THE INTERACTION BETWEEN MSRA AND CSN5/JAB1

By

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The Interaction between Methionine Sulfoxide Reductase A and Csn5/Jab1

ABSTRACT

Oxidative stress can cause toxic outcomes to cells/organisms' survival. To counter this toxicity, multiple cellular mechanisms were evolved. Among them is the methionine sulfoxide reductase (Msr) system. Methionine sulfoxide reductase A (MsrA) plays a key role in protecting cells from oxidative stress. In our previous studies we discovered a novel role of MsrA in the regulation of ubiquitin (Ub) and Ub-like modification of proteins. To further understand the role of MsrA in posttranslational modification of proteins, a yeast-2-hybrid (Y2H) screening was performed to screen for potential substrates for MsrA in brain. Accordingly, one of the major identified substrates was a protein denoted as COP9 signalosome subunit 5 (CSN5, also known as Jab1 or COPS5), containing a unique binding site to MsrA. The COP9 signalosome complex (CSN) is an essential regulator of the ubiquitin conjugation pathway by mediating the removal of Nedd8, an Ub-like protein modifier, from proteins (deneddylation process). Importantly, the Csn5/Jab1 contains a domain that provides the catalytic center of the CSN complex. The known substrates for neddylation and deneddylation (by CSN) are the cullin subunits and their homologues (e.g. Cul-1 of the SCF-type E3 ligase complexes). Furthermore, we showed that MsrA interacts with Csn5/Jab1 in mouse brain following immunoprecipitations and pull-down experiments. This interaction was compromised in brain extracts of *MsrA* knockout (*MsrA* KO) compared with the parent wild type (WT) brains. A decrease in the levels of neddylated Cul-1 was also observed in liver extract of *MsrA* KO mouse, while the levels of Csn5/Jab1 in brain were the same in both the WT and *MsrA* KO strains. These data suggested that MsrA positively regulates Csn5/Jab1 deneddylation activity, presumably via reduction of this protein MetO residue/s. To further study the relationship between neddylation level and MsrA *in vivo*, we used yeast strains with various expression levels of MsrA. These yeast strains were WT, *MsrA* KO and *MsrA* overexpressed (OE) yeast strains. Neddylation levels of yeast under the condition of hydrogen peroxide or human Csn5/Jab1 inhibitor were investigated. The data showed that both oxidative stress and Csn5/Jab1 inhibitor caused inhibition of deneddylation activity of Csn5/Jab1 in the absence of MsrA, while MsrA-containing strains (i.e. WT and OE) showed a strong ability to protect against H_2O_2/Csn5/Jab1 inhibitor induced neddylation. For *in vitro* assays, we used artificial Nedd8 conjugates monitoring deneddylation activity in extracts of brains of WT and *MsrA* KO mice strains as function of incubation time. The acquired data showed that the deneddylation activity was dramatically reduced in the *MsrA* KO compared with the WT brain extracts. In conclusion, the presented data provide direct and indirect evidence to support our hypothesis that MsrA plays an important role in maintaining the Csn5/Jab1 deneddylation activity. Lastly, we investigated the potential role of MsrA in regulating of neddylation levels in brain cancer. Glioblastoma is one of the most aggressive brain cancers. This cancer type demonstrates a relative low level of neddylation compared with normal brain cells. Accordingly, Csn5/Jab1 could be one of the posttranslational regulated proteins that may contribute to this

phenomenon. We have indeed found that the neddylation level was reduced in glioblastoma cells, while the level of Csn5/Jab1 was unchanged compared with normal brain cells. This observation pointed to the possible role of MsrA in enhancing Csn5/Jab1 activity in these cancer cells. This possibility will be further investigated by comparing the MsrA activities in both the normal and glioblastoma cells in the nearest future.

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LIST OF ABBREVIATIONS

COP9: Constitutive photomorphogenic-9 signalosome
CRL: Cullin-RING ubiquitin ligase
CSN: COP9 signalosome
CSN5: COP9 signalosome subunit 5
Cul-1: Cullin 1
Cys: Cystine
IP: Immunoprecipitation
Met: Methionine
MetO: Methionine sulfoxide
MsrA: Methionine Sulfoxide Reductase A
NAE: Nedd8 activating enzyme
NCE: Nedd8 conjugating enzyme
Nedd8: Neural precursor cell expressed developmentally down-regulated 8
ROS: Reactive oxygen species
Rub1: Related to Ubiquitin-1
Ub: Ubiquitin
UBL: Ubiquitin-like molecule
WB: Western blot
Y2H: Yeast two hybrid system
TX: Thioredoxin

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CHAPTER 1: INTRODUCTION

1.1 The Methionine Sulfoxide Reductase System and MsrA

1.1.1 The Methionine Sulfoxide Reductase System

Methionine (Met) is an essential amino acid for most mammals, including humans. It is also one in addition to cysteine (Cys) that is a sulfur-containing amino acid. Methionine has an S-methyl thioester side chain, which is easily to oxidize to methionine sulfoxide (MetO). Oxidized Met residues in proteins can be frequently found in biological samples. These oxidized proteins usually exhibit impaired activity. For example, phosphoglucomutase, chymotrypsin[1] and the pituitary adrenocorticotropin[2] are inhibited as the consequence of methionine oxidation. Remarkably, serum protein α_1-proteinase inhibitor (α_1-antitrypsin) is inactivated due to a critical Met residue oxidation[3].

Oxidative stress is usually driven by environmental and cellular factors. It is manifested by the production of reactive oxygen species (ROS) that in turn can be toxic to the organism. ROS are often generated as a byproduct of cell metabolism[4]. Apoptotic mechanisms and exogenous substances such as chemotherapy drugs could also contribute to ROS generation[5].

To counter the threat of oxidative stress, multiple antioxidant systems have evolved, among them is the methionine sulfoxide reductase (Msr) system. This system is highly conserved through evolution and can be found in mammals as well as in bacteria and archaea[6]. This system is also the only one that can specifically reduce oxidized protein-bound and free methionine. The Msr system consists of two types of enzymes, MsrA and MsrB that reduce methionine sulfoxide in its S and R form, respectively. In most organisms there is one type of MsrA and three types of MsrB (MsrB1/2/3). MsrA is a positive regulator of MsrB expression[7]. In most of biological systems, Msr system utilize reduced thioredoxin as the natural reducing agent. *In vitro* activity assays suggest that the Msr system can use various cellular reduction powers to reduce MetO. For example, reduced forms of either thioredoxin, glutathione, or Dithiothreitol (DTT) can serve as reduction powers for the Msr activity. Through the cycled reduction of MetO to Methionine, the Msr system can scavenge ROS and then alleviate oxidative stress in organisms[8].

1.1.2 Methionine Sulfoxide Reductase A

Methionine sulfoxide reductase A was initially identified in studies on the reactivation of oxidized *Escherichia coli* ribosomal protein L12[9]. It is a highly conserved protein and *MsrA* gene family also shows high homology among different species[10]. The mammalian MsrA was first sequenced by Dr. Moskovitz and was found to be 64% identically homologues to the bacterial form[11]. This observation suggest that the MsrA has an important evolutionary role in most organisms. The *in vivo* reducing ability of MsrA is tightly associated with Thioredoxin/NADPH system. Thioredoxin (Tx) receives an electron from NADPH and by that it is converts to its reduced form. Reduced Tx is the natural electron donor for reducing the Cys

residues of MsrA allowing MsrA to reduce MetO. The reduction of MetO proceeds through nucleophilic attack of the catalytic Cys on the sulfoxide with concomitant protonation of the oxygen. The regeneration of the reductase activity passes through formation of a disulfide bond in the MsrA enzyme, which possess a recycling Cys[12,13]. Recent studies have reported that a subtype of MsrA (which have two recycling Cys) could have a stereospecific oxidase activity in conditions where reducing power is absent[14].

MetO-containing proteins/peptides, as well as free MetO can serve as substrates for MsrA *in vivo* and *in vitro*. Synthetic substrates for MsrA can facilitate *in vitro* evaluation of MsrA activity. For example, 4-dimethylaminoazobenzene-4'-sulfonyl-L-methionine-dl-sulfoxide (DABS-L-MetO) can be reduced by MsrA *in vitro* with the presence of DTT and the product of the reaction can be separated by chromatography using HPLC-C18 column and accordingly the MsrA activity can be quantified[15].

Mammalian MsrA is expressed throughout the body, while the highest expression levels are commonly detected in liver and kidney, followed by brain[16].

1.1.3 The role of MsrA in disease

The absence of MsrA could lead to various cellular malfunctions. The disruption of *msrA* gene in *Saccharomyces cerevisiae* was shown to cause hypersensitivity of the mutated yeast strain to oxidative stress compared to wild-type strain[17]. Likewise, the *MsrA* KO mouse is more vulnerable to oxidative stress and demonstrates several molecular phenotypes that can be linked to age-associated diseases[18]. For example, some characterized phenotypes are associated with Alzheimer's disease (AD)[19] and Parkinson's Disease (PD)[20]. Lack of MsrA can cause other organ or cellular malfunctions that are not directly linked to neurodegeneration. For example, mental health disorders[21,22], heart disease[23], liver toxicity[24] and cancer[25]. Additionally, MsrA is involved in maintaining the basic cochlea structure, in which its deficiency may contribute to hearing loss[26]. In conclusion, MsrA seems to be involved and regulate many cellular processes that affect the integrity and function of the whole organism, a specific organ, or cellular development as function of age and oxidative stress conditions.

1.2 COP9 Signalosome Subunit 5 and neddylation

1.2.1 Csn5/Jab1

Csn5, also known as COPS5 or Jab1, is the fifth subunit of the constitutive photomorphogenic-9 signalosome (COP9). It was initially identified as c-Jun activation domain-binding protein-1 (Jab1)[27].The COP9 signalosome (CSN) is an evolutionary conserved among all eukaryotes, multi-subunit complex with a canonical composition of eight subunits (Csn1–8) found in all multicellular organisms. CSN regulates the activity of the largest family of ubiquitin E3 ligases, Cullin-RING ubiquitin ligases (CRLs). Regulation of CRLs by the CSN involves the catalytic activity of Nedd8 hydrolysis from the cullin scaffold subunit of CRLs through a metalloprotease MPN+/JAMM motif within the catalytic subunit, Csn5/Jab1. In short, CSN promotes deneddylation of Cullin (i.e. Cul-1) and Csn5/Jab1 provide the catalytic center to fulfil this function[28,29].

Csn5/Jab1 contains a c-Jun binding domain (JBD), an Mpr1-Pad1-N-terminal (MPN) domain containing the Zn^{2+}-metalloprotease motif (JAMM) that provides the catalytic center to the complex for the CSN isopeptidase activity[30,31]. Interestingly, although Csn5/Jab1 only exhibits deneddylase activity when it interacts with the other CSN components[32,33], a large portion of the free Csn5/Jab1 is detected in both cytoplasm and nucleus[34], while CSN-associated Csn5/Jab1 is mainly in present in the nucleus. The Csn5/Jab1 mediated deneddylation of Cul-1 act as an upstream regulator of SCF-dependent ubiquitination of numerous substrates, including p27 and IκBα[35].

Recently, researches have suggested that Csn5/Jab1 is overexpressed in cancer[36]. While Csn5/Jab1 is systemically expressed in both tumor tissues and normal tissues, Csn5/Jab1 expression levels in cancers are significantly higher than those in normal tissues. Csn5/Jab1 overexpression was reported in many types of cancers, including hepatocellular carcinoma (HCC)[37], pancreatic cancer[38], breast cancer[39], non-small cell lung cancer (NSCLC)[40], nasopharyngeal carcinoma (NPC)[41], and many others[42].

1.1.2 Neddylation

Neddylation is a posttranslational modification that adds ubiquitin-like protein that is termed as neural precursor cell expressed developmentally down-regulated 8 (Nedd8) to substrate proteins[43]. As a member of Ubiquitin-like molecule (UBL), Nedd8 covalently conjugated to a limited number of cellular proteins in a manner analogous to ubiquitination. In a canonical neddylation process, Nedd8 is activated by Nedd8 Activating Enzyme (NAE)[44]. Nedd8 is then transferred from the NAE via the Nedd8 conjugating enzyme (NCE) and the RING-box protein RBX1 to the Cullin subunit of Cullin-RING E3 ubiquitin ligases (CRL)[45]. RBX1 serves as the E3 ligase for Nedd8 and as an E3 ligase for subsequent ubiquitination reactions[46]. The Cullin subunits of CRLs are the best-studied neddylation substrates. CRLs are stabilized by RBX1-mediated Cullin neddylation at their conserved WHB domain. Neddylation loosens the interaction of RBX1 with the WHB domain and RBX1 can subsequently promote E2-dependent ubiquitination and protein degradation[47]. CRLs are the largest family of multiunit E3 ubiquitin ligases, controlling degradation of about 20% of proteasome-regulated proteins, involving in many aspects of important biological processes[48,49,50]. Over-activation of CRLs leads to cancer growth and development[51]. Nedd8 and enzymes of neddylation pathways are often overexpressed in multiple human cancers, which are associated with cancer progression and poor patient prognosis[52]. Over-activated neddylation pathway also leads to the elevated global neddylation of substrates, to consequently promote degradation of tumor suppressor (e.g. p21 and p27) and facilitate oncogenesis and tumor progression. Thus, regulating neddylation pathways can be a potential anticancer strategy.

1.3 Glioblastoma

Glioblastoma is the most common and most aggressive primary malignant brain tumor in adults[53]. It comprises 15% of all intracranial neoplasms and 60-75% of astrocytic tumors[54]. The initial signs of glioblastoma are usually non-specific, which include headaches, personality

changes, nausea, and symptoms similar to those of a stroke[55]. Glioblastoma is derived specifically from unregulated grown astrocytes, or precursor stem cells that have mutations. The mutations can usually be one or a combination of the following: loss of chromosome 10q, epithelial growth factor receptor (EGFR) amplification, murine double minute 2 (MDM2) overexpression, phosphate and tensin homologue (PTEN) gene mutation, or TP53 mutation[56].

It is very hard to treat glioblastoma. Researchers must face a dilemma of either using therapeutic doses of anticancer agents to enable them to enter the blood brain barrier at therapeutic concentrations or to avoid them due to their tendency to increase the risk of systemic toxicity[57]. As a result, surgery is considered the first stage of glioblastoma treatment. Radiotherapy is also frequently used for the treatment. Chemotherapy has few effects on glioblastoma, but Temozolomide (TMZ) is used together with radiotherapy to increase median survival time of the patients. Other drugs, i.e. EGFR blocker cetuximab did not go beyond phase II trial due to limited effects[58]. Unfortunately, the median survival time of glioblastoma patients following diagnose is still no more than 1 year. Glioblastoma also usually recurs despite maximum treatment and the survival is typically 3 months without treatment[59]. Recent studies[60] showed that Csn5/Jab1 is overexpressed in glioblastoma. We think that our study that unveils interaction between MsrA and Csn5/Jab1 may contribute to the knowledge regarding the development and progression of glioblastoma and help in establishing a novel MsrA-Csn5/Jab1 - based glioblastoma treatment.

1.4 Statement of hypothesis

We hypothesize that MsrA interacts with Csn5/Jab1 and that this interaction positively regulates Csn5/Jab1 function through a reduction of specific MetO residue/s of Csn5/Jab1 that is mediated by MsrA. This function of MsrA maybe relevant to the Csn5/Jab1 function in glioblastoma.

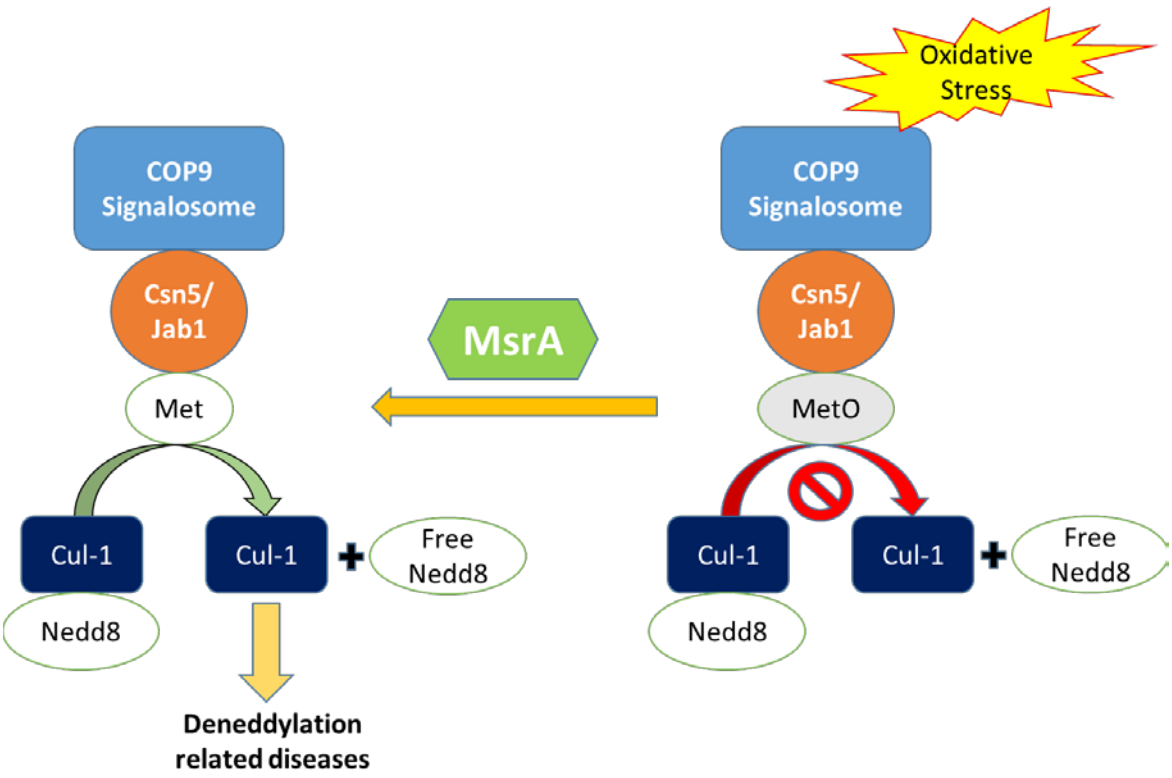

Figure 1. A schematic illustration of the involvement of MsrA in regulating Cul-1 deneddylation under oxidative stress conditions. The presence of external or internal cellular oxidative stress may lead to the formation of reactive oxygen species (ROS) that in turn cause oxidation of Met residue/s of Csn5/Jab1, the core subunit of COP9 Signalosome (CSN). Deneddylation activity of Csn5/Jab1 is inhibited due to the oxidation. As a result, the substrate of Csn5/Jab1, Cul-1 is more neddylated. The presence of MsrA reduces MetO to Met. Thus, it leads to an increase of Csn5/Jab1 deneddylation activity. Enhanced deneddylation activity of Jab1 is associated with various types of diseases, for example, glioblastoma.

CHAPTER 2: MATERIALS AND METHODS

2.1 MsrA interacts with Csn5/Jab1 in brain

2.1.1 Yeast-two-Hybrid Assay Screening for substrates of MsrA

The yeast two-hybrid (Y2H) screen was performed using a full-length MsrA cDNA sequence as bait and an adult human brain cDNA library as the prey [Clontech, product number 630486: 8 Caucasian males ages 43-66 sudden death, 3.2 x 106 independent clones] was performed by the "Next Interactions Inc." company (Richmond CA).

The Y2H procedures were performed similarly to Clontech's protocol of the Matchmaker Gold Yeast Two-Hybrid System. Constructs were made using Clontech's pGBKT7 and pGADT7 plasmids that are components of the Matchmaker Gold Yeast Two-Hybrid System. The MsrA clone was purchased from DNASU Plasmid Repository (https://dnasu.org/DNASU/About.jsp). Constructs encoding the MsrA protein in pGBKT7 ("bait", Trp1 selection marker of Y2HGold yeast strain) and pGADT7 ("prey", Leu2 selection marker, Y187 yeast strain) were mated using the following procedure. An YPD-agar plate was used to streak the S. cerevisiae strains and then they were incubated in YPD media overnight at 30ºC with rotation at 225 rpm. The *S. cerevisiae*/YPD culture was diluted 1:10 with fresh YPD media and incubated for additional 2 hours at 30ºC with rotation at 225 rpm (cell density between 107 and 3.0 $x10^7$ cells per ml). The cells were centrifuged at 580 x g, and the pelleted cells were re-suspended in YPD media and plated onto agar plates containing standard selective media (synthetic dextrose;SD) lacking tryptophan (Trp) and leucine (Leu) and incubated further for five days at 30ºC. Then, colonies from each plate were re-suspended in an Eppendorf tube containing 500 µl of dH_2O; and 5 µl of this solution spotted onto agar plates lacking Trp and Leu or agar plates containing selective media lacking Trp, Leu and histidine (His). Selection for positive clones was done for HIS3 reporter activation (low stringency, SD-Leu, -Trp, and –His (LTH)) and for HIS3 and ADE2 reporter activation (high stringency, SD-Leu, -Trp, -His, and -Adenine (LTHA)). An initial selection for 5 days was performed. The equivalent of 1.07 x 106 diploid cells was selected on SD-LTH and the equivalent of 7.4 x 106 SD-LTHA. We picked the colonies and grew them again for 2-3 day on high stringency medium (SD-LTHA) to ensure robust growth. Fifty-three colonies were finally obtained and prey cDNAs were amplified with colony PCR using a set of flanking primers. The resulting PCR products were then sequenced in the forward direction as follows. Colonies from SD-LTH and SD-LTHA agarose plates were picked into a grid to grow larger. After re-growth, pipet tips were used to transfer each colony into a 30 µL 20 mM NaOH suspension, then boiled for 20 minutes at 99°C in an MJ thermocycler with a hot lid. After cooling, lysates were clarified by centrifugation at >3000 x g for 5 minutes. The supernatants were amplified by PCR with 1 X Mango Polymerase buffer, 10mM NTPs, 10 µM primers NIXO_1838 (5'-gatGAAGATACCCCACCAAACC-3') and NIXO_1839 (5'-cacgatgcacagttgaAGTGAA-3') plus MgCl2 with 0.25 µL Mango Tag DNA polymerase (Bioline)

and 0.05 μL HiFi Tag DNA polymerase (Clontech) in a 50 μL reaction mixture. DNA was amplified with the following protocol: 95°C for 5 minutes, 10 cycles of 95°C for 30 seconds, 68°C for 30 seconds, and 72°C for 3 minutes with the annealing temperature lowered by 1°C per cycle. The reaction was performed for an additional 30 cycles at 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 3 min. The reaction had a final extension of 72°C for 5 minutes and was held at 12°C until the performance of the analysis. 10 μL from each sample were analyzed by gel electrophoresis on a 1% agarose gel in 1X TAE buffer at 115V. The remaining 40 μL were sequenced.

2.1.2 Animal models and brain extracts

The mouse model used in the following experiments were C57 Bl6/129Sv *MsrA* knockout /wild-type mouse (*MsrA* KO/WT) inbreeded for more than 20 generations. The age of the mouse used for making brain extracts were 6 months. For consistency propose, only female mice were used. The brain extracts (n=3, Female, 6 month) were made by homogenization of *post mortem* brains in phosphate buffered saline (PBS) in the presence of proteases inhibitor cocktail (Cat# P8215-1ML, Sigma, St. Louis, MO, USA) and MG132 proteasome inhibitor (Cat# BML-P1102-0005, Enzo) on ice. The insoluble fraction of each extract was separated by high-speed centrifugation (10000 rpm, 10 minutes, 4℃) and then discarded. The supernatants were then collected, and their protein concentration was measured. Bradford protein assay was performed to measure total protein concentration of the extracts. The optical density was read at 595 nm with an iMark microplate reader (BIO-RAD, USA). The protein concentration was then calculated.

2.1.3 GST pull down assay

The fused mammalian Sepharose-bound glutathione-S-transferase (GST)-MsrA protein was used as the “bate” in the GST pull down assay. Brain extracts of WT and *MsrA* KO mice were made as previously described. The *MsrA* KO supernatant was incubated with the Sepharose GST-MsrA resin or with Sepharose-GST only, serving as control (20 μg protein). The incubation was carried out for 2 hours at room temperature followed by overnight incubation at 4ºC. Thereafter, the resins were rinsed with PBS by multiple low speed centrifugations (1000 rpm, room temperature) until no protein was detected in the washing buffer (PBS). The bound proteins moiety of the washed resins was eluted by 2x SDS-gel- electrophoresis buffer (Novex, Carlsbad, CA, USA) and separated by 4-20% SDS-gel-electrophoresis (Cat# XP04200BOX, Invitrogen, Waltham, MA, USA). Anti-Jab1 antibody (MA1-23248, Invitrogen, Waltham, MA, USA) was used as primary antibody in the western blot.

2.1.4 Immunoprecipitation experiments

WT and *MsrA* KO mouse brain extracts were first incubated with anti MsrA antibody (Proteintech Group) (1:100 dilution) for 2 hours on a Glas-Col Rugged Rotator (Cat#099A RD4512 Glas-Col, USA) at room temperature. Protein-G Sepharose (GE Healthcare) was washed with PBS for 3 times to fully remove the solvent. Then, WT and *MsrA* KO mouse brain extracts and anti MsrA antibody mixture were incubated in the presence of protein-G Sepharose

overnight at 4ºC. Thereafter, the resins were washed with PBS by multiple low speed centrifugations (1000 rpm, room temperature) until no protein was detected in the washing buffer (PBS). The bound proteins moiety of the washed resins was eluted by 2x SDS-gel-electrophoresis buffer (Novex, Carlsbad, CA, USA) and subjected to western blot analysis.

2.1.5 Western Blot

All western blots in this study were performed using the following protocol:

The Novex WedgeWell 4-20% Tris-Glycine Gel (Cat#: XP04200BOX, Invitrogen, Waltham, MA, USA) was used to separate proteins. Blue Protein Standard (Cat#: P7706S, BioLabs, Ipswich, MA, USA) was used to show molecular weight of protein bands. The electrophoresis was performed under the condition of 150 V, 76 mA for 70 minutes (Power Supply EC250-90, Thermo-Fisher Scientific, Waltham, MA, USA) in the presence of 500 mL 1X Tris-Glycine SDS running buffer (LC2675, Novex, Carlsbad, CA, USA). Then the gel was transferred to a nitrocellulose membrane (0.45 μm pore size, LC2001, Novex, Carlsbad, CA, USA) in the presence of 1000 mL Tris-Glycine transfer buffer (Prod # 28380, Thermo-Fisher Scientific, Waltham, MA, USA). Thereafter, the transferred nitrocellulose membrane was incubated with 5% (w/v) bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) for one hour. After three washes with PBST (0.5% Tween 20 in PBS), the membrane was incubated with anti-MsrA antibody (Cat #: 14547-1-AP, Proteintech Group, Rosemont, IL, USA) for 1 hour, followed by another three PBST washes and 1 hour incubation with a secondary antibody (Goat anti-rabbit IgG (H+L)-HRP Conjugate, Cat #: 170-6515, Bio-Rad, Hercules, CA, USA). Finally, the membrane was incubated with ECL detection reagent (Cat#: UC279142 ECL Western Blotting Substrate, Pierce). and exposed to an X-ray film. The obtained signals produced by the films were scanned using NIH Image-J program. After the ECL incubation, the nitrocellulose membrane was also washed and subjected to Ponceau S solution (0.1 % (w/v) in 5% acetic acid Ponceau S solution) (Cat#: P3504-10G, Sigma-Aldrich, St. Louis, MO, USA) to show total protein levels of each loaded lane of the gel.

2.2 MsrA promotes Csn5/Jab1 deneddylation activity *in vivo*

Wild-type (WT) and *MsrA* KO (MT) mouse brain and liver extracts (n=3 for each group, Female, 6 month) were obtained as previously described. IP experiments were performed in which the anti-Cul-1 antibody (Cat#: NB100-2616, Novus Biologicals, Centennial, CO, USA) served as the IP antibody and the anti-Nedd8 (Cat#: 0001812M4796, Boster Biological Technology, Pleasanton, CA, USA) served as the primary antibody in the followed western blot analysis and vise-versa. Another IP experiment in which the anti-Jab1 antibody served as IP antibody was performed. This IP experiment was followed by a western blot using anti-Cul-1 antibody (Cat#: NB100-2616, Novus Biologicals, Centennial, CO, USA) as primary antibody. Furthermore, the extracts were also subjected to western blot using Csn5/Jab1 as primary antibody. The protocols of IP and WB were the same as described in section 2.1.4 and 2.1.5.

2.3 MsrA regulates yeast Rub1 modification levels in yeast

Three types of yeast strains (*Saccharomyces cerevisiae*, wild-type (WT), *msrA* null mutant (MT) and *MsrA* overexpressed (OE)) were made according to our previous studies[61,62]. These strains were grown separately in conical flasks in the presence of synthetic minimal media in an incubator shaker at 30°C (C24 Incubator Shaker, New Burnswick Scientific, Edison, NJ, USA). The yeast strains were divided into three groups, while each group consisted of three yeast trains (WT, MT, OE). The first group served as the control group and was grown in the synthetic minimal media only. The second group was exposed to 2 mM of hydrogen peroxide that was added to the medium. The third group was exposed to 1 µM CSN5-i3 (A novel selective human CSN5-i3/Jab1 inhibitor that binds to the catalytic site of the JAMM domain[63]. Kindly provided by Novartis) that was added into the media. All groups were incubated with 150 µg/mL ampicillin at 30°C for 24 hours. At mid-logarithmic phase, the cells were spun down by centrifugation and washed three times with PBS. Then, the cells were resuspended in PBS in the presence of yeast protease inhibitors cocktail (Cat #: P8215- 1ML, Sigma-Aldrich, St. Louis, MO, USA) and MG132 proteasome inhibitor (Cat#: BML-P1102-0005, Enzo, NY, USA), and then extracted by using a glass bead-beater homogenizer apparatus (BioSpec Products, Bartlesville, OK, USA). Following high-speed centrifugation, the resulting supernatants were collected. The protein concentration of each supernatant was determined by Bradford protein assay (Bio-Rad Protein Assay Dye, Cat#: 5000006, Bio-Rad, Hercules, CA, USA). Equal amounts of protein extracts were subjected to Western blot analysis using rabbit anti-Rub1 (a Nedd8 homologue in yeast) as the primary antibody (Cat#: GTX48823, GeneTex Inc, Irvine, CA, USA). Anti-rabbit IgG (Goat anti-rabbit IgG (H+L)-HRP Conjugate (Cat #: 170-6515, Bio-Rad, Hercules, CA, USA) was used as the secondary antibody. The western blot protocol was the same as described in section 2.1.5. The same nitrocellulose membrane was then subjected to a protein staining (Ponceau S), serving as loading control. The major observed bands were scanned and quantified using the Image J program. The rubylation (neddylation in yeast) level was evaluated by the calculated total strength of Rub1-positive bands.

2.4 MsrA alters the kinetics of Csn5/Jab1 deneddylation activity *in vitro*

2.4.1 Nedd8 conjugates

Dr. Julie A. Maupin and her co-workers from University of Florida kindly designed a heterologous expression model of Flag-His_6 tagged Nedd8 in *Haloferax volcanii*. The Nedd8 conjugates from *Haloferax volcanii* was purified and then could be utilized in the following *in vitro* deneddylation assays. The method is described as follows:

2.4.1.1 Design of system for heterologous expression of Nedd8 in *Haloferax volcanii*

An N-terminal tandem Flag-His6- tag was added to the coding sequence for the mature Nedd8 protein (Uniprot accession P29595, residues 1-76) with removal of the original initiator methionine. The recombinant coding sequence was codon optimized for translation in *Haloferax volcanii* using the Codon Optimization On-Line (COOL) web platform[64]. NdeI/BlpI restriction

sites were added and the entire sequence was synthesized by GenScript (Piscataway, NJ) and ligated in pUC57 to create pJAM3518. Flag-His6-Nedd8 was removed from pJAM3518 by NdeI/BlpI digest, gel extracted, and ligated in pJAM202 to create pJAM3519. pJAM3519 was transformed to *E. coli* TOP10, and isolated transformants were patched on LB supplemented with ampicillin. PCR screening by primers HvFW/RV confirmed presence of the plasmid and correct size of the Flag-His_6-Nedd8 insert. Several patches were selected, and integrity of the insert sequence was verified by Sanger sequencing. pJAM3519 was then passaged through a methylation deficient strain of *E. coli* (GM2163) prior to transformation to *H. volcanii*.

2.4.1.2 Purification of Nedd8 conjugates from *Haloferax volcanii*

Note – several strains of *H. volcanii* expressing FHN8 were tested for conjugate formation at small scale, HM1096 produced the highest levels observed.

H. volcanii HM1096 cells expressing pJAM3519 were inoculated at an initial OD of 0.005 in 4 x 700 mL ATCC974 medium supplemented with 50 mM DMSO and 0.2 ug/mL novobiocin. Flasks (2.8L, Fernbach) were incubated at 42°C with 200 rpm orbital shaking for 93 hours. Cultures (4) were pooled and centrifuged at 4,000 x g for 15 minutes, gently resuspended and washed in TBS-2M (2M NaCl, 50 mM Tris-Cl, pH 7.4). The cells were centrifuged again, the supernatant was discarded, and the wet cell pellet (6.28 g) was stored at -80°C until future use. After thawing, the cell pellet was resuspended in 35 mL TBS-2M supplemented with protease inhibitor cocktail (Thermo Scientific, Cat#:A32955) according to the manufacturer, 5 µg/mL DNase I, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, and incubated on ice for 15 minutes. The suspension was passed through a French pressure cell at 20,000 psi five times, supplemented with imidazole at a final concentration of 30 mM, and clarified by centrifugation (13,000 x g, 4°C, 20 min). Clarified supernatant was filtered through a PES membrane (0.45 µm) and applied to a 1 mL HisTrap HP column equilibrated in TBS-2M supplemented with 30 mM imidazole. After loading of the lysate, the column was washed in the same buffer until the A280 trace stabilized, then eluted using TBS-2M supplemented with 500 mM imidazole. All column chromatography steps were performed at a flow rate of 0.5 ml/min. Peak fractions were immediately supplemented with 1 mM EDTA and analyzed by reducing SDS-PAGE (12% acrylamide) and subsequent Coomassie blue staining and western blotting. Selected fractions (2 mL) were pooled and dialyzed at 4°C in TBS-2M with three buffer exchanges over a period of 72 h. The dialyzed sample was quantified by BCA assay at a concentration of 1.3 mg/mL, divided into 100 µL aliquots, snap freeze, and stored at -80°C until future use.

2.4.2 *In vitro* deneddylation activity assay

Equal amounts of brain extracted protein of WT and *MsrA* KO mouse were incubated with Nedd8 conjugates at 37°C. After designated time of incubation, equal volumes of 2X sample buffer were added and immediately heated at 100°C for 5 minutes to terminate the reaction. The reaction solution of different time points were then subjected to western blot using mouse anti-His antibody (Cat#:34610, Qiagen, Venlo, Netherlands) that was used as primary antibody. The western blot protocol was the same as described in section 2.1.5. The deneddylation level was evaluated by the signal strength of lowest molecular mass His-detected positive band.

2.5 Change in Neddylation levels of Cul-1 in glioblastoma postmortem human brains

We have obtained post-mortem human brain specimens (*n*=3) from the University of Kansas Cancer Center. We isolated tissue sections from the malignant portion of the brains that contained glioblastoma tumors, as well as non-tumor sections from adjacent regions that serve as controls (basic information of the subjects is given in **Table 2**). The corresponding tissues were extracted in PBS in the presence of protease inhibitors cocktail (Cat #: P8215- 1ML, Sigma-Aldrich, St. Louis, MO, USA). Following high-speed centrifugation, the supernatants were kept and used for the relevant Western blot analyses. IP experiments were performed to determine the deneddylation activity of Csn5/Jab1 activity towards Cul-1 in these supernatants. For these IP experiments we used the anti-Nedd8 antibody to precipitate neddylated proteins, followed by western blot analysis that specifically probed for neddylated Cul-1 by using mouse anti-Cul-1 antibody (Cat#: NB100-2616, Novus Biologicals, Centennial, CO, USA) that was used as the primary antibody.

CHAPTER 3: RESULTS

3.1 MsrA interacts with Csn5/Jab1 in brain

The result of BlastX screening was analyzed and the following positive clones were identified (**Table 1**). Most prominent prey hits are COP9 signalosome complex subunit 5 (*n*=19), cyclin-I [Homo sapiens] and succinate dehydrogenase [ubiquinone] iron-sulfur subunit [Homo sapiens] are 6 copies each. These strong hits were recurring, i.e. more than once. This showed that we had specific selection and not just random activation. In addition, 6 more hits were unique, i.e. only one copy. The high number of hits obtained for COPS5 (also known as Csn5/Jab1) and the involvement of both MsrA and Csn5/Jab1 in the Ub/Ub-like systems, prompted us to focus on this MsrA interacting target first.

We then performed Y2H screening to find out the binding site of MsrA and Csn5/Jab1. We first investigated and characterized the specific region of interaction between MsrA and Jab1 by using the MsrA as the “bait” and “Csn5/Jab1” as the prey in the Y2H system. Accordingly, we used the MsrA clone from the initial Y2H screening and produced a “prey” library consistent of various Csn5/Jab1 sequences that were sub-cloned into the “prey” vector. The clone for Jab1 was purchased from DANSU Company and its sequence was cut into small fragments that were then sub-cloned into the corresponding “prey” vector (the details for this procedure are proprietary information of “Next Interactions” Inc., Richmond, CA). The first Y2H screening for identifying regions of interaction between MsrA and Jab1 coding segments showed a unique peak of Jab1 at the 5' half that indicates binding; the initial peak was relatively broad, when the mapping was conducted with 600 bp fragments. However, further mapping experiments yielded a sharper peak with a distinct plateau that defines the essential binding site. As shown in **Figure 2** below, the initial screening defined an in-frame sequence of Jab1 (Positions 93-457 of the open reading frame) following a “Sanger” sequence analysis (the underlined sequence). This sequence consists of 7.3% Met residues that is higher than the average Met percentile in proteins (~2%). Further deeper analysis revealed a sharper peak consistent of 6 pairs of Met including Val residues that were aligned as (the percent of Met in this rose to 12.2%) (**Figure 2**, highlighted in yellow). MVM; VMGLM, and MIIM.

To further confirm the interaction between MsrA and Csn5/Jab1, we then used *MsrA* knockout (*MsrA* KO) mouse brain extracts to perform a GST pull down assay. Since there was no endogenous MsrA in *MsrA* KO mouse brain extracts, all proteins which interact with MsrA would be pulled down by GST-MsrA. In **Figure 3A**, one lane containing the GST-MsrA protein was stained for protein with Coomassie Blue to observe the intensity of the major GST-MsrA protein band and the other minor bound proteins. The western blot result indicated that only the lane harboring the GST-MsrA showed a signal corresponding to Csn5/Jab1, while the lane harboring GST alone showed no reaction as expected.

In **Figure 3B**, immunoprecipitation (IP) experiments were performed to further demonstrate the MsrA-Csn5/Jab1 interaction. IP experiments using mouse brain extracts with anti-MsrA

antibody (IP antibody), followed by western blot analysis using a mouse anti-Jab1 antibody as the primary antibody. The results showed that only the lane harboring the GST-MsrA showed a signal corresponding to Csn5/Jab1 (a band with molecular weight of ~36 kDa), while the lane harboring GST alone showed no positive reaction. This result was consistent with our previous Y2H screening result, suggesting an interaction between Csn5/Jab1 and MsrA. The antibody used as the western blot primary antibody was a mouse generated anti-Jab1 antibody. Therefore, in addition to the detected Csn5/Jab1 protein in the WT mouse extracts (and not in the *MsrA* KO mice), two additional reactions with the mouse immunoglobulin light (L) and heavy (H) chains were observed. These results suggested that there was an interaction between Csn5/Jab1 and MsrA in mouse brain.

3.2 MsrA promotes Csn5/Jab1 deneddylation activity *in vivo*

We then focused on investigating the possibility that MsrA would activate Csn5/Jab1 function. We followed the *in vivo* deneddylation activity of Csn5/Jab1 on its substrate Cullin-1 (Cul-1) as function of the presence or absence of MsrA in mouse brain and liver extracts. As mentioned above, MsrA is also highly expressed in liver tissues. The use of liver extracts was also followed to show that the effect of MsrA on Csn5/Jab1 function is general in nature.

IP experiments in which the anti-Cul-1 antibody served as the IP antibody and the anti-Nedd8served as the primary antibody in the followed western blot analysis (and vise-versa) were performed to test our hypotheses. As shown in **Figure 4A a & b**, compared to WT brain, *MsrA* KO brain showed higher level of neddylated Cul-1. Higher Cul-1 neddylation level was also observed in *MsrA* KO liver extracts compared to WT (**Figure 4A c & d**). Higher neddylation levels of Cul-1 in the *MsrA* KO brain and liver suggested an impaired deneddylation activity in the absence of MsrA. This phenomenon was also in agreement with our hypothesis that reduction of specific MetO residue/s of Csn5/Jab1 lead to increase of its activity, as measured by deneddylation of Cul-1. In the absence of MsrA (i.e.as it is in the *MsrA* KO tissue extracts) the activity of Csn5/Jab1 was compromised presumably due to its enhanced Met oxidation, resulting in more neddylation (or less deneddylation) of Cul-1. Furthermore, an active Csn5/Jab1 was supposed to bind to Nedd8 moiety of Cul-1 in before removing it from Cul1 (or any other target protein). Therefore, anti-Csn5/Jab1 antibody could also be used in an IP experiment as a “fishing” probe to isolate protein complexes of neddylated Cul-1 that is bound to Csn5/Jab1 from biological extracts (prior to the removal of Nedd8 by Csn5/Jab1 and its associated cofactors). Indeed, following this approach, the WT brain showed much higher neddylated Cul-1 than the *MsrA* KO (MT), as judged by western blot analysis using anti Cul-1 antibody followed by the IP with anti-Jab1 antibody (**Figure 4A e**). These data confirmed that Csn5/Jab1 was able to bind Nedd8 on Cul-1 more effectively in the presence of MsrA, supporting the hypothesis stated above. We then performed another western blot analysis to investigate whether Csn5/Jab1 levels changed in *MsrA* KO compared with the WT mouse brain extracts. The results showed that the presence/absence of MsrA had no effect on the level of Csn5/Jab1 (**Figure 4 D**). This observation indirectly supports our hypothesis, that MsrA regulates Csn5/Jab1 deneddylation activity through post-translational protein modification and not through changing

Csn5/Jab1expression levels.

3.3 MsrA regulates yeast Rub1 modification levels in yeast

To investigate the *in vivo* regulation of deneddylation in a whole living organism/cells and determine the effect of oxidative stress and Csn5/Jab1 inhibition, we used yeast cells.

Related to Ubiquitin-1 (Rub1) is the yeast homolog of Nedd8. Orthologues of Nedd8/Rub1 are required for vitality in all studied organisms. However, budding yeast (*Saccharomyces cerevisiae*) is a notable exception. In *S. cerevisiae*, neither CSN nor Rub1 are essential for viability[65]. Nevertheless, the CSN complex drives canonical cullin derubylation, yet it is unclear for what mechanistic purpose[66,67]. Thus *S. cerevisiae* is a proper model for our studies. Like in mammalians, the substrate of yeast Csn5/Jab1 is Cdc53-Rub1 (Cullin-Nedd8 in mammals). To investigate whether the Rub1 post-translational modification is Csn5/Jab1 dependent, we used a selective human Csn5/Jab1 inhibitor CSN5-i3 provided by Novartis. We tested the Rub1 modification levels of wild type (WT), MsrA Knockout (MT) and MsrA overexpressed (OE) yeast trains in oxidative stress (H_2O_2) or in the presence of CSN5-i3, respectively. The yeast extracts were obtained via methods described above. The Rub1 modification levels of the yeast strains grown under different conditions was monitored via western blot analysis, as shown in **Figure 5**.

Accordingly, the total rubbylation levels were determined following western blot analysis using anti-Rub1 antibody as the primary antibody (**Figure 5A**) and quantified (**Figure 5B**). An increase of rubbylation level was observed in WT yeast strain in the presence of H_2O_2 (*t*-test, $p<0.05$) while a decrease in the level was observed in CSN5-i3 group compared to control (*t*-test, $p<0.05$) (upper panel). These data suggest that oxidative stress inhibits total deneddylase activity that probably includes also the activity of Csn5/Jab1. However, CSN5-i3 did not show inhibitory effect on Csn5/Jab1 activity, likely due to its decreased affinity towards the yeast homologue of the human Csn5/Jab1. The *MsrA* KO yeast demonstrated a significant increase of rubbylation level under conditions of both H_2O_2 and Csn5/Jab1 inhibitor treated groups, compared to control (*t*-test $p<0.001$) (middle panel). The fact that the CSN5-i3 showed a better inhibitory effect when MsrA is absent suggests that CSN5-i3 may have a higher affinity to a more Met-oxidized state of the yeast form of Csn5/Jab1. In *MsrA* OE yeast, neddylation levels significantly decreased in H_2O_2 and Csn5/Jab1 inhibitor treated groups (*t*-test, $p<0.05$). This phenomenon can be explained by the overexpression of MsrA that can better scavenge the ROS formed by H_2O_2. Environmental oxidative stress could also stimulate *MsrA* promoter to increase MsrA levels in yeast, and especially in the OE strain, leading to further increase of Csn5/Jab1 activity. The failure of CSN5-i3 to inhibit rubylation in the *MsrA* OE strain could also be due to the low affinity of the inhibitor to non-oxidized Csn5/Jab1, which is even better protected from oxidation in the OE strain.

3.4 MsrA alters the kinetics of brain Csn5/Jab1 deneddylation activity *in vitro*.

As we hypothesized, MsrA may regulate Csn5/Jab1 activity by reducing MetO residues of the protein. That is to say, different oxidation levels of Csn5/Jab1 may show different catalytic

kinetics. Thus, we focused on the kinetics of the Csn5/Jab1 deneddylation reaction *in vitro* by co-incubating artificial Csn5/Jab1 substrate, Nedd8 conjugates with WT and *MsrA* KO mouse brain extracts. High amount of His-tagged Nedd8 conjugates (6 µg) were used to get as closely as possible to the effective K_m value to be able to follow the kinetics deneddylation activity (while not overloading the Nedd8 protein per gel lane) in WT and *MsrA* KO brain extracts. Anti-His antibody was used to detect the Nedd8 signal due to its high selectivity compared with the anti-FLAG antibody that seems to have non-specific reactions with mouse brain proteins (data not shown). The levels of the lowest molecular weight Nedd8 (L.M.Nedd8) was monitored to evaluate total deneddylation levels. The results showed that the deneddylation levels of WT and KO groups remained the same at zero time of incubation (T0)(**Figure 6A&B**). However, the *MsrA* KO group exhibited much lower levels of released low-molecular weight Nedd8 compared to the WT as the incubation time increased. The deneddylation level of WT strain rapidly reached the peak after10 minutes of incubation that was much higher than the same peak observed for the *MsrA* KO strain (**Figure 6**B). This big difference in the deneddylation activity between the two strains remained up to the end of the incubation time (30 minutes). Accordingly, it is suggested that the *MsrA* KO brain exhibits much more Met-oxidized Csn5/Jab1 protein compared with the WT brain. This phenomenon is in agreement with our hypothesis that lack of MsrA compromises the deneddylation activity of Csn5/Jab1 through an increase of Csn5/Jab1 MetO levels

3.5 Change in Neddylation levels of Cul-1 in postmortem glioblastoma human brains

As shown in **Table 2**, we obtained post-mortem human brain specimens (*n*=3) from the University of Kansas Cancer Center. We isolated tissue sections from the malignant portion of the brains that contained glioblastoma tumors, as well as non-tumor sections from adjacent regions that serve as controls. IP experiments were performed to determine the deneddylation activity of Jab1 activity towards Cul-1 in brain supernatants. For these IP experiments an anti-Nedd8 antibody was used to precipitate neddylated proteins, followed by western blot analysis to specifically probe for neddylated Cul-1 by using anti-Cul-1 antibody, as the primary antibody. As shown in **Figure 7A&B**, the levels of neddylated of Cul-1 were higher in the control groups compared to the malignant tumor samples following the IP-Western blot analyses. The data suggest that the deneddylation activity of Csn5/Jab1 was higher in the neuroblastoma tumor cells, as expected from its higher expression levels[68]. Interestingly, we did not observe any significant difference between the Csn5/Jab1 levels in tumor and control tissues (**Figure 7D**). One possible explanation is that the number of samples we have is small and there may be differences between the subjects in each study that influence the data. In any event, the lack of Csn5/Jab1 expression differences between the control and malignant groups supports the suggestion that indeed posttranslational modification plays a role in the observed deneddylation differences of Cul-1.

CHAPTER 4: CONCLUSION AND DISCUSSION

The interaction between Csn5/Jab1 and MsrA was confirmed via different methods in this study. An Y2H assay was first used to screen the potential binding proteins of MsrA. Further analysis unveiled several potential binding sites between MsrA and Csn5/Jab1 (**Figure 2**). Then we focused on confirming this binding *in vivo*. An *MsrA* KO mouse model was used to find out whether the interaction between MsrA and Csn5/Jab1 happens naturally in animals. The GST pull down data suggest that there is an interaction between MsrA and Csn5/Jab1 in mouse brain (**Figure 3**). Immunoprecipitations experiments showed that absence of MsrA causes decreased deneddylation level of Cul-1 in brain (**Figure 4A a & b**). *MsrA* KO Cul-1 showed approximately 3 times more neddylation compared to WT (**Figure 4B**). We also found that the same interaction between two proteins exists in liver of mouse, another highly expressed MsrA organ (**Figure 4A c & d**). These data suggest that the MsrA-dependent deneddylation activity (most likely carried out by Csn5/Jab1) is a common feature in several organs. Interestingly, Csn5/Jab1 level remained the same in *MsrA* KO mouse brain compared to WT (**Figure 4D**). This result is in agreement with our hypothesis that MsrA regulates Csn5/Jab1 deneddylation activity via a mechanism of posttranslational modification instead of directly elevating the expression levels of this protein. In **Figure 5**, we discovered that deneddylation was inhibited by oxidative stress in both WT and *msrA* KO yeast strains. However, overexpression of MsrA fully restored oxidative stress induced inhibition of deneddylation. Interestingly, the Csn5/Jab1 inhibitor CSN5-i3 can only inhibit deneddylation activity in *msrA* KO yeast. We suggested that oxidation levels of Csn5/Jab1 could change its affinity to the inhibitor. These results were consistent with our hypothesis that deneddylation activity of Csn5/Jab1 is MsrA dependent. As a proteolytic enzyme, the deneddylation activity of Csn5/Jab1 is supposed to be altered by MsrA catalyzed MetO residues reduction. As we observed in **Figure 6**, the deneddylase kinetics of Csn5/Jab1 was altered in the absence of MsrA. Decreased activity of *MsrA* KO extract to deneddylate artificial Nedd8 conjugate suggest a correlation between the reductase activity of MsrA and Csn5/Jab1 activity. More studies are required to further confirm whether impaired Csn5/Jab1 activity is directly due to the oxidation of Met to MetO in the absence of MsrA. IP and western blot analysis in post mortem human glioblastoma brain tissues showed an increased Csn5/Jab1 activity in tumor samples compared to control, while total Csn5/Jab1 protein levels remained the same (**Figure 7**). Determination of an increase of MsrA activity in glioblastoma cells compared to controls will provide further supportive evidence for the positive role of MsrA in enhancing Csn5/Jab1 activity in cancer.

CHAPTER 5: FUTURE DIRECTION

Proteins are known to exhibit changed activities under different levels of oxidation. Aging[69] or environmental factor-driven oxidative stress may lead to abnormal protein function or even diseases. As a crucial antioxidant enzyme, MsrA plays a key role in maintaining the balance of oxidation-reduction in organisms. Given that MetO was observed in a various of proteins in normal physiological conditions, it is not surprising that MsrA could also regulate the activity of the substrate protein by decreasing protein oxidation levels, in this case, reducing MetO to Met. A few proteins were found out to alter activity due to increased MetO residues. For example, Alpha-antitrypsin is a serine protease inhibitor (serpin) which has surface exposed Mets. Oxidation of Met351 and Met358 in the protein causes a loss of the antineutrophil elastase activity associated with emphysema. Addition of MsrA *in vitro* can partly restore the antielastase activity[70]. In our study, the results clearly showed a positive regulatory role of MsrA to the deneddylation activity of Csn5/Jab1 both *in vivo* and *in vitro*. Considering the main reductase function of MsrA, we hypothesized that MsrA upregulates Csn5/Jab1 activity via a mechanism of reducing MetO residues to Met on Csn5/Jab1 protein. Under natural conditions, a moderate level of oxidative stress is present that makes a portion of Met residues on Csn5/Jab1 oxidized to MetO. This represents the basal level of Csn5/Jab1 activity. Under high oxidative stress conditions, more Met residues are supposed to be oxidized to MetO, leading to inhibition of Csn5/Jab1 deneddylation activity. Under MsrA overexpressed conditions, almost all S-MetO residues are supposed to be reduced to Met, leading to a higher Csn5/Jab1 deneddylation activity than detected in the basal level. Accordingly, the role of MsrA is supposed to maintain the hemostasis of Csn5/Jab1 deneddylation activity

To further confirm this hypothesis, the following experiments will help to illustrate the mechanism of MsrA mediated Csn5/Jab1 activity increase.

1. MsrA activity assay. To fully understand the role of MsrA in glioblastoma, we plan to investigate MsrA activity in tumor tissues. Using DABS-L-MetO as the substrate, the activity in tumor tissues could be quantified by measuring MsrA specific activity. Then we could evaluate the interaction between Csn5/Jab1 deneddylation activity and the activity of MsrA and determine the role of MsrA in neddylation-related disease development. According to our hypothesis, we expect to observe an increased MsrA activity in tumor tissues.
2. GST-Csn5/Jab1 Pull Down Assay. To further confirm the interaction between MsrA and Csn5/Jab1, a GST Pull Down assay using GST-Csn5/Jab1 as bait can be performed following the same IP protocol described in section 2.1.3. We expect that the assay result will also show an MsrA-Csn5/Jab1 interaction
3. Mutagenesis of Csn5/Jab1 Met residues. To fully uncover the binding situation of MsrA-Csn5/Jab1, performing mutagenesis of Csn5/Jab1 Met residues is necessary. According to our preliminary data we have obtained in Y2H screening, the Jab1 binding site to MsrA was identified. Within the 70 amino acids of the brain Csn5/Jab1 binding site there were

three sets of adjacent Met and Val residues as follows:

5'-**MVM**HARSGGNLE**VMGLM**LGKVDGET**MIIM**-3'.

We plan to mutate individually each highlighted Met and Val residue to Ala to reduce hydrophobicity. Thus, initially we will create eight individual mutants of either Val or Met, followed by eight double and eight triple mutants of the Met/Val residues per patch (a grand total of 24 mutants). It is expected that at least one of the mutated patches will reduce the binding of Jab1 to MsrA following the Y2H screening. If no difference in binding will be observed, we will create two additional mutants in which two and all three patches will be fully mutated. For performing the mutagenesis, we will use and follow the protocol provided by the manufacture of the Quick-change site directed mutagenesis kit (Agilent Technologies).

4. Mass Spectrum Analysis of Csn5/Jab1 oxidation levels/sites. It is important to determine the actual Met oxidation level of Csn5/Jab1, as well as their sequence location sites in the presence and absence of MsrA. Thus, we plan to improve our yeast model and analyze the MetO levels of recombinantly expressed Csn5/Jab1. We will overexpress human and yeast recombinant His-tagged Csn5/Jab1 in *MsrA* null mutant and WT yeast strains, respectively. Then, we will grow the cells in expression synthetic media in the presence or absence of H_2O_2 or CSN5-i3. Thereafter, each recombinant Csn5/Jab1 will be affinity purifies from each strain and the relative MetO levels and sequence location will be determined by mass-spectrometry analyses. We predict that the oxidative stress will increase the levels of MetO residue/s of either Csn5/Jab1 form, especially in the *MsrA* null mutant strain. Moreover, it is predicted that the CSN5-i3 will inhibit better the deneddylation activity of the human -recombinant Csn5/Jab1 than the yeast- recombinant Csn5/Jab1, regardless of the growth condition.

Table 1. Count of Hit BlastX results

Hits	Protein name	Species
1	AT-rich interactive domain-containing protein 2	*Homo sapiens*
1	coatomer subunit beta'	*Homo sapiens*
19	COP9 signalosome complex subunit 5	*Homo sapiens*
6	cyclin-I	*Homo sapiens*
1	heat shock 70 kDa protein 4L	*Homo sapiens*
1	phenylalanyl-tRNA synthetase beta chain	*Homo sapiens*
1	protein FAM184A isoform 2	*Homo sapiens*
6	succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial precursor	*Homo sapiens*
1	WD repeat-containing protein 44 isoform 3	*Homo sapiens*

Table 2. Postmortem human brain tissues

Specimen	Tissue review	Gender	Race	Ethnicity	Diagnosis
Malignant frozen tissue from brain (MB)	90%Tumor 1% necrosis	M	White	Not Hispanic or Latino	Glioblastoma, WHO Grade IV
Adjacent nonmalignant tissue from brain (CB)	100% papillar tumor 0% Necrosis	M	White	Not Hispanic or Latino	(Control) Choroid Plexus Papilloma, WHO Grade I
MB	90%-95% Solid Tumor	M	White	Not Hispanic or Latino	Glioblastoma, WHO Grade IV
CB	0% Necrosis	M	White	Not Hispanic or Latino	(Control) Focal Cortical dysplasia, Type IIB
MB	80% Tumor 20% Necrosis	M	White	Not Hispanic or Latino	Glioblastoma, WHO Grade IV
CB	100% Adenoma Tumor 0% Necrosis	M	White	Not Hispanic or Latino	(Control) Adenoma Pituitary gland

-The tissues are listed in pairs of CB and MB that originated from the same individual (CB is the control tissue taken from adjacent region to the tumor location of the MB tissue). WHO, World Health Organization.

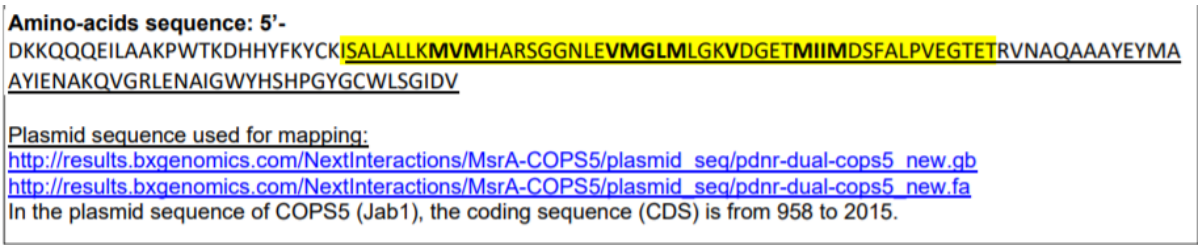

Figure 2. Protein sequence of Jab1 region that binds MsrA. Analysis of the interaction between MsrA and Jab1 using the Y2H system revealed an MsrA-interaction domain in Jab1 protein sequence (underlined). This interaction domain has a peak (highlighted in yellow), containing patches of a Met and Val mixtures sequences that are closely positioned (in bold, the patches are 8-9 amino acids apart)

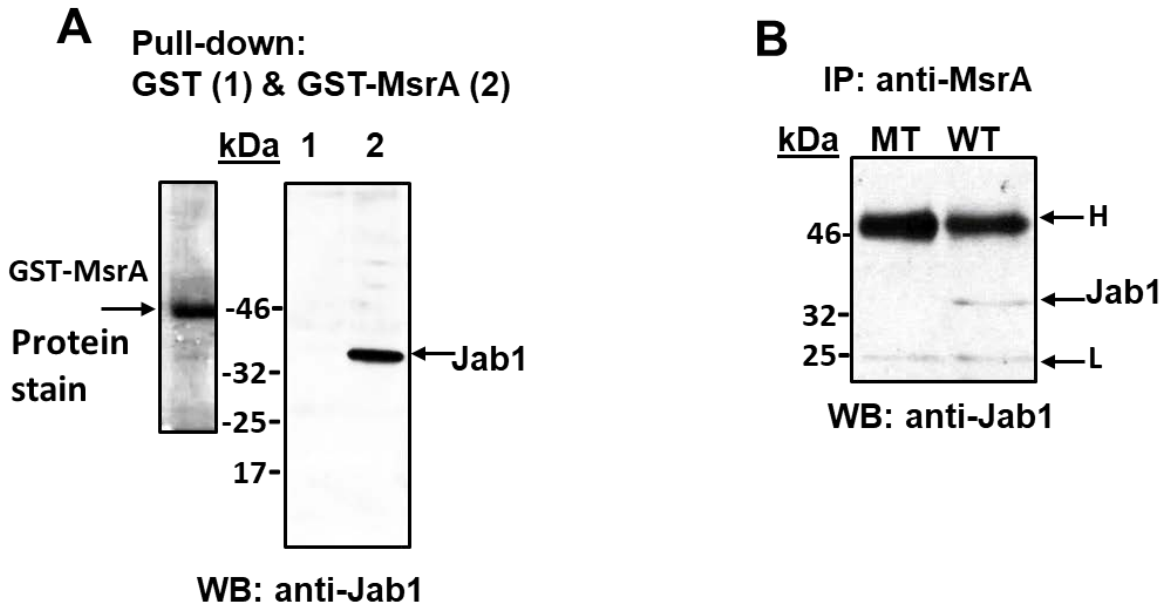

Figure 3. Interaction between MsrA and Jab1 in mouse brain extracts.

A. Pull-down experiments in which recombinant GST-MsrA fused protein is used as a "bait" for binding of brain proteins. MsrA KO brain extracts were used in the pull-down assay. Coomassie Blue staining showed GST-MsrA positive band (left). Jab1 was detected in the lane harboring GST-MsrA (right, lane 2). B. IP experiments using mouse brain extracts with anti-MsrA (IP antibody), followed by western blot analysis using a mouse anti-Jab1 as the primary antibody. kDa, molecular mass markers in kilo-Dalton units; WT, wild-type mouse; MT, MsrA KO mouse; L, light chain of mouse immunoglobin detected in the extract; H, heavy chain of mouse immunoglobulin detected in the extract. This experiment was repeated 3 times and one set of data is represented in the figure.

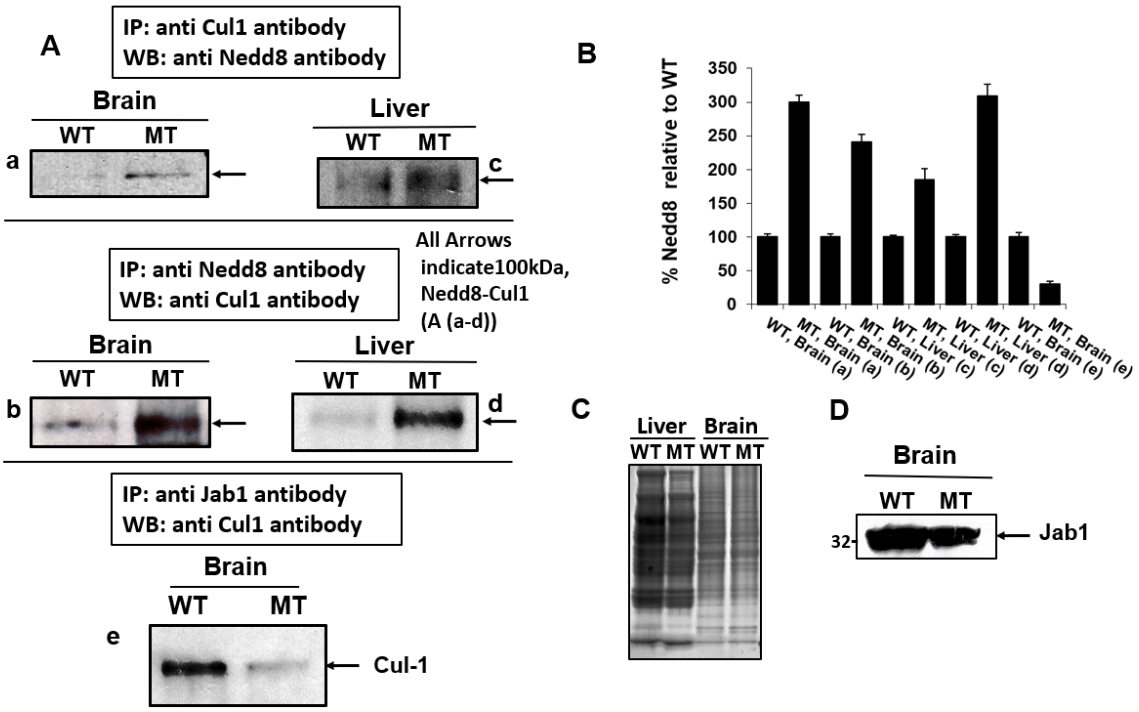

Figure 4. Regulation of Jab1 activity by MsrA in vivo through monitoring Cul-1 neddylation levels in mouse brain and liver extracts. A. a-d. (Mice age, 6 months, *n*=3). Immunoprecipitation (IP) experiments were performed on brain and liver extracts (500µg of protein per extract) using either anti-Cul-1 antibody or anti-Nedd8 followed by western blot analyses using anti-Nedd8 or anti-Cul-1 antibody as the primary antibody, respectively. **e.** anti-Jab1 antibody was used as a"fishing" probe in an IP experiment using brain extracts, followed by western blot analysis with anti-Cul-1. **B.** Quantification of the neddylated band of Cul-1 by Image-J program, following the performed western blot analyses. All the differences in deneddylation levels between the WT and MT pairs were statistically significant as judged by student *t*-test analysis (P<0.01, *n*=3 per strain). **C.** Loading controls for the liver and brain protein levels, following Coomassie blue staining. **D.** Western blot analysis of mouse brain extracts using anti-Jab1 antibody as primary antibody. WT, wild type; M, MsrA KO; WB, Western blot analysis; Arrows in **Aa-e** indicate the position of the neddylated 100kDa Cul-1 (non-neddylated form runs as 90 kDa protein, not detected). WT, wild-type mouse; MT, *MsrA* KO mouse. The shown WB and Coomassie staining experiments are representatives of three repeated experiments that showed.

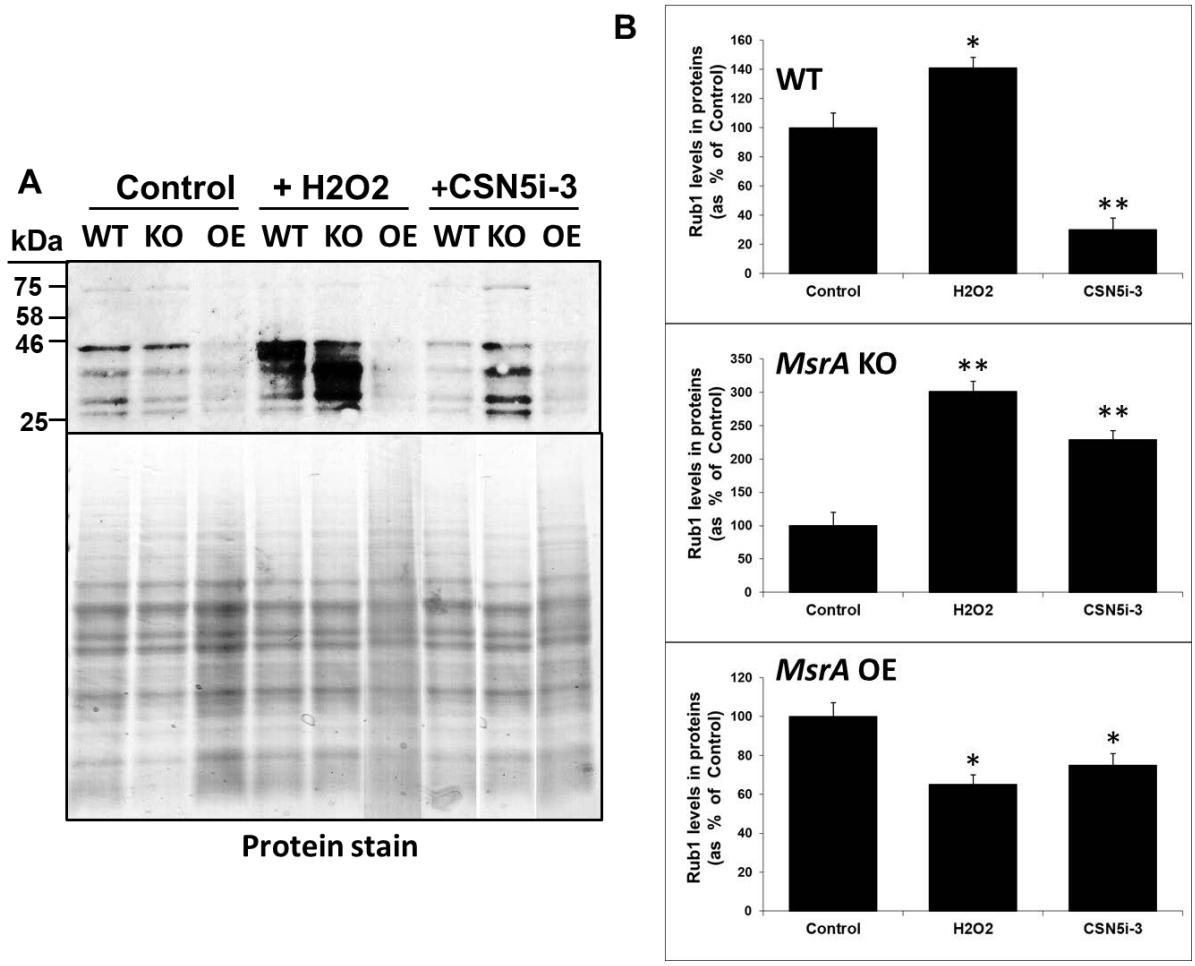

Figure 5. MsrA regulates yeast Rub1 modification levels in yeast. A. Western blot of three different yeast strains (Wild-type, *msrA* knockout and *MsrA* overexpress) in three different conditions (Control, H_2O_2 and CSN5-i3) using anti-Rub1 antibody as primary antibody (upper panel); total protein staining (Ponceau S) as loading control (lower panel). The shown WB and Ponceau S staining experiments are representatives of three repeated experiments. **B.** Quantification of total rubbylation levels. All data are normalized and showed as percentage to control, respectively. Student t-test was used to analysis the data. (n=3; *p<0.05; **p<0.001). WT: Wild-type; KO: *msrA* knockout; OE: *msrA* Overexpress.

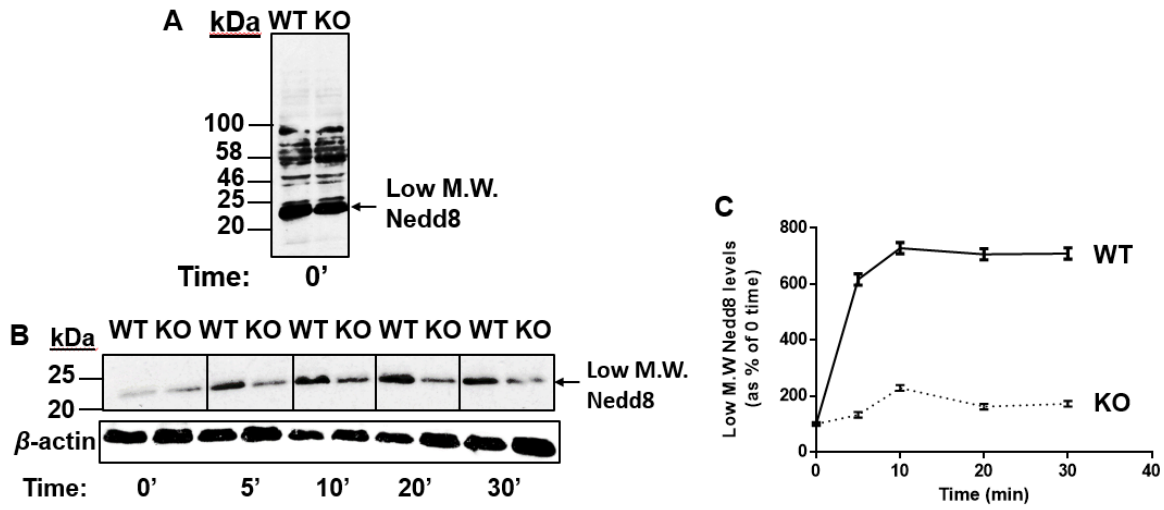

Figure 6. MsrA alters the kinetics of Csn5/Jab1 deneddylation activity in vitro. A. The western blot pattern of control group (time 0’). Total protein neddylation levels were shown. We focused on lowest molecular weight Nedd8 positive bands as they were the products of deneddylation process. **B.** lowest molecular weight Nedd8 positive bands at different time points in WT and *MsrA* KO mouse brain samples (*n*=3, female, 6 month). **C.** Time curve of lowest molecular weight Nedd8 levels. All data were normalized to T0. The shown WB are representatives of three repeated experiments that showed. WT: Wild-type; KO: *MsrA* Knockout; Low M.W.Nedd8: Low molecular weight Nedd8.

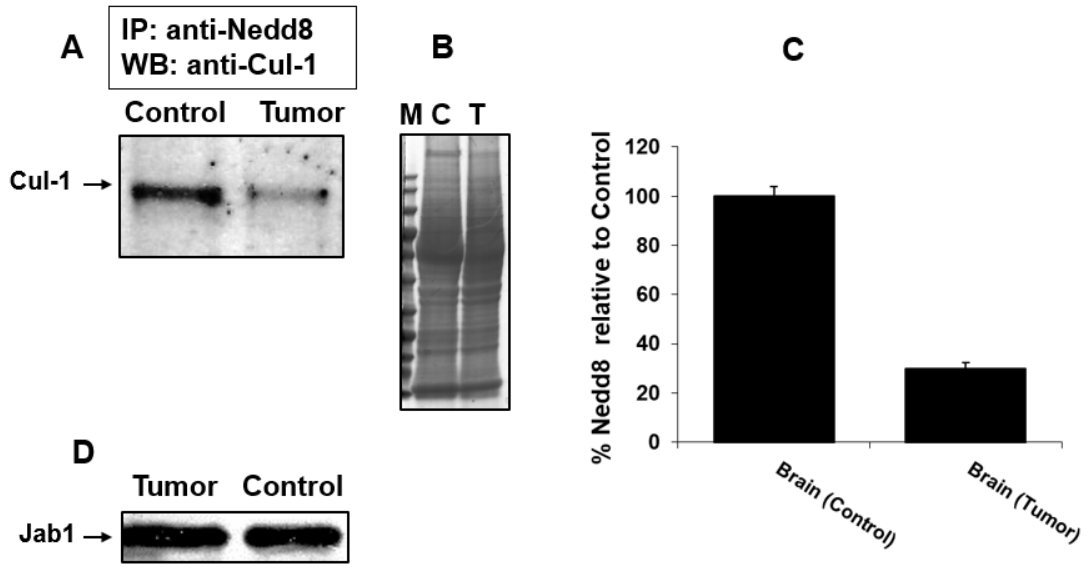

Figure 7. Neddylation levels of Cul-1 as function of the presence of glioblastoma in postmortem human brains. A. For the IP experiments, an anti-Nedd8 antibody was used and for the WB analysis, an antiCul-1 was used as the primary antibody. **B.** Each paired of tissue supernatants (Tumor and Control) were separated on SDS gel/electrophoresis and stained with Coomassie blue stain to confirm the equal protein amounts used. **C.** The relative neddylation levels of each sample was quantified by densitometry analysis of the Cul-1 band (see panel **A**), using the Image-J program. The difference in the Nedd8 levels between the control and Tumor samples was statistically significant as judged by student *t*-test analysis (n=6, $p<0.001$). M, molecular mass-markers (Bio-labs); C, control tissue; T, Tumor sample; the arrow indicated the 100 kDa neddylated Cul-1 band. The data in Panels A and B are representative data of the total six samples that were analyzed and showed a similar pattern of neddylation with respect to their origin (i.e. Control or Tumor tissue). M: Protein marker; C: Control; T: Tumor.

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