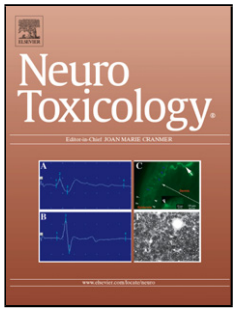


Title: Resveratrol treatment reduces the vulnerability of SH-SY5Y cells and cortical neurons overexpressing SOD1-G93A to Thimerosal toxicity through SIRT1/DREAM/PDYN pathway

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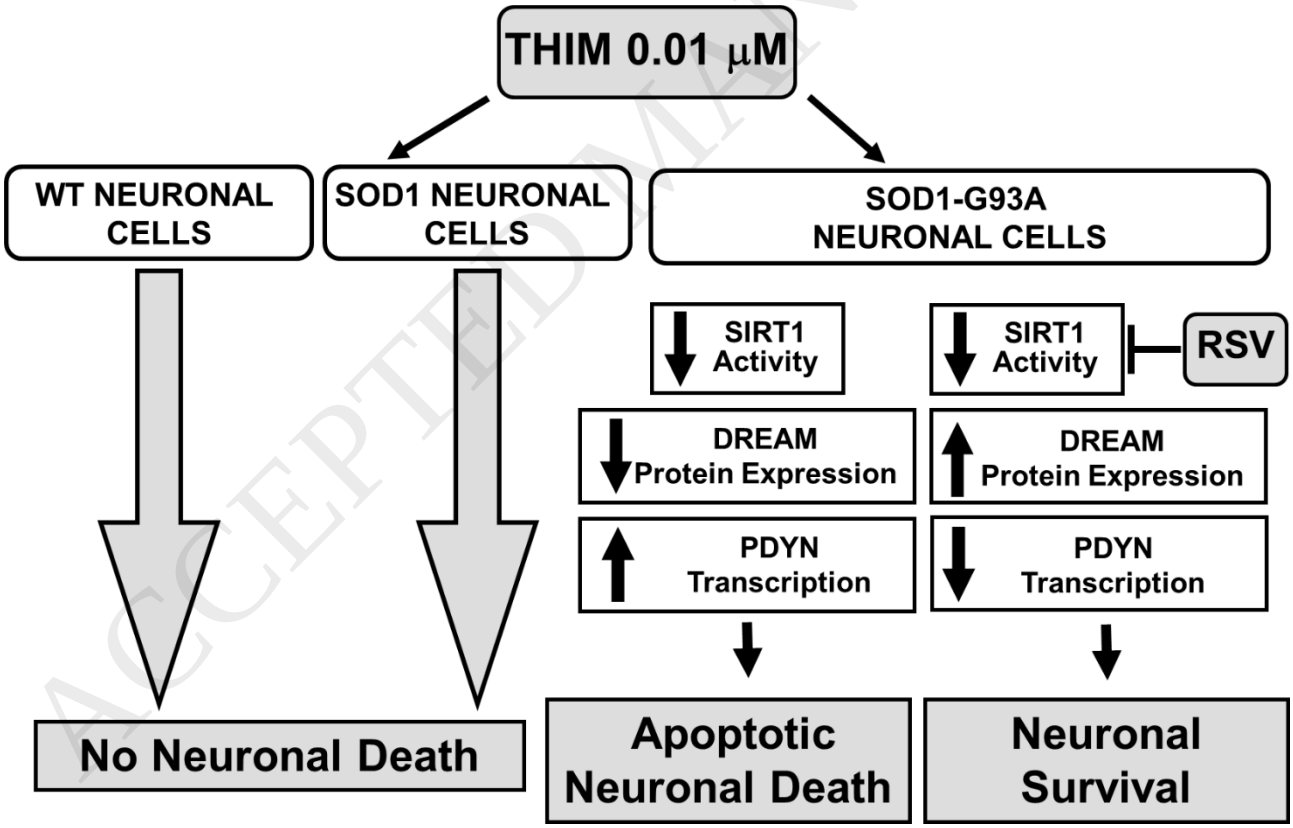


**Highlights**

►Thimerosal, at non-toxic concentration, reduced cell survival in SOD1-G93A cells.

► Thimerosal induced neurotoxicity by activating the SIRT1/DREAM/PDYN pathway

► Resveratrol prevented Thimerosal-induced cell death.

**Abstract**

In humans, mutation of glycine 93 to alanine of $Cu^{++}/Zn^{++}$ superoxide dismutase type-1 (SOD1-G93A) has been associated to some familial cases of Amyotrophic Lateral Sclerosis (ALS). Several evidence proposed the involvement of environmental pollutants that like mercury could accelerate ALS symptoms. SH-SY5Y cells stably transfected with SOD1 and G93A mutant of SOD1 constructs were exposed to non-toxic concentrations (0.01 μM) of ethylmercury thiosalicylate (thimerosal) for 24 hours. Interestingly, we found that thimerosal, in SOD1-G93A cells, but not in SOD1 cells, reduced cell survival. Furthermore, thimerosal-induced cell death occurred in a concentration dependent-manner and was prevented by the Sirtuin 1 (SIRT1) activator Resveratrol (RSV). Moreover, thimerosal decreased the protein expression of transcription factor Downstream Regulatory Element Antagonist Modulator (DREAM), but not DREAM gene. Interestingly, DREAM reduction was blocked by co-treatment with RSV, suggesting the participation of SIRT1 in determining this effect. Immunoprecipitation experiments in SOD1-G93A cells exposed to thimerosal demonstrated that RSV increased DREAM deacetylation and reduced its polyubiquitination. In addition, RSV counteracted thimerosal-enhanced prodynorphin (PDYN) mRNA, a DREAM target gene. Furthermore, cortical neurons transiently transfected with SOD1-G93A construct and exposed to thimerosal (0.5 μM/24 hours) showed a reduction of DREAM and an up-regulation of the prodynorphin gene. Importantly, both the treatment with RSV or the transfection of siRNA against prodynorphin significantly reduced thimerosal-induced neurotoxicity, while DREAM knocking-down potentiated thimerosal-reduced cell survival. These results demonstrate the particular vulnerability of SOD1-G93A neuronal cells to thimerosal and that RSV via SIRT1 counteracts the neurodetrimental effect of this toxicant by preventing DREAM reduction and prodynorphin up-regulation.

**Key words**: SOD1, DREAM, Resveratrol, Neuroprotection, Thimerosal.

## 1 Introduction

Amyotrophic lateral sclerosis (ALS) is an idiopathic neuronal disease of the motor system characterized by degeneration of cortical, brainstem and spinal motor neurons (Kiernan et al. , 2011). The majority of ALS is sporadic with unknown etiology, whereas ~15% is familial with dominant inheritance. Sporadic (sALS) and familial ALS (fALS) are clinically indistinguishable and share several pathogenetic pathways (Paez-Colasante et al. , 2015). Interestingly, mutations in the gene of copper/zinc superoxide dismutase type 1 (SOD1) is the most common cause of fALS and may also be the cause of between 0.7-4% cases of sporadic sALS (Robberecht and Philips, 2013). Notably, several mutant SOD1 transgenic mice have been produced and the SOD1-G93A mouse is the mostly used experimental model for ALS studies (Chen et al. , 2013). It has been

found that neurotoxic agents in specific patients genetically predisposed to ALS can activate the neurodegenerative processes occurring in this pathology (Cox et al. , 2009, Miranda et al. , 2008). The mercurial compounds are classified in three diverse Hg molecular classes: elemental (Hg), inorganic ($Hg^{2+}$), and organic (MeHg) (Aschner and Ceccatelli, 2010). Regarding a possible relationship between Hg exposure and the possibility to develop ALS, it has been reported that clinical symptoms comparable to those characteristic of ALS can develop in patients with continuing accidental Hg exposure or after brief but intense exposure to elemental mercury (Praline et al. , 2007). Furthermore, in SOD1-G93A mice exposed to low concentrations of MeHg the onset of ALS-like phenotype is accelerated (Johnson et al. , 2011). In addition, thimerosal, that belongs to the class of organic mercurial and accumulates in fish, crustaceans, and throughout the food chain in humans (Yu and Pamphlett, 2017), exerts a neurotoxic effect by increasing the epigenetic enzyme histone deacetylase (HDAC)4 expression, that in turn activates an apoptotic pathway (Guida et al. , 2016). Another important target to investigate in the mechanism of neuronal death associated to ALS is the transcriptional factor Downstream Regulatory Element Antagonist Modulator (DREAM), that has been recently found to be up-regulated in SOD1 mice and ALS patients. DREAM is a transcriptional repressor belonging to the superfamily of the neuronal calcium sensors since it is directly regulated via calcium binding (Carrion et al. , 1999). DREAM represses genes involved in neuronal survival, such as $Na^{+}$-$Ca^{2+}$ exchanger isoform 3 (NCX3) (Formisano et al. , 2008), and c-fos, and in neuronal death, such Prodynorphin (PDYN) (Hauser et al. , 2001). On the other hand it has been reported that a neuroprotective effect in ALS can be exerted by Resveratrol (RSV) (3,5,4′-trihydroxystilbene), a well-known Sirtuin 1 (SIRT1) activator (Mancuso et al. , 2014), that **belongs** to the the Class III of HDACs (Conti et al. , 2017). **Here we investigated the particular vulnerability of SH-SY5Y cells stably expressing SOD1-G93A to non-toxic concentrations of MeHg and thimerosal. Furthermore, we identify the mechanism by which Resveratrol, by modulating DREAM and its target gene PDYN, blocks neurotoxicity induced by thimerosal exposure. In the end, we evaluated in rat cortical neurons transiently transfected with SOD1-G93A the effect of thimerosal, alone or in combination with RSV, on neuronal death and its correlation with the activation of SIRT1/DREAM/PDYN pathway.**

## 2 Materials and Methods

### 2.1 Drug and Chemicals

**The stock solutions and catalog numbers** of Methylmercury (II) chloride (MeHg), ethylmercurythiosalicylate (thimerosal), 3-pyridinylmethyl [[4-[[(2-aminophenyl)amino]carbonyl]phenyl]methyl]carbamate (MS275), 3-[5-(3-(3-Fluorophenyl)-3-oxopropen-1-yl)-1-methyl-1H-pyrrol-2-yl]-N-hydroxy-2-propenamide (MC1568), 3,5,4′-trihydroxy-trans-stilbene resveratrol (RSV) and Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132) were already published (Formisano et al., 2008, Guida et al. , 2015a, Guida et al. , 2015b, Guida et al., 2016). Culture media and sera were purchased from Invitrogen. All chemicals were diluted in cell culture medium. For those requiring dilution in dimethyl sulfoxide (DMSO), the final DMSO concentration was 0.1%. DMSO was added to the control cells (vehicle) at the same concentration as that used for treated cells and did not cause cellular toxicity. The primers used for PCR experiments were designed using the Primer Express 3.0 software (Applied Biosystems, Foster City, USA). The siRNAs against **(1)** rat DREAM (siDREAM; SI03061079), **(2)** rat PDYN (siPDYN; SI01959432), **(3)** human DREAM (siDREAM; SI03056354), **(4)** human PDYN

(siPDYN; SI04248216) and the negative control (siCTL; SI01685873) were purchased from Quiagen (Milan, Italy).

### 2.2 Cell Cultures

Human neuroblastoma SH-SY5Y cells and rat embryonic cortical neurons (DIV 7-9), were prepared and cultured as previously reported (Formisano et al. , 2013). **Primary cortical neurons were prepared from 17-day-old Wistar rat embryos (Charles River) and used after 7 days. Briefly: dissection and dissociation were performed in $Ca^{2+}$/$Mg^{2+}$-free PBS containing glucose (30 mM). Tissues were incubated with papain for 10 min at 37°C and dissociated by trituration in Earl's Balanced Salt Solution containing DNase (0.16 U/ml), BSA (10 mg/ml), and ovomucoid (10 mg/ml). Neurons, plated in plastic Petri dishes (Falcon Becton-Dickinson) precoated with poly-D-lysine (20 µg/ml), were grown in MEM/F12 containing glucose, deactivated FBS (5%), horse serum (5%), glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 µg/ml) (Invitrogen). Ara-C (10 µM) was added within 48 hrs of plating to prevent the growth of non-neuronal cells.** The experiments were performed according to the procedures described in experimental protocols approved by the Ethical Committee of the "Federico II" University of Naples. For MTT and LDH assays, cells were plated in 24 well plates at a density of $1x10^4$ cells/well; for qRT-PCR, they were plated in 60 mm plates at a density of $5x10^4$ cells/plate; for Western blot and immunoprecipitation, they were plated in 100 mm plates at $6x10^6$ cells/plate.

### 2.3 Cell transfection.

For stable transfection of SH-SY5Y cells, were used the following constructs: **(1)** pcDNA3.1, **(2)** pF151 pcDNA3.1(+)SOD1WT and **(3)** pF155 pcDNA3.1(+)SOD1-G93A, that were a gift from Elizabeth Fisher (Addgene plasmid # 26397 and # 26401) (Stevens et al. , 2010). The SOD1-G93A construct contains a mutation from GGT to GCT in the codon 93 of the cDNA resulting in the G93A mutation in the protein. These vectors contain a neomycin (G418) resistance gene. Before transfection, SH-SY5Y cells were seeded in six-well plates at a density of $3 \times 10^5$ per well. After 24 hours, cells were transfected with Lipofectamine LTX (15338–100, Invitrogen, Milan, Italy) according to the manufacturer's protocol for six hours with 2 µg of constructs and after that the culture medium was changed with medium containing G418 (100 µg/ml). Untransfected SH-SY5Y cells were used as positive control and died after few days in the selective medium. After 21 days of G418 selection, single clones were isolated and cultured in 24-well plates. Only one clone showed pcDNA3.1, SOD1WT and SOD1-G93A expression. Stable pcDNA3.1, SOD1WT and SOD1-G93A transfected SH-SY5Y cells were named **(1)** EV, **(2)** SOD1 and **(3)**SOD1-G93A. Cerebral cortical neurons (CCNs) were transiently transfected with pcDNA3.1 (Vector) and pcDNA3.1(+)SOD1-G93A constructs (CCNs-SOD1-G93A) in the following amounts: 1 µg for 24 well plates, 4 µg for 60 mm plates, and 8 µg for 100 mm plates, as previously reported (Guida et al. , 2017b). For transient cell transfection anti-sense (AS) and missense (MS) oligonucleotides (ODNs) against human SIRT1 were transfected into SOD1-G93A cells at a concentration of 1 µM as previously reported (Guida et al., 2015a). After ODN transfection, experiments with Thim alone or in combination with RSV were carried out. Briefly, SOD1-G93A cells were incubated for 24 hours with 0.1µM Thim alone or in combination with 1 µM RSV. DREAM and PDYN silencing in SOD1-G93A cells and in CCNs-SOD1-G93A was performed by small interfering RNA (siRNA) as previously reported at the concentration of 50 nM (Guida et al. , 2017a). **Transfection efficiency was almost 20-25%.**

### 2.4 Drug treatments

After 24 hours of cell seeding, EV, SOD1 and SOD1-G93A cells were treated with MeHg (0.1 μM) and Thim (0.01 μM) in RPMI medium containing 1% fetal bovine serum. To study the effects of Thim on neuronal survival, different concentrations of this toxicant were used in SOD1-G93A cells (0.001-0.5 μM) and in CCNs-SOD1-G93A (0.125-1 μM). To evaluate the effects of MS-275 and MC-1568, SOD1-G93A cells were seeded and pre-treated for 2 hours with these drugs at the concentrations of 0.05, 0.5, and 5 μM, respectively. After pre-treatments, cells were exposed to 0.1 μM Thim for 24 hours. Then, the effects of RSV in combination with Thim (0.1μM), were explored by seeding and treating cells for 24 hours with RSV (0.01-1μM) for SOD1-G93A cells, and with RSV (0.33-3μM) for CCNs-SOD1-G93A. For the experiments with MG-132, cells were pre-treated with the proteasome inhibitor MG-132 (at 10 μM) for two hours before thimerosal exposure.

### 2.5 Western Blot Analysis

**Western blot analysis was performed as described elsewhere (Formisano et al. , 2011). In particular, after treatment cells were homogenized in RIPA buffer (50 mM Tris-HCl pH 7.5, NaCl 150 mM, 1% Nonidet P40, 0.1% SDS, 0.5% Sodium deoxycholate) supplemented with PMSF 1 mM, DTT 0.5 mM and protease inhibitor mixture (Roche). Subsequently, samples were cleared by centrifugation, and the supernatants were used for Western blot analysis. Total protein was determined by Bradford's method. Samples (50 μg for DREAM, caspase 3 and 100 μg for SIRT1, SOD1) were loaded per track onto 8% (SIRT1) or 12% (DREAM, procaspase 3, caspase 3, and SOD1) SDS/PAGE gels. Subsequently, the proteins were transferred onto Hybond ECL nitrocellulose membrane (GE Healthcare, Milan, Italy) and blocked with 5% nonfat dry milk (Bio-Rad) in TBS (2mM Tris-HCl and 50 mM NaCl, pH 7.5) 0.1% Tween-20 for 2 hours at room temperature. Next, nitrocellulose membranes were immunoprobed with SOD1 (cod: PA5-27240, 1:1000, polyclonal rabbit antibody; Thermo Fischer Scientific), caspase 3 (Guida et al., 2015b), DREAM (cod: sc-9142, 1:1000, polyclonal rabbit antibody; Santa Cruz Biotechnology), SIRT1 (Guida et al., 2015a) at 4 °C overnight. After, secondary antibody incubations for 1 hour at room temperature. and immunoreactive bands were detected using enhanced chemiluminescence reagent (GE Healthcare). Finally, their optical density was determined using a Chemi-Doc Imaging System (Bio-Rad Laboratories, Hercules, CA). α-Tubulin (Guida et al., 2017a) was used to normalize.**

### Immunoprecipitation

**Immunoprecipitation was performed as previously described (Formisano et al., 2011). Briefly, SH-SY5Y-G93A cells were suspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 100 μM NaF, 1% TRITON, 10% glicerol with 100 μM Na3VO4 and protease inhibitors (1 mM PMSF, and protease inhibitor mixture) and incubated on ice for 20 min. Cell lysates were centrifuged at 13500 rpm for 20 min, after that 1 mg of lysates was incubated with the primary antibody for DREAM (cod: sc-9142 Santa Cruz Biotechnology) for 12 h at 4°C. The immunocomplexes were precipitated with Protein A/G Plus agarose beads (Santa Cruz Biotechnology) for 1 h with continuous mixing, washed three times with lysis buffer and eluted with sample buffer. Immunoprecipitates were resolved by SDS-PAGE gel and transferred to a nitrocellulose membrane. Immunoblot analysis was performed using**

**antibodies for SIRT1 (sc-15404, Santa Cruz Biotechnology) Ubiquitin, Acetylated Lysine (9441, Cell Signaling) and Tubulin as loading control (Guida et al., 2015a, Guida et al. , 2014).**

**2.6 Reverse-Transcription Real-Time PCR**.

**Total RNA isolation and quantitative real-time reverse-transcription PCR were performed as previously reported (Guida et al., 2017b). Total RNA was isolated from cells by use of the TRI-Reagent (Sigma-Aldrich, Milan, Italy), reacted with DNase-I (1U/µl; Sigma-Aldrich, Milan, Italy) for 15 min at room temperature, followed by spectrophotometric quantification. For reverse transcription, 2.0 µg of each RNA extract from each cell group was reverse transcribed by using random hexamers. Total cDNA was amplified by Real-Time PCR (7500 Fast; Applied Biosystems, Monza, Italy).** The primer pairs used for human and rat genes are represented in Table1. Samples were amplified simultaneously in triplicate in one assay run, and normalized for the reference genes Ribosomal Protein L19 (L19) and Hypoxanthine Phospho-Ribosyl-Transferase (HPRT) for human and rat cells (Guida et al., 2015a, Guida et al., 2014), respectively. Changes in mRNAs expression between groups were represented as the mean of the relative quantification (RQ) values, that was calculated as the difference in threshold cycle (ΔCt) between the target gene and the reference gene ($2^{-\Delta\Delta CT}$ =RQ).

**2.7 Lactate dehydrogenase (LDH) assay**

**LDH assay was performed as previously reported (Guida et al., 2017a). LDH levels in the extracellular medium were measured with an LDH assay kit (1000882 Cayman). Because LDH is normally present intracellularly in all cell types, its presence in the extracellular medium indicates a loss of neuronal plasma membrane integrity. The medium was removed and sampled for LDH content.** ~~LDH efflux into the medium was measured~~ SOD1-G93A cortical neurons were transfected with siCTL, siDREAM, siPDYN and, after 24 hours of transfection, exposed to thimerosal, alone or in combination with RSV for others 24 hours. Triton X-100 Sigma-Aldrich (St. Louis, MO, USA) was used as a positive control for cytotoxicity and its value was considered 100%. **LDH content was determined by measuring the absorbance at 490 nm with a spectrophotometer**.

**2.8 Determination of Cell Viability.**

**Cell viability was revealed as previously described (Formisano et al. , 2014)** by using the MTT (Sigma- Aldrich, Milan Italy) staining. Specifically, **after 24 hours of treatment with Thim in CCNs-SOD1-G93A (0.125-1 µM) and in SOD1-G93A cells (0.001-0.5 µM) the medium was removed and cells were incubated in 0.5 mg/ml MTT solution for 2 hours at 37°C. The incubation was stopped by adding 500 µl acidified isopropanol to solubilize the formazan salt, and viability was read by measuring the absorbance at 540 nm with a spectrophotometer.** ~~cells were incubated in 0.5 mg/ml MTT solution for 2 hours at 37°C, after treatment with Thim in CCNs-SOD1-G93A (0.125-1 µM) and in SOD1-G93A cells for 12 and 24 hours (0.001-0.5 µM), respectively.~~

**2.9 Statistical analysis**

**Data were expressed as mean ± standard error. Statistically significant differences between the experimental groups were evaluated by one-way ANOVA, followed by Bonferroni's**

**multiple comparison test using GraphPad Prism 5 software (GraphPad Software, Inc.). The two experimental groups were analyzed with the Student t test. Statistical significance was accepted at the 95% confidence level ($p < 0.05$).**

## 3 Results

### 3.1 Detection of human wild type and G93A mutant of SOD1 in SH-SY5Y cells.

SH-SY5Y cells were stably transfected with: (1) empty vector (EV), (2) human construct of SOD1 wild type (SOD1), and (3) human construct of SOD1 containing G93A mutant (SOD1-G93A), independently. By Western Blot analysis we found that SOD1 protein expression was increased in SOD1 and SOD1-G93A groups compared to EV group (Fig. 1A). Interestingly, after 24 hours seeding cell viability was significantly reduced in SOD1-G93A cells, compared to SOD1 and EV groups (Fig. 1B). These results indicate that exogenic human SOD1 protein was stably expressed in SH-SY5Y cells and that SOD1-G93A cells was more damaged compared to SOD1.

### 3.2 The increased neurotoxicity of thimerosal in SOD1-G93A cells is inhibited by Resveratrol.

EV, SOD1 and SOD1-G93A cells were incubated for 24 hours with non-toxic concentrations of **MeHg (0.1 μM) (Guida et al., 2015b)** and thimerosal (Thim) (0.01 μM) (Guida et al., 2016) (Figs. 2A-C). Notably, Thim (Figs. 2A-C), but not **MeHg (Supplementary Fig. 1A-C),** induced a significant decrease in cell viability in SOD1-G93A group, but not in SOD1 and EV cells. **Importantly, we found that SOD1-G93A SH-SY5Y treated for 24 hours with thimerosal at 0.001–0.5 μM showed a reduction in cell survival in a concentration-dependent manner (Fig. 2D), whereas MeHg significantly reduced cell viability only at 0.5 μM in the same cells (Supplementary Fig. 1D). These experiments indicate that MeHg concentration (0.5 μM) able to induce a significant decrease in cell viability in SOD1-G93A group is the same that occurred in wild-type cells (Guida et al., 2015b), while SOD1-G93A group were more susceptible to Thim non toxic concentration (Guida et al., 2016).** Because 0.1 μM Thim reduced cell survival of 50%, this concentration was adopted for subsequent experiments. To identify the role of distinct classes of HDACs involved in Thim-induced toxicity, SOD1-G93A cells were treated with **HDACs inhibitors:** MS-275 (0.05–5μM) and MC-1568 (0.05–5μM), that are class I and II HDACs **selective inhibitors**, respectively (Formisano et al. , 2015a), and with Resveratrol (RSV) (0.01–1μM), that is an activator of SIRT1, that belongs to class III of HDACs (Guida et al., 2015a). MTT experiments revealed that any amelioration was evaluated after treatment with MS-275 and MC-1568 (Figs. 2E, F), but cell viability reduction was significantly counteracted in SOD1-G93A group treated with RSV at the concentration of 1 μM (Fig. 2G). Interestingly, RSV reduced also Thim-activated cleavage of caspase-3 (Fig. 2H), **whereas in SOD1-G93A group caspase-3 was not cleaved compared to EV group (Supplementary Fig. 1E) indicating that reduction in cell viability, evaluated with the MTT assay (Fig. 1B), is not associated to caspase activation.**

### 3.3 Resveratrol diminished Thim-induced cell death by blocking DREAM protein down-regulation in SOD1-G93A cells.

To identify the role of transcription factor DREAM in Thim-reduced cell viability we evaluated its mRNA and protein expression. As shown in Fig. 3A, 12 and 24 hours of Thim (0.1 μM) did not modify DREAM mRNA expression, whereas its protein level was significantly reduced, compared

to the vehicle (Fig. 3B). Next, to study the role of DREAM in Thim-induced toxicity, its expression was reduced through siDREAM transfection by approximately 40% (Fig. 3C). It is noteworthy that siDREAM potentiated the detrimental effect of Thim, compared to siCTL (Fig. 3D). Furthermore, at 24 hours Thim-decreased DREAM protein expression was blocked by RSV (1 μM) treatment (Fig. 3E). Interestingly, Western Blot analysis showed that Thim, alone or in combination with RSV, did not modify SIRT1 protein expression (Fig. 3F), whereas SIRT1 knocking-down obtained by antisense (AS) SIRT1 transfection (Guida et al., 2015a), completely abolished RSV-induced neuroprotection in SOD1-G93A cells treated with Thim (Fig. 3G). These experiments indicate that Thim modulates SIRT1 activity, but not its expression.

**3.4 Resveratrol reduces Thim-induced DREAM ubiquitination by increasing its deacetylation, that in turn regulates Prodynorphin gene expression in SOD1-G93A cells.**

By immunoprecipitation experiments, we found that SIRT1 was bound in the DREAM immunocomplex and that its binding was not modified when cells were exposed to Thim, alone or in combination with RSV (Fig. 4A). In contrast, Immunoprecipitation for DREAM, followed by western blot with an antibody against acetylated lysine (K Ac.) or ubiquitin, (Ub) showed that DREAM was acetylated and ubiquitinated after 24 hours exposure with 0.1μM Thim (Figs. 4B,C). Thim-induced DREAM acetylation and ubiquitination was significantly reduced when cells were treated with RSV (Figs. 4B,C). These results point out that Thim induced a DREAM acetylation and consequently its reduction via the ubiquitin system. In fact, SOD1-G93A cells treated with the inhibitor of proteasome MG-132 (10μM) prevented DREAM protein down-regulation induced by 24 hours exposure with Thim (Fig. 4D). Intriguingly, transfection of AS SIRT1 significantly reduced RSV effect to block Thim-induced DREAM protein reduction (Fig. 4E). Furthermore, to confirm the functional consequence of DREAM protein reduction induced by Thim, alone or in combination with RSV (1μM), qRT-PCR analysis for PDYN (Hauser et al., 2001) a well-known DREAM target gene was performed. As shown in Figure 5A, RSV significantly reverted Thim-induced PDYN mRNA increase. Particularly, siPDYN, that reduces PDYN mRNA of 78% (Fig. 5B), reduced the neurotoxic effect of Thim, whereas siCTL did not (Fig. 5C).

**3.5 Thim exposure reduced cell survival by activation of SIRT1/DREAM /PDYN pathway in cortical neurons transiently transfected with SOD1-G93A construct.**

**Rat cortical neurons were transiently transfected with human constructs of SOD1, SOD1 containing G93A mutant (SOD1-G93A) and the empty vector (EV), separately. By qRT-PCR we found that after 24 hours of transfection human SOD1 mRNA was expressed in neurons transfected with SOD1 (Supplementary Fig. 2A) and SOD1-G93A (Fig. 6A), but not in those transfected with the EV. Interestingly, 24 hours after transfection cell viability was significantly reduced in CCNs-SOD1-G93A, but not in CCNs-SOD1 and CCNs-EV (Fig. 6B and Supplementary Fig. 2B). Notably, by MTT assay we found that CCNs-SOD1-G93A exposed to Thim (0.125–1 μM/24h) showed reduction in neuronal survival in a concentration-dependent way (Fig. 6C), whereas MeHg significantly reduced cell viability only at 1 μM (Supplementary Fig. 2C). Intriguingly, in CCNs-SOD1 MeHg showed a reduction in cell viability at the same concentration (1μM) occurred in EV cells (Supplementary Fig. 2C). These results are in accordance with those obtained in the SH-SY5Y cells stably transfected with SOD1-G93A construct. Since 0.5 μM Thim determined almost 50% of cell death, this concentration was used for the followed experiments.** Importantly, Thim at 0.5 μM for 24 hours is reported not toxic in neurons that did not express SOD1-G93A construct (Guida et al., 2016). Furthermore, MTT assays demonstrated that in CCNs-SOD1-G93A exposed to Thim neuronal death was significantly reduced in a concentration dependent manner when cells were treated also with RSV (0.33-3 μM), compared to cells exposed to Thim alone (Fig. 6D). In addition, in CCNs-SOD1-G93A, Thim-induced decrease in DREAM protein and increase in PDYN mRNA was

reverted if the cells were co-treated with RSV (3μM) (Figs. 6F and G). Interestingly, as **observed** in SOD1-G93A cells Sirt1 protein levels were unmodified by treatment with Thim, alone or in combination with RSV (Fig. 6E). Then, to study the role of DREAM and PDYN in Thim-reduced neuronal survival, we diminished their expressions by silencing strategy. Specifically, siRNA for DREAM down-regulated its protein expression of almost 50% (Fig. 6H), whereas siPDYN reduced its mRNA level of 38% (Fig. 6I). As shown in Fig. 6L, siDREAM transfection worsened thim-reduced cell survival, whereas siPDYN and RSV treatment were able to counteract Thim-induced cell death. Furthermore, we evaluated LDH efflux, that is a well-known marker of cell death (Formisano et al. , 2015b), in the same experimental conditions. Interestingly, **the effect of Thim to increase LDH release** was reverted by siPDYN and RSV, and incremented by siDREAM transfection (Fig. 6M).

## 4 Discussion

The aim of this study was to validate the hypothesis that the exposure at non-toxic concentrations of Thim could induce neuronal death in an *in vitro* model of ALS. We found that SH-SY5Y neuroblastoma cells transfected with the G93A mutant of SOD1 (SOD1-G93A) are more vulnerable to the neurotoxicant thimerosal compared to cells overexpressing the wild type SOD1 gene (SOD1). In regard of the possible mechanism involved in the neurotoxic effect of Thim, it should be underlined that Thim exposure caused an increase of PDYN, a well-known DREAM target gene and that its knocking-down by siRNA reduced the toxic effect of Thim, thus suggesting that PDYN and DREAM can be involved, as confirmed also by the reduction of the expression of DREAM protein in our experimental condition. This hypothesis is reinforced by the findings showing that the knocking-down of DREAM increased the neurotoxic effect of Thim. **To our knowledge, this is the first evidence demonstrating DREAM role in the neurotoxic effect of Thim and its correlation with ALS physiopathology**. Specifically, our results indicate that in these experimental conditions DREAM is neuroprotective and is in accordance with a recent paper demonstrating that in motoneurons of ALS patients DREAM is involved in ALS physiopathology (Larrode et al. , 2018). Furthermore, we found a potential mechanism by which DREAM protein expression was down-regulated by Thim in SOD1-G93A cells. Thim-induced neuronal death and the decrease in DREAM protein expression were counteracted by the SIRT1 activator RSV, indicating the involvement of this enzyme in Thim-reduced DREAM expression. Notably, DREAM decrease is a consequence of its acetylation followed by its ubiquitination, a regulation in which SIRT1 appears to be involved. Indeed, RSV reduced DREAM protein acetylation and consequently blocked its ubiquitination. Moreover, DREAM decrease was counteracted by both RSV and proteasome inhibitor MG-132. The role of SIRT1 to regulate neuronal survival through DREAM is also confirmed by the effect of RSV, being able to significantly reduce Thim-induced cell death in SOD1-G93A SH-SY5Y and CCNs-SOD1-G93A. Interestingly, SIRT1 knock-down worsened the neurodetrimental effect of Thim. Our results are in line with earlier studies proving that SIRT1 reduces proteasome-mediated N-Myc protein degradation and therefore stabilizes N-Myc protein (Marshall et al. , 2011). Notably, the mechanism by which DREAM is deacetylated involves specifically SIRT1, since its knocking down by siRNA transfection blocked RSV-induced DREAM increase. Furthermore, RSV reverted the effect of Thim to increase the mRNA level of the DREAM target gene PDYN. Interestingly, we found that only the siRNA against PDYN significantly reverted Thim-induced neuronal death in both SOD1-G93A cell types. Since SIRT1 plays an important role in this mechanism, it could be postulated that RSV by blocking Thim-induced DREAM reduction increased its binding on PDYN gene promoter reverting its mRNA up-regulation. Several papers reported that RSV *in vitro* is protective against enviromental and non-enviromental neurotoxicants such as PCB (Guida et al., 2015a), MeHg (Yuntao et al. , 2016) and methamphetamine (Kanthasamy et al. , 2011) and improves motor function and survival in ALS mice (Mancuso et al., 2014). **Regarding the use of**

**cortical neurons to study the effect of Thim in the physiopathology of a motoneuronal disease such as ALS, it has been demonstrated that neurodegenerative processes occur both at motor neurons and cortical neurons level (Geevasinga et al. , 2016). Interestingly,** Thim concentrations able to reduce neuronal survival approximately by 50% are 0.1 μM for SOD1-G93A SH-SY5Y cells and 0.5 μM for CCNs- SOD1-G93A, whereas RSV counteracted Thim toxic effect at 1 μM in SOD1-G93A SH-SY5Y cells and 3 μM in CCNs-SOD1-G93A. These results indicate that CCNs-SOD1-G93A are less vulnerable to Thim neurotoxicity compared to the SOD1-G93A SH-SY5Y cells. **The greater susceptibility of SOD1-G93A SH-SY5Y cells, compared to CCNs-SOD1-G93A, to thimerosal toxicity could be due to the fact that the treatment in SH-SY5Y is performed in low serum culture medium.** Moreover, we demonstrated that RSV reduced Thim-induced neurotoxicity through the histone deacetylase SIRT1, since its knocking-down hampered RSV neuroprotective effects. Indeed, SIRT1 ablation in neurons subjected to an *in vitro* model of brain ischemia (Wang et al. , 2013) or exposed to the neurotoxicant PCBs (Guida et al., 2015a) completely eliminate the neuroprotective effects of RSV. Furthermore, our data are in accordance with the neurotoxic role of PDYN. Indeed, intrathecal injection of PDYN in rats determines reduction of cell bodies associated with loss of motor activity (Long et al. , 1988) and reduced neuronal survival in primary neuronal culture (Hauser et al., 2001). **Regarding Thim concentration ranges tested in the present study, they were already used by other authors in a human neuroblastoma cell line (Herdman et al. , 2006) and in cultured human neurons (Baskin et al. , 2003), where it has been demonstrated that Thim induces DNA breaks, caspase-3 activation, membrane damage and apoptotic cell death. Interestingly, it has been reported that after normal full series of vaccinations containing thimerosal, the infant receives up to 403 μg of thimerosal (equivalent to 200 μg of mercury), that results in administration in a range of 32-52 μg/Kg of mercury depending on sex, weight and age of the infants (Baskin et al., 2003). The concentration ranges that in our study induced cell death in SOD1-G93A SH-SY5Y cells and SOD1-G93A cortical neurons were: 01-0.1 and 0.25-0.5 μM, corresponding respectively to 4.05-40.5 μg/L and 101,25-202.5 μg/L. These concentrations expressed in terms of Hg were: 2.025-20.25 μg/L and 50.625-101.25 μg/L respectively. In our opinion the concentration ranges used in this paper are comparable to those used after normal full series of vaccinations containing thimerosal**. In conclusion, our results demonstrate that neuronal cells expressing human SOD1 carrying G93A mutation are more susceptible to Thim toxicity, through SIRT1/DREAM/PDYN pathway, and indicate a possible role **of this neurotoxicant in the worsening of ALS in genetically vulnerable organisms.**

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**Conflict of interest statement**

There is no potential conflict of interest or competing interest.

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**Figure Legends**

**Fig.1 SOD1 protein expression and cell viability in SH-SY5Y stably expressing the empty vector, SOD1 and SOD1-G93A constructs. (A)** Western Blot of SOD1 in SH-SY5Y stably transfected with the followed constructs: (1) empty vector (EV), (2) wild type human SOD1 (SOD1) and (3) SOD1 containing the G93A mutation (SOD1-G93A). Graphs show quantification of ratio of SOD1 and Tubulin. Bars represent mean ± S.E.M. n = 3 per group. *p≤ 0.05 versus EV. **(B)** Effects of EV, SOD1 and SOD1-G93A constructs on cell survival, measured as MTT assay after 24 hours seeding. Bars represent mean ± S.E.M. n = 4 per group. *p≤ 0.05 versus EV and SOD1.

**Fig.2 Effect of non-toxic concentrations of Thim, alone or in combination with HDACs inibitors, on SOD1-G93A cell survival. (A-C)** Consequence of 24 hours of Thim (0.01μM) exposure on mitochondrial activity in: **(A)** EV, **(B)** SOD1 cells and **(C)** SOD1-G93A cells. Bars represent mean ± S.E.M. n = 4 per group. *p≤ 0.05 versus vehicle (Veh). **(D)** Effect of 24 hours of Thim (0.001, 0.01, 0.1 and 0.5μM) on cell survival, evaluated by MTT assay, in SOD1-G93A cells. Bars represent mean ± S.E.M. n = 4 per group. *p≤ 0.05 versus Veh and Thim 0.001, #p≤ 0.05 versus Veh, Thim 0.001 and 0.01, §p≤ 0.05 versus all. **(E-G)** Effect of 0.1 μM Thim at 24 hours, alone or with 0.05, 0.5, and 5 μM of **(E)** MS-275, or **(F)** MC-1568, or **(G)** 0.01, 0.1, and 1 μM of Resveratrol (RSV) on cell viability in SOD1-G93A cells. Bars represent mean ± S.E.M. n = 4 per group. *p≤ 0.05 versus Veh.,^p≤ 0.05 versus Thim 0.1 μM. **(H)** Western blot of pro-caspase-3, cleaved caspase-3 in SOD1-G93A cells treated with Thim, alone or in combination with RSV (1 μM). Graph shows the quantification of the ratio of cleaved caspase-3 to Tubulin. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus Thim (0.1μM/24h)+vehicle.

**Fig.3 Effect of Thim on DREAM mRNA and protein expression and its role through SIRT1 to regulate Thim-reduced mitochondrial activity, in SOD1-G93A cells. (A, B)** qRT-PCR and Western blot of DREAM in SOD1-G93A cells treated with Thim (0.1μM) for 24 and 48 hours. Graphs show the quantification of the ratio of DREAM to L19 for qRT-PCR and to Tubulin for western blot experiments. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus Veh, §p≤ 0.05 versus all. **(C)** Western blot of DREAM in SOD1-G93A cells transfected with siCTL or with siDREAM. Graphs show the quantification of the ratio of DREAM to Tubulin. Bars represent mean ± S.E.M. n = 3 per group. *p≤ 0.05 versus siCTL. **(D)** Effect of 24 hours of Thim (0.1 μM) exposure on mitochondrial activity in SOD1-G93A cells transfected with siDREAM. Bars represent mean ± S.E.M. n = 4 per group. *p≤ 0.05 versus Veh and siCTL,^p≤ 0.05 versus Thim alone. **(E,F)** Western blot of DREAM and SIRT1in SOD1-G93A cells treated with Thim alone (0.1 μM) or in combination with RSV (1 μM). Graphs show the quantification of the ratio of DREAM or SIRT1 to Tubulin. Bars represent mean ± S.E.M. n = 3 per group. *p≤ 0.05 versus Veh, ^p≤ 0.05 versus Thim alone. **(G)** Effects of 24 hours exposure to Thim 0.1 μM on SOD1-G93A cell survival, as measured by MTT assay, in the following experimental conditions: (1) Veh, (2) MS SIRT1, (3)Thim 0.1 μM, (4) AS SIRT1+Thim 0.1 μM, (5) RSV+Thim 0.1 μM, (6) AS SIRT1+RSV+Thim0.1 μM. Bars represent mean ± S.E.M. n = 4 per group. *p≤ 0.05 versus Veh and MS SIRT1, ^p≤ 0.05 versus Thim alone and #p≤ 0.05 versus RSV 1 μM +Thim0.1 μM.

**Fig.4 Thim-induced DREAM protein reduction in SOD1-G93A cells is determined by its acetylation and ubiquitination. (A-C)** Representative Western blot showing immunoprecipitation (IP) between DREAM and: **(A)** SIRT1, **(B)** acetyl-lysine (K-Ac), **(C)** Ubiquitin (Ub.), in cells exposed to Thim (0.1 µM/24h), alone or in combination with RSV (1 µM). The inputs were aliquots of the extracts before IP. Pre-immune IgG (PI) was used as negative control. **(D)** Western blot of DREAM in SOD1-G93A cells treated with Vehicle and Thim (0.1µM/24h), alone or after 2 hours of pre-treatment with MG-132 (10 µM). Graph shows the quantification of the ratio of DREAM to Tubulin. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus Veh, ^p ≤ 0.05 versus Thim. **(E)** Western blot of DREAM in SOD1-G93A cells in the following conditions: (1) Thim (0.1µM/24h), (2) Thim + MS SIRT1 + RSV (1µM/24h), (3) Thim + AS SIRT1 + RSV (1µM/24h). Graph shows the quantification of the ratio of DREAM to Tubulin. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus Thim, #p ≤ 0.05 versus Thim + MS SIRT1 + RSV (1µM/24h).

**Fig.5 Effect of Thim on PDYN mRNA expression and role of PDYN to determine Thim-reduced neuronal survival, in SOD1-G93A cells. (A)** qRT-PCR of PDYN in SOD1-G93A cells treated with vehicle, Thim (0.1 µM/24h), alone or in combination with RSV (1 µM). Graphs show the quantification of the ratio of PDYN to L19. Bars represent mean ± S.E.M. n = 3-5 per group. *p ≤ 0.05 versus Veh, ^p ≤ 0.05 versus Thim. **(B)** qRT-PCR of PDYN in SOD1-G93A cells transfected with siCTL or with siPDYN. Bars represent mean ± S.E.M. Graphs show the quantification of the ratio of DREAM to L19. n = 3 per group. *p≤ 0.05 versus siCTL. **(C)** Effect of 24 hours of Thim (0.1 µM) exposure on cell viability in SOD1-G93A cells transfected with siCTL and siPDYN. Bars represent mean ± S.E.M. n = 3 per group. *p≤ 0.05 versus vehicle and ^p≤ 0.05 versus Thim, alone or with siCTL.

**Fig.6 Effect of non toxic Thim concentration, alone or in combination with RSV, on cell survival , in CCNs-SOD1-G93A. (A)** qRT-PCR of human SOD1-G93A (hSOD1) in neurons: untransfected (CTL), or transiently transfected with EV and SOD1-G93A constructs. Graph shows the quantification of the ratio of human SOD1 to rat HPRT. Bars represent mean ± S.E.M. n = 4-5 per group. *p≤ 0.05 versus CTL and EV**. (B)** Effects of EV, and SOD1-G93A overexpression on cell survival, measured as MTT assay after 24hrs of transfection. Bars represent mean ± S.E.M. n = 3 per group. *p≤ 0.05 versus CTL and EV. **(C)** Effect of 24 hours of Thim (0.125, 0.25, 0.5 and 1µM) on cell survival, evaluated by MTT assay in CCNs-SOD1-G93A. Bars represent mean ± S.E.M. n = 4 per group. *p≤ 0.05 versus Veh and Thim 0.125 µM/24h, #p≤ 0.05 versus Veh, Thim 0.125 and 0.25µM/24h, §p≤ 0.05 versus all. **(D)** Effect of 0.5 µM Thim at 24 hours, alone or with 0.33, 0.75, 1.5 and 3 µM of RSV on cell viability in CCNs-SOD1-G93A. Bars represent mean ± S.E.M. n = 4 per group. *p≤ 0.05 versus Thim, Thim+RSV 0.33 µM and Thim+RSV 0.75 µM; §p≤ 0.05 versus all. **(E-G)** Western blots of SIRT1 and DREAM and qRT-PCR of PDYN in CCNs-SOD1-G93A: (1) un-treated (CTL), exposed for 24 hours to (2) vehicle (Veh), or Thim (0.5 µM) (3) alone or (4) in combination with RSV (3 µM). Graphs show the quantification of the ratio of SIRT1 and DREAM to Tubulin for western blot experiments and PDYN to HPRT for qRT-PCR. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus CTL and Veh, ^p ≤ 0.05 versus Thim. **(H,I)** Western blot of DREAM and qRT-PCR of PDYN gene in neurons transfected with siCTL or with **(H)** siDREAM and **(I)** siPDYN. Graphs show the quantification of the ratio of DREAM to Tubulin and PDYN to HPRT. n = 3 per group. *p≤ 0.05 versus siCTL. **(J, K)** Neuronal survival evaluated by MTT and LDH assays, in CCNs-SOD1-G93A in the following conditions: (1) exposed to Veh, (2) transfected with siCTL, treated with Thim (3) alone, or after transfection with:

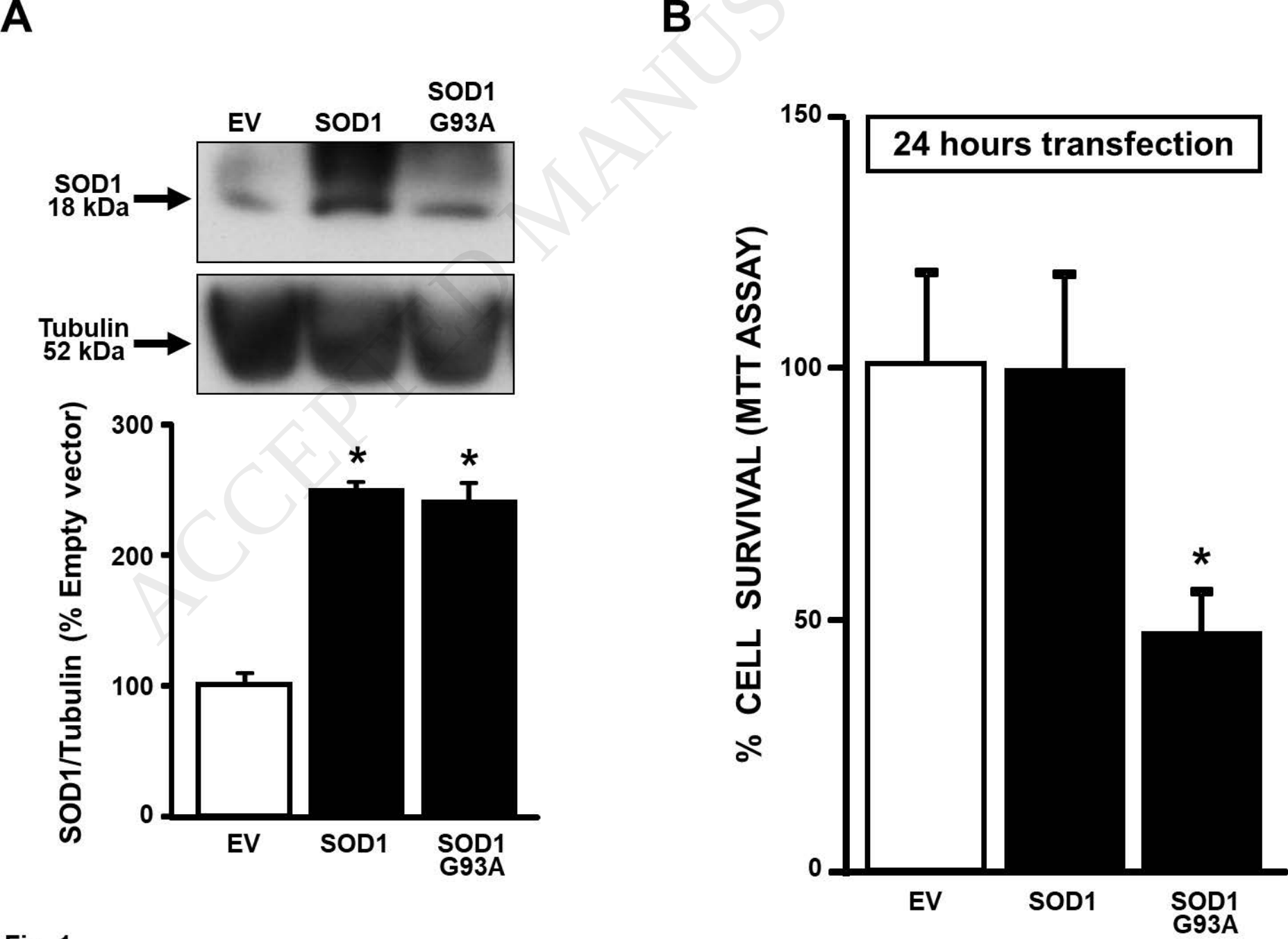


Fig. 1

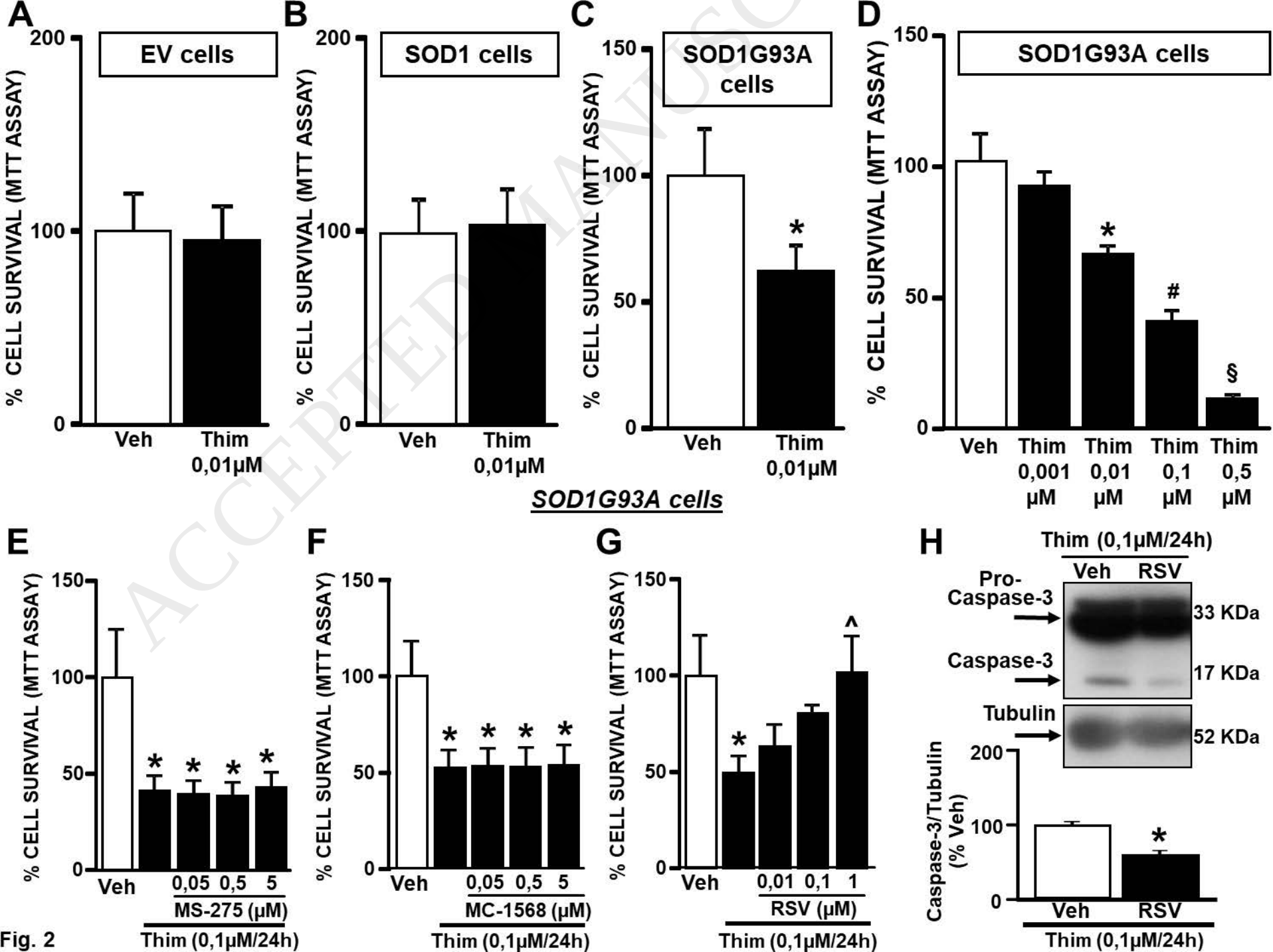


Fig. 2

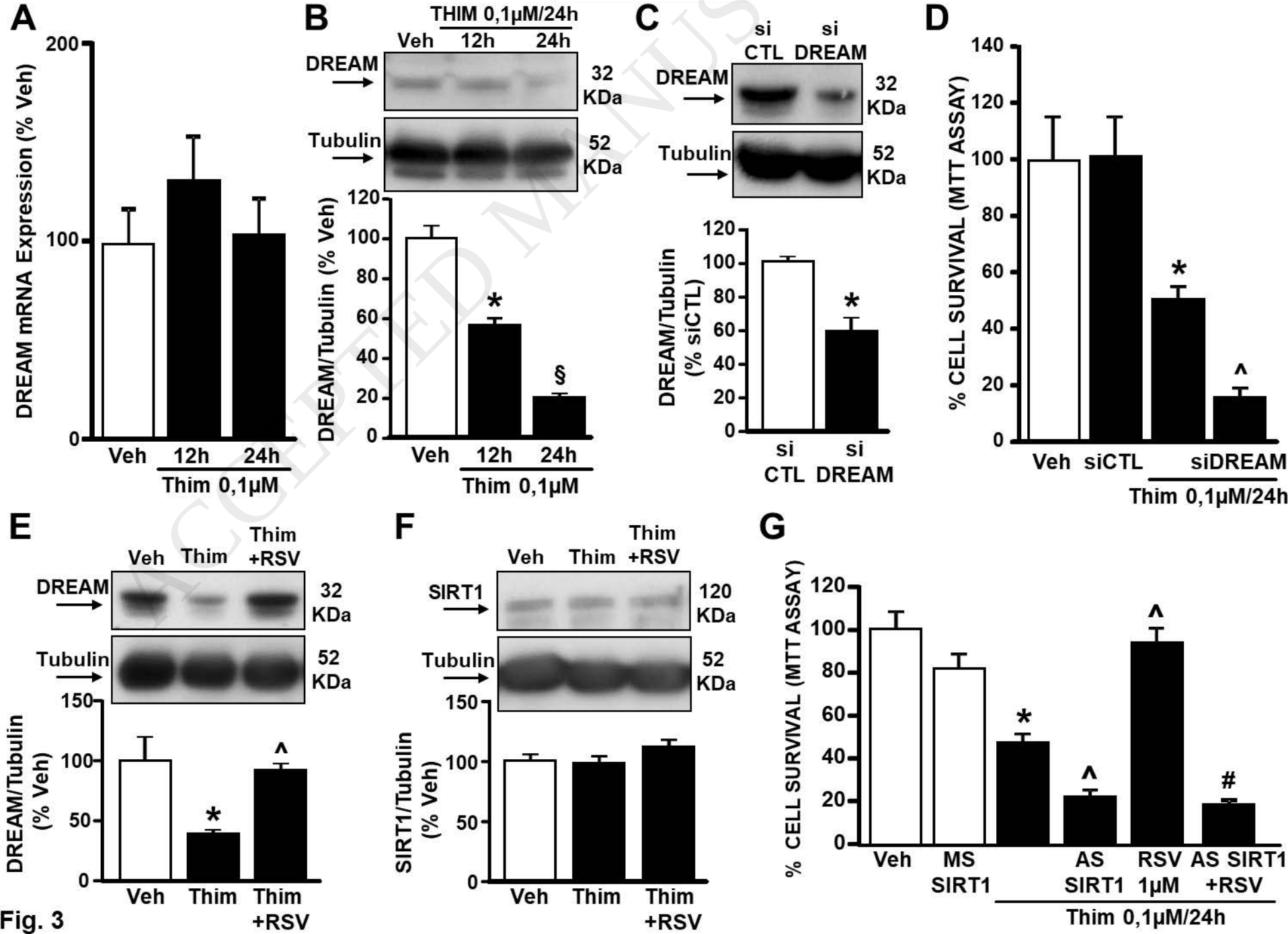


Fig. 3

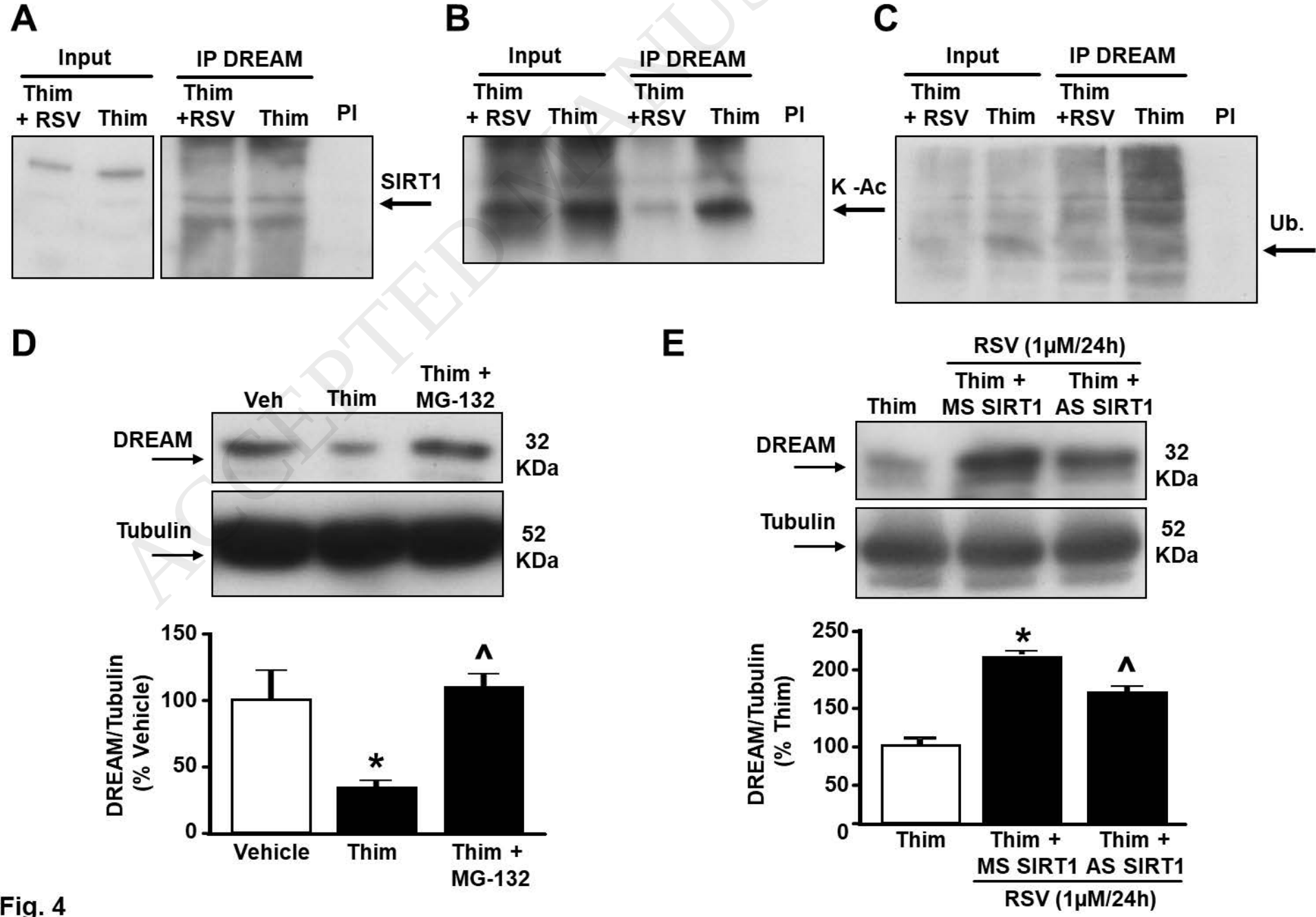


Fig. 4

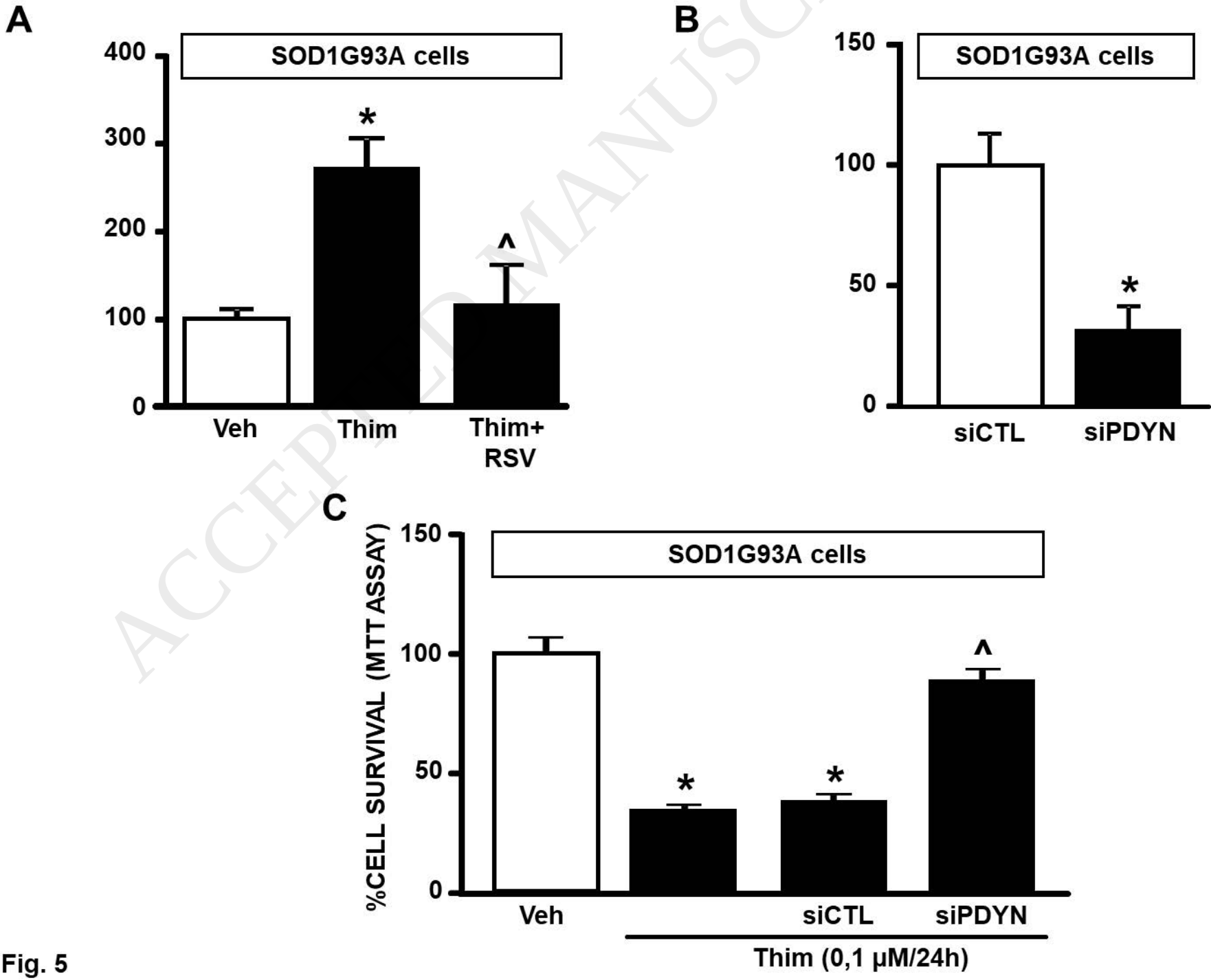


Fig. 5

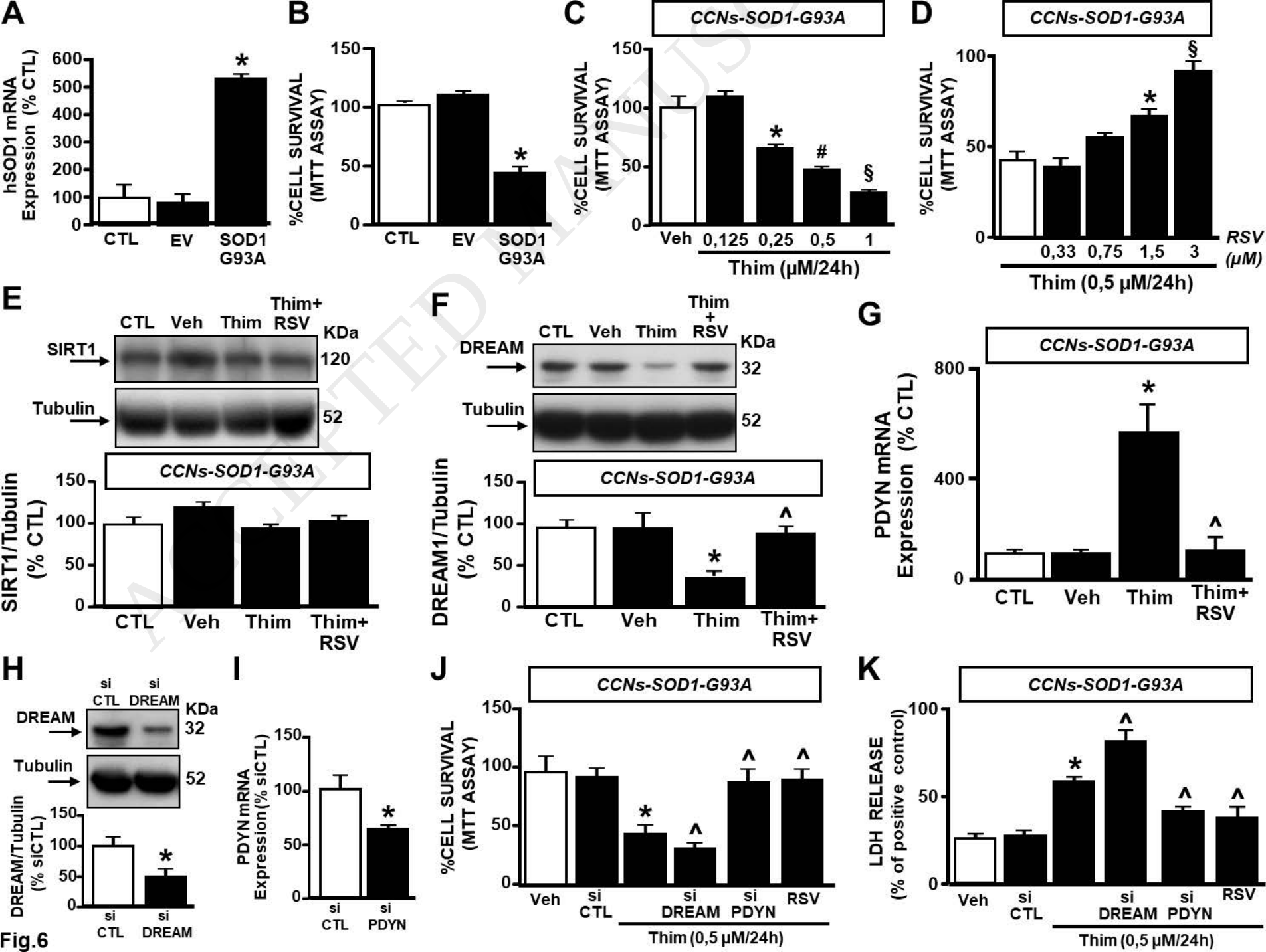
A
hSOD1 mRNA Expression (% CTL)
CTL
EV
SOD1 G93A
B
%CELL SURVIVAL (MTT ASSAY)
C
CCNs-SOD1-G93A
%CELL SURVIVAL (MTT ASSAY)
Veh
0,125
0,25
0,5
1
Thim (µM/24h)
D
CCNs-SOD1-G93A
%CELL SURVIVAL (MTT ASSAY)
0,33
0,75
1,5
3
RSV (µM)
Thim (0,5 µM/24h)
E
CTL
Veh
Thim
Thim+ RSV
KDa
SIRT1
120
Tubulin
52
CCNs-SOD1-G93A
SIRT1/Tubulin (% CTL)
F
DREAM
32
DREAM1/Tubulin (% CTL)
G
CCNs-SOD1-G93A
PDYN mRNA Expression (% CTL)
H
si CTL
si DREAM
DREAM/Tubulin (% siCTL)
I
PDYN mRNA Expression (% siCTL)
si PDYN
J
CCNs-SOD1-G93A
%CELL SURVIVAL (MTT ASSAY)
si DREAM
si PDYN
RSV
Thim (0,5 µM/24h)
K
CCNs-SOD1-G93A
LDH RELEASE (% of positive control)

Fig.6

(4) siDREAM, (5) siPDYN, or (6) co-treated with RSV (3 μM). Bars represent mean ± S.E.M. n = 4 per group. *p≤ 0.05 versus Veh and siCTL, ^p ≤ 0.05 versus Thim alone.

| Gene | GenBank | Primers | PCR product |
|---|---|---|---|
| **human** | XR_922912. | 5′-CAGTCTCTCTACAGGGGCTTT-3′<br>5′-TTTGAAGGTGTCTTCGTCCAC- | **65** |
| **human** | NM_024411. | 5′-CTTGCGGCGCATTCG-3′<br>5′-CGCCATAGCGCTTCTGGTT-3′ | **52** |
| **human** | NM_000454. | 5′-CAGGGCATCATCAATTTCGA-3′<br>5′-TGCTTCCCCACACCTCAC-3′ | **60** |
| **rat** | NM_024411. | 5′-GGGCTCCATGGCAAGGAT-3′<br>5′-AGTTCCGTGTAGCCTTCTTCCA-3′ | **52** |