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Methionine sulfoxide reductase A (MsrA) mediates the ubiquitination of 14-3-3 protein isotypes in brain.

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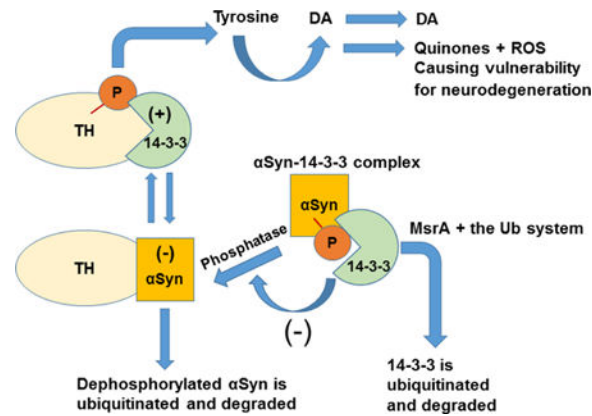
Abstract

The methionine sulfoxide reductase (Msr) system is known for its function in reducing protein-methionine sulfoxide to methionine. Recently, we showed that one member of the Msr system, MsrA, is involved in the ubiquitination-like process in *Archaea*. Here, the mammalian MsrA is demonstrated to mediate the ubiquitination of the 14–3–3 zeta protein and to promote the binding of 14–3–3 proteins to alpha synuclein in brain. MsrA was also found to enhance the ubiquitination and phosphorylation of Ser129 of alpha synuclein in brain. Furthermore, we demonstrate that, similarly to the archaeal MsrA, the mammalian MsrA can compete for capturing ubiquitin using the same active site it contains for methionine sulfoxide binding. Based on our previous observations showing that MsrA knockout mice have elevated expression levels of dopamine and 14–3–3 zeta and our current data, we propose that MsrA-dependent 14–3–3 zeta ubiquitination affects the regulation of alpha synuclein degradation and dopamine synthesis in the brain.

Graphical Abstract

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Keywords

Ubiquitin; 14–3-3 proteins; alpha synuclein; brain

Introduction

Accumulation of aggregated forms of α -synuclein (α .Syn) and phosphorylated tau are associated with the development of the incurable neurodegenerative diseases, Parkinson's disease (PD) and Alzheimer's disease (AD). One family of proteins that regulates the stability and function of these forms of α .Syn and tau are the 14–3-3 proteins that serve in signaling and interact with various protein partners including modifying their function as adaptors that stimulate protein-protein interactions. For example, the isotype 14–3-3 ζ induces oligomerization of its target proteins (*e.g.*, phosphorylated tau) [1,2]. Proteins bound to 14–3-3 ζ are relatively resistant to protein phosphatases [1–3]. Thus, high levels of 14–3-3 ζ are proposed to increase the levels of phosphorylated tau that is a marker associated with AD [1]. Furthermore, binding of 14–3-3 proteins to phosphorylated tyrosine hydroxylase (TH) enhances dopamine (DA) production that can cause the generation of toxic levels of cytosolic DA, quinones, and reactive oxygen species (ROS) [4]. The α .Syn protein binds to 14–3-3 proteins as well as the dephosphorylated form of TH, the latter resulting in reduced TH activity [4]. Thus, the sequestration and regulation of 14–3-3 proteins by α .Syn play an important role in the pathogenesis of neurodegenerative diseases.

MsrA is a stereospecific methionine sulfoxide (MetO) reductase, conserved across all domains of life, and provides antioxidant defense by catalyzing the reduction of S-MetO to methionine [5]. *MsrA* knockout (KO) mice exhibit high levels of DA and 14–3-3 ζ compared with the corresponding wild-type (WT) strain [6]. Recently, we found the archaeal MsrA can direct ubiquitin-like (Ubl) protein modification by a mechanism that is distinct from its MetO reduction/oxidation activity [7]. These observations prompted us to investigate the interaction between the MsrA, 14–3-3 ζ , α .Syn, and the ubiquitin (Ub) system and how these systems may relate to PD in brain.

Specifically, using mouse brain extracts and immunoprecipitation experiments we described positive effect of MsrA on the ubiquitination of 14–3-3 ζ and the MsrA- dependent

interaction between 14–3–3 and α Syn. We also determined that MsrA is able to bind Ub and that the level of its MetO reductase activity is upregulated in the heterozygote and knockout 14–3–3 ζ mouse brain, compared to the control WT strain.

Material and Methods

Key resources

Key resources used in this study, including mouse strains, antibodies, proteins, and reagents, are listed in Supplemental Table S1.

The WT and MsrA KO strains were generated as previously described [8]. The 14–3–3 ζ heterozygotes and homozygotes as well as the corresponding parent WT strain were also generated, as previously described [9]. These strains crossbred for more than 20 generations.

Identification of ubiquitinated 14–3–3 ζ in mouse brain

Whole brains of *MsrA* KO and WT mice were extracted in phosphate buffered saline (PBS) supplemented with a protease inhibitor cocktail and MG132 proteasome inhibitor using a Teflon homogenizer. Equal amounts of the brain extracts [500 μ g of protein, determined by Bradford reagent (Bio-Rad, Hercules, CA)] were incubated with 10 μ g of His-tagged Ub (His-Ub) in the presence or absence of 10 mM ATP and 10 mM MgCl₂ (Mg-ATP) for 30 min at 37°C. At the end of the incubation time, the reaction mixtures were added to 50 μ l of pre-washed His-tag Purification Nickel Resin. After 1 h incubation at room temperature, the mixtures were centrifuged (500 \times g, room temperature), and precipitated protein-bound resin mixtures were washed with PBS. Proteins were eluted from the resin using 1X SDS-PAGE sample buffer supplemented with 0.1 M imidazole and 0.1 M EDTA. The eluates were subjected to Western blot analysis using anti-Ub as the primary antibody. A parallel gel was stained by Coomassie blue R-250 and the ubiquitinated protein bands, observed in the Western blot analysis, were excised from the gel. In-gel tryptic digestion of the acquired protein band was performed, followed by tandem mass-spectrometry (MS/MS) analysis of the resulting peptides for protein and sequence identification of the target protein, as we previously described [10]. Briefly, samples were analyzed by LC-MS/MS using a NanoAcquity chromatographic system (Waters Corp., Milford, MA) coupled to an LTQ-FT mass spectrometer (ThermoFinnigan, Bremen, Germany). Peptides were separated on a reverse phase C18 column. MS/MS spectra were analyzed using Mascot (Matrix Science, London, UK; version 2.3), Sequest (Thermo Fisher Scientific, San Jose, CA; version 27, rev. 12) and X! Tandem (The GPM, thegpm.org; version 2007.01.01.1). X! Tandem was set up to search a subset of the SwissProt_2012 database. Scaffold software (version 3.6, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identification.

Immunoprecipitation experiments

Immunoprecipitation was conducted using extracts (0.5 mg protein) of whole brain from *MsrA* KO and WT mice, prepared as described above. The brain extracts were incubated with the His-Ub (5 μ g) in the presence or absence of 10 mM Mg-ATP. After 20 min incubation at 37°C, the rabbit anti-14–3–3 antibody was added to each reaction mixture and

incubated for an additional 1 h at room temperature. After incubation, each sample was added to pre-washed protein G Sepharose and rotated overnight at 4°C. The protein G beads were washed with PBS (5 × 1 mL) to remove unbound proteins. After washing, the proteins were eluted from the protein G Sepharose using 1X SDS-PAGE sample buffer, containing β -mercaptoethanol, and boiled. The eluates were subjected to Western blot analyses using mouse anti- α Syn antibody or mouse anti-Ub antibody. Immunoprecipitation was also conducted following the same procedure, while alternatively using a mouse anti-Ub antibody and probing the Western blot membrane with either rabbit anti- α Syn antibody or rabbit anti-14-3-3 antibody. In all cases, Western blots were repeated at least three times with essentially the same outcomes. Representative blots are shown.

Phosphorylation of α -synuclein in mouse brain extract. Alpha synuclein (α Syn) of MsrA

KO and WT brain extracts was immunoprecipiated using rabbit anti- α Syn antibody. Following the immunoprecipitation, the precipitates were subjected to Western blot analyses using mouse anti- α Syn antibody or mouse anti- α Syn phospho-Ser129 antibody.

In vitro Parkin and E2 auto-ubiquitination assays

Full length recombinant parkin protein was prepared as described previously [11], except that the solubility factor .GB1 was incorporated into the N-terminus in place of GST. This parkin construct used has an N-terminal GB1 tag giving the construct a molecular weight of 51.7 KDA. This N-terminally tagged Parkin is known to be in an uninhibited form (i.e. activated) form [12].

In addition, parkin has been shown capable of E2-independent ubiquitination but only under conditions where there is a high concentration of the E1 ligase and at pH greater than 8.5 [13]. Those conditions don't apply here. We have also determined that the observed activity is E2-dependent (UbcH7) (results not shown). Accordingly, recombinant proteins were added to a reaction mixture as follows: 5 μ M of MsrA, 10- μ M His-Ub, and 22 nM E1. For the autoubiquitination assay of the E3 ligase parkin, parkin (0.9 mg/mL) and its relevant 1.4 μ M E2 (UbcH7) were added to the reaction mixture. For the E2 autoubiquitination assay, 1.4 μ M E2 (UbcH5a) replaced E3 parkin and E2 UbcH7 in the reaction. The reaction buffer contained 50 mM Tris-HCl (pH 8.0) and 1 mM DTT supplemented with and without 10 mM Mg-ATP. The reaction mixtures were incubated for 30 min at 37°C. At the end of the incubation time, the extracts were diluted to 1X SDS-PAGE sample buffer and subjected to Western blot analysis using anti-Ub monoclonal antibody.

Msr activity assay

Msr activity was determined as previously described [8]. Briefly, the reaction mixture (100 μ l) contained dabsyl-MetO as a substrate (200 μ M), 25 mM Tris-HCl (pH 7.5), 20 mM DTT, and 100 μ g of brain protein extract and 10 μ g for recombinant MsrA. The reaction mixture was incubated for 30 min at 37°C and stopped by the addition of 100 μ l acetonitrile. Thereafter, the sample was subjected to HPLC-based C-18 chromatography to identify the product, dabsyl-methionine. Reaction mixtures containing recombinant MsrA in conjunction with the Ub system (in the presence or absence of E1+ UbcH5a \pm Mg-ATP) were pre-incubated for 15 min at 37°C. The samples were incubated for an additional 15 min, in the

presence of the substrate (dabsyl-MetO) prior to detection of Msr reaction product (dabsyl-methionine) by HPLC. Msr activity was also determined in the whole brain extracts of 14-3-3 ζ heterozygotes and homozygotes and their corresponding wild-type mice, which were homogenized in PBS in the presence of a protease inhibitor cocktail and MG132 proteasome inhibitor.

Results

14-3-3 ζ identified as a high-level ubiquitinated protein in wild type relative to MsrA knockout mice

To examine the impact of MsrA on ubiquitination *in vitro*, equal proteins brain extracts of wild type (WT) and *MsrA* knockout (KO) mice were incubated with His-tagged Ub (His-Ub) in the presence or absence of Mg-ATP. The advantages of using this type of His-Ub pull procedure are the protection against deubiquitinating enzymes, strong avidity, and the low unspecific binding. Proteins covalently modified by the His-Ub were enriched by Ni²⁺ - affinity purification and subjected to anti-Ub Western blot analysis. Protein bands with ubiquitination levels that showed both MsrA and ATP dependency were observed. In particular, an ubiquitinated protein band of ~36 kDa was detected in the WT mouse brain with Mg-ATP that was not detected in the *MsrA* KO or in the absence of Mg-ATP (Figure 1). A parallel gel stained with Coomassie blue R-250 was used to identify the protein bands of interest by tandem mass spectrometry (MS/MS) analysis (data not shown). By this approach, the 36-kDa ubiquitinated protein was identified as 14-3-3 ζ (Figure 2). Moreover, diglycine footprints were found on the lysine of the tryptic peptides (Figure 2), that indicated 14-3-3 ζ was ubiquitinated at lysine residues K138 and K139. The increased molecular mass of 14-3-3 ζ from ~28 kDa to ~36 kDa suggested that one Ub molecule was covalently bound to this protein, as the mass of Ub is ~8 kDa (although it can run at a lower mass in SDS-gel-electrophoresis). Thus, our data (Figures 1 & 2) indicated that 14-3-3 ζ ubiquitination was significantly enhanced in the presence of MsrA.

MsrA influences the interaction between α Syn and 14-3-3 proteins and their levels of ubiquitination

The interrelation between 14-3-3 proteins and α Syn has been already investigated in details demonstrating the following observations: a) Specific 14-3-3 isoforms co-localize with α Syn in a transgenic mouse model of PD [14]; b) 14-3-3 and α Syn from rat brain and the substantia nigra of PD patients co-immunoprecipitated [15, 16]; c) most importantly, large soluble complexes of α Syn and 14-3-3 were observed in human neuronal cells overexpressing α Syn [17]; d) Likewise, the 14-3-3 η isotype was shown to strongly interact with only oligomeric α Syn aggregates but not monomeric α Syn [18]; suggesting that 14-3-3 η may reduce α Syn-mediated cellular toxicity [18]. Consequently, we hypothesize that the binding process of 14-3-3 proteins to α Syn through the production of large complexes is both dependent on 14-3-3 modification (e.g. ubiquitination) and the presence of MsrA.

Accordingly, we first investigated how MsrA affecting not only the ubiquitination levels of 14-3-3 ζ , but how it may also impact the binding of 14-3-3 ζ to α Syn in brain. The 14-3-3 antibody, which recognizes 14-3-3 ζ and other members of the 14-3-3 protein family, was

used for this analysis. In addition, an antibody specific for α Syn and an anti-Ub antibody that recognizes the multiple forms of Ub (mono- and poly-Ub protein conjugates, free poly-Ub chains, and free Ub) were used. First, we immunoprecipitated proteins from the brain extracts of *MsrA* WT and KO mice using mouse anti-Ub antibody and then probed these fractions for 14-3-3 proteins or α Syn by Western blot analysis. As shown in Figure 3A, *MsrA* KO brain showed somewhat lower levels of the ubiquitinated products of both α Syn and 14-3-3 proteins compared with WT brain. The reduction in the molecular mass of the mono-ubiquitinated form of 14-3-3 to the observed less than 36kDa molecular mass protein products (Figure 3A), and together with the observed multiple banding patterns of both proteins, indicates that they are partially degraded. A similar pattern was observed with respect to the ubiquitinated α Syn forms, in which their molecular masses depend on the level of ubiquitination and SDS-resistant oligomers of α Syn (Figure 3A). These results suggest that both α Syn and 14-3-3 proteins are ubiquitinated and degraded more efficiently in the presence of *MsrA in vivo* (note that protease and proteasome inhibitors were present during the brains extraction). The efficiency of using only Western blot analyses for the detection of ubiquitinated 14-3-3 forms is limited. Accordingly, immunoprecipitation experiments were performed to enrich the detection of the ubiquitinated 14-3-3 forms. Immunoprecipitation with anti-14-3-3 antibody suggests that either 14-3-3 proteins, or their protein-binding partners, or both are indeed more ubiquitinated and Ub-dependent degraded in the WT strain (as reflected by the presence of mid to low size molecular mass bands) (Figure 3B). This later result is in agreement with the ability of 14-3-3 and α Syn to produce large soluble complexes [17, 18]. Furthermore, it seems like the profile of the 14-3-3 immunoprecipitates is nearly the same for both anti- α Syn and anti-Ub Western blots, suggesting that the vast majority of the detected protein bands in Figure 3B (lanes 1 and 3) are either ubiquitinated 14-3-3, or ubiquitinated α Syn, or a mixture of both that interact with each other in an *MsrA*-dependent manner. Next, to show the dependency on ATP in this process, a comparative experiment in which the brain extracts were incubated in the presence or absence of Mg-ATP for 30 min at 37°C was performed, prior to the immunoprecipitation by 14-3-3 antibody. The WT brain showed an enhanced degradation and ubiquitination of α Syn in the presence of Mg-ATP. However, in the brain of the *MsrA* KO strain, the levels of the monomer and the ~36kDa form of α Syn were increased by the addition of Mg-ATP, while no mid-to-low molecular mass ubiquitinated proteins were observed (Figure 3C). The level of protein phosphorylation may retard the mobility of any protein in an SDS-gel electrophoresis due to increased polarity. Thus, it is possible that the observed ~36kDa α Syn represents one of the phosphorylated forms of α Syn. Furthermore, the enhanced non-ubiquitinated α Syn band shown in the presence of Mg-ATP in the *MsrA* KO mouse brain extract points to the possibility of an enhanced α Syn stability in the *MsrA* KO versus the WT strain (Figure 3C). This observation correlates with a previous suggestion that 14-3-3 proteins protect the phosphorylated form of α Syn from being dephosphorylated at the presence of high DA levels (like in the case of *MsrA* KO brain) [6]. This function of 14-3-3 is predicated to be an Ub and *MsrA*-independent process, as suggested by the protection of α Syn from degradation in the absence of *MsrA* (i.e. in *MsrA* KO brain and in yeast *msrA* KO overexpressing *MsrA* [19]). Western blot analyses showed equal levels of the loading controls (α Syn, 14-3-3, and β -actin) in each brain extract used for the immunoprecipitation experiments (Figure 3D).

To determine whether α Syn in the *MsrA* KO brain is indeed hyper-phosphorylated compared to the WT strain, we have immunoprecipitated α Syn in each brain extract and probed the precipitates in Western blot analyses using mouse anti- α Syn or rabbit anti- α Syn phospho-Ser129 (the major phosphorylation site that is important for α Syn regulation). As demonstrated in Figure 4B, the relative level of phospho-Ser129 of a ~24 kDa form of α Syn was higher in the *MsrA* KO strain than the WT strain (while both strains had similar expression levels of non-phosphorylated α Syn, as indicated by probing with the anti- α Syn antibody) (Figure 4A). Noteworthy, the extracts used in Figure 4 were the same extracts used in Figure 3 and thus their loading controls are showed in Figure 3D. The level of protein phosphorylation may retard the mobility of any protein in an SDS-gel electrophoresis due to increased polarity. Thus, it is possible that the observed ~24kDa α Syn represents one of the phosphorylated forms of α Syn.

Competition between MsrA and E2 and E3 enzymes on Ub binding

The data presented above suggest that ubiquitination of specific proteins is mediated by MsrA. To determine and evaluate the ability of MsrA to bind Ub, the effect of MsrA on the *in vitro* autoubiquitination of parkin was assessed. Parkin catalyzes E3 Ub ligase activity and is thought to directly or indirectly mediate proteasomal degradation of aggregation-prone proteins, including α Syn. Moreover, mutations in the *Parkin* gene are the most common cause of hereditary parkinsonism [20]. In agreement with previous findings [20], parkin showed a strong autoubiquitination in the presence of E1 and E2 (UbcH7) in the expected 55 kDa region (Figure 5A Lane 3). Surprisingly, this auto ubiquitination disappeared when MsrA was introduced to the system (Figure 5A, Lane 5). Autoubiquitination of MsrA was not observed in this *in-vitro* system (Figure 5A). This result suggests that MsrA inhibits the autoubiquitination of parkin, possibly through competing for binding Ub and/or its intermediates in the process. Similarly, the known auto-ubiquitination of another E2 (UbcH5a) was inhibited upon the addition of MsrA to the reaction mixture, especially in the reaction mixtures containing ATP (compare Lanes 2 and 4 in Figure 5B). UbcH5a is a major E2 protein that is active in a wide-ranging number of *in vitro* ubiquitination reactions and binds Ub non-covalently on a surface that is distinct from its SH-containing active site [21]. Thus, MsrA is found to inhibit not only the ubiquitin-dependent degradation of 14-3-3 and α -Syn (*in vivo*) but also the autoubiquitination of parkin and E2 (UbcH5a) via a direct mechanism (as monitored *in vitro*). It is important to note that no autoubiquitination of the E1 used in this system was detected (data not shown).

Next, we correlated the MetO reductase activity of MsrA with the Ub system. MsrA MetO reductase activity was measured in the presence or absence of the Ub system (E1, E2, and Ub) and ATP. As shown in Figure 6, the MetO reductase activity of MsrA was strongly inhibited when the Ub system was activated by Mg-ATP (vs. absence of Mg-ATP). The complexity of the reaction mixture makes it difficult to determine the linearity of the events that result in either MetO reduction or Ub binding by the MsrA enzyme. However, the presented data clearly demonstrate that the Mg-ATP -dependent processes, yielding activated Ub, can compete with dabsyl-MetO binding by the MsrA's active site (Figure 5A). It is important to note that MsrA was not ubiquitinated under these conditions (data not shown). Therefore, the MsrA activity was not affected by Ub modification to MsrA. Taken together,

our data imply that the active site of the mammalian MsrA for MetO reduction can bind either MetO or the intermediates and/or products of ubiquitination, as it has been shown to be the case for archaeal MsrA [7].

Effect of 14–3–3 ζ ablation on Msr activity in mouse brain

MsrA inhibits the autoubiquitylation of parkin (Figure 5A) and E2 (UbcH7) (Figure 5B) and fosters 14–3–3 ζ ubiquitination (Figure 1) and stabilization (Figure 3A). Accordingly, if 14–3–3 ζ is a major substrate for MsrA-dependent ubiquitination, its ablation will cause the active site of MsrA to be less occupied with autoubiquitylation intermediates and/or products and, therefore, will be more available for MetO reduction activity. Supporting evidence for this hypothesis is shown in Figure 7A, in which the MetO reductase activity of Msr was significantly elevated in brain of the 14–3–3 $\zeta^{-/-}$ and 14–3–3 $\zeta^{-/+}$ mice compared with the 14–3–3 $\zeta^{+/+}$ mouse. Although the MetO reductase activity was the highest in brain of the 14–3–3 $\zeta^{-/-}$ mouse, it was comparable to the 14–3–3 $\zeta^{-/+}$ strain (Figure 7A). The latter observation suggests that a 50 % reduction of 14–3–3 ζ expression is sufficient to elevate MsrA activity by enhancing its MetO reduction activity. The levels of MsrA were also found elevated in the 14–3–3 $\zeta^{-/+}$ and 14–3–3 $\zeta^{-/-}$ strains compared to 14–3–3 $\zeta^{+/+}$ (Figure 7B). Thus, the levels of MsrA protein and MetO reductase activity were increased in parallel by progressive KO of 14–3–3 ζ .

Discussion

In agreement with the role of 14–3–3 proteins described in the introduction section, we have provided supportive evidence demonstrating that lack of MsrA protein inhibits the degradation rate of α Syn in a yeast *ex vivo* system [19], while increasing DA, 14–3–3 ζ , and tau-phosphorylation levels in an *MsrA* knockout (*MsrA* KO) mouse [6,22]. 14–3–3 proteins and α Syn are abundantly expressed in the brain (~1% of total proteins) and are degraded by the Ub system. 14–3–3 proteins interact with many binding partners, including α Syn, affecting cell cycle and transcriptional control, signal transduction, intracellular trafficking, and regulation of ion channels [23]. Furthermore, studies performed on animal models and on post mortem brains of patients with neurodegenerative diseases, like PD and AD, point to the involvement of 14–3–3 proteins in disease-associated processes [23]. In PD, deposition of misfolded and aggregated forms of α Syn in Lewy bodies in the substantia nigra region of brain is associated with the development of this disease. Both 14–3–3 and α Syn can regulate DA levels in the striatum; thus, dysregulation and abnormal structure of either protein may affect DA synthesis (through TH activation). Accordingly, the cellular mechanisms that are responsible to control the levels of each protein (synthesis vs degradation pathways) play a significant role in maintaining normal function of the dopaminergic cell. As with other neurodegenerative diseases, the sporadic form of PD is an age and oxidative stress associated disease. As such, oxidative modification of α Syn and other proteins may lead to posttranslational modifications that are either “repaired” by specific enzymes or degraded via the Ub or autophagy systems. One established oxidative damage repair mechanism is mediated by the Msr system [5]. We and others have shown that lack of MsrA exacerbates the accumulation of oxidized proteins in the cell, reduces cell/organism survival under oxidative stress conditions, and alters the function of specific proteins (e.g., DA D2-

receptor) [5, 24]. Given the dual function of MsrA in the current studies, we propose that MsrA enhances 14–3-3 ubiquitination (Figures 1 & 2), which in term may facilitate the degradation of 14–3-3 through the Ub-proteasome system. MsrA is an ideal candidate for the selection process aiming to either “repair” or “destroy” a protein, as MsrA can “repair” proteins by reducing MetO residues (*e.g.*, of α Syn) or make a targeted protein (*e.g.*, 14–3-3) available for destruction by the Ub system. The increased affinity of the interaction of α Syn with 14–3-3 proteins, due to the presence of MsrA and their increased ubiquitination levels (Figure 3), suggests that MsrA plays an important role in modulating the ubiquitination of both proteins; either directly (*e.g.*, by functioning as an adaptor for the ubiquitination system) or indirectly (through a yet to be discovered protein factor). The 14–3-3 proteins can bind α Syn in the presence of high levels of DA and protect 14–3-3 from being degraded via the Ub system, through binding to a specific phosphorylated Ser129 of α Syn [25]. Thus, it is suggested that the presence of MsrA enhances α Syn degradation by eliminating this protective effect of 14–3-3 ζ through ubiquitination and degradation (Figure 8). Ub and ATP-dependent competition experiments suggest that MsrA can bind either auto-Ub system intermediates/products or MetO residues via its active site (Figures 5 and 6); a phenomenon that agrees with our previous observation in *Archaea* [7]. Lack of 14–3-3 ζ protein in the 14–3-3 ζ KO brain caused an upregulation of MsrA expression and associated activity (Figure 7). The protein 14–3-3 ζ is required for Klotho’s anti-oxidant activity via the ASK1/p38 MAPK pathway, while interacting with thioredoxin [26].

Consequently, enhanced oxidative stress in the 14–3-3 ζ KO brain is hypothesized to cause an upregulation of MsrA expression as a compensatory mechanism. If so, the MsrA may protect against the resulting ROS-mediated damage to proteins by either its MetO reduction activity or by its Ub-modifier activity, or both. A summary of the proposed role of MsrA in DA regulation, with respect to 14–3-3 ζ and α Syn functions is illustrated in Figure 8. Future studies will focus on screening for other Ub related factors and conditions that regulate and mediate the interactions between MsrA and its substrates.

Conclusions

Posttranslational modification to proteins such as oxidation of methionine residues ubiquitination may alter their function and/ or make them more prone to degradation, respectively. Here we show that the enzyme methionine sulfoxide reductase type A (MsrA) is able to function not only as a “repair” mechanism to “oxidative damaged” proteins but also as mediator for the ubiquitination of 14–3-3 proteins and specifically 14–3-3 ζ . Furthermore, the presence of MsrA enhances the phosphorylation levels of α Syn through the ubiquitination and degradation of 14–3-3 ζ . The dual involvement of MsrA in both protein repair and degradation pathways strengthen its important role as a modulator of cellular function under oxidative stress conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- MsrA mediates 14–3–3 protein ubiquitination
- MsrA enhances the interaction between specific forms of 14–3–3 and alpha synuclein
- Phosphorylation levels of alpha synuclein are affected by MsrA

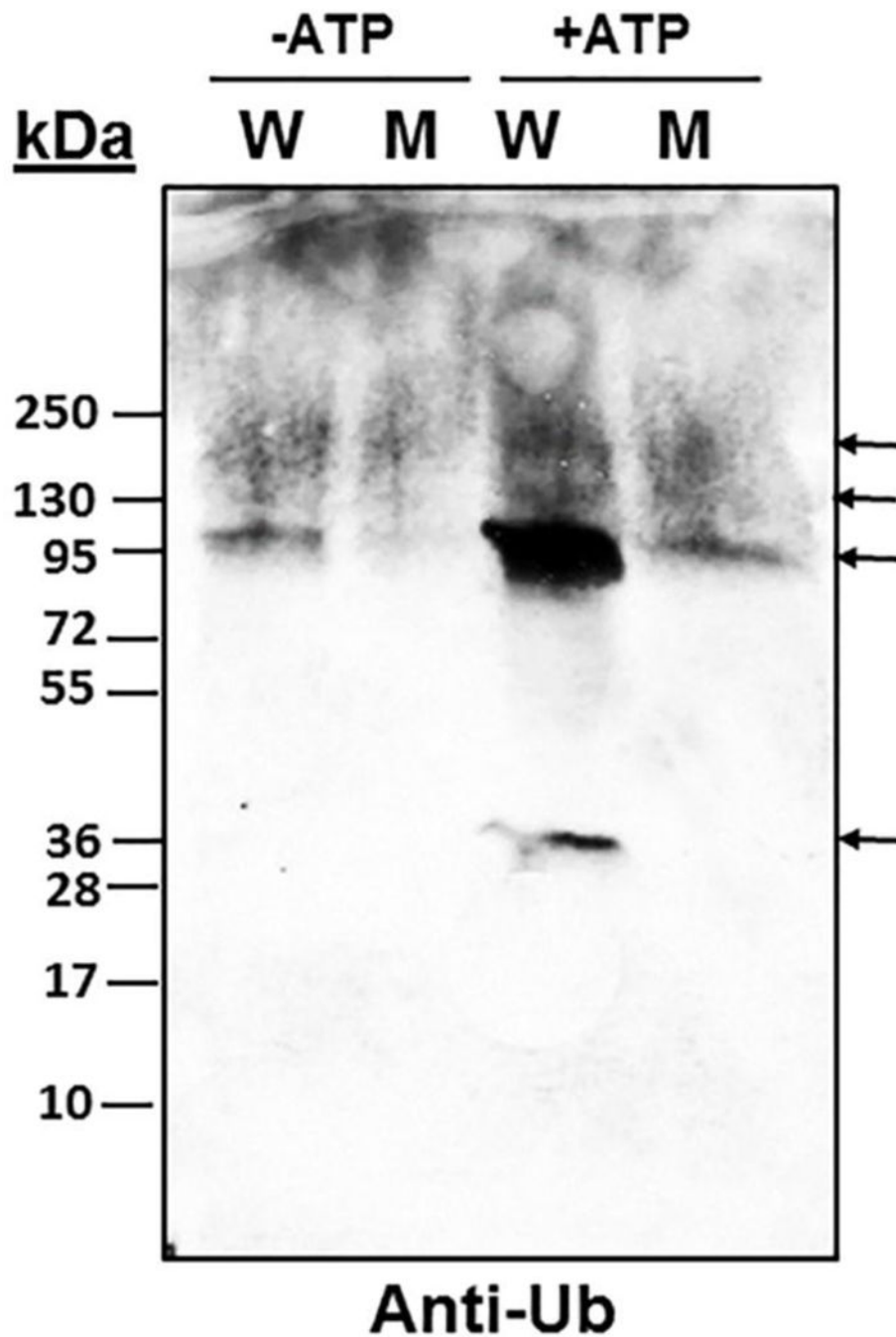


Fig 1. Detection of ubiquitinated proteins in extracts of wild type (W) and *MsrA* knockout (M) mouse 14-3-3 ζ brains.

Brain extracts were prepared in PBS in the presence of protease and proteasome inhibitors. Equal amounts of protein extracts (see loading control gel) were incubated with His-Ub with and without Mg-ATP (10 mM of each) for a period of 30 min at 37°C. The proteins were enriched by Ni²⁺-affinity purification prior to adding SDS-gel-electrophoresis sample buffer, followed by Western blot analysis using anti-Ub antibody. Both Anti-Ub Western blot (WB) and Coomassie blue staining showed the same protein pattern and intensity (the protein-stained gel was subjected to MS/MS analysis and thus is not shown). kDa, molecular

mass markers for both the loading control protein stained gel (Commassie blue staining) and the WB. Arrows indicate the position of major ubiquitinated proteins.

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14-3-3 ζ protein sequence:

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mdknelvqka klaeqaeryd dmaacmksvt eqgaelsnee
rnllsvaykn vvgarrsswr vvssieqkte gaekkkqmar
eyrekietel rdicndvlsl lekflipnas qpeskvyfk
mkgdyryla evaagddkkg ivdqsqqayq eafeiskkem
qpthpirlg1 alnfsvfyye ilnspekacs laktafdeai
aeldtlsees ykdstlimql lrdnltlwt dtqgdeaeag
eggen

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Fig 2. Identification 14-3-3 ζ as the MsrA- and ATP-dependent ubiquitinated protein by mass spectrometry analyses.

The identified 36-kDa ubiquitinated protein band (see Figure 1) was subjected to trypsin digestion followed by mass-spectrometry analyses for protein identification (see “Experimental procedures”). The “ylaevaagddk” sequence indicated in bold was a tryptic peptide detected by MS/MS analysis. The underlined KK residues are the site of ubiquitination detected on the peptide by the presence of a diglycine (-Gly-Gly) remnant of the C-terminal residues of ubiquitin. Either of these lysines was found to be modified and these two lysines could not be distinguished due to their close proximity.

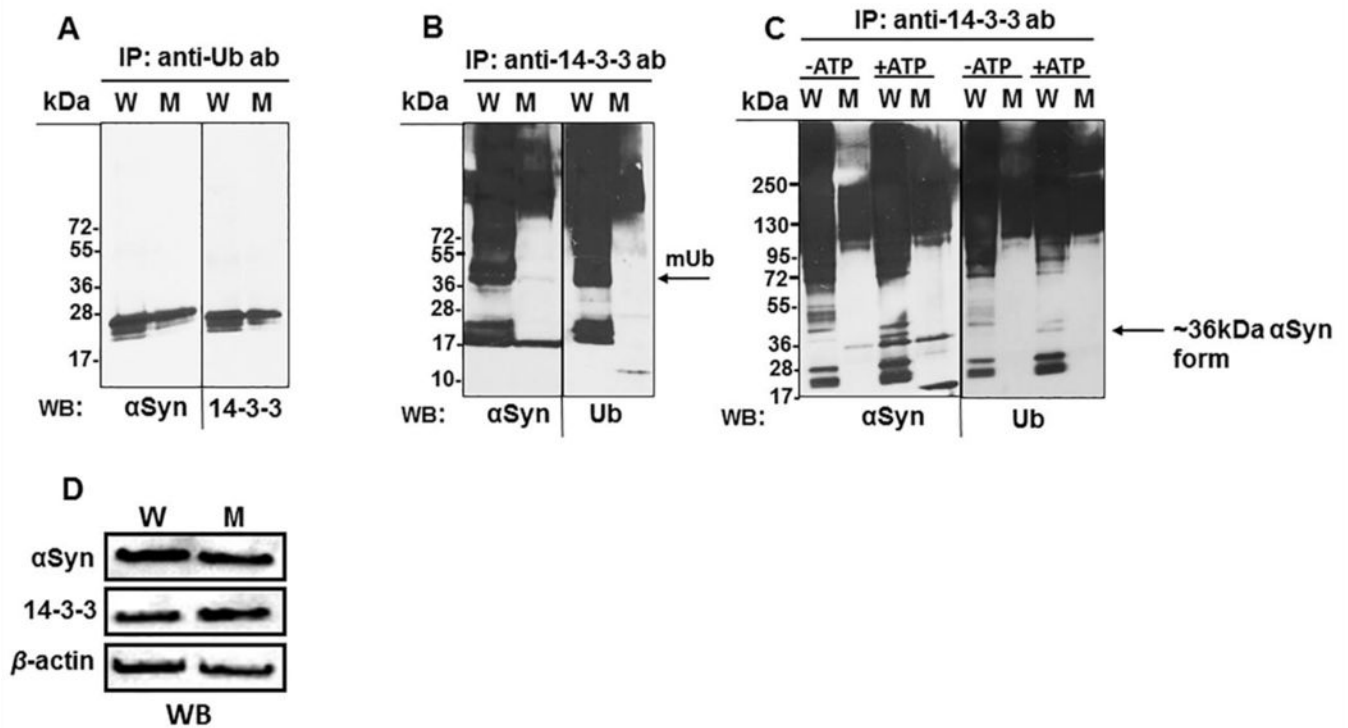


Fig 3. Binding affinity interactions between Ub, α -synuclein (α Syn), and 14-3-3 proteins in extracts of wild type (W) and *MsrA* knockout (M) mouse brains.

Immunoprecipitation (IP) experiments (A-C) were performed on brain extracts, followed by Western blot (WB) analysis using the protein antibodies (mouse anti- α Syn, mouse anti-Ub, and rabbit anti-14-3-3 antibodies) as indicated. The exception was in panel A, where the anti- α Syn antibody was from rabbit (since the precipitating anti-Ub antibody was from mouse). In panel C, the brain extracts were incubated for 20 min at 37°C with the His-Ub (5 μ g) in the presence or absence of 10 mM Mg-ATP, prior to performing the IP procedure (D). Western blot analyses for loading controls of the W and M extracts prior to the immunoprecipitation (mouse anti- β actin antibody, mouse anti- α Syn, and rabbit anti-14-3-3 antibodies were used). kDa, molecular mass markers; mUb arrow, mono ubiquitination band of 14-3-3.

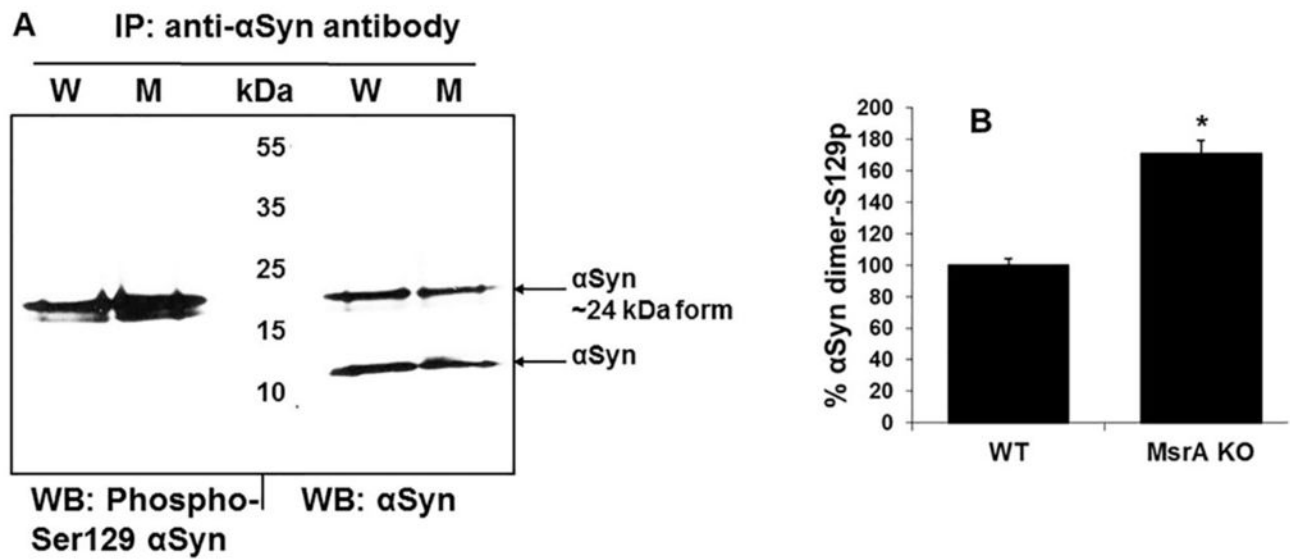


Fig 4. Phosphorylation of α -synuclein (α Syn) in wild type (W) and *MsrA* KO brains (M).

A. Alpha synuclein of each brain extract was immunoprecipitated using rabbit anti- α Syn antibody. Following the immunoprecipitation (IP), the precipitates were subjected to Western blot (WB) analyses using mouse anti- α Syn antibody or rabbit anti- α Syn phospho-Ser129 antibody. **B.** Ratio of the densitometry analyses (using NIH Image-J program) of the phosphorylated α Syn dimer over the total non-phosphorylated α Syn homodimer, presented as percent of wild type (WT) ratio. *, indicates significant statistical differences between the *MsrA* KO and WT group of mice ($P < 0.01$ using *t-test* program). Note, the loading controls for the brain extracts used in this figure are shown in Figure 3D.

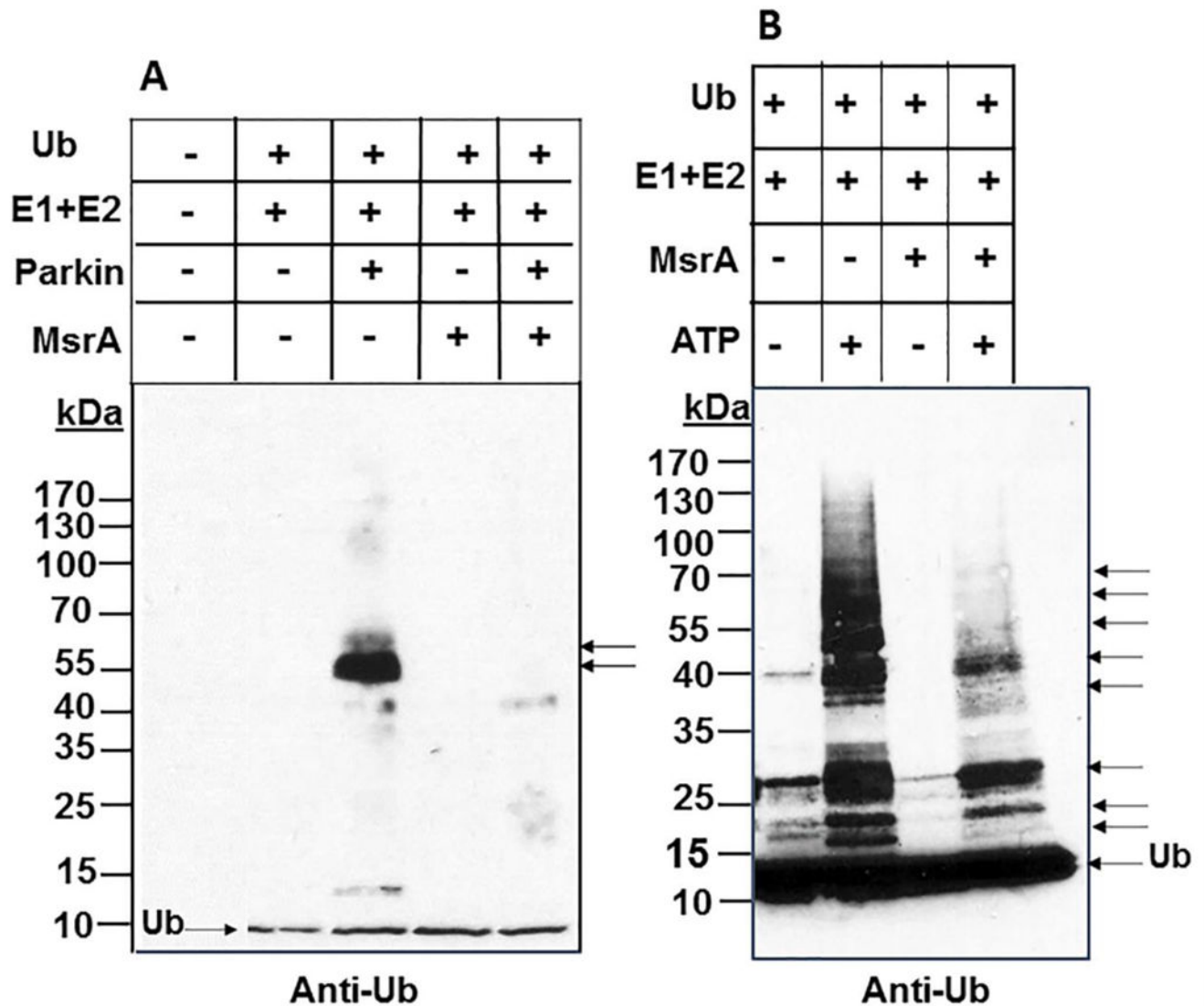


Fig 5. Effect of MsrA on Parkin and E2 (Ubch5a) auto-ubiquitination.

Recombinant proteins were added to reaction mixtures as shown above the images. The reaction mixtures were incubated for 30 min at 37°C. The reactions were stopped by adding 1X SDS-PAGE sample buffer, followed by Western blot analysis using anti-Ub antibody.

Panel A: detection of parkin auto-ubiquitination as indicated by arrowheads. **Panel B:** detection of Ubch5a ubiquitinations as indicated by arrowheads. No E1 auto-ubiquitination was detected when the reaction contained only E1, Ub, and ATP (data not shown). kDa, molecular mass markers; Ub, ubiquitin.

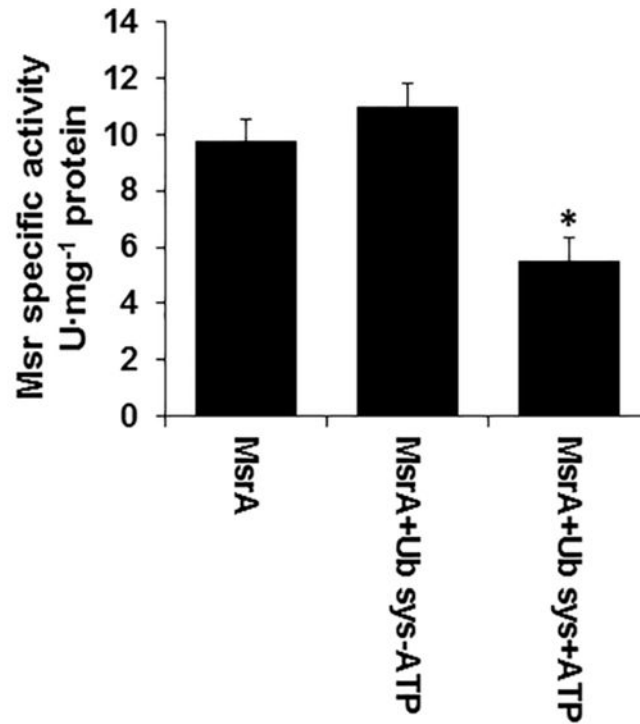


Fig 6. MsrA activity in inhibited in the presence of E1, E2, Ub, and ATP. Recombinant human MsrA was incubated at 37°C for 30 min in the presence or absence of E1+E2+Ub and ± ATP followed by Msr activity determination (according to the related procedure described in “Materials and Methods”). *, indicates significant statistical differences between the MsrA+Ub system (Sys) + ATP group and the other two groups of mice ($P < 0.01$ using one-way Anova program).

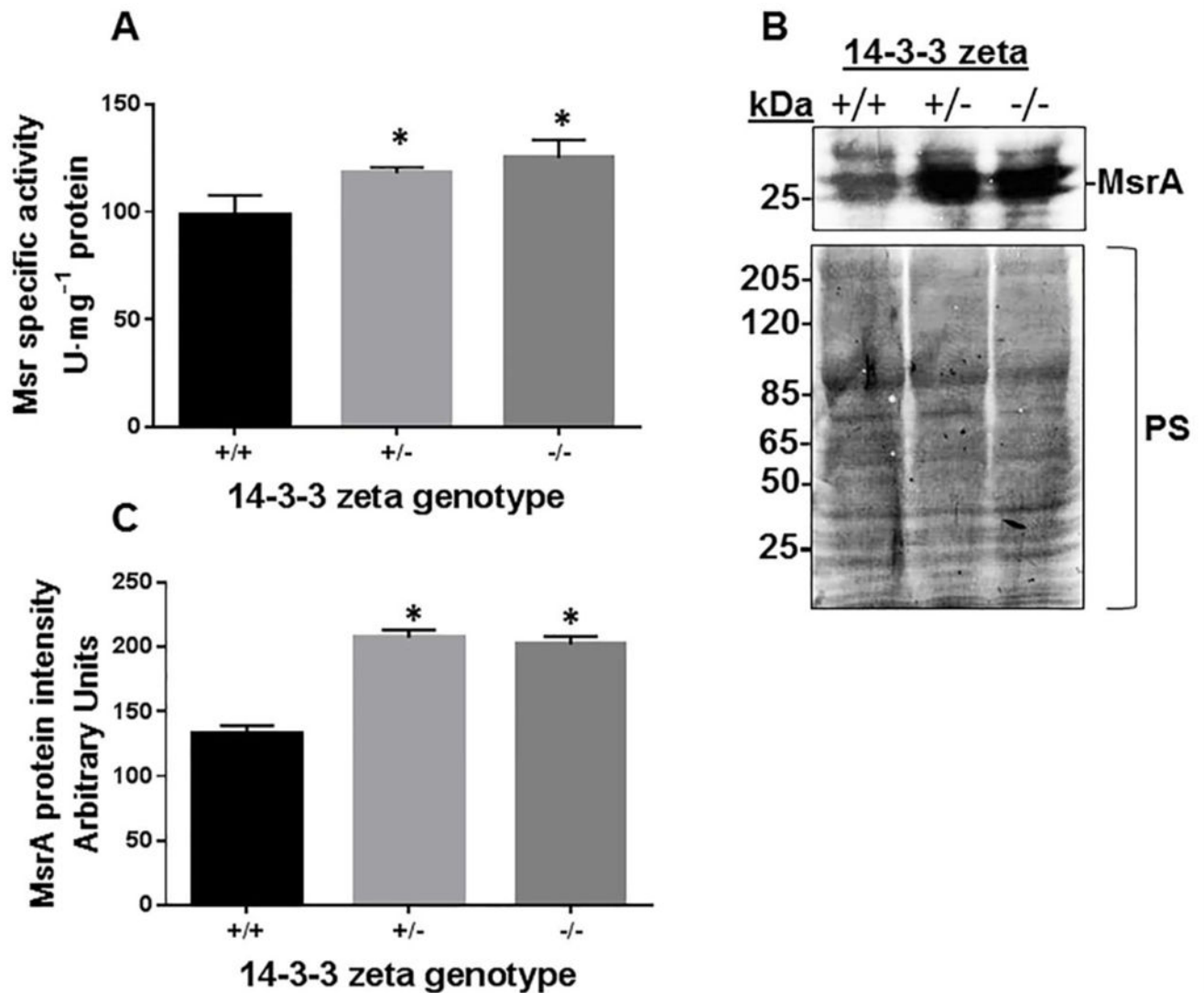


Fig 7. MsrA expression is upregulated in the absence of 14-3-3 ζ in mouse brain.

Protein extracts from mouse brains of three strains (14-3-3 ζ ^{+/+}, 14-3-3 ζ ^{+/-} and 14-3-3 ζ ^{-/-}) were analyzed for Msr activity (A), MsrA expression (through Western blot analysis using anti-MsrA antibody) (B) that was also quantified using NIH Image-J program (C). kDa, molecular mass markers; PS, Ponceau-S staining used for total protein staining; *, indicates significant statistical differences between the 14-3-3 ζ ^{+/+} group and each of the other group of mice ($P < 0.01$ using one-way Anova program).

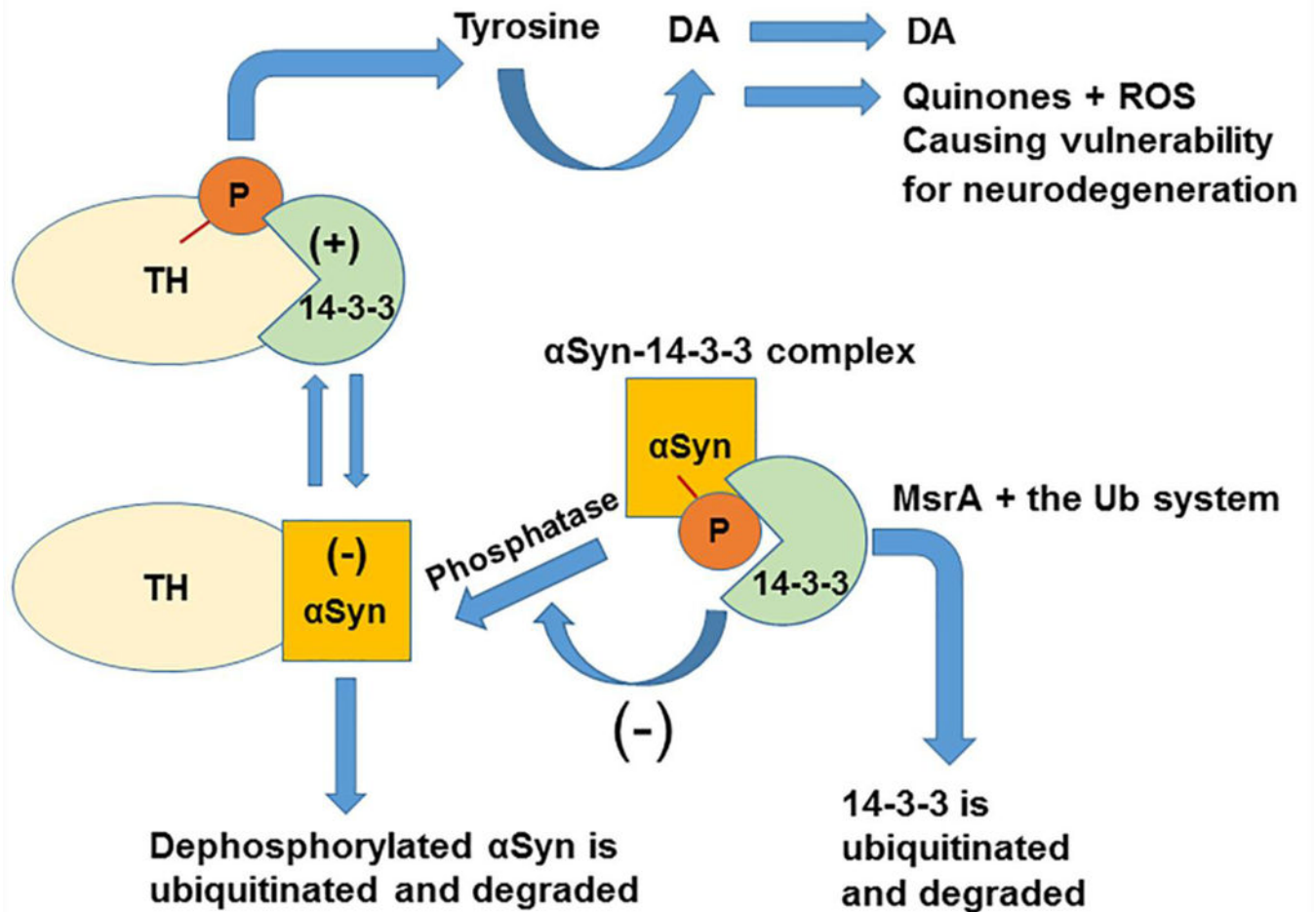


Fig 8. A summary figure describing the Involvement of MsrA in the regulation of 14-3-3 proteins and its effect on synuclein (α Syn) levels and function.

14-3-3 proteins (green) that bind to phosphorylated tyrosine hydroxylase (TH) (yellow), and α Syn (orange) that binds to unphosphorylated TH, differentially regulate TH activity to maintain optimal dopamine levels. In the case of reduced levels of MsrA (e.g. in *MsrA* KO mice), 14-3-3 (e.g. 14-3-3 ζ) is not efficiently degraded by the ubiquitin system and thus preferentially binds to phosphorylated α Syn (P in red, a phosphorus group). This situation provides protection of α Syn from dephosphorylation events, which promote its ubiquitination and degradation. We have provided supportive evidence demonstrating that lack of MsrA protein inhibits the degradation rate of α Syn in a yeast *ex vivo* system [18] and increases the stability of 14-3-3 ζ in mouse brain [6]. It is hypothesized that upon lower levels of MsrA (e.g. the *MsrA* KO brain), 14-3-3 ζ bind strongly to non-aggregated phosphorylated forms of α Syn. This phenomenon leads to a higher stability of α Syn and an enhanced activation of TH, causing an increase in dopamine (DA) formation and release [6]. However, increased levels of ubiquitinated forms of 14-3-3 ζ in WT brain (a phenomenon supported by this paper) seem to bind more strongly to non-monomeric α Syn forms, causing the formation of high molecular mass aggregates of 14-3-3- α Syn complexes. (+), positive regulatory effect of 14-3-3 on TH activity. (-), negative regulatory effect of α Syn on TH

activity and negative regulatory effect of 14-3-3 on α Syn dephosphorylation. ROS, reactive oxygen species.

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