

# SIRT3-SOD2-mROS-dependent autophagy in cadmium-induced hepatotoxicity and salvage by melatonin

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**Keywords:** autophagy, cadmium, hepatotoxicity, melatonin, mitochondrial ROS, SIRT3, SOD2

**Abbreviations:** ACTB, actin;  $\beta$ , Cd, cadmium; CdCl<sub>2</sub>, cadmium chloride; Baf A1, bafilomycin A<sub>1</sub>; GPT/ALT, glutamic-pyruvate transaminase (alanine aminotransferase); H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; LC3, microtubule-associated protein 1 light chain 3; mel, melatonin; mROS, mitochondrial reactive oxygen species; O<sub>2</sub><sup>•-</sup>, superoxide anion; SIRT1, sirtuin 1; SIRT3, sirtuin 3; SOD2, superoxide dismutase 2, mitochondrial; SQSTM1/p62, sequestosome 1; tF-LC3, tandem fluorescent mRFP-GFP-LC3B; 3-MA, 3-methyladenine; 3-TYP, 3-(1H-1,2,3-triazol-4-yl)pyridine.

Cadmium is one of the most toxic metal compounds found in the environment. It is well established that Cd induces hepatotoxicity in humans and multiple animal models. Melatonin, a major secretory product of the pineal gland, has been reported to protect against Cd-induced hepatotoxicity. However, the mechanism behind this protection remains to be elucidated. We exposed HepG2 cells to different concentrations of cadmium chloride (2.5, 5, and 10  $\mu$ M) for 12 h. We found that Cd induced mitochondrial-derived superoxide anion-dependent autophagic cell death. Specifically, Cd decreased SIRT3 protein expression and activity and promoted the acetylation of SOD2, superoxide dismutase 2, mitochondrial, thus decreasing its activity, a key enzyme involved in mitochondrial ROS production, although Cd did not disrupt the interaction between SIRT3 and SOD2. These effects were ameliorated by overexpression of SIRT3. However, a catalytic mutant of SIRT3 (SIRT3<sup>H248Y</sup>) lacking deacetylase activity lost the capacity to suppress Cd-induced autophagy. Notably, melatonin treatment enhanced the activity but not the expression of SIRT3, decreased the acetylation of SOD2, inhibited mitochondrial-derived O<sub>2</sub><sup>•-</sup> production and suppressed the autophagy induced by 10  $\mu$ M Cd. Moreover, 3-(1H-1,2,3-triazol-4-yl)pyridine, a confirmed selective SIRT3 inhibitor, blocked the melatonin-mediated suppression of autophagy by inhibiting SIRT3-SOD2 signaling. Importantly, melatonin suppressed Cd-induced autophagic cell death by enhancing SIRT3 activity in vivo. These results suggest that melatonin exerts a hepatoprotective effect on mitochondrial-derived O<sub>2</sub><sup>•-</sup>-stimulated autophagic cell death that is dependent on the SIRT3/SOD2 pathway.

## Introduction

Cadmium (Cd), a heavy metal, is an environmental and industrial pollutant with high cytotoxicity.<sup>1,2</sup> Atmospheric contamination by the cadmium mining industry and cigarette smoke are the main sources of cadmium exposure to humans.<sup>3,4</sup> The increasing Cd concentration in the environment is cause for great concern, especially in China.<sup>5,6</sup> The liver is an important organ for processing of absorbed nutrients and for detoxification, and it is a main target for Cd.<sup>7</sup> Autophagy has been proposed to play a pivotal role in Cd-mediated hepatotoxicity. Previously, we

demonstrated that excess autophagy may be the primary contributing factor underlying mitochondrial loss, cellular energy depletion, and cell death in Cd-induced hepatotoxicity.<sup>8</sup> These findings highlight the role of autophagy in Cd-induced hepatotoxicity and suggest that the modulation of this process is a viable and novel therapeutic strategy.

The molecular mechanism underlying autophagy has been extensively investigated in recent years. These studies revealed that mitochondrial reactive oxygen species (mROS) generation can induce autophagy. Alterations in mROS and autophagy regulation contribute to many diseases such as cancer,

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atherosclerosis, and neurological disorders.<sup>9</sup> SIRT3 (sirtuin 3) is the primary mitochondrial acetyl-lysine deacetylase that modulates various proteins to control mitochondrial function and mROS generation.<sup>10</sup> SIRT3 primarily regulates mROS clearance by altering the acetylation of SOD2 (superoxide dismutase 2, mitochondrial). More importantly, SIRT3 directly binds and deacetylates SOD2, which increases SOD2 activity and leads to a significant effect on mROS homeostasis and autophagic flux.<sup>11,12</sup>

Melatonin is the major secretory product of the pineal gland. It and its metabolites are best known for their free radical scavenging and antioxidative effects.<sup>13-15</sup> Melatonin is a lipophilic molecule that freely crosses cell membranes and enters cells, and it preserves cellular redox balance by maintaining mitochondrial homeostasis.<sup>16,17</sup> Recent studies have focused on the role of melatonin in the regulation of autophagy in healthy and disease states. For example, melatonin is reported to attenuate methamphetamine-induced autophagy in SK-N-SH cells, rotenone-induced autophagic cell death in HeLa cells, and morphine-induced autophagy in mouse neurons.<sup>18-20</sup> However, the ability of melatonin to protect against Cd-induced autophagic cell death in the human liver remains unknown.

The data presented in the current report indicate that melatonin efficiently protected HepG2 cells against Cd-induced mitochondrial-derived  $O_2^{\bullet-}$ -dependent autophagic cell death by promoting SIRT3-SOD2 signaling both in vitro and in vivo. These data illustrate a new molecular mechanism of melatonin that may be utilized for future clinical treatments of Cd-induced hepatotoxicity.

## Results

### Cadmium induces autophagic cell death in cultured HepG2 cells

To investigate whether autophagy is involved in the cytotoxicity of Cd, we first examined the processing of full-length LC3-I to LC3-II, a hallmark of autophagy, in Cd-treated HepG2 cells. Cd exposure increased the protein levels of LC3-II in a dose-dependent manner (Fig. 1A). Evidence of cadmium-induced autophagy was determined by direct observation of the formation of autophagosomes using electron microscopy (Fig. 1B). SQSTM1/p62 serves as a link between LC3 and ubiquitinated substrates and a reduction in the amount of SQSTM1 reflects an increase in autophagic degradation. We also examined the total cellular quantity of SQSTM1 that was delivered to lysosomes for degradation.<sup>21</sup> Immunoblot analysis showed that Cd treatment decreased the level of SQSTM1 (Fig. 1A), confirming that Cd enhances the autophagic degradation process. Autophagy is a dynamic process of flux. As such, increased levels of autophagosomes can signify either an increase of autophagy, a block in downstream lysosomal processing of these autophagosomes, or both. Bafilomycin A<sub>1</sub> (Baf A1), a specific inhibitor of the vacuolar-type H<sup>+</sup>-ATPase, prevents autophagy at a latest stage by inhibiting the fusion between autophagosomes and lysosomes. To detect autophagic flux, we measured the level of LC3-II and GFP-LC3-positive autophagosomes in the absence or presence of Baf A1. We found that a Baf A1 challenge resulted in increased

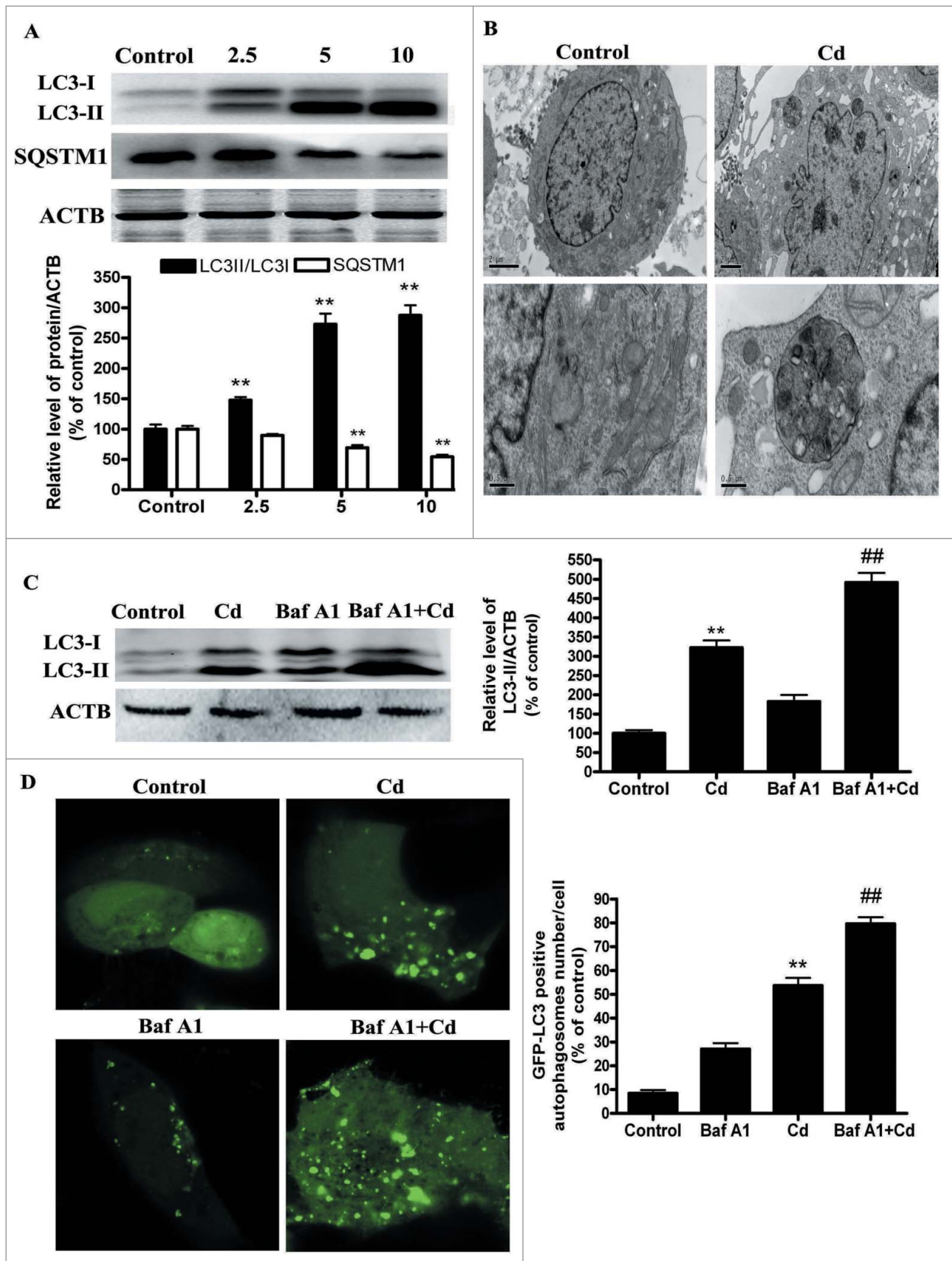
LC3-II expression and GFP-LC3- positive autophagosomes in the cells treated with 10  $\mu$ M Cd (Figs. 1C and D). Moreover, we transfected HepG2 cells with tandem fluorescent mRFP-GFP-LC3B (tf-LC3), a novel marker that allows assessing of autophagic flux, that is, the complete processing of autophagosomes after fusing with lysosomes, using fluorescence microscopy.<sup>22</sup> Cd increased red puncta in the merged image, which indicates formation of autolysosomes (Fig. S1). It is noteworthy that combination treatment of Cd with Baf A1 changed the puncta color to yellow, representing accumulation of autophagosomes resulting from blocked Cd-induced autophagy (Fig. S1). Taken together, these findings demonstrated that Cd treatment induced autophagic flux in HepG2 cells. To further ascertain the role of autophagy in Cd-induced cytotoxicity, 3-MA, an autophagy inhibitor, was employed. As shown in Figures S2A, B and S3A, 3-MA reduced the percentage of autophagy from 250% to 170% and improved cell viability from 56% to 80%. These data indicate that Cd induced autophagic cell death in HepG2 cells. Moreover, *ATG5* siRNA treatment also efficiently protected against Cd-induced cell death (Figs. S2C, D and S3B).

### Mitochondrial-derived $O_2^{\bullet-}$ mediates cadmium-induced autophagy in HepG2 cells

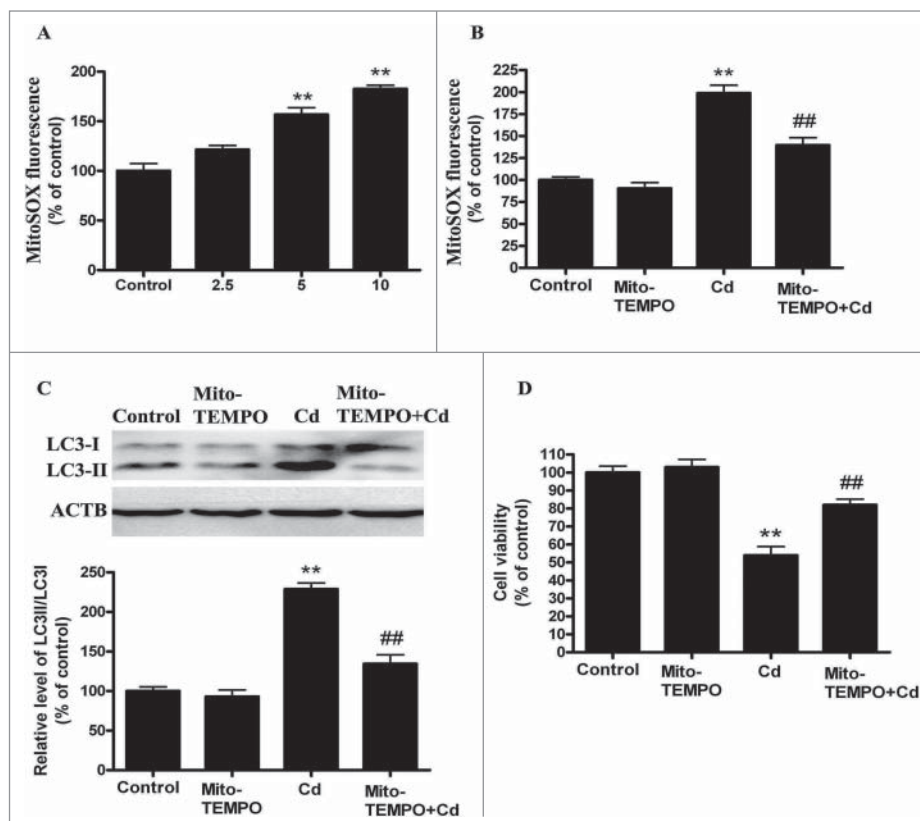
Mitochondrial ROS (mROS) have been implicated in autophagy,<sup>23</sup> and the initial oxygen reduction product generated in mitochondria is superoxide ( $O_2^{\bullet-}$ ), which is quickly dismutated to hydrogen peroxide ( $H_2O_2$ ).<sup>24</sup> Thus, we analyzed the mitochondrial-derived  $O_2^{\bullet-}$  levels in Cd-exposed cells. Mito-SOX-based measurements revealed that mitochondrial-derived  $O_2^{\bullet-}$  levels were significantly increased by 1.58- and 1.84- fold when compared with control cells, respectively (Fig. 2A). Then, HepG2 cells were preincubated with 10  $\mu$ M Mito-TEMPO for 2 h before the 12-h Cd treatment. The mitochondrial antioxidant Mito-TEMPO (mitochondrial-targeted SOD mimetic)<sup>25</sup> enhanced SOD2 activity but not SOD2 levels, suppressed mitochondria-derived  $O_2^{\bullet-}$  generation, and significantly suppressed the Cd-mediated increase in LC3-II expression (Fig. 2B, C and Fig. S4). Moreover, the Cd-induced decrease in cell viability was significantly attenuated after treatment with Mito-TEMPO (Fig. 2D and Fig. S3C). Taken together, these results suggest that the oxidative stress induced by Cd triggers autophagy, and antioxidants, such as Mito-TEMPO, can mitigate mitochondrial oxidative stress and reduce this excess autophagy.

### Cadmium exposure inhibits SIRT3 expression and activity and increases SOD2 acetylation

SOD2, the primary mitochondrial oxidative scavenger, plays a crucial role in the regulation of mROS by catalyzing  $O_2^{\bullet-}$  conversion to  $H_2O_2$ .<sup>26</sup> The effects of cadmium-induced mROS production on SOD2 expression were investigated. Interestingly, Cd treatment significantly decreased SOD2 activity in a dose-dependent manner without changing SOD2 expression levels (Fig. 3A and B). SOD2 activity is tightly regulated by acetylation at its lysine residues.<sup>27</sup> We measured SOD2 acetylation levels with immunoprecipitation and subsequent western blotting using an anti-acetyl-lysine antibody. Cd significantly increased



**Figure 1.** For figure legend, see page 1040.



**Figure 2.** Mitochondrial-derived  $O_2^{\bullet-}$  mediates Cd-induced autophagy in cultured HepG2 cells. (A) Quantification of mitochondrial-derived  $O_2^{\bullet-}$  levels using a fluorescence spectrometer after HepG2 cells were treated with Cd at different concentrations for 12 h. HepG2 cells were preincubated with Mito-TEMPO (10  $\mu$ M) for 2 h and then treated with 10  $\mu$ M Cd, then the mitochondrial-derived  $O_2^{\bullet-}$  levels (B), LC3 level (C) and cell viability (D) were determined. The results are expressed as a percentage of the control, which is set at 100%. The values are presented as the means  $\pm$  SEM, \*\* $p < 0.01$  versus the control group, ## $p < 0.01$  vs. the Cd (10  $\mu$ M) group. (n=6.)

the acetylation levels of SOD2 2.2-, 3.1-, and 3.6-fold, when compared with controls (Fig. 3C). SOD2 is mainly regulated by the deacetylation of specific conserved lysines in a reaction catalyzed by the mitochondrial sirtuin, SIRT3.<sup>27-29</sup> We therefore investigated the effect of Cd on SIRT3 expression and activity. Cd treatment resulted in a significant decrease in SIRT3 mRNA and protein levels (Fig. 4A and B). Our experimental data also showed that SIRT3 activity was reduced in the Cd group when compared with the control (Fig. 4C). SIRT3 and SOD2 functionally and physically interact with each other to form a stable complex that regulates the activity and acetylation of SOD2.<sup>28</sup> The results of the co-immunoprecipitation (Co-IP) affinity isolation assay indicate that Cd treatment does not increase or disrupt the interaction of SIRT3 and SOD2 (Fig. 4D).

### SIRT3-SOD2 modulates cadmium-induced mitochondrial-derived $O_2^{\bullet-}$ accumulation and autophagy in cultured HepG2 cells

To assess if the restoration of SIRT3 levels or activity is sufficient to inhibit Cd-induced autophagy, we overexpressed SIRT3 in HepG2 cells using a transient transfection method. We found that overexpression of SIRT3 attenuated the Cd-induced suppression of SIRT3 protein expression and activity (Fig. S5A and B). Furthermore, overexpression of SIRT3 decreased the expression of acetylated-SOD2, increased SOD2 activity (Fig. 5A and B), and efficiently depressed mitochondrial-derived  $O_2^{\bullet-}$  production in HepG2 cells exposed to 10  $\mu$ M Cd (Fig. 5C). SIRT3-overexpressing cells also exhibited a marked reduction in LC3 expression and an elevation in cell viability (Fig. 5D, E and Fig. S3D).

### SIRT3 deacetylase deficiency does not affect mitochondrial-derived $O_2^{\bullet-}$ accumulation and autophagy in cadmium-treated HepG2 cells

To test if deacetylase activity is required for SIRT3 action in autophagic metabolism, we generated a catalytic mutant of SIRT3 (SIRT3<sup>H248Y</sup>) lacking deacetylase activity. We found that the overexpression of SIRT3<sup>H248Y</sup> attenuated the Cd-induced decrease in SIRT3 protein expression but not SIRT3 activity (Fig. S5A and B). The acetylation of SOD2 and SOD2 activity was unchanged in SIRT3<sup>H248Y</sup>-overexpressing HepG2 cells (Fig. 6A and B). Moreover, the overexpression of SIRT3<sup>H248Y</sup> in HepG2 cells did not block Cd-induced mitochondrial-derived  $O_2^{\bullet-}$  production (Fig. 6C). SIRT3<sup>H248Y</sup> did not attenuate Cd-induced autophagy, but it did restore cell viability when compared with wild-type SIRT3 (Fig. 6D, E and Fig. S3D).

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### Melatonin protects against cadmium-induced autophagic cell death

Melatonin is a mitochondrial antioxidant that aids in the elimination of mROS.<sup>30</sup> We tested whether melatonin protects

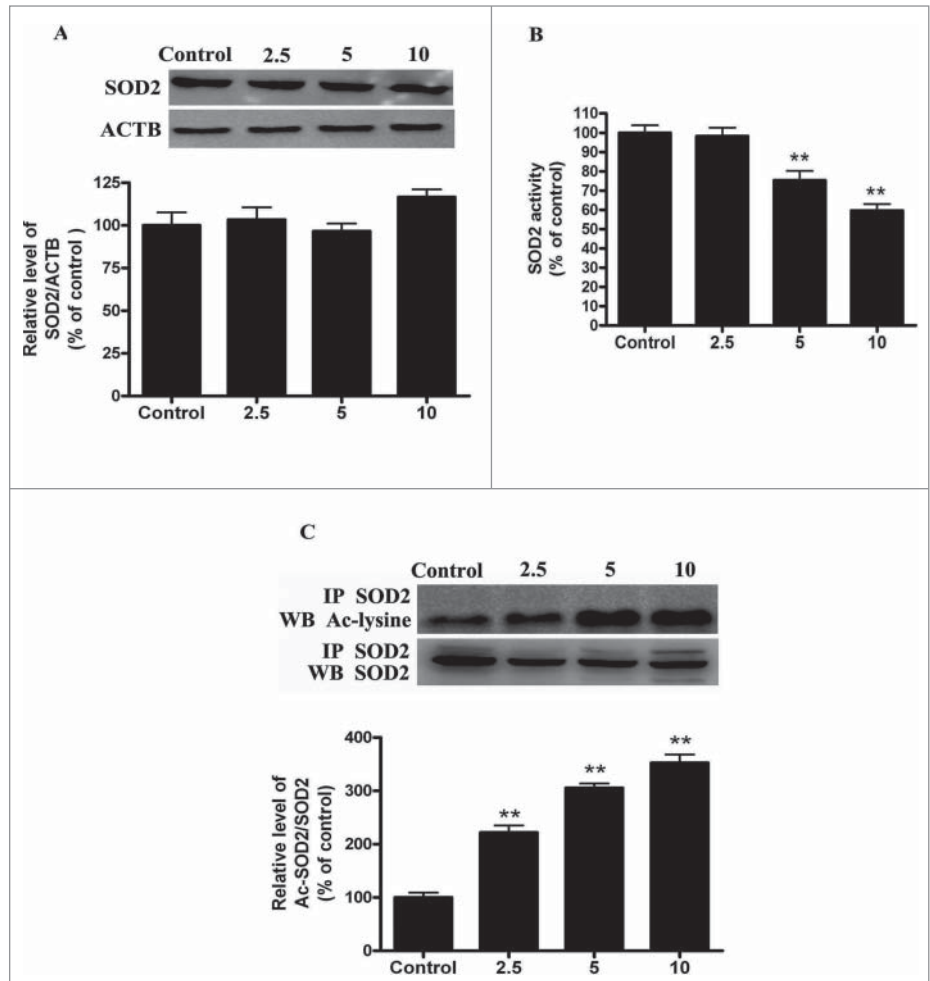
**Figure 1 (See previous page).** Cd induces autophagic cell death in cultured HepG2 cells. (A) A representative immunoblot and quantification analysis of LC3 and SQSTM1 as assayed after HepG2 cells were treated with Cd at different concentrations (0, 2.5, 5, 10  $\mu$ M) for 12 h. ACTB used as an internal standard for protein loading. (B) Electron microscopy revealed an increased number of autophagic vacuoles with or without exposure to 10  $\mu$ M Cd for 12 h. HepG2 cells were incubated with Cd (10  $\mu$ M) in the absence or presence of Baf A1 (10 nM) for 12 h. (C) The expression of LC3-II was quantified by normalization of their densitometry to ACTB. (D) The formation of GFP-LC3 puncta was examined using confocal microscopy and was quantified. The results are expressed as a percentage of the control, which is set at 100%. The values are presented as the means  $\pm$  SEM, \*\* $p < 0.01$  versus the control group, ## $p < 0.01$  vs. the Cd (10  $\mu$ M) group. (n = 4.)

mitochondria against Cd-induced oxidative stress and autophagy. We found that melatonin blocked mitochondrial-derived  $O_2^{\bullet-}$  elevation induced by Cd in HepG2 cells (Fig. 7A). Concomitantly, melatonin restored the levels of LC3-II and cell viability in Cd-treated HepG2 cells to that of untreated controls (Fig. 7B, C and Fig. S3E).

#### Melatonin suppresses cadmium-induced autophagy through a SIRT3-SOD2-dependent mechanism

SIRT3 is involved in the inhibition of Cd-induced autophagy and hepatotoxicity; thus, the relationship between SIRT3 and melatonin in liver cells was investigated. Interestingly, melatonin restored the Cd-mediated reduction in SIRT3 activity without significantly affecting SIRT3 protein levels (Fig. 8A and B). As expected, melatonin significantly blocked the Cd-induced expression of acetylated-SOD2 and restored SOD2 activity (Fig. 8C and D). We used SIRT3 inhibitor (3-TYP) to confirm that melatonin was involved in Cd-induced autophagy and the disruption of SIRT3-regulated mitochondrial-derived  $O_2^{\bullet-}$  production. 3-TYP is a selective SIRT3 inhibitor.<sup>31</sup> Exposure to 3-TYP inhibited melatonin-enhanced SIRT3 activity but did not affect SIRT3 protein expression (Fig. S6A and B). Moreover, 3-TYP pretreatment reversed the protective effects of melatonin on Cd-induced mitochondrial-derived  $O_2^{\bullet-}$  production and autophagic cell death (Fig. 9A–C and Fig. S3F).

As shown in Figures 9D and 9E, melatonin-induced increases in deacetylated-SOD2 expression and SOD2 activity were significantly attenuated by 3-TYP in HepG2 cells exposed to Cd. To determine whether melatonin suppressed Cd-induced autophagy through a SIRT3-SOD2-dependent pathway in vivo, we examined the effects of melatonin in a mouse model. The effects of melatonin on Cd-induced liver damage are presented in Figure 10A. Melatonin successfully reduced GPT/ALT, glutamic-pyruvate transaminase (alanine aminotransferase) levels in the Cd-treated mice. Moreover, melatonin restored the Cd-mediated reduction in SIRT3 activity without significantly affecting SIRT3 protein levels in mice (Fig. S7). Next, the level of SOD2 (acetyl K68), a downstream target of the SIRT3 pathway, was also measured. Melatonin significantly attenuated Cd-induced upregulation of acetylated-SOD2, restored the SOD2 activity and suppressed autophagy (Fig. 10B and C). These data suggest a SIRT3-dependent effect of melatonin on acetylated-SOD2

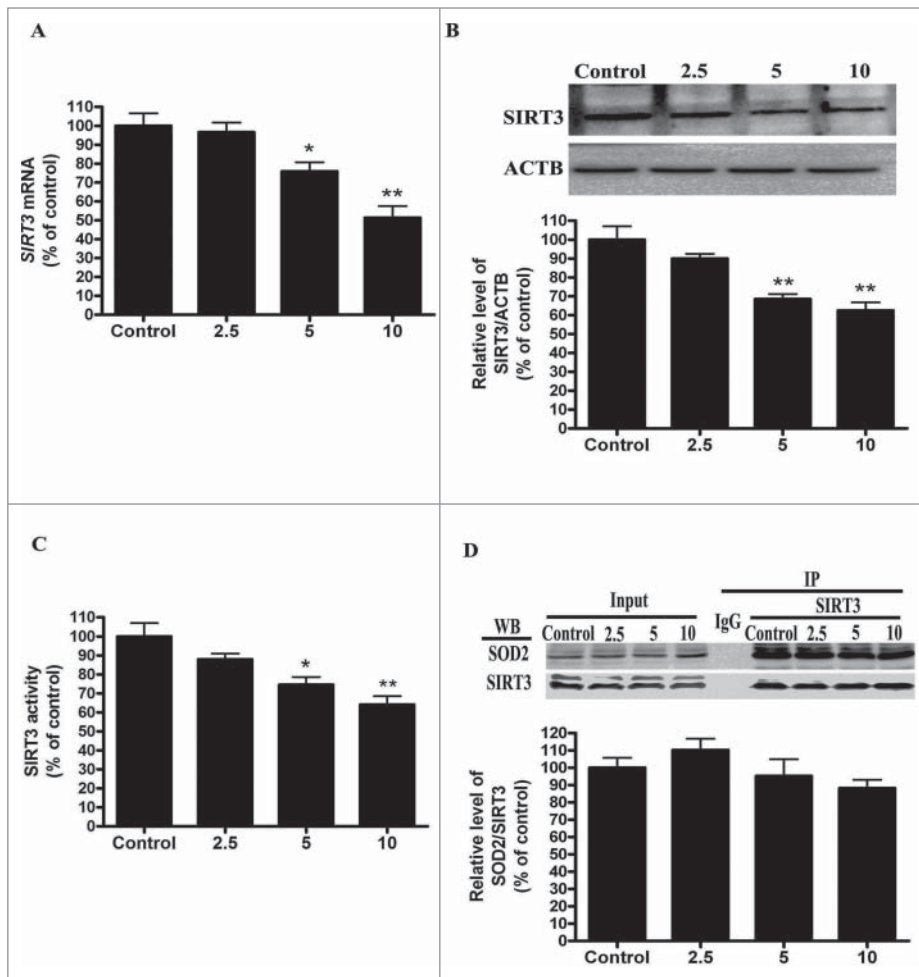


**Figure 3.** Cd exposure increases acetylated-SOD2 expression in HepG2 cells in a dose-dependent manner. (A) Representative immunoblot of SOD2 protein levels in HepG2 cells. (B) SOD2 activity in HepG2 cells (C) Acetylation of SOD2 after Cd exposure was determined by immunoprecipitation with an anti-SOD2 antibody, followed by immunoblot analysis of acetylated-lysine. The results are expressed as a percentage of the control, which is set at 100%. The values are presented as the means  $\pm$  SEM, \*\* $p < 0.01$  versus the control group. (n = 4.)

expression in hepatic cells exposed to cadmium both in vitro and in vivo.

## Discussion

Cd is one of the most toxic metal compounds found in the environment, and it is an important environmental pollutant that can be distributed through the food chain.<sup>32</sup> Melatonin has remarkable antioxidant properties and is a strong candidate for the treatment of Cd-induced hepatotoxicity;<sup>33–38</sup> however, the precise mechanism behind these effects is unclear. The current study is the first to show that melatonin is a feasible treatment option for Cd-induced autophagic cell death. We demonstrated that Cd augmented cellular autophagy, which is dependent on mROS generation in liver cells. We also showed that a reduction in SIRT3 reduced the deacetylation of SOD2, resulting in a loss



**Figure 4.** Cd exposure decreases SIRT3 protein expression and activity in a dose-dependent manner. (A) Quantitative real-time PCR analysis was applied to detect *SIRT3* mRNA levels. (B) Representative immunoblot of SIRT3 protein levels in HepG2 cells. (C) SIRT3 activity was measured based on an enzymatic reaction using a SIRT3 assay kit. (D) Cell lysates were mixed with various concentrations Cd for 12 h. Antibodies against SIRT3 were added to immunoprecipitate the SIRT3-containing complexes, and then immunoblotted for SOD2 or SIRT3. The results are expressed as a percentage of the control, which is set at 100%. The values are presented as the means  $\pm$  SEM, \* $p < 0.05$ , \*\* $p < 0.01$  vs. the control group. (n = 4.)

in SOD2 activity and an elevation in the mROS responsible for Cd-induced autophagy. In addition, our data show that SIRT3 deacetylase activity, rather than SIRT3 expression, may contribute to SOD2 activity in Cd-induced autophagy. Moreover, we determined that melatonin antagonizes Cd-induced autophagic cell death by regulating SIRT3 activity but not influencing its protein expression.

Autophagy may aid in cell survival by removing cells damaged by toxic metabolites and intracellular pathogens. However, autophagy may also promote cell death through excessive self-digestion and degradation of essential cellular constituents.<sup>39</sup> In our previous study, we showed that Cd exposure induced a significant degree of autophagy, which led to cell death and hepatotoxicity in L02 cells.<sup>8</sup> Here, we show that Cd-induced increases in the number of GFP-LC3 puncta resulted from an elevation in autophagic flux rather than an inhibition of autophagosome

degradation in HepG2 cells. In addition, Cd treatment decreased the viability of HepG2 cells, and 3-MA or *ATG5* silencing significantly inhibited this decrease in cell viability. These results provide further evidence that autophagy contributes predominantly to Cd-mediated cytotoxicity in the liver.

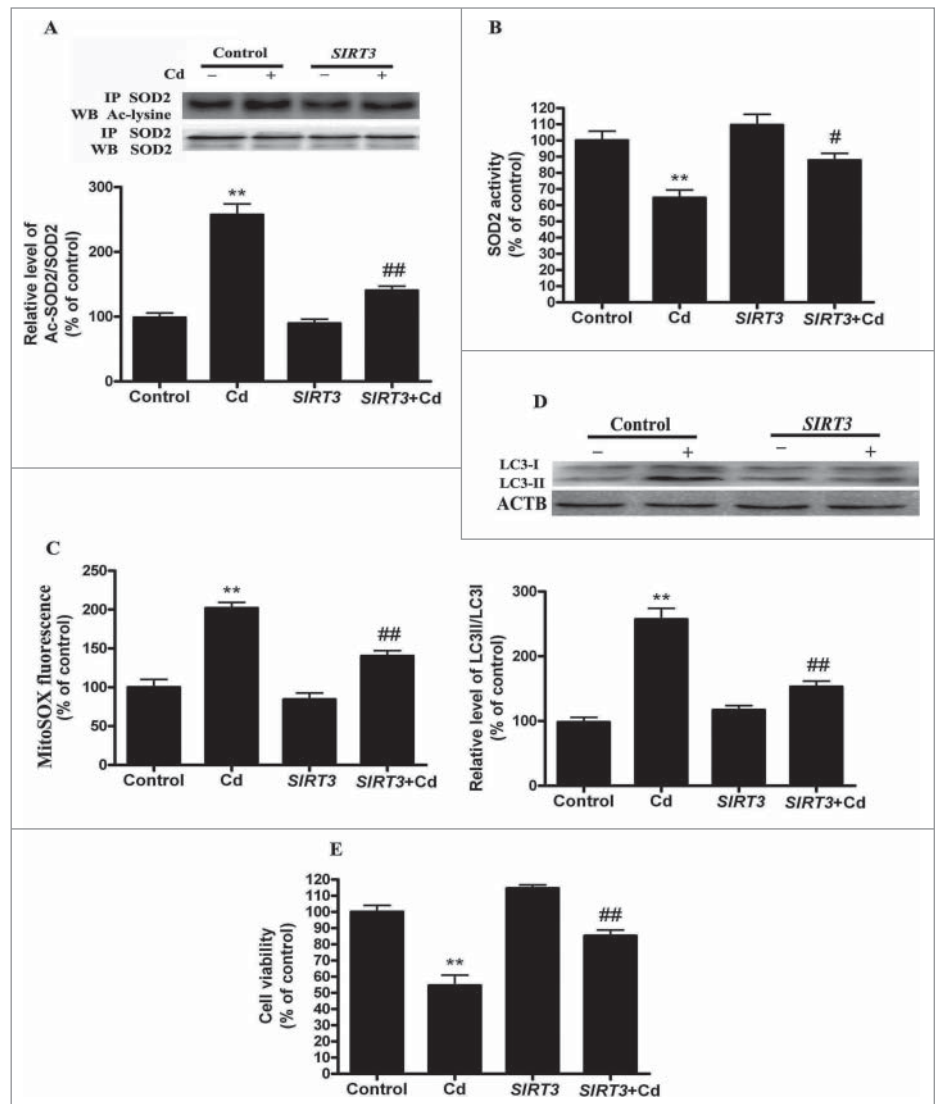
Increased generation of mROS is an important stimulus of autophagy in several diseases.<sup>40-44</sup> Autophagic degradation and removal of damaged oxidized proteins in response to mitochondrial oxidative stress is reportedly beneficial for the cell. Conversely, severe oxidative stress and increasing amounts of mROS may activate signaling pathways that lead to autophagic cell death.<sup>23</sup> Here, we observed that a 12-h Cd exposure promoted mitochondrial-derived  $O_2^{\bullet-}$  levels 2-fold when compared with the control. Treatment with the mitochondrial-targeted SOD mimetic, Mito-TEMPO, mitigated oxidative stress and reduced autophagic cell death, suggesting that mitochondrial-derived  $O_2^{\bullet-}$  accumulation is an important mechanism behind the sensitization of cells to autophagy. Thus, maintaining mitochondrial-derived  $O_2^{\bullet-}$  at tolerable levels may be a viable strategy to treat Cd-induced autophagic cell death. However, a therapeutic window for mitochondrial-derived  $O_2^{\bullet-}$  suppression in this model requires clarification in future studies.  $H_2O_2$  also is an important member of the mROS family. The production of  $O_2^{\bullet-}$  and  $H_2O_2$  is a chain reaction, where  $O_2^{\bullet-}$  is converted to  $H_2O_2$  by SOD.<sup>45</sup> Interestingly, Cd

dose-dependently inhibited the generation of  $H_2O_2$  in HepG2 cells (Fig. S8). Autophagy is involved in human diseases and is regulated by ROS including  $O_2^{\bullet-}$  and  $H_2O_2$ . Our result may confirm that Cd-induced autophagy specifically mediated by  $O_2^{\bullet-}$ , but not  $H_2O_2$ .<sup>46,47</sup>

While mitochondrial oxidative stress is involved in autophagy, the molecular mechanisms by which mitochondrial-derived  $O_2^{\bullet-}$  accumulate remain unknown. Mitochondrial-derived  $O_2^{\bullet-}$  levels are carefully regulated in the cell, and the mitochondria contain specific processes to scavenge and remove mitochondrial-derived  $O_2^{\bullet-}$  to maintain homeostasis. Mitochondria consume over 90% of intracellular oxygen and produce a large flux of mitochondrial-derived  $O_2^{\bullet-}$ ; thus, SOD2 activity is crucial for maintaining mitochondrial-derived  $O_2^{\bullet-}$  balance.<sup>28,48</sup> In the present study, we found that SOD2 activity was significantly reduced in Cd-induced hepatotoxicity. SOD2 is regarded as a

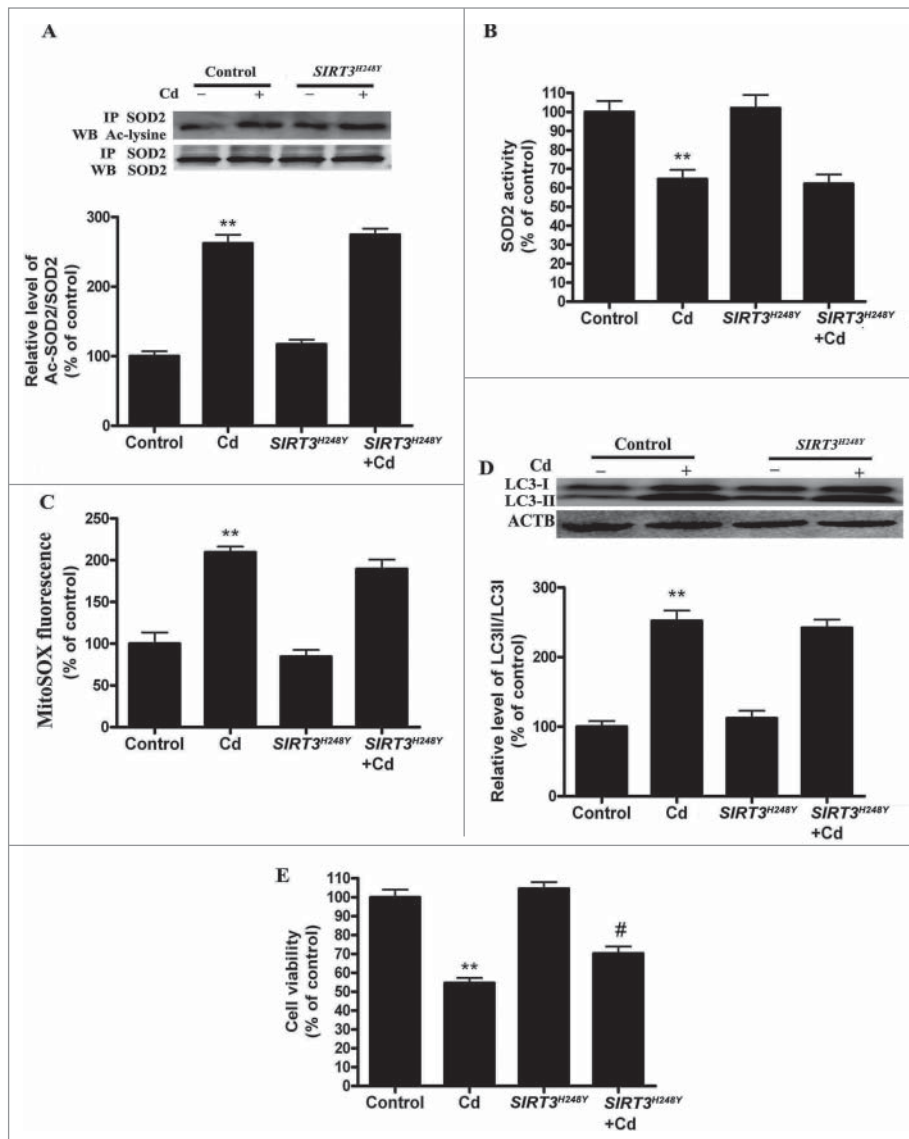
scavenging enzyme, and its activity is thought to be dependent on its mitochondrial levels.<sup>49,50</sup> However, the expression of SOD2 protein was unaffected by Cd treatment. Moreover, regulation of the activity of SOD2 has been mainly reported at the transcriptional level, and the activity of SOD2 is inversely proportional to its acetylation.<sup>27,28,48</sup> Consistent with previous studies, we observed that Cd increased acetylated-SOD2 levels in a dose-dependent manner. We also found that SOD2 activity was significantly reduced. These data suggest that Cd increased mitochondrial-derived  $O_2^{\bullet-}$  through the up-regulation of acetylated SOD2 without affecting SOD2 protein levels.

Lysine acetylation has recently emerged as an important posttranslational modification employed to regulate mitochondrial proteins and autophagy.<sup>51-53</sup> Recently, 3 mitochondrial deacetylation enzymes have been identified, including SIRT3, SIRT4, and SIRT5.<sup>54</sup> SIRT3 is the most robust mitochondrial deacetylase, and it directs biological functions involved in mitochondrial energy production and limits the accumulation of mitochondrial-derived  $O_2^{\bullet-}$ .<sup>55</sup> Deacetylation of SOD2 by SIRT3 regulates SOD2 enzymatic activity, and target lysines have been identified. SIRT3 deacetylates SOD2 in response to ionizing radiation, indicating that SOD2 is a major downstream signal of SIRT3-mediated mitochondrial-derived  $O_2^{\bullet-}$  reduction.<sup>27</sup> Herein, we found that although Cd decreased SIRT3 protein expression and activity, it did not disrupt the interaction between SIRT3 and SOD2, providing new evidence for a likely mechanism by which acetylation modifies the transcription activity of SOD2 in Cd-induced hepatotoxicity. In addition, *SIRT3* overexpression maintained mitochondrial-derived  $O_2^{\bullet-}$  homeostasis and reversed Cd-induced autophagy by deacetylating SOD2 and regulating the antioxidant activity of SOD2. These findings suggest that SIRT3-SOD2-mediated autophagy is an essential mechanism underlying Cd-induced hepatotoxicity. Mechanistically, how Cd decreases SIRT3 levels and activities is unclear, we consider 2 distinct alternatives. First, we found that Cd-mediated decreases in *SIRT3* mRNA levels may contribute to the changes of SIRT3 levels or activity. Second,



**Figure 5.** SIRT3-SOD2 modulates Cd-induced mitochondrial-derived  $O_2^{\bullet-}$  accumulation and autophagy in cultured HepG2 cells. (A) *SIRT3* overexpression induced deacetylation of SOD2 after 10  $\mu$ M Cd treatment. (B) SOD2 activity in HepG2 cells. (C) Mitochondrial-derived  $O_2^{\bullet-}$  production in HepG2 cells. (D) Representative immunoblot of LC3 protein levels in HepG2 cells. (E) Cell viability. The results are expressed as a percentage of the control, which is set at 100%. The values are presented as the means  $\pm$  SEM, \*\* $p < 0.01$  versus control group, # $p < 0.05$ , ## $p < 0.01$  vs. the Cd (10  $\mu$ M) group. (n = 6.)

another new finding shows us that the sequential actions of nuclear SIRT1 (Sirtuin 1) and mitochondrial SIRT3 link nuclear and mitochondrial functions.<sup>56,57</sup> Our previous studies showed SIRT1 progressively decreased in Cd-injured HepG2 cells.<sup>38</sup> Notably, *SIRT1* overexpression increased SIRT3 activity without altering its expression levels in Cd-treated HepG2 cells, which confirmed that communication between SIRT1 (nuclear) and SIRT3 (mitochondrial) may influence Cd-induced hepatotoxicity (Fig. S9). However, it is noteworthy that the activity of SIRT3 can be controlled by the availability of its substrates, post-translational modifications, interactions with other proteins, or changes in its expression levels.<sup>58</sup> Further study is



**Figure 6.** SIRT3 deacetylase deficiency does not affect mitochondrial-derived  $O_2^{\bullet-}$  accumulation and autophagy in Cd-treated HepG2 cells. (A) *SIRT3*<sup>H248Y</sup> overexpression did not induce deacetylation of SOD2 after 10  $\mu$ M Cd treatment. (B) SOD2 activity in HepG2 cells. (C) Mitochondrial-derived  $O_2^{\bullet-}$  production. (D) Representative immunoblot of LC3 protein levels in HepG2 cells. (E) Cell viability. The results are expressed as a percentage of the control, which is set at 100%. The values are presented as the means  $\pm$  SEM, \*\* $p < 0.01$  versus control group, # $p < 0.05$  vs. the Cd (10  $\mu$ M) group. (n = 6.)

needed to identify other mechanisms of Cd-mediated effects on the SIRT3 pathway.

Although *SIRT3*<sup>H248Y</sup> overexpression increased SIRT3 protein levels when compared with wild-type *SIRT3*, *SIRT3*<sup>H248Y</sup> was unable to reverse the Cd-induced increase in mitochondrial-derived  $O_2^{\bullet-}$  and autophagy. These data indicate that SIRT3 activates SOD2 through its deacetylase activity to suppress autophagy. Interestingly, *SIRT3*<sup>H248Y</sup> displayed a certain protective effect in cell death against Cd in HepG2 cells without deacetylase activity. This beneficial role may be explained by a compensation of *SIRT3*<sup>H248Y</sup> overexpression by increased

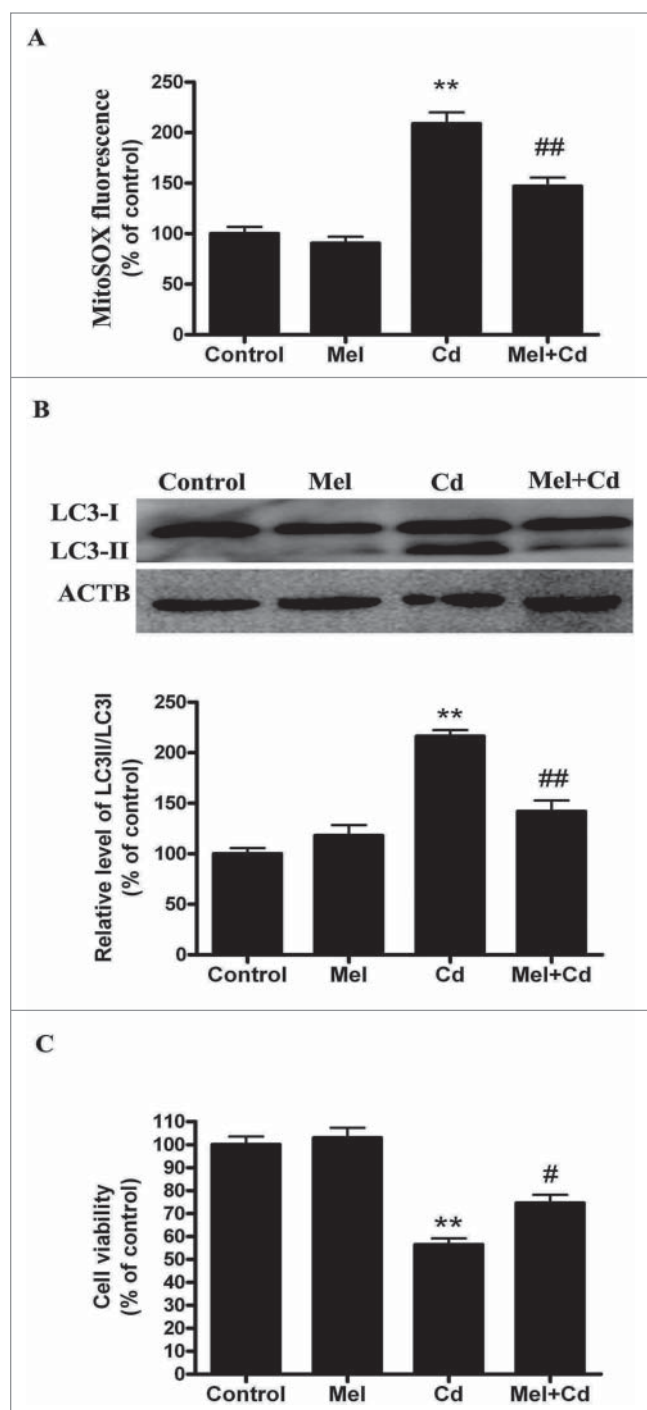
expression of other sirtuins, which leads to a reduction in cell death mechanisms other than autophagy, such as apoptosis, pyroptosis, or necrosis.<sup>59</sup> This hypothesis and the details of these mechanisms need further research.

Cd-induced hepatotoxicity may be the consequence of autophagy, and the antioxidant activity of melatonin was shown to reduce autophagy. Therefore, we tested if melatonin reversed the deleterious effects of Cd by inhibiting autophagy. We found that melatonin attenuated the Cd-induced rise in mitochondrial-derived  $O_2^{\bullet-}$  in cultured cells. Most importantly, melatonin ameliorated the Cd-induced decrease in SIRT3 activity but did not affect its protein levels. Thus, melatonin restored autophagy levels in cultured cells exposed to Cd. Although melatonin and its metabolites modulate the expression of genes involved in detoxification or generation of mitochondrial-derived  $O_2^{\bullet-}$ , melatonin may not change SIRT3 gene expression. Rather, melatonin may only upregulate SIRT3 activity.<sup>60</sup> To identify if the SIRT3-SOD2 pathway mediated the observed protective effects of melatonin, HepG2 cells were pretreated with 3-TYP, followed by melatonin treatment and Cd exposure for 12 h. This study documented that blocking SIRT3 activity with 3-TYP blocked the effects of melatonin on Cd-induced autophagy. Moreover, 3-TYP remarkably reversed the effects of melatonin on acetylated-SOD2 expression and SOD2 activity. Importantly, mice lacking SIRT3, a mitochondrial deacetylase, have increased acetylation and inhibition of many mitochondrial enzymes and complexes, suppressing mitochondrial function.<sup>61</sup> In our study, melatonin

suppressed Cd-induced autophagic cell death in the livers of mice by enhanced SIRT3 activity. These data are consistent with melatonin suppressing mitochondrial-derived  $O_2^{\bullet-}$  production and restoring autophagy levels through the upregulation of the SIRT3-SOD2 pathway both in vitro and in vivo.

In summary, we propose an intriguing mechanism whereby Cd induces hepatotoxicity via mitochondrial-derived  $O_2^{\bullet-}$ -dependent-autophagy. Importantly, melatonin shows a compensatory and protective role in eliminating mitochondrial-derived  $O_2^{\bullet-}$  and suppressing autophagy through the SIRT3-SOD2 pathway both in vitro and in vivo. Taken together, these findings





**Figure 7.** Melatonin suppresses Cd-induced autophagic cell death. (A) Mitochondrial-derived  $O_2^{\cdot-}$  production. (B) A representative immunoblot and quantification analysis of LC3. (C) Cell viability. The results are expressed as a percentage of the control, which is set at 100%. The values are presented as the means  $\pm$  SEM, \*\* $p < 0.01$  vs. the control group, and # $p < 0.05$ , ## $p < 0.01$  vs. the Cd (10  $\mu$ M) group. (n = 6.)

provide new insights into the link between melatonin and autophagy signaling, which could contribute to a better understanding of a melatonin-mediated protective effect in Cd-induced hepatotoxicity (Fig. 11).

## Materials and Methods

### Cell culture

The human hepatocellular carcinoma cell line, HepG2, was purchased from the Cell Bank of the Institute of Biochemistry and Cell Biology (Shanghai, China). The HepG2 cells were cultured in DMEM medium (HyClone, SH30022.01B) that was supplemented with 10% heat-inactivated FBS (HyClone, SV30087.02) and 1% (v/v) penicillin/ streptomycin (Sigma, P4333). Cells were grown in a 5%  $CO_2$  humidified atmosphere at 37°C.

### Cell experimental protocol

The effects of Cd on autophagy in HepG2 cells were evaluated. At 80% confluence, the cells were treated with cadmium chloride ( $CdCl_2$ ; Sigma, 439800) at different concentrations (0, 2.5, 5, 10  $\mu$ M) for 12 h.

Next, we investigated the ability of melatonin to alleviate Cd-induced hepatotoxicity. HepG2 cells were pretreated with 1  $\mu$ M melatonin for 2 h prior to 10  $\mu$ M Cd treatment. Melatonin (Sigma, M5250) was freshly dissolved in ethanol to produce a 10 mM stock solution that was kept at 4°C until use. The stock solution was further diluted in culture medium before being added to the cells. The final ethanol concentration did not exceed 0.1% for cell culture experiments. The vehicle control used for each in vitro assay was 0.1% ethanol.

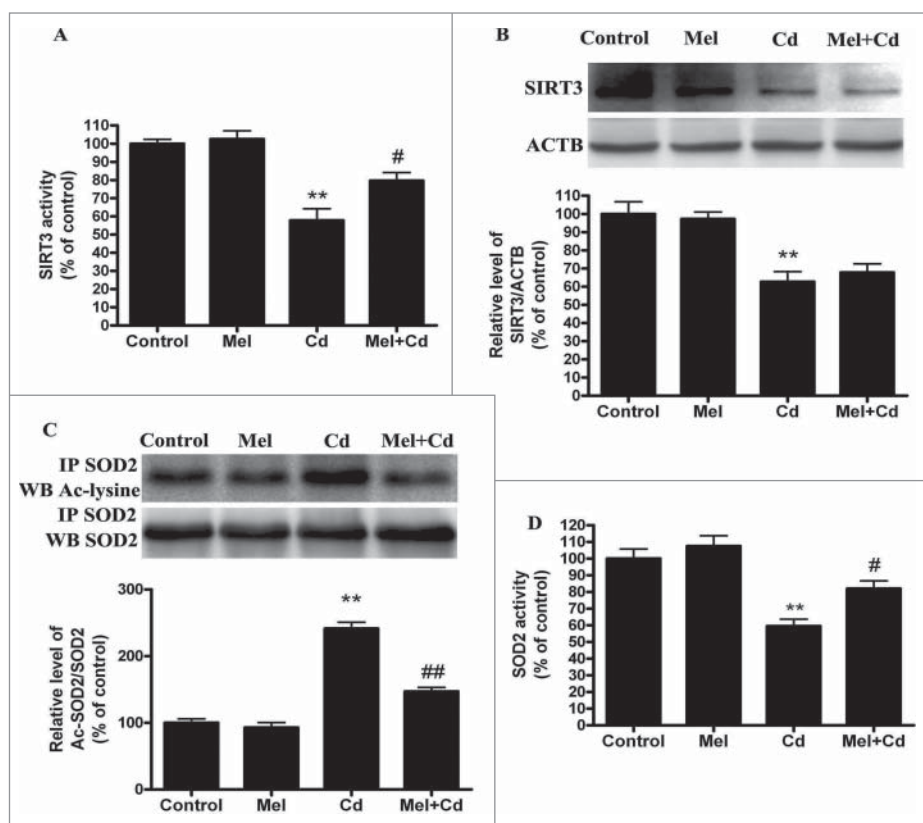
Finally, the role of the SIRT3-SOD2 pathway in hepatic cell protection after melatonin pretreatment was investigated. Cells were randomly divided into the following treatment groups: 10  $\mu$ M Cd; melatonin (1  $\mu$ M) + 10  $\mu$ M Cd; 3-TYP (50  $\mu$ M) + 10  $\mu$ M Cd; and 3-TYP (50  $\mu$ M) + melatonin (1  $\mu$ M) + 10  $\mu$ M Cd. 3-TYP (50  $\mu$ M) had no significant effect on cell viability when compared with the control group (data not shown). 3-(1H-1,2,3-triazol-4-yl)pyridine (3-TYP, CAS: 120241-79-4) was synthesized and characterized by the IDRC innovative drug research center at Chongqing University (Fig. S10–S13) as previously described.<sup>31</sup>

### Animal studies

Thirty-three 2-mo-old C57BL/6 mice were maintained on a 12:12 h light-dark phase and fed ad libitum. They were adapted for 2 wk to the above conditions before experiments. All mice except controls were injected intraperitoneally with Cd (2 mg/kg) for 7 d. Some mice were intraperitoneally injected with melatonin (5 mg/kg) at 2 h before Cd treatment. The control mice received an equal volume of normal saline or equal doses of melatonin.<sup>62</sup> All animal experiments were approved by the Third Military Medical University for Accreditation of Laboratory Animal Care.

### Electron microscopy

We used electron microscopy to morphologically observe the induction of autophagy in Cd-treated HepG2 cells.<sup>8</sup> After being treated with Cd (10  $\mu$ M) for 12 h, cells were washed twice with phosphate-buffered saline (ZSGB-BIO, ZLI-9061) and fixed with ice-cold glutaraldehyde for 30 min at 4°C. Cells were post



**Figure 8.** Melatonin increases SIRT3 activity but not expression, and it inhibits acetylated-SOD2 expression after Cd treatment in vitro. Melatonin enhances SIRT3 activity (A) but not the expression of SIRT3 (B) at 12 h after exposure to 10  $\mu$ M Cd. Melatonin induced deacetylation of SOD2 (C) and increased SOD2 activity (D) after Cd treatment. The results are expressed as a percentage of the control, which is set at 100%. The values are presented as the means  $\pm$  SEM, \*\* $p < 0.01$  versus the control group, and # $p < 0.05$  vs. the Cd (10  $\mu$ M) group. (n = 4.)

fixed in 1% osmium tetroxide for 30 min at 4°C and embedded in LX 122 before being cut and stained with uranyl acetate/lead citrate. Next, the cells were observed using a Hitachi-7500 electron microscope (Hitachi Instrument, Tokyo, Japan).

#### Determination of autophagic cells

To assess autophagy, the cells that overexpressed GFP-LC3 (Cell Biolabs, CBA-401) were examined at 63 $\times$  magnification, and autophagic cells were classified as those with diffuse GFP-LC3 fluorescence or as cells with >20 GFP-LC3 puncta/cell.<sup>63</sup> The cells were treated with either control vehicle or 10  $\mu$ M Cd for 12 h, and then imaged using a Zeiss confocal laser scanning microscope (Carl Zeiss, LSM 780 Confocal Laser Scanning Microscope).

#### Evaluation of fluorescent LC3 puncta

HepG2 cells were transfected with tandem fluorescent mRFP-GFP-LC3B (tf-LC3) (Invitrogen, P36239) in a 35-mm cell culture dish, according to the manufacturer's instructions. Twenty-four h after virus transduction, the cells were treated with either control vehicle or 10  $\mu$ M Cd for 12 h, and then imaged using a Zeiss confocal laser scanning microscope. The number of GFP and RFP dots was determined by manual counting of fluorescent

puncta with a 63 $\times$  objective. At least 50 cells were counted for each experiment.<sup>64,65</sup>

#### RNA interference of *ATG5*

The HepG2 cells ( $1 \times 10^6$ ) were transfected with either 100 nmol/L *ATG5*-targeting small siRNA (Santa Cruz Biotechnology, sc41445) or a control nonspecific siRNA (Santa Cruz Biotechnology, sc37007). 24 h following transfection, the cells were exposed to 10  $\mu$ M Cd for 12 h. The cells were then collected and processed for immunoblotting of LC3B and cell death analyzed.

#### Cell viability and death assay

Cell viability was analyzed using Cell Counting Kit-8 according to the manufacturer's instructions (Dojindo Molecular Technologies, CK04). Briefly,  $1 \times 10^4$  cells were inoculated into 96-well plates. After being treated, 90  $\mu$ l of medium and 10  $\mu$ l of CCK-8 solution were added to each well. The cells were then incubated at 37°C for 2 h. After incubation, the absorption at 450 nm was measured using an Infinite™ M200 Microplate Reader (Tecan, Mannedorf, Switzerland). The results are expressed as a percentage of the control. The cell death was also evaluated

using the trypan blue assay (Beyotime, C0011).<sup>41</sup> HepG2 cells were plated in the 6-well plates ( $5 \times 10^5$  cells per well) and incubated for 24 h. After being treated with Cd or melatonin, the cells were detached with 300  $\mu$ l trypsin-EDTA solution (Beyotime, C0011-2). The mixture of detached cells was centrifuged at 300 g for 5 min. Then, the residue was combined with 800  $\mu$ l trypan blue solution and dispersed. After 3 min staining, cells were counted using an automated cell counter (TC10, Bio-Rad). The dead cells were stained with the blue color. Cell mortality (%) is expressed as percentage of the dead cell number/the total cell number.

#### Determination of mitochondria-derived $O_2^{\bullet-}$ and $H_2O_2$

To assess mitochondrial-derived  $O_2^{\bullet-}$ , HepG2 cells were incubated with culture medium containing 10  $\mu$ M MitoSOX (Invitrogen, M36008) for 20 min at 37°C. The fluorescence intensity was analyzed with an Infinite™ M200 Microplate Reader at an excitation wavelength of 492 nm and an emission wave length of 595 nm.<sup>66</sup> Cellular fluorescence intensity is expressed as the fold change relative to the control. Production of  $H_2O_2$  was measured using the hydrogen peroxide assay kit (Beyotime Company, S0038).<sup>67</sup> The lysis buffer solution supplied in the kit was added at a ratio of 100  $\mu$ l per  $10^6$  cells. Then

the supernatant fractions were gathered by centrifuging at 12 000 g for 5 min for the following tests. All operations were carried out on ice. Then, the test-tubes containing 50  $\mu$ l of the supernatant fractions and 100  $\mu$ l of test solutions were placed at room temperature for 20 min, and the absorbance at 560 nm was instantly measured using an Infinite™ M200 Microplate Reader. The level of H<sub>2</sub>O<sub>2</sub> was calculated according to a standard concentration curve originating from standard solutions using an identical protocol.

#### Measurement of SOD2 enzyme activity

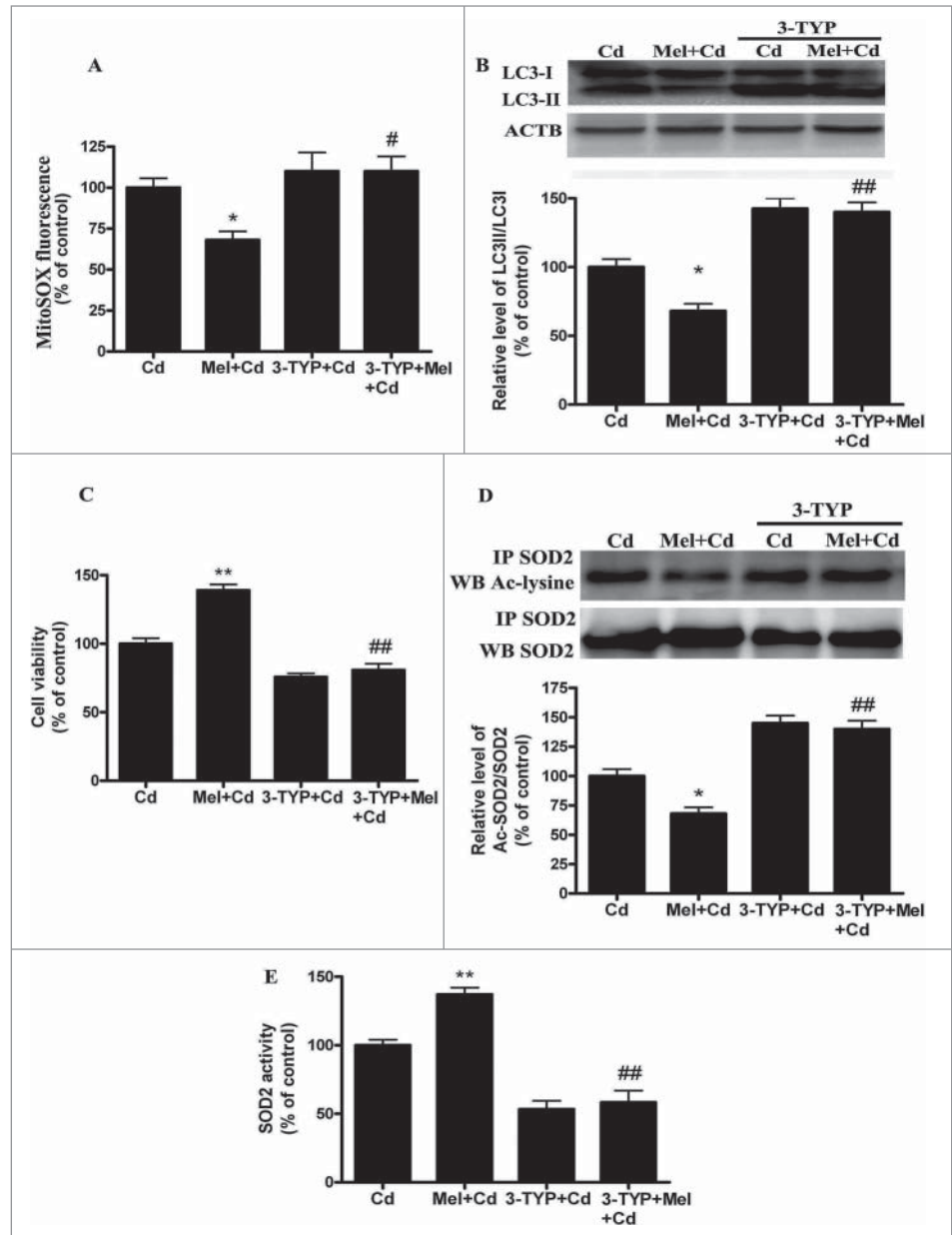
SOD2 enzymatic activity was assayed using a SOD1 and SOD2 Assay Kit with WST-8 (Beyotime Company, S0103) following the manufacturer's instructions. One unit of SOD was defined as the amount of enzyme that inhibits the rate of NBT reduction observed in a blank sample by 50%. The SOD isoforms were identified by adding SOD1 inhibitor A and B to inhibit SOD1 activity (i.e., to detect SOD2). The absorbance at 450 nm was measured using an Infinite™ M200 Microplate Reader.

#### Plasmids and transfection

Three plasmids, pEGFP-N1-*SIRT3*, pEGFP-N1-*SIRT3*<sup>H248Y</sup> and pEGFP-N1-*SIRT1*, were designed by the Invitrogen Corporation, as described previously.<sup>68</sup> HepG2 cells grown in antibiotic-free DMEM on culture dishes for 24 h were transfected with *SIRT3*, *SIRT3*<sup>H248Y</sup>, and control plasmids using Opti-MEM® I reduced serum media and Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, 11668-019). Twenty-four h after transfection, cells were washed and processed for immunoblotting and other studies as described.

#### Real-time PCR analysis to detect *SIRT3* mRNA

All reagents used for real-time PCR were obtained from Life Technologies. The *SIRT3* probes were (5'-GACATTCGGGCTGACGTGAT-3') and (5'-ACCACATG-CAGCAAGAACC TC-3'); the *GAPDH* probes were (5'-

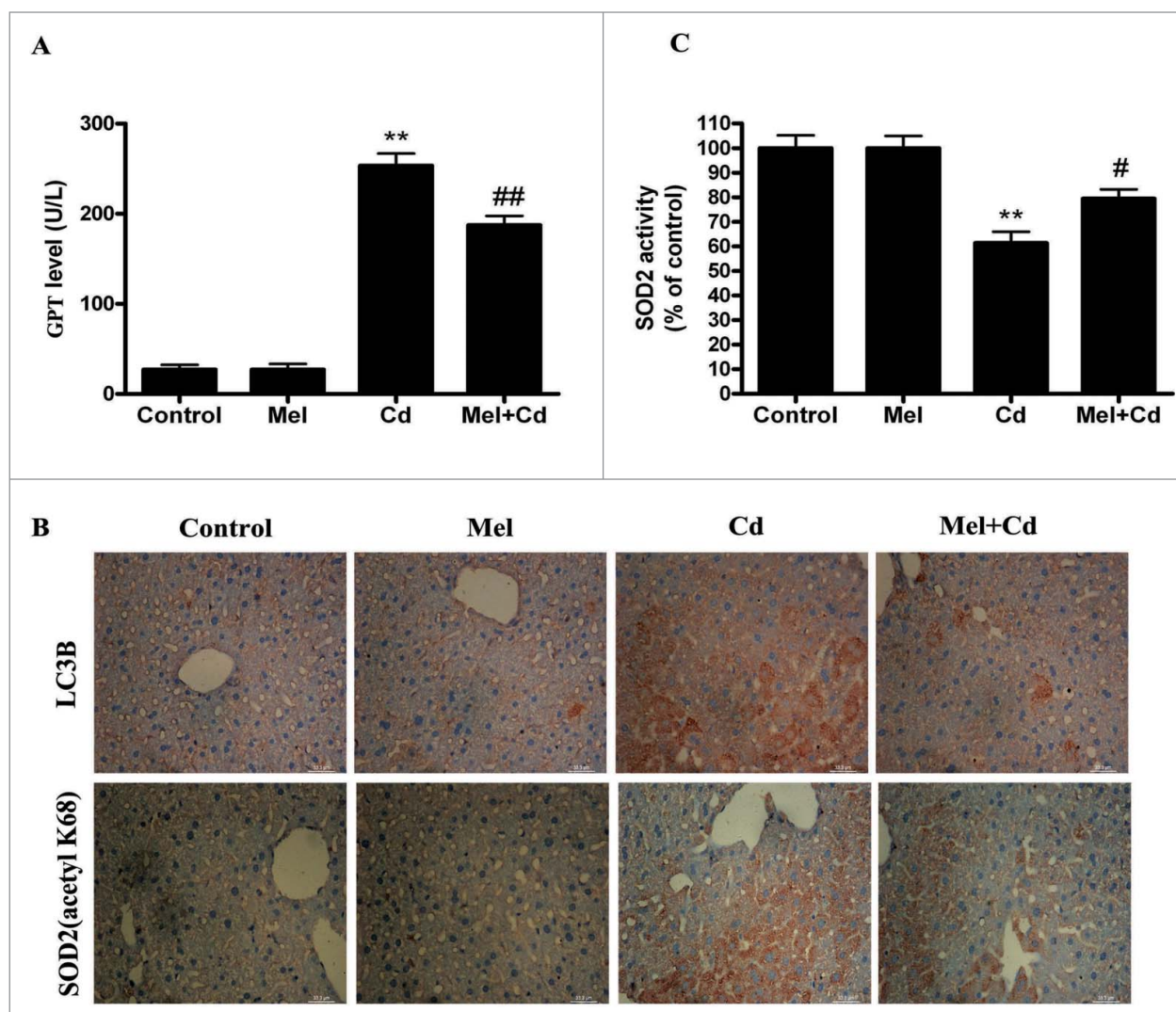


**Figure 9.** 3-TYP pretreatment abolishes the melatonin-suppressed autophagy in Cd-injured HepG2 cells. The effects of melatonin and 3-TYP pretreatment on mitochondrial-derived O<sub>2</sub><sup>•-</sup> production (A), (B) A representative immunoblot and quantification analysis of LC3, (C) cell viability, (D) acetylated-SOD2 expression and (E) SOD2 activity. The results are expressed as a percentage of the Cd group, which is set at 100%. The values are presented as the means  $\pm$  SEM, \**p* < 0.05, \*\**p* < 0.01 versus the Cd (10  $\mu$ M) group, and #*p* < 0.05, ##*p* < 0.01 vs. the Mel + Cd group. (n = 6.)

5'-TGACAACAGCCTCAAGAT-3') and (5'-GAGTCCTTC-CACGATACC-3'). The mean fold change is shown as the natural logarithm of RQ values and the error is estimated by evaluating the 2<sup>- $\Delta\Delta$ Ct</sup> equation using  $\Delta\Delta$ Ct plus standard deviation and  $\Delta\Delta$ Ct minus the standard deviation.

#### SIRT3 activity

SIRT3 enzymatic activity was assayed using a fluorometric kit (Enzo Life Sciences Inc., BML-AK557-0001) following the



**Figure 10.** Melatonin suppresses Cd-induced autophagic cell death by enhancing SIRT3 activity in vivo. (A) Serum GPT activity was measured. (B) Immunohistochemical analysis of LC3B and SOD2 (acetyl K68) expression in liver tissue. (C) SOD2 activity in liver tissue. The results are expressed as a percentage of the control group, which is set at 100 %. The values are presented as the means  $\pm$  SEM, \*\* $p < 0.01$  versus the Cd group, and # $p < 0.05$ , ## $p < 0.01$  vs. the Mel + Cd group. (n = 8.)

manufacturer's instructions with modifications as described.<sup>69</sup> Protein (40  $\mu$ g) was incubated at 37°C for 45 min with specific substrates. Next, 25  $\mu$ l of developer was added and, samples were incubated for an additional 45 min. SIRT3 activity was then measured using an Infinite™ M200 Microplate Reader at 350 nm/450 nm.

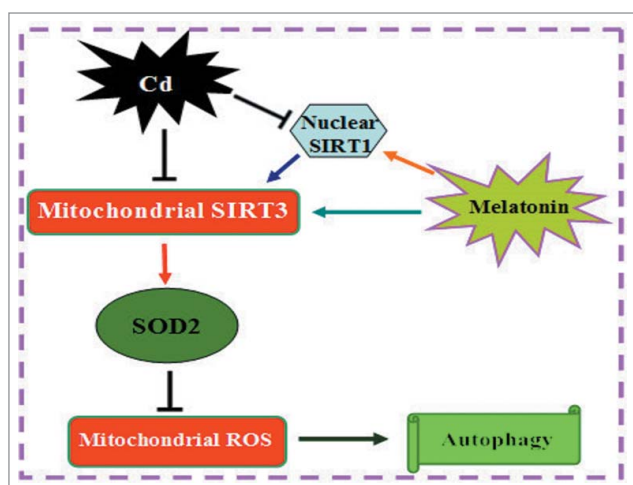
#### Immunoprecipitation

HepG2 cells were treated with Cd and lysed with cell lysis buffer (Beyotime Company, P0013). Lysates were clarified by centrifugation at 12,000 g for 15 min and were used for immunoprecipitation. A total of 2  $\mu$ g of antibody was incubated with 500–1000  $\mu$ g of protein overnight at 4°C. Next, protein A beads (Beyotime Company, P2006) were added and the mixture was incubated overnight at 4°C. After incubation, the beads were

washed 3 times, solubilized in 40  $\mu$ l 3xSDS sample buffer (Cell Signaling Technology, 7722), and analyzed by western blotting.

#### Western blot analysis

The HepG2 cell and the liver tissue lysates were centrifuged for 15 min at 12,000 g, and the resulting supernatant fraction was transferred to a new tube. The protein concentrations were determined using a Bradford protein assay kit (Beyotime Company, P0006). The protein samples were separated by SDS-PAGE. Following protein transfer to PVDF membranes, the membranes were blocked and then incubated overnight at 4°C with antibodies against LC3(1:1000; Sigma, L7543), SQSTM1/p62 (1:1000; Abcam, ab56416), SIRT3 (1:100; Santa Cruz Biotechnology, sc-99143), SOD2 (1:100; Santa Cruz Biotechnology, sc-33254), and ACTB (1:5000; Sigma, A5441) The



**Figure 11.** Melatonin inhibits autophagic cell death by promoting SIRT3-SOD2-mROS pathway in cadmium-induced hepatotoxicity.

membrane was visualized by enhanced chemiluminescence using Super Signal West Pico blotting (Pierce, 34079) detection reagents and exposure to Hyper Performance Chemiluminescence film.

#### Immunohistochemical analyses

Fixed mice livers were embedded in paraffin and were sectioned at a thickness of 3  $\mu\text{m}$ , and immunohistochemical staining was performed with LC3B (1:50; Abcam, ab168831) and SOD2 (acetyl K68) (1:100; Abcam, ab137037) antibody. The sections were mounted in a mounting medium containing glycerol (Beyotime, P0126) and visualized with a light microscope (Leica, DM6000M).

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#### Serum enzyme activity assays

Biochemical evaluation of liver injury was performed by quantifying serum activities of GPT using GPT/ALT test kits (Uscn, SEA207Mu) according to the manufacturer's instructions.

#### Statistical analysis

Data were analyzed using GraphPad Prism-5 software. All of the experimental data are expressed as the mean  $\pm$  SEM, and each experiment was performed a minimum of 3 times. One-way ANOVA was used to determine statistical significance, and  $P < 0.05$  was considered to be statistically significant.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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#### Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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