The role of Kalirin-7 in the pathogenesis of Parkinson Disease and Huntington Disease

Dissertation

zur Erlangung des Grades eines Doktors
der Naturwissenschaften
der Mathematisch-Naturwissenschaftlichen Fakultät
und
der Medizinischen Fakultät
der Eberhard-Karls-Universität Tübingen

vorgelegt
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December 2011

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Table of Contents

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

°C	degree celcius
Aa	amino acid
Ab	antibody
AGERA	agarose gel electrophoresis for resolving aggregates
BS	brain stem
Ctl	control
DMEM	Dulbecco's Minimal Essential Medium
DNA	deoxyribonucleic acid
Cere	cerebellum
CTX	cortex
E. coli	Escherichia coli
EM	electron microscopy
ER	endoplasmic reticulum
G	gravitation constant
G	gram
H	hour
HD	Huntington disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hipp	hippocampus
HRP	horseradish peroxidase
Hsp	heat shock protein
Htt	huntingtin protein
IP	immunoprecipitation
Kb	kilobase
kDa	kilodalton
L	liter
LB medium	lysogeny broth medium
LBs	Lewy Bodies
M	milli
M	molar
Min	minute
N	nano
OB	olfactory bulbs
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	Parkinson disease

PFA	paraformaldehyde
PSD	post synaptic density protein
PK	proteinase K
ROS	reactive oxygen species
Rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulfate
SNCA	α-synuclein
SNpc	substantia nigra pars compacta
STR	striatum
TEMED	N, N, N', N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
U	units
UPS	ubiquitin proteasome system
UV	ultra violet
Wt	wild type
M	micro

GOALS OF THE STUDY

Our lab has identified kalirin-7 as a novel interactor of synphilin-1 using a yeast two hybrid screening. The goal of my thesis was to elucidate if kalirin-7 plays a common role in the pathogenesis of Parkinson disease (PD) and Huntington disease (HD). To answer this question, I designed experiments in three points of view, genetic study, *in vitro* model and *in vivo* model. In genetic study, I tried to identify the potential modifying effects of the kalirin-7 gene on the age-at-onset in HD. In the *in vitro* study, firstly, deletion mutants of both proteins would be used to map the interacting regions. If the interaction between kalirin-7 and synphilin-1 are necessary for both functions, the interference of the interaction might alter the consequences of the signalling output. To investigate whether kalirin-7 is correlated to the neurodegeneration *in vitro*, the kalirin-7 overexpressing or knockout mice should be generated and characterized. Once the animals are available, they would be further cross-bred with PD- or HD- background animals and the impact of kalirin-7 would be examined. Moreover, if kalirin-7 has a significant role in neurodegenerative diseases, there might be detectable abnormalities in patients.

1. INTRODUCTION

1.1 Neurodegenerative diseases

Neurodegenerative diseases are hereditary and sporadic conditions which primarily affect the neurons in the central nervous system (CNS). The most well known disorders include Alzheimer disease (AD), PD, HD, multiple sclerosis, amyotrophic lateral sclerosis (ALS), prion disease, stroke, epilepsy, and others. Some of them share common features, such as protein accumulation, mitochondria dysfunction, defect of proteolytic degradation and aberrant post-translational modifications. Although some pathogenic proteins identified in a certain disease may also be found in other diseases, a direct pathway linking those diseases are still absent. Therefore, analysis of crucial proteins that are recruited in various disorders would help to figure out the whole picture of the pathogenesis and novel therapies.

1.2 Parkinson Disease

PD is the second most common neurodegenerative disease in the world which is only superseded by Alzheimer disease. PD is named after James Parkinson, a British physician who first described six cases of the clinical features of this disorder in “An Essay on the Shaking Palsy” in 1817 (Parkinson, 1817). Nowadays, approximately 5 million people are suffering from PD all over the world which affects about 0.3% of the total population, 1-2% of those aged over 60 years and rising to nearly 3-5% for those over 85 (De Lau and Breteler 2006; Van Den Eeden et al. 2003). The lifetime risk of developing PD in men is 2.0%, which is higher than the 1.3% in women (Elbaz et al. 2002). With an increase of the aging population, the prevalence of PD will grow dramatically in the decades to come and cause considerable social and economical problems. Therefore it is urgent to improve therapies that are able to delay the progression and reduce disability.

problems. Therefore it is urgent to improve therapies that are able to delay the progression and reduce disability.

1.2.1 Clinical picture

The four dominant motor manifestations of PD include resting tremor, rigidity (increased muscular tone), bradykinesia (slowness of movement) and gait dysfunction, which lead to the Parkinsonian phenotype (parkinsonism). Parkinsonism can be caused by the neuropathological condition of PD (idiopathic PD [iPD]) while other neurodegenerative or non-degenerative disorders, including parkinsonism induced by cerebrovascular injury and medication-induced parkinsonism may also share similar clinical features. In cases of “neuropathologically confirmed PD”, approximately 90% of the patients are sporadic and the remaining 10% are inherited. As PD progresses, asymmetric tremor (of the thumb or wrist) starts. According to the age of the patient at the time of onset, predominant clinical features and progression rate, PD may be divided into two subtypes: a tremor-predominant form, which is often observed in younger patients, and a postural imbalance and gait disorder (PIGD) that is frequently often observed in older patients. The clinical diagnosis is focused on motor symptoms; however, recent studies have shown that PD has a broader impact on the nerve system (Chaudhuri and Schapira 2009) and is also associated with many nonmotor features, including olfactory dysfunction, disordered sleep, constipation, depression, dementia and psychosis, all of which affect the health-related quality of life.

1.2.2 Pathological features

The pathological hallmark of PD are dopaminergic neuron loss within the pars compacta of the substantia nigra (SNpc) and accumulation of cytoplasmic inclusions known as Lewy bodies (LBs) (Moore et al. 2005), as well as extracellular synuclein deposits. Dopaminergic neurons in the SNpc project to the striatum, which consists

of the caudate and putamen nuclei, and therefore the loss of nigral cells results in depletion of striatal dopamine. As shown in FIGURE 1.1, the signal output from globus pallidus to thalamus and cortex is dysinhibited due to the loss of nigral cells and thereby destroys the initiation and execution of movements (Rodriguez-Oroz et al. 2009). By the time the clinical repercussions of PD are recognized, 60% of neurons

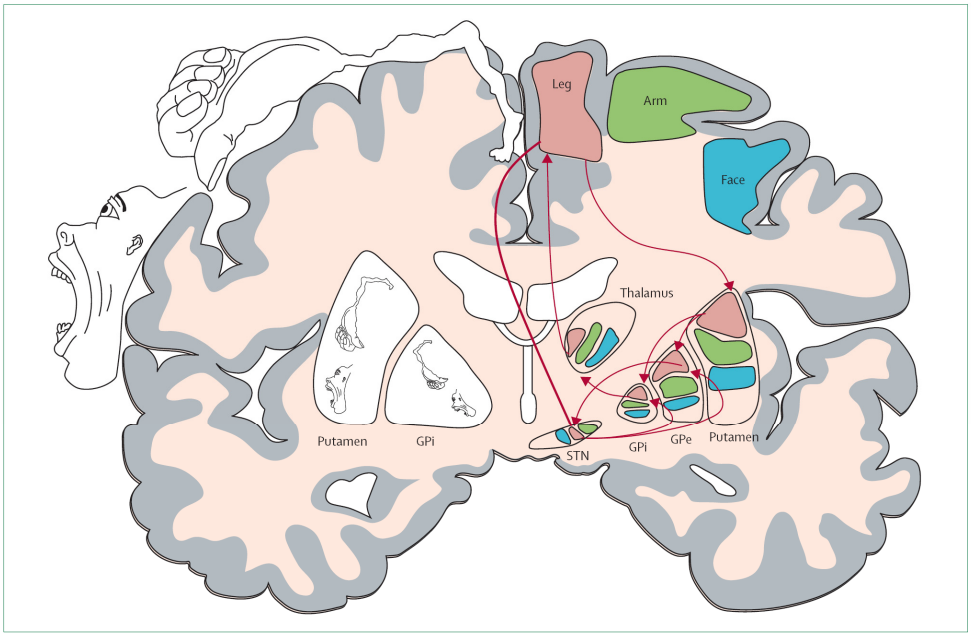


FIGURE 1.1 Anatomy of neuronal circuits involving the basal ganglia, thalamus, and cortex and their degeneration in PD
The motor circuit is organized throughout the loop which affects leg, arm and face movements. In a healthy brain, SNpc provides excitatory dopaminergic input to the putamen. The putamen inhibits GPi which subsequently inhibits the thalamus. The thalamus then projects excitatory input to the motor cortex. However, the function is attenuated in PD patients. The degeneration within SNpc leads to increased inhibition of the thalamocortical projection. The alternative indirect pathway, including GPe and STN, is inhibited by the SNpc dopaminergic input, which functions to repress movements, but the activity is enhanced in PD. GPe= *globus pallidus pars* externa. GPi= *globus pallidus pars* interna. SNpc=*substantia nigra pars* compacta. STN= subthalamic nucleus. (Taken from Rodriguez-Oroz et al. 2009)

in SNpc are lost and lead to 80% depletion of striatal dopamine. In addition to the degeneration of dopaminergic neurons in SNpc and the loss of striatal dopamine, approximately 30-50% of non-dopamine cells are lost in the final stage of PD. Those cells include monoaminergic cells (Zarow et al. 2003), cholinergic cells (Hilker et al. 2005) and hypocretin cells (Thannickal et al. 2007) which are associated with

cognitive deficits, gait problems and sleep disorders in PD patients. Furthermore, as the disease progresses, other brain regions are also affected. Braak and colleagues have proposed a staging procedure for PD (FIGURE 1.2) which suggests LB pathology

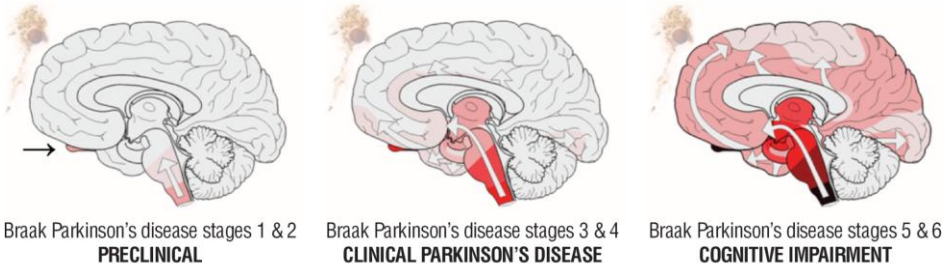


FIGURE 1.2 Progression of PD pathology
Schematic outline of the major stages of PD pathology proposed by Braak et al., 2003. They hypothesized that pathologic changes are first noted in the olfactory and lower brainstem, and then spread to dopaminergic neurons in the SNpc later. (Taken from Olanow et al. 2009)

spreading from the lower brainstem (stage 1) to the neocortex (Braak et al. 2003) Although α-synuclein containing LB and Lewy neuritis (FIGURE 1.3) are present in both sporadic and familial PD patients, it is still controversial whether Lewy pathology is detrimental or protective.

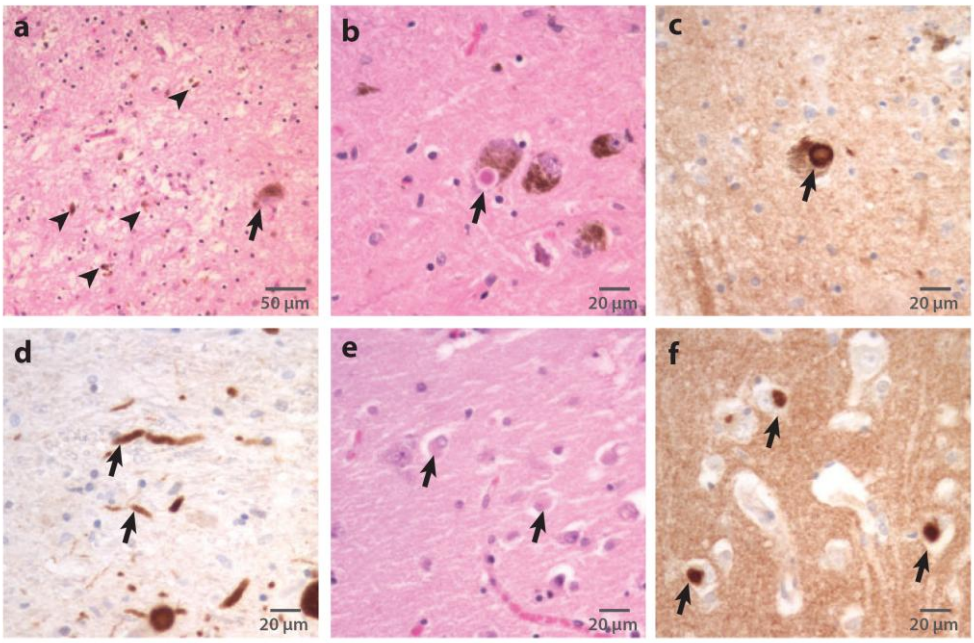


FIGURE 1.3 Pathology of PD
(a) Depletion of dopaminergic neurons (arrow, remaining neuron), reactive gliosis and neuromelanin in phagocytic cells (arrowheads). (b) Brainstem-type LB (arrow) in pigmented dopaminergic neuron. (c) α-synuclein positive brainstem-type LB (arrow). (d) Lewy neurites (arrows). (e) Cortical-type LB (arrows). (f) α-synuclein positive cortical-type LB (arrow). (Adapted from Shulman et al. 2011)

1.2.3 Genetic and non-genetic causes with PD

PD has been historically considered as a sporadic disorder until an outbreak of MPTP-induced parkinsonism was found in the 1980s (Langston et al. 1983). Indeed, about 90% of PD cases are sporadic and 10% have a genetic origin. The genetic forms are more often observed in young-onset of PD (Simuni 2007). With the help of linkage analysis and association studies, which could identify rare genetic variants and susceptible loci, several PD genes have been identified. A decade after the first identification of a PD gene, six genes have been clearly proven to be associated with the monogenic forms of parkinsonism. Of those, *SNCA* (α-synuclein) and *LRRK2* (leucine-rich repeat kinase 2) are considered responsible for autosomal dominant PD, whereas *Parkin*, *PINK1* (phosphatase and tensin (PTEN)-induced putative kinase 1), *DJ-1* and *ATP13A2* cause recessively inherited parkinsonism (Klein and Lohmann-Hedrich 2007). Recently, two novel genes, *VPS35 (*vacuolar protein sorting 35) (Vilariño-Güell et al. 2011; Zimprich et al. 2011) and *EIF4G1* (translation initiation factor 4-gamma) (Chartier-Harlin et al. 2011) have been found in a Swiss (*VPS35*), an Austrian (*VPS35*) and a multi-incident family (*EIF4G1*), respectively. Additional susceptibility loci have been identified, but with an as yet unestablished relevance to the disease. Moreover, age, environment and unknown genetic factors may interact and play roles in the pathogenesis of PD. The genes related to PD are listed in TABLE 1.1 and outlined below.

TABLE 1.1 Selected genetic causes of PD

Acronym	Map position	Gene	Mutations
PARK1	4q21	*SNCA*	A30P, E36K, A53T
PARK2	6q23–27	*Parkin*	>100 mutations (point mutations, exonic rearrangements)
PARK3	2p13	Unknown	not identified
PARK4	4p21	*SNCA*	A30P, E36K, A53T
PARK5	4q14	*UCH-L1*	One mutation in one single family

Acronym	Map position	Gene	Mutations
PARK6	1p35–36	*PINK1*	>40 point mutations, rare large deletions
PARK7	1p36	*DJ-1*	>10 point mutations, large deletions
PARK8	12q12	*LRRK2*	>50 variants, >16 of them pathogenic
PARK9	1p36	*ATP13A2*	>5 point mutations
PARK10	1p32	Unknown	not identified
PARK11	2q36–37	Unknown	7 missense variants
PARK12	X	Unknown	not identified
PARK13	2p13	*HtrA2/Omi*	2 missense variants
not assigned	5q23.1-q23.3	*Synphilin-1*	1 missense mutation in two patients
not assigned	2q22-q23	*Nurr1*	3 mutations
not assigned	15q25	*POLG*	one family with heterozygous mutation
not assigned	16q12	*VPS35*	> 2 point mutations in two families
not assigned	3q26-q28	*EIF4G1*	> 5 point mutations in one family

PD-associated genes:

✧ PARK1/4: α-Synuclein

The *SNCA* gene which encodes α-synuclein protein was first mapped to chromosome 4q21 in the *PARK1* locus. In addition to the three mutations (A53T, A30P and E46K) found in the *SNCA* gene (Kruger et al. 1998; Polymeropoulos et al. 1997; Zarranz et al. 2004), genomic duplication or triplication of the *SNCA* gene in the *PARK4* locus led to an increase of disease manifestation (Chartier-Harlin et al. 2004; Singleton et al. 2003). Later on, α-synuclein has been shown to be a major component of LB (Spillantini et al. 1997) which first linked the familial form of the disease to the neuropathological lesion of PD in the general population. α-synuclein is a protein of 140 amino acid which is abundantly expressed in neurons and associated with synaptic vesicle membranes. Interestingly, the oligomer species of α-synuclein is considered to be detrimental (Serpell et al. 2000; Waxman and Giasson 2009).

✧ PARK2: Parkin

Several mutations in the *Parkin* gene were reported as a cause (10-20%) of early-onset parkinsonism (Kitada et al. 1998). Parkin is an E3-ubiquitin ligase that targets specific substrate proteins to proteasomal degradation (Shimura et al. 2000). Further, mitochondrial function (Clark et al. 2006) and post-modifications (Lavoie et al. 2005) of parkin have been shown to associate with the development of sporadic parkinsonism.

✧ PARK5: Ubiquitin C-terminal hydrolase L1 gene (UCH-L1)

Two mutations were found in *UCH-L1* gene (Leroy et al. 1998; Maraganore et al. 1999). UCH-L1 is a component of ubiquitin-proteasome system, which is involved in α-synuclein degradation. As a monomer, it hydrolyzes poly-ubiquitin chains and subsequently promotes the degradation of α-synuclein via proteasome. When UCH-L1 forms dimers, it ligates ubiquitin to α-synuclein and accordingly prevents the degradation of α-synuclein (Liu et al. 2002). The S18Y variant of UCH-L1 showed reduced ligase activity but comparable hydrolase activity which led to less accumulation of α-synuclein (Liu et al. 2002).

✧ PARK6: PTEN-induced kinase 1 (PINK1)

Mutations and deletions in *PINK1* account for 1-8% of PD patients (Tan et al. 2006; Valente et al. 2004). PINK1 is a protein kinase localized in mitochondria and is ubiquitously expressed in the brain (Gandhi et al. 2006). It can phosphorylate several mitochondrial proteins and modulate their functions (Sim et al. 2006). Mutant PINK1 leds to a decrease of mitochondrial membrane potential under stress conditions (Abou-Sleiman et al. 2006; Clark et al. 2006).

✧ PARK7: DJ-1

Mutations in the *DJ-1* gene are associated with early-onset parkinsonism (~1%) (Bonifati et al. 2003). The protein function has been proposed as an intracellular

sensor for oxidative stress (Lev et al. 2009) and as a part of a novel E3 ligase, working together with parkin and PINK1 (Xiong et al. 2009). Mutant DJ-1 led to an impaired dopamine synthesis by up-regulation of the human tyrosine hydroxylase and an impaired ability to protect against oxidative stress (Ramsey and Giasson 2008; Zhong et al. 2006).

✧ PARK8: Leucine-rich repeat kinase 2 (LRRK2)

LRRK2 is the second causal gene linked to autosomal dominantly inherited PD (Zimprich et al. 2004b). Mutations in the *LRRK2* gene showed high frequency (>2%) in sporadic late-onset parkinsonism (Gilks et al. 2005; Tomiyama et al. 2006). LRRK2 is a large protein containing 51 exons (Zimprich et al. 2004a) and multiple functional domains, including a kinase domain which is dependent on the GTPase function of the Roc domain and the multiple protein-protein interaction regions, which implies that it has a function as a scaffold protein (Mata et al. 2006).

✧ PARK9: ATP13A2

Mutations in a predominantly neuronal P-type ATPase gene (*ATP13A2*) have been found in atypical parkinsonism, namely Kufor-Rekeb syndrome (KRS) (Williams et al. 2005). The mutant ATP13A2 was unstable and lead to relocation of the protein into the endoplasmic reticulum and was then degraded by the proteasome, whereas wildtype ATP13A2 was localized in the lysosome membrane (Ramirez et al. 2006).

✧ PARK13: HtrA serine peptidase 2 (HtrA2)/Omi

Two gene variants were observed in PD (Strauss et al. 2005). HtrA2/Omi is a serine protease located in the intermembranous space of the mitochondria. The release of the protein from damaged mitochondria can activate proapoptotic proteins (Hegde et al. 2002). However, the protein function is still controversial since protease inactivating mutations in Omi/ HtrA2 were associated with a high risk for developing PD (Strauss et al. 2005).

Other susceptible genes and risk factors for developing PD

✧ NR4A2/Nurr-1:

The *Nurr1* gene was mapped to chromosome 2q22-23. Nurr1 is a transcription factor that belongs to the orphan nuclear receptor superfamily, an essential protein involved in the development and maintenance of the dopaminergic system (Zetterström et al. 1997). Some variants of the *Nurr1* gene have been identified to be associated with PD (Grimes et al. 2006; Le et al. 2003).

✧ POLG:

The *POLG1* gene encodes for the mitochondrial DNA polymerase gamma (*pol* γ), which is important for mitochondrial maintenance. Mutations found in the *POLG1* gene (Davidzon et al. 2006; Tiangyou et al. 2006) led to a gradual accumulation of secondary deletions in mitochondrial DNA and subsequently resulted in respiratory chain dysfunction (Hudson and Chinnery 2006).

✧ Synphilin-1:

One missense mutation was found in two sporadic German PD patients (Marx et al. 2003). Synphilin-1, a protein of 919 amino acids, was identified as the first interactor of α-synuclein (Engelender et al. 1999). It contains several domains propagating protein-protein interactions, including ankyrin-like repeats and a coiled-coil domain, facilitating a role of synphilin-1 as a potential adapter molecule that could link α-synuclein to other intracellular proteins, which might be involved in vesicle transport, cytoskeletal functions, mitochondrial dysfunction and other mechanisms. Apart from interacting with α-synuclein, synphilin-1 also interacts with other proteins involved in the pathogenesis of PD, linking synphilin-1 to the ubiquitin-proteosome system and aggregation pathways. Specifically, synphilin-1 interacts with and is ubiquitinated by different E3 ligases, such as dorfin (Ito et al. 2003), SIAH 1 and SIAH2 (Liani et al. 2004; Nagano et al. 2003), marking synphilin-1

for the degradation via the UPS. Phosphorylation by the synphilin-1 interactor GSK3β is thereby a regulator of ubiquitylation that leads to a decrease of synphilin-1 ubiquitylation and inclusion formation (Avraham et al. 2005). The interaction with S6 ATPase/Tbp7 (Marx et al. 2007) and the negative regulator of ubiquitin-like proteins NUB1 (Tanji et al. 2006) provides another link to proteasomal degradation. Parkin, which is most frequently mutated in early onset autosomal recessive forms of PD, is another interacting E3 ligase (Chung et al. 2001); however, it ubiquitinates synphilin-1 via K63-linked ubiquitin chains, thereby increasing the aggregation rate of the synphilin protein (Lim et al. 2005).

✧ Glucocerebrosidase (GBA)

Gaucher's disease is a genetic disease caused by a hereditary deficiency in the gene encoding the lysosomal enzyme glucocerebrosidase (*GBA*). Due to the aberrant lipid accumulation in cells and certain organs, patients show diverse manifestations, such as hepatosplenomegaly, thrombocytopenia, anemia, bone disease and neurologic involvement. It is noticed that patients with Gaucher's disease have parkinsonism and therefore linked *GBA* mutations to PD (Sidransky et al. 2009).

✧ Non-genetic risk factors:

Many studies have been devoted to investigate the correlation between PD and environmental factors, personal habits and gender. Although the data were not consistent due to various populations and complex influences, it has been suggested that PD was associated with smoking, coffee drinking, excess uptake of food, obesity, cholesterol level, exposure to a toxic environment etc. (Tanner 2010).

1.2.4 Pathogenetic mechanisms of PD

In the past decade, numerous inherited mutations found in familial forms of PD have shed light on the molecular mechanisms that are shared by the familial and sporadic form of the disease. According to the biochemical function of the identified

genes, several pathogenetic pathways have been identified, including abnormal protein aggregation, ubiquitin-proteasome dysfunction, oxidative stress, mitochondrial dysfunction and aberrant kinase activity. The spacious functions of those mutated proteins all lead to the age-associated dysfunction and death of dopaminergic neurons. Accordingly, the hypothesized pathways should fit together, as illustrated in FIGURE 1.4 (Farrer 2006; Schulz 2008).

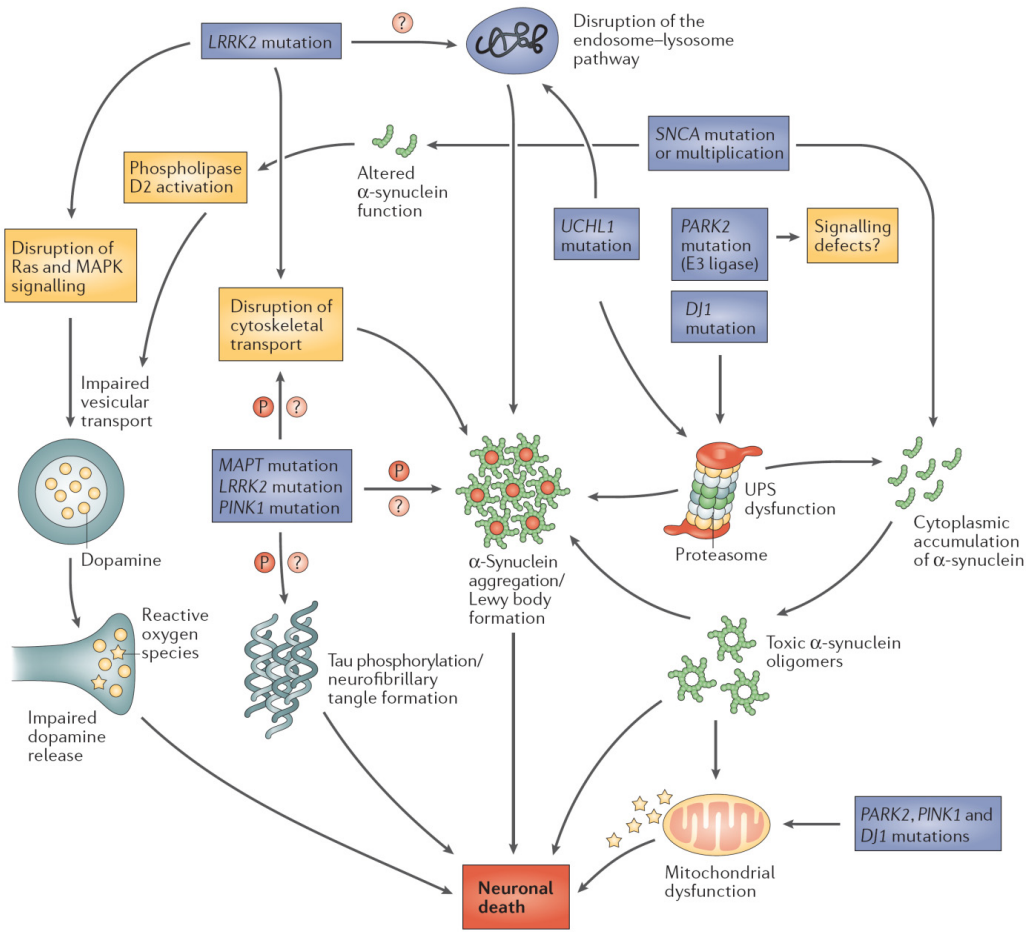


FIGURE 1.1 Pathways proposed to be involved in PD
Missense mutations and genomic multiplication of the *SNCA* gene which encodes α-synuclein lead to increased susceptibility of the mutant protein to aggregate. Altered α-synuclein function abrogates the inhibition of phospholipase D2, an enzyme involved in lipid-mediated signaling cascades and vesicle trafficking. Impaired neurotransmitter release may lead to an increase of ROS and trigger neural cell death. Mutations in parkin, DJ-1 and UCHL1 disrupt the normal UPS function and therefore impair the neuronal function to clear α-synuclein aggregation. Furthermore, mutations in PINK1 cause mitochondrial dysfunction and mutations in LRRK2 disrupt cytoskeletal transport and signaling cascades which all lead to abnormal cell death. (Taken from Farrer 2006)

✧ *Impairment of UPS and autophagic apparatus:*

The discovery of mutations in *parkin* gene first interpreted a direct role of UPS in PD. Parkin encodes an ubiquitin E3 ligase (Shimura et al. 2000) which is essential for the last step of ubiquitin tagging. Another suspective gene, *UCH-L1*, encodes the ubiquitin-C-terminal hydrolase-L1 to degrade the poly-ubiquitin chains and release mono-ubiquitin (Liu et al. 2002). Mutations in those genes may cause a dysfunction of UPS and an accumulation of aggregated α-synuclein.

✧ *Mitochondrial dysfunction and oxidative stress:*

Defects in mitochondrial complex I was the first hypothesis of PD pathogenesis based on the reproduction of parkinsonism with selsective dopaminergic neuronal loss induced by mitochondrial complex I inhibitors, MPTP (Seniuk et al. 1990), and rotenone (Casarejos et al. 2006). The mutations found in *PINK1* (Clark et al. 2006) and the *DJ-1* (Meulener et al. 2005; Yang et al. 2005) genes revealed the involvement of mitochondrial impairment in PD, as well as polymorphisms found in the *Htra2/Omi* gene (Strauss et al. 2005). Recent studies showed that PINK1 and its interactor Parkin worked in a common pathway to regulate mitochondrial function (Clark et al. 2006; Park et al. 2006). The dysfunction of mitochondrial electron transport chain leads to cell death cascades, including oxdative stress, impaired energy metabolism and proteasomal dysfunction (Caneda-Ferrón et al. 2008).

✧ *Aberrant kinase activities:*

Several aberrant kinase activities have been pointed to be involved in the pathogenesis of PD. Mutations found in *LRRK2* gene, especially in its MAPKKK domain, seem to increase kinase activity (Macleod et al. 2006) and lead to a progressive reduction in neurite length and branching (West et al. 2007). Furthermore, PINK1 encodes a serine/threonine kinase and plays a potential role in cell cycle regulation (Unoki and Nakamura 2001). The knowndown of PINK1 renders

cells more susceptible to apoptosis (Mackeigan et al. 2005) which proposes a neuroprotective role of PINK1 in regulating the programmed cell death pathway.

✧ *Proteolytic processing and abnormal protein aggregation*

A common feture of Lewy bodies are aggregates containing α-synuclein. Therefore, it is important to understand the proteolytic processing of α-synuclein. Earlier study showed that inhibition of calpain proteolysis attenuated MPTP-induced loss of nigral dopamine neurons (Crocker et al. 2003). Calpain I leads to the formation of high-molecular weight aggregation consisting α-synuclein fragment (Dufty et al. 2007). However, whether α-synuclein is neuroprotective or neurotoxic remains controversial.

1.2.5 Therapeutic strategies

The ultimate therapeutic goal with PD is to halt or stop the progression of the neurodegeneration. Several existing and developing therapeutic strategies are described below.

I. Symptomatic therapies:

✧ Levodopa is the first choice of treatment and most widely used drug in PD, which dramatically improves the symptoms. L-dopa is metabolized into dopamine by dopa-decarboxylase with an efficiency of 1-5% in the dopaminergic neurons. However, the remaining L-dopa is metabolized elsewhere and its auto-oxidation property cause a wide variety of side effects, such as wearing-off symptoms after treatment with levodopa.

✧ Dopamine agonists stimulate dopamine receptors to mimic dopamine effect and are often used in combination with levodopa. Available dopamine agonists include bromocriptine, paramipexole, ropinirole, cabergoline lisuride and rotigotine. After chronic treatment, the dopamine receptors become less sensitive and the symptoms progressively increase.

✧ COMT (catecholamine-*O*-methyltransferase) inhibitors are used to maintain the levodopa plasma level and prolong the duration of the effect. Two drugs, tolcapone and entacapone, are on the market.

✧ MAO-B inhibitors inhibit monoamine oxidase-B (MAO-B) which breaks down dopamine in striatal synapses and thereby is thought to slow the degeneration of dopaminergic neurons. Two drugs, selegiline and rasagiline, are on the market.

II. Surgical treatment:

Deep brain stimulation (DBS) by implanting a small electrical device called impulse generator (IPG) is a way to inactivate a specific part of the brain without destroying other regions. The IPG can be easily programmed by sending continuous electrical pulses to the target areas, thus and blocking the impulses that trigger tremors. According to the stimulators, the device should be replaced every three to five years. With the help of DBS, the need for levodopa could be reduced and therefore attenuate the side effects of the drug.

III. Non-motor treatment (Chaudhuri and Schapira 2009):

Several non-motor features, as described below, are normally associated with PD and sometimes even more problematic than the classic motor signs.

✧ Neuropsychiatric symptoms range from depression, anxiety, apathy, cognitive impairment, psychosis, sleep dysfunction to insomnia. Antidepressants and drugs for dopaminergic therapy are used for treatment. Furthermore, the symptoms can be improved with the help of DBS of the SNpc.

✧ Autonomic symptoms include urinary bladder dysfunction, nocturia and sexual dysfunction. Dopamine agonists combined with DBS of the subthalamic nucleus alleviate symptoms.

✧ Gastrointestinal symptoms: It is proposed that constipation, a common non-motor feature of PD, is due to a severe loss of central and colonic dopaminergic neurons. Apomorphine and duodopa have been used to eliminate symptoms.

✧ Sensory symptoms include unexplained pains and visual dysfunction. Clinically, dopaminergic therapy would bring relief.

IV. Protective therapies:

Although the therapeutic strategies mentioned above could ease the early stage of the disease, there are still no proven drug treatments that can bring the degenerative process of PD to a halt. Therefore, novel strategies including gene therapy, neurotransplantation and delivery of neurotrophic factors based on molecular studies are urgently developed. Specific targets are selected for trial, such as NMDA antagonists, AMPA receptor agents, secretonergic receptor antagonists, cannabinoid receptor modulators, noradrenergic re-uptake inhibitors and anti-inflammatory drugs.

1.3 Huntington Disease

HD, also known as Huntington chorea, is one of the most common autosomal-dominant neurodegenerative disorders caused by an expansion of an instable CAG repeat in the *HTT* gene resulting in a polyglutamine tract near the amino terminus of the huntingtin protein (htt) (Macdonald et al. 1993). The symptoms and hereditary features of HD have been noticed in the 19th century and the disorder was named after George Huntington, who in 1872 vividly described a classic account of the condition in *The Medical and Surgical Reporter* (Huntington 1872). To date, the worldwide prevalence of HD is 5-10 cases per 100,000 people, but there are large geographic differences according to ethnicity (Walker 2007). In general, in Caucasian populations the prevalence is much higher than in most Asian populations. Nearly two decades after the announcement of the crucial gene, the precise pathological mechanisms are still barely known. Accordingly, further molecular pathway studies and transgenic animal models might provide an insight into other causative factors and efficient treatments.

1.3.1 Clinical picture

The onset of HD can occur between the age of 1 and 80 years. Before the disease progresses, sufferers are apparently healthy and no detectable clinical abnormalities are observed (Myers 2004). When otherwise healthy individuals show subtle changes of personality, cognition and motor control, they might be in a prediagnostic phase of HD. The classical symptoms of HD include distinct chorea, incoordination, motor impersistence, slowed saccadic eye movements, bradykinesia and rigidity (Watts and Koller 1997), and the latter two in particular in the late stage. Furthermore, cognitive dysfunction, especially in executive functions, speech deterioration, psychiatric symptoms, manic-depressive behavior, and suicide can

develop during the course of HD (Craufurd and Snowden 2002; Robins et al. 2000). On the other hand, the juvenile form of HD (onset before the age of 20 years) shows prominent slowed saccadic eye movements, seizure, decline in cognitive function, changes in behavior and oral motor function etc (Kremer 2002; Macdonald et al. 1993).

1.3.2 Pathological progression

The pathological hallmark of HD is strikingly selective with gradual cell loss and atrophy in the caudate and putamen (striatum). According to the grading system developed by Vonsattel et al. (1985), HD cases are classified into five grades of severity (0-4) based on the striatal degeneration in post-mortem brains. Grade 0 shows no obvious differences from a normal brain, but with 30-40% neuronal loss in the caudate nucleus. Grades 1 and 2 are associated with gross striatal atrophy and 50% of neuronal loss, as well as astrogliosis. Grades 3 and 4, display severe atrophy and nearly 95 % of neuronal loss in the striatum (Vonsattel. et al. 1985). But there is not only restriction in the striatum, other nonstriatal brain structures are also affected, including the cerebral cortex, globus pallidus, thalamus, substantia nigra, white matter, cerebellum and hypothalamus (Kassubek et al. 2004; Vonsattel and Difiglia 1998). Another pathological characteristic of HD is the presence of nuclear and cytoplasmic inclusions which contain mutant htt protein (Davies et al. 1997; Difiglia et al. 1997). Protein aggregates are found in dystrophic neurites in striatal and cortical neurons (Sapp et al. 1999). These inclusions appear after symptoms start in some transgenic animal models (Menalled et al. 2003). There is evidence that not the inclusions but rather the intermediate stages of polyglutamine aggregates are the cause of cellular dysfunction (Mukai et al. 2005).

1.3.3 Genetics and genetic modifiers of HD

The gene for HD (*HTT*) was discovered in 1993 (Macdonald et al. 1993) and it was mapped to chromosome 4p16.3 with a stretch of CAG trinucleotide repeats located near the 5'-end in exon 1. Normal alleles at this site contain 10 to 35 CAG repeats encoding a polyglutamine tract to the N-terminus of the protein htt. When CAG expansions reach 39 or more, it is associated with the development of HD and is transmitted through autosomal dominant inheritance. In the juvenile form of HD, the expansions are usually more than 60.

The number of CAG repeats in the *HTT* gene is the primary determinant of the onset of the disease; however, it only accounts for approximately 42-73% of the variance in age-at-onset (AAO) with HD, as shown in Fig 1.5 (Brinkman et al. 1997; Stine et al. 1993). Similar to several other neurodegenerative disorders, the detrimental gene alone does not fully determine the AAO in the course of the disease. Environmental factors, although not specifically defined yet, may contribute to different manifestations of the disease. The remaining variations of AAO may be due to modifier genes and seem to be strongly heritable (Wexler et al. 2004). Several genetic modifiers of HD have been identified so far, including huntingtin-associated protein-1 (HAP1) (Metzger et al. 2008), ubiquitin C-terminal hydrolase 1 (UCHL1) (Metzger et al. 2006; Naze et al. 2002) GluR6 subunit of kainate receptor (GRIK2) (Zeng et al. 2006), the adenosinergic A2A receptor (ADORA2A)(Dhaenens et al. 2009; Taherzadeh-Fard et al. 2010), autophagy-related protein 7 (Atg7) (Metzger et al. 2010), the peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) (Che et al. 2011; Taherzadeh-Fard et al. 2009; Weydt et al. 2009) and NMDA receptor (Arning et al. 2005; Saft et al. 2011). These modifiers with their respective functions contribute to different aspects of pathogenesis in HD.

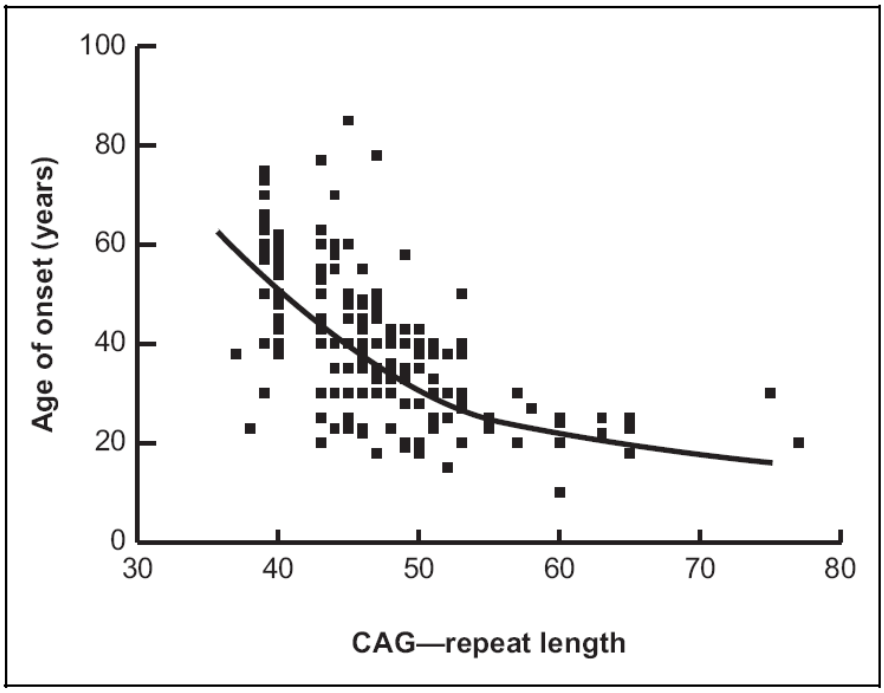


FIGURE 1.5 The correlation between the number of CAG repeats and the age of onset of HD

The association is most significant in younger patients with high CAG repeats (>60). (Taken from Huntington's disease. 3rd ed New York, NY: Oxford University Press; 2002:124, figure 5.3.4)

1.3.4 Pathogenic mechanisms of HD

Most available evidence shows that HD arises mainly from the gain of function of mutant htt protein due to the expanded polyglutamine stretch, but the loss of function of htt could also contribute to disease pathogenesis. The presence of the expanded polyglutamine stretch in htt protein leads to the conformational change. It is suggested that truncated htt protein starts to aggregate by forming dimers, trimers and oligomers and this process requires a specific concentration of protein and a polyglutamine tract with at least 37 residues. Furthermore, the rate of aggregation is positively correlated to the length of glutamine repeats. How does polyglutamine aggregation lead to neuronal dysfunction in HD? The studies of mechanisms have so far not shown definite results; nevertheless, some pathological pathways have been identified. The postulated intracellular pathogenesis of HD is proposed in FIGURE 1.6.

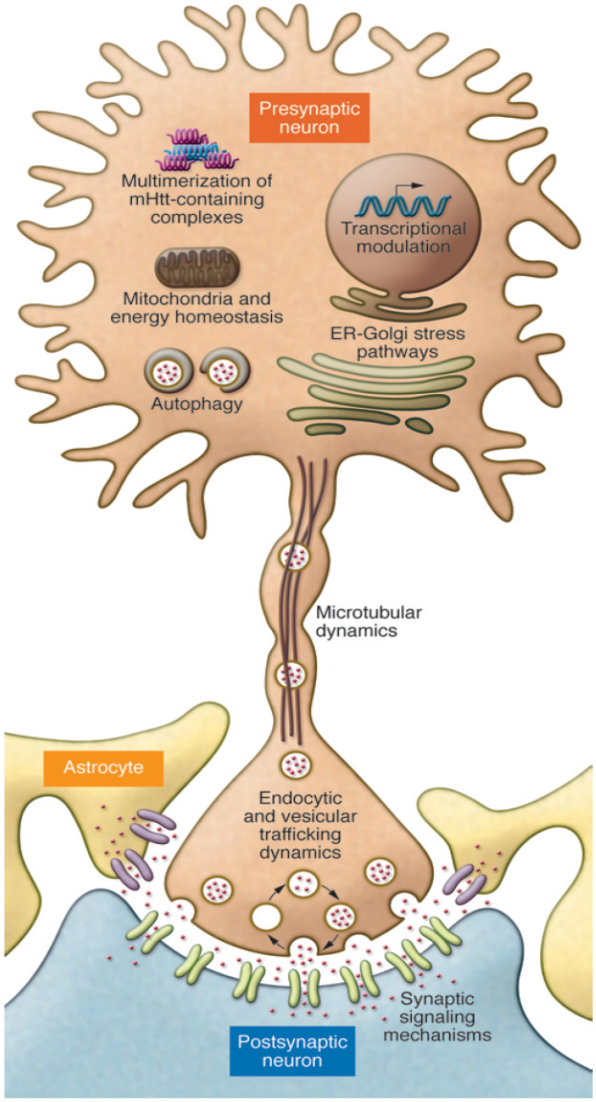


FIGURE 1.1 Postulated cellular pathogenesis of HD

The major mechanisms associated with the HD pathogenesis are illustrated here. The mutant htt with an expanded polyglutamine repeat undergoes a conformational change and interferes with cellular equilibration. These mechanisms include multimerization of mHtt-containing complexes, transcriptional perturbation, ER-Golgi stress pathways, mitochondria and energy homeostasis, microtubular dynamics, endocytic and vesicular trafficking dynamics, autophagy, and synaptic signaling pathways. mHtt= mutant huntingtin protein. (Taken from Munoz-Sanjuan et al., 2011)

✧ *Proteolytic cleavage of huntingtin and protein aggregation:*

Growing evidence from post-mortem brain, animal models, cell culture and biochemical studies of HD indicate that mutant htt cleavage resulting in an N-terminal fragment containing the polyglutamine expansion could be a key step in HD pathogenesis (Difiglia et al. 1997; Landles et al. 2010). Several enzymes have been demonstrated to cleave mutant htt, including caspases (Graham et al. 2006; Kim et al. 2001), calpain (Goffredo et al. 2002; Southwell et al. 2011) and aspartic endopeptidase (Lunkes et al. 2002). One cleavage site at residues 586 is predicted

for caspase 6 proteolysis, which produces a 80kDa peptide fragment. Transgenic mice expressing full length mutant *Htt* gene with alterations at position 586 (making them resistant to caspase 6 cleavage at that position) had strikingly attenuated behavioural and neuropathological HD phenotype (Graham et al. 2006).

✧ *Perturbation of transcription:*

The toxicity of mutant htt was proposed by the redistribution of the cleaved protein from cytoplasm to nuclear compartment (Martindale et al. 1998). Numerous transcription factors have been proven to interact with the expanded polyglutamine tract of mutant htt and further cause cytotoxicity. Those proteins include TBP (Schaffar et al. 2004), CREB [cyclic-adenosine monophosphate (cAMP) response element (CRE) binding protein]-binding protein (Steffan et al. 2000), specific protein-1 (Sp1) (Li et al. 2002), and the TBP-associated factor (TAF)II130 (Dunah et al. 2002). Furthermore, htt affects the transcriptional regulation of BDNF (Zuccato et al. 2001; Zuccato et al. 2003) and mediates the expression of PGC-1α (peroxisome proliferator-activated receptor γ coactivator-1α) (Cui et al. 2006; Weydt et al. 2006) which is important for the activity of cortico-striatal synapses and energy metabolism, respectively.

✧ *Elevated oxidative stress and mitochondrial dysfunction:*

Mitochondria are the major source of ROS (reactive oxygen species) production and also targets for ROS damage. Especially in the brain, mitochondria have more lipids with polyunsaturated acyl, which are more sensitive to oxidative damage. Excessive uptake of calcium or generation of ROS induces activation of mitochondrial permeability and subsequent release of calcium and proapoptotic factors into the cytosol (Orrenius 2004). Evidence has shown that HD transgenic mice had an increase in striatal lipid peroxidation (Pérez-Severiano et al. 2000) which can perturb mitochondrial homeostasis.

✧ *Impairment of the UPS and autophagic apparatus:*

The co-localization of polyglutamine aggregates with ubiquitin and proteasome subunits in cell models (Wyttenbach et al. 2000), transgenic mice (Difiglia et al. 1997) and human post-mortem brains (Davies et al. 1997) was observed. Two hypotheses regarding the UPS dysfunction in HD have been proposed: the sequestration of UPS components into the aggregates might affect UPS activity; the expanded polyglutamine proteins may "stick" in the proteasome and block the entry of other substrates (Imarisio et al. 2008). However, these hypotheses need further investigation. In addition to UPS, autophagy is an alternative pathway for the degradation of misfolded proteins. Mutant htt can interfere with cargo recognition and compromise autophagic clearance (Martinez-Vicente et al. 2010). An overburdened autophagy may release lysosomal enzymes and other toxic factors that promote cell death (Wang et al. 2006).

✧ *Disruption of vesicular trafficking and neurotransmitter release:*

Htt protein plays a role in cytoskeletal motor functions, including vesicle transport and recycling (Caviston and Holzbaur 2009), via association with HAP1 and dynein which regulates both anterograde and retrograde transport (Mcguire et al. 2006). The expanded polyglutamine of mhtt disrupts this function. For example, mutant htt impaired association between motor proteins and microtubules and thus attenuated BDNF transport, which results in loss of neurotrophic support (Gauthier et al. 2004).

✧ *Increased excitotoxicity and inflammation:*

Excessive activation of glutamate receptors, such as NMDA receptors, by excitatory amino acids leads to numerous deleterious consequences, including impairment of calcium buffering, generation of free radicals, activation of the mitochondrial permeability transition and secondary excitotoxicity (Dong et al. 2009). Studies have shown that mutant htt is associated with NMDA receptors via PSD-95

and thus enhances the activity of the receptors, which cause an increase in cell death (Liang et al. 2005; Song et al. 2003; Zhang et al. 2008). On the other hand, some inflammatory proteins such as complement proteins and clusterin are upregulated (Hodges et al. 2006) and microglia are activated in HD patients (Tai et al. 2007). This suggests that abnormal immune activation could play a role in the pathogenesis of HD.

1.3.5 Therapeutic strategies

To date, there is no specific medication to stop or reverse the course of HD. Most medicines can only keep movement and emotional symptoms under control, such as antipsychotic and antidepressive drugs. Since the major part of that medication is not specific, many side effects, including fatigue, restlessness and hyperexcitability may occur. Based on pathogenetic mechanisms of HD, two strategies have been used to develop new drugs: reducing toxicity by reducing mutant htt and blocking mutant htt-induced toxicity (Johnson and Davidson 2010). To accomplish the first end, antisense oligonucleotides and siRNAs that are able to silence mutant alleles of *HTT* have been delivered via infusion or viral vectors into the brain (Difiglia et al. 2007; Harper et al. 2005; Smith et al. 2006). Furthermore, reducing the amount of mutant htt protein and enhancing its clearance is an alternative way to silence HD gene expression. To prevent cell toxicity induced by mutant htt, some therapeutic strategies have been designed to target specific molecular pathways (Ross and Tabrizi 2011).

1.4 Kalirin

1.4.1 Properties and protein domains

Kalirin is encoded by the *kalirin (KALRN)* gene on chromosome 3q21.2 that generates several isoforms by alternative splicing and usage of multiple promoters (Johnson et al. 2000). Among them, kalirin-7, 9, and -12 are major isoforms, which have unique carboxyl terminal ends. Rat kalirin-7, corresponding to isoform 2 of the human *kalirin* gene, is the major splice variant in the adult brain. Kalirin-7 contains a Sec14p domain, spectrin-like domains, a single RhoGEF domain, a pleckstrin homology domain (PH) and terminates in a postsynaptic density-95 (PSD-95)/Discs large/zona occludens-1 (PDZ) binding motif (Alam et al. 1997) which binds to PSD-95 (Penzes et al. 2001b), as illustrated in Fig 2.1 A. Sec14p domains which facilitate protein-protein interactions are responsible for lipid binding. The DH and PH domains function as guanine nucleotide exchange factor (GEF), which catalyzes the guanine nucleotide exchange, thereby activating downstream proteins.

Kalirin-7 is a 190kDa, brain-specific guanine nucleotide exchange factor (GEF) for Rho-like small GTPases, which was first identified as an interactor of peptidylglycine α-amidating monooxygenase (PAM) (Alam et al. 1997). Kalirin-7 expression is not detectable at birth and increases after P7-10, while kalirin-9 and -12 are expressed early in postnatal development (Ma et al. 2003). Kalirin-7 is restrictedly expressed in the cerebral cortex and hippocampus, especially in the spine-like structures and is undetectable outside the brain (Ma et al. 2001).

1.4.2 Protein functions, interactors and involved signaling pathways

With multiple domains and an abundance of interactions with other proteins, kalirin-7 can integrate various signaling inputs and modulate dendritic spine maturation, plasticity and dynamics. The major pathways are illustrated in Fig. 1.7.

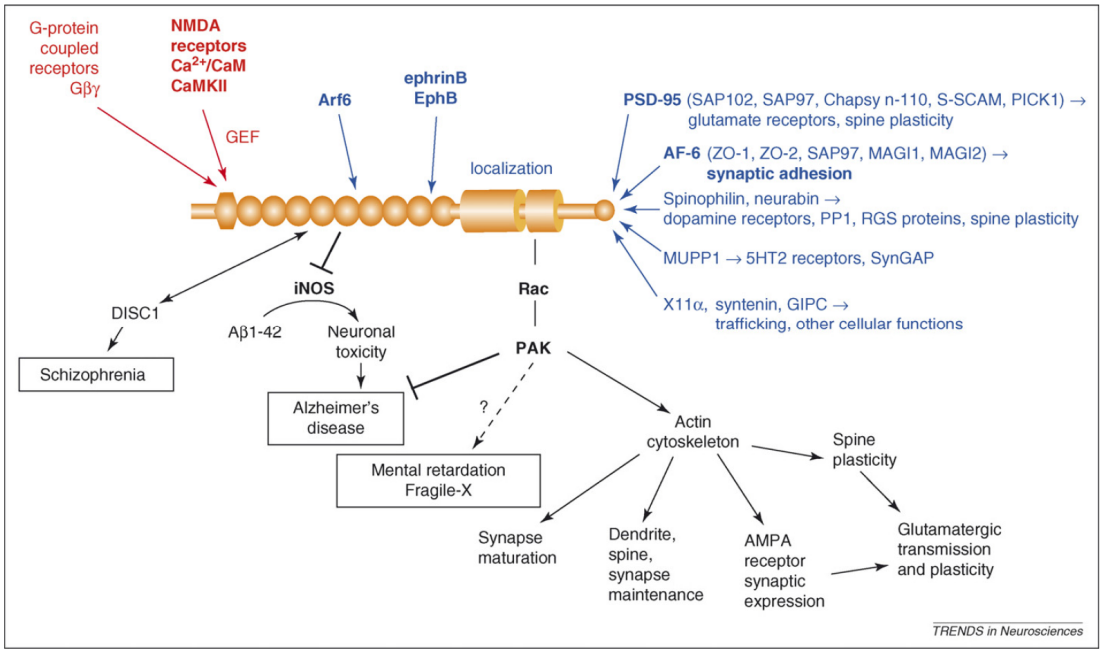


FIGURE 1.1 Major signaling pathways regulated or targeted by kalirin-7
Signaling inputs that modulates kalirin-7 GEF activity are shown in red, namely NMDAR and G-protein- coupled receptors. EphB, Arf6 and PDZ domain-containing proteins regulate kalirin-7 localization (blue). The major signaling output of kalirin-7 is through Rac1/PAK (black). (Taken from Penzes and Jones, 2008)

✧ *Spine maturation: EphB/kalirin-7/PAK pathway*

In young hippocampal neurons, EphB2 activates kalirin-7 and leads to the recruitment of kalirin-7 to synapses (Penzes et al. 2003). Moreover, ephrinB1-induced spine morphogenesis requires kalirin-7 GEF activity, Rac1 activation and p21-activated kinase (PAK) phosphorylation. B-type ephrins and their EphB receptors which are localized in the presynaptic and postsynaptic membrane, respectively, have been proposed to regulate synapse formation and functional plasticity (Dalva et al. 2007).

✧ *Activity-dependent spine structural and functional plasticity:*

When NMDAR is stimulated, kalirin-7 is subsequently phosphorylated by CaMKII, and thereby enhances GEF activity of the protein. The activation of kalirin-7 is essential for NMDAR-dependent Rac1 activation, which results in spine enlargement and maintenance, increased GluR1 in spines and enhanced AMPAR-mediated

synaptic transmission (Xie et al. 2007). A recent study has shown that Disrupted-in-Schizophrenia 1 (DISC1) anchored kalirin-7 and regulated the access of kalirin-7 to Rac1 and controlled the duration and intensity of Rac1 activation in response to NMDAR activation (Hayashi-Takagi et al. 2010).

✧ *Adhesion signaling and spine stability:*

Upon engagement of N-cadherin adhesion protein, kalirin-7 is recruited to the complex and interacts with AF-6, an actin-binding scaffolding protein, via its PDZ domain (Xie et al. 2008). The subsequent activation of Rac1 and PAK regulates postsynaptic actin rearrangements and therefore modulates spine plasticity, maturation and stability.

✧ *Protein signaling networks:*

Kalirin-7 is enriched in postsynaptic densities (PSD) and its C terminus interacts with various PDZ domain-containing proteins, thereby regulating several signaling pathways (Penzes et al. 2001b). For example, kalirin-7 interacts with PSD-95, SAP102, SAP97, Chapsyn-110, S-SCAM and PICK-1, which are involved in glutamate receptor trafficking or assembly of glutamate receptor-associated signaling complexes. Other proteins, such as AF-6, SAP97, ZO-1, MAGI-1, MAGI-2, MUPP1 and spinophilin are supposed to mediate adhesion signaling. Furthermore, kalirin-7 is regulated by the small GTPase Arf6 and EFA6 (its activator) through a Rac1-dependent pathway to modulate the stability of early and mature spines (Koo et al. 2007). On the other hand, kalirin-7 is able to amplify those signaling cascades that result in higher signaling specificity and temporal efficiency (Penzes and Jones 2008).

✧ *Other interactors:*

Shortly after the identification of PAM, Colomer et al. (Colomer et al. 1997) found huntingtin-associated protein 1 (HAP1) as a novel interactor of kalirin-7, linking Kalirin to HD. However, there have been no subsequent studies implicating the

contribution of kalirin-7 to HD. Furthermore, kalirin-7 interacts with inducible nitric oxide synthase (iNOS) through its spectrin-like domain. Overexpression of kalirin-7 reduces the amount of NOS activity by forming a stable heterodimer with iNOS, and thereby prevents iNOS homodimerization which is crucial for NO production (Ratovitski et al. 1999).

✧ *Post-translational modification:*

Two phosphorylation sites of kalirin-7 have been identified. It was possible to phosphorylate Thr95 and Thr1590 by calcium/calmodulin dependent protein kinase II (CaMKII) (Xie et al. 2007) and prolin-directed kinase Cdk5 (Xin et al. 2008), respectively, both of which are essential for GEF activity.

1.4.3 Implication of kalirin-7 in diseases

Recently, several genome-wide association studies have revealed genetic associations of the *kalirin* gene with coronary artery disease (Wang et al. 2007), ischemic stroke (Krug et al. 2010) and schizophrenia (Hayashi-Takagi et al. 2010). Furthermore, kalirin-7 is supposed to be involved in Alzheimer disease and schizophrenia due to an interaction with nitric oxide synthase (iNOS) (Ratovitski et al. 1999; Youn et al. 2007) and disruption in schizophrenia 1 (DISC1) (Hayashi-Takagi et al. 2010), respectively. Direct implication of kalirin-7 in PD or HD has not been described yet.

1.4.4 Animal models

Transgenic mice lacking the terminal exon unique to kalirin-7 (kalirin-7 knockout) show decreased anxiety-like behavior and a deficiency in hippocampal long-term potentiation (Ma et al. 2008). Moreover, the absence of kalirin-7 reveals compensatory upregulation of other larger kalirin isoforms. On the other hand, the total kalirin knockout mice show specific reductions in cortical Rac1 signaling and density as well as deficits in working memory, sociability and prepulse inhibition

(Cahill et al. 2009). These animal models suggest that kalirin-7 might play an essential role in the synaptic structure and function, which cause cognitive dysfunction.

1.5 Mechanisms that clear misfolded or aggregate-prone proteins

The most prominent pathology in many neurodegenerative diseases, including PD, HD and AD is the abnormal aggregates in the brain. The appearance of these misfolded proteins suggests an abnormal machinery of cellular homeostasis. Newly synthesized proteins must overcome several obstacles on their way to become functional molecules. Monomeric proteins fold through a series of folding intermediates. During the process, hydrophobic domains of proteins sometimes lead to inappropriate association and protein aggregation. The ultimate fate of a protein is either correct folding or aggregation. Misfolding can occur due to particular mutations, modification, mistakes during translation, unequal synthesis of subunits, pH, temperature, ionic strength and redox environment (Garcia-Mata et al. 2002).

To avoid an excess of inescapable misfolded proteins, cells have several quality control systems to minimize misfolding and to dispose those proteins before aggregation (Wickner et al. 1999). Firstly, molecular chaperones assist with the folding of newly synthesized proteins and prevent inter- and intra-molecular interactions among misfolded polypeptides to avoid aggregate formation. Secondly, in eukaryotic cells, the ubiquitin-proteasome system (UPS) and autophagy-lysosome pathway serve as two main routes to clean misfolded proteins and organelles (Rubinsztein 2006). Proteasomes are barrel-shaped protein complexes containing a 20S central complex and two 19S lid complexes. Most short-lived proteins are modified with ubiquitin and targeted for proteasomal degradation via a series of enzymatic reactions, including E1 (ubiquitin-activating enzyme), E2

(ubiquitin-conjugation enzyme) and E3 (ubiquitin ligase) enzymes. The proteins are degraded into oligopeptides and released into the cytoplasm or nucleoplasm where they can be digested into amino acids (Korolchuk et al. 2010). In contrast to the UPS, autophagy is capable of degrading long-lived and various cytoplasmic proteins. Autophagy is initiated by the formation of double-membrane-bounded structures, called autophagosomes or autophagic vacuoles and the process is regulated by a set of Atg genes. Autophagosomes are transported along microtubules in a dynein-dependent manner and fuse with lysosomes where their contents is degraded by acidic lysosomal hydrolases.

1.5.1 Aggresome pathway in protein degeneration

Aggresomes, organelles that serve as storage compartments for misfolded proteins when molecular chaperones and the UPS fail to repair damaged or misfolded proteins, can also be actively involved in refolding or degradation of abnormal proteins. The aggresomal particles are then transported toward the microtubule organizing center (MTOC) which is dependent on retrograde dynein-dependent trafficking along microtubules (Fig. 1.8). However, so far the basic molecular mechanisms involved are poorly understood. Evidence indicates that besides the dynein motor complex aggresomes associate with histone deacetylase 6 (HDAC6) (Kawaguchi et al. 2003), parkin, ataxin-3, and ubiquilin-1 for recognition and transportation (Olzmann et al. 2007) and with chaperones, ubiquitination enzymes, proteasomes and autophagic components to facilitate the clearance of aggregated proteins (Garcia-Mata et al. 2002). Finally, aggresomes are proposed to be engulfed by autophagosomes and proteins are degraded as mentioned above.

1.5.2 Role of HDAC6 in the aggresome pathway and neurodegenerative diseases

HDAC6 is a member of the class II HDAC family which is known for its two catalytically active deacetylase domains and an ubiquitin interacting domain

(Grozinger et al. 1999; Seigneurin-Berny et al. 2001). It is of particular importance in the aggresomal protein degradation because it can bind both polyubiquitinated misfolded proteins and dynein proteins and thereby recruits protein cargos to dynein motors for transport to aggresomes (Kawaguchi et al. 2003). In addition, HDAC6 controls major cell response pathways related to cytotoxic accumulation of protein aggregates. Boyault et al. have shown that HDAC6 sensitizes ubiquitinated protein aggregates and consequently induces cellular chaperones by triggering the dissociation of HDAC6/HSF1 (heat-shock factor 1)/HSP90 (heat-shock protein 90) complex and subsequently inducing HSF1 activation (Boyault et al. 2007b). Another important factor that determines the processing of polyubiquitinated misfolded proteins is the cellular concentration of HDAC6 and its partner, p97/VCP. Ubiquitinated proteins are in a favor of aggresome formation when HDAC6 is in excess, whereas an abundance of p97/VCP results in the release of HDAC6 and delivery of those proteins to proteosomal degradation (Boyault et al. 2007a).

Emerging evidence has focused on HDAC6 in neurodegenerative diseases. The role of HDAC6 in AD is supported via its association with tau protein (Ding et al. 2008). In the brain of AD patients, the protein level of HDACs was significantly decreased and microtubule stability was reduced in neurons containing neurofibrillary tangle (NFT) (Hempen and Brion 1996). Therefore, HDAC6 α-tubulin deacetylase activity may be involved in the pathogenesis of AD. In a cell model of HD, HDAC6 was required for aggresome formation and autophagic degradation (Iwata et al. 2005). On the other hand, an increase of tubulin acetylation mediated by HDAC6 inhibition can enhance the recruitment of kinesin-1 and dynein and thereby rescue the brain-derived neurotrophic factor (BDNF) transport deficit from the cortex to the striatum (Dompierre et al. 2007). In HDAC6 knock-down *Drosophila melanogaster*, the retinal degeneration was exacerbated whereas the overexpression of HDAC6 decreased

aggregate load and ameliorated retinal degeneration (Pandey et al. 2007). In addition to the polyglutamine diseases, overexpression of HDAC6 was protective in *Drosophila melanogaster* models of Parkinson disease (Du et al. 2010) and Alzheimer disease (AD) (Pandey et al. 2007). HDAC6 has been found to localize to LBs in PD patients (Kawaguchi et al. 2003) and glial cytoplasmic inclusions in multiple system atrophy (MSA) (Miki et al. 2011). In cell models, HDAC6 was preferentially bound to K63-linked polyubiquitinated DJ-1 and facilitated the transport of DJ-1 to the MTOC (Olzmann et al. 2007). Moreover, parkin could recruit ubiquitin-binding deacetylase HDAC6 and p62 and subsequently assembled autophagy machinery to clear impaired mitochondria (Lee et al. 2010).

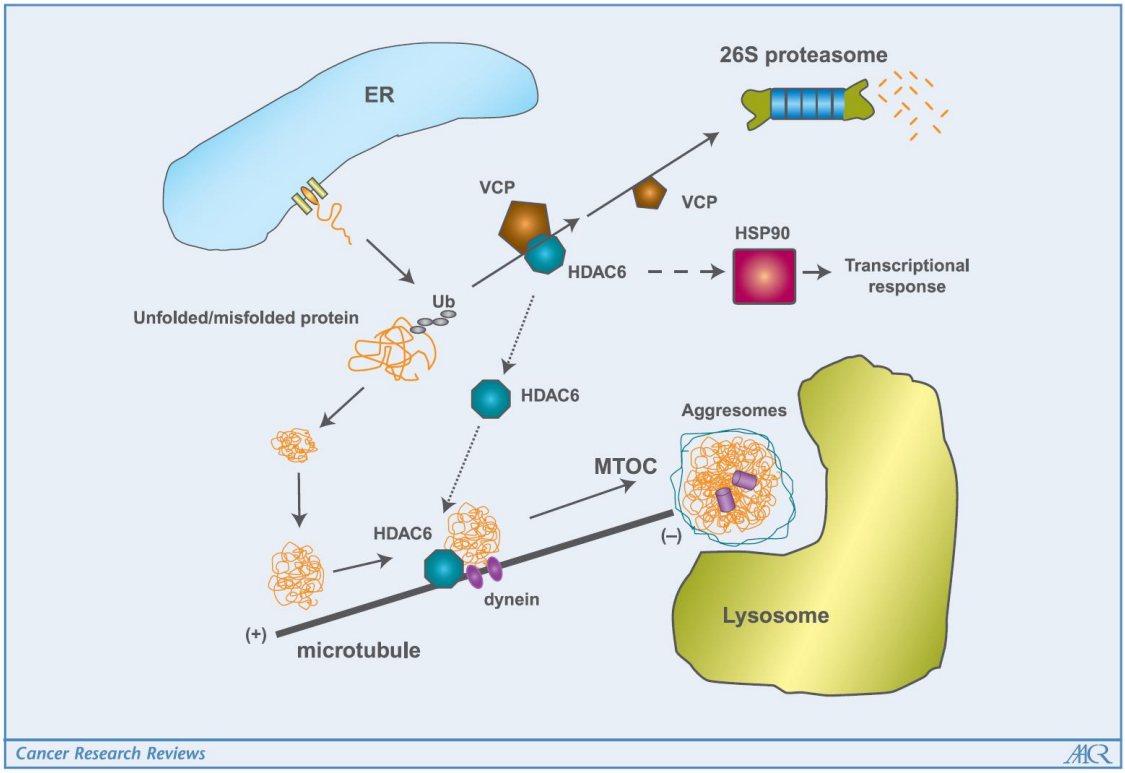


FIGURE 1.8 Model of the aggresome pathway
Unfolded or misfolded proteins can originate from translating polysomes, from proteins retrotranslocated from the ER to the cytosol, or from proteins damaged by stress. When the proteasomes are not able to degrade those misfolded/unfolded proteins, aggresomes are formed. The transportation of aggresomes to the MTOC is microtubule dependent with the help of dynein motor complex. HDAC6 presents as a link between ubiquitinated proteins and dynein complex. (Taken from Rodriguez-Gonzalez et al., 2008)

2. RESULT

2.1 Genetic analysis of polymorphisms in the *kalirin* gene

To explore the potential modifying effects of the *kalirin* gene on the AAO in HD, we chose eleven single nucleotide polymorphisms (SNPs) that were published in the NCBI SNP database at the start of this study. According to our hypothesis, via interaction with HAP-1, abnormal kalirin function may contribute to spine pathogenesis in HD. Therefore, in this study we focused on SNPs that could potentially influence protein function or expression based on their positions in functional domains or regulatory regions. Accordingly, ten of the selected SNPs are non-synonymous and are located in spectrin-like domains (rs111472457 in exon 8, rs35057827 in exon 9, rs13074913 in exon 11, rs61745397 in exon 13, rs112304715 in exon 14), the Rho GEF domain (rs77832285 in exon 20, rs2289838 in exon 25) and downstream of the pleckstrin homology domain (rs1062749 in exon 32), respectively (Figure 2.1). SNP rs10934657 is in the 5' untranslated region (5'UTR), a predicted promoter region. Accession numbers and alleles of the analyzed SNPs are shown in TABLE 2.1.

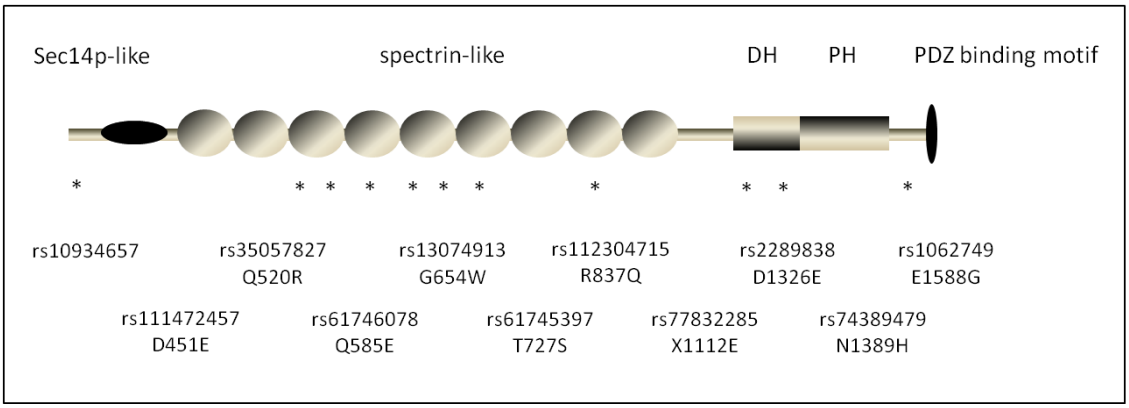


FIGURE 2.1 Domain structure of the kalirin gene
The isoform 2 of the kalirin gene contains 34 exons and five domains, Sec14p-like domain, spectrin-like domains, Dbl-homology (DH), pleckstrin homology (PH) domains and a PDZ domain-binding motif (STYV). The localizations of the examined eleven SNPs are highlighted at the respective positions.

TABLE 2.1 Overview of the SNPs studied

SNP ID	Chromosome position	Alleles[a]	Gene location	SNP type	Protein position	Controls (CEPH)		HD patients				
						Allele frequency		Genotype frequency			Allele frequency	
	chromosome 3	*(1/2)*				*1*	*2*	*1-1*	*1-2*	*2-2*	*1*	*2*
rs10934657	123812836	C / T	5'-UTR	Non-coding		0.8	0.2	487	174	19	0.844	0.156
rs111472457	124048782	G / T	exon 8	non-synonymous	D451E	1	0	-	-	-	-	-
rs35057827	124053260	G / A	exon 9	non-synonymous	Q520R	1	0	-	-	-	-	-
rs61746078	124066099	G / C	exon 10	non-synonymous	Q585E	1	0	-	-	-	-	-
rs13074913	124113985	T / G	exon 11	non-synonymous	G654W	1	0	-	-	-	-	-
rs61745397	124117557	T / A	exon 13	non-synonymous	T727S	1	0	-	-	-	-	-
rs112304715	124132486	A / G	exon 14	non-synonymous	R837Q	1	0	-	-	-	-	-
rs77832285	124165034	G / T	exon 20	non-synonymous	X1112E	1	0	-	-	-	-	-
rs2289838	124181433	G / T	exon 25	non-synonymous	D1326E	1	0	-	-	-	-	-
rs74389479	124196161	C / A	exon 27	non-synonymous	N1389H	1	0	-	-	-	-	-
rs1062749	124211666	G / A	exon 32	non-synonymous	E1588G	1	0	-	-	-	-	-

Chromosome positions were obtained from NCBI single nucleotide polymorphism (SNP) browser (http://www.ncbi.nlm.nih.gov/SNP/)

Reference sequence for coding SNPs: NM_003947.4

The studied distribution of genotypes for SNPs were consistent with that expected for Hardy-Weinberg distribution P=0.4716

a Genotype and allele frequency of nucleotide substitutions are described as 1 (wild type allele) or 2 (variant allele)

2.1.1 Screening of polymorphism frequency in healthy control samples

We first screened these SNPs in 60 control samples (CEPH) to monitor the allele frequencies of each polymorphism. SNP rs10934657 in the 5'UTR region (C>T) was polymorphic with a frequency ≥ 1% in HD samples and controls whereas the ten coding SNPs were monomorphic in our cohort (TABLE 2.1). Therefore, SNP rs10934657 was selected for further genotyping of HD patients.

2.1.2 Analyzing the modifier effect of the *kalirin* gene in HD patients

To examine the effect of the polymorphic changes on disease onset, genotypes in SNP rs10934657 were determined in a total of 680 HD patients. Genotyped allele frequencies are listed in TABLE 2.1. The genotype frequencies of SNP rs10934657 in our European population was consistent with the HapMap-CEU population studies reported in the International HapMap project (www.hapmap.org) (C: 0.8; T: 0.2). In order to identify a possible modifying effect of SNP rs10934657 on the AAO of the analysed HD patients, an analysis of covariance together with the influence of the expanded HD allele was applied. Analysing the effect of the expanded CAG repeat in the HD gene itself, R^2 in the statistical model reaches a value of 0.5394 (TABLE 2.2). This indicates that the expanded HD alleles accounts for about 53% of the variance in the AAO, which is in good accordance to other studies (Brinkman et al. 1997; Stine et al. 1993). However, SNP rs10934657 in the promoter region of the *kalirin* gene did not affect the AAO of the disease (P=0.9713) in the examined HD population.

TABLE 2.2 Effect of SNP rs10934657 on AAO in HD (Analysis of covariance)

Variable	R^2	ΔR^2	p-value	Least significant number of patients
HTT CAG	0.5394		<0.0001*	7
HTT CAG + SNP rs10934657	0.5394	0	0.9713	70065

2.2 Interaction of Synphilin-1 and Karlirin-7: functional relevance

2.2.1 Interaction of Synphilin-1 with Kalirin-7 in vitro and in vivo

N-terminus of synphilin-1 and spectrin domain of kalirin-7 are crucial for binding

The protein conformation is a critical criterion to influence the protein function, especially when they form a protein complex. Therefore, it is important to know how the protein-protein interacts. To confirm the interaction and to define the critical interacting domain of kalirin-7, co-immunoprecipitation experiments were performed using FLAG-tagged kalirin-7 or various deletion mutants and V5-tagged full length synphilin-1. The results showed that synphilin-1 co-precipitated with full-length kalirin-7 and that spectrin domains III and IV are necessary and sufficient for the interaction with synphilin-1 (Fig. 2.2 A). The kalirin-binding domain on synphilin-1 was determined by co-immunoprecipitation with different V5-synphilin-1 mutants. These data demonstrate that III-IV of spectrin-like repeats of kalirin-7 and the N terminus of synphilin-1 (aa 1-348) (Fig. 2.2 B) are crucial for their interaction.

Synphilin-1 and kalirin-7 are colocalized in the cytoplasm and interact *in vivo*

To investigate whether kalirin-7 co-localizes with synphilin-1 in cells, we transiently overexpressed FLAG-kalirin-7 and V5-synphilin-1 for 6 hours. Laser scanning microscopy revealed that both proteins localize to the cytoplasm and show a similar cellular distribution (Fig. 2.3). To validate the interaction *in vivo* we immunoprecipitated synphilin-1 from mouse brain tissue and probed the immunoprecipitates with the KALRN antibody (Abcam) which recognizes isoform 2 of the kalirin protein (190 kDa), corresponding to the major adult isoform in rat brain, kalirin-7. The observed co-precipitation of kalirin-7 confirmed that endogenous synphilin-1 and kalirin-7 interact also in an *in vivo* situation (Fig. 2.4).

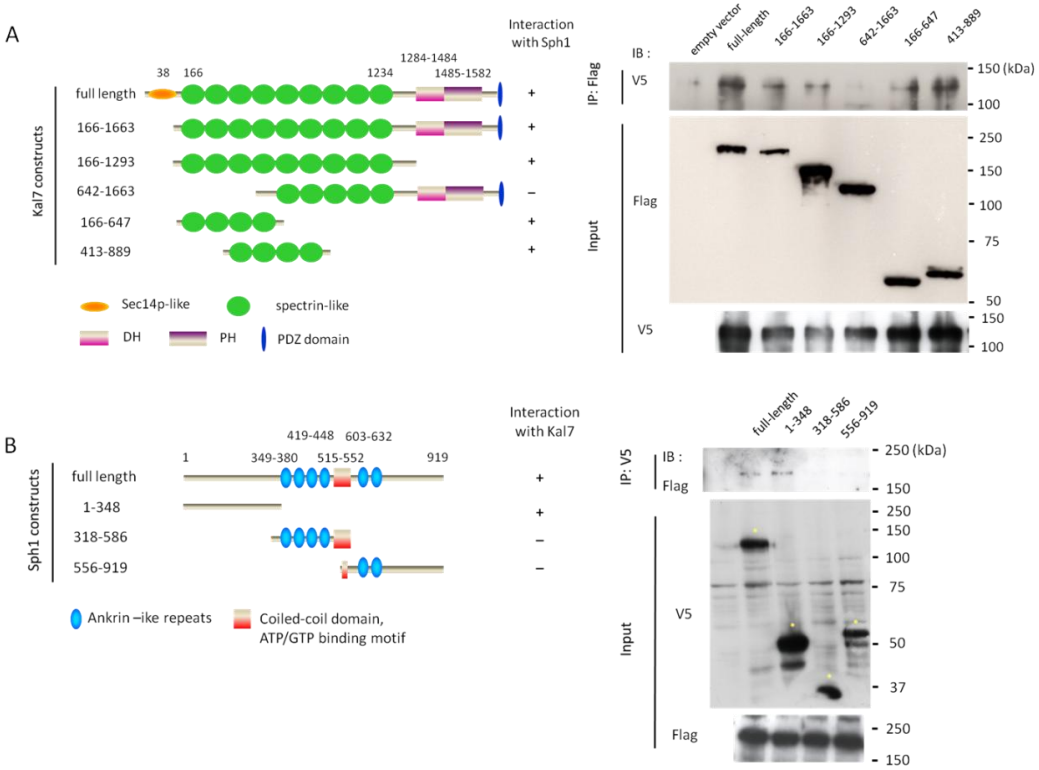


FIGURE 2.2 Kalirin-7 interacts with synphilin-1 *in vitro*

(A) Mapping of the interacting domain in the kalirin-7 protein. FLAG-kalirin-7 constructs as shown in the diagram were co-transfected with V5-synphilin-1 in HEK293 cells. 24 h after transfection, cells were subjected to immunoprecipitation with anti-V5 agarose beads and subsequently kalirin-7 and synphilin-1 immunoreactivities were monitored applying anti-FLAG- or anti-V5 antibodies, respectively. IP indicates antibodies used for pulling down target proteins. IB indicates antibodies used for detection in western blot. The figure shows that kalirin-7 co-immunopreciptates with synphilin-1 and that spectrin-repeats III and IV of the kalirin-7 protein are crucial for the interaction. (B) Mapping of the binding region in synphilin-1. The indicated V5-synphilin-1 constructs were co-transfected with FLAG-kalirin-7, which was precipitated with anti-FLAG antibodies. The precipitates were then probed with anti-V5 antibodies. The deletion mapping revealed that amino acids 1-348 of the synphilin-1 protein are crucial for the binding of kalirin-7.

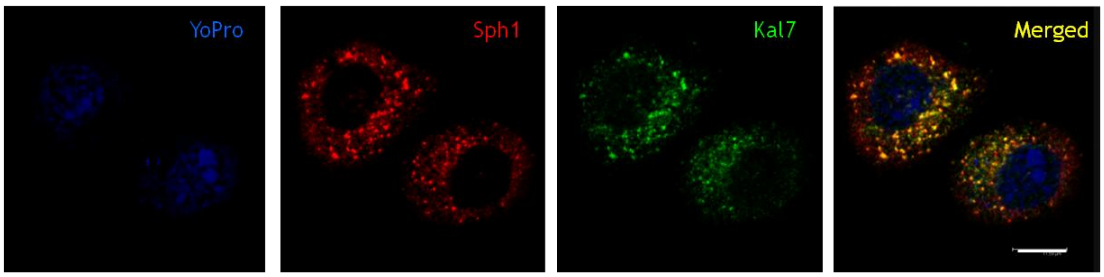


FIGURE 2.3 Overlapping localization of synphilin-1 and kalirin-7 in cell culture

HEK293 cells were transiently transfected with synphilin-1 and kalirin-7 constructs and stained with anti-V5 and anti-FLAG antibodies. The counterstaining was stained with YoPro dye. The confocal sections demonstrate that both proteins display a cytosolic localization. Sph1, synphilin-1; Kal7, kalirin-7. *Scale bar*, 10 µm.

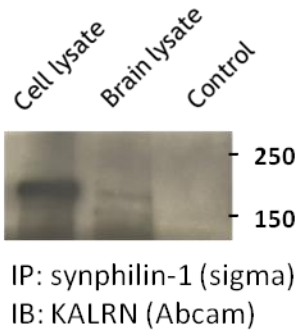


FIGURE 2.4 Interaction of endogenous synphilin-1 and kalirin-7 from mouse brain lysate
Synphilin-1 was precipitated from whole-brain tissues of a wild type mouse with an anti-synphilin-1 antibody. The precipitates were probed with a kalirin-7-specific antibody. The *in vivo* co-immunoprecipitation revealed that endogenous synphilin-1 interacts with kalirin-7.

2.2.2 Kalirin-7 promotes the formation of synphilin-1 perinuclear inclusions

In order to evaluate the functional relevance of the newly discovered interaction, we first focused on the ability of synphilin-1 to promote inclusion formation (Engelender et al. 1999; O'farrell et al. 2001; Xie et al. 2010). This property was chosen, as it allows to hypothesize a functional interaction of both proteins. Synphilin-1 containing inclusions are often characterized as aggresomes, whose formation is dependent on retrograde dynein-dependent trafficking along microtubules. This intracellular traffic can be regulated by Rho-GTPases (Segev 2011) and importantly, kalirin proteins are neuronal Rho-GDP/GTP exchange factors (GEFS) and may thus be involved in this process.

To investigate the functional effect of kalirin-7 on synphilin-1 induced inclusion formation, synphilin-1 tagged with HcRed was transiently overexpressed in HEK293 cells. 48 h after transfection, 43.2% of transfected cells formed cytoplasmic small aggregates, whereas 1.3% showed perinuclear aggregates (Fig. 2.5 A and B). Importantly, co-expression of FLAG-kalirin-7 resulted in a dramatic increase in the percentage of perinuclear synphilin-1 positive aggregates. 56.9% of double transfected cells formed perinuclear aggregates, but only 2.7% of transfected cells

showed cytoplasmic small aggregates (Fig. 2.5 B). Of note, HcRed alone expressed from the same vector showed a diffused pattern. Moreover, the total number of inclusions dropped from in average 5.3 to 1.8 per cell (Fig. 2C). Similar results were obtained in the HN-10 cell line, a mouse hippocampal cell line (Fig. 2.5 A, e and f). These data clearly demonstrate that co-expression of kalirin-7 and synphilin-1 causes a dramatic relocation of synphilin-1 cytoplasmic small inclusions to a single prominent, perinuclear inclusion.

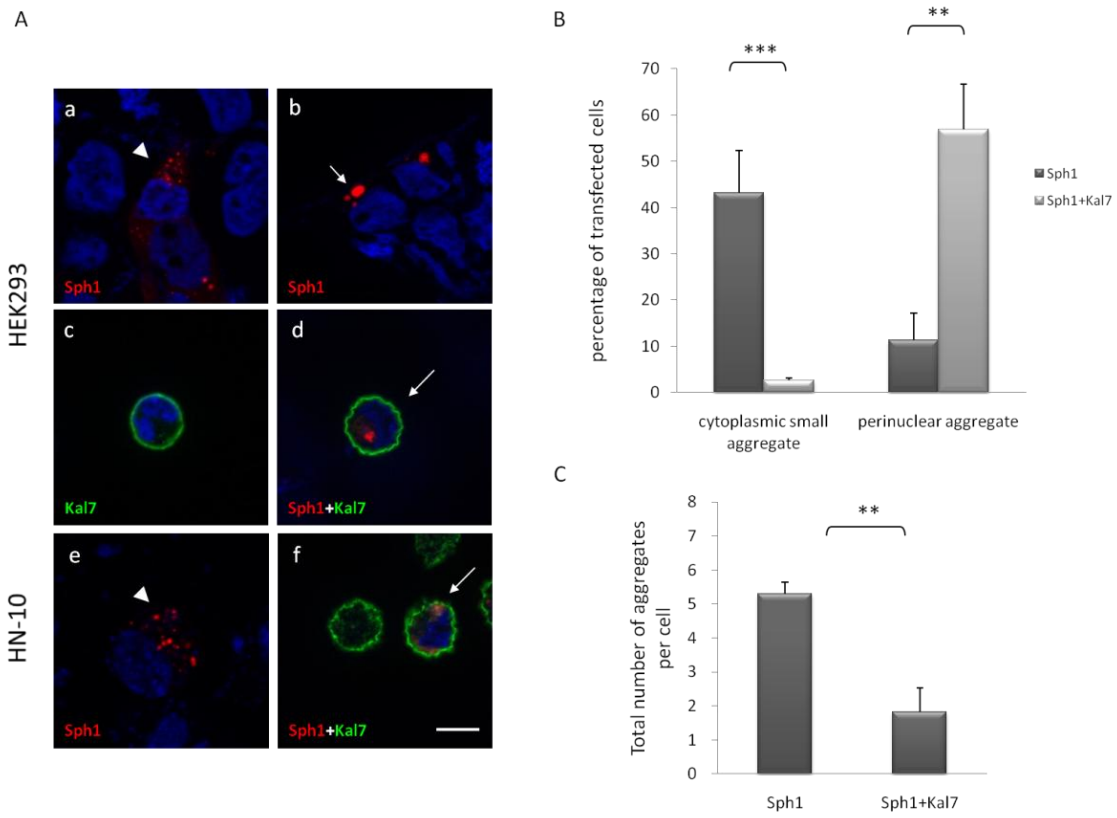


FIGURE 2.5 Kalirin-7 alters synphilin-1-induced inclusion formation
(A) When HEK293 cells were transfected with HcRed-synphilin-1 alone (a,b), FLAG-kalirin-7 (c) or both expression constructs (d) two types of inclusions were observed: small cytoplasmic aggregates (arrowhead; a) and perinuclear aggregates (arrow; b, d). The same experiment was performed in HN-10 cell line and two types of aggregates were observed, e and f. *Blue*, DAPI. *Scale bar*, 10µm. (B) Quantitative analysis of the experiment described in (A). HcRed-synphilin-1 was expressed without or with FLAG-kalirin-7 for 48 h. Cells were fixed and immunostained with anti-FLAG antibodies. Cells with cytoplasmic small aggregates, perinuclear aggregates or soluble synphilin-1 were counted. Results represent the average of three independent experiments. (C) Total numbers of aggregates per cell (cytoplasmic and perinuclear) were counted applying ApoTome confocal fluorescent microscopy. Over 100 cells were counted for each condition. The asterisks indicate statistical significance ($^{**}P \leq 0.005$; $^{***}P \leq 0.001$). *Error bars*, S.E.

2.2.3 Synphilin-1 perinuclear inclusions exhibit features of aggresomes

The morphology and localization of the perinuclear inclusions appeared similar to that of aggresomes (Garcia-Mata et al. 2002; Kopito 2000). It has been reported that aggresomes are formed via the retrograde transport of aggregated proteins on microtubules (Kopito 2000). Several features were used to identify aggresomes, including microtubule-dependency, recruitment of chaperones and ubiquitination enzymes, and the increase of protein insolubility.

Therefore, we first assessed the effects of microtubule-depolymerizing drugs on kalirin-7 mediated synphilin-1 perinuclear inclusion formation in HEK293 and HN-10 cells. When microtubules were disassembled by the microtubule poisons, nocodazole and colchicine, the aggregation patterns of HcRed-synphilin-1 switched from perinuclear aggregates into small cytoplasmic aggregates (Fig. 2.6 A). The effect of nocodazole and colchicine on microtubule depolymerization was monitored using β-tubulin as an indicator. Disruption of microtubules led to a decrease in the percentage of perinuclear inclusions from 44.1% to 11.4% (nocodazole) and 10.6% (colchicine), respectively (Fig. 2.6 B).

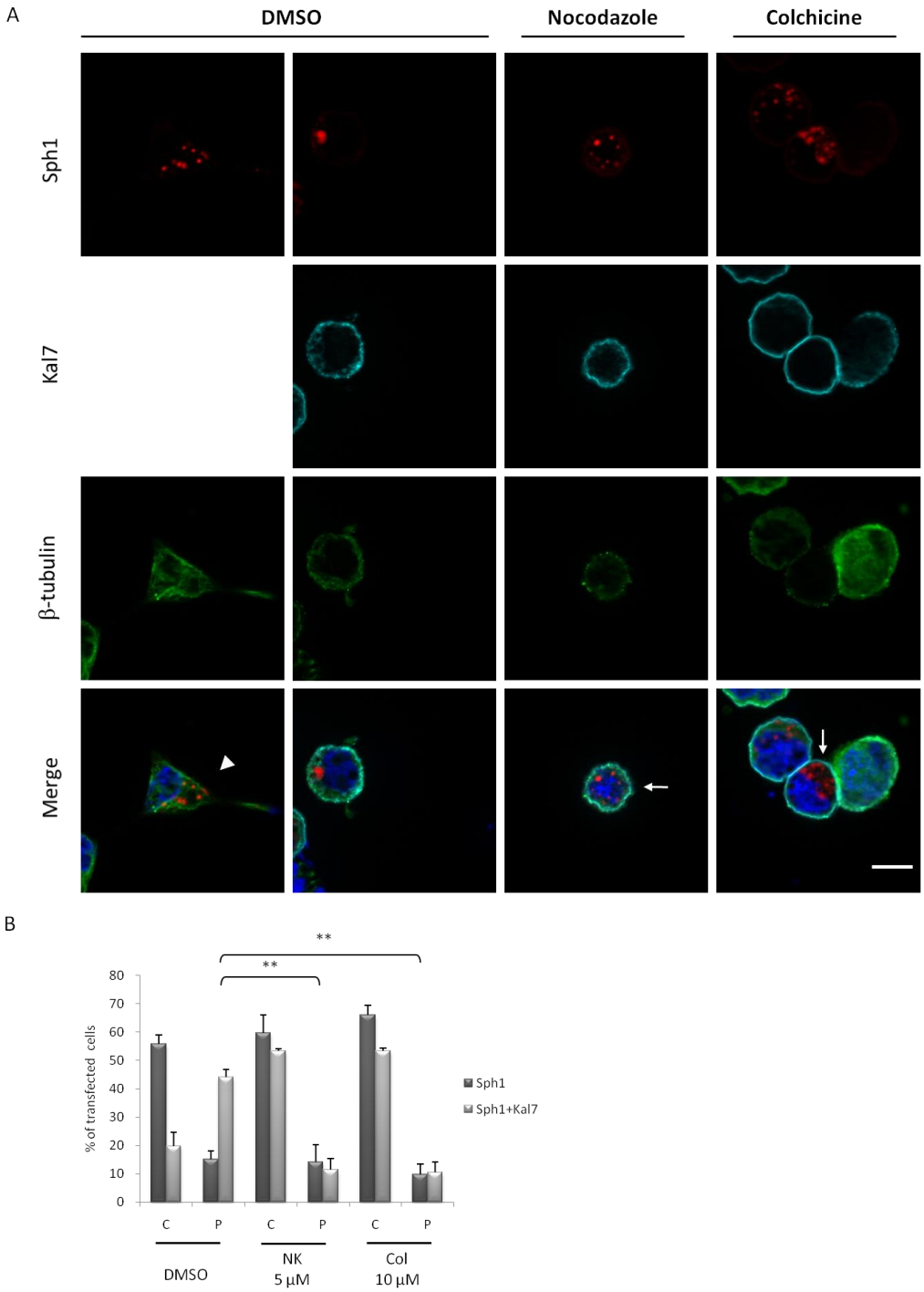


FIGURE 2.6 Kalirin-7 mediates perinuclear synphilin-1 inclusion formation in a microtubule-dependent manner

(A) HEK293 cells were cotransfected with HcRed- synphilin-1 and FLAG-kalirin-7. After 36 h cells were incubated with DMSO, 5µM nocodazole or 10µM colchicine for 12 h before being subjected to immunofluorescence with anti-FLAG and anti- β-tubulin antibodies. Cells expressing HcRed-synphilin-1 alone served as controls (arrowhead). In cells treated with nocodazole or colchicine, more cytoplasmic small aggregates (arrows) were formed. (B) Quantification shows that nocodazole and colchicine inhibited the kalirin-7-mediated formation of synphilin-1-containing perinuclear inclusions. P, perinuclear aggregates; C, cytoplasmic small aggregates. The asterisks indicate statistical significance ($^{**}P \leq 0.005$). *Error bars*, S.E.

To further confirm whether these inclusions were indeed aggresomes, cells were stained with antibodies against γ-tubulin, a centrosome component that is typically found in aggresomes (Olzmann et al. 2007). Immunostaining confirmed a-co-localization of synphilin-1-containing perinuclear aggregates with the centrosome (Fig. 2.7). Other widely used markers of aggresomes, including vimentin, ubiquitin and Hsp27, were also used to characterize these inclusions. We found that the perinuclear aggregates of double transfected cells were positive for γ-tubulin, ubiquitin and Hsp27 and were surrounded by a compact cage of the intermediate filament protein vimentin (arrows in Fig. 2.7) and therefore fulfill all criteria of aggresomes.

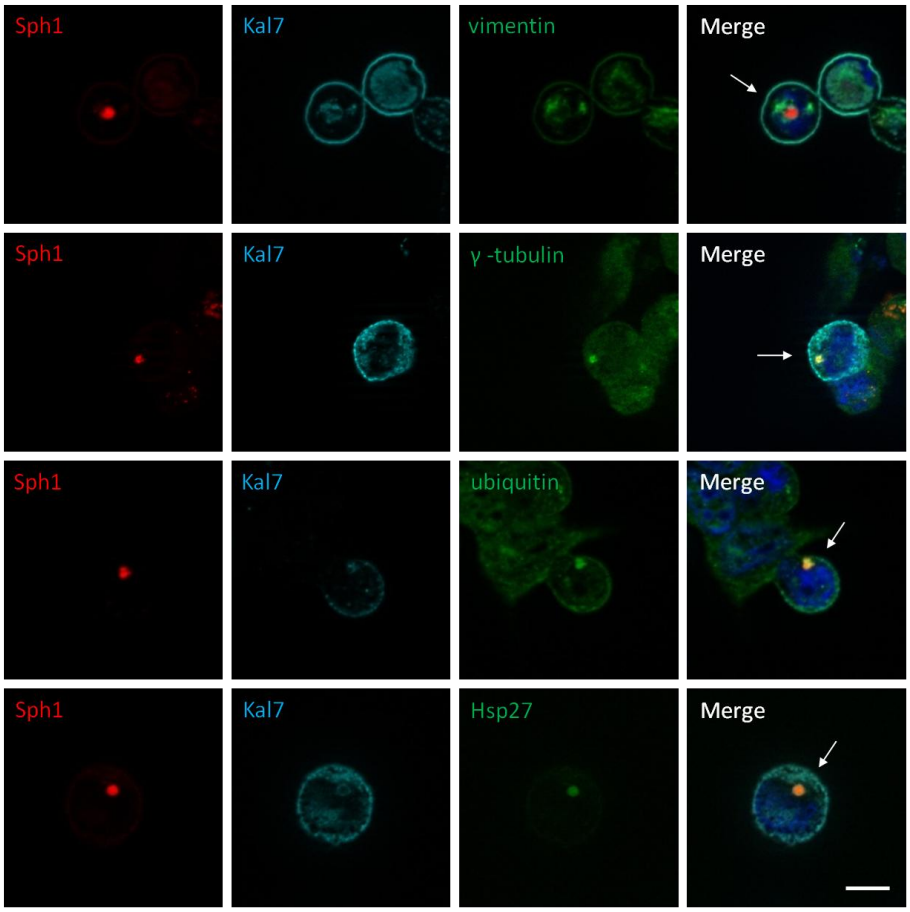


FIGURE 2.7 Characterization of synphilin-1-containing aggregates as aggresomes
HEK293 cells coexpressing HcRed-synphilin-1 and FLAG-kalirin-7 were fixed 48 h post-transfection and subsequently stained with indicated antibodies. Arrows indicate the colocalization between synphilin-1 inclusions and γ-tubulin, ubiquitin and Hsp27 while intermediate filament protein vimentin forms a cage surrounding a pericentriolar core of aggregates. Merged images are shown to the right. *Blue*, DAPI. *Scale bar*, 10 µm.

Misfolded and aggregated proteins in aggresomes are often largely insoluble in non-denaturing detergents (Johnston et al. 2002). To investigate if recruitment of synphilin-1 inclusions to aggresomes by kalirin-7 alters its solubility, cell lysates were separated into detergent soluble and insoluble fractions, and examined by Western blotting. Synphilin-1 was mainly detected in the soluble fraction either in the presence or absence of kalirin-7. However, synphilin-1 was also accumulated in the detergent insoluble fraction under coexpression of kalirin-7 (Fig. 2.8 A and B). Taken together, these data demonstrate that the recruitment of synphilin-1 to the perinuclear aggregates mediated by kalirin-7 is dependent on intact microtubules and the perinuclear inclusions containing synphilin-1 are aggresomes.

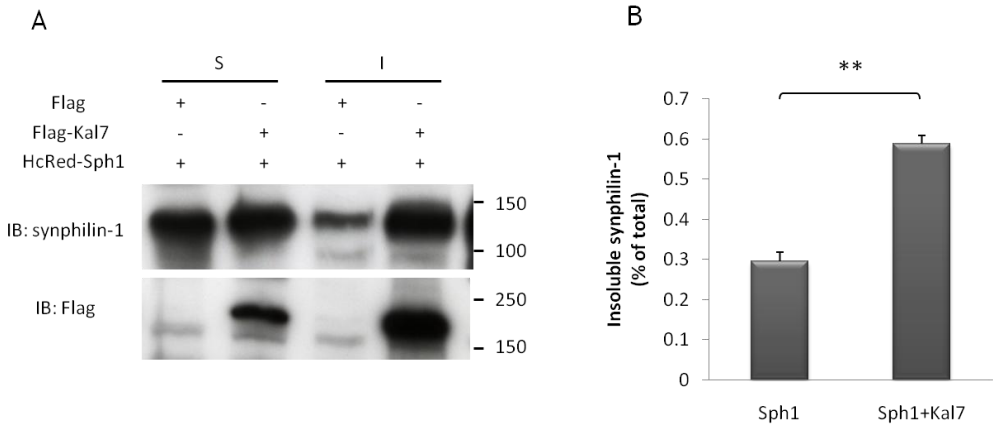


FIGURE 2.8 Kalirin-7 increases the insolubility of synphilin-1 aggregates
(A) Lysates from transfected HEK293 cells were separated into detergent-soluble (S) and detergent-insoluble (I) fractions. Samples were subjected into a 8% SDS-PAGE and probed with the antibody against synphilin-1. (B) To compare the relative level of insoluble synphilin-1, the intensity of the HcRed-synphilin-1 band in the detergent-insoluble fraction was quantified and normalized to the total level of HcRed-synphilin-1 in the corresponding cell lysate. The asterisks indicate statistical significance ($^{**}P \leq 0.005$). *Error bars*, S.E.

2.2.4 Kalirin-7 renders synphilin-1 inclusions susceptible to degradation

To quantify synphilin-1 inclusions biochemically, both for HcRed-synphilin-1 expression alone and for co-expression with FLAG-kalirin-7, a newly described method, Agarose gel electrophoresis for resolving aggregates (AGERA), was used (Weiss et al. 2008). It is a sensitive biochemical method used to reliably quantify aggregate load and size, as previously demonstrated for the huntingtin protein *in vitro* and *in vivo* (Fox et al. 2010; Zheng et al. 2010).

The analyses showed that the amount of synphilin-1 aggregates increased in a time-dependent manner (Fig. 2.9). Notably, in accordance with the results from the immunofluorescence stainings, co-expression of FLAG-kalirin-7 resulted in less aggregates than for HcRed-synphilin-1 expression alone at 48 h after transfection. The same result was obtained 72 h post-transfection.

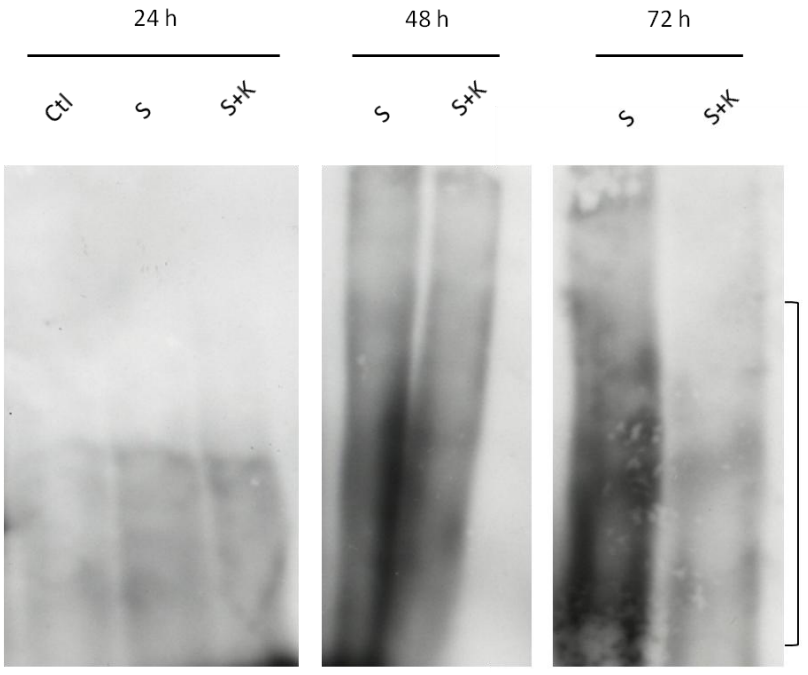


FIGURE 2.9 Kalirin-7 decreases synphilin-1-induced aggregates in biochemical analysis
HEK293 cells were transfected with HcRed-synphilin-1 alone or cotransfected with FLAG-kalirin-7. Cells were lysed 24, 48 or 72 h after transfection, fractionated by AGERA on 2% agarose gels and analyzed by western blotting with an antibody recognizing synphilin-1 aggregates. S, HcRed-synphilin-1; K, FLAG-kalirin-7.

Next, we monitored the effect of kalirin-7 on the temporal and spatial changes of synphilin-1 aggregates in the transition period between cytoplasmic small aggregates and perinuclear aggresomes in living cells. For this purpose we performed time-lapse imaging, which has been used to investigate fundamental cellular processes and the formation of huntingtin aggregates in a cell model of HD as well as α-synuclein aggregates (Arrasate et al. 2004; Opazo et al. 2008) . HcRed-synphilin-1 was coexpressed with EGFP empty vector as control or with EGFP-kalirin-7, respectively. Long-term monitoring revealed that cytoplasmic small aggregates were stable whereas aggresomes disappeared at the end of observation (Fig. 2.10 A). The total number of steady state synphilin-1 aggregates was significantly reduced at 67h post-transfection when kalirin-7 was co-overexpressed (Fig. 2.10 B). These results indicate that kalirin-7 increases the susceptibility of synphilin-1 inclusions to be degraded.

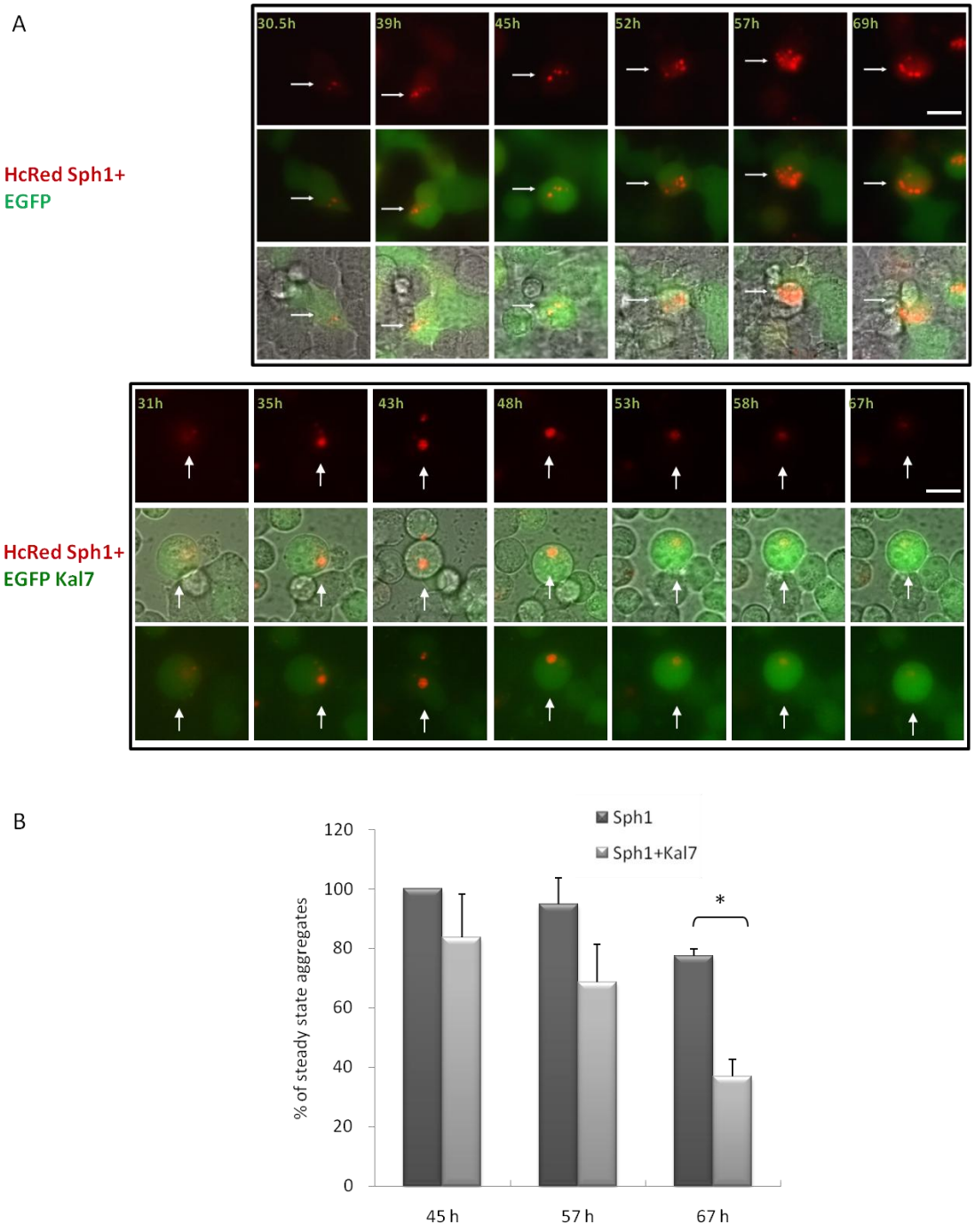


FIGURE 2.10 Kalirin-7 decreases synphilin-1-induced aggregates in live cell analysis
(A) Long-term time-lapse imaging. HEK293 cells were transfected with HcRed-synphilin-1 and empty EGFP vector (upper chart) or EGFP-kalirin-7 (lower chart) and observed by live cell imaging fluorescent microscopy (Cell Observer Z1, Zeiss, Germany) at 37 °C using ApoTome optical slides. Time-points indicate hours post-transfection. Images were merged from red, green and phase contrast channels. Arrows indicate the cell traced over the experimental time. *Scale bar*, 10 µm. (B) Quantification of the time-lapse imaging shows that aggregate numbers are reduced when FLAG-kalirin-7 is coexpressed. For each batch of experiment, at least 30 cells were tracked. Results represent the average of three independent experiments. The asterisks indicate statistical significance (*$P \leq 0.05$). *Error bars*, S.E.

2.2.5 Kalirin-7 mediated aggresome response is not dependent on its GEF activity

To test if the observed enhancement of aggresome formation by kalirin-7 is dependent on its Rho-GEF activity as outlined above, we tested whether various dominant negative Rho-like small GTPases mutants, such as RhoG F37A, Rac1 T17N and Rheb D60K can inhibit the kalirin-7-mediated recruitment of synphilin-1 aggregates into aggresomes. To this end, the percentage of cytoplasmic aggregates and perinuclear aggregates was determined in transfected cells. However, none of the tested Rho-like small GTPase mutants, nor a GEF knockout construct led to significant changes in the percentage of cytoplasmic and perinuclear aggregates (TABLE2.3), indicating that the enhanced formation of aggregates does not depend on GTP-GDP exchange activity of kalirin-7.

TABLE 2.3 Percentage of cells with cytoplasmic small aggregates or perinuclear aggregates in HEK293 cells transfected with different small GTPase competitive constructs

Constructs	% of cytoplasmic small aggregates	% of perinuclear aggregates
Sph1	43.2 ± 9.05	11.3 ± 5.86
Sph1+Kal7	2.0 ± 0.49	56.9 ± 9.70
Sph1+Kal7+RhoG F37A*	6.31 ± 8.26	44.17 ± 7.65
Sph1+Kal7+Rac1 T17N*	8.71 ± 3.99	61.36 ± 3.21
Sph1+Kal7+Rheb D60K*	16.01 ± 4.26	42.47 ± 4.33
Sph1+Kal7 dGEF**	7.51 ± 5.34	59.77 ± 6.30

Numbers in the TABLE represent the mean ± standard deviation.

* Cells were co-transfected with HcRed-synphilin-1, FLAG-kalirin-7 and RhoG F37A/Rac1 T17N/Rheb D60K (dominant negative RhoG/Rac1/Rheb constructs).

** Cells were co-transfected with HcRed-synphilin-1 and an inactivated GEF domain of kalirin-7.

2.2.6 Kalirin-7 has no effect on microtubule stability

To examine the effect of kalirin-7 on microtubule stability biochemically, the cell was lysed in MT-stabilizing buffer containing 0.5% Nonidet P-40 and the cellular tubulin was fractionated into polymeric and protomeric pools. When the cells were treated with nocodazole, most of the tubulin was disrupted and detected in the supernatant. In contrast, when the cells were treated with taxol, tubulin was stabilized and recovered in the pellet fraction (Fig. 2.11). When HEK293 cells were transfected with synphilin-1, kalirin-7 or both constructs, the distribution of polymeric and protomeric tubulin did not change. The same results were obtained in the nocodazole or taxol treated cells, indicating that kalirin-7 or the synphilin-1/kalirin-7 complex did not affect microtubule stability.

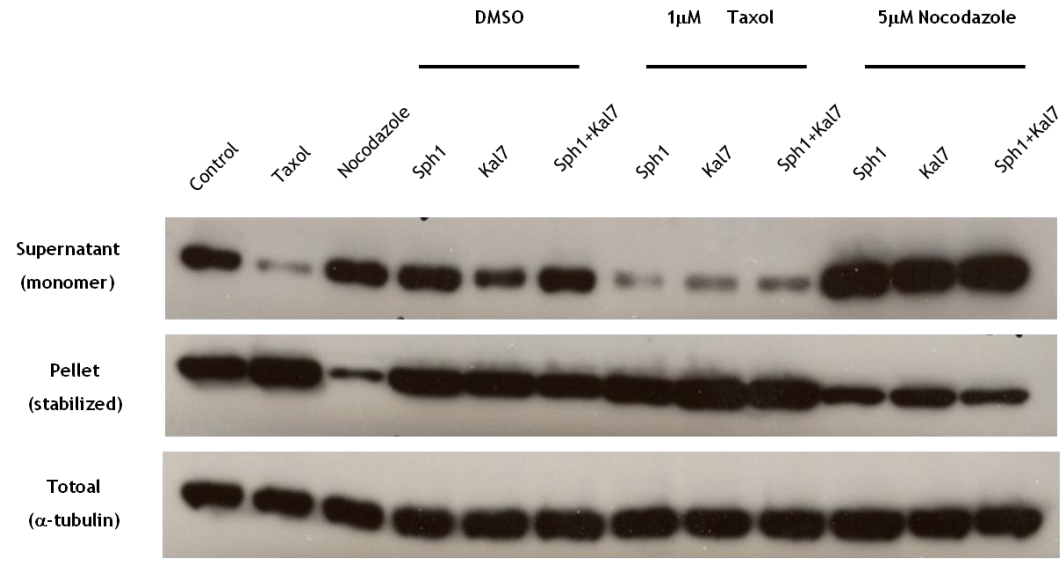


FIGURE 2.11 Kalirin-7 has no effect on microtubule stability
HEK293 cells expressing V5-synphilin-1 alone or V5-synphilin-1 plus FLAG-kalirin-7 were lysed in MTS buffer. Immunoblots showing the amount of tubulin in the pellet and supernatant in control cells, cells treated with Taxol for 18 h or Nocodazole for 18 h.

2.2.7 Recruitment of synphilin-1 aggregates into aggresomes by kalirin-7 is HDAC6 dependent

To determine the molecular basis of the observed kalirin-dependent aggresome formation, we focused on other aspects of aggresome formation and function. It is

known that polyubiquitinated proteins are transported into aggresomes for degradation and/or storage. This process is mediated by histone deacetylase 6 (HDAC6) which binds both polyubiquitinated misfolded proteins and dyneins, thereby recruiting protein cargo to dynein motors for transport to aggresomes (Kawaguchi et al. 2003). We therefore tested whether HDAC6 is involved in kalirin-7 mediated synphilin-1 aggresome formation.

To examine this, we first treated cells with trichostatin A (TSA) which inhibits three classes of HDACs or sodium butyrate (NaBu), a broad deacetylase inhibitor which does not affect HDAC6 activity (Guardiola and Yao 2002). As shown in Fig. 2.12 A, treatment with TSA, but not NaBu, resulted in an increase of small cytoplasmic aggregates when HcRed-synphilin-1 and FLAG-kalirin-7 were coexpressed. The percentage of perinuclear aggregates in the double transfected cells decreased from 44.1% to 17.03% after treatment with 1µM TSA (Fig. 2.12 B). Thus TSA significantly inhibited the kalirin-7-mediated recruitment of synphilin-1 aggregates into aggresomes. HDAC6 contains two deacetylase domains that are both required for normal deacetylase activity (Zhang et al. 2006). To further explore whether kalirin-7-induced aggresome response is dependent on catalytic activity of HDAC6, WT and double mutant (H216A/H611A) HDAC6 were triple transfected with synphilin-1 and kalirin-7 to compete with endogenous HDAC6 protein (Fig. 2.14 A). The percentage of cytoplasmic small aggregates in the HDAC6 mutant triple transfected cells significantly increased from 19.79% to 53.49% (Fig. 2.14 B), while HDAC6 WT did not affect the aggresome response. These data underline that kalirin-7 stimulates the recruitment of synphilin-1 into aggresomes in an HDAC6-dependent manner. The deacetylase activity of WT and H216A/H611A mutant HDAC6 was confirmed by the level of acetylated α-tubulin. As shown in Fig

2.13, WT HDAC6 displayed lower levels of acetylated α-tubulin while the mutant construct blocked the effect. There was no difference in α-tubulin staining.

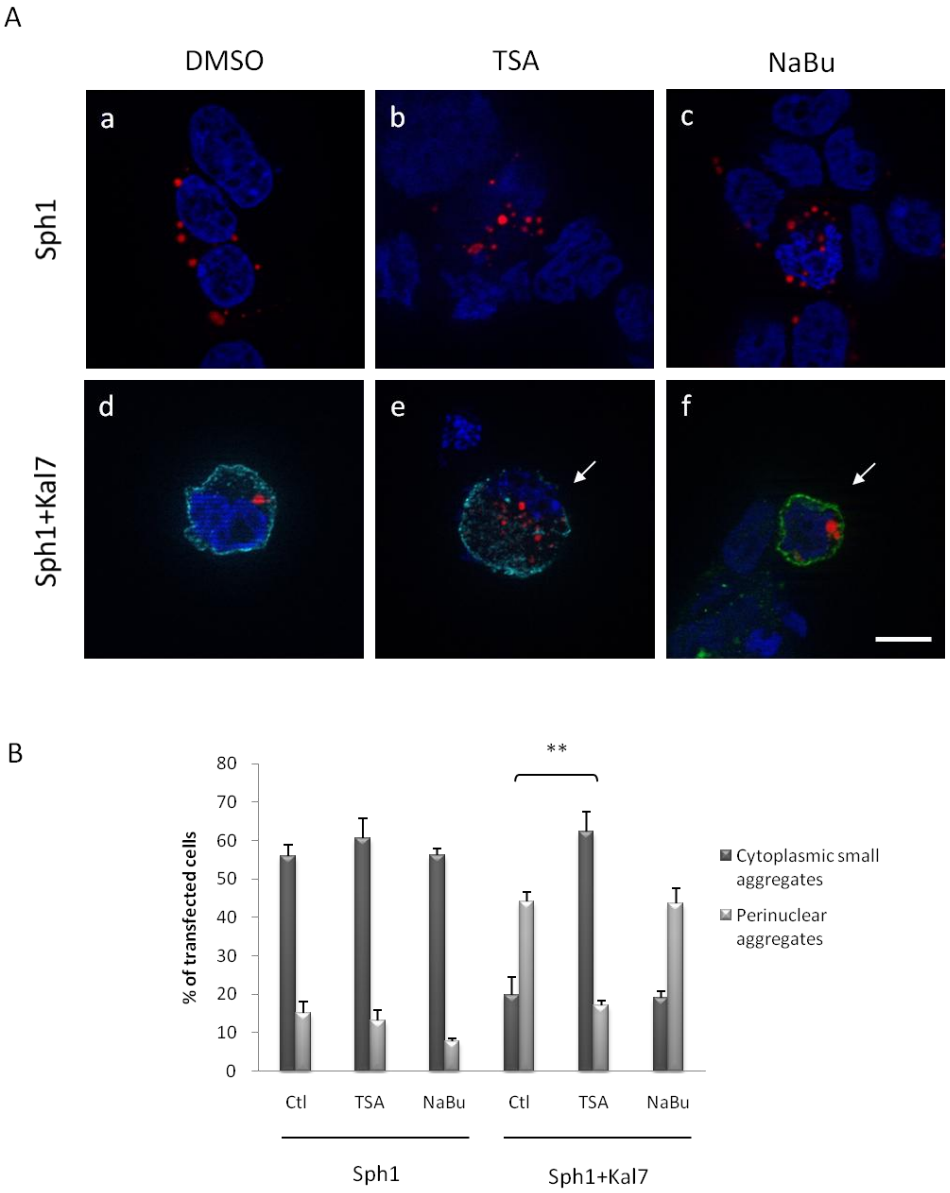


FIGURE 2.12 Kalirin-7-mediated recruitment of synphilin-1 inclusions into aggresomes is blocked by the HDAC inhibitor trichostatin A (TSA), but not sodium butyrate (NaBu)
(A) HEK293 cells expressing HcRed-synphilin-1 alone (a,b,c) or coexpressing HcRed-synphilin-1 and FLAG-kalirin-7 (d,e,f) were incubated in the presence of DMSO (a,d), 1μM TSA (b,e) or 5mM NaBu (c,f) for 18 h before being fixed and immunostained with anti-FLAG antibodies. The arrow indicates synphilin-1 cytoplasmic small aggregates. *Blue*, DAPI. *Scale bar*, 10 μm. (B) Quantification shows that treatment with the HDAC6 inhibitor TSA counteracts the recruitment of synphilin-1 into aggresomes mediated by kalirin-7, whereas the broad deacetylase inhibitor NaBu does not exert such an effect. The asterisks indicate statistical significance (**$P \leq 0.005$). *Error bars,* S.E.

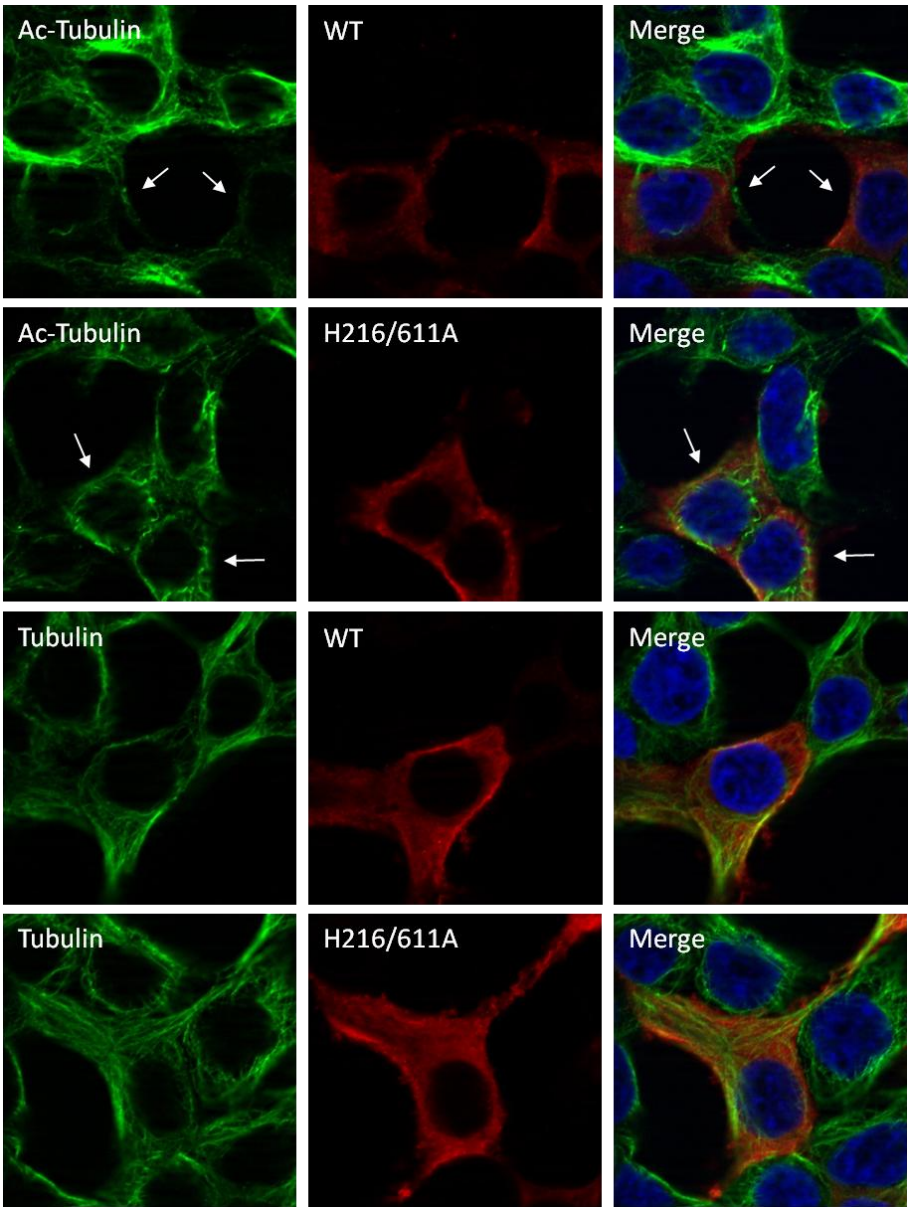
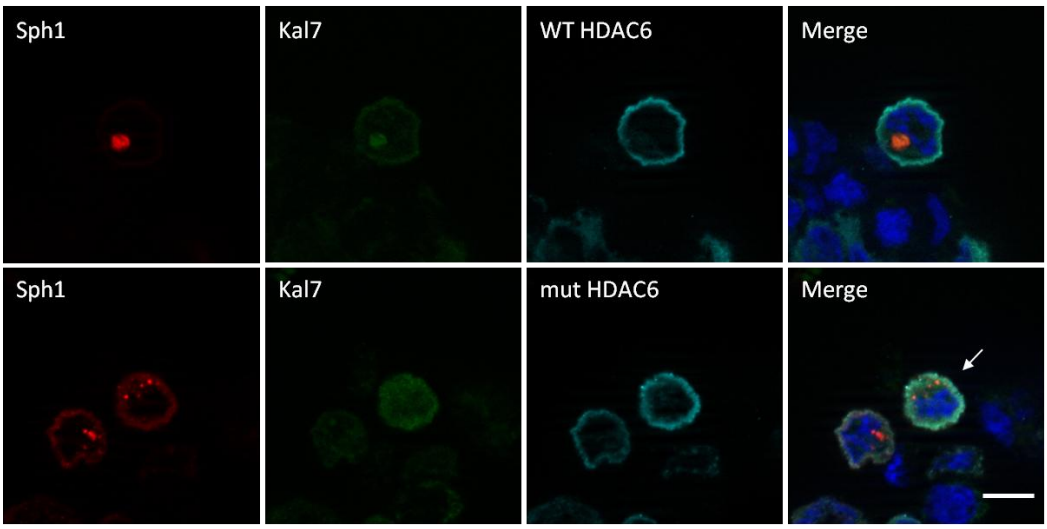


FIGURE 2.13 HDAC6 H216A/H611A double mutant inhibits the deacetylase activity of the wild type protein

Expression vectors encoding FLAG-tagged wild type or H216A/H611A HDAC6 were transiently transfected into HEK298 cells as indicated. Cells were fixed 24 h after transfection and immunofluorescence stainings for FLAG, acetyl-tubulin or tubulin were performed. Arrows indicate the staining of acetyl-tubulin in HDAC6 WT or double mutant transfected cells. Blue, DAPI. *Scale bar*, 10µm.

A



B

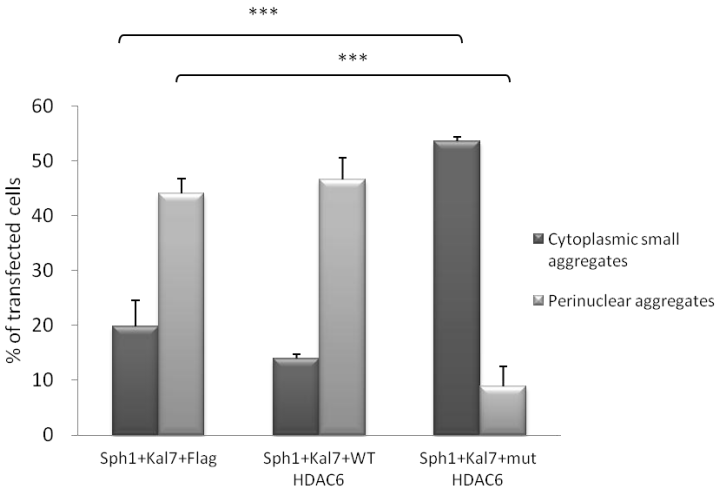


FIGURE 2.14 An HDAC6 deacetylase-dead mutant rescues the formation of synphilin-1 containing aggresomes mediated by kalirin-7

(A) HEK293 cells were triple-transfected with HcRed-synphilin-1, EGFP-kalirin-7 and FLAG-WT HDAC6 or FLAG-H216A/H611A mutant HDAC6. After 48 h cells were fixed and stained with anti-FLAG antibodies. Only cells coexpressing mutant HDAC6 form more cytoplasmic small aggregates (arrows). (B) Quantification shows that mutant HDAC6 inhibited the kalirin-7-mediated perinuclear synphilin-1 inclusion formation. The asterisks indicate statistical significance ($^{***}P \leq 0.001$). *Error bars*, S.E.

Furthermore, kalirin-7 is reported to interact with HAP1, which is associated with p150 ^{Glued} and regulates retrograde and anterograde trafficking of cargos (Rong et al. 2007; Wu et al. 2010). Apart from binding to ubiquitinated proteins, HDAC6 catalyzes the removal of acetyl groups of α-tubulin and plays an important role in microtubule-dependent intracellular trafficking (Zhang et al. 2003). Given this involvement in intracellular trafficking, we next tested if kalirin-7 and HDAC6 act in a common complex to transport synphilin-1 into aggresomes. To address this hypothesis, kalirin-7 and HDAC6 were subjected to coimmunoprecipitation. As shown in Fig. 2.15 A, kalirin-7 interacted with HDAC6 *in vitro*. In addition, interactions between synphilin-1 and HDAC6 (Fig. 2.15 B), synphilin-1 and HAP-1 (Fig. 2.16 A), synphilin-1 and p150 ^{Glued} (Fig. 2.16 B) were also observed.

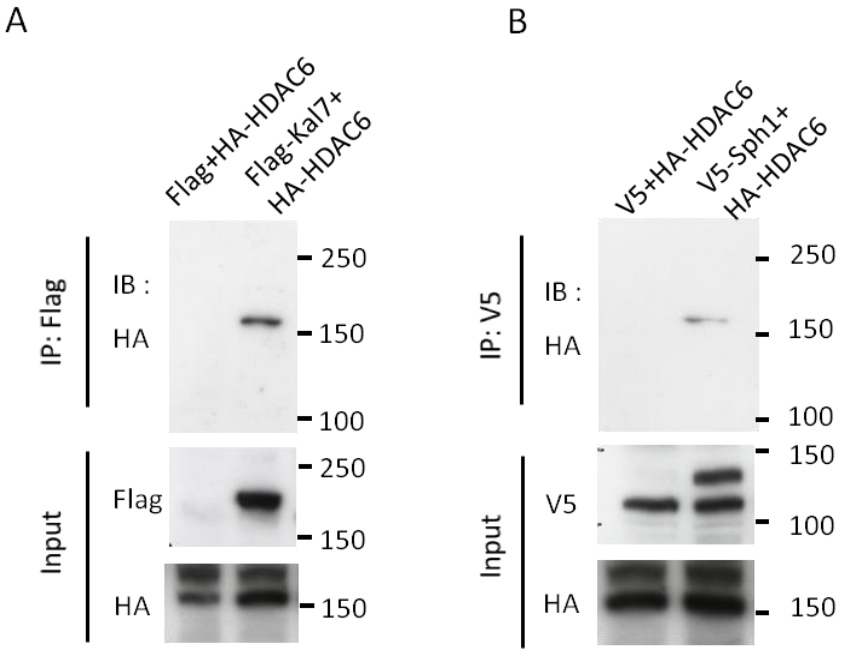


FIGURE 2.15 Kalirin-7 and synphilin-1 are interactors of HDAC6
(A, B) An interaction of FLAG-kalirin-7, V5-synphilin-1 and HA-HDAC6 was examined by coimmunoprecipitation experiments. 24 h after transfection, the cells were subjected to immunoprecipitation with anti-FLAG or anti-V5 conjugated agarose beads, respectively. The precipitates were probed with HA antibodies to detect HDAC6 and revealed an interaction of HDAC6 with both kalirin-7 and synphilin-1.

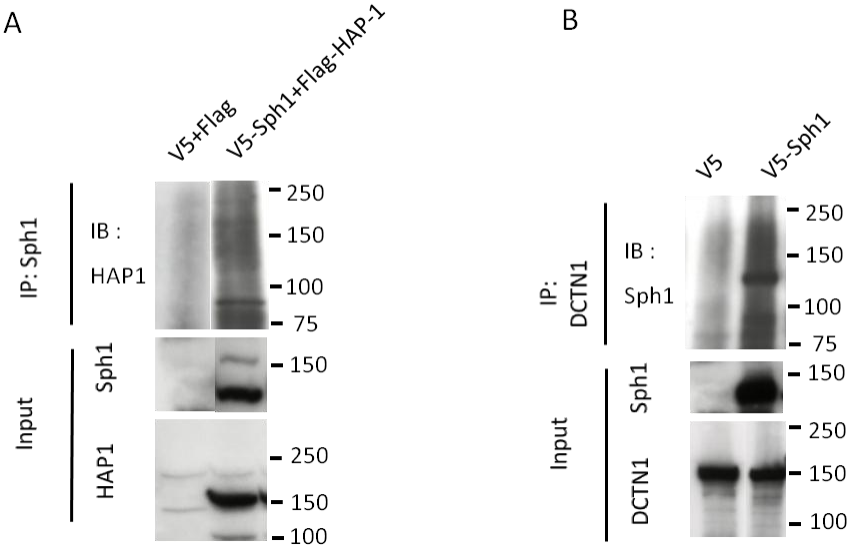


FIGURE 2.16 HAP-1 and dynactin p150Glued are interactors of synphilin-1

Binding of HAP-1, p150Glued and synphilin-1 was examined by co-immunoprecipitation respectively. 24 h after transfection, cells were subjected to immunoprecipitation with anti-synphilin-1 agarose beads (A) and probed with HAP-1 or anti-DCTN1 coupled with A/G agarose beads and probed with synphilin-1 (B).

Next, we evaluated if kalirin-7 would affect the acetylation level of α-tubulin. The changes in tubulin acetylation were quantified by measuring the fluorescence intensity of individual cells after immunostaining for acetylated tubulin in HEK cells overexpressing empty vector, FLAG-kalirin-7, HcRed-synphilin-1 or both constructs. While TSA treatment resulted in higher acetylation levels in comparison to controls, the presence of kalirin-7 led to a significant decrease of the acetylation level of α-tubulin (Fig. 2.17 A and B), indicating that kalirin-7 activates the deacetylation activity of HDAC6. This probably represents the molecular basis for the kalirin-mediated effect on the microtubule-dependent recruitment of synphilin-1 inclusions into aggresomes.

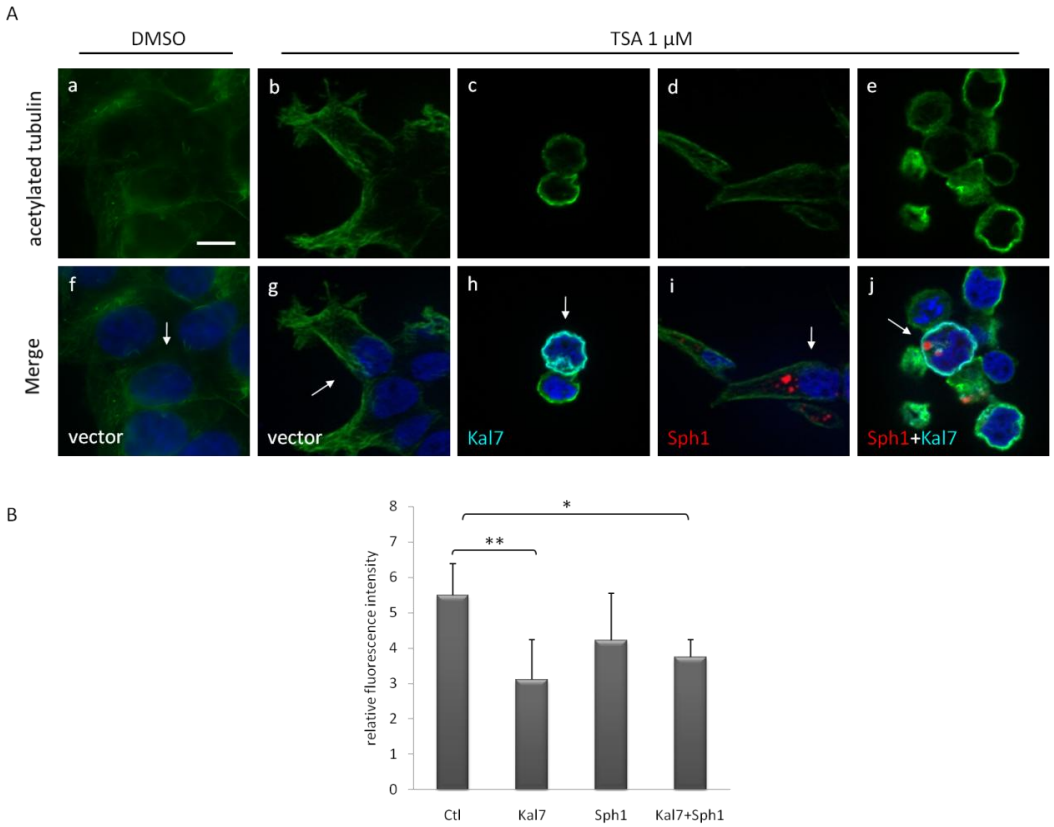


FIGURE 2.17 Decreased tubulin acetylation in kalirin-7 expressing cells under TSA treatment

(A) Cells transiently overexpressing FLAG-kalirin-7 (c, h), HcRed-synphilin-1 (d, i), both constructs (e, j) or empty vectors (a, b, f, g) were immunostained for acetylated tubulin (a-e, green) and kalirin-7 (h, j, light blue) after DMSO or 1µM TSA treatment. The arrows show examples of cells selected for further quantification. *Blue*, DAPI. *Scale bar*, 10 µm. (B) Acetylated tubulin levels were quantified by the fluorescence signal of individual cells, as described in Materials and Methods. The relative fluorescence intensity was measured in controls (vector transfection with TSA treatment) as well as in FLAG-kalirin-7-, HcRed-synphilin-1- or double-transfected cells. The asterisks indicate statistical significance ($^{**}P \leq 0.005$). *Error bars*, S.E., n=100.

2.2.8 Synphilin-1 interactor study by mass spectrometry analysis

Synphilin-1 has been proposed to anchor α-synuclein to the vesicle membrane (Ribeiro et al. 2002) and in this study we found that synphilin-1 is transported along the microtubule. Therefore we were interested in whether synphilin-1 serves as a cargo or whether it interacts with motor proteins to facilitate transportation. To this end, mass spectrometry analysis was conducted. Using a newly developed tandem affinity purification method (Gloeckner et al. 2009), the native synphilin-1

multiprotein complex was isolated. The quality and quantity of the purified protein from HEK293 and PC12 cells are shown in Fig. 2.18 A and B. After the second purification step with the FLAG resin, the background of unspecific proteins was reduced. However, the purified protein from PC12 cells was barely detected. The protein expression and specificity was also detected on the western blot, which showed a high overexpresssion of TAP-tagged synphilin-1 in HEK293 and PC12 cell lines (Fig. 2.18 C and D). The newly identified potential interactors are shown in TABLE 2.4, including three heat shock proteins (HSPA1B heat shock 70kDa protein 1A/1B, HSPA8 heat shock 71kDa protein 8 and HSPA5 heat shock glucose-regulated protein), PPP1CA protein phosphatase 1 and Acetyl-CoA carboxylase.

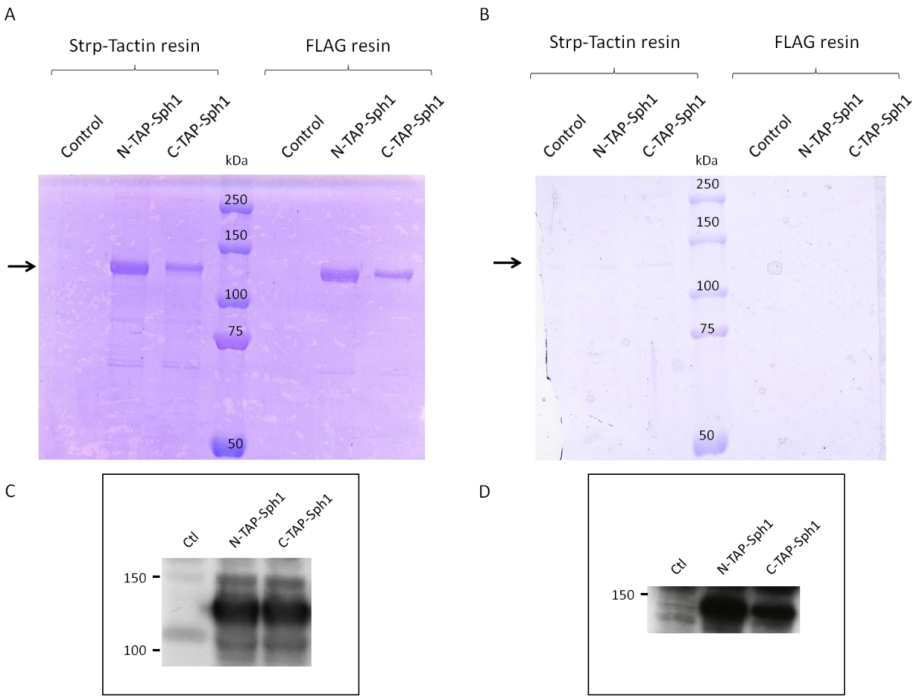


FIGURE 2.18 Purification of TAP-tagged synphilin-1 protein from HEK293 and PC12 cell lines

(A) N-, C- terminal SF-TAP-tagged synphilin-1 and empty vector (control) were overexpressed in HEK293 for 48 h. The cell lysates containing synphilin-1-SF-TAP fusion protein was purified by a two-step elution procedure: Strep-Tactin resin and anti-FLAG resin. 20 µl of the corresponding lysate was kept and denatured in Laemmili buffer. The samples were subjected into SDS-PAGE and stained with Coomassie blue to visualize the protein as indicated by the arrow (~130kDa). The same experiment was repeated in PC12 cell line (B). The expression of fusion proteins from HEK293 and PC12 cell lines was detected in Western blot (C, D).

TABLE 2.4 Synphilin-1 interactors identified by MS analysis

Identified Proteins	Accession Number	Molecular Weight	Score		
			TAP control	Sph1-CTAP	Sph1-NTAP
Heat shock 70 kDa protein 1A/1B n=3 Tax=Hominidae RepID=HSP71_HUMAN	P08107	70 kDa	13 (3)	27 (31)	26 (32)
Serine/threonine-protein phosphatase PP1-alpha catalytic subunit n=5	P62136	38 kDa	0 (0)	17 (20)	15 (20)
Heat shock cognate 71 kDa protein n=8 Tax=Eutheria RepID=HSP7C_HUMAN	P11142 (+1)	71 kDa	8 (2)	22 (23)	20 (25)
78 kDa glucose-regulated protein n=1 Tax=Homo sapiens RepID=GRP78_HUMAN	P11021 (+1)	72 kDa	3 (0)	17 (28)	19 (25)
Acetyl-CoA carboxylase 1 OS=Homo sapiens GN=ACACA PE=1 SV=2		266 kDa	0	22	22

2.2.9 Morphological changes of kalirin-7 and its variants

The spectrin-repeat domain and GEF domain of kalirin-7 have been postulated to modulate cytoskeletal rearrangement and various signal transduction pathways (Kapiloff 2002; Penzes and Jones 2008). In Fig. 2.3 and Fig. 2.5 A we saw dramatically different distribution of kalirin-7 at 6 h and 48 h post-transfection. To rule out an artificial effect of the overexpressed protein, we monitored the distribution of full length kalirin-7 as well as various mutants over time. 6 h after overexpression, kalirin-7 was diffusely distributed in the cytoplasm and the morphology of the cell was similar to non-transfected cells (Fig. 2.19). However, the protein seemed to concentrate to the nucleus upon the time of transfection and the morphology of the cell became round (Fig. 2.19). Next, we observed the morphological changes of kalirin-7 deletion mutants. The results were obtained 24 h post-transfection. Full length and GEF-inactive kalirin-7 transfected cells showed round morphology and the protein was mainly located around the nucleus (Fig. 2.20). Nevertheless, kalirin-7 deletion mutants containing different parts of spectrin-repeats domains showed a diffused distribution (Fig. 2.20).

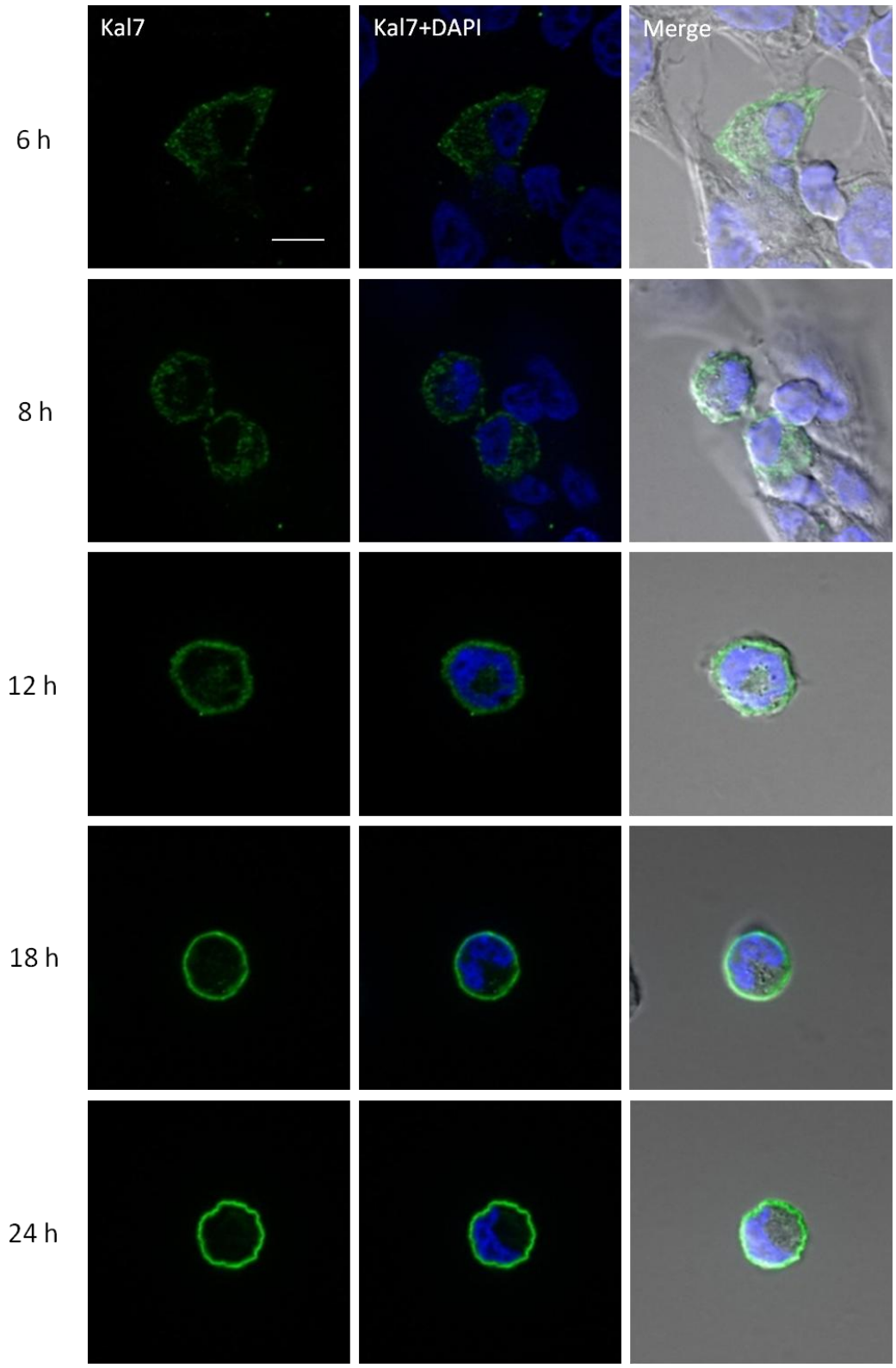


FIGURE 2.19 Time course of Kalirin-7 overexpression patterns
FLAG-kalirin-7 was overexpressed in HEK293 cells and fixed at five different time points indicated above. The samples were stained with FLAG antibody to visualize kalirin-7 expression. Merged images are shown to the right. Kalirin-7 was diffusely distributed in the cytoplasm at 6 h post-transfection whereas the protein was observed in the periphery of the nucleus 12 h after transfection. *Green*, kalirin-7; *Blue*, DAPI. *Scale bar*, 10 µm.

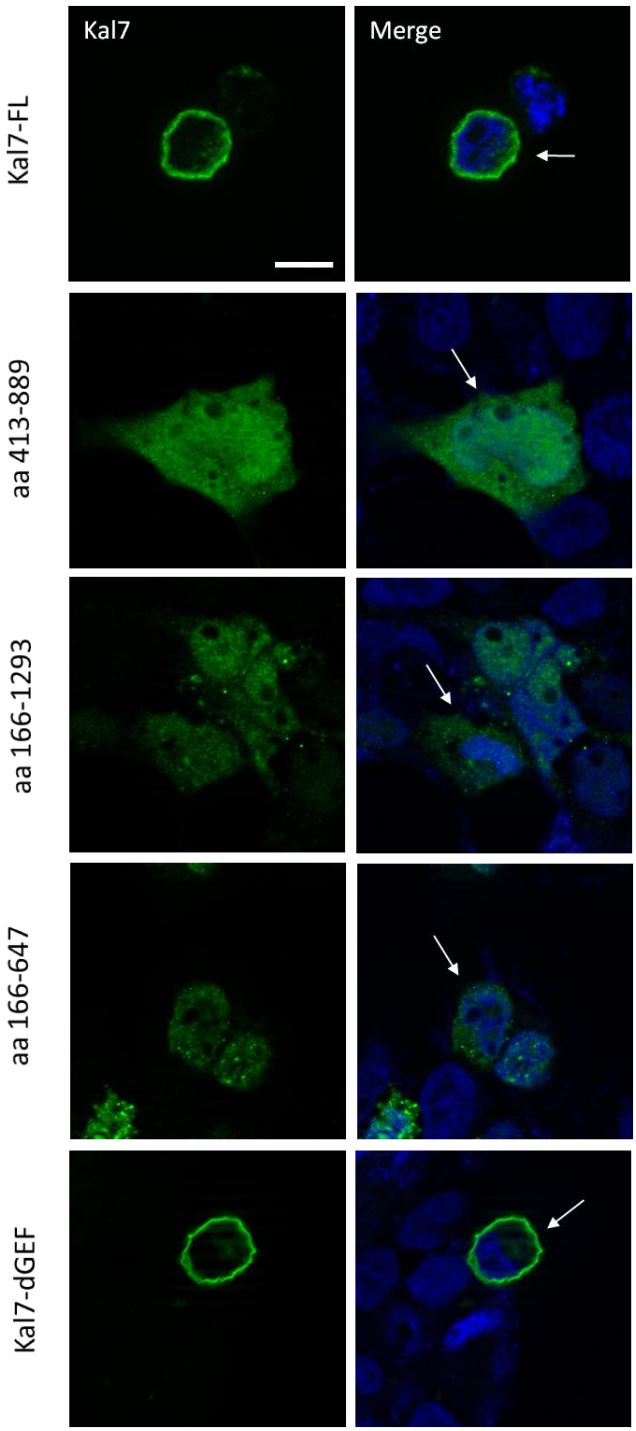


FIGURE 2.20 Different protein distribution of kalirlin variants
HEK293 cells transfected with various FLAG-kalirin-7 mutants indicated above were fixed 24 h post-transfection and subsequently processed for immunocytochemistry to visualize protein distribution. Merged images are shown to the right. Arrows indicate the protein distribution of kalirin-7 variants: spectrin-repeats domains of kalirin-7 (aa 413-819, aa 166-1293 and aa 166-647) showed distributed pattern; full length and dGEF of kalirin-7 were found around the nucleus. *Green*, kalirin-7; *Blue*, DAPI. *Scale bar*, 10 µm.

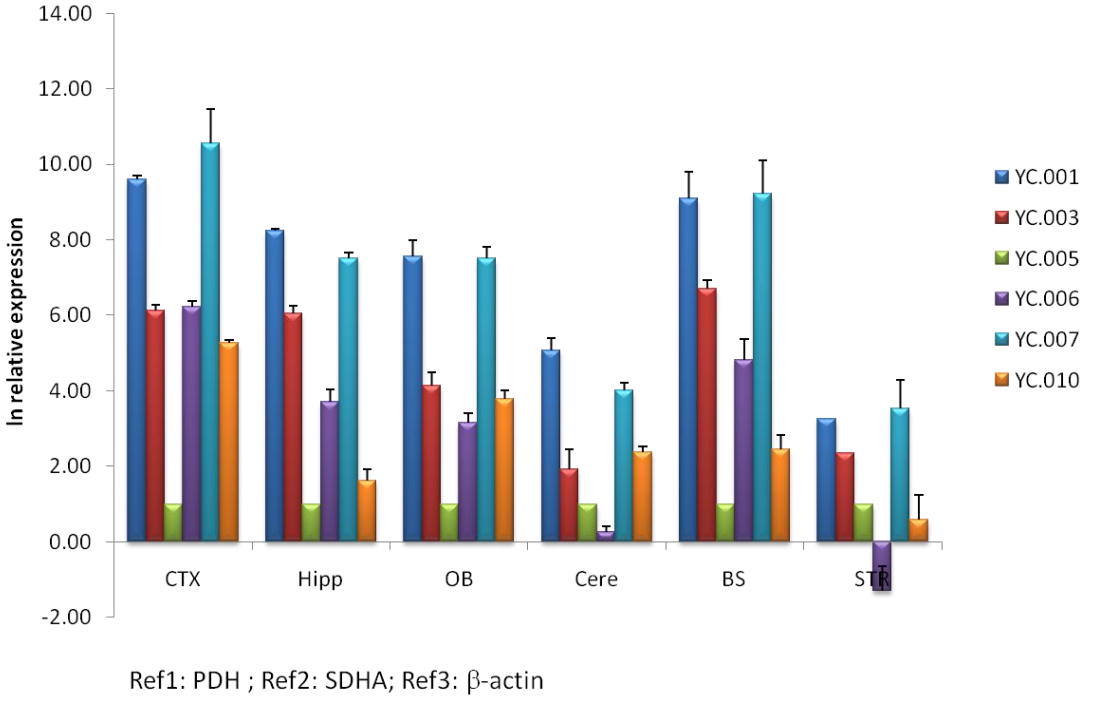
2.3 Generation of human kalirin-7 transgenic animals and human kalirin-7 specific antibody

2.3.1 Generation of human kalirin-7 transgenic mouse lines

Human kalirin-7 cDNA with a 5'-HD promoter and a 3'-poly A tail was cloned as described in the materials and methods section. The DNA construct was precipitated with 70% EtOH and sent to Dr. Zimmermann (IBF-Interfakultäre Biomedizinische Forschungseinrichtung, Heidelberg) for microinjection. 253 oocytes were injected and transferred into 9 pseudo pregnant dams. Out of 66 newborn pups 10 founders were identified by PCR. These founders mated with C57Bl/6N mice to bring them on a congenic C57Bl/6N background. Out of these 10 potential lines (hereafter called HD-Kal7), 6 lines showed germline transmission of the construct.

2.3.2 mRNA expression of kalirin-7 transgenic mice

To confirm the mRNA expression level of the transgene in HD-Kal7 transgenic mice, total mRNA was isolated and the human kalirin-7 mRNA was quantified using qRT-PCR technique. The results are shown in Fig. 2.21. Line 1 and 7 exhibited relatively higher expression of the transgene in all brain regions, including cortex (CTX), hippocampus (Hipp), olfactory bulbs (OB), cerebellum (Cere), brain stem (BS) and striatum (STR).



Ref1: PDH ; Ref2: SDHA; Ref3: β-actin

FIGURE 2.21 mRNA expression of human kalirin-7 in HD-Kal7 transgenic mice
The brain was dissected into CTX, Hipp, OB, Cere, BS and STR and subjected to qRT-PCR to quantify the human kalirin-7 mRNA expression. Different lines were shown in various colors. The relative expression was normalized by the line which has the lowest expression in each region, line YC.005. PDH, SDHA and β-actin were taken as reference genes for loading control. Results were the average of three independent experiments.

2.3.3 Protein expression of kalirin-7 in kalirin-7 transgenic mice

Second, we compared the protein expression level of human kalirin-7 in each transgenic line. It has been reported that kalirin-7 is highly enriched in cerebral cortex, hippocampus and Purkinje cells of the cerebellum (Ma et al. 2001). Therefore, we first examined human kalirin-7 expression level in the cortex and hippocampus of adult mice using a Kalirin-spectrin antibody (MP-KAL) which detects four isoforms of kalirin, including isoform 2 at the size of 190 kDa (Fig. 2.22 A and B). It's known that the protein size of kalirin-7 in human and mouse is nearly the same owing to the 98% homology of amino acid sequence. We expected that the higher expression of human kalirin-7 protein could be separated from the endogenous one in mouse. Unfortunately, we did not find any differences between WT and Tg animals.

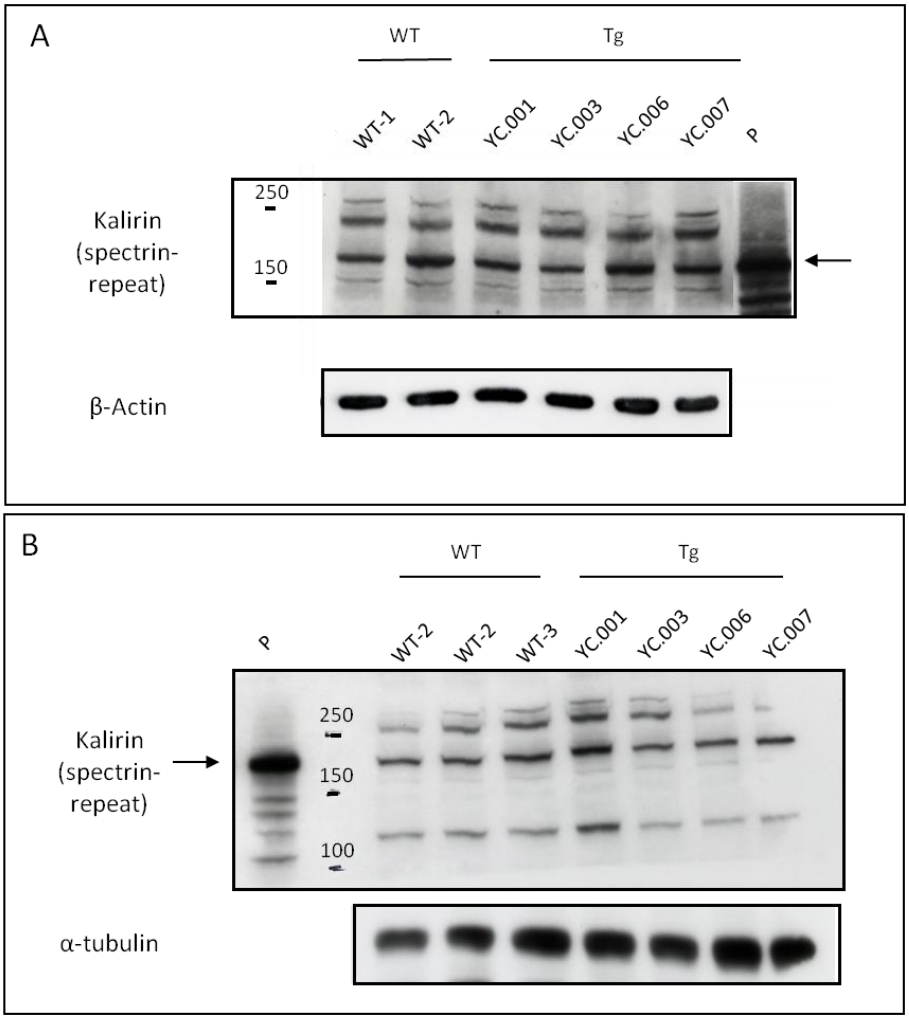


FIGURE 2.22 Protein expression of human Kal-7 in adult HD-Kal7 transgenic mice in cortex (A) and hippocampus (B)

The brain lysates were prepared as described in M&M. The samples were analyzed by western blotting and the protein expression was detected with Kalirin-spectrin antibody (MP-KAL). The arrow points out the specific band of kalirin-7 (190kDa) whereas other isoforms at the size of 370 and 420 kDa were also visible. β-Actin and α-tubulin served as loading controls. WT and Tg indicate wild type and Kal7 transgenic mice, respectively. P refers to the cell lysate overexpressing FLAG-tagged kalirin-7, shown as a positive control.

The expression of kalirin in cerebral cortex during postnatal development has been evaluated previously (Penzes et al. 2001a) and kalirin-7 is detectable only until postnatal day 15 whereas kalirin-9 and kalirin-12 are prevalent at postnatal day 2 (P2). Therefore, we tested whether human kalirlin-7 is already overexpressed at an early stage of development while endogenous kalirin-7 is not expressed. The results showed that the human kalirin-7 was detectable neither at P2 (Fig. 2.23 A) nor at P6 (Fig. 2.23 B).

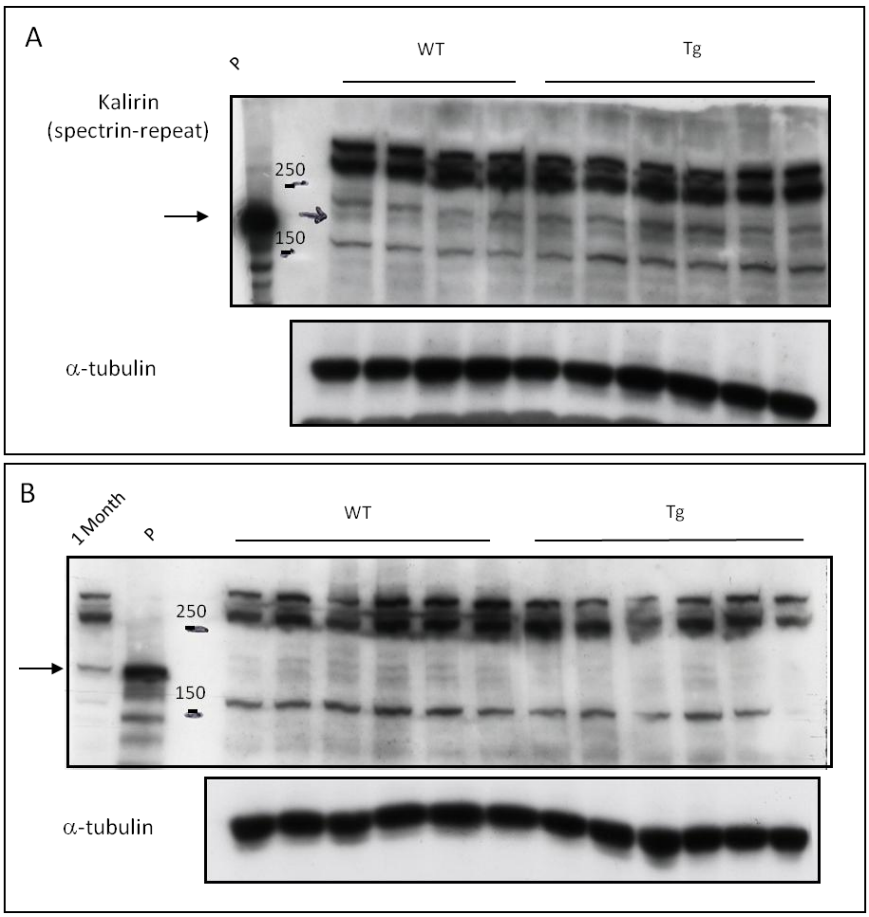


FIGURE 2.23 Kalirin-7 protein expression of P2 (A) and P6 (B) HD-Kal7 transgenic pups
Pups were sacrificed two days and six days after birth, respectively. The brain lysates were prepared as described in M&M. The samples were analyzed by western blotting and the protein expression was detected with Kalirin-spectrin antibody (MP-KAL). The arrow points out the specific band of kalirin-7 (190kDa). α-tubulin served as loading control. WT and Tg indicate wild type and Kal7 transgenic mice, respectively. P refers to cell lysate overexpressing FLAG-kalirin-7, shown as a positive control.

2.3.4 Generation and characterization of human specific kalirin-7 antibody

The commercial available antibodies against kalirin and their specificity are listed in TABLE 2.5. All of them show cross-reactivity between human and mouse and therefore they might not be suitable for detecting human specific kalirin-7. According to the NCBI database, sequences blast of kalirin-7 between *Homo sapiens* and *Mus musculus* reveals 9 additional amino acids in humans (Fig. 2.24). The sequences were confirmed by us via sequencer. Thereafter we generated a new antibody using this short stretch, SLFHATSLQ, which is located at position 942 to 950

in the spectrin-repeat domain, as an epitope. This work was done in collaboration with Dr. Kalbacher (Interfacultary Institute for Biochemistry, University of Tuebingen).

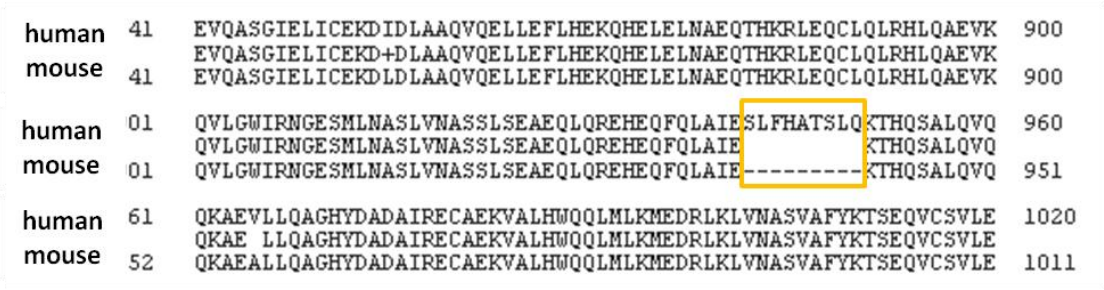


FIGURE 2.24 The blast of amino acid sequences between *Homo sapiens* and *Mus musculus* The first row indicates the sequence of kalirin isoform 2 of *Homo sapiens*, NP_003938.1 and the second row indicates the sequence of kalirin isoform 2 of *Mus musculus*, NP_01157740.1. The sequences were aligned with protein blast in NCBI. The frame shows 9 additional aa in *Homo sapiens* used as epitope of human specific kalirin-7 antibody.

TABLE 2.5 List of commercial available kalirin antibodies

Name	Epitope	Cross reactivity	Manufacturer	Note
anti-Kalirin (MP-KAL)	aa 517-976 of rat Kalirin protein (JH2582)	Mouse Human	Millipore (Upstate)	115, 190, 370, 420 kDa
anti-KALRN (BS-KAL)	aa 1591-1605 of human KALRN protein (KEPLQLPKTPAKQRN)	Mouse Human	Biosciences	
anti-KALRN (NV-KAL)	C-terminus of human KALRN (RWHLGPGDPFSTYV)	Mouse Human Rat	Novus	
anti-KALRN (Ab-KAL)	aa 1650-1663 of human KALRN protein (RWHLGPGDPFSTYV)	Mouse Human	Abcam	
anti-Duo (SC-Duo)	C-terminus of Duo of human origin (RWHLGPGDPFSTYV)	Mouse Rat	Santa Cruz	

The human specific kalirlin-7 antibody that we generated is a polyclonal antibody purified from rabbit serum. To characterize the specificity and affinity of our new antibody, kalirin-7 was first immunoprecipitated by available kalirin antibodies to enrich the protein concentration and then detected with our human specific kalirin-7

Ab. The first part was tested in HEK293 cells overexpressing FLAG-kalirin-7. Cell lysates were precipitated with FLAG Ab, human specific kalirin-7 Ab, kalirin-spectrin Ab from Millipore (MP-KAL) and C-terminus kalirin-7 Ab from Abcam (Ab-KAL), respectively and probed with FLAG or human specific kalirin-7 Ab (Fig. 2.25). The capacity of antibodies to pull down overexpressed FLAG-kalirin-7 was FLAG > C-terminus kalirin-7 (Ab-KAL) > kalirin-spectrin (MP-KAL) > human specific kalirin-7 Ab. Notably, human specific kalirin-7 Ab recognized the epitope in precipitates pulled down by the FLAG Ab and the C-terminus kalirin-7 Ab, but not when the other two Abs were used for pull-down (Fig. 2.25). This data indicates that human specific kalirin-7 Ab is able to detect human kalirin-7 protein only when the protein is highly concentrated.

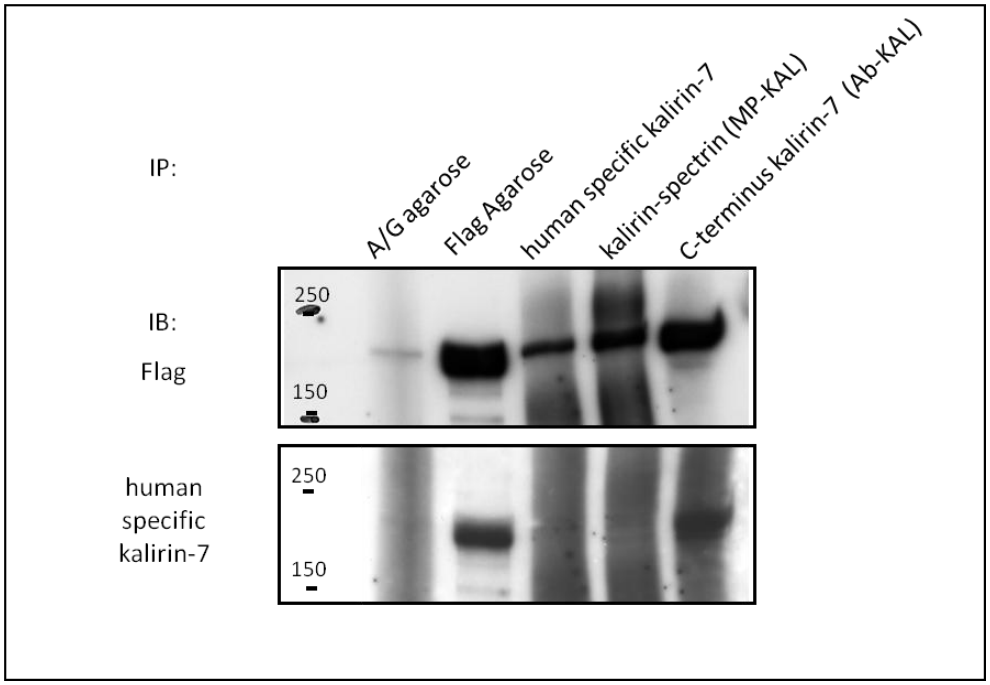


FIGURE 2.25 The specificity and affinity of human specific kalirin-7 Ab in a cell-based model

HEK 293 cells were transfected with FLAG-kalirin-7 for 24 h and lysed in RIPA buffer. Lysates were immunoprecipitated with various antibodies, FLAG Ab, human specific kalirin-7 Ab, kalirin-spectrin Ab (MP-KAL) and C-terminus kalirin-7 Ab (Ab-KAL). A/G agarose was used as control. Immunoprecipitates were analyzed with anti-FLAG and anti-human specific kalirin-7 antibodies.

In the second part, we precipitated kalirin-7 protein from mouse brain lysates. Since the overexpressed human kalirin-7 in transgenic mice was not tagged, we pulled down the protein with the C-terminus kalirin-7 Ab (Ab-KAL), which showed the second highest precipitation affinity in the cell model. The brain lysates and precipitates were analyzed by C-terminus kalirin-7 Ab (Ab-KAL) (Fig. 2.26 A) and human specific kalirin-7 Ab (Fig. 2.26 B). The C-terminus kalirin-7 Ab could clearly detect the endogenous kalirin-7 and transgenic human kalirin-7 in brain lysates as well as in precipitates from C-terminus kalirin-7 Ab (Fig. 2.26 A). However, human kalirin-7 Ab was not able to pull down kalirin-7 protein. Furthermore, human kalirin-7 Ab did not show the potential to recognize the kalirin-7 protein neither in the brain lysates nor precipitates. Although the new Ab did not work in Western blot, it might function in brain sections. To test this possibility, we stained brain slices of transgenic mice with human kalirin-7 Ab. However, there was no obviously different staining between wild type and HD-Kal7 transgenic mice (Fig. 2.27).

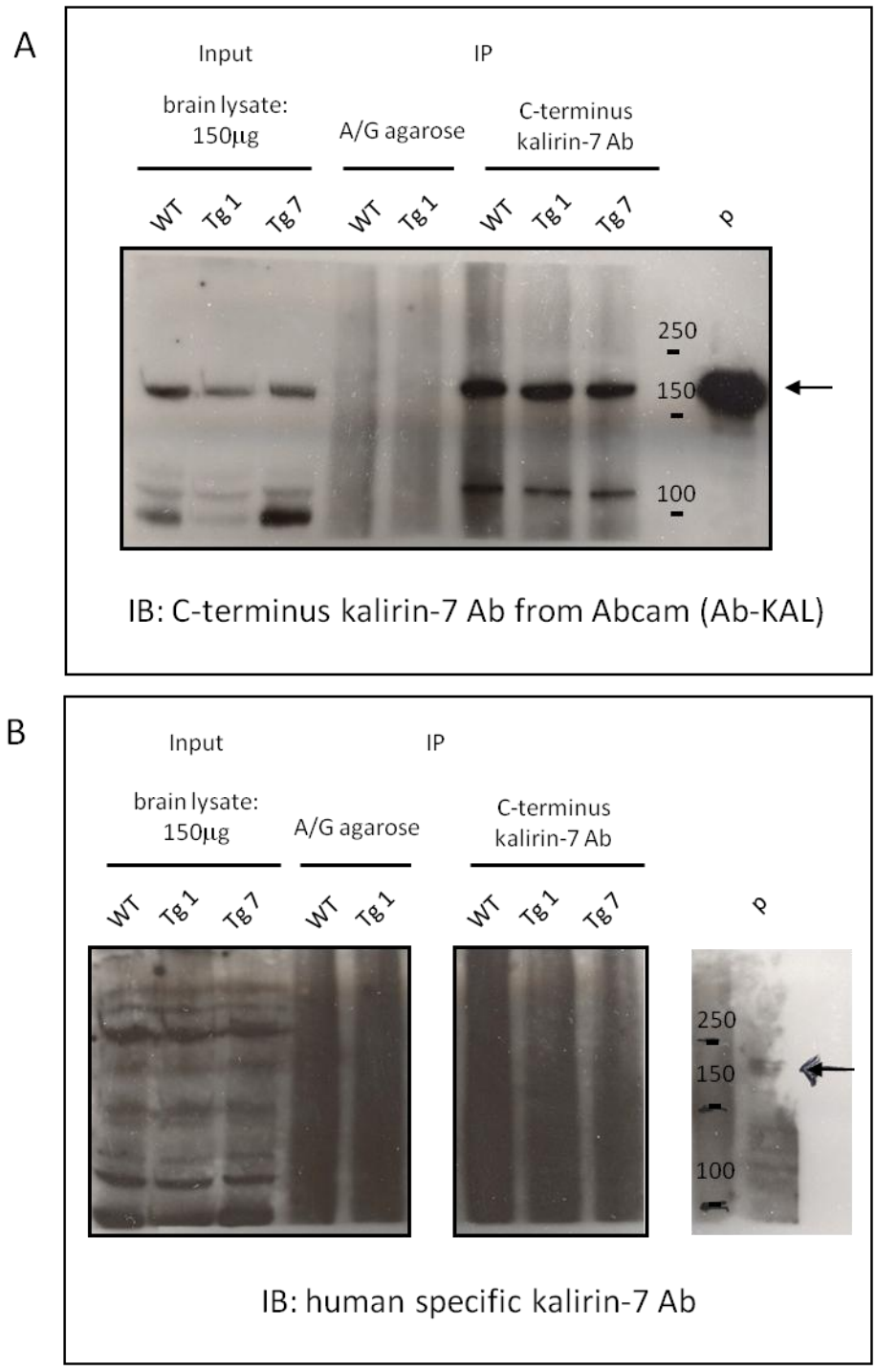


FIGURE 2.26 The specificity and affinity of human specific kalirin-7 Ab in brain lysates
500µg of brain lysates in TNES buffer from cortex of WT, transgenic line 1 (Tg1) and transgenic line 7 (Tg7) were immunoprecipitated with human specific kalirin-7 Ab and kalirin-spectrin Ab (MP-KAL). Immunoprecipitates were probed with anti-kalirin-spectrin Ab (Ab-KAL) (A) and human specific kalirin-7 Ab (B). Arrows indicate the predicted size of human kalirin-7 protein. P refers to cell lysate overexpressing FLAG-kalirin-7, shown as a positive control.

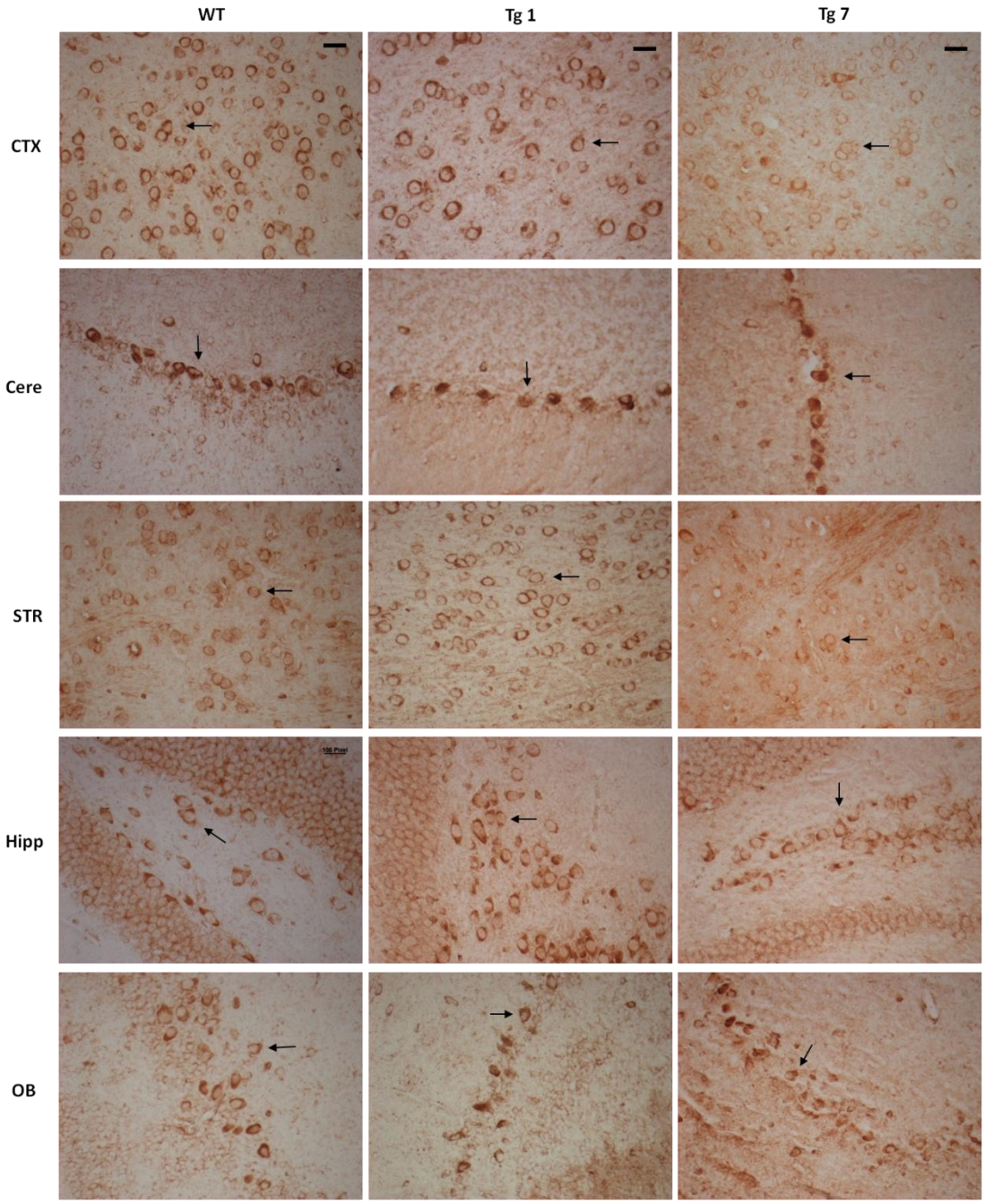


FIGURE 2.27 The specificity of human specific kalirin-7 Ab in brain tissue slices
The samples were prepared as described in M&M. The slices were stained with human specific kalirin-7 Ab diluted in 1:100. Photos from different regions were taken using fluorescence microscopy. Arrows indicate the immuno-staining which show similar patterns in WT and Tg samples. *Scale bars*, 20 µm.

3. DISCUSSION

3.1 Genetic analysis of polymorphisms in the *kalirin* gene

This is the first study that examines an association of the *kalirin* gene with AAO in HD. Previous studies have established a connection between *kalirin* variants and susceptibility to schizophrenia, Alzheimer disease, adult attention deficit hyperactivity disorder (ADHD), coronary artery disease and ischemic stroke (Hayashi-Takagi et al. 2010; Krug et al. 2010; Lesch et al. 2008; Li et al. 2006; Wang et al. 2007; Youn et al. 2007). In the present study, we hypothesized that isoform 2 of the *kalirin* gene, corresponding to rat kalirin-7, the predominant *kalirin* isoform in an adult brain, may also contribute as a novel genetic modifier for HD based on its role in spine plasticity and its interaction with HAP-1.

Although the length of the expanded CAG tract in the *HD* gene is the main determinant of the HD phenotype, the manifestation of the disease is also modified by other risks, such as environmental or genetic factors. To date, two strategies have been applied in order to identify genetic modifiers, the genome-wide approach and the candidate gene approach. The genome-wide studies are based on genetic linkage to search for specific chromosome regions, which might be associated with an alteration of age at neurological onset, including the HD-MAPS project which identified 6q23-24 as an association region (Li et al. 2006) and the Venezuela pedigree-study (Gayán et al. 2008). The identified genomic regions in both studies were relatively large and it is difficult to detect specific modifiers which are now analyzed with a combination of densely spaced SNPs and copy number probes. On the other hand, association studies investigating candidate genes that are suspected to be involved in HD pathogenesis provide a straightforward option to identify these modifiers, although they are not comprehensive. Recent studies have demonstrated several genetic modifiers related to various mechanisms implicated in HD pathology,

such as metabolic impairment, transcription dysregulation, oxidative stress and excitotoxicity (Arning et al. 2008; Arning et al. 2007; Metzger et al. 2006; Metzger et al. 2008). Among them, the polymorphism T441M of HAP-1 showed an 8-year delay in AAO probably due to a tighter interaction of HAP-1 with mutant huntingtin (htt) protein and thus ameliorated htt-mediated toxicity (Metzger et al. 2008). HAP-1 is associated with huntingtin, dynactin p150/kinesin light chain (KLC), endosomal organelles and BDNF, suggesting its role in intracellular trafficking and endocytosis.

Here we have examined polymorphisms in the *kalirin* gene due to its crucial role in spine plasticity and its interaction with HAP-1. Recently, several genome-wide association studies (GWAS) had revealed genetic associations of the *kalirin* gene with several diseases. An intronic SNP rs9289231 has been associated with an early onset of coronary artery disease in an American white population (Wang et al. 2007), while intronic SNPs rs11712039, rs17286604 and rs11712619 have been associated with ischemic stroke in a small Portuguese population (Krug et al. 2010). A recent GWAS in a Japanese population showed that a missense mutation in the *kalirin* gene, P2255T (ss250607859), may be a genetic risk factor for schizophrenia (Hayashi-Takagi et al. 2010). In the present study we did not include any intronic SNPs, since we concentrated on potential functional changes on the protein level. Also SNP ss250607859 was excluded from our analyses, as it affects different isoform of the *kalirin* gene.

Among the eleven SNPs screened in our study, we found only SNP rs10934657 to be polymorphic in our European control cohort. However, in an analysis of covariance, there was no significant effect (P=0.9713) of this SNP on the AAO in our cohort of European HD patients. Also, tests for additional factors such as sex or ethnicity (German or Italian ancestry), did not reveal a significant effect either. Further power analysis was performed to determine the population size that would

be required to show potentially significant effects of rs10934657. Our calculations revealed a minimum of 70065 samples, indicating that this SNP is very unlikely to have a considerable impact on AAO in HD.

In summary, although the genetic variations of the *kalirin* gene investigated here showed no effect on the AAO in HD, this does not exclude other SNPs of the *kalirin* gene as susceptible genetic modifiers.

3.2 Functional relevance of interaction between Synphilin-1 and Karlirin-7

Synphilin-1 is linked to the pathogenesis of Parkinson disease by its presence in Lewy bodies (Murray et al. 2003), association with α-synuclein, its relation to the UPS both as a substrate and a mediator of degradation (Chung et al. 2001; Lee et al. 2002; Marx et al. 2007), and its involvement in synaptic vesicle trafficking (Ribeiro et al. 2002). Furthermore, overexpression of synphilin-1 and non-Aβ component (NAC) portion of α-synuclein leads to the formation of inclusion bodies (Engelender et al. 1999). We have previously identified periphilin as an interactor of synphilin-1 which displayed overlapping expression patterns in cells, animals and Lewy bodies of PD patients (Soehn et al. 2010). In this thesis, I describe the interaction between synphilin-1 and the brain specific Rho GEF kalirin-7. We have found that kalirin-7 enhanced the recruitment of synphilin-1 inclusions into aggresomes and their subsequent degradation. The action of kalirin-7 was not related to its well-known GEF activity but rather an HDAC6-mediated process.

3.2.1 Interaction of Synphilin-1 with Kalirin-7

The interacting domains of synphilin-1 and kalirin-7 were narrowed down to the N-terminus of the synphilin-1 protein (aa 1-348) and the spectrin domains III and IV in the kalirin-7 protein (aa 413-642). The interacting domain in the synphilin-1

protein therefore differs from other synphilin-1 interactors, which bind mainly to the central part of the protein, namely α-synuclein, parkin and dorfin (Ito et al. 2003; Kruger 2004; Xie et al. 2010), or to the C-terminus of synphilin-1, including acidic phospholipids and periphilin (Soehn et al. 2010; Takahashi et al. 2006). Solely the minimal binding region of the E3 ligases SIAH-1 and SIAH-2 is also located in the N-terminus (Liani et al. 2004; Nagano et al. 2003). A recent NMR study proposed that the central coiled-coil domain (CC) of synphilin-1 forms a dimer and facilitates the self-aggregation of synphilin-1 and the inclusion formation with α-synuclein (Xie et al. 2010). In addition, the central ankyrin-like domains (ANK) encompass an aggresome-targeting signal (ANK1) and an aggregation-promoting segment (CC-ANK2) (Zaarur et al. 2008). Stereologically, with the N-terminal stretch of the synphilin-1 protein being implicated in the interaction with kalirin-7, the central synphilin-1 domains would be available for the recruitment of other protein complexes and the integration of further signaling pathways.

3.2.2 Synphilin-1 aggregates and their relevance to cellular responses

In this thesis I have demonstrated that synphilin-1 predominantly forms small cytoplasmic aggregates while perinuclear aggregates increase when kalirin-7 is coexpressed (Fig. 2.5 B). The presence of several marker proteins identified these perinuclear inclusions as aggresomes and we were able to show that synphilin-1 containing aggresomes are readily degraded, while small cytoplasmic inclusions are more stable (Fig. 2.9 and 2.10)

Some data have been published in the literature concerning the nature of synphilin-1 aggregates and their relevance. Synphilin-1 is known to form multiple cytoplasmic aggregates in naïve cells, whereas in the presence of the proteasome inhibitor MG132 aggresomes are formed (Tanaka, M. et al. 2004; Wong et al. 2008;

Zaarur et al. 2008). In contrast to certain aggregating polypeptides, *e.g.* glial fibrillary acidic protein (GFAP) which can form multiple aggregates but is not targeted to the aggresome, synphilin-1 has a specific aggresome targeting signal and thus can be recruited to aggresomes (Zaarur et al. 2008). Some studies have dissociated aggresome formation from cell death regarding synphilin-1 and huntingtin proteins, indicating that aggresomes serve as a protective cellular response when chaperones and UPS machineries fail to clean misfolded proteins (Tanaka, Mikiei. et al. 2004; Taylor et al. 2003).

There is evidence that shows that these synphilin-1 aggresomes are ubiquitin positive. Consistent with previous reports, we also observed a colocalization of synphilin-1 and ubiquitin in aggresomes triggered by kalirin-7 (Fig. 2.6). The ubiquitination of synphilin-1 has been proposed to play a role in its aggregation and aggresome formation (Lim et al. 2006) and this process was mediated by SIAH-1 and SIAH-2, protein-ubiquitin E3 ligases which interact and ubiquitinate synphilin-1 (Liani et al. 2004) as well as parkin, a protein-ubiquitin E3 ligase which promotes K63-linked ubiquitination of synphilin-1 (Lim et al. 2005). SIAH-1 and SIAH-2 were proposed to promote synphilin-1 degradation through the UPS while parkin-mediated non-classical K63-linked ubiquitination promoted the formation of LB-like inclusions (Liani et al. 2004; Lim et al. 2005). Evidence indicated that these ligases are associated with synphilin-1 degradation and formation of inclusion bodies, nevertheless, the mechanisms are still obscure.

Another interesting issue which has been debated for a decade was synphilin-1 and cell viability. In cell models, wild type synphilin-1 displayed protective effects against pharmaceutical treatment, proteasome inhibitor and rotenone (Li et al. 2010; Marx et al. 2003; Marx et al. 2007; Tanaka et al. 2004) while the R621C variant of synphilin-1 had the opposite effect. In a yeast model, both wild-type and R621C

mutant synphilin-1, triggered cell death in ageing cells (Büttner et al. 2010). Recent *in vivo* studies overexpressing synphilin-1 in mice brains also exhibited inconsistent results. One group revealed that synphilin-1 protein was polyubiquitinated and partially insoluble and the transgenic animals showed mild motor impairment, but no signs of neurodegeneration were detected (Jin et al. 2008). WT and R621C synphilin-1 transgenic mice generated by our group showed cell loss in the *cerebellum* and reduction of motor skill learning and motor performance (Nuber et al. 2010). Overexpression of synphilin-1 in mice by means of adenoviral vector induction induced degeneration of dopaminergic neurons in the *substantia nigra* (Krenz et al. 2009). Furthermore, synphilin-1/A53T α-synuclein double transgenic mice attenuated A53T α-synuclein-induced neuronal degeneration (Smith et al. 2010). Therefore, whether synphilin-1 is trophic or detrimental to cell viability is presently not clear.

3.2.3 Impact of kalirin-7 on synphilin-1 aggresome formation

Central to our study was the question how kalirin-7 could contribute to the transport of synphilin-1 into aggresomes. We first focused on the GEF activity of kalirin-7 for Rho GTPases, especially Rac1 and RhoG (May et al. 2002; Penzes et al. 2000), as a Ras family GTPase, Rheb, was recently shown to modulate aggresome formation (Zhou et al. 2009) which might be a potential effector of kalirin-7 although their interaction has not yet been investigated. To determine whether the GEF activity of kalirin-7 mediated the recruitment of synphilin-1 into aggresomes, we tested dominant-negative variants of Rho-like small GTPases and a dead mutant of the kalirin-7 GEF domain to block or attenuate kalirin-7-GEF activity, respectively. Unexpectedly, our results showed that the transportation of synphilin-1 into aggresomes is GEF-independent (TABLE 2.3). Indeed, kalirin-7 is known to also have

GEF-independent functions, *e. g.* the induction of lamellipodia does not require GEF activity (Schiller et al. 2005), and comparably its effect on aggresome formation appears to act through GEF-independent pathways or in combination with accessory factors. In search for the downstream target of kalirin-7 in terms of aggresome formation, we next focused on HDAC6.

HDAC6 is a histone deacetylase which has been shown to concentrate in Lewy bodies of PD patients (Kawaguchi et al. 2003) and there is increasing evidence showing that HDAC6 plays an essential role in aggresome formation via its ubiquitin binding and its deacetylase activity. HDAC6 binds to ubiquitinated proteins, including CFTR (Kawaguchi et al. 2003) and huntingtin (Iwata et al. 2005), through a C-terminal ubiquitin-binding zinc finger domain (ZnF-UBP). HDAC6 also binds to the dynein motor complex through a segment between two catalytic domains (Kawaguchi et al. 2003) facilitating the transport of cargo proteins by the dynein motor complex towards the minus end of microtubules to the microtubule-organizing center (MTOC). Furthermore, HDAC6 has been shown to mediate the deacetylation of α-tubulin in several studies (Boyault et al. 2007a; Hubbert et al. 2002; Matsuyama et al. 2002; Zhang et al. 2003) and the inhibition of HDAC6 has been proposed to regulate the dynamics of the microtubule network (Zilberman et al. 2009). A recent *in vivo* study demonstrated that the depletion of *Hdac6* in the R6/2 mouse model resulted in an increase of tubulin acetylation, but had no effect on the onset and progression of the HD-related phenotype (Bobrowska et al. 2011). Another HDAC6-deficient mouse model which had hyperacetylated tubulin and increased Hsp90 acetylation also showed apparently normal microtubule organization and stability. These data suggest that HDAC6 might play a crucial role in a specific pathway without being detrimental to normal mammalian development.

Here, we observed that TSA (but not NaBu), as well as a dominant negative HDAC6

mutant conferring loss of catalytic activity counteracted the kalirin-7-mediated recruitment of synphilin-1 aggregates into aggresomes (Fig. 2.12 and 2.14). Importantly, kalirin-7 decreased α-tubulin acetylation induced by TSA (Fig. 2.16), further corroborating an effect of kalirin-7 on HDAC6 mediated protein transportation and aggresome formation. The potential role of kalirin-7 in microtubule-dependent trafficking furthermore supported by the interaction of kalirin-7 with HAP-1 (Colomer et al. 1997), which in turn is associated with the microtubule-dependent trafficking proteins dynactin p150 (Glued) (Engelender et al. 1997; Li et al. 1998) and kinesin light chain 2 (Mcguire et al. 2006). Compatible with a concerted action of kalirin-7 and HDAC6, we have been able to show that kalirin-7 and synphilin-1 both interact with HDAC6 (Fig. 2.15), indicating that they act in a common protein complex. All in all, one possible explanation for the efficient transport of synphilin-1 into aggresomes is that the deacetylation of α-tubulin increases the dynamics of microtubules thereby accelerating microtubule-based transport (Matsuyama et al. 2002; Tran et al. 2007). The proposed pathway of kalirin-7-mediated synphilin-1 aggresome formation is illustrated in Fig. 3.1. Nevertheless, the relationship between tubulin acetylation and microtubule-based transport is controversial from various studies, for instance, Dompierre et al. showed that tubulin acetylation has no effect on microtubule dynamics in COS7 cells (Dompierre et al. 2007) while Reed et al. found an association between tubulin acetylation and microtubule stability promoting the recruitment of kinesin and dynein motors to microtubules and cargo transport (Dompierre et al. 2007; Reed et al. 2006). These data suggest that the regulation of tubulin acetylation and microtubule-based transport may depend on the cellular conditions (Verhey and Gaertig 2007).

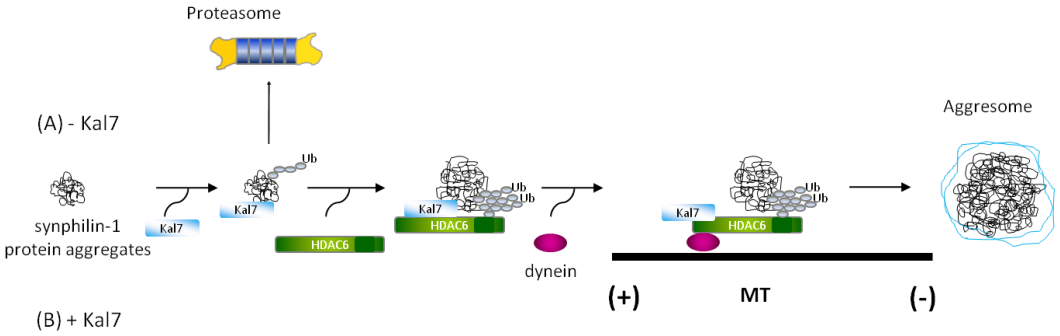


FIGURE 3.1 Proposed pathway of kalirin-7-mediated synphilin-1 aggresome formation
(A) Under normal conditions, misfolded synphilin-1 is mainly accumulated in cytoplasmic small aggregates. (B) When kalirin-7 is co-overexpressed, it facilitates the recruitment of HDAC6 and the dynein motor complex and acts on microtubule dynamics by stimulating the deacetylase activity of HDAC6, thereby increasing the transportation of synphilin-1 into aggresomes.

Corresponding to AGERA and live imaging experiments, aggresomes in synphilin-1 and kalirin-7 double overexpressing cells were readily degraded (Fig. 2.9 and 2.10). Indeed, in the aggresome-autophagy pathway proposed by Olzmann et al. (Olzmann and Chin 2008), aggresomes are substrates of autophagy and seem to be engulfed by autophagosomes, which then fuse to lysosomes, resulting in misfolded protein degradation by lysosomal hydrolases. The molecular mechanisms on how the cells recognize aggregated proteins and target them to the aggresomes are still unknown. Emerging studies highlighted this issue. Meriin et al. suggested that the cellular signaling such as activation of mitogen-activated protein kinase kinase (MEKK1) could increase formation of aggresome (Meriin et al. 2001), indicating that phosphorylation of downstream substrates might be critical although the signaling pathway has not been identified so far. Another study identified parkin-mediated K63-linked polyubiquitination as a signal for misfolded proteins (DJ-1) to interact with HDAC6 and thereby linked proteins to the dynein motor complex and transported them to aggresomes (Olzmann et al. 2007). Moreover, an aggresome-targeting segment found in synphilin-1 and huntingtin serves as a native signal that targets aggregation-prone polypeptides to aggresomes (Zaarur et al.

2008). In our study, a polyubiquitination of misfolded synphilin-1 and native protein signal segment might contribute to the synphilin-1 aggresome response upon kalirin-7 overexpression. Still, we cannot rule out the possibility that kalirin-7 activates other cellular signaling which recruits machinery for aggresome formation.

Kalirin-7 is phosphorylated by Cdk5 which attenuates the morphology of dendritic spines (Xin et al. 2008). On the other hand, the Cdk5 activity has been reported to suppress the formation of inclusions via disruption of microtubules which is considered an essential process (Kaminosono et al. 2008). Therefore, we explored whether overexpression of kalirin-7 could indirectly influence Cdk5 activity and microtubule stability that consequently affects the transport of synphilin-1 aggregates. The results indicated (Fig. 2.11) that kalirin-7 had no direct effect on microtubule stability. However, we cannot exclude that the assay was not sensitive enough to detect the changes. The morphological changes of kalirin-7 in HEK cells from diffused patterns in the cytoplasm to the concentrated form around the nucleus were observed at various time points (Fig. 2.19), which is in consistence with previous studies (Schiller et al. 2008). They demonstrated that the Sec14/spectrin repeat region of kalirin-7 contributes to the structural integrity of the round cell (Schiller et al. 2008) and the same results were obtained by different kalirin-7 variants as shown in Fig. 2.20. Proteins with spectrin repeats are well connected between the plasma membrane and the actin cytoskeleton, such as erythrocyte spectrins and membrane stability (De Matteis and Morrow 2000).

Besides the HDAC6 dependence of the kalirin-7-mediated aggresome formation, the kalirin-7 interactor HAP-1 may act as an additional mediator or cofactor of kalirin-7 action. Recently, evidence supported the role of HAP1 in the endocytic trafficking of membrane receptors. For instance, overexpression of HAP-1 could modulate the trafficking of the epidermal growth factor receptor (EGFR) (Li et al.

2002), NGF receptor TrkA (Rong et al. 2006) and $GABA_AR$ (Sheng et al. 2006) which in turn inhibits the degradation of internalized receptors. In its capacity as interactor of the microtubule motor protein subunits that facilitates cargo transport along microtubules, HAP-1 could also play a role in the transport of synphilin-1 aggregates into aggresomes.

In summary, this study demonstrates for the first time an interaction between synphilin-1 and kalirin-7 that leads to the recruitment of synphilin-1 into aggresomes in a GEF-independent but HDAC6-dependent manner. As synphilin-1 also interacts with dynactin and HAP-1 (Fig. 2.16), the protein complex consisting of α-synuclein, synphilin-1, kalirin-7, HAP-1, huntingtin, HDAC6 and dynactin is supposed to form a functioning network. This is also the first report linking kalirin-7 to microtubule dynamics. Importantly, this novel interaction and its impact on aggresome formation, a fundamental mechanism in the pathogenesis of PD, links the HAP-1 interactor kalirin-7 to PD for the first time. The implication of kalirin-7 as a pathogenesis factor to PD will potentially allow for the investigation of novel therapeutic targets in the treatment of PD.

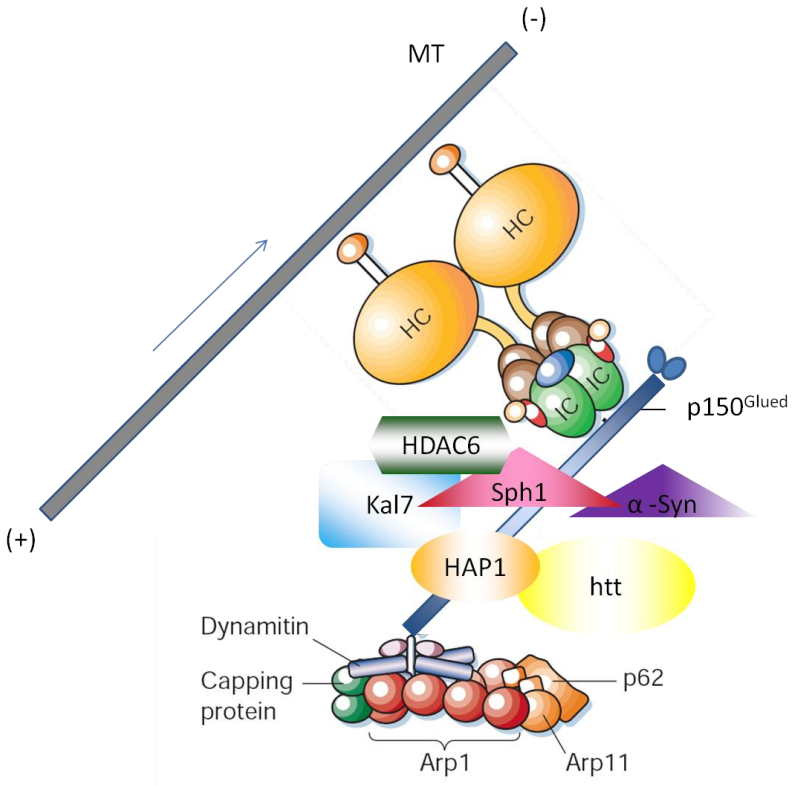


FIGURE 3.2 Illustration of proposed novel kalirin-7 working complex
Kalirin-7 is known to interact with HAP1 which is associated with huntingtin protein (htt). The novel interactions of kalirin-7 identified in the study, synphilin-1 (Sph1) and HDAC6, linked the protein to aggresome pathway and PD pathogenesis through a microtubule-dependent manner. (Modified after Schliwa and Woehlke 2003)

3.2.4 Novel synphilin-1 interactors

Our aim was to identify novel microtubule-related proteins that might directly or indirectly associate with synphilin-1. To identify protein-protein interaction it is important to preserve the native state of the proteins of interest to assure detection of novel interactors instead of nonspecific proteins (Phizicky and Fields 1995). To this end, we chose together with the group of Prof. Ueffing (Eye Research Institute Tübingen) the tandem affinity purification (TAP) tag method, an efficient and large-scale purification which also conserves native protein (Butland et al. 2005) to investigate novel interactors of synphilin-1. As shown in TABLE 2.4, three heat shock proteins which could facilitate correct protein folding (P08107) (Mayer 2010), function as ATPase in the disassembly of clathrin-coated vesicles during transport of

membrane components (P62136) (Hannan et al. 1998) and regulate folding and assembly of proteins in the ER (P11142) (Nishikawa et al. 2005) were observed. Indeed, inhibition of Hsp90 triggers the aggresome response of synphilin-1 (Zaarur et al. 2008), indicating the involvement of heat shock proteins in the synphilin-1 protein complex under heat shock condition (Xiong et al. 2009). The other novel interactors are: PPP1CA protein phosphatase 1, one of the three catalytic subunits of protein phosphatase 1 (PP1) that regulates cell division, glycogen metabolism, protein synthesis and viral transcription (Liu et al. 2011; Xiao et al. 2010); Acetyl-CoA carboxylase (ACC) belongs to a complex multifunctional enzyme system which is involved in fatty acid synthesis and energy homeostasis (Hardie 1989; Wolfgang and Lane 2006). Herein, we used the TAP method to identify synphilin-1 interactors in HEK 293 cells, however, none of the proteins observed in the study had neither a direct connection to the microtubule-associated pathway, nor to the well known interactors, such as α-synuclein or parkin. Concerning the nature of the TAP approach, it often only retains the tight interactions (Piehler 2005). Therefore if synphilin-1 interacts with microtubule-related proteins transiently or loosely, the interaction will not be detected here. Furthermore, the origin of different cell types, such as neuronal and non-neuronal cells may also influence the results.

3.3 Generation of human Kalirin-7 overexpressing transgenic mice and of human Kalirin-7 specific antibodies

Our *in vitro* study showed that kalirin-7 interacts with synphilin-1 and enhances the transportation of synphilin-1 aggregates into aggresomes, which implicated the potential role of kalirin-7 in the pathogenesis of PD. Furthermore, although the functional relevance between kalirin-7 and HAP-1 is still under investigation, we cannot rule out the contribution of kalirin-7 in HD pathogenesis. Because *in vitro*

results were not always able to represent *in vivo* function, we engineered a mouse model overexpressing the human kalirin-7 and aimed at cross-breeding tg-kal7 with PD (synphilin-1 tg and α-synuclein/synphilin-1 tg) (Nuber et al. 2010) and HD animal models (YAC128, R6/2) (Mangiarini et al. 1996; Slow et al. 2003). We intended to compare animals overexpressing kalirin-7 or with a knockout of the protein under a PD or HD tg animal background in order to investigate the role of kalirin-7 in these diseases, especially on aggregate formation, synaptic function and cognitive dysfunction.

To this end, transgenic mice containing human kalirin-7 (isoform 2 of *KALRN* gene) were generated. Owing to high homology (95%) of the mouse and human RNA sequence, only the primer pair mapped to the end of C-terminal and specific polyA tail was unique to transgenic animals. The mRNA profile showed that line 1 and 7 had relatively higher expression (Fig. 2.21); however, the intensity of protein expression regarding WT and transgenic could not be distinguished (Fig. 2.23 and 2.24) due to the nature of protein homology (98%) and the lack of proper antibodies (Table 2.5). The kalirin-7 antibody with an SLFHATSLQ epitope had only a poor capacity to recognize kalirin-7 from brain lysate (Fig. 2.26) and could not be applied to immunohistochemistry staining (Fig 2.27) which limited the progress of the project. Generation of new antibodies against different epitopes of human kalirin-7 is the most urgent issue. Moreover, other small molecule tagged kalirin-7 overexpressing mice should be generated.

3.4 SUMMARY AND OUTLOOK

Huntington disease (HD) is caused by an expanded CAG repeat in the *HD* gene. Although the length of the CAG repeat strongly correlates with the age-at-onset (AAO), AAO in HD individuals may differ dramatically in spite of similar expanded CAG repeat lengths. Additional genetic or environmental factors are thought to influence the disease onset. To identify such a genetic modifier, we analyzed single nucleotide polymorphisms (SNPs) in the *kalirin* (*KALRN*) gene. Eleven SNPs in the *kalirin* gene were selected and SNP rs10934657 was examined in an association study in 680 independent European HD patients. However, the results did not reveal an association between the analyzed *kalirin* polymorphisms and the AAO in HD.

In the second part of this study, we investigated the effect of kalirin-7 on synphilin-1 aggregate formation. Coexpression of kalirin-7 and synphilin-1 caused a dramatic relocation of synphilin-1 cytoplasmic small inclusions to a single prominent, perinuclear inclusion. These perinuclear inclusions were located in aggresomes according to the colocalization with microtubule organization center markers and their formation was microtubule-dependent. Furthermore, kalirin-7 increased the susceptibility of synphilin-1 inclusions to be degraded as shown by live imaging and quantification of aggregates. The GEF activity of kalirin-7 is the most prominent function of the protein, however, kalirin-7-mediated synphilin-1 aggresome response was not dependent on its GEF activity since various dominant negative small GTPases could not inhibit the formation of aggresomes. Interestingly, the aggresome response was blocked by HDAC6 catalytic mutants and the HDAC inhibitor trichostatin A (TSA), but not by sodium butyrate (NaBu). Moreover, kalirin-7 decreased the level of acetylated α-tubulin in response to TSA, which suggests an effect of kalirin-7 on HDAC6-mediated protein transportation and aggresome

formation. In summary, we demonstrated that kalirin-7 leads to the recruitment of synphilin-1 into aggresomes in a HDAC6-dependent manner and also links kalirin-7 to microtubule dynamics. Our results implicate an important role of a GEF protein in the pathogenesis of PD and provide a link to the huntingtin pathway by binding the huntingtin associated protein 1 (HAP1) interactor.

Kalirin-7 has been proposed as an important regulator for synaptic structure and function which is associated with cognitive processes in kalirin-7 knockout mice. In the third part of this study, we tried to generate and characterize kalirin-7 overexpressing mice to further crossbreed them with PD and HD animal models and evaluate the impact of kalirin-7 in these two diseases *in vivo*. Unfortunately, we were unsuccessful in characterizing and establishing the kalirin-7 animal model due to the lack of proper antibodies, including the one we generated, which was supposed to be human kalirin-7 specific. To further generate a kalirin-7 animal model, a stronger promoter and a small tag might have to be included.

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Plasmids

TABLE 4.1 List of cloned constructs used

Insert	Vector	Tag
HDAC6	pcDNA3.1/V5-HisA	HA
Kal7 (full length)	pBS	-
Kal7 (full length)	pBS	FLAG
Kal7 (full length)	pcDNA3.1(-)	FLAG
Kal7 (aa 166-1663)	pcDNA3.1(-)	FLAG
Kal7 (aa 166-1293)	pcDNA3.1(-)	FLAG
Kal7 (aa 642-1663)	pcDNA3.1(-)	FLAG
Kal7 (aa 166-647)	pcDNA3.1(-)	FLAG
Kal7 (aa 413-889)	pcDNA3.1(-)	FLAG
Kal7 dGEF	pcDNA3.1(-)	FLAG
Kal7 (full length)	pEGFPN1	EGFP
Sph1 (full length)	pcDNA3.1(-)	V5
Sph1 (aa 1-348)	pcDNA3.1(-)	V5
Sph1 (aa 318-586)	pcDNA3.1(-)	V5
Sph1 (aa 556-919)	pcDNA3.1(-)	V5
Sph1 (full length)	pcDNA3.2(-)	HcRed

TABLE 4.2 Construct from other institution

Plasmid Name	Description	Source/Reference
pEGFP-RhoG-F37A	EGFP tagged	Fort et al., 2000
pEGFP-Rac1-T17N	EGFP tagged	Fort et al., 2000
pcDNA3-HA/Rheb D60K	C-terminal tagging	Zhou et al., 2009
pcDNA3-FLAG/HDAC6 WT	C-terminal tagging	Lee et al., 2010
pcDNA3-FLAG/HDAC6 H216/611A	C-terminal tagging	Lee et al., 2010

4.1.2 Oligonucleotides

All oligonucleotides (primers) are synthesized by Metabion in a concentration of 100 µM.

TABLE 4.3 Oligonucleotides used for cloning

Kalirin-7 Tg animal construct		RE site
polyA (sense)	*CCCGGG*aacttgtttattgcagcttataa	SmaI
polyA	ggtacc*CCGCGG*taagatacattgatgagttt	KpnI/Sac

Kalirin-7		
aa 166-1663		
sense	ag*TCTAGA*ccaccATGgattacaaggatgacgacgataag TCCcagctgacggaggag	XbaI
antisense	gcat*GCGGCCGC*taaacgtaagtggactctgtctggagtagttc	NotI
aa 166-1293		
sense	ag*TCTAGA*ccaccATGgattacaaggatgacgacgataag TCCcagctgacggaggag	XbaI
antisense	at*GCGGCCGC*taCTCTGTCTGGAGTAGTTC	NotI
aa 642-1663		
Sense	cgg*CATATG*ccaccATGgattacaaggatgacgacgataag tctgtttccttccacac	NdeI
antisense	gcat*GCGGCCGC*taaacgtaagtggactctgtctggagtagttc	NotI
aa 166-647		
sense	ag*TCTAGA*ccaccATGgattacaaggatgacgacgataag TCCcagctgacggaggag	XbaI
antisense	at*GCGGCCGC*TAgtggaaggaaacagacatgtc	NotI
aa 413-889		
sense	ag*CTCGAG*ccaccATGgattacaaggatgacgacgataag ATCctcgccatgtctgctg	XhoI
antisense	at*GCGGCCGC*ctaGCACTGCTCTAGCCGCTTAT	NotI
Kalirin-7		
sense	GTGTCCCAAAGAAAGCCgcagcgGCCATGCATGTCAGCATGCTGG	
antisense	CCAGCATGCTGACATGCATGGCcgctgcGGCTTTCTTTGGGACAC	
Synphilin-1		
aa 1-348		
sense	at*GGATCCcccacc*ATGgtaagcctatccctaaccctctcctcggtctcgattctacgATGGAAGCCC CTGAA	BamHI
antisense	at*GCGGCCGC*ttaTTCGTCGTGAATTTTGTCTAG	NotI
aa 318-586		
sense	at*GGATCC*ccaccATGgtaagcctatccctaaccctctcctcggtctcgattctATGacgctgcatatg agcctgaa	BamHI
antisense	at*GCGGCCGC*ttaCTTGCCCTCTGATTTCTGGG	NotI
aa 556-919		
sense	at*GGATCC*ccaccATGgtaagcctatccctaaccctctcctcggtctcgattctATGgctaccttggatg catac	BamHI
antisense	at*GCGGCCGC*ttaTTATGCTGCCTTATTCTTTCCT	NotI

TABLE 4.4 Oligonucleotides used to identify kalirin-7 Tg animal

Genotype	
Sense	GATGTGCAGTGAAGGTGGTC
Antisense	ATCCTGGCTGACCTCTGTGT
qRT-PCR	
Sense	AGTGGACAGTGACAAGGATGG
Antisense	GG AACTTGTTTATTGCAGCTT

TABLE 4.5 Oligonucleotides and conditions used for fragment length analysis

SNP	Sequence (5' → 3')	PCR product (bp)	Annealing temperature	Restriction Enzyme
rs10934657				
sense	TGGCAAGAGGGAGAGG***A***G	139	55.2	AluI
antisense	CTTCCTCCTCTGTAAACCAGAGAGA			
rs111472457				
sense	CATCCGAGATGCAAGACCTAGA	156	58.5	AluI
antisense	CCGTGAGGGATTCGGAGT			
rs35057827				
sense	GCATGAGGTGTTACATCACCAGC***C***AC	123	60.9	BtgI
antisense	CAATCCAGTCCAACACCTGCT			
rs13074913				
sense	TCTACAAGGCAGCTCGACAC	136	58	MscI
antisense	AGGTCTTCCATCCATG***G***CC			
rs61745397				
sense	GCCAGGGACTCGGCTGGA	154	60.6	BamHI
antisense	TCACCTCGATGGTGTACTGC			
rs112304715				
sense	CAGCAGGGACAGGATCTGCAC***T***	170	62.5	SpeI
antisense	AGCCGCTTATGAGTCTGCTCT			
rs77832285				
sense	TCCTGAGTGAGCTCCTGCATAT	110	59	NdeI
antisense	GCTCGAACACCACATATTGC			
rs2289838				
sense	AGCCCGGAAGAAAGAATTTA	168	58.2	AluI
antisense	TGGATGTTGCCAAAGATGATA***A***G			
rs1062749				
sense	CTGCAAATTCGCCTTGTGGT	162	55.2	SacI
antisense	GCTGAAGTGGCTCCT***T***TAGAGCT			

4.1.3 Enzymes

TABLE 4.6 List of enzymes used

Name	Manufacturer
BioTherm™ Taq DNA Polymerase	GeneCraft
Expand Long Template PCR System	Roche
OneTaq™ DNA Polymerase	New England Biolabs
Restriction enzymes	New England Biolabs
Shrimp Alkaline Phosphatase (SAP)	Roche
T4 DNA ligase/ 10x ligase buffer	Fermentas

4.1.4 Antibodies

TABLE 4.7 List of primary antibodies used

Name	Dilution WB	Dilution IF	Dilution IHC	Manufacturer	Prod. No.
Anti-α-Tubulin mouse mAb	1:1000	-		Calbiochem	CP06
Anti-ß-Actin mouse mAb (clone AC-15)	1:20000	-		Sigma	
Anti-γ-Tubulin mouse mAb	-	1:100		Sigma	T6557
Anti-acetylated tubulin mouse mAb (clone 6-11-B1)	1:4000	1:100		Sigma	T7451
Anti-FLAG mouse mAb (clone M2)	-	1:400		Sigma	F3165
Anti-FLAG rabbit pAb (clone M2)	1:500	1:200		Sigma	F7425
Anti-HA rabbit pAb	1:500	-		Sigma	H6908
Anti-HSP27 goat pAb (clone C-20)	-	1:200		Santa Cruz	sc-1048
Anti-Kalirin rabbit pAb (MP-KAL)	1:500	-		Millipore	07-122
Anti-KALRN goat pAb (Ab-KAL)	1:500	-		Abcam	ab52012
Anti-Kalirin-7 rabbit pAb	1:500	-	1:50-1:500	Dr. Kalbacher	
Anti-Synphilin-1 rabbit pAb	1:500	-		Sigma	S5946
Anti-ubiquitin rabbit pAb	-	1:300		BostonBioche	UCM-310
Anti-V5 mouse mAb	1:1000	1:200		Invitrogen	V8012
Anti-vimentin (clone S-20)	-	1:200		Santa Cruz	sc-7558

TABLE 4.8 List of secondary antibodies used

Name	Dilution WB	Dilution IF	Manufacturer
donkey anti-goat-Cy2	-	1:400	Santa Cruz
donkey anti-mouse-Cy2	-	1:300	Jackson laboratories
donkey anti-rabbit-Cy2	-	1:300	
donkey anti-rabbit-Cy5	-	1:300	
sheep anti-mouse IgG, HRP-linked whole Ab	1:3000	-	Amersham Biosciences
donkey anti-rabbit IgG, HRP-Linked F(ab')2 Fragment	1:3000	-	Amersham Biosciences

4.1.5 Buffers and Solutions

All buffers and solutions are dissolved in ddH_2O if there unless noted otherwise.

TABLE 4.9 The composition of buffers and solutions used

DNA gel electrophoresis	
10x agarose blue buffer	0.01% Bromophenol blue
	8% Ficoll
	1x TBE
10x Tris-Borate-EDTA buffer (TBE)	890mM Tris
	890mM Boric acids
	20mM EDTA
Bacterial culture	
LB medium (LB), pH7.5, autoclaved; 4°C	25g LB Broth
LB agar plates, pH7.5, autoclaved; B101	25g LB Broth
	7.5g Yeast extract
	7.5g Agar-Agar
Tfb1, pH 5.8, adjust with Acetic acid	10% Glycerol
	30mM Potassium acetate
	100mM RbCl
Tfb2, pH 6.8, adjust with NaOH	10% Glycerol
	10mM MOPS
	10mM RbCl
Western blot	
5X SDS-PAGE sample buffer	1% Bromophenol blue
(5X Laemmli buffer)	10% Glycerin
	5% β-Mercaptoethanol
	10% SDS
	0.5M Tris-HCL pH 6.8
10x SDS-PAGE running buffer	192mM Glycin
	1% SDS
	25mM Tris base
SDS-PAGE stacking gel buffer	0.5M Tris (pH 6.8)
	10% SDS
SDS-PAGE separating gel buffer	1.5M Tris (pH8.8)
	10% SDS
10x Transfer buffer	25mM Tris base
	192mM Glycin
1x Transfer buffer	10% 10x Transfer buffer
	10% Methanol
Tris buffered saline/ Tween20 (TBST)	50mM Tris (pH7.4)
	150mM NaCl
	0.1 (v/v) Tween 10
Stripping buffer, pH2.0	0.2M Glycin
	1% SDS
Blocking solution	5% (w/v) non-fat milk powder/TBST

Cell culture	
Cell freezing medium	DMEM
	10% DMSO
Medium for HEK293 cells	DMEM
	10% FBS
	100U/ml Penicillin
	100µg/ml Streptomycin
Medium for HN-10 cells	DMEM GlutaMAX
	10% FBS
	100U/ml Penicillin
	100µg/ml Streptomycin
Lysate	
Cell lysate: RIPA lysis buffer, pH8.0	1mM EDTA
	150mM NaCl
	0.25% NaDeoxycholate
	1% NP40
	50mM Tris (pH8.0)
	Complete protease inhibitor cocktail
Brain tissue lysate: TNES lysis buffer, pH 7.4	2mM EDTA
	100mM NaCl
	1% (v/v) NP-40
	50mM Tris pH 7.5
	Complete protease inhibitor cocktail
AGERA	
AGERA loading buffer	150 mmol/L Tris–HCl pH 6.8
(2× nonreducing Laemmli sample buffer)	33% glycerol
	1.2% SDS
	1% bromophenol blue
	1mg/ml BSA
HEPES buffer	10mM EDTA
	50mM HEPES (pH7.0)
	0.5% Na-cholate
	150mM NaCl
	1% NP40
	0.1% SDS
Immunicytochemistry	
4% Paraformaldehyde (PFA), pH7.4, 4°C	4% paraformaldehyde
	2.5mM NaOH in PBS
Blocking solution	10% NDS in PBS
PBS, pH7.4	10 mM Na2HPO4
	2 mM NaH2PO4
	150mM NaCl
Mowiol mounting solution, -20°C	2.5% DABCO in 20% Mowiol solution
Immunohistochemistry	
Citrate buffer	9ml 100 mM Citrate acid
	41ml 100 mM sodium citrate
	add to 450 ml
Blocking solution	5% NGS in PBS+0.3%Triton X-100

4.1.6 Chemicals

TABLE 4.10 List of chemicals and toxins used

Reagent	Manufacturer
30% Acrylamide and bis-acrylamide solution (29:1)	Bio-Rad
Agar-Agar	Roth
Agarose	Invitrogen
Ammonium persulfate	Sigma
Ampicillin	Roth
Ampuwa water	Fresenius Kabi
Anti-FLAG M2 Affinity Agarose Gel produced in mouse	Sigma
Anti-V5 Agarose Affinity Gel antibody produced in mouse	Sigma
Bacto™ Agar	Becton Dickinson Labware
Bacto™ Trypton	Becton Dickinson Labware
Bacto Yeast Extract	Becton Dickinson Labware
β-Mercaptoethanol	Sigma
Boracic acid	Roth
Bradford Reagent	Bio-Rad
Bromophenol blue	Sigma
BSA (100x BSA 10 mg/ml)	New England Biolabs
Complete Mini, EDTA-free Protease Inhibitor Cocktail	Roche
DAB (Diaminobenzidin)	Sigma
DABCO (1,4-Diazabicyclo[2.2.2]octane)	Sigma
DAPI (4',6-Diamidino-2-phenyindole)	Sigma
dNTPs (dATP, dCTP, dGTP, dTTP), 100mM	Invitrogen
DMSO (Dimethyl sulfoxide)	Sigma
D-PBS (Dulbecco's Phosphate-Buffered Saline)	Invitrogen
DNA 1kb ladder	Fermantas
D-MEM (Dulbecco's Modified Eagle Medium)	Invitrogen
D-MEM with GlutaMAX™ I	Invitrogen
Ethanol	Merk
Ethidium bromide solution 1%	Sigma
FBS (Fetal Bovine Serum)	Invitrogen
Ficoll	Sigma
Glycerin	Roth
Glycin	Roth
Igepal CA-630	Sigma
Isofluoran	Halocarbon
Isopropanol	Merk

Reagent	Manufacturer
Kanamycin	Roth
KCl (Potassium chloride)	Merk
Mowiol 4-88	Merk
NaAc (Sodium acetate)	Merk
NaCl (Sodium chloride)	Merk
NaOH (Sodium hydroxide)	Roth
NDS (normal donkey serum)	Dianova
NGS (normal goat serum)	Dianova
Oligonucleotides	Metabion
Opti-MEM	Invitrogen
PBS (Phosphate buffered saline)	Invitrogen
Penicillin-Streptomycin, liquid	Invitrogen
PFA (Paraformaldehyde)	Merk
Poly-L-Lysin solution, 0.01%	Sigma
Precision PlusProtein Standard Dual Color	Bio-Rad
Protein G agarose	Millipore
QIAZOL lysis reagent	Qiagen
PVDF membrane	Millipore
SDS (Sodium dodecyl sulfate)	Roth
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Roth
TritonX-100	Roth
Trypsin-EDTA (1x), 0,25% Trypsin	Invitrogen
Tween20	Merk
Toxin	
Colchicine	Sigma
Nocodazole	Sigma
NaBu (Sodium butyrate)	Enzo life sciences
TSA (Trichostatin A)	Sigma

4.1.7 Kits

TABLE 4.11 List of kits used

Name	Manufacturer
ECL™ Western Blotting Detection Reagents	Amersham Biosciences
High Pure PCR Template Preparation Kit	Roche
Hyperfilm ECL high performance chemiluminescence	GE Healthcare
Light Cycler TaqMan Master	Roche Diagnostics
Lipofectamine™ 2000	Invitrogen
Plasmid Wizard Plus SV Minipreps-Kit	Promega
QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
QuantiTect ReverseTranscription Kit	Qiagen
QuantiTect SYBR Green PCR Kit	Qiagen
QuickChange II Site-Directed Mutagenesis kit	Stratagene
RNeasy Mini Kit	Qiagen
Transcriptor First Strand cDNA Synthesis Kit	Roche

4.1.8 DNA samples

4.1.8.1 Huntington patients

A total of 680 unrelated European HD patients were analyzed. Among them 320 patients were of German descent and 171 patients were of Italian descent. The remaining 189 patients were from other European countries (Che et al. 2011; Metzger et al. 2008; Metzger et al. 2010). Age-at-onset (AAO) was determined as the time point when motor or cognitive symptoms were first noticed. The mean AAO was 44.0 years (SD 13.0, age range 5-80 years). CAG repeat lengths in the *HD* gene had been tested in all patients and CAG numbers had been standardized in a reference laboratory. The number of the expanded CAG repeats ranged from 39 to 90.

4.1.8.2 Control DNA

All healthy individuals are samples of the Centre d'Etude du Polymorphisme Human cohort (CEPH).

4.1.8.3 Ethics

All participating individuals gave informed consent according to the Declaration of Helsinki. An ethics proposal was approved by the ethical review committee of the Medical Department of the University of Tuebingen (39/2003).

4.2 Methods

4.2.1 Cell culture

4.2.1.1 Culture conditions and passaging

HEK293 (human embryonic kidney cell line) and HN10 (mouse hippocampal cell line) cells were maintained in Dulbecco's modified Eagle medium (DMEM) and DMEM-Glutamax medium (Gibco), respectively, supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco) at 37 °C in an atmosphere of 5% CO_2 in a Binder CB210 incubator. The cells were harvested when 80% confluence was reached and split for further culture or used for experiments. Briefly, cells were washed with 5ml PBS, and 1ml Trypsin-EDTA was applied to detach cells by incubating at 37 °C for 2 min. 9 ml medium was then used to stop the reaction and cells were counted. For subculturing, 1:10 dilution of cells was added into a new flask. For western blot analysis and immunofluorescent imaging, 8 x 10^5 and 1 x 10^5 cells per well were cultured in 6-well plates, respectively.

4.2.1.2 Transient transfection

Plasmid DNA was transiently transfected into HEK293 and HN10 cells using Lipofectamine^{TM}2000 according to manufacturer's instructions. Plasmid DNA and Lipofectamine reagent were mixed in a ratio of 1:2. For single transfection, 1 µg DNA was applied per construct per well (6-well plates) while a total amount of 2 µg DNA was used for double and triple transfection.

4.2.1.3 Cryopreservation of culture cells

For long-term storage of cell lines, cells were grown in 75 cm^2 flask until 90% confluence was reached and then trypsinized by Trypsin/EDTA and pelleted by centrifugation for 5 min at 1200 rpm. The pellet was resuspended in 1 ml cell freezing medium and aliquoted in -1 °C/min cryotubes before frozen at -80 °C. To re-cultivate the frozen cells, cells were rapidly thawed in 37 °C water bath. The cell suspension was washed with 10 ml culture medium and centrifuged for 5 min at 1200 rpm. The pellet was resuspended in fresh medium and transferred into a new cell culture flask.

4.2.2 Molecular biology

4.2.2.1 Construction of DNA plasmids

pHD-Kal7 construct used for generating transgenic mice was manipulated as in Fig. 4.1. In brief, the vector containing the HD promoter was obtained by XmaI/KpnI

digestion from pBS_HDpro_HMJD1_polyA. A polyA tail with proper restriction sites was amplified by PCR and subcloned into the HD promoter vector. The kalirin-7 cDNA insert was obtained by NotI/SpeI digestion from pBMT117c_Duo followed by a blunting procedure and subcloned into pBS_HDpro_polyA. The linear DNA fragment for microinjection was generated by SacII digestion of pBS_HDpro-Kal7polyA. The fragment was subcloned into pcDNA3.1 (-) to check kalirin-7 overexpression in cell culture.

All other constructs listed below were generated by amplification of inserts with proper restriction sites and tags through PCR and subcloning into pcDNA3.1 (-), pEGFPN1 or pHcRed1-N1/1. Briefly, all kalirin-7 deletion variants were amplified containing FLAG tag and 5'XbaI/3'NotI restriction sites and subcloned into pcDNA3.1 (-) with SpeI/NotI sites. All synphilin-1 deletion variants were amplified containing V5 tag and 5'BamHI/3'NotI restriction sites and subcloned into pcDNA3.1 (-) with BamHI/NotI sites. All sequences of the inserts were confirmed by sequencing and enzyme digestion.

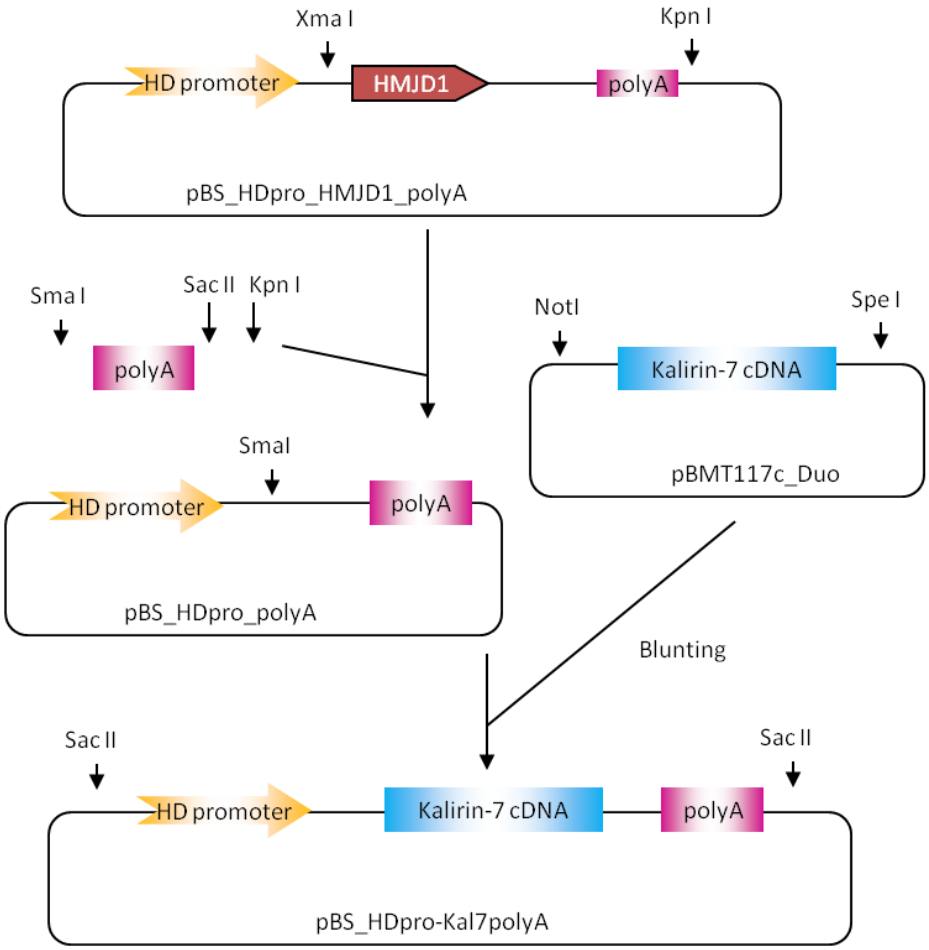


FIGURE 4.1 Construction of pHD-Kal7 construct used for generating transgenic mice

4.2.2.2 Polymerase chain reaction (PCR), Agarose gel electrophoresis and DNA gel extraction

PCR protocols used for amplification of insert, DNA mutagenesis, genotyping of kalirin-7 tg mice and SNP screening and are listed in TABLE 4.12. The PCR products for inserts were separated on a 0.6-1% agarose gel according to the fragment size and visualised with UV followed by excision and purification using QIAquick gel extraction kit. The genotyping and SNP screening PCR products were separated on a 2% agarose gel. The kalirin-7 N1454A/D1455A GEF dead mutant was cloned using Stratagene QuikChange II Site-Directed Mutagenesis Kit according to manufacturer's instructions.

4.2.2.3 Enzymatic modification of DNA

Both vector and insert DNA were digested with indicated restriction enzymes in a 20 µl reaction volume for 2 h according to manufacturer's instruction. Digested vectors were subsequently dephosphorylated with 1 µl Shrimp Alkaline Phosphatase (SAP) for 30 min at 37 °C. Vectors and inserts were purified using gel extraction before ligation. The protocol of ligation is listed in TABLE 4.13, overnight at 4 °C. The ligation products were transformed into DH5α competent cells.

TABLE 4.12 The composition of DNA ligation reaction

Ligation reaction	
Vector	optional
Insert	optional
10x Ligation Buffer	1.5µl
10x PEG	1.5µl
dNTPs (10mM)	0.5µl
T4 DNA ligase	1µl
ddH_2O	add to 15µl

4.2.2.4 Preparation of chemically competent cells

The chemically competent cells, DH5α bacteria from Invitrogen were picked up and cultured in 20 ml LB medium, and underwent 225 rpm shaking overnight at 37 °C. 5 ml of bacteria from the overnight culture was incubated with 500 ml LB medium and was shaken at 225 rpm at 37 °C until O.D.600 reached 0.45. The bacteria were aliquoted into 50 ml Falcon tubes and placed on ice for 5 min before they were centrifuged for 5 min at 1500 ×g at 4°C. The supernatant was discarded and the pellet was resuspended in 10 ml sterilized ice-cold Tfb1-buffer and left on ice for 5 min. Then, the bacteria were centrifuged for 5 min at 1500 × g at 4 °C and resuspended in 4 ml sterilized ice-cold Tfb2-buffer for 15 min on ice. The DH5α

bacteria were aliquoted in Eppendorf tubes in a volume of 200 µl, frozen in liquid nitrogen and stored immediately at -80 °C.

4.2.2.5 Transformation

10 µl of the ligation product was incubated with 100 µl of DH5α bacteria on ice for 30 min. The heat shock procedure was performed at 42 °C for 45 sec and placed on ice for 2 min. Then 1 ml of LB medium was added, followed by 225 rpm shaking at 37 °C for 1 h. The bacteria were pelleted by 3000 rpm centrifugation for 30 sec and the supernatant was discarded. The pellets were resuspended in 50 µl of LB medium and cultivated on LB agar plates containing appropriate selective antibiotics overnight at 37 °C.

4.2.2.6 Purification and validation of plasmid DNA

A single colony of plasmid DNA from an LB plate or glycerol stock was incubated in 2 ml of LB medium containing appropriate selective antibiotics and shaken at 250 rpm overnight at 37 °C. The overnight culture was purified with Plasmid Wizard Plus SV Miniprep-Kit from Promega according to manufacturer's instructions. In brief, 2 ml of mini-prep culture was centrifuged at 12,000 rpm in a table top centrifuge for 1 min. The supernatant was discarded and the pellet was resuspended in 250 µl of cell resuspension buffer followed by 250 µl of cell lysis buffer until the solution became clear and 350 µl of cell neutralization buffer was added. The mixture was centrifuged at 14,400 rpm for 10 min and the plasmid DNA was purified through the spin column. The plasmid DNA was then eluted with 50 µl ddH_2O and the concentration was determined by BioPhotometer (Eppendorf).

To validate the cloned constructs, DNA was digested with restriction enzymes and subsequently underwent DNA sequencing. For long-term storage of vectors and successful clones, the glycerol stock was prepared by mixing 90 µl of 87 % glycerol and 410 µl of LB culture and stored at -80 °C.

TABLE 4.13 List of PCR protocols used for various reactions

PCR amplification for insert		Program			
Template	1µg	Segment	Temperature	Time	Cycles
10x Reaction Buffer	2.5µl	Denaturation	94°C	5min	1
dNTPs (10mM)	0.5µl	Denaturation	94°C	30sec	
Primer (forward), 10 µM	1µl	Annealing	variable	30sec	35
Primer (reverse), 10 µM	1µl	Extension	72°C	variable	
pfu DNA polymerase	0.3µl	Final extension	72°C	10min	1
ddH_2O	add to 25µl	Storage	10°C	∞	
PCR protocol for mutagenesis		Program			
Template	1µg	Segment	Temperature	Time	Cycles
10x Reaction Buffer	2.5µl	1	95°C	30sec	1
dNTPs (10mM)	0.5µl	2	95°C	30sec	
Primer (forward), 10 µM	1µl		55°C	1min	18
Primer (reverse), 10 µM	1µl		68°C	10min	
pfu DNA polymerase	0.3µl	3	10°C	∞	1
ddH_2O	add to 25µl				
PCR protocol for genotyping		Program			
DNA	1µg	Segment	Temperature	Time	Cycles
10x Reaction Buffer	2.5µl	Denaturation	94°C	5min	1
dNTPs (10mM)	0.5µl	Denaturation	94°C	30sec	
Primer (forward), 10 µM	1µl	Annealing	variable	30sec	35
Primer (reverse), 10 µM	1µl	Extension	68°C	40sec	
OneTaq™ DNA Polymerase	0.125µl	Final extension	68°C	5min	1
ddH_2O	add to 25µl	Storage	10°C	∞	
PCR protocol for genotyping		qRT-PCR Program			
DNA	1µg	Segment	Temperature	Time	Cycles
10x Reaction Buffer	2.5µl	Denaturation	95°C	15min	1
dNTPs (10mM)	0.5µl	Amplification	94°C	15sec	
Primer (forward), 10 µM	1µl		55°C	30sec	55
Primer (reverse), 10 µM	1µl		75°C	30sec	
OneTaq™ DNA Polymerase	0.125µl	Melting curve	95°C	15sec	
ddH_2O	add to 25µl		65°C	20sec	1
			95°C		
		Cooling	40°C	30sec	1
		Storage	10°C	∞	
PCR protocol for SNP		Program			
DNA	5µl	Segment	Temperature	Time	Cycles
5x Reaction Buffer	5µl	Denaturation	94°C	5min	1
dNTPs (10mM)	0.5µl	Denaturation	94°C	30sec	
Primer (forward), 10 µM	1µl	Annealing	variable	30sec	35
Primer (reverse), 10 µM	1µl	Extension	68°C	40sec	
OneTaq™ DNA Polymerase	0.125µl	Final extension	68°C	5min	1
Q solution	optional	Storage	10°C	∞	
ddH_2O	add to 25µl				

4.2.3 Protein biochemistry

4.2.3.1 Protein isolation and measurement of protein concentration

The cells were washed three times with ice cold PBS and lysed in RIPA buffer containing complete protease inhibitor cocktail for 30 min on ice. Afterward, the cell debris was centrifuged for 15 min at 13,000 rpm. Protein concentration was determined with the Bradford assay (Bio-Rad) according to the manufacturer's instructions. For AGERA experiment, samples were lysed in HEPES buffer for 30 min on ice and the following procedures were the same.

4.2.3.2 SDS-PAGE and immunoblotting

Samples for western blot were prepared in Laemmli sample buffer and cooked for 5 min at 95 °C. Proteins were resolved on different percentage of polyacrylamide gels. Firstly, proteins went through the 4 % stacking gel with 60 V of power till they reached the same interface and they were separated according to their molecular weight on the separating gel with the voltage of 120 V. The composition of both gels is listed in TABLE 4.14. After the bromophenol blue running front reached the bottom of the gel, the SDS-PAGE was transferred onto a PVDF membrane using the wet blotting method (80 V for two hours or 35 V for overnight at 4 °C). After blotting, the membrane was washed with TBST and blocked by incubation with 5% skim milk in TBST (blocking solution). The membrane was incubated with primary antibodies (antibodies used are listed in TABLE 4.6) overnight at 4 °C in blocking solution, followed by an incubation with HRP-conjugated secondary antibodies (TABLE 4.7) for 1 h at RT. Protein signals were visualized using ECL chemiluminescence reagent or ECL high performance chemiluminescence reagent on Hyperfilm. For densitometric analysis National Institutes of Health ImageJ software version 1.6.0 was used. For further antibodies probing, the membrane was washed with TBST and ddH_2O and stripped by incubation in stripping buffer for 15 min at RT. Then it was washed with TBST for several times before blocking.

TABLE 4.14 The composition of SDS-PAGE gels

Separating gel	6%	7.5%	10%	12%	Stacking gel	4%
29:1 acrylamide	1ml	1.25ml	1.67ml	2ml	Stacking gel buffer	1.3ml
Separating gel buffer	1.5ml	1.5ml	1.5ml	1.5ml	29:1 acrylamide	0.2ml
ddH_2O	2.5ml	2.25ml	1.83ml	1.5ml	ddH_2O	0.1ml
10% APS	50µl	50µl	50µl	50µl	10% APS	10µl
TEMED	10µl	10µl	10µl	10µl	TEMED	4µl

4.2.3.3 Coimmunoprecipitation

For immunoprecipitation, a total of 500 µg protein lysate was incubated with 30 µl (1:1 slurry) protein A/G agarose beads, FLAG-agarose beads or V5-agarose beads overnight at 4 °C. After three times washing with 500 µl PBS, 15 µl of 5× Laemmli buffer was added and then incubated at 95 °C for 5 min. Proteins were separated on 7.5% SDS-PAGE and transferred onto a PVDF membrane.

4.2.3.4 Agarose Gel Electrophoresis for Resolving Aggregates (AGERA) analysis

The AGERA protocol was modified from Weiss et al. 2008. Briefly, cells were lysed in HEPES buffer (pH 7.4), 30 min on ice and then centrifuged at 4 °C for 15 min at 13,000 rpm. The protein concentration was measured by Bradford assay. 2× nonreducing Laemmli sample buffer was supplied and incubated at 95 °C for 5 min. Samples were run on a 2% agarose gel in SDS-PAGE running buffer at 100 V until the bromophenol blue running front reached the bottom of the gel. Then the gel was transferred onto a PVDF membrane with a Semi dry blotter PEDGASUS system (Luebeck) with a constant current 350 mM for 1 h. The remaining steps of immunoblotting were performed as described above.

4.2.4 Fluorescent imaging

4.2.4.1 Fluorescence microscopy, immunostaining and quantification

Cells were cultured on glass coverslips coated with poly-L-lysine (Sigma), fixed with 4% paraformaldehyde (pH 7.4) in PBS at RT or ice cold methanol (for γ-tubulin staining) at 4 °C for 15 min. After three rinses with PBS, cells were permeabilized and blocked with 0.05% Triton-X 100/10% NDS at RT for 1 h. This was followed by incubation in various primary antibodies (TABLE 4.6) overnight at 4 °C and secondary antibodies conjugated with Cy2 or anti-rabbit Alexa Fluor-647 (TABLE 4.7) diluted in 1:400 at 37 °C for 1 h. DAPI (4´,6´-diamidino-2-phenylindole) was used as counter staining. Samples were mounted with Mowiol /DABCO for imaging. Confocal images were taken with an AxioImager microscopy equipped with an ApoTome Imaging System (Carl Zeiss) using a 63× objective. Images were pseudocolored, merged and processed with AxioVision 4.8 software (Carl Zeiss) and ImageJ software. The level of acetylated tubulin was determined by Image J software using the Analyze, Measure option. Using the Freehand Line tool, the whole territory of each cell was outlined, and the sum of the gray values of the pixels in the selection (integrated density) was analyzed by subtracting the background.

4.2.4.2 Live cell imaging

Cells were grown in Lab-Tek®II chambered coverglasses (#155382, Nalge Nunc International). EGFP-tagged kalirin-7 and/or HcRed-tagged synphilin-1 were

transfected and monitored at the time points indicated. Time-lapse imaging was acquired using an inverted Zeiss Axiovert microscope (Zeiss Plan-Apochromat) with a 63× oil immersion objective equipped with an incubation chamber (37 °C and 5% CO2) at defined positions every 2-3 h for 72h post-transfection. Images were exported to JPG files using Zeiss Axio Vision 4.8 software (Zeiss, Germany).

4.2.5 Generation and characterization of kalirin-7 transgenic animal

4.2.5.1 Genomic DNA isolation and genotyping

The mice were anesthetized with isofluoran and the tail (~0.5 cm) was chopped. DNA isolation was conducted by High Pure PCR Template Preparation Kit (Roche) according to manufacturer's instruction. Briefly, the samples were digested in 200 µl of Tissue Lysis Buffer plus 40 µl of Proteinase K (10 mg/ml) at 55 °C until tissues were completely digested, then mixed well with 200 µl Binding Buffer and 100 µl isopropanol, centrifuged at 13,000 × g for 5 min. The supernatant was transferred into High Filter Tube and washed with 500 µl Inhibitor Removel Buffer and twice with 500 µl Wash Buffer. The DNA was eluted with 200 µl Elution Buffer and stored at 4 °C for later analysis.

4.2.5.2 RNA isolation and cDNA synthesis

The tissue was lysed and homogenized in 1:10 (mg/ml) QIAZOL lysis buffer and left in RT for 10 min. The lysate was centrifuged 13,000 rpm at 4 °C for 15 min, and one volume of 70 % EtOH was added to the supernatant. After transferring into the filter tubes provided by RNeasy Mini Kit, washed with 700 µl RW1 buffer and 500 µl RPE. Total RNA was eluted with 50 µl RNase-free ddH_2O.

For cDNA synthesis, first the genomic DNA was eliminated by incubating template RNA in gDNA Wipeout Buffer for 2 min at 42 °C and kept on ice immediately. The reverse transcription reaction was conducted by adding 1 µl Reverse Transcriptase, 4 µl 5x RT Buffer, 1 µl RT Primer Mix and 14 µl template RNA (gDNA eliminated) and incubated at 42 °C for 30 min. After inactivating reverse transcriptase at 95 °C for 3 min, cDNA concentration was measured and stored at 4 °C for further analysis.

4.2.5.3 Quantitative Realtime-PCR (qRT-PCR)

To quantify mRNA expression of transgenic animals, QuantiTect SYBR Green PCR Kit (Qiagen) was used and programmed by LightCycler 480 in 384er format. The SYBR green I dye binds to double-strand amplicons during the PCR amplification and leads to a conformational change, which emits more fluorescence. The excitation and emission spectra of SYBR green are at 494 nm and 521 nm according to manufacturer's instruction. The protocol and program are listed in TABLE 4.12.
Each batch of experiment should include a standard curve, which estimates the

efficiency of the reaction, and the optimal efficiency is 2.

4.2.5.4 Protein isolation from mouse brain

To extract protein from tissue, the sample was homogenized 1:10 (mg/µl) using a dounce tissue grinder in TES-complete buffer containing 1 % NP-40 (TNES) and kept on ice for 30 min. After a centrifugation step (13,000 rpm at 4 °C for 30 min), 10 % volume of glycerol was added and the protein concentration was measured by Bradford method or the supernatant was stored at -80 °C.

4.2.5.5 Paraffin section and Immunohistochemistry staining

The mice were perfused with 4 % PFA and the brain was post-fixed in 4 % PFA overnight. Dehydration and transfer in paraffin was done using the automatic tissue processor (Leica TP 1050) and the protocol was as follows: Ethanol: 1x 70% → 2x 96% → 3x 100%; then in: 3x Xylol → 3x Paraffin at 60 °C/ 1.5 h for each reaction. 7 µm thick paraffin sections were cut with the Microtom Leica RCI2155, floated in warm water bath (42 °C-46 °C) and attached on the slices. The paraffin was eliminated with xylol and ethanol and ready for staining. The slices were washed twice with PBS and incubated in Citrate-Buffer 5 min for three times. The endogenous peroxidase of tissue was removed by incubating in 0.3 % H_2O_2 for 20 min. In the following steps, slices were washed with PBS in between. The slices were blocked with 5 % NGS/0.3 % Triton-X100 RT for 2 h, incubated with primary antibody at 4 °C overnight and secondary antibody RT for 1 h. Then the slices were incubated with Avidin-Biotin-complex RT for 1 h, and the protein signal was visualized by Diaminobenzidin (DAB) staining.

4.2.6 Statistical analysis

Statistical significance ($p < 0.05$) was processed with paired, two-sided Student's *t* test. Error bars indicate s.e.m. Asterisks indicate significance, $^{*}P \leq 0.05$, $^{**}P \leq 0.005$, $^{***}P \leq 0.001$.

5. REFERENCES

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