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c-Abl Stabilizes HDAC2 Levels by Tyrosine Phosphorylation Repressing Neuronal Gene Expression in Alzheimer's Disease

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SUMMARY

In Alzheimer's disease (AD), there is a decrease in neuronal gene expression induced by HDAC2 increase; however, the mechanisms involved are not fully elucidated. Here, we described how the tyrosine kinase c-Abl increases HDAC2 levels, inducing transcriptional repression of synaptic genes. Our data demonstrate that (1) in neurons, c-Abl inhibition with Imatinib prevents the AβO-induced increase in HDAC2 levels; (2) c-Abl knockdown cells show a decrease in HDAC2 levels, while c-Abl overexpression increases them; (3) c-Abl inhibition reduces HDAC2-dependent repression activity and HDAC2 recruitment to the promoter of several synaptic genes, increasing their expression; (4) c-Abl induces tyrosine phosphorylation of HDAC2, a posttranslational modification, affecting both its stability and repression activity; and (5) treatment with Imatinib decreases HDAC2 levels in a transgenic mice model of AD. Our results support the participation of the c-Abl/HDAC2 signaling pathway in the epigenetic blockade of gene expression in AD pathology.

INTRODUCTION

In AD the expression of neuronal genes decreases (Ginsberg et al., 2012). Although the molecular mechanisms are not fully understood, recent evidence suggests the contribution of epigenetic changes, such as histone acetylation, in this process (Fischer et al., 2007; Ricobaraza et al., 2009; Govindarajan et al., 2011; Gräff et al., 2012). In fact, the increase in histone acetylation induced by treatment with histone deacetylase (HDAC) inhibitors prevents cognitive deficit and behavioral impairments in AD mice models. This effect was associated with increased expression of genes involved in synaptic plasticity and memory consolidation in the hippocampus (Fischer et al., 2007; Francis et al., 2009; Kilgore et al., 2010; Zhang and Schluesener, 2013). Thus, the evidence suggests that HDACs are involved in AD pathology.

HDACs are a group of enzymes that remove the acetyl groups on lysine residues of histones, inducing gene repression (Bannister and Kouzarides, 2011; Kouzarides, 2007). HDAC2, a member of the Rpd3/Hda1 family (class I), is the catalytic subunit with deacetylase activity of multiprotein repressor complexes, such as Sin3A/Sin3B (Laherty et al., 1997), NuRD (Zhang et al., 1998), and CoREST (You et al., 2001). HDAC2 seems to be relevant in the differentiation, function, and pathology of neurons (Guan et al., 2009; Jawerka et al., 2010; Gräff et al., 2012). HDAC2 is expressed at low levels in neuronal progenitors during brain development, but its expression is induced during the process of neuronal differentiation, constituting the main HDAC class I in the adult brain (MacDonald and Roskams, 2008). *Hdac2* knockout (KO) mice do not exhibit obvious brain defects (Montgomery et al., 2007); however, when expression of *Hdac2* is absent in neurons, they show improved associative learning and increased synaptic plasticity (Guan et al., 2009; Morris et al., 2013). Moreover, these mice show increased expression of the synaptic marker synaptophysin and more dendritic spines in the CA1 region than wild-type mice (Guan et al., 2009). In contrast, HDAC2-overexpressing mice show cognitive impairments, decreased synaptic plasticity, fewer dendritic spines, and lower expression of synaptophysin than wild-type mice (Guan et al., 2009). Furthermore, HDAC2, but not HDAC1, is preferentially recruited to the promoters of neuronal-related genes (Guan et al., 2009).

These results support a main role of HDAC2 in transcriptional repression in adult neurons. In fact, HDAC2 is involved in the control of BDNF-induced genes (Nott et al., 2008), and increases in HDAC2 levels trigger the repression of CAMKII and CREB gene expression (Saiyed et al., 2011). Additionally, treatment with antipsychotics such as clozapine increases HDAC2 levels, which in turn reduces metabotropic glutamate receptor 2 (mGlu2) expression (Kurita et al., 2012).

On the other hand, the evidence shows that HDAC2 is increased in AD and that its activation worsens neuronal and

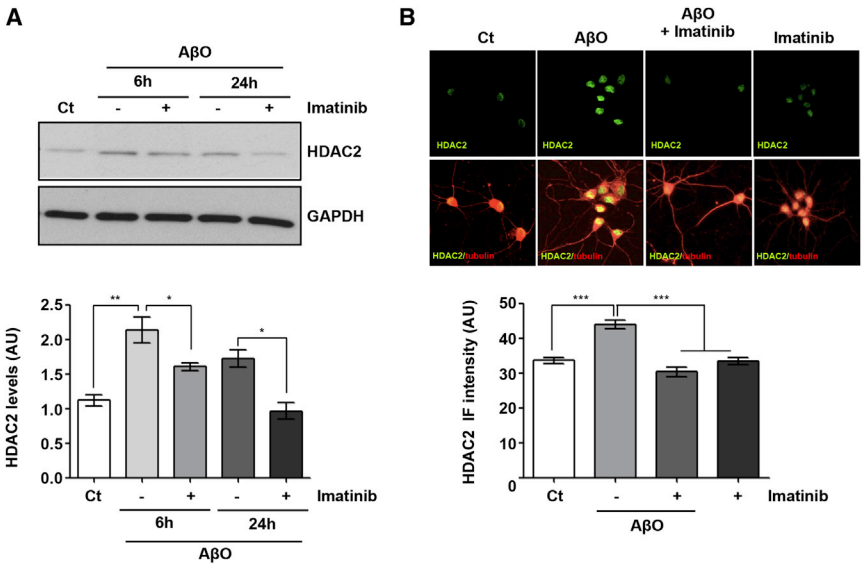

Figure 1. Imatinib Treatment Prevents the Increase in HDAC2 Levels Induced by AβO in Hippocampal Neurons

(A) Hippocampal neurons (7 DIV) were treated either with vehicle (control), AβO 5 μM, or AβO 5 μM plus Imatinib 5 μM for 6 or 24 hr. Representative western blot of HDAC2 protein levels and quantification normalized against GAPDH levels (n = 3).

(B) Representative immunofluorescence and quantification of HDAC2 (green) and tubulin (red) levels in neurons after 24 hr of imatinib treatment (n = 50–75 neurons per group). Results were obtained from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; values are mean ± SEM.

synaptic function. p25 transgenic mice, an AD mice model (Cruz et al., 2003; Cruz et al., 2006), show an increase in HDAC2 levels, but not in HDAC1 and HDAC3 levels, in the CA1 area of the hippocampus, (Gräff et al., 2012). Moreover, these AD animals have higher HDAC2 recruitment at promoters of key genes related with memory, learning, and synaptic plasticity compared to wild-type animals. Interestingly, the reduction of HDAC2 levels in p25 transgenic mice induces histone acetylation of the promoters, which activates the expression of the neuronal genes, restores synaptic plasticity, and alleviates cognitive deficit. Furthermore, other AD animal models, such as 5XFAD and APPswe/PSEN1ΔE9 mice, as well as rats injected with Aβ fibrils into the hippocampus, show increased HDAC2 levels in the cortex and hippocampus (Gräff et al., 2012; Bie et al., 2014). Finally, postmortem analysis of patients with varying degrees of nonfamiliar AD show increased HDAC2 levels in the hippocampus, whereas HDAC1 and HDAC3 levels remain unchanged (Gräff et al., 2012).

Thus, the evidence suggests that HDAC2 activity is increased in AD models; however, the molecular mechanisms triggering the increase in HDAC2 are not fully understood. Interestingly, COS-1 cells that overexpress the c-Abl tyrosine kinase exhibit decreased levels of acetylated histones H3 and H4. This effect is prevented with the HDAC paninhibitor TSA (Aoyama et al., 2011).

Interestingly, the c-Abl tyrosine kinase has been involved in AD pathology. We demonstrated that c-Abl is activated in both in vitro and in vivo AD models (Alvarez et al., 2004; Cancino et al., 2008). Treatment with Imatinib, a c-Abl-specific inhibitor, reduces neuronal loss, τ phosphorylation, cognitive impairments, and Aβ deposition in APPswe/PSEN1ΔE9 mice (Cancino et al., 2008, 2011). Recently, we demonstrated that c-Abl activation is involved in synapse loss and long-term potentiation inhibition induced by Aβ oligomers (AβO) (Vargas et al., 2014). In contrast, overexpressing the active form of c-Abl in adult mice brains induces severe neurodegeneration and inflammation (Schlatterer et al., 2011). Furthermore, patients with a clinical history of AD exhibit increased c-Abl activation in the hippocampal CA1 area (Jing et al., 2009). c-Abl is also involved in the neurodegenerative processes triggered by diseases such as Niemann Pick type C (Alvarez et al., 2008), Parkinson, (Ko et al., 2010), and amyotrophic lateral sclerosis (Katsumata et al., 2012).

The focus of this study is to elucidate the molecular mechanisms of HDAC2-triggered gene repression in AD models. Our findings show that c-Abl regulates the activity and protein levels of HDAC2 by tyrosine phosphorylation, suggesting that c-Abl might regulate neuronal gene expression through HDAC2.

RESULTS

c-Abl Regulates HDAC2 Levels in Neurons

In agreement with previous reports, we observed that AβOs induce an increase in HDAC2 levels in primary culture of hippocampal neurons (Gräff et al., 2012). Neurons at 7 DIV, exposed to 5 μM AβO for 6 hr and 24 hr, presented higher HDAC2 protein levels than controls (Figure 1A), which localized mainly in the nucleus (Figure 1B). Interestingly, cotreatment with the c-Abl inhibitor Imatinib prevented the AβO-induced increase in HDAC2 levels (Figures 1A and 1B). These results suggest that activation of c-Abl by Aβ aggregates (Alvarez et al., 2004; Cancino et al., 2008) could contribute to HDAC2 upregulation in AD models.

Imatinib also induced a reduction of HDAC2 levels in the hippocampal-like cell line HT22 and in HeLa cells. HT22 cells were treated with different concentrations of Imatinib or vehicle (DMSO). c-Abl inhibition significantly decreases HDAC2 protein levels (Figure 2A), although surprisingly, *HDAC2* mRNA levels do not change significantly (Figure 2D). Similar results were obtained in HeLa cells treated with Imatinib (Figures S1A and S1B available online).

To confirm that the effect of Imatinib over HDAC2 levels is mediated by c-Abl inhibition, we downregulated c-Abl expression using a shRNA against c-Abl (Figure 2B), and also overexpressed c-Abl using recombinant c-Abl-GFP (Figure 2C). HeLa cells were transfected with a plasmid expressing a shRNA against c-Abl or a scrambled shRNA, and HDAC2 levels were evaluated 48 hr later. The reduction in c-Abl levels was

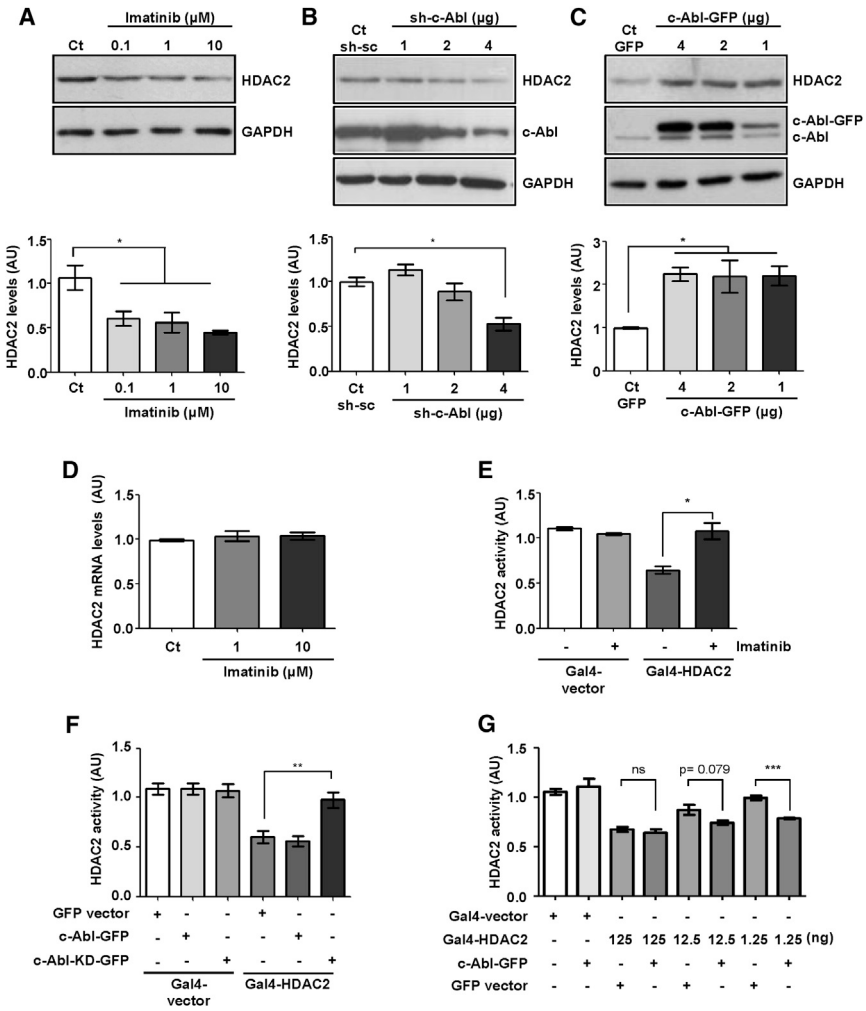

Figure 2. c-Abl Increases Protein Levels and Repression Activity of HDAC2

(A and B) Representative western blot and quantification of (A) HDAC2 protein levels of HT22 cells treated with Imatinib (0.1, 1, and 10 μM) for 24 hr (n = 3) and (B) HDAC2 expression of HeLa cells transfected with a plasmid expressing a shRNA against c-Abl or shRNA scramble as a control (n = 3).

(C) HT22 cells transfected with a plasmid expressing c-Abl-GFP or an empty vector (n = 3).

(D) Quantitative PCR of *HDAC2* mRNA in HT22 cells treated with Imatinib (1 and 10 μM) by 24 hr (n = 3). For HDAC2 repression activity, cells were transfected with a plasmid expressing Gal4-TK-luciferase, together with Gal4-HDAC2 or Gal4-vector (negative control). Luciferase activity was normalized against renilla.

(E–G) HDAC2 repression activity assays were as follows: (E) HT22 cells were treated with Imatinib 10 μM for 24 hr after transfection (n = 5). (F) HT22 cells cotransfected with Gal4-TK-luciferase and Gal4-HDAC2 plasmids plus the expression plasmids for c-Abl (c-Abl-GFP) or dominant-negative mutant of Abl (Abl-KD-GFP) or empty vector (GFP) (n = 3). (G) HT22 cells cotransfected with the Gal4-TK-luciferase and c-Abl plasmids (c-Abl-GFP) plus decreasing quantities of Gal4-HDAC2 plasmid. Results were obtained from three or more independent experiments. *p < 0.05; **p < 0.01; values are mean ± SEM.

associated with a significant decrease in HDAC2 levels (Figure 2B). Conversely, overexpression of c-Abl in HT22 cells caused a significant increase in HDAC2 levels (Figure 2C). Similar results were obtained in HeLa cells, where c-Abl overexpression increased HDAC2 protein levels while mRNA levels remained constant (Figures S2A and S2B).

Next, we evaluated the effects of c-Abl inhibition over HDAC2 function following HDAC2 transcriptional repressor activity. We used a plasmid expressing a Gal4-HDAC2 fusion protein, which represses the luciferase expression of a reporter plasmid with a Gal4 binding site upstream of the thymidine kinase promoter (Gal4-TK-luciferase) (Tsai and Seto, 2002). HDAC2 repression activity was assessed as the decrease in luciferase activity in cells transfected with the Gal4-HDAC2 recombinant plasmid versus a Gal4 empty vector. The treatment of HT22 cells and HeLa cells with Imatinib reduced Gal4-HDAC2-induced repression of luciferase gene in both cells types (Figures 2E and S3A). To rule out a direct effect of Imatinib over HDAC2 deacetylase activity, we conducted an in vitro assay measuring the deacetylase activity of immunoprecipitated Flag-HDAC2. The results showed that Imatinib treatment did not change the deacetylase activity of HDAC2 (Figure S3B). Thus, our data suggest that c-Abl inhibition decreases the transcriptional repressor activity of HDAC2.

To confirm that c-Abl regulates the HDAC2-dependent transcriptional repression, we cotransfected the Gal4-TK-luciferase plus Gal4-HDAC2 plasmids with either c-Abl-GFP, c-Abl–KD-GFP (kinase death dominant-negative form of c-Abl), or a GFP empty vector. The results show that Abl-KD-GFP expression inhibited the transcriptional repressor activity of Gal4-HDAC2 (Figure 2F), which is consistent with the Imatinib effect over HDAC2 activity. We were able to detect an increase in the HDAC2-dependent transcriptional repression induced by c-Abl-GFP using lower quantities of the Gal4-HDAC2 plasmid (1.25 ng; Figure 2G) than those used in the previous experiment (0.5 μg; Figure 2F). These results demonstrate that c-Abl regulates HDAC2 levels and that c-Abl inhibition decreases the transcriptional repression activity of HDAC2.

It has been described that the HDAC2 recruitment on promoters of key synaptic genes mediates their repression, affecting neuronal function and worsening cognitive decline in AD models (Gräff et al., 2012; Bie et al., 2014). We evaluated the recruitment of HDAC2 on some of these neuronal promoter genes by chromatin immunoprecipitation assay. Figure 3A shows that in the HT22 cells, HDAC2 is recruited to the promoters of *Synaptotagmin*, *NR2a* (*Grin2a*), and *GluR1* (*Gria1*). Interestingly, HDAC2 recruitment on the promoters of *Synaptotagmin*, *NR2a*, and *GluR1* was significantly reduced with Imatinib

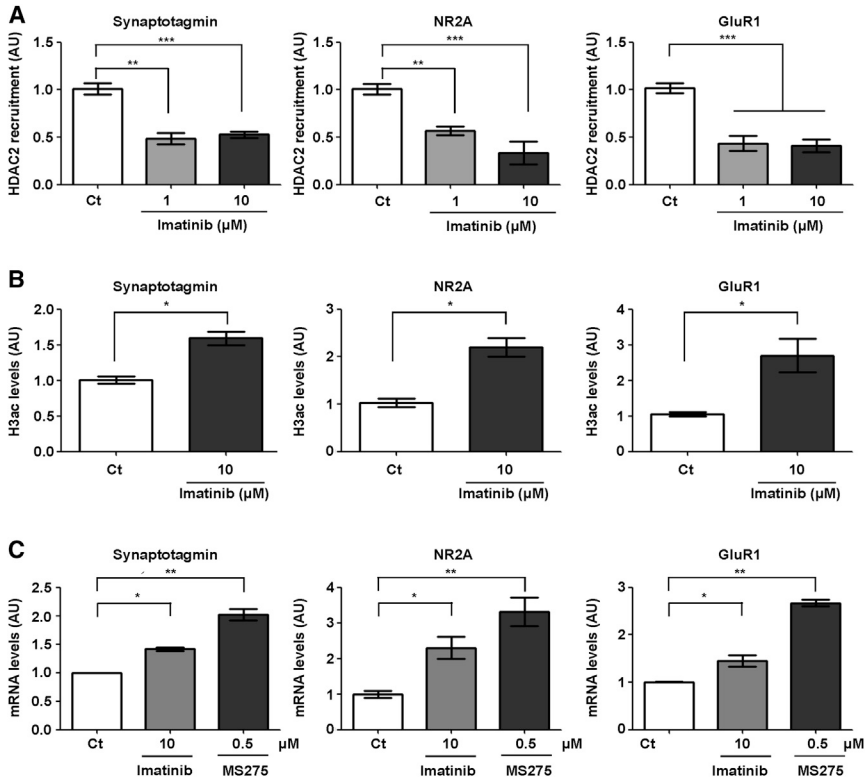

Figure 3. Imatinib Treatment Increases the Expression of Key Synaptic Genes

(A) Quantitative PCR results of the promoter of *Synaptotagmin*, *NR2a*, and *GluR1* from HDAC2-immunoprecipitated chromatin from HT22 cells treated with Imatinib for 24 hr (n = 3).

(B) Quantitative PCR results of the promoter of *Synaptotagmin*, *NR2a*, and *GluR1* from histone H3-acetylated immunoprecipitated chromatin from HT22 cells treated with 10 μM Imatinib for 24 hr (n = 3).

(C) Quantitative PCR results of *Synaptotagmin*, *NR2a*, and *GluR1* mRNA expression in HT22 cells treated with 10 μM Imatinib or 0.5 μM MS275 for 24 hr (n = 5). Results are from three or more independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; values are mean ± SEM.

treatment (Figure 3A). Likewise, Imatinib treatment significantly increased histone H3 acetylated (H3ac) levels at the promoter of these genes (Figure 3B), a result that is in agreement with the decrease of HDAC2 binding. Also, HeLa cells treated with Imatinib showed an increase in the acetylation of histone H3; concordantly, the overexpression of c-Abl decreases histone H3-acetylated levels (Figures S4A and S4B).

We next evaluated the role of c-Abl over the expression of HDAC2-regulated synaptic genes, assessing their mRNA levels in HT22 cells treated with Imatinib. The results show that Imatinib treatment significantly increases the mRNA levels of *Synaptotagmin*, *NR2a*, and *GluR1* (Figure 3C). These results are in agreement with the reduction of HDAC2 recruitment on their promoters under c-Abl inhibition.

c-Abl Mediates HDAC2 Tyrosine Phosphorylation

c-Abl-dependent phosphorylation regulates the stability of several proteins such as p73 (Tsai and Yuan, 2003), c-Jun (Gao et al., 2006), ERα (He et al., 2010), and C/EBPβ (Li et al., 2009), preventing their proteosomal degradation. We predicted that a similar mechanism could be involved in the regulation of HDAC2 levels by c-Abl. To test this idea, we cotransfected HT22 cells with a Flag-HDAC2 expression plasmid plus c-Abl-GFP, c-Abl-KD-GFP, or GFP-empty vectors. Twenty-four hours later we immunoprecipitated HDAC2 from whole-cell lysates using an anti-Flag antibody, and evaluated tyrosine phosphorylation levels. We found that c-Abl overexpression induced tyrosine phosphorylation of Flag-HDAC2, while a kinase-dead c-Abl prevented it (Figure 4A). Moreover, using pull-down assays, we show that c-Abl directly interacts with HDAC2. Endogenous c-Abl and HDAC2 present in cell lysates bind to HDAC2-GST and c-Abl-GST, respectively (Figure 4B). We next performed HDAC2 in vitro phosphorylation assays using recombinant human c-Abl (T315I). We found (1) increases in tyrosine phosphorylation levels in HDAC2-GST by western blot (Figure 4C) and (2) increases in the radioactivity incorporated in HDAC2-GST when using [γ-^{32}P]-ATP, by autoradiography (Figure 4D), and the cpm-incorporated in HDAC2-GST compared to CrkII-GST control (percentage of radioactivity, Figure 4E). Then, herein we demonstrated that c-Abl phosphorylates HDAC2.

Next, we explored which residues on HDAC2 could be phosphorylated by c-Abl. We used the online plataform Netphos 2.0 to identify tyrosines included in the c-Abl consensus phosphorylation site YX_{1-5}(P/F) with the highest phosphorylation probability (Cujec et al., 2002). The in silico analysis revealed that tyrosines 73, 222, and 238 on HDAC2 could be phosphorylated by c-Abl. We then performed site-directed mutagenesis, changing these tyrosines to phenylalanines, and evaluated tyrosine phosphorylation levels of these mutants by cotransfection of c-Abl-GFP expression plasmids with Flag-HDAC2, Flag-HDAC2Y73F, Flag-HDAC2Y222F, or Flag-HDAC2Y238F. The three HDAC2 mutants (immunoprecipitated using an anti-Flag antibody) showed a similar reduction in the phosphotyrosine signal with respect to wild-type Flag-HDAC2 (Figure 5A), suggesting that tyrosines 73, 222, and 238 could be phosphorylated by c-Abl. Moreover, the in vitro analysis of deacetylase activity showed that the three mutants have similar enzymatic activity compared to HDAC2 wild-type (Figure 5B).

Surprisingly, when we evaluated the role of the three potential phosphorylated tyrosines over HDAC2 function by cotransfecting the Gal4-TK-luciferase plasmid plus Gal4-HDAC2 or the mutants Gal4-HDAC2Y73F, Gal4-HDAC2 Y222F, or Gal4-HDAC2 Y238F, we found that the mutant on tyrosine 222 did not exhibit repression activity, while Gal4-HDAC2 and the mutants Gal4-HDAC2 Y73F and Gal4-HDAC2 Y238F maintained their repression activity (Figure 5C). In the same line, the double and triple

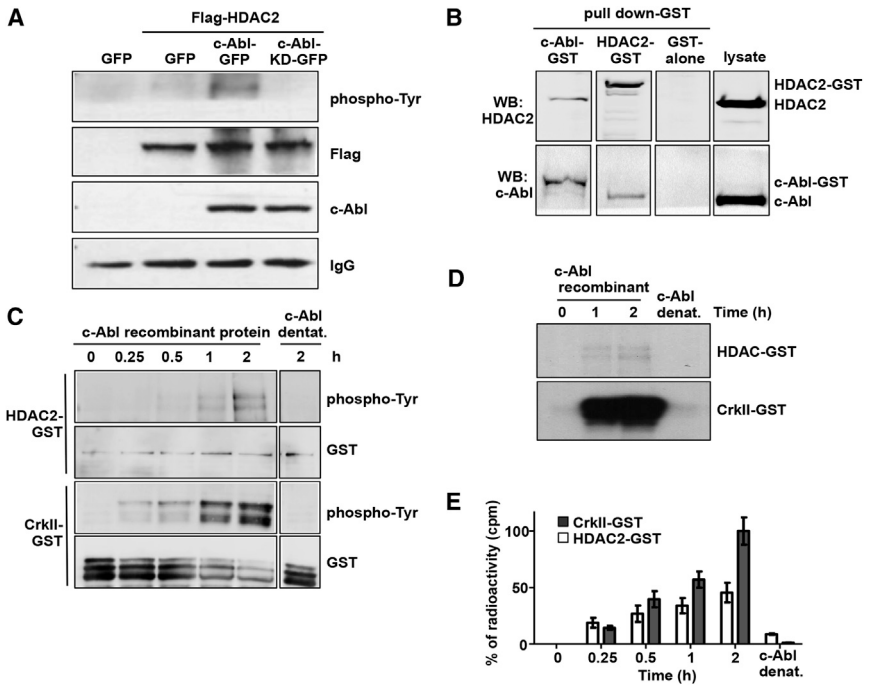

Figure 4. HDAC2 Is Phosphorylated by c-Abl Kinase

(A) HDAC2 tyrosine phosphorylation: HeLa cells were transfected with a Flag-HDAC2 expression plasmid, plus expression plasmids for either c-Abl WT (c-Abl-GFP), Abl-KD (c-Abl-KD-GFP), or the control vector (GFP). HDAC2-Flag was immunoprecipitated and evaluated using anti-phospho-Tyr and anti-Flag antibodies Representative western blot (n = 3).

(B) Pull-down assay: purified HDAC2-GST, c-Abl-GST, or GST proteins were incubated with glutathione agarose beads and HT22 whole-cell lysates for 12 hr.

(C–E) After the bead washing, the c-Abl and HDAC2 binding was evaluated by western blot (C, D, and E). In vitro phosphorylation assays were performed incubating HDAC2-GST or CrkII-GST (phosphorylation positive control) proteins with recombinant c-Abl kinase (T315I) and ATP (0.5 μCi [γ-^{32}P]-ATP or cold ATP 200 μM) for different times (0–120 min). Then the HDAC2-GST or CrkII-GST proteins were pulled down, washed, and analyzed by (C) western blot analysis using anti-phospho-Tyr and anti-GST antibodies, (D) autoradiography, or (E) counting the radioactivity incorporated (cpm) in a scintillation counter. The HDAC2 radioactivity was normalized with respect to CrkII phosphorylation; values are mean ± SEM.

mutants that included tyrosine 222, Gal4-HDAC2 Y73F-Y222F, Gal4-HDAC2 Y222F-Y238F, and Gal4-HDAC2 Y73F-Y222F-Y238F did not present repression activity, but the double mutant without the tyrosine 222 mutation, Gal4-HDAC2 Y73F-238F exhibited repression activity on luciferase expression (Figure 5C). Our results suggest that tyrosine 222 could be relevant for the transcriptional repression of HDAC2. Considering that the three mutants have similar deacetylase activity, we believe that the minor repression activity of HDAC2 Y222F is not mediated by a decrease in its enzymatic activity.

Consistent with these results, Gal4-HDAC2Y222F mutant protein levels were always lower than wild-type Gal4-HDAC2, Gal4-HDAC2Y73F, and Gal4-HDAC2Y238F protein levels (Figure 5D). A similar result was observed for Flag-HDAC2Y222F protein levels compared to wild-type Flag-HDAC2, Flag-HDAC2Y73F, and Flag-HDAC2Y238F constructs (Figure S5A) and in the triple mutants that include Y222F change (Figure S5A) Our results suggest that c-Abl mediates the phosphorylation on HDAC2-tyrosine 222, increasing its stability and therefore the repression of genes controlled by this protein.

c-Abl tyrosine phosphorylation prevents the polyubiquitination and proteosomal degradation of several proteins. Therefore, we evaluated the effects of c-Abl activity modulation over HDAC2 ubiquitination and proteosomal degradation. We cotransfected HT22 cells with an ubiquitin-HA expression plasmid plus the expression plasmids for Flag-HDAC2 or Flag-HDAC2Y222F or an empty vector, and then we treated them with Imatinib. Our results showed that in wild-type HDAC2-transfected cells c-Abl inhibition induced higher HDAC2 polyubiquitination levels compared with cells treated with vehicle (Figure 6A). Additionally, HDAC2Y222F showed higher polyubiquitination levels than wild-type HDAC2, but Imatinib did not increase HDAC2Y222F polyubiquitination (Figure 6A). Our results indicate that the phosphorylation on tyrosine 222 of HDAC2 by c-Abl prevents HDAC2 polyubiquitination.

In order to establish whether c-Abl regulates proteasomal degradation of HDAC2, we downregulated c-Abl by transfecting HeLa cells with a shRNA against c-Abl or a control shRNA plasmid. Then cells were treated with the proteasome inhibitor MG132 (5 μM) or vehicle alone. As previously described, when c-Abl was downregulated we found lower HDAC2 levels compared to control cells. Interestingly, MG132 treatment prevented the decrease in HDAC2 levels (Figure 6B). Moreover, MG132 prevented the decrease of the HDAC2-dependent transcriptional repression induced by c-Abl–KD-GFP (Figure 6C). These results show that c-Abl activity prevents HDAC2 proteasomal degradation, probably through HDAC2- tyrosine phosphorylation modulating the HDAC2-dependent transcriptional repression.

c-Abl Activation in AD Models Stabilizes HDAC2 and Increases Its Recruitment to the Promoter of Several Neuronal Genes

Next, we evaluated whether AβO treatment, which activates c-Abl, also promotes HDAC2 tyrosine phosphorylation. We observed that HDAC2 immunoprecipitated from hippocampal neurons treated with AβOs presented higher levels of tyrosine phosphorylation compared to the control, while cotreatment with Imatinib prevented this posttranslational modification (Figure 7A). This result sustains that AβO-activated c-Abl induces tyrosine phosphorylation on HDAC2 in neurons. To evaluate whether HDAC2 phosphorylation by c-Abl induced by AβOs

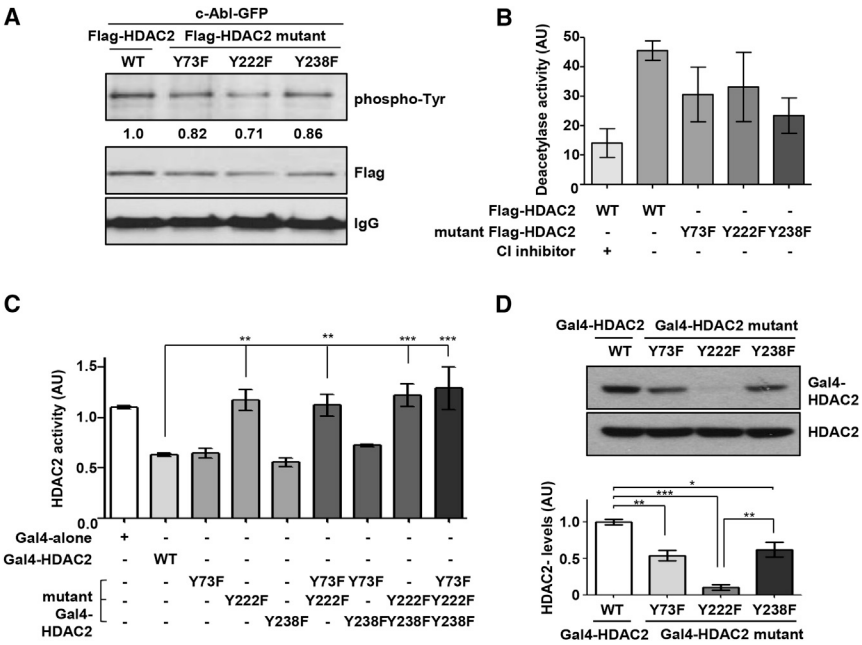

Figure 5. HDAC2 Is Phosphorylated by c-Abl in the Tyrosine 222, Affecting Its Repression Activity

(A) Tyr-phosphorylation analysis for HDAC2 and the mutant versions Y73F, Y222F, and Y238F: HT22 cells were transfected with the plasmids encoding Flag-HDAC2 or the mutant versions Flag-HDAC2 Y73F, Flag-HDAC2 Y222F, or Flag-HDAC2 Y238F plus c-Abl or an empty vector. The proteins containing the Flag epitope were immunoprecipitated, and western blot was probed with anti-phospho-Tyr and Flag antibodies (n = 3). The ratio of anti-phospho-Tyr/anti-Flag signal of western blot quantification is shown.

(B) HDAC2 enzymatic activity assay Flag-containing proteins were immunoprecipitated from HT22 cells transfected with Flag-HDAC2 or Flag-HDAC2 Y73F, Flag-HDAC2 Y222F or Flag-HDAC2 Y238F mutant versions, and the deacetylase activity was evaluated. The deacetylase activity was normalized by HDAC2-Flag immunoprecipitated levels (n = 3). CI inhibitor: CI994, HDAC2, and HDAC1 inhibitor.

(C) HDAC2 repression activity assay: luciferase activity was assessed in HT22 cells transfected with the Gal4-TK-luciferase plasmid together with Gal4-HDAC2 or the different HDAC2 mutant versions (n = 3).

(D) Gal4-HDAC2Y73F, Gal4-HDAC2Y222F, and Gal4-HDAC2Y238F mutant expression: anti-HDAC2 antibody representative western blot and quantification of HT22 cells transfected with Gal4-HDAC2 or the mutant versions. Results are from three independent experiments. *p < 0.05; values are mean ± SEM.

treatment has functional consequences, we analyzed HDAC2 recruitment at the promoter of several HDAC2 target genes. Figure 7B shows that AβOs treatment caused an increase in HDAC2 recruitment to the promoters of *Synaptotagmin*, *NR2a*, *GluR1*, and *Synaptophysin* genes, which was prevented by c-Abl inhibition with Imatinib. Thus c-Abl might be regulating HDAC2-dependent transcriptional repression of key functional neuronal genes in AD.

We next studied the c-Abl/HDAC2 pathway in vivo in the APPswe/PSEN1ΔE9 transgenic mice model of AD. As it had been previously described, 7-month-old transgenic mice presented higher HDAC2 levels compared to wild-type mice (Bie et al., 2014). In agreement with our in vitro results (Figures 1A and 1B), intraperitoneal treatment with Imatinib (25 mg/Kg) for 2 weeks, decreased HDAC2 levels in the AD mice to levels similar to control mice (Figure 7C). In addition, Imatinib treatment reduced HDAC2 levels in the CA1 hippocampus region and cortex of older transgenic mice (10-month-old) (Figure 7D). Altogether, our results suggest that the inhibition of c-Abl could be an effective strategy for preventing gene repression mediated by HDAC2 in AD.

DISCUSSION

In AD the increase in HDAC2 levels and activity has been linked to the worsening of neuronal and synaptic function. Gene repression, mediated by HDAC2, could be contributing to synapse dysfunction and memory impairment in AD (Gräff et al., 2012); however, the mechanisms that mediate the increase in HDAC2 levels in AD are largely unknown. Our findings demonstrate that c-Abl activity increases HDAC2 levels, inducing H3 histone deacetylation and transcriptional repression of key synaptic genes. Herein, we describe that HDAC2 is phosphorylated by c-Abl on tyrosine 222, affecting its stability and the repression activity of HDAC2. Also, our results suggest that c-Abl/HDAC2 signaling activation may contribute to the decrease in gene expression and memory decline in AD pathology. c-Abl induces chromatin structural changes through histone modifications that include H4K16 hypoacetylation, which decreases gene expression (Aoyama et al., 2011). Although the mechanisms underlying this phenomenon are unclear, it was demonstrated that c-Abl tyrosine kinase activity is involved. Here, we show that HDAC2 tyrosine phosphorylation by c-Abl could be a new key mechanism that contributes to chromatin structural changes and transcriptional repression control in neurons.

Our first finding, that Imatinib prevents the increase of HDAC2 levels induced by AβOs in hippocampal neurons, allowed us to establish a connection between HDAC2 and c-Abl. Previously, we demonstrated that Aβf and AβOs induce activation of c-Abl in hippocampal neurons (Alvarez et al., 2004; Vargas et al., 2014). In addition, c-Abl is overactivated in AD patients (Jing et al., 2009) and in the brain of AD animal models (Cancino et al., 2008, 2011). In this scenario, it is possible that c-Abl activated by Aβ contributes to the increase in HDAC2 levels in AD.

To demonstrate the link between c-Abl activity and the increment of HDAC2, we used different cell types and approaches; our results show (1) a dose-dependent decrease of HDAC2 levels in the presence of Imatinib, a specific c-Abl inhibitor, (2) a reduction in HDAC2 levels when we used a short hairpin RNA against c-Abl to reduce c-Abl levels, and (3) an increment in HDAC2 levels when we overexpressed c-Abl. Altogether these

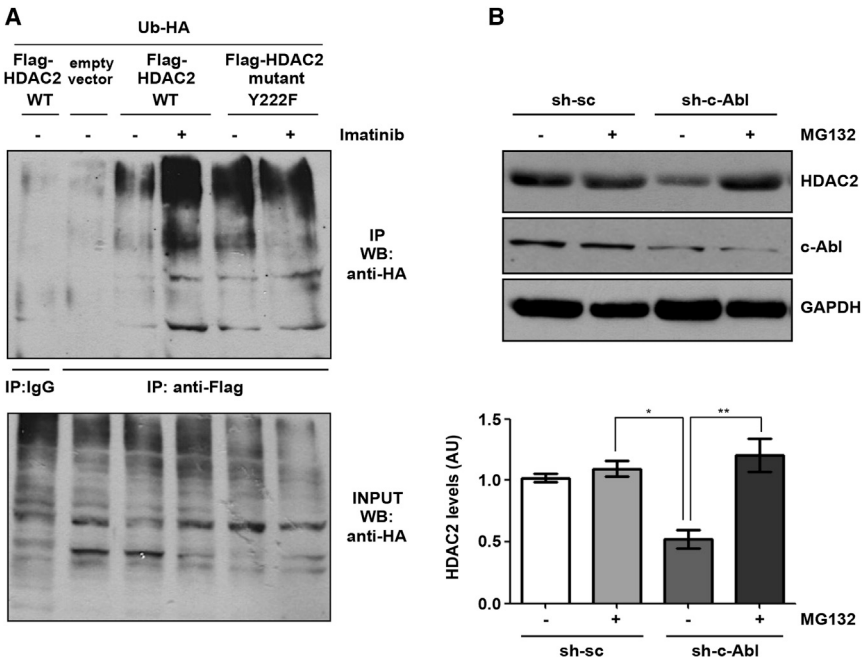

Figure 6. c-Abl Inhibition Induces the Polyubiquitination and Proteasome Degradation of HDAC2

(A) Ubiquitination analysis of HDAC2: HT22 cells were transfected with plasmids expressing Flag-HDAC2 or the mutant version Flag-HDAC2 Y222F, together with ubiquitin-HA; additionally the cells were treated with Imatinib or vehicle. Anti-HA western blot of the anti-ubiquitin-immunoprecipitated proteins.

(B) Proteasome inhibition prevents decrease in HDAC2 protein levels. HeLa cells were transfected with a plasmid expressing an shRNA against c-Abl or scramble shRNA as a control and treated with the proteasome inhibitor MG132 10 μM (n = 3). Representative western blot and quantification.

(C) HDAC2 repression activity assay: luciferase activity of HT22 cells transfected with the plasmid expressing Gal4-TK-luciferase plus Gal4-HDAC2 or Gal4-vector. Twenty-four hours after transfection, the cells were treated with MG132 10 μM for 24 hr (n = 3). Results are from three independent experiments. *p < 0.05, values are mean ± SEM.

results support the idea that the effect of Imatinib on HDAC2 is mediated by c-Abl inhibition. In agreement with a role for c-Abl on the modulation of HDAC2 levels, the i.p. administration of Imatinib reduced HDAC2 levels in APPswe/PSEN1ΔE9 mice. Interestingly, Imatinib treatment also reduces cognitive impairments in AD models (Cancino et al., 2008); similar results were observed in AD mice models treated with HDAC inhibitors (Fischer et al., 2007; Francis et al., 2009; Kilgore et al., 2010; Zhang and Schluesener, 2013) and in the CK-p25 transgenic mice injected with short-hairpin RNAs against HDAC2 into the hippocampus area (Gräff et al., 2012). Although c-Abl activity has been linked to apoptosis, synaptic loss, and cytoskeletal alterations (Cancino et al., 2008, 2011; Vargas et al., 2014), the c-Abl /HDAC2 downstream signaling pathway could also be contributing to neuronal gene repression and the development of cognitive impairments in AD.

HDAC2 has been described as an epigenetic repressor, especially relevant in the reduction of neuronal gene expression in neurodegenerative models (Gräff et al., 2012; Bie et al., 2014). Using a HDAC2 activity assay, we showed that effects of Imatinib on HDAC2 levels functionally correlate, decreasing HDAC2-dependent transcriptional repression activity. Also, Imatinib did not affect the catalytic activity of immunoprecipitated HDAC2 in an in vitro activity assay, eliminating the possibility that Imatinib directly inhibits HDAC2 catalytic activity. Moreover, the kinase-dead c-Abl (a dominant-negative form of c-Abl) also reduced HDAC2 repression activity. This result confirms that c-Abl inhibition mediates the effects of Imatinib on HDAC2-dependent transcriptional repression activity. On the other hand, confirming the role of c-Abl, a significant increase of HDAC2 repression activity was observed with the c-Abl-GFP overexpression.

Consistent with a c-Abl-dependent regulation of HDAC2 activity, c-Abl inhibition by Imatinib increased the levels of acetylated histone H3 on the promoter of the *NR2a*, *Synaptotagmin*, and *GluR1* genes, an effect that was associated with the decrease in HDAC2 recruitment on the same promoters. Moreover, Imatinib promoted *NR2a*, *Synaptotagmin*, and *GluR1* gene expression. Thus, activation of c-Abl signaling contributes to the HDAC2 recruitment on neuronal gene promoters, triggering histone deacetylation and decreasing the transcription rate of these genes.

c-Abl signaling is activated in hippocampal neurons exposed to Aβ and in AD animal models (Alvarez et al., 2004; Cancino et al., 2008). Interestingly, we found that hippocampal neurons exposed to AβOs show increased HDAC2 recruitment on the promoter of the *NR2a*, *Synaptotagmin*, *Synaptophysin*, and *GluR1* genes. This increase was prevented by Imatinib. Moreover, the APPswe/PSEN1ΔE9 mouse model presents higher HDAC2 levels and nuclear signal compared to wild-type mice,

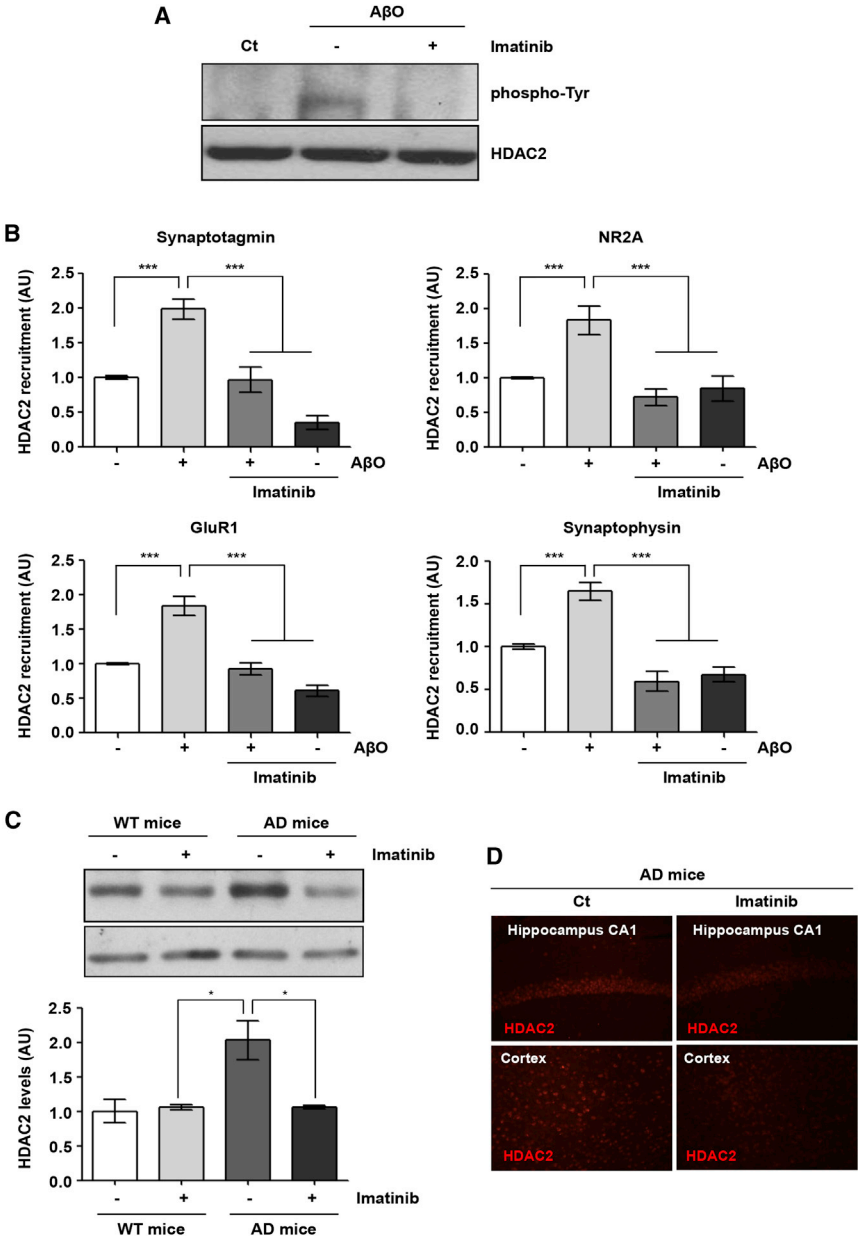

Figure 7. Imatinib Treatment Prevents the HDAC2 Recruitment to the Promoter and the Increase of HDAC2 Levels in AD Models

(A) HDAC2 tyrosine phosphorylation: HDAC2 was immunoprecipitated from hippocampal neurons (7 DIV) treated with AβO for 1 hr plus 5 μM Imatinib and evaluated by western blot using anti-phospho-Tyr and anti-HDAC2 antibodies (n = 3).

(B) Quantitative PCR results of HDAC2 immunoprecipitated chromatin at the promoter of *GluR1*, *NR2a*, *Synaptophysin*, and *Synaptotagmin* from hippocampal neurons treated with vehicle, 5 μM AβO, 5 μM AβO plus 5 μM Imatinib, or 5 μM Imatinib alone for 24 hr (n = 3).

(C) Representative western blot images and quantification of HDAC2 levels in extracts of brain from 7-month-old wild-type and APPswe/PSEN1ΔE9 transgenic mice treated with Imatinib at a dose of 25 mg/Kg or vehicle for 2 weeks (n = 5).

(D) Representative immunofluorescence pictures depicting HDAC2 levels in the CA1 area of APPswe/PSEN1ΔE9 transgenic mice treated with 25 mg/Kg of Imatinib or vehicle (n = 3). Results are from three or more independent experiments. *p < 0.05; values are mean ± SEM.

while the AD mice treated with Imatinib present lower HDAC2 levels very similar to the wild-type mice. p25 transgenic mice and other AD mouse models also show an increase in HDAC2 levels, higher HDAC2 recruitment on the promoters, and lower expression of key neuronal genes compared to wild-type mice (Guan et al., 2009; Gräff et al., 2012). In addition, the reduction of HDAC2 levels using an shRNA against HDAC2 restored the expression of the genes analyzed (Gräff et al., 2012).

Our results indicate that c-Abl regulates HDAC2 levels and HDAC2 binding to the promoter of several neuronal genes, decreasing their expression. Although HDAC2 plays a key role in gene repression, this protein lacks a DNA binding domain. In fact, HDAC2 requires its assembly into a large multiprotein repressor complex such as mSin3A, NuRD, and CoREST (Haberland et al., 2009; Gräff and Tsai, 2013) to affect gene expression. Thus, c-Abl effects on HDAC2 levels and promoter recruitment could be mediated by different mechanisms: (1) c-Abl can directly affect HDAC2 levels, or (2) c-Abl can affect an HDAC2 partner in the repressor complex preventing the correct HDAC2 binding to the promoter of genes, inducing its degradation.

Although we cannot rule out the possibility that c-Abl targets other repressor complexes related to HDAC2 affecting their assembly, our results suggest that c-Abl can directly modify HDAC2 levels. Preliminary data show that the HDAC2 Y222F interacts with corepressor complex CoREST (data not shown). Future work is required to evaluate the potential role of c-Abl regulating the expression or function of other transcription regulatory proteins or repressor complexes.

Additionally, c-Abl activity does not affect *HDAC2* mRNA levels, although it increases HDAC2 proteins levels, thereby suggesting that c-Abl regulates HDAC2 levels by a posttranslational mechanism. In this context, it has been shown that HDAC2 is regulated by posttranslational modifications such as serine phosphorylation (Tsai and Seto, 2002), polyubiquitination (Krämer et al., 2003), acetylation (Adenuga and Rahman, 2010), nitrosylation (Nott et al., 2008), and nitration (Osoata et al., 2009).

Interestingly, among HDAC2 posttranslational modifications, tyrosine phosphorylation was not previously described. We found that c-Abl induces HDAC2 tyrosine phosphorylation. By

in silico analysis we identified tyrosines 73, 222, and 238 as the three tyrosines in HDAC2 with the highest probability of phosphorylation by c-Abl.

Remarkably, only the HDAC2 mutant in tyrosine 222 was deficient for repression activity when it was evaluated in an in vivo cell assay. Therefore, although tyrosines 73 and 238 could be targets of c-Abl phosphorylation, they are not linked to the c-Abl effect on HDAC2-dependent gene repression. However, in an in vitro assay using immunoprecipitated HDAC2, the HDAC2 mutants—tyrosines 73, 222, and 238—showed similar catalytic activity as wild-type HDAC2, indicating that the amino acidic change Y222F in HDAC2 does not disturb HDAC2 catalytic activity. Indeed, tyrosine 222 is not localized in the HDAC2 active site pocket or in the Zn^{2+} binding site (Finnin et al., 1999; Vanommeslaeghe et al., 2005). Thus, the lower repression activity of HDAC2Y222F is not caused by deficient catalytic activity. Interestingly, the Y222 and the c-Abl phosphorylation motif $YX_{1-5}P$ in HDAC2 (Cujec et al., 2002) is conserved in different species, such as mice and human (Figure S6A). Additionally, this motif is partially conserved in other members of the family, such as HDAC1 and HDAC3 (Figure S6B), suggesting that c-Abl could phosphorylate and regulate these proteins. Indeed, we have preliminary data suggesting that c-Abl regulates HDAC1 levels (data not shown).

Polyubiquitination of HDAC2 mediated by Ubc8 E2 conjugase and RLIM E3 ligase induces its proteosomal degradation (Krämer et al., 2003). Also, the evidence indicates that tyrosine phosphorylation by c-Abl stabilizes proteins, preventing their proteasomal degradation. The transcription factors p73 (Tsai and Yuan, 2003), Yap1 (Levy et al., 2008), c-Jun (Gao et al., 2006), ERα (He et al., 2010), and C/EBPβ (Li et al., 2009) are phosphorylated by c-Abl inducing their transcriptional function. Three observations allow us to infer that tyrosine 222 phosphorylation by c-Abl prevents the polyubiquitination of HDAC2, preventing its proteosomal degradation: (1) that c-Abl inhibition induces the polyubiquitination of HDAC2, (2) that HDAC2Y222F shows higher polyubiquitination levels than wild-type HDAC2, and (3) that c-Abl inhibition does not change the polyubiquitination levels of HDAC2 Y222F, while affecting the polyubiquitination of wild-type HDAC2. This could explain why HDAC2 Y222F shows lower protein levels than wild-type HDAC2, HDAC2 Y73F, and HDAC2 Y238F mutants, and also the decreased levels of wild-type HDAC2 when c-Abl is inhibited. On the other hand, the proteosomal inhibitor MG132 prevents the decrease of HDAC2 levels in c-Abl knockdown cells as well as the HDAC2 repression activity in HT22 cell overexpressing GFP-Abl-KD. Thus, our data suggest that the decline in c-Abl activity and HDAC2 phosphorylation promotes the ubiquitination and proteosomal degradation of HDAC2. Although the mechanism involved is unknown, the evidence indicates that the tyrosine phosphorylation of c-Jun and p73 prevents the interaction with E3 ligase Itch (Tsai and Yuan, 2003; Gao et al., 2006). RLIM and Mule, HDAC2 E3 ligases, are interesting candidates to be evaluated in the future (Krämer et al., 2003; Zhang et al., 2011). Osoata et al. described that tyrosine 253 nitration also induces proteosomal degradation of HDAC2; however, tyrosine 222 was not evaluated in this study (Osoata et al., 2009). On the other hand, our analysis did not include tyrosine 253, because this residue presents a lower probability of c-Abl phosphorylation than tyrosines 73, 222, and 238. Further work is required to elucidate how phosphorylation and nitration of tyrosine residues are contributing to the ubiquitination and the stability of HDAC2. Also, we cannot rule out the possibility that tyrosine 222 is regulating other posttranslational modifications of HDAC2, which could increase the stability or activity of this protein.

Tyrosine phosphorylation of HDAC2 by c-Abl appears to be especially relevant for neuronal gene expression in neurons exposed to AβO damage in an AD in vitro model. In spite of the several posttranslational modifications identified in HDAC2, only one published work studied a posttranslational modification of HDAC2 in neurons (Nott et al., 2008). In this study the authors observed that BDNF induces the nitrosylation of HDAC2 in cysteine residues, triggering HDAC2 release from chromatin and promoting the transcription of neurotrophin regulated genes (Nott et al., 2008). Here we show that c-Abl, through tyrosine phosphorylation of HDAC2, induces gene repression. Thus, the nitrosylation of cysteine and the phosphorylation of tyrosine in HDAC2 could be antagonistic mechanisms for the control of gene expression mediated by HDAC2.

In addition, HDACs regulate proteins other than histones through deacetylation (Yang and Seto, 2008). In fact, HDAC2 induces Klf5 deacetylation in response to a RAR agonist, which decreases the expression of p21 (Zheng et al., 2011). Furthermore, HDAC2 mediates the deacetylation of the transcription factor Gata4, decreasing the transactivation of genes associated with cell proliferation (Trivedi et al., 2010). Also, HDAC2 induces p53 deacetylation, inhibiting its transactivation function and reducing p53-dependent apoptotic responses (Brandl et al., 2012). Thus, by modulating protein deacetylation, the c-Abl/HDAC2 pathway could regulate several cellular processes beyond those of histones and effects at the transcriptional level evaluated in this study.

Finally, our results reveal a new mechanism of gene expression control in neurons through the regulation of HDAC2 levels by c-Abl. This mechanism, induced in neurons by damage, links c-Abl activation with the reduction of neuronal and synaptic gene expression, contributing to synapse dysfunction and memory impairments in neurodegenerative diseases. The inhibition of c-Abl kinase could be a strategy for preventing the deleterious effects of increased HDAC2 levels in AD as well as in other neurodegenerative diseases.

EXPERIMENTAL PROCEDURES

Please refer to the Supplemental Experimental Procedures for more details.

Animals

Transgenic B6C3-Tg(APPswePSEN1ΔE9)85Dbo/J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Rats and mice were maintained in the Animal Care Facility following the Guide for the Care and Use of Laboratory Animals published by the NIH (Publication 86-23). The animal protocols used were reviewed and approved by the animal studies board at our institution. The APPswe/PSEN1ΔE9 mice and wild-type mice received an i.p. injection of 25 mg/kg Imatinib or saline every 2 days for 2 weeks.

Primary Culture of Rat Hippocampal Neurons

Rat hippocampal cultures were prepared as described previously, with modifications (Kaech and Banker, 2006; Alvarez et al., 2004).

HDAC2 Repression Activity Assay

Plasmids directing the synthesis of Gal4-HDAC2 (wild-type or tyrosine mutants) and luciferase reporters were transfected into cells using Lipofectamine 2000 reagent (Life Technologies, Carlsbad, US). After 48 hr of transfection, cells were collected, and luciferase activity was determined with the Dual Luciferase Reporter Assay System (Promega, E1910).

Tyrosine Phosphorylation Assay

Protein extracts were obtained from hippocampal cultures, HT22, and HeLa cells, lysed in RIPA buffer plus protease and phosphatase inhibitors. The immunoprecipitations were performed using anti-Flag or anti-HDAC2 antibodies, and tyrosine phosphorylation of HDAC2 was evaluated with the antibody anti-phospho-Tyr 4G10 (Millipore).

Chromatin Immunoprecipitation and Gene Expression Analysis

Chromatin immunoprecipitation and gene expression analysis were performed as described in the Supplemental Experimental Procedures.

Statistical Analysis

Mean and SEM values and the number of experiments are indicated in each figure. Statistical analysis was performed by one-way ANOVA, followed by Bonferroni posttest using Prisma Software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article at http://dx.doi.org/10.1016/j.molcel.2014.08.013.

AUTHOR CONTRIBUTIONS

This research was designed by M.G.-Z. and A.R.A. Experiments were planned and performed as follows: M.G.-Z. and A.V., ChIP and ubiquitination assays; M.G.-Z., P.C., and S.Z., assays in hippocampal neurons; L.D.E., D.C., and M.G.-Z., APPswePSEN1ΔE9 transgenic mice assays; E.S. and M.G.-Z., HDAC2 tyrosine phosphorylation, HDAC2 repression activity, and site-directed mutagenesis assays. The manuscript was written by M.G.-Z., L.D.E., S.Z., and A.R.A.

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