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C-ABL ESTABILIZA LOS NIVELES DE HDAC2 POR FOSFORILACIÓN EN TIROSINA REPRIMIENDO LA EXPRESIÓN DE GENES NEURONALES EN LA ENFERMEDAD DE ALZHEIMER.

C-ABL STABILIZES HDAC2 LEVELS BY TYROSINE PHOSPHORYLATION REPRESSING NEURONAL GENE EXPRESSION IN ALZHEIMER DISEASE

Tesis entregada a la Pontificia Universidad Católica de Chile en cumplimiento parcial de los requisitos para optar al Grado de Doctor en Ciencias con mención en Biología Celular y Molecular.

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A mis padres por su sacrificio, amor y consejos

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ABBREVIATIONS

AICD: APP Intracellular Domain

AD: Alzheimer's Disease

APP: Amyloid Precursor Protein

AraC: Citosina-β-D-arabinofuranosido

Aβ: Amyloid-beta

AβOs: Aβ oligomers

BDNF: Brain derived neurotrophic factor

BSA: Bovine serum albumin

Cdk5: Cyclin-dependent kinase 5

DIV: Days *in vitro*

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

FAD: Familial Alzheimer's disease

GAB2: GRB2-associated-binding protein 2

GFP: Green Fluorescent Protein

GLUR1: Glutamate receptor 1

H3ac: Histone H3 acetylated

H4ac: Histone H4 acetylated

HAT: Histone Acetyltransferase

HDAC: Histone Deacetylase

HIV: Human immunodeficiency virus

KO: Knockout

LTP: Long Term Potentiation

mRNA: messenger RNA

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

NAD: Nicotinamide adenine dinucleotide

NR2A: N-methyl D-aspartate receptor subtype 2A

PBS: Phosphate buffered saline

RIPA: Radioimmunoprecipitation assay

RNA: Ribonucleic acid

SDS: Sodium dodecyl sulfate

SH: Src Homology

shRNA: Short hairpin RNA

RESUMEN

La Enfermedad de Alzheimer (EA) es un desorden neurodegenerativo caracterizado por un deterioro cognitivo progresivo. El sello distintivo de los cerebros afectados con la enfermedad de Alzheimer es la presencia de agregados proteicos insolubles. En este sentido, la hipótesis de la cascada del amiloide plantea que la acumulación y agregación del péptido Aβ desencadena un conjunto de mecanismos que conducen a la disfunción y la apoptosis de las neuronas.

Entre los mecanismos descritos, uno que ha despertado gran interés es la disminución en la expresión de genes neuronales producto del incremento en los niveles de HDAC2. Esta enzima cataliza la deacetilación de las histonas, lo que provoca que la cromatina adquiera una conformación cerrada que es transcripcionalmente inactiva. Actualmente, la evidencia indica que HDAC2 esta involucrada en el deterioro cognitivo y en la disfunción sináptica que caracteriza a la EA. A pesar de que se ha descrito extensamente que el incremento en los niveles de HDAC2 tiene un papel negativo en el desarrollo de la EA, los mecanismos moleculares involucrados en este incremento no están completamente dilucidados.

Interesantemente, se ha demostrado en modelos *in vitro* que la tirosina quinasa c-Abl esta implicada en la represión de genes por un mecanismo epigenético, el cual sería dependiente de la actividad de las HDACs. En neuronas, la tirosina quinasa c-Abl es un actor clave en los procesos neurodegenerativos. En efecto, resultados de nuestro laboratorio han demostrado que la c-Abl es activada y participa en la muerte neuronal, la disfunción y la pérdida sináptica en modelos de la EA.

Considerando estos antecedentes, la hipótesis de esta tesis es:

"La tirosina quinasa c-Abl incrementa los niveles de HDAC2 y regula la expresión de genes neuronales"

Para probar nuestra hipótesis, modificamos la actividad de c-Abl utilizando distintos aproximaciones *in vitro* e *in vivo* y en ellos evaluamos los niveles y actividad de HDAC2. Primero evaluamos los niveles de HDAC2 en neuronas hipocampales expuestas a oligómeros de Aβ, un modelo que presenta un incremento en la actividad de c-Abl. Interesantemente, el tratamiento con oligómeros de Aβ incrementa los niveles de HDAC2; y de forma concordante con nuestra hipótesis, el pre-tratamiento con Imatinib, un inhibidor de c-Abl, previene el incremento en los niveles de HDAC2. Además, para confirmar el rol de c-Abl en la regulación de los niveles de HDAC2, transfectamos células con el plásmido que expresa c-Abl o un shRNA en contra de c-Abl; mientras la sobrexpresión de c-Abl causa un incremento significativo en los niveles de HDAC2, la reducción de los niveles de c-Abl muestra una disminución significativa de los niveles de HDAC2.

Posteriormente, evaluamos el rol de c-Abl en la regulación de la actividad como represor transcripcional de HDAC2. Nuestros resultados muestran que el tratamiento con Imatinib y la transfección con el plásmido de expresión para Abl-KD (forma dominante negativa de c-Abl) reducen la actividad como represor transcripcional de HDAC2. Asimismo, la sobreexpresión de c-Abl incrementa la actividad como represor transcripcional de HDAC2. En conjunto nuestros resultados confirman el rol de c-Abl sobre la actividad como represor transcripcional de HDAC2.

Luego estudiamos mediante Inmunoprecipitación de Cromatina si c-Abl regula el reclutamiento de HDAC2 en los promotores de algunos genes sinápticos seleccionados. Los resultados demuestran que el tratamiento con Imatinib reduce el reclutamiento de HDAC2 en el promotor de los genes evaluados; además demuestra que esta reducción va

acompañada por un incremento en el nivel de acetilación de la histona H3 en estos mismos promotores. Concordante con estos resultados, observamos que el tratamiento con Imatinib incrementa la expresión de los genes estudiados. En conjunto, nuestros resultados demuestran que c-Abl por medio de HDAC2 regula la expresión de genes neuronales.

El próximo paso fue dilucidar el mecanismo molecular involucrado en la regulación de HDAC2 por c-Abl. Nuestros resultados demostraron que c-Abl induce la fosforilación en tirosina de HDAC2, una modificación postraduccional que no había sido descrita para HDAC2 y que previene tanto la ubiquitinación como la degradación vía proteosoma de HDAC2. En conjunto nuestros hallazgos sugieren que la fosforilación en tirosina de HDAC2 es el mecanismo por el cual c-Abl incrementa los niveles de esta proteína.

También analizamos el efecto de Imatinib sobre el reclutamiento de HDAC2 en neuronas hipocampales tratadas con oligómeros de Aβ. Interesantemente, Imatinib redujo el reclutamiento de HDAC2, inducido por oligómeros Aβ, sobre los promotores de los genes sinápticos evaluados. Finalmente estudiamos el ratón transgénico APPswe/PSEN1ΔE9 un modelo de la EA, que presenta un incremento en los niveles de HDAC2. En este modelo el tratamiento intraperitoneal con Imatinib previene el incremento en los niveles de HDAC2, resultado que confirma *in vivo* el rol de c-Abl en el incremento de los niveles de HDAC2.

En resumen, la activación de c-Abl incrementa los niveles de HDAC2 mediante la fosforilación en tirosina de esta proteína, lo que finalmente induce la represión en la expresión de genes neuronales. Así, nuestros resultados sugieren una nueva vía de señalización formada por c-Abl/HDAC2 que participa en la represión de genes neuronales en modelos de la EA; además propone a c-Abl como un blanco terapéutico para prevenir los efectos negativos producidos por la actividad de HDAC2 en EA.

ABSTRACT

Alzheimer's disease is a neurodegenerative disorder characterized by progressive cognitive decline. The main histopathological hallmark of Alzheimer's disease brains is the presence of insoluble proteinaceous aggregates. In this sense, the amyloid cascade hypothesis proposes that accumulation and aggregation of Aß peptide initiate a set of mechanisms that lead to neuronal dysfunction and apoptosis.

Among the mechanisms described, one that has aroused great interest is the decrease in neuronal gene expression through an increase in HDAC2 (Histone Deacetylase 2) levels. This enzyme catalyzes histone deacetylation, causing chromatin to acquire a closed conformation that is transcriptionally inactive. Currently, the evidence indicates that HDAC2 is involved in the cognitive decline and synaptic dysfunction that characterizes AD. Although it has been widely reported that increased HDAC2 levels have a negative role in AD development, the molecular mechanisms involved in this increase have not been fully elucidated.

Interestingly, it has been shown using *in vitro* models that the c-Abl tyrosine kinase is implicated in gene repression through an epigenetic mechanism, which might be dependent on HDAC activity. In neurons, c-Abl is a principal actor in neurodegenerative processes. In fact, results from our laboratory have demonstrated that c-Abl is activated and participates in neuronal death, synaptic loss and synaptic dysfunction in AD models.

Considering these antecedents, the hypothesis of this thesis is the following:

"The c-Abl tyrosine kinase increases HDAC2 levels and regulates the expression of neuronal genes."

To test our hypothesis, we modified c-Abl activity using different *in vitro* and *in vivo* approximations and then evaluated HDAC2 levels and activity.

First, we evaluated HDAC2 levels in hippocampal neurons exposed to Aβ oligomers, a model that shows increased c-Abl activity; concordant with our hypothesis, pretreatment with Imatinib, a c-Abl inhibitor, prevented the Aβ oligomer-induced increase in HDAC2 levels. In addition, to confirm the role of c-Abl in HDAC2 regulation, we transfected cells with a plasmid expressing c-Abl or an shRNA against c-Abl, with their respective controls. While c-Abl overexpression significantly increased HDAC2 levels, c-Abl knockdown significantly decreased these levels.

Subsequently, we evaluated the role of the c-Abl with regard to HDAC2 function following HDAC2 transcriptional repressor activity. Our results showed that Imatinib treatment and Abl-KD (a dominant-negative form of c-Abl) overexpression inhibited HDAC2 repression activity, which confirmed the role of c-Abl over HDAC2 function. Concordantly, c-Abl overexpression increased HDAC2 repressor activity. Together, our results confirm the role of c-Abl over HDAC2 transcriptional repressor function.

Then, we used chromatin immunoprecipitation to assess whether c-Abl regulates HDAC2 recruitment to the promoters of selected synaptic genes. The results showed that Imatinib treatment reduced HDAC2 recruitment to the promoters of the synaptic genes evaluated, and this event correlated with increases in histone H3 acetylation. Furthermore, Imatinib treatment increased the expression of the selected genes. Altogether, these results suggest that the c-Abl regulates gene expression in neurons through HDAC2.

The next step was to elucidate the molecular mechanism involved in HDAC2 regulation by c-Abl. Our results demonstrated that c-Abl induces the tyrosine phosphorylation of

HDAC2, a posttranslational modification not previously described for HDAC2, which prevented both HDAC2 ubiquitination and proteasomal degradation. Thus, our results support the idea that the tyrosine phosphorylation of HDAC2 is a mechanism by which c-Abl promotes protein level increases.

Additionally, Imatinib prevented the HDAC2 recruitment to the promoter of selected synaptic genes in Aβ oligomer-treated neurons. Finally, we assessed APPswe/PSEN1ΔE9 transgenic mice, an AD model that presents with higher HDAC2 levels than wild-type mice. In this model, intraperitoneal treatment with Imatinib prevented the increase in HDAC2 levels, which confirmed *in vivo* the role of c-Abl in the increased HDAC2 levels.

In summary, c-Abl activation increases HDAC2 levels through the tyrosine phosphorylation of this protein, which ultimately induces neuronal gene repression. Thus, our results suggest a novel cell-signaling pathway of c-Abl/HDAC2, which is involved in the repression of neuronal genes in AD. Furthermore, these results propose c-Abl as a therapeutic target to prevent the adverse effects of HDAC2 activity in AD.

INTRODUCTION

4.1 Alzheimer's disease

Alzheimer's disease (AD) is the principal cause of dementia in the elderly. It is estimated that 42.3 million individuals will be diagnosed with dementia worldwide by the year 2020, and that number may double in the next 20 years (Ballard et al., 2011), leading to a high social and economic impact. AD is characterized by progressive neuronal death, synaptic loss, cytoskeleton alterations, dystrophic neurites and axonal degeneration (Mattson et al., 2004; Krstic D. et al., 2013), which underlie memory loss and cognitive decline (Mattson et al., 2004; Castellani et al., 2010). The main risk factor for AD development is age. In fact, it is estimated that after the age of 65 years, AD incidence increases twofold every five years, and the prevalence increases exponentially with age (Castellani et al., 2010).

The histopathological hallmark of AD brains is the presence of two types of proteinaceous aggregates (Mattson et al., 2004): i) the senile plaques that are extracellular deposits of fibrils and amorphous aggregates of a proteolytic fragment of the amyloid precursor protein (APP), known as Aβ peptide; and ii) the neurofibrillary tangles that are intracellular fibrillar aggregates of hyperphosphorylated tau protein (Mattson et al., 2004; Ballard et al., 2011; Krstic D. et al., 2013). These aggregates accumulate in regions associated with learning, memory and emotional behavior.

The amyloid hypothesis proposes that Aβ peptide accumulation is the main pathological cause that leads to neurodegeneration in AD (Mattson et al., 2004; Hardly et al., 2002). The genetic analysis of familial AD supports the amyloid hypothesis. Indeed, it has been demonstrated that the mutations in genes that increase APP processing and Aβ peptide

generation are linked with disease development (Hardy et al., 2006). However, the cases of familial AD associated with *App*, *Presenilin 1* and *Presenilin 2* genes account for only 2% of the reported clinical cases (Bertram et al., 2008). In contrast, the vast majority of AD cases correspond to the sporadic form of the disease, which has not been linked to one particular gene; rather, these cases are associated with an array of risk alleles joined with lifestyle and environmental factors. Among these alleles, a number of different polymorphisms in several genes such as *Apolipoprotein E, Angiotensin I converting enzyme 1, GAB2*, and *Transferrin* have been correlated with the risk of developing Alzheimer's disease (Bertram et al., 2008; Krstic D. et al., 2013).

The Aβ peptide neurotoxicity depends on its aggregation (Walsh et al., 2007), which is a polymerization reaction that produces species such as Aβ oligomers and amyloid fibrils (Kaden et al., 2012). Amyloid fibrils are neurotoxic in *in vitro* and *in vivo* models (Geula et al., 1998; Deshpande et al., 2006). However, the association between amyloid fibril accumulation and dementia severity is controversial; while some groups have demonstrated a clear correlation between the two (Cummings et al., 1995; Dickson 1995), other groups have not found such a correlation (McKee et al., 1991; Benett et al., 1993).

Although the relationship between Aβ plaques and dementia is not clear, studies have demonstrated a positive correlation between soluble Aβ oligomers (AβOs) and neurodegeneration in AD patients (Lue et al., 1999; McLean et al., 1999). Indeed, soluble Aβ oligomers induce neuronal death (Lambert et al., 1998), synaptic loss (Shresta et al., 2006), interrupt hippocampal synaptic plasticity *in vitro* (Lambert et al., 1998; Townsend et al., 2006) and *in vivo* (Walsh et al., 2002) and induce cognitive impairment in rodents (Clearly et al., 2005; Lesné et al., 2006).

The neurodegenerative process in AD triggers several mechanisms of damage such as the following: synaptic failure; neurotrophin and neurotransmitter depletion; mitochondrial dysfunction; oxidative stress; insulin pathway alterations; deregulated neuronal calcium homeostasis; inflammation; reduction in axonal transport; and aberrant cell cycle reentry (Querfurth et al., 2010). Recently, an analysis of AD patient brains revealed changes in gene expression profiles. In fact, the CA1 hippocampal regions of AD patients show increases in the expression of glial markers, such as interleukin 1β, advanced glycosylation end product-specific receptor, tumor necrosis factor receptor 1A, heat-shock protein 60, heat-shock protein 70 and heat-shock protein 90 (Ginsberg et al., 2012). Most likely, this induction is linked to the neuroinflammation associated with the disease. In addition, the CA1 hippocampal region shows a downregulation of neuronal genes. Several neuronal key genes, such as the glutamate receptors (GRIA1, GRIA2, GRIN2B), synaptic-related markers (synaptophysin, synaptotagmin 1, α-synuclein, synaptobrevin 1, synaptopodin, syntaxin 4A, vesicle-associated membrane protein, associated protein B, and vacuolar proton pump homolog1) and neurotrophin receptor (TrkB) are downregulated in AD patients (Ginsberg et al., 2012). Although, these changes could be the result of the neuronal loss that characterizes the neuropathological process; currently, evidence suggests that the decrease in the expression of neuronal genes is mediated by epigenetic mechanisms (Fisher et al., 2007; Ricobaraza et al., 2009; Govindarajan et al., 2011; Gräff et al., 2012).

4.2 Epigenetic Mechanisms in Alzheimer's disease

The term "epigenetic" has several meanings depending on the field of study. Professor Adrian Bird of Edinburgh University proposed a global definition, which indicates that epigenetic is *"the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states"* (Bird et al., 2007). Epigenetic mechanisms are highly

regulated and define the three-dimensional structure of chromatin in response to the environment. The changes in chromatin structure can generate two types of structures: one type is low-compacted chromatin, which is associated with active transcription, referred to as euchromatin; the other type is highly compacted chromatin, which is transcriptionally inactive, commonly called heterochromatin (Li et al., 2007). The epigenetic mechanisms that determined these chromatin structures are as follows: i) DNA methylation; ii) histone posttranslational modification; and iii) histone variants (Jakovcevski et al., 2012).

Interestingly, the brains of patients with senile or presenile dementia of Alzheimer's show a decrease in the euchromatin fraction compared with control elderly individuals (Crapper et al., 1979). In addition, the chromatin isolated from AD brains present with higher levels of DNA associated with dinucleosomes and more compacted DNA-nucleosome structures than controls (Lewis et al., 1981). Interestingly, the analysis of the 5' upstream DNA sequence of neurofilament light chain gene in AD patients showed a more compacted chromatin structure than age-matched controls (Lukiw et al., 1990). These results suggest that the chromatin in AD brains preferentially has a heterochromatin structure, which is associated with reduced gene expression.

The posttranslational modifications of histone residues are the main mechanism of chromatin structure regulation (Zentner et al., 2013). To date, several posttranslational modifications have been described, such as lysine acetylation, methylation and ubiquitination, arginine methylation and serine phosphorylation (Kouzarides et al., 2007). The acetylation of lysine residues is the first covalent modification to be identified in histones, which are associated with euchromatin (Allfrey et al., 1964; Marushige et al., 1976; Li et al., 2007). Lysine acetylation of histones increases gene transcription, mediating the neutralization of the positive charge of lysine residues, decreasing the

interaction between DNA and nucleosomes, and facilitating the access of the transcriptional machinery to DNA (Kouzarides et al., 2007; Zentner et al., 2013). Histone acetylation is a dynamic modification catalyzed by two enzymes families: i) the Histone Acetyl Transferases (HATs), which catalyze the addition of acetyl groups on lysine residues of histones and ii) the Histone Deacetylase (HDACs), which removes the acetyl groups from the lysine residues of histones and non-histone proteins (Kouzarides et al., 2007; Yang et al., 2008-1, Zentner et al., 2013).

Remarkably, the temporal lobes of AD patients exhibit a reduction in the acetylation of histones compared with aged controls (Zhang et al., 2012). A similar trend was observed in the hippocampus of AD mice (16-month old Tg2576) compared with control animals (Ricobaraza et al., 2009). Additionally, other AD mice (CK-p25 transgenic mice) presented with lower levels of histone H3 and H4 acetylation in the hippocampus than control animals. Surprisingly, the cognitive deficit of these AD animals decreased with exposure to an enriched environment, and this event correlated with increases in histone H3 acetylation. In addition, treatment with the HDAC inhibitor sodium butyrate reduces cognitive deficits and increases the expression of the synaptic marker synaptophysin (Fisher et al., 2007). Concordantly, the treatment of AD mice with non-selective HDAC inhibitors prevents cognitive deficits (Francis et al., 2009; Kilgore et al., 2010) and behavioral impairment (Zhang et al., 2013). These effects are associated with the following: i) a decrease in Aβ deposition in the cortex and hippocampus (Zhang et al., 2013); ii) the acetylation of histones in the cortex and hippocampus (Ricobaraza et al., 2009; Kilgore et al., 2010; Govindarajan et al., 2011); and iii) increases in the expression of genes involved in synaptic plasticity and memory consolidation in the hippocampus

(Ricobaraza et al., 2009; Govindarajan et al., 2011). Altogether, these results support the role of HDACs in the neurodegenerative process in AD.

4.3 HDACs functions in the adult brain

Until now, 18 enzymes have been identified as having deacetylase activity, and they are divided into four classes. Class I, II and IV members include eleven enzymes also known as the classical HDACs or the Rpd3/Hda1 family. These enzymes are characterized by a Zn^{2+} in the catalytic site (Yang et al., 2008-2; Haberland et al., 2009). The class III or sirtuin family has seven members, and their activity is NAD^{+} dependent (Duan et al., 2013). The protein deacetylation catalyzes by these enzymes can regulate different functions in the cell, while histone deacetylation represses gene expression (Bannister et al., 2011; Kouzarides et al., 2007; Zentner et al., 2013). The deacetylation of other proteins regulates activity and subcellular distribution (Yang et al., 2008-1).

The class I HDACs (the group that includes HDAC1, HDAC2, HDAC3 and HDAC8) play a crucial role in the nervous system. In fact, during mouse brain development, HDAC1 is primarily expressed in neuronal progenitors and glia, but its expression decreases in differentiated neurons (McDonald et al., 2008). Although the mice that overexpress HDAC1 in the nervous system do not show changes in LTP and associative or spatial memory (Guan et al., 2009), the loss of function induces aberrant cell cycle reentry and DNA damage in primary cortical neurons (Kim et al., 2008). In addition, HDAC1 overexpression in hippocampus facilitates the extinction of fear memory in mice (Bahari-Javan et a., 2012). However, the focal deletion of HDAC3 in the mouse hippocampus promotes long-term memory, an effect that is accompanied by increases in c-fos and Nr4A2 expression (McQuown et al., 2011). In addition, HDAC3 overexpression induces cell death in rat cerebellar granule neurons and cortical neurons; in contrast, HDAC3

knockdown prevents cell death in response to high concentrations of potassium (Bardai et al., 2011).

Interestingly, HDAC2 is expressed at low levels in neuronal progenitors during brain development but its expression is induced through neuronal differentiation, as it is the main HDAC in the adult brain.. Although HDAC2-knockout mice show a severe cardiac malformation, and its phenotype is lethal in P1, they do not exhibit obvious brain defects (Montgomery et al., 2007). Interestingly, nervous system-specific HDAC2-deficient mice present with improved associative learning and increased synaptic plasticity (Guan et al., 2009; Morris et al., 2013). Furthermore, HDAC2-KO mice have increased expression of synaptophysin, a synaptic marker, and a higher number of dendritic spines in the CA1 region neurons than wild-type mice (Guan et al., 2009). In contrast, HDAC2-overexpressing mice show cognitive impairments, decreased synaptic plasticity, reduced dendritic spine numbers and a lower expression of synaptophysin than wild-type mice (Guan et al., 2009). Furthermore, HDAC2 but not HDAC1 is recruited preferentially to the promoters of neuron-related genes (Guan et al., 2009). These results support a foremost role of HDAC2 in transcriptional repression in adult neurons. In fact, HDAC2 is involved in the control of BDNF-induced gene expression, through the S-nitrosylation of HDAC2, inducing its release from the promoter of BDNF-regulated genes, Fos, Vgf and Egr-1, triggering increases in H3 and H4 histone acetylation and the transcriptional activation of these genes (Nott et al., 2008).

4.4 The HDAC2 is involved in Alzheimer's disease neurodegeneration

Interestingly, some studies using HDAC inhibitors have suggested that class I HDACs could be involved in AD pathology (Fisher et al., 2007; Zhang et al., 2013). In fact, the treatment of AD transgenic mice with entinostat (MS275), an HDAC1, HDAC2 and

HDAC3 inhibitor, reduces neuroinflammation and β-amyloid deposition and prevents decreases in nesting behavior (Zhang et al., 2013). These results suggest that HDAC1, HDAC2 or HDAC3 could be deregulated in neurodegenerative conditions. Interestingly, the p25 transgenic mouse, an AD model (Cruz et al., 2003, Cruz et al., 2006), shows increased levels of HDAC2, but not of HDAC1 and HDAC3, in the CA1 area of the hippocampus (Gräff et al., 2012). Equally important; these animals have increased HDAC2 recruitment at the promoter of genes related to memory, learning and synaptic plasticity than wild-type animals, these changes have been associated with reduced acetylation of histone H4 in neuronal gene promoters and neuronal gene repression.

On the other hand, reduced HDAC2 levels induce histone acetylation of promoters, which activates the expression of the neuronal genes analyzed. Furthermore, the reduction in HDAC2 levels alleviates the cognitive deficit and restores synaptic plasticity in p25 transgenic mice. Concordantly, the AD mouse model 5XFAD presents with increased HDAC2 levels in the cortex and hippocampus without changes in HDAC1 and HDAC3 levels. In addition, the postmortem analysis of the hippocampus of patients with varying degrees of non-familiar AD showed increased HDAC2 levels, but no changes in HDAC1 and HDAC3 levels (Gräff et al., 2012). Also, HDAC2 has been implicated in the HIV-associated neurocognitive disorder; treatment with the HIV protein TAT induces an increase in HDAC2 levels that triggers the repression of CamkIIa and Creb genes (Saiyed et al., 2011). Additionally, the treatment with atypical antipsychotics, such as clozapine, increases HDAC2 levels, mediating the reduction of the metabotropic glutamate receptor 2 (mGlur2) expression (Kurita et al., 2012).

Thus, the evidence suggests that increased HDAC2 levels worsen both the neuronal and synaptic functions in AD. Therefore, HDAC2 inhibition or the control of HDAC2 activity

could be a strategy to ameliorate the pathology progression and to improve the cognitive skills of AD patients. Although treatment with HDAC inhibitors has demonstrated to be effective against the cognitive decline and neuronal gene repression in AD mouse models (Fisher et al., 2007; Francis et al., 2009; Ricobaraza et al., 2009; Kilgore et al., 2010; Govindarajan et al., 2011), currently, there are no available selective inhibitors for HDAC2 (Gräff et al., 2013).

HDAC2 activity is regulated by several post-translational modifications such as serine phosphorylation (Tsai et al., 2002), polyubiquitination (Krämer et al., 2003), sumoylation (Brandl et al., 2012), acetylation (Adenuga et al., 2010), nitrosylation (Nott et al., 2008) and nitration (Osoata et al., 2009). HDAC2 phosphorylation in serine increases its enzymatic activity and promotes the formation of transcriptional repressor complexes (Tsai et al., 2002). Additionally, treatment with valproic acid triggers the poly-ubiquitination and proteasomal degradation of HDAC2 through the E2 ubiquitin-conjugating enzyme Ubc8 and the E3 ubiquitin ligase RLIM (Krämer et al., 2003). The E3 ubiquitin ligase Mule mediates HDAC2 polyubiquitination in response to sodium butyrate (Zhang et al., 2011). Furthermore, HDAC2 nitration at tyrosine 253 induces its proteasomal degradation (Osoata et al., 2009). Also, the exposition of human bronchial epithelial cells to cigarette extracts induces HDAC2 acetylation, although the role of this modification is unclear (Adenuga et al., 2010). In neurons, as previously described, the nitrosylation of HDAC2 at cysteine residues causes its release from chromatin and promotes the transcription of genes regulated by neurotrophins (Nott el al., 2008). However, little is known regarding the signaling pathways that increase HDAC2 levels in AD.

4.5. c-Abl tyrosine kinase

The *abl1* gene encodes for a non-receptor tyrosine kinase called c-Abl; this protein in addition to Arg are members of the Abl family. The Abl1 gene was first identified as the cellular homolog of the oncogene in Abelson murine leukemia virus genome (Goff et al., 1980; Greuber et al., 2013). Later, it was identified as the oncogene associated with the development of chronic myelogenous leukemia (CML), which is an oncogene formed by the translocation between chromosome 9 and 22, generating the Bcr-Abl fusion protein, also known as Philadelphia chromosome (Ben-Neriah et al., 1986; Greuber et al., 2013).

The Abl family, along with members of the Src and Tec family of tyrosine kinases, is organized into three-homology domains: i) SH1 (tyrosine kinase catalytic core); ii) SH2 (to dock to phosphorylated tyrosine residues); and iii) SH3 (which binds to sequences rich in proline). These domains are conserved among the three families; however, the domains beyond the Src homology domains distinguish c-Abl from other tyrosine kinases (Hantschet et al., 2004; Greuber et al., 2013). In fact, the vast carboxy-terminal portion of c-Abl has proline-rich motifs to interact with the SH3 domains of other proteins, such as p53 (Goga et al., 1995), and motifs to interact with globular (G) and filamentous (F)-actin, (Van Etten et al., 1994) and DNA (David-Cordonier et al., 1999). Furthermore, c-Abl has three nuclear localization signals and one nuclear export signal, which allow a dynamic transport between the cytosol and the nucleus in response to different stimuli (Hantschet et al., 2004; Greuber et al., 2013). In addition, alternative splicing produces two c-Abl isoforms: isoform 1b, which is myristoylated in the N-terminal region and is associated with membranes, while isoform 1a is cytosolic (Hantschel et al., 2003) (Figure 1).

Figure 1

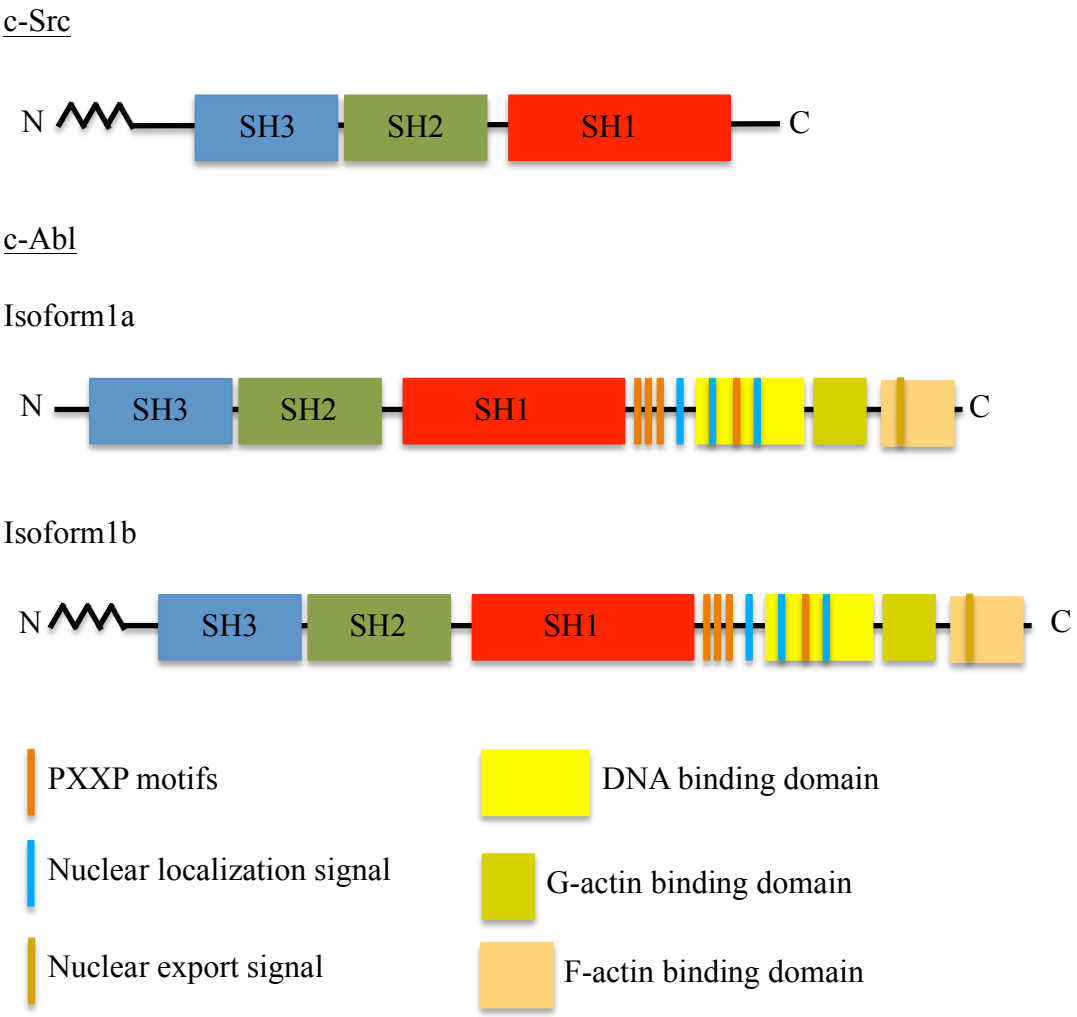

Figure 1. Molecular structure of c-Abl. The c-Abl tyrosine kinases similar to Src has a modular structure arranged in three domains: SH1 (tyrosine kinase domain), SH2 (to dock to phosphorylated tyrosine residues) SH3 (binds to sequences rich in proline). Besides, in the carboxyl-terminal region c-Abl has proline-rich motifs (PXXP motifs) and binding domains to DNA, G-actin and F-actin. Also, c-Abl has two isoforms by alternative splicing; c-Abl 1b isoform can be myristoylated, which is crucial for c-Abl acquires an inactive conformation.

In the cell, c-Abl activity is tightly controlled. Usually, c-Abl acquires an autoinhibited conformation, where the SH3 domain binds to the polyproline-containing linker sequence that connects the SH2 and the kinase domains. Thus, the SH2 domain can interact with the kinase domain (SH1), forming an SH3–SH2–SH1 clamp structure (Barilá et al., 1998; Greuber et al., 2013). Additionally, the myristoylation of c-Abl prevents the activation of this kinase; the myristoyl group binds to the hydrophobic pocket in the kinase domain, docking the SH2 domain against the kinase domain (Hantschel et al., 2003).

On the other hand, tyrosine phosphorylation induces c-Abl activity. Tyrosine 412 within the catalytic domain and tyrosine 245 within the SH2-kinase domain linker are phosphorylated, which activates c-Abl (Hantschet et al., 2004). In fact, the mutation of tyrosine 412 to phenylalanine (Y412F) produces a drastic reduction in catalytic activity, whereas the mutation of the tyrosine 245 to phenylalanine (Y245F) partially reduces c-Abl activity (Brasher et al., 2000). These phosphorylation reactions can be catalyzed by members of the Src family kinases or by transphosphorylation between c-Abl proteins (Brasher et al., 2000).

Tyrosine phosphorylation by c-Abl is key in the regulation of multiple proteins, influencing different cellular process. For example the phosphorylation of the postsynaptic density 95 (PSD95) by c-Abl modulates its synaptic clustering (Perez de Arce et al., 2010), KAT5 phosphorylation by c-Abl initiates the ATM response to DNA damage (Kaidi et al., 2013), and BMP receptor IA phosphorylation by c-Abl promotes osteoblast expansion through p16 downregulation (Kua et al., 2012). Likewise, tyrosine phosphorylation by c-Abl increases the stability of several proteins such as p73 (Tsai et al., 2003), Yap1 (Levy et al., 2008), c-Jun (Gao et al., 2006), ERα (Xu et al., 2010) and C/EBPβ (Li et al., 2003). Specifically, c-Abl activity induces the interaction of p73 with Yap1, preventing its

interaction with the E3 ligase Itch and its proteasomal degradation (Tsai et al., 2003; Levy et al., 2008). In the same way, c-Jun tyrosine phosphorylation by c-Abl prevents the interaction with Itch, preventing its polyubiquitination and proteasomal degradation (Gao et al., 2006).

In addition, c-Abl participates in several cellular functions, including the following: cytoskeleton dynamics, cell proliferation, cell differentiation, cellular adhesion, migration, cell cycle arrest, response to stress, and apoptosis (Estrada et al., 2011; Greuber et al., 2013). The cellular functions of c-Abl are dependent on its subcellular localization. Cytoplasmic c-Abl participates in cell proliferation, differentiation, cytoskeleton dynamics and receptor endocytosis (Sirvent et al., 2008), whereas nuclear c-Abl is involved in cell cycle arrest and apoptosis (Wen et al., 1994; Sridevi et al., 2013). The subcellular localization of c-Abl is regulated through the phosphorylation of threonine 735, a modification that promotes its interaction with 14-3-3 protein, thus sequestering c-Abl in the cytoplasm. However, in response to DNA damage, 14-3-3 proteins are phosphorylated by JNK, releasing c-Abl, which then translocates to the nucleus (Yoshida et al., 2005).

Reflecting on the key role of c-Abl in cellular functions, the knockout mice for this protein display high perinatal mortality, with abnormal spleen, thymus, and eye development, T and B cell lymphopenia and osteoporosis (Schwartzberg et al., 1991; Tybulewicz et al., 1991; Li et al., 2000; Kua et al., 2012). Interestingly, the double knockout of c-Abl and Arg shows interrupted neurulation and death in the embryonic stage (Koleske et al., 1998). However, the conditional deletion of c-Abl in neurons and glia precursors over an Arg-knockout background does not cause brain abnormalities, but causes defects in the cerebellum development and motor coordination (Qiu et al., 2010).

Interestingly, it has been described that the tyrosine kinase activity of c-Abl might also regulate chromatin dynamics; COS-1 cells overexpressing c-Abl exhibit decreased acetylation of histone H3 and H4, decreased lysine-4 trimethylation of histone H3 and an increases in trimethylated histone H3 at lysine 9 (Aoyama et al., 2011). These epigenetic modifications produce a heterochromatin structure, which limit the accessibility of the transcriptional machinery to DNA. Thus, these data suggest that the c-Abl activity could promote gene repression.

4.6 Role of c-Abl in neurodegenerative processes

The phenotype of the c-Abl and Arg double knockout mice supports the role of Abl family in neuronal physiology. Indeed, c-Abl plays a key role in several neuronal processes, such as neurogenesis, neuronal migration (Koleske et al., 1998), neurite outgrowth (Zukerberg et al., 2000), synaptic plasticity (Moresco et al., 2003; Perez de Arce et al., 2010), dendrogenesis (Jones et al., 2004) and cytoskeleton dynamics (Woodring et al., 2002).

Additionally, c-Abl has been implicated in neuronal death in AD. Our laboratory showed that the c-Abl is activated *in vitro* and *in vivo* using AD models. In primary cultures of hippocampal neurons exposed to Aβ fibrils, c-Abl activation mediates neuronal death through the activation of the p73 transcription factor. Interestingly, treatment with the c-Abl inhibitor Imatinib prevents the neuronal death induced by Aβ fibrils (Alvarez et al., 2004). Furthermore, c-Abl modulates the activity of the APP intracellular domain (AICD), which participates in the transcriptional induction and the apoptotic response in neurodegenerative models (Vásquez et al., 2009). AD animal models, rats exposed to hippocampal injections of Aβ fibrils and transgenic APPswe/PSEN1ΔE9 mice, show activated c-Abl/p73 signaling. In addition, intraperitoneal administration of Imatinib in these AD mouse models prevents neuronal loss and cognitive deficits and decreases Aβ

deposition (Cancino et al., 2008). Concordantly, the overexpression of constitutively active c-Abl in adult mouse brains induces severe neurodegeneration and inflammation, while the overexpression of an active form of Arg does not produce an obvious phenotype (Schlatterer et al., 2011). Moreover, patients with clinical history of Alzheimer's disease dementia show active c-Abl in the hippocampal area CA1 (Jing et al., 2009).

c-Abl inhibition by Imatinib treatment reduces tau phosphorylation in APPswe/PSEN1ΔE9 mice. In fact, c-Abl through the Cdk5 phosphorylation induces tau phosphorylation (Cancino et al., 2011). In addition, c-Abl participates in synaptic loss and dysfunction in response to Aβ oligomers, an effect that is prevented by Imatinib treatment (Vargas et al., 2014). Finally, c-Abl increases Aβ production, most likely to promote the amyloidogenic processing of APP (Estrada et al., 2014).

c-Abl is involved in other neurodegenerative processes, beyond those described in AD. The Niemann-Pick type C disease mouse model shows increases in both levels and activity of c-Abl. Imatinib treatment in this model had the following effects: i) prevented apoptosis in the cerebellum; ii) improved the locomotor function and iii) increased survival of animal models of this disease (Alvarez et al., 2008). Also, mice treated with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a chemical model of Parkinson's disease, exhibit an increase in c-Abl activity. The substantia nigra and striatum from Parkinson's disease patients show an increase in c-Abl activity. In addition, the conditional deletion of c-Abl in neurons of mice prevents the MPTP-induced loss of dopaminergic neurons (Ko et al., 2010). Furthermore, SOD1 G93A transgenic mice, a model of amyotrophic lateral sclerosis, also show increases in both levels and activity of c-Abl in the lumbar spinal cord. The treatment of the ALS mice with dasatinib, a second-generation c-Abl inhibitor,

increases survival and prevents motor neuron loss, locomotor deficits and weight loss (Katsumata et al., 2012).

In summary, the evidence supports a role for HDAC2 in epigenetic repression in AD; however the mechanisms involved in HDAC2 regulation are not fully elucidated. With this background, the focus of our research was on elucidating the molecular mechanisms that trigger the HDAC2-mediated gene repression in Alzheimer's disease models. Interestingly, c-Abl, a tyrosine kinase involved in neurodegenerative processes, participates in the epigenetic control of gene expression, most likely by HDAC regulation, which suggests a role for c-Abl in HDAC2 regulation. In fact, our findings suggest that c-Abl activity participates in gene repression in AD through increasing HDAC2 levels and activity.

HYPOTHESIS

"The c-Abl tyrosine kinase increases HDAC2 levels and regulates the expression of neuronal genes".

AIMS

6.1 Goal

To demonstrate that c-Abl tyrosine kinase increases HDAC2 levels and regulates the expression of neuronal genes.

6.2 Specifics Aims

6.2.1. To demonstrate that c-Abl tyrosine kinase increases HDAC2 levels.

6.2.2. To investigate the mechanism by which c-Abl regulates both HDAC2 levels and activity.

6.2.3 To demonstrate that the c-Abl/HDAC2 signaling pathway regulates the expression of neuronal genes.

PAPER ACCEPTED IN MOLECULAR CELL

c-Abl stabilizes HDAC2 levels by tyrosine phosphorylation repressing neuronal gene expression in Alzheimer Disease

c-Abl stabilizes HDAC2 levels by tyrosine phosphorylation repressing neuronal gene expression in Alzheimer Disease

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Running Title: c-Abl induces neuronal gene repression.

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: i) in neurons, c-Abl inhibition with Imatinib prevents the AβO-induced increase in HDAC2 levels ii) c-Abl knockdown cells show a decrease in HDAC2 levels, while c-Abl overexpression increases them, iii) c-Abl inhibition reduces HDAC2-dependent repression activity and HDAC2 recruitment to the promoter of several synaptic genes, increasing their expression iv) c-Abl induces tyrosine phosphorylation of HDAC2, a novel previously unknown posttranslational modification, affecting both its stability and repression activity v) 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.

Highlights

- c-Abl inhibition prevents the increase of HDAC2 in AD models.
- c-Abl inhibition decreases HDAC2-dependent gene repression.
- Tyrosine phosphorylation of HDAC2 by c-Abl regulates its stability and activity.
- c-Abl activity inhibits the ubiquitination and proteasomal degradation of HDAC2.

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 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 et al., 2013). Thus, the evidence suggests that HDACs are involved in AD pathology.

Histone deacetylases (HDACs) are a group of enzymes that remove the acetyl groups on lysine residues of histones, inducing gene repression (Bannister et al., 2011; Kouzarides et al., 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 (McDonald et al., 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 its activation worsens neuronal and 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 non-familiar 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 pan-inhibitor 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, tau 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 Alzheimer Disease 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 Aβ oligomers (AβO) induce an increase in HDAC2 levels in primary culture of hippocampal neurons (Gräff et al., 2012). Neurons at 7DIV, exposed to 5 μM AβO, for 6 h and 24 h, presented higher HDAC2 protein levels than controls (Figure 2A), which localized mainly in the nucleus (Figure 2B). Interestingly, co-treatment with the c-Abl inhibitor Imatinib, prevented the AβO-induced increase in HDAC2 levels (Figure 2A and 2B). These results suggest that activation of c-Abl by Aβ aggregates (Alvarez et al., 2004; Cancino et al., 2008), could contribute to HDAC2 up regulation 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 3A), although surprisingly, HDAC2 mRNA levels do not change significantly (Figure 3D). Similar results were obtained in HeLa cells treated with Imatinib (Figure 9 S1A &S1B).

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 3B), and also overexpressed c-Abl using recombinant c-Abl-GFP (Figure 3C). HeLa cells were transfected with a plasmid expressing a shRNA against c-Abl or a scrambled shRNA, and HDAC2 levels were evaluated 48 h later. The reduction in c-Abl levels was associated with a significant decrease in HDAC2 levels (Figure 3B). Conversely, overexpression of c-Abl in HT22 cells caused a significant increase in HDAC2 levels (Figure 3C). Similar results were obtained in HeLa cells, where c-Abl overexpression increased HDAC2 protein levels while mRNA levels remained constant (Figure 10 S2A &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 et al., 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 (Figure 3E & Figure 11 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 11 S3B). 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 co-transfected 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 3F), 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 3G) than those used in the previous experiment (0.5 µg; Figure 3F). These results demonstrate that c-Abl regulates HDAC2 levels and that c-Abl inhibition decreases the transcriptional repression activity of HDAC2.

Figure 2

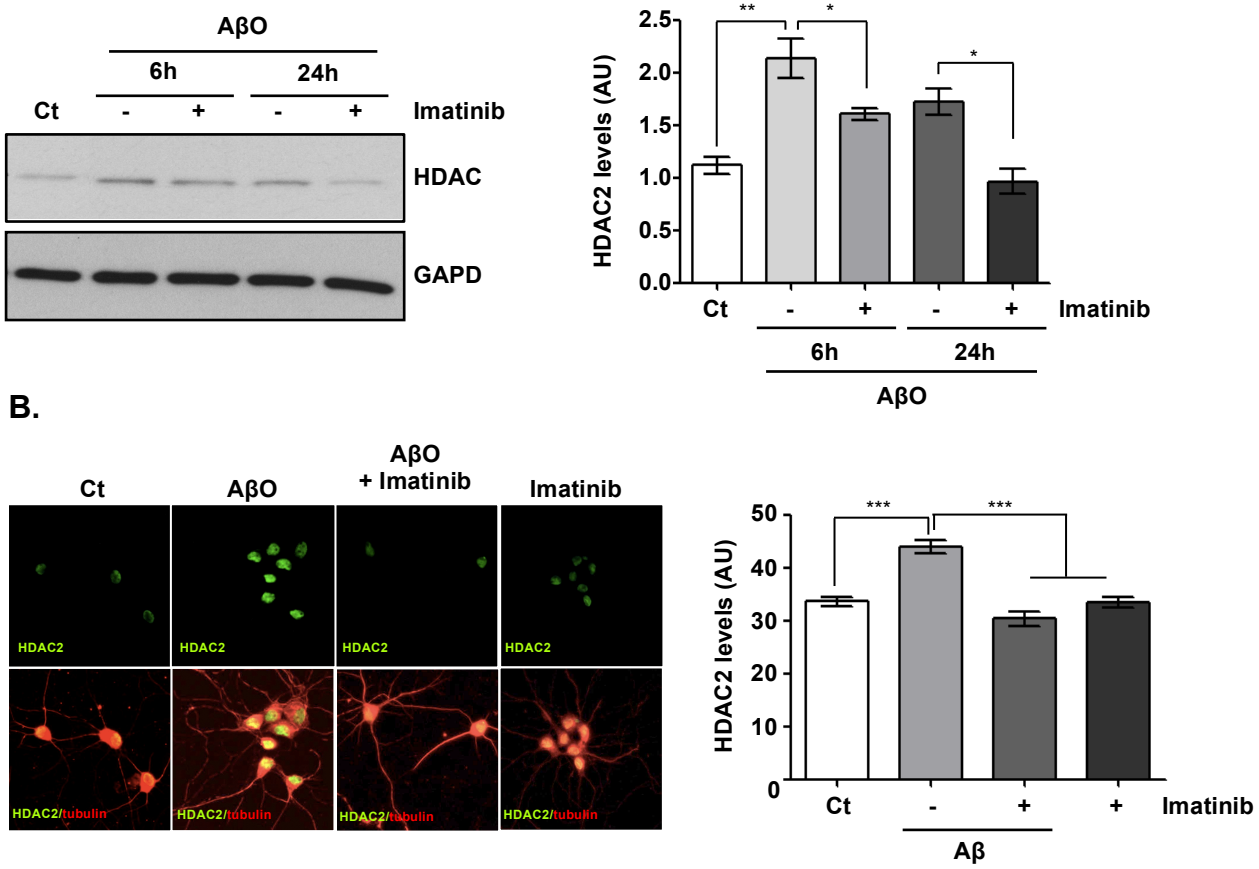
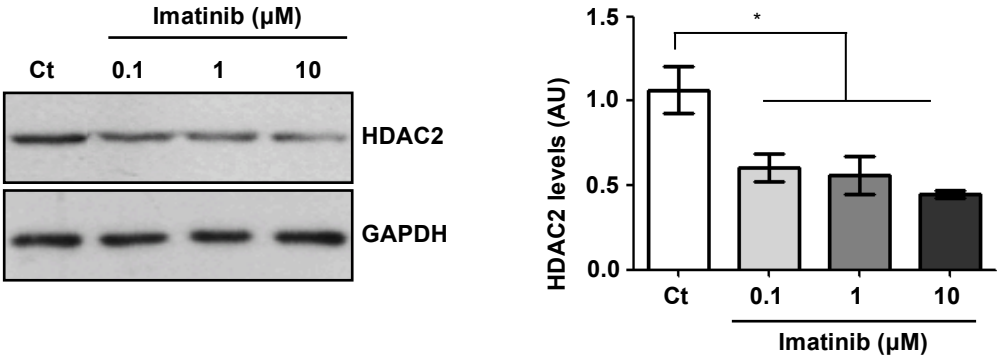


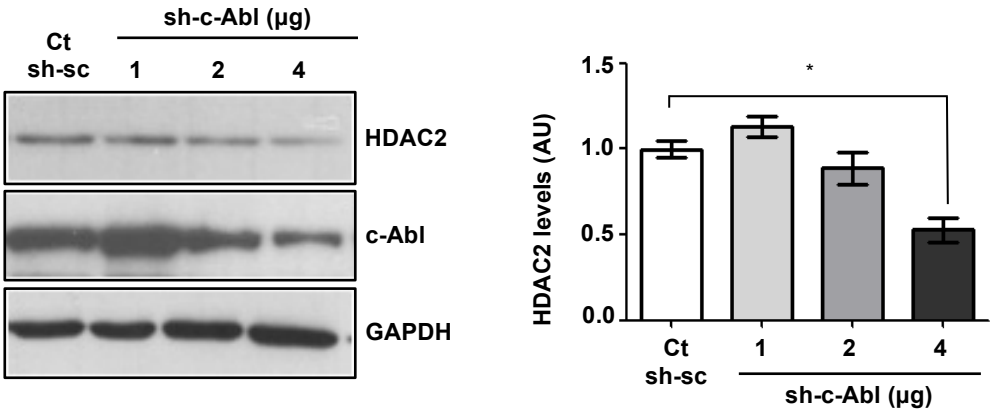
Figure 2. **Imatinib treatment prevents the increase in HDAC2 levels induced by AβO in Hippocampal neurons.** (A) Hippocampal neurons (7DIV) were treated either with vehicle (control), AβO 5 μM or AβO 5 μM plus Imatinib 5 μM for 6 or 24 h. Representative western blot of HDAC2 protein levels and quantification normalized against GAPDH levels (n=3). (B) Representative immunofluorescence and quantification of HDAC2 (green) and tubulin (red) levels in neurons after 24 h 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 ± s.e.m

Figure 3

A.


Imatinib (μM)
Ct
0.1
1
10
HDAC2
GAPDH
HDAC2 levels (AU)
1.5
1.0
0.5
0.0
*
Ct
0.1
1
10
Imatinib (μM)

B.


sh-c-Abl (μg)
Ct
sh-sc
1
2
4
HDAC2
c-Abl
GAPDH
HDAC2 levels (AU)
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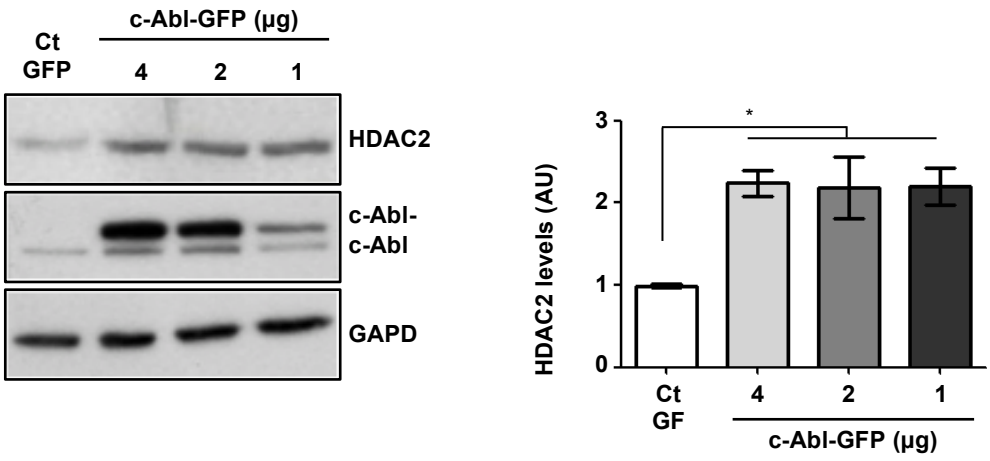
1.0
0.5
0.0
*
Ct
sh-sc
1
2
4
sh-c-Abl (μg)

C.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GFP
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2
1
HDAC2
c-Abl-
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0
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Ct
GF
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2
1
c-Abl-GFP (μg)

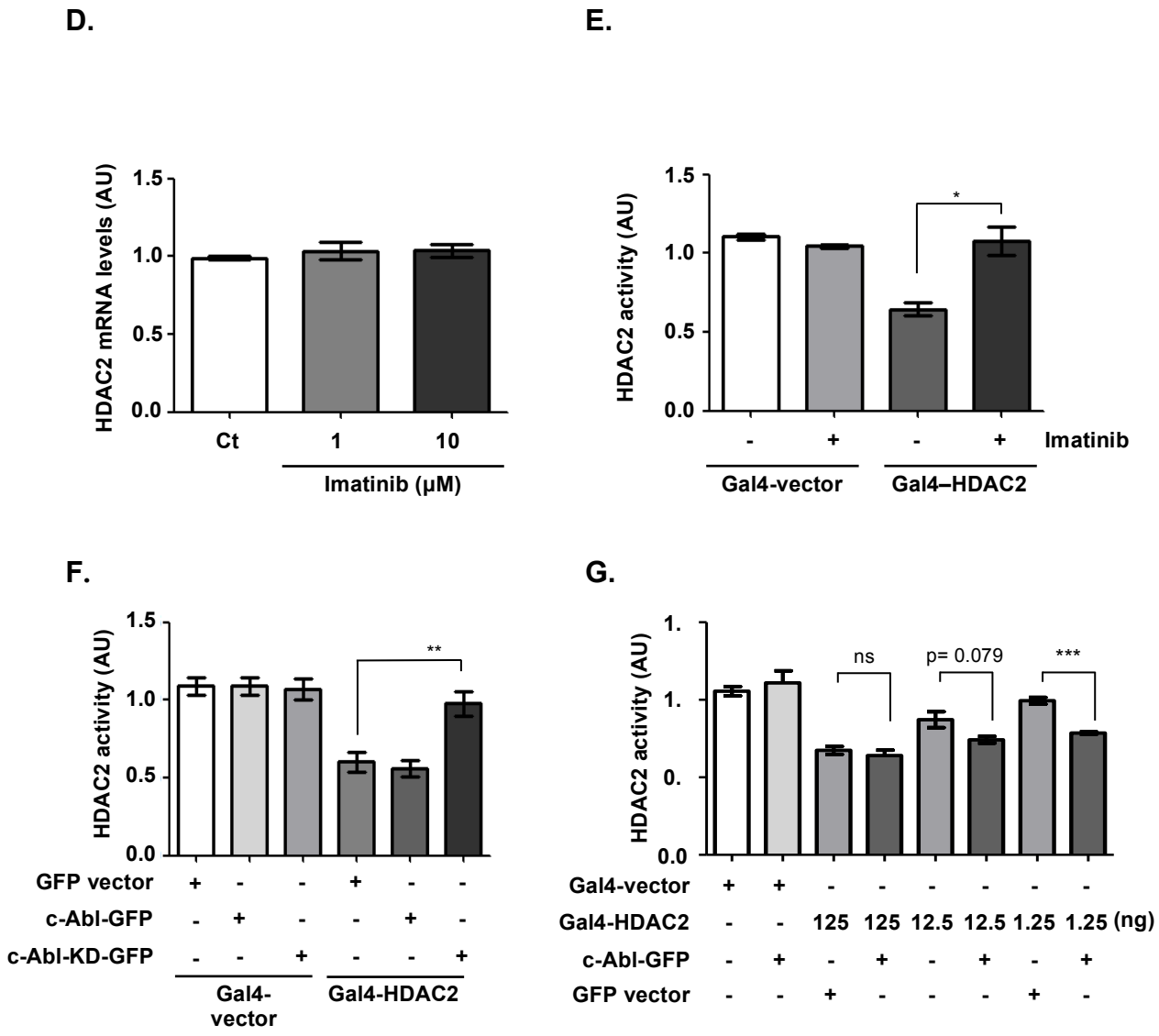


Figure 3. **c-Abl increases protein levels and repression activity of HDAC2.**

(A) Representative western blot and quantification of HDAC2 protein levels of HT22 cells treated with Imatinib (0.1, 1 and 10 μM) for 24 h (n=3). (B) Representative Western Blot images and quantification of 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 h (n=3). HDAC2 repression activity: Cells were transfected with a plasmid expressing Gal4-TK-luciferase, together with Gal4-HDAC2 or Gal4-vector (negative control).

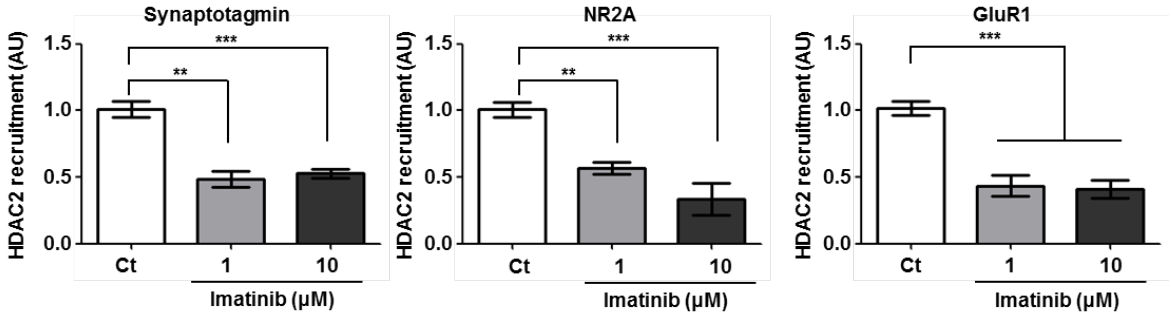
Luciferase activity was normalized against renilla. HDAC2 repression activity assay of: (E) HT22 cells were treated with Imatinib 10 μM for 24h after transfection (n=5). (F) HT22 cells co-transfected with Gal4-TK-luciferase and Gal4-HDAC2 plasmids plus the expression plasmids for: c-Abl (c-Abl-GFP) or dominant negative mutant of Abl (Abl-KD-GFP) or empty vector (GFP), (n=3). (G) HT22 cells co-transfected 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 ± s.e.m

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 4A 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 treatment (Figure 4A). Likewise, Imatinib treatment significantly increased Histone H3 acetylated (H3ac) levels at the promoter of these genes (Figure 4B), 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 (Figure 12 S4A &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 4C). These results are in agreement with the reduction of HDAC2 recruitment on their promoters under c-Abl inhibition.

Figure 4

A.



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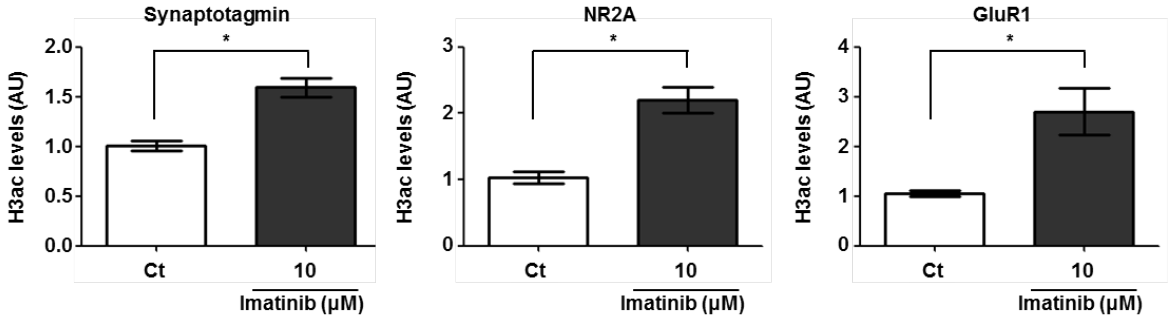


C.

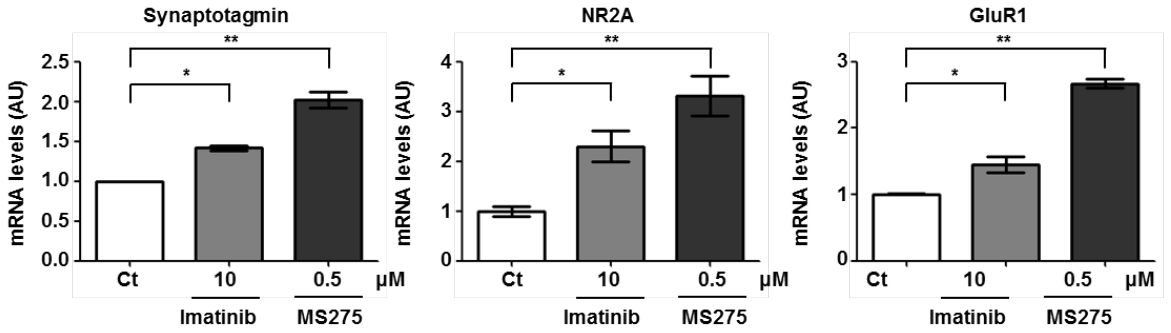


Figure 4. **Imatinib treatment increases the expression of key synaptic genes.** (A) Quantitative PCR results of the promoter of Synaptotagmin, NR2a and GluR1 from HDAC2 immunoprecipitated-chromatin from HT22 cells treated with Imatinib for 24 h (n=3). (B) Quantitative PCR results of the promoter of Synaptotagmin, NR2a and GluR1 from Histone H3 acetylated immunoprecipitated-chromatin from HT22 cells treated with 10 μM Imatinib for 24 h (n=3). (C) Quantitative PCR results of Synaptotagmin, NR2a and

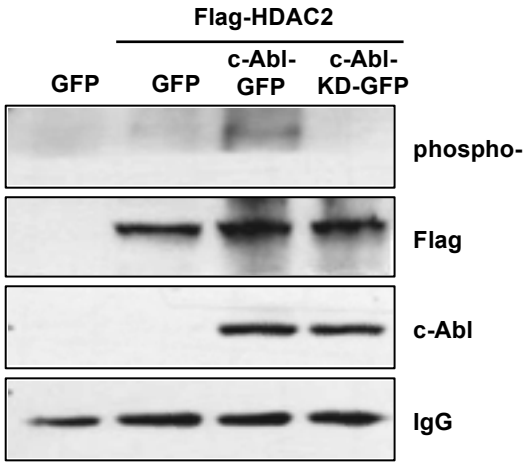
GluR1 mRNA expression in HT22 cells treated with 10 μM Imatinib or 0.5 μM MS275 for 24 h (n=5). Results are from three or more independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$, values are mean ± s.e.m

c-Abl mediates HDAC2 tyrosine phosphorylation.

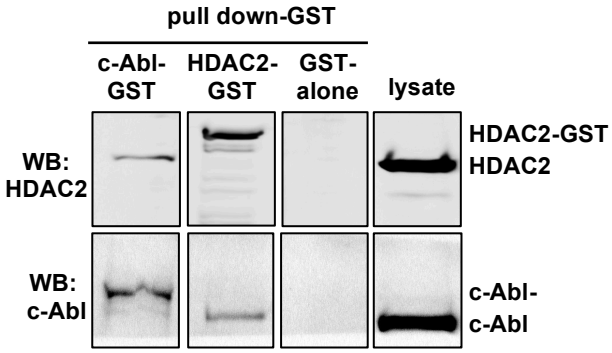
c-Abl dependent phosphorylation regulates the stability of several proteins such as p73 (Tsai et al., 2003), c-Jun (Gao et al., 2006), ERα (He et al., 2010) and C/EBPβ (Li et al., 2009), preventing their proteosomal degradation. We predicted that a similar mechanism could be involved in the regulation of HDAC2 levels by c-Abl. To test this idea we co-transfected HT22 cells with a Flag-HDAC2 expression plasmid plus, c-Abl-GFP, c-Abl-KD-GFP or GFP-empty vectors. 24 h 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 5A). 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 5B). We next performed HDAC2 *in vitro* phosphorylation assays using recombinant human c-Abl (T315I). We found: i) increases in tyrosine phosphorylation levels in HDAC2-GST by western blot (Figure 5C) and ii) increases in the radioactivity incorporated in HDAC2-GST when using [γ-^{32}P]-ATP, by autoradiography (Figure 5D) and the cpm-incorporated in HDAC2-GST compared to CrkII-GST control (% of radioactivity, Figure 5E). Then, herein we demonstrated for the first time, that c-Abl phosphorylates HDAC2.

Figure 5

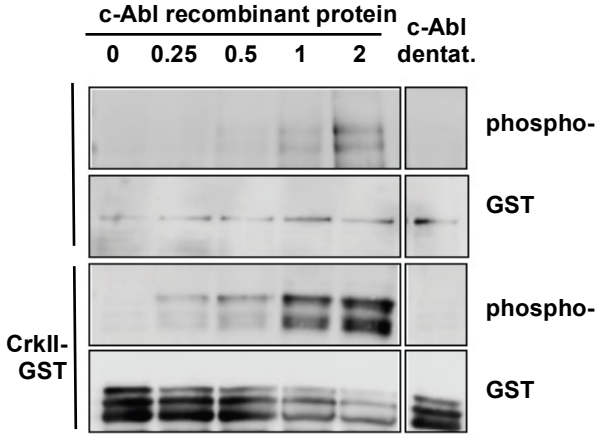
A.


Flag-HDAC2
GFP
GFP
c-Abl-GFP
c-Abl-KD-GFP
phospho-
Flag
c-Abl
IgG

B.


pull down-GST
c-Abl-GST
HDAC2-GST
GST-alone
lysate
WB: HDAC2
HDAC2-GST
HDAC2
WB: c-Abl
c-Abl-
c-Abl

C.


c-Abl recombinant protein
0
0.25
0.5
1
2
c-Abl dentat.
phospho-
GST
phospho-
CrkII-GST
GST

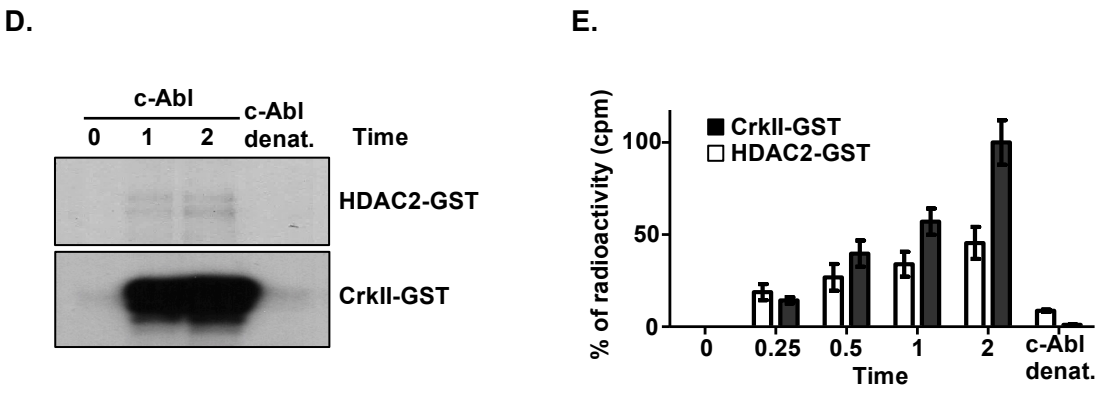


Figure 5**. HDAC2 is phosphorylated by c-Abl kinase. (**A) HDAC2 tyrosine phosphorylation: HeLa cells were transfected with a Flag-HDAC2 expression plasmid, plus expression plasmids for either c-Abl WT (c-Abl-GFP), Abl-KD (c-Abl-KD-GFP) or the control vector (GFP). HDAC2-Flag was immunoprecipitated and 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 h. After the beads washing, the c-Abl and HDAC2 binding was evaluated by western blot (C, D & 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 pull 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 respect CrkII phosphorylation.

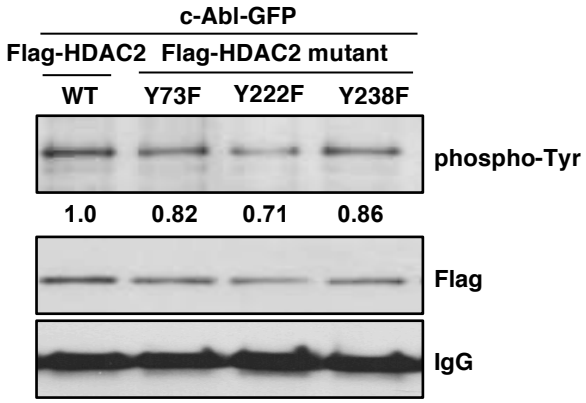
Next, we explored which residues on HDAC2 could be phosphorylated by c-Abl. We used the online plataform Netphos 2.0 to identify tyrosines included in the c-Abl consensus phosphorylation site with the highest phosphorylation probability (Cujec et al., 2002). The *in silico* analysis revealed that tyrosine 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 co-transfection 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 phospho-tyrosine signal respects to wild type Flag-HDAC2 (Figure 6A), suggesting that the tyrosine 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 6B).

Surprisingly, when we evaluated the role of the three potential phosphorylated tyrosines over HDAC2 function by co-transfecting the Gal4-TK-Luciferase plasmid plus Gal4-HDAC2 or the mutants Gal4-HDAC2Y73F, Gal4-HDAC2 Y222F, 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 6C). In the same line, the double and triple 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 6C). 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.

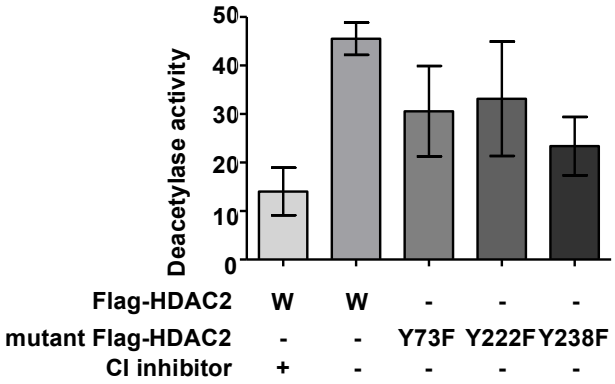
Consistent with these results, Gal4-HDAC2Y222F mutant protein levels were always lower than wild type Gal4-HDAC2, Gal4-HDAC2Y73F and Gal4-HDAC2Y238F protein levels (Figure 6D). A similar result was observed for Flag-HDAC2Y222F protein levels compared to wild type Flag-HDAC2, Flag-HDAC2Y73F and Flag-HDAC2Y238F constructs (Figure 13 S5A) and in the triple mutants that include Y222F change (Figure 13 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.

Figure 6.

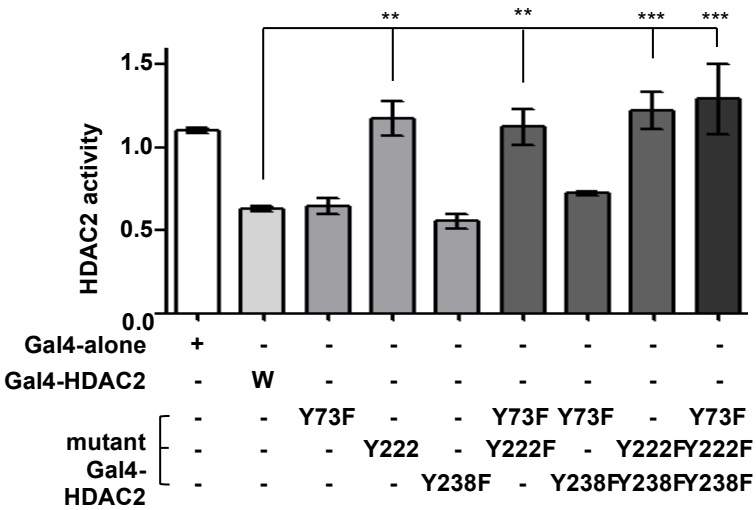
A.


c-Abl-GFP
Flag-HDAC2
Flag-HDAC2 mutant
WT
Y73F
Y222F
Y238F
phospho-Tyr
1.0
0.82
0.71
0.86
Flag
IgG

B.


Deacetylase activity
50
40
30
20
10
0
Flag-HDAC2 W W - - -
mutant Flag-HDAC2 - - Y73F Y222F Y238F
CI inhibitor + - - - -

C.


**
**
***
***
HDAC2 activity
1.5
1.0
0.5
0.0
Gal4-alone + - - - - - - - -
Gal4-HDAC2 - W - - - - - - -
mutant Gal4-HDAC2
- - Y73F - - Y73F Y73F - Y73F
- - - Y222 - Y222F - Y222F Y222F
- - - - Y238F - Y238F Y238F Y238F

D.

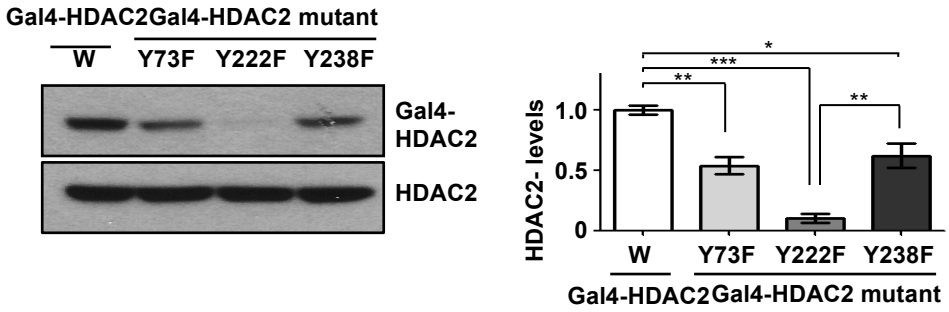


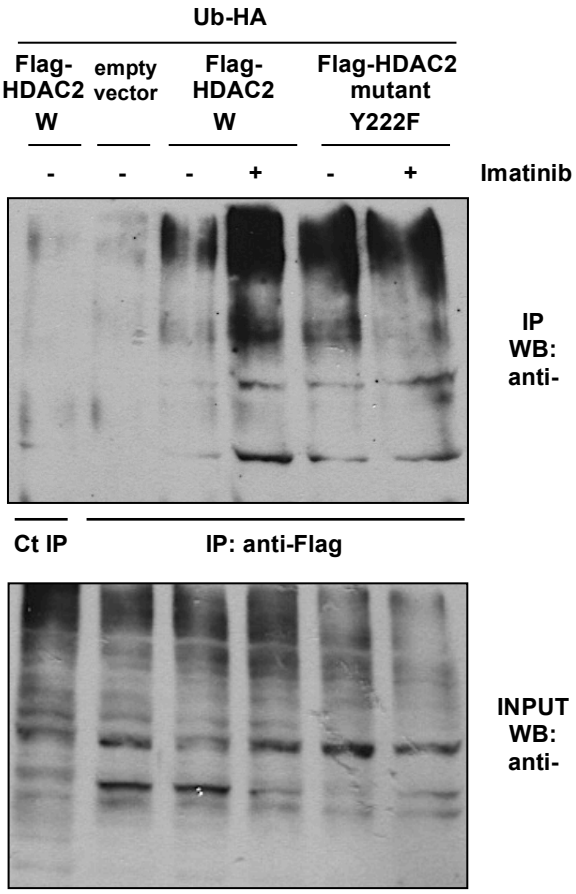
Figure 6. **HDAC2 is phosphorylated by c-Abl in the tyrosine 222, affecting its repression activity.**(A) Tyr-phosphorylation analysis for HDAC2 and the mutant versions Y73F, Y222F and Y238F: HT22 cells were transfected with the plasmids encoding Flag-HDAC2 or the mutant versions Flag-HDAC2 Y73F, Flag-HDAC2 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 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 assed 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 ± s.e.m.

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 7A). Additionally, HDAC2Y222F showed higher polyubiquitination levels than wild type HDAC2, but Imatinib did not increase HDAC2Y222F polyubiquitination (Figure 7A). Our results indicate that the phosphorylation on tyrosine 222 of HDAC2 by c-Abl, prevents HDAC2 polyubiquitination.

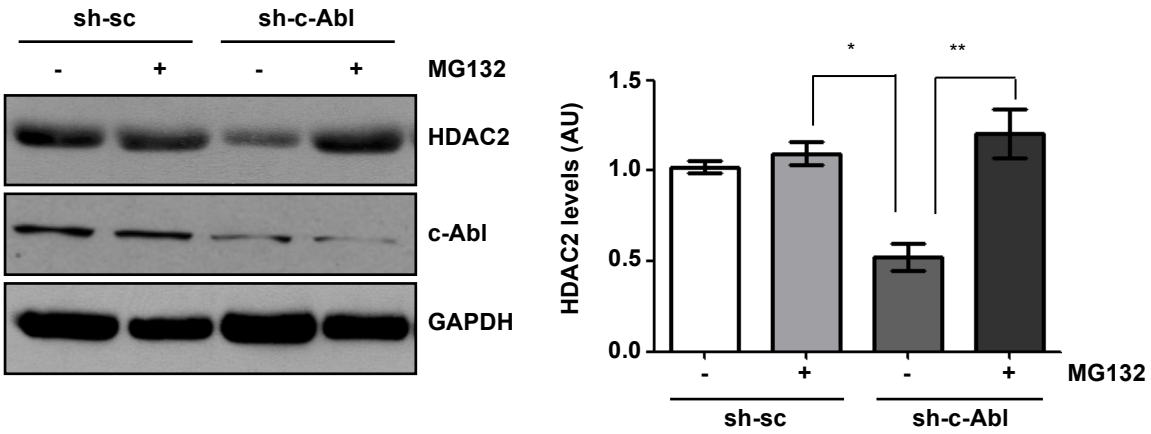
In order to establish whether c-Abl regulates proteasomal degradation of HDAC2 we downregulated c-Abl by transfecting HeLa cells with a shRNA against c-Abl or a control shRNA plasmid. Then, cells were treated with the proteasome inhibitor MG132 (5 μM) or vehicle alone. As previously described, when c-Abl was downregulated we found lower HDAC2 levels compared to control cells. Interestingly, MG132 treatment prevented the decrease in HDAC2 levels (Figure 7B). Moreover MG132 prevented the decrease of the HDAC2-dependent transcriptional repression induced by c-Abl–KD-GFP (Figure 7C). These results show that c-Abl activity prevents HDAC2 proteasomal degradation probably through HDAC2- tyrosine phosphorylation modulating the HDAC2 dependent transcriptional repression.

Figure 7.

A.


Ub-HA
Flag-HDAC2 W
empty vector
Flag-HDAC2 W
Flag-HDAC2 mutant Y222F
- - - + - +
Imatinib
IP WB: anti-
Ct IP
IP: anti-Flag
INPUT WB: anti-

B.


sh-sc
sh-c-Abl
- + - +
MG132
HDAC2
c-Abl
GAPDH
HDAC2 levels (AU)
1.5
1.0
0.5
0.0
*
**
- + - +
MG132
sh-sc
sh-c-Abl

C.

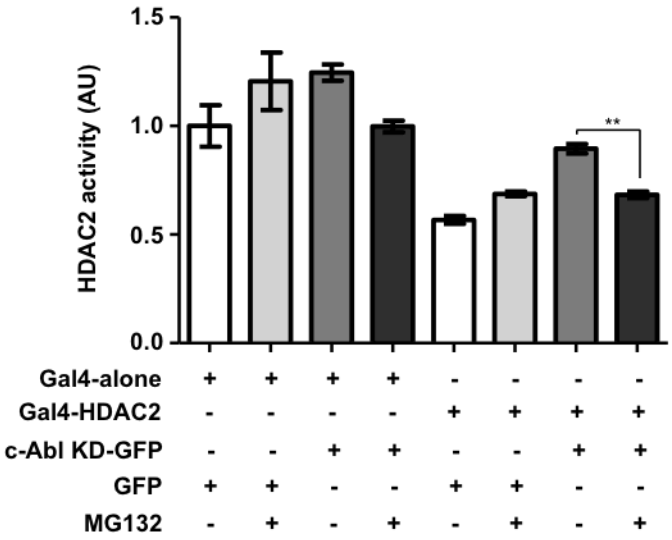


Figure 7**. c-Abl inhibition induces the polyubiquitination and proteasome degradation of HDAC2. (**A) Ubiquitination analysis of HDAC2: HT22 cells were transfected with plasmids expressing Flag-HDAC2 or the mutant version Flag-HDAC2 Y222F, together with Ubiquitin-HA; additionally the cells were treated with Imatinib or vehicle. Anti-HA western blot of the anti-Ubiquitin immunoprecipitated proteins (B) Proteasome inhibition prevents HDAC2 protein levels decrease. 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 10μM (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. 24h after transfection, the cells were treated with MG132 10μM for 24h (n=3). Results are from three independent experiments. * $p<0.05$, values are mean ± s.e.m

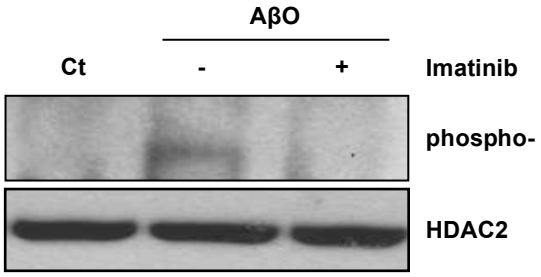
c-Abl activation in AD models stabilizes HDAC2 and increases its recruitment to the promoter of several neuronal genes.

Next, we evaluated whether AβOs treatment, that 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 co-treatment with Imatinib prevented this posttranslational modification (Figure 8A). This result sustains that AβOs –activated c-Abl induces tyrosine phosphorylation on HDAC2 in neurons. To evaluate whether HDAC2 phosphorylation by c-Abl induced by AβOs treatment has functional consequences we analyzed HDAC2 recruitment at the promoter of several HDAC2 target genes. Figure 8B shows that AβOs treatment caused an increase in HDAC2 recruitment to the promoters of *Synaptotagmin, NR2a*, *GluR1* and *Synaptophysin* genes, that 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.

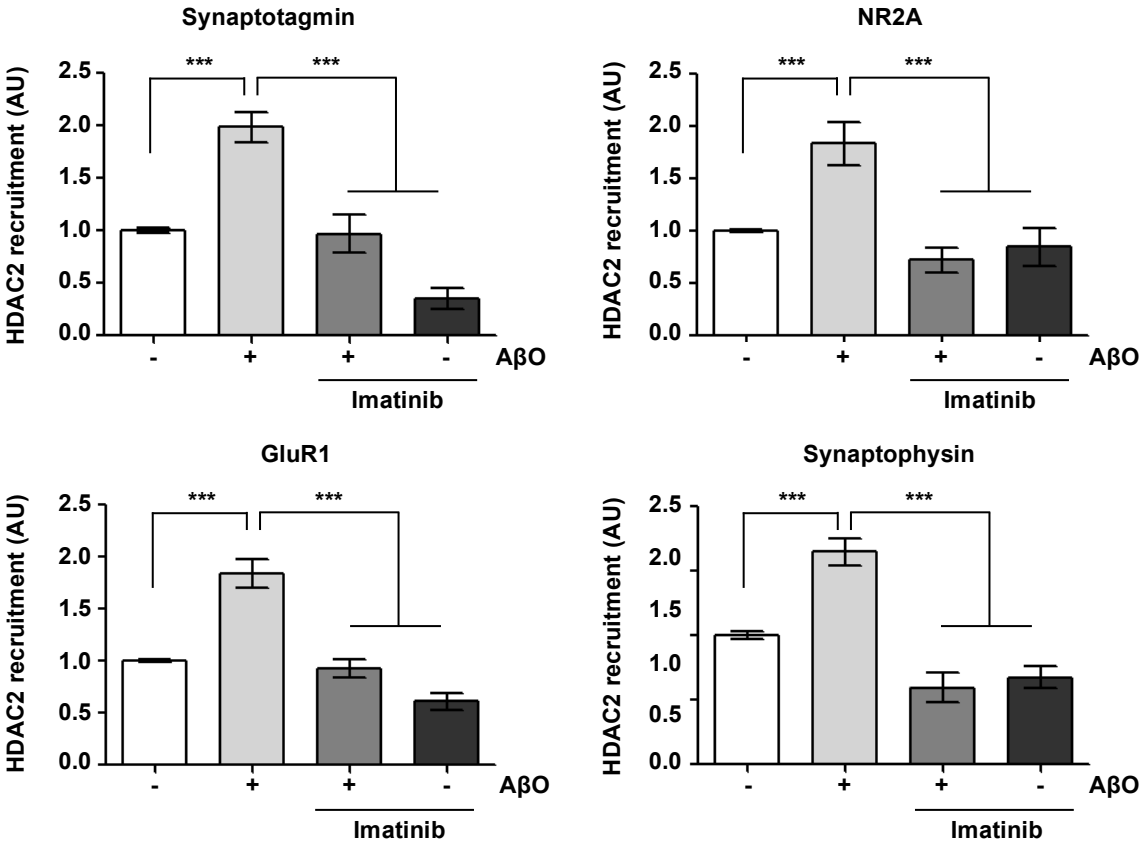
We next studied the c-Abl/HDAC2 pathway *in vivo* in the APPswe/PSEN1ΔE9 transgenic mice model of AD. As it had been previously described, 7 month-old transgenic mice presented higher HDAC2 levels compared to wild type mice (Bie et al., 2014). In agreement with our *in vitro* results (Figure 2A & 2B), intraperitoneal treatment with Imatinib (25 mg/Kg) for two weeks, decreased HDAC2 levels in the AD mice to levels similar to control mice (Figure 8C). In addition, Imatinib treatment reduced HDAC2 levels in the CA1 hippocampus region and cortex of older transgenic mice (ten month-old) (Figure 8D). Altogether, our results suggest that the inhibition of c-Abl could be an effective strategy for preventing gene repression mediated by HDAC2 in AD.

Figure 8.

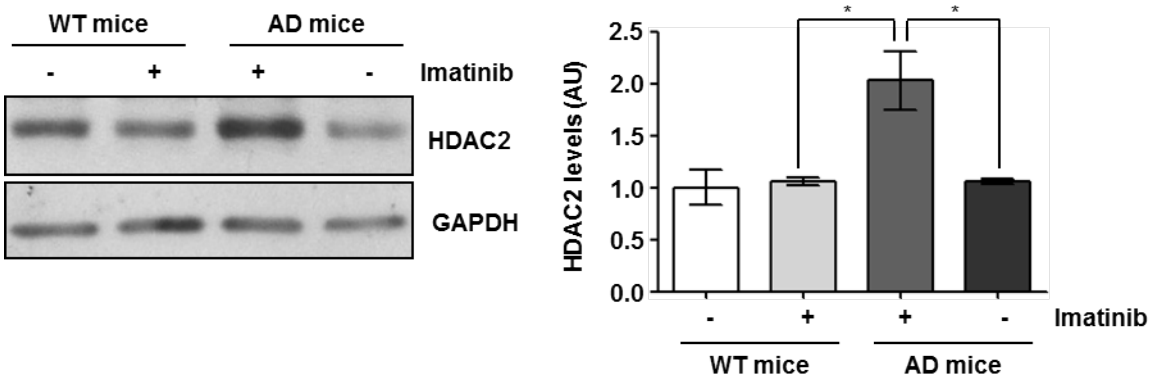
A.


AβO
Ct
-
+
Imatinib
phospho-
HDAC2

B.


Synaptotagmin
NR2A
GluR1
Synaptophysin
HDAC2 recruitment (AU)
2.5
2.0
1.5
1.0
0.5
0.0
***
***
-
+
+
-
AβO
Imatinib

C.



D.

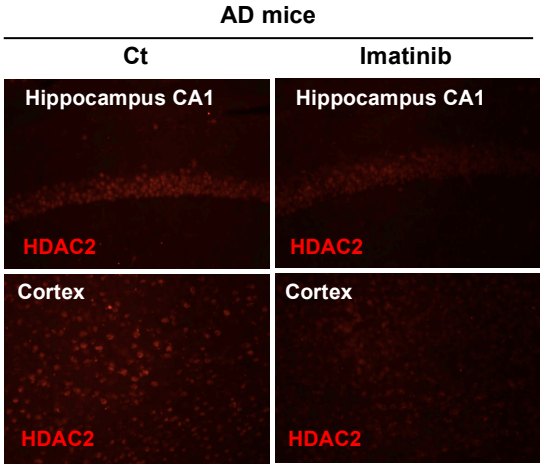


Figure 8**. Imatinib treatment prevents the HDAC2 recruitment to the promoter and the increase of HDAC2 levels in AD models. (**A) HDAC2 Tyrosine phosphorylation: HDAC2 was immunoprecipitated from Hippocampal neurons (7DIV) treated with AβO for 1h plus 5 μM Imatinib and evaluated by western blot using 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 h (n=3). (C) Representative Western blot images and quantification of HDAC2 levels in extracts of brain from seven month-old wild type and APPswe/PSEN1ΔE9 transgenic mice treated with Imatinib at a dose of 25 mg/Kg or

vehicle for two 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)

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 over-activated 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: i) a dose dependent decrease of HDAC2 levels in the presence of Imatinib, a specific c-Abl inhibitor, with lower inhibitory activity on other tyrosine kinases such as c-kit, PDGFR, CSF1R and DDR1/2 (Greuber et al., 2013); ii) a reduction in HDAC2 levels when we used a short hairpin RNA against c-Abl to reduce c-Abl levels, and iii) an increment in HDAC2 levels when we overexpressed c-Abl. Altogether these 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 (Fisher et al., 2007; Francis et al., 2009; Kilgore et al., 2010; Zhang et al., 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, 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, 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 et al., 2013), to affect gene expression. Thus,

c-Abl effects on HDAC2 levels and promoter recruitment could be mediated by different mechanisms; i) c-Abl can directly affect HDAC2 levels or ii) 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 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 shows that the HDAC2 Y222F interacts with co-repressor complex CoREST (data not shown). Future works 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 inhibition by Imatinib does not affect HDAC2 mRNA levels and consistently, c-Abl overexpression does not change 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 et al., 2002), polyubiquitination (Krämer et al., 2003), acetylation (Adenuga et al., 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 — tyrosine 73, tyrosine 222 and

tyrosine 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) are conserved in different species such as mice and human (Figure 14 S6A). Additionally this motif is partially conserved in other members of the family such as HDAC1 and HDAC3 (Figure 14 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 et al., 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: i) that c-Abl inhibition induces the polyubiquitination of HDAC2, ii) that HDAC2Y222F shows higher polyubiquitination levels than wild type HDAC2, and iii) 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 Y238Fmutants; and also the decreased levels of wild type HDAC2 when c-Abl is inhibited. On the other hand, the protesomal 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, preventing polyubiquitination and proteasomal degradation (Tsai et al., 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 AβOs damage in an AD *in vitro* model. In spite of the several posttranslational modifications identified in HDAC2, only one published work studied a post-translational modification of HDAC2 in neurons (Nott el 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 el 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 et al., 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 that 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 Alzheimer Disease as well as in other neurodegenerative diseases.

Experimental Procedures

Animals: Transgenic B6C3-Tg(APPswePSEN1ΔE9)85Dbo/J mice were purchased from Jackson Laboratory (Bar Harbor, USA). Rats and mice were maintained in the Animal Care Facility following the Guide for the Care and Use of Laboratory Animals published by NIH, USA (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 two weeks.

Primary culture of rat hippocampal neurons: Rat hippocampal cultures were prepared as described previously with some modifications (Kaech et al., 2006, Alvarez et al., 2004). Hippocampi from Sprague Dawley rats at embryonic day 18 were removed, dissected in Ca^{2+}/Mg^{2+}-free HBSS, and rinsed twice with HBSS. Then, the tissue was resuspended in HBSS containing 0.25% trypsin and incubated for 15 min at 37°C. After three rinses with HBSS, the tissue was mechanically dissociated in DMEM (Invitrogen), supplemented with 10% horse serum (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin. Dissociated hippocampal cells were seeded onto poly-L-lysine-coated. Cultures were maintained at 37°C in 5% CO2 for 2 h before the plating medium was replaced with Neurobasal growth medium (Invitrogen) supplemented with B27 (Invitrogen), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. At day 2, cultured neurons were treated with AraC 2μM for 24 h; this method resulted in cultures highly enriched for neurons (approximately 5% glia).

Cell Culture: HT22 cells were kindly donated by Elena Pasquale and HeLa cells were purchased from ATCC (CCL-2). HT22 and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/ml streptomycin.

Transfection: Cells were seeded at a density of 25,000 cells/cm^2 and transfected using Lipofectamine 2000 (Life Technologies, 11668019), according to the instructions of the manufacturer. Cells were harvest 24 o 48h after transfection.

Antibodies and Reagents: Mouse anti-HDAC2 3F3 ChIP grade (ab51832) and rabbit anti-HDAC2 ChIP grade (ab7029) were purchased from Abcam (Cambridge, United Kingdom). Mouse anti-c-Abl (sc-23), rabbit anti-c-Abl (sc-13) and mouse anti-GAPDH (sc-32233) were purchased from Santa Cruz biotechnology (Dallas, United States of America). Rabbit anti-acetyl-Histone H3 (06-599), anti-phosphotyrosine (phospho-Tyr) antibody 4G10 (05-321) and Abl (T315I) Protein were purchased from Millipore (Billerica, United States of America). Imatinib mesylate (13139) was a purchased from Cayman Chemical Company (Ann Arbor, United States of America). [γ-^{32}P]ATP was purchased from PerkinElmer (Waltham, United States of America)

Immunoblot analysis: Treated cells were washed with PBS and immediately lysed with radioimmunoprecipitation assay (RIPA) supplemented with protease inhibitors cocktail (Roche) and 1 μM Na_3VO_4. Also, Whole brain extract samples (30 mg) were then homogenized in RIPA buffer. The homogenates were maintained on ice for 30 min and then they were centrifuged at 10,000g for 10 min. Proteins were resolved in SDS-PAGE, transferred to Nitrocellulose membrane (Thermo Scientific), and reacted with primary antibodies against HDAC2, c-Abl, GAPDH, phospho-Tyr, Histone H3 and Histone H3 acetylated. The reactions were followed by incubation with horseradish peroxidase labeled secondary antibodies (Thermo Scientific), and developed using the ECL technique (Thermo Scientific). The proteins levels were quantified using ImageJ software. HDAC2 levels were normalized against GAPDH levels. In the same way, phospho-Tyr levels were normalized against Flag levels and Gal-HDAC2 levels were normalized against HDAC2 levels.

Aβ oligomers preparation: Human synthetic Aβ1–42 (Genemed Biotechnologies) was suspended in 1,1,1,3,3,3 hexafluoro-2-propanol (Sigma-Aldrich, St. Louis, MO, US). The peptide samples were vortexed to obtain a homogeneous solution, aliquoted into microfuge tubes and lyophilised. The Aβ1–42 peptide aliquots were resuspended to 200 mM in nanopure water and vortexed briefly. Aggregation was allowed to proceed for 12 h at 4 ºC in accordance with Vargas et al., 2014.

Tyrosine phosphorylation assay. Protein extract was obtained from hippocampal cultures, HT22 cells and HeLa cells lysed in RIPA buffer containing a mixture of protease and phosphatase inhibitors. The immunoprecipitations were performed using anti-Flag or antic-HDAC2 antibody. Complexes were isolated using protein G Agarose. The tyrosine phosphorylation levels of HDAC2 were evaluated by immunoblotted with phosphotyrosine (pTyr) antibody (Millipore Bioscience Research Reagents).

Immunofluorescence procedures: Hippocampal neurons were seeded onto poly-Lysine coated coverslips in 24-well culture plates at a density of 3.0 x 10^4 cells per well. Cells were rinsed twice with PBS, fixed with 4% paraformaldehyde in PBS for 20 min, and permeabilized for 10 min with 0.2% Triton X-100 in PBS. After rinses twice with PBS, the cells were incubated in 3% BSA in PBS for 30 min at room temperature, followed by an overnight incubation at 4°C with primary antibodies against HDAC2 and β-Tubulin. The cells were washed four times with PBS and then incubated with anti-mouse Alexa 488 and anti-rabbit Alexa 555 antibodies (Life Technologies, Carlsbad, United States of America) for 1h at room temperature. The fluorescence images were captured with an Olympus BX51 microscope and analyzed and quantified with ImageJ software.

Histological analysis, mice were perfused with 4% paraformaldehyde in PBS. Brains were removed and post-fixed overnight at 4°C, placed in 20% sucrose in PBS at 4°C overnight, and then cut in 25 μm thick sagital sections using a cryostat (Leika) at -20°C. Slices were permeabilized with 0.1% Triton X-100, blocked in 5% BSA in PBS and incubated

overnight with the antibody rabbit anti-HDAC2 (ab7029) in 5% BSA in PBS. The primary antibody was visualized with anti-rabbit Alexa-Fluor 555.

Plasmids: GFP, c-Abl-GFP and c-Abl-KD-GFP were kindly donated by Dr. Zhi-Min Yuan (Department of Genetics and Complex Diseases, Harvard School of Public Health). pM18S Flag-HDAC2, pGal4-HDAC2, p-Gal4E1B-TK-Luciferase were previously described (Tsai et al., 2002). pGEX HDAC2-GST, pGEX CrkII-GST and pGEX vector were previously described (Yang et al., 1997; Perez de Arce et al., 2010)

Site-directed mutagenesis: HDAC2 Tyr mutants were generated by PCR using the proofreading Pfu polymerase (Stratagene, Santa Clara, US), followed by DpnI (New England Biolabs, Massachusetts, United States of America) digestion of the methylated parental plasmid. Oligonucleotides used are described in the table

Table 1. Primers used for Site-directed mutagenesis

Mutations	Primers	Sequences 5'-3'
Y73F	Forward	TACCACAGCGATGAGTTTATCAAGTTTCTACGATCA
	Reverse	TGATCGTAGAAACTTGATAAACTCATCGCTGTGGTA
Y222F	Forward	GCTGGAAAGGGAAAATTCTATGCTGT
	Reverse	GACAGCATAGAATTTTCCCTTTCCAG
Y238F	Forward	GATGGTATAGATGATGAATCATTTGGACAAATTTTTAAGCC
	Reverse	GGCTTAAAAATTTGTCCAAATGATTCATCATCTATACCATC

HDAC deacetylase activity assay: Plasmids directing the synthesis of Flag-HDAC2 (wild type or tyrosine mutants) or empty vector were transfected into cells using Lipofectamine 2000 reagent (Life Technologies, Carlsbad, United States of America). 24 h after transfection the cells were lysed in buffer containing 50mM Tris-HCl pH: 7.4, 150mM

NaCl, 1% Triton and 1mM EDTA, and the proteins with Flag-tag were immunoprecipitated using 1μg of anti-Flag antibody. HDAC deacetylatse activity was evaluated in the immunocomplex using HDAC-Glo I/II Assay (Promega, G6420) and normalized with the input.

Quantitative Real-Time PCR: Total RNA from HT22 cells was extracted using Trizol (Life Technologies, 15596), and reverse-transcribed into cDNA using iScript RT Supermix (Bio-Rad, 1708840). Real-time quantitative PCR assays were performed in triplicate using iQ SYBR Green supermix (Bio-Rad, 170-8882) and exon specific primers (primers described in the table) in a CFX96 real-time PCR Detection system (Bio-Rad). The relative quantities of cDNA were calculated by using the comparative CT method. Data were derived from three independent amplifications.

Table 2. Primers sequences used for exon amplification.

Gene	Forward 5'-3'	Reverse 5'-3'
Sinaptotagmin I	AGACACTGGTGATGGCTGTG	GGCCAAAATCCACGGTGTTC
NR2a	CAGCCACGACGTGACAGAA	TAACGCCACCACGTTCACAT
Glur1	TACATTGAGCAACGCAAGCC	GGTTTACGGGACCTCTCAGG
HDAC2	TCGAGCATCAGACAAACGGA	GCAACATTCCTACGACCTCCT
GAPDH	AAGGACACTGAGCAAGAGAG	GGGATGGAAATTGTGAGGGA

HDAC2 repression activity assay: 2.5 x 10^4 HT22 cells/cm^2 were seeded 18 h before transfection. Plasmids directing the synthesis of Gal4-HDAC2 (wild type or tyrosine mutants) or empty vector and luciferase reporters were transfected into cells using Lipofectamine 2000 reagent (Life Technologies, Carlsbad, United States of America). Each transfection contained 2.5 μg of plasmids Gal4-HDAC2/1 x 10^6 cells plus 5 μg of luciferase reporter/1 x 10^6 cells and 0.1 μg of renilla reporter /1 x 10^6 cells to normalize the

transfection. 48 hours after transfection, cells were collected, and luciferase activity was determined with the Dual Luciferase Reporter Assay System (Promega, E1910).

Chromatin Immunoprecipitation: ChIP was performed with HT22 cells and Rat hippocampal neurons (7DIV). Cells were rinsed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min and rinsed with 0.125M Glycine in PBS. Then, the cells were lysed in Buffer Lysis I (50mM HEPES, 3mM $MgCl_2$, 20mM KCl and 0.1% NP-40) they were disrupted with a Dounce Homogenizer. After, the samples were sonicated using a Bioruptor (Diagenode B01010002), with a cycle of eight pulses of 30 seconds (30s between pulses) at 20 KHz frequency 8 times at 4°C. The resulting whole-cell extract was diluted with 50mM HEPES, 140 mM NaCl, 1mM EDTA and 1% Triton and incubated with 25µL of Protein A (sc-2001, Santa Cruz Biotechnology) or G agarose (sc-2002, Santa Cruz Biotechnology) according to the primary antibody isotype for 4 hours at 4°C. After, the samples were centrifuged at 4°C the supernatant was incubated overnight at 4°C with 4 µg of mouse anti-HDAC2 3F3 ChIP grade antibody (ab51832) or Rabbit anti-acetyl-Histone H3 (06-599). Later, 50 µL of Protein A or G, according to the antibody isotype, was added and incubated another 4 hours at 4ºC in a rotator, and the pellet was washed four times with 1M HEPES, 140 mM NaCl, 1mM EDTA and 1% Triton. The immunocomplex were eluted by incubation at 65 °C for 15 min in 50mM Tris pH = 8.0, 1mM EDTA, 1% SDS and NaCl 200mM. Immunocomplex and whole-cell extract were treated with RNAseA, (Qiagen, 19101) proteinase K (Life Technologies 25530) and then the DNA was purified using the Qiagen DNA purification kit (Cat# 28106). The enrichment of DNA fragment was evaluated by Real Time PCR analysis, which was performed using iQ SYBR Green supermix (Bio-Rad, 170-8882) and primers to the promoters of the genes analyzed (primers are described in the table), in a CFX96 real-time PCR Detection system (Bio-Rad). The relative quantities of immunoprecipitated DNA fragments were calculated by using Pfaffl method (Pfaffl et al., 2001).

Table 3. Primers sequences used for promoter amplification in *Mus musculus*.

Promoter	Forward 5'-3'	Reverse 5'-3'
Sinaptotagmin I	CCGGGAAGGCTAAGACCAAA	CACAGGAAAATGGGGCTCGT
NR2a	CCGGAGTGGAACAGAAAGCT	CGCGACTCTCAGACCTCATC
Glur1	CAGTTAATCTGGCTGTCAGTC	CTTGTTTCTCTTGGAAGGGGAG

Table 4. Primers sequences used for promoter amplification in *Rattus novergicus.*

Promoter	Forward 5'-3'	Reverse 5'-3'
Sinaptotagmin I	CCTGAGGAGAGGGGTTTAGG	CTGAACAGGTTGAGGGCATT
NR2a	GGATAGACTGCCCCTGCA	TCGGCTTGGACTGATACGTG
Glur1	GGAGGAGAGCAGAGGGAGAG	CTTCCTGCAATTCCTTGCTTGC

In vitro phosphorylation assay. Kinase assay mixtures contained 25 mM HEPES, pH 7.25, 100 mM NaCl, 5 mM MgCl2, 5% glycerol, 100 ng/μL of bovine serum albumin, 1 mM sodium orthovanadate, GST–HDAC2 or GST–CrkII, 5 μM ATP, and 0.5 μCi of [γ-^{32}P]ATP. After a 5 min preincubation at 30°C, 25 μL reactions were initiated by the addition of 10 nM c-Abl T315I kinase. As negative controls of phosphorylation, we used c-Abl T315I kinase denatured by heat. All reaction mixtures were incubated at 30°C for different periods of time, and the reaction mixtures were stopped on phosphocellulose P81 paper (Millipore) and washed three times with 0.75% v/v phosphoric acid. Alternatively, the reactions were realized without 0.5 μCi of [γ-^{32}P]ATP and terminated by the addition of ice-cold SDS sample buffer, the tyrosine phosphorylation levels were evaluated by Western Blot using an anti-phosphotyrosine (phospho-Tyr) antibody 4G10.

Pull Down Assay: GST-HDAC2, GST-CrkII or GST bind to Glutaion agarose beads were incubated by 12h with 500 µg of HT22 whole cell lysate. After the beads were washed three times with PBS and then evaluated by Western Blot.

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 post-test using Prisma Software.

Author Contributions

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

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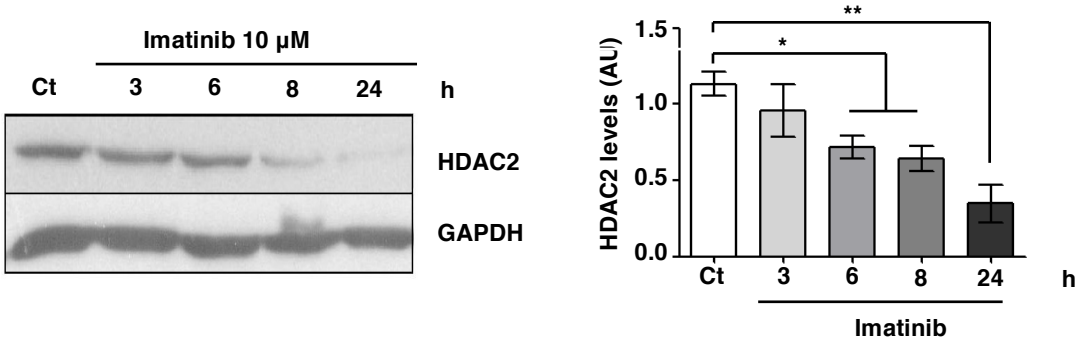
Supplemental Data

Figure 9 S1

A.



B.

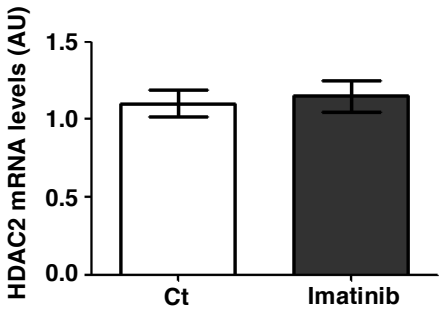
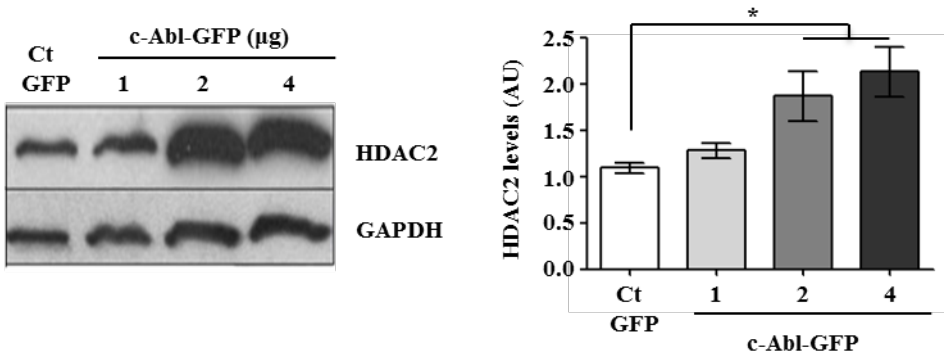


Figure 9 S1. **Imatinib treatment decreases HDAC2 levels in HeLa cells.** A. Representative Western Blot images and quantification of HDAC2 expression of HeLa cells treated with Imatinib 10 µM for 3, 6, 8 or 24 h (n=3). B. Quantitative PCR results of HDAC2 mRNA expression in HeLa cells treated with Imatinib 10 µM for 24h (n=3).

Figure 10 S2

A.



B.

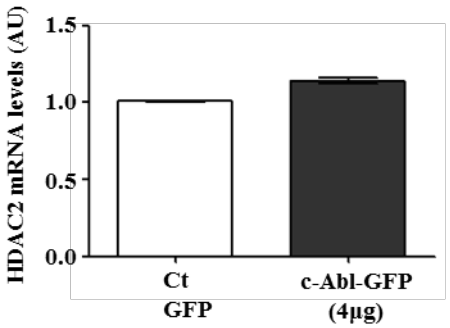
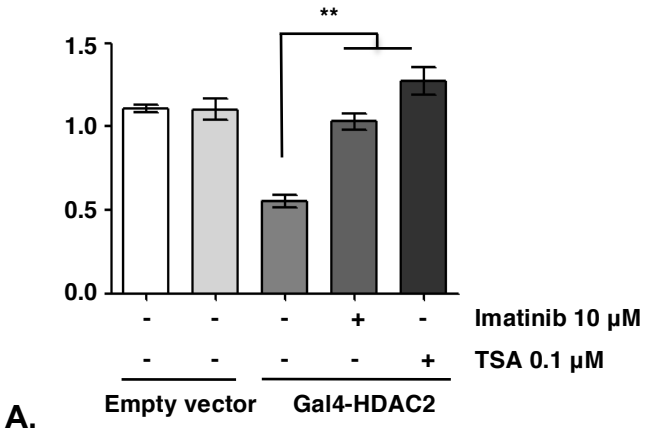


Figure 10 S2. **c-Abl increases protein levels but not mRNA levels of HDAC2.** A. Representative Western Blot images and quantification of HDAC2 expression in HeLa cells transfected with a plasmid expressing c-Abl-GFP or empty vector (n=3). B. Quantitative PCR results of HDAC2 mRNA expression in HeLa cells transfected with a plasmid expressing c-Abl-GFP or empty vector (n=3).

Figure 11 S3



A.

B.

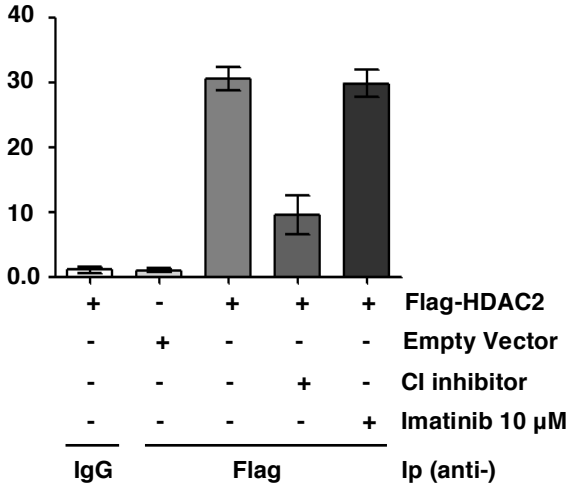
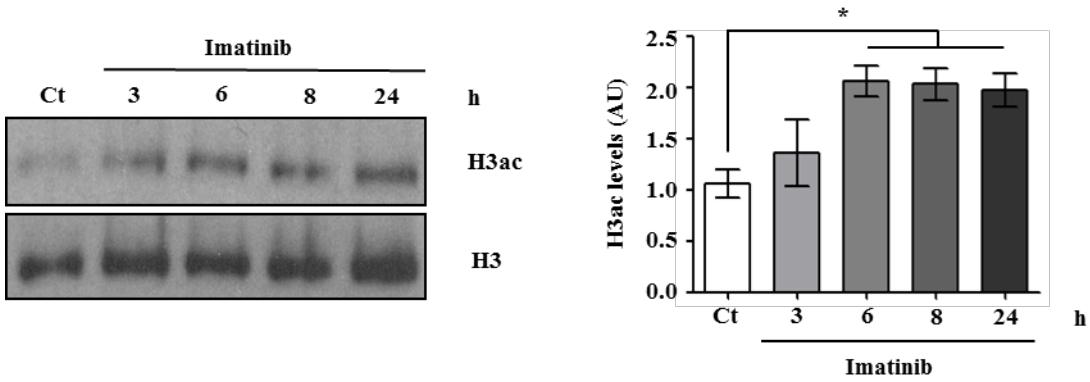


Figure 11 S3**. c-Abl increases the repression activity of HDAC2.** A. HDAC2 repression activity assay. Luciferase activity was normalized against renilla from HeLa cells transfected with the plasmid expressing Gal4-TK-luciferase along with Gal4-HDAC2 or the empty vector. 24 h after transfection, the cells were treated with Imatinib 10 µM or TSA 0,1 µM for 24h (n=3). B. HDAC2 enzymatic activity assay. Deacetylase activity was evaluated on Flag-immunoprecipitated proteins from HT22 cells transfected with the plasmids to Flag-HDAC2. Deacetylase activity from Flag-immunoprecipitated proteins were normalized against the deacetylase activity of the input and Flag levels (n=3). CI inhibitor: CI994, HDAC2 and HDAC1 inhibitor.

Figure 12 S4

A.



B.

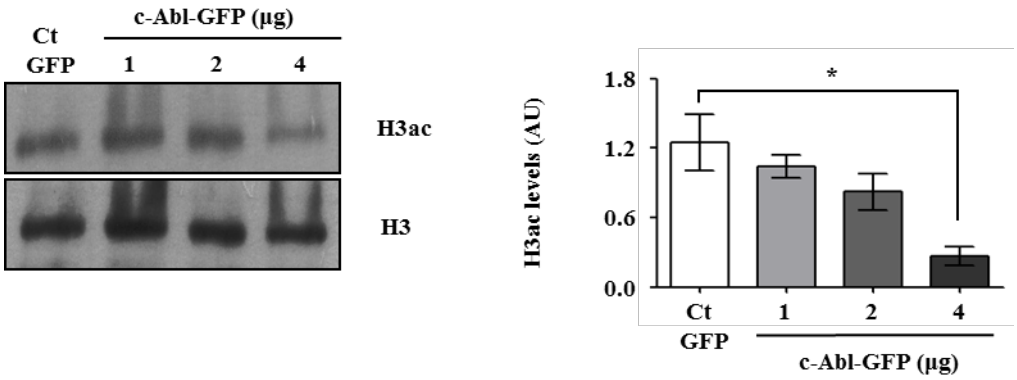


Figure 12 S4. **c-Abl activity induces histone H3 deacetylation**. Representative Western Blot images and quantification of acetylated Histone H3 normalized to Histone H3 expression of HeLa cells treated with Imatinib 10 µM for 3, 6, 8 or 24 h (n=3). Representative Western Blot images and quantification of acetylated Histone H3 normalized to Histone H3 expression in HeLa cells transfected with a plasmid expressing c-Abl-GFP or empty vector (n=3).

Figure 13 S5

A.

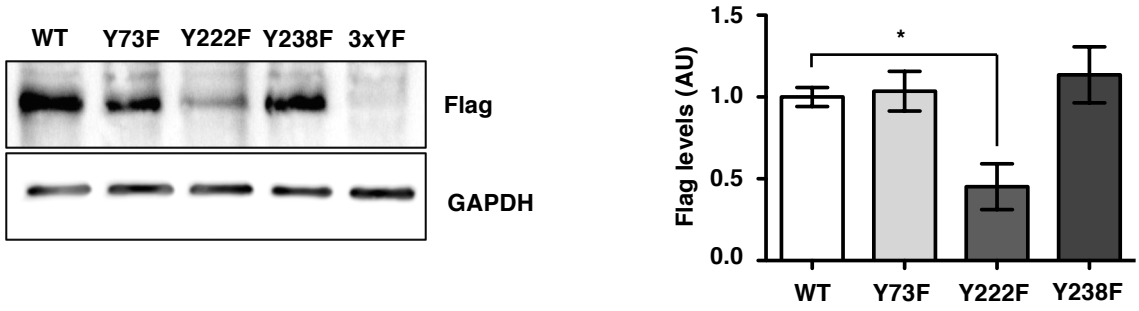


Figure 13 S5. **Flag-HDAC2 Y222F showed lower expression levels than Flag-HDAC2.** Representative Western Blot images and quantification Flag levels normalized to GAPDH expression of HT22 cells transfected with Flag-HDAC2 and the mutant versions Flag-HDAC2 Y73F, Flag-HDAC2 Y222F, Flag-HDAC2 Y238F and Flag-HDAC2 Y73F-Y222F-Y238F (3xYF) (n=3).

Figure 14 S6

A.

Mus musculus (Y222)	IGAGKGK**Y**YAVNF**P**M
Homo sapiens (Y222)	IGAGKGK**Y**YAVNF**P**M

B.

HDAC2 (Y222)	IGAGKGK**Y**YAVNF**P**M
HDAC1 (Y221)	IGAGKGK**Y**YAVNY**P**L
HDAC3 (Y216)	VGAESGR**Y**YCLNV**P**L

Figure 14 S6. **The motif of phosphorylation by c-Abl on the tyrosine 222 of HDAC2 is conserved in different species and partially conserved in other HDACs.** A. The amino acid sequence (215-229 residues) of mouse and human HDAC2 are aligned. The tyrosine 222 (red) and the proline 228 (bold letter) formed the motif YX_5P of phosphorylation by c-Abl. B. The amino acid sequence of human HDAC2 (215-229 residues), HDAC1 (214-228 residues) and HDAC3 (209-223 residues) are aligned. The tyrosine 222 of HDAC2, 221 of HDAC1 and 216 of HDAC3 formed the motif YX_5P of phosphorylation by c-Abl along with the proline 228 of HDAC2, 227 of HDAC1 and 215 of HDAC3.

DISCUSSION

In recent years, the role of HDAC2 in AD has generated great interest (Fisher et al., 2007; Ricobaraza et al., 2009; Govindarajan et al., 2011; Gräff et al., 2012). In fact, the evidence support that the increases in HDAC2 levels induce histone deacetylation on neuronal gene promoters, which causes gene repression that contributes to neuronal and synaptic dysfunction in AD (Gräff et al., 2012; Bie et al., 2014). However, the mechanisms that underlie the increases in HDAC2 levels in AD are unknown. Our findings demonstrate that c-Abl activity increases HDAC2 levels, inducing H3 histone deacetylation and transcriptional repression of neuronal genes. Additionally, our results indicate that HDAC2 is phosphorylated on tyrosine by c-Abl, a posttranslational modification that increases HDAC2 levels. Thus, our results support the notion that c-Abl/HDAC2 signaling activation may contribute to gene repression in AD models.

8.1. c-Abl in the control of HDAC2 levels.

Our first finding, that hippocampal neurons treated with AβOs exhibit an increase HDAC2 signal in the nucleus, is not new. Gräff et al. previously described increased HDAC2 levels induced by AβOs (Gräff et al., 2012). In addition, AD animal models exhibit higher HDAC2 levels than control animals (Gräff et al., 2012; Bie et al., 2014). However, unexpectedly, treatment with the c-Abl kinase inhibitor Imatinib prevented the increase of HDAC2 levels in hippocampal neurons exposed to AβOs in an AD mouse model. Previously, it has been demonstrated that Aβ peptide, fibrils and oligomers induce c-Abl activation in hippocampal neurons (Alvarez et al., 2004, Vargas et al., 2014) and that c-Abl is overactivated in the AD brain (Jing et al., 2009) and AD animal models (Cancino et al.,

2008, 2011). Furthermore, in hippocampal neurons, Aβ peptides induce the nuclear targeting of c-Abl (Alvarez et al., 2004), the cell compartment where HDAC2 is located (Yang et al., 2008-2; Gräff et al., 2013). Thus, the reduction of AβO-induced HDAC2 levels by Imatinib treatment suggests that the activation of c-Abl could contribute to the increased HDAC2 levels in AD.

To demonstrate the link between the c-Abl activity and HDAC2 levels, we used different experimental approaches. First, the pharmacological inhibition of c-Abl using Imatinib decreased HDAC2 levels in both HT22 and HeLa cells. Imatinib was developed as a specific c-Abl inhibitor, but evidence has shown that it has inhibitory activity on other tyrosine kinases, such as c-kit, PDGFR, CSF1R and DDR1/2 (Greuber et al., 2013). Therefore, it was necessary confirm the role of c-Abl in the regulation of HDAC2. Thus, we reduced c-Abl levels in HeLa cells using a short hairpin RNA against c-Abl, which reduced HDAC2 levels. Concordant with our hypothesis, c-Abl overexpression increased HDAC2 levels. Altogether, these results support a role for c-Abl in the increase in HDAC2 levels.

Interestingly, both Imatinib (Cancino G. et al., 2008) and HDAC inhibitors (Fisher et al., 2007; Francis et al., 2009; Ricobaraza et al., 2009; Kilgore et al., 2010; Govindarajan et al., 2011) reduce cognitive impairment in AD animal models. Until now, the cognitive decline associated with c-Abl has been linked to neuronal apoptosis, synaptic loss and cytoskeletal alterations (Cancino et al., 2008, 2011, Vargas et al., 2014). However, now, our results propose that c-Abl through HDAC2 could be a new mechanism that contributes to neuronal gene repression and cognitive decline in AD.

8.2 c-Abl/ HDAC2 signaling represses gene expression.

Using a HDAC2 repression activity assay, we showed that Imatinib reduces HDAC2 repression activity. Moreover, transfection with a kinase-dead c-Abl form (cAbl-KD, which acts a dominant negative form of c-Abl) also reduced HDAC2 repression activity, confirming that c-Abl inhibition mediates the effects of Imatinib on HDAC2 repression activity. Concordantly, when we reduced the amount of Gal4-HDAC2 transfected, c-Abl overexpression increased HDAC2 repression activity. In addition, an *in vitro* enzymatic activity assay demonstrated that Imatinib did not affect the catalytic activity of immunoprecipitated HDAC2, eliminating the possibility that Imatinib directly inhibits HDAC2 catalytic activity. Altogether, the results suggest that the c-Abl activity increases HDAC2 repression activity.

In accord with the role of c-Abl in HDAC2 repression activity, in HeLa cells, Imatinib and c-Abl knockdown induced an increase in acetylated histone H3 (H3ac) levels, while cells that overexpress c-Abl presented with lower H3ac levels than control cells. Previously, it has been described that nuclear c-Abl overexpression induces the deacetylation of histone H3 and H4 by an unknown mechanism; interestingly, treatment with TSA, an HDAC inhibitor, prevents the drop in histone H3ac and H4ac levels by the c-Abl overexpression (Aoyama et al., 2011). In conclusion, the evidence connects the c-Abl activity with a “higher HDAC activity," which causes the deacetylation of histone H3 and H4. Thus, our results support that the “higher HDAC activity" induced by c-Abl is caused by the upregulation of the HDAC2 levels, which could be the mechanism that induces histone deacetylation and gene repression.

Through c-Abl inhibition in HT22 cells, Imatinib increased histone H3ac levels on the promoter of the NR2a, Synaptotagmin and GluR1 genes, and the effect was associated

with a decrease in HDAC2 recruitment to the very same promoters. Moreover, Imatinib promoted NR2a, Synaptotagmin and GluR1 gene expression. In addition, c-Abl inhibition prevented HDAC2 recruitment to the promoters of NR2a, Synaptotagmin, Synaptophysin and GluR1 in hippocampal neurons exposed to Aβ, a model where c-Abl signaling is activated (Alvarez et al., 2004; Cancino et al., 2008). Thus, our results support that the activation of c-Abl contributes to HDAC2 recruitment to neuronal gene promoters, triggering histone deacetylation and decreasing the transcription rate of these genes.

Moreover, the AD mouse model APPswe/PSEN1ΔE9 presented higher HDAC2 levels and nuclear signal than wild-type mice. Interestingly, the AD mice treated with Imatinib exhibited lower HDAC2 levels than APPswe/PSEN1ΔE9 treated with saline, and the levels were very similar to wild-type mice. Other AD models, such as p25 transgenic mice, along with increased HDAC2 levels, also exhibit a higher HDAC2 recruitment to promoters and a lower expression of key neuronal genes than wild-type mice. Furthermore, the reduction of HDAC2 levels using an shRNA against HDAC2 restored the expression of the genes analyzed (Gräff et al., 2012). Hence, the evidence supports that the c-Abl inhibition in AD animal models prevents the HDAC2-mediated reduction in the expression of neuronal genes.

Although it has been widely described that HDACs induce gene repression, HDACs can regulate other cellular processes by deacetylation of non-histone proteins (Yang et al., 2008-1). In fact, HDAC2 deacetylates the following: i) the Klf5 transcription factor in response to RAR agonist, decreasing the expression of p21 (Zheng et al., 2011); ii) the GATA4 transcription factor, decreasing the transactivation of genes associated with cell proliferation (Trivedi et al., 2010); and iii) the p53 transcription factor, reducing the p53-dependent apoptotic response (Brandl et al., 2012). Therefore, the c-Abl/HDAC2 pathway,

besides regulating gene expression by histone deacetylation, might regulate other cellular processes through protein deacetylation.

Despite the fact that HDAC2 plays a key role in gene repression, this protein does not contain a DNA-binding domain. In fact, HDAC2 requires its incorporation into large multiprotein transcriptional complexes such as mSin3A, NuRD, and CoREST (Haberland et al., 2009; Gräff et al., 2013) to alter gene expression. Although c-Abl most likely directly affects HDAC2 levels or activity, we cannot discard the possibility that c-Abl inhibition regulates HDAC2 repressor complex partners to prevent the correct HDAC2 binding to promoters and induce its degradation. The repressor complexes are multiprotein, and many of their components have not been identified; furthermore, the composition of the complexes changes with cell environment and cell type. Until now, the role of the multiprotein transcriptional complexes in neuronal function has not been diligently addressed (Nott et al., 2008; Guan et al., 2009; Saiyed et al., 2011; Gräff et al., 2012; Morris et al., 2013). Thus, more research is necessary to clarify this point. Another issue that should be investigated is the role of the DNA binding domain of c-Abl in the repression activity of HDAC2. Although this domain has been described as essential to mouse development, its function is not fully understood. It is possible to hypothesize that the DNA binding domain of c-Abl participates in the recruitment of HDAC2 to DNA.

8.3 HDAC2 is phosphorylated at tyrosine by c-Abl.

c-Abl inhibition by Imatinib reduced HDAC2 protein levels; however, it did not affect HDAC2 mRNA levels. Conversely, c-Abl overexpression did not change HDAC2 mRNA levels, but increased HDAC2 proteins levels. These data suggest that c-Abl regulates HDAC2 levels through a posttranslational mechanism; this idea that is supported by *in vitro* analysis, which indicated that c-Abl and HDAC2 can interact. In this context, it has

been described that HDAC2 is regulated through posttranslational modifications, such as serine phosphorylation (Tsai et al., 2002), polyubiquitination (Krämer et al., 2003), acetylation (Adenuga et al., 2010), nitrosylation (Nott et al., 2008) and nitration (Osoata et al., 2009).

The evidence indicates that the tyrosine phosphorylation by c-Abl stabilizes proteins, avoiding proteasomal degradation. In fact, transcription factors, such as p73 (Tsai et al., 2003), Yap1 (Levy et al., 2008), c-Jun (Gao et al., 2006), ERα (Xu et al., 2010) and C/EBPβ (Li et al., 2003), are phosphorylated on tyrosine residues by c-Abl, which induces transcriptional function. Here using different approaches, we found that the c-Abl induces the phosphorylation of tyrosine of HDAC2. Furthermore, *in silico* analysis identified that tyrosines 73, 222 and 238 are the three residues of HDAC2 with the highest probability of phosphorylation by c-Abl. The co-expression of c-Abl with wild-type HDAC2 or HDAC2 mutated in tyrosine 73, 222 or 238 showed that HDAC2 mutants have lower tyrosine phosphorylation levels than wild-type HDAC2, suggesting that these tyrosines are phosphorylated by c-Abl.

Remarkably, only the HDAC2 mutated in tyrosine 222 showed a deficit in repression activity, indicating that although tyrosine 73 and tyrosine 238 could be targets of c-Abl, their phosphorylation is not related to the effects of c-Abl on HDAC2-dependent gene repression. Interestingly, *in vitro* assays using immunoprecipitated HDAC2 indicated that the mutant HDAC2 proteins (either at tyrosine 73, 222 or 238) have very similar catalytic activity to HDAC2 wild-type, indicating that the amino acidic change of Y222F in HDAC2 does not disturb HDAC2 catalytic activity. With respect to the catalytic activity, although tyrosine 222 is in the catalytic domain of HDAC2, this residue neither participates in the catalytic mechanism proposed (Figure 15), nor forms part of the structure of the active site

pocket nor is a Zn^{2+} ligand (Finnin et al., 1999; Vanommeslaeghe et al., 2005). Interestingly, the Y222 and the c-Abl phosphorylation motif $YX_{1-5}P$ in HDAC2 (Cujec et al., 2002) are conserved in different species, such as *Mus musculus* and humans (www.uniprot.org). In addition the structural analysis of HDAC2 showed that the Y222 is exposed on the protein surface and is therefore a residue available for phosphorylation by c-Abl (www.uniprot.org). In summary, these results are in agreement with the notion that c-Abl affects HDAC2 levels and not HDAC2 activity.

The results obtained suggest that the decrease in repression activity of HDAC2 Y222F is not mediated by a deficiency in its catalytic activity. In fact, the analysis of protein levels of HDAC2 wild-type and the mutants revealed that the HDAC Y222F protein levels were lower than HDAC2 wild-type and the mutants in the tyrosine 73 and 238.

Previously, our lab described that the treatment with Aβ fibers produces a significant increase in c-Abl kinase activity at 30 min and 60 min (Alvarez et al., 2004). Interestingly, neurons treated with Aβ oligomers for 1 h showed an increased in the tyrosine phosphorylation of HDAC2, an effect prevented by Imatinib. Although we do not know the role of the tyrosine phosphorylation in neurons exposed to Aβ oligomers, our results suggest that the tyrosine phosphorylation of HDAC2 is involved in the increase in HDAC2 levels and the gene repression in neurodegenerative conditions.

Figure 15

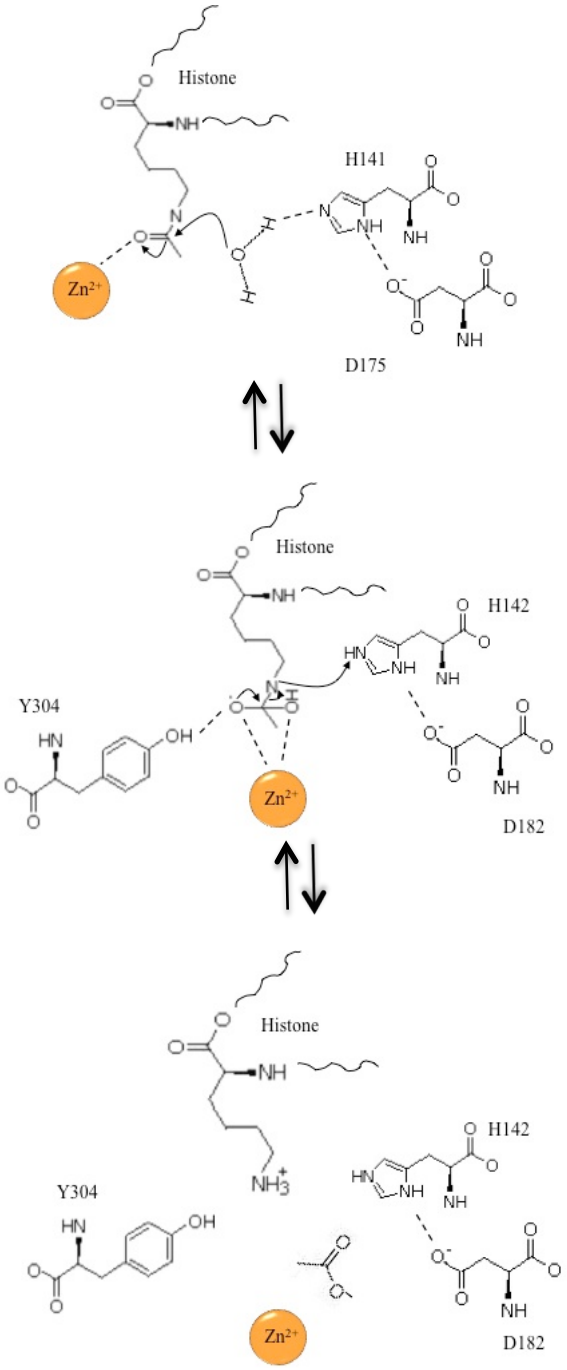


Figure 15. Catalytic mechanism proposed for HDAC2. The current model proposes that the Zn^{2+} in the active site pocket increase the electrophilicity in the carbonyl group of the acetylated lysine residue. By another hand, the nucleophilicity of the residues of D175 and H141 of HDAC2 increase the negative charge of the water. This polarization triggers the

nucleophilic attack of the water on the carbonyl group of the acetylated lysine residue, and the Y304 residue stabilizes the oxyanion intermediary. Finally, the carbon-nitrogen bond of the intermediary would break, and the nitrogen accept a H^+ from D182-H142 charge relay, yielding acetate and lysine deacetylated as products (Adapted from Finnin et al., 1999).

8.4 c-Abl prevents the polyubiquitination and the proteasomal degradation of HDAC2.

The polyubiquitination of proteins has been classically defined as a modification that induces proteasomal degradation, although recently, it has been implicated in other cellular processes, such as endocytosis, protein traffic, gene expression, kinase activation and the DNA damage response (Pickart et al., 2004). However, evidence indicates that tyrosine phosphorylation by c-Abl stabilizes proteins, avoiding its ubiquitination and proteasomal degradation. For example, the transcription factors p73 (Tsai et al., 2003), Yap1 (Levy et al., 2008), c-Jun (Gao et al., 2006), ERα (Xu et al., 2010) and C/EBPβ (Li et al., 2003) are tyrosine phosphorylated by c-Abl, which induces their stabilization and transcriptional function.

Three observations support the notion that c-Abl prevents the proteasomal degradation of HDAC2: i) c-Abl inhibition induced HDAC2 ubiquitination; ii) HDAC2 Y222F showed higher ubiquitination levels than wild-type HDAC2; and iii) c-Abl inhibition did not change the ubiquitination levels of HDAC2 Y222F, although it increased the ubiquitination levels of wild-type HDAC2. The evidence allows us to infer that the tyrosine 222 phosphorylation by c-Abl prevents HDAC2 ubiquitination. Previously, it has been described that the polyubiquitination of HDAC2 mediated by the Ubc8 E2 conjugase and RLIM E3 ligase induces its proteasomal degradation (Krämer et al., 2003). Indeed, the proteasome inhibitor MG132 prevented both the decrease in HDAC2 levels in c-Abl-knockdown cells and the reduction in the repression activity in Abl-KD-transfected cells. Thus, the results suggest that the decrease in c-Abl activity and HDAC2 phosphorylation promotes the ubiquitination and proteasomal degradation of this protein.

Although the mechanism involved is unknown, the evidence indicates that the phosphorylation of c-Jun and p73 prevents the interaction with the E3 ligase Itch, avoiding the polyubiquitination and the proteasome degradation of these proteins (Tsai et al., 2003; Gao et al., 2006). Thus, HDAC2 could be regulated through a similar mechanism. RLIM and Mule have been described as HDAC2 E3 ligases, whereby both proteins are interesting candidates to be evaluated in future work (Krämer et al., 2003; Zhang et al., 2011).

Previously, the role of tyrosine residues in the proteasome degradation of HDAC2 has been already described. In fact, Osoata et al. reported that the tyrosine 253 nitration also induces the proteasomal degradation of HDAC2; however, tyrosine 222 was not evaluated in this study (Osoata et al., 2009). Nonetheless, our analysis did not include tyrosine 253 because this residue presents a lower probability of phosphorylation by c-Abl than the tyrosines 73, 222 and 238. Thus, further work is required to elucidate how the phosphorylation and the nitration on tyrosine residues contribute to HDAC2 ubiquitination and stability. Additionally, we cannot rule out the possibility that tyrosine 222 regulates other posttranslational modifications of HDAC2, which could increase the stability or activity of this protein.

In summary, the tyrosine phosphorylation of HDAC2 by c-Abl could be especially relevant for neuronal gene expression in neurons exposed to Aβ in an AD *in vitro* model. Despite the several posttranslational modifications identified in HDAC2, only one study has described a post-translational modification of HDAC2 in neurons (Nott el al., 2008). BDNF induces the nitrosylation of HDAC2 on cysteine residues, triggering its release from chromatin and promoting the transcription of neurotrophin-regulated genes (Nott el al., 2008). In contrast, here, we described that c-Abl through the tyrosine phosphorylation of HDAC2 induces gene repression. Thus, the HDAC2 nitrosylation of cysteine residues and

the HDAC2 phosphorylation of tyrosine residues could be antagonistic mechanisms to control HDAC2-mediated gene expression. To understand how HDAC2 regulates gene expression in neurons, it will be necessary to know how the different posttranslational modifications in this protein, which are downstream of distinct signal pathways, can together regulate the HDAC2 effects on gene expression in different cellular contexts.

CONCLUSIONS

The results obtained allow us to propose a new cell-signaling pathway, where c-Abl regulates neuronal gene expression through the control of HDAC2 levels. In fact, in neuronal-like cells, the inhibition of c-Abl activity along with decreased HDAC2 levels and its repression activity also reduces the HDAC2 recruitment to the promoter of synaptic plasticity-related genes, which triggers their transcriptional activation. In addition, in AD models, such as the primary culture of hippocampal neurons treated with Aβ oligomers and in the APPswe/PSEN1ΔE9 mice, the pharmacologic inhibition of c-Abl prevented the increase in HDAC2 levels compared with the controls. Interestingly, Imatinib prevented HDAC2 recruitment to the promoter of synaptic plasticity-related genes in hippocampal neurons treated with Aβ oligomers. Thus, our results suggest that the c-Abl mediates the control of HDAC2 levels and triggers the transcriptional repression of neuronal genes in Alzheimer's disease models.

With respect to the mechanism involved, our findings support the possibility that c-Abl induces the tyrosine phosphorylation of HDAC2. Furthermore, both the *in silico* and *in vitro* analyses demonstrated that c-Abl phosphorylates HDAC2 on tyrosines 73, 222 and 238. Interestingly, in analyzing each of the HDAC2 mutants, we found that only a mutation in tyrosine 222 exhibited a decrease in repression activity and in protein levels, although its catalytic activity was not changed significantly compared with HDAC2 wild-type. In addition, the mutation in the tyrosine 222 and Imatinib treatment induced HDAC2 ubiquitination. Furthermore, treatment with a proteasome inhibitor prevented the reduction of HDAC2 levels in c-Abl-knockdown cells. Altogether, these results support the idea that

HDAC2 phosphorylation on Y222 by c-Abl increases HDAC2 levels by preventing its ubiquitination and proteasomal degradation.

In summary, the results support a fundamental role of c-Abl in the control of HDAC2 levels and the transcriptional repression of genes associated with synaptic function in AD (Figure 16).

Figure 16

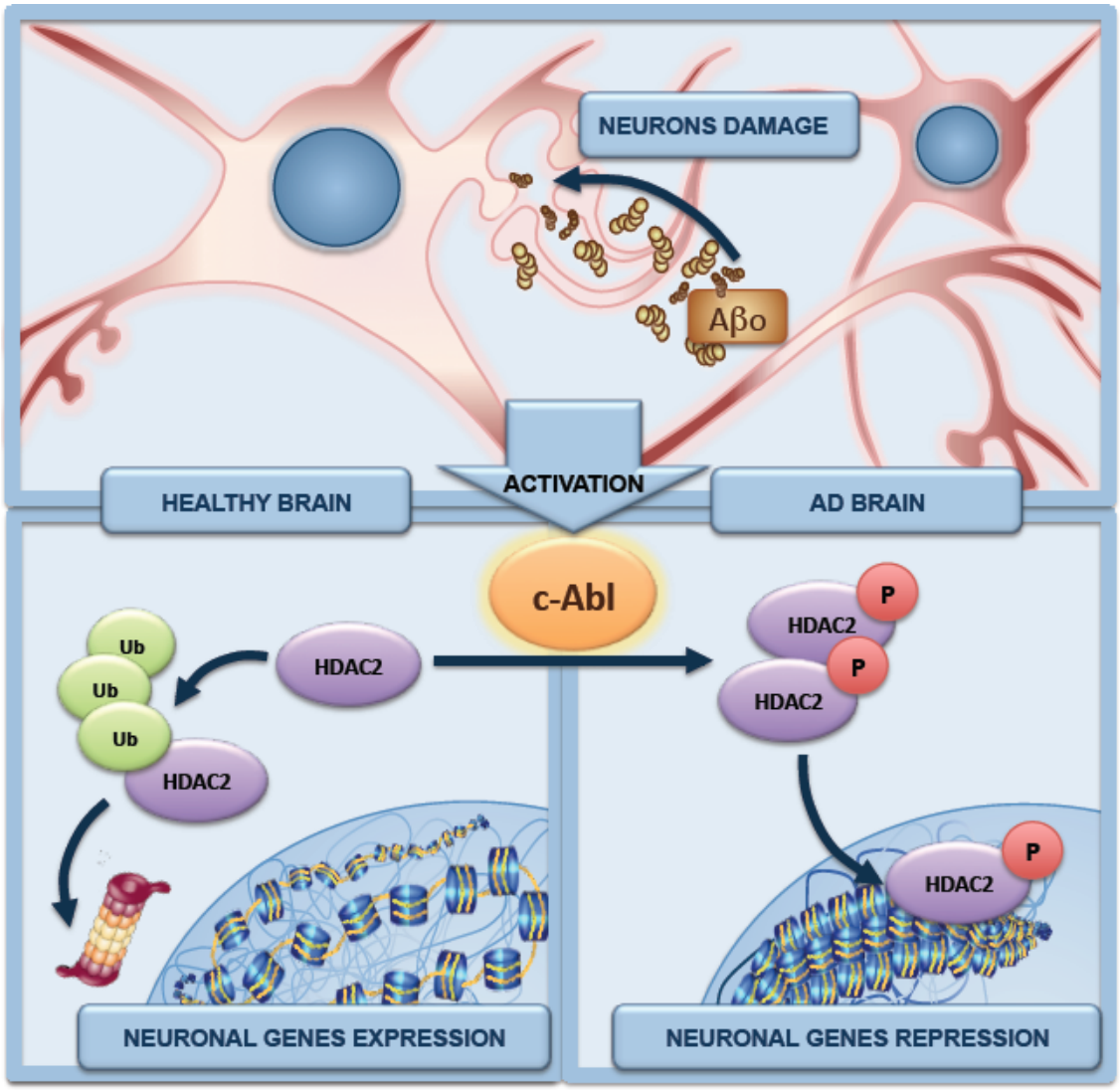


Figure 16. c-Abl increases HDAC2 levels through tyrosine phosphorylation, which induces gene repression. The cellular stress, as the induced by Aβ oligomers, triggers c-Abl activation and its nuclear translocation. Once there c-Abl phosphorylate HDAC2, which prevents its proteasome degradation and increases HDAC2 levels. In the nucleus, the increases in HDAC2 levels induce histone deacetylation in the promoter of synaptic plasticity genes. These changes stimulate that the chromatin acquire a heterochromatin conformation, which hinders the access of the transcriptional machinery to DNA that finally induces gene repression.

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