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Prolyl-peptidyl isomerase, Pin1, phosphorylation is compromised in association with the expression of the HFE polymorphic allele, H63D

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ABSTRACT

There is substantial interest in HFE gene variants as putative risk factors in neurodegenerative diseases such as Alzheimer disease (AD). Previous studies in cell models have shown the H63D HFE variant to result in increased cellular iron, oxidative stress, glutamate dyshomeostasis, and an increase in tau phosphorylation; all processes thought to contribute to AD pathology. Pin1 is a prolyl-peptidyl *cis/trans* isomerase that can regulate the dephosphorylation of the amyloid and tau proteins. Hyperphosphorylation of these later proteins is implicated in the pathogenesis of AD and Pin1 levels are reportedly decreased in AD brains. Because of the relationship between Pin1 loss of function by oxidative stress and the increase in oxidative stress in cells with the H63D polymorphism it was logical to interrogate a relationship between Pin1 and HFE status. To test our hypothesis that H63D HFE would be associated with less Pin1 activity, we utilized stably transfected human neuroblastoma SH-SY5Y cell lines expressing the different HFE polymorphisms. Under resting conditions, total Pin1 levels were unchanged between the wild type and H63D HFE cells, yet there was a significant increase in phosphorylation of Pin1 at its serine 16 residue suggesting a loss of Pin1 activity in H63D variant cells. To evaluate whether cellular iron status could influence Pin1, we treated the WT HFE cells with exogenous iron and found that Pin1 phosphorylation increased with increasing levels of iron. Iron exposure to H63D variant cells did not impact Pin1 phosphorylation beyond that already seen suggesting a ceiling effect. Because HFE H63D cells have been shown to have more oxidative stress, the cells were treated with the antioxidant Trolox which resulted in a decrease in Pin1 phosphorylation in H63D cells with no change in WT HFE cells. In a mouse model carrying the mouse equivalent of the H63D allele, there was an increase in the phosphorylation status of Pin1 providing *in vivo* evidence for our findings in the cell culture model. Thus, we have shown another cellular mechanism that HFE polymorphisms influence; further supporting their role as neurodegenerative disease modifiers.

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1. Introduction

Alzheimer disease (AD) is a neurodegenerative disease that results in cognitive deficiencies along with neuropathological changes including accumulation of amyloid-beta (Aβ) plaques and neurofibrillary tangles (NFT) [1–3]. Numerous studies have discovered that biometals such as iron, copper, and zinc can directly impact AD pathological markers [4–6]. Iron has been shown to directly regulate amyloid precursor protein (APP) synthesis via an iron responsive element in the 5′ UTR of APP mRNA [7]. Furthermore, iron and other metals have been found in neuritic plaques and also influence amyloid-beta (Aβ) aggregation [5,8–10]. The aggregation of tau protein leading to NFT pathology is also impacted by iron [11,12]. It is clear from these data that metal homeostasis in the brain is essential for healthy brain aging and that identification of genetic and environmental factors that may disrupt the biometal homeostasis is critical to identifying pathogenic mechanisms leading to neurodegenerative processes.

One such factor that combines gene and environment interaction is *HFE*. The gene is located on chromosome 6 and has been investigated in numerous genetics studies as a possible risk factor for developing or modifying AD onset [13]. HFE protein is a major histocompatibility class-1 like molecule that is reported to be involved in iron regulation [14] and innate immunity [15,16]. One of the reported functions of HFE protein is to complex with transferrin receptor (TfR) at the cell membrane to decrease TfR affinity for iron uptake [14]. When the H63D HFE variant is expressed, the ability to limit iron uptake is lost, resulting in increased cellular iron [14,17]. The elevated cellular iron levels associated with the H63D can lead to oxidative stress [17,18] and exacerbate the inflammatory response of macrophages [19]. Recently, we have reported that cells carrying the H63D HFE have alterations in glutamate homeostasis and tau phosphorylation [20,21]. These

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proposed functions of HFE and the data on H63D HFE are directly relevant to AD pathogenesis mechanisms that involve iron, oxidative stress, and neuroinflammation.

The prolyl-peptidyl isomerase Pin1 can affect numerous cellular mechanisms, such as protein localization, protein interactions, protein dephosphorylation, transcription activity, enzymatic activity, protein stability, and cell cycle regulation [22–24]. Of specific interest to AD, Pin1 can regulate the phosphorylation of APP and tau at the threonine 668 and threonine 231 residues, respectively [25–28]. A deficiency in Pin1 expression and/or activity appears to lead to the accumulation of Aβ fragments and NFT formation suggesting that this enzyme could be a key regulator of proteins involved in AD pathology [26,28,29]. Moreover, a study investigating mild cognitively impaired (MCI) patients found Pin1 to be oxidatively modified and suggested that this change could impact progression to develop AD [30]. Given the interest in and potential for H63D variants of HFE to increase the risk of AD [13], we examined the impact of H63D on Pin1 in a cell line and newly developed animal model. Specifically, we hypothesized that the presence of H63D HFE would be associated with decreased Pin1 activity.

2. Materials and methods

2.1. Chemicals and antibodies

Cell culture reagents including DMEM/F12, DMEM, pen/strep/glutamine and Geneticin were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum was purchased from Gemini Bio-Products (West Sacramento, CA, USA). DC protein assay was obtained from Bio-Rad (Hercules, CA, USA). A rabbit polyclonal Pin1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A rabbit polyclonal Pin1 (serine 16) antibody was purchased from Cell Signaling Technology (Beverly, MA, USA).

2.2. Cell culture

Human neuroblastoma SH-SY5Y cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). Human neuroblastoma SH-SY5Y cells were stably transfected to express wild type and H63D HFE forms as previously reported along with a vector alone control [31]. We have previously reported that these cells were chosen because endogenous expression of HFE could not be detected. The transfected cells were maintained in DMEM/F12 media supplemented with 10% FBS, 1% antibiotics (pen–strep–glutamine), 1× nonessential amino acids, and 1.8 g/L sodium bicarbonate. Cells were differentiated with 10 μM all-*trans* retinoic acid (Sigma-Aldrich, St. Louis, MO, USA) over six days [32]. To evaluate cellular iron effects, cells were treated with ferric ammonium citrate (FAC) or desferrioxamine (DFO) over 48 h (Sigma-Aldrich, St. Louis, MO, USA) [17].

2.3. Cell lysate preparation

Cells were lysed with RIPA buffer supplemented with 1% Triton X-100 and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Phosphatase inhibitor (Sigma-Aldrich, St. Louis, MO) was included in cell lysis buffer for phosphorylation protein detection. Cell extracts were spun at $8000 \times g$ for 10 min. Total protein levels were determined by Bio-Rad DC protein assay.

2.4. Enzyme-linked immunoabsorbant assay (ELISA)

The phosphorylation of APP at threonine 668 was determined using a DuoSet IC ELISA assay (R & D Systems, Minneapolis, MN, USA). An immobilized capture antibody for multiple APP isoforms that binds phosphorylated and unphosphorylated protein was used to coat the wells of a 96 well microplate overnight. Phosphorylated APP threonine 668 protein standards were added to achieve a standard curve to determine the specific amount of phosphorylated APP in unknown HFE cell samples. A biotinylated detection antibody recognizing APP threonine 668 was used to detect phosphorylated APP using standard streptavidin-HRP. The ELISA assay plates were read at 450 nm and 540 nm to correct for optical imperfections. All solutions used throughout were provided or prepared according to the manufacturer's recommendations. This ELISA experiment was performed using samples in triplicate per genotype at two dilution concentrations along with the known standards for the phosphorylated APP threonine 668 proteins, resulting in a total of six samples for analysis.

A monoclonal antibody specific for human tau phosphorylated residue threonine 231 was coated onto the wells of the microtiter strips provided (Invitrogen, Carlsbad, CA, USA). Standards of known phosphorylated tau threonine 231 proteins were processed to achieve a standard curve to determine the specific amount of phosphorylated protein in the unknown HFE cell samples. The ELISA assay plate was read at 450 nm. This ELISA experiment was performed using samples in triplicate per genotype at two dilution concentrations along with the known standards for the phosphorylated tau threonine 231 proteins, resulting in a total of six samples for analysis.

2.5. Western blot

Cells lysates were obtained as described above. Twenty-five to forty μg total protein was equally separated by electrophoresis in a 4–20% 12-well Criterion gel (Bio-Rad, Hercules, CA). Protein was then transferred to a nitrocellulose membrane and blocked for 1 h at room temperature in TBS-T with 5% nonfat milk or 1.5% BSA (phosphorylated protein detection). Membranes were probed with primary antibodies in TBS-T with 5% nonfat milk overnight at 4 °C. The membranes were incubated with antibodies specific for total Pin1 (1:1000), Pin1 serine-16 (1:500), and β-actin (1:5000). HRP-conjugated secondary antibodies were added in 5% nonfat milk for 1 h at room temperature. Protein signals were obtained by chemiluminescence and visualized by CCD camera. All western blot experiments were repeated at least twice with a minimum of four different cultures per genotype per experiment, resulting in a total of eight samples for analysis. The bands on the western blot were quantified by densitometry using Fuji MultiGauge analysis software.

2.6. Mouse model

H67D knock-in mice of mixed C57BL6×129SvEv genetic background were developed similar to the H67D mouse model of Tomatsu et al. [33]. Homozygote H67D and wild type mice were generated by intercrossing H67D heterozygote mice. Genotype was confirmed by PCR analysis. All of these mice were maintained under normal housing conditions in accordance with Penn State University's IUCAC policy for animal use, which is in agreement with the NIH Guide for the Care and Use of Laboratory Animals. Male and female mice at 6 months of age were examined. Protein for biochemical analysis was obtained from mice brain removal after the mice were anesthetized.

2.7. Statistical analysis

The Student's *t*-test was used for analyzing HFE variant comparisons when one variable was being determined. Experimental data where samples were treated with various agents (i.e. iron or desferrioxamine) and compared with controls were analyzed by one-way analysis of variance. Differences among the means were considered statistically significant when the p value was <0.05. If overall $p<0.05$, Tukey's multiple comparison post-hoc analysis was performed. Data are presented as the mean ± S.E.; GraphPad Prism software (version 4.0) was utilized to perform the statistical analysis.

3. Results

3.1. HFE effects on Pin1 levels and phosphorylation

The first study was designed to examine total Pin1 protein expression as a function of genotype. No significant differences were found (Fig. 1). The function of Pin1 can be altered by its phosphorylation state at its serine 16 residue, which is located at the center of the phosphorylated serine/threonine-proline binding pocket [34,35]. Therefore we used the analysis of phosphorylated Pin1 as a surrogate marker for Pin1 activity. The phosphorylation of Pin1 at its serine 16 residue was significantly increased (30%) in cells expressing the H63D variant ($p<0.01$) compared to the vector and wild type HFE cells (Fig. 1). These data would suggest that there is approximately a 30% decrease in the activity of Pin1 in H63D cells.

3.2. The impact of cellular iron on Pin1

Because HFE is involved in regulating cellular iron status and we have shown that there is more iron in the labile iron pool in cells carrying the H63D variant, the second study was to determine if iron could impact Pin1 phosphorylation status. Upon, treating the wild type HFE cells with ferric ammonium citrate (FAC), total Pin1 protein expression was unaffected (Fig. 2A) but the phosphorylation of Pin1 at serine 16 increased in a dose-dependent manner (Fig. 2B). However, any differences in Pin1 phosphorylation in the H63D carrying cells treated with FAC did not reach statistical significance at $p<0.05$ at the same concentrations of iron provided to WT cells (Fig. 2B).

3.3. Removal of iron affects Pin1 phosphorylation in HFE H63D cells

To continue to evaluate the sensitivity of Pin1 phosphorylation to iron availability, WT and H63D cells were treated with the iron chelator desferrioxamine (DFO). Pin1 phosphorylation was unaffected in the WT HFE cells by iron chelation but was decreased with 10 μM DFO treatment ($p<0.01$) in the H63D HFE cell lines (Fig. 3).

3.4. Antioxidant affects Pin1 phosphorylation in HFE H63D cells

Increased cellular iron can result in oxidative stress. There is evidence of oxidative stress impacting the cellular activity of Pin1 [30,36,37] and we have shown increased indices of oxidative stress in H63D expressing cells [17]. Therefore, we treated the cells expressing the HFE variants with Trolox, a vitamin E analog to decrease cellular oxidative stress. Pin1 phosphorylation decreased approximately 20% with Trolox treatment in H63D cells ($p=0.0257$), but was unchanged in the control WT expressing cells treated with Trolox (Fig. 4).

3.5. HFE and Pin1 Alzheimer's disease protein substrates

Pin1 has been shown to regulate the phosphorylation of AD related proteins [25,26,28]. Therefore, APP phosphorylation at its threonine 668 residue was measured in HFE cells by enzyme-linked immunoabsorbant assay (ELISA). APP phosphorylation was increased in vector cells by approximately 65% ($p<0.01$) compared to cells expressing wild type HFE (Fig. 4). Cells expressing the H63D variant had 50% less APP threonine 668 phosphorylation ($p<0.05$) with respect to WT HFE cells (Fig. 5). Tau threonine 231 phosphorylation was evaluated by ELISA and was decreased in vector cells ($p<0.01$) by 42% and H63D expressing cells ($p<0.05$) by 33% compared to cells expressing WT HFE (Fig. 5).

3.6. Pin1 levels and phosphorylation in an H67D transgenic mouse model

To further assess the impact of H63D HFE on Pin1, we examined Pin1 levels and phosphorylation *in vivo*. We evaluated the H67D HFE mice, which express an HFE point mutation H67D that is homologous to human H63D [33]. Total Pin1 levels were not changed among the mice expressing an H67D allele compared to wild type mice (Fig. 6). Pin1 phosphorylation levels at its serine 16 residue were significantly increased in H67D homozygous mice ($p<0.05$) and H67D heterozygous mice ($p<0.05$) compared to wild type mice, respectively (Fig. 6). These data further support our cell culture findings demonstrated in this manuscript.

4. Discussion

The H63D variant of HFE gene is under examination as a risk factor for neurodegenerative diseases [13]. We have established a cell model in which to directly examine the impact of HFE polymorphisms on an otherwise homogenous genetic background in a controlled culture condition. The controlled conditions are essential to understanding

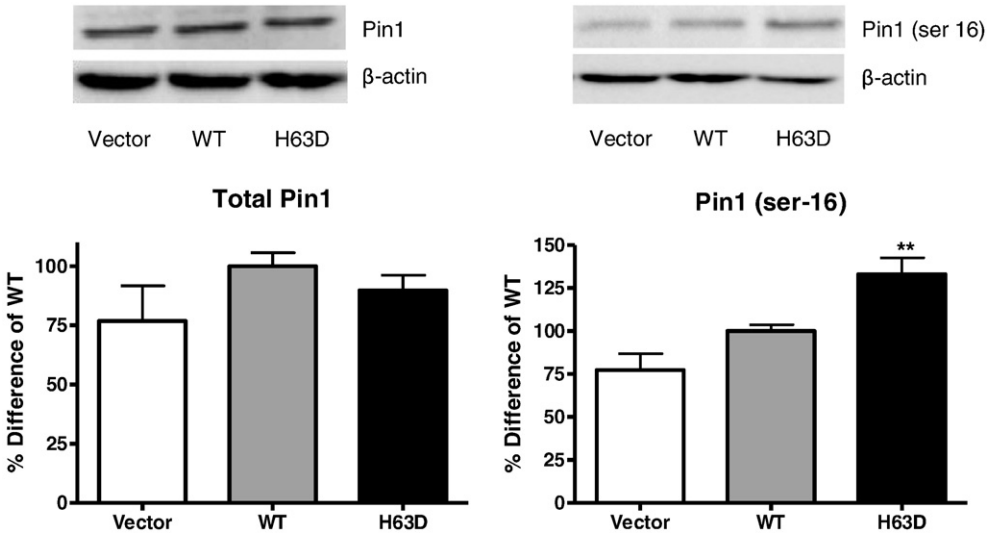

Fig. 1. Pin1 levels and phosphorylation. Expression of total Pin1 protein and Pin1 activity determined by its serine 16 phosphorylation was measured in an HFE polymorphism stably transfected SH-SY5Y cell line by western blot. The data show that there were no significant differences in total Pin1 protein levels. Pin1 phosphorylation was significantly increased in H63D expressing cells ($p<0.01$) compared to cells containing wild type HFE. Experiments were performed with a minimum of four different cultures per genotype. Representative western blot images are shown with graphs displaying differences in expression determined by densitometric analysis. One-way ANOVA was performed to analyze the data followed by Tukey's post-hoc analysis. Data are represented as mean ± S.E. The symbol ** ($p<0.01$) indicates a significant difference from wild type HFE.

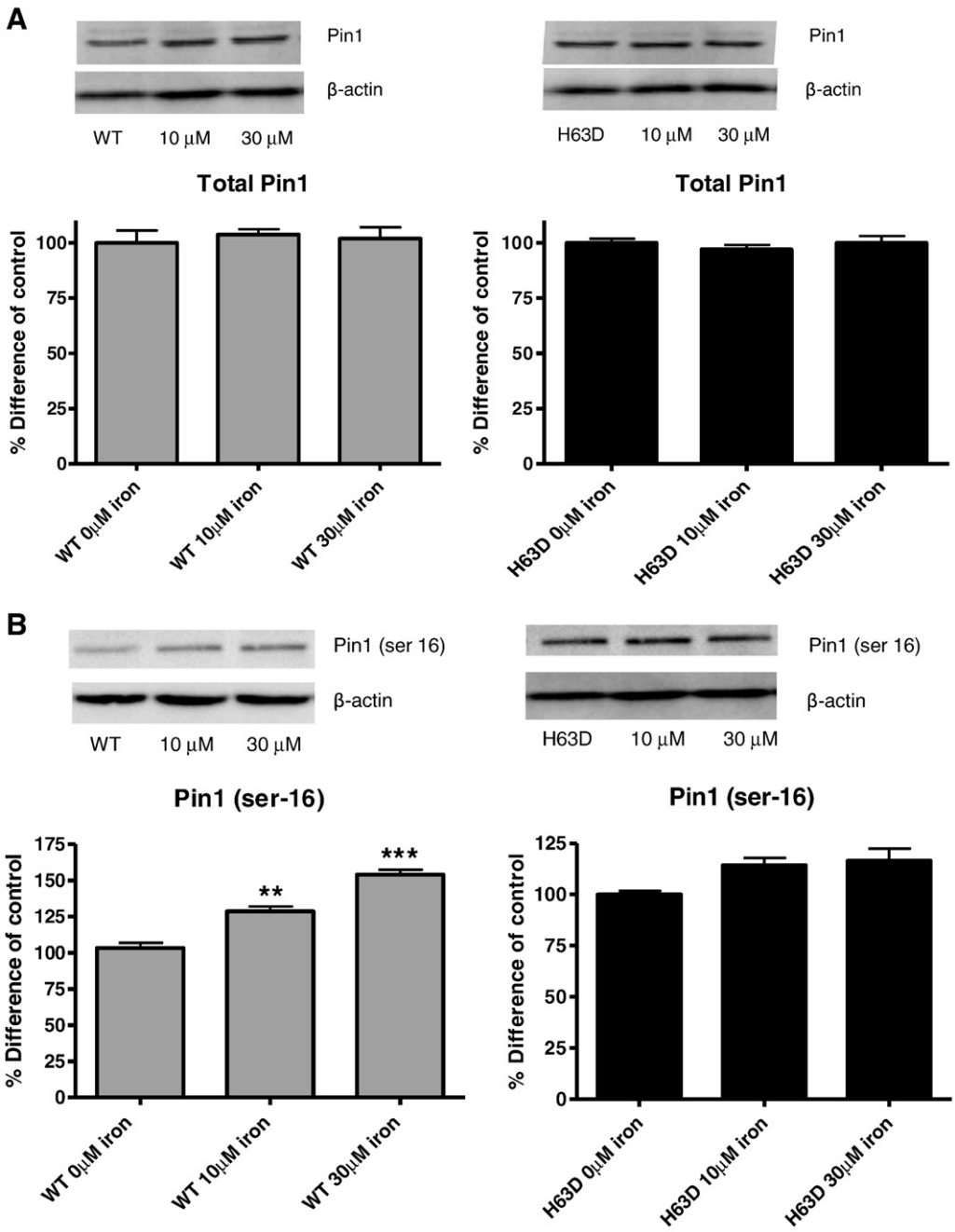

Fig. 2. Cellular iron effects on Pin1 expression and phosphorylation. We added increasing amounts of ferrous ammonium citrate (FAC) to cells expressing wild type and H63D HFE. Total Pin1 protein levels were not changed with iron treatments (A). Pin1 activity decreased with increasing amounts of iron in a dose-dependent fashion in wild type cells (10 μM, $p<0.01$) and (30 μM, $p<0.001$) as indicated by an increase in Pin1 serine 16 phosphorylation (B). Additionally, we further challenged the H63D cells by treating them with iron in the form of FAC. H63D expressing cells did not achieve a statistical significant increase in Pin1 serine 16 phosphorylation with iron treatments (B). Experiments were performed with a minimum of four different cultures per genotype. Representative western blot images are shown with graphs displaying differences in expression determined by densitometric analysis. One-way ANOVA was performed to analyze the data followed by Tukey's post-hoc analysis. Data are represented as mean ± S.E. The symbols ** ($p<0.01$) and *** ($p<0.001$) indicate significance from the respective non-treated group.

the contribution of HFE gene variants to neurodegenerative disease because of the likelihood of gene/environment interaction given the availability of iron in the environment. Our working hypothesis for H63D HFE gene variants and neurodegenerative disorders is that the H63D allelic variants does not in itself cause disease but creates a permissive or enabling cellular milieu for pathogenic agents. We have previously reported that expression of the H63D HFE variant in stably transfected SH-SY5Y cells results in increased cellular stress [17], altered glutamate homeostasis [21], and increased tau phosphorylation [20]. Oxidative stress and glutamate excitotoxicity are indirectly thought to contribute to neurodegeneration in AD whereas tau phosphorylation is more directly implicated as part of the pathogenesis of AD [38,39]. In this study, we extend these observations to show that Pin1, an enzyme responsible for regulating phosphorylation of amyloid and tau, is altered in cells expressing H63D HFE and in a mouse model expressing H67D the human equivalent of the H63D gene variant.

Total Pin1 expression levels were found to be unchanged between the HFE polymorphisms and the transfection vector control. Pin1 phosphorylation at serine 16 was significantly increased in cells that expressed the H63D variant compared to wild type HFE cells implying that Pin1 activity is decreased. We expanded the cell culture results

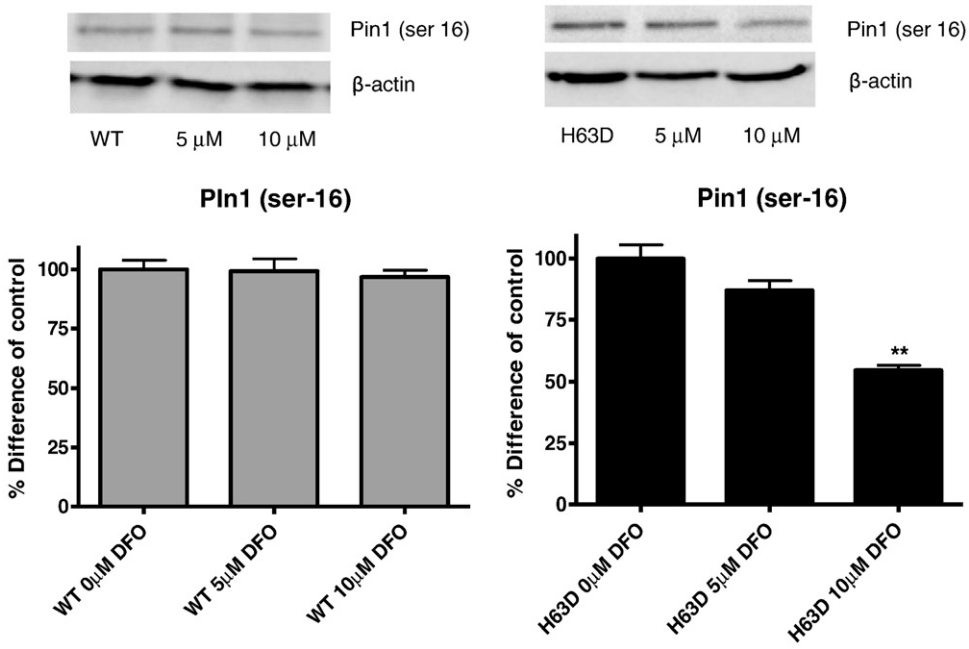

Fig. 3. Iron chelation and Pin1 phosphorylation. Increasing amounts of the iron chelator desferrioxamine (DFO) were added to cells expressing wild type and H63D HFE. Pin1 activity was measured by western blot via phosphorylation of Pin1 at serine 16. Pin1 activity was not altered when WT cells were treated with DFO. DFO treatment of H63D cells resulted in an increase in Pin1 activity as indicated by a decrease in phosphorylation of Pin1 at serine 16. At 5 μM DFO, there was not a significant difference compared to the non-treated H63D group. Upon treating the H63D cells with 10 μM DFO, there was a significant decrease ($p<0.01$) in Pin1 phosphorylation. Experiments were performed with a minimum of four different cultures per genotype. Representative western blot images are shown with graphs displaying differences in expression determined by densitometric analysis. One-way ANOVA was performed to analyze the data followed by Tukey's post-hoc analysis. Data are represented as mean ± S.E. The symbol ** ($p<0.01$) indicate significance from the respective non-treated group.

into evaluating total Pin1 and phosphorylated Pin1 in a mouse model expressing the equivalent of the H63D HFE polymorphism. The data from the H67D HFE mice studies showed similar results to the cell model as total Pin1 levels were unchanged and Pin1 phosphorylation levels increased with expression of an H67D allele.

To determine the cause of the increased phosphorylation of Pin1, we examined the cellular iron status and oxidative stress because of the previous literature indicating a relationship between oxidative stress and H63D and the vulnerability of Pin1 to oxidative stress. H63D HFE cells have more labile iron in the cytosol [17,19] than WT HFE cells. To determine if the higher iron content was potentially related to the increased phosphorylation of Pin1, we treated the wt cells with increasing amounts of iron which resulted in a dose-dependent increase in phosphorylation of Pin1. We also challenged the H63D HFE cells with more iron to determine if we could further increase the phosphorylation of Pin1 but increasing amounts of iron did not significantly increase Pin1 phosphorylation suggesting a ceiling effect. Chelating iron resulted in a reduction of Pin1 phosphorylation in the H63D HFE cells but not in the WT HFE expressing cells. The cellular iron effects on Pin1 based on HFE

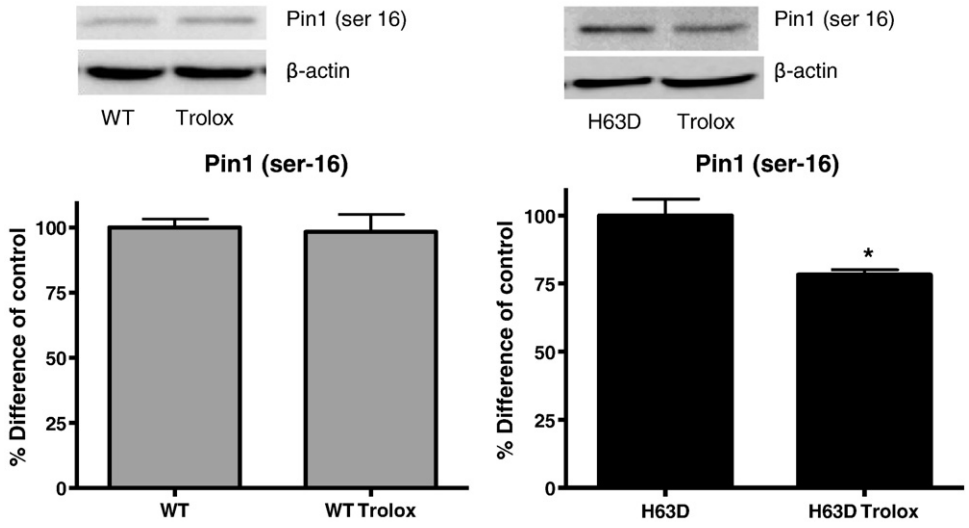

Fig. 4. Trolox treatment and Pin1 phosphorylation. HFE polymorphism stably transfected SH-SY5Y cells expressing wild type and H63D variant were treated for 72 h with 200 μM Trolox, a water-soluble vitamin E analog to assess the effect of oxidative stress on Pin1 activity determined by western blot. Trolox treatment had no effect on WT HFE cells. There was an increase in Pin1 activity upon treating H63D cells with Trolox as evidenced by a decrease in Pin1 serine 16 phosphorylation ($p=0.0257$). Student's *t*-test was performed to analyze the data; data are represented as mean ± S.E. The symbol * ($p<0.05$) indicates a significant difference from baseline H63D HFE.

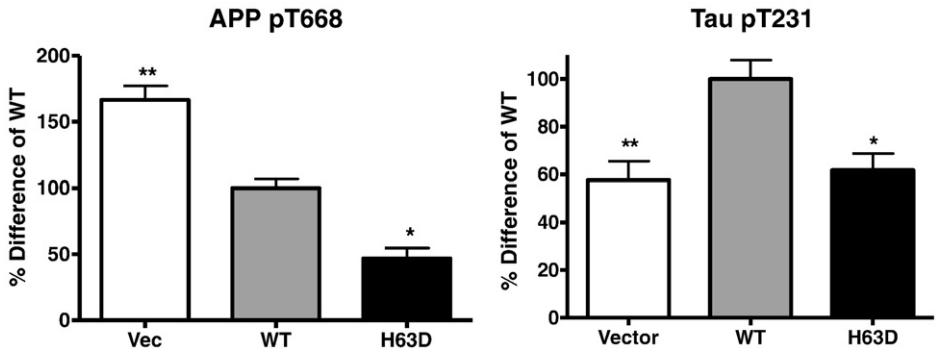

Fig. 5. HFE effects on Alzheimer disease Pin1 substrates. The phosphorylation of amyloid and tau proteins at threonine 668 (t668) and threonine 231 (t231), respectively, has been shown to be impacted by Pin1 activity. APP t668 protein levels were increased in vector cells ($p<0.01$) compared to wild type HFE cells determined by an ELISA assay. APP t668 levels were decreased in H63D expressing cells ($p<0.05$) compared to WT HFE cells. Tau t231 protein levels were decreased in vector ($p<0.01$) and H63D cells ($p<0.01$) with respect to cells expressing wild type HFE. The vector only cells are a control for transfection, but because the cells do not express detectable HFE [17] they are not the appropriate comparison for the effect of the H63D allelic variant. Experiments were performed with a minimum of four different cultures per genotype. One-way ANOVA was performed to analyze the data followed by Tukey's post-hoc analysis. Data are represented as mean ± S.E. The symbols * ($p<0.05$) and ** ($p<0.01$) indicate a significant difference from wild type HFE.

genotype are consistent with a higher labile iron pool in the H63D cells that is available to alter phosphorylation status of Pin1 and can be mimicked by elevating the iron status in the WT HFE cells.

The iron effect on Pin1 could be indirect due to oxidative stress through the Fenton reaction [4,40]. Higher indices of oxidative stress associated with the H63D HFE allele have been demonstrated in our cell line and human patient data [17,18,41]. To determine the role of oxidative stress, we treated the HFE polymorphism carrying cells with the antioxidant Trolox, a vitamin E analog. In the WT HFE cells, there was not a change in Pin1 phosphorylation following Trolox exposure but Trolox treatment in the H63D cells resulted in a decrease in Pin1 serine 16 phosphorylation. These data suggest that Pin1 activity, under resting conditions in the presence of H63D HFE is affected by oxidative stress. Our data are consistent with the findings of other groups [30,36,37] showing that Pin1 can be impacted by oxidative stress.

The increase in Pin1 phosphorylation at serine 16 in association with the H63D HFE mutation could lead to cellular changes associated with AD, specifically the inability to dephosphorylate APP and tau proteins [25,27,42]. Pin1 is an intracellular regulator of amyloid and tau protein phosphorylation at the APP threonine 668 and tau threonine 231 amino acid residues, which appear to be important to the pathological generation of Aβ plaques and tangles [26,28,29]. To evaluate the consequence of altered Pin1 function in H63D variant cells, we determined the phosphorylation of APP threonine 668 and tau threonine 231 levels in HFE expressing cells. Surprisingly, we found a significant decrease in APP phosphorylation in H63D cells compared to cells expressing wild type HFE. Furthermore, a significant reduction at the tau threonine 231 residue occurred in the H63D expressing cells, consistent with previous findings [20]. These data are not consistent with the reduction in Pin1 activity in H63D variant cells. The apparent inconsistency may be explained by our previous report of a reduction in cyclin-dependent kinase 5 (cdk-5) expression and activity in H63D polymorphism expressing cells [20]. Cdk-5 has been shown to regulate the phosphorylation of these proteins at the

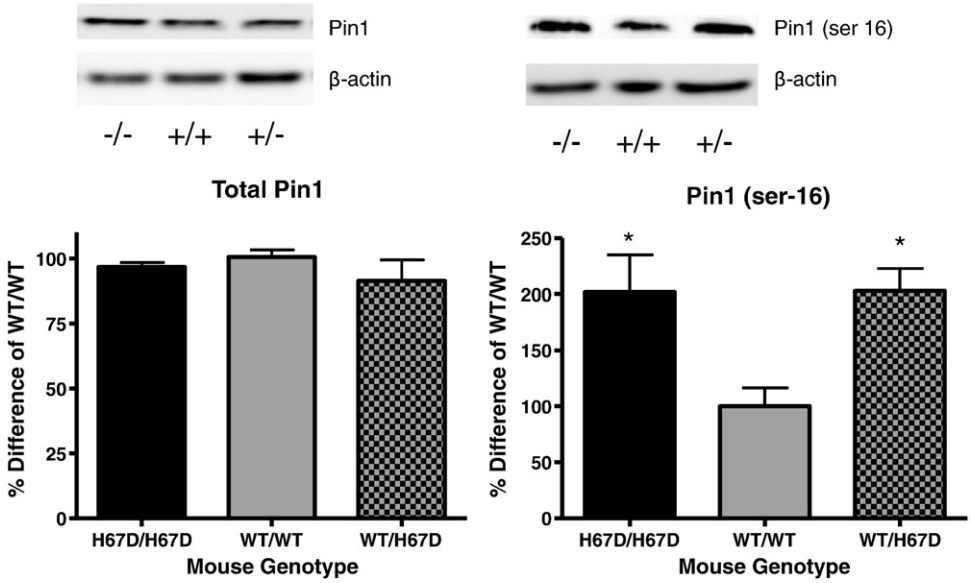

Fig. 6. Pin1 levels and phosphorylation in an H67D transgenic mouse model. Total Pin1 protein and phosphorylation levels were determined in 6 month old H67D knock-in mice. The mouse H67D point mutation is homologous to the human H63D polymorphism. There were no significant differences in total Pin1 protein levels among homozygous H67D/H67D (−/−), heterozygous H67D (+/−), and wild type (+/+) HFE mice. Pin1 phosphorylation levels at its serine 16 residue were significantly increased in H67D homozygous mice ($p<0.05$) and in H67D heterozygous mice ($p<0.05$) compared to wild type mice, respectively. Experiments were performed with a minimum of three mice per genotype ($n=3$). Data are represented as mean ± S.E. The symbol * ($p<0.05$) indicates a significant difference from the WT (+/+) HFE mice. Representative immunoblot images are shown with graphs displaying differences in expression determined by densitometric analysis. One-way ANOVA was performed to analyze the data followed by Tukey's post-hoc analysis. Data are represented as mean ± S.E. The symbol * ($p<0.05$) indicates a significant difference from wild type HFE mice.

threonine 668 and threonine 231 specific sites of APP and tau, respectively [38,43–46]. Furthermore, the indirect evidence of decreased Pin1 activity data in the H63D cells are consistent with the increased GSK-3β activity associated with expressing the H63D variant [20]. Min et al. showed that lithium inhibition of GSK-3β resulted in an increase in Pin1 activation suggesting that regulating GSK-3β may affect Pin1's ability to dephosphorylate its substrates such as tau [47]. These data, in association with our findings that Trolox effects on Pin1 in this study are affected by H63D HFE expression are compelling evidence that the HFE polymorphism should be considered when evaluating treatment strategies in neurodegenerative diseases. We conclude that the discovery of HFE, implicated as a putative risk factor for neurodegenerative disease such as AD can impact Pin1 is clinically meaningful and given the abundance of iron in the diet and environment, further investigation is warranted into the gene–environment interaction between HFE polymorphisms and iron.

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