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Prions and autophagy: Effect of lithium on prion infection and role of basal autophagy in primary prion infection

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I. SUMMARY

I.A ENGLISH VERSION

The formation of an abnormally folded, protease resistant isoform of the host-encoded cellular prion protein (PrP^{c}) is thought to be the responsible mechanism for prion diseases. This disease-associated agent, designated PrP^{Sc}, is derived from PrP^{c} by a post-translational conformational change, and the presence of PrP^{c} is essential for development of prion disease. The exact physiological function of PrP^{c} is unclear, although roles in copper transport and neuroprotection are possible. Prominent examples of prion diseases, also referred to as transmissible spongiform encephalopathies (TSEs), comprise scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and Creutzfeldt-Jakob disease (CJD) in humans. Prion diseases are incurable, fatal neurodegenerative diseases associated with neuronal cell death, spongiform vacuolation and accumulation of PrP^{Sc}. In prion-infected cultured cells and in neurons derived from brain biopsy samples of TSE affected humans and animals the appearance of multi-vesicular bodies and autophagic vacuoles has been reported. In macroautophagy (here referred to as autophagy), autophagosomes can engulf cytosolic macromolecules and deliver them to lysosomes for degradation. The clearance of aggregate-prone proteins, such as mutant huntingtin fragments or mutant forms of α-synuclein causing Huntington`s and Parkinson`s disease, respectively, can be mediated by autophagy. Recently, it has been seen that the drug Glivec (also known as imatinib) is a potent anti-prion compound that is also able to induce autophagosome formation and autophagy.

Intrigued by this finding, in the first part of this work the potential of induced autophagy in degrading PrP^{Sc} was examined. In persistently prion-infected mouse neuroblastoma (ScN2a) and mouse fibroblast (ScL929) cell lines, treatment with lithium induced autophagy and provoked reduction of PrP^{Sc}. Reduced PrP^{Sc}-levels were also observed for other autophagy-inducing compounds. Pharmacological inhibition of autophagy counter-acted the anti-prion effect of lithium demonstrating for the first time that activated autophagy is mediating reduction of PrP^{Sc}. Moreover, lithium can reduce the level of PrP^{c} in an autophagy-dependent manner, probably leading to less conversion of PrP^{c} into PrP^{Sc} due to a decrease of substrate for prion conversion. Treatment of prion-infected mice with rapamycin, a widely used autophagy-inducing compound, showed a small but significant prolongation of survival times, indicating that such drugs may have therapeutic potential.

Besides the potential of compound-induced autophagy in degrading PrP^{Sc} in persistently prion-infected cells, the role of basal constitutive autophagy in primary prion infection is elusive, as both beneficial and deleterious roles are possible. On the one hand it might be conceivable that basal autophagy is inhibiting primary prion infection by elimination of infectious PrP^{Sc}/prions, on the other hand a vice versa scenario might be the case in which basal autophagy generates smaller PrP^{Sc}-seeds, known to be more infectious, thereby enhancing primary prion infection. Therefore, in the second part of this work, the role of basal autophagy in primary prion infection was investigated. In PrP^{Sc}-susceptible N2a clones, autophagosome formation was increased when newly converted PrP^{Sc} was detected upon primary prion infection. In contrast, increased autophagosome formation was not observed in a PrP^{Sc}-unsusceptible N2a clone (cells that do not convert endogenous PrP^{c} into PrP^{Sc}) upon primary prion infection, indicating that autophagosome formation accompanies and supports primary prion infection. Furthermore, wild-type mouse embryonic fibroblasts (MEFwt) do propagate PrP^{Sc} much more efficiently upon primary prion infection compared to autophagy-deficient MEFs ($MEFATG5^{-/-}$). In turn, reintroduction of *Atg5* in $MEFATG5^{-/-}$ rendered cells more susceptible to primary prion infection, indicating that basal autophagy is subsidizing primary prion infection.

Taken together, these data indicate that pharmacologically up-regulated autophagy results in enhanced clearance of PrP^{Sc} in cells persistently infected with prions. Moreover, concerning primary prion infection situation, a physiological basal activity of autophagy promotes primary prion infection whereas disturbance of this autophagy activity results in less efficient primary prion infection.

I.B DEUTSCHE VERSION

Die Bildung einer abnormal gefalteten, Protease-resistenten Isoform des vom Wirt kodierten zellulären Prion Proteins (PrP^{c}) wird als der verantwortliche Mechanismus für Prion Erkrankungen betrachtet. Dieses krankheits-assoziierte Agens, welches PrP^{Sc} genannt wird, stammt durch eine post-translationale Konformationsänderung von PrP^{c} ab und die Anwesenheit von PrP^{c} ist essentiell für die Entwicklung von Prion Erkrankungen. Die genaue physiologische Bedeutung von PrP^{c} is unbekannt, mögliche Funktionen sind Kupfertransport und Neuroprotektion. Bedeutende Beispiele für Prion Erkrankungen, welche auch als transmissible spongiforme Enzephalopathien (TSEs) bezeichnet werden, umfassen Scrapie bei Schafen, bovine spongiforme Enzephalopathie (BSE) bei Rindern und Creutzfeldt-Jakob Erkrankung (CJD) im Menschen. Prion Erkrankungen sind unheilbar, und daher tödliche, neuronale Erkrankungen welche mit neuronalem Zelltod, spongiformer Vakuolierung und Akkumulation von PrP^{Sc} einhergehen. In mit Prionen infizierten kultivierten Zellen und in Neuronen, welche aus Hirn-Biopsie Materialien von TSE erkrankten Menschen und Tieren stammen, wurde das Auftreten von multi-vesikulären Einschlußkörperchen und autophagischen Vakuolen berichtet. Während des Prozesses der Makroautophagie (welche hier als Autophagie bezeichnet wird), können zytosolische Makromoleküle durch Autophagosomen aufgenommen werden und zum Abbau zu Lysosomen transportiert werden. Die Beseitigung von Proteinen welche zu Aggregationsbildung neigen, wie beispielsweise mutante Huntingtin Fragmente oder mutante Formen von α-synuclein, die Huntington- bzw. Parkinson-Erkrankungen auslösen können, kann durch Autophagie bewerkstelligt werden. Kürzlich konnte gezeigt werden, daß die Arznei Glivec (auch als imatinib bekannt) ein potentes anti-Prionen Präparat ist, welches gleichzeitig in der Lage ist die Bildung von Autophagosomen und Autophagie zu induzieren.

Inspiriert durch diese Entdeckung wurde im ersten Teil dieser Arbeit das Potential von induzierter Autophagie PrP^{Sc} zu degradieren untersucht. Behandlung mit Lithium induzierte Autophagie und reduzierte PrP^{Sc} in persistent mit Prionen infizierten murinen Neuroblastomzellen (ScN2a) und murinen Fibroblastenzellen (ScL929). Verringerte PrP^{Sc} Menge wurde auch nach Behandlung mit anderen Autophagie induzierenden Substanzen festgestellt. Zum ersten Mal konnte gezeigt werden daß aktivierte Autophagie den Abbau von PrP^{Sc} bewerkstelligt, da pharmakologische Inhibierung von Autophagie dem anti-Prionen Effekt von Lithium entgegenwirkt. Des weiteren kann Lithium in einer Autophagie-abhängigen Weise den Gehalt an PrP^{c} reduzieren, was vermutlich zu weniger Konversion von

PrP^{c} in PrP^{Sc} führt, da weniger Substrat für die Prionen-Konversion vorhanden ist. Behandlung von Prionen-infizierten Mäusen mit Rapamycin, eine oft verwendete Autophagie induzierende Substanz, bewirkte eine kleine aber signifikante Verlängerung der Überlebenszeit, was ein Hinweis darauf ist daß solche Substanzen therapeutisches Potential besitzen.

Neben dem Potential von pharmakologisch induzierter Autophagie PrP^{Sc} in persistent mit Prionen infizierten Zellen zu degradieren ist die Rolle von basaler konstitutiver Autophagie bei der primären Prionen Infektion ungelöst, da sowohl eine fördernde als auch eine inhibierende Rolle denkbar ist. Auf der einen Seite ist es möglich daß basale Autophagie die primäre Prionen Infektion durch Eleminierung von PrP^{Sc}/Prionen inhibiert, auf der anderen Seite ist der umgekehrte Fall denkbar, daß basale Autophagie kleinere PrP^{Sc} Keime produziert, welche bekannt dafür sind hoch infektiös zu sein, und somit die primäre Prionen Infektion fördert. Deshalb wurde im zweiten Teil dieser Arbeit die Rolle von basaler Autophagie während der primären Prionen Infektion untersucht. In PrP^{Sc} empfänglichen N2a Klonen wurde vermehrte Autophagosomen Bildung beobachtet wenn neu konvertiertes PrP^{Sc} nach primärer Prionen Infektion detektiert wurde. Im Gegensatz dazu wurde in einem PrP^{Sc} unempfänglichen N2a Klon (Zellen die endogenes PrP^{c} nicht in PrP^{Sc} konvertieren) nach primärer Prionen Infektion keine Autophagosomen Bildung beobachtet, was ein Hinweis darauf ist daß Autophagosomen Bildung die primäre Prionen Infektion begleitet und unterstützt. Des weiteren propagieren wild-typ murine embryonische Fibroblasten (MEFwt) nach primärer Prionen Infektion PrP^{Sc} sehr viel effizienter als Autophagie-defiziente MEFs ($MEFATG5^{-/-}$). Die Wiedereinführung von *Atg5* in $MEFATG5^{-/-}$ veränderte die Zellen hingehend zu größerer Empfänglichkeit für die primäre Prionen Infektion, was ein Anzeichen dafür ist daß basale Autopahgie die primäre Prionen Infektion fördert.

Zusammenfassend zeigen diese Daten daß pharmakologische Hochregulierung von Autophagie in vermehrter Degradierung von PrP^{Sc} in persistent mit Prionen infizierten Zellen resultiert. Darüber hinaus, die Situation bei der primären Prionen Infektion betreffend, fördert eine basale physiologische Autophagie-Aktivität die primäre Prionen Infektion während eine Veränderung dieser Autophagie-Aktivität zu weniger effizienter primärer Prionen Infektion führt.

II. INTRODUCTION

II.A THE PRION PROTEIN

II.A.1 HISTORICAL BACKGROUND

First written descriptions of transmissible spongiform encephalopathies (TSEs) or prion diseases occurred in the middle of the 18th century. It was termed scrapie because this fatal disease was observed in sheep which had the tendency to scrape and to rub off their wool as a result of the disease. In 1759 an interesting article appeared in the German literature, from which the following paragraph is quoted in its entirety: "Some sheep also suffer from scrapie, which can be identified by the fact that affected animals lie down, bite at their feet and legs, rub their backs against posts, fail to thrive, stop feeding and finally become lame. They drag themselves along, gradually become emaciated and die. Scrapie is incurable. The best solution, therefore, is for a shepherd who notices that one of his animals is suffering from scrapie, to dispose of it quickly and slaughter it away from the memorial lands, for consumption by the servants of the nobleman. A shepherd must isolate such an animal from healthy stocks immediately because it is infectious and cause serious harm to the flock" (Leopoldt 1759). In 1936 the French scientists Cuillé and Chelle successfully transmitted scrapie to two healthy sheep by intraocular inoculation of brain or spinal cord tissue from an affected animal (Cuillé and Chelle 1936). The transmissible nature of the scrapie agent was thereby established without any doubt. In 1954 Sigurdson suggested a "slow virus" as the causative agent due to incubation times of the disease being as long as 20 years. Then in 1966, Gajdusek successfully transmitted Kuru to chimpanzees by injecting them brain tissue from people who had died of Kuru (Gajdusek et al. 1966, 1967). Kuru occurred in the Fore homeland in Papua New Guinea where the Fore people practiced ritual endo-cannibalism. Transmission and epidemiological studies proved that the Fore people contracted the disease through the act of cannibalism and that Kuru and scrapie have neuropathological similarities. One year later in 1968, Gibbs and Gajdusek showed that Creutzfeld-Jakob Disease (CJD) was infectious in the same way as Kuru (Gibbs et al. 1968). CJD is a human neurodegenerative disease similar to scrapie and was first described by Gerhard Creutzfeldt and Alfons Jakob in the early 20's of the 20th century.

In 1966 the British scientist Alper exposed scrapie-infected brain material to radiation, which usually destroys nucleic acids, and found that it could still transmit scrapie (Alper et al. 1966). This fact excluded the hypothesis that a virus could be the causative agent of the disease. In

line with these results, a British female mathematician named Griffith made the assumption that the causative agent might be a protein folded in an abnormal way (Griffith 1967). Then in 1982, Stanley B. Prusiner proclaimed that the scrapie agent contained no nucleic acid, because he had subjected the extract of scrapie infected hamster brain to procedures that destroy DNA and RNA with the result that the extract still had the power to cause scrapie (Prusiner 1982). In contrast, when the brain was exposed to agents known to destroy proteins, it became less or not infectious. Prusiner proposed the definition "prion" as a proteinaceous infectious particle that lacks nucleic acid and causes the disease. Purification of infectious brain material revealed a 27-30 kDa protein. Further studies revealed the fact that the proteins are produced normally in the brains of mammals by the prion protein gene (*PRNP*) (Chesebro et al. 1985; Robakis et al. 1986a; Sparkes et al. 1986). This protein was called PrP^{c} (cellular prion protein) (Prusiner et al. 1987). Further research revealed that disease-causing prions (PrP^{Sc}, for scrapie-associated prion protein) consist mainly, if not entirely, of an abnormally folded isoform of the normal, host-encoded PrP^{c} (Cohen et al. 1994; Prusiner 1998; Collinge 2001; Aguzzi and Polymenidou 2004; Weissmann 2004). PrP^{c} and PrP^{Sc} have the same primary structure but have drastically different biochemical properties (Prusiner 1991).

II.A.2 PRION DISEASES

Long incubation times, a short symptomatic phase, the always fatal progression of the disease and, usually, the lack of preclinical diagnostic are main features of prion diseases. Clinical symptoms consist of progressive motor dysfunction, cognitive impairment, and cerebral ataxia. The brains of diseased individuals are highly abnormal and share the following histopathological hallmarks: spongiform vacuolation, severe neuronal loss, strong astrogliosis, mild microglia activation, and accumulation of misfolded protein deposits (DeArmond and Prusiner 1995; Prusiner 1998; Aguzzi and Polymenidou 2004; Weissmann 2004; Collinge 2005). In addition, amyloid plaques (consisting of ordered proteinaceous deposits with high β-sheet content) occure during prion disease (Clinton et al. 1992; Bessen et al. 1997), though cases of TSEs lacking such plaques have been reported (Collinge et al. 1995a; Tateishi et al. 1995). Prion pathology shares several profound similarities with other protein misfolding and neurodegenerative diseases like Alzheimer's, Huntington's and Parkinson's disease (Aguzzi and Haass 2003; Chiti and Dobson 2006). Nevertheless, prions are unique as they are not only able to replicate their conformation but are also naturally and experimentally transmissible within and to some extend between species (Weissmann et al. 1996; Prusiner 1998). Below, human and animal prion diseases are discussed in detail.

II.A.2.1 HUMAN PRION DISEASES

This section summarizes the known human prion diseases, which are characterized by a wide range of clinical symptoms comprising weight loss, insomnia, depression, memory problems, confusion, headache, and general pain sensations. Neurological features include ataxia, extrapyrimidal signs, cortical blindness, and finally dementia.

Human prion diseases can be divided into three etiological categories: sporadic, acquired, and inherited (Prusiner 1998; Collinge 2001). Inherited prion diseases represent about 10 % of all cases. Autosomal dominant pathogenic coding mutations in *PRNP*, of which over 30 distinct types are recognized, are responsible for these cases (Collinge 2001; Kovacs et al. 2002; Wadsworth et al. 2003; Mead 2006). In sporadic (about 90 % of all cases) or acquired prion diseases (very rare, less than 5 %) no such pathogenic *PRNP* mutations are present. **Table 1** gives an overview of the human prion diseases.

Table 1. Human prion diseases. Taken from (Gilch et al. 2008).

Etiology	Disease and Frequency	Mechanism of Transmission / Infection
Acquired	Kuru (pandemic in the 1950s, nowadays Virtually distinct); iatrogenic Creutzfeldt-Jakob Disease (CJD) (< 5 %); variant CJD (vCJD) (total so far > 215 cases)	infection through environmental exposure to prions; exogenous
Genetic	familiar or genetic CJD (~ 10 %); Gerstmann-Sträussler-Scheinker (GSS) syndrome; fatal familiar insomnia (FFI)	mutations in the *PRNP* gene (more than 30 different mutations are known); endogenous
Sporadic	sporadic CJD (~ 1 case per million per year worldwide, ~ 90 %)	apparently spontaneous formation of PrP^{Sc}; endogenous

Creutzfeldt-Jakob disease (CJD) was first described by Gerhard Creutzfeldt and Alfons Jakob in the early 1920s. Classical (sporadic) CJD is a rapidly progressive, multifocal dementia, usually with myoclonus, leading to death within 6-12 months of disease onset (Gambetti et al. 2003) and occurs at a rate of 1-2 cases per million population per year across

the world, with an equal incidence in men and women (Brown et al. 1987; Collins et al. 2006). The peak incidence is between 55 and 65 years of age (Collins et al. 2006). Iatrogenic CJD is acquired by prion exposure of individuals during neurosurgical procedures such as implantation of human dura matter, corneal craft implantation, or treatment with human cadaveric pituitary extracts (Brown et al. 1992; Brown et al. 2000b). Less than 300 cases of iatrogenic CJD are reported. Although there is no pathogenic *PRNP* mutation in sporadic or acquired CJD, a common PrP polymorphism at residue 129, where either methionine (M) or valine (V) can be encoded, is regarded as a key determinant of genetic susceptibility to acquired and sporadic prion disease, the large majority of which occur in homozygous individuals (Collinge et al. 1991; Palmer et al. 1991; Windl et al. 1996). Familial CJD (fCJD) belongs to inherited prion diseases which are based on autosomal dominant mutations of *PRNP*.

Fatal familial insomnia (FFI) was proposed in 1986 to describe an illness involving five members of a large Italian family (Lugaresi et al. 1986). Substitution of asparagine for aspartic acid (D178N) at codon 178 and coexisting methionine at the polymorphic codon 129 of *PRNP* at the same allele are crucial for developing FFI (Medori et al. 1992), though sporadic FFI with no causative mutation at codon 178 of *PRNP* has been reported (Montagna et al. 2003). In contrast, fCJD segregates with the D178N mutation when combined with valine at codon 129 (Zerr et al. 1998). Core clinical features are profound disruption of the normal sleep-wake cycle, with prominent insomnia, sympathetic over-activity, diverse endocrine abnormalities and impaired attention.

The typical clinical features of **Gerstmann-Sträussler-Scheinker syndrome (GSS)** are slowly progressive cerebellar ataxia, beginning in the fifth or sixth decade (but with onsets as early as age 25 years reported), accompanied by cognitive decline (Masters et al. 1981). GSS share the distinctive and defining neuropathological feature of widespread, multicentric amyloid plaques, which are immunoreactive for PrP. Mutation P102L of *PRNP* has first been reproducibly associated with disease development (Hsiao et al. 1989). GSS is now linked to seven *PRNP* mutations and forms part of the phenotypic spectrum of inherited prion diseases.

A well-known example of acquired prion disease in humans is **Kuru**, transmitted by cannibalism among the Fore linguistic group of the Eastern Highlands in Papua New Guinea (Gajdusek 1977). The disease mainly occurred in women and young children (of both sexes) due to the fact that they ate the brain and internal organs of dead relatives. The epidemic of Kuru is thought to have originated when a case of sporadic CJD, known to occur at random in all populations, occurred in a member of this population and was, as were most deceased

individuals, eaten. Besides dietary exposure as route of transmission, inoculation with brain or other tissue via cuts or sores was also likely (Prusiner et al. 1982a). The central clinical feature is progressive cerebellar ataxia but, in contrast to CJD, dementia is often absent. The Fore called the disease the "laughing death" because in its later stages it caused fits of giggling. The introduction of Christianity and subsequent prohibition of such ancient customs resulted in virtual elimination of Kuru, albeit some cases are still reported (Collinge 2001).

Variant CJD (vCJD) was first reported in the United Kingdom in 1996 (Will et al. 1996). This human prion disease is caused by the same prion strain that causes BSE in cattle (Collinge et al. 1996; Bruce et al. 1997; Hill et al. 1997b; Asante et al. 2002). The disease develops as a result of dietary exposure to cattle BSE and therefore the possibility was raised that a major epidemic will occur in the United Kingdom and in other countries (Cousens et al. 1997; Ghani et al. 1998; Collinge 1999). In vCJD, main clinical features are behavioural and psychiatric disturbances, in some cases there are marked sensory phenomena (notably dysaesthesia or pain in the limbs or face) (Zeidler et al. 1997; Hill et al. 1999). In detail, patients suffer of depression, anxiety, withdrawal, delusions, emotional lability, aggression, insomnia, and auditory and visual hallucinations. In most of the patients, a progressive cerebellar syndrome develops, with gait and limb ataxia. Dementia usually develops later in the clinical course. By striking contrast with classic CJD, patients with variant disease are much younger (median age at death 29 years) and beside the central nervous system (CNS), PrP^{Sc} is also found in the spinal cord and in immune cells in peripheral tissues (Spencer et al. 2002). Illness duration is usually longer than in classic CJD, with a median of 14 months (Knight 2006). Death in an akinetic-mute state is a typical outcome. All clinical cases to date are homozygous for methionine at *PRNP* codon 129 (Hill et al. 1997a; Hill et al. 1999) and only recent findings revealed that susceptibility to vCJD infection is not confined to the methionine homozygous *PRNP* genotype (Llewelyn et al. 2004; Peden et al. 2004). To date, more than 215 cases of vCJD are reported. Another characteristic of vCJD is the fact that the infectious agent is abundant in the lymphoreticular system and many other organs beside the CNS (Wadsworth et al. 2001), potentially increasing the risk of horizontal spread. Therefore, vCJD transmission via blood transfusion (secondary vCJD) was likely and appeared (Llewelyn et al. 2004; Peden et al. 2004; Hewitt et al. 2006; Wroe et al. 2006). In February 2009, the "Health Protection Agency" (HPA, London, UK) confirmed the first case of vCJD transmission via blood products (Factor VIII) in a patient with haemophilia.

II.A.2.2 ANIMAL PRION DISEASE

The most familiar prion diseases in animals are scrapie in sheep and goat, bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease (CWD) in deer and elk. In addition, a few other animal prion diseases have been reported. This section summarizes the known animal prion diseases. **Table 2** gives an overview of the animal prion diseases.

Table 2. Animal prion diseases.

Disease	Species	Mechanism of Transmission / Infection
Scrapie	sheep and goat	vertical and horizontal transmission; sporadic
BSE (bovine spongiform encephalopathy)	cattle	ingestion of contaminated meat and bone meal; very rarely sporadic
CWD (chronic wasting disease)	deer and elk	vertical and horizontal transmission; oral transmission (e.g. faeces, salivary)
TME (transmissible mink encephalopathy)	mink	apparently ingestion of contaminated food (produced from sheep and cow)
FSE (feline spongiform encephalopathy)	cat and big cat	ingestion of BSE contaminated food
EUE (exotic ungulate encephalopathy)	exotic hoofed animals	ingestion of BSE contaminated food

Scrapie was first described in the 18th century. In 1936, scrapie was experimentally transmitted to goats, providing prove for the infectious nature of the agent according to the Koch's postulates (Cuillé J 1936). Affected sheep and goat show the typical symptoms for prion disease which are in general spongiosis, gliosis and neuronal loss. Scrapie has a wide spread distribution with a variable and generally imprecisely known prevalence in Europe, North America and Japan.

The first case of **bovine spongiform encephalopathy (BSE)** or "mad cow disease" was recognized in the United Kingdom (UK) in 1986 by British veterinarians (Wells et al. 1987).

Since the recognition of BSE more than 180,000 cattle have clinically developed the disease in the UK with its epidemic peak in 1992. Ninety-nine per cent of known BSE cases were in cattle born in the UK (Beghi et al. 2004). Beside the UK, BSE has been identified in cattle in most European countries and more recently in some countries outside of Europe. In Germany the first officially reported case of BSE was reported in November 2000. Affected cattle show diffuse cellular degeneration with spongiosis and astrocytic gliosis, typical for TSEs. Death occurs within six months from initial symptoms. It is suggested that the reason for the outbreak of the epidemic is the use of contaminated meat-and-bone-meal (MBM) as a high protein supplement feed for cattle. MBM contains, among other things, brain material from sheep and cattle. One possibility for the infection of cattle is feeding them with scrapie containing MBM (Prusiner et al. 1991; Wilesmith and Wells 1991). Another possibility is that BSE occurs sporadically in very low frequency in cattle and tissue from such a case was incorporated into MBM to seed the epidemic due to changes in the rendering processes introduced in the UK in the late 1970s (Wilesmith and Wells 1991; Fraser 2000). As a consequence of the epidemic, in 1988 feeding of MBM to cattle and sheep was banned in the UK and reinforced in 1996 with a total ban on feeding mammalian proteins to any farmed animals. This feed ban was introduced across the EU in 2001. Today only few BSE cases are reported but because of the long incubation time of the disease new epidemic peaks cannot be fully excluded in other countries.

Chronic wasting disease (CWD) is a prion disease affecting deer and elk in North America (Williams and Young 1980). In some areas of Colorado it is suggested that the disease is a common phenomenon in wild deer and elk (Spraker et al. 1997). The mechanism of transmission is not clearly revealed but lateral spread is unprecedented (Miller et al. 1998). From initial symptoms (weight loss, behavioural alterations, lowered head, flaccid hypotonic facial muscles), death occurs in deer after 7-8 months (Williams and Young 1980), elk may survive longer.

Transmissible mink encephalopathy (TME) was initially recognized in Wisconsin and Minnesota in 1947 and has sporadically appeared in countries including Canada, Finland, Russia and former East Germany (Marsh and Hadlow 1992). Transmission to mink is probably caused by feeding them with scrapie-contaminated meat (Marsh and Bessen 1993). Initial symptoms are behavioural changes including increased aggressiveness and hyperesthesia which progresses to ataxia, occasionally tremors or circling, and compulsive biting of self or objects (Rhein et al. 1974). Initial symptoms to death last from one week to several months (Marsh and Hadlow 1992).

Feline spongiform encephalopathy (FSE) has been described in captive cheetahs, pumas, an ocelot, and tigers from zoological collections in Great Britain (Kirkwood and Cunningham 1994; Williams ES 2001). Histopathology revealed spongiform degeneration in the neuropil of the brain and spinal cord with the most severe lesions localized to the medial geniculate nucleus of the thalamus and the basal nuclei (Ryder et al. 2001).

II.A.3 PRION GENE STRUCTURE

PRNP (the PrP gene) is located on the short arm of the human chromosome 20 and in the same region in the mouse chromosome 2 (Robakis et al. 1986b; Sparkes et al. 1986). *PRNP* is highly conserved in evolution and so far it was analyzed in more than 70 species (Schatzl et al. 1995; Wopfner et al. 1999; Strumbo et al. 2001; Suzuki et al. 2002; Rivera-Milla et al. 2003). Besides mammals, *prnp* is found in marsupials (Windl et al. 1995), birds (Harris et al. 1993), amphibians (Strumbo et al. 2001) and prion-related proteins have been identified in fish (Premzl et al. 2003; Rivera-Milla et al. 2003). The promoter region of *PRNP* has no TATA-Box but it contains multiple copies of GC-rich repeats, potentially for binding of transcription factors of the SP-family (e.g. SP-1) and AP-1 (McKnight and Tjian 1986). A GC-rich domain near the promoter region is a typical feature for so-called “house-keeping” genes (Basler et al. 1986). All known PrP genes consist of one (e.g. in hamster, humans, tamar wallaby) or two (e.g. in rat, mouse, bovine, sheep) short exons at the 5’-end and one bigger exon at the 3’-end, the latter coding for the entire open reading frame (ORF) of the prion protein (Hsiao et al. 1989; Gabriel et al. 1992; Schatzl et al. 1995). Therefore, since the entire protein-coding region is contained within one exon, the possibility of generating different proteins by alternative splicing of the mRNA is excluded (Basler et al. 1986; Westaway et al. 1994). PrP-mRNA is between 2.1 and 4 kb in length and codes for a protein of approximately 250 amino acids (aa), depending on the species.

II.A.4 FUNCTION OF THE PRION PROTEIN

Highest expression-levels of PrP are seen in the CNS, in particular in association with synaptic membranes (Kretzschmar et al. 1986). Moreover, PrP is also widely expressed in cells of the immune system (Dodelet and Cashman 1998). The fact that *PRNP*/*prnp* is found in a wide range of different species (s. II.A.3) indicates an obviously important biological function of the protein (Schatzl et al. 1995; Shmerling et al. 1998; Wopfner et al. 1999).

Nevertheless, though *PRNP* is highly conserved, the function of the cellular prion protein, PrP^c, is still enigmatic. Hope was pinned on the use of PrP^c knock-out mice to unveil the function of the protein, but no clear phenotype was observed (Bueler et al. 1992). Importantly, knock-out mice were completely resistant to prion disease following inoculation and did not replicate prions (Bueler et al. 1993). However, subtle phenotypical alterations of PrP^c knock-out mice were in abnormalities in synaptic physiology (Collinge et al. 1994) and in circadian rhythms and sleep (Tobler et al. 1996). Reduction of slow after-hyperpolarizations evoked by trains of action potentials was reported as a further physiological phenotype for PrP^c knock-out mice (Colling et al. 1996).

One possible role for PrP^c might be as a receptor for an extracellular ligand. PrP^c has been shown to bind to laminin (Graner et al. 2000) and the 37-kDa/67-kDa laminin receptor precursor (LRP/LR) (Hundt et al. 2001). LRP might act as the cellular receptor for the recycling/catabolism of endogenous PrP^c, and might also interact with PrP^c molecules present at the surface of other cells, thus contributing to cell communication and survival. Furthermore, more recent studies have reported evidence for direct interactions between LRP/LR and PrP^{Sc} in mediating binding of exogenous PrP^{Sc} to enterocytes (Morel et al. 2005) and baby hamster kidney (BHK) cells (Vana and Weiss 2006), implying that LRP/LR might have a role in the initial infection process. Other important intracellular PrP-interactors include heat shock protein 60 (Hsp60) (Edenhofer et al. 1996), cochaperone Hsp70/Hsp90, organizing protein/stress-induced protein 1 (hop/STI1) (Martins et al. 1997), neural cell adhesion molecule (NCAM) (Schmitt-Ulms et al. 2001), Bcl-2 (Kurschner and Morgan 1996), and sub2 (Spielhaupter and Schatzl 2001). Zinc, manganese and nickel cations also bind to PrP^c, but with lower affinities than copper (Pan et al. 1992; Brown et al. 2000a; Jackson et al. 2001). The binding is co-operative and occurs via histidines of the N-terminal region of PrP^c. The fact that mice devoid of PrP^c harbour 50% lower copper concentrations in synaptosomal fractions compared to their wild-type counterparts suggests that PrP^c could regulate the copper concentration in the synaptic region of the neuron (Brown et al. 1997). In addition, PrP^c might play a role in the re-uptake of copper into the presynaptic cells (Kretzschmar et al. 2000). Antioxidant activity has also been attributed to PrP^c, as it has been shown that PrP^c features copper/zinc-dependent superoxide dismutase 1 (SOD1) activity (Brown et al. 1999).

In summary, though an exact function for PrP^c remains elusive, diverse potential physiological functions of PrP^c have been suggested in health and disease: Alterations in circadian rhythm (Tobler et al. 1996), hippocampal neuronal function (Collinge et al. 1994), spatial learning (Criado et al. 2005), brain copper and cuproenzyme levels (Brown et al.

1997), oxidative tissue damage (Shyu et al. 2005), and phagocytosis and inflammatory response (de Almeida et al. 2005). Furthermore, function of PrP^{c} has been implicated in haematopoietic-stem-cell renewal (Zhang et al. 2006), neural-stem-cell differentiation (Steele et al. 2006), stress response (Nico et al. 2005), and there have also been claims and counter-claims regarding the modulation of cellular apoptosis by PrP^{c} (Roucou and LeBlanc 2005). Extending the array of putative functions, PrP^{c} has very recently been suggested to be a mediator of amyloid β (Aβ)-oligomer-induced synaptic dysfunction (Lauren et al. 2009).

II.A.5 STRUCTURAL AND BIOCHEMICAL CHARACTERISTICS OF PRP^{C} AND PRP^{SC}

A wide body of data now supports the idea that prions consist principally or entirely of an abnormal isoform of the host-encoded prion protein, designated PrP^{Sc}. PrP^{Sc} is derived from PrP^{c} by a posttranslational mechanism (Borchelt et al. 1990; Caughey and Raymond 1991). For many years, the idea that the aa sequence specifies one biologically active conformation of a protein had been accepted (Anfinsen 1973). However, when purified PrP^{Sc} and PrP^{c} were isolated, the secondary structure of the two PrP isoforms sharing the same aa sequence were compared by applying circular dichroism and infrared spectroscopy and were found to be different (Caughey et al. 1991; Safar et al. 1993). PrP^{c} contains about 42% of α-helices and 3% β-sheet, whereas PrP^{Sc} is composed of about 30% α-helices and 45% β-sheet (Gasset et al. 1993; Pan et al. 1993; Pergami et al. 1996). The C-terminal domain (aa 121-231) of mouse PrP was analyzed by nuclear magnetic resonance (NMR) (Riek et al. 1996) and consists of three α–helices (aa 144-154, 175-193 and 200-219) and two small antiparallel β–sheets (aa 128-131 and 161-164) (**Figure 1**). When the structure of mature "full-length" PrP (aa 23-231) was analyzed it was evident that the C-terminal part contained the complete globular part of the structure, whereas the N-terminus (aa 23-120) was more or less flexible and had no defined secondary structure (Riek et al. 1997). In mammals the N-terminus is known to contain a region normally formed by repetition of five consecutive, eight residues long peptides, rich in glycine, proline and histidine (octarepeats).

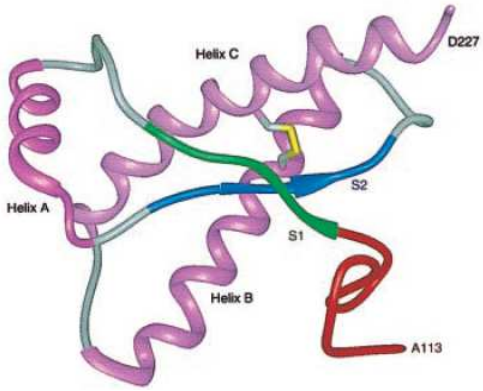


Figure 1. NMR structure of recombinant Syrian hamster PrP^{c} (aa 90-231). The color scheme is as follows: three α-helices in pink, two β-sheets in blue and green, disulfide bond in yellow, conserved hydrophobic region in red and loops in grey (Prusiner 1998).

When digested with proteinase K (PK), PrP^{c} is completely degraded. In contrast, PrP^{Sc} lacks only the N-terminal domain (up to aa 23-90) upon digestion with proteinase K, the rest of the molecule is resistant (Prusiner et al. 1984; Oesch et al. 1985) generating a well defined resistant core of an apparent mass of 27-30 kDa, termed PrP27-30 (Turk et al. 1988). A further difference between the two isoforms is the detergent insolubility of PrP^{Sc}, whereas PrP^{c} is detergent soluble. **Table 3** summarizes the different structural and biochemical properties of PrP^{c} and PrP^{Sc}.

Table 3. Structural and biochemical properties of PrP^{c} and PrP^{Sc}.

Characteristics	PrP^{c}	PrP^{Sc}
Infectivity	no	yes
Secondary structure	mainly α-helical	mainly β-helical
Half-life time	2-6 h	24 h or longer
PK-digestion	sensitive	partially resistant (PrP27-30)
Detergent solubility	soluble	insoluble

The insolubility of PrP^{Sc} excludes a structural analysis by X-ray cristallography or NMR spectroscopy, though electron diffraction data and two-dimensional crystals revealed that PrP^{Sc} might adopt left-handed β-helices that associate in turn to form trimers. The trimeric model accommodates the PrP sequence from residues 89–175 in a β-helical conformation with the C terminus (residues 176–227), retaining the disulfide-linked α-helical conformation observed in the normal cellular isoform (Wille and Prusiner 1999; Wille et al. 2002; Govaerts et al. 2004). Another different model for the organisation of individual PrP molecules within

amyloid fibrils was recently provided by analyzing recombinant human PrP90-231 with site-directed spin labelling (SDSL), coupled with EPR spectroscopy. Thereby, it seems that the core of the amyloid maps to the C-terminal part of the protein, and that residues within this region form single-molecule layers that stack on top of each other with parallel, in-register alignment of β–strands (**Figure 2**) (Cobb et al. 2007).

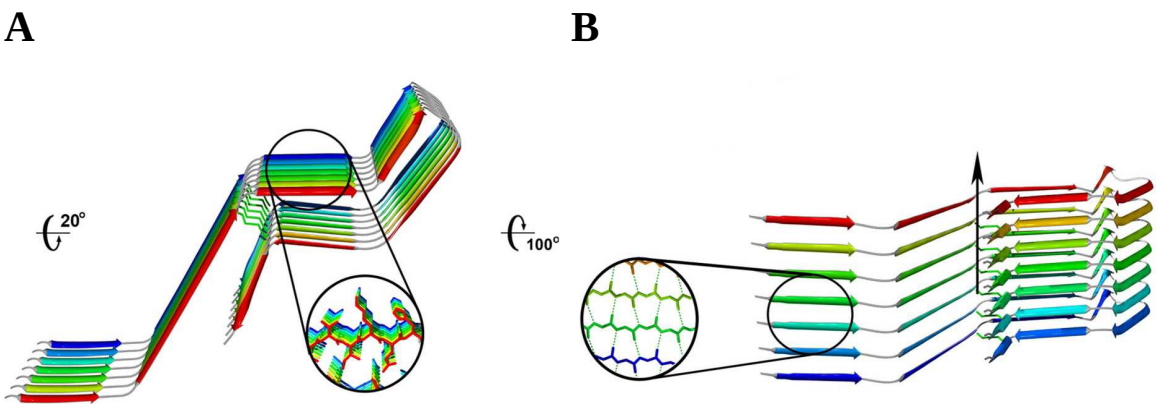


Figure 2. Model of PrP alignment in amyloid structures. (A) Nearly planar PrP monomers in the amyloid with in-register stacking of β-strands. **(B)** Intermolecular hydrogen bonding between the β-strands shown in rotated structure. The arrow indicates the long axis of the fibril (Cob et al. 2007).

II.A.6 CELL BIOLOGY AND LIFE-CYCLE OF PRP

PrP^c is expressed in neurons with its highest concentration (Kretzschmar et al. 1986). The protein follows an axonal pathway (Borchelt et al. 1994) and localizes mainly at synaptic (Fournier et al. 1995) or presynaptic ends (Herms et al. 1999). Although the highest levels are seen in the CNS, the protein is found in most tissues and is widely expressed in cells of the immune system (Dodelet and Cashman 1998).

Translation of PrP-mRNA results in a primary product of 254 aa in rodents and 253 aa in humans. The first 22 aa function as a signal peptide for translocation into the endoplasmatic reticulum (ER) and are co-translationally cleaved off (Oesch et al. 1985). In the C-terminal end of the polypeptide, another signal peptide is replaced in the ER by a glycosylphosphatidylinositol (GPI) anchor. For stabilizing the conformation of the protein a single disulphide bond is built between two cysteine (Cys) residues (Cys-178 and Cys-213 in mice and Cys-179 and Cys-214 in humans). A further post-translational modification results in the possible addition of 2 N-linked carbohydrate chains at asparagines (Asn)-180 and Asn-

196 in mice and Asn-181 and Asn-197 in humans (Haraguchi et al. 1989). Therefore, the prion protein can exist in non-, mono-, or di-glycosylated forms in the cell. After cleavage of the N- and C-terminal signal peptides, the mature prion protein comprises 208-209 aa (**Figure 3A**).

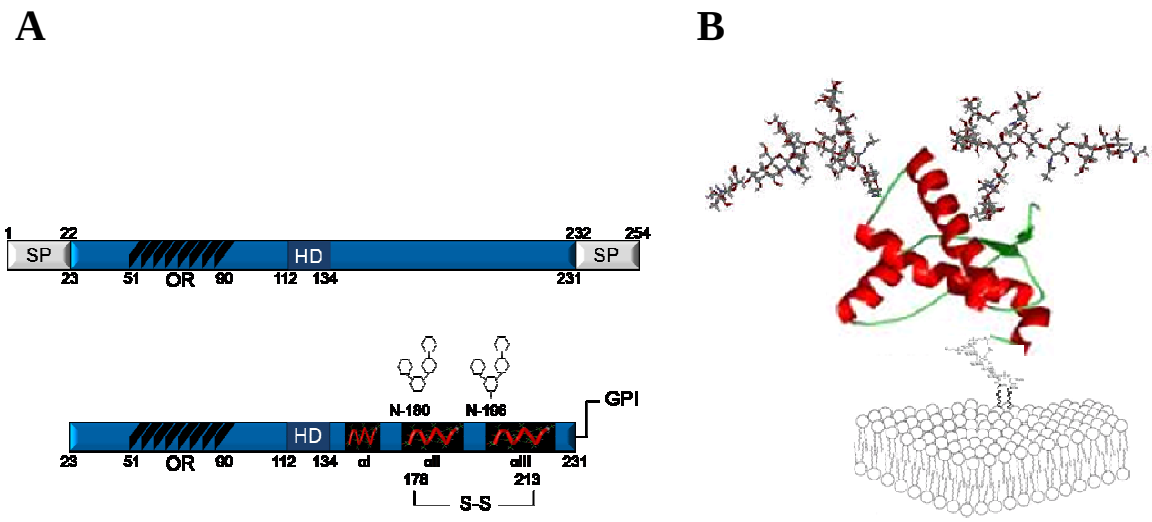


Figure 3. Primary structure of PrP^c, post-translational modifications and localization of PrP^c at the cell surface. (A) The N- and C-terminal signal peptides are cleaved from the translation product and a glycosylphosphatidylinositol (GPI) anchor is attached to the C-terminal end of the protein. The molecule can be N-glycosylated twice (CHO), and a disulfide bond (S-S) is built. The final product consists of 209 aa. **(B)** Mature PrP^c is attached to the extra-cellular surface of the plasma membrane via its GPI-anchor.

Upon post-translational modifications, mature PrP^c follows the secretory pathway through ER and Golgi compartment and is attached to the outer leaflet of the cellular plasma membrane by the GPI-anchor (Borchelt et al. 1990; Taraboulos et al. 1990; Caughey 1991; Taraboulos et al. 1992) (**Figure 3B**).

When PrP^c is not properly folded inside the ER, such "misfolded PrP" gets degraded by the ER-associated degradation (ERAD) (Ma and Lindquist 2001) (**Figure 4**). In contrast, mature PrP^c segregates to the cell surface where it is localized in "lipid rafts" (Madore et al. 1999) which are specialized and organized domains in the membrane rich in cholesterol and sphingolipids (Simons and Ikonen 1997). From here internalization seems to occur through clathrin-mediated endocytosis (Sunyach et al. 2003), though it might also be mediated by caveolin-related endocytosis and transport (Prado et al. 2004) or in rafts (Taraboulos et al. 1995). The localization of PrP^c at the cell surface is thought to be essential for subsequent conversion into PrP^{Sc} (Taraboulos et al. 1990; Caughey 1991) as it is suggested that

conversion of PrP^{c} to PrP^{Sc} takes place in caveolae-like domains (CLDs) or lipid rafts close to the plasma membrane along the endocytic pathway (Borchelt et al. 1992; Taraboulos et al. 1995; Vey et al. 1996; Kaneko et al. 1997). After internalization, PrP^{c} is transported to endosomes. Here, either it can be recycled (e.g. transported back to the cell surface) (Vey et al. 1996) or it might be degraded in acidic compartments (lysosomes). Targeting PrP^{c} to lysosomes for degradation might also be mediated by autophagosomes which are thought to fuse with endosomes or multi vesicular bodies (MVBs) (Liou et al. 1997; Berg et al. 1998). Autophagosomes are cytosolic, double membrane vesicles which are part of a process called autophagy, an ubiquitous cellular bulk degradation process which has been shown to mediate several important functions in health and disease [see II.B.3 and reviewed in (Mizushima et al. 2008)].

Concerning conversion of PrP^{c} to PrP^{Sc}, it has been shown that glycosaminoglycans (GAGs) such as heperan sulfates, present on the outer leaflet of the plasma membrane, may bind to PrP^{c}, associate with PrP^{Sc} *in vivo* and support PrP^{Sc} formation and internalization (Wong et al. 2001; Ben-Zaken et al. 2003; Hijazi et al. 2005; Horonchik et al. 2005). In addition, the low pH-value of late endosomes or lysosomes may enhance denaturation and refolding of the prion protein and therefore can also represent a compartment for prion replication (Taraboulos et al. 1992; Arnold et al. 1995; Marijanovic et al. 2009).

In regard to degradation and clearance of PrP^{Sc} the main compartment in the cell is thought to be the lysosome (Taraboulos et al. 1992; Ertmer et al. 2004) in which the amino terminus of nascent PrP^{Sc} is truncated by acidic proteases (Caughey and Raymond 1991; Taraboulos et al. 1992). Furthermore, it seems possible that leakage of PrP^{Sc}-containing late endosomes or lysosomes cause PrP^{Sc} to accumulate in the cytosol forming aggresomes (Kristiansen et al. 2005) which have been shown to impair proteasomal function (Kristiansen et al. 2007). Clearing aggresomes, and thereby PrP^{Sc}, might be accomplished by autophagy.

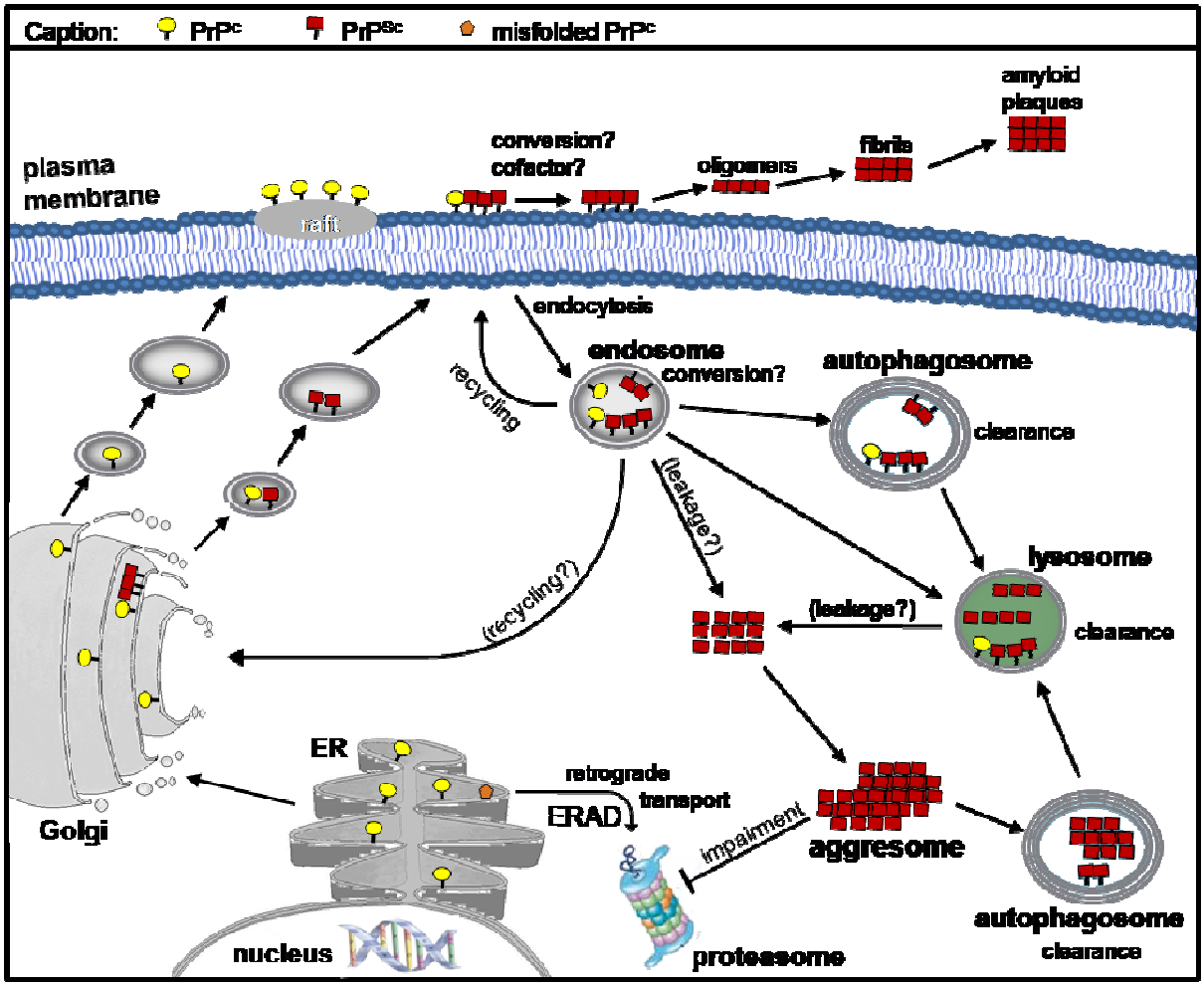


Figure 4. The life-cycle of PrP and potential sites for PrP^{Sc} biogenesis and degradation. PrP^{c} follows the secretory pathway through ER and Golgi, is attached to the outer leaflet of the plasma membrane and localizes in lipid rafts. Then PrP^{c} can be internalized and either transported to acidic compartments for degradation or recycled. Main sites for conversion of PrP^{c} to PrP^{Sc} are the cell surface and during internalization in endosomes or in lysosomes. Clearance of PrP^{Sc} takes place in lysosomes or might be accomplished by autophagy. Detailed description is found in the main text and is also reviewed in (Krammer et al. 2009).

II.A.7 MECHANISMS OF PRION CONVERSION

The precise mechanism by which infectious PrP^{Sc} proteins induce host PrP^{c} molecules to undergo conformational change and create new PrP^{Sc} molecules is currently undetermined. Most notably, it is unknown whether any molecules other than PrP^{Sc} and PrP^{c} are required to produce new prions *in vivo*. The structural and chemical dynamics of the conversion process have also remained obscure. For instance, it has not been fully determined whether the intramolecular disulfide bridge in PrP^{c} remains intact during conformational change into PrP^{Sc}, or whether this bond must break and reform to permit structural rearrangement of the protein.

In PrP knock-out mice it could be shown that the existence of PrP^{c} is essential for replication of the causative agent PrP^{Sc} (Bueler et al. 1992; Bueler et al. 1993). Neither it is possible to infect PrP knock-out mice nor can they transmit the disease (Bueler et al. 1993). The protein-only hypothesis propagates that PrP^{c} is converted into the infectious isoform PrP^{Sc} by direct interaction of PrP^{c} with PrP^{Sc}. Conversion of the two short β–sheet structures and the first α–helix into a large β–sheet formation is the major event which is responsible for conversion of PrP^{c} into the pathogenic isoform PrP^{Sc}. The remaining two α–helices and the disulfide bond need to be preserved for PrP^{Sc} to be infectious (Hornemann et al. 1997; Prusiner 1998; Wille et al. 2002).

A suggested, currently widely accepted model for PrP^{Sc} biogenesis is the nucleated polymerization model, in which PrP^{Sc} aggregates are generated by a crystallization-like process (Serio et al. 2000; Caughey 2003; Soto et al. 2006) (**Figure 5**). This so-called "nucleation" step is very slow, the energy barrier is supposed to be very high and the equilibrium lies for thermodynamic reasons on the side of PrP^{c} (Cohen et al. 1994). PrP^{Sc} is then able to generate nuclei (small oligomers) (Brown et al. 1990; Come et al. 1993; Jarrett and Lansbury 1993; Caughey et al. 1995). In the second, much faster "seeding" step, PrP^{Sc}-nuclei act as seeds to recruit native proteins into the growing aggregates forming protofibrils. These protofibril might break up forming new PrP^{Sc} seeds resulting in an exponential rise in amyloid formation. Currently it is unacquainted whether certain cofactors are needed for each or some steps in the PrP^{Sc} or amyloid formation, respectively.

To relate the above described model to the different forms of prion diseases, mutations in *PRNP* render the prion protein more aggregate-prone leading to genetic prion disease, whereas spontaneous formation of PrP^{Sc} from normal, physiological PrP^{c} occurs very rare leading to sporadic forms of prion disease. The exogenous addition of PrP^{Sc} seeds might induce conversion of the host-encoded PrP^{c} resulting in acquired prion disease.

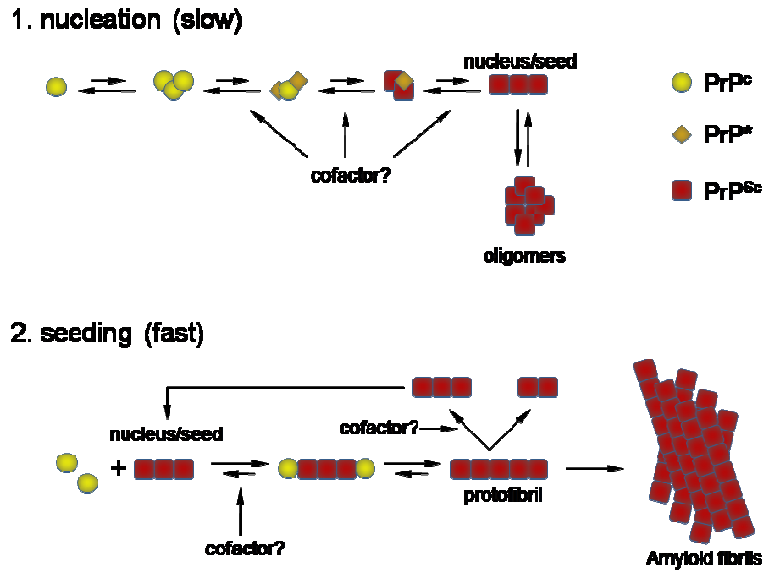


Figure 5. Prion replication according to the nucleated polymerization model. PrP^{Sc} and amyloid formation occurs in two steps. In the first step, the so-called "nucleation", PrP^{Sc}-nuclei are formed which act as seeds in the second step (seeding), recruiting new template (PrP^{c}) for subsequent conversion and amyloid formation. Detailed description is found in the main text and is also reviewed in (Gilch et al. 2008).

II.A.8 PRION STRAINS AND SPECIES BARRIER

The prion or protein-only hypothesis postulates that pathogenic and infectious prions, PrP^{Sc}, are only made of protein and are derived from a normal prion protein isoform PrP^{c} (Prusiner et al. 1982). One of the biggest challenges for this theory is to explain the existence of multiple strains of the infectious agent PrP^{Sc} in the absence of informational nucleic acid. Mammalian prion strains are classically defined in terms of their differing incubation times and the different profiles of pathological lesions that they produce in the CNS of recipient organisms. Differences in the glycosylation pattern in SDS-PAGE and different products upon PK-digestion (Collinge et al. 1996) as well as strain specific degrees of PK-resistance (Safar et al. 1998) support the idea of the existence of different PrP^{Sc} strains.

Prion strains cannot be encoded by differences in PrP primary structure, as they can be serially propagated in inbred mice with the same *prnp* genotype. Furthermore, strains can be re-isolated in mice after passage in intermediate species with different PrP primary structures (Bruce et al. 1994). Serial propagation of two distinct strains of transmissible mink encephalopathy prions (designated hyper [HY] and drowsy [DY]) in hamsters showed different physiochemical properties of the accumulated PrP^{Sc} in the brains of affected hamsters (Bessen and Marsh 1992, 1994). This supports the idea that strain specificity may be

encoded by PrP itself, since HY and DY show strain-specific migration patterns on polyacrylamide gels following limited proteolysis. Furthermore, different phenotypes of CJD have been identified and associated with different human PrP^{Sc} types (Collinge et al. 1996; Parchi et al. 1996). In summary, different prion strains seem to be originated in different three-dimensional conformations of PrP^{Sc} and the different properties of the prion strains are likely encoded in different folds of PrP^{Sc} (Telling et al. 1996; Scott et al. 1997).

Transmission of prion diseases between different mammalian species is restricted by a "species barrier" (Pattison 1965). When passaging prions from species A to species B, those that develop the disease have much longer and more variable incubation periods than those that are seen with transmission of prions within the same species. Additionally, an altered neuropathological distribution is observed (Schatzl 2003). However, when the disease has been established in the foreign host and is passaged through further individuals, an adaptation to the new host is observed with a corresponding decrease in incubation times of the disease (Kimberlin and Walker 1979). Transgenic mice expressing hamster PrP were, unlike wild-type mice, highly susceptible to infection with Sc237 hamster prions (Prusiner et al. 1990). This led to the assumption that the species barrier, and thereby transmission of the disease, is mainly encoded by the primary sequence (Scott et al. 1989; Scott et al. 1992; Scott et al. 1993; Telling et al. 1994; Schatzl et al. 1995; Priola et al. 2001). Another indication supporting the hypothesis that prion propagation proceeds most efficiently when the interacting PrP^{Sc} and PrP^{c} are of identical primary structure is that acquired or sporadic CJD mostly occurs in individuals homozygous at polymorphic residue 129 of PrP (Collinge et al. 1991; Palmer et al. 1991; Collinge 2001). Moreover, in heterologous systems, that means in systems in which two different strains of prion proteins are co-expressed, only the prion protein homologous to the pathogenic isoform is converted into the PK-resistant, pathogenic form. Studies with chimeric prion protein revealed that a defined sequence region (aa 112-187) is responsible for sequence specificity (Scott et al. 1992; Priola et al. 1995; Priola and Chesebro 1995).

An interesting connection between the two phenomenon species barrier and different prion strains was observed in human prion disease transmission studies. While classical CJD prions could efficiently be transmitted to transgenic mice expressing human PrP^{c}, they encounter a significant barrier for transmission to wild-type mice (Collinge et al. 1995b; Hill et al. 1997b). On the other hand, vCJD prions transmit readily to wild-type mice, whereas their transmission to transgenic mice expressing human PrP^{c} is relatively inefficient (Hill et al. 1997b). Therefore, as prions comprised of PrP with identical aa sequence but corresponding to

different TSE strains may be characterized by pronounced differences with respect to transmissibility, the term “transmission barrier” might be more appropriate than “species barrier” (Collinge 1999).

II.A.9 THERAPEUTIC APPROACHES

Long incubation times, a short symptomatic phase, the fatal progression of the disease, and the lack of a preclinical diagnostic are main features of prion diseases. These are reasons for the fact that finding prion therapeutics is a tough challenge.

In various model systems, numerous substances and compounds with anti-prion activity have been identified [reviewed in (Gilch et al. 2008)]. Prominent representatives among these are sulfated glycans like pentosan polysulfate or dextrane sulfate 500 known to interfere with binding of PrP to GAGs (Farquhar and Dickinson 1986; Ladogana et al. 1992), related compounds like Congo Red (Caughey and Race 1992; Ingrosso et al. 1995) which induces reduction of cell surface PrP^{c} (Caspi et al. 1998), or suramin that has been seen to cause aggregation of PrP^{c} and re-routing to lysosomes (Gilch et al. 2001). Other potent anti-prion compounds are polyene antibiotics like amphotericin B that bind to cholesterol (Mange et al. 2000) and tetracyclic compounds which may induce structural changes in proteins by direct interaction (Caughey et al. 1998). In addition, phosphorus containing dendrimers which are known to induce proteolytic digestion of PrP^{Sc} by causing disaggregation at acidic pH (Solassol et al. 2004) and lysosomotropic agents like quinacrine (Doh-Ura et al. 2000; Korth et al. 2001) have been elucidated as anti-prion compounds. On nucleic acid level, the use of small interfering RNAs (siRNA) targeting PrP mRNA have been observed to abrogate PrP^{Sc} accumulation in cell culture (Daude et al. 2003) and successful knock-down of PrP^{c} expression in livestock has been demonstrated (Golding et al. 2006). In line with this, prolonged incubation times were detected in prion-infected chimeric mice expressing short hairpin RNA (shRNA) in neural cells directed against PrP^{c} mRNA (Pfeifer et al. 2006). Another approach to reduce levels of the infectious agent is targeting the tertiary structure of PrP^{Sc}. Thereby, beta-sheet breaker peptides have been seen to reverse structural transitions occurring in misfolded PrP^{Sc} (Soto et al. 2000). Furthermore, direct binding of RNA aptamers to PrP^{c} has been seen to interfere with PrP^{Sc} biogenesis (Proske et al. 2002) which has also been demonstrated for peptide aptamers (Gilch et al. 2007).

Considering immunology, although there is no obvious humoral immune response stimulated in prion disease due to autotolerance (Porter et al. 1973), various elements of the immune system have been manipulated and these experiments have suggested potential strategies for

prion therapeutics. Early experiments assessed the efficacy of both immunostimulation and immunosupression, but since the experimental demonstration that antibodies can be raised to the prion protein, efforts have been focused on the therapeutic application of antibodies or of stimulating an antibody response. Initial attempts to produce anti-prion antibodies included immunization with purified prions (Bendheim et al. 1984) or scrapie-associated fibrils (SAFs) (Kascsak et al. 1987), with a greater response observed in prion knock-out mice (Prusiner et al. 1993) compared to wild-type mice. With appropriate adjuvants, it is also possible to use recombinant cellular prion protein as an immunogen in both knock-out (Krasemann et al. 1996; Williamson et al. 1996; Korth et al. 1997; Beringue et al. 2003; White et al. 2003) and wild-type mice (Souan et al. 2001; Sigurdsson et al. 2002; Gilch and Schatzl 2003; Schwarz et al. 2003). The *in vivo* immunization experiments (active and passive) suggest a possible use of anti-PrP antibodies as a therapy for prion diseases (Heppner et al. 2001; Sigurdsson et al. 2002; Gilch and Schatzl 2003; Gilch et al. 2003; White et al. 2003; Weissmann and Aguzzi 2005). Alternatively, antibodies raised against the putative cellular prion receptor LRP have been seen to inhibit prion conversion in cell culture models (Zuber et al. 2008).

In summary, as prion disease are fatal neurodegenerative diseases and up to now a striking therapeutic intervention targeting TSEs are lacking, the search for efficient therapy and/or prophylaxis in prion disease is challenging and essential. As TSEs manifest in the CNS, compounds destined for therapy in prion disease have to efficiently cross the blood-brain-barrier (BBB). So far almost all anti-prion compounds effective in *in vitro* assays suffer from rather ineffective crossing of the BBB *in vivo* and subsequently fail in therapy. This main problem might be suppressed by investigating proper delivery of drugs based on pharmacokinetics and through direct intracerebral application (Gilch and Schatzl 2003). Additionally, the intelligent combination of different compounds with additive or even synergistic effects may be useful in therapy. Last but not least, due to the short symptomatic phase of prion diseases, efforts have to be made to improve earlier diagnosis and preclinical diagnostics the basis to therapeutically intervene early enough before disease manifests.

II.B AUTOPHAGY

II.B.1 TYPES OF AUTOPHAGY

Autophagy is a cellular degradation process involved in intracellular turnover of proteins and cell organelles and is a highly conserved process that occurs in all species and cell types studied so far (Shintani and Klionsky 2004; Levine 2005). Currently, autophagy processes are sub-divided into three main pathways: chaperone-mediated autophagy (CMA), micro- and macroautophagy (**Figure 6**). Of these, CMA is as yet only described in mammals and degrades only soluble proteins in a selective manner whereas micro- and macroautophagy have the potential to degrade large structures through selective and non-selective processes and occur in a wide range of different species.

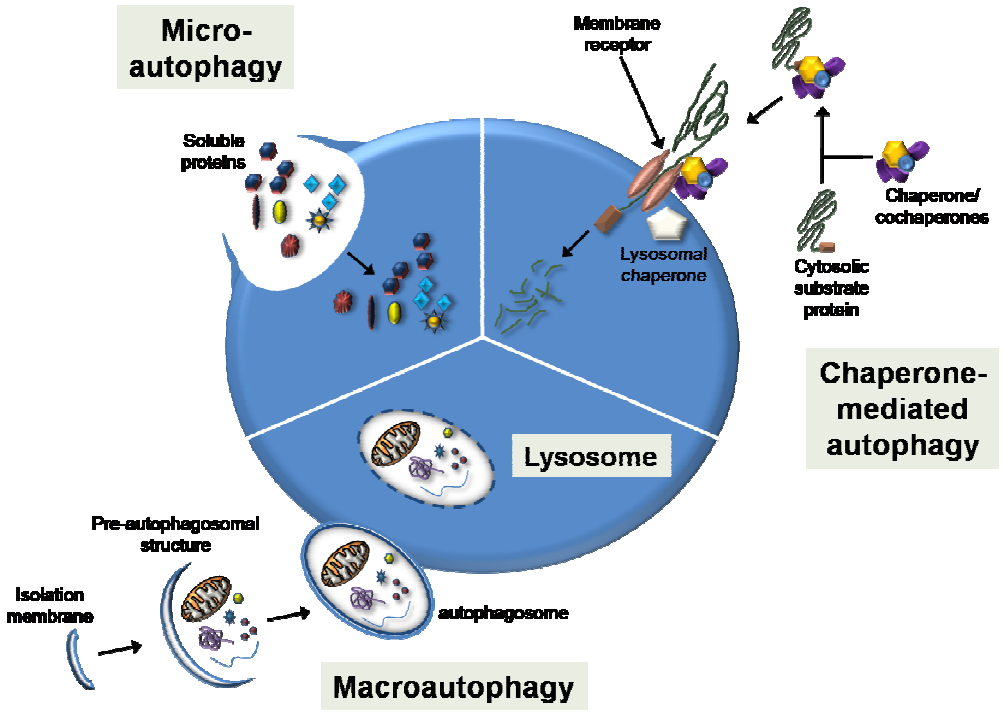


Figure 6. Different types of autophagy. Illustration of the three known main types of autophagy: chaperone-mediated autophagy (cytosolic chaperones target proteins destined for degradation to the lysosome), microautophagy (lysosome deforms and engulfs cytosol for degradation), and macroautophagy (autophagosomes containing substrate for degradation fuse with lysosomes). A detailed description of the different types is found in the main text.

Protein degradation by CMA involves a protein translocation system through a proteinaceous pore in the lysosomal membrane that is formed by lysosomal-associated membrane protein

2A (LAMP-2A) (Dice 2007). In the cytosol, a member of the family of chaperones, namely heat shock cognate 70 (Hsc70), recognizes substrate proteins by a specific motif (KFERQ) and unfolds the substrate protein with the help of Hsp70 co-chaperones (like Hsp40 and Hsp90) (Chiang and Dice 1988; Dice 1990). Hsc70-cargo complex then binds to LAMP-2A and Hsc73 in the lysosomal lumen which pull the target protein into the lysosome where it gets degraded (Agarraberes and Dice 2001; Bandyopadhyay et al. 2008). In contrast to CMA, microautophagy is a rather unspecific process, in which the lysosome deforms and engulfs whole areas of cytosol containing soluble proteins which are then degraded inside the lysosome (Marzella et al. 1981; Ahlberg and Glaumann 1985). Macroautophagy is a bulk degradation pathway and the only known intracellular mechanism capable of degrading large protein aggregates and/or damaged organelles. In the initial step of macroautophagy, an isolation membrane of uncertain origin forms [recent evidence suggest that it might be derived from the ER (Axe et al. 2008)]. This membrane elongates and forms a double membrane vesicle, called autophagosome, which engulfs substrate destined for degradation (Mizushima et al. 2002; Wang and Klionsky 2003). The autophagosome then fuses with lysosomes and the content of the autophagosome gets degraded (Dunn 1990b, a).

Besides the above described three main types of autophagy, other specialized forms exist, such as mitophagy (direct targeting of mitochondria to lysosomes) (Kanki and Klionsky 2008), pexophagy (selective degradation of peroxisomes) (Dunn et al. 2005), xenophagy (degradation of intracellular bacteria and viruses) (Levine 2005), piecemeal microautophagy of the nucleus (partial sequestration and degradation of the nucleus) (Roberts et al. 2003), reticulophagy (selective autophagy of the ER) (Bernales et al. 2007), and macrolipophagy (regulation of cellular lipid content by autophagy) (Singh et al. 2009).

This work will focus on macroautophagy and therefore macroautophagy will be referred to as autophagy from here on.

II.B.2 AUTOPHAGIC SIGNAL TRANSDUCTION

II.B.2.1 MTOR-DEPENDENT AND -INDEPENDENT INDUCTION OF AUTOPHAGY

In mammalian cells, autophagy induction can be regulated by mammalian target of rapamycin (mTOR). Under physiological nutrient-rich conditions, class I phosphoinositide 3-kinase (PI 3K) is stimulated in response to the binding of a ligand to a receptor (such as the insulin receptor) (**Figure 7**). Class I PI 3K then allows membrane binding and subsequent activation of Akt/protein kinase B (PKB) which inhibits tuberous sclerosis complex (TSC1/2) (Codogno

and Meijer 2004). Inhibition of TSC1/2 in turn stabilizes Rheb which stimulates mTOR, a negative regulator of autophagy (Noda and Ohsumi 1998). Hence, inhibition of mTOR (e.g. by rapamycin or lack of growth factors) results in activation of autophagy.

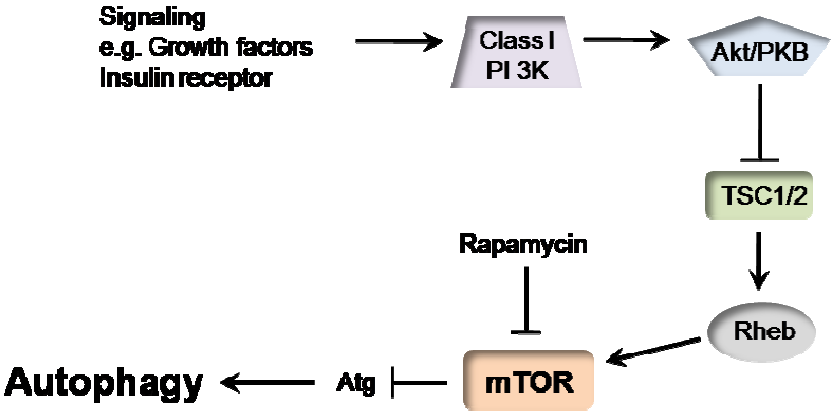


Figure 7. mTOR-dependent induction of autophagy. Activation of Akt/PKB by class I PI 3K inhibits TSC1/2 complex. Rheb is then able to activate mTOR, resulting in inhibition of autophagy. In contrast, inhibition of mTOR (e.g. by rapamycin) results in induction of autophagy. Detailed description is found in the main text.

Besides regulation of autophagy by mTOR, pathways regulating autophagy in an mTOR-independent manner have been described. Beclin 1 (the mammalian ortholog of yeast Atg6) was originally discovered as a Bcl-2-interacting protein (Liang et al. 1998) and was the first human protein shown to be indispensable for autophagy (Liang et al. 1999). Beclin 1 acts as an obligatory allosteric activator of class III phosphoinositide 3-kinase (PI 3K), which phosphorylates phosphatidylinositol to generate phosphatidylinositol 3-phosphate (PI3P) (Kametaka et al. 1998) (**Figure 8**). PI3P is involved in the nucleation of pre-autophagosomal structures. Interactors of beclin 1 include UVRAG, Ambra-1 and Bif-1 (also called endophilin B1) and knock-out studies performed on human and mouse cells indicate that UVRAG, Ambra-1 and Bif-1 are essential for the activation of autophagy (Liang et al. 2006; Fimia et al. 2007; Takahashi et al. 2007). A binding partner of beclin 1, which is inhibiting the autophagy-inducing potential, represents Bcl-2 (Pattingre et al. 2005). Proteins that contain BH3 domains or small molecules that mimic BH3 domains can bind to the BH3-binding groove of Bcl-2 and completely disrupt the interaction of Bcl-2 and beclin 1 (Maiuri et al. 2007b). Moreover, C-Jun-N'-terminal kinase 1 (JNK1)-mediated phosphorylation of Bcl-2 during starvation can disrupt its interaction with beclin 1 (Wei et al. 2008). Disrupting the interaction of beclin 1 and Bcl-2 has been seen to activate autophagy.

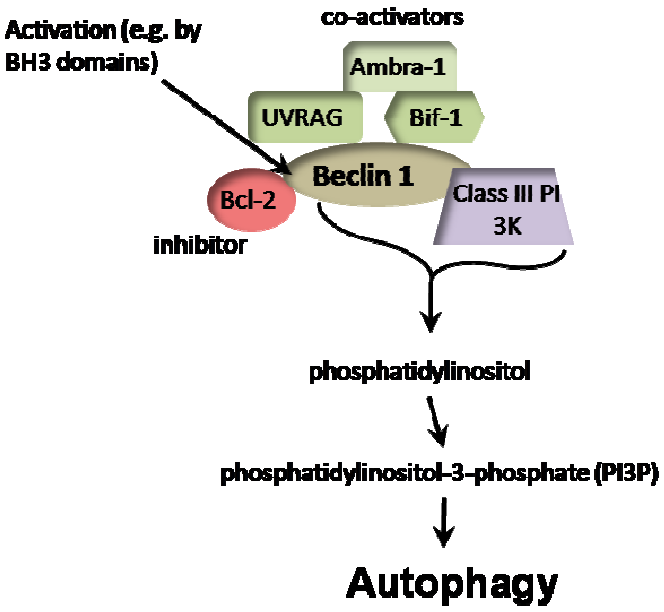


Figure 8. Beclin 1-dependent induction of autophagy. In concert with class III PI 3K, beclin 1 can induce autophagy by generating PI3P. Beclin 1 interacts with several co-activators including Ambra-1, UVRAG and Bif-1, as well as with the inhibitor Bcl-2. Activation of autophagy can be achieved by disrupting the binding of Bcl-2 with beclin 1 (e.g. with molecules containing BH3 domains). Detailed description is found in the main text.

Recently, a cyclical mTOR-independent pathway regulating autophagy has been described (Williams et al. 2008; Sarkar et al. 2009) (**Figure 9**). Thereby, cyclic AMP (cAMP) can activate phospholipase C-ε activity through Epac which results in elevated levels of inositol 1,4,5,-triphosphate (IP_3). IP_3 then increases intracytosolic Ca^{2+} levels, thereby regulating calpain activity, which in turn regulates the cleavage and activity of $G_{S\alpha}$ that subsequently generates cAMP, forming a loop.

Activation of this cyclic pathway inhibits autophagy and thus pharmacological interference (inhibition) results in induction of autophagy. In line with this, lithium has been seen to activate autophagy by reducing intracellular IP_3 levels (Sarkar et al. 2005).

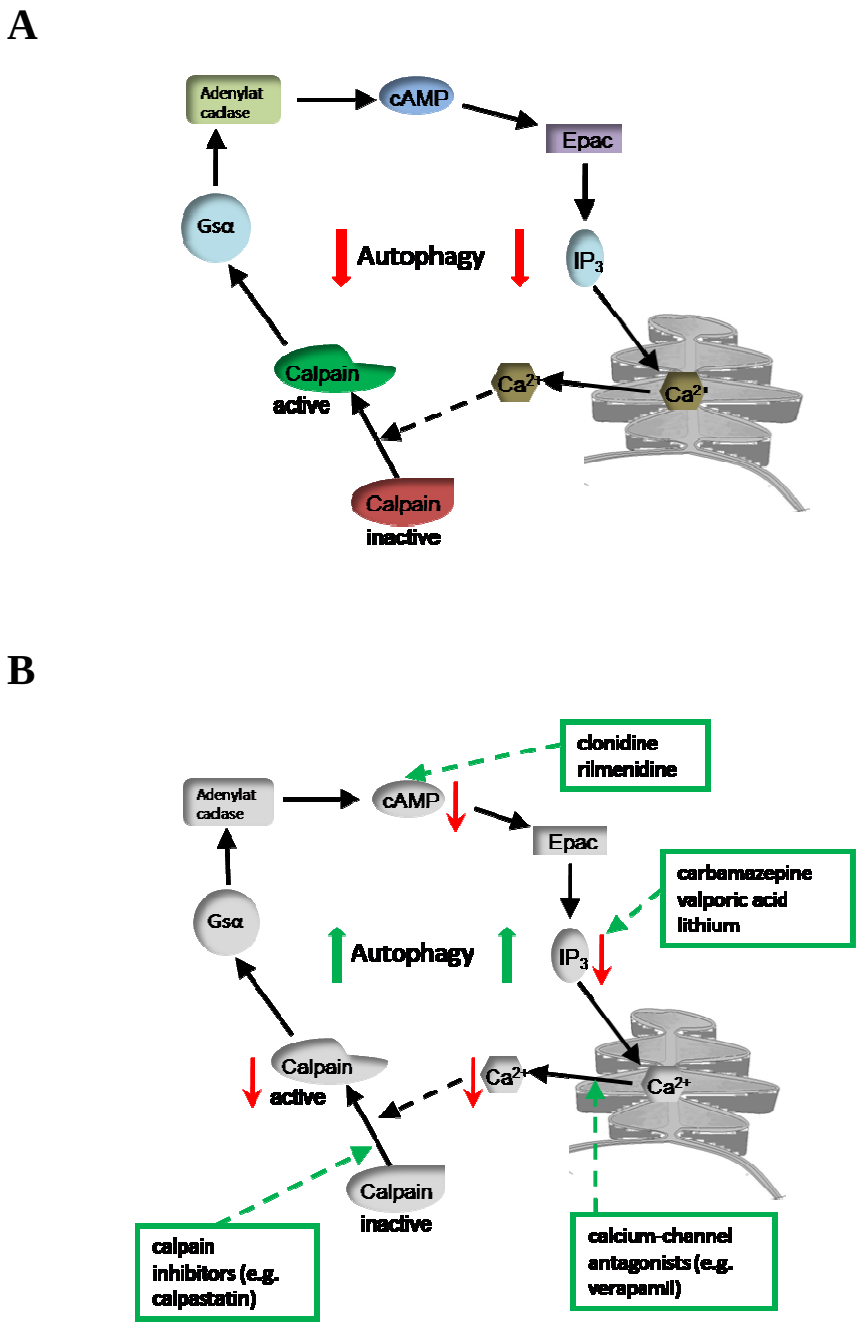


Figure 9. Cyclical mTOR-independent regulation of autophagy. (A) Increased levels of cAMP results in elevated levels of IP_3 which increase intracytosolic Ca^{2+} levels and activation of calpain. In turn, calpain is able to induce adenylate cyclase with results in increased levels of cAMP, forming a loop which negatively regulates autophagy. **(B)** The cyclical autophagy pathway possesses multiple drug targets for chemically inducing autophagy. Decreased levels/activity of calpain, cAMP, IP_3, and intracytosolic Ca^{2+} has been seen to disrupt the cyclical autophagy pathway, thereby inducing autophagy. Reviewed in (Sarkar et al. 2009).

II.B.2.2 AUTOPHAGOSOME FORMATION

The core and unique event upon induction of autophagy is autophagosome formation, in which two ubiquitin-like (Ubl) proteins participate. Microtubule-associated protein 1 light

chain 3 (LC3), the mammalian homolog of Atg8, is a Ubl that undergoes proteolytic processing by the Atg4 protease to reveal a glycine residue with the help of the E1-like enzyme Atg7, which is also activating the Ubl Atg12 (Kametaka et al. 1996; Kim et al. 1999; Tanida et al. 1999; Yuan et al. 1999) (**Figure 10**). Atg8 is then transferred to the E2-like enzymes Atg3 and is conjugated to phosphatidylethanolamine (PE) (Ichimura et al. 2000).

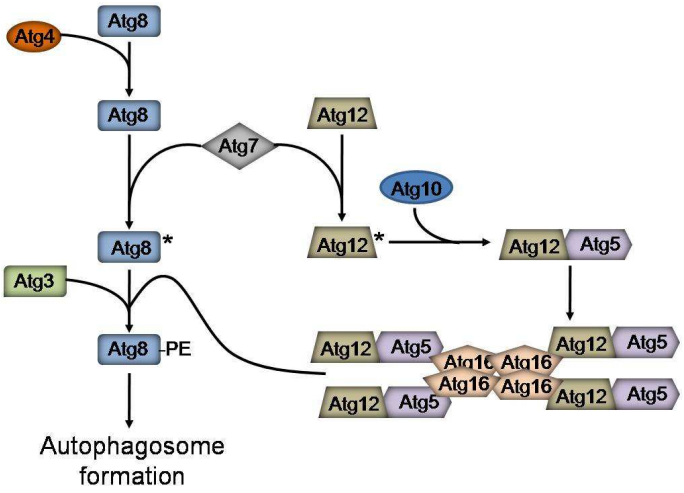


Figure 10. Autophagy-related (Atg) proteins induce autophagosome formation. Activated Atg12 covalently binds to Atg5 and forms in concert with Atg16 a tetrameric complex. With the help of the E2-like enzyme Atg3 and the tetrameric complex, Atg8 (LC3) is lipidated by addition of phosphatidylethanolamine (PE), leading to autophagosome formation. Detailed description is found in the main text.

In parallel, activated Atg12 is covalently attached to Atg5 by the action of another E2-like enzyme, Atg10 (Mizushima et al. 1998; Shintani et al. 1999). A third protein, Atg16, multimerizes and links the Atg12-Atg5 conjugate to form a tetrameric complex (Mizushima et al. 1998; Kuma et al. 2002). This tetrameric complex recognizes the isolation membrane (or its membrane source), leading finally to formation of autophagosomes, and Atg12 functionally recruits Atg3, such that Atg16 complex acts as a scaffold for LC3 lipidation (Fujita et al. 2008). Lipidated LC3 (also termed LC3-II) then localizes on the membrane of autophagosomes (Kabeya et al. 2000).

II.B.3 ROLE OF AUTOPHAGY IN HEALTH AND DISEASE

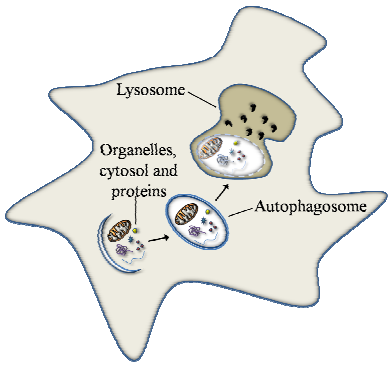
Underlining the significance of autophagy as a cellular bulk degradation process, a broad spectrum of different functions and modulatory roles of autophagy ranging from cellular physiology to disease have been elucidated in recent years. In several fields, both protective

and detrimental functions have been observed. **Figure 11** summarizes the most prominent roles of autophagy in health and disease. Thereof, examples are described in detail in the main text below.

Infection and immunity
Pro: Bacteria and viruses can be degraded in autophagosomes and antigens can be processed for MHC class II presentation [reviewed in (Virgin and Levine 2009)]
Autophagy might prevent development of inflammatory Bowel`s disease [reviewed in (Xavier et al. 2008)]
Con: Some microbes can utilize autophagosomes for nutrient supply and can use autophagosomes as a compartment for replication [reviewed in (Orvedahl and Levine 2009)]

Neurodegeneration
Pro: Autophagy can clear aggregate-prone proteins
Con: Accumulating autophagosomes can be compartments for processing of the amyloid precursor protein in Alzheimer`s disease [reviewed in (Mizushima et al. 2008)]

Ageing
Pro: Autophagy can remove damaged organelles and decelerate the rate at which tissues age [reviewed in (Vellai 2009)]



Heart disease
Pro: Autophagy can be helpful in ischemia
Con: Autophagy is noxious during reperfusion [reviewed in (Gustafsson and Gottlieb 2008)]

Liver disease
Pro: Autophagy can clear misfolded/toxic proteins in liver disease
Con: Highly increased autophagy may cause liver damage
[reviewed in (Yin et al. 2008)]

Cancer
Pro: Autophagy can act in tumor suppression by removing damaged organelles and possibly growth factors
Con: Autophagy can help cancer cells to survive in nutrient-limited environments [reviewed in (Mizushima et al. 2008)]

Cellular homeostasis and development
Pro: Autophagy supplies cells with metabolic substrates, helps bypass starvation periods and growth factor depletion and contributes to normal cellular development [reviewed in (Levine and Kroemer 2008)]

Cell survival
Pro: Autophagy can act as a pro-survival mechanism
Con: Autophagy is linked to cell death [reviewed in (Bossy et al. 2008)]

Figure 11. Physiological and pathophysiological roles of autophagy. Besides an important factor in regulating cellular physiological functions, such as homeostasis, metabolic stress and development, protective (pro) and deleterious (con) functions of autophagy are associated with several diseases in higher eukaryotes. Controversial roles of autophagy are observed in cancer and cell survival. Dysfunctional autophagy can lead to disease and in some cases, such as infection with microbes, heart- and liver disease, autophagy can promote both cyto-protective and cyto-toxic effects. Taken from (Mizushima et al. 2008).

II.B.3.1 AUTOPHAGY IN CELLULAR HOMEOSTASIS, STRESS INDUCED DIFFERENTIATION AND DEVELOPMENT

Autophagy is physiologically activated as an adaptive, pro-survival catabolic process in response to different forms of metabolic stress, including nutrient deprivation, growth factor depletion and hypoxia. Thereby, autophagy functions as a recycling process generating free amino and fatty acids. Yeast *Atg22* recently has been identified as a vacuolar permease required for the efflux of amino acids resulting from autophagic degradation, hinting at the molecular basis for the recycling function of autophagy (Mizushima and Klionsky 2007). Autophagy-produced free amino acids can in turn be incorporated in the tricarboxylic acid cycle (TCA) to maintain cellular ATP synthesis. Moreover, in the absence of growth factors, which are often required for nutrient uptake, autophagy can be activated and function as a survival mechanism (Lum et al. 2005). Stress induced differentiation and development is also linked to autophagy. In *Caenorhabditis elegans* (*C. elegans*), for example, under condition of limited food and autophagy induction, *C. elegans* reversibly arrest in an alternative third larval stage (Riddle et al. 1997) and essential roles of autophagy in early development in *Drosophila*, *C. elegans* and mice have been suggested (Juhasz et al. 2003; Melendez et al. 2003; Qu et al. 2003; Yue et al. 2003).

II.B.3.2 AUTOPHAGY IN CELL SURVIVAL AND CELL DEATH

The role of autophagy in cyto-protection and cyto-toxicity is a controversial matter. Evidences are observed for both autophagy as a modulator of cell death and autophagy as a pro-survival mechanism.

Arguing for a function of autophagy in cell death, autophagosomes accumulate during a non-apoptotic form of cell death known as type II cell death (Clarke 1990), which is also described as autophagic cell death (Gozuacik and Kimchi 2004; Yu et al. 2004). Non-apoptotic cell death is blocked by siRNA-mediated knock-down of essential autophagy genes (Yu et al. 2004) and in response to etoposide or staurosporine, $Bax^{-/-}$ and $Bak^{-/-}$ mouse embryonic fibroblasts (MEFs) undergo non-apoptotic cell death accompanied by large-scale autophagosome formation, a process which can be blocked by siRNA targeting essential members of the autophagic machinery (Shimizu et al. 2004). Several other studies have shown altered cell death following *ATG* knock-down *in vitro* (Maiuri et al. 2007a) and a few reports reveal similar findings *in vivo* (Berry and Baehrecke 2007; Koike et al. 2008; Samara et al. 2008), supporting a role of autophagy in cell death. Moreover, autophagy can lead to

cell death, possibly through activating apoptosis (Scott et al. 2007). Despite these findings, no model system exist so far which convincingly shows that physiological or even pathological cell loss in mammalian cells *in vivo* is executed by autophagy (Kroemer and Levine 2008).

In contrast to the above described hypothesis that autophagy is a modulator of cell death, several facts hint at a pro-survival function of autophagy and suggest that increased levels of autophagosomes found in dying or dead cells results from a failed survival attempt by the cell. Supporting a pro-survival role, autophagy controls the number and quality of organelles and manages elimination of superfluous, aged and damaged organelles, functions contributing to cell survival. Moreover, autophagy is essential for keeping cellular homeostasis in balance and acts as a pro-survival process in response to different forms of stress, including growth factor depletion, hypoxia, ER stress, microbial infection and disease characterized by the accumulation of protein aggregates (s. II.B.3.1). In addition, several autophagy genes have been identified in screens for mutants defective in survival on nitrogen-poor media (Tsukada and Ohsumi 1993; Thumm et al. 1994; Harding et al. 1995; Levine and Klionsky 2004), autophagy has been seen to play a crucial role in eliminating damaged proteins during oxidative stress (Xiong et al. 2007), and autophagy has been observed to protect cells from caspase-independent cell death (Colell et al. 2007). In animal model systems lacking essential autophagy genes, increased numbers of apoptotic cells are detected, arguing for a cyto-protective role of autophagy (Takacs-Vellai et al. 2005; Qu et al. 2007).

In conclusion, not least for the fact that autophagy and apoptosis are strongly linked and can regulate or modify the activity of each other in different ways (Pattingre et al. 2005; Yousefi et al. 2006; Maiuri et al. 2007b; Maiuri et al. 2007a), the exact role of autophagy in cell survival and cell death remains elusive.

II.B.3.3 AUTOPHAGY AND CANCER

Due to the uncertain role of autophagy in cell survival and cell death (s. II.B.3.2), it is obviously a conflicting subject whether autophagy has the potential to promote or prevent cancer.

The fact that autophagy is able to recycle nutrients to maintain cellular energy homeostasis and to degrade toxic cytoplasmic constituents helps to keep cells alive during nutrient and growth factor deprivation and other stressful conditions such as disturbed oxygen and redox homeostasis (s. II.B.3.1), supporting a pro-cancer role of autophagy. Recent studies have shown that inhibition of autophagy enhances cyto-toxicity of cancer chemotherapeutic compounds and, consequently, clinical trials are in progress to disrupt autophagic degradation

to maximize the effects of cancer cyto-toxic agents (Abedin et al. 2007; Amaravadi et al. 2007; Carew et al. 2007; Maiuri et al. 2009). In contrast to the above described role of autophagy in cancer development, overexpression of the autophagy protein beclin 1 can induce autophagy in mice and inhibit tumour growth, and heterozygous deletion of *beclin 1* makes mice more susceptible to development of spontaneous tumours (Qu et al. 2003; Yue et al. 2003; Liang et al. 2006). In line with this, *beclin 1* is monoallelically deleted in 40-75 % of cases of human breast, ovarian and prostate cancer (Kondo et al. 2005). In addition, several commonly mutated or epigenetically silenced tumour suppressor genes stimulate autophagy and a couple of generally stimulated oncogenes inhibit autophagy (Botti et al. 2006). A proposed explanation for the anti-cancer potential of autophagy is that autophagy genes (like *beclin 1* and *Atg5*) may prevent genotoxic stress and DNA damage (limiting chromosome instability), including oncogenic mutations, and hence may function as protectors of the genome (Mathew et al. 2007; Levine and Kroemer 2008). Last but not least, supporting a protective function of autophagy in cancer, various autophagy-inducing drugs are used for several years in cancer therapy, including rapamycin (among other mTOR inhibitors) and Glivec (Ertmer et al. 2007).

In summary, the exact role of autophagy in cancer remains to be determined, tightly connected to the controversial functions of autophagy in cell survival and cell death (s. II.B.3.2). A recent hypothesis suggests that autophagy, though a cyto-protective pro-survival event, may disrupt and prevent tumour growth by preventing necrotic cell death, a process that might aggravate local inflammation and thereby increasing tumour growth rate (Mathew et al. 2007; Mizushima et al. 2008).

II.B.3.4 AUTOPHAGY IN INFECTION AND IMMUNITY

Several years ago, the autophagy protein beclin 1 was identified as a protein with anti-viral function in mice (Liang et al. 1998) and later on autophagy genes were shown to be critical for successful innate immune response to fungal, bacterial and viral pathogens in plants (Liu et al. 2005), demonstrating a significant role of autophagy in anti-microbial defense. In *in vitro* infection studies it was shown that direct degradation of invading pathogens (like bacteria, protozoa, viruses and fungi) can be accomplished by autophagy [reviewed in (Orvedahl and Levine 2009)], a specific process now termed xenophagy [s. II.B.1 and (Levine 2005)]. Another important connection between autophagy and the immune system represent toll-like receptors (TLRs), a front line of defense against invading pathogens (Akira et al. 2006). TLRs are able to induce autophagy, the autophagy machinery can be used to deliver

viral genetic material to endosomal TLRs for efficient induction of type I interferon, and TLRs may interact in the recruitment of autophagy proteins to phagosomal membranes (Lee et al. 2007; Sanjuan et al. 2007; Xu et al. 2007; Delgado et al. 2009). In terms of antigen presentation by major histocompatibility complex (MHC), growing evidence suggests a role of autophagy in the MHC class II presentation of endogenously synthesized peptides (Lunemann and Munz 2009). Moreover, a recent study also suggests involvement of the autophagy protein *Atg5* in MHC class I antigen presentation (English et al. 2009). Several signals involved in the immune response positively regulate autophagy (Munz 2009; Orvedahl and Levine 2009), whereas on the other hand some cytokines have been seen to negatively regulate autophagy (Harris et al. 2007). Furthermore, paradoxically to the above described connection of TLRs and autophagy in anti-microbial defense, some studies have revealed that absent or hypomorphic expression of autophagy genes in certain cell types can result in enhanced production of type I interferon or pro-inflammatory molecules such as interleukin (IL)-1β and IL-18 among others (Jounai et al. 2007; Saitoh et al. 2008; Tal et al. 2009). In line with this, a polymorphism in the autophagy gene *Atg16L1* is associated with susceptibility to inflammatory bowel disease (such as Crohn`s disease) (Massey and Parkes 2007; Barrett et al. 2008), and mice with genetically engineered mutations in *Atg16L1* have identified critically important functions of this autophagy protein in innate immunity (Cadwell et al. 2008; Saitoh et al. 2008).

Besides the above described role of autophagy in anti-microbial defense (e.g. in pathogen recognition by TLRs or antigen presentation by MHC), some pathogens have evolved strategies to outsmart autophagy. It was reported that intracellular pathogens can modulate signaling pathways of autophagy or block membrane trafficking events required for autophagy-mediated pathogen delivery to lysosomes (Levine and Deretic 2007). Moreover, autophagy-dependent dynamic membrane rearrangements have been seen to be co-opted by pathogens for replicative advantages (Kirkegaard et al. 2004). Notably, microbial evasion of autophagy may be essential for microbial pathogenesis, a phenomenon that has been seen for example for encephalitis induced by herpes simplex virus (HSV), which specifically inhibits beclin 1 by a virus neurovirulence factor (Orvedahl et al. 2007).

II.B.3.5 AUTOPHAGY AND NEURODEGENERATION

In several neurodegenerative diseases, including Parkinson`s disease, polyglutamine expansion disease (such as Huntington`s disease), amyotrophic lateral sclerosis (ALS), and some forms of frontotemporal dementia (FTD) *in vitro* and *in vivo* studies revealed that up-

regulated autophagy acts as a cellular defense mechanism by degrading aggregate-prone proteins causing disease (Berger et al. 2006; Rubinsztein 2006; Fornai et al. 2008b; Winslow and Rubinsztein 2008). Emphasizing the physiological importance of the autophagic machinery in preventing neurodegeneration, autophagy deficiency (impaired autophagic flux) and consequential accumulation of autophagosomes has been seen to increase levels of toxic aggregate-prone proteins and has therefore been described as a secondary disease mechanism contributing to development of neurodegenerative diseases such as motor neuron disease (MND) and lysosomal storage disorders (Ravikumar et al. 2005; Settembre et al. 2008). In line with the above described beneficial role of autophagy in preventing neurodegeneration, mice with neural-tissue specific autophagy deficiency (knock-out of essential autophagy genes) develop symptoms of neurodegeneration without expression of any disease-associated mutant proteins (Hara et al. 2006; Komatsu et al. 2006). The exact mechanism by which autophagy is targeting aggregate-prone proteins for degradation is uncertain and the question whether autophagy specifically recognizes toxic proteins or is degrading such proteins in an undirected bulk degradation process remains to be resolved. One hypothesis suggests p62/sequestosome-1 (SQSTM1) as a possible adaptor molecule targeting aggregate-prone proteins to autophagosomes, as almost all aggregated proteins are decorated with ubiquitin and SQSTM1 has binding domains for both LC3 (located on autophagosomes) and ubiquitin (Bjorkoy et al. 2005; Pankiv et al. 2007). Another study recently suggested a posttranslational modification process which actively targets proteins for degradation by autophagy. Thereby, autophagic clearance of mutant huntingtin can be achieved by acetylation at lysine residue 444 (K444) (Jeong et al. 2009).

Concerning Alzheimer`s disease, the role of autophagy seems to be more conflicting. On the one hand, it has been seen that the autophagic machinery plays a neuroprotective role against Aβ-induced neurotoxicity (Hung et al. 2009), on the other hand studies have linked elevated levels of autophagy with increased amyloid precursor protein (APP) processing and Aβ peptide generation, and Aβ peptide production has been seen to take place inside autophagosomes (Yu et al. 2005). Therefore, it is hypothesized that impaired autophagic flux provides a novel and significant site for Aβ peptide production.

II.B.4 AUTOPHAGY AND PRIONS

Two decades ago, autophagic vacuoles were for the first time identified in neurons in experimental models of TSEs in mice and hamsters (Boellaard et al. 1989; Boellaard et al. 1991) and the appearance of multi-vesicular bodies and autophagic vacuoles was observed in

prion-infected cultured cells (Schatzl et al. 1997). More recently it has been reported that autophagic vacuoles are not only formed in neuronal perikarya but also in neurites and synapses in experimentally induced scrapie, CJD and GSS syndrome (Liberski et al. 2004) and autophagic vacuoles have been identified in many synapses in all categories of human transmissible encephalopathies (Sikorska et al. 2004). An interesting correlation between TSEs and autophagy was observed in studies on scrg1 (scrapie responsive gene 1), which has been seen to be up-regulated in brains of mice infected with scrapie and BSE, as well as in the brain of a patient with sporadic CJD (Dandoy-Dron et al. 1998; Dron et al. 1998; Dandoy-Dron et al. 2000). In the CNS of scrapie-infected mice, up-regulated scrg1 is associated with autophagic vacuoles, which are observed at the terminal stage of disease (Dron et al. 2005), and, as a consequence, it was suggested that scrg1 might be used as a marker to identify neuronal autophagy in TSEs (Dron et al. 2006). Besides autophagy in TSEs, the involvement of the physiological form of the prion protein (PrP^{c}) in the autophagy pathway has recently been described. Increased expression level of LC3-II was observed in Zürich I *Prnp*$^{-/-}$ hippocampal neuronal cells compared to wild-type control cells under serum deprivation and this up-regulation of LC3-II was retarded by reintroduction of PrP^{c} into *Prnp*$^{-/-}$ cells (Oh et al. 2008). As such retardation was not detectable for PrP^{c} lacking the octapeptide region, it was suggested that the octapeptide region of PrP^{c} may play a crucial role in control of autophagy in neuronal cells mediated by PrP^{c}. Concerning the role of autophagy in prion disease, it was proposed that autophagy may contribute in the formation of spongiform change, a pathological hallmark in TSE affected brains, and may be activated by apoptosis (Liberski et al. 2002; Liberski et al. 2004; Liberski et al. 2008). In contrast, support for a protective role of autophagy in prion disease was described in studies concerning a member of the galactin family of proteins, namely galactin-3. Reduced levels of the lysosomal activation marker LAMP-2 was observed in prion-infected galactin-3$^{-/-}$-mice and, interestingly, in brain tissue of prion-infected wild-type and galactin-3$^{-/-}$-mice, lower mRNA levels of autophagy markers *beclin-1* and *Atg5* have been detected compared to mock-infected control brains (Mok et al. 2007). Therefore, it was suggested that endo-/lysosomal dysfunction in combination with reduced autophagy may contribute to development of prion disease. Previously we could show that Glivec (STI571, imatinib), a drug used to treat chronic myelogenous leukemia, is activating lysosomal degradation of PrP^{Sc} (Ertmer et al. 2004) and is a potent inducer of autophagosome formation (Ertmer et al. 2007). In a scrapie-infected mouse model, Glivec treatment at an early phase of peripheral scrapie infection delayed both the neuroinvasion of pathological PrP^{Sc} and the onset of clinical disease (Yun et al. 2007). Yet, drug application,

either intraperitoneally or intracerebroventricular, provoked no PrP^{Sc} clearance effects in the CNS, probably due to problems crossing the BBB.

II.C OBJECTIVE

For several years it is known that autophagy plays a protective role in many neurodegenerative diseases, including Parkinson`s and Huntington`s disease, by clearing aggregate-prone proteins causing disease. Besides the finding that Glivec, a potent anti-prion compound, is able to induce autophagosome formation, not much is known about the role of autophagy in clearing PrP^{Sc}. Therefore, in this study it should be analyzed whether autophagy-inducing compounds are able to reduce pathological PrP^{Sc} (and levels of PrP^{c} respectively) in persistently prion-infected cells, and whether activated autophagy is directly mediating reduction of PrP^{Sc}. Moreover, *in vivo* studies with prion-infected mice should reveal whether autophagy-inducing compounds might have therapeutic potential in prion disease.

Besides the role of autophagy in persistent prion infection scenarios, the role of basal autophagy in primary prion infection is subject-matter of this study. Concerning primary prion infection, different roles of basal autophagy are possible. On the one hand autophagy might inhibit primary prion infection by clearing PrP^{Sc}, on the other hand it is conceivable that autophagy might support primary prion infection by breaking up larger PrP^{Sc}-aggregates forming smaller PrP^{Sc}-seeds, which are known to be more efficient templates for prion conversion. To elucidate the role of autophagy in primary prion infection, different PrP^{Sc}-susceptible and –unsusceptible mouse neuroblastoma (N2a) cells should be analyzed for autophagosome formation upon inoculation with prions. Furthermore, mouse embryonic fibroblasts (MEF), either wild-type (MEFwt) or autophagy-deficient ($MEFATG5^{-/-}$), should be prion-infected to shed light into the role of autophagy in primary prion infection.

III. MATERIALS AND METHODS

III.A MATERIALS

III.A.1 CHEMICALS

7-AminoActinomycin D (7-AAD)	Becton Dickinson, Heidelberg
Error! Hyperlink reference not valid.	Sigma-Aldrich, Munich
Ammonium peroxodisulfate (APS)	Roth, Karlsruhe
Ammonium chloride (NH_4Cl)	Roth, Karlsruhe
Bacillol Plus	Roth, Karlsruhe
Bacto Agar	Becton Dickinson, Heidelberg
Bacto Trypton	Becton Dickinson, Heidelberg
Bacto Yeast extract	Becton Dickinson, Heidelberg
Bromphenole Blue	Sigma-Aldrich, Munich
Calcium chloride ($CaCl_2$)	Roth, Karlsruh
Dimethhylsulfoxide (DMSO)	Sigma-Aldrich, Munich
Ethanol p. a. 99 %	Roth, Karlsruh
Ethylen diamine tetraacetate, sodium salt (EDTA)	Roth, Karlsruh
Gelatine 40 % solution	Sigma-Aldrich, Munich
Glucose	Roth, Karlsruh
Glycerol	Roth, Karlsruh
Glycine	Roth, Karlsruh
HCl 37 % (w/w)	Roth, Karlsruh
Hexadimethrine bromide (Polybrene)	Sigma-Aldrich, Munich
Hoechst 33342, trihydrochloride, trihydrate	Sigma-Aldrich, Munich
Isopropanol p. a.	Roth, Karlsruh
Lithium acetate ($LiCH_3COO$)	Sigma-Aldrich, Munich
Lithium bromide (LiBr)	Sigma-Aldrich, Munich
Lithium carbonate (Li_2CO_3)	Sigma-Aldrich, Munich
Lithium chloride (LiCl)	Sigma-Aldrich, Munich
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich, Munich
Magnesium sulfate ($MgSO_4$)	Sigma-Aldrich, Munich
Methanol p. a.	Roth, Karlsruh
N,N,N`,N`-Tetramethylenediamine (TEMED)	Sigma-Aldrich, Munich

N-Lauryl-sarcosine (Sarcosyl)	Sigma-Aldrich, Munich
Pefabloc SC (AEBSF)	Roche, Mannheim
Permafluor	Beckman Coulter, Marseille, France
Phosphate buffered saline (PBS)	Invitrogen, Karlsruhe
Protogel Ultra Pure 30 %; Acrylamide	National Diagnostics, Atlanta, GA, USA
Potassium chloride (KCl)	Sigma-Aldrich, Munich
Re-Blot Plus Strong Solution	Millipore, Temecula, CA, USA
Roti-Histofix	Roth, Karlsruh
Saponin	Sigma-Aldrich, Munich
Skim Milk Powder	Roth, Karlsruh
Sodium azide (NaN_3)	Sigma-Aldrich, Munich
Sodium chloride (NaCl)	Roth, Karlsruh
Sodium deoxycholate (DOC)	Roth, Karlsruh
Sodium Dodecylsulfate (SDS)	Roth, Karlsruh
Sodium hydroxide (NaOH)	Sigma-Aldrich, Munich
Trehalose	Sigma-Aldrich, Munich
Tris-hydroxy-methyl-aminomethan (Tris)	Roth, Karlsruh
Triton X-100	Roth, Karlsruh
Trypan Blue	Sigma-Aldrich, Munich
Tween-20	Roth, Karlsruh

III.A.2 BUFFERS AND SOLUTIONS

Buffers and solutions are listed in their applied concentration in the context of the described methods.

III.A.3 ANTIBIOTICS AND BACTERIA

Ampicillin (Amp)	Sigma-Aldrich, Munic
E. coli XL1 Blue strain	Stratagene, Amsterdam, Netherlands
Penicillin/Streptomycin (Pen/Strep)	Invitrogen, Karlsruhe

III.A.4 ENZYMES

Proteinase K (PK)	Roth, Karlsruh
RNase A (for Maxiprep)	Qiagen, Hilden
RNase-free DNase set	Qiagen, Hilden
Trypsin-EDTA	Invitrogen, Karlsruhe

III.A.5 INHIBITORS

Table 4. Inhibitors used in this work.

Substance	Description
3-methyladenine (3-MA)	Purchased from Sigma (Munich, Germany); stored at room-temperature; dissolved in cell culture medium to a final concentration of 10 mM;
Bafilomycin A1	Purchased from Sigma (Munich, Germany); 10 µM stock-solution in H_2O; stored at -20 °C;
Rapamycin	Purchased from Sigma (Munich, Germany); 1 mM stock-solution in DMSO; stored at -20 °C;
Glivec	Purchased from Novartis (Zürich, Switzerland); 10 mM stock-solution in DMSO; stored at -20 °C;

III.A.6 ANTIBODIES

Table 5. Antibodies used in this work (IF: immunofluorescence; WB: Western blot; FACS: fluorescence-activated cell sorting; HRP: horseradish peroxidase).

Primary antibody	Source and reference	Specificity	Application	Dilution
Anti-ß-actin	Mouse monoclonal (Sigma-Aldrich Chemie GmbH, Steinheim, Germany)	Actin	WB	1:10000
Anti-LC3	Mouse monoclonal (nanoTools Antikörper-technik GmbH & Co. KG, Teningen, Germany)	LC3 (microtubule-associated protein 1 light chain 3)	WB	1:1500
Anti-ATG5	Mouse monoclonal (nanoTools Antikörper-	ATG5 (Autophagy related gene 5),	WB	1:800

	technik GmbH & Co. KG, Teningen, Germany)	ATG5/ATG12 protein complex		
Anti-lamp-1	Host species: rat; reactive with mouse (Santa Cruz Biotechnolgy; Santa Cruz, CA, USA)	Lamp-1	IF	1:100
4H11	Mouse monoclonal (Ertmer et al. 2004)	PrP of various species (including mouse); no linear epitope defined	WB FACS	1:1000 1:10
3F4	Mouse monoclonal (Barry et al. 1986; Barry and Prusiner 1986; Kascsak et al. 1987; Rogers et al. 1991); Kindly provided by Michael Baier (Robert Koch Institut, Berlin, Germany)	Epitope in Syrian hamster and human PrP; aa 109-112 (MKHM)	WB	1:5000
Anti-GFP	Rabbit polyclonal (Santa Cruz Biotechnology; Santa Cruz, CA, USA)	Green fluorescent protein (GFP)	WB	1:5000
Secondary antibody	**Source**	**Specificity**	**Application**	**Dilution**
HRP-conj. anti-IgG	Sheep (GE Healthcare, Freiburg)	Mouse IgG	WB	1:7500
HRP-conj. anti-IgG	Sheep (GE Healthcare, Freiburg, Germany)	Rabbit IgG	WB	1:7500
Cy2-conj. anti-IgG	Donkey (Dianova, Hamburg, Germany)	Mouse IgG	FACS	1:100
Cy3-conj. anti-IgG	Donkey (Dianova, Hamburg, Germany)	Rat IgG	IF	1:200

III.A.7 OLIGODESOXYNUCLEOTIDES

Primers (PrP-forward primer and PrP-reverse primer, purchased from Roth, Karlsruhe) and probe (TaqMan probe, purchased from MWG, Ebersberg) for RT-PCR were VIC- and TAMRA-labeled. Oligodesoxynucleotides were purified by HPLC (high performance liquid chromatography) and sent in lyophilized form.

Table 6. Oligodesoxynucleotides used in this work.

Name	Sequence
PrP-forward primer	GCGGTACATGTTTTCACGGTAGTA
PrP-reverse primer	GAGCAGGCCCATGATCCA
TaqMan probe	CGGTCCTCCCAGTCGTTGCCAAAA

III.A.8 PLASMIDS

Table 7. Plasmids used in this work.

Plasmid	Description and source
pcDNA3.1-wtPrP	Mammalian expression vector (Invitrogen, Karlsruhe) in which the open reading frame of wild-type murine PrP (wtPrP) is inserted (under the control of the CMV promoter)
GFP-LC3	LC3 cDNA inserted into the *BglII* and *EcoRI* sites of pEGFP-C1, a GFP fusion protein expression vector (Kabeya et al. 2000)
GFP-pWPT	Lentiviral GFP fusion protein expression vector (purchased from addgene; www.addgene.org)
ATG5-pWPT	Lentiviral expression vector encoding for ATG5; Construct was obtained by replacing GFP of the plasmid GFP-pWPT with ATG5 (kindly provided by Prof. Hans-Uwe Simon; Department of Pharmacology, University of Bern, CH-3010 Bern, Switzerland)
pCMV-dR8.2 dvpr	Packaging vector for producing lentiviral particles (purchased from addgene; www.addgene.org)
pMD2.G	Envelope vector for producing lentiviral particles (purchased from addgene; www.addgene.org)

III.A.9 EUCARYOTIC CELL LINES

Table 8. Cell lines used in this work.

Cell line	Description	Source
N2a	Murine neuroblastoma cell line	ATCC CCL-131 (Butler et al. 1988)
ScN2a	N2a cells both persistently infected with RML prion strain and overexpressing 3F4-epitope-tagged murine PrP	(Scott et al. 1992)
ScL929	Murine fibroblast cell line persistently infected with 22L prion strain	ATCC CCL-1 (Vorberg et al. 2004)
MEFwt	Wild-type (wt) mouse embryonic fibroblast cell line	(Kuma et al. 2004)
MEFATG5$^{-/-}$	ATG5 knock-out (autophagy-deficient) mouse embryonic fibroblast cell line	(Kuma et al. 2004)
HEK293FT	Cell line based on the 293T cell line (a human embryonic kidney line transformed with adenovirus E1a and carrying a temperature sensitive T antigen co-selected with neomycin)	(Graham et al. 1977; Harrison et al. 1977; Naldini et al. 1996)
Kl17; Kl21	PrP^{Sc}-susceptible N2a clones (cells were obtained by curing prion-infected ScN2a cells using pentosan polysulphate; subsequently cells were sub-cloned and tested for PrP^{Sc}-susceptibility)	Kindly provided by Dr. Ina Vorberg (Institute of Virology, Technical University of Munich)
Kl35	PrP^{Sc}-unsusceptible N2a clone (cells were obtained by curing prion-infected ScN2a cells using pentosan polysulphate; subsequently cells were sub-cloned and tested for PrP^{Sc}-unsusceptibility)	Kindly provided by Dr. Ina Vorberg (Institute of Virology, Technical University of Munich)

III.A.10 CELL CULTURE MEDIA AND SUPPLEMENTS

OptiMEM with Glutamax	Invitrogen, Karlsruhe
D-MEM with Glutamax	Invitrogen, Karlsruhe
Fetal calf serum	Invitrogen, Karlsruhe
Penicillin/Streptomycin (Pen/Strep)	Invitrogen, Karlsruhe

III.A.11 KITS

ECL Plus	GE Healthcare, Freiburg
Fugene 6 Transfection Kit	Roche, Mannheim
Lipofectamine 2000	Invitrogen, Karlsruhe
NucleoSpin RNA II Kit	Macherey-Nagel, Düren
Plasmid Maxi Kit	Qiagen, Hilden
QuantiTect Probe TR-PCR Kit	Qiagen, Hilden

III.A.12 INSTRUMENTS AND ACCESSORIES

Autoclave V95	Systec, Wettenberg
Blotting transfer units:	
ECL Semi-Dry Transfer Unit	GE Healthcare, Freiburg
Semi-Dry PowerPac 200	Biorad Laboratories, Munich
Centrifuges:	
Beckman Avanti	Beckman Coulter, Krefeld
Beckman TL-100 ultracentrifuge	Beckman Coulter, Krefeld
Eppendorf 5417R	Eppendorf-Nethaler-Hinz, Köln
Sigma 4K15	Sigma-Aldrich, Munich
CO_2 Incubator	Heraeus, Hanau
Cover slips and slides	Marienfeld, Bad Mergentheim
Cryotubes	Corning Inc., Corning, NY, USA
Developer (Typon Medical)	Protec Medizintechnik GmbH, Oberstenfeld
Elektrophoresis power supplies:	
Consort E835	Consort, Turnhout, Belgium
EPS 3500 XL	Pharmacia Biotech, Upsala, Sweden
Eppendorf tubes (1.5 or 2 ml)	Eppendorf-Nethaler-Hinz, Köln
FACS-polystyrene tubes	Becton Dickinson, Heidelberg

Falcon tubes (15 or 50 ml)	Falcon, Le Pont de Claix, France
Filters:	
0.22 µm	Millipore, Temecula, CA, USA
0.45 µm	Millipore, Temecula, CA, USA
Flow cytometer EPICS® XL	Beckman Coulter, Krefeld
Fuchs-Rosenthal hemocytometer	Roth, Karlsruhe
Gloves:	
SemperCare powder free	Semperit Austria, Vienna, Austria
SemperCare nitrile	Semperit Austria, Vienna, Austria
Hybond-P PVDF membrane	GE Healthcare, Freiburg
Microscopes:	
Axiovert 40C	Carl Zeiss Jena, Göttingen
Olympus CKX41	Olympus Deutschland GmbH, Hamburg
LSM510 confocal laser microscope	Carl Zeiss Jena, Göttingen
Minitron incubator	Infors AG, Bottmingen
Perfect blue double gelsystem	Peqlab Biotechnologie GmbH, Erlangen
pH-Meter	WTW, Weilheim
Pipets (0.5-10µl, 10-100 µl, 100-1000µl)	Eppendorf-Nethaler-Hinz, Köln
Pipet tips:	
SafeSeal-Tips (20 µl, 100 µl, 1000µl)	Biozym, Hessisch Oldendorf
Stripette (5 ml, 10 ml, 25 ml)	Corning Inc., Corning, NY, USA
Pipetus	Hirschmann Laborgeräte, Eberstadt
ScanJet 4100C scanner	Hewlett-Packard GmbH, Böblingen
Spectrophotometer	GE Healthcare, Freiburg
TaqMan machine	Applied Biosystems, Darmstadt
Thermomixer compact	Eppendorf-Nethaler-Hinz, Köln
Tissue culture dishes and plates	Falcon, Le Pont de Claix, France
Vortex mixer	Neolab Migge, Heidelberg
Waterbath	GFL, Burgewede
Whatman paper	Schleicher & Schüll, Dassel
X-ray films Kodak Biomax MS	Sigma-Aldrich, Munich

III.A.13 COMPUTING PROGRAMS

Abobe Photoshop 7.0	Abobe Systems Inc., USA
Abobe Acrobat 7.0	Abobe Systems Inc., USA
ImageQuant TL	GE Healthcare, Freiburg
Prism 4 software	Graphpad Software, San Diego, CA, USA
Zeiss LSM Image Viewer	Carl Zeiss Jena GmbH, Göttingen

III.B METHODS

III.B.1 BIOLOGICAL SAFETY

Genetical engineering of organisms was accomplished under biosafety level 2 according to the Genoa genetic engineering law of 01.01.2004. Biologically contaminated materials and solutions were collected separately and inactivated according to the lab operating instructions. Inactivation of prions is subject to special regulations. Solutions were incubated with 1 M NaOH for 24 hours. Both liquid and solid prion waste were autoclaved for 60 min at 134 °C and 3 bar.

III.B.2 MOLECULAR BIOLOGICAL METHODS

III.B.2.1 QUANTIFICATION OF NUCLEIC ACID

Concentration of DNA or RNA was determined by measuring the absorbance at a wave length of 260 nm using a spectrophotometer (GE Healthcare). If diluted in $H_2O_{dest.}$, an absorbance of 1 for a double-stranded (ds) DNA preparation corresponds to 50 µg/ml DNA, for RNA an absorbance of 1 corresponds to 40 µg/ml. The amount of DNA or RNA in a sample was calculated according to the Beer-Lambert equation:

$A = \varepsilon \cdot c \cdot l$

A = absorbance (OD unit)

ε= molar extinction coefficient [$(\mu g/ml)^{-1}\ cm^{-1}$]

[$\varepsilon_{(dsDNA)} = 0.020\ (\mu g/ml)^{-1} cm^{-1}$]

c = concentration (µg/ml)

l = path length (cm)

As aromatic aa, especially tryptophan, have their absorption maximum at OD_{280}, the values at 280 nm were determined to estimate the protein content in the solution. The DNA or RNA sample was considered pure if the ratio of OD_{260}/OD_{280} was 1.8 or higher.

III.B.2.2 AMPLIFICATION OF PLASMID DNA

III.B.2.2.1 PREPARATION OF CHEMICALLY COMPETENT BACTERIA

Buffers and Solutions:

Luria-Bertani-(LB) medium	1 % (w/v) (10 g/l) Bacto Trypton
	0.5 % (w/v) (5 g/l) Bacto Yeast extract
	1 % (10 g/l) NaCl
	ad 1000 ml $H_2O_{dest.}$
$MgCl_2$ solution	100 mM in $H_2O_{dest.}$
$CaCl_2$ solution	100 mM in $H_2O_{dest.}$

A single colony of *E. coli* XL1 Blue strain (III.A.3) was transferred to 5 ml LB medium without antibiotics and incubated over night at 37 °C with constant shaking (180 rpm). The next day, 1 ml of this culture was transferred to 100 ml LB medium and further cultivated at 37 °C with constant shaking. After the solution had reached an optical density measured at a wavelength (λ) of 600 nm (OD_{600}) of 0.6-0.8, cells were cooled on ice for 10 min prior to sedimentation at 2600 x *g* (Sigma 4K15 centrifuge) at 4°C. The pellet was resuspended in 50 ml ice cold sterile $MgCl_2$ solution (100 mM) and incubated for 30 min on ice before the centrifugation step was repeated. The supernatant was discarded and bacteria were resuspended in ice cold sterile $CaCl_2$ solution, chilled on ice for 30 min and centrifuged as described above. The cell pellet was resuspended in 2 ml ice cold $CaCl_2$ solution and incubated for 24 h on ice. Finally, after addition of 2.5 ml sterile ice cold $CaCl_2$ solution and 0.5 ml glycerol, 100 µl aliquots in 1.5 ml Eppendorf tubes were prepared and immediately frozen at -80 °C.

III.B.2.2.2 HEAT SHOCK TRANSFORMATION OF *E. COLI* WITH PLASMID DNA

Buffers and Solutions:

SOC-Medium	2 % (w/v) (20 g/l) Bacto Trypton
	0.5 % (w/v) (5 g/l) Bacto Yeast extract
	10 mM NaCl
	2.5 mM KCl

	2 mM $MgCl_2$
	10 mM $MgSO_4$
	20 mM Glucose
	in $H_2O_{dest.}$
LB-agar	1 % (w/v) (10 g/l) Bacto Trypton
	0.5 % (w/v) (5 g/l) Bacto Yeast extract
	1 % (w/v) (10 g/l) NaCl
	1.5 % (w/v) (15 g/l) Bacto Agar
	ad 1000 ml $H_2O_{dest.}$

An aliquot (50 – 100 µl) of chemically competent *E. coli* (III.B.2.2.1) was thawed on ice and 1 µl plasmid DNA (III.A.8) was added and incubated on ice for 30 min. Bacterial cells were then warmed to 42 °C for 90 sec and immediately cooled on ice for 2 min. Addition of 400 µl pre-warmed SOC medium was followed by an incubation step at 37 °C with constant shaking (180 rpm) for 45 min. An aliquot of the bacteria was then transferred to 100 ml LB medium supplemented with 50 µg/ml ampicillin and grown over night at 37 °C in a shaking incubator (minitron incubator). Another aliquot of the bacteria was plated on an agar plate containing 50 µg/ml ampicillin and incubated over night at 37 °C and subsequently stored at 4 °C.

III.B.2.2.3 ISOLATION OF PLASMID DNA

To isolate high-copy plasmid DNA in a preparative scale the Qiagen Plasmid Maxi Kit was used according to the manufacturers` instructions. Eluted DNA was precipitated in 0.7 volumes of isopropanol and desalted with 70 % ethanol. Air-dried DNA pellets were redissolved in sterile 200 - 400 µl $H_2O_{dest.}$ and the concentration was determined (III.B.2.1).

III.B.2.3 ISOLATION OF TOTAL CELLULAR RNA

Cells were grown on 6 cm culture dishes. For isolation of total cellular RNA the NucleoSpin RNA II Kit was used according to the manufacturers` instructions. All extraction steps were carried out in an RNase free environment with sterile RNase-free pipet tips and test tubes and in a PrP-DNA free laminar flow in order to avoid any contaminations. As TaqMan real-time (RT)-PCR is very sensitive to small DNA amounts, additional DNase digestion was performed in order to eliminate any genomic DNA contaminations. Extracted RNA was eluted in 50 µl RNase-free water and the concentration was determined (III.B.2.1). For RT-PCR (III.B.2.4), volume of each sample was adjusted to 100 ng RNA/µl.

III.B.2.4 REAL-TIME (RT)-PCR

RT-PCR was carried out in a TaqMan machine (Applied Biosystems, Darmstadt) using isolated total cellular RNA (III.B.2.3) in order to analyze cellular PrP-mRNA copy numbers. For RT-PCR the QuantiTect Probe TR-PCR Kit (Qiagen, Hilden) was used. Primers and probe are described elsewhere (III.A.7).

RT-PCR reaction mix was as follows:

RT-Mastermix	16 µl
RNase free water	6.3 µl
PrP-forward primer	0.9 µl (10 pmol/µl)
PrP-reverse primer	0.9 µl (10 pmol/µl)
TaqMan probe	0.6 µl (10 pmol/µl)
RT-mix	0.3 µl
RNA	5 µl (100 ng/µl)
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Total volume	30 µl

For quantification of mRNA copy number, dilution series of pcDNA3.1-wtPrP (III.A.8) were prepared in sterile $H_2O_{dest.}$ starting from 10^6 copies and ending at 10^1 copies (power series) and served as a standard for RT-PCR. Copy numbers of mRNA were determined by comparison with the standard curve.

III.B.3 PROTEIN BIOCHEMICAL METHODS

III.B.3.1 PREPARATION OF POSTNUCLEAR LYSATES

Buffers and Solutions:

3 x SDS sample buffer	90 mM Tris-HCl (pH 6.8 ad)
	7 % (w/v) SDS
	30 % (v/v) glycerol
	20 % (v/v) ß-mercaptoethanol
	0.01 % (w/v) bromphenol blue
	in $H_2O_{dest.}$
Lysis buffer	100 mM NaCl
	10 mM Tris-HCl (pH 7.5 ad)
	10 mM EDTA

	0.5 % Triton X-100
	0.5 % DOC
	in $H_2O_{dest.}$
Pefabloc SC	1 % stock solution in $H_2O_{dest.}$
TNE	50 mM Tris-HCl (pH 7.5 ad)
	150 mM NaCl
	5 mM EDTA
	in $H_2O_{dest.}$

Adherent cells were washed once with PBS and were subsequently covered with 1 ml lysis buffer at room temperature (RT) for 10 min. Total lysates were transferred to 1.5 ml reaction tubes for separation of cell debris by centrifugation for 1 min at 20800 x *g* at RT (Eppendorf 5417R centrifuge). The supernatants were transferred into a fresh 1.5 ml reaction tube and 20 µl of 1 % Pefabloc stock solution were added before precipitation with 5-10 vol. methanol over night at -20 °C. Samples were then centrifuged at 2600 x *g* for 25 min (Sigma 4K15 centrifuge) at 4 °C and the resulting pellet was resuspended in adjusted volume of TNE and 3 x SDS sample buffer (1/2 x volume) was added. Samples were heated to 95 °C for 5 min and placed on ice for Western blot analysis.

III.B.3.2 PROTEINASE K (PK)-DIGESTION

Buffers and Solutions:

Pefabloc SC	1 % stock solution in $H_2O_{dest.}$
Proteinase K (PK)	1 mg/ml stock solution in H_2O_{dest}

Aliquots of post-nuclear lysates (III.B.3.1) were incubated for 30 min at 37 °C with 20 µg/ml PK. The digestion was stopped by addition of the protease inhibitor Pefabloc. Samples were precipitated with methanol (5 x volume) over night at -20 °C or subjected to detergent solubility assay (III.B.3.3). When precipitated with methanol, samples were centrifuged for 25 min at 2600 x *g* (Sigma 4K15 centrifuge) at 4 °C and pellets were subsequently resuspended in an adjusted volume of TNE. 3 x SDS sample buffer (1/2 x volume) was added and samples were heated to 95 °C for 5 min and placed on ice for Western blot analysis.

III.B.3.3 DETERGENT SOLUBILITY ASSAY

Buffers and Solutions:

N-Lauryl sarcosine	10 % stock solution in $H_2O_{dest.}$

Detergent solubility assay was performed for detection of PrP^{Sc} upon inoculation of cells with prion-infected brain homogenate (III.B.4.8). Aliquots of PK-digested, postnuclear lysates (III.B.3.2) were supplied with N-Lauryl sarcosine to 1 % and ultracentrifuged at 100000 x *g* for 1 h at 4 °C (Beckman TL-100 ultracentrifuge). Subsequently, insoluble fraction (pellet) was resuspended in an adjusted volume of TNE and 3 x SDS sample buffer (1/2 x volume) was added. Then, samples were heated to 95 °C for 5 min and placed on ice for Western blot analysis.

III.B.3.4 SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Buffers and Solutions:

4 x Lower gel solution	1.5 M Tris-HCl (pH 8.8 ad) 0.4 % (w/v) SDS in $H_2O_{dest.}$
4 x Upper gel solution	0.5 M Tris-HCl (pH 6.8 ad) 0.4 % (w/v) SDS in H_2Odest.
APS stock solution	10 % (w/v) in $H_2O_{dest.}$
3 x SDS sample buffer	90 mM Tris-HCl (pH 6.8 ad) 7 % (w/v) SDS 30 % (v/v) glycerol 20 % (v/v) ß-mercaptoethanol 0.01 % (w/v) bromphenol blue in $H_2O_{dest.}$
10 x SDS electrophoresis buffer	250 mM Tris 2.5 M Glycine 1 % (w/v) SDS in $H_2O_{dest.}$

Protocol for SDS-PAGE gels (for 4 gels):

Resolving gel (12.5 %)	Protogel acrylamide solution 51.8 ml
	Lower gel solution 30.8 ml
	$H_2O_{dest.}$ 40.6 ml
	TEMED 180 µl
	APS 384 µl
Stacking gel (5 %)	Protogel acrylamide solution 5.6 ml
	Upper gel solution 8.4 ml
	$H_2O_{dest.}$ 19.8 ml
	TEMED 60 µl
	APS 336 µl
High range protein molecular weight marker	GE Healthcare, Freiburg

Before SDS-PAGE analysis, samples were boiled at 95 °C for 5 min and subsequently placed short-term on ice for cooling. Proteins were separated on denaturing SDS gels containing 12.5 % acrylamide according to their molecular mass. The amount of postnuclear lysate (III.B.3.1) loaded on gel (volume ranging between 5 – 50 µl) depended on the concentration of the protein(s) which shall be detected. A molecular weight marker (3.5 µl) was additionally loaded on gel. Electrophoresis was accomplished under constant current (30 mA per gel) until the tracking dye reached the bottom of the resolving gel.

III.B.3.5 WESTERN BLOT (IMMUNOBLOT)

Buffers and Solutions:

Blotting buffer	20 % (v/v) Methanol
	3 g Tris
	14.4 g Glycine
	ad 1000 ml $H_2O_{dest.}$
10 x Tris-buffered saline Tween-20 (TBST)	100 mM Tris HCl (pH 8.0 ad)
	100 mM NaCl
	0.5 % (v/v) Tween-20
	in $H_2O_{dest.}$
Blocking buffer	5 % Skim milk powder
	in 1 x TBST

The Western blot technique allows the detection of proteins separated by SDS-PAGE (III.B.3.4) by specific antibodies (III.A.6). Transfer of proteins from the polyacrylamide gel onto a PVDF membrane was accomplished by an electrophoretic, semi-dry method. The membrane was activated by a short incubation in methanol and equilibrated in $H_2O_{dest.}$ before being placed on three layers of Whatman paper soaked in blotting buffer. The gel was then transferred on the membrane and covered with three additional Whatman papers soaked in blotting buffer. When the "Semi-Dry transfer unit" (GE Healthcare) was used, a current of approx. 101 mA per gel (calculated as follows: size of the gel in cm^2 x 0.8 mA) was applied for a period of 1 hour. When the "Semi-Dry PowerPac 200" (Biorad Laboratories) was used, proteins from the gel were transferred on the membrane by constant voltage (18 V) for 30 min (60 min for two gels). Afterwards, the membrane was incubated in blocking buffer at RT for 30 min. The primary antibody (III.A.6) was then diluted in 1 x TBST buffer, added to the membrane and incubated on a horizontal shaker at 4 °C over night. After seven rinsing steps with 1 x TBST for 5 min each, the secondary antibody (III.A.6) in 1 x TBST was added for 30 min at RT. After additional seven washing steps for 5 min each, the membrane was rinsed in $H_2O_{dest.}$. For detection of antibody-protein complexes, the membrane was covered for 3 min with an appropriate mixture of ECL solutions, prepared according to the manufacturers` instructions. Subsequently, the membrane was quickly dried between two Whatman papers before exposition to X-ray films for signal detection.

III.B.3.6 BAND INTENSITY QUANTIFICATION BY IMAGEQUANT TL

Intensities of signals detected by Western blot analysis were quantified using a ScanJet 4100C scanner (Hewlett-Packard) and the Image Quant TL software (GE Healthcare).

III.B.3.7 STATISTICAL ANALYSIS

Statistical analysis was performed with Prism Software (Graphpad Software, San Diego, CA, USA) using either the unpaired two-tailed *t*-test for pairwise comparisons or the One-way ANOVA analysis with Tukey post-test for multiple comparisons. Statistical significance was expressed as follows: ns: not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

III.B.4 CELL BIOLOGICAL METHODS

III.B.4.1 THAWING OF CELLS

Thawing of frozen cells (stored in cryovials in liquid nitrogen) was achieved in a water bath at 37 °C. Cells were then resuspended in 10 ml appropriate, pre-warmed culture medium and subsequently centrifuged for 10 min at 200 x *g* at 20 °C (Sigma 4K15 centrifuge) in order to remove toxic DMSO in the cryoconservation medium. The supernatant was discarded and the cell pellet was resuspended in 10 ml fresh culture medium and then plated on a 10 cm cell culture dish.

III.B.4.2 CULTIVATION AND PASSAGING OF CELLS

Cells were cultivated on appropriate culture dishes in a humidified 5 % CO_2 atmosphere at 37 °C. The cell lines N2a, ScN2a, Kl17, Kl21 and Kl35 were maintained in Opti-MEM (III.A.10), ScL929, MEFwt, MEFATG5$^{-/-}$ and HEK293FT cell lines were maintained in D-MEM (III.A.10). If not otherwise stated, cell culture medium was always supplemented with 10 % fetal calf serum (FCS) and 1 % Pen/Strep (III.A.3). Passaging of cells was carried out using 10 cm culture dishes. Confluent adherent cells were rinsed once with 5 ml PBS and then detached using 500 µl Trypsin/EDTA solution. Cells were subsequently suspended in 10 ml appropriate culture medium and a desired volume of this cell suspension was transferred to a new 10 cm culture dish supplemented with 10 ml appropriate cell culture medium.

III.B.4.3 CRYOCONSERVATION OF CELLS

For storage of cells, 75-80 % confluent adherent cells were detached from the culture dish (III.B.4.2) and suspended in 10 ml appropriate culture medium. Then cell suspension was centrifuged for 5 min at 200 x *g* (Sigma 4K15 centrifuge) at 4 °C and cell pellet was resuspended in 3-4 ml cryoconservation medium [culture medium (Opti-MEM/D-MEM supplemented with 10 % FCS and 1 % Pen/Strep) with addition of 10 % FCS and 10 % DMSO]. Aliquots in cryotubes (1 ml) were then immediately placed at -80 °C for at least 24 h before being transferred to liquid nitrogen for long-term storage.

III.B.4.4 DETERMINATION OF CELL NUMBERS

Cultivated cells were detached from cell culture dish (III.B.4.2) and resuspended in 10 ml new culture medium. Subsequently cells were transferred to a 15 ml Falcon tube. From this cell-

suspension a 1:10 dilution was prepared in appropriate culture medium and 20 µl were analyzed in a Fuchs-Rosenthal hemocytometer. The counting chamber consists of cubes with a defined total volume. The number of cells in 4 diagonally lying squares was counted. The following equation was used to determine the total number of cells *(N_{total})* per ml:

$$N_{total} = \frac{Cells_{counted}}{Squares_{counted}} \times F_{diltuion} \times F_{volume},$$

with $F_{dilution}$ representing the dilution factor (here 10) and F_{volume} representing the volume factor (here 5000).

III.B.4.5 TREATMENT OF CELLS WITH CHEMICAL COMPOUNDS

To guarantee equal distribution of chemical compounds in cell culture medium, compounds destined for treatment of cells (III.A.1 and III.A.5) were foremost diluted to desired concentration in the appropriate cell culture medium. Then cells were directly plated and cultivated in such modified medium.

If not otherwise stated, the following concentrations of chemical compounds were applied for treatment of cells:

3-MA	10 mM
Bafilomycin A1	200 nM
Glivec	10 µM
$LiCH_3COO$	10 mM
LiBr	10 mM
Li_2CO_3	10 mM
LiCl	10 mM
NaCl	10 mM
Rapamycin	200 nM
Trehalose	100 mM

III.B.4.6 TRANSIENT TRANSFECTION OF CELLS

For ScN2a cells, transient transfections of recombinant plasmids were carried out using the FuGENE transfection Kit (Roche), for HEK293FT cells, the Lipofectamine 2000 transfection

reagent (Invitrogen) was used. Transient transfections were carried out according to the manufacturers` directions.

III.B.4.7 TRANSDUCTION OF CELLS

III.B.4.7.1 PRODUCTION OF RECOMBINANT LENTIVIRUS

In order to produce recombinant lentiviral particles, 2 x 10^6 HEK293FT cells (III.A.9) were plated in a 6 cm culture dish. The next day, cells were transiently co-transfected (III.B.4.6) with 3 µg each lentiviral envelope vector, lentiviral packaging vector and either the lentiviral plasmid ATG5-pWPT or GFP-pWPT (III.A.8). Further 48 h later, medium of HEK293FT cells containing lentiviral particles was filtered (0.45 µm) to remove cellular debris and either stored at -80 °C or immediately used for transduction of cells (III.B.4.7.2).

III.B.4.7.2 TRANSDUCTION OF CELLS WITH RECOMBINANT LENTIVIRUS

For lentiviral transduction of MEFATG5$^{-/-}$ cells (III.A.9), 1 x 10^5 cells were plated in a 6-well culture dish. The next day, cells were incubated with 4 g/ml Polybrene (Sigma, Munich) for 2 h before exposure to 2 ml medium containing lentiviral particles over night (III.B.4.7.1). Then, normal cell culture medium was added. When cells reached confluency, an aliquot of cells was transferred to a 10 cm dish for analyzing expression of the recombinant protein by Western blot (III.B.3.5) and another aliquot of the cells was transferred to a further 10 cm culture dish for cryoconservation (III.B.4.3).

III.B.4.8 PRION INFECTION OF CELL LINES

III.B.4.8.1 PREPARATION OF BRAIN HOMOGENATE

The mouse-adapted scrapie strain 22L was a kind gift of Prof. Dr. M. Groschup [Friedrich-Loeffler-Institut, Bundesforschungsinstitut für Tiergesundheit (FLI), Insel Riems] and was propagated in CD1 or C57B1/6 mice. Brains of un-infected animals (mock) were used as a negative control. To prepare brain-homogenates, brains were homogenized in PBS. Homogenate [10 % (wt/vol)] was stored at -80 °C. Brains were stored at -80 °C.

III.B.4.8.2 INOCULATION OF CELLS WITH BRAIN HOMOGENATE

For prion infection of cells, 1 x 10^5 cells (III.B.4.4) were cultivated in a 12-well culture dish. 24 h later, medium was removed and cells were incubated with brain homogenate (1 % dilution) of 22L prion-infected mice or mock-brain (un-infected). Therefore, culture medium was removed and cells were overlaid with 450 µl appropriate serum free culture medium and

50 µl of 10 % brain homogenate. After 5 h incubation, fresh culture medium was added and further 24 h later, medium was removed and cells were washed once with PBS before fresh culture medium was added to the cells. Confluent cells were then detached from the 12-well culture dish (III.B.4.2) and all cells were transferred to a 6-well plate. This procedure was continued as far as cells reached 10 cm dishes. From this scale forth, confluent cells were transferred in a 1:16 dilution to a new 10 cm culture dish.

For detection of PrP^{Sc} upon prion infection, cells were lysed and an aliquot of the cell lysate was subjected to PK-digestion (III.B.3.2) followed by detergent solubility assay (III.B.3.3). Then samples were analyzed by Western blot analysis (III.B.3.5) using anti-PrP monoclonal antibody (mAb) 4H11, or 3F4 mAb respectively (III.A.6), for detection of PrP^{Sc}.

III.B.4.9 TRYPAN BLUE ASSAY

To assess viability of cells upon 10 mM lithium treatment, trypan blue assay was performed. Thereby, treatment of cells with trypan blue allows detection of the number of viable cells by staining unviable cells. Cells were cultured for 48, 72 or 96 h in medium containing 10 mM lithium or no lithium treatment was performed. Then cells were mixed in a 1:2 ratio with trypan blue and subsequently stained and unstained cells were counted. Percentage of viable cells was calculated by determining the ratio of unstained (viable) cells to stained (not viable) plus unstained cells. In each experiment at least 600 cells were counted.

III.B.4.10 CONFOCAL LASER MICROSCOPY ANALYSIS (IMMUNOFLUORESCENCE)

Buffers and Solutions:

Blocking solution	0.2 % Gelatine in PBS
Hoechst staining solution	2 µg/ml Hoechst in PBS
Permeabilizing solution	0.3 % Triton X-100 in PBS
Quenching solution	50 mM NH_4Cl
	20 mM Glycine in PBS
Roti-Histofix	10 % solution

Immunofluorescence is a technique allowing the localization of specific proteins in cells by specifically binding antibodies. There are two major types of immunofluorescence staining methods: 1) direct immunofluorescence staining in which the primary antibody is labeled with fluorescence dye, and 2) indirect immunofluorescence staining in which a secondary antibody is labeled with a fluorescence dye and is used to detect a primary antibody. Besides staining

with antibodies, proteins exhibiting auto-fluorescence can be detected by immunofluorescence per se without any antibody stainig [e.g. GFP (green fluorescent protein)].

Cells were plated on a 6 cm dish supplemented with sterile glass cover slips. After 24 h, cells were transfected (III.B.4.6) with GFP-LC3 (III.A.8) and either left untreated or treated with specific substances (III.B.4.5) for 24 h. Then, cover slips were removed and transferred into a 12-well plate, washed three times with PBS, and fixed in 500 µl Roti-Histofix (10 %) for 30 min at RT. Subsequently, cells were quenched using NH_4Cl/glycine solution, permeabilized with 0.3 % Triton X-100 and blocked by incubation with blocking solution. Each step was performed for 10 min at RT and terminated by three times rinsing steps with PBS. Then, cells were incubated with the primary antibody anti-lamp-1 (III.A.6) in a humid chamber for 45 min at RT, washed three times with PBS and incubated with the secondary antibody Cy3 (III.A.6) in a dark, humid chamber for 30 min at RT. Again, cells were rinsed three times with PBS and nuclei were stained by incubation with Hoechst DNA staining solution for 10 min at RT in the dark. Finally, cover slips were overlaid with mounting medium (Permafluore) on the slides and dried over night at 4 °C. Analysis was carried out using a LSM 510 confocal laser microscope (Zeiss).

For determination of the ratio of GFP-LC3 expressing cells which show induction of autophagosome formation upon lithium treatment (Figures 13 and 22), a total number of 300 GFP-LC3-positive cells were counted in each experiment. Mean number of lithium-treated cells showing autophagosome formation was expressed as percentage of untreated cells showing autophagosome formation.

III.B.4.11 FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

Buffers and Solutions:

EDTA solution	1 mM in PBS
FACS buffer	2.5 % fetal calf serum (FCS)
	0.05 % NaN_3 in PBS
Quenching solution	50 mM NH_4Cl
	20 mM Glycine in PBS
Roti-Histofix	10 % solution
Saponin solution	5 % stock solution
Saponin buffer	0.1 % saponin in FACS buffer

Flow cytometry allows the analysis of single cells in suspension with respect to their size, granularity or fluorescence. In order to detect total PrP^c expression, cells were plated in a 6 cm dish over specific periods and detached using EDTA solution. Approximately 4 x 10^5 cells were transferred to FACS tube and sedimented at 300 x *g* (Sigma 4K15 centrifuge) at 4 °C for 2 min. After sequential treatment for 10 min each at RT with Roti-Histofix and quenching solution, cells were resuspended in saponin buffer. Subsequently, cells were incubated with the primary antibody 4H11 (III.A.6) for 45 min on ice (diluted in saponin buffer). Cells were washed three times with saponin buffer and the secondary antibody Cy2 (III.A.6) was added for 45 min in the dark (diluted in saponin buffer). Cells were washed again for three times using saponin buffer and then resuspended in 500 µl FACS-buffer. Flow cytometry was performed on a flow cytometer EPICS® XL (Beckman Coulter, Krefeld).

III.C ANIMAL EXPERIMENTS

Mice were intracerebrally infected using a brain homogenate obtained from a terminally ill scrapie strain 139A-infected mouse as inoculum as previously described (Mok et al. 2006; Riemer et al. 2008). Rapamycin and lithium treatment commenced 100 days post infection. Both drugs were administered via the chow pellets at dosages of 0.2 % (w/w) for lithium and 25 mg per kg body weight per day for rapamycin. Animal experiments were carried out in collaboration with Dr. Michael Baier (Project Neurodegenerative Diseases, Robert-Koch-Institut, Berlin, Germany).

IV. RESULTS

IV.A AUTOPHAGY INDUCTION MEDIATES REDUCTION OF PrP^{Sc}

In several neurodegenerative diseases, including Parkinson`s disease, polyglutamine expansion disease (such as Huntington`s disease), amyotrophic lateral sclerosis (ALS), and some forms of frontotemporal dementia (FTD) *in vitro* and *in vivo* studies revealed that up-regulated autophagy acts as a cellular defense mechanism by degrading aggregate-prone proteins causing disease (Berger et al. 2006; Rubinsztein 2006; Fornai et al. 2008b; Winslow and Rubinsztein 2008). In this part of the study it was analyzed whether induction of autophagy can mediate reduction of PrP^{Sc}.

IV.A.1 LITHIUM INDUCES AUTOPHAGY IN PRION-INFECTED CELLS

Lithium has been shown to induce autophagy, thereby reducing α–synucleins and mutant forms of huntingtin in experimental systems (Sarkar et al. 2005; Sarkar et al. 2008). In order to analyze whether lithium chloride (LiCl) is inducing autophagy in prion-infected cells, murine neuroblastoma cells persistently infected with RML (Rocky Mountain Laboratories) prions (ScN2a) were utilized. Used LiCl concentration for treatment of cultured cells was 10 mM as such concentration has been seen to be sufficient for autophagy induction and reduction of mutant aggregate-prone proteins (Sarkar et al. 2005). Upon induction of autophagy, post-translationally processed microtubule-associated protein 1 light chain 3 (LC3-I) is converted into LC3-II. An increase in the level of LC3-II can be used as a marker for autophagy induction, as the amount of LC3-II associated with autophagosome membranes correlates with the extent of autophagosome formation (Kabeya et al. 2000).

ScN2a cells were either untreated or treated with LiCl for 24 h and analyzed for endogenous levels of LC3-II (**Figure 12A**). An increased amount of LC3-II (relative to actin signals) was observed in cells treated with LiCl (**Figure 12B**). To exclude that the observed increase in LC3-II upon lithium treatment does result from impaired autophagosome-lysosome fusion (also leading to an increased level of LC3-II), the amounts of LC3-II were measured in the presence and absence of bafilomycin A1, which blocks autophagosome-lysosome fusion (Yamamoto et al. 1998). Cells were either untreated or treated with LiCl in the presence and absence of bafilomycin A1 and analyzed for LC3-II (**Figure 12C**). The used dose of bafilomycin A1 had achieved a ceiling effect in terms of its ability to induce LC3-II (data not shown). Cells co-treated with LiCl and bafilomycin A1 showed an increased amount of

LC3-II (relative to actin signals) compared to cells treated with bafilomycin A1 alone (**Figure 12D**), indicating that LiCl treatment results in induction of autophagic flux in ScN2a cells.

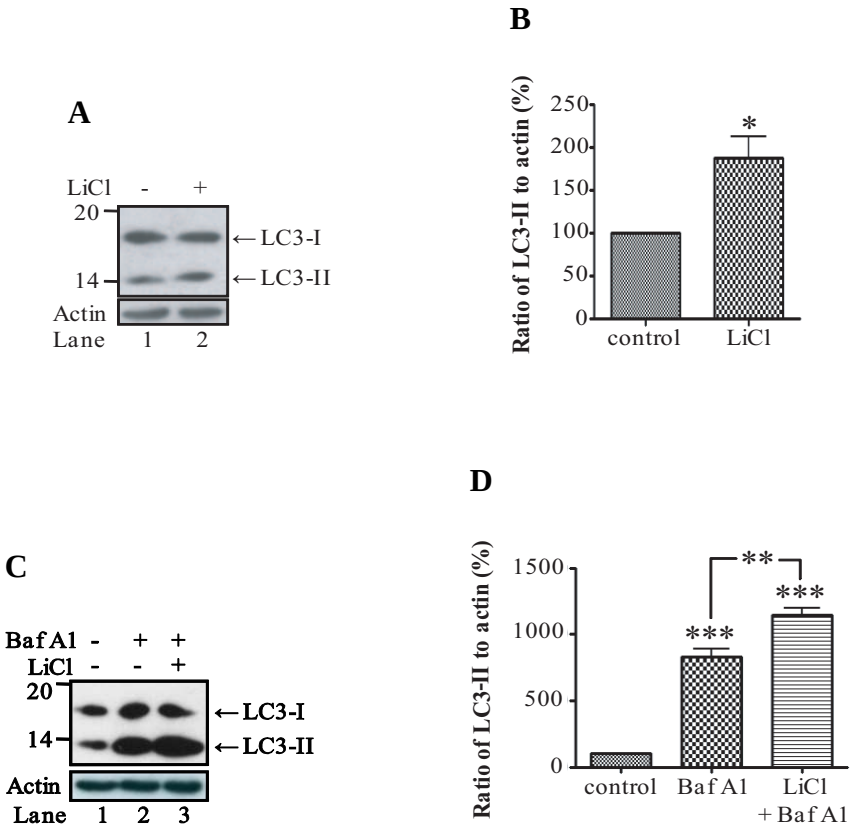


Figure 12. Lithium chloride (LiCl) induces autophagy in ScN2a cells. (A) To measure induction of autophagy upon LiCl treatment, ScN2a cells were either untreated or treated with 10 mM LiCl for 24 h and analyzed by immunoblotting using anti-LC3 monoclonal antibody (mAb). **(B)** An increased amount of LC3-II was observed upon lithium treatment. Values represent the mean ± SEM of three independent experiments. **(C)** To confirm induction of autophagy by lithium, ScN2a cells were treated with or without 10 mM LiCl for 24 h with simultaneous bafilomycin A1 (Baf A1; 200 nM) treatment for 4 h prior to lysis of cells. Cells were subsequently probed using anti-LC3 mAb. **(D)** Higher amounts of endogenous LC3-II were detected in ScN2a cells co-treated with LiCl and Baf A1 compared to Baf A1 alone, indicating induction of autophagy upon lithium treatment. Values represent the mean ± SEM of three independent experiments.

To further investigate the above described induction of autophagy by lithium, confocal laser microscopy was performed. In immunofluorescence analysis induction of autophagy can be monitored by appearance of auto-fluorescing GFP-LC3 puncta, representing autophagosome formation. Compared to electron microscopy, LC3-based assessment of autophagosome number by immunofluorescence appeared to be both more sensitive and specific (Kabeya et

al. 2000). ScN2a cells were transfected with GFP-LC3, treated for 24 h with LiCl, Glivec, or left untreated, and subsequently analyzed by confocal microscopy. Glivec is a tyrosine kinase inhibitor that has been shown by us to activate autophagosome formation and to induce autophagy (Ertmer et al. 2007).

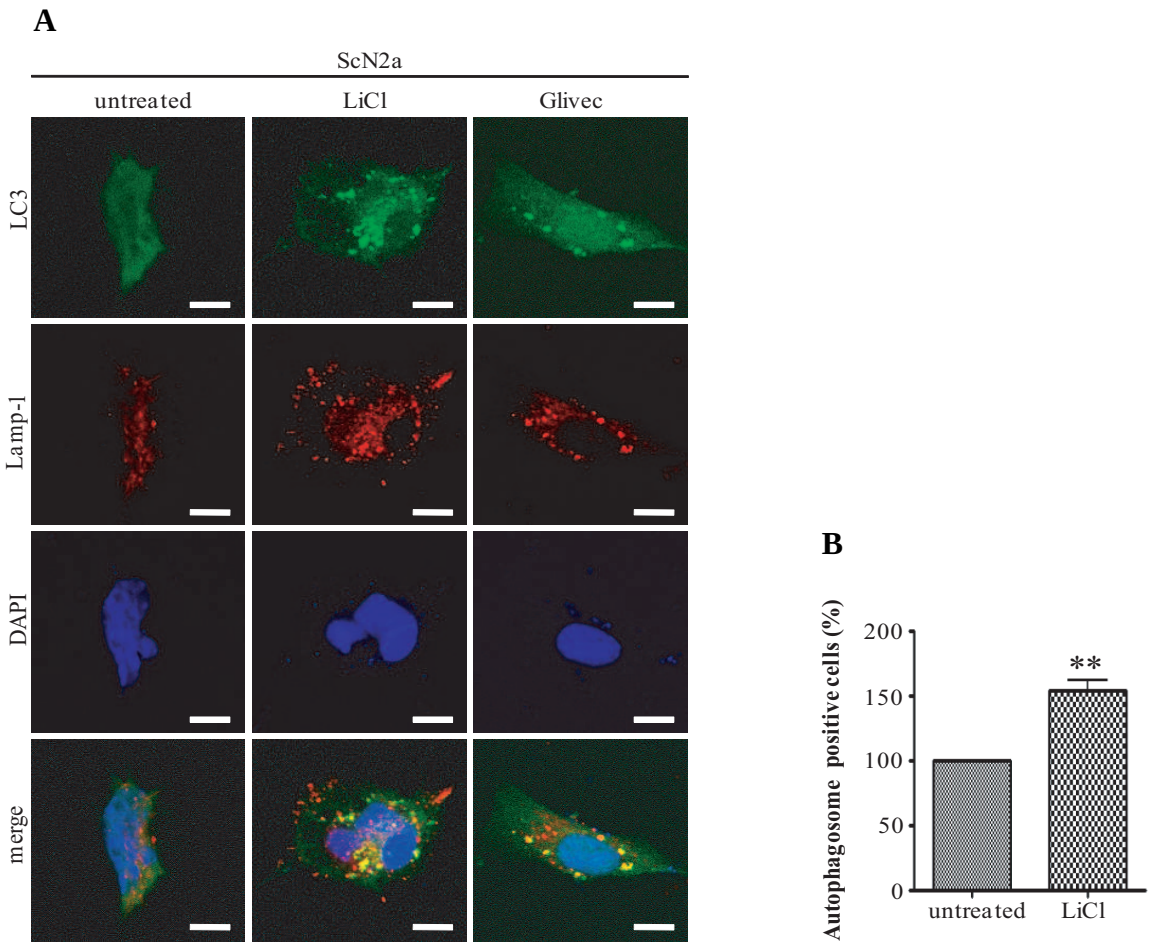


Figure 13. Autophagosome formation is induced upon LiCl treatment. (A) ScN2a cells were transfected with GFP-LC3 and treated with 10 mM LiCl for 24 h. As a control, cells were treated with 10 µM Glivec for 24 h. Cells were stained with anti-lamp-1 and Cy2-conjugated secondary antibody and analyzed by confocal microscopy. Bar on the lower right of each panel indicates 10 µm. **(B)** The ratio of GFP-LC3 expressing cells showing autophagosome formation was determined for lithium-treated and untreated cells and the number of lithium-treated cells is expressed as percentage of untreated cells. Values represent the mean ± SEM of three independent experiments.

In **Figure 13A** (upper panels) LiCl- and Glivec-treated cells with GFP-LC3 puncta are shown, indicating autophagosome formation. The overlay (**Figure 13A**, lower panels) provides evidence for co-localization of lysosomes (stained with lamp-1) and autophagosomes, probably representing autophago-lysosomes. Upon LiCl treatment, the number of cells

showing autophagosome formation, indicating autophagy induction, was increased when compared to untreated cells (**Figure 13B**). This observation correlates with data obtained in immunoblot analysis (compare to **Figures 12A and B**).

IV.A.2 LITHIUM ENHANCES CLEARANCE OF PRP^{SC} IN NEURONAL AND NON-NEURONAL CULTURED CELLS

In order to analyze whether LiCl is affecting the level of cellular PrP^{Sc}, ScN2a cells were either untreated or treated with LiCl for 24 h, 48 h, 72 h, and 96 h (**Figure 14A**). A time-dependent reduction of PrP^{Sc} was observed in LiCl-treated cells (**Figure 14B**). Similar results were obtained in N2a cells persistently infected with 22L prions (data not shown). As a control, ScN2a cells were either untreated or treated with 10 mM NaCl for 96 h (**Figure 15A**). In contrast to LiCl, NaCl was not affecting the level of PrP^{Sc} (**Figure 15B**). Of note, toxic effects were not observed when cells were treated with LiCl under these conditions (**Figure 15C**).

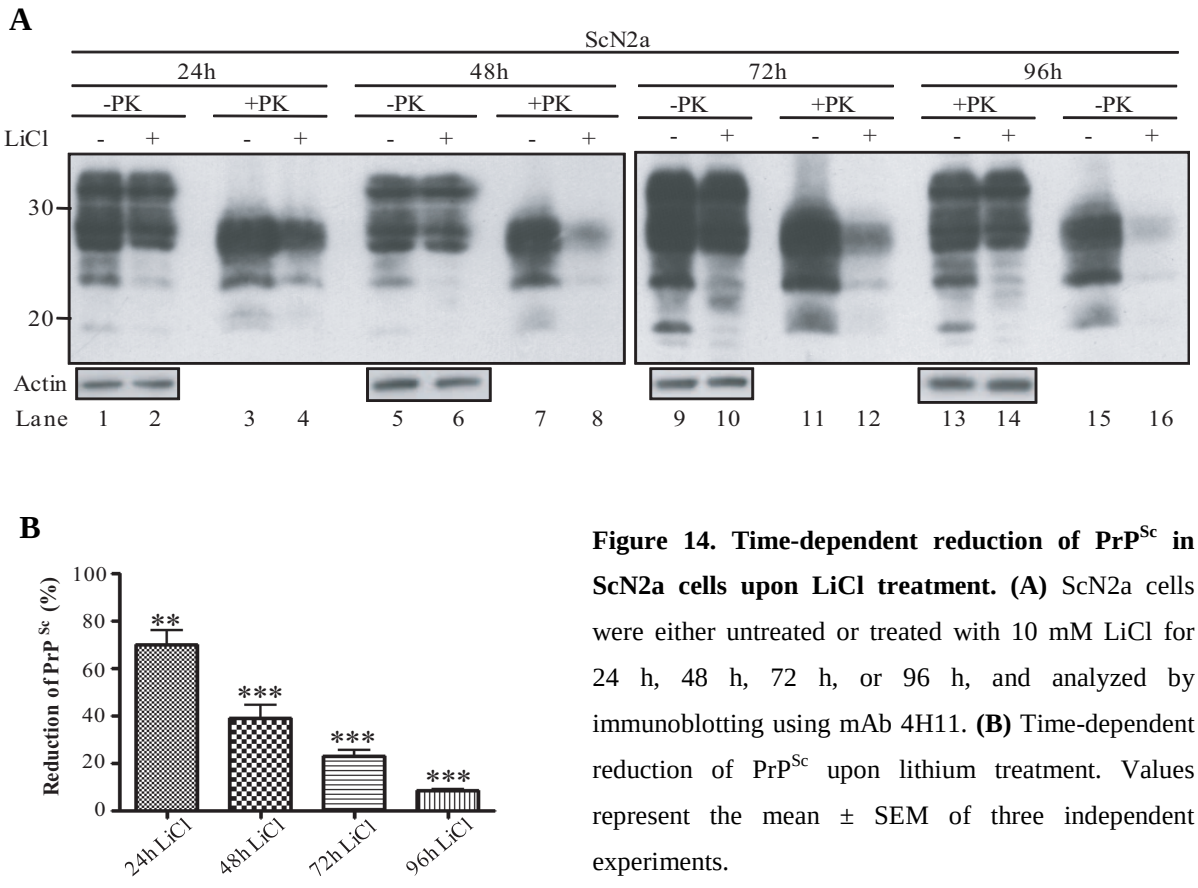


Figure 14. Time-dependent reduction of PrP^{Sc} in ScN2a cells upon LiCl treatment. (A) ScN2a cells were either untreated or treated with 10 mM LiCl for 24 h, 48 h, 72 h, or 96 h, and analyzed by immunoblotting using mAb 4H11. **(B)** Time-dependent reduction of PrP^{Sc} upon lithium treatment. Values represent the mean ± SEM of three independent experiments.

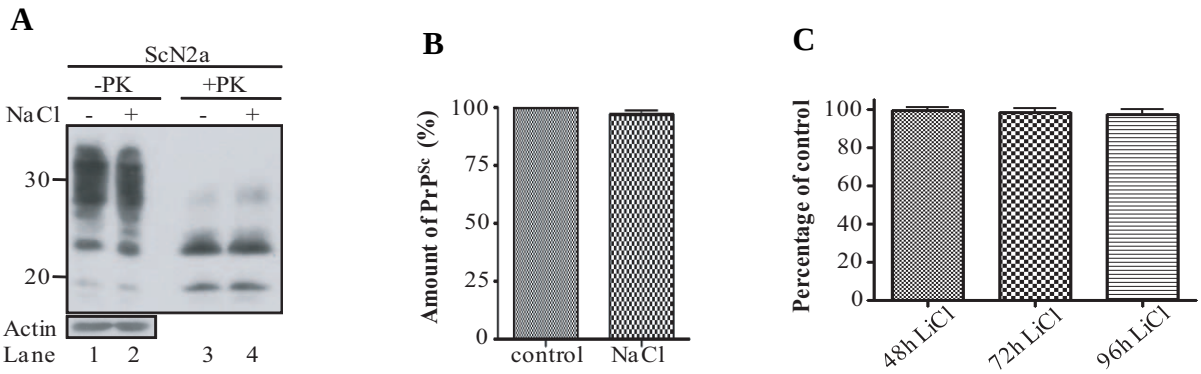


Figure 15. NaCl is not affecting cellular PrP^{Sc} level and LiCl treatment is not toxic for cells. (A) ScN2a cells were treated with or without 10 mM NaCl for 96 h and probed with mAb 4H11. **(B)** NaCl did not affect the level of PrP^{Sc}. Values represent the mean ± SEM of three independent experiments. **(C)** Trypan blue assay to analyze whether 10 mM LiCl is toxic for cells. The percentage of viable cells treated with 10 mM LiCl (for 48 h, 72 h, or 96 h) was calculated and is expressed as percentage of viable, mock-treated cells (control) for each time point. Values represent the mean ± SEM of three independent experiments.

To analyze the effect of lithium in long-term treatment, ScN2a cells were either untreated or treated with 10 mM LiCl for 10 days (d) or 15 d, respectively. Enhanced clearance of PrP^{Sc} was observed both after 10 and 15 d (**Figure 16**).

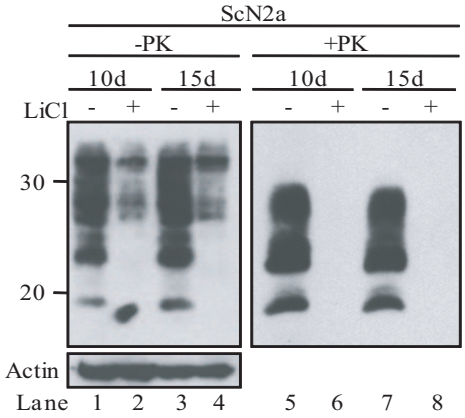


Figure 16. Long-term LiCl treatment enhances reduction of PrP^{Sc}. (A) ScN2a cells were either untreated or treated with 10 mM LiCl for 10 days (d) or 15 d and analyzed by immunoblotting using mAb 4H11.

Next, we investigated the effect of LiCl on PrP^{Sc} in non-neuronal mouse fibroblast cells, persistently infected with 22L prions (ScL929). LiCl enhanced the clearance of PrP^{Sc} in ScL929 cells, as observed after 48 h, 96 h, 10 d, and 15 d (**Figure 17**), yet the reduction of PrP^{Sc} upon LiCl treatment was slightly less pronounced compared to that in ScN2a cells (compare to **Figures 14A, B and 16**).

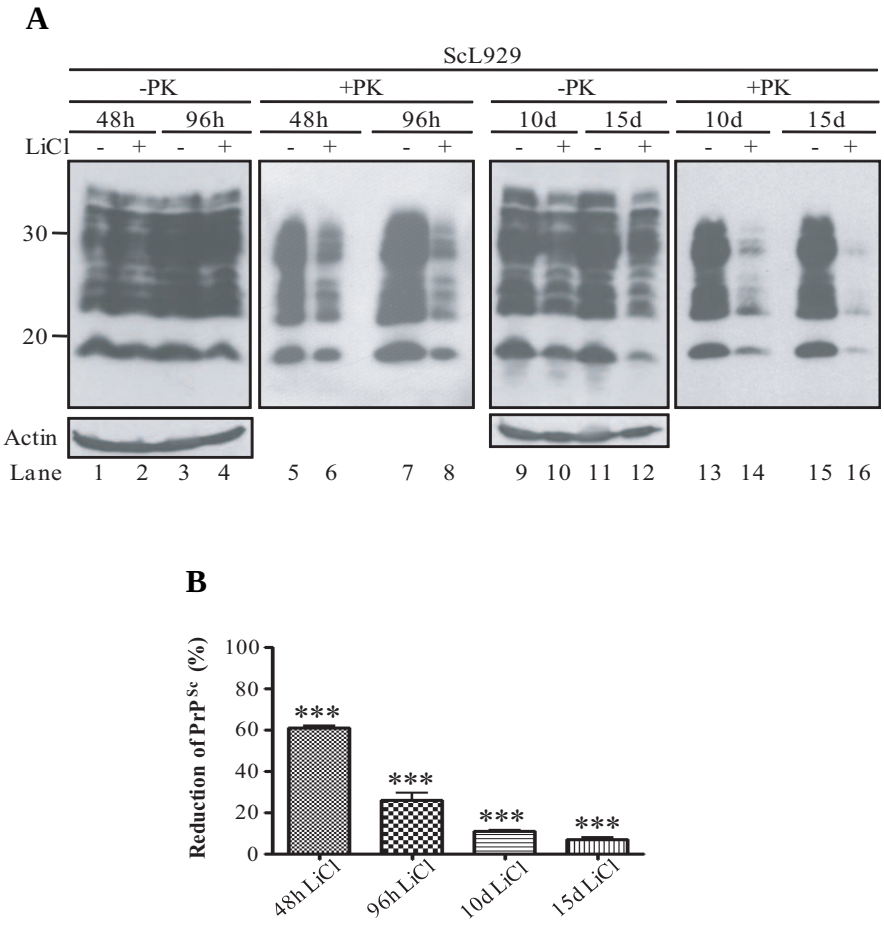


Figure 17. LiCl reduces PrP^{Sc} in non-neuronal cells. (A) Non-neuronal prion-infected cells (ScL929) were either untreated or treated with 10 mM LiCl for 48 h, 96 h, 10 d, or 15 d, and analyzed by immunoblotting using mAb 4H11. **(B)** A time-dependent reduction of PrP^{Sc} was observed in ScL929 cells. Values represent the mean ± SEM of three independent experiments.

Previously it has been shown that PrP^{Sc} can be released into cell culture medium by exosomes (Fevrier et al. 2004; Vella et al. 2008). In order to analyze whether the observed reduction of PrP^{Sc} in LiCl-treated cells is based on such exportation of PrP^{Sc}, we looked for PrP^{Sc} in serum-depleted medium of ScN2a cells treated with LiCl. Slightly less PrP^{Sc} was detected in medium of LiCl-treated cells compared to control cells (**Figure 18**), supporting the idea that

the observed LiCl-induced cellular reduction of PrP^{Sc} is rather based on intracellular degradation than on exportation into the cell culture medium. Taken together, our results show that treatment of prion-infected neuronal and non-neuronal cells with LiCl enhances degradation and clearance of PrP^{Sc}.

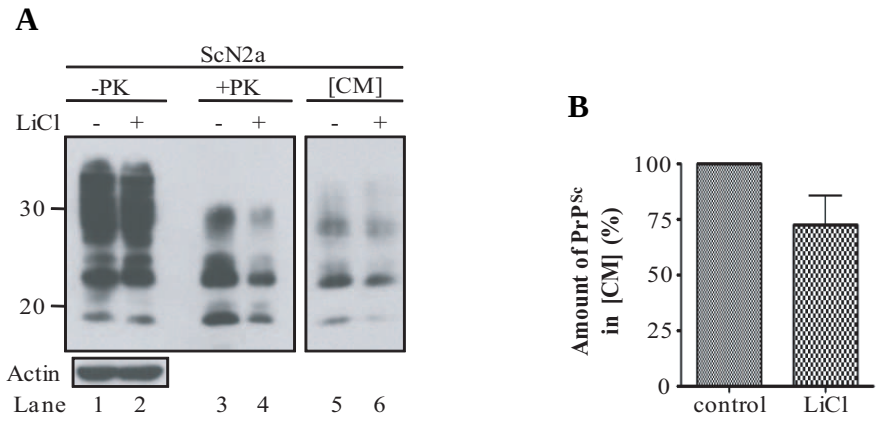


Figure 18. LiCl-induced reduction of PrP^{Sc} is not due to exportation of PrP^{Sc} into the medium. (A) ScN2a cells were treated with or without LiCl for 48 h in serum-depleted conditioned medium [CM]. PrP in cell lysate and medium was detected in immunoblot using mAb 4H11. **(B)** PrP^{Sc} in media is compared in lithium-treated and untreated cells (control). Values represent the mean ± SEM of three independent experiments.

IV.A.3 LITHIUM-INDUCED AUTOPHAGY MEDIATES REDUCTION OF PRP^{SC}

Next, we analyzed whether the above described induction of autophagy by lithium is indeed responsible for reduction of PrP^{Sc}. For this purpose 3-methyladenine (3-MA), widely used to inhibit autophagy, was used (Blommaart et al. 1997). ScN2a cells were either untreated, treated with 3-MA or LiCl alone, or co-treated with 3-MA and LiCl for 24 h and analyzed for PrP^{Sc} levels (**Figure 19A**). Reduction of PrP^{Sc} was alleviated in cells co-treated with 3-MA and LiCl compared to cells treated with LiCl alone (**Figure 19B**), indicating that induction of autophagy by LiCl is mediating enhanced degradation of PrP^{Sc}.

Lithium has been shown to mediate induction of autophagy in an mTOR- independent manner although it has also been seen to possess inhibitory effects on autophagy by activating mTOR (Sarkar et al. 2008). In order to counteract the autophagy-inhibitory effect of lithium, we applied co-treatment of cells with LiCl and rapamycin, a known activator of autophagy by inhibiting mTOR. Cells were either untreated, treated with rapamycin or LiCl alone, or co-treated with rapamycin and LiCl for 48 h and subsequently analyzed for PrP^{Sc} (**Figure 19C**

and D). A significantly increased reduction of PrP^{Sc} was detected in co-treated cells compared to cells treated with rapamycin alone, but the reduction was less pronounced in comparison to treatment with LiCl only. Similar results were obtained with ScL929 cells (data not shown).

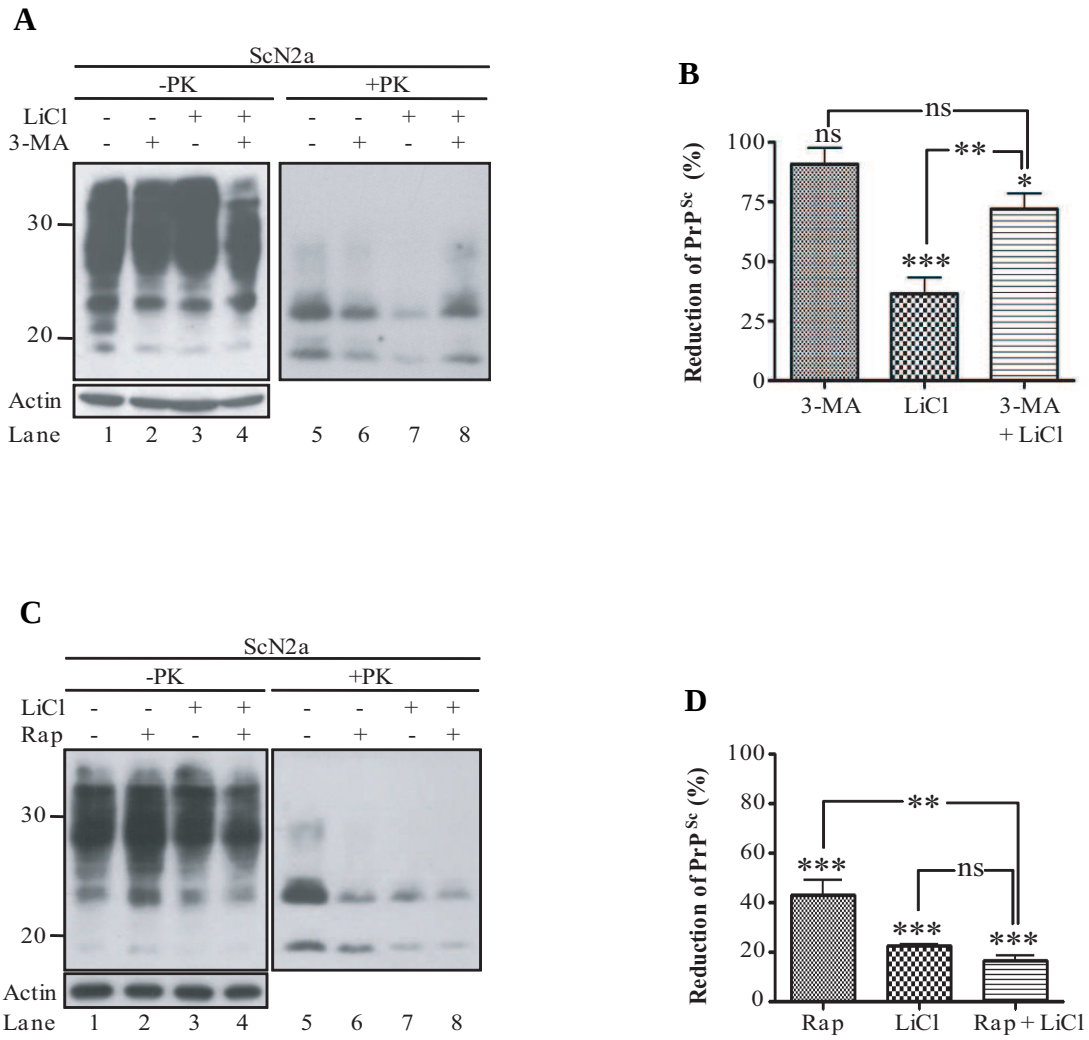


Figure 19. Reduction of PrP^{Sc} is mediated by lithium-induced autophagy. (A) ScN2a cells were either untreated, treated with 10 mM 3-MA or 10 mM LiCl, or co-treated with 3-MA and LiCl for 24 h and analyzed by immunoblotting using mAb 4H11. **(B)** Less reduction of PrP^{Sc} was observed in cells co-treated with 3-MA and LiCl compared to cells treated with LiCl alone. Values represent the mean ± SEM of three independent experiments. **(C)** ScN2a cells were either untreated, treated with 0.2 µM rapamycin or 10 mM LiCl, or co-treated with rapamycin and LiCl for 48 h and analyzed by immunoblotting using mAb 4H11. **(D)** Co-treatment of cells with rapamycin and LiCl enhanced clearance of PrP^{Sc} compared to either compound alone. Values represent the mean ± SEM of three independent experiments.

IV.A.4 AUTOPHAGY-INDUCING COMPOUNDS REDUCE PRP^{Sc}

To verify whether the ability of reducing cellular levels of PrP^{Sc} is a general phenomenon of autophagy-inducing compounds, several autophagy inducers were utilized. Besides LiCl, the autophagy activators Glivec (Ertmer et al. 2007), rapamycin (Noda and Ohsumi 1998) and trehalose were applied. Trehalose has been seen in the recent past to enhance clearance of mutant forms of huntingtin and α–synucleins by inducing autophagy (Sarkar et al. 2007). ScN2a cells were either left untreated or treated with Glivec, rapamycin, trehalose, or LiCl for 48 h and subsequently analyzed for PrP^{Sc} levels. All autophagy-inducing compounds used in this study reduced cellular levels of PrP^{Sc} (**Figure 20**). Furthermore, as was the case for lithium, we could demonstrate in another study that induction of autophagy by trehalose is responsible for reduction of PrP^{Sc}, underlining that induction of autophagy mediates reduced levels of cellular PrP^{Sc} (data not shown) (Aguib*, Heiseke* et al. 2009; * contributed equally).

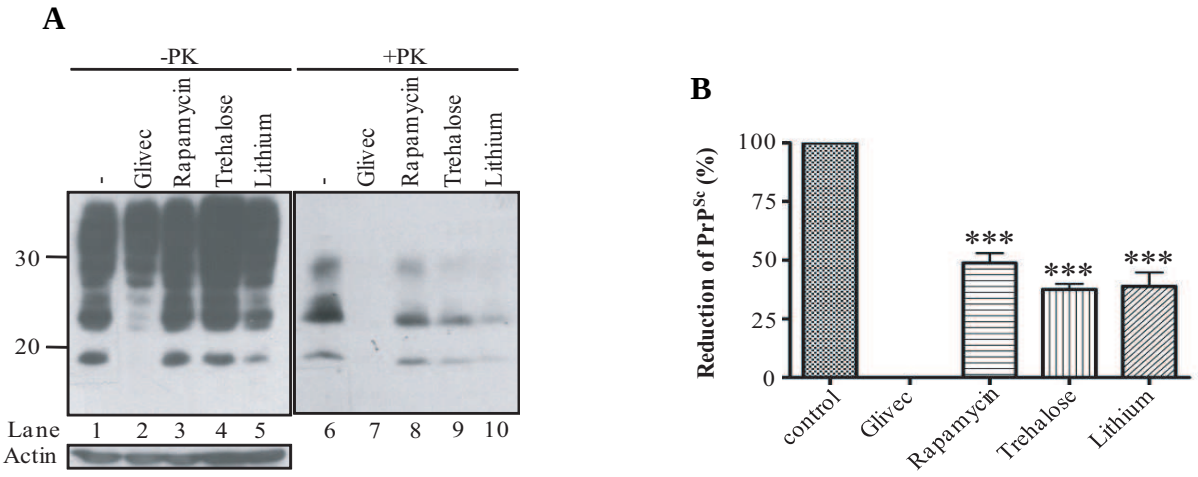


Figure 20. Reduction of PrP^{Sc} upon treatment of cells with different autophagy-inducing compounds. (A) ScN2a cells were either left untreated or treated with 10 µM Glivec, 200 nM rapamycin, 100 mM trehalose, or 10 mM lithium for 48 h and cell lysates subsequently analyzed by SDS-PAGE using anti-PrP mAb 4H11. **(B)** Less PrP^{Sc} is observed in compound-treated cells compared to untreated control cells. Values represent the mean ± SEM of three independent experiments.

IV.A.5 SEVERAL LITHIUM SALTS REDUCE PRP^{SC} AND INDUCE AUTOPHAGY

Next we tested whether other lithium salts, besides the classically administered drug LiCl, also have the potential to reduce PrP^{Sc} and to induce autophagy. ScN2a cells were either untreated or treated with LiCl, lithium carbonate (Li_2CO_3), lithium bromide (LiBr), or lithium

acetate ($LiCH_3COO$) for 96 h. **Figure 21A** shows that all lithium salts used here possess the ability to reduce PrP^{Sc}.

To analyze whether these lithium salts also induce autophagy, cells were either untreated or treated with Li_2CO_3, LiBr, or $LiCH_3COO$ in the presence and absence of bafilomycin A1 and analyzed for endogenous levels of LC3-II (**Figure 21B**). All used lithium salts induced autophagy similar to the level of induction observed upon LiCl treatment (**Figure 21C**).

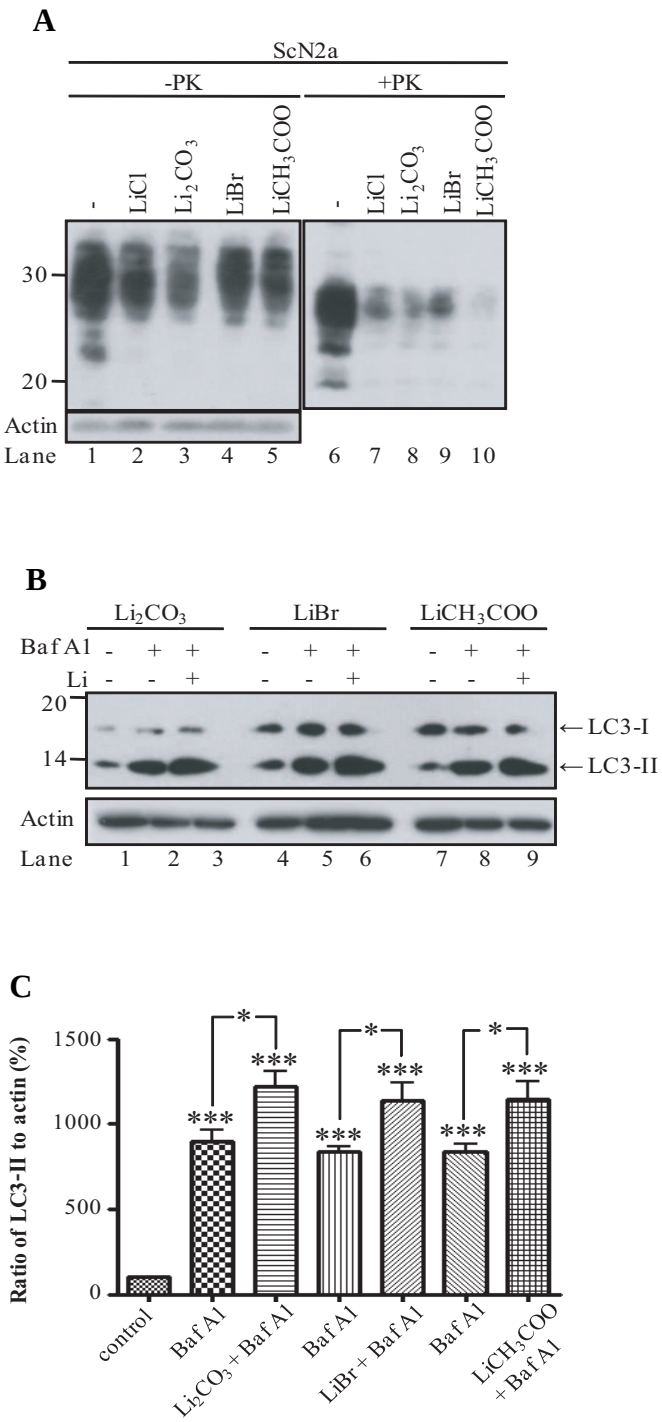


Figure 21. Different lithium salts reduce PrP^{Sc} while inducing autophagy. (A) ScN2a cells were either untreated or treated with 10 mM LiCl, 10 mM Li_2CO_3, 10 mM LiBr, or 10 mM $LiCH_3COO$ for 96 h and analyzed by immunoblotting using mAb 4H11. **(B)** To monitor induction of autophagy, ScN2a cells were either untreated or treated with 10 mM Li_2CO_3, 10 mM LiBr, or 10 mM $LiCH_3COO$ for 24 h with simultaneous bafilomycin A1 (Baf A1; 200 nM) treatment for 4 h prior to lysis of cells. Cells were subsequently probed using anti-LC3 mAb. **(C)** Higher amounts of endogenous LC3-II were detected in ScN2a cells co-treated with the different lithium salts and Baf A1 compared to Baf A1 alone. Values represent the mean ± SEM of three independent experiments.

These results were confirmed by confocal microscopy which revealed that treatment with lithium salts increased the number of cells showing autophagosome formation when compared to untreated cells (**Figure 22**).

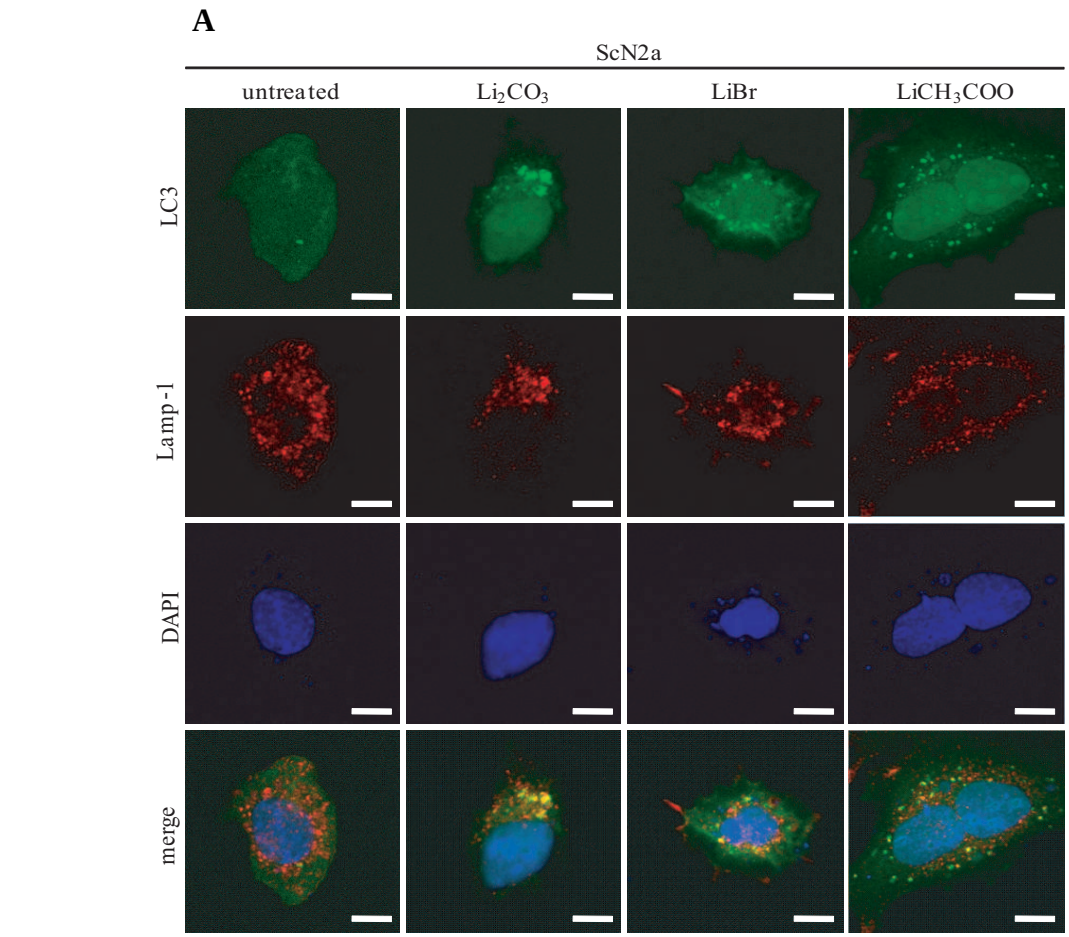


Figure 22. Autophagosome formation induced by different lithium salts. (A) ScN2a cells were transfected with GFP-LC3 and subsequently either untreated or treated with 10 mM Li_2CO_3, 10 mM LiBr, or 10 mM $LiCH_3COO$ for 24 h. Cells were stained with anti-lamp-1 and Cy2-conjugated secondary antibody and analyzed by confocal microscopy. Bar on the lower right of each panel indicates 10 µm. **(B)** The ratio of GFP-LC3 expressing cells showing autophagosome formation was determined for untreated cells and cells treated with the different lithium salts and the number of lithium-treated cells is expressed as percentage of untreated cells. Values represent the mean ± SEM of three independent experiments.

IV.A.6 LITHIUM REDUCES THE LEVEL OF PRP^C

To analyze whether LiCl treatment affects PrP^c, N2a cells were either untreated or treated with LiCl for 96 h or 15 d, respectively. Subsequently, cells were subjected to FACS analysis (**Figures 23A and B**). Slightly lower levels (20% reduction) of PrP^c for both 96 h and 15 d LiCl treatments compared to untreated cultures were observed. Next we investigated whether the observed reduction of PrP^c is regulated at the mRNA level and performed quantitative real-time (RT)-PCR. Total mRNA was isolated from N2a cells which were either untreated or treated with LiCl for 96 h and the number of PrP mRNA copies was compared (**Figure 23C**). As a control, mRNA was analyzed without preceding RT reaction to exclude DNA contaminations (data not shown).

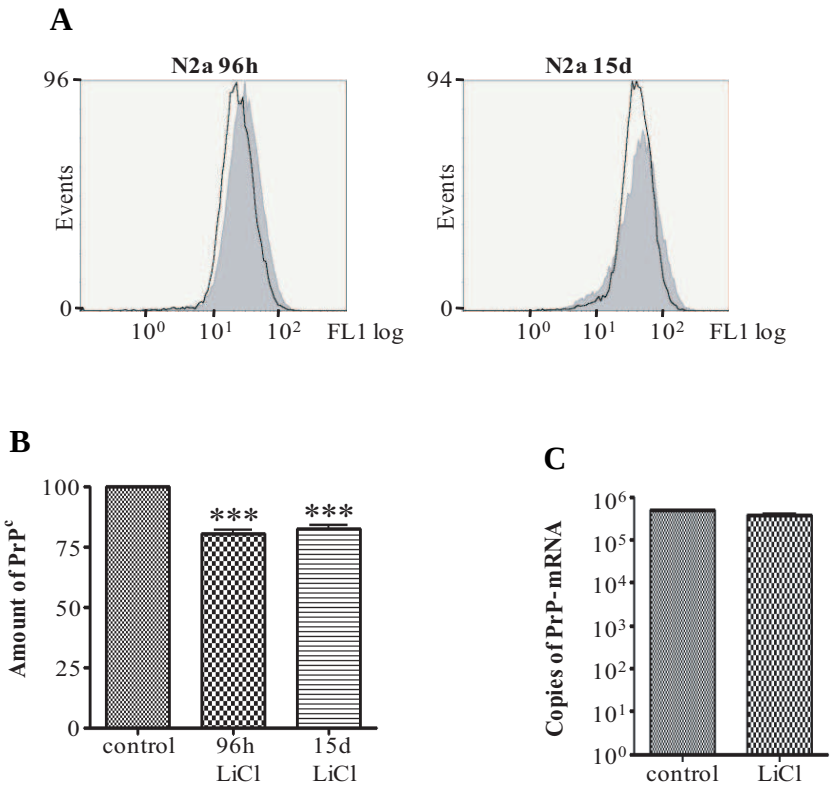


Figure 23. Lithium reduces the level of PrP^c. (A) N2a cells were either untreated or treated with 10 mM LiCl for 96 h (left-hand panel) or 15 d (right-hand panel), respectively. Expression of PrP^c was investigated by FACS analysis using mAb 4H11 and Cy2-conjugated secondary antibody. FL1 represents the fluorescence intensity in untreated (gray peak) and lithium-treated (black lined peak) cells, plotted against the number of cells (events). For each experiment 15000 events were measured. **(B)** Reduced levels of PrP^c were detected in cells treated with 10 mM LiCl for 96 h or 15 d, respectively. Values represent the mean ± SEM of three independent experiments. **(C)** Real-time RT-PCR was performed with RNA prepared from N2a cells either untreated or treated with 10 mM LiCl for 96 h. Copy numbers of PrP mRNA are shown and represent the mean ± SEM of three independent experiments.

To further shed light on the mechanism underlying the reduction of PrP^c we used mouse embryonic fibroblasts (MEF). Wild-type MEF (MEFwt) or autophagy-deficient MEF ($MEFATG5^{-/-}$) were either untreated or treated for 15 d with LiCl. Subsequently, cells were investigated for endogenous PrP^c levels by FACS analysis (**Figure 24A**). In contrast to MEFwt, which reveal reduction of PrP^c upon LiCl treatment, no reduced level of PrP^c was obtained in LiCl-treated $MEFATG5^{-/-}$ (**Figure 24B**). In summary, these results indicate that LiCl mediates reduction of PrP^c in an autophagy-dependent manner.

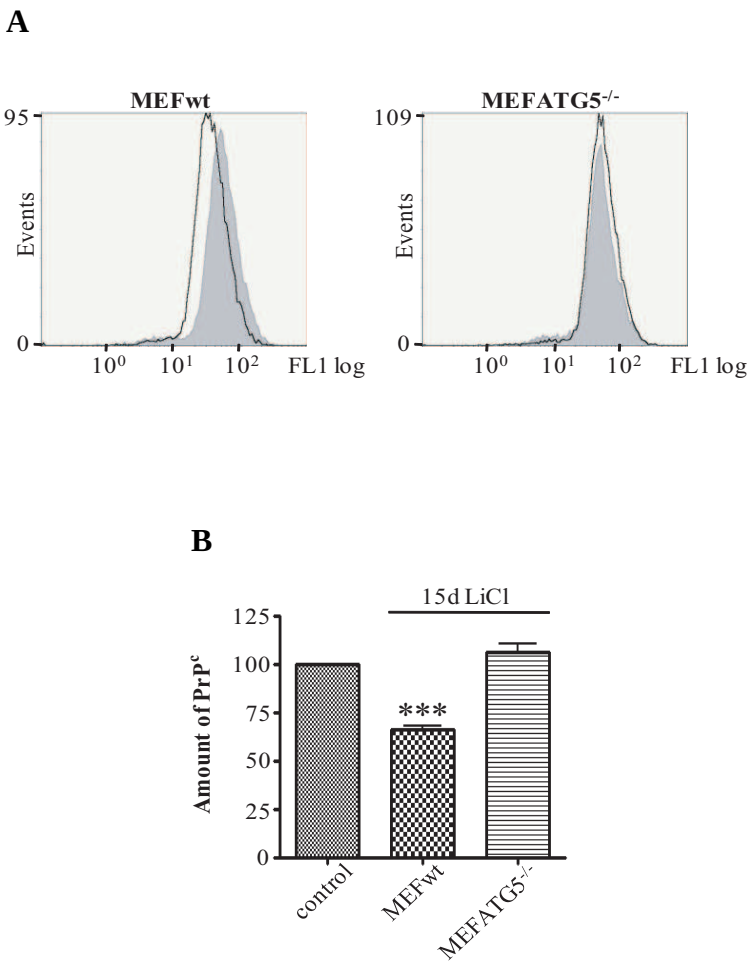


Figure 24. Lithium reduces the level of PrP^c in an autophagy-dependent manner. (A) MEFwt (left-hand panel) and $MEFATG5^{-/-}$ (right-hand panel) were either untreated or treated with 10 mM LiCl for 15 d. Expression of PrP^c was investigated by FACS analysis using mAb 4H11 and Cy2-conjugated secondary antibody. FL1 represents the fluorescence intensity in untreated (gray peak) and lithium-treated (black lined peak) cells, plotted against the number of cells (events). For each experiment 15000 events were measured. **(B)** No reduction of PrP^c was detected in LiCl-treated $MEFATG5^{-/-}$, whereas MEFwt exhibit less PrP^c when treated with LiCl. Values represent the mean ± SEM of three independent experiments.

IV.A.7 THERAPEUTIC POTENTIAL OF AUTOPHAGY INDUCTION IN PRION DISEASE AS ASSAYED IN BIOASSAY

In order to assess the therapeutic anti-prion efficacy of autophagy-inducing compounds, lithium and rapamycin were utilized in *in vivo* experiments. Mice were intracerebrally infected with prions and lithium or rapamycin was orally given to mice starting at day 100 post infection, mimicking a preclinical therapeutic intervention situation (Mok et al. 2006; Riemer et al. 2008). Treatment of prion-infected mice with rapamycin showed a small but significant therapeutic effect ($p<0.05$). In contrast, no significant prolongation of survival times was observed upon administration of lithium (**Figure 25**). However, survival times in the lithium group showed an increased spread compared to controls (53 versus 25 days), which may have masked a therapeutic effect of lithium. In summary, results obtained with rapamycin indicate that such drugs may have therapeutic potential.

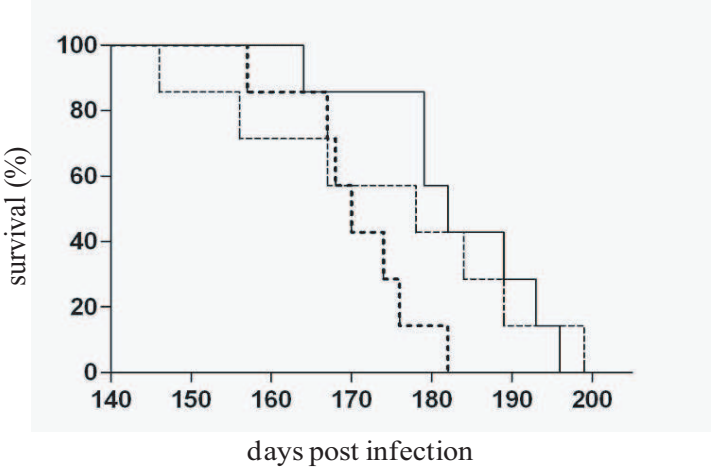


Figure 25. Survival times of lithium- and rapamycin-treated mice. Oral treatment with either lithium or rapamycin was initiated at day 100 post intracerebral infection with prion strain 139A. Solid line depicts rapamycin-treated mice (mean 183.1±10.8 days, $p<0.05$ versus controls); thin broken line, lithium-treated mice (mean 174.1±18.8 days, $p>0.05$ versus controls); bold broken line, untreated controls (mean 170.6±7.9 days). Group sizes were n=7. Of note, survival times in the lithium group showed for unknown reasons an increased spread compared to controls (53 versus 25 days), which may have masked a small therapeutic effect of lithium.

IV.B ROLE OF BASAL AUTOPHAGY IN PRIMARY PRION INFECTION

In the first part of the study it could be demonstrated that pharmacologically induced autophagy results in reduced levels of cellular PrP^{Sc}. In the following part of the study, the role of basal, physiological autophagy in primary prion infection is subject matter of analysis. Several different roles of autophagy in primary prion infection are conceivable. First, it might be possible that autophagy inhibits primary prion infection. Second, a vice versa scenario might be true, which means that autophagy promotes primary prion infection. And last but not least a dual role of autophagy seems to be possible: a moderate basal rate of autophagy promotes primary prion infection and a strong autophagy activation results in reduced susceptibility to primary prion infection.

IV.B.1 AUTOPHAGOSOME FORMATION IS ACTIVATED UPON PRIMARY PRION INFECTION

In order to analyze the role of basal autophagy in primary prion infection, PrP^{Sc}-susceptible (Kl17 and Kl21) and PrP^{Sc}-unsusceptible (Kl35) N2a clones were utilized and incubated with brain homogenate either infected with 22L prions or mock, as a control. Used N2a clones are overexpressing mouse PrP^{c} with the 3F4 epitope. Therefore the endogenous 3F4-tagged PrP^{c} acts as a substrate and is incorporated into newly synthesized PrP^{Sc} upon incubation with prion-infected brain homogenate. Only this newly formed endogenous PrP^{Sc} is selectively detected in immunoblot analysis by mAb 3F4, which does not bind to PrP molecules derived from the brain homogenate in the inoculum.

Upon inoculation with brain homogenate (either mock or 22L prion-infected), cells were lysed and analyzed 5, 8, 14, and 20 days post infection. An aliquot of the lysate was PK-digested, ultracentrifuged, and analyzed by immunoblotting using mAb 3F4 for detection of newly formed PrP^{Sc} (III.B.4.8). Another aliquot of the same lysate was left undigested and analyzed by immunoblotting using anti-LC3 mAb for detection of autophagosome formation. In **Figures 26, 27 and 28** immunoblotting signals obtained with mAb 3F4 and anti-LC3 mAb are depicted for PrP^{Sc}-susceptible (Kl17 and Kl21) and PrP^{Sc}-unsusceptible (Kl35) N2a clones. In PrP^{Sc}-susceptible clones a significantly increased amount of LC3-II was observed when newly converted PrP^{Sc} was detected compared to mock treated cells, indicating autophagosome formation upon primary prion infection (**Figures 26 and 27**). In contrast, in

the PrP^{Sc}-unsusceptible clone (Kl35), neither such significantly increased amount of LC3-II nor newly converted PrP^{Sc} was detected (**Figure 28**), indicating that autophagosome formation supports primary prion infection.

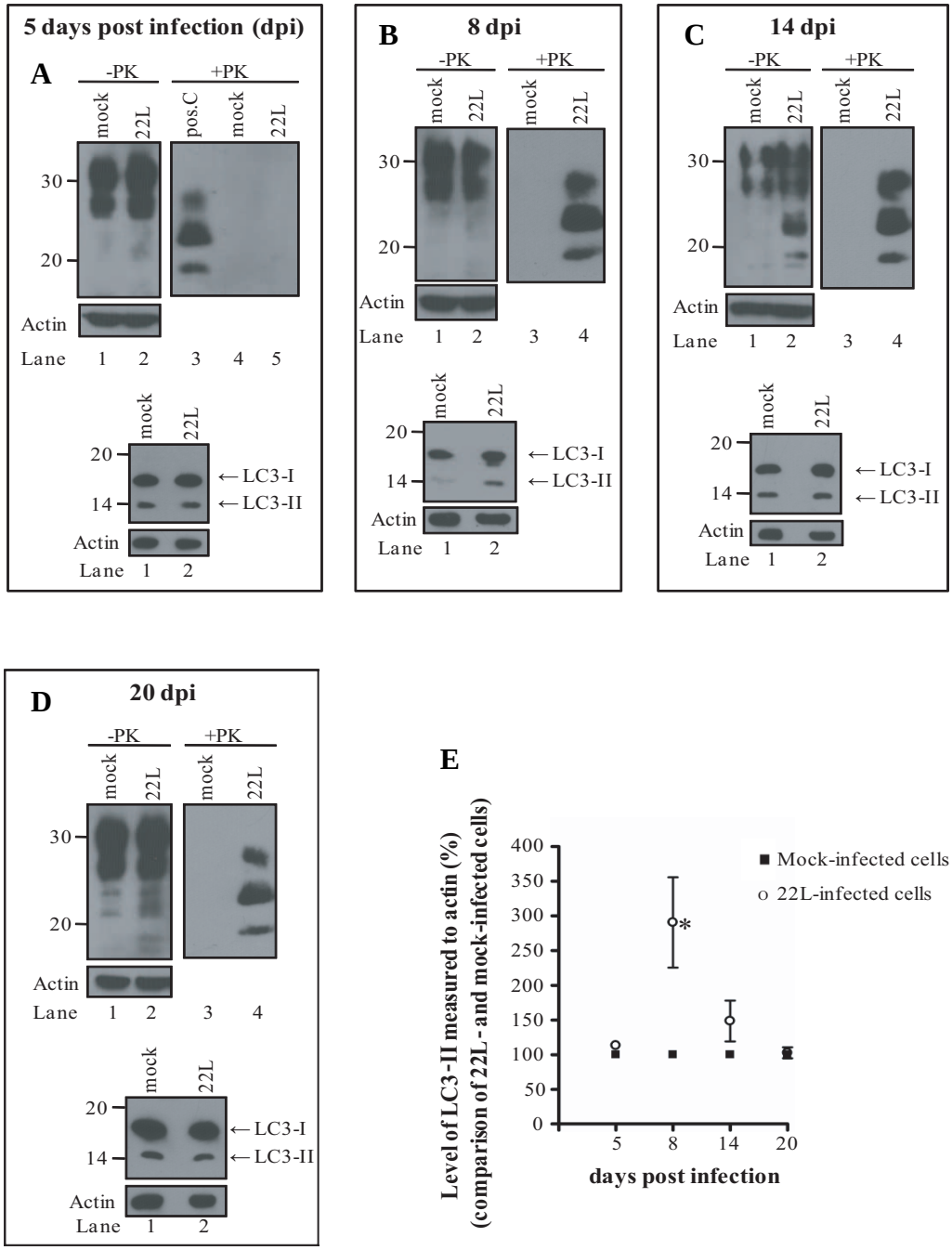


Figure 26. Increase in autophagosome formation upon primary prion infection in PrP^{Sc}-susceptible clone. **(A-D)** PrP^{Sc}-susceptible N2a clone (Kl17) was either inoculated with 22L prion-infected or mock brain homogenate. After 5, 8, 14, and 20 days post infection (dpi) cells were lysed and an aliquot of the lysate was PK-digested, ultracentrifuged, and analyzed by immunoblotting using mAb 3F4 for detection of newly converted PrP^{Sc}. Lysate from N2a cells propagating 3F4-tagged PrP^{Sc} was used as a positive control (pos.C) for PrP^{Sc}. An aliquot of the same lysate was left undigested and probed with anti-LC3 mAb for detection of autophagosome formation. **(E)** Increased amount of LC3-II, indicating autophagosome formation, was observed in cells

inoculated with 22L prions when newly converted PrP^{Sc} was detected for the first time (8 dpi). Values represent the mean ± SEM of three independent experiments.

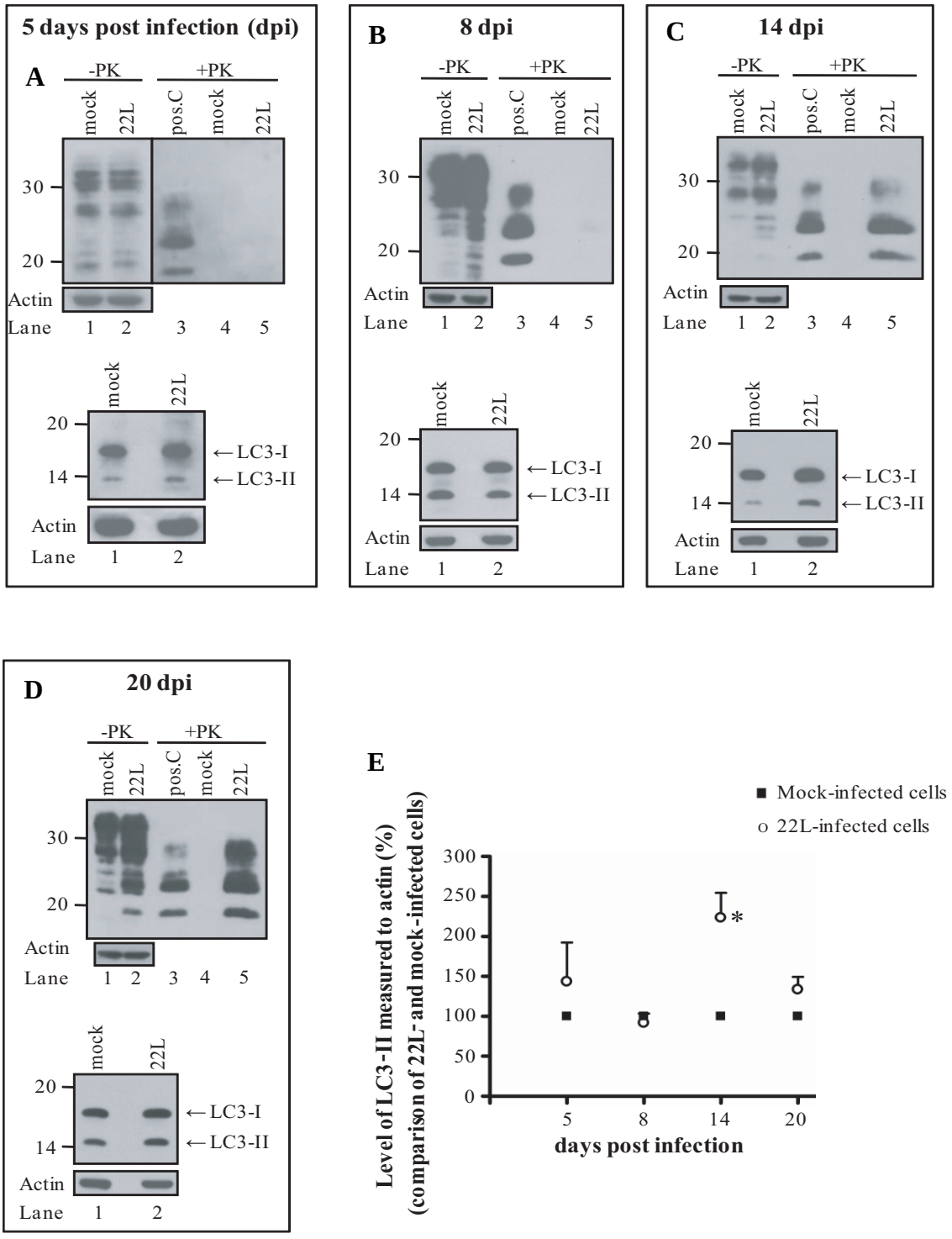


Figure 27. Increase in autophagosome formation upon primary prion infection in PrP^{Sc}-susceptible clone. (A-D) PrP^{Sc}-susceptible N2a clone (Kl21) was either inoculated with 22L prion-infected or mock brain homogenate. After 5, 8, 14, and 20 days post infection (dpi) cells were lysed and an aliquot of the lysate was PK-digested, ultracentrifuged, and analyzed by immunoblotting using mAb 3F4 for detection of newly converted PrP^{Sc}. Lysate from N2a cells propagating 3F4-tagged PrP^{Sc} was used as a positive control (pos.C) for PrP^{Sc}. An

aliquot of the same lysate was left undigested and probed with anti-LC3 mAb for detection of autophagosome formation. **(E)** Increased amount of LC3-II, indicating autophagosome formation, was observed in cells inoculated with 22L prions when newly converted PrP^{Sc} was detected for the first time (14 dpi). Values represent the mean ± SEM of three independent experiments.

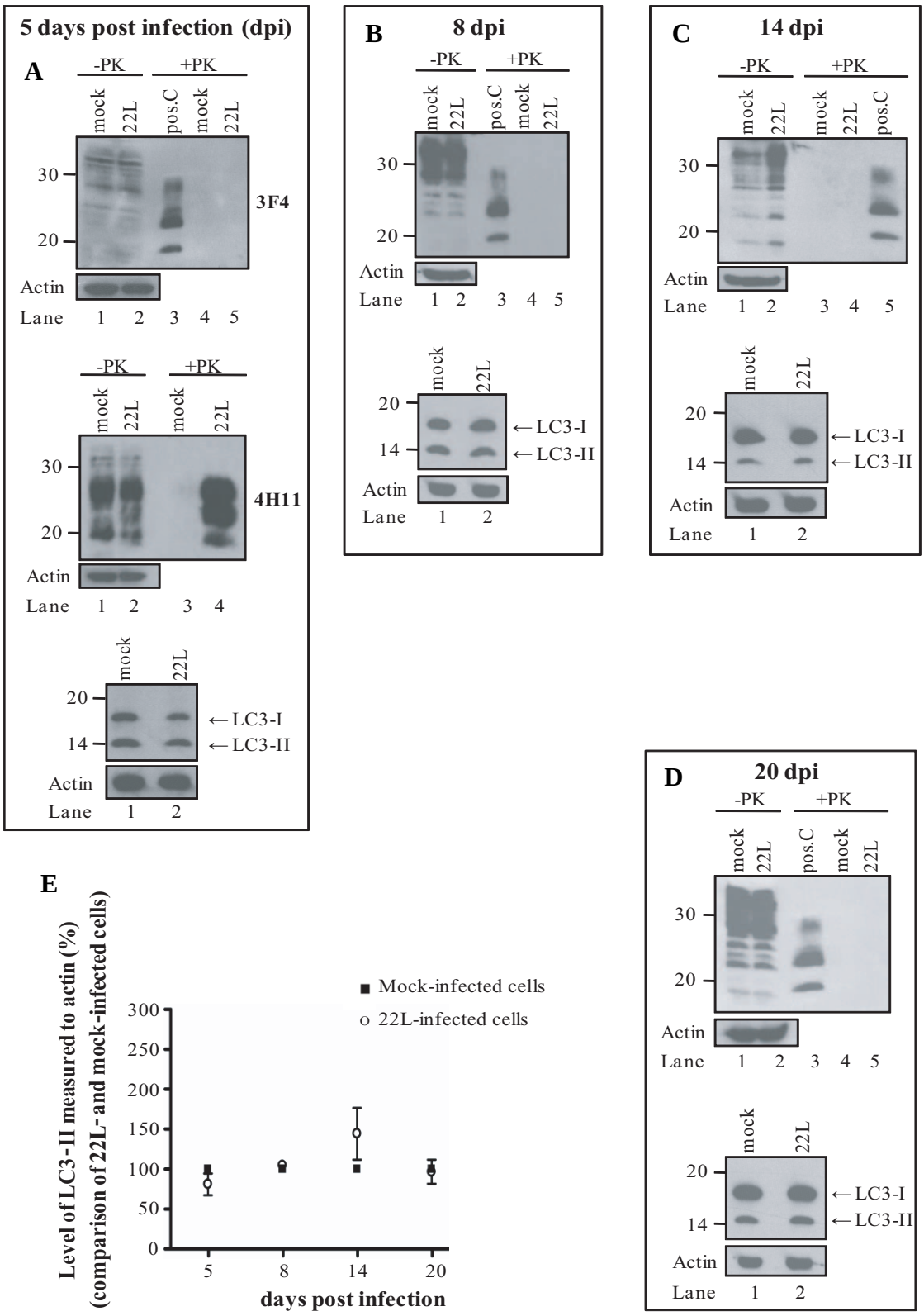


Figure 28. Absence of increased autophagosome formation when cells are not generating newly converted PrP^{Sc}. (A-D) PrP^{Sc}-unsusceptible N2a clone (Kl35) was either inoculated with 22L prion-infected or mock brain homogenate. After 5, 8, 14, and 20 days post infection (dpi) cells were lysed and an aliquot of the lysate was PK-digested, ultracentrifuged, and analyzed by immunoblotting using mAb 3F4 for detection of newly converted PrP^{Sc}. Lysate from N2a cells propagating 3F4-tagged PrP^{Sc} was used as a positive control (pos.C) for PrP^{Sc}. An aliquot of the same lysate was left undigested and probed with anti-LC3 mAb for detection of autophagosome formation. To verify that PrP^{Sc} was present in the used 22L prion-infected brain homogenate, a lysate of cells (5 dpi) was additionally stained using anti-PrP mAb 4H11 (which detects PrP^{Sc} in the inoculum). Neither newly converted PrP^{Sc} nor increased amount of LC3-II, indicating autophagosome formation, was detected in 22L prion-infected cells at each time point analyzed. **(E)** Significantly increased amount of LC3-II, indicating autophagosome formation, was not observed in mock- and 22L prion-infected cells (PrP^{Sc}-unsusceptible Kl35) at each time point analyzed. Values represent the mean ± SEM of three independent experiments.

Figure 29 summarizes data described in **Figures 26, 27 and 28**. In PrP^{Sc}-unsusceptible N2a clone (Kl35), which is not able to convert endogenous PrP^{c} into PrP^{Sc}, significantly increased amounts of LC3-II were not observed. In contrast, in PrP^{Sc}-susceptible N2a clones (Kl17 and Kl21) significantly increased amounts of LC3-II, indicating autophagosome formation, were detected when newly converted PrP^{Sc} was detected for the first time. This finding reveals that autophagosome formation is up-regulated upon primary prion infection and might be an important cellular factor regulating susceptibility to primary prion infection.

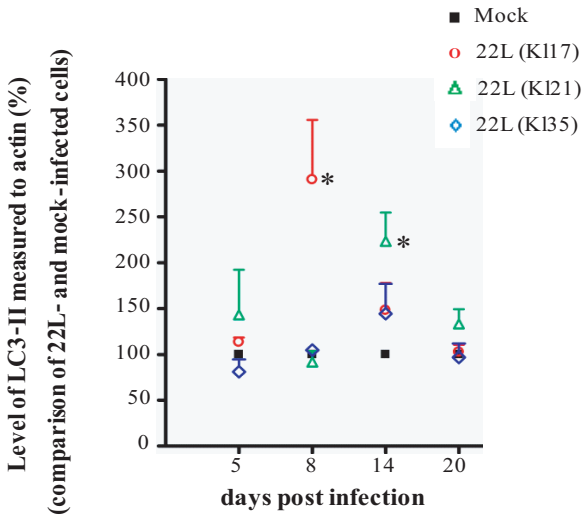


Figure 29. Increased autophagosome formation upon primary prion infection. Upon primary prion infection of PrP^{Sc}-susceptible N2a clones, Kl17 (red circle) and Kl21 (green triangle), increased amounts of LC3-II, indicating autophagosome formation, were observed in cells inoculated with 22L prions when newly converted PrP^{Sc} was detected for the first time. In contrast, PrP^{Sc}-unsusceptible N2a clone, Kl35 (blue square), was not able to convert endogenous PrP^{c} into PrP^{Sc} upon inoculation with 22L prions and did not show significantly increased amounts of LC3-II when compared to mock treated cells.

IV.B.2 AUTOPHAGY-DEFICIENT MOUSE EMBRYONIC FIBROBLASTS (MEFS) ARE LESS SUSCEPTIBLE TO PRIMARY PRION INFECTION COMPARED TO WILD-TYPE MEFS

To analyze the role of basal autophagy in primary prion infection in more detail, mouse embryonic fibroblasts (MEFs), either wild-type (MEFwt) or autophagy-deficient (MEFATG5$^{-/-}$), were utilized. When MEFwt and MEFATG5$^{-/-}$ cells were inoculated with 22L prion-infected brain homogenate, or mock brain as a control, significantly less susceptibility to primary prion infection was observed for autophagy-deficient MEFATG5$^{-/-}$ cells compared to their autophagy-competent counterparts MEFwt, which propagate PrP^{Sc} much more efficiently upon primary prion infection (**Figure 30**).

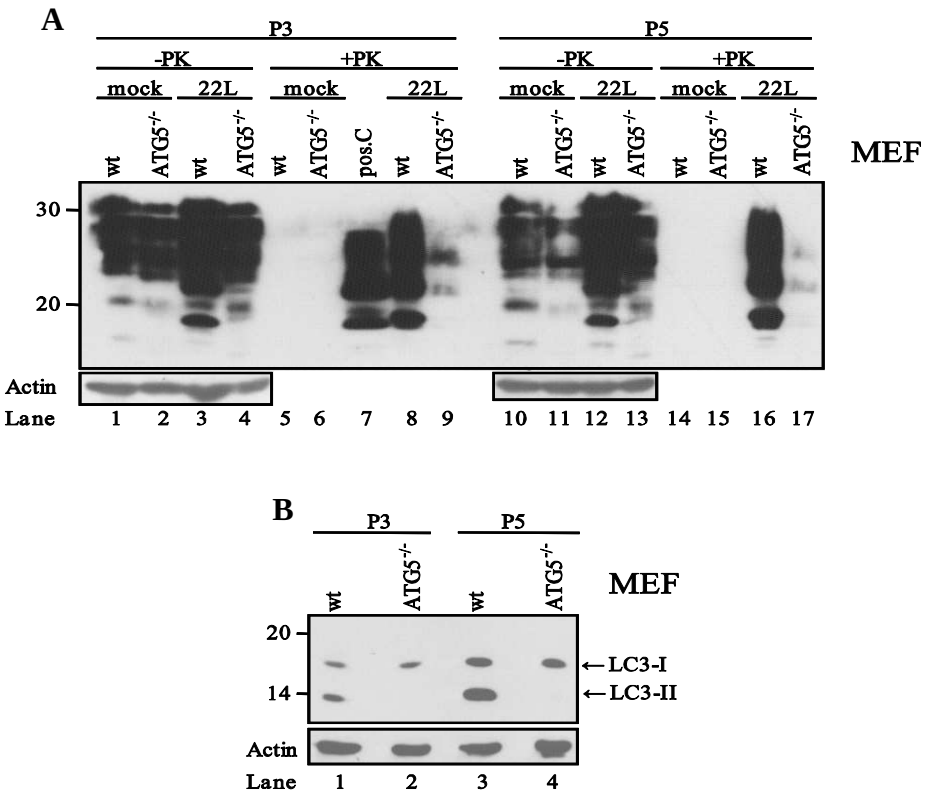


Figure 30. MEFATG5$^{-/-}$ cells are much less susceptibility to primary prion infection compared toMEFwt cells. (A) MEFwt and MEFATG5$^{-/-}$ cells were infected with 22L prions, or mock as a control, and analyzed at passage 3 (P3) and P5 by immunoblotting using anti-PrP mAb 4H11. Lower amount of PrP^{Sc}, and accordingly less susceptibility to primary prion infection, was detected in MEFATG5$^{-/-}$ compared to MEFwt cells at P3 and P5 (compare lanes 8, 9 and 16, 17). Lysate of prion-infected cells was used as positive control for PrP^{Sc} (pos.C). **(B)** PK-undigested lysates (-PK) from P3 and P5 of 22L prion-infected MEFwt and MEFATG5$^{-/-}$ cells were probed with anti-LC3 mAb. LC3-II signals were detected in MEFwt whereas MEFATG5$^{-/-}$ cells are not able to convert LC3-I into LC3-II, verifying autophagy-deficiency.

IV.B.3 BASAL AUTOPHAGY ENHANCES PRIMARY PRION INFECTION

Indicative for a role of basal autophagy in enhancing primary prion infection, autophagy-deficient MEFATG5$^{-/-}$ are less susceptible to primary prion infection compared toMEFwt cells (IV.B.2). For this reason, in order to shed light into the role of basal constitutive autophagy in primary prion infection, MEFATG5$^{-/-}$ cells were transduced with lentivirus coding for *Atg5* to restore autophagy-competence (MEFATG5). Subsequently cells were tested for susceptibility to primary prion infection.

IV.B.3.1 REINTRODUCTION OF *ATG5* IN MEFATG5$^{-/-}$ CELLS

MEFATG5$^{-/-}$ cells were transduced with lentivirus coding for ATG5 in order to restore autophagy-competence. Transduced cells were named MEFATG5. In **Figure 31A**, expression of ATG5 is detected by immunoblotting in MEFATG5 cells, demonstrating successful transduction of cells with *Atg5*. Furthermore, in contrast to their autophagy-deficient progenitor cells MEFATG5$^{-/-}$, MEFATG5 cells possess the ability to convert LC3-I into LC3-II, representing restored ability to form autophagosomes and autophagy competence, observed upon serial passaging of cells (**Figure 31B**).

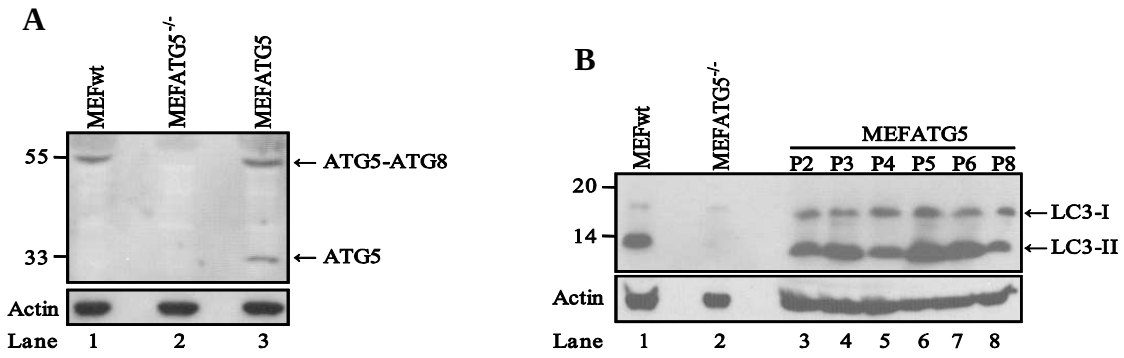


Figure 31. Autophagy-competence of MEFATG5 cells. (A) Upon lentiviral reintroduction of *Atg5* in MEFATG5$^{-/-}$, cells were analyzed for ATG5 expression by immunoblotting using anti-Atg5 mAb. Whereas ATG5 signals are lacking in MEFATG5$^{-/-}$ cells, ATG5-ATG8 complex is detected both in MEFwt and in lentivirally transduced MEFATG5 cells. Single ATG5 band (33kDa) is detected in MEFATG5 cells only (lane 3). **(B)** MEFATG5 cells were serially passaged and lysed after passage (P) 2, 3, 4, 5, 6, and 8 and analyzed by immunoblotting using anti-LC3 mAb. In contrast to MEFATG5$^{-/-}$, both MEFwt and MEFATG5 cells are able to convert LC3-I into LC3-II, indicating autophagosome formation and autophagy competence.

To analyze the ability of autophagosome formation in more detail, transduced MEFATG5, MEFwt, and MEFATG5$^{-/-}$ cells were transfected with the plasmid GFP-LC3, treated with

Glivec in order to induce autophagosome formation, and analyzed by confocal microscopy (**Figure 32**). GFP-LC3 puncta were observed in high amounts in the cytosol of MEFwt and MEFATG5 cells, representing autophagosome formation and autophagy-competence. In contrast, autophagy-deficient MEFATG5$^{-/-}$ cells exhibit mainly diffuse expression of GFP-LC3 in the cytosol and are therefore not able to form autophagosomes.

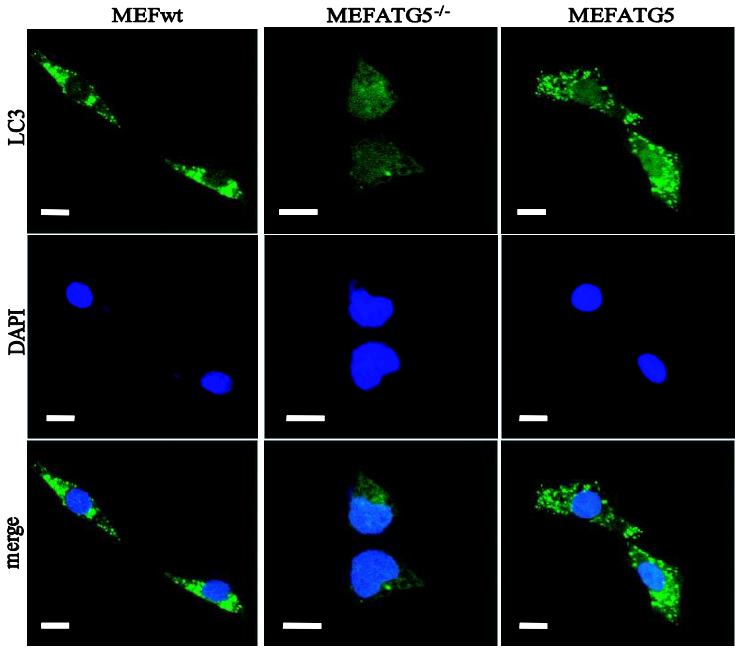


Figure 32. MEFATG5 cells are able to induce autophagosome formation. MEFwt, MEFATG5$^{-/-}$, and transduced MEFATG5 cells were transfected with GFP-LC3, treated with Glivec to induce autophagosome formation, and analyzed by confocal microscopy. GFP-LC3 puncta in the cytosol, representing autophagosome formation and autophagy-competence, was observed in MEFwt and MEFATG5 cells. In contrast, MEFATG5$^{-/-}$ cells showed mainly diffuse GFP-LC3 expression in the cytosol.

IV.B.3.2 INCREASED SUSCEPTIBILITY TO PRIMARY PRION INFECTION UPON REINTRODUCTION OF *ATG5* IN MEFATG5$^{-/-}$ CELLS

As MEFwt are more prone to primary prion infection than MEFATG5$^{-/-}$ cells, transduced autophagy-competent MEFATG5 cells (IV.B.3.1) were inoculated with 22L prion-infected brain homogenate, or mock brain as a control, to analyze weather reintroduction of autophagy-competence renders cells more susceptible to primary prion infection. As a control, MEFATG5$^{-/-}$ cells were transduced with lentivirus coding for GFP to exclude influence of lentivirus on primary prion infection. Cells were named MEFGFP. **Figure 33** demonstrates that up to 100 % of MEFATG5$^{-/-}$ cells transduced with lentivirus coding for GFP express the recombinant protein.

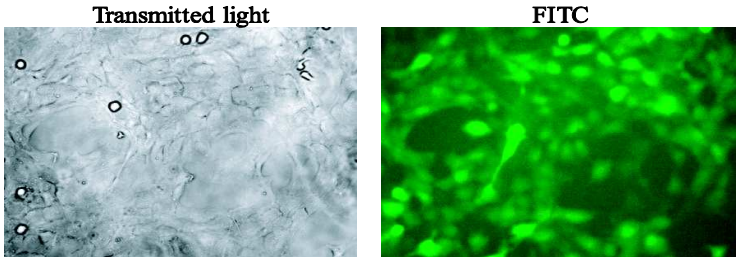


Figure 33. GFP-expression in transduced MEFGFP cells. MEFATG5$^{-/-}$ cells were transduced with lentivirus coding for GFP (MEFGFP). MEFGFP cells served as a control in primary prion infection. GFP expression in MEFGFP cells was analyzed in a fluorescence microscope and is shown in the right hand panel. Left hand panel depicts transmitted light. Up to 100 % of MEFGFP cells express GFP.

Subsequently, MEFwt, MEFATG5$^{-/-}$, MEFGFP, and MEFATG5 cells were inoculated with 22L prion-infected brain homogenate, or mock brain as a control, and analyzed for PrP^{Sc} at P3 and P5 (**Figures 34A and B**). Additionally, lysates were probed with anti-GFP and anti-LC3 mAb`s to control GFP expression and autophagy-competence (**Figures 34C and D**). At both time points analyzed, the amount of PrP^{Sc} was significantly increased in MEFATG5 cells when compared to either MEFATG5$^{-/-}$ or MEFGFP control cells (**Figures 34E and F**), indicating that basal autophagy promotes primary prion infection.

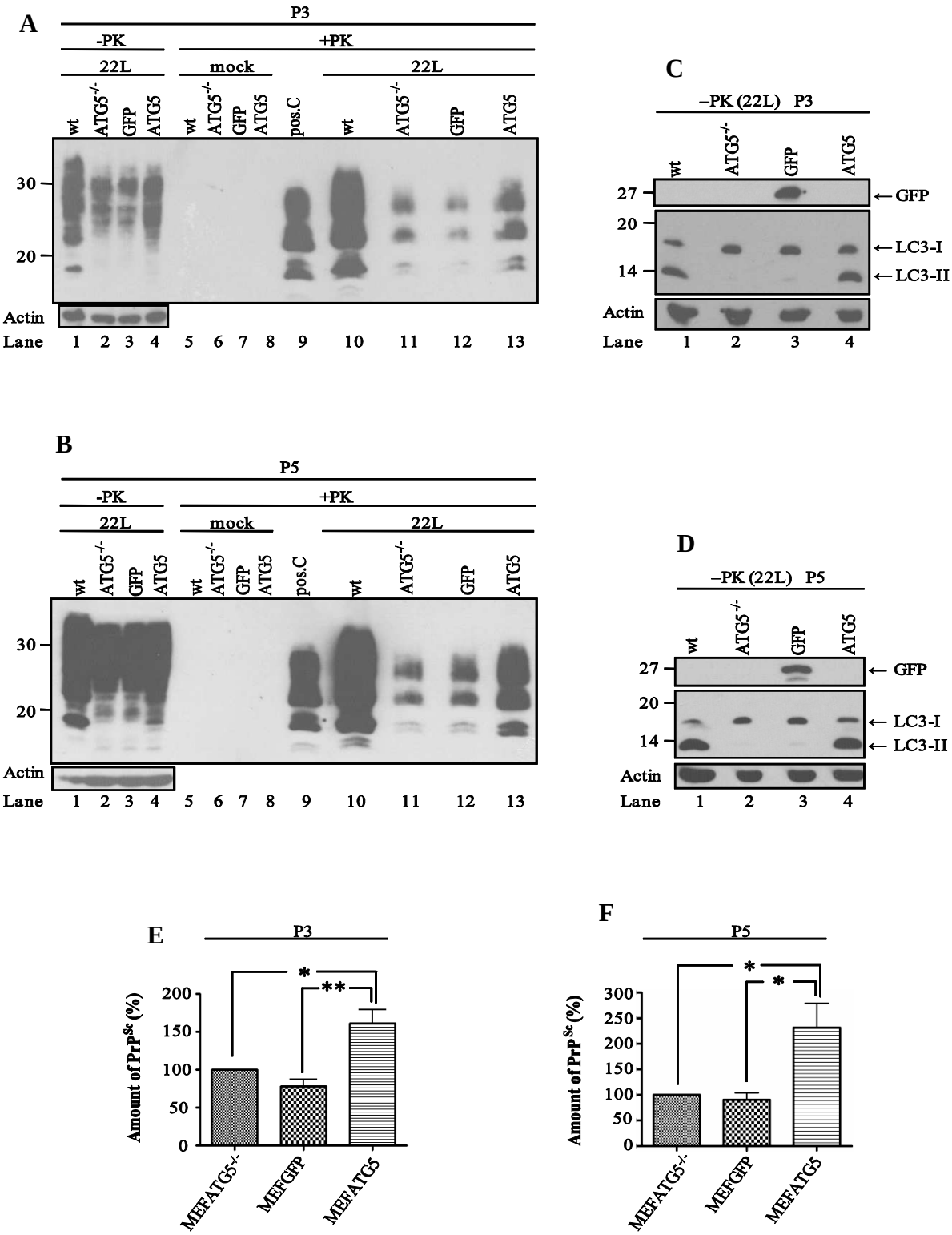


Figure 34. Basal autophagy promotes primary prion infection. (A, B) MEFwt, MEFATG5$^{-/-}$, MEFGFP, and MEFATG5 cells were infected with 22L prions, or mock as a control, and analyzed at passage 3 (P3) and P5 by immunoblotting using anti-PrP mAb 4H11. Increased amounts of PrP^{Sc} were detected in MEFATG5 compared to both MEFATG5$^{-/-}$ and MEFGFP cells at P3 and P5 (compare lanes 11, 12, and 13 of each panel). Lysate of prion-infected cells was used as positive control for PrP^{Sc} (pos.C). **(C, D)** PK-undigested lysates (-PK) from P3 and P5 of 22L prion-infected MEFwt, MEFATG5$^{-/-}$, MEFGFP, and MEFATG5 cells were probed with anti-LC3

mAb. LC3-II signals were detected in autophagy-competent MEFwt and MEFATG5 whereas MEFATG5$^{-/-}$ and MEFGFP cells are not able to convert LC3-I into LC3-II, verifying autophagy-deficiency. Additionally, lysates were probed with anti-GFP polyclonal Ab to verify GFP expression in MEFGFP cells. **(E, F)** Significantly increased amounts of PrP^{Sc} were detected in autophagy-competent MEFATG5 compared to both MEFATG5$^{-/-}$ and MEFGFP cells at P3 and P5 upon primary prion infection, indicating that basal autophagy promotes primary prion infection. Values represent the mean ± SEM of three independent experiments.

V. DISCUSSION

V.A. AUTOPHAGY INDUCTION MEDIATES REDUCTION OF PrP^{Sc}

In the recent past, induction of autophagy has been seen to accelerate reduction of aggregate-prone proteins causing several neurodegenerative diseases. Therefore, the first part of this study focused on whether induction of autophagy might be a potential new pathway for degradation of PrP^{Sc}, the pathogenic agent thought to be responsible for development of prion disease. In order to induce autophagy, the compound lithium was utilized which has been shown to mediate reduction of mutant forms of α–synucleins and huntingtin fragments causing Parkinson`s and Huntington`s disease, respectively.

Lithium treatment of prion-infected neuronal and non-neuronal cells revealed an intense reduction of PrP^{Sc}. Immunoblot analysis and confocal laser microscopy showed that lithium is inducing autophagy in prion-infected cells. Evidence for the first time that induction of autophagy is responsible for enhanced degradation of PrP^{Sc} was obtained by use of 3-methyladenine (3-MA), a known potent inhibitor of autophagy, which blocked the anti-prion effect of lithium. Simultaneous application of lithium and rapamycin, a drug widely used to induce autophagy, enhanced the anti-prion effect compared to either compound alone. Furthermore, several lithium salts possess both anti-prion effects and the ability to induce autophagy. To a minor extent as compared to the degradation of PrP^{Sc}, FACS analysis revealed that lithium treatment also slightly, but significantly, reduced the levels of PrP^{c} in an autophagy-dependent manner. This provides evidence that besides direct degradation of cellular PrP^{Sc}-aggregates by induction of autophagy, lithium may also limit the substrate for conversion into PrP^{Sc}, which in addition contributes to the observed anti-prion effect. Besides, several other autophagy-inducing agents were utilized for treatment of prion-infected cells. Thereby, it could be demonstrated that all these compounds used in this study possess potent anti-prion effects, verifying that induction of autophagy in general results in reduced levels of cellular PrP^{Sc} in persistently prion-infected cells. Finaly, treatment of prion-infected mice with rapamycin prolonged survival times, demonstrating that autophagy-inducing drugs may have therapeutic potential in treatment of prion disease.

V.A.1. LITHIUM REDUCES PrP^{Sc}

Fifty years ago the clinical studies by Mogens Schou revealed the effectiveness of lithium chloride in treatment of manic-depressive illnesses (MDI, bipolar affective disorder). Since

that time a variety of benefits in the treatment of mood disorders, including acute anti-manic and anti-depressant effects, anti-depressant potentiating effects, long term prophylactic effects, and probably also anti-suicidal effects (Goodwin and Jamison 1990; Baldessarini et al. 1999) were attributed to lithium. Several neuroprotective effects of lithium have been described *in vitro* and *in vivo*. In primary neuronal cultures and in neuroblastoma cells, lithium significantly decreased prion peptide-induced cell death (Perez et al. 2003). Moreover, cell death induced by the pathogenic amyloid β (Aβ) peptide, which is deposited in the brain of Alzheimer's disease patients (Haass 2004), is significantly reduced in cultured neurons treated with lithium (Alvarez et al. 1999). With regard to levels of neuroprotective bcl-2 in rat cerebellar granule cells (Chen and Chuang 1999), a protein found to inhibit neuronal cell death by decreasing the generation of reactive oxygen species has been demonstrated to be increased upon lithium administration (Kane et al. 1993). Recently, *in vivo* studies of Amyotrophic Lateral Sclerosis (ALS) have revealed that lithium stimulates the biogenesis of mitochondria in both the central nervous system (CNS) and the spinal cord and, furthermore, induces neurogenesis and neuronal differentiation (Fornai et al. 2008a). Furthermore, lithium has been seen both to slow progression of ALS in human patients and to induce autophagy (Fornai et al. 2008b).

In line with the above described neuroprotective effects of lithium, the study here reveals for the first time that lithium is significantly enhancing the cellular clearance of the pathological isoform of the prion protein (PrP^{Sc}) in neuronal and non-neuronal cell lines in a time-dependent manner (**Figures 14, 16 and 17**). Of note, viability of lithium treated cells was not affected under the used conditions (**Figure 15**).

V.A.2. INDUCTION OF AUTOPHAGY BY LITHIUM MEDIATES REDUCTION OF PRP^{SC} IN CELLS PERSISTENTLY INFECTED WITH PRIONS

Induction of autophagy, mediated by lithium, has been seen to accelerate the clearance of mutant huntingtin and α-synucleins (Sarkar et al. 2005). The beneficial effect of up-regulated autophagy has also been described for other diseases associated with aggregate-prone proteins, such as Alzheimer`s disease, forms of motor neuron disease caused by mutations in superoxide dismutase 1 (SOD1), and forms of peripheral neuropathy caused by mutations in peripheral myelin protein 22 (PMP22) (Fortun et al. 2003; Berger et al. 2006; Kabuta et al. 2006). Evidence for the neuroprotective effect of autophagy was observed in mice deficient in autophagy specifically in the CNS (Hara et al. 2006; Komatsu et al. 2006). These mice

spontaneously developed symptoms of neurodegenerative disease without any disease causing agent.

Concerning prion disease we could show in the recent past that the drug Glivec is a potent inducer of autophagosome formation (Ertmer et al. 2007). Furthermore, Glivec has potent anti-prion activity (Ertmer et al. 2004), although direct correlation of autophagy and reduction of PrP^{Sc} was lacking. This study here provides evidence that autophagy is induced in lithium treated prion-infected cells (**Figures 12 and 13**). Moreover, by co-treatment of prion-infected cells with 3-MA, a widely used inhibitor of autophagy, it could be demonstrated for the first time that lithium-induced autophagy is mediating clearance of prion-disease associated PrP^{Sc} (**Figure 19**).

The mechanism of lithium-induced autophagy has been shown to be based on inhibition of inositol monophosphatase (IMPase) in an mTOR-independent manner. Down-regulation of IMPase activity results in decreased levels of free inositol, leading to lowered myo-inositol-1,4,5-triphosphate (IP_3) levels (Sarkar et al. 2005). IP_3 and the stimulation of its receptor (IP_3R) have been seen to suppress autophagy (Criollo et al. 2007). Another target of lithium is glycogen synthase kinase-3β (GSK-3β) which, in contrast to the above described autophagy-inducing properties of lithium, results in inhibition of autophagy, as GSK-3β is inhibiting mTOR under physiological conditions (Sarkar et al. 2008). As a consequence of these antithetic influences of lithium on the level of autophagy activity, *in vivo* co-treatment with lithium and rapamycin in a Huntington`s disease fly model revealed enhanced neuroprotective effects than treatment with either compound alone (Sarkar et al. 2008). In line with these results this study here demonstrates that enhanced clearance of PrP^{Sc} is obtained when prion-infected cells are co-treated with lithium and rapamycin compared to either drug alone (**Figure 19**).

Multi-vesicular bodies (MVBs) are known to fuse with autophagosomes (Liou et al. 1997; Berg et al. 1998) and MVBs have also the ability to fuse with the plasma membrane to be released as exosomes into the extracellular medium (Johnstone 1992). Exosomes have been seen to deliver prions into cell culture medium (Fevrier et al. 2004). In this study, less PrP^{Sc} was detected in serum-depleted medium of lithium treated cells compared to control cells, excluding that induction of autophagy might result in increased exportation of PrP^{Sc} into the extracellular space by induced release of exosomes containing PrP^{Sc} (**Figure 18**). Reduced cellular levels of PrP^{Sc} upon lithium treatment are therefore rather based on intracellular clearance mediated by the autophagic degradation machinery than on exportation into the cell culture medium.

Besides the classically administered drug lithium chloride, a pilot clinical study recently revealed that lithium carbonate treatment slows progression of ALS in human patients and induces autophagy (Fornai et al. 2008b). Our study provides evidence that several lithium salts are able to induce autophagy in prion-infected cell lines and possess prion clearing effects (**Figure 21**). Whether this is also the case for other aggregate-prone proteins, such as mutant huntingtin or α-synucleins, remains to be established.

V.A.3. LITHIUM REDUCES THE AMOUNT OF PRP^C

It has been shown that $Prnp^{0/0}$ mice remain healthy upon prion inoculation and that PrP^c is absolutely required for susceptibility to prion infection (Bueler et al. 1993). In line with this, Mallucci and colleagues demonstrated that also post-natal PrP knockout does not result in neurodegeneration, ruling out loss of PrP function as a primary pathogenic mechanism in prion disease and validating therapeutic approaches targeting PrP (Mallucci et al. 2002; Mallucci et al. 2007). Our FACS analysis showed that also the level of PrP^c is reduced upon lithium treatment in an autophagy-dependent manner (**Figures 23 and 24**). In lithium-treated, autophagy-deficient fibroblasts ($MEFATG5^{-/-}$) reduction of PrP^c was not observed, whereas the level of PrP^c was reduced in wild-type fibroblasts upon lithium treatment. PrP^c localizes via a glycosylphosphatidylinositol (GPI)-anchor at the outer leaflet of the plasma membrane in cholesterol- and sphingolipid-rich microdomains (Taraboulos et al. 1995) and can move laterally to detergent-soluble domains within the plasma-membrane for subsequent internalization (Sunyach et al. 2003). Autophagosomes are thought to fuse with endosomes or MVBs among other compartments (Liou et al. 1997; Berg et al. 1998). Therefore, it is possible that upon internalization PrP^c is in reach of the autophagic degradation machinery. In the recent past it has been shown that reduction of PrP^c by either shedding of the protein from the membrane or by down-regulation of PrP^c reduces conversion of PrP^c into its pathogenic isoform PrP^{Sc} by limiting the amount of PrP^c substrate available for conversion (Marella et al. 2002; Parkin et al. 2004; Aguib et al. 2008; Heiseke et al. 2008). Thus, reduction of PrP^c by lithium-induced autophagy may indirectly contribute to reduction of PrP^{Sc} by autophagy. As a note of caution, reduced levels of PrP^c upon treatment of cells with other autophagy-inducing compounds were not observed indicating that this phenomenon may be compound-specific and that the exact molecular mechanism remains to be deciphered (Yasmine Aguib, personal communication).

V.A.4. SEVERAL AUTOPHAGY INDUCERS REDUCE PrP^{Sc} IN CELLS PERSISTENTLY INFECTED WITH PRIONS

Rapamycin, which is a lipophilic, macrolide antibiotic, induces autophagy by inactivating mTOR, and as such serves as an autophagy enhancer (Berger et al. 2006). It has been seen that rapamycin attenuated toxicity of mutant huntingtin fragments in cells (Ravikumar et al. 2002), transgenic *Drosophila*, and mouse models (Ravikumar et al. 2004). Besides, autophagy induction mediated by rapamycin decreased toxicity in flies expressing both wild-type tau and mutant tau that cause FTD and also reduced aggregate-prone proteins with polyalanine expansion mutations causing diseases like oculopharyngeal muscular dystrophy (Berger et al. 2006). In line with these neuroprotective effects of rapamycin in several neurodegenerative diseases, lower amounts of pathogenic PrP^{Sc} were detected in ScN2a cells when treated with the autophagy inducer rapamycin (**Figure 20**). Another autophagy-inducing compound used in this study was trehalose which has been seen in experimental systems to mediate clearance of mutant Huntingin and α–synuclein by induction of autophagy (Sarkar et al. 2007). Similar reduction of PrP^{Sc} levels was observed in ScN2a cells treated with trehalose when compared with rapamycin treated cells (**Figure 20**). Furthermore, in a different study we were able to demonstrate by both pharmacological and genetical inhibition of autophagy that induction of autophagy mediated by trehalose is responsible for reduction of PrP^{Sc}. These findings underline that clearance of disease-associated PrP^{Sc} seems to be a general phenomenon of activated autophagy in persistently prion-infected cells in a compound independent manner. Moreover, treatment of prion-infected cells with 3-MA or wortmannin, drugs widely used to inhibit autophagy, resulted in increased levels of PrP^{Sc}, suggesting a physiological role of autophagy in degradation of PrP^{Sc}. This increased PrP^{Sc}-level might result in an enhanced conversion of PrP^{c} into PrP^{Sc}, subsequently leading to more cellular PrP^{Sc} (Aguib*, Heiseke* et al. 2009; * contributed equally). This phenomenon goes in line with other studies which demonstrated that interruption of autophagy by either 3-MA treatment or siRNA knock-down of autophagy genes increased polyglutamine aggregation associated with Huntington`s disease (Ravikumar et al. 2002; Iwata et al. 2005; Shibata et al. 2006). Of note, previous studies which demonstrated clearance of aggregate-prone proteins as mediated by induction of autophagy exclusively concerned cytosolic toxic proteins. Therefore, the exact molecular mechanisms how induced autophagy accelerates reduction of PrP^{Sc} are still incompletely understood, in particular as the very vast majority of PrP^{Sc}/prions reside within endosomal

and lysosomal vesicles. On the other hand this clearly shows that not only cytosolic materials are prone to autophagic degradation.

V.A.5. mTOR-DEPENDENT VERSUS mTOR-INDEPENDENT AUTOPHAGY INDUCTION

Recently, a cyclical mTOR-independent pathway regulating autophagy has been described (Williams et al. 2008). With regard to the therapeutic efficacy of pharmacologically induced autophagy in prion disease scenarios, the fact may be important by which pathway induction is mediated, i.e. mTOR-dependent or -independent. Treatment of prion-infected cells with either lithium, Glivec or trehalose, compounds inducing autophagy in an mTOR-independent manner, all revealed significantly reduced levels of PrP^{Sc} (**Figure 20**). With respect to rapamycin, which specifically blocks mTOR and therefore induces autophagy in an mTOR-dependent manner, clearance of PrP^{Sc} was also observed in prion-infected neuronal cells, verifying that both pathways can be involved in reduction of PrP^{Sc}. Moreover, rapamycin let to a small but significant prolongation of survival times in prion-infected mice (**Figure 25**). On the other hand, in preliminary cell culture studies, we observed that an analogon of rapamycin (kindly provided by Novartis Pharma AG, CH-4002, Basel, Switzerland) is lacking anti-prion efficacy in concentrations which are sufficient to induce autophagy (Yasmine Aguib, personal communication), pointing to a less efficient PrP^{Sc} degradation potential for mTOR-dependent induction of autophagy. Differing impact on prion clearance efficacy might also be true for different drugs inducing autophagy in an mTOR-independent manner as such induction of autophagy might be mediated through different non-overlapping pathways with unequal PrP^{Sc} clearance properties. Furthermore, to combine the two major routes for autophagy induction, co-treatment with lithium and rapamycin enhanced neuroprotective effects in a Huntington`s disease fly model (Sarkar et al. 2008) and co-treatment here of prion-infected cells with both substances increased reduction of PrP^{Sc} compared to either compound alone (**Figure 19**). Combining mTOR-dependent and –independent compounds in order to amplify anti-prion efficacy of autophagy induction might represent a powerful strategy in treatment of prion diseases and will be subject of further *in vitro* and *in vivo* studies. In summary, further analysis will shed light onto the possible therapeutic potential and efficacy of the different autophagy-inducing pathways. Overall, the anti-prion efficiency of drugs inducing autophagy in an mTOR-dependent manner remains to be compared to drugs inducing autophagy mTOR-independently in order to reveal similarities, differences, and

additive or even deleterious effects of both pathways with respect to PrP^{Sc} clearance properties.

V.A.6. IS THERE THERAPEUTIC POTENTIAL OF AUTOPHAGY INDUCTION IN TREATMENT OF PRION DISEASE?

In recent years, *in vivo* studies in animals and even in humans demonstrated that induction of autophagy results in reduction and attenuated toxicity of aggregate-prone proteins causing neurodegenerative disease, accompanied by expanded survival time of disease-affected organisms. Moreover, in contrast to activated autophagy, neural-tissue specific loss of autophagy in mice (knock-out of essential autophagy genes) has been seen to result in development of neurodegenerative disease without any disease-associated mutant proteins, suggesting a neuroprotective role of autophagy per se (Hara et al. 2006; Komatsu et al. 2006). In line with this, a recent study showed that the liver-specific knock-out of *Atg7*, an essential autophagy-related gene, leads to an increase in the propensity of protein aggregation in the liver of mice (Komatsu et al. 2005). Concerning neuroprotection mediated by induced autophagy, in a genetical ALS animal model marked neuroprotection accompanied by both delayed disease onset and duration with augmented life span was observed when mice were treated with lithium. These effects were concomitant with activated autophagy, attenuated astrogliosis and elevated amount of mitochondria in motor neurons. Furthermore, lithium treatment delayed disease progression in human patients affected with ALS (Fornai et al. 2008b; Fornai et al. 2008a). With regard to rapamycin as a potential drug in therapy against neurodegeneration, rapamycin attenuated toxicity of mutant huntingtin fragments in transgenic *Drosophila* and mouse models (Ravikumar et al. 2004), decreased toxicity in flies expressing both wild-type tau and mutant tau that cause FTD, and enhanced clearance of aggregate-prone proteins with polyalanine expansion mutations *in vivo* (Berger et al. 2006). The protective effects of rapamycin in these situations appeared to be autophagy-dependent, as its protective ability was abolished when autophagy activity was interrupted, either by siRNA knock-down of *Atg12* or when mutant proteins causing disease were expressed on a genetic background with 50 % loss of the key autophagy gene *Atg1* (Berger et al. 2006; Pandey et al. 2007).

In order to assess the therapeutic potential of autophagy-inducing compounds in prion disease scenarios, mice were intracerebrally infected with prions and lithium or rapamycin was orally given to mice starting at day 100 post infection, mimicking a preclinical therapeutic

intervention situation (Mok et al. 2006; Riemer et al. 2008). Treatment of prion-infected mice with rapamycin showed a small but significant therapeutic effect ($p<0.05$). In contrast, we observed no significant prolongation of survival times upon administration of lithium (**Figure 25**). However, survival times in the lithium group showed of unknown reasons an increased spread compared to controls (53 versus 25 days), which may have masked a small therapeutic effect of lithium. The lack of significantly prolonged survival time in prion-infected mice treated with lithium might also be due to the fact that the anti-prion effect of lithium was observed in quite high concentrations in cell culture (10 mM), which might not be obtained in the CNS in the *in vivo* situation. Furthermore, for both drugs it is possible that treatment was initiated not early enough to have a clearer effect on disease progression. Besides, as a rational combination treatment approach with lithium and rapamycin revealed greater protection against neurodegeneration in a Huntington`s disease fly model compared with either compound alone (Sarkar et al. 2008), it seems reasonable to combine these two drugs for treatment of prion disease in order to enhance survival times of mice.

Previously, our group showed that Glivec, a drug used to treat chronic myelogenous leukemia, is activating lysosomal degradation of PrP^{Sc} (Ertmer et al. 2004) and is at the same time a potent inducer of autophagy and/or autophagosome formation (Ertmer et al. 2007). In prion-infected mice, Glivec treatment at an early phase of peripheral infection delayed both the neuroinvasion of PrP^{Sc} and the onset of clinical disease (Yun et al. 2007). Unfortunately, drug application at time points when neuroinvasion was already accomplished provoked no clear PrP^{Sc} clearance effects in the CNS, probably due to ineffective BBB crossing of the drug.

In another study, we were utilizing trehalose, which has been seen to accelerate clearance of mutant forms of huntingtin and α–synucleins in experimental systems (Sarkar et al. 2007). Our studies revealed that treatment of prion-infected mice with trehalose did not prolong incubation times, but clearly showed effects on the appearance of PrP^{Sc} in spleens (Aguib*, Heiseke* et al. 2009; * contributed equally). Depending on when trehalose treatment was started, peripheral accumulation of PrP^{Sc} was delayed. As was the case with Glivec treatment, this probably also reflects that the process of neuroinvasion was decelerated. Noteworthy, in terms of trehalose this finding was not unexpected as the anti-prion effect in cell culture was highly dose-dependent and the effective anti-prion concentration of 100 mM is probably not achievable in brain tissue.

Further *in vivo* studies using compounds which induce autophagy at low concentrations and are at the same time able to cross the BBB will have to reveal the effectiveness of autophagy

induction in treatment of prion disease. Nevertheless, the results obtained with rapamycin indicate that such drugs might represent a novel avenue for therapy in prion disease.

V.B. ROLE OF BASAL AUTOPHAGY IN PRIMARY PRION INFECTION

In neuronal and non-neuronal cultured cells persistently infected with prions, reduced levels of PrP^{Sc} were observed when autophagy was pharmacologically induced (see V.A.). In contrast to the role of activated autophagy in persistent infection situations, the second part of this study focused on the role of basal, physiological autophagy in primary prion infection. When PrP^{Sc}-susceptible N2a clones were primary prion-infected, increased amounts of the autophagosomal marker protein LC3-II were detected when cells propagated newly converted PrP^{Sc} for the first time. In contrast, in a PrP^{Sc}-unsusceptible N2a clone, which is unable to convert and propagate PrP^{Sc}, autophagosome formation was not observed upon primary prion infection. This phenomenon indicates that autophagosome formation is induced when cells are able to convert endogenous PrP^{c} into PrP^{Sc} upon primary prion infection.

To further shed light into the role of basal constitutive autophagy in primary prion infection, autophagy-incompetent MEFs ($MEFATG5^{-/-}$) and their wild-type counterparts (MEFwt) were freshly prion-infected. Much less efficient susceptibility to prions was observed in $MEFATG5^{-/-}$ when compared to MEFwt cells. In turn, reintroduction of ATG5 into $MEFATG5^{-/-}$ rendered cells more susceptible to prions, indicating that basal autophagy enhances primary prion infection.

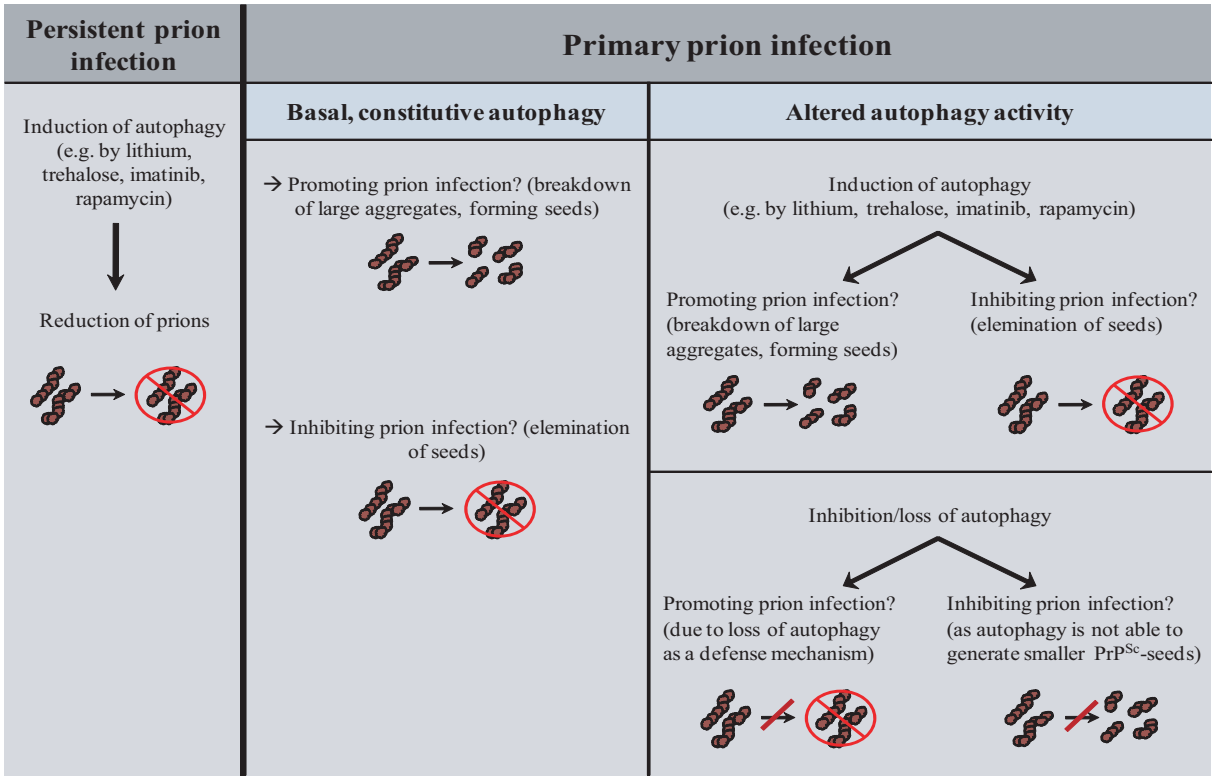
V.B.1. MULTIPLE ROLES OF AUTOPHAGY IN PRIMARY PRION INFECTION?

The first part of this study gave evidence that induction of autophagy accelerates reduction of PrP^{Sc} in cells persistently infected with prions. However, concerning primary prion infection, several different functions of autophagy are conceivable which will be discussed in this chapter (**Table 9**).

Under physiological conditions, when cells are maintaining a basal constitutive rate of autophagy, which is present ubiquitously, different impacts on primary prion infection are possible. On the one hand, basal autophagy might promote primary prion infection due to breakdown of large aggregates forming smaller PrP^{Sc}-seeds, which are known to be more

efficient templates for prion conversion (Silveira et al. 2005). On the other hand, a vice versa scenario might be true, meaning that basal autophagy accomplishes degradation of such highly infectious PrP^{Sc}-seeds, thereby inhibiting primary prion infection. Besides constitutive autophagy, alterations of autophagy activity (increased or decreased activity), e.g. by compounds inducing or inhibiting autophagy or knock-down and knock-out of essential autophagy genes, might influence primary prion infection. Similar to the different possibilities of basal autophagy, induction of autophagy might either result in enhanced or inhibited primary prion infection, depending on whether induced autophagy is producing higher levels of infectious PrP^{Sc}-seeds or mediates elimination of such seeds. Derogation of autophagy activity by either inhibition or loss of autophagy function (knock-down or knock-out of autophagy genes) also might have different outcomes. Decreased autophagy activity might promote primary prion infection, because autophagy as a putative defense mechanism is lacking and therefore might not function in elimination of PrP^{Sc}. In contrary, inhibition or loss of autophagy might also counter-act primary prion infection as autophagy might be unable to produce highly infectious PrP^{Sc}-seeds.

Table 9. Putative different functions of autophagy in primary prion infection



V.B.2. AUTOPHAGOSOME FORMATION IS ACTIVATED UPON PRIMARY PRION INFECTION

Since quite recently, proof for existence of basal, constitutive autophagy in neurons has mainly been elusive because, in contrast to other cells, autophagosomes are barely detectable in neurons by electron microscopy or by confocal microscopy using fluorescent labeled protein reporters (Mizushima et al. 2004; Nixon et al. 2005). However, attesting the existence of autophagy as a basal constitutive process in neural tissue, it has been demonstrated a couple of years ago that autophagy is an essential pro-survival process in neurons (Hara et al. 2006; Komatsu et al. 2006). Moreover, in contrast to the assumption that autophagy is present in neurons only in quite low activity due to curtness of observed autophagosomes in healthy brain, studies with primary cortical neurons revealed a relatively active role of autophagy with a quick turn-over of autophagosomes (Boland et al. 2008).

In neurodegenerative diseases, lysosomes and autophagosomes have been seen to proliferate in striatal neurons expressing mutant huntingtin (Kegel et al. 2000) and in Huntington`s (Sapp et al. 1997) and Alzheimer`s disease brains (Nixon et al. 2000), although such morphological studies in neurodegenerative disease cannot distinguish between a role for autophagy in cytoprotection or cell death. Concerning Alzheimer`s disease it has been reported that autophagy is induced and impaired leading to accumulation of autophagosomes in diseased brains of affected organisms. Furthermore it was proposed that autophagosomes represent a novel site for toxic Aβ peptide production because purified autophagosomes from such brains contain APP, β-cleaved APP, and are highly enriched in presenilin 1, nicrastin, and presenilin 1-dependent γ-secretase activity (Yu et al. 2005). On the other hand, in contrary to the above described role of autophagy in Alzheimer`s disease, it has been seen that the autophagic machinery plays a neuroprotective role against Aβ-induced neurotoxicity (Hung et al. 2009).

In prion disease, the appearance of autophagosomes in brains of disease affected organisms and also in cultured cells persistently infected with prions has been reported in several studies (Boellaard et al. 1989; Boellaard et al. 1991; Schatzl et al. 1997; Liberski et al. 2004; Sikorska et al. 2004). Concerning the role of autophagy in prion disease, it was proposed that autophagy may contribute in the formation of spongiform change, a pathological hallmark in TSE affected brains, and may be activated by apoptosis (Liberski et al. 2002; Liberski et al. 2004; Liberski et al. 2008). However, in contrast to the assumption that autophagy in general may play a disease-promoting role, it is also quite conceivable that the observed increase in autophagic vacuoles in TSEs is due to cellular activation of the autophagic machinery as a

defense mechanism, leading to degradation of prions. Nevertheless, proof of evidence for a beneficial or deleterious role of basal autophagy in prion disease is still missing.

In contrast to previous studies which focused on monitoring autophagy, or appearance of autophagic vacuoles, respectively, in persistently prion-infected cells or in model organisms in which disease already is manifested, this study also focused on monitoring autophagy in primary prion infection situations. An increase in LC3-II, the autophagosomal marker protein, was observed in PrP^{Sc}-susceptible neuronal cells upon primary prion infection (**Figures 26, 27 and 28**). Such increase in autophagosome formation was only detected when cells initially propagated newly converted PrP^{Sc} and was not a constant phenomenon as at later time points the level of LC3-II seceded back to normal levels. Moreover, in cells which are not able to convert endogenous PrP^{c} into PrP^{Sc} (i.e. PrP^{Sc}-unsusceptible cells) increased levels of LC3-II were not detected (**Figures 28 and 29**). This phenomenon indicates that autophagy, or at least autophagosome formation, enhances primary prion infection. A potential explanation for this might be that autophagosomes/the autophagic machinery is breaking up larger PrP^{Sc}-aggregates forming smaller PrP^{Sc}-seeds which are known to be more efficient templates for prion conversion (Silveira et al. 2005). Thereby, autophagosomes/autophagy might enhance the process of primary prion infection. In turn, it might also be conceivable that PrP^{Sc}-unsusceptible cells do possess such ability less pronounced and might therefore be less susceptible to primary prion infection compared to PrP^{Sc}-susceptible cells. Of note, the observed increase in autophagosome formation upon primary prion infection cannot originate as a mere cellular response to inoculation with (PrP^{Sc})-aggregates as PrP^{Sc}-unsusceptible cells (which are autophagy competent) do not show an increase in autophagosome formation, though also incubated with prions. It will be subject of further analysis whether cells in general exhibit an increase in autophagosome formation/autophagy activity when inoculated with PrP^{Sc}-aggregates. For this purpose, however, time points very shortly after inoculation of cells with PrP^{Sc}-aggregates containing material have to be analyzed, as the time points which were used in this study are probably too late for such analysis.

From the above described results it is not possible to discriminate whether the detected increase in autophagosome formation upon primary prion infection is due to autophagosome accumulation or increase in autophagy activity. Due to the fact that in Alzheimer`s disease accumulation of autophagosomes has been reported and autophagosomes are suggested to represent a compartment for toxic Aβ peptide generation (Yu et al. 2005), it might also be possible that increased levels of autophagosome formation upon primary prion infection are caused by impaired autophagic flux which might promote prion infection. Arguing for the

opposite, that the observed autophagosome formation upon primary prion infection points to an activation of the autophagic machinery as a whole intact process, in preliminary data we could observe that cells treated with bafilomycin A1, a widely used drug to inhibit autophagosome-lysosome fusion resulting in accumulation of autophagosomes, are less susceptible to primary prion infection compared to control cells (Yasmine Aguib, personal communication). This finding indicates that mere autophagosome accumulation might not be responsible for increased susceptibility to primary prion infection. Therefore, the detected increase in autophagosome formation in PrP^{Sc}-susceptible cells can be interpreted as an increase in autophagic activity in general and not as impaired autophagy or autophagic flux, respectively.

V.B.3. BASAL AUTOPHAGY PROMOTES PRIMARY PRION INFECTION

Neural tissue specific knock-out of autophagy in mice results in development of neurodegenerative symptoms without expression of any mutant protein (Hara et al. 2006; Komatsu et al. 2006). In both *Atg5* and *Atg7* knock-outs, considerable accumulation of polyubiquitylated proteins, which appeared as inclusion bodies whose size and number increased with aging, was observed among other histological alterations. Another study demonstrated that inactivation of autophagy in transgenic *C. elegans* (expressing polyQ expansion tracts) increased the accumulation of disease associated protein aggregates and enhanced their toxicity, indicating protective effects of autophagy in preventing disease caused by polyQ expansion proteins (Jia et al. 2007).

In contrast to the above described protective effects of autophagy in preventing protein aggregation causing neurodegenerative disease, autophagy-deficient MEFATG5$^{-/-}$ cells were much less susceptibility to primary prion infection as compared to MEFwt cells (**Figure 30**). Furthermore, to verify whether this phenomenon is based on autophagy deficiency, *Atg5* was stably reintroduced into MEFATG5$^{-/-}$ cells to restore autophagy competence (**Figures 31 and 32**). Strikingly, such reconstituted MEFATG5 cells exhibited significantly increased susceptibility to primary prion infection compared to their autophagy-deficient counterparts (**Figure 34**), indicating that basal autophagy promotes primary prion infection. Due to the fact that prions are infectious and in primary prion infection cells are exposed to infectious aggregates derived from the extracellular space, which is in contrast to other neurodegenerative disease mainly characterized by accumulation and formation of aggregates consisting of mutant proteins expressed from the cell, the role of autophagy in prion disease might be different compared to other neurodegenerative diseases. Furthermore, in the above

mentioned *Atg5* and *Atg7* conditional knock-outs, the level of ubiquitin-positive proteinaceous aggregates increased over time. Accordingly, we cannot exclude that at later stages of prion disease, basal autophagy might also have beneficial effects in protein quality control by preventing PrP^{Sc}-aggregate formation, like obviously is the case for polyubiquitylated proteins that accumulate in inclusion bodies. We rather show here that the initial infection process is enhanced by a basal constitutive autophagy rate.

The first part of this discussion describes that pharmacological induction of autophagy is mediating reduction of PrP^{Sc} in persistently prion-infected cells (see V.A.). With regard to the primary prion infection process, preliminary results showed that cells treated with autophagy-inducing drugs are much less susceptible to primary prion infection compared to untreated controls that exhibit a basal constitutive autophagy rate (Yasmine Aguib, personal communication). This phenomenon indicates that disturbance of the physiological basal autophagy rate, both by knock-out and by pharmacological induction, results in alleviated susceptibility to primary prion infection. It seems conceivable that absence of autophagy might result in less ability of cells to generate smaller PrP^{Sc}-seeds, whereas strong induction of autophagy might lead to degradation of such highly infectious PrP^{Sc}-seeds, both phenomenon leading to less efficient primary prion infection. In line with these results, the temporary increase in autophagosome formation (only when newly converted PrP^{Sc} was detected) observed in PrP^{Sc}-susceptible cells upon primary prion infection (V.B.2), might therefore reflect a short-time moderate physiological elevation of autophagy in order to break up larger PrP^{Sc}-aggregates forming smaller PrP^{Sc}-seeds.

Concerning again primary prion infection of MEF cells, reconstituted MEFATG5 cells exhibited increased amounts of PrP^{Sc} upon primary prion infection when compared to their autophagy-deficient $MEFATG5^{-/-}$ counterparts. Though, MEFATG5 cells were not as susceptible to primary prion infection when compared to MEFwt cells (**Figure 34**). A possible explanation for this phenomenon might be that slightly increased amounts of ATG5 were detected in reconstituted MEFATG5 compared to MEFwt cells (single ATG5 band at 33 kDa is lacking in MEFwt) (**Figure 31A**), indicating slight overexpression of ATG5 in MEFATG5 cells. Overexpression of ATG5 has been seen to increase autophagy (Yousefi et al. 2006). As mentioned above, pharmacological autophagy induction results in less efficient primary prion infection. Therefore, this slightly elevated autophagy activity in MEFATG5 cells might be the reason that reconstituted MEFATG5 do not propagate as much PrP^{Sc} upon primary prion infection compared to MEFwt (**Figure 34**). A further reason for the different amounts of PrP^{Sc} in reconstituted MEFATG5 and MEFwt cells might by the fact that MEFwt

and MEFATG5$^{-/-}$ cells are derived from different mice and thus might possess different cellular factors influencing primary prion infection aside from autophagy. Nonetheless, data presented here verify that reconstituted MEFATG5 cells exhibit a sufficient level of basal autophagy rate to provide enhanced susceptibility to primary prion infection compared to autophagy-deficient MEFATG5$^{-/-}$ cells. Further studies in which ATG5 is overexpressed in reconstituted MEFATG5 cells in higher levels, sufficient to clearly induce autophagy beyond a basal constitutive autophagy rate, will have to reveal whether this results in inhibition of primary prion infection, as was the case for pharmacological autophagy induction during primary prion infection.

In summary, a basal moderate rate of autophagy seems to promote primary prion infection, whereas alterations of autophagy activity (either knock-out or pharmacological induction) have inhibitory effects on primary prion infection. To elucidate the exact mechanism of how basal autophagy promotes primary prion infection (e.g. by break down of large PrP^{Sc}-aggregates forming smaller PrP^{Sc}-seeds) remains to be determined and will be subject of further analysis. Another challenge will be to establish reliable *in vivo* models for studying prion infection and autophagy side by side. Faced with early lethality in *Atg* knock-out mice (Hara et al. 2006; Komatsu et al. 2006), one way to go might be crossing the available conditional knock-out mice, which are neuron-specifically floxed, with alternative Cre deleter mice to gain both postnatal knock-out and prolonged life time which then allows performing standard prion incubation time assays.

VI. Abbreviations

3-MA	3-methyladenine
A	Ampere
aa	Amino acids
Aβ	Amyloid β
ALS	Amyotrophic Lateral Sclerosis
APP	Amyloid precursor protein
APS	Ammoniumpersulfate
Asn	Asparagine
Atg	Autophagy related proteins
Baf A1	Bafilomycin A1
BBB	Blood-brain-barrier
BSE	Bovine spongiform encephalopathy
c	Concentration
°C	Degree Celsius
CJD	Creutzfeldt-Jakob disease
CLD	Caveolae-like domains
cm	Centimeter
CMA	Chaperone-mediated autophagy
CNS	Central nervous system
CWD	Chronic wasting disease
Cys	Cysteine
d	Day
Da	Dalton
Dest.	Destilled
DNA	Desoxyribonucleic acid
DNAase	Desoxyribonuclease
DOC	Sodium deoxycholate
dpi	Days post infection
DY	Drowsy
EDTA	Ethylenediamine-N,N,N´,N´-Tetraacetate
ER	Endoplasmatic reticulum
ERAD	ER-associated degradation
et al.	And others (´et alii´)
EUE	Exotic ungulate encephalopathy
FACS	Fluorescence activated cell sorting
fCJD	Familiar CJD
FCS	Fetal calf serum
FFI	Fatal familiar insomnia
FITC	Fluoresceine isothiocyanate
FTD	Frontotemporal dementia
FSE	Feline spongiform encephalopathy
g	Gram; acceleration of gravity

GAG	Glycosaminoglycan
GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol
GSS	Gerstmann-Sträussler-Scheinker
h	Hour
Hsc	Heat shock cognate
Hsp	Heat shock protein
HY	Hyper
Ig	Immune globuline
k	Kilo
Kb	Kilo base pairs
kDa	Kilodalton
l	Liter
LAMP	Lysosomal-associated membrane protein
LB	Luria-Bertani
LC3	Microtubule-associated protein 1 light chain 3
LiBr	Lithium bromide
$LiCH_3COO$	Lithium acetate
LiCl	Lithium chloride
Li_2CO_3	Lithium carbonate
LR	Laminin receptor
LRP	Laminin receptor precursor
m	Meter, mili
M	Methionine; molar
mAb	Monoclonal antibody
MBM	Meat-and-bone-meal
MEF	Mouse embryonic fibroblast cell line
MEFwt	Wild-type MEF
$MEFATG5^{-/-}$	Autophagy-deficient MEF
MEM	Minimal essential medium
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
MVB	Multi vesicular body
n	Nano
N2a	Mouse neuroblastoma cell line
NaCl	Sodium chloride
NaN_3	Sodium azide
NH_4Cl	Ammoniumchloride
NMR	Nuclear magnetic resonance
OD	Optical density
ORF	Open reading frame
p	Piko
P	Passage
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PI 3K	Class I phosphoinositide 3-kinase
PK	Proteinase K
PRNP	Prion protein gene
PrP	Prion protein
$PrP^{0/0}$	PrP knock-out
PrP^{c}	Cellular non-pathogenic form of the prion protein
PrP^{Sc}	Pathogenic form of the prion protein
PVDF	Polyvinyl difluoride
RNA	Ribonucleic acid
RNAase	Ribonuclease
rpm	Rounds per minute
RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
ScL929	Prion-infected mouse fibroblast cell line
ScN2a	Prion-infected mouse neuroblastoma cell line
SEM	Standard error of the mean
SDS	Sodium dodecyle sulfate
siRNA	Small interfering RNA
SOD1	Superoxide dismutase
TBST	Tris buffered saline tween-20
TEMED	N,N,N´,N´-Tetramethylethylendiamin
TME	Transmissible mink encephalopathy
Tris	Tris-(hydroxymethyl-)aminomethan
TSE	Transmissible spongiform encephalopathy
µ	Micro
UK	United Kingdom
V	Valine; Volt
vCJD	Variant CJD
wt/vol	Weight by volume
% (v/v)	Volume percentage
% (w/v)	Weight percentage

VII. REFERENCE LIST

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VIII. PUBLICATIONS

Heiseke A, Schöbel S, Lichtenthaler SF, Vorberg I, Groschup MH, Kretzschmar H, Schätzl HM, Nunziante M. The novel sorting nexin SNX33 interferes with cellular PrP formation by modulation of PrP shedding. **Traffic**. 2008 Jul;9(7):1116-29. Epub 2008 Apr 18.

Heiseke A, Aguib Y, Riemer C, Baier M, Schätzl HM. Lithium induces clearance of protease resistant prion protein in prion-infected cells by induction of autophagy. **J Neurochem**. 2009 Apr;109(1):25-34. Epub 2009 Feb 20.

Aguib Y*, **Heiseke A***, Gilch S, Riemer C, Baier M, Schätzl HM, Ertmer A. Autophagy induction by trehalose counteracts cellular prion infection. **Autophagy**. 2009 Apr;5(3):361-9. Epub 2009 Apr 19. (*** authors contributed equally**)

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	Ab Mai 2007: Promotion in der Arbeitsgruppe von Prof. Dr. Hermann Schätzl am Institut für Virologie, TUM (Betreuer: Prof. Dr. Dr. Johann Bauer, Inhaber des Lehrstuhls für Tierhygiene, TUM)