Alternative fates of newly formed PrP^{Sc} upon prion conversion on the plasma membrane

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Running title: Alternative fates of newly formed PrP^{Sc}
Summary

Prion diseases are fatal neurodegenerative diseases characterised by the accumulation of misfolded prion protein (PrPSc) in the brain. They are caused by the templated misfolding of normal cellular protein, PrPC, by PrPSc. We have recently generated a unique cell system in which epitope-tagged PrPC competent to produce bona fide PrPSc is expressed in neuroblastoma cells. Using this system we demonstrated that PrPSc forms on the cell surface within minutes of prion exposure. Here, we describe the intracellular trafficking of newly formed PrPSc. After formation in GM1-enriched lipid microdomains at the plasma membrane, PrPSc is rapidly internalised to early endosomes containing transferrin and cholera toxin B subunit. Following endocytosis, PrPSc intracellular trafficking diverges: some is recycled to the plasma membrane via Rab11 labelled recycling endosomes; the remaining PrPSc is subject to retromer-mediated retrograde transport to the Golgi. This pathway leads to lysosomal degradation and we show that this is the dominant PrPSc degradative mechanism in the early stages of prion infection.

Key words: prions, intracellular trafficking, retrograde transport, lysosomes
**Introduction**

Many neurodegenerative diseases result from the aggregation of misfolded proteins. Indeed, their shared mechanism provides the basis for a new class of pathologies: the protein misfolding disorders. These include Alzheimer’s, Huntington’s and Parkinson’s diseases, as well as prion diseases. Prion diseases are the prototypical protein misfolding disorders, as their pathogenesis is associated solely with aberrant misfolding of a host cellular protein, the normal cellular PrP (PrP^C) to abnormal conformers (PrP^Sc), in agreement with the “protein-only” hypothesis (Prusiner, 1982). PrP^C is a glycosphatidylinositol (GPI) - linked peripheral membrane protein found principally at the plasma membrane. It is monomeric and rich in \( \alpha \)-helical structure whereas the disease-associated PrP^Sc is characterised by an increase in \( \beta \)-sheets, detergent insolubility and partial resistance to proteolysis. Neuropathologically, prion diseases are associated with severe neuronal loss, marked gliosis, spongiform change and accumulation of PrP^Sc in the brain. A major gap exists in our understanding of how conversion of PrP^C to PrP^Sc causes neuronal dysfunction and death. Various mechanisms have been proposed including synaptic impairment (Senatore et al., 2012), ubiquitin-proteasome system (UPS) dysfunction (Kristiansen et al., 2007), endoplasmic reticulum (ER) stress (Hetz et al., 2003; Rane et al., 2008), toxicity due to the presence of PrP species in the cytosol (Chakrabarti and Hegde, 2009; Kristiansen et al., 2005; Ma et al., 2002), and unfolded protein response induction and translational arrest (Moreno et al., 2012). To assess the contribution of these mechanisms to prion-induced toxicity, it is essential to know the exact cellular trafficking pathways of PrP^Sc. That is, where is it formed and what cellular compartments does it traffic to within the prion-infected cell.

Numerous intracellular compartments have been proposed as the initial site of prion conversion (i.e. PrP^Sc formation): the endo-lysosomal system (Borchelt et al., 1992; Magalhaes et al., 2005), the endosomal recycling compartment (ERC) (Marijanovic et al., 2009) and the trans-Golgi network (TGN)/Golgi/ER (Beranger et al., 2002). Critically, these studies relied on the use of chronically-infected cell lines, which require serial sub-passage to ensure inocula-derived PrP^Sc had been eliminated. These studies are further complicated by the lack of PrP^Sc-specific antibodies, making fixation and processing to remove PrP^C prior to immunostaining necessary (Kristiansen et al., 2005; Veith et al., 2008). As a result, details of the initial stages of prion conversion and early trafficking events remained elusive. We recently generated a unique cell system in which epitope-tagged PrP^C is expressed in PrP
knockdown neuroblastoma cells. The PrP-224AlaMYC chimera supports prion replication and results in the production of a bona fide epitope-tagged PrPSc (Goold et al., 2011). Using anti-MYC antibodies in conjunction with formic acid treatment to remove PrPC (Goold et al., 2011; Kristiansen et al., 2005), we were able to distinguish newly formed PrPSc generated in the recipient cell from both cellular PrPC and inocula-derived PrPSc species (Goold et al., 2011; Kristiansen et al., 2005). Our initial studies have unequivocally demonstrated that prion infection of cells is extremely rapid, occurring within one minute of prion exposure, and that the plasma membrane is the initial site of prion conversion. Further, de novo produced PrPSc formed at the plasma membrane is rapidly endocytosed and trafficked to a perinuclear compartment. Cells fixed shortly after prion exposure host PrPSc in a diffuse cellular pattern, reflecting its transition through an early endosomal compartment. A short time later, the cells have assumed a characteristic phenotype with PrPSc found primarily at the plasma membrane and in the perinuclear region, which is densely packed with organelles, including early endosomes, recycling endosomes, the TGN and Golgi. This steady-state distribution is maintained thereafter as the cells continue to stably propagate PrPSc.

Here, we extend our previous work by taking advantage of the PrP-224AlaMYC cell system to map the intracellular trafficking of PrPSc following its initial formation at the plasma membrane. We show that newly formed PrPSc colocalises with cholera toxin B subunit (CTB), a well characterised marker of GM1-enriched membrane microdomains, at and near the cell surface. PrPSc is endocytosed to early endosome-associated protein 1 (EEA1), transferrin (Tf) and CTB-labeled organelles. PrPSc is then segregated into two pathways: it can be recycled back to the plasma membrane via a Rab11-positive recycling compartment or rapidly sorted to the TGN and Golgi apparatus. We show that the retromer complex mediates PrPSc trafficking to the TGN. Further, we provide evidence that PrPSc reaching the Golgi is rapidly transferred to lysosomes and degraded, suggesting that this pathway is the major degradative mechanism in the early stages of prion infection.

Results

**PrPSc colocalises with transferrin and cholera toxin B following endocytosis**

To analyse PrPSc intracellular transport in detail, we compared its distribution shortly after its formation with the distribution of three well-defined trafficking cargoes - Tf, CTB and dextran. These fluorescently-labelled molecules are predominantly found in the ERC (Tf), in
GM1-enriched membrane microdomains and along the retrograde pathway (CTB), and within the endo-lysosomal system (dextran) (Baravalle et al., 2005; Lencer and Tsai, 2003). PrP-224AlaMYC cells were labelled with the individual cargoes and exposed to RML prions for 2 min prior to fixation. Cells were then treated with formic acid and immunostained with anti- MYC antibodies, a process that allows specific visualisation of de novo produced PrP\textsuperscript{Sc} by confocal microscopy. After 2 min exposure to prions, colocalisation was observed with all three cargoes, but most notably with CTB (Fig. 1). PrP\textsuperscript{Sc}/CTB colocalisation was observed at and near the plasma membrane and also in more perinuclear compartments. PrP\textsuperscript{Sc} also co-distributed with Tf in this compartment (Fig. 1).

**Newly formed PrP\textsuperscript{Sc} traffics through early endosomes, recycling endosomes, the TGN and the Golgi apparatus**

Colocalisation with CTB and Tf suggests PrP\textsuperscript{Sc} undergoes retrograde transport and may be trafficked to recycling endosomes. To explore this further we measured the extent of PrP\textsuperscript{Sc} colocalisation with well-defined organelle markers. PrP-224AlaMYC cells were exposed to RML prions and fixed at serial time-points up to 16 min. Cells were then processed to reveal PrP\textsuperscript{Sc} and the different organelle markers for confocal microscopy. The proportion of total PrP\textsuperscript{Sc} that colocalised with each organelle marker was determined. Colocalisation of PrP\textsuperscript{Sc} with the early endosomal marker EEA1 and the recycling endosomal marker Rab11 was observed at early time points (Fig. 2A,B). Later, PrP\textsuperscript{Sc} colocalisation with the TGN marker TGN46 and the Golgi marker GM130 was observed (Fig. 2A,B). Little colocalisation with the ER marker protein disulphide isomerase (PDI) was observed (Fig. 2C,D and Fig. S1). This apparent codistribution between PDI and PrP\textsuperscript{Sc} is likely to be due to our inability to distinguish juxtaposed signals in the crowded perinuclear region of the cell. This non-specific colocalisation accounted for less than 5% of that measured for EEA1 after 16 min. A low, yet consistent, level of colocalisation of PrP\textsuperscript{Sc} with LAMP1, a late endosome and lysosomal marker, was also observed (Fig. 2C,D). Overall, our data suggests that PrP\textsuperscript{Sc} moves from its site of formation at the plasma membrane through the cell periphery to the ERC, TGN and Golgi apparatus. This implies at least two trafficking routes are followed by PrP\textsuperscript{Sc}; that is, the recycling and the retrograde pathways.

**PrP\textsuperscript{Sc} is a cargo for retrograde transport**

Close association with CTB and high colocalisation with TGN46 and GM130 markers suggests that PrP\textsuperscript{Sc} undergoes retrograde transport to the TGN and Golgi. However, the close
juxtaposition of several types of organelles in the perinuclear region of PrP-224AlaMYC cells makes a precise localisation difficult. Therefore, we added Brefeldin A (BFA) to chronically prion-infected PrP-224AlaMYC cells to break up the Golgi apparatus into dispersed vesicles termed ‘ministacks’ (Alcalde et al., 1992). BFA treatment enabled us to visualise the degree of colocalisation of PrPSc with GM130 with a higher spatial resolution. Distinct vesicles labelled with both PrPSc with GM130 were clearly visible (Fig. 3A). In an independent experiment, we added BFA prior to RML prion exposure and examined the effect on PrPSc distribution. A gross disruption in PrPSc distribution was observed, with a more diffuse localisation pattern, indicating delivery to the perinuclear region was inhibited (Fig. S2A,B). This effect occurred within 16 min of prion exposure, well before any reduction in PrPSc levels induced by BFA were observed (Fig. S2C). BFA also inhibited accumulation of CTB in the perinuclear region indicating that retrograde trafficking of this cargo was inhibited (Fig. S3A). This suggests that the trafficking of PrPSc to the perinuclear region is dependent on an intact Golgi and/or ER and is consistent with the retrograde trafficking of PrPSc. To test this hypothesis we used Retro-2, a small molecule inhibitor of retrograde transport (Stechmann et al., 2010). Consistent with published data, we found no detectable changes in EEA1, Rab11, TGN46 or GM130 distribution in PrP-224AlaMYC cells following Retro-2 treatment (data not shown). However, colocalisation of CTB with a Golgi marker was reduced indicating that retrograde trafficking of this cargo was inhibited (Fig. S3A). Retro-2 treatment of PrP-224AlaMYC cells prior to prion exposure did not affect prion infection or change the gross distribution of PrPSc in the infected cells. However, PrPSc colocalisation with GM130 was reduced in treated cells relative to controls, indicating that transport to the Golgi was inhibited (Fig. 3B,C). This suggests that PrPSc is trafficked via the retrograde pathway from endosomes to the Golgi, a process which has been shown to be mediated by the retromer complex (Bonifacino and Hurley, 2008)

To confirm this hypothesis, we targeted retromer function with RNAi directed against one of its core subunits, VPS35. This treatment reduced VPS35 expression in PrP-224AlaMYC cells by approximately 70%, as determined by western blot (Fig. S3B) and confirmed by immunofluorescence staining (Fig. 3D). We tested its functional role in PrPSc trafficking by quantifying the extent of colocalisation with TGN46, in VPS35 RNAi and mock-treated PrP-224AlaMYC cells in the early minutes of prion exposure. Gross PrPSc and TGN46 distribution were unchanged by this treatment (Fig. 3D). However, we found that colocalisation between PrPSc and TGN46 was significantly reduced by the downregulation of
VPS35 relative to control after 4 min prion exposure (Fig. 3E). By 16 min, no difference in colocalisation was detected. This partial effect is likely to be due to the incomplete VPS35 knockdown, which in turn affects the kinetics of transport.

**A fraction of PrPSc is recycled to the plasma membrane**

If our hypothesis that the plasma membrane is a major site of prion conversion is correct, then prion propagation (i.e. PrPSc formation in the absence of exogenous inocula) would require recycling of at least a portion of the internalised PrPSc to the cell surface. To test this hypothesis, we used phosphoinositol-specific phospholipase C (PI-PLC), an enzyme that cleaves GPI-linked proteins from their membrane anchor (Enari et al., 2001). Chronically-infected PrP-224AlaMYC cells were treated with PI-PLC at 4°C for 30 min to cleave surface PrP (PrPc and PrPSc) from cells. Since this temperature shift inhibits vesicular trafficking (including recycling to the plasma membrane), intracellular PrP remains largely unaffected. Cells were washed with cold media, shifted to 37°C and the reappearance of cell surface PrPSc monitored. Nearly all of the control cells incubated at 4°C in the absence of PI-PLC showed plasma membrane PrPSc (Fig. 4A,B). As expected, PI-PLC effectively removed most of the PrPSc from the surface of the cells whilst leaving PrPSc in the perinuclear region (Fig. 4A,B). Interestingly, after 10 min at 37°C more cells showed membrane PrPSc and by 30 min at 37°C, the proportion had nearly reached control levels (Fig. 4A,B). Overall levels of PrPSc were reduced by PI-PLC treatment even after 30 min incubation at 37°C. This is probably due both to the initial reduction in PrPSc cleaved from the plasma membrane and also due to the reduction of the overall level of PrPc at the cell surface, the substrate for further PrPSc formation, which will not have returned to steady state in the timespan of the assay (Nunziante et al., 2003).

This data confirms the recycling of PrPSc and supports our hypothesis that the plasma membrane is a major site of prion conversion. We used Retro-2 to assess if passage through the TGN is necessary for PrPSc recycling via the ERC. Control PrP-224AlaMYC cells or cells pretreated with Retro-2 were exposed to prions and fixed after 4 min when the effect of Retro-2 is significant (Fig. 3C). Cells were then processed to reveal PrPSc and Rab11 and the proportion that colocalised was determined. No significant difference in the colocalisation was detected (Fig. 4C,D). This indicates that the presence of PrPSc in the ERC was not sensitive to Retro-2 and is therefore not dependent on retrograde transport of PrPSc.
Newly formed PrPSc is targeted for lysosomal degradation

We previously showed that prion infected cells assume a steady state PrPSc distribution within minutes of prion infection suggesting that PrPSc is formed at the plasma membrane and internalised continuously. In addition, we observed that PrPSc levels remained relatively unchanged within 2-24 h from prion infection (Goold et al., 2011) indicating that PrPSc formation and degradation kinetics are balanced. This implies that PrPSc degradation is rapid and continuous in the early stages of prion infection. At steady state, a low level of PrPSc is found colocalised with LAMP1 (Fig. 2C,D), suggesting that PrPSc is delivered to lysosomes for degradation. To explore this further, we investigated the effects of lysosomal protease inhibitors (leupeptin, E64d and peptatin A) on PrPSc distribution. PrP-224AlaMYC cells were pre-treated with lysosomal protease inhibitors for 15 min, then exposed to RML prions for 180 min, fixed and processed to reveal PrPSc. In agreement with previously published data (Luhr et al., 2004), treatment with the cysteine protease inhibitors leupeptin and E64d caused an increase in PrPSc levels, whereas treatment with peptatin A did not (Fig. S4).

In view of these findings, we probed routes that PrPSc may follow to reach the late endosome/lysosomal system. Delivery via the endo-lysosomal system akin to the trafficking of the epidermal growth factor receptor (Dikic, 2003) would provide the most direct route. However, when we compared the colocalisation of PrPSc with TGN46 and Rab7, it showed an increased accumulation of PrPSc within the TGN relative to the late endosome at early time points, indicating that the trafficking through the TGN represents the major transport route for PrPSc (Fig. 5A,B). To explore this further we inhibited lysosomal PrPSc degradation using leupeptin and E64d, combining the two reagents to maximise their effects. Leupeptin and E64d were added to PrP-224AlaMYC cells prior to prion infection and the distribution of PrPSc was analysed. Under control conditions, PrPSc is found principally in the perinuclear region and at the plasma membrane by 4 min, and this steady-state distribution is maintained thereafter (Fig. 5D). A similar PrPSc distribution is observed under leupeptin and E64d treatment at 4 min, with most of the intracellular PrPSc found close to the nucleus (Fig. 5C). However, by 16 min, PrPSc shows a more widespread distribution that overlaps closely with LAMP-1 (Fig. 5C,D). Quantifying the colocalisation of PrPSc with LAMP1 supports this observation: colocalisation is relatively low, (similar to control conditions) up to 4 min following RML prion addition in the presence of leupeptin and E64d (Fig. 5E); at 16 min post infection, this colocalisation is significantly increased relative to controls. This suggests that PrPSc is delivered to lysosomes after transit through the perinuclear region (i.e.
endosomes and the TGN). In support of this, BFA treatment, known to inhibit retrograde transport (Fig. S2, S3), prevents the lysosomal accumulation of PrP<sup>Sc</sup> caused by combined leupeptin and E64d treatment (Fig. S5).

To further test the role of retrograde transport of PrP<sup>Sc</sup> in its lysosomal delivery and degradation, we assayed PrP<sup>Sc</sup> levels in acutely-infected PrP-224AlaMYC cells after treatment with Retro-2. PrP-224AlaMYC cells were pre-treated with Retro-2 then exposed to prions for 180 min, fixed and processed to reveal PrP<sup>Sc</sup> and EEA1 or LAMP1. This treatment resulted in the accumulation of PrP<sup>Sc</sup> in the perinuclear region of the cell, coincident with EEA1 immunostaining (Fig. 6A). The intensity of PrP<sup>Sc</sup> immunostaining was then quantified from confocal images obtained from three independent experiments. This analysis showed a significant increase in pixel intensity relative to control cells (Fig. 6D). Interestingly, the increased PrP<sup>Sc</sup> levels did not result in an increase in its colocalisation with LAMP1 (Fig. 6E). This data suggests Retro-2 treatment retards PrP<sup>Sc</sup> delivery to the lysosome and thereby inhibits its degradation (Fig. 6A,D). A similar paradigm was used to directly analyse the role of lysosomes in PrP<sup>Sc</sup> degradation. We assayed PrP<sup>Sc</sup> levels in acutely-infected PrP-224AlaMYC cells after combined treatment with leupeptin and E64d. This analysis showed a robust increase in pixel intensity relative to control cells indicating an accumulation of PrP<sup>Sc</sup> under these conditions (Fig. 6B,D). PrP<sup>Sc</sup> distribution was altered such that it was more widely spread through the cell. An increase in colocalisation with LAMP1 was also noted, indicating that PrP<sup>Sc</sup> builds up in lysosomes (Fig. 6E).

To assess the contribution of autophagy and the UPS to PrP<sup>Sc</sup> breakdown we used Bafilomycin A (Baf A) and 3-methyladenine (3-MA), two reagents commonly used to inhibit autophagy (Klionsky et al., 2008) and the proteasome inhibitors epoxomicin and MG262. PrP-224AlaMYC cells were pre-treated with the inhibitors then exposed to prions for 180 min, fixed and processed to reveal PrP<sup>Sc</sup>, which was then quantified from confocal images. Baf A treatment caused a significant increase PrP<sup>Sc</sup> levels whereas 3-MA, epoxomicin and MG262 had little effect (Fig. 6D). The efficacy of proteasomal inhibition was confirmed by using the proteasome activity probe MV151 (Verdoes et al., 2006). Little probe was incorporated into the proteasome in the presence of epoxomicin or MG262 (Fig S6A). No changes in PrP<sup>C</sup> or 20S levels were detectable (Fig. S6B). Baf A treatment caused a redistribution of LC3, a protein marker of the autophagosomal membrane, into discrete puncta indicating a block in autophagic flux (Fig. 6C). A pronounced increase in PrP<sup>Sc</sup> levels
in the perinuclear region was also induced, which was quite distinct from the distribution observed after leupeptin/E64d treatment (Fig. 6B,C). No increase in lysosomal colocalisation was observed under these conditions (Fig. 6B,E) and little colocalisation with LC3 was detected (Fig. 6C).

**PrP<sup>Sc</sup> is targeted for lysosomal degradation in chronically-infected cells**

We were interested to compare PrP<sup>Sc</sup> degradation in cells acutely-infected with prions to that observed in chronically-infected cells that have been propagating PrP<sup>Sc</sup> for several passages. Therefore, we assayed PrP<sup>Sc</sup> levels in chronically-infected PK1 (ScPK1) cells treated for 180 min with the lysosomal, autophagy and UPS inhibitors used above. Western blot analysis of proteinase K (PK) digested lysates showed a significant increase in PrP<sup>Sc</sup> levels following treatment with leupeptin and E64d, Baf A, 3-MA and epoxomicin (Fig. S7A,B,C). MG262 treatment resulted in a small but non statistically significant increase in PrP<sup>Sc</sup> levels (Fig. S7B,C). None of the treatments increased PrP<sup>C</sup> levels in the time course of our experiments (Fig. S7A,B). The effects of 3-MA and epoxomicin indicate that autophagy and the proteasome play additional roles in PrP<sup>Sc</sup> degradation in ScPK1 cells.

To quantify the proportion of plasma membrane PrP<sup>Sc</sup> that is targeted for degradation we performed surface labelling experiments. ScPK1 cells were treated with cell impermeable NHS-sulpho-biotin at 4°C for 30 min, a procedure that specifically labels cell surface proteins (Gottardi et al., 1995). Cells were analysed immediately or returned to 37°C for a chase period of up to 6 h. Lysates from surface-labelled cells were digested with PK and detergent insoluble material was recovered by centrifugation. Immunoblotting showed the PrP<sup>Sc</sup> was recovered in the insoluble material (Fig. S8). Probing the membranes with NeutrAvidin-HRP revealed biotinylated bands that migrated with the same apparent molecular weight as PK-resistant PrP (Fig. S8). Uninfected cells did not contain these bands nor did unbiotinylated ScPK1 cells (Fig. S8 and Fig. 7A). We were also able to detect PK-resistant PrP in NeutrAvidin affinity chromatography eluates, albeit with low efficiency because of the difficulty in solubilising PrP<sup>Sc</sup> (data not shown). Probing similar eluates not treated with PK showed the Tf receptor was labelled and captured efficiently but that ERK1/2 was not (data not shown). Together, our data shows we were able to effectively label and detect surface PrP<sup>Sc</sup>. 
We then analysed the levels of labelled PrPSc in our pulse-chase experiments (Fig. 7). Little change was seen in the levels of total PrP or PK-resistant PrP (PrPSc) over a 6 h time course (Fig. 7A). However, the levels of biotinylated PrPSc decrease appreciably in the same time frame (Fig. 7B). Addition of leupeptin and E64d in the chase media reduced but did not completely abolish the loss of biotinylated PrPSc relative to control conditions (Fig. 7C,D). As expected, total levels of PrPSc were increased by leupeptin and E64d treatment (Fig. 7C,D). Our experiments show 60% of surface PrPSc is degraded in 3 h, with a large proportion processed in the lysosome. The rest is presumably en route to this organelle, sorted through the recycling pathway or becomes resistant to degradation. However, this quantification may be an under-estimate of the true level of degradation because surface-labelled PrPC may well be a substrate for prion conversion during the chase period, as previously observed for surface-iodinated PrP (Caughey and Raymond, 1991).

**Discussion**

To gain a better understanding of how PrPSc formation causes cytotoxicity, it is essential to know where PrPSc is formed and to what cellular compartments it traffics to within prion-infected cells. In earlier work, we used our PrP-224AlaMYC cell system to demonstrate that PrPSc is formed at the cell surface and rapidly endocytosed to a perinuclear region (Goold et al., 2011). Here, we extend this work by characterising in more detail the trafficking pathways utilised by newly formed PrPSc. We have performed a precise spatio-temporal analysis of PrPSc intracellular distribution following prion infection using established methods to visualise de novo PrPSc (Goold et al., 2011). In the absence of PrPSc-specific antibodies, we employed immunodetection of formic acid-resistant MYC-tagged PrP to produce a detailed trafficking itinerary of newly formed PrPSc and elaborate further on the kinetics of prion propagation (Borchelt et al., 1992; Marijanovic et al., 2009; Veith et al., 2008). Data presented here suggests that PrPSc forms at the plasma membrane and is then endocytosed to early endosomes containing CTB and Tf. Following endocytosis, a proportion of PrPSc is recycled to the plasma membrane via the ERC, whilst the remainder undergoes retrograde transport to the TGN and the Golgi apparatus. Recycling then provides further PrPSc at the plasma membrane, for template-seeded PrPSc conversion, whereas retrograde transport leads to lysosomal delivery and PrPSc degradation. The PrPSc trafficking kinetics described here in cells support previously observed immunohistochemistry analyses, where PrPSc has been detected on the plasma membrane (Jeffrey et al., 1994), in the Golgi (Barmada...
and Harris, 2005) and endo-lysosomal system (Arnold et al., 1995) in the prion-infected mouse brain.

Shortly after prion infection, we observed extensive colocalisation of newly formed PrP\textsuperscript{Sc} with CTB, at and near the plasma membrane (Fig. 1), suggesting that PrP\textsuperscript{Sc} may enter the cell in GM1-enriched membrane microdomains (Lencer and Tsai, 2003). This conclusion is in agreement with previous studies of PrP\textsuperscript{Sc} endocytosis. Pharmacological manipulation of the lipid content and organisation of membrane microdomains has been shown to disrupt PrP\textsuperscript{Sc} trafficking and propagation (Marella et al., 2002). In addition, PrP\textsuperscript{Sc} is known to segregate with membrane microdomain fractions (Naslavsky et al., 1997; Pimpinelli et al., 2005). It is, however, important to acknowledge the possibility of cell type-specific differences in PrP\textsuperscript{Sc} trafficking. For example, the endocytosis of PrP\textsuperscript{C} and PrP\textsuperscript{Sc} fibrils in sensory neurons appears to be dependent upon clathrin-coated pits (Jen et al., 2010; Parkyn et al., 2008). This apparent heterogeneity in PrP\textsuperscript{Sc} trafficking in different cell-types could also be explained by differences in the uptake of newly formed PrP\textsuperscript{Sc} versus the more mature PrP\textsuperscript{Sc} fibrils purified from end-stage prion infected mouse brain that have been previously used to study endocytosis.

Following endocytosis, internalised PrP\textsuperscript{Sc} is mainly segregated by two intracellular trafficking pathways: recycling to the cell surface via the ERC and retrograde transport to the TGN/Golgi. We have previously observed colocalisation of PrP\textsuperscript{Sc} with Tf in perinuclear regions (Goold et al., 2011) and detailed kinetic analysis demonstrated colocalisation with Rab11 early in the course of prion infection (Fig. 2). These findings suggest that PrP\textsuperscript{Sc} is rapidly transferred to recycling endosomes. In support of this hypothesis, we observed that a proportion of PrP\textsuperscript{Sc} is recycled to the plasma membrane from an internal compartment (Fig. 4A,B). Evidence of rapid PrP\textsuperscript{Sc} recycling has important implications for sustained prion propagation as it replenishes plasma membrane PrP\textsuperscript{Sc} and thus facilitates further seeded-misfolding of PrP\textsuperscript{C} at the cell surface. This hypothesis is consistent with the work of Marijanovic et al (2009), who demonstrated the importance of the ERC to PrP\textsuperscript{Sc} formation. However, our data does not rule out further PrP\textsuperscript{C} prion conversion in the ERC as both PrP\textsuperscript{C} and PrP\textsuperscript{Sc} traverse this compartment. Our data does not differentiate between PrP\textsuperscript{Sc} formed at the cell surface from that formed within the recycling pathway. Indeed, the rapid return of plasma membrane PrP\textsuperscript{Sc} to control levels suggests PrP\textsuperscript{Sc} may form within intracellular
compartments during recycling and supports the hypothesis that the ERC plays an important part in prion conversion.

Detailed spatio-temporal analysis revealed a sequential build-up of PrP Sc in the endosomal system, followed by the TGN and Golgi, consistent with trafficking along the retrograde transport route. However, we found that the presence of PrP Sc in recycling endosomes was independent of retrograde transport (Fig. 4C,D). This suggests that the PrP Sc trafficking pathway diverges at an early point into a recycling/propagative pathway and a retrograde/degradative pathway. We confirmed that PrP Sc reaches the Golgi using BFA, a reagent that destabilises the Golgi/ER interface to generate Golgi ministacks (Alcalde et al., 1992). BFA is known to exhibit pleiotropic effects within the cell. For instance, BFA-induced disruption of the secretory pathway has previously been shown to inhibit PrP delivery to the cell surface and hence reduce prion propagation (Taraboulos et al., 1992). BFA has also been shown to inhibit retrograde transport of two well-defined cargos: shiga toxin (Mallard et al., 1998) and CTB (Fig. S3A). We exploited the latter effect to show that transfer of newly formed PrP Sc to the perinuclear region is dependent upon an intact Golgi and/or ER. To confirm this finding, we pre-treated cells with Retro-2, a small molecule inhibitor of retrograde transport (Stechmann et al., 2010), and observed a block of PrP Sc and CTB trafficking to the Golgi (Fig. 3B,C and Fig. S3A). Further, inhibition of retromer function by RNAi-mediated knockdown of one of its core subunits (VPS35) delayed PrP Sc transfer to the TGN (Fig. 3D,E). This data indicates that the retrograde transfer of PrP Sc to the TGN is mediated at least in part by the retromer complex. Taken together, this data demonstrates that PrP Sc is rapidly trafficked to the Golgi via retrograde transport, a route also followed by other GPI-linked proteins (Nichols et al., 2001).

The ultimate destination for many retrograde transport cargos is the ER, and indeed PrP Sc has previously been shown to build up in this compartment, albeit in a cell system overexpressing Rab6A (Beranger et al., 2002). In our system, we find little evidence for PrP Sc accumulation in the ER, at least in the timespan analysed in our experiments (Fig. 2C,D,S1). Rather than transfer from the Golgi to the ER, our data suggests that PrP Sc is instead routed to the lysosome for degradation: (1) more PrP Sc accumulates in the TGN relative to the late endosome at early time points (Fig. 5A,B); (2) leupeptin and E64d treatment resulted in an early accumulation of PrP Sc in the perinuclear region, followed by a more peripheral distribution with a high degree of LAMP1 colocalisation (Fig. 5C-E); (3) BFA treatment
abrogates lysosomal accumulation of PrPSc (Fig. S5); (4) Retro-2 treatment produces a significant increase in PrPSc levels, causing an accumulation in the perinuclear region and not in lysosomes (Fig. 6A,E). Although our data cannot rule out the trafficking of a proportion of PrPSc through the endo-lysosomal system, taken together our observations indicate that retrograde transport plays an important role in the lysosomal delivery and degradation of PrPSc, and are consistent with previous work in sensory neurons, where purified PrPSc was shown to accumulate in a perinuclear compartment prior to trafficking to the lysosome (Jen et al., 2010).

The absence of detectable PrPSc in the ER and evidence of PrPSc delivery to the lysosome from the Golgi apparatus suggests that PrPSc may be a substrate of Golgi quality control. This mechanism clears misfolded or aggregated protein from the Golgi and transfers them to the lysosome for degradation (Anelli and Sitia, 2008; Arvan et al., 2002). This pathway has previously been implicated in the clearance of misfolded PrP isoforms generated by inherited prion disease mutations or pharmacological manipulation of PrP trafficking in neuronal cells (Ashok and Hegde, 2009; Gilch et al., 2001). The results presented here extend these findings to infectious PrP isoforms, where PrPSc formed following inocula exposure at the cell surface enters the Golgi via the retrograde pathway before being targeted for degradation. We propose that Golgi quality control is necessary to maintain PrPSc at a constant level in the acute phase of prion infection. To support this we showed a robust increase in PrPSc levels in as little as three hours of leupeptin/E64d treatment following acute prion infection. Interestingly, Baf A treatment also caused an increase in PrPSc levels after three hours (Fig. 6D). However, the lack of PrPSc in LC3-labelled puncta suggests little PrPSc is targeted for macroautophagy at this stage of prion infection (Fig. 6C). Consistent with this, 3-MA, an inhibitor of autophagosome sequestration (Klionsky et al., 2008), had little effect on PrPSc levels under these conditions (Fig. 6D). These findings indicate the primary effect of Baf A under these conditions is to block PrPSc progression to the lysosome, probably trapping it in an early endosomal compartment (Baravalle et al., 2005). Inhibition of the UPS in this paradigm did not result in PrPSc accumulation (Fig. 6D). Taken together, these data indicate the Golgi/lysosome route represents the primary pathway for PrPSc breakdown during the acute phase of prion infection.

As cells advance towards a state of chronic infection, the appearance of cytosolic or large aggregated forms of PrPSc may stimulate other degradative pathways, including the UPS and
macroautophagy, which have previously been shown to regulate PrP<sup>Sc</sup> levels (Aguib et al., 2009; Nunziante et al., 2011). Our demonstration that treatment of ScPK1 cells with inhibitors of autophagy and the proteasome results in PrP<sup>Sc</sup> accumulation is consistent with this hypothesis (Fig. S7). The increase in PrP<sup>Sc</sup> levels after as little as three hours in the presence of lysosomal, autophagy and proteasome inhibitors emphasises the dynamic nature of its metabolism. It also demonstrates the delicate equilibrium that exists between PrP<sup>Sc</sup> formation and degradation in these cells, which is clearly visualised by our surface labelling experiments (Fig. 7). Our data is consistent with previously published work showing the degradation of surface PrP<sup>Sc</sup> (Caughey and Raymond, 1991). However, not all surface PrP<sup>Sc</sup> was degraded in both sets of experiments. Our data suggests some of the PrP<sup>Sc</sup> is recycled but it is possible a proportion of the PrP<sup>Sc</sup> is resistant to degradation, as indicated by metabolic labelling experiments (Borchelt et al., 1990; Caughey and Raymond, 1991). This may be due to alternative PrP<sup>Sc</sup> trafficking pathways not detected in our experiments using acutely-infected cells. In chronically-infected cells PrP<sup>Sc</sup> that escapes degradation may be routed to compartments, such as aggresomes, known to accumulate aggregated proteins (Kristiansen et al., 2005). Such differences in the trafficking and metabolism of PrP<sup>Sc</sup> between acute and chronic phases of prion infection may also be inferred by the differential contributions made by the lysosome, macroautophagy and the UPS to PrP<sup>Sc</sup> degradation (Fig. 6 and S7). Overall, our data suggests a large proportion of the PrP<sup>Sc</sup> formed at the cell surface is endocytosed, recycled or targeted for degradation in a few minutes.

Elucidation of the PrP<sup>Sc</sup> trafficking pathway has relevance to other proteinopathies associated with the endocytosis of misfolded proteins that access the cytosol such as amyloid-beta (A<sub>β</sub>), superoxide dismutase 1 and α-synuclein (Munch et al., 2011). Indeed, striking similarities exist between some aspects of A<sub>β</sub> metabolism and that of PrP<sup>Sc</sup>. In particular, the retrograde trafficking route and involvement of lysosomes in A<sub>β</sub> degradation and toxicity have been widely reported (Muhammad et al., 2008; Small and Gandy, 2006). It has been suggested that destabilisation of the lysosomal limiting membrane in response to luminal accumulation of aggregated A<sub>β</sub> may contribute to cytotoxicity. A similar mechanism has also been postulated in prion diseases (Kovacs et al., 2007; Zhang et al., 2003). Accumulation of PrP<sup>Sc</sup> in lysosomes may lead to membrane destabilisation and the leakage of luminal contents. This mechanism could provide a route through which PrP<sup>Sc</sup> gains access to the cytosol, a likely prerequisite for reported sources of cytotoxicity, including proteasomal inhibition and aberrant interactions with other cytosolic proteins (Chakrabarti and Hegde, 2009; Kristiansen
et al., 2005; Kristiansen et al., 2007). Whilst PrP-224AlaMYC and related neuroblastoma cell lines have proved invaluable in studying prion infection and trafficking, they are limited in their application to studies of cytotoxicity as they stably propagate prions without noticeable effect on cell viability (Weisssmann, 2004). Indeed, previous attempts to study prion-associated toxicity in neuroblastoma subclones, have required the addition of exogenous stressors (Kristiansen et al., 2005; Nunziante et al., 2011). Identification of the trafficking compartments at the interface between prion infection and prion cytotoxicity demands the use of a post-mitotic cell line expressing PrP-224AlaMYC, which is currently under development.
Materials and methods

Cell culture and chemicals

PrP-224AlaMYC cells were generated as described previously (Goold et al., 2011) and routinely cultured in dual selection media (puromycin and G418) in OptiMEM, FCS (10%) and penicillin/streptomycin. ScPK1 cells were prepared as described (Klohn et al., 2003). Tissue culture media, labelled cargo proteins and AlexaFluor-conjugated antibodies were purchased from Invitrogen. Retro-2 was obtained from Merck. Other chemicals were purchased from Sigma. The primary antibodies used in this study are detailed in table S1.

Cellular prion infection

Brain homogenates were prepared from end-stage RML prion-infected CD-1 mice as described (Klohn et al., 2003). These were diluted to 0.1% in media, and added to cells grown to 70% confluence that were seeded on tissue culture plastic or poly-L-lysine coated glass coverslips. Chronically prion-infected PrP-224AlaMYC cells were prepared by exposing cells grown on plastic to RML prions for 16 h then washing the cells three times with fresh media and passaging the cells for at least seven days in the absence of external prions prior to use. Cells were washed and fixed with pre-warmed formaldehyde (3.7% in PBS) for 13 min at 37°C.

Cell manipulations

Cells were treated with vehicle, BFA (10 µg/ml), retro-2 (20 µM), leupeptin (200 µM), E64d (2 µM), pepstatin A (20 µM), epoxomicin (1 µM), MG262 (1 µM), bafilomycin A (20 nM) and 3-MA (10mM). Cells were transfected with RNAi oligonucleotides using Dharmafect according to the manufacturer’s instructions. A mixture of four RNAi oligonucleotides targeting VPS35 (Dharmacon ON-TARGETplus kit) was added at 100 nM for 72 h prior to use. PI-PLC (Sigma) treatment was carried out using 0.5 U/ml PI-PLC for 30 min at 4°C. The cells were fixed at 4°C for 10 min then transferred to 37°C for a further 10 min prior to washing or transferred to 37°C and fixed as described above. Texas red labelled transferrin (5 µg/ml), AlexaFluor555-CTB (10 µg/ml) or AlexaFluor555-Dextran (10,000 kDa, 2.5 mg/ml) was added to the media for 30 min prior to prion exposure. Proteasome activity probe MV151 (1 µM) was added to the cells for 60 min prior to fixation.
**Immunofluorescence analysis**

Cells were routinely prepared for immunofluorescence analysis with 5 min pre-treatment with 98% formic acid to remove host PrP<sup>C</sup> and expose PrP<sup>Sc</sup> as described previously (Goold et al., 2011). Formic acid treated cells were permeabilised with cold methanol for 10 min at -20°C then washed with PBS, prior to immunostaining as described previously (Goold et al., 2011). Fluorescence images were acquired using a Zeiss LSM510 META confocal microscope with a plan-Apochromat 63 x/1.40 oil DIC objective. Control uninfected cells were processed for each experiment and LSM settings were chosen to give minimal background staining. The same settings were used to examine all infected cells in that experiment. Colocalisation coefficients were calculated using the colocalisation tool in the Zeiss LSM software version 4.2. Background colocalisation levels were determined using uninfected cells and subtracted from values obtained from prion-infected cells. The intensity of intracellular MYC staining was determined using Volocity software (Improvision). At least 25 cells in four independent experiments were analysed per condition. Differential interference contrast images were obtained and merged with confocal images to allow an accurate determination of the proportion of cells that showed membrane staining.

**Biochemical analysis**

Cell lysates were prepared for biochemical analysis as described previously (Goold et al., 2011). Surface labelling was performed using NHS-sulpho-biotin (Thermo Scientific). Cells were washed twice in ice cold PBS with 0.5 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub> then incubated on ice with 1 mg/ml NHS-sulpho-biotin in PBS with 0.5 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub> for 30 min. The reaction was quenched with 50 mM glycine, 0.5% BSA in PBS, then cells were washed once with TBS and harvested immediately or treated with warmed media and returned to 37°C for the chase period. Harvested cells were washed with PBS then resuspended in lysis buffer (PBS, 0.5% Triton X-100, 0.5% sodium deoxycholate). The protein concentration of the lysates was adjusted to 1 mg/ml and aliquots were methanol precipitated for total PrP analysis or digested with PK (10 µg/ml, 90 min at 37°C) for the analysis of PrP<sup>Sc</sup>. To enrich for detergent-insoluble proteins the PK digested samples were centrifuged at 20,000 g for 30 min at 4°C. The pellet was washed once with lysis buffer and once with methanol. The pellet was then air dried and resuspended in SDS sample buffer. Biotinylated proteins were detected with NeutrAvidin-HRP (Invitrogen) used at a 1:1000
dilution. ScPK1 lysates were incubated with NeutrAvidin-agarose (Thermo Scientific) according to the manufacturer’s instructions.

**Statistical analysis**

Data are expressed as mean ± standard error of the mean (SEM), unless otherwise stated. Data were compared by two-tailed $t$-tests and considered significantly different when $P<0.05$. Degree of significance is expressed as follows: $P<0.05*$; $P<0.01**$; unless otherwise specified.

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References


Figures

Fig. 1 Newly formed PrP\textsuperscript{Sc} colocalises with cholera toxin B and transferrin in prion infected cells

PrP-224AlaMYC cells were incubated with labelled cholera toxin B (CTB), transferrin (Tf) and dextran (Dex) and then exposed to prions for 2 min. Cells were fixed and processed to reveal PrP\textsuperscript{Sc} and imaged by confocal microscopy. Nuclei were visualised with DAPI (blue). Individual channels and merged confocal images are shown, scale bar 10 \( \mu \text{m} \). PrP\textsuperscript{Sc} (green) co-localises with all cargos (red) but most notably with CTB.

Fig. 2 PrP\textsuperscript{Sc} colocalises with markers for early endosomes, recycling endosomes, the trans Golgi network and Golgi.

A) PrP-224AlaMYC cells were fixed after 2 and 16 min exposure to prions and processed to reveal PrP\textsuperscript{Sc} and organelle markers EEA1 (early endosomes), Rab11 (recycling endosomes), TGN46 (TGN) and GM130 (Golgi). Nuclei were visualised with DAPI (blue). Individual channels and merged confocal images are shown, scale bar 10 \( \mu \text{m} \). After 2 min PrP\textsuperscript{Sc} (green) is found at the plasma membrane, the cell periphery and in the perinuclear region and colocalises with EEA1 and Rab11 and less well with TGN46 and GM130 (red). After 16 min exposure to prions intracellular PrP\textsuperscript{Sc} accumulates close to the nucleus where it colocalises with EEA1, Rab11, TGN46 and GM130. B) The proportion of PrP\textsuperscript{Sc} that co-localises with the indicated organelle markers was quantified at various time points following prion addition (mean ± SEM, n=4). A transition from early/recycling endosomes to the TGN and Golgi is revealed. C) PrP-224AlaMYC cells were fixed after 4 min exposure to prions and processed to reveal PrP\textsuperscript{Sc} (green) and organelle markers protein disulphide isomerase (ER; red) and LAMP1 (late endosomes and lysosomes; red). Nuclei were visualised with DAPI (blue). Individual channels and merged confocal images are shown, scale bar 10 \( \mu \text{m} \). Little colocalisation with the ER was observed but a low level of PrP\textsuperscript{Sc} was consistently found in lysosomes. D) The proportion of PrP\textsuperscript{Sc} that colocalises with protein disulphide isomerase and LAMP1 after 4 min exposure to prions was quantified (mean ± SEM, n=4).
Fig. 3 PrPSc is a substrate for retrograde transport

A) Chronically-infected PrP-224AlaMYC cells were treated with vehicle or Brefeldin A (BFA) for 15 min prior to fixation and processed to reveal PrPSc (green) and GM130 (red). Nuclei were visualised with DAPI (blue). Individual channels and merged confocal images are shown, scale bar 10 μm. BFA treatment induces the formation of dispersed Golgi ministacks, some of which contain PrPSc, which appear yellow in the merged image (arrow). B) PrP-224AlaMYC cells were treated with vehicle or Retro-2 prior to exposure to prions for 4 min and fixation. Cells were processed to reveal PrPSc (green) and GM130 (red). Nuclei were visualised with DAPI (blue). Individual channels and merged confocal images are shown, scale bar 10 μm. No gross effects on PrPSc or GM130 distribution were observed. C) The proportion of PrPSc that co-localises with GM130 in the presence and absence of Retro-2 was quantified at various time points following prion addition (mean ± SEM, n=4). Retro-2 inhibits PrPSc transfer to the Golgi. D) PrP-224AlaMYC cells were mock transfected or transfected with siRNA oligos directed against Vps35. Cells were exposed to prions for 4 min, fixed and processed to reveal PrPSc (green), VPS35 or TGN46 (red). Nuclei were visualised with DAPI (blue). Individual channels and merged confocal images are shown, scale bar 10 μm. RNAi treatment reduced VPS35 expression but had no gross effects on PrPSc or TGN46 distribution. E) The proportion of PrPSc that co-localises with TGN46 after mock or VPS35 RNAi transfection was quantified at various time points following prion addition (mean ± SEM, n=3). VPS35 knockdown retarded PrPSc delivery to the TGN.

Fig. 4 PrPSc is recycled to the plasma membrane

A) Chronically-infected PrP-224AlaMYC cells were incubated for 30 min at 4°C in control conditions or in the presence of PIPLC then fixed immediately or transferred to 37°C for the indicated times. Cells were processed to reveal PrPSc (green) and nuclei were visualised with DAPI (blue). Merged confocal images are shown, scale bar 10 μm. After incubation at 4°C, PrPSc was found at the plasma membrane (arrowhead) and in the perinuclear region (arrow). PIPLC effectively removes PrPSc from the cell surface under these conditions but does not affect intracellular PrPSc (arrow). Incubation at 37°C results in the recycling of internal PrPSc to the cell surface (arrowheads). B) Quantification of the proportion of cells with membrane PrPSc (mean ± SEM, n=3). C) PrP-224AlaMYC cells were treated with vehicle or Retro-2 prior to exposure to prions for 4 min and fixation. The cells were processed to reveal PrPSc (green) and Rab11 (red). Nuclei were visualised with DAPI (blue). Individual channels and
merged confocal images are shown, scale bar 10 μm. D) The proportion of PrP^Sc that co-localises with Rab11 in the presence and absence of Retro-2 was quantified 4 min following prion addition (mean ± SEM, n=4). Retro-2 did not affect PrP^Sc trafficking through the recycling endosome.

Fig. 5 Newly formed PrP^Sc is trafficked to lysosomes following retrograde transport.
A) PrP-224AlaMYC cells were fixed after 2, 4 and 16 min exposure to prions and processed to reveal PrP^Sc (green) and Rab 7 (red). Nuclei were visualised with DAPI (blue). Individual channels and merged confocal images are shown, scale bar 10 μm. Intracellular PrP^Sc localisation shows a typical perinuclear accumulation. Rab7-labelled late endosomes show a more widespread distribution. B) The proportion of PrP^Sc that co-localises with Rab7 and TGN46 was quantified after various times following prion exposure (mean ± SEM, n=4). More PrP^Sc accumulates in the TGN at early time points. C) PrP-224AlaMYC cells were treated with leupeptin and E64d for 15 min then exposed to prions for 2, 4 and 16 min prior to fixation and processing to reveal PrP^Sc (green) and LAMP1 (red). Nuclei were visualised with DAPI (blue). Individual channels and merged confocal images are shown, scale bar 10 μm. After 4 min PrP^Sc is observed concentrated in the perinuclear region (arrow). After 16 min PrP^Sc is shifted to the cell periphery in puncta that often co-inside with lysosomes, appearing yellow in the merged image. No major effects on LAMP1 distribution were observed. D) PrP-224AlaMYC cells were treated with vehicle or leupeptin and E64d as in panel (C). The proportion of cells showing a pronounced perinuclear PrP^Sc distribution was quantified (mean ± SEM, n=4). E) PrP-224AlaMYC cells were treated as in panel (D) and the proportion of PrP^Sc that colocalises with LAMP1 was quantified after various times following prion exposure (mean ± SEM, n=4). In the presence of leupeptin and E64d, PrP^Sc builds-up firstly in the perinuclear region and shows relatively low co-localisation with LAMP1 before assuming a more peripheral distribution that shows a higher level of co-localisation with LAMP1.

Fig. 6 Newly formed PrP^Sc is degraded in lysosomes
A) PrP-224AlaMYC cells were treated with vehicle or Retro-2 for 15 min then exposed to prions for 180 min prior to fixation and processing to reveal PrP^Sc (green) and EEA1 (red). Nuclei were visualised with DAPI (blue). Individual channels and merged confocal images are shown, scale bar 10 μm. Retro-2 induces PrP^Sc accumulation in the perinuclear region. B)
PrP-224AlaMYC cells were treated with vehicle, leupeptin and E64d or Baf A for 15 min then exposed to prions for 180 min prior to fixation and processing to reveal PrPSc (green) and LAMP1 (red). Nuclei were visualised with DAPI (blue). Individual channels and merged confocal images are shown, scale bar 10 μm. Leupeptin and E64d treatment increases the intensity and changes the distribution of PrPSc immunostaining, causing a build-up in the cell periphery, coincident with LAMP1 (arrow). Baf A treatment also increases PrPSc levels but an accumulation in the perinuclear region, distinct from LAMP1, is induced. C) PrP-224AlaMYC cells were treated with Baf A for 15 min then exposed to prions for 180 min prior to fixation and processing to reveal PrPSc (green) and LC3 (red). Nuclei were visualised with DAPI (blue). Individual channels and merged confocal images are shown, scale bar 10 μm. Baf A treatment induces the formation of distinct LC3-labelled puncta and causes PrPSc accumulation near the nucleus. D) Quantification of the pixel intensity per cell after vehicle (control), Retro-2, leupeptin and E64d, Baf A, 3-methyladenine (3-MA), epoxomicin (epox), or MG262 treatment. Data is normalised to control levels (mean ± SEM, n=4). E) Quantification of the colocalisation of PrPSc with LAMP1 after treatment with vehicle, Retro-2, leupeptin and E64d or Baf A (mean ± SEM, n=4).

Fig. 7 Surface PrPSc is rapidly degraded
A) ScPK1 cells were surface labelled with NHS-sulpho-biotin at 4°C for 30 min and cells were harvested immediately or returned to 37°C for a chase period of up to 6 h. Cell lysates were analysed directly or after limited PK digestion and centrifugation to enrich for PrPSc (+PK). ScPK1 cells not exposed to NHS-sulpho-biotin but processed in parallel were included as a control (-Biotin). Western blots were probed with anti-PrP antibodies or with NeutrAvidin-HRP to visualise biotinylated proteins. B) Western blots similar to those in panels (A) were quantified to determine total PrPSc and biotinylated PrPSc levels (mean ± SEM, n=3). Biotinylated PrPSc levels are rapidly reduced. Total PrPSc levels are unchanged. C) ScPK1 cells were surface labelled with NHS-sulpho-biotin at 4°C for 30 min and cells were harvested immediately or returned to 37°C for a chase period of 180 min in control media or in media containing leupeptin/E64d before analysis. Samples were analysed as in panel (A). PK1 cells processed in parallel were included as a control. D) Western blots similar to those in panels (C) were quantified to determine total PrPSc and biotinylated PrPSc levels (mean ± SEM, n=4). Inclusion of leupeptin and E64d in the chase media reduced but did not completely abolish the loss of biotinylated PrPSc. Total levels of PrPSc were increased by this treatment.