

# A chemical screen to identify inducers of the mitochondrial unfolded protein response in *C. elegans*

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**Keywords:** *C. elegans*, chemical screen, ethidium bromide, mitochondrial unfolded protein response, methacycline hydrochloride, statin

We previously showed that inhibition of the mevalonate pathway in *C. elegans* causes inhibition of protein prenylation, developmental arrest and lethality. We also showed that constitutive activation of the mitochondrial unfolded protein response, UPR<sup>mt</sup>, is an effective way for *C. elegans* to become resistant to the negative effects of mevalonate pathway inhibition. This was an important finding since statins, a drug class prescribed to lower cholesterol levels in patients, act by inhibiting the mevalonate pathway, and it is therefore possible that some of their undesirable side effects could be alleviated by activating the UPR<sup>mt</sup>. Here, we screened a chemical library and identified 4 compounds that specifically activated the UPR<sup>mt</sup>. One of these compounds, methacycline hydrochloride (a tetracycline antibiotic) also protected *C. elegans* and mammalian cells from statin toxicity. Methacycline hydrochloride and ethidium bromide, a known UPR<sup>mt</sup> activator, were also tested in mice: only ethidium bromide significantly activate the UPR<sup>mt</sup> in skeletal muscles.

## Introduction

Statins are a class of cholesterol lowering drugs taken by tens of millions of patients worldwide. They act by inhibiting the enzyme HMG-CoA reductase, which is rate limiting in the mevalonate pathway of cholesterol biosynthesis. In spite of their wide use, many effects of statins remain poorly understood. For example, patients on statin regimen frequently complain of muscle pains or, rarely, rhabdomyolysis,<sup>1</sup> and statins have immunosuppressive effects;<sup>2</sup> both these effects are thought to be unrelated to cholesterol lowering and are poorly understood. Besides cholesterol, other outputs of the mevalonate pathway include dolichol-phosphate (important for protein N-glycosylation), coenzyme Q (a soluble anti-oxidant that is important in the mitochondrial electron transport chain) and prenyl groups (small lipids that may be covalently attached to the C-terminus of small GTPases or other proteins, and hence target them to membranes).<sup>3-5</sup> Inhibiting the mevalonate pathway could therefore have consequences not only on cholesterol levels, but also on mitochondrial function, protein glycosylation or small GTPases.

*C. elegans* is an ideal model organism to investigate the effects of statins that are unrelated to cholesterol because its mevalonate

pathway is conserved with that in mammals, except for the important fact that the output branch leading to cholesterol synthesis is absent from the worm.<sup>5</sup> We previously showed that statins cause loss of protein prenylation, activation of the endoplasmic reticulum unfolded protein response (UPR<sup>er</sup>), developmental arrest and lethality in *C. elegans*, that these effects are strictly due to on-target inhibition of HMG-CoA reductase and that they can be alleviated by activating the mitochondrial unfolded protein response (UPR<sup>mt</sup>) either by mutations or by treatment with ethidium bromide or paraquat.<sup>6-9</sup>

The mitochondrial unfolded stress response is the mitochondria's primary defense mechanism against stress. Upon stress, UPR<sup>mt</sup> activation results in expression of mitochondrial chaperones such as HSP6, HSP60 and proteases (CLPP1, LON1) to maintain mitochondrial proteostasis either by proper folding or degrading unfolded proteins.<sup>10,11</sup> Suppression of this stress response either by knocking down ATFS-1, a key UPR<sup>mt</sup> regulator, or by inhibiting the mevalonate pathway, reduces the ability of mitochondria to cope with stressors.<sup>7,8,12</sup> Conversely, activation of the UPR<sup>mt</sup> protects mitochondria against stress and promotes survival.<sup>7,9</sup> Identifying factors or molecules that can activate the UPR<sup>mt</sup> may therefore lead to novel therapeutic

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approaches in diseases or clinical conditions where mitochondria are stressed or otherwise challenged.<sup>13</sup> With this objective, we performed a chemical screen and identified 4 drugs that specifically activate UPR<sup>mt</sup> in *C. elegans*. Additionally, one of the compounds, methacycline hydrochloride (a tetracycline antibiotic) also protected *C. elegans* and mammalian cells from statin toxicity. We also found that ethidium bromide is a potent *in vivo* UPR<sup>mt</sup> activator in mouse muscle tissue.

## Results

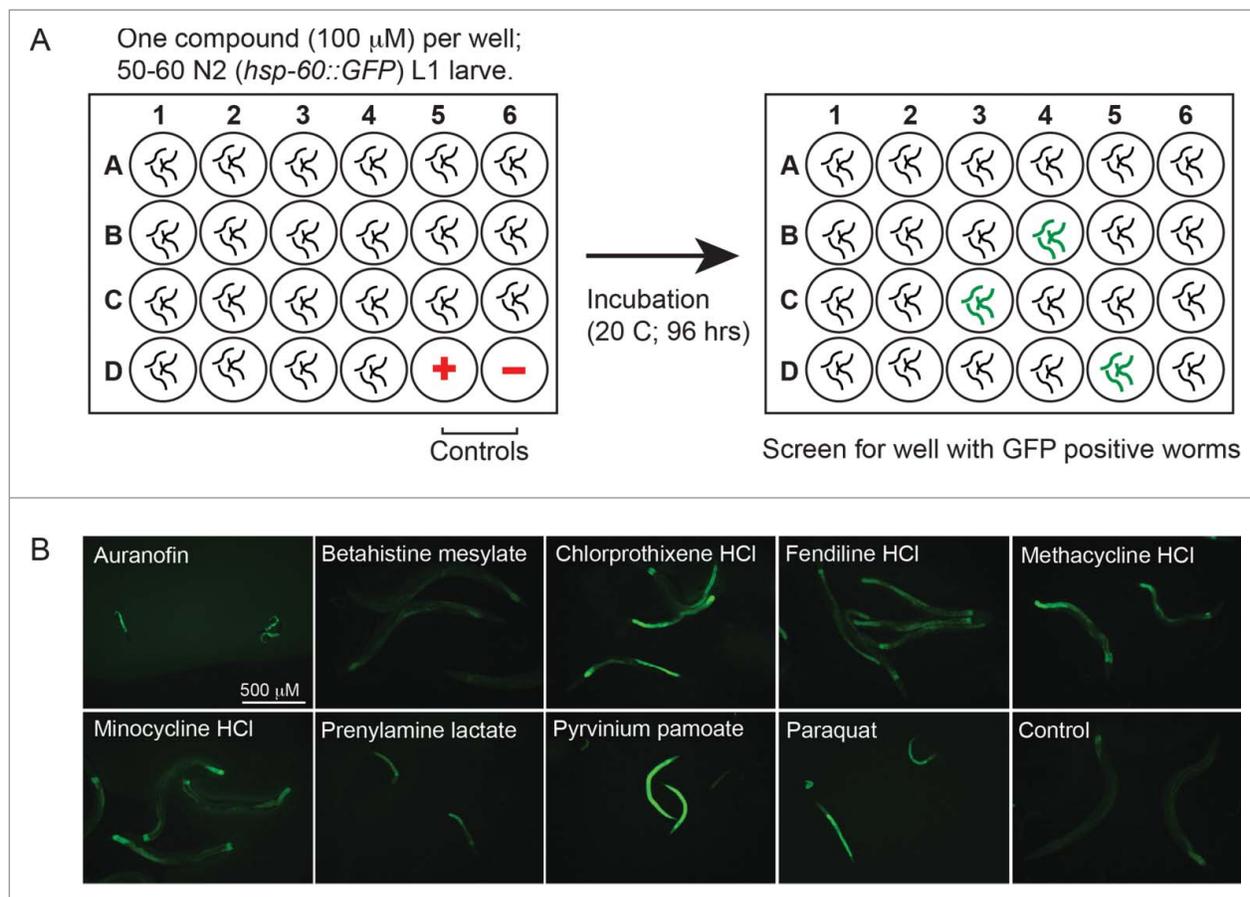
### Chemical screen identifies UPR<sup>mt</sup> activators

The Prestwick Chemical library includes over 1 200 compounds that have previously been approved for use in humans by the US. Food and Drugs Administration (FDA). These were screened at a concentration of 100  $\mu$ M following a published protocol.<sup>14</sup> to identify compounds that cause activation of the *hsp-60::GFP* transgene, a reporter of UPR<sup>mt</sup> activation<sup>15</sup> (Fig. 1A). The oxidative agent paraquat is a known activator of the UPR<sup>mt</sup><sup>15,16</sup> and was used as a positive control in the screen. Eight compounds reproducibly

scored positive (Table 1, Fig. 1B). When re-tested over a range of concentrations, we found that only 4 of the 8 compounds specifically caused activation of the UPR<sup>mt</sup> reporter without also activating high levels of the *hsp-4::GFP*, a UPR<sup>er</sup> reporter (Fig. 2, Fig. S1). These were: minocycline hydrochloride and methacycline hydrochloride (2 tetracycline antibiotics), chlorprothixene hydrochloride (a D2 dopamine receptor antagonist) and auranofin (a gold salt used as an antirheumatic agent).

### ATFS-1 is required for UPR<sup>mt</sup> activation

ATFS-1 is a leucine zipper transcription factor that contains a mitochondrial targeting signals at its N terminus and a nuclear localization signal at its C terminus.<sup>7,12,17-19</sup> It is the primary activator of the UPR<sup>mt</sup> in response to mitochondrial stress. None of the newly identified UPR<sup>mt</sup>-activating compounds could bypass the requirement for ATFS-1 since their ability to induce *hsp-60::GFP* was either completely abolished or greatly reduced in the *atfs-1(gk3094)* null mutant (Fig. 3). The most likely explanation for the ATFS-1 dependency is that the compounds activate the UPR<sup>mt</sup> by causing mitochondrial stress, hence activating ATFS-1.



**Figure 1.** Screen for UPR<sup>mt</sup> activators. (A) shows the schematic of the chemical screen to identify UPR<sup>mt</sup> activator. (B) shows drugs identified in chemical screen that activate of *hsp60::GFP* reporters, with the oxidative agent paraquat used as a positive control.

**Table 1** List of hits from the UPR<sup>mt</sup> activator screen and their therapeutic functions.

COMPOUND	THERAPEUTIC CLASS	BIOLOGICAL EFFECT
Auranofin (a gold salt)	Analgesic	antirheumatic agent
Bethahistine mesylate	vasodilator	histamine H3 receptor antagonist
Chlorprothixene HCl	antipsychotic, antiemetic	D2 dopamine receptor antagonist
Fendiline HCl	antianginal	calcium channel blocker
Methacycline HCl	antibacterial	tetracycline antibiotic
Minocycline HCl	antibacterial	tetracycline antibiotic
Prnylamine lactate	antianginal	calcium channel blocker
Pyvinium pamoate	antihelminthic, anticancer	androgen receptor inhibitor

### Methacycline hydrochloride protect from statin toxicity

We previously showed that activation of the UPR<sup>mt</sup>, either by mutation or using chemical agents that stress mitochondria (ethidium bromide, paraquat), renders worms, yeast and mammalian cells resistant to otherwise lethal doses of statins.<sup>7</sup> Of the 4 specific UPR<sup>mt</sup>-activating compounds isolated in our screen, one induced statin resistance in *C. elegans*: the tetracycline antibiotic methacycline hydrochloride (Fig. 4A-E). The gold salt auranofin appeared toxic to *C. elegans* in this assay, and minocycline hydrochloride and chlorprothixene hydrochloride provided no protection.

### Ethidium bromide, but not methacycline hydrochloride, induces the UPR<sup>mt</sup> in mice

Methacycline hydrochloride, identified in the present screen, and ethidium bromide, a known activator of the UPR<sup>mt</sup> in mammalian cells which we previously showed protects these cells from the statin effects,<sup>7</sup> were chosen for further studies in mammalian cells and whole mice. We found that methacycline hydrochloride confers a weak protection against statin toxicity in mouse 3T3 cells; this protection was inferior to that obtained using ethidium bromide, (Fig. 4F). Finally, we tested 2 doses of ethidium bromide and of methacycline hydrochloride for their ability to induce the UPR<sup>mt</sup> in mice treated using a one-time intraperitoneal injection. Ethidium bromide caused *in vivo* induction of UPR<sup>mt</sup> response genes in skeletal muscle tissues tested using quantitative PCR, with the higher dose of 50 mg/kg being more effective than the 10 mg/kg dose (Fig. 5A-B). Methacycline hydrochloride caused no significant induction of the UPR<sup>mt</sup> response genes in mouse skeletal muscles at either of the 2 doses tested, although there was a tendency toward increased expression and significance may have been obscured due to high variability among the treated samples (Fig. 5C-D).

## Discussion

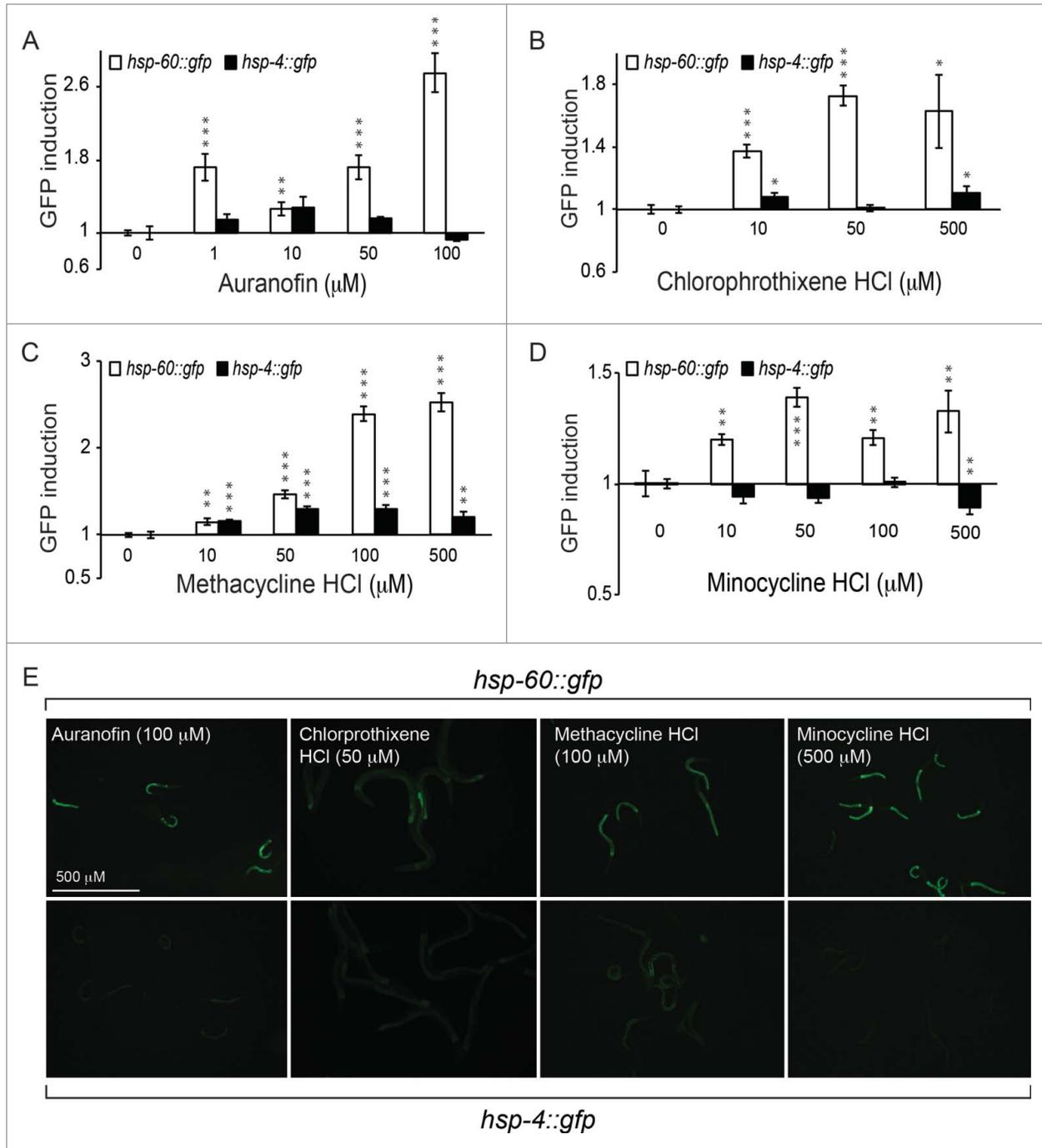
By screening a library of over 1 200 FAD approved compounds, we identified 4 compounds that activate specifically the UPR<sup>mt</sup> in *C. elegans* without also activating the UPR<sup>er</sup>. One of these compounds, the tetracycline antibiotic methacycline also protected *C. elegans* from the toxic effects of statins. The three other UPR<sup>mt</sup> inducers failed to protect from statins and there may be several reasons for this. For example, the UPR<sup>mt</sup> may be

insufficiently induced, the compounds may have toxic effects or the compounds may fail to enter into specific cell types where UPR<sup>mt</sup> activation is required.

Interestingly, methacycline hydrochloride conferred some protection against statins to mouse 3T3 cells, but did not induce the UPR<sup>mt</sup> *in vivo* when provided by intraperitoneal injection. Ethidium bromide was more potent than methacycline hydrochloride both in terms of protecting NIH-3T3 cells from the adverse effects of statins and in inducing UPR<sup>mt</sup> *in vivo*. This is to our knowledge the first instance of a drug treatment that activates the UPR<sup>mt</sup> *in vivo*. Note too that ethidium bromide is used in veterinary medicine to treat trypanosome infections at doses similar to those used in the present study (10-15 mg/kg).<sup>20</sup> Thus, at present, if a situation arise where the UPR<sup>mt</sup> must be activated *in vivo*, there is a possibility that ethidium bromide could be a useful option.

One of the compounds identified as UPR<sup>mt</sup> activators is a tetracycline antibiotics. This is not surprising since this antibiotic class inhibits translation in prokaryotes and therefore is likely to interfere with this process in mitochondria, hence trigger the UPR<sup>mt</sup>. This is also in agreement with the fact that RNAi knock-down of mitochondrial ribosomal subunits, e.g. *mrsps-5*, also trigger UPR<sup>mt</sup> activation.<sup>21</sup> More generally, others have also shown that antibiotics, e.g., doxycycline or chloramphenicol<sup>21</sup> and antimycin,<sup>22</sup> are potent activators of the UPR<sup>mt</sup>. Similarly, auranofin was recently found to have broad-spectrum bactericidal activities and act as an inhibitor of thioredoxin reductase.<sup>23</sup> It therefore likely has direct negative effects on mitochondria that lead to UPR<sup>mt</sup> activation. More puzzling is the identification of chlorprothixene hydrochloride as a UPR<sup>mt</sup> activator. This compound inhibits several types of receptors (e.g. D2 dopamine receptors,<sup>24</sup> serotonin receptors<sup>25</sup>) is a likely inhibitor of acid sphingomyelinase,<sup>26</sup> can be used to reverse antibiotic resistance due to its ability to inhibit efflux pumps,<sup>27</sup> and inhibits the growth of mycobacteria probably by impairing their membrane transport functions.<sup>28</sup> It is therefore possible that it too act directly on mitochondria biology, though this is at present mere speculation.

One of our goals with the present study was to try and identify compounds that could efficiently induce the UPR<sup>mt</sup> *in vivo*. Ultimately, such compounds could have clinical uses, for example to increase patient tolerance against unusually high doses of statins deployed to treat tumors with activated small GTPases. Such a line of thought emerges from two observations: 1) statins are promising anti-cancer drugs because the mevalonate pathway is



**Figure 2.** Four drugs specifically activate the UPR<sup>mt</sup>. Treatment of 4 drugs specifically activates UPR<sup>mt</sup> reporters (*hsp60::GFP*) but not the UPR<sup>er</sup> reporter (*hsp4::GFP*). (A-D) GFP induction of UPR<sup>mt</sup> reporters and UPR<sup>er</sup> reporters on various doses of drugs. (E) shows corresponding images. The bars show the average  $\pm$  SEM ( $n > 20$ ). \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  in paired Student's t-test.

essential for the production of the prenyl lipid groups responsible for the membrane association of small GTPases such as RAS,<sup>29</sup> and 2) we have previously showed that statin toxicity is primarily due to insults to mitochondria and can be abrogated by activating the UPR<sup>mt</sup> in *C. elegans*, yeast and mammalian cells.<sup>7,9</sup> Even though the present study failed to identify a compound more

potent than ethidium bromide, it did demonstrate the feasibility of the approach: a larger scale screen would likely identify potent UPR<sup>mt</sup> activators with useful *in vivo* efficacy. In particular, the main challenge of *in vivo* UPR<sup>mt</sup> activation is that all compounds identified so far seem to act by causing some form of mitochondria toxicity. Even ethidium bromide, a known mutagen, induces

the UPR<sup>mt</sup> by impairing mitochondrial DNA replication and transcription and is therefore very unlikely to see any clinical applications in humans.<sup>30,31</sup> The real breakthrough would be to identify compounds that trigger the UPR<sup>mt</sup> without causing mitochondrial toxicity. Such compounds could, in theory, act by binding and impairing the mitochondrial localization signal of ATFS-1 or act via a completely new mechanism.

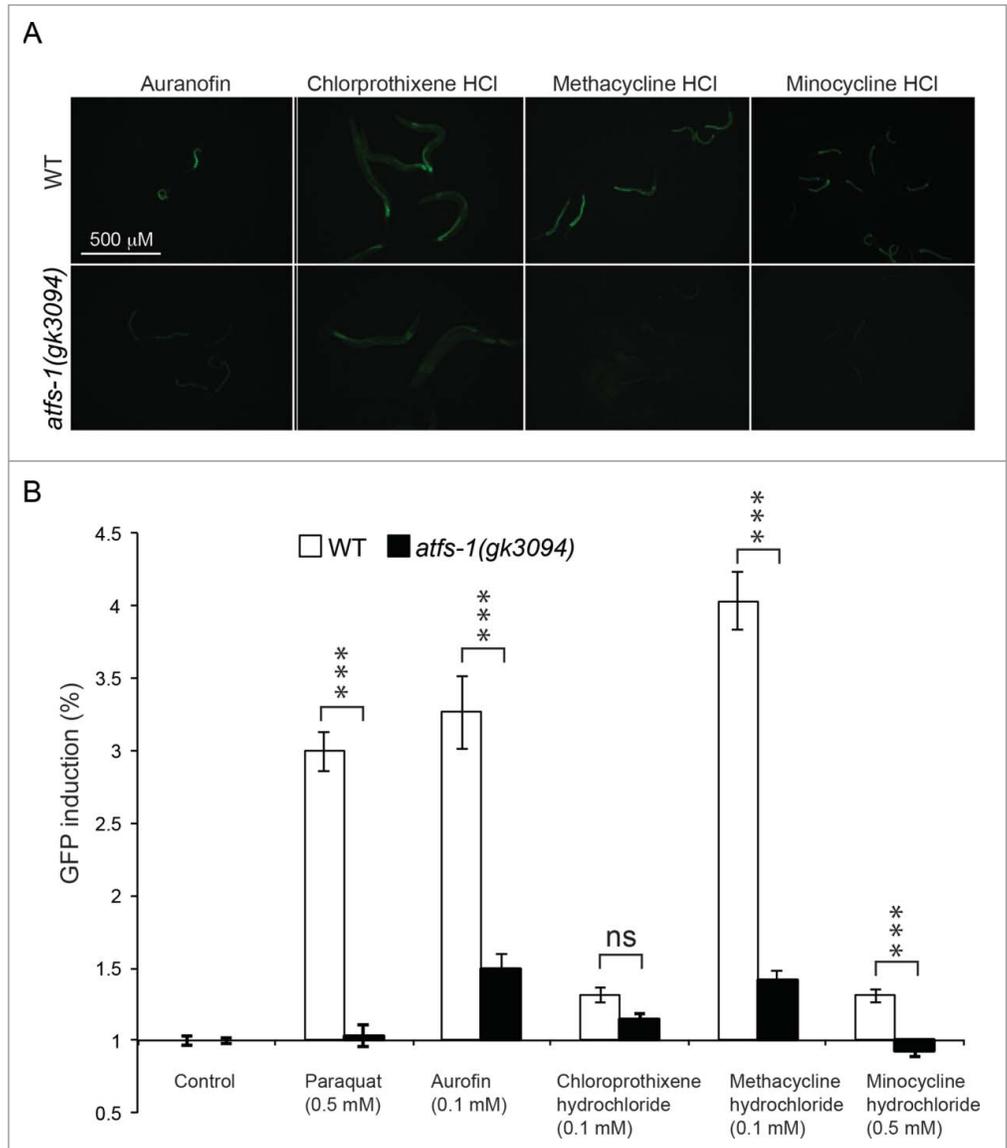
## Materials and Methods

### Nematode strains and maintenance

All strains are maintained at 20°C unless otherwise stated and the Bristol strain N2 was used as wild-type (WT).<sup>32</sup> The following strains were obtained from the *Caenorhabditis* Genetics Center: *zcls4[phsp4::GFP]*, *zcls9[hsp-60::GFP]*, and *atfs-1(gk3094)*.

### Chemical library screen

The protocol for the chemical library screen adapted from a previously described method.<sup>14</sup> Compound-containing plates from the Prestwick chemical library were thawed and 10  $\mu$ l of each compound (final conc of 100  $\mu$ M) was added into the well of 24-well plates (one drug per well). Additionally, each plate contained one negative control well with DMSO (10  $\mu$ l) and one positive control well with paraquat (0.5 mM) or tunicamycin (10  $\mu$ g/ml), depending on the reporter used. The chemical library drug plates were re-sealed and stored at -20°C. Molten nematode growth media (NGM; 1 ml) was added to each drug-containing well and mixed thoroughly by shaking, then allowed to cool. 50  $\mu$ l of dead OP50 bacterial culture was added as food and allowed to dry for 2 hrs in sterile conditions. Finally, synchronized L1 larvae (50-80 worms) carrying either the *zcls9* or *zcls4* transgenes were added, and the plates were sealed and incubated in dark at 20°C for 96 hrs. Each well was then scored for the presence of GFP-positive worms using a stereoscope equipped for epifluorescence, and possible hits were retested.



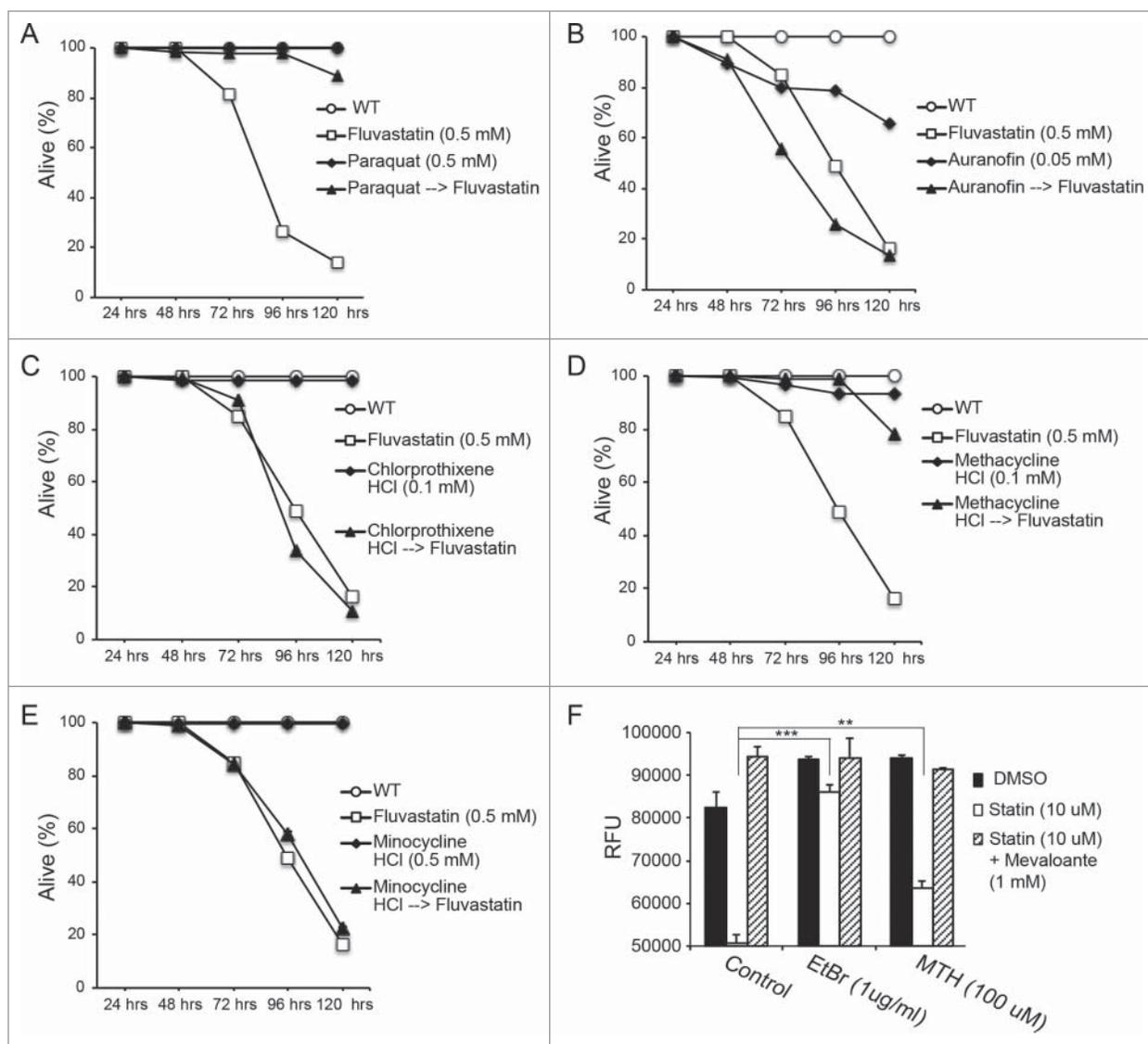
**Figure 3.** Activation of UPR<sup>mt</sup> after drug treatment is mediated through ATFS-1. (A) The drug mediated activation of UPR<sup>mt</sup> reporter (*hsp60::GFP*) is suppressed in the *atfs-1(gk3094)* mutants compared to wild type. (B) GFP quantification of the drug treatments. The bars show the average  $\pm$  SEM (n > 20). \*\*\*  $p < 0.001$ ; ns (not significant) in paired Student's t-test.

### Drug treatment and GFP intensity measurement

Fluvastatin (brand Lescol; Novartis) plates were prepared as described in a previous study.<sup>6</sup> The following compounds were also used: mevalonolactone (Sigma), ethidium bromide (Sigma), paraquat (Sigma) and tunicamycin (Sigma). GFP images were acquired 96 hrs after placing synchronized L1 larvae on drug plates. Images were acquired using a Zeiss Axio Scope A1 to measure their GFP levels and all images for a single experiment were taken with the same excitation intensity and exposure time. The GFP intensity was measured with the Image J software (NIH, USA).

### Mitochondrial UPR pre-induction experiment

Pre-induction of the UPR<sup>mt</sup> using paraquat was performed as previous described.<sup>7</sup> In brief, the synchronized L1 larvae



**Figure 4.** Pre-activation of mitochondria UPR using methacycline hydrochloride and paraquat confers statin resistance in worms. (A-E) Worms pre-treated with methacycline hydrochloride and paraquat (positive control) but not other 3 compounds are viable and grow into fertile adults when subsequently cultivated on 0.5 mM fluvastatin. (F) The mammalian fibroblast cell line NIH 3T3 shows better viability in the presence of 10  $\mu$ M fluvastatin when it has been pre-treated with 1  $\mu$ g/ml ethidium bromide (EtBr) or methacycline hydrochloride (MTH) (100  $\mu$ M); bars show the average readout from the Presto Blue Cell Viability assay  $\pm$  SEM (n > 5 wells; \*\*\*, p < 0.001). Note that the effect of statins on NIH-3T3 cell viability is entirely due to the inhibition of HMG-CoA reductase since they are completely abrogated by the inclusion of mevalonate in the culture medium.

were placed on paraquat (500  $\mu$ M) plates for 24 hrs and then transferred to either control or fluvastatin (0.5 mM) plates. The viability of the worms was then measured every 24 hrs until 96 hrs post-paraquat treatment.

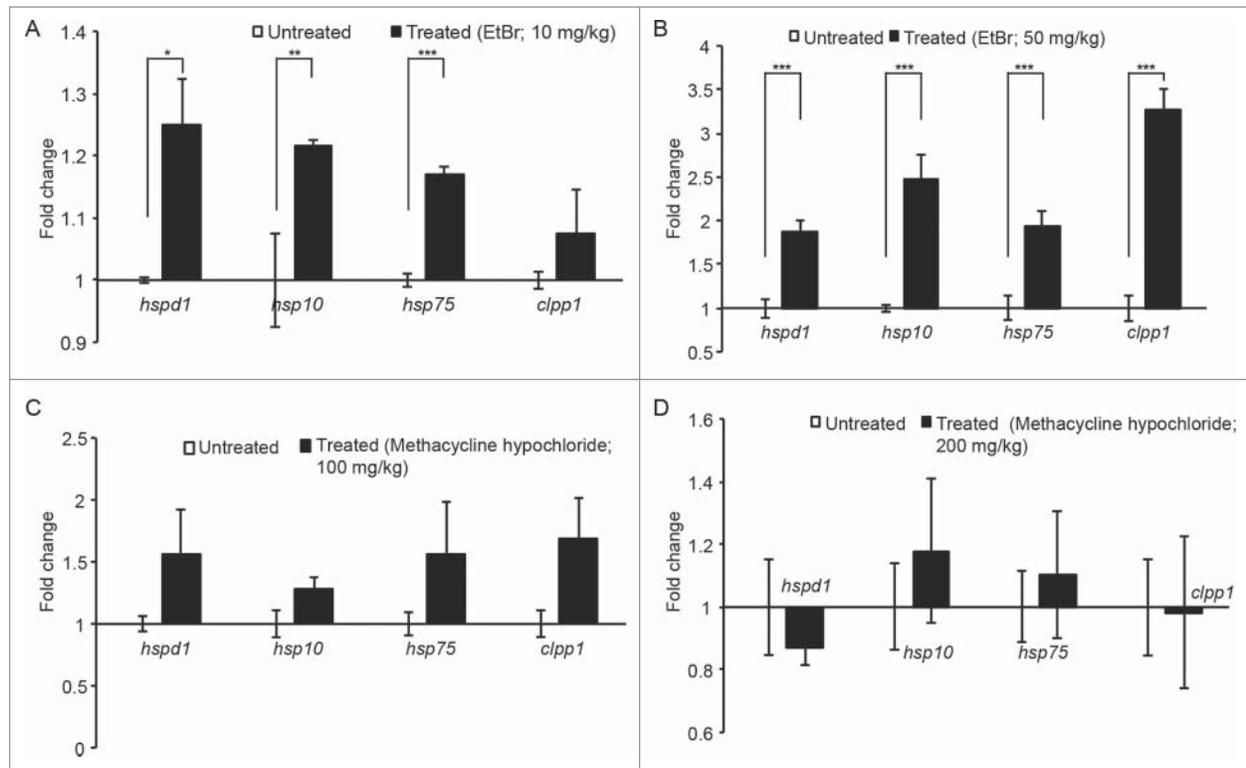
#### Mammalian cell culture

The 3T3 mouse embryonic fibroblast cells were maintained in DMEM with high glucose (Gibco) and 10% fetal bovine serum (FBS). The UPR<sup>mt</sup>-mediated statin resistance assay was performed as described in a previous study.<sup>7</sup> In summary, ~2 000 3T3 cells were seeded per well on 96-well plates (TPP Nordic Biolab) and allowed to grow for 24 hrs. These cells were then treated with

media containing EtBr (1  $\mu$ g/ml) for 48 hrs and were challenged with fluvastatin (10  $\mu$ M) or fluvastatin (10  $\mu$ M) plus mevalonolactone (1 mM) for 48 hrs. Cell viability was measured using the Presto Blue Cell Viability Reagent (Invitrogen) as recommended by the manufacturer.

#### Mouse experiments

BALB/c mice strains aged between 8 to 10 weeks old were used to evaluate the potency of candidate drugs to activate UPR<sup>mt</sup> response *in vivo*. The drugs were administered only once through the intraperitoneal (IP) route and not more than 50  $\mu$ l drug dissolved in saline or DMSO was injected per



**Figure 5.** Intraperitoneal administration of ethidium bromide activates UPR<sup>mt</sup> in muscle. Balb/c mice were injected through the intraperitoneal route, with 2 different concentration of ethidium bromide (10 mg/kg; 50 mg/kg) and methacycline hydrochloride (100 mg/kg; 200 mg/kg). The quadriceps muscle from mouse hind limb was isolated after 16 hrs of drug administration. The muscle RNA was isolated and expression of mitochondrial specific chaperones and protease (*hspd1*, *hsp10*, *hsp75* and *clpp1*) were measured using QPCR. The bars show the average  $\pm$  SEM of 3 mice per group (\*\*\*,  $p < 0.001$ ).

mice. Each animal was weighted and observed for any signs of toxicity during and after the administration of the drug. The mice were weighted and sacrificed 24 hrs after drug administration. The quadriceps muscles from mice hind leg was isolated and stored in RNA stabilizing solution (RNAlater $\rightarrow$  from Ambion).

#### RNA isolation from muscle tissue and Quantative PCR (QPCR)

The RNA from muscle tissue was isolated using a Qiagen RNeasy mini spin column kit and following the manufacturer's instructions (Qiagen). Briefly, ~50-100 mg of the muscle tissue was homogenized with a mechanical homogenizer in 1 ml of TRIzol reagent (Invitrogen 15596-026). The homogenized tissue samples were centrifuged to pellet debris, and a chloroform extraction of the supernatant was performed to remove proteins prior to loading the column for RNA purification. DNase treatment of the RNA sample was also carried out to eliminate trace DNA contaminations.

cDNA from the isolated muscle tissue RNA was then synthesized using an ABI high-capacity cDNA RT kit and the QPCR was performed using a 5  $\times$  HOT FIREPOL EvaGreen qPCR Mix; Cat no. 08-36-00008) from SOLIS BIODYNE. The CT value of the target genes in each sample is normalized with the CT value of the internal house-keeping gene (Gusb). The

normalized expression value of target genes for each individual sample (treated and untreated) for the same drug treatment was also normalized to the average expression value of the untreated samples. The bar graphs therefore represent the mean fold change in expression of the individual target genes in treated and untreated group for a same treatment group (n=3).

The following primer pairs were used to monitor the expression of UPR<sup>mt</sup> response genes:

Gusb-F: CCGATTATCCAGCGAGTATG  
 Gusb-R: CTCAGCGGTGACTGGTTCG (Gusb was used as a reference house-keeping gene)  
 mCLPP1/F: GCCTTGCCGTGCATTTCTC  
 mCLPP1/R: CTCCACCACTATGGGATGA  
 omHSP10/FOR1: AGTTTCTTCCGCTCTTTGACAG  
 omHSP10/REV1: TGCCACCTTTGGTTACAGTTTC  
 omHSP75-F4: CCTGGGACCAAATAATCATCCA  
 omHSP75-R4: CCATTAAGGTACAAGGGGAAGC  
 omHSPD1-FOR1: CACAGTCCTTCGCCAGATGAG  
 omHSPD1-REV1: CTACACCTTGAAGCATTAAAGGCT

#### Statistics

Unless stated otherwise, data points in graphs and columns in histograms show the average (n > 20), error bars show the SEM, and significant differences were determined using Student's t-test.

## 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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