Molecular Bases of Disease:

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J. Biol. Chem. published online June 21, 2013

JBC Papers in Press. Published on June 21, 2013 as Manuscript M113.482091
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M113.482091

The Parkinson's disease protein DJ-1 binds metals and protects against metal induced cytotoxicity

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*Running title: *Metal binding by DJ-1 and protection against cytotoxicity*

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Keywords: DJ-1, metals, cytotoxicity

Background: DJ-1 is associated with recessive early onset Parkinson's disease
Results: DJ-1 acts as a metal binding protein, binding copper and mercury, and DJ-1, but not mutated clinical variants, protects cells against metal induced cytotoxicity.
Conclusion: Small genetic alterations in a central Parkinson's disease protein can sensitize cells to metal induced cell death.
Significance: Understanding the effect of exogenous factors in the etiology of Parkinson's disease and other neurodegenerative disorders.

Abstract

Progressive loss of motor control due to reduction of dopamine producing neurons in the substantia nigra pars compacta and decreased striatal dopamine levels are the classically described features of Parkinson's disease (PD). Neuronal damage progresses also to other regions of the brain and additional non-motor dysfunctions are common. Accumulation of environmental toxins, such as pesticides and metals, are suggested risk factors for the development of typical, late onset PD, while genetic factors seem to be substantial in early onset cases. Mutations of DJ-1 are known to cause a form of recessive early onset Parkinson's disease, highlighting an important functional role for DJ-1 in early disease prevention. This study identifies human DJ-1 as a metal binding protein able to evidently bind copper as well as toxic mercury ions *in vitro*. The study further characterizes the cytoprotective function of DJ-1 and PD mutated variants of DJ-1, in respect to induced metal cytotoxicity. The results show that expression of DJ-1 enhances the cells protective mechanisms against induced metal toxicity, and that this protection is lost for DJ-1 PD mutations; A104T and D149A. The study also shows that oxidation site mutated DJ-1 C106A retains its ability to protect cells. We also show that concomitant addition of dopamine exposure sensitizes cells to metal induced cytotoxicity. We also confirm that redox-active dopamine adducts enhances metal-catalyzed oxidation of intracellular proteins *in vivo* by use of live-cell imaging of redox sensitive S3roGFP. The study indicate that even a small genetic alteration can sensitise cells to metal induced cell death, a finding that may revive the interest in exogenous factors in the etiology of PD.

Parkinson's disease (PD) is the second most common neurodegenerative disease, classically described as a progressive movement disorder, and with increased prevalence in the aging population. Selective degeneration of dopamine producing neurons in the substantia nigra pars compacta (SNpc) and depletion of striatial dopamine levels are the cardinal features of PD (1). It has been estimated that 50-60% of the SNpc dopaminergic neurons are irreversibly lost and about 80–85% of the dopamine content of the striatum is depleted by the time of clinical diagnosis (2). However, there are also widespread alterations in other brain regions observed in a predominant caudal to rostral progression that may influence the development of non-motor symptoms, such as pain, sleep disorders, cognitive decline or depression (3,4). Since the progression of the disease is slow, but irreversible, the underlying factors resulting in cell death need to be clarified to enable new treatment strategies for the affected patients.

PD etiology remains obscure and appears in most instances sporadic in nature. Environmental risk factors including prolonged pesticide exposure, lead or manganese intoxication have been observed to cause parkinsonian symptoms (5-8). Altered zinc, copper and iron levels have been found specifically in the substantia nigra of PD patients compared to controls (9). Increased levels of mercury measured in blood or urine have also been associated with an increased risk of PD (10). However, several studies of occupational exposure to metals have been inconclusive in relation to PD development, as previously reviewed (11). A combination of genetic components that could sensitise cells to environmental risk factors and induced cell death at an older age has been discussed but evidence are still scarce.

Several genetic lesions, directly associated with early onset PD, have given clues about molecular mechanisms affected early in the disease progression. (12-17). Mutations of the *PARK7* gene, which encodes the protein DJ-1, have been linked to forms of early onset recessive autosomal PD (12,18,19). Even though *PARK7* mutation carriers are considered to be rare, their presence highlights the molecular function of DJ-1 as a crucial component for disease prevention.

DJ-1 is a predominantly cytosolic, homodimeric protein, ubiquitously expressed in both brain and peripheral tissue (20,21). DJ-1 has been reported to be a multifunctional protein with putative roles in, for example, ras-dependent cell transformation (22), neuroprotection (23,24), fertility (25), control of protein–RNA interactions (26) and modulation of androgen receptor signaling (27,28), or acts as a protein chaperone (29) and protease (29-31). DJ-1 has also been shown to interact with other known PD linked proteins, PINK1 and Parkin (32,33). A clear consensus on what DJ-1 actually does within the cells has not been established. DJ-1 deficient mice display dopamine re-uptake dysfunction but lack any overt sign of neurodegeneration, suggesting that loss of DJ-1 alone is insufficient to induce cell death and that additional extrinsic stress factors are required (23,34,35). Multiple studies have demonstrated that DJ-1 is responsive to oxidative stress and that DJ-1 may act as an antioxidant, protecting cells against reactive oxygen species (ROS) (23,36-39). In human cells, DJ-1 residue C46, C53 and C106 have been shown to be oxidized upon hydrogen peroxide treatment (40). The C106 being the most sensitive to oxidation and form a cystein-sulfinic acid upon exposure to ROS. DJ-1 has further been described as an atypical peroxiredoxin-like peroxidase that scavenges mitochondrial H_2O_2 through oxidation of C106 (34). Oxidation of C106 has also been suggested to be important for DJ-1's relocalization to mitochondria in response to oxidative stress (36,41).

In a previous study we showed that DJ-1 interacts with Cu/Zn-superoxide dismutase 1 and that this interaction can be enhanced by titration of copper ions, a known ligand for the superoxide dismutase 1 enzyme (42). Considering this and the fact that increased metal exposure or deficient metal regulation as well as mutations of DJ-1 are underlying factors for PD development, we sought to find out whether DJ-1 itself could be influenced by directly interacting with metals. We also characterized the effects of metal induced cytotoxicity, in relation to DJ-1, by utilizing a cell model system deficient of DJ-1 or with wild type

(WT) or PD mutated variants of human DJ-1 protein expression.

EXPERIMENTAL PROCEDURE

Special reagents – Polyclonal goat anti-DJ-1 (AB4150, Abcam), monoclonal mouse anti-actin AC-40 (A3853), Dopamine-hydrochloride (H8502) and MG132 (C2211) were from Sigma-Aldrich. Rabbit anti-DJ-1-N was a gift from Dr. Darren Moore at the Brain Mind Institute, EPFL, Switzerland. MEF cells from WT and DJ-$1^{-/-}$ mice were obtained from Dr. Huaibin Cai, National Institute on Aging, NIH, Bethesda, USA.

Cloning – The human *PARK7* gene was cloned and inserted into bacterial expression vectors pET28a as previously described (42). For mammalian cell expression *PARK7* was amplified with DJ1-F-XhoI (5'-ATCTCGAGATGGC TTCCAAAAGAGCTC-3') and DJ1-R-KpnI (5'- AT GGTACCCTAGTCTTTAAGAACAAGTGG -3') and inserted into pCDNA3.1 (-) hygro. Restriction sites added to the primers are underlined. *PARK7* point mutations were generated by site-directed mutagenesis. The sequence of redox-sensitive S3roGFP was kindly provided by Dr. C. Plieth (University if Kiel, Germany). The reporter was generated from the smGFP sequence described by (66) using previously published technology (52), with additional cysteine mutations at the positions C149 and C202 that decreased the dependence of the fluorescence to intracellular pH variations. Neither S3roGFP carry S65T mutation that was previously shown to be pH sensitive. For our experiments the sequence of S3roGFP was PCR amplified and cloned into pcDNA 3.1/Hygro (+) vector (Invitrogen) using forward primer 5'-TTAGGTACCATGAGTAAAGGAGAAGAACTTTTCAC TG-3' and reverse primer 5'- ATAGCGGCCGCTTT ATTTGTATAGTTCATCCATGCCATG-3' carrying *KpnI* and *NotI* sites respectively (as underlined). All constructs were verified by DNA sequencing at Genome Enterprise Limited, Norwich, UK or at Macrogen, the Netherlands.

Cell culturing and generation of stable cell lines – MEF WT and DJ-1 knockout cells (48) were cultured in DMEM supplemented with 7% heat inactivated bovine calf serum (Gibco), 2 mM glutamine, 1% non-essential amino acids (Sigma-Aldrich), 50 U/ml penicillin, 50 µg/ml streptomycin, in 5% CO_2 atmosphere at 37 °C. Cells were transfected using Lipofectamine 2000™ according to manufactures description (Invitrogen). Stable DJ-1 re-expression cell lines were generated by transfecting DJ-$1^{-/-}$ cells with pCDNA3.1 Hygro(-) empty vector or pCDNA3.1 Hygro(-) vector containing WT or point mutated human *PARK7* genes. Stable cell lines were selected using 800 µg/ml hygromycin B and subcultured for three weeks. All cell culture reagents not specifically specified were from Invitrogen.

Immunofluorescent imaging – Immunocyto-chemical staining was carried out as described previously (67). Briefly, cells growing on glass coverslips were washed in PBS, fixed in 4% buffered paraformaldehyde for 20 minutes followed by another fixation and permeabilization step in 100% methanol for 5 minutes at -20 °C. Samples were blocking in 5% BSA (w/v), PBS, 0.1% Tween-20 (v/v) for 1 hour at room temperature prior to incubation over night at 4 ˚C with primary antibody rabbit anti-DJ-1-N, raised against human DJ-1 N-terminal residues 1-13 as previously characterized (21,68). 4µg/ml anti-rabbit Alexa-488 secondary antibody was used for 1 hour at room temperature prior to nuclear staining using Hoechst 33342 and mounting in Mowiol mounting media. Images were taken using an inverted Nikon A1R confocal laser scanning microscope using a 60x oil objective and 4x zoom. Fluorescence images of DJ-1-Alexa488 and Hoechst 33342 nuclear stained cells were acquired at 450/50 nm and 525/50 nm, using same laser intensities and detection settings for all images to enable sample comparison. Maximum intensity scans are shown.

Cytotoxicity assay – 1.0 x 10^4 cells were seeded per well in a 96-well transparent flat bottom polystyrene non-coated tissue culture plate (3603, Corning Inc.). The cells were grown for 24 hours prior to addition of stresses, and grown another 18-24 hours prior to assessment of cell survival. All metal salts were dissolved in water and sterile filtered immediately prior to use. The cell media pH was not altered by addition of any of the used chemicals. Cell cytotoxicity was analysed by use of the water soluble tetrazolium salt, WST-1 (Roche), according to the manufacturer instruction and measured using a Thermo Scientific Multiskan Ascent at 450 nm wavelength, and 630 nm as reference. Reference values including metal

treatment without cells were subtracted from all data sets and compared to non-treated control cells, set to 100% survival. Mean values for multiple repeats ± SEM values are shown. Cell survival data obtained using WST-1 was confirmed by microscopy. Approximate LC_{50} values obtained from the combined data sets are indicated in the figure legends of each experiment.

Protein induction and purification – *E. coli* Rosetta(DE3)pLysS (Novagen) were transformed with pET28a harbouring the WT human *PARK7* gene. Protein expression was performed for 20 h in auto-induction ZYM-5052 media at 28 °C (69). Expressed protein was initially isolated with TALON affinity resin (Clontech) and eluted by thrombin (GE healthcare) cleavage of the engineered N-terminal thrombin cleavage site in PBS, pH 7.5. The eluate was incubated with Benzamidin Sepharose (GE healthcare) to remove residual thrombin, following another TALON affinity resin purification step to ensure complete removal of un-cleaved protein and maximized protein purity as verified by SDS–PAGE (Fig. 1A). The purified protein was further prepared for mass spectrometric analysis (70). LC-MS/MS sequencing of the trypsinized protein was performed using an Acquity UPLC coupled Q-TOF Micro (Waters, Milford, MA) and peptide elution with acetonitrile in either 0.1% ammoniumhydroxide or 0.1% formic acid environment. Combination of generated MS/MS data resulted in a 97.4% sequence coverage matching human DJ-1 using Swissprot database and Mascot (Matrix Sciences) search algorithm. Peptide mass tolerance 100 ppm, fragment tolerance 0.5 Da, for $[M+2H]^{2+}$, $[M+3H]^{3+}$ and $[M+4H]^{4+}$. Sequencing of the most N-terminal DJ-1 peptide revealed an addition of three amino acids (Gly-Ser-His) originating from the engineered N-terminal thrombin cleavage site.

X-ray fluorescence – For XRF analysis recombinant human DJ-1 WT protein was dialysed for 24 hours against a 20 mM MOPS buffer at pH 7.2 containing 1 mM metal salt, followed by another dialysis for 24 hours in the same buffer without metal salt. 5 mM ascorbic acid was included in the first dialysis buffer to reduce Cu(II) to Cu(I). Both dialysis steps were done at RT at a 1:1000 (v/v) protein to buffer ratio. XRF data was collected on the ESRF protein crystallography beamlines from drops of protein or buffer frozen in liquid nitrogen, using 30 second exposure time for the automatic procedure implemented in MxCuBE (71). Data was normalized on the Compton peak maximum and smoothed.

Atomic absorption spectroscopy – For AAS analysis 0.1 mg/ml WT or point mutated recombinant human DJ-1 were dialysed for 24 hours against in AAS binding buffer (20 mM MOPS buffer, pH 7.2, 150 mM NaCl, 5 mM ascorbic acid, 10% glycerol) containing 10 µM CuCl, followed by a second dialysis for 24 hours in the same buffer without metal salt. The recombinant protein the samples were diluted 6x in dH_2O by a Hamilton microlab 500 dispenser system prior to AAS analysis by us of a Perkin Elmer AAnalyst 300. AAS data was normalized to copper standard curve and correlated to final DJ-1 protein concentrations, assessed by the Bradford method.

Microscale thermophoresis – Copper (I, II) and mercury binding affinities to WT recombinant human DJ-1 or point mutated human DJ-1 variants were measured by MST using a Monolith NT.115 instrument (NanoTemper Technologies, Germany). The proteins were fluorescently labeled by use of the Monolith NT L001 labeling assay according to manufacturer's description (NanoTemper Technologies, Germany). Serial dilutions of metal ligands spanning a concentration of 10^{-2} – 10^{6} nM, were mixed in binding buffer (20 mM MOPS buffer, pH 7.2, 150 mM NaCl, 8% glycerol (supplemented with 5 mM ascorbic acid for reduction of Cu(I)) containing NT-647 labeled protein and collected in standard MST sample capillaries. Thermophoresis was analysed by use of 30% LED and 50% laser intensity for all samples, and average K_D values were calculated according to observed mobility shifts from multiple repeats.

Live-cell confocal laser scanning microscopy of redox-sensitive GFP – For the *in vivo* measurements of cytosolic reactive oxygen species dynamics, MEF cells were grown in tissue culture treated 8-well µ-Slide chambers with optical plastic bottom (Ibidi). After 24h cells were transfected with pcDNA-S3roGFP using Lipofectamine 2000 (Invitrogen). Cultures were allowed to express S3roGFP for 24h prior imaging. Shortly before each experiment

culturing media of cells was replaced by HBSS (Thermo Scientific) and all further manipulations were carried out in HBSS. Imaging was done on Nikon A1R confocal laser scanning microscopy system mounted on a Nikon Ti-E inverted epifluorescence microscope, equipped with a 408-nm diode laser and 488-nm multiline Ar-ion laser. Correct temperature and air/CO_2 levels were maintained within the top stage electric incubation chamber with heated glass cover mounted on the microscope stage and connected to humidity module and manual air/CO_2 mixer (UNO, Okolab). Images were taken with Nikon Plan Apo VC 60x/1 40 Oil objective and NIS-elements AR 4.00.07 software of several different XY focal planes for 60 and 90 minutes at 1 and 2 min intervals respectively. Specimen was excited in line-scanning mode with 408 nm and 488 nm wavelengths and emission was collected at 525/550 nm. To minimize the fluorescence bleaching laser power was set at the lowest intensity and was equal for both excitation wavelengths. All other parameters that were shown to affect the resulting ratio were always kept the same for every experiment. Pixel intensities of images were controlled to vary between 500 and 3000 to avoid both saturation and false ratios due to insufficient brightness of the reporter. Metal treatments were added at the min 6 of the experiment and imaging was continued 50 sec later with almost no delays. All quantitative measurements of S3roGFP fluorescence intensity were done on NIS-elements by defining the region of interest copying the shape of the cell and the average intensities were determined for each time point. Prior to this the mean pixel intensity of the background was subtracted from every image using background region of interest.

Statistical analysis – Test of sample homogeneity of variance was performed by the Levene test. Due to non-homogenous sample distribution, the non-parametric Kruskal-Wallis test was performed for test of significance. In tested groups where the null hypothesis could be rejected, the differing pairs were identified by use of the Mann-Whitney U-test. Statistically significant probability values ($P < 0.05$) are indicated with asterisk. All statistical analysis was done using SPSS version 18.0.

RESULTS

DJ-1 is a metal binding proteins that binds copper and mercury – To investigate whether DJ-1 could directly interact with metals we first cloned the human *PARK7* gene into a bacterial expression vector system and purified large amounts of high purity recombinant human DJ-1 protein (Fig. 1A). The metal binding ability of DJ-1 was assessed by dialysing the native purified protein against buffer containing metal salts ($MgCl_2$, $ZnSO_4$, $NiSO_4$, $AlCl_3$, CuCl, $CuCl_2$, $MnCl_2$, $FeCl_2$, $FeCl_3$, or $HgCl_2$) followed by a second dialysis to wash out residual metals. X-ray fluorescence (XRF) analysis of the metal exposed protein revealed evident amounts of both copper as well as mercury bound to the human DJ-1 protein (Fig. 1B). Both copper (I) and copper (II) oxidation states were tested and DJ-1 bound copper was identified by XRF as a single peak. In correlation to the internal titanium peak, a weaker signal for manganese bound to DJ-1 was also observed while the other tested metal salts were negative. Although mercury is reactive and has high relative ion exchange selectivity for a cation exchange adsorbent, both manganese and copper have similar selectivities as iron, zinc, and magnesium. The observed metal bindings were therefore considered to be specific. XRF analysis of the metal containing dialysis buffers against protein and back soak were done to confirm metal peak identity (Fig 1B).

DJ-1, but not the clinical DJ-1 variants A104T and D149A, protects against copper and mercury induced cytotoxicity – At the cellular level, metal concentrations are regulated through processes of absorption, distribution, biotransformation, and excretion (43). For metal absorption in intact tissue, copper (II) is reduced to copper (I) by metalloreductases and copper (I) is delivered across the cells plasma membrane by high affinity copper transporters (44). Iron, manganese as well as non-essential heavy metals may cross the plasma membrane through binding to transferrin and divalent metal transporter 1 (45,46). Intracellular concentrations of essential transition metals are normally maintained within a narrow range, through a complex system of metal binding proteins. Alteration of intracellular transition metal homeostasis is cytotoxic and has been implicated in neurodegenerative diseases, such

as PD (43,47). We performed a set of cytotoxicity tests to evaluate whether DJ-1's metal binding property could serve a role in the cells ability to maintain non-toxic metal homeostasis. To specifically assess the role of DJ-1 we made use of primary mouse embryonic fibroblasts (MEFs) isolated from WT or DJ-1 homozygous knockout mice (48). WT and DJ-$1^{-/-}$ MEF cells were exposed to increasing concentrations (0-1000 µM) of metals for 24 hours before assessment of cell survival. An obvious reduction of cell survival was detected when exposing MEF DJ-1 knockout cells to either copper or mercury, compared to WT cells (Fig. 2A, B).

To ascertain that the observed reduction in cell survival was due to the expression of DJ-1 protein alone, we re-introduced the human DJ-1/PARK7 gene into the DJ-$1^{-/-}$ MEF cells by transfection and selected monoclonal stably re-expressing DJ-1 cell lines. By doing this we also sought to find out if the observed phenotypes could be rescued by re-expression of WT DJ-1 in the knockout background. In addition to WT human DJ-1 we also included DJ-1 mutant A104T and D149A, previously identified in familiar forms of PD (18,19). We also included oxidation site mutated DJ-1 C106A, which lacks the major oxidation site of DJ-1, and have been shown to have reduced cell protective function against mitochondrial induced ROS (36,41). Approximately equal DJ-1 protein expression was seen when comparing endogenously expressed DJ-1 in MEF WT cells to the selected human DJ-1 re-expression cell lines by western blotting (Fig. 3A). Visualization of WT DJ-1 and the mutated DJ-1 variants in intact cells was performed by immunocytochemical staining using an antibody raised against the N-terminal part of human DJ-1. Both WT and the mutated DJ-1 variants retained the same sub-cellular localization, characterised as predominantly soluble, cytosolic localization (Fig. 3B, supplementary figure S1). A low level of DJ-1 could also be seen in the nucleus while other types of organelle localization were not apparent. Re-introduction of human DJ-1 WT expression in the knockout cells significantly rescued cell survival in case of both copper and mercury exposure (Fig. 3C, D), highlighting a potential role for DJ-1 involvement in regulation of metal homeostasis and protection against metal induced cytotoxicity. In the case of PD mutated DJ-1 A104T and D149A, both mutants showed a significant reduction in cell survival compared to DJ-1 WT upon exposure to copper or mercury, whereas the oxidation site mutated DJ-1 C106A retained similar protective features as DJ-1 WT (Fig. 3E, F). The results indicate that the PD mutated DJ-1 variants are not functionally active in response to these stresses. Interestingly, these results also highlight a potential dual role for DJ-1 as a mitochondrial antioxidant and a cytoplasmic metal binding protein, since the oxidation site mutated DJ-1 C106A variant maintained the ability to protect cells exposed to metal stresses (Fig. 3E, F).

Clinically important DJ-1 mutant variants show altered metal binding potential – To further investigate whether the reduced cytoprotective function of DJ-1 PD mutants was a result of altered metal binding ability, recombinant human DJ-1 A104T, D149A and C106A were produced and purified without affinity tag (Fig 4A). The *in vitro* metal binding ability of the DJ-1 proteins were again assessed by dialysing the native purified proteins against buffer containing copper salt, followed by a second dialysis without metal salt to wash out residual metal. To obtain quantitative data for the total amount of copper bound to DJ-1 after 24 hour exposure, atomic absorption spectroscopy (AAS) was used instead of XRF. The AAS analysis affirmed the presence of copper bound to DJ-1 WT, and detected approximately 3 µM copper per µM DJ-1 (Fig. 4B). The analysis also confirmed that all other tested point mutated DJ-1 proteins retained their copper binding ability (Fig. 4B). Surprisingly the copper binding to PD mutant A104T was higher than WT, while copper binding to C106A was significantly reduced. These alterations were not large but still statistically significant due to small sample to sample variations. A 6xHis tagged DJ-1 WT protein was included in the analysis as a positive control for copper binding, while no residual copper was found in dialysed metal binding buffer lacking DJ-1 protein after washing (Fig. 4B). Since the described AAS analysis only reflects the equilibrium of copper bound to DJ-1 at the end point of the dialysis, we also determined the initial binding affinities of the metal ligands. For this purpose we made

use of the microscale thermophoresis (MST) technology, that is based on direct movement of particles in a temperature gradient, also known as the Ludwig-Soret effect (49). Any change of the size, charge and hydration shell of biomolecules due to binding to interacting molecules results in a relative change of movement along the temperature gradient and is used to determine ligand to protein binding affinities. In our study the dissociation constant (K_D) of monovalent copper (I) was found to be in the range of 4-5.7 µM for all DJ-1 variants, except A104T that showed a ~200-fold enhancement in binding affinity (Fig. 4C). The binding affinity of copper (II) was also enhanced in DJ-1 mutant A104T and D149A compared to WT DJ-1, while DJ-1 C106A was not found to bind divalent copper (II). In case of mercury binding, WT DJ-1 and D149A showed a K_D value of 0.06-0.12 µM respectively, while A104T and C106A were not found to bind mercury. These data point out that DJ-1 residues A104-C106 may be important for divalent mercury binding and in case of C106 also divalent copper binding (Fig. 4D). Moreover our data indicate that copper (I) binds to another region of the protein, not directly affected by the studied point mutations. Our AAS study of total amount of copper bound to DJ-1 after 24 hour exposure reveals that copper is found in a 3:1 ratio bound to DJ-1. This analysis would suggest that the second copper binding site would reside within the interface of the two DJ-1 proteins in the dimeric complex (Fig. 4D). A smaller amplitude in the thermophoresis shift was observed for A104T compared to WT DJ-1, which indicates that the A104T mutant may have lower thermostability, which could be linked to the altered binding affinity observed here.

Copper and mercury-induced cytotoxicity protecting is compromised in response to dopamine but does not involve the proteasome – In this study we also attempted to find an answer to the key question of PD, which is why dopaminergic neurons are the main target for degeneration. In parallel to normal aging, neurodegeneration of the dopaminergic cells of the SNpc has been linked to various types of cellular injury due to toxin exposure, oxidative stress, mitochondrial dysfunction, or dysfunction of the ubiquitin proteasome system, as recently reviewed (50). A key reason to why in particular dopamine producing neurons are vulnerable is likely their production of dopamine itself. It is known that products of dopamine degradation are highly toxic for cellular environment. Dopamine metabolism and oxidation into dopamine quinone have been suggested to enhance ROS production, which needs to be controlled by various intrinsic and extrinsic antioxidants. For this reason, in addition to the cytotoxic effects observed in relation to metal exposure, we analyzed additional stresses such as dopamine induced oxidative stress and proteasome inhibition in our cell system. Exposing DJ-1 WT or DJ-$1^{-/-}$ cells to increasing concentration of dopamine showed a significant reduction of cell survival in the DJ-1 deficient cells (Fig. 5A), whereas cells exposed to the cell-permeable proteasome inhibitor MG132 showed no change in survival rates either with or without expressed DJ-1 (Fig. 5B). Since MEF cells lack the neuron-specific dopamine transporters used for active and rapid re-uptake of dopamine from the synaptic cleft, the observed effect seen in this cell model system relies on passive diffusion through organic cation transporters, where the driving force is supplied by the electrochemical gradient of the transported cation (51). Our analysis on dopamine induced cytotoxicity showed a highest no-observed-effect concentration (NOEC) value of 33 µM in both WT and DJ-$1^{-/-}$ cells. However a significant change in LC_{50} value of 95 µM for the DJ-$1^{-/-}$ cells compared to 185 µM for the DJ-1 WT cells were observed after 24 hours exposure (Fig. 5A). To further analyse a possible cumulative effect of dopamine exposure and metal induced cytotoxicity we exposed our cell model system to non-toxic concentrations (6.6 µM or 33 µM) of dopamine in combination with metal stresses and analysed cell survival (Fig. 5C-M). In case of combined copper and dopamine exposure (Fig. 5C, D-H) a clear dopamine dose dependent reduction of cell survival was observed in both DJ-$1^{-/-}$ and DJ-1 re-expression cells. The dopamine dose dependent reduction of cell survival was less evident in DJ-$1^{-/-}$ cells or PD mutated DJ-1 re-expression mutants, which already showed reduced survival rates. In the case of combined mercury and dopamine exposure a similar interrelation in dose

dependent cytotoxicity between DJ-$1^{-/-}$, PD mutated DJ-1 A104T and D149A, and DJ-1 WT and C106A re-expression cells were observed (Fig. 5C, I-M). With non-toxic dopamine exposure, the LC_{50} values for copper dropped to the same levels for DJ-1 WT and C106A re-expression cells as for the DJ-$1^{-/-}$ cells (Fig. 5C). For combined dopamine and mercury exposure, both WT and C106A retained a certain degree of cytoprotection compared to DJ-$1^{-/-}$, and PD mutated DJ-1 variants (Fig. 5C). The observed reduction in cell survival due to metal-induced toxicity, dopamine and combined metal and dopamine induced toxicity could possibly be linked to DJ-1's proposed antioxidative function. As shown here, DJ-1's ability to protect cells from copper- and mercury-induced cytotoxicity is dramatically reduced upon combined non-toxic dopamine exposure, while DJ-1 is not involved in cytoprotective mechanism linked to non-oxidative insults in the form of inhibition of proteasomal degradation.

Dopamine enhances metal induced oxidative stress and DJ-1 contributes to redox homeostasis – Most cellular compartments maintain a reducing environment under normal conditions. The intracellular redox state can however be influenced by internal factors, like mitochondrial hydrogen peroxide production, or external factors like pesticide- or metal-catalysed ROS production. To enable dynamic measurements of the cellular redox environment in real-time upon our stress conditions, we made use of the reduction-oxidation sensitive green fluorescent protein (roGFP) technology (52). When imaged by live-cell confocal laser scanning microscopy (CLSM) the roGFP probe exhibits significant opposing shifts in the fluorescence intensities at two excitation maxima, 408 nm and 488 nm. The shift in the fluorescence intensity is a result of the formation of an oxidation-dependent intramolecular disulphide bridge. The formation of the disulphide bridge is thus directly responsive to the redox state within the analysed cellular compartment in which roGFP is expressed, which enables real-time ROS production monitoring at 408/488 nm. In our hands the used cytosolic S3roGFP probe exhibited clear changes in fluorescence ratio arising directly from changes in intracellular redox state (Fig.6 A, B, supplementary movie M1). Treating DJ-$1^{-/-}$ or DJ-1 WT expressing cells with up to 100 µM dopamine alone did not result in any effect on oxidative conditions in the cells during one hour of imaging (Fig. 6C). However, exposing the cells to 100 µM CuCl resulted in an evident enhancement of the intracellular oxidative state of the cells (Fig. 6D, E, (blue line)). Interestingly, exposing the cells to a non-toxic concentration of 33 µM dopamine together with 100 µM CuCl, resulted in almost immediate and dramatic surge of intracellular oxidation (Fig. 6D, E, (red line)). The oxidative stress observed upon combined copper and dopamine exposure was both faster and larger in magnitude in the DJ-$1^{-/-}$ cells compared to DJ-1 WT. Similar S3roGFP experiments were performed using 5 µM $HgCl_2$ treatments together with 33 µM dopamine. On the contrary to copper treatment experiment where oxidation can be explained by enhanced Fenton reaction, mercury exposure alone shifted the intracellular redox status slightly towards a more reduced state (Fig. 6F, G, (blue line)). Interestingly, combined mercury exposure with dopamine treatment resulted again in an obvious enhancement of intracellular oxidation in the DJ-$1^{-/-}$ cells, that was absent in DJ-1 WT cells (Fig. 6F, G (red line)). The live-cell measurements of the intracellular redox state were done during a relative short time span of 90 minutes, due to the cytotoxicity of the treatments. The results clearly indicate that the presence of non-toxic concentration of dopamine enhances metal induced oxidative stress and that DJ-1 is important for the maintenance of the cells redox buffering capacity.

DISCUSSION

Our study identifies human DJ-1 as a metal binding protein able to evidently bind copper and mercury *in vitro*. We also show that expression of DJ-1 protects the cells against mercury and copper induced cytotoxicity and that PD mutated DJ-1 A104T and D149A have lost this function. To our knowledge this is the first report identifying DJ-1 as metal binding protein involved in metal homeostatic processes and able to protect cells from induced metal cytotoxicity.

Previous cross sectional as well as longitudinal population based studies of heritability of PD in

twins have reported no or a very low genetic component in typical, late onset PD (53-55). It has therefore been suggested that environmental factors are most important in typical, late onset PD, while genetic factors are substantial in early onset PD (53). The primary environmental effectors believed to be involved are toxic pollutants such as pesticides and toxic metals, causing brain inflammation and oxidative damage to neurons (56,57). Mercury, in particularly the lipophilic methylmercury, bioaccumulates in the food chain and is stored in biological tissue, especially in the brain (58). Dose-response association between PD and blood mercury levels has been reported (10). Alterations in brain iron homeostasis has also been linked to PD, where up to two-fold elevation of iron levels specifically in the SNpc and lateral globus pallidus in comparison to age-matched controls has been reported, as reviewed in (47,59). Chronic occupational exposure to manganese or copper, individually or combinations of lead, iron and copper, has been associated with PD (7). Since both copper and iron are capable of catalyzing free radical formation via the Fenton reaction excess accumulation or deficient regulation of these metals can induce production of ROS and trigger cell death. Our study indicates that even a small genetic alteration can sensitize cells to metal induced cell death. This finding support the assumption that a combination of genetic predisposition may sensitise cells to environmental toxin induced cell death, and subsequent susceptibility for disease development.

As previously outlined, mutation of the human DJ-1/PARK7 gene has been associated with familial early onset PD. In some patients the DJ-1/PARK7 gene is deleted (12). In the case of the DJ-1 point mutations M26I and L166P, the mutations alter the proteins' stability leading to enhanced degradation of DJ-1 and can therefore be considered clear loss of function mutations (60). The reasons for PD development in other DJ-1 point mutation carriers are less clear. The ability to dimerize and expression levels of the studied PD mutants A104T and D149A are comparable to non-mutated DJ-1 and do not *per se* support a loss of function hypothesis (60,61). However, circular dichroism analysis have revealed reduced termostability of mutant A104T and D149A, indicating secondary structure changes (62). DJ-1 A104 and D149 are completely conserved residues between a broad spectrum of species, highlighting a potentially important structure-function relationship. In our cell model system both the A104T and the D149A mutant forms are normally expressed as compared to WT DJ-1. However, our data show that both PD mutants have lost their ability to protect cells against copper and mercury induced stresses, resembling DJ-1 deficient cells. These results indicate that the mutants are not functionally active in terms of cytoprotection. Mutating DJ-1 cysteine 106 to alanine has been shown to abolish DJ-1's ability to protect cells from MPP^+ and rotenone induced oxidative stress as a result of mitochondrial damage (36,41). Our data show that C106A mutated DJ-1 retains its cytoprotective function against induced metal exposure, indicating that DJ-1s ability to function as an antioxidant through C106 oxidation is not needed for metal induced stress protection. Exposing cells to non-toxic concentrations of dopamine enhances the cytotoxic effect of metal exposure. Oxidised dopamine itself has been shown to bind directly to cysteine residues in proteins (63). It has also been suggested that dopamine bound to proteins may be involved in redox alteration under oxidative stress, generating redox-active cysteinyl-dopamine adduct that enhance metal-catalysed oxidation of proteins (64). Our live-cell analysis of redox sensitive S3roGFP confirms these suggestions and provide direct *in vivo* evidence of this enhanced intracellular oxidation.

The observation of enhanced cell toxicity upon combined metal and dopamine treatment may also be interesting from a pharmacological perspective. The dopamine concentrations used in this study were not higher than the blood plasma dopamine concentrations measured in PD patients on conventional levodopa therapy, where mean plasma levodopa concentration ranging between 2.3-35.9 μM (0.45-7.07 μg/ml) and peak concentrations up to 69.7 μM (13.75 μg/ml) has been reported (65). Our data shows that non-toxic concentration (6.6-33 μM) of dopamine in combination with metals dramatically enhances ROS production and

reduced cell survival in comparison to metals alone. While this *in vitro* study hints that individual bodily accumulation of toxic metals could accelerate cell death upon dopamine replacement therapy, the relevance of this observation to the *in vivo* situation in PD patients cannot be assessed without further studies in clinical material. We hope that our findings may revive the interest in exogenous factors in the etiology of PD and encourage new research aiming for a better understanding of metal homeostatic processes and their role in the etiology of idiopathic PD.

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Acknowledgements – We thank Dr. Darren Moore at the Brain Mind Institute, EPFL, Switzerland for providing the rabbit anti-DJ-1-N antibody. We thank Dr. Huaibin Cai, National Institute on Aging, NIH, Bethesda, USA for MEF cells from WT and DJ-$1^{-/-}$ mice. We also thank Dr. C. Plieth, University of Kiel, Germany for the sequence of redox-sensitive S3roGFP.

FOOTNOTES

*This work was funded by the Western Norway Regional Health Authority (Helse Vest project grant no. 911575).

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The abbreviations used are: PD, Parkinson's disease; SNpc, substantia nigra pars compacta; ROS, reactive oxygen species; MEF; mouse embryonic fibroblasts; AAS, atomic absorption spectroscopy; XRF, X-ray fluorescence; MST, microscale thermophoresis; NOEC, no-observed-effect concentration; CLSM, confocal laser scanning microscopy

FIGURE LEGENDS

FIGURE 1. Human DJ-1 purification and metal binding analysis. A. Purified recombinant human DJ-1 analysed by SDS-PAGE and Coomassie G250 stained for detection of total protein. High purity non-tagged human DJ-1 used for XRF indicated at arrow. B. Combined XRF spectra of recombinant human DJ-1 exposed to metal containing buffer (coloured solid line), metal containing buffer only (dotted line) and backsoak buffer without added metal salt (black solid line). Prominent DJ-1 metal binding peaks for Cu (I), Cu (II) and Hg (II) were detected as well as a weaker signal for Mn (II). Asterisk (*) marks the internal titanium peak originating from the XRF instrument.

FIGURE 2. Metal exposure and analysis of cell survival. Cell survival data comparing WT and DJ-$1^{-/-}$ MEF cells exposed to increasing concentration of metals for 24 hours. All values were compared to non-treated control set to 100% survival and plotted against the logarithmic scale for the used metal concentrations. A. CuCl, WT LC_{50} = 70 µM, DJ-$1^{-/-}$ LC_{50} = 30 µM. B. $HgCl_2$, WT LC_{50} = 15 µM, DJ-$1^{-/-}$ LC_{50} = 4 µM. Statistically significant change in cell survival compared to DJ-$1^{-/-}$ cells indicated with *, $P < 0.05$.

FIGURE 3. Characterization and comparison of WT or PD mutated DJ-1 re-expression cells to metal stresses. A. Western blot of wild type, DJ-$1^{-/-}$ MEF cells and stable DJ-1 re-expression MEF cells expressing human DJ-1 variants as indicated. Equal protein expression was detected using protein specific antibodies raised against human DJ-1, and for actin as loading control. B. Immunocytochemical staining of DJ-$1^{-/-}$ MEF cells or DJ-1 re-expression cells expressing human DJ-1 variants as indicated. Green = DJ-1, blue = nucleus. Analysed DJ-1 variants show equal sub-cellular localization, predominantly soluble and cytoplasmic. Scale bars, 10 µm. -1˚ = minus primary antibody. C-D. Cell survival data comparing empty vector transfected DJ-$1^{-/-}$ cells or stably re-expressing human DJ-1 WT cells exposed to increasing concentration of metal stresses for 24 hours. C. CuCl, WT LC_{50} = 65 µM, DJ-$1^{-/-}$ LC_{50} = 32 µM. D. $HgCl_2$, WT LC_{50} = 7 µM, DJ-$1^{-/-}$ LC_{50} = 3 µM. E-F. Cell survival data comparing DJ-$1^{-/-}$ or re-expression cells expressing human DJ-1 WT, PD mutated human DJ-1 A104T, D149A or the oxidation site mutated human DJ-1 C106A. Cells exposed to increasing concentration of CuCl (E) and $HgCL_2$ (F) metal stresses for 24 hours before cell survival analysis. Statistically significant increase in cell survival compared to DJ-$1^{-/-}$ cells indicated with *, $P < 0.05$.

FIGURE 4. Point mutated DJ-1 metal binding analysis. A. Western blot analysis, using anti-6xHis and anti-DJ-1 specific antibodies of purified recombinant human DJ-1 WT and point mutated DJ-1 A104T, DJ-1 D149A, DJ-1 C106A and DJ-1 WT-6xHis tag as positive control. B. AAS analysis of total amount of copper bound to recombinant human DJ-1 proteins, as shown in A, after 24 hour exposure time. Statistically significant changes in total copper binding compared to WT DJ-1, are indicated with asterisks, ** $P < 0.01$, *** $P < 0.001$. C. Initial binding affinities for metal binding to DJ-1 recombinant human DJ-1 proteins, as shown in A. Calculated average MST dissociation constants (K_D) for recombinant human DJ-1 WT and point mutated variants using Cu (I), Cu(II) and Hg(II) as ligands. Lack of detected binding indicated with - sign. D. Crystal structure model of dimeric human DJ-1 with the studied mutation sites indicated with *, and potential metal binding areas indicated within squares (dotted line). Protein structure model adopted from PDB ID: 2R1V.

FIGURE 5. Comparison of WT or DJ-1 deficient cell survival in response to dopamine, MG132 or combined dopamine and metal induced stress. A. Cell survival data comparing DJ-$1^{-/-}$ and WT DJ-1 expressing MEF cells exposed to increasing concentrations of dopamine. Highest NOEC of dopamine observed at 33 µM in both DJ-1 knockout and DJ-1 WT expressing cells. WT LC_{50} = 185 µM, DJ-$1^{-/-}$ LC_{50} = 95 µM. Statistically significant change in cell survival indicated with *, $P < 0.05$. B. No change is cell survival was observed upon treatment with proteasome inhibitor MG132. C. Table of LC_{50} values calculated from combined dopamine and metal induced cell toxicity data shown in D-M.D-M. Cell survival data comparing DJ-$1^{-/-}$ or cells stably re-expressing human DJ-1 WT, A104T, D149A or C106A, exposed to 0, 6.6 or 33 µM dopamine in combination with increasing concentration of copper (D-H) or mercury (I-M) stresses for a total of 18 hours. Dopamine dose dependent reduction of cell survival was observed in re-expressing DJ-1 WT and DJ-1 C106A cells, as well as in DJ-$1^{-/-}$, DJ-1 A104T and D149A re-expression cell lines exposed to copper or mercury.

FIGURE 6. Real-time analysis of oxidative stress levels by use of CLSM live-cell imaging of redox-sensitive S3roGFP. A. CLSM live-cell imaging experiment showing redox-sensitive S3roGFP 408/488 nm ratios in transfected MEF DJ-1 WT cells. The cells were exposed to 0.5 mM H_2O_2 to mimic enhanced oxidative stress conditions 5 minutes after initiating image acquisition. After 35 minutes imaging acquisition, the cells were reduced by treatment with 2 mM DTT. A clear change in the 408/488 nm ratio were observed upon oxidation and reduction of the imaged cells. B. CLSM images of S3roGFP expressing cells treated as shown in A. The observed redox-dependent change in 408/488 nm ratio is visualized by pseudo-colouring according to included ratio scale bar 0-1.2 (blue; reduced, oxidised; red). Image size scale bar, 20 µm. C. CLSM live-cell imaging experiment showing S3roGFP 408/488 nm ratios in DJ-$1^{-/-}$ and DJ-1 WT expressing cells exposed to 100 µM dopamine, arrow. Mean value, +/- SEM for five cells per cell type are shown. D. S3roGFP 408/488 nm ratios in DJ-1 WT expressing cells exposed to 100 µM CuCl, with or without addition of 33 µM dopamine. Control indicates non-exposed cells. Mean value, +/- SEM for ten cells per treatment are shown. E. S3roGFP 408/488 nm ratios in DJ-1-/- cells treated as in D. Mean value, +/- SEM for ten cells per treatment are shown. F. S3roGFP 408/488 nm ratios in DJ-1 WT expressing cells exposed to 5 µM $HgCl_2$, with or without addition of 33 µM dopamine. Mean value, +/- SEM for 12-17 cells per treatment are shown. G. S3roGFP 408/488 nm ratios in DJ-1-/- cells treated as in F. Mean value, +/- SEM for 13-19 cells per treatment are shown. Cells that died during the analysis were excluded from the shown quantified data.

Figure 1

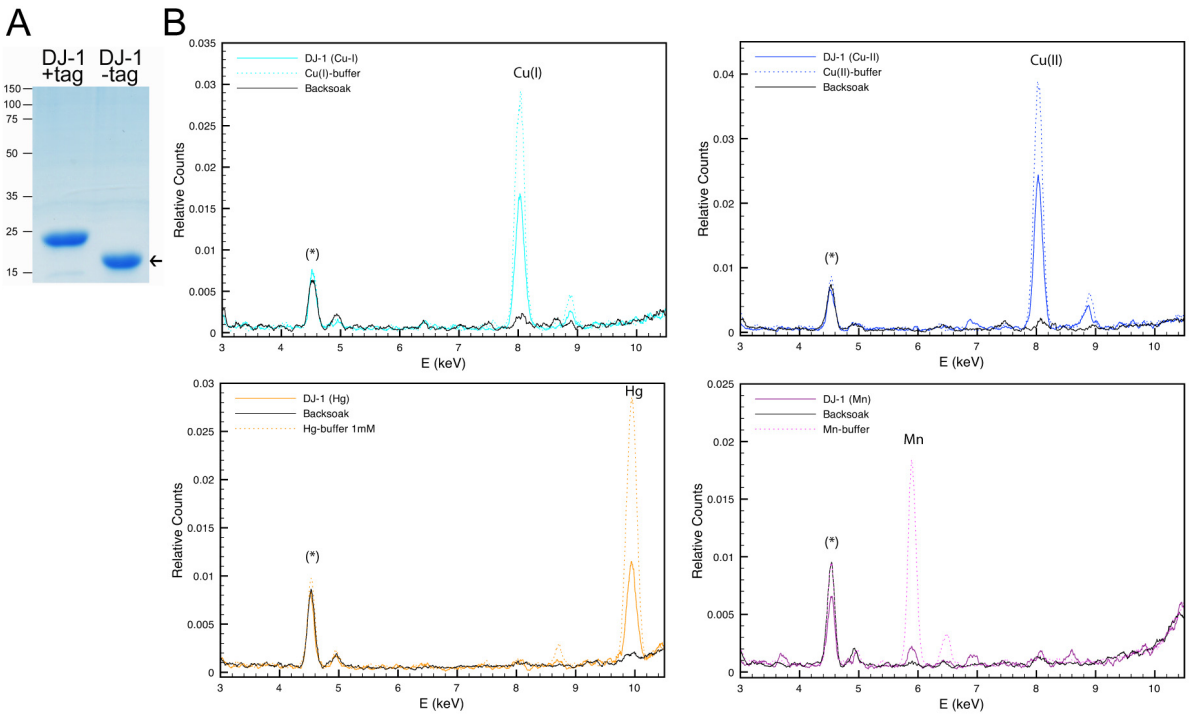


Figure 2

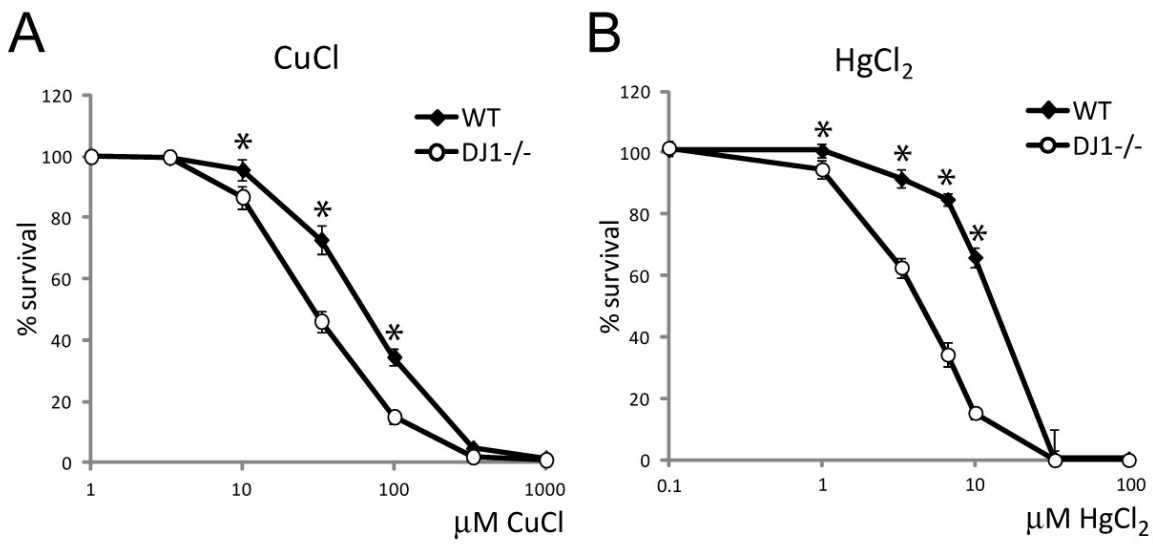


Figure 3

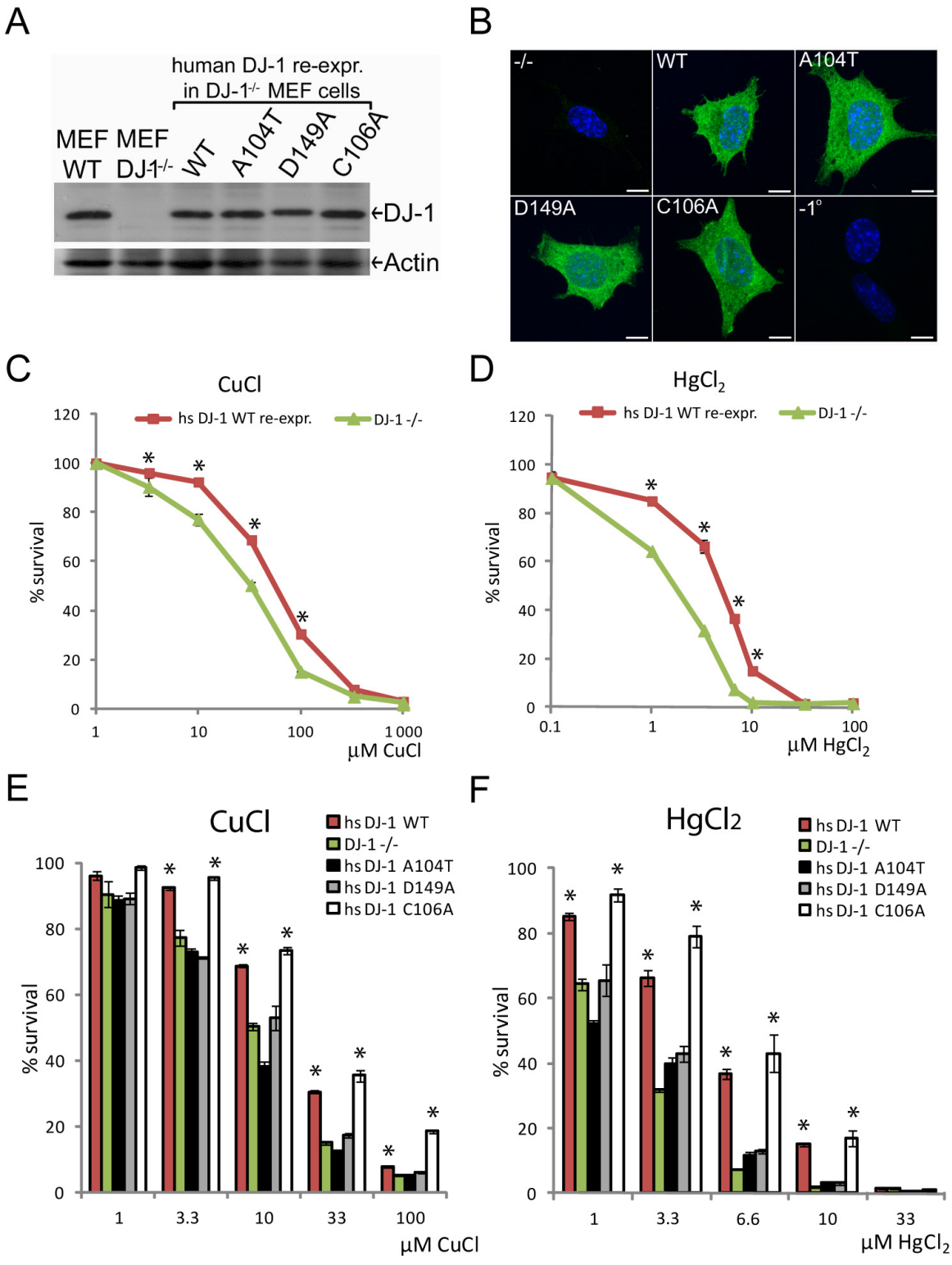
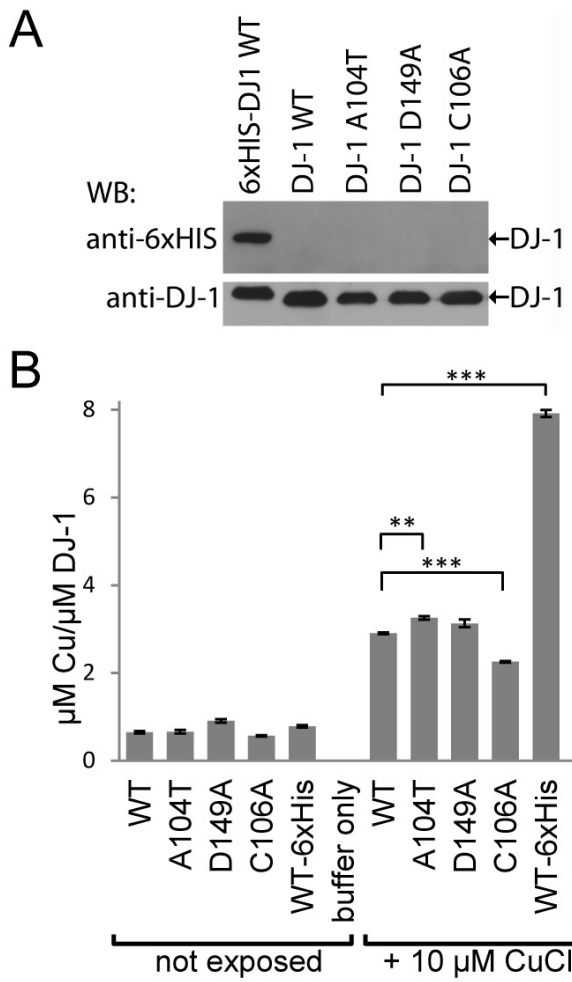


Figure 4



C

ligand	MST K_D values [μM] WT	A104T	D149A	C106A
Cu^{+}	5.66	0.02	4.00	4.35
Cu^{2+}	3.10	0.05	0.67	-
Hg^{2+}	0.06	-	0.12	-

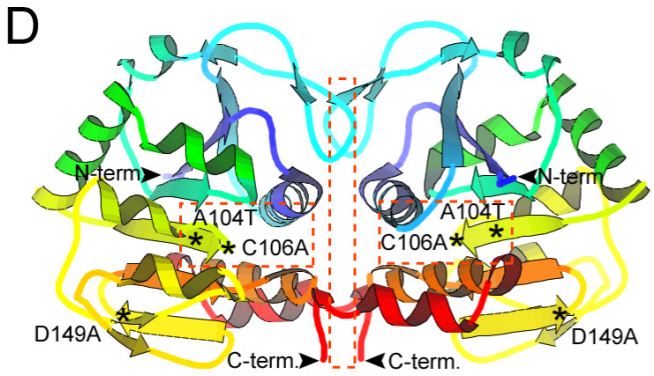
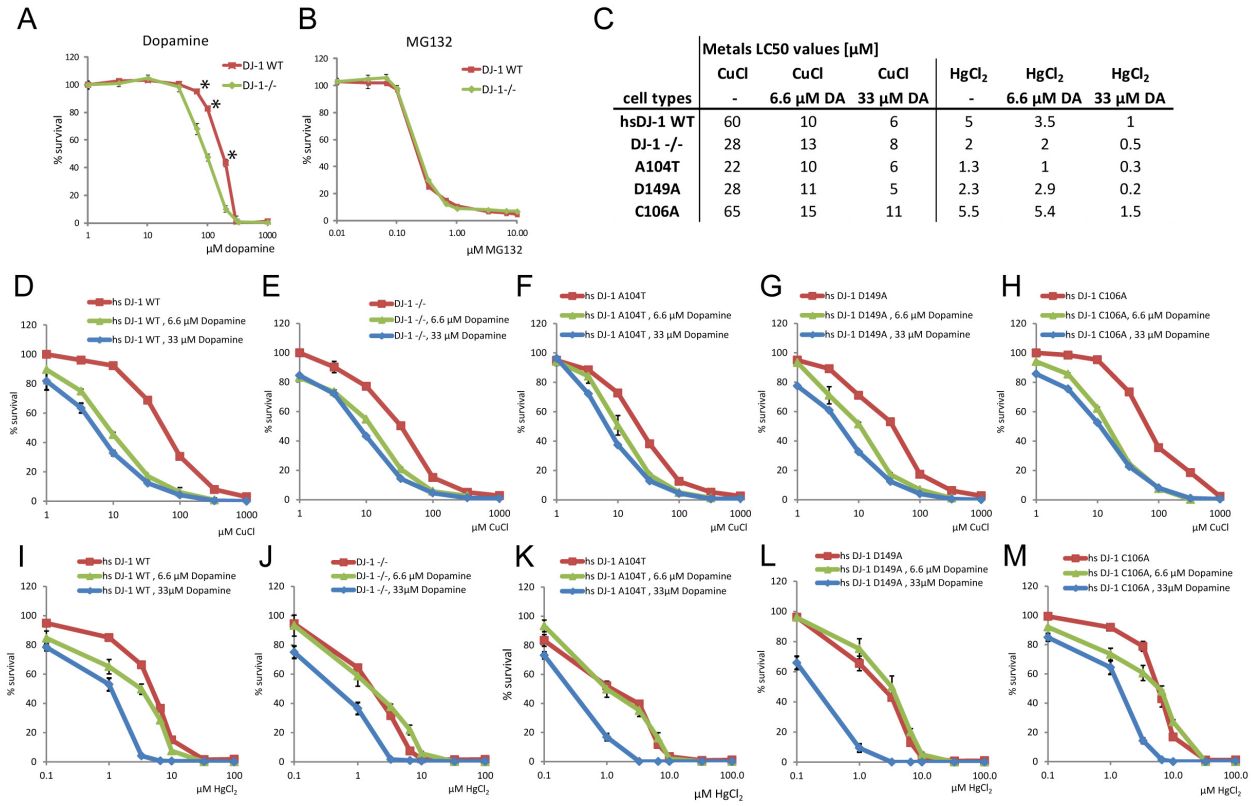


Figure 5



	Metals LC50 values [µM]					
	CuCl	CuCl	CuCl	$HgCl_2$	$HgCl_2$	$HgCl_2$
cell types	-	6.6 µM DA	33 µM DA	-	6.6 µM DA	33 µM DA
hsDJ-1 WT	60	10	6	5	3.5	1
DJ-1 -/-	28	13	8	2	2	0.5
A104T	22	10	6	1.3	1	0.3
D149A	28	11	5	2.3	2.9	0.2
C106A	65	15	11	5.5	5.4	1.5

Figure 6