THE PARKINSON'S DISEASE ASSOCIATED DJ-1 PROTEIN IS MODIFIED BY SUMO-2/3

Candidate: Flavia Mazzarol

Supervisor: Prof. Stefano Gustincich

INTRODUCTION

1 PARKINSON' S DISEASE

1.1 Brief history of Parkinson's Disease

Parkinson's Disease (PD) was originally observed by James Parkinson in 1817. In his monograph "Essay on the Shaking Palsy" the British physician described the core clinical features of the disease then named *paralysis agitans*: " *Involuntary tremolous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forewords and to pass from a walking to a running pace: the senses and the intellect being ininjured*".

In the 1950s large efforts were spent to characterize biochemically this pathology, mostly by the Nobel Prize 2000 Arvid Carlsson. He demonstrated that Levodopa reverses the parkinsonian state induced by Reserpine in rabbits (1957), and that Dopamine (DA) is present in the brain (1958). Finally he discovered that PD patients showed a massive loss of DA (1960) which can be clinically reverted by the employment of Levodopa (1968). Furthermore, typical neuropathological hallmarks were described as the loss of dopaminergic neurons in the *Substantia Nigra* and the presence of intraneuronal inclusions called Lewy Bodies (LBs) in the surviving ones. However, despite this early description, the etiology of PD is still unclear and the neurodegenerative process is irreversible.

1.2 Clinical characteristics of PD

Nowadays PD is the second most common progressive neurodegenerative disorder after Alzheimer's Disease (AD). It affects 1-2 % of all individuals

above the age of 65 years old, increasing to 4-5% by the age of 85. Old age is the greatest risk factor of PD, indeed the onset is extremely rare before age 40. Men are slightly more affected than woman. PD is a slowly progressive neurodegenerative disorder, which begins insidiously, gradually worsens in severity and usually affects one side of the body before spreading to involve the another side. PD is characterized by six cardinal features: tremor at rest, rigidity, bradikinesia, hypokinesia and akinesia, flexed posture of neck, trunk and limbs, loss of postural reflexes and freezing phenomenon. The early symptoms of PD are usually alleviated by the treatment with Levodopa and DA agonists. As PD advances from year to year, late symptoms such as flexed posture, loss of postural reflexes and freezing phenomenon, don't respond to the Levodopa treatment. Furthermore, bradykinesia, that responded to Levodopa in the early stage of PD, worsens and does not respond to Levodopa any more. While motor symptoms dominate PD clinical features, many patients show other non-motor symptoms. These include fatigue, depression, anxiety, sleep disturbances, constipation, bladder and other autonomic disturbances (sexual and gastrointestinal), sensory complaints, decreased motivation, apathy and a decline in cognition that can progress to dementia.

1.3 Neuropathological features of PD

PD is due to the relatively selective loss (70-90%) of dopaminergic neurons in the *Substantia Nigra pars compacta* (SNc), which leads to a profound reduction in striatal dopamine (DA). The loss of dopaminergic neurons is asymmetric, slow and progressive as the disease itself. These neurons project into *striatum caudate* and *putamen nuclei*, that anatomically form *basal ganglia* (**figure 1**). Voluntary movements originate at motor cortex level: signals are sent through the encephalic trunk (*mesencephalon, pons*

and medulla) up to the spinal cord. These signalling pathways are controlled by different sub-cortical structures (*thalamus, putamen and subthalamic nuclei*) that modulate movements. Basal ganglia dopaminergic neurons depletions provoke movement control dysfunction and initiate the characteristic symptoms of PD [Dauer and Przedborski, 2003].

With the progressive loss of dopaminergic neurons, there is a corresponding decrease of DA content in both the *Substantia Nigra* and the *striatum*. The loss of the nigrostriatal pathway can be detected during life using PET and SPECT scanning, showing a progressive reduction of fluoro-DOPA (FDOPA) and DA transporter ligand binding in the *striatum*.

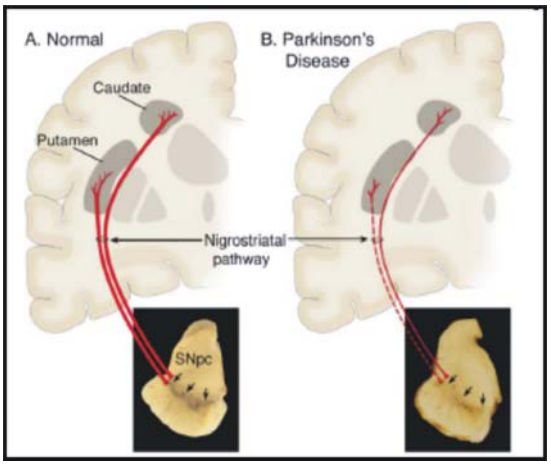


Fig 1: Schematic rapresentation *of Substantia Nigra* and pathways towards caudate and putamen nuclei in physiological (left) and PD conditions (right) (Dauer *et al.*, 2003).

The neuronal loss is accompanied by an increase in glial cells and loss of neuromelanin, pigment normally contained in dopaminergic neurons. LBs and dystrophic neuritis, also called Lewy Neurites (LNs), are present in some of the remaining dopaminergic neurons and are the typical pathological hallmark of PD. Classically, LBs are round eosinophilic inclusions composed of a halo of radiating fibrils and a less defined core (**figure 2**). Both LBs and LNs are composed by the accumulation of cytoplasmic aggregates containing a variety of proteins, of which α-synuclein is a major component.

Interestingly, LBs are also present in other diseases that are associated with dementia (LBs dementia) [Bove *at al.*, 2005, Gibb *et al.*, 1987].

The role of LBs in PD neurodegenerative process, if they could be perpetrators or protective, remains a matter of fierce dabate.

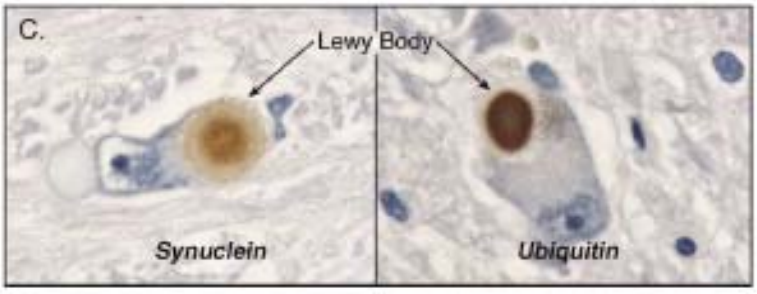


Fig 2: Immunohistochemical staining of Lewy Bodies found in dopaminergic neurons of PD patients in *Substantia Nigra pars compacta*. Unsoluble fibrous component of aggregates are detected with anti-α- synuclein antibody and anti-ubiquitin (Dauer *et al.*, 2003).

1.4 Etiology of PD

1.4.1 Sporadic Forms

PD is a multifactorial disease caused by both genetic and environmental factors. The cause of sporadic PD is unknown, but the environmental hypothesis was dominant for much of the 20th century. The finding that people intoxicated with 1-methyl-4-phenyl-1,2,3,4-tetrahydropyrydine (MPTP) develop a syndrome nearly identical to PD (Langston *et al.*, 1983) is a prototypic example of how an exogenous toxin can mimic the clinical and pathological features of PD. Human epidemiological studies have implicated residence in rural environment and related exposure to herbicide (i.e. Paraquat) and pesticides (i.e. Rotenone) with an elevated risk for PD. Instead, cigarette smoking and coffee drinking are inversely correlated to the risk for PD development (Hernan *et al.*, 2002).

1.4.2 Genetic forms

In recent years, linkage analyses and positional cloning of an increasing number of genes that are linked to inherited forms of PD have provided new insights into PD pathogenesis. Although 90% of all PD cases are sporadic, 10 % of all cases are characterized by a positive familiar history. Therefore, in the last ten years great efforts has been spent to identify the PD genes. Up to now 13 monogenic PD forms have been described, (labelled from PARK 1 to PARK 13): in nine cases it was possible to identify the genes involved (**Table 1**).

TYPE	LOCUS	GENE	TRANSMISSION	LEWY BODIES
PARK 1	4q21-23	α-synuclein	AD	+
PARK 2	6p25.2-27	Parkin	AR	-
PARK 3	2p13	Unknown	AD	+
PARK 4	4p14-16.3	α-synuclein	AD	+
PARK 5	4p14	UCH-L1	AD	?
PARK 6	1p35-36	PINK-1	AR	+
PARK 7	1p36	DJ-1	AR	?
PARK 8	12p11.2-13.1	LRRK2	AD	+
PARK 9	1p36	ATP13A2	AR	?
PARK 10	1p32	Unknown	NP	?
PARK 11	2q36-37	Unknown	AD	?
PARK 12	Xq23-25	Unknown	XR	?
PARK13	2p13	Omi-HtrA2	AD?	?

Table 1: Genetic *loci* and genes identified in PD.

1.5 Pathogenesis of PD

Whatever is the initial insult that provokes the dopaminergic neurodegeneration, studies of toxic PD models and of the biological function of the genes implicated in inherited forms of PD suggest two major hypotheses regarding the pathogenesis of the disease:

1. misfolding and aggregation
2. mitochondrial dysfunction and the consequent oxidative stress.

These two hypotheses are here discussed.

1.5.1 Misfolding and aggregation of proteins

The abnormal protein deposition in brain tissue is a feature of several age-related neurodegenerative disorders. Although the compositions and locations are different from disease to disease, this common feature indicates that the deposition itself could be toxic.

As anticipated, LBs, the tipical hallmark of surviving dopaminergic neurons, are cytoplasmic aggregates, mainly containing α-synuclein. This natively unfolded protein has a great propensity to aggregate both *in vitro* and *in vivo*, especially in the presence of several herbicide and pesticide. Moreover, two PD-linked mutants (Ala30Pro and Ala52Thr) show a great propensity to form protofibrils.

Cells respond to misfolded proteins by inducing chaperone activities. When misfolded proteins cannot be properly refolded, they are targeted for proteosomal degradation by polyubiquitination. It is interesting to note that LBs are immunoreactive against Chaperone proteins (i.e. Hsp-70), Ubiquitin, and components of the 26S of the proteosome system.

Protein aggregates may interfere with intracellular trafficking in neurons or sequestrate proteins important for cell survival. This hypothesis considers LBs as perpetrators although till now there is no clear correlation between inclusion formation and neuronal cell death. Alternatively, LBs could be considered as protectors. The increasing demand of protein degradation, hypothesis proposes that LBs form late in the disease after cells have tried all their the consequent failure of the Ubiquitin Proteosome System (UPS) to process abnormal proteins, and the resulting accumulation of misfolded proteins, lead to the formation of aggregates that can be removed by autophagy.

In inherited PD cases, pathogenic mutations could be directly involved by inducing abnormal and toxic protein conformations (i.e. α-synuclein) or interfering with the processes that normally recognize misfolded proteins.
Mutations in Parkin were first linked to autosomal recessive juvenile Parkinsonism (ARJP) and are the most frequent cause of autosomal recessive PD. Parkin is an E3 ubiquitin protein ligase that is upregulated under unfolded protein stress.
Working in concert with the E1 activating enzyme and the E2 conjugating enzyme, Parkin can ubiquitinate specific substrates (such as Cycline E, α-synuclein, Pael Receptor and Synphilin-1) thereby targeting them for proteasomal degradation. Considering that the majority of the patients with parkin mutation lacks LBs pathology, it has been suggested that parkin may promote LBs formation (Mizuno *et al.*, 2001), mostly because in sporadic PD patients these inclusions are immunoreactive for some of parkin substrates. Even if parkin fails to directly ubiquitinate α-synuclein (Chung *et al.*, 2001), it interacts with the α-synuclein interacting protein synphylin I, and through its interaction it may promote LBs formation. Alternatively, parkin-mediated neurodegeneration may proceed through a mechanism that is distinct from those that occur in the cases of PD with LBs.
The linkage with PD of PARK5 gene also supports the notion that derangements in the UPS may contribute to DA neuronal death. PARK5 encodes for UCH-L1, which hydrolyzes ubiquitin from polymeric chains and from ubiquitinated proteins producing monomeric units. UCH-L1 has also been found in the LBs of patients with sporadic PD. This finding provides an additional evidence for the involvement of aberrant ubiquitinilation and aggregates formation in the PD pathogenesis.

1.5.2 The oxidative stress hypothesis

The hypothesis that oxidative stress plays a role in the pathogenesis of PD was fuelled by the discovery that MPTP blocks the mitochondrial electron transport chain by inhibiting complex I (Nicklas *et al.*, 1987).

MPTP is highly lipophilic, and it crosses the blood brain barrier within minutes (Markey *et al.*, 1984). Once in the brain, MPTP is oxidized to 1-methyl-4 phenyl-2,3-dihydropyridinium ($MPDP^+$) by monoamine oxidase B (MAO B) in glia and serotonergic neurons and then is spontaneously oxidazed to MPP^+. Due to its high affinity for the DA Transporter (DAT), it is selectively accumulated in dopaminergic neurons, where it causes toxicity and neuronal death through complex I inhibition (**figure 3**). Similar toxic effects are produced by the common herbicide 1,1'-dimethyl-4,4'-5 bipyridinium (Paraquat) coupled with the amministration of the fungicide manganese ethylenepistithiocarbamate (Maneb). While Paraquat, which is structurally similar to MPTP, blocks the mitochondrial complex I, Maneb inhibits the mitochondrial complex III. Derangements in complex I cause α-synuclein aggregation, which further contributes to the demise of Dopaminergic neurons (Dawson and Dawson, 2003). Furhermore, genetic defects in the mitochondrial complex I could contribute to cell degeneration in PD through decreased ATP synthesis. Selective decrease in the activity of mitochondrial complex I has also been found in the *Substantia Nigra* of PD patients (Shapira *et al.*, 1990). A consequence of the mitochondrial dysfunction is oxidative stress and *viceversa*. Ninety-five percent of the molecular oxygen is metabolized within the mitochondria by the electron-transport chain: thus mitochondria are highly exposed and damaged by oxidative stress and this leads to a more intense and perpetuating cycle in which reactive oxygen species (ROS) are generated.

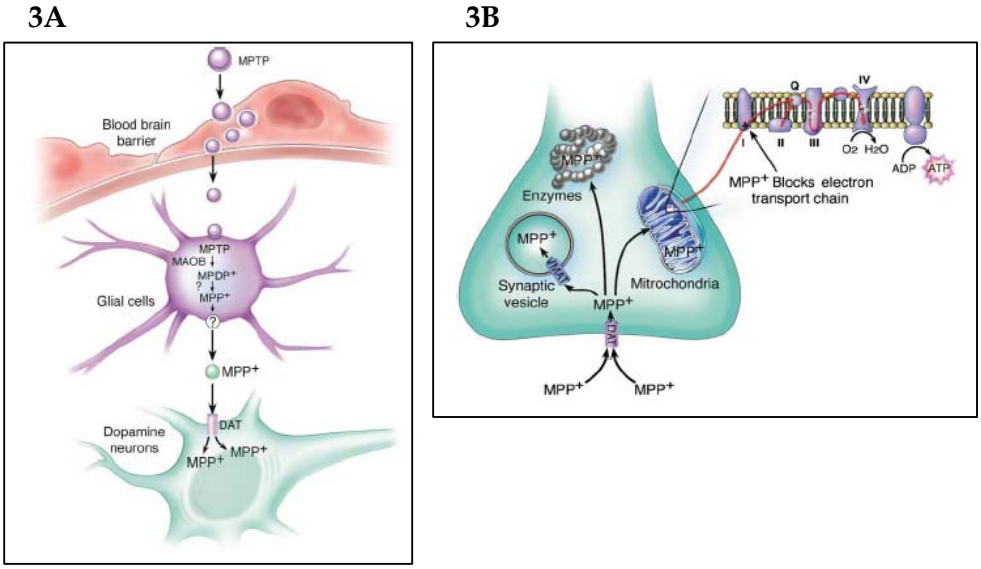


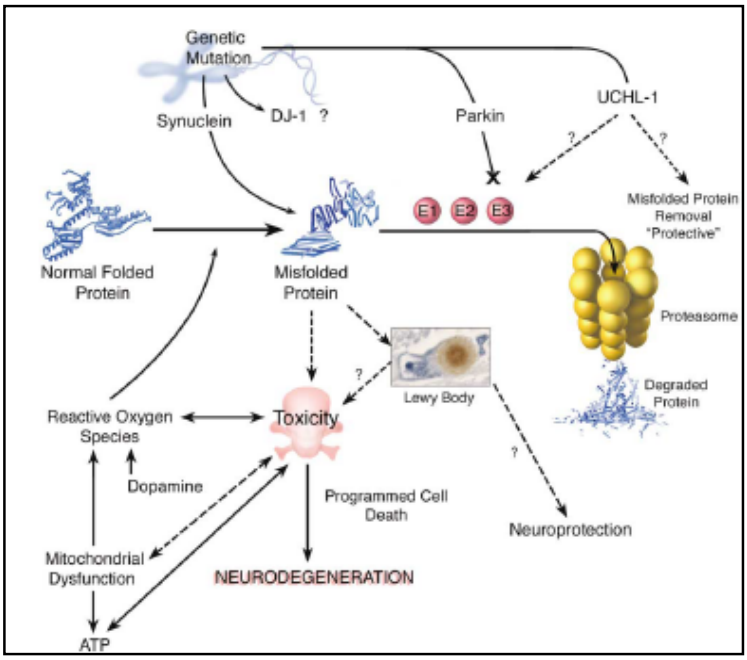
Fig 3: A) Schematic representation of the MPTP metabolism and B) of MPP^+ intracellular pathway (Dauer *at al.*, 2003).

ROS cause functional alterations in proteins, lipids and DNA. Lipid damage leads to loss of membrane integrity and ions permeability, promoting excitotoxicity (Halliwell, 1992). Although ROS levels cannot be directly measured, the assessment of their reaction products and of the resulting damage in *post mortem* tissues is as an indirect index of their levels (Foley and Riederer, 2000).

Many factors lead to oxidative stress in SNpc. The reduced content of the anti-oxidant glutathione in the SNpc of PD brains lowers the capability to clear H_2O_2. Moreover, the increased free iron level can promote OH^* formation and DA oxidation into toxic dopamine-quinone species (Graham *et al*, 1978). The impairment of the complex I system may be central in PD pathogenesis of DA neurons since its defects and inhibitors cause dopaminergic cell death and induce the formation of filamentous inclusions containing α-synuclein. A complex interaction between mitochondrial dysfunction, oxidative stress and the propensity to form aggregates occur (**figure 4**). Complex I inhibitors causes a reduction in the

proteosomal activity (Shen *et al.*, 2004) and conversely proteosomal inhibition can cause mitochondrial damage (Hoglinger *et al.*, 2003). Further studies are needed to understand the biochemical pathways involved in dopaminegic neurons cell death, including the interaction between oxidative stress and aggregates formation through UPS impairment.

Fig 4: Mechanism of neurodegeneration: linking oxidative stress and mitochondrial damage to UPS impairment and aggregates formation (Dauer *et al.*, 2003).



2 DJ-1 IN PARKINSON'S DISEASE

After the identification of the PARK-7 locus by van Dujin *et al.*, Bonifati *et al.* have shown mutations in the DJ-1 gene in two PARK-7 linked families. The Dutch family displays a large homozygous deletions of exons 1-5 of the DJ-1 gene and an Italian kindred harbors a single missense mutation at an highly conserved position, Leu166Pro.

Till now different mutations affecting DJ-1 (including missense, truncating and splice-site mutations and large deletions) have been linked to autosomal recessive PD. The homozygous deletions found in PARK-7 patients represent a natural knock out, indicating that the loss of function is pathogenic. Several studies indicate that the mutant Leu166Pro is unstable and thus degraded through the UPS (Miller *et al.*,2003; Olzmann *et al.*, 2004; Blackinton *et al.*, 2005). It is worth noting that position 166 is localized in the penultimate C-terminal α-helix near the dimer interface and the mutation to proline is predicted to interrupt the helix (Tao *et al.*, 2003; Moore *et al.*, 2003; Olzmann *et al.*, 2004). The Leu166Pro mutant doesn't dimerize but is rapidly degraded as monomer. Interestingly, other studies indicate that Leu166Pro tends to form multimeric aggregates (Baulac *et al.*, 2004; Macedo *et al.*, 2003; Olzmann *et al.*, 2004). The Leu166Pro monomer has been recently shown to possess a different conformation from the wild type DJ-1 and the data computational analysis indicates that Leu166 is not located at the subunit surface involved in the dimerization. Thus the different conformation of Leu166Pro monomeric units might affect the protein-protein interaction repertory (Herrera *et al.*, 2007).

The pathogenic role of other mutants remains to be established.

Some of them might be simple polymorphisms that predispose to PD, as demonstrated for the Arg98Gln.

Although DJ-1 mutations account for only a small fraction of early onset PD (1-2%), they are the second most frequent cause of recessive forms of PD (Heidrich K *et al.*, 2004), after PARK2-linked families.

Clinically, DJ-1-dependent PD is characterized by early onset of parkinsonism (the average age is the early 30s), slow disease progression and good initial response to Levodopa.

Psychiatric and behavioural disturbances (including severe anxiety and psychotic episodes) are reported in both original DJ-1 families (Abou-Sleiman *et al.*, 2004). Further analysis is necessary to investigate whether this aspect is more frequent in DJ-1 patients than in other PD forms.

2.1 DJ-1 gene and protein distribution

DJ-1 gene spans 24 kb and contains seven exons, the first two are non-coding and subject to alternative splicing in mRNA. (Taira *et al.*, 2001) It encodes a small 189-aminoacid protein that is ubiquitously expressed and widely distributed in brain and other tissues. It is conserved through different species (Bandopadhyay and Cookson, 2004) and determination of crystal structure of human DJ-1 has demonstrated that it exists in homodimeric form, essential to retain its biological function (Olzmann *et al.*, 2004). In human brain, DJ-1 has a marked astrocytic expression (Bandopadhyay *et al.*, 2004), while neuronal labelling is very weak. It never localizes at LBs or LNs (Bandopadhyay *et al.*,2005). On the contrary, in the murine brain DJ-1 presents both a neuronal and glial expression.

Bader *at al.* found DJ-1 expression in murine neurons of different neurotransmitter phenotypes and in all glial types, such as astrocytes, microglia, and oligodendrocytes. The high DJ-1 expression is not confined to a single anatomical area, considering that positive immunoreactivity was found in cortical areas, hippocampus, olfactory bulb, amygdale, thalamus, locus coeruleus, caudate, putamen, globus pallidus and the

deep nuclei of the cerebellum (Shang *et al*., 2004; Bader at al., 2005). Within the *Substantia Nigra*, DJ-1 is localized in both neuronal and glial cells. At the cellular level DJ-1 immunoreactivity is found in both cytoplasm and nucleus, and in the mitochondrial matrix and intermembrane space (Zhang *et al.*,2005). This staining is increased in oxidative stress conditions suggesting that oxidation promotes the mitochondrial localization on DJ-1 (Blackinton at al., 2005).

2.2 DJ-1 functions

The exact role of DJ-1 protein in health and disease remains mostly unknown. DJ-1 is involved in various cellular processes including cellular transformation, regulation of RNA stability, transcriptional activation and in oxidative stress response.

Initial computational analysis revealed that DJ-1 belongs to the ThiJ-PfpI Superfamily containing a ThiJ domain. This domain has been first described in the Large Glutamine Amidotransferase superfamily (GAT), enzyme that is involved in the Thiamine synthesis in bacteria. However it is also present in chaperones, catalases and proteases. This heterogeneity of functions of the members of the ThiJ-PfpI superfamily limits the ability to predict the cellular role of the human ortholog. Structural data suggests that DJ-1 conformation seems to be unfavorable for catalytic activity. Furhermore, the catalytic triad Cys-His-Asp/Glu is not conserved across evolution. Olzmann *et al*., reported that DJ-1 exhibits protease activity instead of a molecular chaperone function, which is abrogated by the mutation of Cys106Ala. Up to know the general consensus is on the lack of catalase or protease activity.

Structural comparisons between DJ-1 and Hsp31, a member of the ThiJ-PfpI family, may point to a chaperone activity (Lee *et al*., 2003). Shendelman *et al.* showed that DJ-1 acts as a redox-regulated chaperone. It

is activated in an oxidative cytoplasmic environment and inhibits the formation of α-synuclein aggregates *in vitro* and *in vivo*. These data suggests that DJ-1 can mitigate the molecular insults downstream ROS burst suppressing the early step of protein aggregation.
Zhou *et al.* have recently confirmed that DJ-1 chaperone activity prevents α-synuclein fibrillation, but only when Cys106 is oxidized to sulfinic acid (Cys106-SO_2H), while the native DJ-1 and the highly oxidized form of DJ-1 resulted to be ineffective. These results suggest that the oxidation level can affect its structure thus impairing its normal function.

An intriguing hypothesis links DJ-1 to oxidative stress response. DJ-1 undergoes a pI shift from 6.2 to 5.8 upon Reactive Oxigen Species (ROS) exposure, indicating that it may function as an indicator of oxidative stress (Taira *et al.*, 2004). Cys 106 is the most sensitive among all the three cystein residues (i.e. Cys 46, Cys 53 and Cys 106). Oxidation induces a mitochondrial relocalization of DJ-1 and protection against cell death is abrogated in Cys106Ala but not by Cys46Ala or Cys53Ala (Canet-Aviles *et al.*, 2004). Two *post mortem* studies of brain samples from PD brains found that acidic isoforms of DJ-1 are more abundant in PD compared to controls (Bandopadhway *et al.*, 2004, Choi *et al.*, 2006). The accumulation of acidic isoforms of DJ-1 monomer is followed by the enrichment of basic isoforms of DJ-1 dimer in PD/AD brains due to protein carbonylation (Choi *et al.*, 2006).
DJ-1 displays a protective role against ROS-induced cell death. DJ-1 knock out by short RNA interfering rendered SH-SY5Y susceptible to both hydrogen peroxide (H_2O_2) and 1-methyl-4-phenylpyridinium (MPP^+) (Yokota *et al.*, 2003; Taira *et al.*, 2004). While overexpression of wild type DJ-1 protects from oxidative stress, proteasomal inhibition and ER stress (Yokota *et al.*, 2003; Taira *et al.*, 2004), cells harboring Leu166Pro DJ-1

mutant become susceptible to death induced by hydrogen peroxide and do not show oxidized forms of DJ-1.

Moreover, a number of studies performed on DJ-1 deficient mouse model supports this hypothesis.

Dopaminergic neurons derived from *in vitro* differentiated (Martinat *et al.*, 2004) DJ-1-deficient embryonic stem cells showed a decreased survival and an increased sensitivity to oxidative stress. Comparing the responses to H_2O_2 of primary cortical neurons from brains of DJ-1 +/+, DJ-1 +/- and DJ-1 -/- mice embryos exposed to H_2O_2 (Kim *et al.*, 2005), DJ-1 -/- deficient neurons showed a 20% increase in cell death compared to wild type DJ-1 +/+ neurons, whereas an intermediate amount of cell death was observed in DJ-1 +/- neurons suggesting a gene-dosage effect.

A similar study analyzed the effect of pesticide rotenone on dopaminergic neurons. This pesticide inhibits mitochondrial complex I, increasing ROS. Upon rotenone treatment, the number of surviving dopaminergic neurons in DJ-1 deficient mice is decreased by 30% compared to control DJ-1 +/+ neurons. In addition, it has been shown that DJ-1 knock-out mice are more vulnerable to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrindine (MPTP) and restoration of DJ-1 expression in DJ-1 deficient mice via adenoviral vector delivery mitigated cell death (Kim *et al.*, 2005).

Several DJ-1 KO were produced independently but none of them showed degeneration of dopaminergic neurons *per se*. Although they showed an age and task-dependent motor deficits, and a marked reduction in evoked dopamine overflow in the striatum (Goldberg *et al.*, 2005; Chen *et al.*, 2005), dopaminergic neuronal loss was present only when they were treated with drugs that cause toxicity through mitochondrial complex I inhibition.

A complementary approach to elucidate DJ-1 role in PD is the study of its homologs in *Drosophila,* DJ-1 α and DJ-β. DJ-1 α shares 56 % of homology with human DJ-1 and DJ-β shares 52 % identity and they are characterized

by distinct temporal and spatial expression pattern. Menzies *et al.* found that DJ-1 α is expressed predominantly in testis, with high expression level only in the late stages of development, whereas DJ-β is ubiquitously present, with no relevant changes in expression during the development, closely resembling the human DJ-1 expression pattern. *Drosophila* lacking DJ-1 activity are fertile, have a normal life span and normal number of dopaminergic neurons. Meulener *et al.* indicated that double knock out (DKO) is not deleterious, but it is much more sensitive to agents that induce oxidative stress (i.e Paraquat and Rotenone). Loss of DJ-β expression results in an increased survival of dopaminegic neurons due to a compensatory up-regulation of DJ-1 α in the brain, which is also associated to a decreased sensitivity to Paraquat (Menzies *et al.*, 2005). Yang *at al*. analyzed a DJ-1 α knock out fly strain: the specific inhibition of DJ-1 α in dopaminergic neurons leads to a decrease of TH- positive neuron number, to elevated ROS accumulation and hypersensitivity to oxidative stress. These data clearly indicate a protective role of DJ-1 mostly in oxidative stress response.

DJ-1 activity is also studied through the analysis of its protein-protein interaction network.

The anti-apoptotic function of DJ-1 is exerted through its interaction with the death protein Daxx. DJ-1 sequesters Daxx in the nucleus, preventing its binding and consequential activation of ASK-1 (Apoptosis Signal Regulating Kinase 1) (Junn *et al.*, 2005). DJ-1 is also a transcriptional co-regulator of several transcription factors acting on various promoters, functioning as a co-activator and as a co-repressor. Xu *et al.* demonstrated that DJ-1 interacts with the nuclear proteins p54nrb and pyrimidine tract-binding protein associated splicing factor (PSF), two multifunctional regulators of transcription and RNA metabolism, highly expressed in brain. DJ-1 blocks the transcriptional silencing and apoptosis induced by PSF binding p54nrb and antagonizing the effect of PSF. It has been

proposed that mutations that can attenuate the nuclear function of DJ-1 predispose to neurodegeneration. Interestingly, DJ-1 can activate the transcription at the Tyrosine Hydroxylase promoter (TH), the rate-limiting enzyme for dopamine biosynthesis (Jeong *et al.*, 2006). DJ-1 prevents the post translational modification of PSF by the Small Ubiquitin Modifier SUMO-1. Mutations that abolish the sumoylation of PSF relieve the transcriptional repression of the TH promoter by PSF. Interestingly; the same study demonstrated that DJ-1 regulates sumoylation *in vivo.* Lymphoblast from PD patients (both carring deletion of exon 1-5 and the missense mutation Leu166Pro) have a slight reproducible increase of SUMO-1-modified high molecular weight complexes, compared to patients with wild type DJ-1 or carring the non-pathogenic mutation Arg98Gln. These data indicate that DJ-1 loss results in an accumulation of SUMO-1-modified PSF, leading to a decreased DA synthesis. It is worth noting that this effect is specific for SUMO-1 conjugated proteins, while it does not affect SUMO2/3 conjugated proteins. (Zhong *et al.*, 2006)

On the other hand it has been shown that DJ-1 is indispensable for the stabilization of the transcriptional regulator NrfII. In unstimulated conditions, its expression is maintained at low level by Keap-1, that targets NrfII to protein degradation. Upon exposure to oxidative stress, NfrII is translocated to the nucleus, where it forms heterodimers with other TFs inducing the expression of antioxidant genes whose promoters contain the ARE (Antioxidant Responsive Element) [Clements *et al.*, 2006].

DJ-1 is also studied in Cancer. DJ-1 was first discovered as a novel oncogene in cooperation with activated *ras* (Nagakubo *et al.*,1997) and its overexpression has been reported in several cancers including breast, lung and prostate (Kim *et al.*, 2005).

Shinbo *et al.* found that DJ-1 is bound to p53 *in vitro* and *in vivo* and that this binding is stimulated by UV radiation. Moreover, DJ-1 restores p53 transcriptional activity inhibited by Topors (Shinbo *et al.*, 2005a), which

sumoylates both DJ-1 and p53. DJ-1 is sumoylated by SUMO-1 on the conserved Lys 130 and its post translational modification is induced by UV radiation in a p53-independent manner The mutant DJ-1 Lys130Arg abrogates cell growth-promoting activity with activated *ras* (Shinbo *et al.*, 2005b).

Furthermore, Kim *et al.* 2005 demonstrated that DJ-1 modulates the phosphatidylinositol 3-kinase survival pathway by negatively regulating the function of the tumor suppressor gene PTEN. Abstrakt, an RNA helicase, expressed ubiquitously in all tissues, was also found to stimulate the transforming activity of DJ-1 in rat 3Y1 cells transfected with DJ-1 and activated ras (Sekito *et al.*, 2005)

By interacting with PIASxalpha and with DJBP, proteins predominantly expressed in testis, DJ-1 was also able to alter their DJ-BP binding to the Androgen Receptor (AR) (Takahashi *et al.*, 2001).

PIASxalpha directly binds to AR in a testosterone-dependent manner and it negatively modulated the AR transcription activity by recruiting the histone-deacetylase complexes. DJ-1 antagonizes this inhibition by the abrogation of the complex and restoring the AR activity.

2.3 DJ-1 in Neurodegeneration

Immunohistochemical staining for DJ-1 labels Tau inclusions found in several neurodegenerative disorders, including Alzeheimer's disease (AD), Progressive Supranuclear Palsy (PSP), Frontotemporal Dementia linked to chromosome 17 (FTDM-17), and Pick's Disease (PiD) (Bandopadhyay *et al.*, 2004; Neumann et a., 2004; Rizzu *et al.*, 2004). Furthermore, DJ-1 antibodies readily label α-synuclein glial cytoplasmic inclusions observed in patients with Multisystem Atrophy (MSA) (Neumann *et al.*, 2004) and PD. As with tau and α-synuclein, a fraction of DJ-1 protein becomes markedly insoluble in brain from AD, PiD and MSA compared to controls. (Neumann *et al.*, 2004; Rizzu *et al.*, 2004). Moore *et al.* monitored the level of detergent-soluble DJ-1 in human *post mortem* cingulated cortex tissue. They found a dramatic increase of DJ-1 levels in the detergent-insoluble fraction in PD and Dementia with Lewy Bodies (DLB), indicating that in pathological conditions DJ-1 can undergo a biochemical modification. These data support the notion that different neurodegenerative diseases might share a common mechanism in which DJ-1 role needs to be elucidated.

3 SUMOYLATION

Small Ubiquitin Modifiers (SUMO), an highly conserved protein family found in all eukaryotes, has been shown to be responsible of post translational modification. It belongs to the Ubiquitin Like Proteins (UBLs) superfamily, since it shares structural similarity with ubiquitin (Bayer *et al.*, 1998) and is mechanistically involved into the transfer of small protein moieties onto various substrates, modulating a growing number of cellular pathways.

SUMOs shares only 18% sequence identity with Ubiquitin and their molecular weights are around 11 kDa, but they could appear larger on SDS-PAGE adding around 20 kDa to most substrates. While yeast and invertebrates contain a single SUMO gene, vertebrates contain four different genes: SUMO-1 (also known as sentrin, PIC1, GMP1, Ubl1 and Smt3c), SUMO-2 (sentrin-3, Smt3a), SUMO-3 (sentrin-2, Smt2b) and SUMO-4. SUMO-1 mainly exists in conjugated form, whereas SUMO-2 and SUMO-3 mostly in free form. SUMO-2 and SUMO-3 share ~95% sequence identity and are 50 % identical to SUMO-1 (**figure 5**). They are generally regarded as being functional equivalent, being the main differences clustered at their C-terminal region. At the level of their N-terminal they contain the ψKXE sequences, which can serve as SUMO attachment sites, allowing the formation of SUMO chain *in vitro* and *in vivo*. This sequence is lacking in SUMO-1, inhibiting the formation of poly-SUMO-1 chain. Recently, another member of the superfamily, SUMO-4, has been identified. Its messenger is shown to be mainly expressed in kidney cells (Bohren *et al.*, 2004). However this intronless gene has been suggested to be one of numerous SUMO-3 pseudogenes, as the expression of the endogenous protein has yet not been demonstrated. Further studies are required to resolve this issue and establish the functional role of SUMO-4.

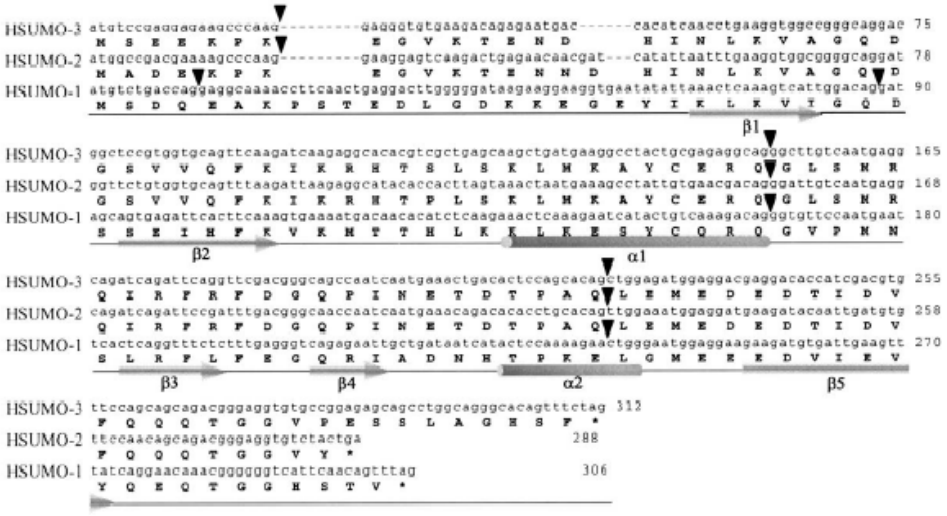


Fig 5: Aligment of nucleotide and encoded amino acid sequences of human SUMO 1/ 2/3. The inverted triangles indicate the position of introns (Su *et al.*, 2002).

3.1 Mechanism of SUMO conjugation

SUMO protein conjugates are formed as the result of serial reactions with no apparent discrimination among all SUMOs (**figure 6**).

SUMOs form from inactive precursors: the maturation occurs by a carboxy-terminal proteolityc cleavage that exposes di-glycine residues.

This reaction is catalyzed by the heterodimer E1-activating enzyme Aos/Uba2.

In detail, the E1 activating enzyme uses ATP to form a bound SUMO-adenylate via the di-glycine of the SUMO protein. Then, a cystein in the active site of Uba2 subunits interacts with the SUMO-adenylate, releasing AMP and forming an high energy thioester bond. The sumo moiety is then transferred in a second step to the E2-conjugating enzyme, Ubc9, via a trans-esterification reaction, forming the Ubc9-SUMO thioester complex through Cys93.

Ubc9, the only SUMO-conjugating enzyme in yeast and invertebrates and most likely in vertebrates as well, is able to recognize the substrate protein

itself. It subsequently catalyzes the formation of isopeptide bond between the Gly of SUMO and the ε-amino group of the target lysine residue. The lysine is usually found within a SUMO modification consensus motif, ψKXE/D (where ψ is a large hydrophobic residue and X any residue), the major element from target proteins that bind directly to Ubc9 (Lin D.*et al.*, 2002; Johnson *et al.*, 2004).

Although all targets interact with Ubc9, an efficient conjugation requires the presence of the additional factor E3-ligase. Even if it doesn't increase the rate of sumo-conjugation *in vitro*, it has been shown to be an important regulator of the modification *in vivo.*

Indeed, it might increase the affinity of Ubc9 for a specific target through the recognition of the surrounding regions near the SUMO consensus motif, contributing mechanistically to conjugation.

Three different E3-SUMO ligases have been discovered: the PIAS (Protein Inhibitor of Activated Stat) family, RanB2/Nup358 and the polycomb group protein PC2.

RanBP2 is required for the sumoylation of RanGAP-1 and it is not known if it may promote the modification of other proteins as well. PC2 belongs to the PcG proteins, a multimeric complex involved in histone methylation activity and in transcriptional repression. Four mammalian genes encoding PIAS have been also described: PIAS1, PIAS3, PIASx and PIASy. PIAS3 have a splice variant called KChaP, and PIAS also produces two isoforms derived from alternative splicing, designated PIASxα and PIASxβ. PIAS1 and PIAS3 are found in all cell types, whereas PIASx and PIASy appear to be expressed primarily in testis. However, all PIAS localize to intranuclear dots, which are, at least in part, PML nuclear bodies.

Further studies are required to characterize the substrate specificity of the PIAS proteins, but the emerging evidence is that they have different

substrates specificities (Miyauchi *et al.*, 2002) and promote attachment of different SUMO isoforms.

The pattern of SUMO conjugation is dynamic and changes during the cell cycle and in response to various stimuli. Since sumoylation is involved in different cellular pathway, its not surprising that it is a reversible process. The deconjugation is catalyzed by SUMO-cleaving enzymes (called isopeptidases or SENPs), that have at least two functions in this process. They remove SUMO from proteins, and providing an additional source of free SUMO to be used to modify other targets. Both these sources of free SUMO are likely to be critical in maintaining normal levels of SUMO conjugation, considering that cellular pools are very low.

Up to now seven different SENPs protein have been identified: they have different N-terminal domains and different cellular localization, suggesting that they may desumoylate different proteins.

SENP6 (SUSP1) is primarily found in the cytoplasm, SENP1 localizes to foci in the nucleus and the nuclear rim, SENP3 (SMT3IP1) and SENP5 are localized in nucleolus. For SENP2 (Axam, SMT3IP2/Axam2, SuPr-1) there are at least three different isoforms derived from alternatively splicing that mainly differ at the level of the N-terminal: this extended region of the SENP2/Axam allow to bind the nucleoplasmic side of the nuclear pore complex, while Axam2/SMT3IP2 localizes to the cytoplasm and SuPr1 lacks these N-terminals localizing to PML nuclear bodies.

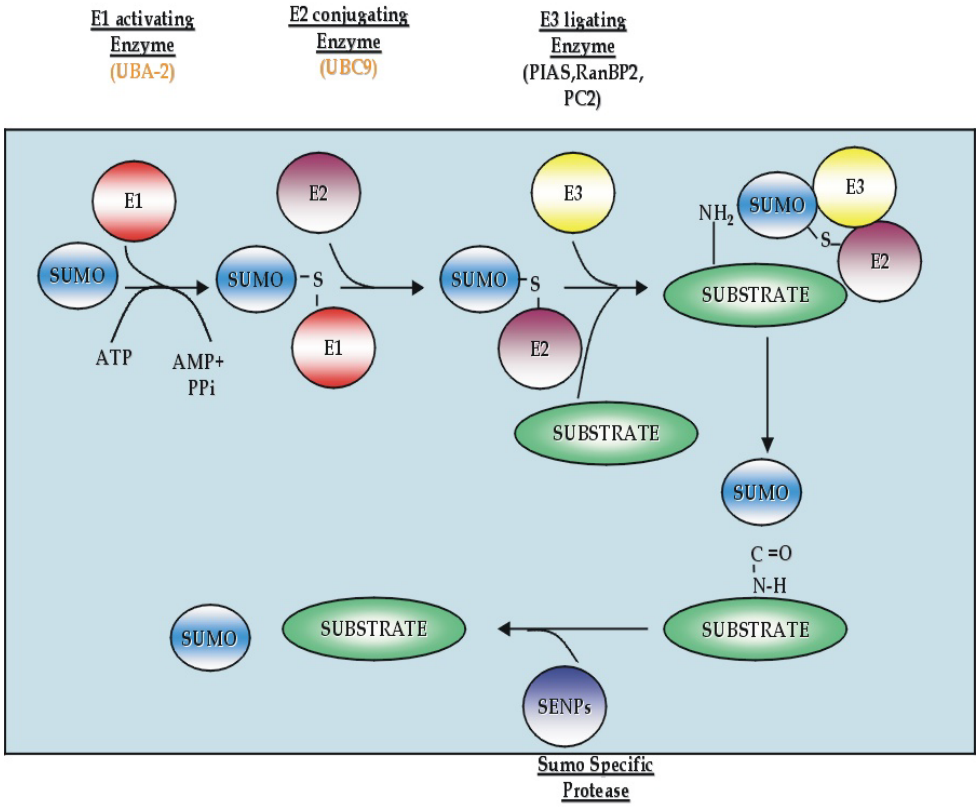


Fig 6: Schematic representation of SUMO conjugation and deconjugation.

3.2 Substrate specificity in SUMOylation

As anticipated, Sumo is attached to most substrates by a lysine residue, belonging to ψ*K*XE/D consensus sequences. However additional determinants are involved in substrate selection. Inside the consensus sequence, the glutamic acid is the most highly conserved position for the lysine. In some cases even the conservative Glu to Asp mutation significantly reduces sumoylation (Sapetschning *et al.*, 2002), although few ψKXD sequences are sumoylated.

Considering that the ψ*K*XE/D sequence is very short, it can be easily found in many proteins, most of which are probably not modified by SUMO. On the other hand, increasing experimental data show that 30% of real sumoylation sites don't follow this consensus and several proteins are also modified at other sites than ψ*K*XE/D.

The replication processivity factor PCNA presents two sumoylation sites, one with the consensus sequence and the other with the T*K*ET sequence (Hoege *et al.*, 2002). In addition it has been reported that TEL, PML, Smad4 and the Epstein Barr virus BZLF1 protein sumoylation site are respectively T*K*ED, A*K*CP, V*K*YC and V*K*FT, while in Axin both lysines in a G*K*VE*K*VD sequence are sumoylated. Moreover, some sumoylated proteins (i.e. Mdm2, Daxx, CREB, and CTB-2) do not contain a ψ*K*XE/D and others are still sumoylated when all consensus sites are mutated (Rangasamy *et al.*, 2000; Jang M.S. *et al.*, 2002; Miyauchi *et al.*, 2002; Comerford *et al.*, 2003;).

It's still not known how these "non consensus sites" are recognized. It has been hypothesized that different E1-activating enzyme can cleave and activate distinct SUMOs pools upon specific cellular pathway.

3.3 Substrates and functions of SUMO protein modification

Since its discovery in 1996, SUMO has received a high degree of attention because of its intriguing and essential functions, and because its substrates include a variety of biomedically important proteins such s p53, c-Jun, PML and Huntingtin.

SUMO modification alters the activity and the ability of the target proteins to interact and plays an important role in diverse processes such as chromosome segregation and cell division, DNA replication and repair, nuclear import, protein targetting to and formation of certain subnuclear structures.

3.3.1 Role of SUMO in cytosol/nucleus trafficking

The mammalian protein RanGAP1 is identified as the first substrate for SUMO-1 modification (Matunis *et al.*, 1996). RanGAP1 is a GTPase-activating protein for the small nuclear Ras-Realated GTPase Ran, whose function is essential for transport of proteins into the nucleus across the nuclear pore complex. Here RanGAP1 is highly concentrated and forms a stable complex with RanBP2, a component of cytoplasmic filaments in the nuclear pore complex. The interaction between RanGAP1 and RanBP2 requires Sumoylation. The replacement of Lys-526 by Arg in RanGAP1 prevents the localization of the protein to the nuclear rim, resulting in its cytoplasmic accumulation (Mahajan *et al.*, 1997). Sumoylation may induce conformational changes in the C-terminal of RanGap1 that exposes or creates a binding surface for RanBP2.

In support of this hypothesis, it has been shown that the nuclear import of a number of proteins (i.e. CtBP, the bovine papillomavirus E1 protein, Elk-

1) depends on their modification with SUMO (Lin *et al.*, 2003; Rangasamy D. *et al.*, 2000, Salinas *et al.*,2004). However, there are also examples of proteins (i.e. TEL and DdMEK-1) whose export from the nucleus dependson their sumoylation (Wood *et al.*, 2003; Sobko *et al.*, 2002).

3.3.2 Role of SUMO in protein targeting to subnuclear structures

PML is a RING-finger protein with tumor suppressor activity. In the majority of the patients with acute promyelocytic leukaemia, the PML gene has undergone a fusion with the retinoic acid receptor α gene (RARα) by a chromosomal translocation, yielding a chimeric PML/RARα protein.

PML is enriched in subnuclear-matrix associated structures, called the PML nuclear bodies, or ND10 (Nuclear Domain 10) or PODs (PML Oncogenic Domains). The SUMOylated PML is preferentially targeted to the nuclear bodies, whereas the unmodified form remains in the nucleoplasmic fraction.

SUMOylation is also required for the nuclear bodies localization of several other proteins, including Sp100, Daxx and CBP. Interestingly, nuclear bodies fail to form when mutant PML that cannot be SUMOylated is expressed into PML -/- cells. This indicates a role of SUMO modification of PML in the formation of PODs and in recruiting other nuclear body proteins. With the exception for Sp3 (Ross *et al.*, 2002), the localization of most proteins to PML bodies (i.e Sp100, Daxx, Topors, Lef1, Hsf2) doesn't depend on their SUMOylation (Jang M.S. *et al.*, 2002; Sternsdorf T. *et al.*, 1999; Weger S .*et al.*,2003; Goodson M.L. *et al.*, 2001).

Interestingly, PML is also modified by SUMO-2/3 (Kamitani *et al.*, 1998).

Both the conjugation and the olygomerization of SUMO-3 to PML are essential for its stable nuclear localization. Since the modification occurs

on Lys160 for both SUMO-1 and SUMO-2/3, it has been proposed that SUMO-1 may act as SUMO-3 polymeric chain terminator (Fu *et al.*, 2005).

SUMO modification of homeodomain-interacting protein kinase 2 (HIPK2) and TEL induces formation of the nuclear speckles, a different subnuclear structure. HIPK2 is a nuclear protein kinases that acts as corepressor of homeodomain transcription factors. TEL is a transcription factor required for hematopoiesis within the bone marrow. The SUMOylated TEL localizes to the nuclear speckles in a cell cycle-specific manner (Chakrabarti *et al.*,2000) and in both cases mutations of lysines residue in the SUMO acceptor site impair the specific nuclear spleckle formation.

3.3.3 Role of SUMO in the regulation of transcriptional factors

As anticipated, the PML sumoylation is followed by the recruitment of specific proteins to the nuclear bodies, which are involved in the modulation of transcriptional activity.

P53 is a target for SUMO-1 modification (Gostissa *et al.*, 1999). Upon exposure of cells to UV, SUMO-1 is covalently attached at Lys 386, that is located in the C-terminal of p53. The modification of this region induces p53 transcriptional and apoptotic activity.

Similar results were also recently obtained with SUMO-2/3, which can modify p53 at the same Lys of SUMO-1, activating its transcriptional activity (Li *et al.*, 2006).

Sumoylation was initially associated to transcriptional inhibition, as for c-Jun and AR. The mutations of the sumoylation sites in these proteins (Lys to Arg substitutions) were shown to enhance their transcriptional activity, suggesting a negative modulation by sumoylation.

An increasing number of transcriptional repressors and activators whose activity is regulated by sumoylation are identified. Considering that the effect of sumoylation differs according to the specific target, it is probably involved in the transcriptional regulation by multiple mechanisms.

3.3.4 SUMO as an inhibitor of ubiquitin-mediated degradation

It has been shown that sumoylation plays an additional role in antagonizing ubiquitin conjugation, as demonstrated for NF-kB, Mdm2, and Huntingtin.

NF-kB, a transcription factor involved in the regulation of the immune function, inflammatory response, cell adhesion and growth control, is kept inactive by its inhibitory protein, IkBs, in unstimulated cells. Upon stimulation by effectors (i.e. proinflammatory cytokines, phorbol esters, oxidants) IkBs is first phosphorylated; then upon poly-ubiquitination it is rapidly targeted for degradation by the 26S proteasome. Interestingly, SUMO-1 can be conjugated at the same ubiquitination sites with a consequent stabilization against degradation (Desterro *et al.*, 1998).

3.3.5 SUMO and the cell cycle

Mutations in the SUMO conjugation system in *S. Cerevisiae* have revealed the importance of this protein modification for normal execution of cell cycle. Mutants deficient in SUMO conjugation accumulate at G2/M in the cell cycle with duplicated DNA content, unseparated sister chromatids and undivided nuclei (Biggins S. *et al.*, 2001; Dieckhoff P., 2004). Many SUMO targets have been discovered, such as Top2, Pds5, PCNA, septins in yeast, as well as TOPII in mammalian cells. These proteins are sumoylated in a cell cycle-controlled manner indicating that SUMO

modification serves to synchronize the function of many of its substrates with the cell cycle. Substrates are modified in different phases of cell cycle: for example, PCNA is modified in S phase (Hoege *et al.*,2002), whereas septins in G2/M (Johnson *et al.*, 1999). How the modification of these substrates is regulated respect to the cell cycle it is still not clear.

3.3.6 SUMO in DNA damage repair

Genetic data obtained with yeast mutants defective in the SUMO cycle implicate SUMO modification in the DNA damage response. In mammals PCNA provides a direct link of SUMO to the DNA repair processes. Upon lethal exposure of methyl methansulfonate PCNA is modified by the attachment of SUMO in the S-phase of the cell cycle. Interestingly, the same Lys that is acceptor of SUMO, may be also Ubiquitinated, but this process doesn't target PCNA for degradation but it activates PCNA in DNA repair processes. Moreover, preventing this modification, as in Lys164Arg, it provokes an increase in the DNA damage sensitivity of cells that are unable to ubiquitinate this site (Hoege *et al.*,2002). Alternatively sumoylation may restrict PCNA to engage in DNA replication, inhibiting DNA repair and inducing mutagenesis (Haracska L *et al.*, 2004) .

3.4 Proteomic approach to SUMO-2/3 substrates

The identification of the entire spectrum of proteins modified by sumoylation is required to better define the range of cellular events regulated by sumoylation. It is not clear, however, whether different SUMO family members have a unique cellular role or they act in a redundant manner. Further investigations are needed especially for SUMO-2 and SUMO-3, since they share 95% of homology and since their substrates are largely unknown.

Table 2 shows a list of SUMO-2/3 protein substrates known so far. They are divided according to the method of identification. As "tagged" we refer to a proteomic approach from stable transfected cell lines with SUMO-2 and/or SUMO-3 cDNAs fused in frame with the tagged sequence used for the purification (HA/6x-His tag). In "ectopic", transient co-transfections of SUMO-2/3 and potential cDNA targets are carried out. As "endogenous", we indicate proteins that are identified as sumoylated within living cells with no experimental manipulations. Very few endogenous SUMO-2/3 targets are known.

SUMO-2 MODIFIED PROTEINS

	PROTEIN NAME	tagged	ectopic	endogenous
DNA REPAIR	DNA-damage-binding protein 2	a		
HELICASE	Probable Helicase with zinc finger motif	a		
	DEAH box polypeptide 30	a		
METABOLISM	α-Macroglobuli receptor associated protein precursor	a		
	Malonyl-CoA-acyl carrier protein transcylase	a		
	2-Oxoglutatarate dehydrogense E component	a		
SIGNALLING	Protein Kinase CLK3	a		
TRANSCRIPTIONAL REGULATOR	Nuclear co-repressor 2	a		
	AT-rich interactive domain-containing protein 1B	a		
	Homeobox protein Meis1	a		
	PML	a	d	
	CCAAT/Enhancer binding proteinβ		f	
	P53		g	
	pRB		g	
	c-Myb		i	
	Zinc finger protein RIf	a		
CONDUCTANCE	Erythrocyte band 7 integral membrane protein	a	b	
GTPase	Ran GTPase-activating protein 1 (GTPase)	a, b	b	
UNKNOWN	Ataxin-2	a, b		
	Ataxin like protein	a, b		
RNA-BINDING PROTEINS	hnRNP M	a, b		
	SART 1	b		
NUCLEIC ACID BINDING	Topisomerase II			h
UNFOLDED PROTEINS	Tau		j	
	α-synuclein		j	

Table 2A: Schematic list of SUMO-2 modified proteins known so far. a) Vertegaal *et al.*, 2006; b) Vertegaal *et al.*, 2004; c) Acosta *et al.*, 2005; d) Kamitani *et al.*, 1998; e) Fu *et al.*, 2005; f) Eaton *et al.*, 2004; g) Li *et al.*, 2006; h) Azuma *et al.*, 2005; i) Šramko *et al.*,2006; j) Dorval *et al.*, 2006.

SUMO-3 MODIFIED PROTEINS

	PROTEIN NAME	tagged	ectopic	endogenous
CYTOSKELETON	Actin α cardiac	c		
	Actinin, α 2 fragment	c		
	Actinin α	c		
TRANSCRIPTIONAL REGULATOR	Bone marrow zinc finger protein	c		
	Homeodomain protein DLX-2	c		
	KIAA1969	c		
	TRAF6-binding zinc finger proetin	c		
	TFIIA-42	c		
	TIF1-β; KAP-1	c		
	TYY1	c		
	Zinc finger proteins(141; 15; 91)	c		
	PML		d	
	CCAAT/Enhancer binding proteinβ		f	
	P53		g	
	pRB		g	
	c-Myb		i	
NUCLEIC ACID BINDING	Topoisomerase II			h
	Fibrillarin	c		
	Histone 2A	c		
GTPase	NFAR	c		
CHAPERONE	HSP 90	c		
RNA-BINDING PROTEINS	Nucleophosmin (NPM)	c		
	PSF-1, isoform 2	c		
METABOLISM	Splicing Factor 3B	c		
SIGNALLING	Poly(ADP-ribose)polymerase 1 (PARP-1)	c		
GTPase	Ran (GTP-binding nuclear protein Ran)	c		
	Ran GAP1			
UNFOLDED PROTEINS	Tau		j	
	α-synuclein		j	

Table 2B: Schematic list of SUMO-3 modified proteins known so far. . a) Vertegaal *et al.*, 2006; b) Vertegaal *et al.*, 2004; c) Acosta *et al.*, 2005; d) Kamitani *et al.*, 1998; e) Fu *et al.*, 2005; f) Eaton *et al.*, 2004; g) Li *et al.*, 2006; h) Azuma *et al.*, 2005; i) Šramko *et al.*,2006; j) Dorval *et al.*, 2006.

3.5 SUMOylation and Oxidative stress

Various stress (heat shock, oxidative and osmotic stress) have been shown to increase global SUMO-conjugation, in particular for SUMO-2 and SUMO-3. It has been also reported that ROS (reactive oxygen species) at low concentration lead to a rapid diseapperance of most SUMO-conjugates. This is due to direct and reversible inhibition of SUMO conjugating enzymes through the formation of di-sulfides bonds involving the catalytic Cysteins of the SUMO E1 subunits Uba 2 and the E2 conjugating enzyme Ubc9. (Bossis and Melchior., 2006). These findings add SUMO proteins and the SUMO conjugating enzyme to the list of specific direct effectors of H_2O_2 and implicated ROS as Key regulators of

Sumoylation-Desumoylation equilibrium. ROS, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH) are constantly produced in the human body by metabolism and are also generated intracellularly after exposure to UV, ionization radiation, chemiotherapeutic agents. The deregulation of cellular redox status is a causal agent in numerous degenerative disorders such as Alzheimer disease (AD), Amyotrophic Lateral Sclerosis (ALS) and Parkinson' s disease (PD).

3.6 SUMOylation and neurodegeneration

Considering that SUMO pathway controls a number of important regulatory molecules, it can promote both the cell survival and dysfunction leading to cell death. While most studies focused on its role in normal cell metabolism, several recent reports have implicated this pathway in neurodegeneration.

Since many SUMO acceptors proteins are nuclear, is not surprising that first evidences linking SUMOylation to neurodegeneration were discovered on disorders characterized by intranuclear protein aggregates. Pountney *et al.* presented evidence that SUMO-modified proteins accumulate in the Intranuclear aggregates of Neuronal Intranuclear Inclusion Disease (NIID), even if the identity of the SUMO-modified protein is still to be determined. Increasing sumoylation has been observed in brain tissue from Spinocerebellar Ataxia type 3 (SCA3) and in Dentatorubral-pallidoluysian atrophy (DRPLA) (Terashima *et al.*, 2002; Ueda *et al.*, 2002). In a neuronal model of DRPLA, co-expression of wild type SUMO-1 with PolyQ-Atrophin-1 significantly accelerates the formation of nuclear aggregates and promotes apoptosis. On the other hand, expression of the conjugation-deficient SUMO promoted cell

survival compared to the mutant PolyQ protein alone or in the presence of wild type SUMO-1.

One proposed mechanism involves the sequestration of SUMO-modified proteins in the nucleus or their accumulation via non-covalent binding motifs. The recruitment of SUMO monomers or sumoylated substrates into inclusions could further exacerbate the sequestration of cellular components critical for neuronal survival.

An interesting hypothesis suggests that competition between SUMO and Ubiquitination may avoid the degradation of mutant atrophin-1. This theory was first demonstrated for a pathogenic fragment of Huntingtin (Htt) (Steffan *et al*., 2004). Expression of a permanent, non hydrolyzable Htt-SUMO conjugate in a neuronal cell line resulted in a more stable protein and potentially increases the level of toxic oligomers. The Lys modified by SUMO-1 is also the acceptor site for Ubiquitin, leading to a competition between the two modifiers. In the same model, Htt displayed an increased transcriptional repression. Therefore, the sumoylation occuring in the N-terminal domain of the protein may mask the cytoplasmic retention signal and promote nuclear localization.

The cellular models for HD and DRPLA have suggested an exacerbating role of sumoylation in neurodegeneration. The opposite has been proposed for another polyglutamine disease, the Spinal Bulbar Muscular Atrophy (SBMA). In *Drosophila,* expression of the catalytic-deficient C175S mutant form of the SUMO-activating enzyme (E1) subunit Uba2 greatly enhances degeneration. Similar observation is obtained for the Machado-Joseph-Disease (MJD), suggesting that down-regulation of SUMO modification is detrimental to cells.

Synucleinopathies are a group of neurodegenerative diseases characterized by accumulation of filamentous aggregates of α-synuclein in cytoplasmic inclusions. This group includes PD, Dementia with Lewy Bodies (DLB) and Multiple System Atrophy (MSA). SUMO-1

immunoreactivity has been reported in MSA oligodendroglial cytoplasmic inclusions as well as punctuated staining at the nuclear membrane level. Similar to the poly-Q diseases the colocalization raised the question of the identity of the targets within aggregates. It has been recently reported that α-synuclein is substrate for SUMO-1, and less for SUMO-2/3, providing a direct link for the pathogenic deposit. (Dorval *et al.*, 2006)

Since, several genes involved in the pathogenesis of PD are closely linked to the Ubiquitin proteasome system, recent data on the interplay between SUMOylation and Ubiquitination are of major interests.

Parkin is an Ubiquitin E3 ligase that is involved in the turnover of the SUMO E3 ligase RanBP2, catalyzing its ubiquitination and promoting proteasome degradation. More recently a functional non-covalent interaction between Parkin and SUMO-1 has been reported, which results into parkin nuclear localization and auto-ubiquitination.

In the case of Alzheimer's Disease (AD), SUMO-3 overexpression reduced Aβ production whereas expression of SUMO mutants that cannot be conjugated or form polymeric chains increases the production of amyloidogenic peptides (Li *et al.*, 2003). In contrast, a more recent study investigating the effect of overexpression of the three major SUMO isoforms on the APP processing pathway showed that SUMO-3 increases the generation of Aβ (Dorval *et al.*, 2007).

Intriguingly, the misregulation of SUMO conjugation may result in loss/gain of function of proteins that contribute to a diminished survival of neurons.

In PD, α-synuclein and DJ-1 are substrates for SUMOylation (Dorval *et al.*, 2006; Shinbo *et al.*, 2005b). DJ-1 is modified at Lysine 130 and this modification is stimulated by both PIASxα and PIASy, confirming that they may act as E3 ligase for DJ-1. Previously, it was reported that DJ-1 acts as a positive regulator for the Androgen Receptor (AR) by sequestering PIASxα (Takahashi *et al.*, 2001), which functions as negative

regulators for AR by preventing AR DNA-binding activity. While the role of Sumoylation has been investigated in the DJ-1 transforming activity, Shinbo *et al.* have found that the PD-related DJ-1 Leu166Pro can be improperly sumoylated as compared to its wild type counterpart. This was evident by the presence of higher molecular weight DJ-1/SUMO positive bands and suggested that the mutant protein may be polysumoylated on selected lysine or sumoylated on multiple lysines.

The SUMO conjugation on DJ-1-Leu166Pro may promote aggregation leading to high molecular weight species, insolubility, change in subcellular localization and instability (**figure 7**).

The DJ-1-Leu166Pro mutant and to less extent, the DJ-1 Lys30Arg are more sensitive to oxidative stress induced by UV radiation.

This implicates a role of DJ-1-SUMOylation in the response to cellular insults but the relationship between SUMO, DJ-1 and oxidative stress appears increasingly complex.

In conclusion, SUMO modification may be involved in neurodegeneration by different mechanisms: the modification of a substrate can affect its subcellular localization and sumoylated substrates can sequestrate critical proteins in virtue of the Sumo Binding Motif (SBM). SUMO can then contribute to the formation of inclusions in the case of a decrease solubility of the protein, as well as, it may antagonize the proteasomal degradation and protein turnover.

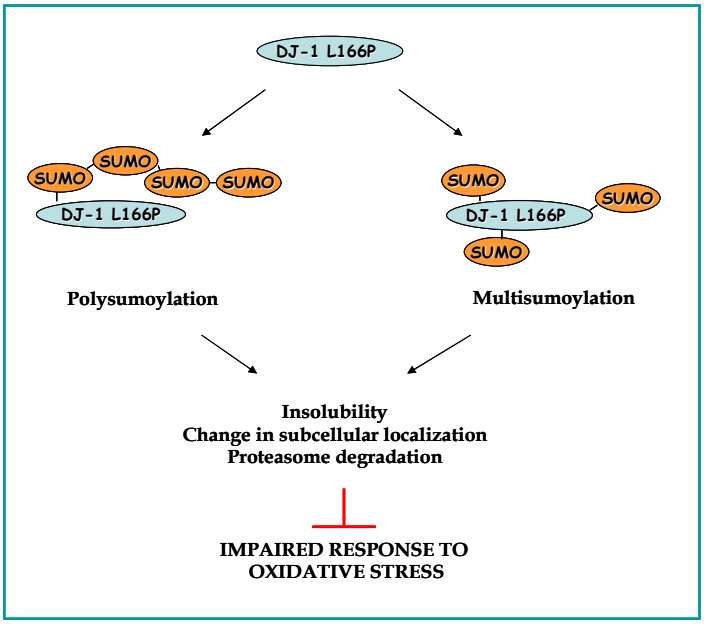


Figure 7: SUMO effects on DJ-1 and its relationship to oxidative stress: the modification could interfere with DJ-1 function and may lead to cellular sensitivity to oxidative stress (Dorval *et al.*, 2007).

MATERIALS AND METHODS

1 CELL LINES AND CULTURE CONDITIONS

HEK293T (Human Embryonic Kidney) and COS-7 (African Green Monkey SV40-transformed kidney fibroblast cell line) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% *foetal* bovine serum (FBS), 100 μg/ml Streptomycin and 100 U/mL Penicillin at 37°C in a humidified CO_2 incubator. SH-SY5Y (Human neuroblastoma cell line), were cultured in F-12 Nutrient Mixture (Ham) and EMEM (modified Eagle's medium) in a volume ratio 1:1, 15% FBS, 1% NEAA (Non Essential Aminoacid), 2 mM Glutamine and 100 μg/ml Streptomycin and 100 U/mL Penicillin at 37°C in a humidified CO_2 incubator.

2 PLASMIDS

2.1 Cloning wild type SUMO-2 and wild type SUMO-3 into pcDNA3-vector

cDNAs for SUMO-2 and SUMO-3 coding sequences (cds), gift of Prof G. Del Sal (LNCIB, Trieste) were amplified by Polymeric Chain Reaction (PCR) using specific primers with *Bam*HI and *Eco*RI restriction sites on the 5′ and 3′ primers respectively.

Primers.

Wild Type SUMO-2.

Forward: 5′ -GGG GAT CCA TGG CCG ACG AAA AGC CCA AG-3′

Reverse: 5′-AAG AAT TCT CAG TAG ACA CCT CCC GTC TG-3′

Wild Type SUMO-3

Forward: 5′ GGG GAT CCA TGT CCG AGG AGA AGC CCA AG-3′

Reverses 5′- AAG AAT TCC TAG AAA CTC TGC CCT GCC AG-3′

After PCR amplification and removal of primers and enzyme by the PCR purification kit (Qiagen), each PCR product was digested by *Bam*HI and *Eco*RI for 2 hours at 37°C. After another step of purification of the digested fragments by PCR purification kit, each of them has been cloned directionally *Bam*HI and *Eco*RI in the vector pcDNA3-HA, digested with the same restriction enzymes.

2.2 PCR Site-Directed Mutagenesis of SUMO-2 and SUMO-3: creating the unconjugable forms

The wild type SUMO-2 and wild type SUMO-3 cds were further used to obtain unconjugable SUMO-2 and SUMO-3 proteins with the strategy of PCR Site-Directed Mutagenesis. In particular the di-Gly at the C-terminal motif was converted into a di-Ala motif.

One PCR reaction was required for SUMO-2.

Primers

Unconjugable SUMO-2 (SUMO-2GG92/93AA)

Forward: 5′ -GGG GAT CCA TGG CCG ACG AAA AGC CCA AG-3′

Reverse: 5′-AAG AAT TCT CAG TAG ACA GCT GCC GTC TG-3′

For the unconjugable form of SUMO-3, two PCR reactions with were required to amplify two different overlapping fragments. They were purified by Gel Extraction kit (Qiagen) and subsequently annealed with a third PCR. The first PCR amplifies SUMO-3 from nucleotide 1 to nucleotide 294 with the forward primer previously described inserting the *Bam*HI restriction site at 5′ end. The reverse primer (GG90/91AA) was used to introduce the mutations. On the template obtained, purified by Gel Extraction, a second PCR was performed with the same forward primer and a second reverse primer (*Eco*RI) to introduce the *Eco*RI

restriction site. The PCR product fragment was further subcloned into the pcDNA3-HA vector.

Primers

Unconjugable SUMO-3 (SUMO-3GG91/921AA)

Forward: 5′ GGG GAT CCA TGT CCG AGG AGA AGC CCA AG-3′

Reverse GG90-91AA: 5′-CAG GCT GCT CTC CGG CAC AGC TGC CGT CTG-3′

Reverse *Ecor*RI: 5′-AAG AAT TCC TAG AAA CTC TGC CCT GCC AGG CTG CTC TCC GGC AC-3′

2.3 PCR Site-Directed Mutagenesis of DJ-1: strategy to obtaine different DJ-1 Lysine mutants replaced by Arginine

cDNAs encoding the DJ-1 mutants K4R, K12R, K32R, K41R, K62-63R, K89-93R, K99R, K122R, K130R, K132R, K148R, K175R, K182R, K187R were generated by PCR Site Directed mutagenesis and the respective cDNAs were cloned by *Eco*RI and *Xba*I restriction into a 2X-Flag-pCDNA3 vectors. The following sets of overlapping primers were used to introduce the mutation.

Primers

DJ-1 K4R:

Forward: 5′-ATA TAG AAT TCG CTT CCA GAA GAG CTC TGG-3′

DJ-1 K12R:

Forward: 5′-TCC TGG CTA GAG GAG CAG A-3′

Reverse: 5′-TCT GCT CCT CTA GCC AGG A-3′

DJ-1 K32R:

Forward: 5′-GCT GGG ATT AGG GTC ACC GTT-3′

Reverse: 5′-ACC GGT GAC CCT AAT CCC AGC-3′

DJ-1 K41R

Forward: 5′-TGC AGG CCT GGC TGG AAG AGA C -3′

Reverse: 5′-ACT GTA CTG GGT CTC TTC CAG C-3′

DJ-1 K62/63R

Forward: 5′-CCT TGA AGA TGC AAG AAG AGA G-3′

Reverse: 5′-ATG GTC CCT CTC TTC TTG CAT C-3′

DJ-1 K89/93R

Forward: 5′-GCT GTG AGG GAG ATA CTG AGG GAG CAG-3′

Reverse: 5′-CTG CTC CCT CAG TAT CTC CCT CAC AGC-3′

DJ-1 K99R

Forward: 5′-GAA AAC CGG AGG GGC CTG ATA-3′

Reverse: 5′-TAT CAG GCC CCT CCG GTT TTC-3′

DJ-K122R

Forward: 5′-TGG AAG TAG AGT TAC AAC ACA C-3′

Reverse: 5′-GTG TGT TGT AAC TCT ACT TCC A-3′

DJ-1 K130R

Forward: 5′-CAC ACC CTC TTG CTA GAG ACA AAA TGA TG-3′

Reverse: 5′-CAT CAT TTT GTC TCT AGC AAG AGG GTG TG-3′)

DJ-1 K132R

Forward: 5′-CTT GCT AAA GAC AGA ATG ATG AAT-3′

Reverse: 5′-ATT CAT CAT TCT GTC TTT AGC AAG-3′

DJ-1 K148R

Forward: 5′-GAA TCG TGT GGA AAG AGA CGG C-3′

Reverse: 5′-GAA TCA GGC GTC CTC TTT CCA C-3′

DJ-1 K175R

Forward: 5′- TGA ATG GCA GGG AGG TGG C-3′

Reverse: 5′-GCC ACC TCC CTG CCA TTC A-3′

DJ-1 K182R

Reverse: 5′-GCG CGT CGA CGT CTT TAA GAA CAA GTG GAG CCC TCA-3′

DJ-1 K187R

Reverse: 5′ GCG CGC TCT AGA CTA GTC TCT AAG AAC AAG TGG-3′

To amplify the whole DJ-1 cDNA in which Lys were replaced by Arg, two external sets of primers were used. The forward, (5′-ATA TAG AAT TCG CTT CCA AAA GAC CTC TGG-3′) introduced the *Eco*RI restriction site, and the reverse one the *Xba*I site (5′-GCG CGC TCT AGA CTA GTC TTT AAG AAC AAG TGG-3′). Overlapping PCR products were purified by Gel Extraction, subjected to annealing, and finally to a third PCR using external primers.

3 TRANSFECTIONS

Transient transfections were performed by standard calcium phosphate precipitation method. A transfection mix was prepared by 0.25 mM $CaCl_2$, plasmid DNA, HEPES Buffered Saline Solution (2X Stock Solution: 140 mM NaCl, 1, 5 mM $Na_2HPO_4*2H_2O$, 50 mM HEPES pH7.1). The $CaCl_2$/DNA solution was added dropwise to the 2X HEPES Buffered Saline Solution while gently vortexing. The mixture was incubated at room temperature (R.T.) for 45 minutes, and then it was gently added to cells. HEK293T, seeded 24 hrs before transfection at 50-60% confluency, were further incubated with precipitates over night (O.N.). Precipitates were removed washing with Phosphate Buffered Saline (PBS) and transfected cells were further incubated for 24 hrs with fresh medium. For the sumoylation assay, 300.000 HEK293T were seeded the day before on 6-multiwell plate and transfected with 3 μg of total DNA: 1 μg 2X-Flag-DJ1, 1μg HA-SUMO-2/3 in conjugable or unconjugable forms and 1 μg HA-Ubc9 (gift of Dr. L. Collavin) in different combinations. Similar experiments were initially perfomed with GFP-SUMO-1 and the truncated unconjugable form GFP-SUMO-1 Δ6 provided by Dr. Collavin L. and Prof.

Del Sal G. All the 2X-Flag-DJ-1 lysines mutants were co-transfected with HA-Ubc9 and HA-SUMO-3 conjugable/unconjugable in ratio 1:1:1.
For the immunoprecipitation experiments 2.000.000 HEK293T were seeded on 10-cm^2 dishes and 21 μg of total DNA were transfected (7 μg of DNA for each plasmid). The same amount of cells was transfected with 7-10 μg of HA-Ubc9 to demonstrate its interaction with endogenous DJ-1. After 48 hrs the expression of exogenous proteins was analyzed by Western Blot Analysis or Immunoprecipitation experiments.

4 TREATMENTS

Titration and time course experiments were done on 90 % confluent SH-SY5Y cells using different drugs that rapresent an *in vitro* neurochemical model of PD. 600.000 cells were seeded 24 hours before.
Treatments were performed as follows

- H_2O_2 : 0.01 mM, 0.1 mM, 1 mM, 5 mM, 10 mM, 100 mM for 30 minutes and 1 mM, 10 mM for 1 hour;
- MG-132: 0.25 μM, 0.5 μM and 5 μM MG-132 for 1 hour and 24 hours using as control the respective amount of the vehicle, DMSO;
- Dopamine (DA) and 6-OHDA (6-OHDA): 10 μM, 100 μM, 250 μM, 500 μM for 24 hours and 500 μM and 1mM for 1 hour.
- 1-methyl-4-phenylpyridinium (MPP^+): 50 mM for 1 hour.

5 PROTEIN ANALYSIS

5.1 Western Blotting Analysis

For the Sumoylation analysis, transfected HEK293T and SH-SY5Y in 6-multiwell were washed carefully with ice-cold PBS supplemented with 10 mM NEM (N-Ethylmaleimide) and 10 mM IAA (Iodoacetamide) and lysed directly into 200 μl of hot 2X Laemmly Buffer (50 mM Tris-HCl pH 6.8, 100 β-mercaptoethanol, 2% SDS, 1% Glycerol, 0.1% Bromophenol blue). Each sample was boiled for five minutes and sonicated. 30 μl were loaded in 10-12% SDS-polyacrilamide gel (SDS-PAGE). The resolved proteins were blotted onto nitrocellulose transfer membrane (Schleicher & Schuell) at 100 V for 1 hour. Membranes were blocked in TBS 0.1% Tween-20 (TBST) with 5% dried milk for 1 hour at room temperature (R.T.) and then incubated O.N. with the primary antibody at the working diluition in TBST-milk.
The following primary antibodies were used:

- Home-made rabbit polyclonal anti-DJ-1 1:1000
- Mouse monoclonal anti-Flag (Sigma) 1:4000
- Mouse monoclonal anti-Hemoagglutinin (HA, kindly provided by Dr L. Collavin) 1:1000
- Mouse monoclonal anti-Actin (Sigma) 1:5000
- Rabbit Monoclonal anti-SUMO-2/3 (Zymed) 1:250

The following secondary antibodies were used:

- HRP conjugated goat anti-mouse antibody (DAKO) 1:1000
- HRP conjugated goat anti-rabbit antibody (DAKO) 1:2000

After washes with TBST (five minutes each), protein bands were detected by HRP/hydrogene peroxide catalyzed oxidation of luminol by an enhanced chemiluminescence system (ECL Westren blotting detection reagents, Amersham Biosciences) and autoradiography.

5.2 Immunoprecipitation

To confirm DJ-1 SUMO-3ylation through immunoprecipitation, HEK293T transfection was performed as described. After 36-48 hours, cells were washed carefully with ice-cold PBS/NEM/IAA and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris HCL pH 7.5, 0.5 % NP-40, 0.5% Deoxycholic Acid, 0.1% SDS) supplemented with Protease Inhibitors (Roche), NEM (Sigma) and IAA (Sigma). After centrifugation, the protein concentration of the supernatant was determined with Bradford method using a calibration curve built with standard amounts of Bovine Serum Albumine (BSA). Around 3-4 mg of proteins were subjected for each immunoprecipitation and each control. Immunoprecipitation of exogenous 2X-Flag-DJ-1 was carried out using 40 µl of Flag Resin (Sigma) for 2 hours at 4°C in rocking. For experiments in HEK293T, in *Substantia Nigra* and in *Striatum*, immunoprecipitations were carried out using the RIPA lysis buffer. 4 mg of protein were incubated with 2 µg of the immunoprecipitating antibodies (Rabbit IgG as control and anti DJ-1 home made or anti SUMO-2/3). The incubation was carried out for 2 hours followed by an incubation at 4°C with 40 µl of Sepharose A (Amersham) for 1 hour. Samples were washed four times with PBS or 0.5% NP-40/PBS, the resin was pelleted and dried out with a syringe. Samples were further boiled with 20 µl of 2X Laemmli Buffer and subjected to SDS/PAGE on 10 % PAA gels.

5.3 Isoelectrofocusing

HEK293T cells were lysed in 7 M urea, 2 M Thiourea, 4% w/v CHAPS, 1.2% v/v DeStreak Reagent, and 1% v/v IPG buffer 3–10 (Amersham Biosciences). IEF was performed on linear immobilized pH gradients (7

cm long/pH 4-7) according to the manufacturer's instructions (Amersham Biosciences), loading 1 mg of total proteins, and using the paper bridge method and the IPGphor IEF System (Amersham Biosciences). At the end of IEF, the IPG strips were frozen (-80°C). Before running the second dimension, strips were equilibrated 2 x 15 min in 6 M urea, 50 mM Tris/HCl pH 8.8, 30% v/v glycerol, 2% w/v SDS and 1% (w/v) DTT (first equlilibration) or 2.5% (w/v) IAA (second equilibration). Second dimension electrophoresis was carried out on a 15% SDS polyacrylamide gel under constant current (20 mA for 60 min then 60 mA). Proteins were then transferred to nitrocellulose membrane and revealed using the enhanced chemiluminescence technique.

6 IMMUNOFLUORESCENCE STAINING

6.1 Immunocytochemistry

Cells grown onto 13-mm poly-D-lysine-coated coverslips, once removed the medium and washed in PBS, were fixed in 4% Paraformaldehyde (PFA) at Room Temperature (R.T.) for 10 minutes. After fixation, cells were rinsed twice with PBS and first quenched with Glycine 0.1M/PBS for 5'. Cells were permealized with 0.1% Triton X-100/PBS for 10 minutes and the nonspecific immunoreactivity were blocked for 30 minutes using 0.2% BSA (bovine serum albumine)-10% NGS (normal goat serum) at R.T. Both primary and secondary antibodies were incubated in 0.2% BSA/PBS.

The following antibodies were used:

- Mouse monoclonal anti-Flag (Sigma) 1: 2000, 1 hour
- Mouse monoclonal anti-HA 1:1000, 1 hr
- Home-made rabbit anti DJ-1 1:500, 1 hr
- Rabbit anti-SUMO-2/3 (Zymed) 1:50, 3 hrs.

After washes, cells were incubated with secondary antibodies.

The following antibodies were used for 1 hour:

- AlexaFluor 488-conjugated goat anti-rabbit (Invitogen) 1:500
- AlexaFluor594-conjugated goat anti-mouse (Invitrogen) 1:500

DNA was stained with Hoechst33258 DAPI (Sigma) added during the incubation of the secondary antibody (1:2000). After washing in PBS and water, coverslips was mounted on slides using Vectashield mounting medium (Vector).

6.2 Immunohistochemistry

12 week-old mice were anesthetized and intracardially perfused with 4% PFA in PBS. Brains were dissected and postfixed for 1 hr at R.T. in 4% PFA. Serial coronal sections (40 μm) were cut at the vibratome, collected and preserved at 4°C in 0,1% Sodium Azide.

The slices were incubated for 1 hour in blocking solution (NGS 10%, BSA 0,2%; Fish Gelatin 0, 1% in PBS) and then with the primary antibody, at the appropriate concentration, in a solution for antibody incubation.(BSA 0, 2%, Fish Gelatin 0,1%, 0,1% Triton-X-100 in PBS) for 16-18 hours at R.T.

The following primary antibodies were used:

- Mouse monoclonal anti-Tyrosine Hydroxylase (TH Diarosin) 1:2000
- Mouse monoclonal anti-NeuN (Chemicon) 1:300
- Mouse monoclonal anti-GFAP (Sigma) 1:1000
- Rabbit polyclonal anti-SUMO-2/3 (Zymed) 1:100

After three washes with 0,2%BSA/PBS of 10 minutes each, the slices were incubated with the secondary antibody for 1hour at R.T. in the solution used for the primary antibody incubation.

The following secondary antibody were used for 1 hour:

- AlexaFluor 488-conjugated goat anti-rabbit (Invitogen) 1:500
- AlexaFluor594-conjugated goat anti-mouse (Invitrogen) 1:500

Nuclei were labeled with Hoechst33258, DAPI (Sigma) added 1:2000 during the incubation of the secondary antibody. Slices were washed three times with PBS and mounted in vectashield mounting medium (Vector).
Slides were examined using a Leica Confocal Microscope using independent excitation for all channels. Omission of primary antibody was used to evaluate non-specific fluorescence and in all cases gave no signal.

7 IMMUNOAFFINITY PURIFICATION OF A POLYCLONAL ANTI-DJ-1 ANTIBODY RAISED IN RABBIT

7.1 Purification of GST- DJ-1 fusion protein

The full length cDNA of DJ-1 human protein was expressed in fusion with a glutathione-S-Transferase (GST) domain. E. Coli BL21 competent cells were transformed with pGEX-4T-1 vector containing the GST fusion protein. Before performing large scale purifications, protein expression was previously checked in the laboratory under different induction conditions to establish the optimal one. A single colony of transformed cells was inoculated O.N. at 37°C in 20 mL of LB broth with Ampicilline (Amp). Then the culture was diluted into fresh LB in order to obtain a staring A600 of 0.2 and then it was grown to a final A600 of 0.8. The fusion protein was induced by adding IPTG to a final concentration of 1mM and growing the culture for an additional 2-3 hours at 30°C. Then bacteria were pelleted at 6000 rpm at 4 °C for 10 min and pellets were stored at

80°C. The pellet was lysed in PBS supplemented with Protease Inhibitor (Roche) and sonicated on ice for 30''and incubated on ice for 5 minutes.
To separate the GST-fusion protein from all other proteins, the supernatant was incubated with glutathione resin (Glutathione Sepharose 4B, Amersham Biosciences, 1mL for 100 mL of culture) for 2-4 hours at 4 °C. The GST fusion protein unbound to the Glutathione Sepharose and all other proteins were washed away with three washes in PBS. The GST protein was then eluited from the Glutathione Sepharose with 15 mM of reduced GST (GSH) in 50 mM Tris pH 8. The eluted GST fusion protein was quantified in SDS PAGE with standard amounts of BSA labeled with Coomassie staining.

7.2 Immunization of rabbit with purified GST-DJ- 1

The GST-DJ-1 purified protein was used for male rabbit immunization They were performed by Dr. Marco Stebel from the animal facility of the University of Trieste (Italy) with standard procedure. The protocol included 10 boosts with 100-150 μg of GST-DJ-1 fusion protein. Blood samples were collected either before immunization and after each single booster (1st-10 st bleads).

7.3 Purification of the anti-DJ-1 antibody

The GST-DJ-1 purified protein was used for coupling reaction and affinity purification of the antibody.
GST and GST-DJ-1 were immobilized on CNBR-Activated Sepharose 4B. (Amersham Biosciences). 1 g of dried resin was weighed (1g freeze-dried powder gives about 3.5 mL final volume of medium) and suspended in 1mL HCl. Several washes with 1mM HCl were made with a total volume of 200 mL. GST and GST-DJ-1 were dialyzed in Coupling Buffer (0.1 M

$NaHCO_3$ pH 8.3, 0.5 M NaCl). About 2.5 mL of coupling solution and 5 mg of proteins either GST and GST-DJ-1 per mL of wet resin were used according to the manufacturer's protocol. Coupling solution was incubated with the resin overnight rocking at 4° C. The excess of ligand was washed away with 5 medium volume of copling buffer. Unbound active groups were blocked with 0.1 M Tris HCl buffer pH 8 rocking for 2 hours at R.T. The resin was washed with 3 cycles of alternating pH with 5.0 medium volumes of each buffer. Each cycle consisted of a wash of 0.1 M acetate pH 4 buffer 0.5 M NaCl followed by a wash of 0.1 M Tris HCl pH 8.0 buffer 0.5 M NaCl. The GST and GST-DJ-1 were transferred and packed onto two different columns. They were washed with buffer used to elute the antibodies in order to remove any antigen. Columns were washed with 10 volumes of 10 mM Tris HCl pH 7.5, then with 10 volumes of 10 mM Tris HCl ph 8.8 and several washes with 10 mM Tris HCl pH 7.5 until reached pH 7.5. 5mL of rabbit serum were passed through the GST column in order to bind and to remove the GST-Antibodies. The flow through was collected and passed to the GST-DJ-1 column. Both the columns were washed with 20 volumes of 10 mM Tris-HCl pH 7.5 containing 0.5 M NaCl. Anti-GST and anti-GST-DJ-1 were eluted by 100 mM Glycine pH 2.5. Eluates were collected in tubes containing 1M Tris PH 8.0 for equilibrating the antibody solution to pH 7.5. The pH of both columns was brought to pH 8.8 by washing with 10 mM Tris pH 8.8. The concentration of the antibodies were determined by measuring absorbance at 280 nm and using the following formula: Concentration (mg/mL)=A 280/1.028. Columns were washed extensively with 10 mM Tris pH 7.5 until the pH is reached and stored at 4°C with 0.02% sodium azide. The specificity of the affinity-purified anti-DJ-1 antibody was tested using the competition assay.

7.4 Competition Assay

1, 3, 10 μg of purified GST-DJ-1 were incubated with the anti-DJ-1 antibody for 2 hrs at 37°C. The mixture was further used for western blot analysis or immunofluorescence.

AIM OF THE PROJECT

Homozygous deletions and missense mutations in the DJ-1 gene result into a EOARPD indicating that DJ-1 is involved in neuroprotection. Investigations of the biochemical and biological function of genes that cause inherited forms of Parkinson's Disease are essential to elucidate the events that lead to dopaminergic neurodegeneration.

To dissect the molecular events related to DJ-1 function, the laboratory of Prof. Stefano Gustincich has isolated a group of DJ-1 interactors from a human *foetal* brain cDNA library. Among others, Small Ubiquitin-like MOdifier 1 (SUMO-1), SUMO-activating enzyme (Uba2), and SUMO-conjugating enzyme (Ubc9) were identified as DJ-1 interactors. DJ-1 has been recently reported to be SUMOylated by SUMO-1 at lysine-130 (Shinbo *et al.*, 2006) and to be involved in global protein SUMOylation (Zhong *et al.*, 2006).

Here I study the modification of DJ-1 by SUMO-2/3 *in vivo* and *in vitro*.

RESULTS

1 PRODUCTION AND PURIFICATION OF ANTI-DJ-1 ANTIBODY

1.1 Purification of GST-DJ-1 fusion protein and rabbit immunization

The full length DJ-1 was previously subcloned in our laboratory into a pGEX4-T1 vector and expressed in bacteria in fusion with GST. A large scale GST-DJ-1 was produced from 500 mL of induced BL21.
The GST-DJ-1 purified protein was further used for the immunization of two rabbits. Immunizations and blood sampling were performed by Marco Stebel from the animal facility of the University of Trieste (Italy). Ten different boosts were collected.

1.2 Purification of anti-DJ-1 antibody

Two affinity columns were prepared, one coupled to the GST alone and the other to GST-DJ-1. GST and GST-DJ-1 proteins were previously purified from induced BL21 bacteria using the Glutation Sepharose Resin and dialyzed in Coupling buffer, as described in Material and Methods.
The yield of purified GST and GST-DJ-1 protein was estimated in **figure 8.**
The 7^{th} bleeding sera was first diluited 1:2 in equilibration buffer, 100 mM Tris HCl pH 7.5, then applied into the GST column to bind and eliminate the anti-GST antibody. The flow through was then loaded into the GST-DJ-1 column in order to purify the anti-DJ-1 antibody. Specific antibodies were eluted from the column in acid conditions (Glycine 100 mM pH 2.5). The pH was brought to 8 by adding Tris HCl pH 8 and checking the pH with strips.

The concentration of the eluted antibody was determined through spectrophotometric measures and the presence of contaminants or degradation were checked by SDS-PAGE and further Blue-Coomassie staining (data not shown).

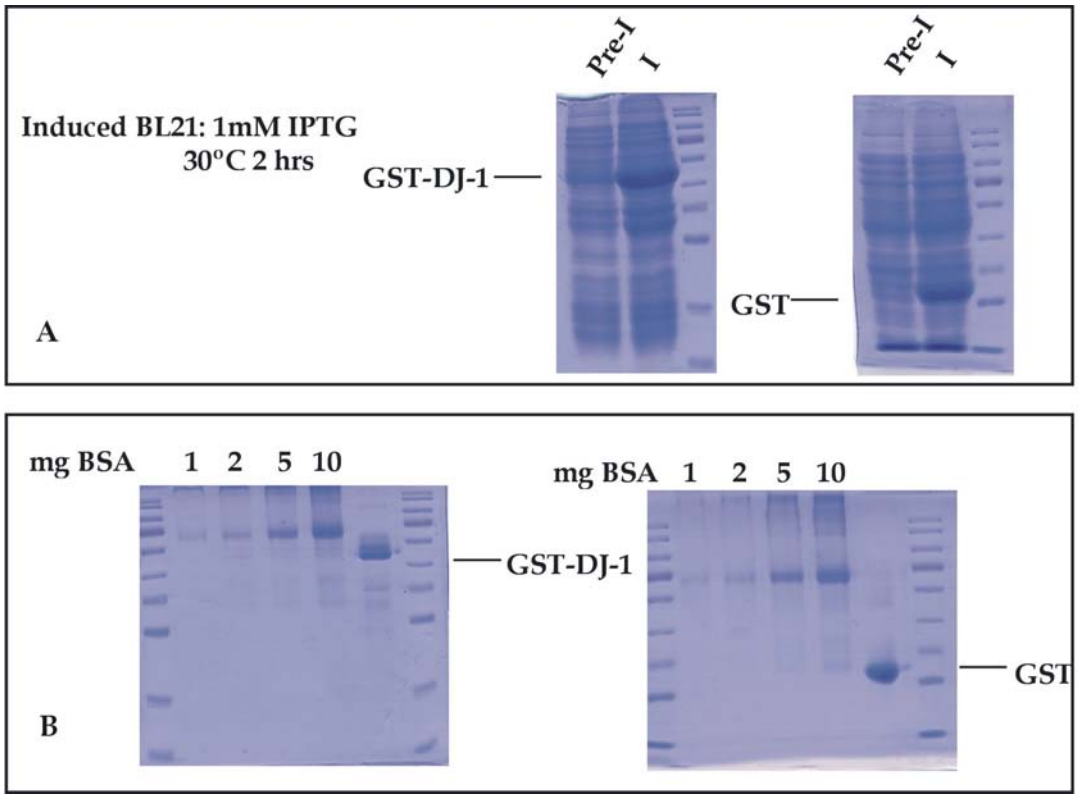


Fig 8: A) Induction and purification of GST and the GST fusion protein DJ-1 and B) quantization towards standard amounts of BSA.

1.3 DJ-1 is expressed in SH-SY5Y cells

The purified serum was tested at different diluitions in Western Blotting experiments on SH-SY5Y cell lysates. Twenty μg of total lysate were loaded for each lane on SDS-PAGE and after blotting, membranes were incubated with 1:100, 1:200, 1:500, 1:1000 and 1:2000 diluitions of the purified anti DJ-1 antibody and with 1:100 of unpurified serum. A band corresponding to DJ-1 was detected at the expected molecular weight of 20

kDa. The anti-DJ-1 was subsequently used at 1: 1000 diluition. Results are shown in **figure 9A.**

Competition experiments were also performed to prove the specificity of the detected band. Briefly, the anti-DJ-1 antibody was incubated with 0, 1 and 3 μg of antigen (GST-DJ-1) for 2 hours at 37°C before the Western blot detection. The 24 kDa band detection was abolished completely by using 1μg of antigen (GST-DJ-1) as indicated in **figure 9B.**

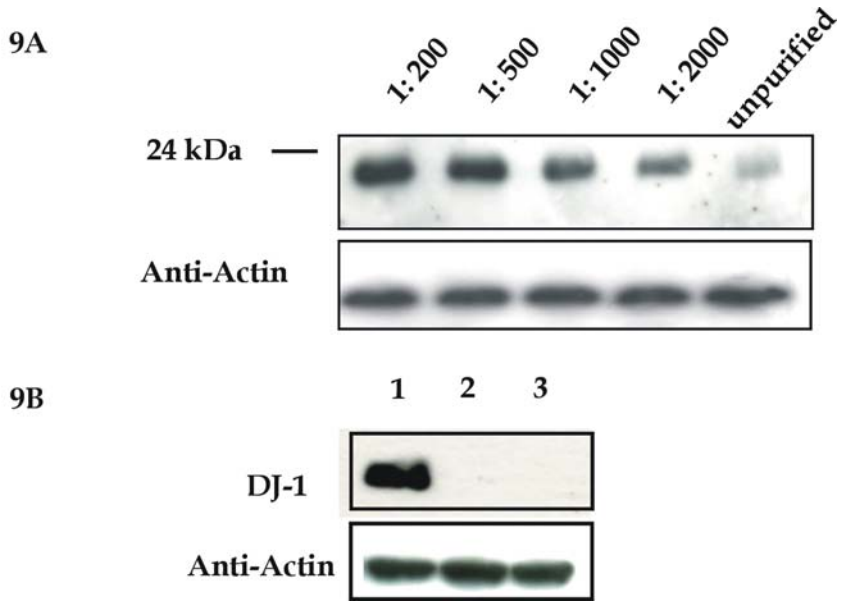


Fig 9: DJ-1 is expressed in SH-SY5Y cells. A) Different diluitions of the anti-DJ-1 antibody purified from 7th serum on 20 μg of SH-SY5Y cell extracts. B) Competition experiments with 0, 1 and 3 μg of GST-DJ-1 respectively to lane 1, 2, 3.

Similar experiments were performed in immunofluorescence. The purified antibody was incubated at the working solution 1:500 with 1 μg of GST-DJ-1 and the cellular localization was further analized at the confocal microscope. The detection of the DJ-1 cellular localization is abrogated in competition experiments (**Figure 10).**

It has been reported that DJ-1 is localized in various subcellular compartments of the cells, mainly in the cytoplasm but also in the nucleus and in the mitochondria (Bonifati *at al.,* 2003).

This anti-DJ-1 antibody detected the cytoplasmic and the nuclear localization, while specific mitochondrial localization was not observed after the PFA fixation.

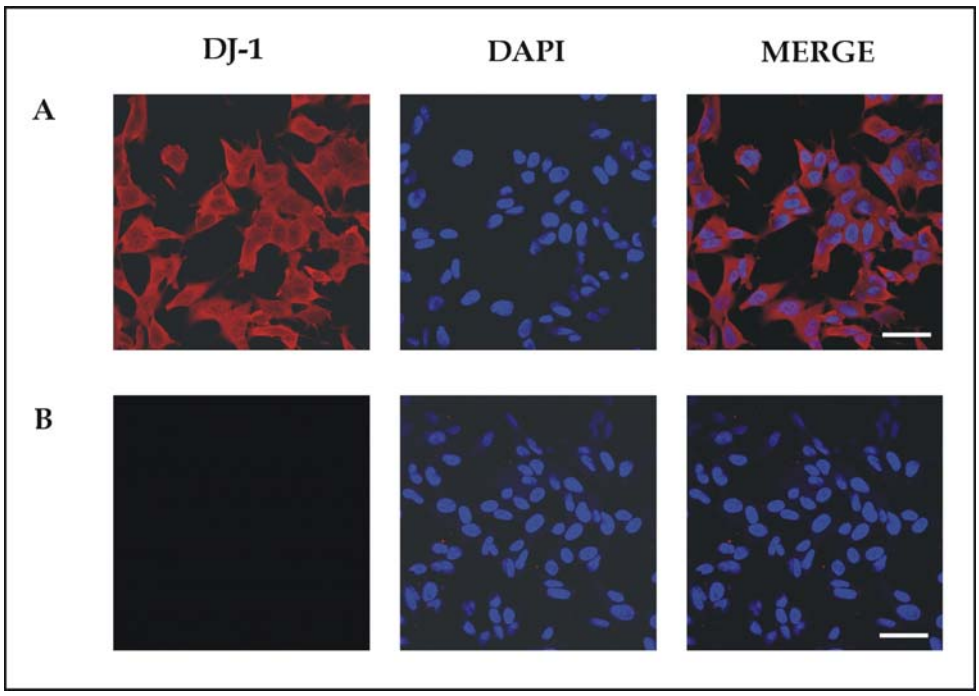


Fig 10: A) nuclear and cytoplasmic DJ-1 localization on SH-SY5Y B) competition experiment using 1 μg of GST-DJ-1 with the working diluition of 1:500 of the home-made anti-DJ-1 indicating the specificity of cellular localization. Scale bar 45 μm.

2 DJ-1 LOCALIZATION IN MOUSE BRAIN

This anti-DJ-1 antibody was used to analyze the localization of endogenous DJ-1 in mouse brain, with special enphasis to the *Substantia Nigra*, brain region in which dopaminergic cells degenerate in Parkinson's Disease. Mouse brain coronal slices stained with anti-DJ-1 antibody presented two different cellular staining: a marked astrocytic expression

and a neuronal labelling. To demonstrate the expression of DJ-1 in both type of cells, double Immunofluorescence experiments were performed.

As shown in **figure 11** DJ-1 co-localizes with glial cells stained with anti-GFAP (Glial Fibrillary Acidic Protein) antibody, which is used as a glial marker. It worth noting that in glial cells the localization of DJ-1 is both nuclear and cytosolic. Other GFAP-negative cells, assumed to be neurons, displayed a predominant cytoplasmic localization.

Further investigations confirmed the DJ-1 is expressed in dopaminergic cells, by using an antibody specific for these neurons, the anti-TH (Tyrosine Hydroxylase).

As shown in **figure 12** the double Immunofluorescence using the anti DJ-1 and anti-TH antibodies indicated the expression of DJ-1 in dopaminergic cells. This was confirmed by the cytofluorogram (**figure 13**) obtained by plotting for each point of the cell the fluorescence green on the *x* axis and the fluorescence red on the *y* axis. Co-localization is indicated from the yellow points in the centre of this graph.

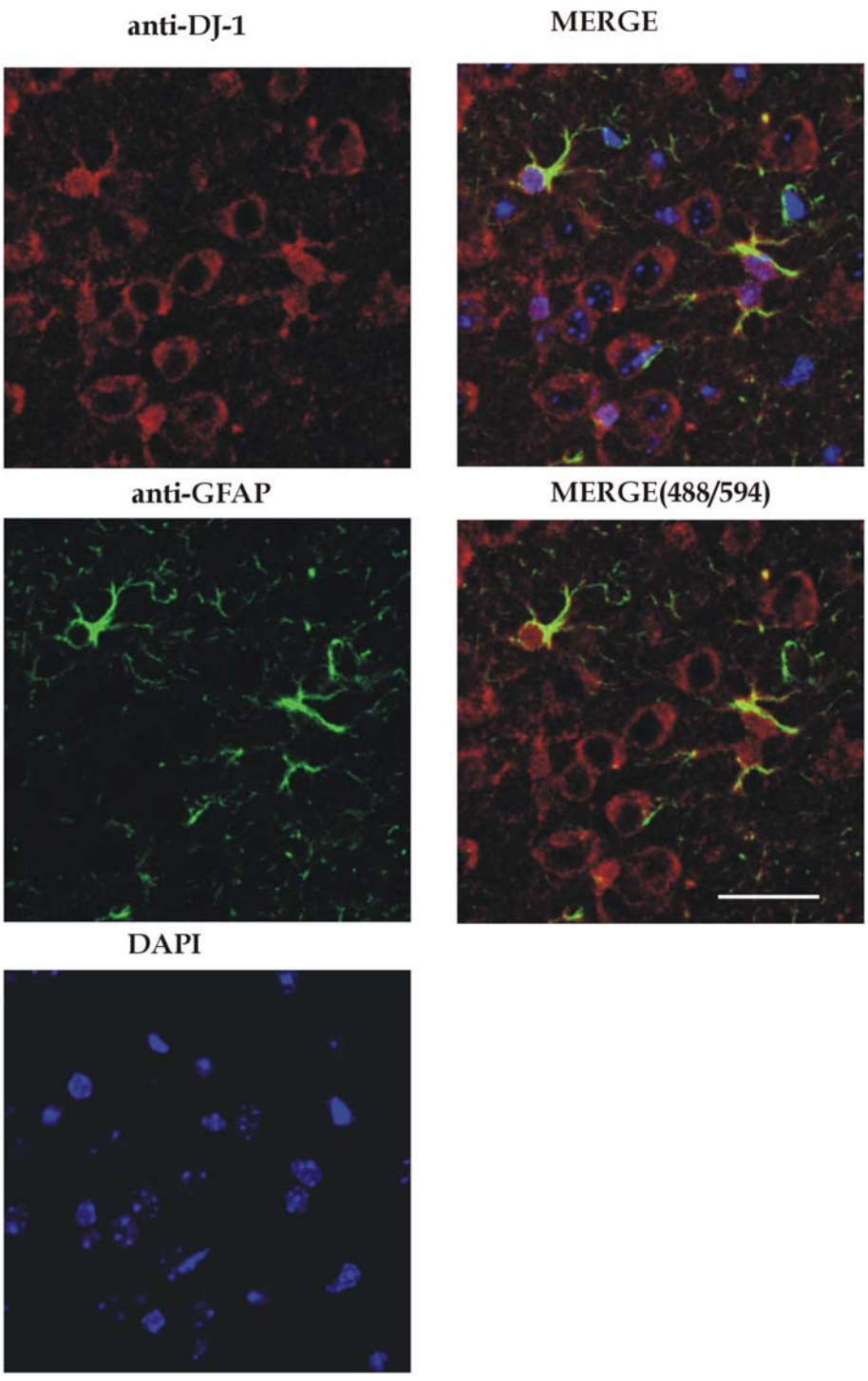


Figure 11: Immunocytochemistry of midbrain sections with anti-GFAP and anti-DJ-1 antibodies. Scale bar 25 μm.

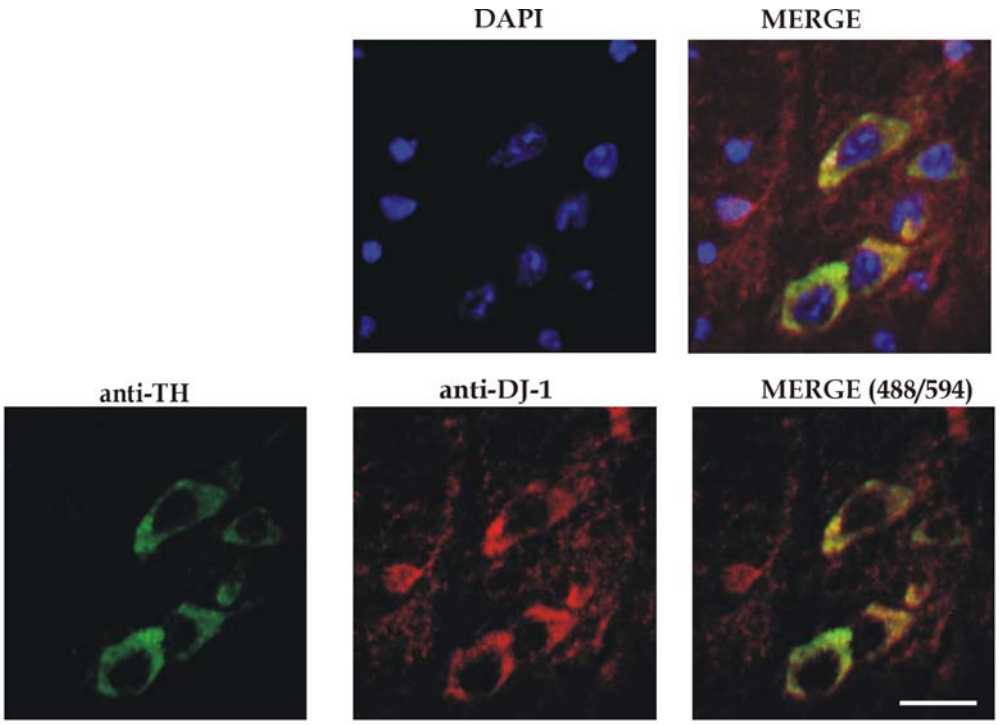


Figure 12: Immunocytochemistry of midbrain sections with anti-TH and anti-DJ-1 antibodies. Scale bar 15 μm.

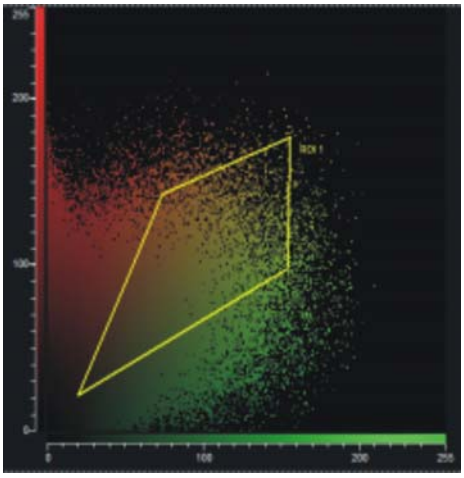


Figure 13: Cytofluorogram analysis of figure 12 stained with anti-TH and anti-DJ-1 antibodies confirmed the DJ-1 localization in dopaminergic neurons.

3 CONFIRMING THE YEAST TWO HYBRID SCREENING

3.1 DJ-1 interacts with Ubc9

Ubc9 is the only SUMO-conjugating enzyme in yeast and in vertebrates. It doesn't discriminate between SUMO-1, SUMO-2, SUMO-3. To confirm the data from the Yeast Two Hybrid Screening, the interaction between endogenous DJ-1 and the transfected Ubc9 was analyzed in HEK293T cell by co-immunoprecipitation assay.

HEK293T cells were transfected with pCDNA3-HA-Ubc9 (kindly provided by Dr. Licio Collavin) and, after 48 hours, cells were lysed and protein extracts were subjected to immunoprecipitation using the home-made polyclonal anti-DJ-1 antibody. The western blot was revealed using an anti-HA antibody. As shown in **figure 14,** the overexpressed HA-Ubc9 co-immunoprecipitates with the endogenous DJ-1. No background was observed in the control immunoprecipitation using anti-Rabbit IgG. The interaction between endogenous DJ-1 and ectopic Ubc9 was confirmed *in vitro* in mammalian cell lines.

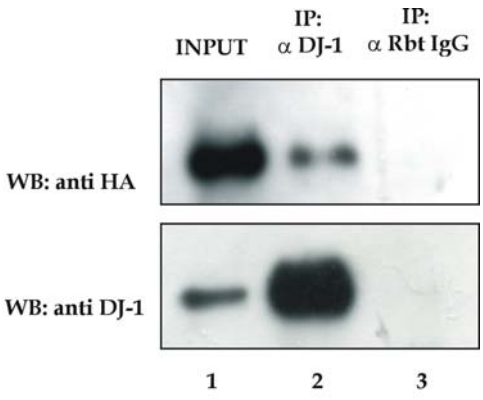


Fig 14: Endogenous DJ-1 co-immunoprecipitate with transfected HA-Ubc-9. Lane 1, total input protein (20 μg); Lane 2, immunoprecipitation with anti-DJ-1 antibody; Lane 3, immunoprecipitation with Rabbit IgG.

4 DJ-1 IS SUMO-2/3YLATED

4.1 Endogenous DJ-1 is covalently modified by endogenous SUMO-2/3 in HEK293T

To determine whether DJ-1 is modified by SUMO-2/3 in addition to SUMO-1 (Shinbo *et al.*, 2004), immunoprecipitation experiments were performed from HEK293T cells using a polyclonal antibody anti-SUMO 2/3 (Zymed), the polyclonal antibody anti-DJ-1 home-made and affinity purified and a Rabbit IgG as a negative control. Since SUMO-2 and SUMO-3 share 95% homology, the available commercial antibodies don't discriminate between these two isoforms.

Immunoprecipitations were loaded into a 10% PAA gel and the slower migrating DJ-1 form was recognized probing the western blot with the anti-DJ-1 antibody. The apparent molecular weight of the immunoprecipitated DJ-1 is consistent with the addition of a single SUMO-2/3 molecule, as shown in **figure 15**. The membrane stripped and incubated with the anti-SUMO-2/3 antibody detected the same bands (data not shown). This experiment suggests that endogenous DJ-1 is modified by endogenous SUMO -2/3 in mammalian cell lines.

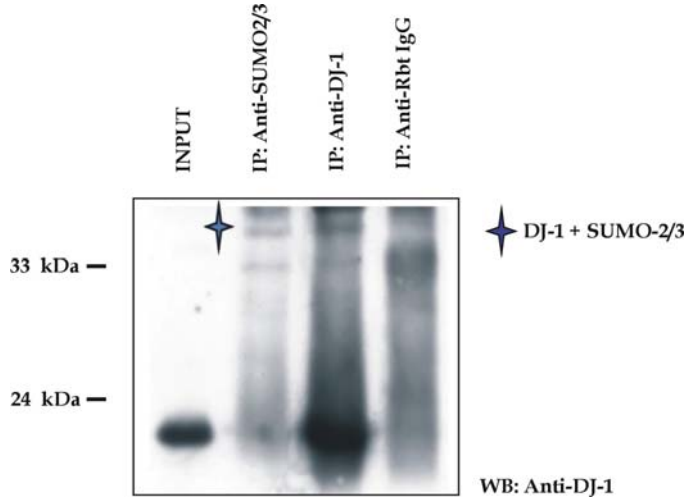


Figura 15: Immunoprecipitation of endogenous DJ-1 modified by endogenous SUMO-2/3 in HEK293T.

4.2 Colocalization of DJ-1 and SUMO-2/3 in SH-SY5Y

SUMO-2/3 (Su *et al.*, 2002) are mostly localized in the nucleus. Because of the high degree of homology between SUMO-2/3, these proteins are virtually identical and share similar protein distribution. Immunofluorecence experiments were performed on SH-SY5Y to investigate the cellular localization of SUMO-2/3. DJ-1 was detected using a monoclonal antibody (Zymed) and SUMO-2/3 a polyclonal one (Zymed). Anti-mouse 488 was used to detect the DJ-1 localization and anti-rabbit 594 for SUMO-2/3. This immunofluorescence experiment indicated that a partial co-localization between DJ-1 and SUMO-2/3 occurs in the nucleus **(figure 16).**

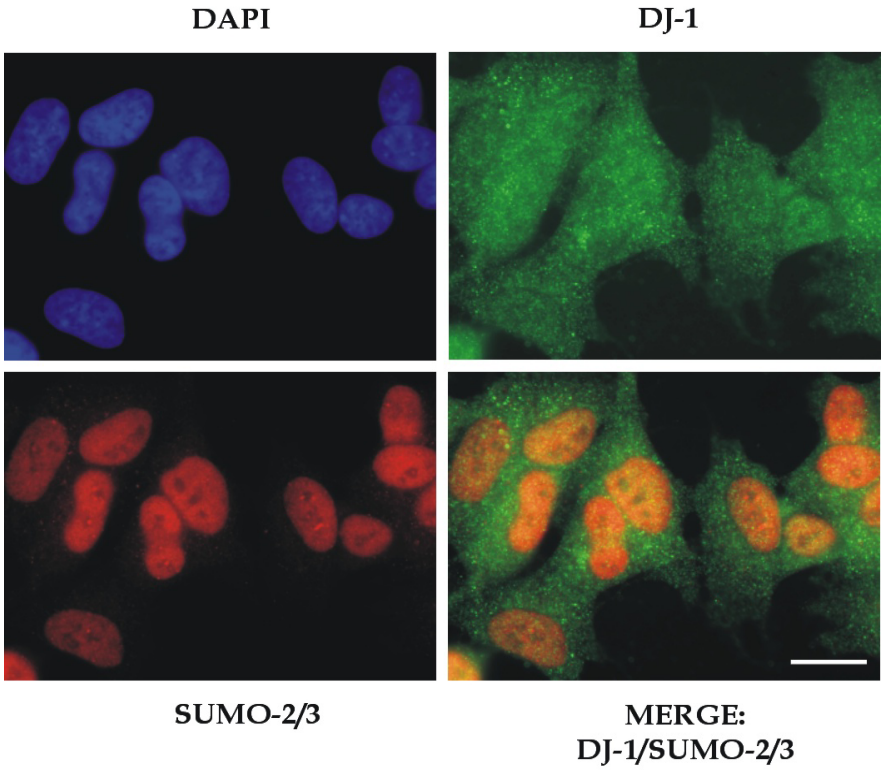


Figure 16: Immunofluorescence of endogenous SUMO-2/3 and DJ-1 and their nuclear co-localization in SH-SY5Y.Scale bar 15 µm.

4.3 DJ-1 is modified by SUMO-2/3 in *Striatum* and *Substantia Nigra*

In order to validate the DJ-1 modification by SUMO-2/3 and its importance in Parkinson's Disease, Immunoprecipitation experiments were performed from *Striatum* and *Substantia Nigra* total protein extracts. These specific mouse brain regions were chosen since PD is characterized by the degeneration of nigrostriatal dopaminergic pathway.

Endogenous DJ-1 was immunoprecipitated using the home-made polyclonal antibody. As control, Rabbit IgG was used to immunoprecipitate the same amount of protein. Immunoprecipitations and inputs (100 μg) were subsequently loaded into a 10% PAA gels and western blots were revealed with both the anti-DJ-1 and the anti-SUMO-2/3 antibodies.

These experiments confirmed that DJ-1 is subjected to a covalent post translational modification *in vivo*, which is responsible of the detection of a DJ-1 species of ~15 kDa higher molecular weight. Revealing the western blot with SUMO-2/3 confirmed that the modification is due specifically to the addition of a single SUMO-2/3 mojety.

It is worth noting that the covalent post translational modification of DJ-1 by SUMO-2/3 occurs in both *striatum* and *substantia nigra* with the same efficiency.

Results are shown in **figure 17** and blots are representative of at least three separate experiments.

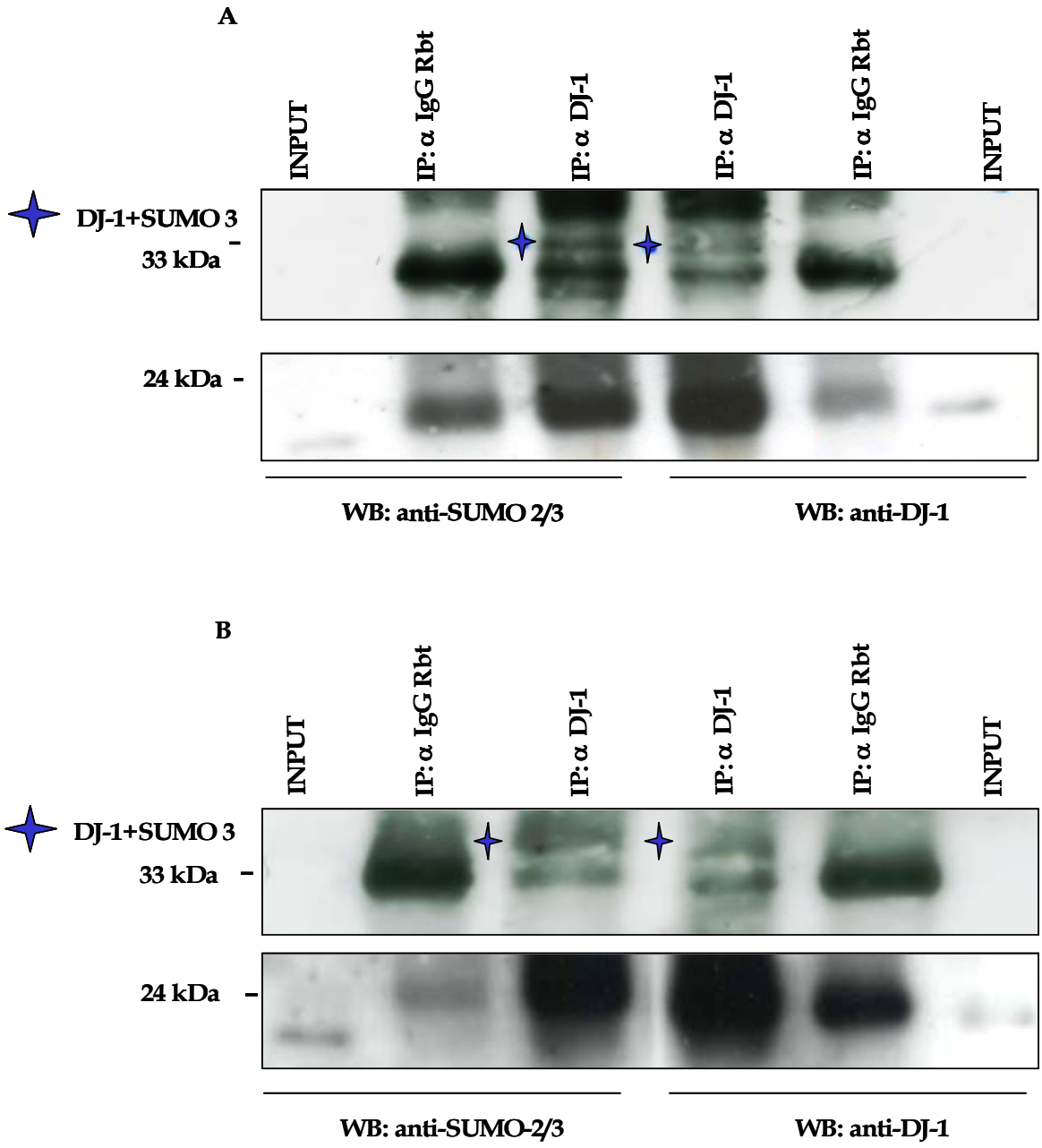


Figure 17: A) DJ-1 is SUMO-2/3ylated *in vivo* in *Substantia Nigra* and in B) *striatum* isolated from mouse brain. In the short exposure inputs of free SUMO-2/3 and DJ-1 were detected; the long exposure was essential to detect the immunoprecipitated fraction of SUMO-2/3ylated DJ-1.

4.4 Confirmation of DJ-1 modification by SUMO-2/3

DJ-1 SUMOylation by SUMO-2/3 was then monitored using additional techniques in order to provide a definitive evidence for DJ-1 sumoylation. On this purpose, two different approaches have been used.

1. The post translational modification of DJ-1 by covalent attachment of SUMO-2/3 was studied using isoelectrofocusing on endogenous protein extracts from HEK293T.

2. Transient co-transfection experiments of DJ-1 and of the SUMO-2/3 conjugation pathway

4.4.1 Isoelectrofocusing.

Two-dimensional gel electrophoresis experiments were performed on HEK293T in collaboration with Prof. G. Manfioletti and Dr. R. Sgarra from University of Trieste.
Protein extracts were prepared as described in "material and methods" and 1 mg of protein was resolved by isoelectric focusing on 7-cm immobilized pH gradient strip (4-7). Second dimensional separation was performed by electrophoresis on 15% SDS-PAGE. Then western blots were analyzed with the home-made anti-DJ-1 antibody.
It was previously demonstrated that DJ-1 exists as a homodimeric protein that migrates on SDS gels as a 20 kDa monomer because of denaturation by SDS (Olzmann *et al.*, 2004; Rizzu *et al.*, 2004). Furthermore, immunoblot analysis revealed that at least three different distinct isoforms of DJ-1 monomer exist. They have the same apparent molecular weight of 20 kDa but different isoelectric points (6.1; 6.4; 6.6). In our analyses we were able to detect DJ-1 at the expected molecular weight. IEF allowed us to

evidence two maily forms (poorly focalized due to overload effects): one at ~ pI 6.0 and the other at ~ pI 6.6. These results were in good agreement with both the predicted values of 20 kDa/pI 6.33 for the DJ-1 monomer (calculated by www.expasy.org) and the data present in literature (Taira *et al.*, 2004; Choi *et al.*, 2006).

Two gels were loaded independently, one was revealed with the purified anti-DJ-1 antibody, the other one with the anti-SUMO-2/3 antibody.

In addition to the monomeric forms of DJ-1, an immunoreactive spot at about 35 kDa and at a more acidic pI (5.8) form was observed with the anti-DJ-1 antibody. This spot perfectly matched with that one recognized by the anti-SUMO-2/3 antibody, indicating clearly that endogenous DJ-1 is SUMO-2/3ylated in HEK293T.

This result supported the theoretical analysis done with the *in silico* program on www.expasy.org that allows to determine the pI/mw. Human SUMO-2/3 have a theoretical pI of 5.34, instead human SUMO-1 a theoretical pI of 5.19. The addition of one moiety of SUMO-2/3 leads to a shift both in the pI with a consequent acidification and in the DJ-1 molecular weight as previously demonstrated. Depending on the algorithm used to perform *in silico* analysis, the predicted shift in the pI upon SUMO-2/3 DJ-1 modification is between 5.47-5.85.

The spot detected in these experiments and confirmed to be DJ-1-modified by SUMO-2/3 displayed a pI shift according to the *in silico* prediction analysis.

Results are shown in **figure 18** and are representative of at least three separate experiments.

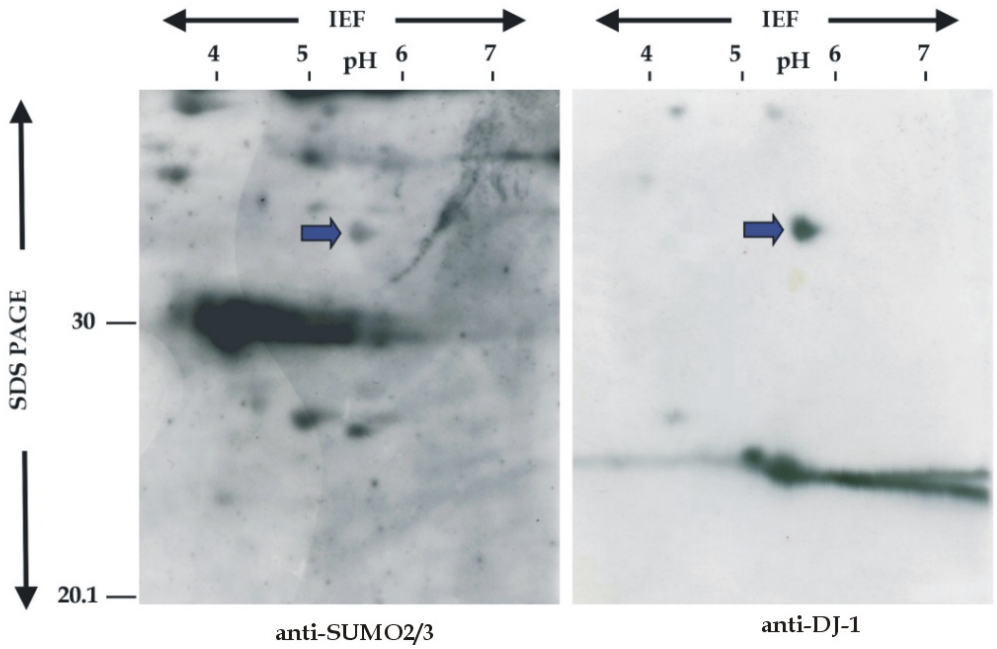


Figure 18: Bidimensional gel analysis of whole protein extracts from HEK293T. Gels were revealed with anti-DJ-1 and anti-SUMO-2/3 antibodies. A modified form of DJ-1 with a shift in the molecular weight and in the pI was detected. The same spot was recognized also by the anti-SUMO-2/3 antibody, indicating that endogenous DJ-1 is modified by SUMO-2/3 in HEK293T.

4.4.2 Cloning and characterization of HA-SUMO-2 and HA-SUMO-3 conjugable and unconjugable.

To further confirm the modification of DJ-1 by SUMO-2/3, *in vitro* experiments were performed.

First, constructs encoding tagged SUMO-2 and SUMO-3 were generated by subcloning the entire SUMO-2 wild type cDNA and SUMO-3 wild type cDNA into a pcDNA3-HA vector. Both cDNAs (kindly provided by Prof. Giannino Del Sal, University of Trieste, Italy) were fused to the HA epitope at the N-terminal, maintaining the C-terminal accessible for its processing and the consequent protein activation with the exposition of the di-Glycine motif.

The unconjugable form of SUMO-2 and SUMO-3 were generated by PCR-Site Directed Mutagenesis using specific designed primers that could convert the di- Glycine motif at the C-terminal into a di-Alanine motif. The cDNA obtained was consequently subcloned into the same pcDNA3-HA vector. In the **figure 19** the procedure is briefly summarized.

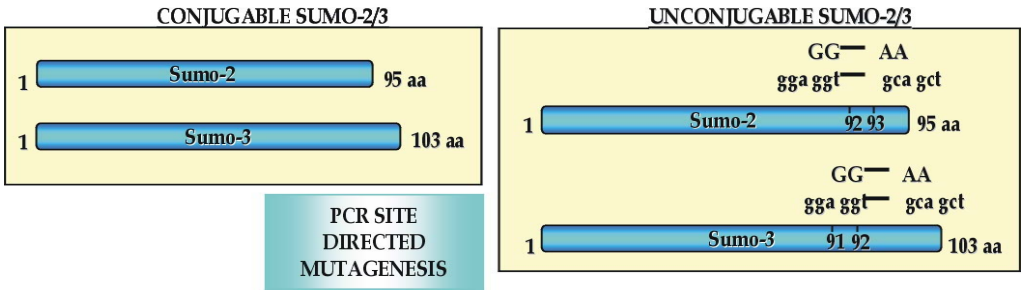


Figure 19: Schematic representation of PCR Site-Directed Mutagenesis on SUMO-2 and SUMO-3 in order to convert the di-Gly motif into di-Ala motif creating the unconjugable form of the SUMOs proteins.

Expressions of wild type SUMO-2 and SUMO-3 fusion proteins and of their unconjugable forms were tested in transient transfection experiments followed by both Western blot analysis and Immunofluorescence using the monoclonal antibody anti-HA.

In **figure 20** the expression was tested by western blot analysis in a 15% PAA gel of both conjugable and unconjugable forms of HA-SUMO-2 and HA-SUMO-3. The fusion proteins are detected at the predicted molecular weight, 20 kDa. Interestingly, for HA-SUMO-3, two bands were detected. It was deduced that the slower migrating form is the inactive protein, while the faster migrating form is the activated one. The activation leads to the exposition of a di-Gly motif due to the cleavage of a peptide of 12 amino acid.

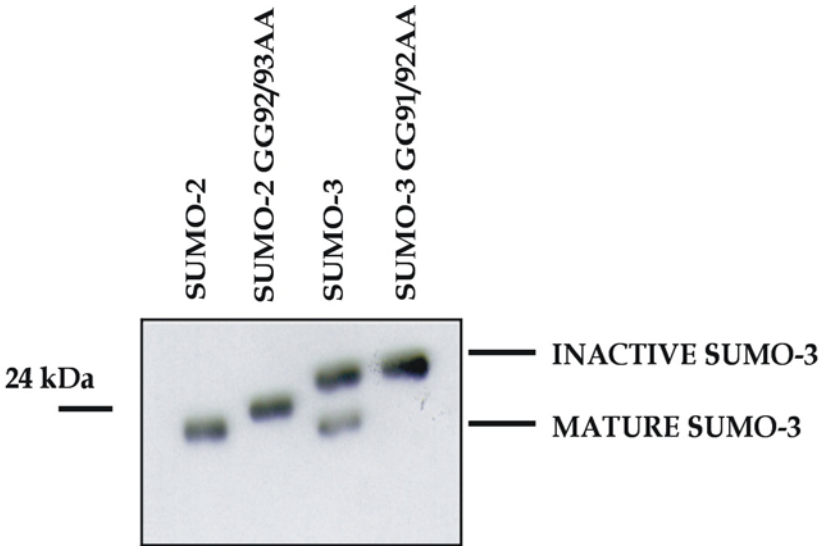


Figure 20: Expression analysis of both HA-SUMO-2 and HA-SUMO-3 in both conjugable and unconjugable forms. The western blot was revealed using the anti-HA antibody.

.

According to this result, the inactivated HA-SUMO-3 runs at the same molecular weight of the unconjugable HA-SUMO-3.

The SUMO-2 activated/inactivated proteins cannot be appreciated because only 2 amino acids are released after its activation.

The expression the HA-SUMO-2 and HA-SUMO-3 fusion proteins in both conjugable and unconjugable forms were also tested in immunofluorescence, as shown in **figure 21A and figure 21B** respectively, using the anti-HA antibody.

The predominant cellular localization of HA-SUMO-2 in the conjugable form was nuclear, while the unconjugable form presented a marked cytoplasmic localization. Similar results were obtained transfecting the conjugable HA-SUMO-3 in HEK293T: both nuclear and also cytoplasmic localization were detectable. The corresponding unconjugable form displayed a cellular localization similar to the wild type form.

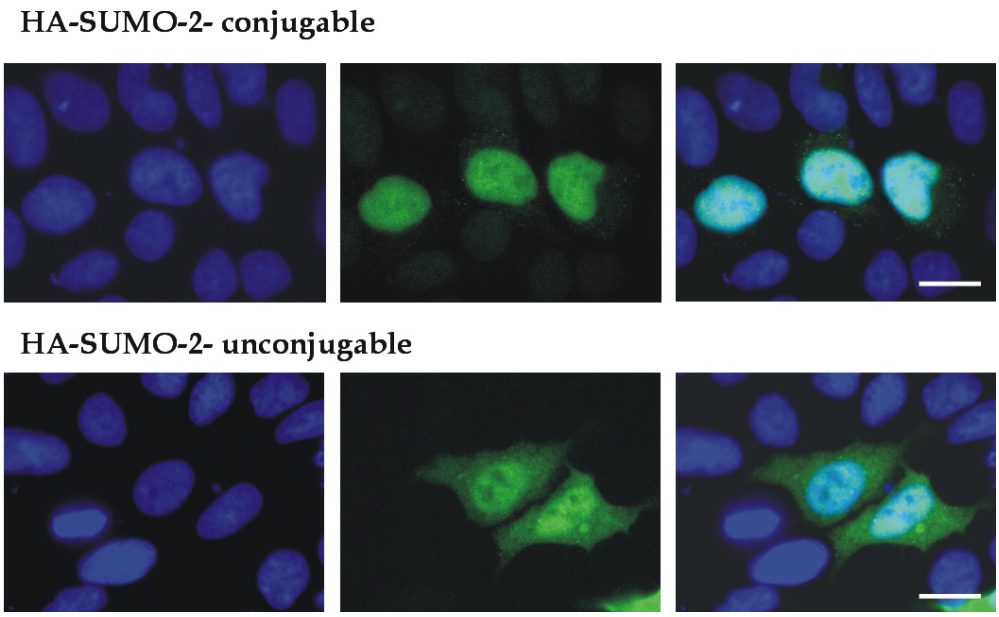


Figure 21A) Immunofluorescence on HEK293T after transient transfection with HA-SUMO-2 in both conjugable and unconjugable forms. The cellular localization was detected using an anti-HA antibody. Scale bar 15 μm.

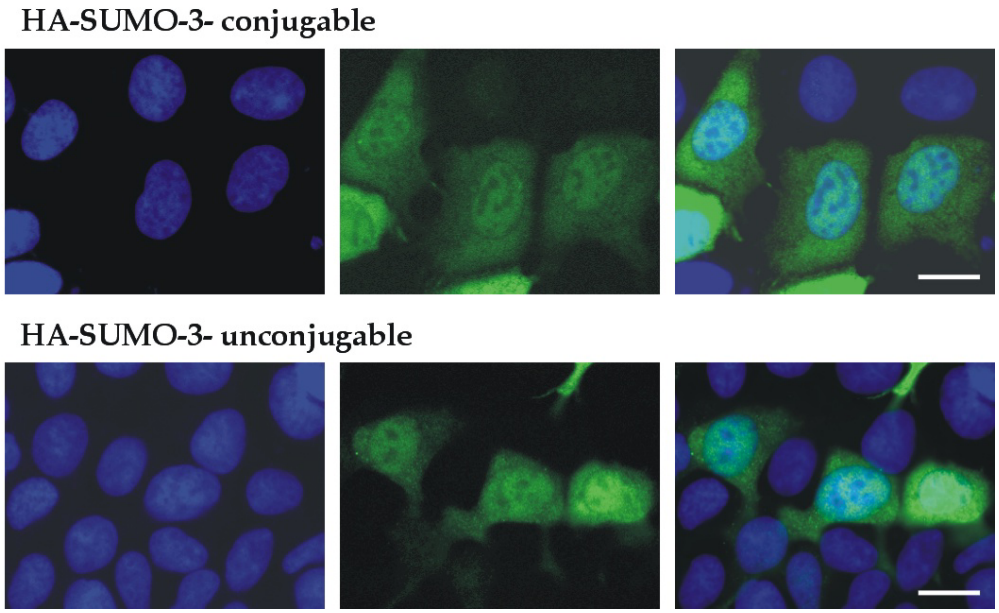


Fig 21B) Immunofluorescence on HEK293T after transient transfection with conjugable and unconjugable HA-SUMO-3. The cellular localization was detected using anti-HA antibody. Scale bar 15 μm.

4.4.3 Validation of the conjugable and unconjugable forms of HA-SUMO-2 and HA-SUMO-3 proteins

The abilility of HA-SUMO-2/HA-SUMO-3 wild type to be efficiently conjugated to endogenous cellular proteins was tested in transient co-transfection experiments with the E2-conjugating enzyme, HA-Ubc9. Western blotting experiments were revealed using the antibody anti-HA. Consistent with previous reports (Gostissa *et al.*, 1999), a high molecular weight smear was observed. This result indicates that several endogenous cellular proteins could be covalently modified by conjugation to the transfected tagged SUMO-2 (**figure 22A**) and SUMO-3 (**figure 23A**) in the presence of HA-Ubc9.

These unconjugable forms were also validated through western blotting analysis: when these cDNAs were transfected in the presence of the E2-conjugating enzyme, the conjugation of exogenous unconjugable HA-

SUMO-2/3 protein to endogenous cellular protein was totally abrogated (**figure 22 and 23 B**).

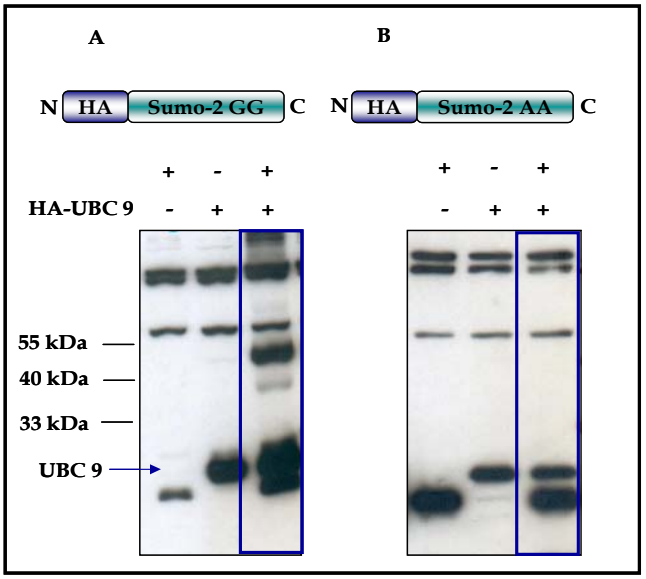


Figure 22: Sumoylation analysis of A) HA-SUMO-2 wild type and B) HA-SUMO-2 unconjugable in the presence of HA-Ubc9. The western blot was revealed using an anti-HA antibody.

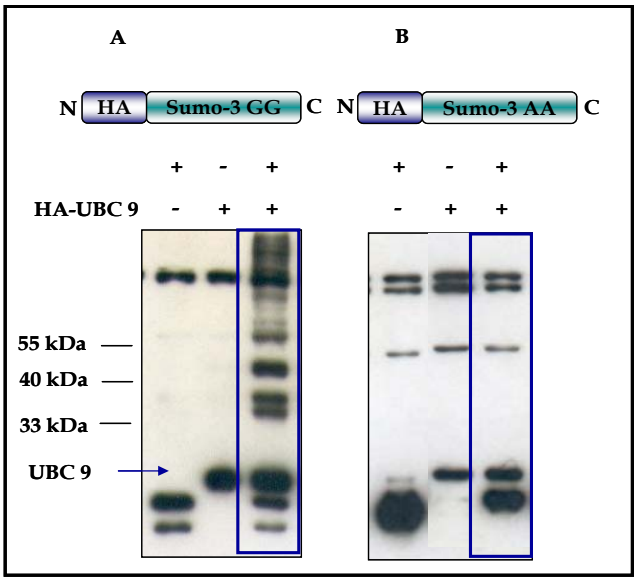


Figure 23: Sumolation analysis of A) HA-SUMO-3 wild type and B) HA-SUMO-3 unconjugable in the presence of HA-Ubc9. The western blot was revealed using an anti-HA antibody.

These results indicated that the use of unconjugable forms of HA-SUMO-2 and HA-SUMO-3 are good internal controls to demonstrate that a protein of interest is sumoylated.

Specific experiments were performed with tagged DJ-1 to demonstrate its covalent post translational modification in transient co-transfection experiments.

4.5 Confirming the post translational modification of DJ-1 by covalent attachment of SUMO-2/3 using Western Blot analysis

To confirm that DJ-1 can be modified by SUMO-2 and SUMO-3, DJ-1 sumoylation was analyzed in transient transfections. HEK293T were transfected with equimolar amounts of the following plasmids: 2X-Flag-DJ-1, HA-SUMO-2/3 in both conjugable and unconjugable forms and HA-Ubc9.

The following transfections were performed: 2X-Flag-DJ-1; 2X Flag DJ-1 + HA-Ubc9; 2X-Flag-DJ-1 + HA-Ubc9 + HA-SUMO-2/3 conjugable; 2X Flag DJ-1 + HA-Ubc9 + HA-SUMO-2/3 unconjugable; 2X-Flag-DJ-1 + HA-SUMO-2/3 conjugable and, finally, HA-Ubc9 + HA-SUMO-2/3 conjugable.

Thirty-six hours after transfection, cells were lysed in hot SDS sample buffer and analyzed by Western blotting using the anti-Flag, anti-DJ-1 (data not shown) and anti-HA antibodies. Only when the 2X-Flag-DJ-1 was co-expressed with HA-SUMO-2/3 in the conjugable form and HA-Ubc9, a more slowly migrating band was visible, as shown in **figure 24**. The molecular weight (~ 42 kDa) was consistent with a form of transfected 2X Flag DJ-1 covalently modified by a single moiety of HA-SUMO-2 or HA-SUMO-3. The DJ-1 modification was observed only when co-transfected with the HA-Ubc9.

The specificity of the modification was confirmed by the transfection of unconjugable HA-SUMO-2 and HA-SUMO-3. No shifted DJ-1 cross-reactive bands were observed.

Anti-HA antibody detected a ladder of bands representing the HA-SUMO-2/3 conjugated cellular proteins in cells transfected with HA-SUMO-2/3 and HA-Ubc9.

Figure 24 shows representative results of at least three separate experiments for each blot and demonstrates that SUMO-2/3 proteins efficiently modify DJ-1 in transient transfections. .

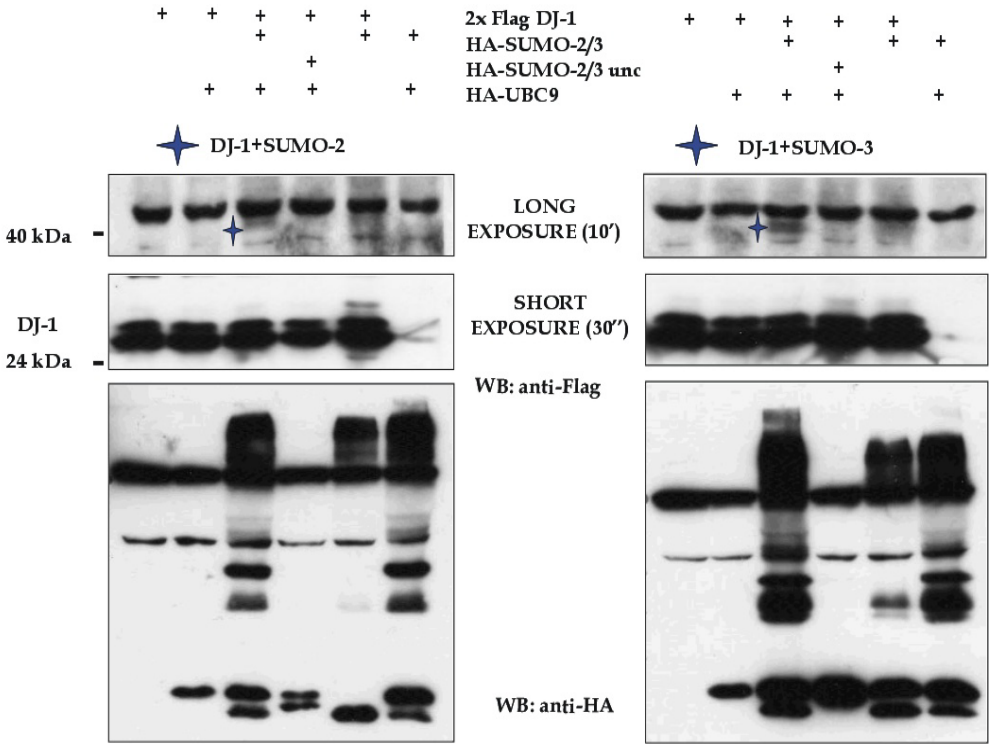


Figure 24: Sumoylation analysis of DJ-1 in transient transfection with HA-SUMO 2 (left panel) and HA-SUMO 3 (right panel). Westen blotting were revealed with anti-Flag to detect transfected DJ-1 and with anti-HA to detect the SUMO-2/3 protein conjugation. The band at 42 kDa detected with anti Flag was also recognized by anti-DJ-1 (data not shown).

Furthermore, since SUMO-2 and SUMO-3 presents 98% identity in their activated form (except for two amino acid), only HA-SUMO-3 was used for this study in transient transfection. The same result was obtained with 6Xmyc DJ-1; the use of different tags didn't affect the reproducibility of DJ-1 SUMO-2/3ylation after transient transfection, as shown in **figure 25.**

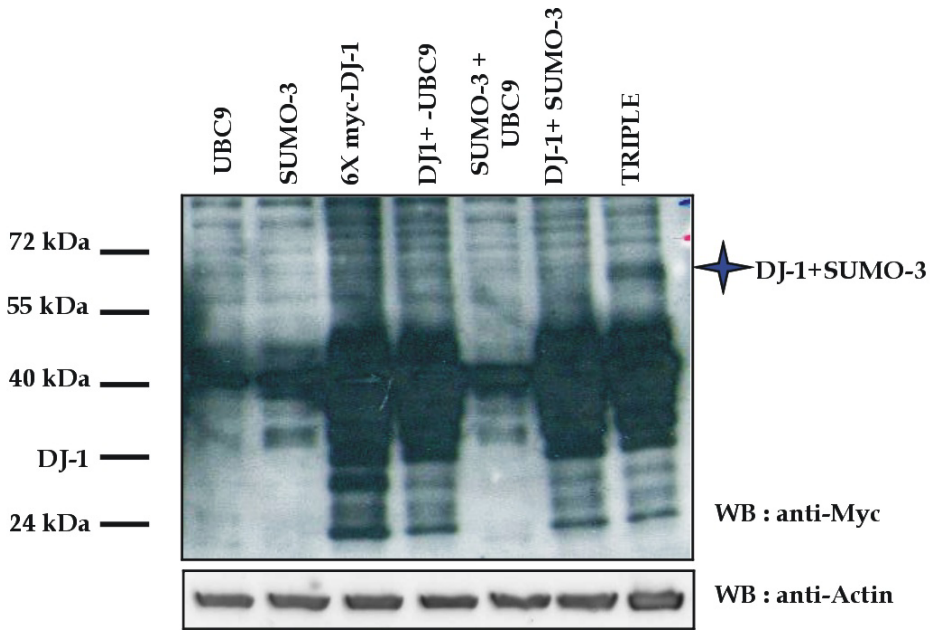


Figure 25: SUMOylation analysis of 6X-myc-DJ-1. The tag doesn't affect the reproducibility of the result.

4.6 Confirming the post translational modification of DJ-1 by covalent attachment of SUMO-2/3 using immunoprecipitation

Immunoprecipitation experiments were performed to confirm that the band detected at 42 kDa was DJ-1 sumoylated by SUMO-2/3. HEK293T were transiently trasfected with 2x-Flag-DJ-1 alone; 2x-Flag-DJ-1 + HA-SUMO-3 conjugable and HA-Ubc9; 2x-Flag-DJ-1 + HA-SUMO-3 unconjugable and HA-Ubc9. Since SUMO-2 and SUMO-3 are identical in their processed form, all the experiments were performed with HA-SUMO-3.

Thirty-six hours post-transfection, cells were lysed and DJ-1 was immunoprecipitated using a Flag-Resin and analyzed by western blotting with an anti-Flag antibody. As shown in **figure 26** the band at 42 kDa was specifically immunoprecipitated by the resin and recognized by the antibody, indicating that the transfected DJ-1 is covalently modified only when the conjugable form of HA-SUMO-3 is co-transfected.

To further demonstrate that the modification was due to SUMO-3ylation, the membrane was stripped and probed with anti-HA. The antibody recognized a band of the same size.

The same membrane was re-incubated after stripping with the affinity purified anti-DJ-1: as expected, the band at 42 kDa was recognized confirming that transfected DJ-1 is modified by exogenous SUMO-3 in the presence of Ubc9.

Figure 26 shows representative results of at least three separate experiments.

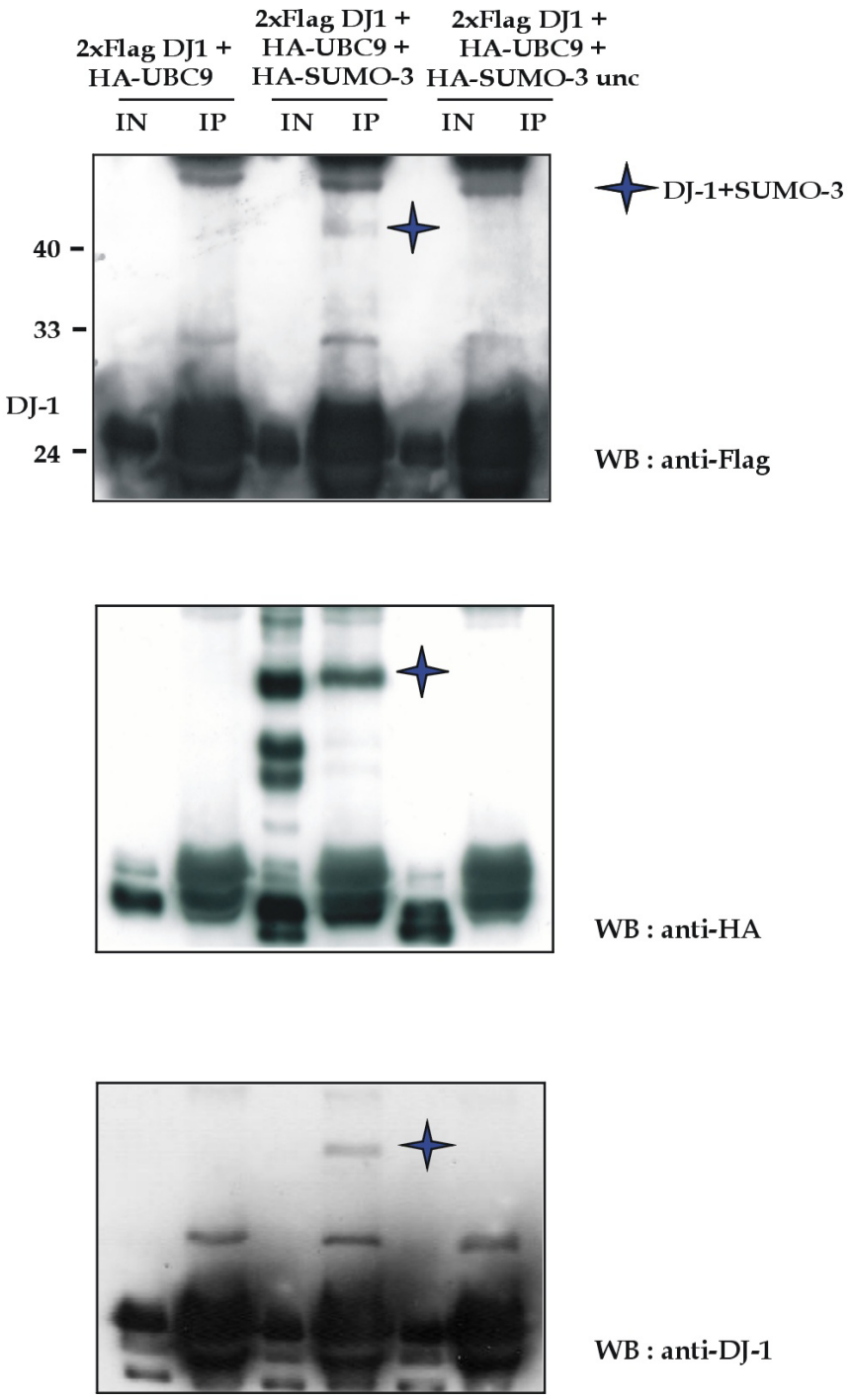


Figure 26: Confirmation of DJ-1 modification by SUMO-3. Whole protein extract from HEK293T transfected with 2X-Flag-DJ-1; 2X-Flag-DJ-1, HA-SUMO-3 and HA-Ubc9; 2X-Flag-DJ-1, HA-SUMO-3 unconjugable and HA-Ubc9 was subjected to immunoprecipitation with Flag Resin. Western Blot was revealed with anti-Flag, anti-HA and anti-DJ-1 antibody after sequential stripping.

4.7 Identification of the Sumoylated Lysines in DJ-1

4.7.1 *In silico* SUMOylation prediction programs

SUMO attachment to target proteins occurs at specific lysine residue, which are in most cases embedded in the consensus sequence ψ-K-X-E (ψ is a hydrophobic amino acid) (Johnson S.M.,2004) or ψ-K-X-E/D(Melchior F., 2003) as described in the Introduction.

To identify the lysine that is involved in DJ-1 modification, a sequence analysis by SUMO plot was performed (available at www.expasy.org)

This program is a tool for *in silico* sumoylation sites prediction. It showed that Lys 182 lies within the canonical sequence ψ-K-X-E indicating an high probability to be a SUMO acceptor. The same program indicated Lysines 41, 62, 63 and 148 with a low probability motives.

The accumulating experimental data show that about ~23% of real sumoylation sites don't follow the standard motif, leading to both false positive and false negative sumoylation site predictions. These unexpected features introduce difficulties into the sumoylation analysis.

Interestingly, it was recently published that Lys 130 is the acceptor for SUMO-1 modification (Shinbo *et al.,* 2005).

A new program, SUMOsp, was recently developed. 239 experimentally verified sumoylation sites were considered for the data set. The final prediction system showed satisfying sensitivity and specificity (Xue *et al.*, 2006). This program can be freely accessed from: http: //bioinformatics.Icd-ustc.org/sumosp/.

The SUMOsp program indicates that the most probable Lys in DJ-1 is 62, (performing an analysis with an high cut off), then Lysines 4 and 182 and Lysines 4, 32, 62, 89, 93, 175, 182 and 187. These *in silico* analysis are summarized in **figure 27.**

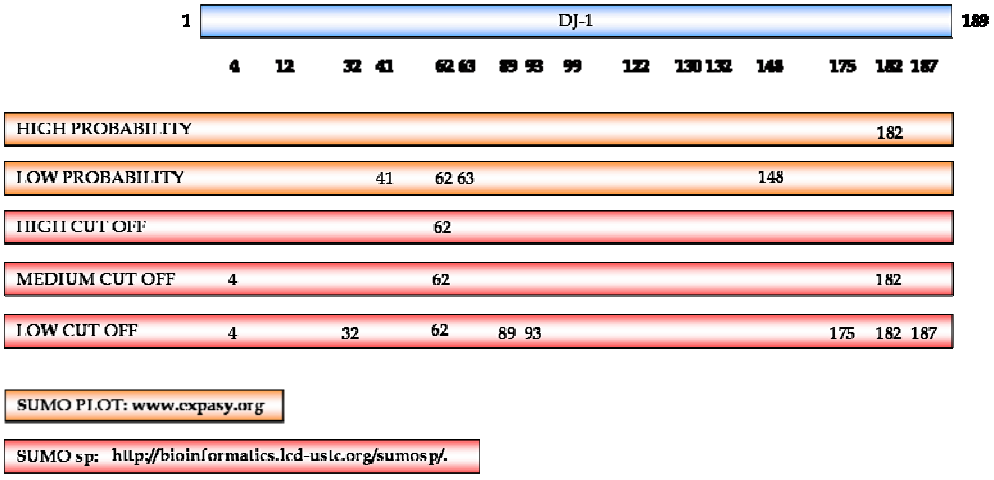


Figure 27: Schematic representation of the results for *in silico* prediction analysis programs used to determine potential lysines.

4.7.2 Characterization of DJ-1 lysines mutants

To verify which residues are *in vivo* acceptors of SUMO-3, point mutations were introduced into the 2X-Flag-DJ-1 in order to convert Lys into Arg without altering the total charge of the protein and, consequently, it's structure.

Lysines 62/63 and Lysines 89/93 were mutated together according to the protocol followed in section "Material and Methods".

The following clones have been created using the strategy of the PCR Site-Directed Mutagenesis:

- 2X-Flag-DJ-1 Lys4Arg (DJ-1K4R)
- 2X-Flag-DJ-1 Lys12Arg (DJ-1K21R)
- 2X-Flag-DJ-1 Lys32Arg (DJ-1K32R)
- 2X-Flag-DJ-1 Lys41Arg (DJ-1K41R)
- 2X-Flag-DJ-1 Lys62/63Arg (DJ-1K62/63R)
- 2X-Flag-DJ-1 Lys89/93Arg (DJ-1K89/93R)

- 2X-Flag-DJ-1 Lys99Arg (DJ-1K99R)
- 2X-Flag-DJ-1 Lys122Arg (DJ-1K122R)
- 2X-Flag-DJ-1 Lys130Arg (DJ-1K130R)
- 2X-Flag-DJ-1 Lys132Arg (DJ-1K132R)
- 2X-Flag-DJ-1 Lys148Arg (DJ-1K148R)
- 2X-Flag-DJ-1 Lys175Arg (DJ-1K175R)
- 2X-Flag DJ-1 Lys182Arg (DJ-1K182R)
- 2X-Flag-DJ-1 Lys187Arg (DJ-1K187R)

These clones were first tested in transient transfections in both Western Blot (**figure 28A**) and Immunofluorescence (**figure 28B**) using the anti-Flag antibody. As expected, all DJ-1 clones displayed the same rate of expression in transient transfection and they shared the same cytoplamic and nuclear localization as the wild type form of 2X-Flag-DJ-1. **Figure 28A** and **figure 28B** show representative results of at least three separate experiments.

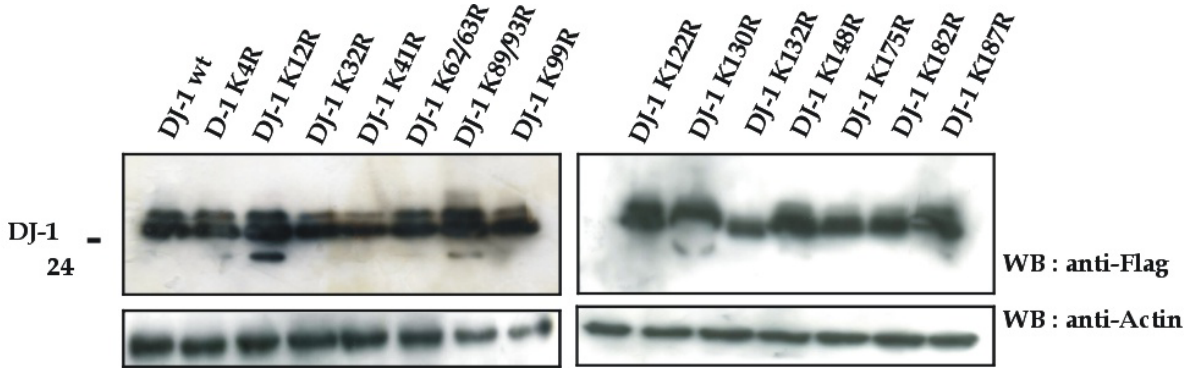


Fig 28: DJ-1 Lysines mutant expression by Western blot with the anti-Flag antibody.

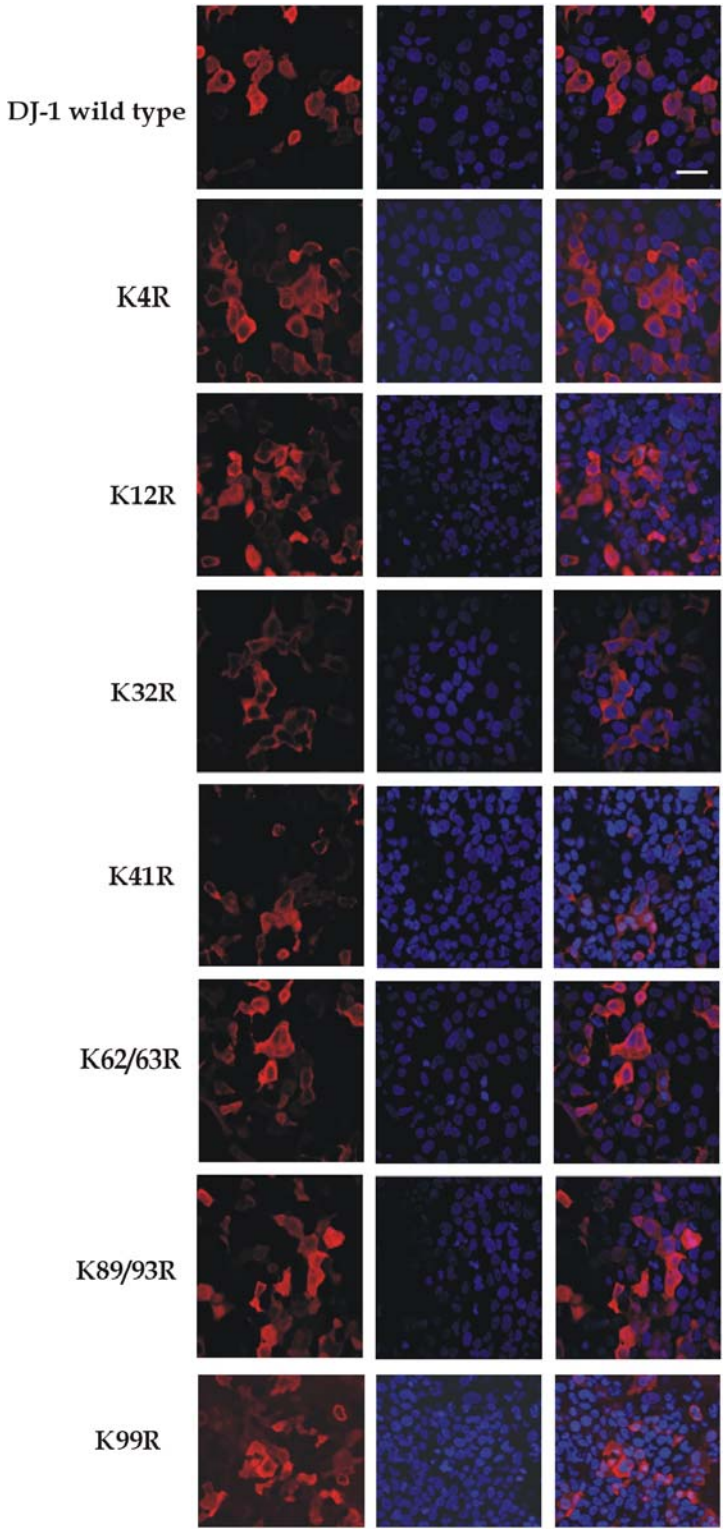
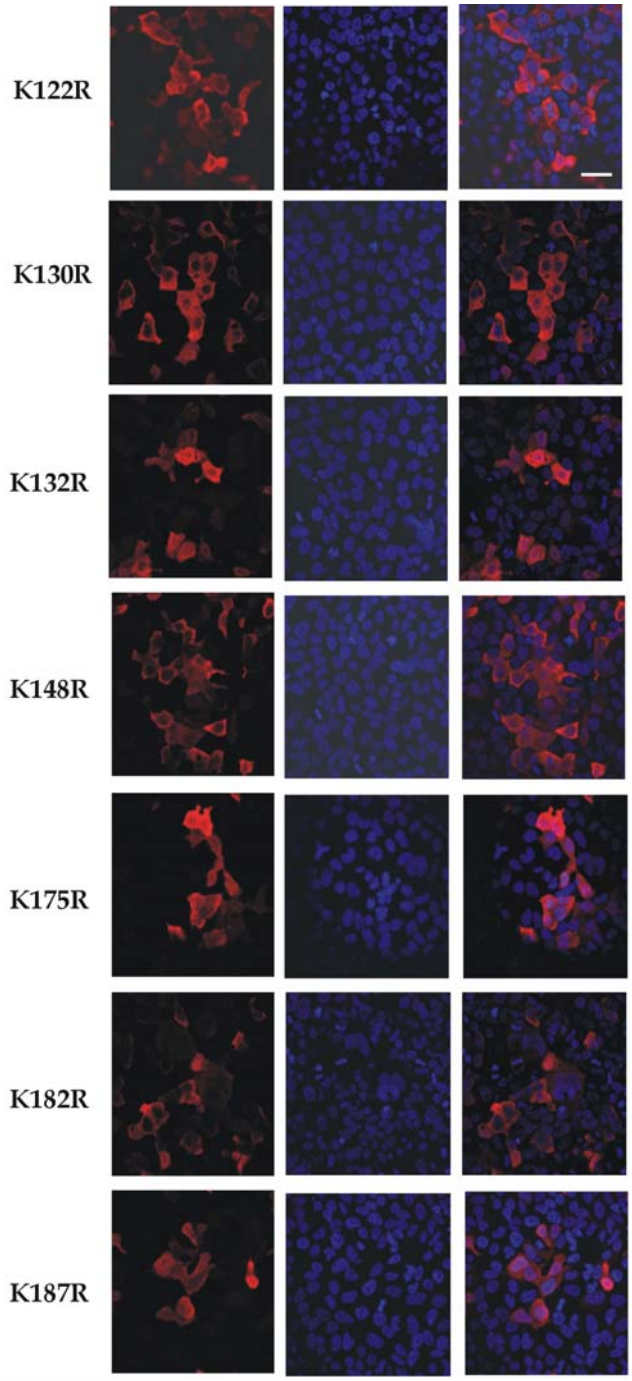


Fig 28B: DJ-1 Lysines mutant expression by Immunofluorescence with the anti-Flag antibody. Scale bar 45 μm.


K122R
K130R
K132R
K148R
K175R
K182R
K187R

4.8 SUMOylation analysis of DJ-1 lysines mutants

To investigated which lysines are sites of SUMO-3 conjugation, HEK293T were co-transfected with HA-SUMO-3 in the conjugable form, HA-Ubc9 and wild type 2X-Flag-DJ-1, as well the 2X-Flag-DJ-1 in which Lys were changed into Arg. As a negative control, co-trasfection of 2X-Flag-DJ-1 wild type, HA-Ubc9 and HA-SUMO-3 GG91/92AA was performed.

Cells were lysed directly into hot 2X Laemmly Buffer and proteins were subjected to SDS-Page and Western Blot analysis using the anti-Flag antibody.

As shown in **figure 29** none of the DJ-1 single/double Lysines mutants abrogated the DJ-1 SUMO-3ylation, indicating that more than one lysine can be sumoylated alternatively by SUMO-3.

The same gel was stripped and probed with anti-HA antibody to evaluate the transfection efficiency of the HA-SUMO-3 conjugation in the presence of the E2-conjugating enzyme HA-Ubc9.

Since DJ-1 is modified by SUMO-1 on Lys 130 (Shinbo *et al.*, 2005), the SUMO-3 modification on DJ-1 occurs on different Lys respect to SUMO-1 and more than one Lys can be modified alternatively by SUMO-3.

It is also worth noting that in the case of DJ-1 the *in silico* prediction analyses were insufficient *per se* to identify the target Lys for both SUMO-1 and SUMO-3.

Figure 29 shows representative results of separate experiments for each Lys/Arg mutant.

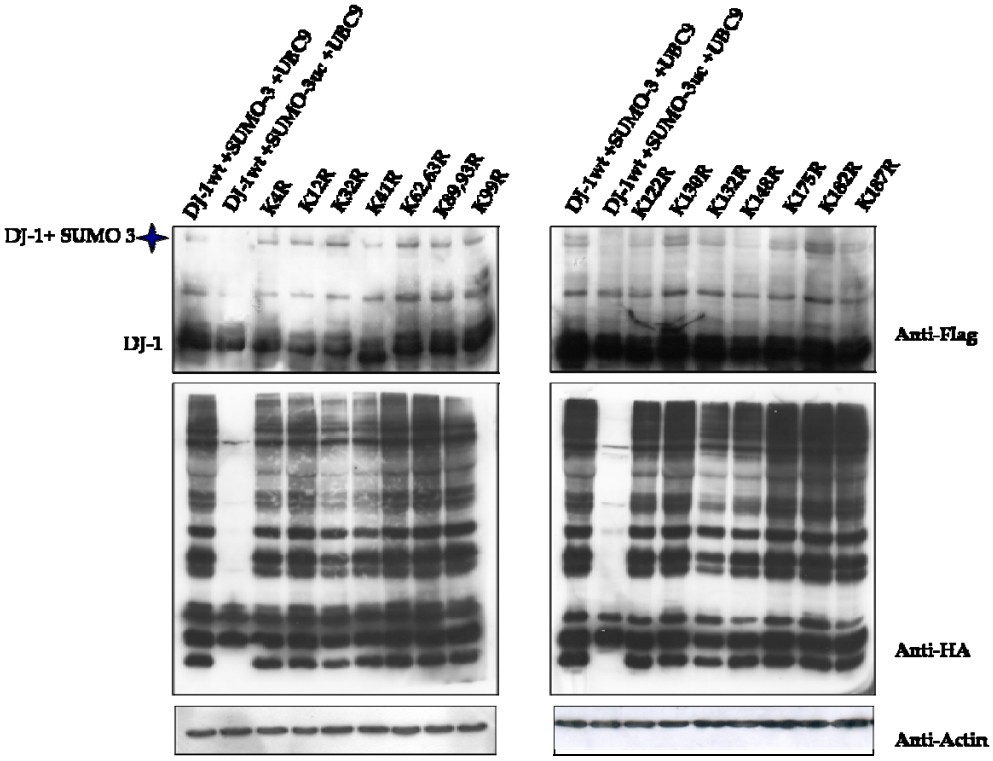


Figure 29: Sumoylation analysis of DJ-1 Lys mutants. None of the Lys X Arg was able to abrogate the sumoylation indicating that more than one Lys can be sumoylated alternatively.

The *in silico* mapping, using both SUMO plot and SUMOsp server, indicated that Lys 182 and Lys 62 were the most probable lysines involved in sumoylation. Analysis of lysine sumoylation within DJ-1 revealed that none of single and double lysines mutations significantly affected the DJ-1 SUMO-3 conjugation. Since the combined approach of Single Lysines PCR Site-Directed Mutagenesis and *in silico* prediction was unsuccessful, multiple Lysines were changed into Arginines in the same polypeptide.

To chose among the different combinations we took into account the *in silico* prediction programs and the conservation of Lysines during evolution.

Two different clones were created, the 2X-Flag-Lys 62/63/130/182-Arg DJ-1 and (DJ-1 4M) the 2X-Flag-Lys 4/62/63/130/182/187-Arg DJ-1 (DJ-1 6M): the Lys 130 was considered because it's known to be acceptor for SUMO-1 (Shinbo *et al.*, 2005); Lysines 62/63 are acceptors by SUMO plot; Lys 182 is the most probable amino acid according to SUMO plot; Lys 62 is the potential SUMO modification site for SUMO sp with an high cut off analysis; Lys 4 and Lys 187 are predicted to be SUMO acceptor with a middle cut. These last two amino acids are also highly conserved during evolution.

Preliminary experiments tested the level of expression of these DJ-1 mutants respect to the DJ-1 wild type and their cellular localization. No significative changes were appreciated in both western blot analysis (**figure 30A**) and immunofluorescence using the anti-Flag antibody (**figure 30B**).

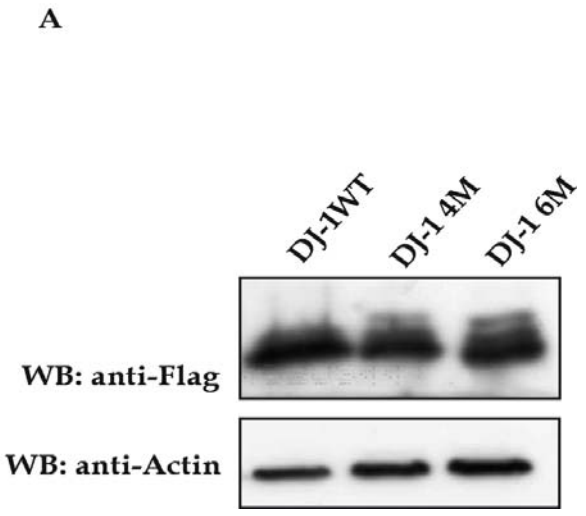


Figure 30A: Analysis expression of DJ-1 4M (Lys 62/63/130/182 Arg) and DJ-1 6M (Lys 4/62/63/130/182/187Arg) respect to the wild type in western blot using anti-Flag antibody.

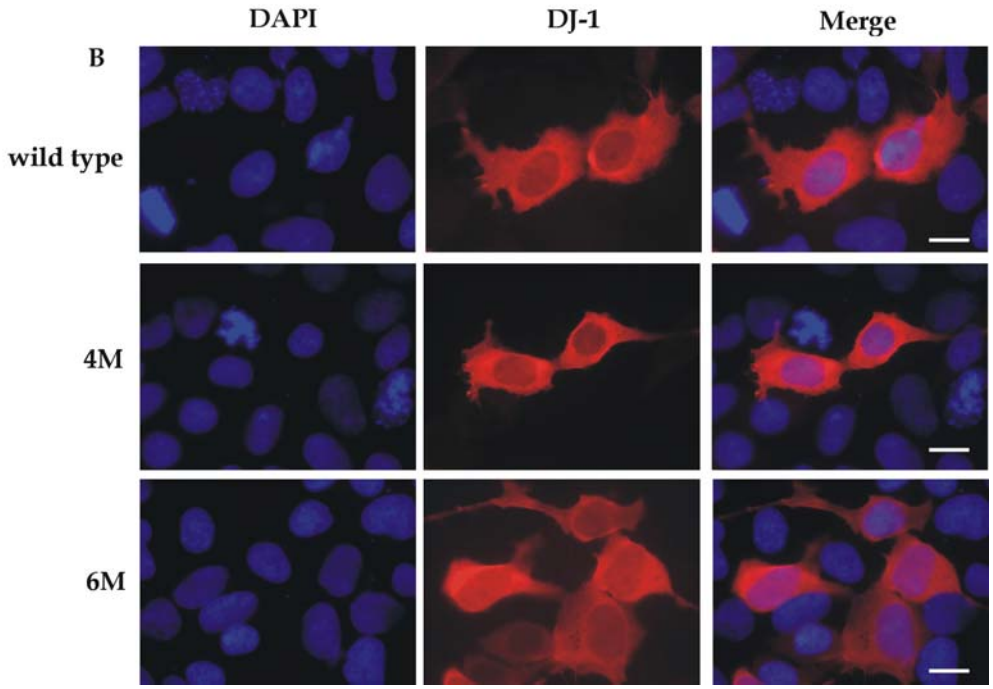


Figure 30B: Analysis expression of DJ-1 4M (Lys 62/63/130/182 Arg) and DJ-1 6M (Lys 4/62/63/130/182/187Arg) respect to the wild type in immunofluorescence using anti-Flag antibody. Scale bar 10 μm.

Subsequently, HEK293T were transfected with the DJ-1 multiple mutants, HA-SUMO-3 and HA-Ubc9 and total cellular proteins were subjected to SDS-PAGE and blotting analysis with the anti-Flag antibody in order to analyze the sumoylation pattern. Notably, the multiple DJ-1 mutants still retained sumoylation indicating that multiple Lys residues are not SUMO-3 acceptors within DJ-1 and excluding again that the Lys 130 is the major sumoylation site for SUMO-3.

Result shown in **figure 31** are representative of at least three different experiments.

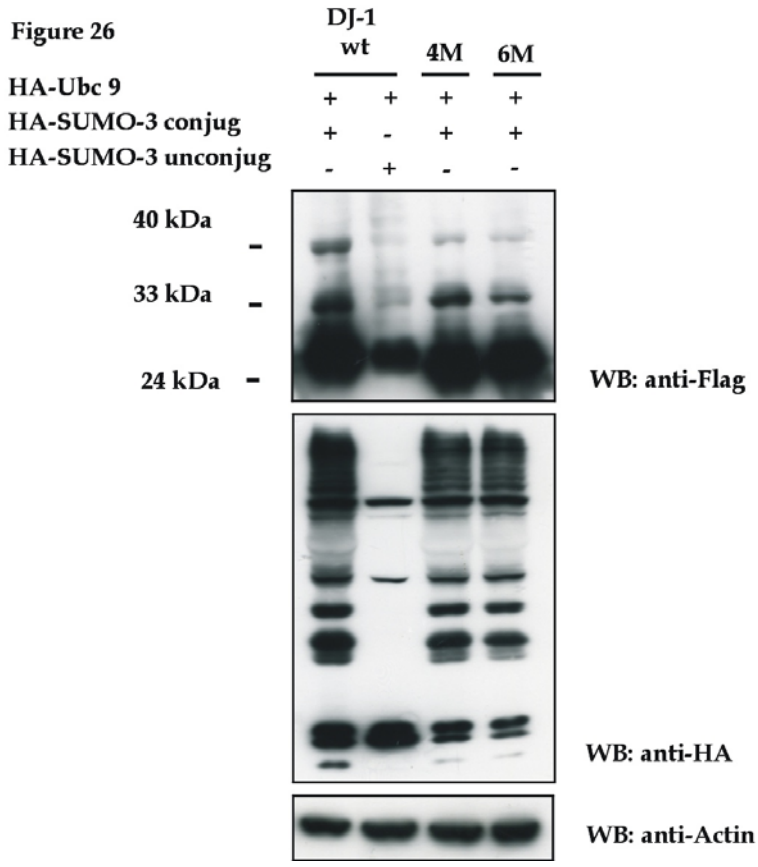


Figure 31: Sumoylation analysis of DJ-1 4M and DJ-1 6M respect to the wild type with anti-Flag and anti-HA antibody.

In collaboration with Prof. P. Carloni and Dr A. Jezierska, a computational approach was addressed to identify the lysines responsible of SUMO-3ylation. Docking and molecular dynamics (MD) calculations were runned between DJ-1 wild type and SUMO-3. The crystal structure of SUMO-3 was still not resolved but was deduced from that of SUMO-2. MD calculations pointed to a role of Lys 130 and Lys 132. In order to investigate whether this new combination could affect sumoylation, a new DJ-1 mutant was created by PCR Site-Directed Mutagenesis. The expression was tested in transient transfection in both western blot (**figure 32A**) and immunofluorescence (**figure 32B**) analyses. Unfortunately, DJ-1 Lys 130/132 Arg retained the SUMOylation status. Results repeated in triplicate are shown in **figure 33**.

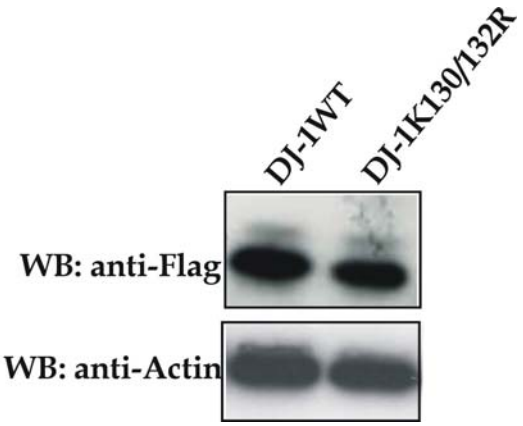


Figure 32A: Expression analysis of DJ-1 Lys 130/132 Arg respect to the wild type protein with anti-Flag antibody.

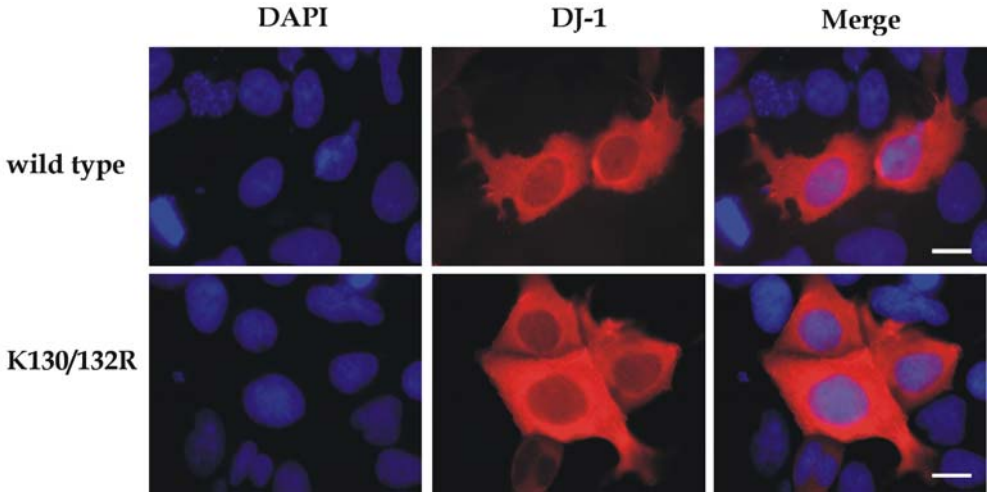


Figure 32B: Subcellular localization analysis of DJ-1 Lys 130/132R with anti-Flag antibody. Scale bar 10 μm.

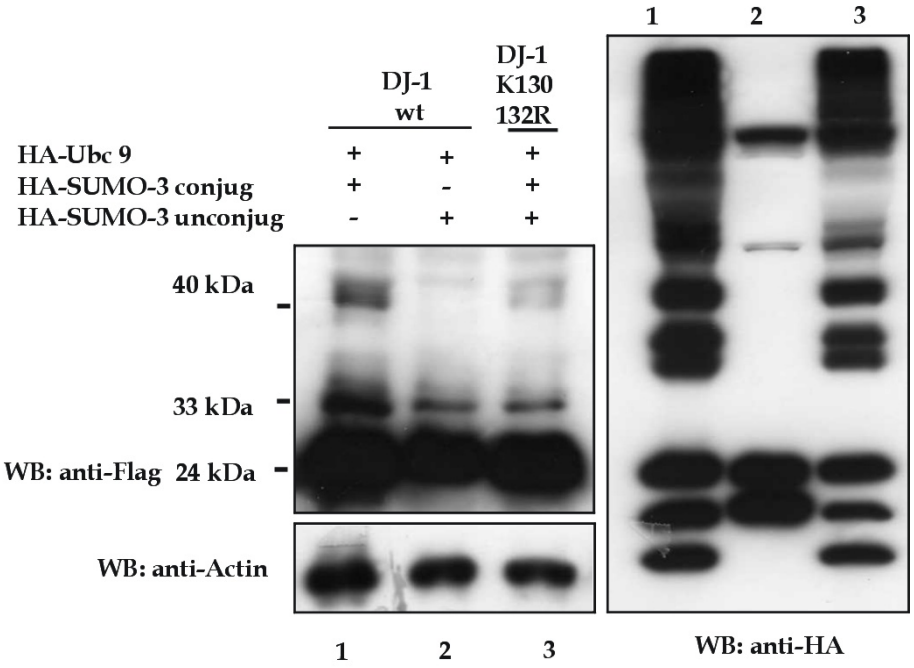


Figure 33: Sumoylation analysis of DJ-1 Lys 130/132 Arg respect to the wild type protein with anti-Flag and anti-HA antibodies.

4.9 SUMOylation analysis of DJ-1 pathological mutants

SUMO-3ylation was analyzed for some DJ-1 mutant proteins associated to familiar form of PD. A particular attention was focused on the DJ-1 Leu166Pro (L166P) and DJ-1Met26Iso (M26I).

HEK293T were transfected with 2X-Flag-DJ-1-Leu166Pro or 2X-Flag-DJ-1 Met26Iso in the presence of the HA-Ubc9 and of the conjugable form of HA-SUMO-3. As negative control, cells were transfected in the presence of the HA-SUMO-3 GG91/92AA instead of the wild type protein.

As shown in **figure 34**, co-transfection of the mutant 2X-Flag-DJ-1-Leu166Pro, HA-Ubc9 and HA-SUMO-3 in the conjugable form led to the detection of hyper-sumoylated form of DJ-1-LeuL166Pro respect to the wild type DJ-1 protein. A stronger signal was detected for the band at 42 kDa while a band at 60 kDa band was also visible.

These results indicated that the amount of 2X-Flag-DJ-1-Leu166Pro covalently modified by SUMO-3 is higher respect to the wild type protein and that or it is polysumoylated by a poly-SUMO chain or multi-sumoylated on different Lysines. The nature of the modification is still not known and further experiments are required to investigate this aspect.

The 2X-Flag-DJ-1 Met26Iso displayed a modification comparable to the 2X-Flag DJ-1 wild type protein (**figure 34**).

Results are representative of at least three separate experiments.

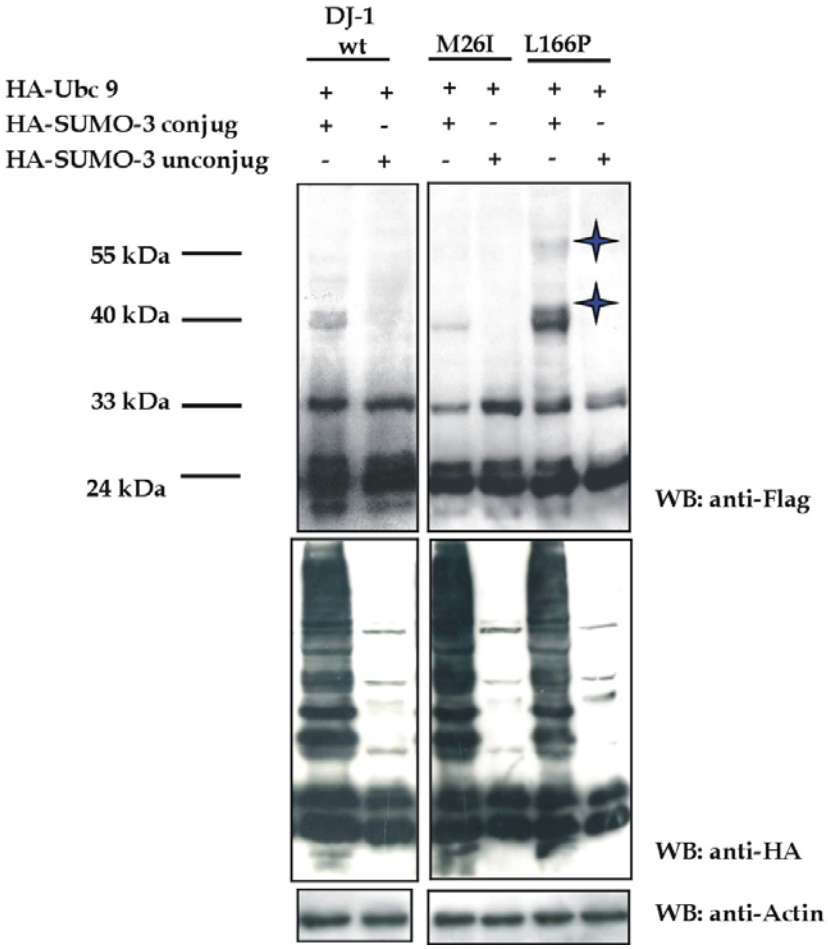


Figure 34: SUMOylation analysis of DJ-1 pathological mutants with anti-Flag and anti-HA antibodies. The DJ-1-Leu 166Pro is Hypersumoylated respect to the wild type protein and DJ-1-Met26Iso

4.10 SUMOylation analysis of DJ-1 functional mutants

SUMO-3ylation was analyzed for some functional DJ-1 mutants, which carried the point mutation Cys53Ala (C53A) and Cys106Ala (C106A). As described in the introduction, several studies indicated that reactive Cysteines play a critical role in the function and/or regulation of DJ-1 activity, which is consequently abrogated by mutagenizing Cys to Ala.

HEK293T were transfected with 2X-Flag-DJ-1 Cys53Ala or 2X-Flag-DJ-1 Cys106Ala in the presence of the E2-conjugating enzyme, HA-Ubc9, and the conjugable form of HA-SUMO-3. As negative control, cells were transfected in the presence of the unconjugable HA-SUMO-3.

As shown in figure 35, DJ-1-Cys53Ala and Cys106Ala displayed a sumoylation comparable to the wild type form. Results are representative of at least three separate experiments.

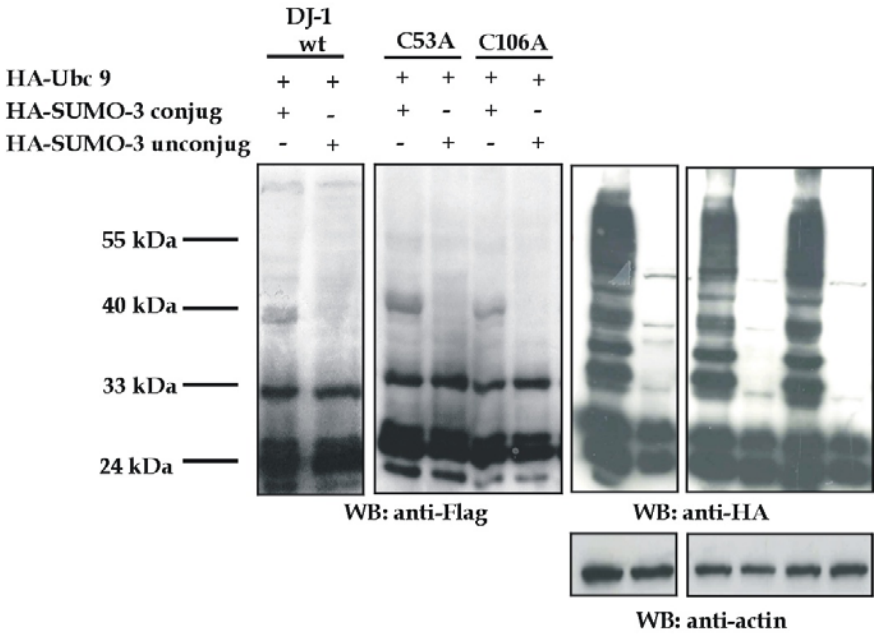


Figure 35: SUMOylation analysis of DJ-1 functional mutants with anti-Flag and anti-HA antibodies.

5 MAY OXIDATIVE STRESS REGULATE DJ-1 SUMO2/3YLATION? LINKING DJ-1 AND SUMO2/3 TO A COMMON PATHWAY

Treatments with drugs that mimic *in vitro* a neurochemical model of PD have been performed in human neuroblastoma cell lines. For the mitochondrial dysfunction and oxidative stress, hydrogen peroxide, DA, 6-OHDA, were analyzed. For the proteosomal impairment, MG-132 was used. The global endogenous sumoylation and the DJ-1 expression were analyzed.

5.1 Treatments of SH-SY5Y cells with Hydrogen Peroxide (H_2O_2)

Confluent neuroblastoma cell lines have been treated with different increasing concentrations of hydrogen peroxide for 30 minutes. The initial goal was to follow the observation by others that oxidative stress induced SUMO-2/3 conjugation at high concentration of H_2O_2 (Manza *et al.*, 2004; Saitoh and Hinchey, 2000; Zhou *et al.*, 2004) in mammalian cell lines. Cells were lysed in Laemmly Buffer and whole protein extracts have been analyzed into a 6%-12% PAA gels. Western Blots were probed with antibody anti-SUMO-2/3 (Zymed), anti DJ-1 (home-made) and anti-Actin (Sigma). The protein analysis using a 6-12% PAA gel was necessary to observe both the high molecular masses conjugates to SUMO-2/3 and the free form of SUMO-2/3. Results shown in **figure 36** are representative of three separate experiments. SH-SY5Y treated with H_2O_2 showed changes in the conjugation of SUMO-2/3. Global SUMO-2/3ylation was induced in SH-SY5Y treated with 100 mM H_2O_2, while a decrease in their free form was showed.

Interestingly, lower concentration (1mM for 1 hr) induced a severe reduction in the level of SUMO-2/3 conjugation as demonstrated by Bossis and Melchior, 2006.

These experiments indicated that different amount of hydrogen peroxide concentration affected SUMOylation differently. From the literature it's now clear that low hydrogen peroxide concentration (1mM for 30 minutes) prevents Ubc9-SUMO thioester formation and induces Uba2-Ubc9 crosslink through a disulfide bond (Bossis and Melchior, 2006).

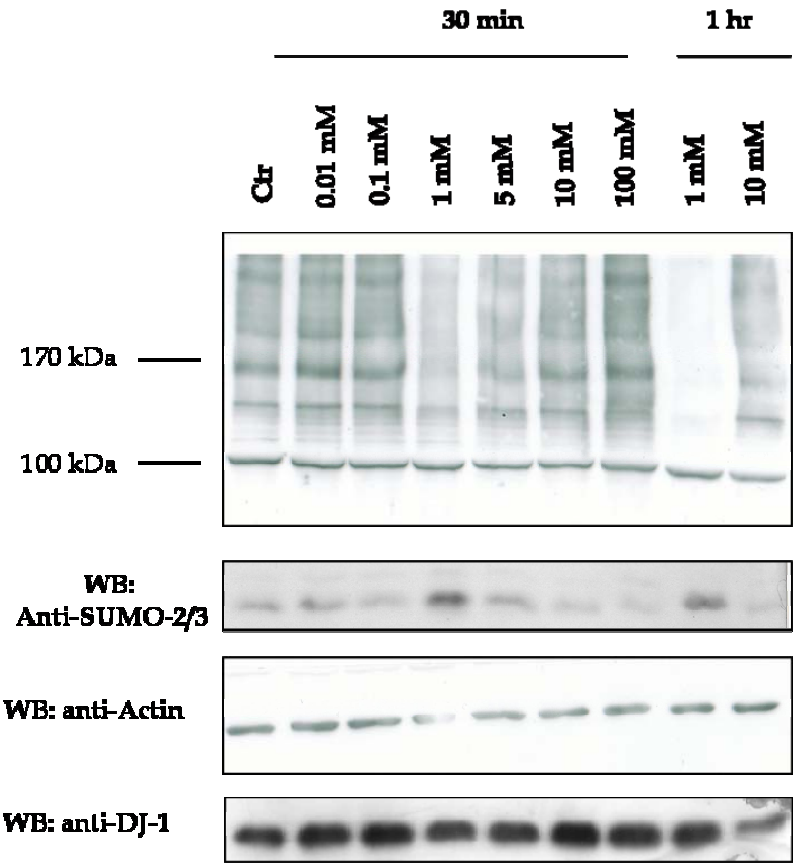


Figure 36: SUMO-2/3 and DJ-1 expression analysis after treatments with different amount of hydrogen peroxide for 30 minutes or 1 hr.

Global SUMOylation after oxidative stress treatments was also analyzed in immunofluorecence experiments using an anti SUMO-2/3 antibody. As for the western blot, confluent cells were treated with chosen amount of

H_2O_2 (0.1 mM and 100 mM for 30 minutes and 1mM for 1 hr). It was observed that when the SUMO-2/3ylation is inhibited or induced, the SUMO-2/3 proteins relocalized from the nucleus to the cytoplasm. Results are shown in **figure 37** and are representative of at least three separate experiments.

The physiological importance of this phenomenon is still under investigation.

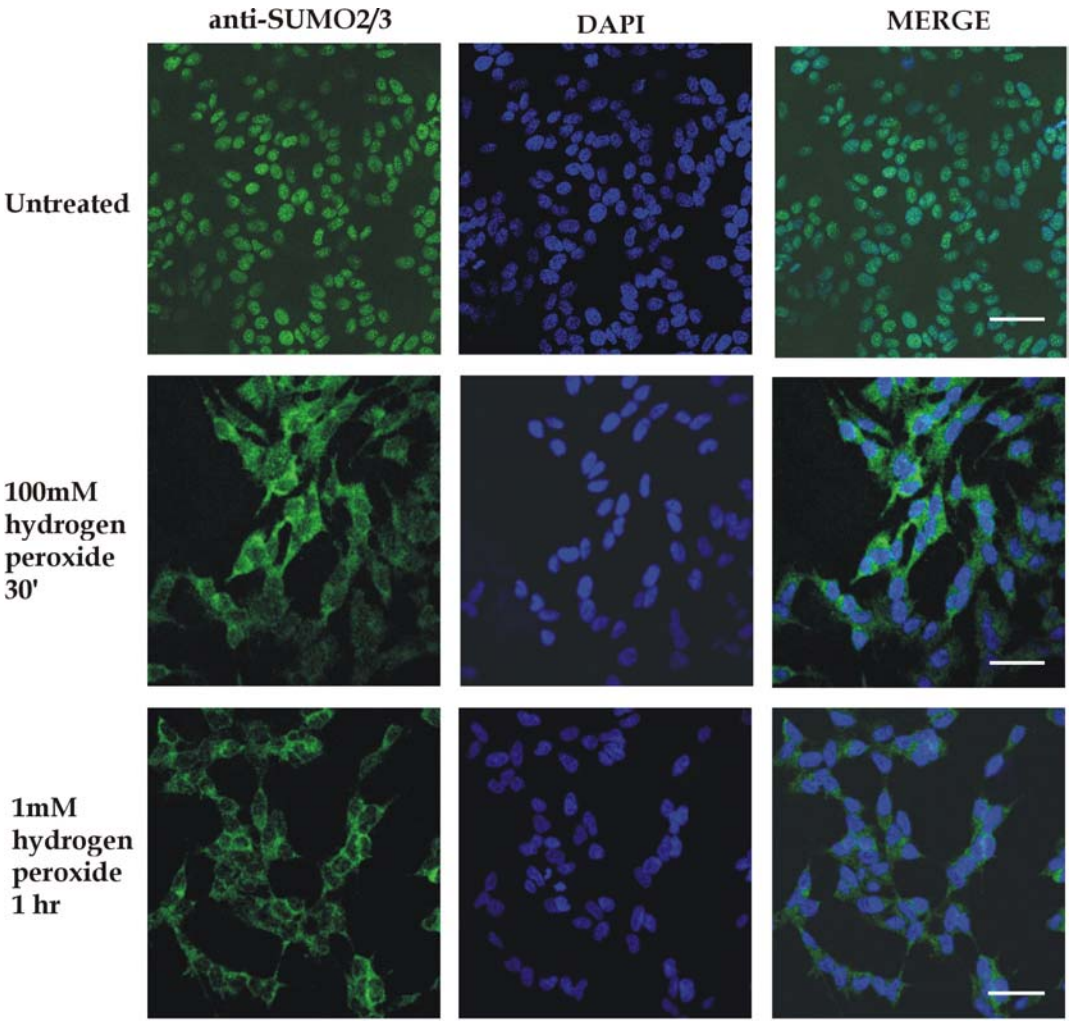


Figure 37: Immunofluorescence of SH-SY5Y untreated, treated with 100 mM H_2O_2 for 30 minutes and 1mM H_2O_2 for 1 hr. Re-localization from the nucleus to the cytoplasm was detected for both treatments. Scale bar 45 μm.

Similar results were also obtained with COS-7 cell lines. Treating with 100 mM hydrogen peroxide, SUMO-2/3ylation was massively induced (**figure 38A**) and the endogenous proteins relocalized from the nucleus to the cytoplasm (**figure 38B**) in some limited group of cells**.** COS-7 were also transfected with HA-SUMO-2 and HA-SUMO-3 and treated with hydrogen peroxide: both proteins relocalize from nucleus to cytoplasm as indicated in **figure 39.**

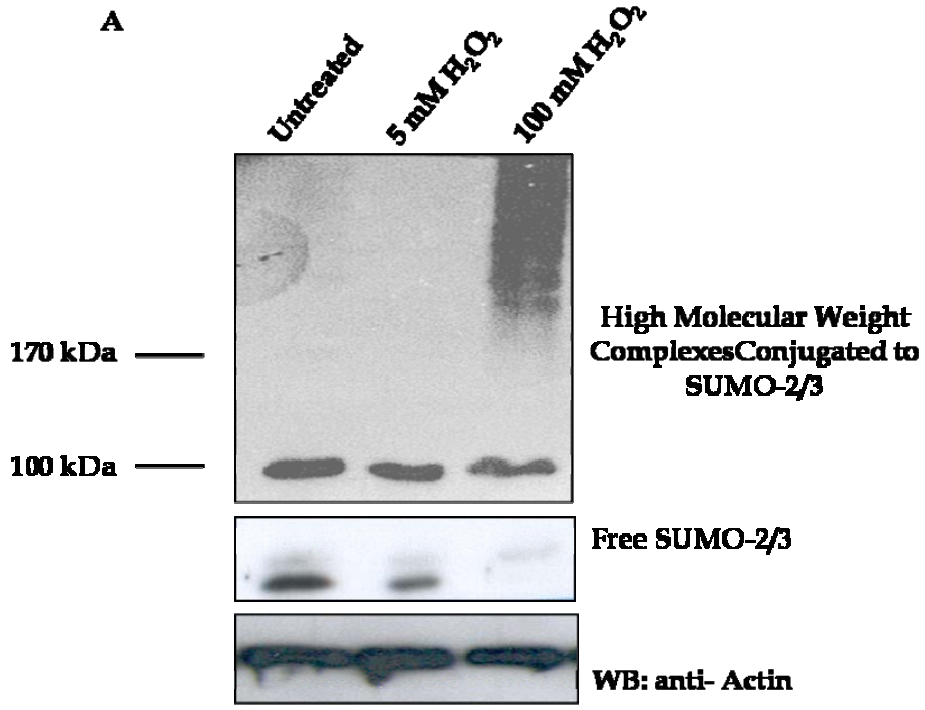


Fig 38A: Western blot SUMOylation analysis after hydrogen peroxide treatments on COS-7 at two different concentrations: 5 mM and 100 mM. Both treatments were carried out for 30 minutes. Results were compared to untreated cells.

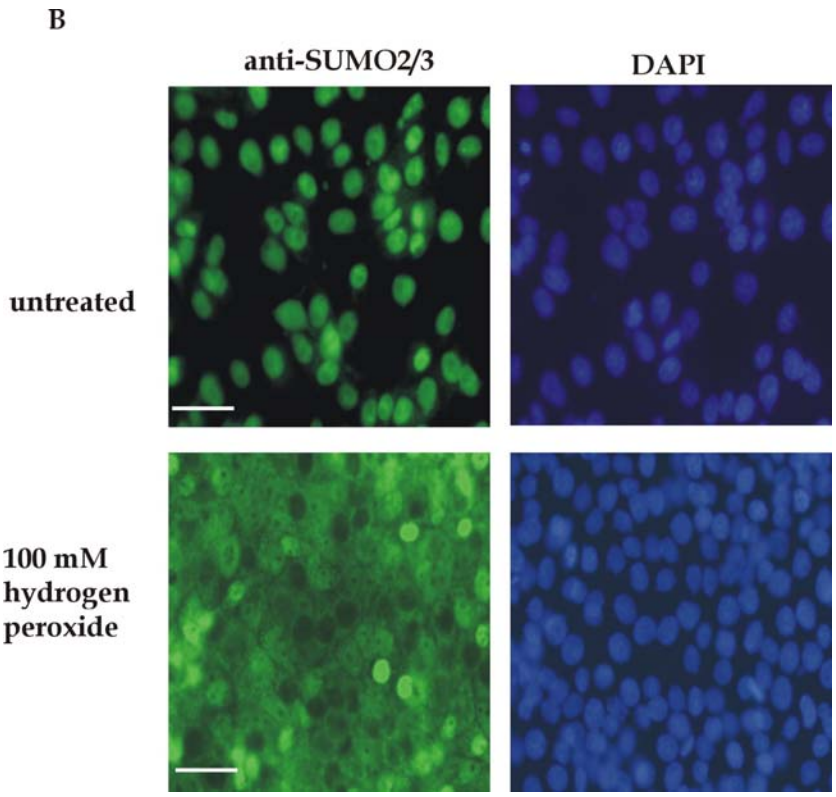


Fig 38:B. Immunofluorescence with anti-SUMO-2/3 antibody of untreated COS-7 and treated with 100 mM H_2O_2 for 30 minutes. The nucleus/cytoplasm relocalization in a limited group of cells was detected. Scale bar 45μm.

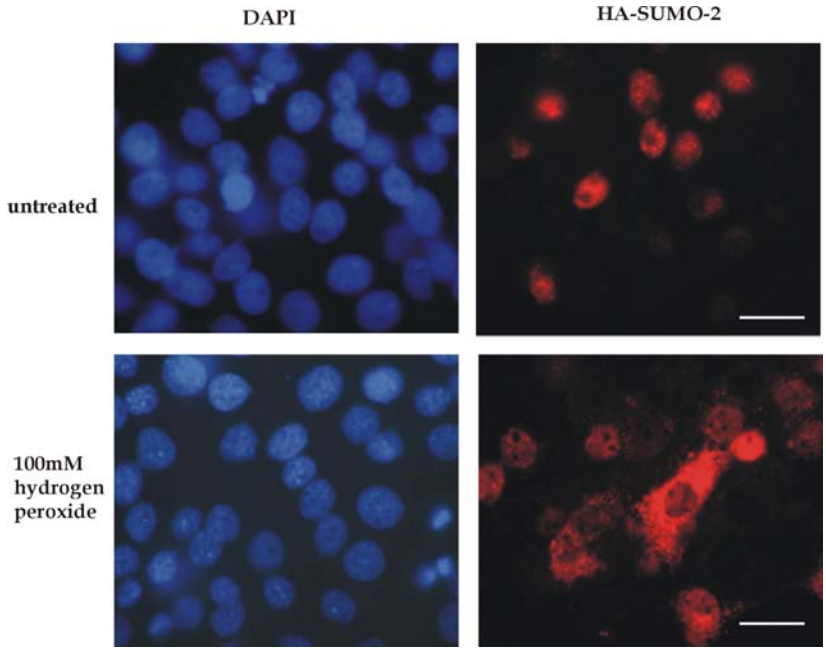


Fig 39A: Immunofluorescence with anti-HA of ectopic HA-SUMO-2 upon 100 mM H_2O_2 for 30 minutes. Scale bar 20 μm.

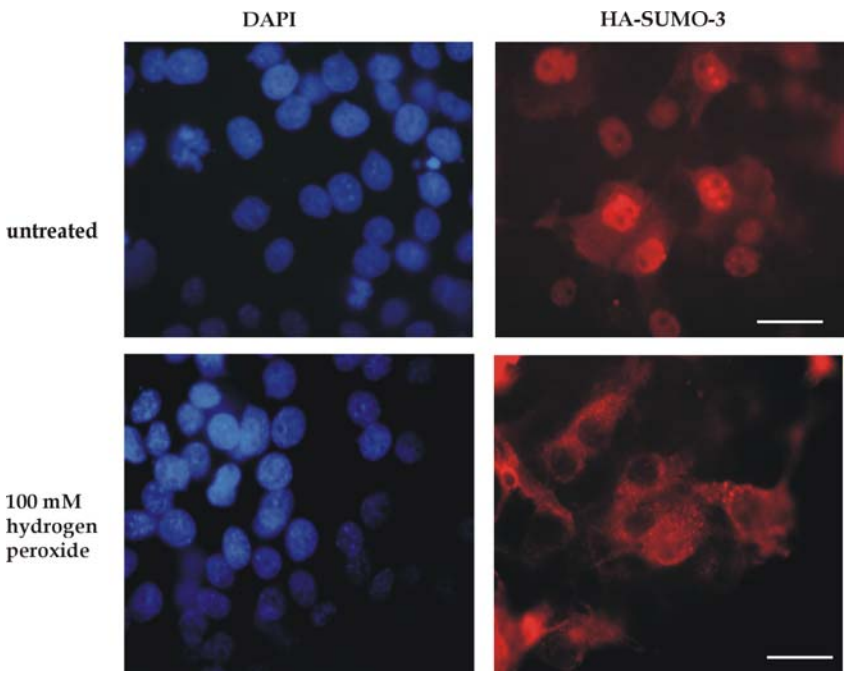


Fig 39B: Immunofluorescence with anti-HA of ectopic HA-SUMO-3 upon 100 mM H_2O_2 for 30 minutes. Scale bar 20 μm.

5.2 Treatments of SH-SY5Y cells with the proteasome inhibitor MG-132

SUMOylation was then analyzed after having treated confluent SH-SY5Y with 0.25, 0.5 μM, 5 μM MG 132, an inhibitor of proteosomal function, for both 1 hr and 24 hrs. Untreated cells and cells treated with the vehicle (DMSO) were considered controls. As shown in the **figure 40A** at 1 hr of treatments for each concentration, no changes in the SUMO-2/3 conjugation were detected, while for treatments performed at 24 hrs a massive SUMO-2/3 conjugation inhibition was observed (**figure 40B**). For DJ-1 no expression change was detected at 1 hr, while at 24 hrs, DJ-1 expression is decreased respect to the vehicle. Results shown in **figure 40** are representative of three independent experiments.

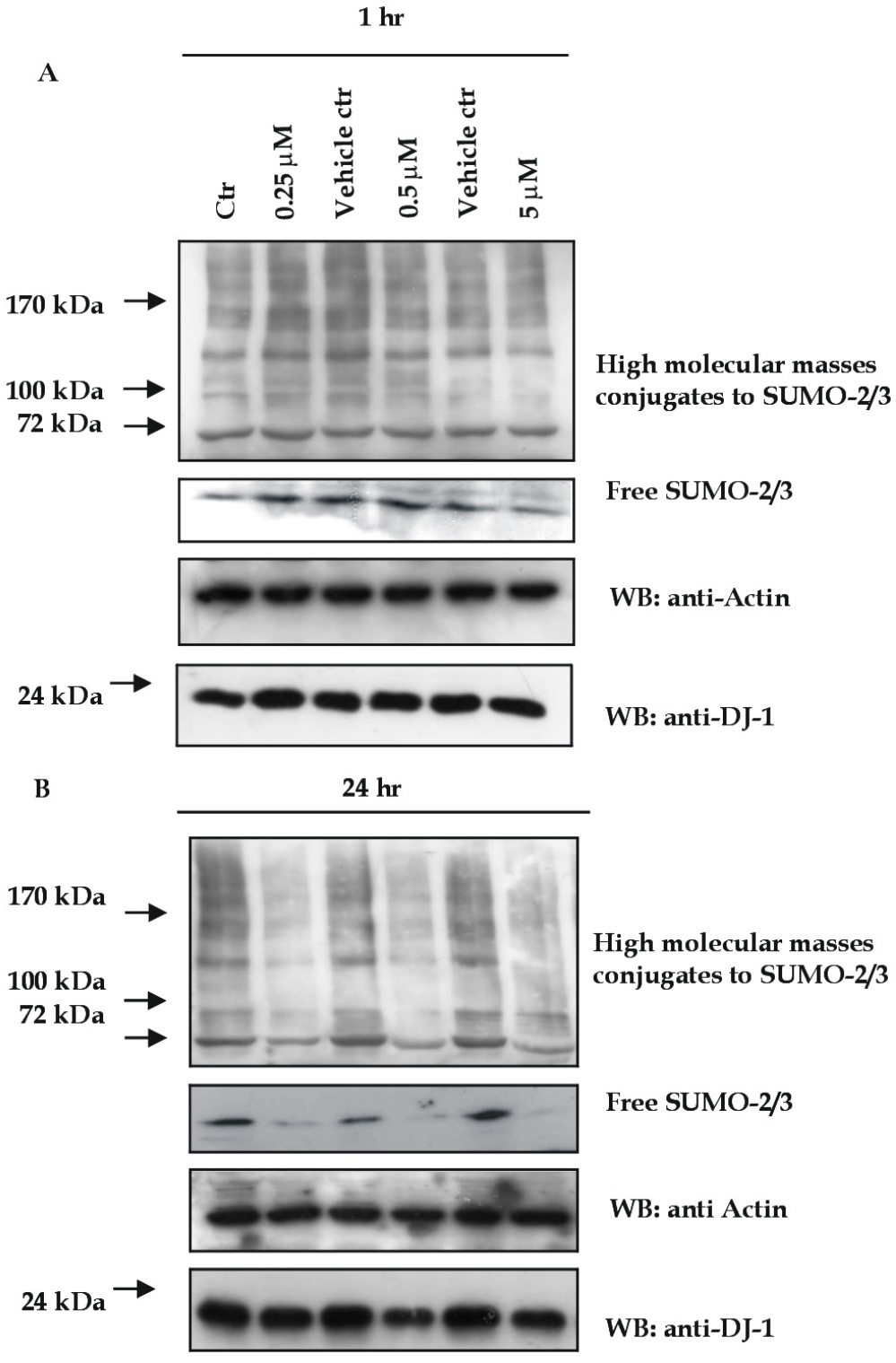


Figure 40) Treatments with different concentration of MG-132 at 1 hr (A) and 24 hr (B) on SH-SY5Y. Western blots were revealed for anti-SUMO-2/3 and anti-DJ-1 antibodies.

5.3 Treatments of SH-SY5Y cells with the HNE

Confluent SH SY5Y cells were also treated with HNE, a drugs that induces lipid peroxidation. It was demonstrated that at 100μM for 30 minutes it was able to induce, as for treatments with 100 mM H_2O_2, a massive SUMO-3 conjugation (Manza *et al.*, 2004). A titration experiment was performed and the data was confirmed. Treatments performed in our laboratory found an increase in the high molecular masses conjugates to SUMO-2/3 in a dose dependent manner (A.Chesi, personal communication). In these conditions DJ-1 showed a decreased expression in a dose dependent manner. The experiment was repeated in triplicates and **figure 41** shows the representative result.

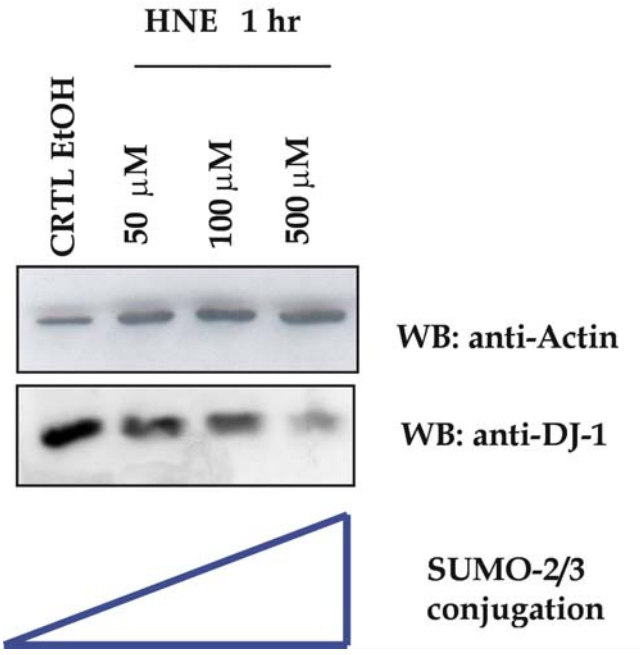


Figure 41: DJ-1 expression analysis after increasing concentration of HNE treatments.

5.4 Treatments of SH-SY5Y cells with the MPP^+

Confluent SH SY5Y has been treated with the Parkinsonian toxin MPP^+ since previous results showed that SUMO-2/3 conjugation decreased in a dose dependent manner (A.Chesi, personal communication). In these conditions, DJ-1 showed an increase in protein expression.

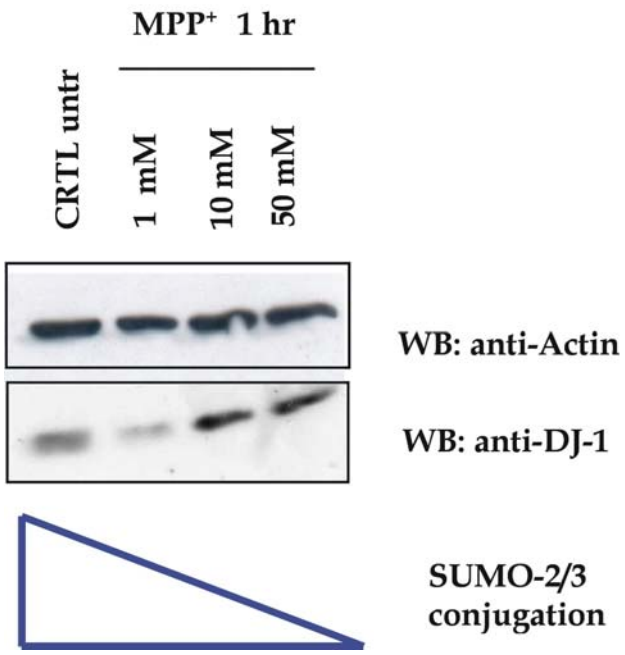


Fig 42: DJ-1 expression analysis after increasing concentration of MPP^+ treatments.

5.5 Treatments of SH-SY5Y cells with the Dopamine and 6-OHDA

Confluent SH-SY5Y cells were treated with DA at different concentrations: 10, 100, 250, 500 μM and 1mM for 24 hr; 500μM and 1mM for 1hr. Both SUMO-2/3 conjugation and DJ-1 expression were analyzed in western blot. Experiments were repeated in triplicates and representative results are shown in **figure 43.** DA didn't affect the global SUMO-2/3 conjugation

since no differences were detectable respect to the untreated sample. Similar results were obtained treating SH-SY5Y cells with 6-OHDA (data not shown). Furthermore, immunofluorescence analysis didn't display SUMO-2/3 relocalization detected after the hydrogen peroxide and the MPP^+ treatments (**figure 44**).

The level of DJ-1 was induced at 24 hr with 100, 250, 500 μM DA and at 1 hr with 1 mM DA treatment.

All immunofluorescence and western blot analyses are representative of at least three separate experiments.

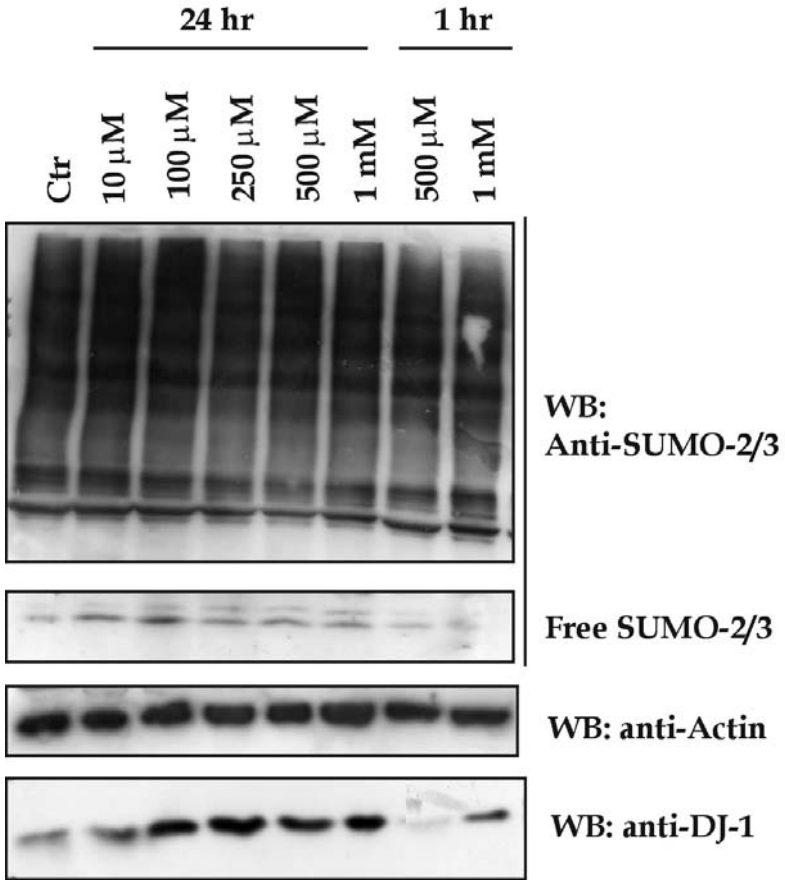


Fig 43: SUMO2/3 protein conjugation and DJ-1 expression analyses after treatments with DA.

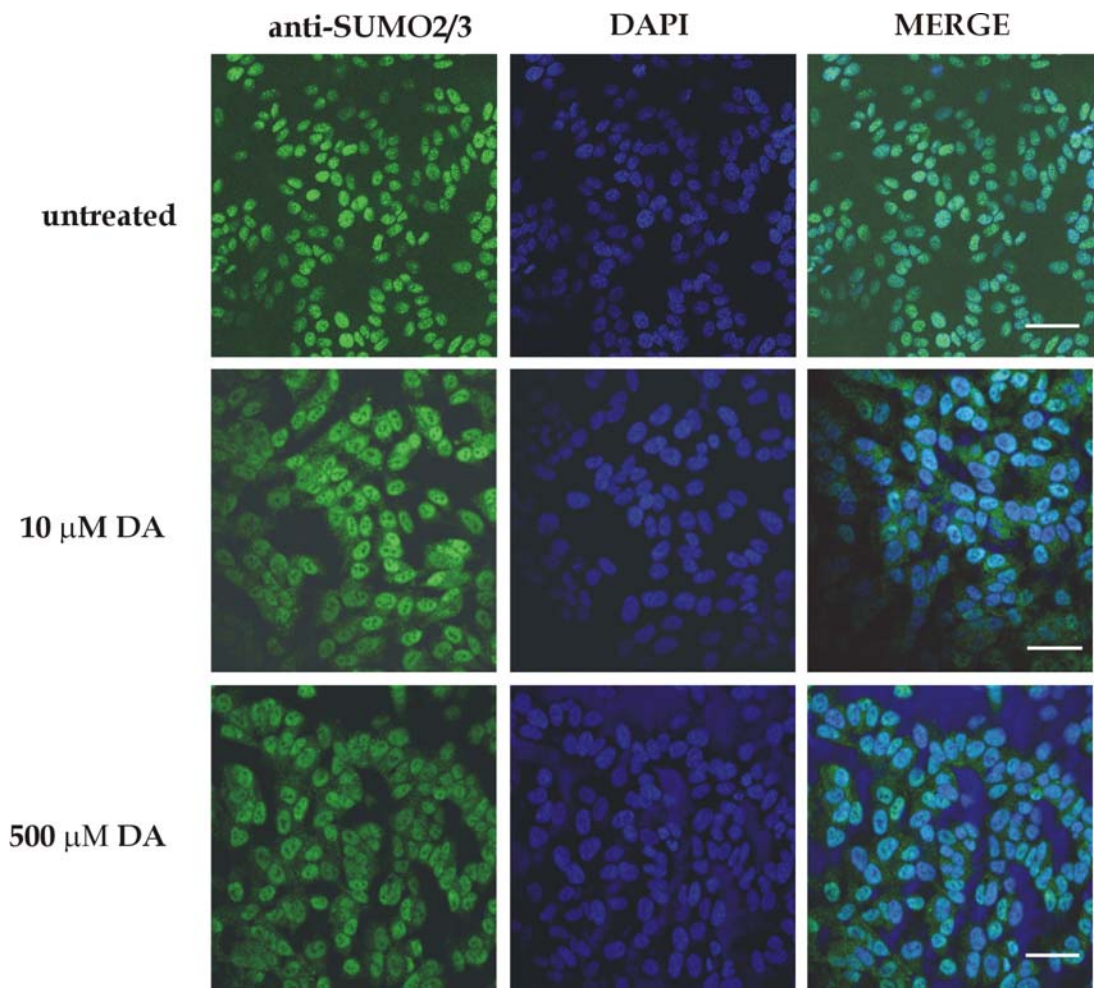


Fig 44: Immunofluorescence with anti-SUMO-2/3 on SH-SY5Y treated with 10 and 500 μM DA. Scale bar 45 μm.

6 *IN VIVO* CHARACTERIZATION OF SUMO-2/3 POSITIVE CELLS

6.1 Neuronal SUMO-2/3 expression analysis

The expression of endogenous SUMO-2/3 protein was investigated in *substantia nigra* by immunofluorescence experiments.

Mouse brain coronal slices were stained with anti-SUMO-2/3, anti-NeuN antibodies and nuclei were counterstained with DAPI. The anti-NeuN antibody recognizes a neuron-specific nuclear protein and reacts with mostly neuronal cell types with a primarily staining in the nucleus and a lighter staining in the cytoplasm.

As shown in the **figure 45**, SUMO-2/3 expression was detected in neurons of the midbrain. As expected, other regions, such as *hippocampus,* were positive for SUMO-2/3 (**figure 46**). SUMO-2/3 protein showed a clear nuclear expression in all NeuN positive cells analyzed, in accordance to the cellular localization detected in mammalian cell lines.

6.2 SUMO-2/3 expression analysis in *substantia nigra* dopaminergic cells

SUMO-2/3 expression was analyzed in the dopaminergic neurons, using the specific labelling with the anti-TH antibody. SUMO-2/3 is expressed at in the nucleus of dopaminergic neurons. Results are shown in **figure 47**

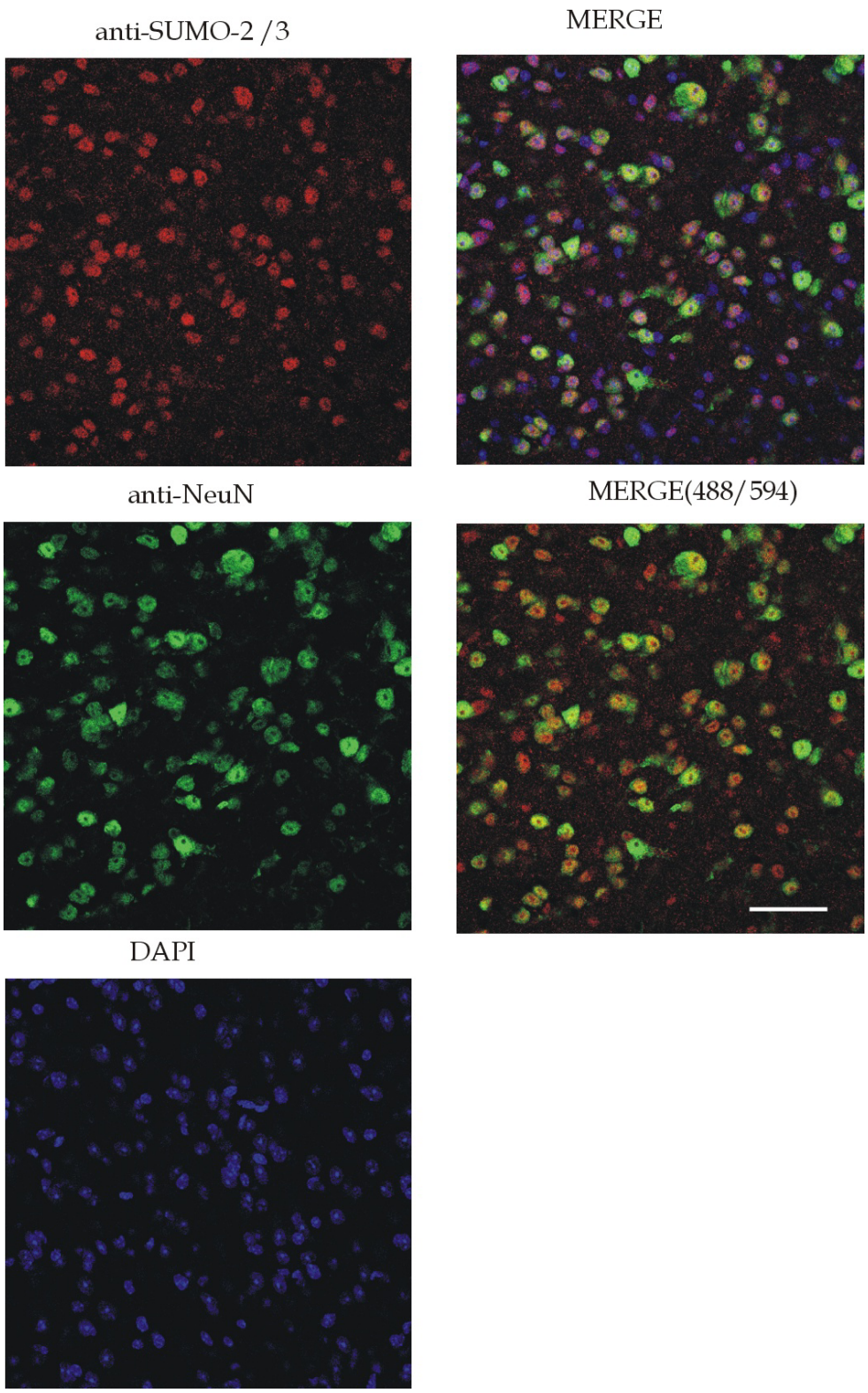


Fig 45: Immunocytochemistry of midbrain sections with anti-NeuN and anti-SUMO-2/3 antibodies. Nuclei were stained with DAPI. Scale bar 45 µm

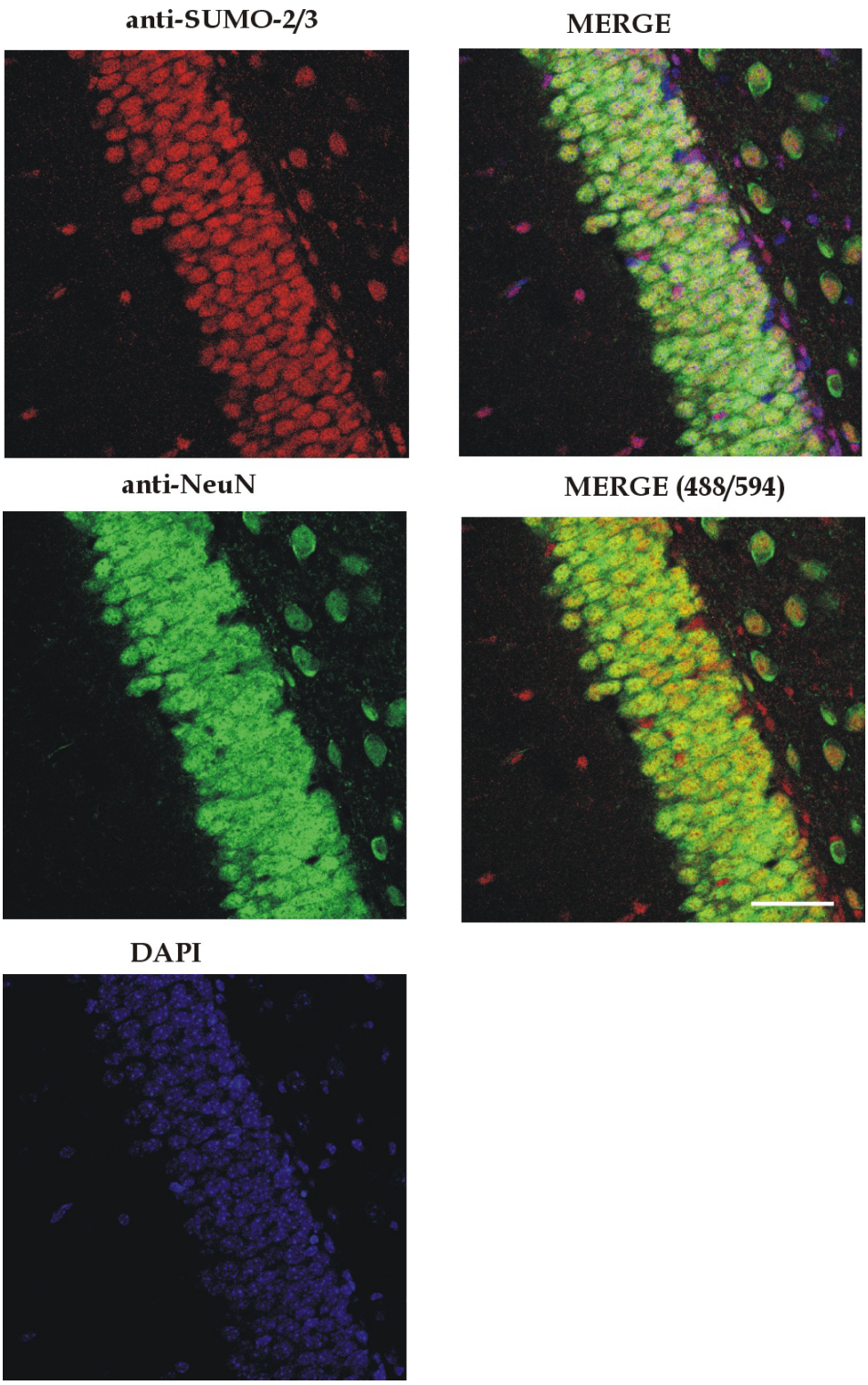


Fig 46: Immunocytochemistry of hippocampal section with anti-SUMO-2/3 and anti-NeuN antibodies. Nuclei were stained with DAPI. Scale bar 45 µm.

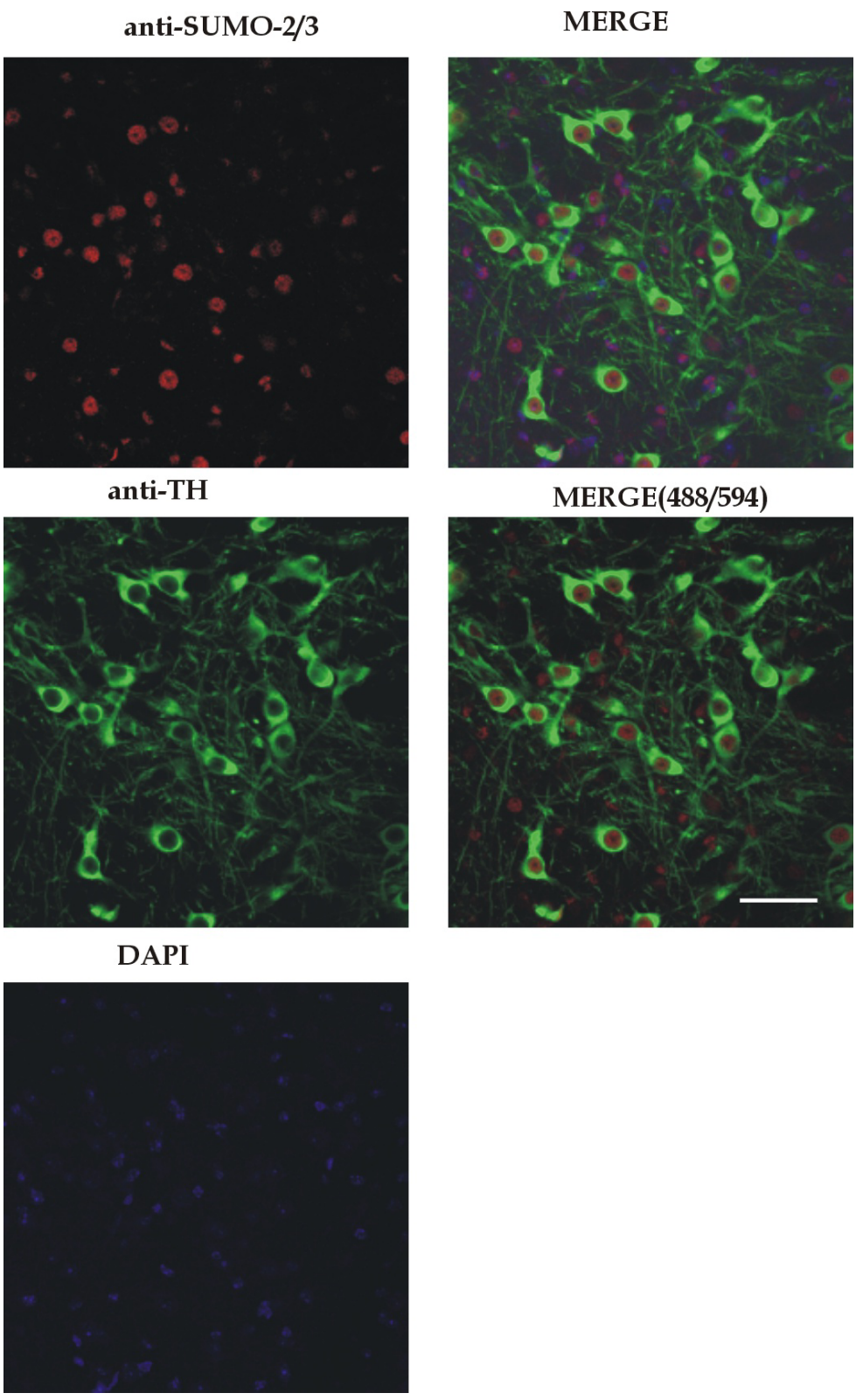


Fig 47: Immunocytochemistry of midbrain section with anti-SUMO-2/3 and anti-TH antibodies. Nuclei were stained with DAPI. Scale bar 45 µm

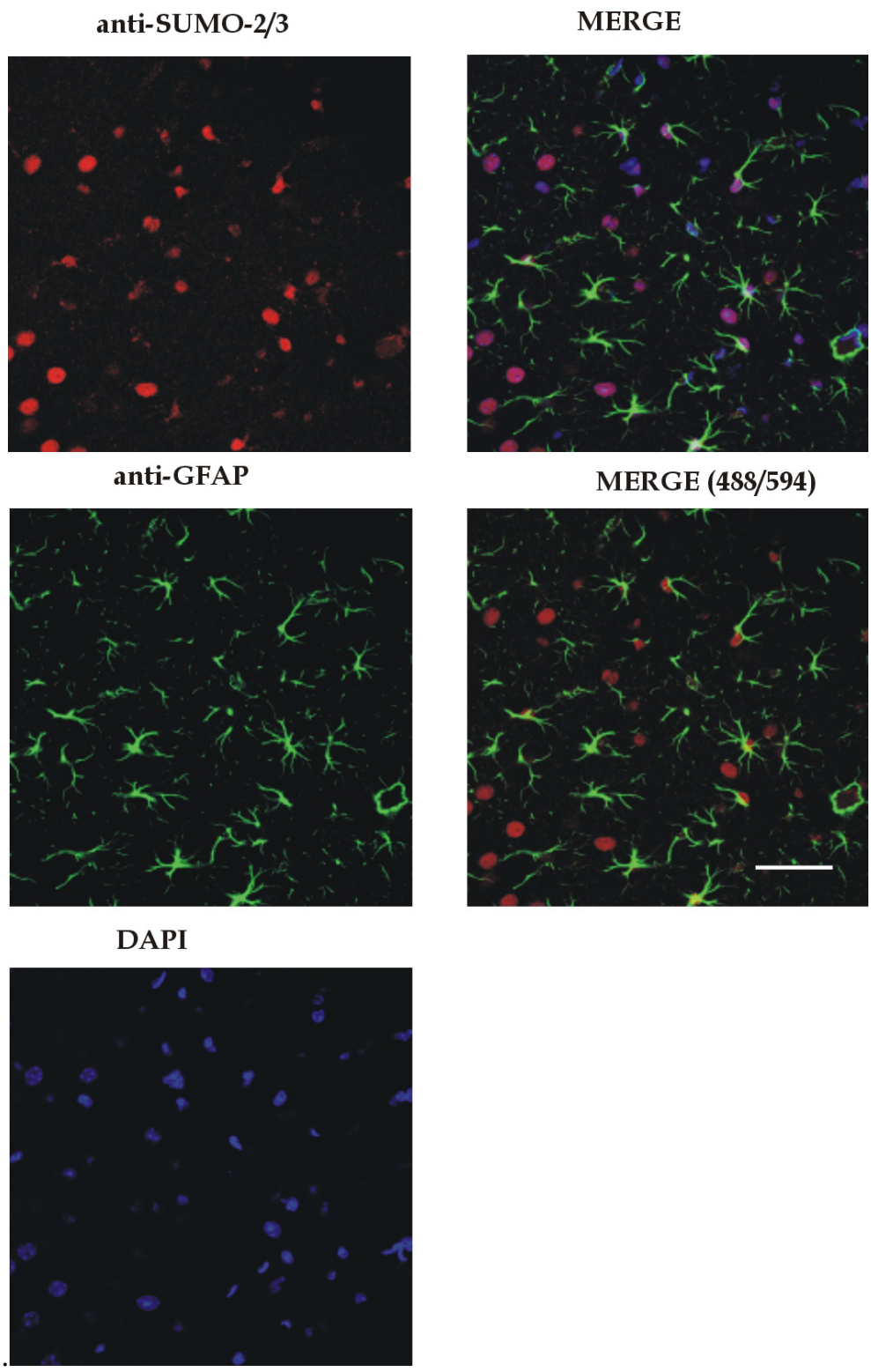


Figure 48: Immunocytochemistry of midbrain sections labeled with anti-SUMO-2/3 and anti-GFAP antibodies. Nuclei were stained with DAPI. Scale bar 45µm

6.3 SUMO-2/3 expression analysis in glial cells

Since some positive SUMO-2/3 cells were NeuN negative, mouse brain coronal slides were stained with anti-GFAP antibody, a glial marker.
Indeed SUMO-2/3 is present at the nuclear level also in astrocytes, as shown in **figure 48.**

6.4 Downregulation of SUMO-2/3 expression upon MPTP treatments

The expression of SUMO-2/3 was then investigated in mouse *substantia nigra* upon MPTP acute treatments. Mice received one injection of MPTP every two hours for a total of four doses over an 8 h period in one day. Slices were made 6 hrs and 12 hrs after the last injection.
As shown in **figure 49**, in the *substantia nigra* of MPTP-treated mice SUMO 2/3 was downregulated in its expression in all cells of the midbrain and not only in the dopaminergic neurons. This experiment was repeated five times. Because the signal strength for SUMO-2/3 in *substantia nigra* MPTP-treated mice was lower than the staining in untreated conditions, slices were also analyzed by increasing the photomultiplier tube voltage on this channel, confirming the almost complete absence of staining.
All images were collected with the same level of photomultiplier tube voltage in 594 channel. Results shown in **figure 49** are representative of five independent and separate experiments.

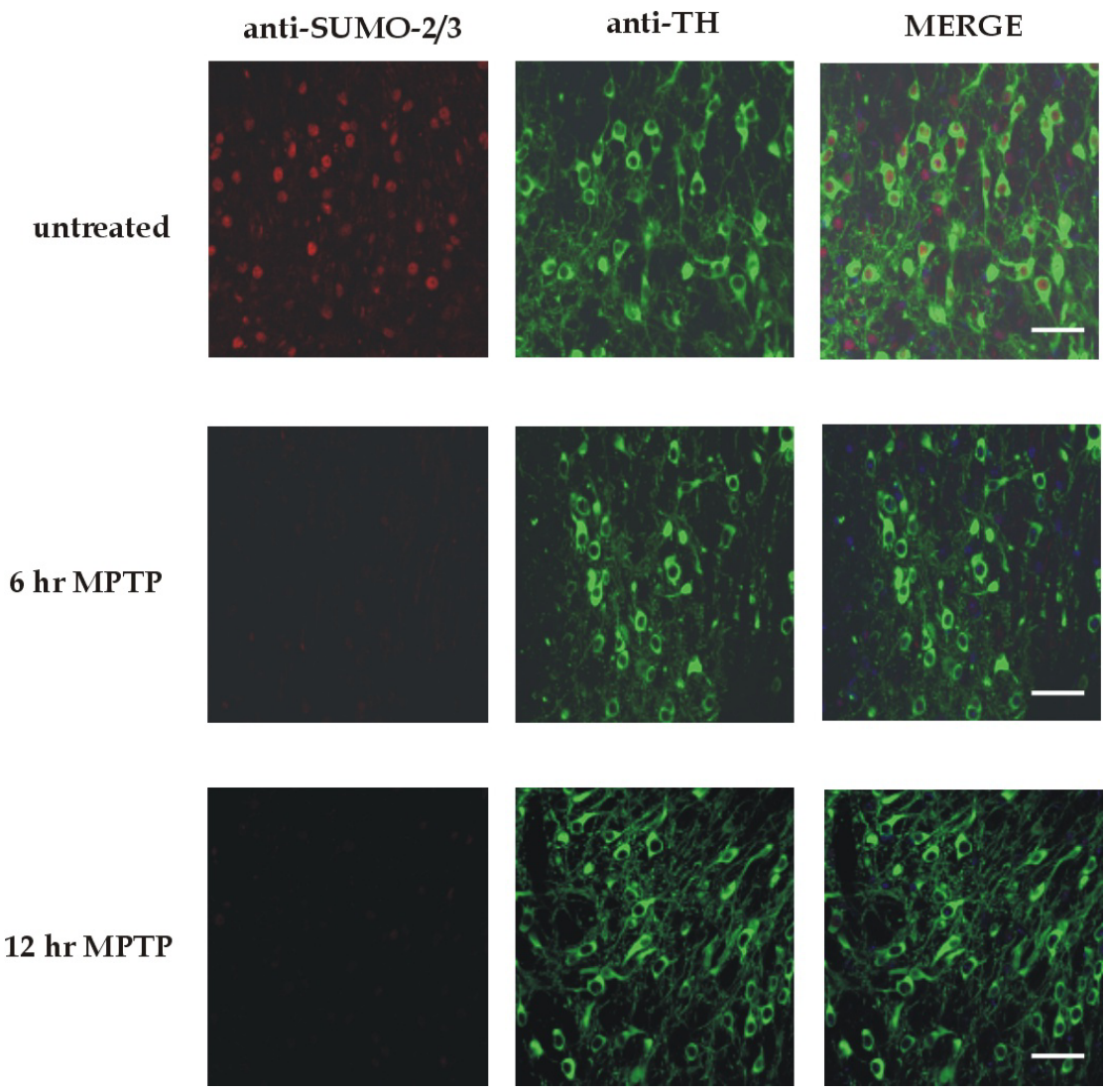


Figure 49: Immunocytochemistry of midbrain sections labelled with anti-TH and anti-SUMO-2/3. ***Substantia Nigra*** **was isolated from untreated mice and treated with MPTP after 6 and 12 hours the last injection. Scale bar 45 μm.**

DISCUSSION

Parkinson's Disease (PD) is the second most common neurodegenerative disorder after Alzheimer Disease (AD). The etiology of the majority of PD cases is still unknown, but it is likely to be a combination of both genetic and environmental factors. The recent identification of genetic loci and genes linked to familiar PD have consistently advanced the knowledge on the molecular mechanisms involved in the pathogenesis.

Recently it has been found that mutations in DJ-1 are the second cause of EOARPD after Parkin (Bonifati *et al.*, 2003). A neuroprotective role of DJ-1 has been proposed but its exact function is still unknown.

To study the molecular mechanism of DJ-1-mediated protection against the nigrostriatal degeneration, an Yeast Two Hybrid Screening has been performed in the laboratory of Prof. S. Gustincich.

The prey consisted of components of an yeast expression library synthesized from cDNAs of the human *foetal* brain. In the screening, eleven different interactors have been identified and confirmed in yeast. One of these clones encoded DJ-1 itself, a finding that proved the validity of the screening and consistent with reports that DJ-1 may act as a homodimer.

Among all the DJ-1 interactors, this thesis focused on the DJ-1 modification by SUMO, since Small Ubiquitin Modifiers 1 (SUMO-1), SUMO-activating enzyme Uba2 and SUMO conjugating enzyme Ubc9 were fished out.

While we were investigating the potential DJ-1 modification by SUMO-1, Shinbo *et al.* reported that DJ-1 is sumoylated by SUMO-1 at Lys 130, while Junn *et al.* reported that SUMO-1, Uba2 and Ubc9 are interactors of

DJ-1 in YIIH using a human adult brain cDNA library. These findings validated the quality and the specificity of the screening method.
We first tried to detect SUMO-1ylated DJ-1, however our efforts were unsuccessful (data not shown).
Since HEK293T is commonly used to analyze SUMO conjugation, all our experiments were carried out in this cell line. To detect DJ-1 modification by SUMO-1, experiments were repeated in similar conditions to those published, but the post translational modification of DJ-1 was never detectable. Shinbo *et al.* examined the DJ-1 SUMO-1ylation in human H1299 (human lung cancer cell line) and they detected SUMOylation on both endogenous DJ-1 and with ectopic Flag-DJ-1 co-transfected with T7-SUMO-1 and Ubc-9-HA. Furthermore, the same author reported that HA-PIASxα and HA-PIASγ bind DJ-1 and they function as E3-SUMO-1 ligases (Takahashi *et al.*, 2001).
Since a different cell line was used in the paper of Shinbo *et al.*, we may suggest that a different cell background could alter the effects of ectopic protein expression and the consequent detection of the post translational modification. Furthermore, GFP-SUMO-1 was transfected instead of a T7-SUMO-1. GFP-SUMO-1 can modify different substrates as demonstrated for GATA-1 (Collavin *et al.*, 2003) and p53 (Gostissa *et al.*, 1999) but in the case of DJ-1 a steric hindrance may occur.

This thesis demonstrated that DJ-1 is modified *in vitro* and *in vivo* by SUMO-2/3. Interestingly these two proteins were not isolated from the YIIH screening: probably they are not present in the library or they are weakly expressed. No experiments have been done to validate these hypotheses.
The YIIH found Uba2 and Ubc9 as DJ-1 protein interactors. It is important to note that Ubc9 doesn't discriminate between SUMO-1, SUMO-2 and SUMO-3 (Tatham *et al.*, 2001) and that it is the only E2-conjugating

enzyme known in vertebrates. Several papers investigated protein SUMOylation after having fished out enzymes belonging to the SUMO conjugation pathway, as Ubc9 or PIAS (Giorgino *et al.*, 2000; Pan *et al.*, 2006; Martin *et al.*, 2007)

We confirmed that DJ-1 interacted with ectopic HA-Ubc9 *in vitro*, opening the intriguing hypothesis that DJ-1 could be modified by SUMO moieties different from SUMO-1.

Unfortunately, it is still not known the subcellular compartment in which DJ-1/HA-Ubc9 interaction takes place. Cellular fractionation and immunoprecipitation are necessary to explore this aspect. The DJ-1/Ubc9 interaction may have also a biological function independent on the E2-conjugating activity, as demonstrated for the transcriptional factor SOX-4 (Pan *et al.*, 2006) and for the GLT4, GLT1 glucose transporter (Giorgino *et al.*, 2000).

This study demonstrated that DJ-1 is modified by SUMO 2/3 *in vitro.* The post translational modification was detected both on endogenous proteins and overexpressing the SUMOylation pathway and DJ-1 in HEK293T.

The SUMO-2/3 ylation of DJ-1 was further shown *in vivo* in adult mice *substantia nigra* and in *striatum*, the two brain regions involved in the PD dopaminergic nigrostriatal degeneration.

It will be interesting analyzing this modification in other brain regions and in different tissues. The DJ-1 SUMOylation may exert a tissue-specific biological function.

To detect endogenous DJ-1-SUMO-2/3 modification, immunoprecipitation experiments were essential, but different technical difficulties have been met. First of all, the amount of modified DJ-1 was very low; this aspect could be due to at least two different reasons. First, the Sulfhydryl alkylating agent inhibitor *N*-ethylmaleimide (NEM) is not specific for blocking the process of desumoylation, since it covalently modifies Cys residues in proteins, irreversibly inhibiting the formation of

Cys linkage. Second, the amount of DJ-1 that is modified in a specific moment could be very low, similarly to the majority of the sumoylated proteins identified *in vivo*.

A great improvement in the detection of the DJ-1 modification was obtained after the affinity purification of the anti-DJ-1 antibody. The home-made anti-DJ-1 antibody enriched the amount of immunoprecipitated DJ-1 respect to the commercial available ones, allowing the detection of modified DJ-1 fraction. To further validate the endogenous DJ-1 modification by SUMO-2/3, a proteomic approach was specifically designed.

The proteomic approach is generally used to overcome the difficulty of identifying low-abundant sumoylated proteins. Cell lines stable transfected with tagged SUMO are characterized by a dramatic higher sumoylated protein levels than that of untransfected cells (**Table 2**) (Li *et al.*, 2004; Rosas-Acosta *et al.*, 2005, Vertegaal *et al.*, 2006). Cell lysates are then used for immunoprecipitation or affinity chromatography purification and the isolated sumoylated proteins are trypsinized and identified by Mass Spectrometry (MS).

In this thesis we used the bidimensional electrophoresis to confirm that endogenous DJ-1 is modified by SUMO2/3 *in vitro*. Furthermore, we have tried to confirm it using Mass Spectrometry. Experiments were designed in the following way: several immunoprecipitations were loaded together using anti-DJ-1 antibody and anti-IgG as control. Two SDS-PAGE were processed separately. The first was subjected to Blue Coomassie and Silver Staining and the second was blotted and revealed with anti-DJ-1 antibody. This last one was used as positive control of immunoprecipitated DJ-1, using the enhanced chemiluminescence system. This gel was further used as reference for the first one: the region in which the slower migrating band was detected would be cut and subjected to trypsinization. The main problem of this approach was that no differentially-immunoprecipitated

bands using the anti-DJ-1 antibody was detected with Blu Coomasie and Silver Staining respect to that performed with anti-IgG. These results indicated that the amount of endogenous DJ-1 modification was too low to be compatible to a proteomic approach such as Mass Spectrometry.

To confirm DJ-1 SUMO-2/3ylation, we resorted to Isoelectrofocusing (IEF). Since the amount of modified DJ-1 is low, we were obliged to overload the strips with 1mg of proteins. Although the overloading explained the weak focalization of DJ-1 itself, it allowed the detection of SUMO-2/3-DJ-1. The perfect matching between the spot of modified DJ-1 and that one recognized by anti-SUMO-2/3 removed any possible doubts about endogenous DJ-1 SUMO-2/3ylation in HEK293T and confirmed the results previously obtained by Immunoprecipitation.

It is worth noting that preliminary experiments in which 300 μg of total cell lisates were subjected to IEF demonstrated a perfect focalization as previously shown (Taira *et al.*, 2004; Choi *et al.*, 2006) but didn't allow the detection of modified DJ-1 (data not shown).

In a second approach, largely used in literature, we analyzed DJ-1 sumoylation after overexpressing SUMO-2/3, Ubc9 and DJ-1 itself. A first analysis was focused on the characterization of the SUMO-2 and SUMO-3 conjugation pathways.

The conflicting designation of SUMO-2 and SUMO-3 in number of publications has led to confusion on nomenclature. This work used the original notation described in the National Center for Biotechnology Information database: (The SUMO-2 and SUMO-3 accession number are respectively AAIO7854 and AAHO8420.1)

To improve SUMO 2/3 conjugation, the co-transfection of Ubc9 was essential, while the transfection of wild type SUMO-2 or SUMO-3 alone induced a weak conjugation. Two forms of SUMO-3 were detected: the slower migrating form is the unactivated protein, the other is the activated one that has been subjected to the cleavage of the C-terminal. Since the

activation of SUMO-2 released only 2 amino acids, these two forms could not be detected. Using the strategy of PCR Site-Directed Mutagenesis the unconjugable forms of SUMO-2 and SUMO-3 have been created: the C-terminal di-Gly motif has been converted into a di-Ala motif and this mutation abrogated the possibility of ectopic SUMO-2 and SUMO-3 activation and conjugation to endogenous or overexpressed substrates. Truncating forms of SUMO, lacking the C-terminal Gly required for attachment to substrates (SUMO1/2/3Δ6), were also used.

The SUMO-2 and SUMO-3 protein localization was further analyzed. SUMO-2 in the conjugable form displayed mostly a nuclear and a nuclear bodies localization while the unconjugable forms partially relocalized in the cytoplasm. As reported for SUMO-1, SUMO-2 conjugation may also be involved in transcriptional regulation or in protein nuclear import.

Wild type SUMO-3 displayed an increased cytoplasmic localization respect to wild type SUMO-2. This indicated the possibility that SUMO-3 could modify different targets respect to SUMO-2, according to a different subcellular localization. Furthermore, since these two proteins share 95% of homology, they could modify the same targets but under different cellular events, leading to a different biological function. The different subcellular localization of SUMO-2 and SUMO-3 was according to that one reported by Su. *et al.*, 2002.

This work confirmed that DJ-1 could be conjugated to the SUMO family members, SUMO-2 and SUMO-3, in ectopic expression. The co-transfection of the E2-conjugating enzyme Ubc9 was essential to detect the DJ-1 modification. This result indicated that the amount of DJ-1 that is modified in physiological conditions is very low. Furthermore, it is still an open question whether this modification can be stabilized or induced upon specific cellular conditions, such as oxidative stress. In our experimental conditions DJ-1 was modified by both SUMO-2 and SUMO-3.

Since the efficiency of SUMO-3 conjugation was higher than that of SUMO-2, we carried out experiments on SUMO-3 overexpression.

A big challenge of this thesis was the identification of the Lys responsible of DJ-1 modification. Sumoylation of proteins has been shown to occur at specific Lys residues, which are in most cases embedded in a consensus sequence (I/L/V)*K*XE, where X represents any amino acid.

Several proteome-scale analyses have been performed to delineate the potential sumoylated substrates, but the exact sumoylation sites still remain to be identified. Only a small fraction of the substrates, often less that 1%, is sumoylated *in vivo* at any given time (Johnson, 2004). Therefore *in silico* identification of SUMO substrates with their respective sites are fundamental for understanding the mechanism of sumoylation-related regulations in eukaryotic cells and suggesting potential candidates for further drug design.

In this work *in silico* programs were insufficient to predict the sumoylation consensus sequence, most probably because they are designed for SUMO-1 conjugation: the pathway of SUMO-2/3 activation and conjugation and their substrates have still to be analyzed and compared to those modified by SUMO-1.

Very few substrates have been till now identified as targets for SUMO-2/3 modification *in vivo*: p53 (Li *et al.*, 2006), PML (Fu *et al.*, 2005), c-Myb, (Sramko *et al.*, 2006) Topoisomerase II (Azuma *et al.*, 2005), C/EBPβ (Eaton at al., 2003), Tau and α-synuclein. (Dorval *et al.*, 2006) All these substrates are also modified by SUMO-1.

Several questions are still open on the specificity of the sumoylation site. 1) Are the Lys responsible for SUMO 1 conjugation the same as for SUMO 2/3? 2) Which is the molecular mechanism that allow SUMO-2/3 modification of a specific substrate respect to SUMO-1?

It is known that in p53, SUMO-2/3 and SUMO-1 share the same conjugating site at Lys 386, and the same occurs for C/EBPβ in which the

major site of conjugation of SUMO-2/3 as well for SUMO-1 is Lys 173. Similar interplay occurs for c-Myb. In contrast, PML has three mayor sentrinization sites for SUMO -1: Lys 65, Lys 160 and Lys 490, while SUMO-3 is conjugated to Lys 160 and is essential for a stable nuclear localization of PML.

SUMO-1 is the most studied Ubiquitin like Modifier, but for some substrates the identification of Lys responsible of SUMOylation was very challenging. PCNA has 18 Lys residues which were individually changed to Arg. Only the combination K127/164R specifically altered the pattern of SUMO conjugates. It is worth noting that Lys 127 lies within a postulated SUMO-modification site, while Lys 164 is a non-consensus SUMO conjugation site, but is highly conserved from yeast to human (Hoege *et al.*, 2002).

Daxx was initially indicated to be sumoylated at Lys 630 and Lys 631 by Jang *et al.*, 2002, while a more recent paper indicated that 15 Lys have to be mutated together in order to completly abolish SUMOylation (Lin *et al.*, 2006). The authors argued as possible discrepancy the different cell line used in these works, but they didn't indicated the criteria by which they mutated specific Lysines. The Glucocorticoid (GC) receptor has also two major modification sites, Lys 277 and Lys 293, but faint bands were still visible suggesting that some sumoylation may occur on other Lys than those conforming to the consensus sequence ψKXE (Tian *et al.*, 2002).

It may be concluded that although in the majority of cases SUMOylation can occour on consensus motif predicted by *in silico* program, both false positives and false negatives may occur. Molecular mechanisms that regulate activation and conjugation have to be analyzed to solve these issues.

In the case of DJ-1, Lysines responsible of SUMO-3 conjugation are still unknown. Unlike other substrates, SUMO-3ylation occurs on different Lys

respect to SUMO-1 and since no single mutant abrogated the SUMO-3ylation, more than one Lys can be sumoylated alternatively.
A provocative hypothesis is that ectopic DJ-1 could be modified differently than the endogenous protein. Since all experiments are done overexpressing also SUMO-3 and Ubc9, the cell could lose the specificity of the modification in a satured cellular environment for SUMOylation. Alternatively, SUMO may have a random access on sumoylation sites normally hidden by the proper protein folding.

Further experiments are necessary to investigate the biological function of the hypersumoylated pathological mutant DJ-1 Leu166Pro (DJ-1 L166P). The hypersumoylation could be due to a multisumoylation on different Lys or to a di-SUMO chain modification on a single Lys (multisumoylation).
We may hypothesize that Sumoylation probably may occurs on monomeric DJ-1 L166P, since it is known that this mutant protein cannot form dimers. Furthermore, sumoylation could compete with Ubiquitination inhibiting the degradation of DJ-1 L166P by UPS. The SUMO-2/3-DJ-1 L166P may further accumulate into High Molecular Weight Complexes with the consequent protein sequestration important for cell survival. This hypothesis suggests that the pathological mutation DJ-1 L166P may lead to an unexpected gain of function.
A similar improper SUMOylation of DJ-1 L166P was observed by Shinbo *et al.* A ladder of bands was detected indicating multi or poly SUMO-1 conjugation. Since SUMO-1 cannot form poly SUMO-chain, it is most probable that the misfolded DJ-1-L166P could expose multiple Lys residue further resulting in multisumoylation.

To understand the biological function of DJ-1 SUMO 2/3ylation, biochemical analyses have to be carried out. Controversial results have

reported that DJ-1 works as a protease (Olzmann *et al.*, 2004), or as a chaperone (Shendelmann *et al.*, 2004; Zhou *et al.*, 2006). DJ-1 has three Cys at amino acid number 46, 53, 106 and it has been reported that oxidation of Cys 106 is essential to exert its full activities (Takahashi *et al.*, 2004; Zhou *et al.*, 2006). Abnormal oxidation of DJ-1 has been found in patients with PD and in patients with AD (Bandopadhyay *et al.*, 2004; Choi *et al.*, 2006) and these findings indicated that the oxidation status of DJ-1 modulates its function.

In this thesis, it has been demonstrated that functional mutants DJ-1 Cys53Ala and DJ-1 Cys106Ala were sumoylated as the wild type protein. It will be interesting analyzing the SUMOylation of these functional mutants upon oxidative stress conditions. Most importantly, it will be crucial assessing whether this modification could alter DJ-1 biological and biochemical activity.

Since DJ-1 and SUMO-2/3 are oxidative stress sensor proteins, an intriguing hypothesis is that oxidative stress conditions could regulate DJ-1 modification. This thesis has demonstrated that different treatments can induce or inhibit the global SUMO 2/3 conjugation, confirming previous results (Saitoh *et al.*, 2000; Manza *et al.*, 2004; Bossis *et al.*, 2006).

Isoelectrofocusing may be used to analyze the endogenous SUMO 2/3 DJ-1 modification. Furthermore, with this approach, the DJ-1 pI shift upon stress condition would be a positive control (Taira *et al.*, 2004; Choi *et al.*, 2006). To understand better the biochemical conjugation pathway, the amount of free radicals produced by each treatments could be measured with 2′,7′-Dichlorofluorescein Diacetate (DCFH-DA) assay. The induction or the inhibition of the SUMO-2/3 conjugation pathway is probably regulated by the intracellular level of hydrogen peroxide.

An alternative approach may take advantage of the transient transfection: ectopic expression of DJ-1 could be analyzed after different treatments, and compared to SUMO-3 conjugable or unconjugable overexpression.

This experiment may indicate whether upon conditions in which global SUMO-2/3ylation is induced or inhibited, DJ-1 SUMOylation is affected. In this thesis some preliminary results indicated that, in ectopic overexpression of 2X-Flag-DJ-1, HA-SUMO-3 and HA-Ubc9 and upon oxidative stress, DJ-1 is hypersumoylated since a ladder of bands were specifically detected with the anti-Flag antibody (data not shown). This result has to be confirmed. Experiments on tagged DJ-1 stable cell lines transfected with the SUMO-3 conjugation machinery and subjected to oxidative stress conditions may also be performed.

In this thesis SUMO-2/3 was also investigated by immunofluorescence in *Substantia Nigra* isolated from acute MPTP-treated mice. A downregulation of the signal was detected respect to the nuclear localization in untreated mice. Interestingly, the downregulation of SUMO-2/3 detection was not limited to dopaminergic neurons, since MPTP is specifically internalized into them, but also to other cell types. Further investigations are necessary to elucidate this phenomenon. SUMO-2/3 may also aggregate in HMW complexes inhibiting the detection in brain tissue. To explore this possibility, experiments have to be supported by Western blot analysis. A crucial experiment will be the demonstration that global sumoylation could be affected by a Parkinson disease-like treatment, such as MPTP, and that DJ-1 SUMO-2/3lation could be affected as well with a consequent alteration of its physiological activity.

REFERENCES

Abou-Sleiman P.M., Healy D.G., Wood N.W. (2004) Causes of Parkinson's Disease: genetics of DJ-1. *Cell Tissue Res.* 318, 185-188

Azuma Y., Arnaoutov A., Dasso M. (2003) SUMO-2/3 regulates topoisomerase II in mitosis. *J Cell Biol*. 163(3):477-487.

Bader V., Ran Zhu X., Lubbert H., Stichel C.C. (2005) Expression of DJ-1 in the adult mouse CNS. *Brain Res.*1041,102-111

Bandopadhyay R. and Cookson M.R. (2004) Evolutionary and functional relationships within the DJ-1 superfamily. *BMC Evolutionary Biology*

Bandopadhyay R., Kingsbury A.E., Cookson M.R., Reid A.R., Evans I.M., Hope A.D., Pittman A.M, Lashley T., Canet-Aviles R., Miller D.W., McLendon C., Strand C., Leonard A.J., Abou-Sleiman P.M., Healy D.G., Ariga H., Wood N.W., de Silva R., Revesz T., Hardy J.A., Lees A.J. (2004) The expression of DJ-1 (PARK7) in normal human CNS and idiopathic Parkinson's Disease. *Brain*. 127, 420-430

Bandopadhyay R., Miller D.W., Kingsbury A.E., Jowett T.P., Kaleem M.M., Pittman A.M., de Silva R., Cookson M.R., Lee A.J. (2005) Development, characterization and epitope mapping of novel monoclonal antibodies for DJ-1 (PARK7) protein. *Neurosci. Lett*. 383, 225-230

Bayer P., Arndt A., Metzger S., Mahajan R., Melchior R., Jaenicke R., Becker J. (1998) Structure determination of the small ubiquitin-related modifier SUMO-1. *J. Biol. Chem.* 280(2), 275-286

Baulac S., LaVoie M.J. Strahle J., Schiossmacher M.G. Xia W. (2004) Dimerization of Parkinson's disease-causing DJ-1 and formation of high molecular weight comnplexes in human brain. *Mol. Cell. Neurosci*. 27, 236-246

Betarbet R., Canet-Aviles R.M., Sherer T.B., Mastroberardino P.G., MvLendon C.m Kim J-H., Lund S., Na H-M., Taylor G., Bence N.F., Boo Seo B., Ynag T., Yagi A., Klinefelter G., Cookson M.R. and Greenamyre T. (2006) Intersecting pathways to neurodegeneration in Parkinson's Disease: effects of the pesticide rotenone on DJ-1, α-synuclein, and the Ubiquitin-proteasome system. *Neurobiol. Disease*.22(2) 404-420

Biggins S., Bhalla N., Chang A., Smith D.L. Murray A.W. (2001) Genes involved in sister chromatin separation and segregation in the budding yeast *Saccharomyces Cerevisiae*. *Genetics* 159, 453-470

Blackinton J., Ahmad R., Miller D.W., van der brug M.P. Canet-Aviles R.M. Hague S.M., Kalem M., Cookson M.R. (2005) Effects of DJ-1 mutations and polymorphism on protein stability and subcellular localization. *Brain. Res. Mol*. 134, 76-83

Bohren K.M., Nadkami V., Song J.H., Gabbay K.M., Owerbach D. (2004) A M55V polymorphism is a novel SUMO gene (SUMO-4) differentially activates hear shock transcription factors and is associated with susceptibility to type I diabetes mellitus. *J.Biol. Chem.* 278 (26); 27233-27238

Bonifati V., Oostra B.A and Heutink P. (2003) Linking DJ-1 to neurodegeneration offers novel insights for understanding the pathogenesis of parkinson's disease. *J. Mol. Med.* 82, 163-167

Bonifati V., Rizzu P., van Baren M.J., Schaap O., Breedveld G.J., Krieger E., Dekker M.C., Squiteri F., Ibanez P., Joosse M., van Dongen J.W., Vnacore N., van Swieten J.C., Brice A., Meco G., van Duijn C.M., Oostra B.A., Heutink P (2003) Mutation in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science* 299, 256-259

Bossis G., Melchior F. (2006) Regulation of SUMOylation by reversible oxidation of SUMO conjugating enzymes. *Mol Cell*. 21(3):349-357.

Bove J., Prou D., Perier C., Przedborski S. (2005) Toxin-induced models of Parkinson's Disease. NeuroRx 2(3): 484-494

Bretaud S., Allen C., Ingham P. and Bandmann O. (2006) p53-dependent neuronal cell death in a DJ-1 deficient zebrafish model of Parkinson's disease. J. *Neuroch*. 100(6), 1626-1635

Canet-Aviles R.M., Wilson M.A. Miller D.W. Ahmad R., McLendon C., Bandyppadhyay S., Baptista M.J., Ringe D., Petsko G.A and Cookson M.R. (2004) The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic-acid-driven mitochondrial localization. *Proc. Natl. Acad. Sci.USA* 101 (24), 9103-9108

Chakrabarti S.R. Sood R., Nandi S., Nucifora G. (2000) Post translational modification of TEL and TEL/AML1 by SUMO-1 and cell cycle-dependent assembly into nuclear bodies. *Proc. Natl. Acad. Sci.* USA 97, 13281-13285

Chen L., Cagniard B., Mathews T., Jones S., Koh H.C., Ding Y., Carvey P.M., Ling Z., Kang U.J and Zhuang X. (2005) Age dependent motor

deficits and dopamingergic dysfunction in DJ-1 null mice. *J.Biol. Chem.*280 (21):418-426

Choi J., Sullards M.C., Olzmann J.A., Rees H.D., Weintraub S.T., Bostwick E., Georing M., Levey A.J., Chin L.S., Lin L. (2006) Oxidative stress damage of DJ-1 is linked to Parkinson and Alzheimer disease. *J.Biol.Chem.* 281(116), 10816-10824

Chung K.K., Zhang Y., Lim K.L., Tanaka Y., Huang H., Gao J., Ross C.A., Dawson V.L., Dawson T.M. (2001) Parkin ubiquitinates the alpha-synuclein-interracting protein, synphilin-1: implication for Lewy-body formation in Parkinson's disease. *Nat. Med.* 7(10) 1144-1150

Clements C.M., McNally R.S., Conti B.J., Mak T.W., Ting J.P-Y. (2006) DJ-1, a cancer-and Parkinson's disease-associated protein, stabilizes the antioxidant transcriptional master regulator Nrf2. *Proc. Natl. Acad. Sci.USA* 103(41) 15091-15096

Collavin L., Gostissa M., Avorio F., Secco P., Ronchi A., Santoro C., Del Sal G. (2004) Modification of the erythroid transcription factor GATA-1 by SUMO-1. *Proc. Natl. Acad. Sci.* USA 101(24):8870-5.

Comerford K.M., Leonard M.O., Karhausen. J., Carey R., Colgan S.P., Taylor C.T. (2003) Small ubiquitin-related modifier-1 modification mediates resolution of CREB-dependent responses to hypoxia. *Proc. Natl. Acad. Sci.* USA 100, 986-991

Dauer W and Przedborki S. (2003) Parkinson's disease: mechanism and models. *Neuron* 39, 889-909. Review

Dawson V.L. Dawson V.L., (2003) Molecular pathways of neurodegeneration in Parkinson's Disease. *Science* 302, 819-822

Desterro J.M. Rodriguez M.S., Hay R.T. (1998) SUMO-1 modification of IkBα inhibits NF-KB activation. *Mol.Cell*. 2, 233-239

Dieckhoff P., Bolte M., Sancak Y., braus G.H., Irninger S. (2004) Smt3/SUMO and Ubc9 are required for efficient APC/C-mediated proteolysis in budding yeast. *Mol. Microbiol.* 51, 1375-1387

Dorval V., Fraser P.E. (2006) Small Ubiquitin Modifier (SUMO) modification of natively unfolded proteins tau and α-synuclein. *J. Biol. Chem*. 281, 9919-9924

Dorval V., Mazzella M.J., Mathews P.M., Hay R.T., Fraser P.E. (2007) Modulation of Aβ generation by small ubiquitin-like modifiers does not require conjugation to target proteins. *Biochem. J*. 404, 309-316

Eaton E.M., Sealy L. (2003) Modification of CCAAT/Enhancer-binding protein-β by the small ubiquitin modifier (SUMO) family members SUMO-2 and SUMO-3. *J.Biol. Chem.* 278(35), 33416-33421

Foley P., Riederer P. (2000) Influence of neurotoxins and oxidative stress on the onset and progression of Parkinson's Disease. *J. Neurol*. 247 (Suppl. 2) II82-II94

Fu C., Ahmed K., Ding H., Ding X., Lan J., Yang Z., Miao Y., Zhu Y., Shi Y., Zhu J., Huang H., Yao X. (2005) Stabilization of PML nuclear localization by conjugation and oligomerization of SUMO-3, *Oncogene.* 24(35), 5401-5413

Gibb W.R., Esiri M.M. Lees A.J. (1987) Clinical and pathological features of diffuse cortical Lewy body disease (Lewy Body Dementia) *Brain* 110; 1131-1153

Giorgino F., de Robertis O., Laviola L., Montone C., Perrini S., McCowen K., Smith R.J. (2000) The sentrin-conjugating enzyme mUbc9 interacts with GLUT4 and GLUT1 glucose transportes and regulates transporter level in skeletal muscle cells. *Proc. Acad. Natl. Sci*. USA 97(3) 1125-1130

Goldberg M.S., Pisani A., Haburcak M., Vortherms T.A., Kitada T., Costa C., Tong Y., Martella G., Tscherter A., Martins A., Bernardi G., Roth B.L., Pothos E.N., Calabresi P., Shen J. (2005) Nigrostriatal dopaminergic deficits and hypokinesia caused by inactivation of the familiar Parkinsonism-linked gene DJ-1. *Neuron.* 45, 489-496

Goodson M.L., Hong Y., Rogers R., Matunis M.J., Park-Sarge O.K., Sarge K.D., (2001) SUMO-1 modification regulates the DNA-binding activity of heat shock transcription factor 2, a promyelleukemia nuclear body associated transcription factor. *J. Biol. Chem.* 276, 18513-18518

Gostissa M., Hengstermann A., Fogal V., Sandy P., Schwarz S.E., Scheffner M., Del Sal G. (1999) Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1. *EMBO J.* 18, 6462-6461

Graham D.G., Tiffany S.M., Bell W.R. Jr, Gutknecht W.F. Autooxidation versus covalent binding of quinines as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds towards C1300 neuroblastome cells *in vitro.* (1978) *Mol. Pharmacol.* 14(4): 644-653

Halliwell B. (1992) Oxigen radicals as key mediators in neurological disease: fact or fiction? *Ann. Neurol*. 32 S10-S35

Haracska L., Torres-ramon C.A., Johnson R.E., Prakash S., Prakash L. (2004) Opposing effects of ubiquitin conjugation and SUMO modification of PCNA on replicational bypass of DNA lesions in *Saccharomycies Cerevisiae .Mol. Cell. Biol.* 24, 4267-4274

Heidrich K., Djarmati A., Schafer N., Hering R., Wellenbrock C., Weiss P.H.(2004) DJ-1 (PARK-7) mutations are less frequent than Parkin (PARK2) mutations in early onset Parkinson's disease. *Neurology* 62, 389-394

Hering R., Karsten M.S., Tao X., Bauer A., Woitalla D., Mietz E., Petroviz S., Bauer P., Müller T., Schöls L., Klein C., Berg D., Meyer P.T., Schulz J.B., Wollnok B., Tong L., Krüger R. and Riess O. (2004) Novel homozygous pE64D mutation in DJ-1 in early onset Parkinson's Disease (PARK7) *Human Mutation* 24: 321-329

Hernan M.A. Takkouche B., Caamano-Isorna F., Gestal-Otero J.J. (2002) A meta-analysis of coffee drinking, sigarette smoking, and the risk of Parkinson's disease. *Ann. Neurol*. 52 (3):276-284

Herrera F.E., Zucchelli S., Jezierka A., Scotto Lavina Z., Gustincich S., Carloni P. (2007) On the oligomeric state of DJ-1 protein and its mutants associated with parkinson disease. A combined computational and in vitro study. *J.Biol. Chem*. 282(34):24905-24914

Hod Y., Pentyala SN., Whyard T.C. El-Maghrabi M.R. (1999) Identification and characterization of a novel protein that regulates RNA-protein interaction. *J. Cell. Biochem.* 72, 435-444

Hoege C., Pfander B., Moldovan G.L. Pyrowolakis G., Jentsch S. (2002) RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO.*Nature* 419, 135-141

Hoglinger G.U., Carrad G., Michel P.P., Medja F., Lombes A., Ruberg M., Friguet B., Hirsch E.C. (2003) Dysfunction of mitochondrial complex I and the proteasome: interaction between two biochemical deficits in a cellular model of Parkinson's Disease. *J. Neurochem* 86(5): 1297-1307

Honbou K., Suzuki N.N. Horiuchi M., Niki T., Taira T. Ariga H. (2003) The crystal structure of DJ-1, a protein related to male fertility and parkinson's disease. *J. Biol. Chem*. 278, 31380-31384

Inden M., Taira T., Kitamura Y., Yanagida T., Tsuchiya D., Takata K., Yanagisawa D., Nishimura K., Taniguchi T., Kiso Y., Yoshimoto K., Agatsuma T., Koide-Yoshida S., Iguchi-Ariga S.M-M. Shimohama S. and Ariga H. (2006) PARK7 DJ-1 protects against degeneration of nigral dopaminergic neurons in Parkinson's disease rat model. *Neurobiology of Disease* 24(1) 144-158

Jackson-Lewis V, Przedborski (2007) Protocol for the MPTP mouse model of Parkinson's Disease. *Nature Protocol.* 2(1) 141-151

Jacowek M.J., Nixon K., Hogg E., McNeill T., Petzinger G. (2004) Tyrosine Hydroxylase and Dopamine transporter expression following MPTP

induced neurodegeneration of mouse nigrostriatal pathway. *J. Neurosci. Res.* 76, 539-550

Jang M.S. Ryu S.W., Kim E. (2002) Modification of Daxx by small ubiquitin-related modifier-1. *Biochem. Biophys. Res. Commun*. 295 , 495-500

Jeong H., Kim M.S., Kwon J., Kim KS., Seol W., (2006) Regulation of the transcriptional activity of the tyrosine hydroxilase gene by androgen receptor. *Neurosci Lett.* 396 (1): 57-61

Jin J., Li G.J., Davis J., Zhu D., Wang Y., Pan C. and Zhang J. (2006) Identification of novel proteins interacting with both DJ-1 and α-synuclein. *Molec. Cell Proteomics.*

Johnson E.S., Blobel G. (1999) Cell cycle-regulated attachment of the ubiquitin related protein SUMO to the yeast septins. *J.Biol. Chem*. 147,981-994

Johnson E.S. (2004) Protein modification by SUMO. *Ann.Rev.Bioche.*73, 355-382

Junn E., Taniguchi H., Jeong B.S., Zhao X., Ichijo H., Mouradian M.M. (2005) Interaction of DJ-1 with Daxx inhibits apoptosis signal-regulating kinase 1 activity and cell death. *Proc. Natl. Acad. Sci. USA* 102, 9691-9696

Kamitani T., Kito K., Nguyen H.P. Wada H., Fukuta-Kamitani T., Yeh E.T. (1998) Identification of three major sentrinization sites in PML. *J. Biol. Chem.*273, 26675-26682

Kim R.H., Peters M., Jang Y., Shi W., Pintilie M., Flectcher G.C. (2005) DJ-1 a novel regulator of the tumor suppressor PTEN. *Cancer Cell* 7, 263-273

Langstom J.W., Ballard P., Tetrud J.W. Irwin I. (1983) Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science,* 219 (4587): 5411-5417

Lee S.J., Kim S.J. Kim I.K., Ko J., Jeong C.S., Kim G.H., Park C., Kang S.O., Suh P.G., Lee H.S., Cha S.S. (2003) Crystal structures of human DJ-1 and Escherichia coli Hsp31, which share an evolutionary conserved domain. *J. Biol. Chem*. 278(44): 552-559

Li T., Santockyte R., Shen R-F., Tekle E., Wang G., Yang D.C. Chock P.B. (2006) Expression of SUMO-2/3 induced senescence through p53-and pRB-mediated pathways. *J. Biol. Chem*. 281(47):36221-36227.

Li Y., Wang H., Wang S., Quon D., Liu Y-W, Cordell B (2003) Positive and negative regulation of APP amyloidogenesis sy Sumoylation. *Proc. Natl. Acad. Sci*. USA 100, 259-264

Lin D., Tatham B., Yu S., Kim S., Hay R.T., Chen Y.(2002) Identification of a substrate recognition site on Ubc9. *J.Biol.Chem.* 277, 21740-21748

Lin D.Y., Huang Y.S., Jeng C.J., Kuo H.Y., Chang C.C., Chao T.T., Ho C.C., Chen Y.C., Lin T.P., Fang H.I., Hung C.C. Suen C.S., Maul G.G., Shih H.M. (2006) SUMO-interacting motif in Daxx SUMO modification, subnuclear localization, and repression of sumoylated transcription factors. *Mol Cell*.;24(3):341-354.

Lin X., Sun B., Liang M., Liang Y.Y. Gast A., Hildebrand J, Brunicardi F.C. Melchior F. (2003) Opposed regulation of corepressor CtBP by SUMOylation and PDZ binding. *Mol. Cell* 11, 1389-1396

Macedo M.G., Anar B., Bronner I.F., Cannella M., Squitieri F., Bonifati V., Hoogeveen A., Heutink P., Rizzu P. (2003) The DJ-1L166P mutant protein associated with early onset Parkinon's Disease is unstable and forms higher order protein complexes. *Hum. Mol. Genet.* 12(21), 2807-2816

Mahajan R., Delphin C., Guan T., Gerace L., Melchior F. (1997) A small ubiquitin-related polypeptide involved in the targeting RanGAP1 to nuclear pore complex RanBP2. *Cell*. 88, 97-107

Manza L.L., Codreanu S.G., Stamer S.L., Smith D.L., Welles K.S., Roberts R.L., Liebler D.C. (2004) Global shifts in protein sumoylation in response to electrophile and oxidative stress. *Chem Res Toxicol*. 17(12):1706-1715.

Markey S.P., Johannessen J.P., Chiueh C.C., Burns R.S., Herkenham M.A. (1984) Intraneuronal generation of pyridinium metabolite may cause drug-induced parkinsonism. *Nature* 311(5985):464-467

Martin S., Nishimune A., Mellor J.R., Henley J.M. (2007) SUMOylation regulates kainate-receptor-mediated synaptic transmission. *Nature*. 447(7142):321-325.

Martinat C., Shendelman S., Jonason A., Leete T., Beal M.F., Yang L., Floss T., Abeliovich A. (2004) Sensitivity to oxidative stress in DJ-1 deficient dopamine neurons: an ES-derived cell model of primary of primary parkinsonism. *Plos biology*. 2(11), 1754-1763

Matunis M.J. Coutavas E., Blobel G.(1996) A novel ubiquitin-like modification modulates the partioning of the Ran-GTPase-activating protein Ran-GAP1 between the cytosol and the nuclear pore complex. *J.Biol.Chem*. 135, 1457-1470

McInerney-Leo A., Hadley D., Gwinn-Hardy K., Hardy J. (2005) Genetic testing in Parkinson's Disease. *Movement Disorder* 20 (1), 1-10

Melchior F., Schergaut M., Pichler A. (2003) SUMO: ligases, isopeptidases and nuclear pores. *Trends Biochem Sci*.11:612-8. Review.

Menzies F.M., Yenisetti S.C., Min K.T. (2005) Roles of *Drosophila* DJ-1 in survival of dopaminergic neurons and oxidative stress. *Curr. Biol*.
15, 1578-1582

Meulener M., Whitworth A.J., Armstrong-Gold C.E., Rizzu P., Heutink P., Wes P., Pallank L.J. and Bonini N. (2005) *Drosophila* DJ-1 mutants are selectively sensitive to environmental toxins associated with Parkinsons's Disease. *Curr. Biol*. 15, 1572-1577.

Meulener M.C., Xu K., Thomson L., Ischiropoulos H., Bonini N. (2006) Mutation analysis of DJ-1 in *Drosophila* implicates functional inactivation by oxidative damage and aging. *Proc. Acad. Natl. Sci*. USA 103(33), 12517-12522

Miller D.W., Rili A., Hague S., Baptista M.J. Canet-Aviles R., McLendon C., Carter D.M. Zhu P-P., Stadler J., Chandran J., Klinefelter G.R., Blackstone C., Cookson M.R. (2003) L166P mutant DJ-1, causative for recessive Parkinson's disease, is degraded through the Ubiquitin-Proteasome System. *J. Biol. Chem.* 278(38): 36588-36595

Miyauchi Y., Yogosawa S., Honda R., Nishida T., Yasuda H. (2002) Sumoylation of Mdm2 by protein inhibitor of activated STAT (PIAS) and RanBP2 enzymes. J. *Biol. Chem*. 277, 50131-50136

Mizuno Y., Hattori N., Mori H., Suzuki T., Tanaka K. (2001) Parkin and Parkinson's Disease. *Curr. Opin. Neurol*. 14, 477-482

Moore D.J., Zhang L., Dawson T.M and Dawson V. (2003) A missense mutation (L166P) in DJ-1 linked to familiar Parkinson's disease, confers reduced protein stability and impairs homo-oligomerization. *J.Neurochem*.87, 1558-1567

Moore D.J. , Zhang L., Troncoso J., Lee M.K., Hattori N., Mizuno Y., Dawson T.M. and Dawson V.L. (2005) Association of DJ-1 and parkin mediated by pathogenic DJ-1 mutations and oxidative stress. *Hum. Mol. Genet.* 14 (1), 71-84

Nagakubo D., Taira T., Kitaura H., Ikeda M., Tamai K., Iguchi-Ariga S.M. and Ariga H. (1997) DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras. *Biochem. Biophys. Res. Commun.* 231, 509-513

Neumann M., Muller V., Gorner K., Kretzschmar H.A., Haass C., Kahle P.J. (2004) Pathological properties of the Parkinson's disease-associated protein DJ-1 in alpha-synucleopathies and taupathies:relevance for multiple system atrophy and Pick's disease. *Acta Neuropathol*. 107, 489-496

Nicklas W.J., Vyas I., Heikkila R.E. (1987) Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a

metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. *Life Sci*. 1; 36(26): 2503-2508

Ooe H., Maita C., Maita H., Iguchi-Ariga S.M.M., Ariga H. (2006) Specific cleavage of DJ-1 under an oxidative condition. *Neuroscience Lett.* 406, 165-168

Olzmann J.A., Brown K., Wilkinson K.D., Rees H.D., Huai Q., Ke H ., Lewey A.I., Li L., Chin L.S. (2004) Familiar parkinson's disease-associated L166P mutation disrupts DJ-1 protein folding and function. *J. Biol. Chem.* 279, 8506-8515

Olzmann J.A., Bordelon J.R., Muly C.E., Rees H.D., Lewey A.I., Li L., Chin L.-S. (2007) Selective enrichment of DJ-1 protein in primate striatal neuronal processes: implication for parkinson's disease. *J. Comp. Neurol*. 500, 585-599

Pan X., Li H., Zhang P., Jin B., Man J., Tian L., Su G., Zhao J., Li W., Liu W., Gong W., Zhou T., Zhang X. (2006) Ubc9 interacts with SOX4 and represses its transcriptional activity. *Biochem. Biophys. Res. Commun.* 344, 727-734

Pountney D.L., Huang Y., Burns R.J., Haan E., Thompson P.D., Blumbergs P.C., Gai W.P. (2003) SUMO-1 marks the nuclear inclusions in familiar neuronal intranuclear inclusion disease. *Exp. Neurol*. 184, 436-446

Rangasamy D., Woytek K., Khan S.A., Wilson V.G. (2000) SUMO-1 modification of bovine papillomavirus E1 protein is required for intracellular accumulation, *J.Biol. Chem*. 275, 37999-38004

Rizzu P., Hinkle D.A., Zhukareva V., Bonifati V., Severijnen L.A., Martinez D., Ravid R., Kamphorst W., Eberwine J., Lee V. M-Y., Trojanowski J.Q. Heutink P. (2004) DJ-1 colocalizes with Tau inclusions: a link between Parkinsonism and Dementia. *Ann. Neurol.* 55, 113-118

Ross S., Best J,L., Zon L.I., Gill G. SUMO-1 modification represses Sp3 transcriptional activation and modulates its subnuclear localization. *Mol. Cell.* 10, 831-842

Saitoh H., Hinchey J., 2000 Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3.*J.Biol.Chem.* 275(9), 6252-6258

Salinas S., Briancon-Marijollet A., Bossis G., Lopez M.A., Pierchaczyk M., Jariel-Encontre I., Debant A., Hipshing R.A. (2004) SUMOylation regulates nucleo-cytoplasmic shuttling of Elk-1.J.Biol.Chem. 165(6), 767-773

Sapetsching A., Rischitor G., Braun H., Doll A., Schergaut M.,Melchior F., Suske G. (2002) Transcription factor Sp3 is silenced through SUMO modification by PIAS1. *EMBO J.*21, 5206-5215

Sekito A., Taira T., Niki T., Iguchi-Ariga S.M.M., Ariga H. (2005) Stimulation of transforming activity of DJ-1 by Abstract, a DJ-1 binding protein. *International Journal of Oncology.*26, 685-689

Shang H., Lang D., Jean-Marc B., Kaelin-Lang A. (2004) Localization of DJ-1 m-RNA in the mouse brain. *Neurosci. Lett.* 367, 273-277

Shapira A.H., Cooper J.M., Dexter D., Clark J.B., Jenner P., Marsden C.D. (1990) Mitochondrial complex I deficiency in Parkinson's Disease. *J. Neurochem*. 54, 823-827

Shen J., Cookson M.R. (2004) Mitochondria and dopamine: new insights into recessive parkinsonism. *Neuron*.5; 43(3):301-304

Shendelman S., Jonason A., Matinat C., Leete T., Abeliovich A. (2004) DJ-1 is a redox-dependent molecular chaperone that inhibits α-synuclein aggregate formation. *Plos Biology* 2 (11), 1-10

(a) Shinbo Y., Taira T., Niki T., Iguchi-Ariga S.M.M and Ariga H. (2005) DJ-1 restores p53 trasncriptional activity inhibited by Topors/p53BP. *Int. J. Oncol* 26(3):641-648

(b) Shinbo Y, Niki T., Taira T., Ooe H., Takahashi-Niki K., Maita C., Seino C., Iguchi-Ariga S.M.M., Ariga H. (2005) Proper SUMO-1 conjugation is essential to DJ-1 to exert its full activities.*Cell.Death.Differ.*13(1), 96-108

Sobko A., Ma H., Firtel R.A. (2002) Rgulated SUMOylation and ubiquitination of DdMEK1 is required for proper chemotaxis. *Dev. Cell* 2, 745-756

Šramko M., Markus J., Kabat J., Wolff L., Bies J. (2006) Stress-induced inactivation of the c-Myb transcription factor through conjugation of SUMO2/3 proteins. *J. Biol. Chem.* 281(52), 40065-40075

Steffan J.S., Agrawal N., Pallos J., Rockabrand L.C., Trotman N., Slepko N., Illes T., Lukacsovich Y., Zhu Y., Cattaneo E., Pandolfi P.P., Thompson L.M.

Marsh J.L. (2004) Sumo modification of Huntingtin and Huntington's disease pathology. *Science* 304, 100-104

Sternsdorf T., Jensen K., Reich B., Will H. (1999) The nuclear dot protein sp100, characterization of domains necessary for dimerization, subcellular localization, and modification by small ubiquitin-like modifiers. J. Biol. Chem. 274, 12555-12566

Su H-L., Li S.L. (2002) Molecular features of human ubiquitin-like SUMO genes and their encoded proteins. *Gene,* 296, 65-73

Taira T., Takahashi-Niki K, Kitagawa R., Iguchi-Ariga S.M., Ariga H. (2001) Molecular cloning of human and mouse DJ-1 genes and identification of Sp1-dependent activation of the huan DJ-1 promoter. *Gene* 263, 285-292

Taira T., Saito Y.,Niki T., Iguchi-Ariga S.M.M, Takahashi K., Ariga H. (2004) DJ-1 has a role in antioxidative stress to prevent cell death. *EMBO Rep*. 5, 213-218

Takahashi-Niki K., Niki T., Taira T., Iguchi-Ariga S.M.M and Ariga H. (2004) Reduced anti-oxidative stress activities of DJ-1 mutants found in Parkinson's disease patients. *Bioch. Biophys.Research. Comm.*320, 389-397

Takahashi K., Taira T., Niki T., Seino C., Iguchi-Ariga S.M.M. and Ariga H. (2001) DJ-1 positively regulates the androgen receptor by impairing the binding of PIASx to the receptor. *J. Biol. Chem.* 276 (40) 37556-37563

Tang B., Xiong H., Sun P., Zhang Y., Wang D., Hu Z., Zhu Z., Ma H., Pan Q., Xia J-H., Xia K. and Zhang Z. Association of PINK-1 and DJ-1 confers

digenic inheritance of early-onset Parkinson's disease. *Hum. Mol. Genet.* 15 (11), 1816-1825

Tao X., Tong L. (2003) Crystal Structure of human DJ-1, a protein associated with early onset parkinson's disease. *J. Biol. Chem.* 278, 31372-31379

Tatham M.H., Jaffay E., Vaughan O.A., Desterro J.M., Botting C.H., Naismith J.H., Hay R.T (2001) Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. *J. Biol. Chem*. 2001 276(38):35368-35374.

Terashima T., Kaway H., Fujitani M., Maeda K., Yasuda H. (2002) SUMO-1 co-localized with mutant atrophin-1 with expanded polyglutaminea accelerate intranuclear aggregation and cell death. *Neuroreport* 13, 2359-2364

Tian S., Poukka H., Palvimo J.J., Janne O.A. (2002) Small ubiquitin-related modifier-1 (SUMO-1) modification of the glucocorticoid receptor. *Biochem J*. 367(Pt 3):907-911.

Ueda H., Goto J., Hashoda H., Lin X., Oyanagi K., Kawano H, Zoghobi H.Y. Kanazawa I., Okazawa H. (2002) Enhanced SUMOlation in polyglutamine disease. *Biochem Biophys Res. Commun.* 293, 307-313

Weger S., Hammer E., Engstler M.(2003) The DNA topoisomerase I binding protein topors as a novel cellular target for SUMO-1 modification: characterization of domains necessary for subcellular localization and sumolation. *Exp. Cell. Res.* 290, 13-27

Wood L.D., Irvin B.J. Nucifora G., Luce K.S., Hiebert S. W. (2002) Small Ubiquitin modifier conjugation regulates nuclear export of TEL, a putative tumor suppressor. *Proc. Natl. Acad. Sci. U.S.A*. 100, 3257-3262

Xu J., Zhong N., Wang H., Elias J.E., Kim C.Y., Woldman I., Pifl C., Gygi S., Geula C., and Yanker B.A. (2005) The parkinson's disease-associated DJ-1 protein is a transcriptional co-activator that protects against neuronal apoptosis. *Hum .Mol.Genet.* 14 (9)., 1231-1241

Xue Y., Zhou F., Fu C., Xu Y. and Yao X. (2006) Sumo sp: a web server for SUMOylation site prediction. *Nucl.Acid.Research.* 34, 254-257

Yanagida T., Takata K., Inden M., Kitamura Y., Taniguchi T., Yoshimoto K., Taira T. and Ariga H. (2006) Distribution of DJ-1, Parkinson's disease-related protein PARK7, and its alteration in 6-hydroxydopamine-treated hemiparkinsonian rat brain. *J. Pharmacol Sci* 102, 243-247

Yang Y., Gehrke S., Haque E., Imai Y., Kosek J., Yang L., Flint Beal M., Nishimura I., Wakamatsu K., Ito S., Takahashi R., Lu B. (2005) Inactivation of Drosophila DJ-1 leads to impairments of oxidative stress response and phosphatidylinositol 3-kinase/Akt signalling. *Proc. Natl. Acad. Sci*. 102 (38), 13670-13675

Yokota T., Sugawara K., Ito K., Takahashi R., Ariga H., Mizusawa H. (2003) Down regulation of DJ-1 enhances cell death by oxidative stress, ER stress, and proteasome inhibition. *Biochem. Biophys. Res. Commun*. 312, 1342-1348

Zhang L., Shimoji M, Thomas B, Moore D.J., Yu S.W. Marupudi N.I., Torp R., Torgner I.A., Ottersen O.P., Dawson T.M., Dawson VL. (2005)

Mitochondrial localization of the Parkinson's Disease related protein DJ-1:implications for pathogenesis. *Hum. Mol. Genet.*14(14): 2063-2073

Zhong N., Kim C.Y. Rizzu P., Geula C., Porter D.R. Pothos E.N., Squitieri F., Heutink P. and Xu J. (2006) DJ-1 transcriptionally up-regulates the human tyrosine hydroxylase by unhibiting the sumoylation of PSF. *J.Biol.Chem.* 281 (30), 20940-20948

Zhou W., Ryan J.J., Zhou H. (2004) Global analyses of sumoylated proteins in Saccharomyces cerevisiae. Induction of protein sumoylation by cellular stress. *J. Biol Chem.*279(31):32262-32268

Zhou W., and Freed C.R. (2005) DJ-1 up-regulates glutathione syntheis during oxidative stress and inhibits A53T α-synuclein toxicity. *J.Biol. Chem.* 280 (52), 43150-43158

Zhou W., Zhu M., Wilson M.A., Petsko G.A. and Fink A.L. (2006) The oxidation state of DJ-1 regulates its chaperone activity toward α-synuclein. *J.Mol.Biol.* 356, 1036-1048.