consequences of binding between pro-PrP & FLNa

To study the contribution of pro-PrP to PDAC biology, we downregulated PrP expression in these cell lines using PrP-specific shRNA. Reducing PrP expression in the PDAC cell lines did not alter the expression level of FLNa; however, it did drastically change the spatial distribution of FLNa [87]. In control cells, FLNa is mainly present close to the inner-membrane leaflet in the leading edges. In PrP downregulated cells, FLNa is rare in the leading edges, and more concentrated in the cytosol. It appears that without PrP, FLNa is unable to reach the inner membrane leaflet.

As expected, downregulation of PrP also alters the organization of the actin filaments. *In vitro*, the PrP downregulated cell lines proliferate more slowly and are less invasive than control cells. *In vivo*, their growth in nude mice is inhibited. Thus, binding of pro-PrP to FLNa enables the PDAC cell lines to proliferate faster, to be more invasive *in vitro* and to have a more rapid growth rate *in vivo*. The precise mechanism by which binding of pro-PrP alters the function of FLNa is not clear. Binding of pro-PrP may physically remove FLNa from its normal environment and, thus, render it unable to interact with its normal partners. Alternatively, binding of pro-PrP may compete for binding sites on FLNa that are normally occupied by its interaction partners. Furthermore, expression of pro-PrP may also disrupt the normal function of GPI-anchored PrP, which then contributes to the biology of the PDAC cell lines.

At the molecular level, PrP downregulated cell lines have reduced levels of p-cofilin-1, a critical regulator of the actin filament polymerization. On the other hand, the levels of p-Rac1, a Rho-GTPase; p-ERK-1/2 and p-MEK-1, two serine/threonine kinases in the MAPK pathway; and p-Fyn, a *Src* family tyrosine kinase, are markedly increased in the PrP downregulated cells. Therefore, reducing the expression of PrP in the PDAC cell lines appears to have effects on multiple signal transduction pathways. More than 40 proteins have been reported to bind FLNa [88–90]. It is almost certain that binding of pro-PrP to FLNa will have rippling effects on the binding of FLNa to some of its binding partners, such as integrins, which are known to play critical roles in cellular adhesion, invasion and migration.

The reason why the GPI-PSS of PrP is not removed in the PDAC cell lines is not known. The GPI anchor-modification process is complex, and protein- and cell-type dependent [57–59]. A mutation in the *PRNP* gene is not the cause; sequencing of *PRNP* in six PDAC cell lines revealed no mutation. It should be noted that the GPI-PSS of PrP is intrinsically inefficient compared with other GPI-anchored proteins [61]. Thus, a slight defect in the GPI anchor assembly machinery in PDAC may have a more dramatic effect on PrP than other GPI-anchored proteins with a more efficient GPI-PSS, such as CD55, which is GPI-anchored in the PDAC cell lines. Pro-PrP is a precursor of mature PrP. In cell lines that express a GPI-anchored PrP, pro-PrP is undetectable. Therefore, in these cell lines, either the processing of pro-PrP or the removal of unprocessed pro-PrP is very efficient. On the other hand, a defect in the quality-control system in the endoplasmic reticulum or in the removal of the unprocessed pro-PrP, presumably by the proteasomal degradation machinery, may also contribute to the accumulation of pro-PrP.

In the PDAC cell lines, PrP is also not glycosylated. It is known that the presence of the *N*-linked glycans on PrP is not required for GPI anchor modification [96]. On the other hand, the presence of a GPI anchor has been reported to influence the glycosylation of Thy-1, a

GPI-anchored protein [97]. Thus, failure to remove the GPI-PSS may cause PrP to not be glycosylated. The lack of *N*-linked glycans may then alter the metabolism or transit of pro-PrP, contributing to its accumulation in the PDAC cell lines.

## PrP is a poorer prognosis marker in human PDAC

We also determined whether our findings in PDAC cell lines have clinical relevance. For this purpose, we investigated the expression of PrP in normal human pancreas, in pancreas with pancreatitis, in pancreas with preneoplastic lesions, such as PanIN-1, -2 and -3, and in pancreas with PDAC by immunohistochemical staining. In normal human pancreas, only islet cells demonstrated PrP immunoreactivity; neither acinar nor ductal epithelial cells stained for PrP. PrP was also undetectable in the duct cells in chronic pancreatitis, and PanIN-1 and -2. Approximately 13% PanIN-3 specimens showed weak PrP staining. Among the 83 PDAC cases examined, 34 (41%) showed strong PrP staining. All PDAC tumor cells reacted strongly with the anti-GPI-PSS antiserum. Thus, as in the PDAC cell lines, PrP exists as pro-PrP in human PDAC lesions [88].

Most interestingly, we found that the expression of pro-PrP is associated with poorer clinical outcome. Patients with intratumoral pro-PrP had a median survival time of 360 days, while patients without PrP expression in their tumors had a mean survival time of over 1000 days. Furthermore, this association is independent of other factors, such as age and gender of the patient, as well as the size or differentiation stage of the tumor. It is clear that PrP expression is not essential for PDAC initiation because only 41% of the PDAC cases have detectable PrP. However, the presence of PrP is associated with poorer clinical outcome, suggesting that, similar to PDAC cell lines, PDAC tumors with PrP have a growth advantage and, thus, are more aggressive.

Little is known about the regulation of PrP expression, either at the gene or protein level in the pancreas. *PRNP* has been reported to be upregulated in BxPC 3, Capan 1 and five other PDAC cell lines [78]. However, other gene profiling studies have not identified *PRNP* as a contributing factor in human PDAC [73–75,98]. The reasons for these discrepancies are not known. More than 90% of PDAC cases have a *KRAS* mutation and inactivation of *CDKN2A*, but, only 41% of the PDAC cases have detectable PrP. Therefore, it is unlikely that a mutation in *KRAS* or inactivation of *CDKN2A* by itself is responsible for the activation of PrP expression in PDAC. On the other hand, gene profiling is much more sensitive than immunohistochemical staining; it is possible that immunohistochemical staining may not be able to detect PDAC cases that express low levels of PrP. Alternatively, other genetic anomalies, such as mutation in *TP53* and/or *DPC-4*, which are found in approximately 50% of PDAC cases, must have occurred to facilitate the expression of PrP in PDAC. It is interesting to note that in the promoter region of *PRNP*, there is a potential p53 binding site [99], and PrP regulates p53 function [33].

So far, only resectable PDAC cases have been studied. Nonresectable PDAC is rarely available. If our interpretation that expression of PrP provides PDAC cells with growth advantages is correct and PrP-positive PDAC is more aggressive, then we will expect a larger proportion of the nonresectable PDAC to express PrP.

## PrP & FLNa in human melanomas & other tumors

Filamin A is an integrator of cell signaling and mechanics and, thus, participates in many biological responses [88–90]. However, despite its importance in cellular biology, FLNa is dispensable for cell-autonomous survival. Some human melanoma cell lines, such as M2 and -3 do not express FLNa [100]. These cells lack actin fiber bundles and, thus, are impaired in their cellular migration *in vitro*. This phenotype is revised in A7 cells, which are



derived from M2 cells after the transfection of human FLNa [101]. This pair of isogenic cell lines has been used extensively to study the functions of FLNa [102–105]. Biological responses, such as signal transduction and cellular migration, observed in A7 cells but not in M2 cells, have been attributed to the functions of FLNa [101–105].

More recently, we found that both M2 and A7 cells express pro-PrP. Similar to the PDAC cell lines, in A7 cells, pro-PrP binds FLNa; binding of pro-PrP to FLNa modulates cellular behaviors, such as cell spreading and migration [Li CY, Yu S, Nakamura F *et al.*, Pro-prion promotes melanoma cells spreading and migration by binding to FLNa and modulates its interaction with integrin  $\beta$ 1. Submitted]. Reduction of pro-PrP expression in A7 cells inhibits A7 cell spreading and migration. Therefore, in A7 cells, FLNa does not act alone. It is the binding between pro-PrP and FLNa that influences the function of FLNa in A7 cells.

Whether PrP is a marker in human melanoma tumorigenesis has never been studied. In normal human skin, only epithelial cells and sporadic mononuclear cells within the dermis demonstrated weak PrP immunoreactivity [106]. However, it is known that not all melanoma cell lines express FLNa [100,101]. Interestingly, the patterns of FLNa expression in melanocytes in nevi and in melanomas vary, depending on the location of the lesion – whether it is in the epidermis or dermis. It was postulated that FLNa might be important in cell–cell adhesion, and in tumor cell motility during tissue invasion from the epidermis to the dermis [107]. Therefore, immunostaining for pro-PrP and FLNa in melanoma biopsies may provide new insights into the role these molecules play in human melanoma tumorigenesis.

Hepatocarcinoma (HCC) is rare in the USA, but is one of the most common tumors worldwide. The majority of HCC cases are secondary to either a viral infection (hepatitis B or C viruses), or cirrhosis caused by toxic agents or alcohol consumption [108]. A PrP species with molecular mass similar to pro-PrP is detected in five human HCC cell lines. All five HCC cell lines also express FLNa. Small-cell lung carcinoma is generally thought to be a neuroendocrine tumor originating from neuroendocrine cells in bronchi [109]. In three human small-cell carcinoma cell lines, FLNa, but not PrP, was undetected. This observation is consistent with our finding in patient biopsies; neuroendocrine tumors originating from the lung did not express PrP (not shown). On the other hand, neither PrP nor FLNa was detected in two human neuroblastoma cell lines. The numbers of cell lines we have studied were rather small, nevertheless, these findings suggest that the contribution of PrP and FLNa to tumor biology does not apply to all tumor types.

## Binding of pro-PrP to FLNa as a potential target for therapeutic intervention in human cancers

The PrP binds nucleic acid [38]. When a recombinant PrP (rPrP) is mixed with an expression plasmid *in vitro*, the rPrP/DNA complex is taken up by cells – leading to gene expression – in a  $\text{Ca}^{2+}$ -dependent manner [110]. Interestingly, rPrP, by itself, is also taken up by cells. However, cells do not take up rPrP $\Delta$ KKRPPK, which lacks the KKRPPK motif. When mixed with DNA, a pentapeptide KKRPPK, but not KKKKKK, is sufficient for DNA internalization and expression. Recently, we found that if the cells were incubated with the peptide for a longer time (>1 h), the peptide can enter the cells without  $\text{Ca}^{2+}$  [Li CY, Yu S, Nakamura F *et al.*, Pro-prion promotes melanoma cells spreading and migration by binding to FLNa and modulates its interaction with integrin  $\beta$ 1. Submitted].

We have previously reported that the *in vitro* copurification of pro-PrP and FLNa was inhibited in the presence of a synthetic peptide corresponding to the GPI-PSS of PrP [87]. Based on the aforementioned findings, we hypothesized that if we link a KKRPPK motif to

the N-terminus of the PrP GPI-PSS, the peptide, KKRPK-PrP-GPI-PSS, might be able to enter cells, and compete for the binding of pro-PrP to FLNa. Indeed, the synthetic peptide is able to block the migrations of melanoma cell line A7 [Li CY, Yu S, Nakamura F *et al.*, Prion promotes melanoma cells spreading and migration by binding to FLNa and modulates its interaction with integrin  $\beta$ 1. Submitted], as well as a hepatocarcinoma cell line (Figure 4) in a wound-healing assay. The inhibition is peptide-concentration dependent. These results provide the 'proof of principle' that the binding of FLNa to pro-PrP could be a target of therapeutic intervention in human cancers that have both pro-PrP and FLNa.

## Conclusion & future perspective

Our finding that a protein, which was infamous owing to the role it plays in mad cow disease, is involved in human pancreatic cancer is totally unexpected. We hypothesize that the fatal attraction between pro-PrP to FLNa is the underlying mechanism by which PrP-positive PDAC cells have a growth advantage. As an integrator of cell signaling and mechanics, many proteins have been reported to bind FLNa. It is almost certain that binding of pro-PrP would have disrupted the interaction between FLNa and some of these proteins. Identifying these proteins will provide insights into the mechanisms by which expression of pro-PrP modulates tumor cell biology. In addition, identifying the underlying mechanisms that cause the retention of the GPI-PSS on PrP in cancer cell lines will help us understand the cell biology of the GPI-anchor modification pathway and the roles it plays in tumor biology. Furthermore, since high levels of soluble PrP are detected in the culture supernatants of the PDAC cell lines [88], therefore, soluble pro-PrP may be present in the circulation or body fluid of patients with PDAC. Detection of pro-PrP may provide an early and noninvasive method for detecting PDAC. In addition, prevention of the interaction between pro-PrP and FLNa could provide a novel target for therapeutic intervention in PDAC. Finally, our finding that pro-PrP is also detected in some other tumors, such as melanoma and hepatocarcinoma, suggests that the aberrant processing of prion is not only limited to pancreatic cancer. On the other hand, the absence of either PrP or FLNa in other tumors, such as small-cell lung carcinomas and neuroblastomas, suggests that an interaction between pro-PrP and FLNa do not occur in all tumor types.

### Executive summary

#### Prion protein exists as the prion pro-protein in pancreatic cancer cell lines

- Instead of being a glycosylated and glycosylphosphatidylinositol (GPI)-anchored protein, the prion protein (PrP) in pancreatic ductal cell adenocarcinoma (PDAC) cell lines are neither glycosylated nor GPI-anchored.
- The GPI peptide signal sequence of PrP contains a filamin A (FLNa)-binding motif and, thus, binds FLNa.
- The fatal attraction between pro-PrP and FLNa disrupts the normal functions of FLNa and, thus, provides the PDAC cells with a growth advantage.

#### Expression of PrP is a poorer prognosis marker in human pancreatic cancer

- In normal human pancreas, only islet cells have detectable PrP. PrP is not detectable in precancerous lesions such as PIN-I and -2.
- Approximately 13% of PIN-3 cases have detectable PrP. The frequency is increased to approximately 40% in PDAC. As in the PDAC cell lines, the PrP in the tumor is pro-PrP.
- PDAC patients, whose PDAC tumors had PrP, had a much shorter survival.

- Detection of pro-PrP may provide an early noninvasive diagnostic marker for PDAC.

#### **Prp & FLNa in human melanomas & other tumors**

- Pro-PrP is detected in some but not all human tumor cell lines: melanoma (n = 3), hepatocarcinoma (n = 5).
- Small-cell lung carcinomas and neuroblastomas do not express PrP.
- Some melanomas do not express FLNa.

#### **Binding of pro-PrP to FLNa as a potential target for therapeutic intervention**

- A PrP-GPI peptide signal sequence synthetic peptide with cell-penetrating capacity blocks the interaction between PrP and FLNa and, thus, inhibits the migration of tumor cells in a wound-healing assay.

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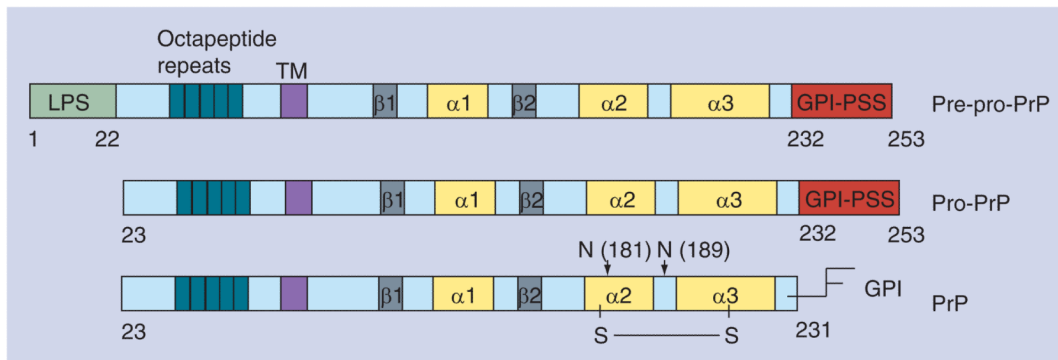
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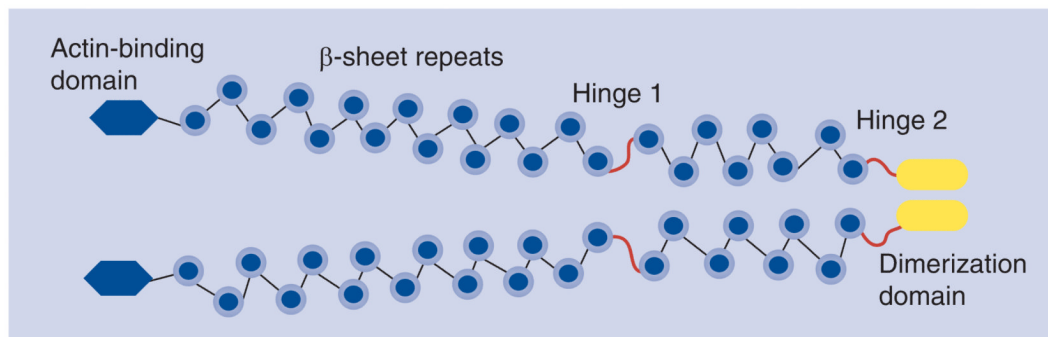
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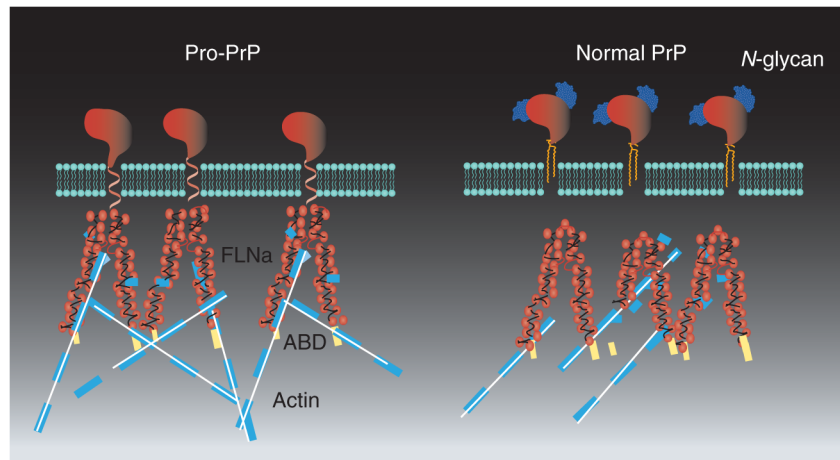
**Figure 1. The synthesis and processing of PrP from pre-pro-PrP to pro-PrP to mature, glycosylated, GPI-anchored PrP**

GPI: Glycosylphosphatidylinositol; LPS: Leader peptide signal sequence; N: *N*-linked glycans; PrP: Prion protein; PSS: Peptide signal sequence; S-S: Disulfide bridge; TM: Putative transmembrane domain.



**Figure 2. Dimeric filamin A**

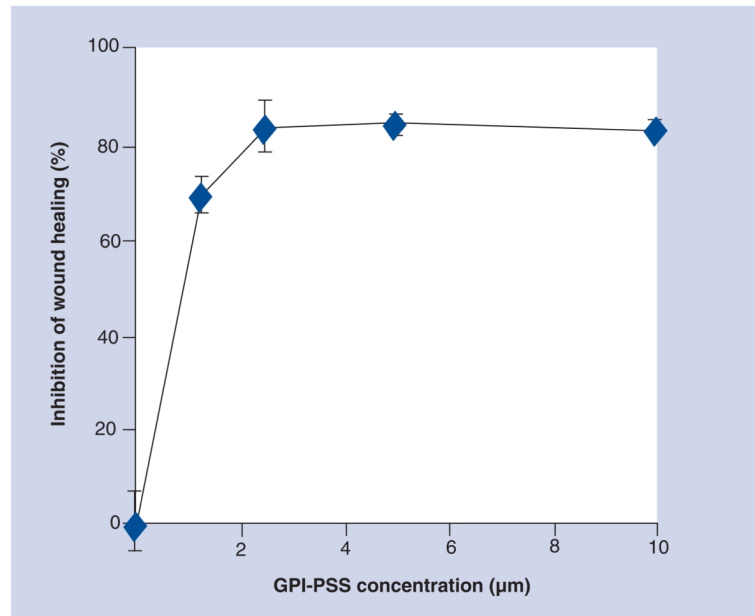
At the N-terminus of each monomeric unit, there is an actin-binding domain, followed by 24  $\beta$ -sheet Ig-like domain. Domain 24 is the self-association domain. The two hinge regions provide flexibility to permit high-angle binding of the F-actin filament.



**Figure 3. Cell surface pro-PrP and normal, glycosylated, GPI-anchored PrP**

Cell surface pro-PrP is using the GPI-PSS as a transmembrane domain. The GPI-PSS transverse the membrane and interacts with FLNa. The ABD of FLNa binds actin filaments. The normal PrP is GPI-anchored and glycosylated.

ABD: Actin-binding domain; GPI: Glycosylphosphatidylinositol; PrP: Prion protein; PSS: Peptide signal sequence.



**Figure 4. Inhibition of hepatocarcinoma cell migration with the KKRPK-PrP-GPI-PSS synthetic peptide**

Various concentrations of the synthetic peptide were added at the beginning of the wound-healing assay. At 24 h after the assay, the areas of the wound were imaged on a microscope equipped with a digital camera system, and quantified. Inhibition of cell migration was determined by comparing the healed area of nontreated cells with the healed area of cells treated either with the KKRPK-PrP-GPI-PSS peptide or the KKRPK-control peptide. GPI: Glycosylphosphatidylinositol; PrP: Prion protein; PSS: Peptide signal sequence.

**Table 1**

Key terms in prion biology.

Term	Definition
GPI anchor	A GPI anchor that some proteins use to attach to the outer membrane leaflet of a cell
Pre-pro-PrP	A precursor of the mature prion protein that contains the N-terminal peptide signal sequence as well as the C-terminal GPI anchor peptide signal sequence
Pro-PrP	A precursor of the mature prion protein, in which the N-terminal peptide sequence has been removed but that retains the C-terminal GPI anchor peptide sequence. This sequence is removed prior to the addition of the GPI anchor
PrP	Mature prion protein, which is a highly conserved, widely expressed glycoprotein that is tethered to the outer cell surface by a GPI anchor
PrP <sup>Sc</sup>	Scrapie prion, an abnormal, rogue isoform of the normal PrP that is pathogenic and infectious
Filamin A	A cytolinker, which links cell surface receptors to actin filaments. Filamin A is an integrator of the cell signaling and cell mechanical force

GPI: Glycosylphosphatidylinositol; PrP: Prion protein; PrP<sup>Sc</sup>: Scrapie prion protein.