BBA - Molecular Basis of Disease 1867 (2021) 166089

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

c-Abl activates RIPK3 signaling in Gaucher disease

M.J. Yañez [a,f,*], F. Campos [a], T. Marín [a], A.D. Klein [d], A.H. Futerman [e], A.R. Alvarez [b,c], S. Zanlungo [a,*]

[a] Department of Gastroenterology, Faculty of Medicine, Pontificia Universidad Católica de Chile, Santiago, Chile
[b] Department of Cell & Molecular Biology, Biological Sciences Faculty, Pontificia Universidad Católica de Chile, Santiago, Chile
[c] CARE UC Pontificia Universidad Católica de Chile, Santiago, Chile
[d] Centro de Genética y Genómica, Facultad de Medicina, Clínica Alemana Universidad del Desarrollo, Santiago, Chile
[e] Department of Biomolecular Sciences, Weizmann Institute of Science, Rehovot, Israel
[f] School of Medical Technology, Health Sciences Faculty, Universidad San Sebastian, Sede Los Leones, Santiago, Chile

ARTICLE INFO

Keywords:
Tyrosine kinase c-Abl
Receptor interacting serine/threonine kinase 3 (RIPK3)
Gaucher disease (GD)
Lysosomal storage disorders (LSD)
Necroptosis
Death

ABSTRACT

Gaucher disease (GD) is caused by homozygous mutations in the *GBA1* gene, which encodes the lysosomal β-glucosidase (GBA) enzyme. GD affects several organs and tissues, including the brain in certain variants of the disease. Heterozygous *GBA1* variants are a major genetic risk factor for developing Parkinson's disease. The RIPK3 kinase is relevant in GD and its deficiency improves the neurological and visceral symptoms in a murine GD model. RIPK3 mediates necroptotic-like cell death: it is unknown whether the role of RIPK3 in GD is the direct induction of necroptosis or if it has a more indirect function by mediating necrosis-independent. Also, the mechanisms that activate RIPK3 in GD are currently unknown.

In this study, we show that c-Abl tyrosine kinase participates upstream of RIPK3 in GD. We found that the active, phosphorylated form of c-Abl is increased in several GD models, including patient's fibroblasts and GBA null mice. Furthermore, its pharmacological inhibition with the FDA-approved drug Imatinib decreased RIPK3 signaling. We found that c-Abl interacts with RIPK3, that RIPK3 is phosphorylated at a tyrosine site, and that this phosphorylation is reduced when c-Abl is inhibited. Genetic ablation of c-Abl in neuronal GD and GD mice models significantly reduced RIPK3 activation and MLKL downstream signaling. These results showed that c-Abl signaling is a new upstream pathway that activates RIPK3 and that its inhibition is an attractive therapeutic approach for the treatment of GD.

1. Introduction

Lysosomal storage diseases (LSDs) are a cluster of neuro-visceral inherited disorders caused by genetic variants in genes encoding for lysosomal hydrolases, their activators, or their transporters. For each type of disorder, there is an accumulation of specific substrates within this organelle, which triggers cellular dysfunction and clinical manifestations. In one group of LSDs, the sphingolipidoses, there is an accumulation of sphingolipids due to the deficiency of enzymes and transporters involved in their metabolism [1].

Gaucher disease (GD, OMIM #230800, ORPHA355) is a rare metabolic disorder and one of the most common LSDs. It is an autosomal recessive condition resulting from loss of function variants in the *GBA1* gene, causing reduced acid β-glucosidase activity (GBA, also called glucocerebrosidase, glucosylceramidase EC: 4.2.1.25). GBA catalyzes the hydrolysis of glucosylceramide (GlcCer) into ceramide and glucose [2]. Atypical GD due to GBA activator (saposin C) deficiency shows similar GlcCer accumulation [3]. Its incidence rate varies in each population, ranging from 1/40,000 births in the general population to 1/800 births in Ashkenazi Jews [4,5]. The disease is divided into three forms, although it is recognized as a continuum of phenotypes (PMID: 23114583). Type 1 GD is the most frequent, affecting internal organs without evident neurological impairment. Type 2 and type 3 GD are associated with neurological involvement [6].

Recently, the timeline of neurological progression was reported in a murine model of neuronopathic GD (the $Gba^{flox/flox}$; nestin-Cre mouse, nGD) [7]. Systematic analysis of the nGD mouse brain highlighted the presence of periadventitial Gaucher cells (enlarged macrophages containing undegraded GlcCer) along with particular patterns of microglial activation, astrogliosis, and neuronal loss, in addition to non-specific

* Corresponding authors at: Facultad de Medicina, Pontificia Universidad Católica de Chile, Avenida Libertador Bernardo O'Higgins 340, Santiago 8331010, Chile.
E-mail addresses: msyanez@uc.cl (M.J. Yañez), szanlungo@uc.cl (S. Zanlungo).

https://doi.org/10.1016/j.bbadis.2021.166089
Received 19 October 2020; Received in revised form 15 January 2021; Accepted 21 January 2021
Available online 4 February 2021

gray and white matter gliosis [8].

Several brain areas and pathways may be responsible for the neurological manifestations observed in nGD. Microglial activation and astrogliosis correlate spatially and temporally with selective neuronal loss. The most affected brain areas include cortical layers III and V, and the hippocampal CA2-4 [9]; while other brain regions, such as hippocampal CA1, remain unaffected even at late stages of the disease [9]. Consistent with the human neuropathology, cortical layer V is particularly compromised in Gaucher mice. Moreover, in Gaucher mice, there is microglial activation in cortex layer V, although this event occurs later in disease progression [10].

Little is known about the molecular mechanisms involved in cellular dysfunction in the GD brain. Cell death seems to be caspase-independent but mediated by necroptosis triggering [11]. Necroptosis is an orchestrated cell death pathway. It can be triggered by Fas, IFN receptors (IFNRs), TLRs, intracellular RNA- or DNA-sensing molecules, and tumor necrosis factor receptor 1 (TNFR1), among others [12]. Activation of necroptosis by death receptor ligands or deficient conditions requires the intact kinase activity of receptor-interacting protein 1 (RIPK1) [13]. Pharmacological inhibition of the RIPK1 kinase by necrostatin-1 halts the initiation of necroptosis and increases cell survival [14]. Upon stimulation of necroptosis, receptor-interacting protein 3 (RIPK3), RIPK1 [15–17], and mixed lineage kinase domain-like (MLKL) are activated by phosphorylation [18,19]. Forced dimerization of RIPK3 can initiate its autophosphorylation and activation of MLKL leading to necrosis [20,21]. However, significant activation of RIPK3 kinase is critical for mediating necroptosis or inflammatory engagement independent of necrosis and basal levels of RIPK3 kinase activity seems to be necessary for arresting apoptosis [16,17,22–24].

Interestingly, genetic ablation of RIPK3 significantly improved clinical signs in GD mice, showing increased survival times and motor coordination, as well as reduced hepatic injury [11]. However, the molecular mechanisms that activate the RIPK3 pathway in GD are unknown.

The pharmacological properties of the available RIPK3 inhibitors are not optimal for the clinical treatment of the disease [25,26]. The development of potent RIPK3 inhibitors with long half lifes may take several years. Instead, we propose to study the activation mechanism of RIPK3 in GD models. The tyrosine kinase c-Abl is an attractive pathway. This kinase is involved in other neurodegenerative diseases such Alzheimer [27], Parkinson [28], and amyotrophic lateral sclerosis [29]. Previously, we described that c-Abl is activated in Niemann-Pick type C (NPC) disease and that Imatinib, a c-Abl inhibitor, reduces apoptosis, improves neurological symptoms, and increases the survival of NPC mice [30]. Moreover, oxidative stress is the main upstream stimulus that activates the c-Abl pathway and neuronal apoptosis in NPC models [31].

Here we show that the c-Abl kinase is activated in different GD neuronal models and that c-Abl interacts with RIPK3.

We also found that inhibition of c-Abl decreases RIPK3 signaling in different GD models and that c-Abl mediates its phosphorylation at a Tyr residue: a RIPK3 activation site not previously described. We observed that c-Abl genetic ablation prevents RIPK3 activation and its downstream signaling in GD cells.

In summary, in the context of lysosomal Gaucher disease, our results demonstrate that activation of the c-Abl pathway triggers neuronal death in Gaucher disease through RIPK3, making this pathway a potential therapeutic target for treating GD.

2. Results

2.1. The c-Abl pathway is activated in different GD models

To study the possible molecular mechanisms involved in RIPK3 activation and its downstream signaling in GD, we first established GD pharmacological models in the neuronal cell line SHSY5Y using conduritol b epoxide (CBE), an irreversible competitive inhibitor of GBA.

SHSY5Y cells were treated with 150 μM of CBE for 2, 7, and 14 days. As expected, treatment significantly decreased GBA activity (Supplementary Fig. 1A). We confirmed that CBE induced a GD phenotype by measuring glucosylceramide (GlcCer) levels, which were increased (Supplementary Fig. 1B). As a control, we also measured GBA activity in fibroblasts from GD patients (Supplementary Fig. 1C)- which was almost undetectable.

We analyzed the levels of the activated phospho-c-Abl in the pharmacological GD models induced by CBE in SHSY5Y cells and cortical neurons from wild-type mice embryos. In SHSY5Y cells treated with CBE we observed increased levels of c-Abl tyrosine kinase phosphorylated at Tyr 412 (phospho-c-Abl) suggesting that c-Abl is activated (Fig. 1A), whereas total c-Abl protein levels were unchanged (Fig. 1B). Thus, in this pharmacological GD model, we found c-Abl kinase activation associated with glucosylceramide (GlcCer) accumulation. A similar trend was observed when primary cultures of cortical neurons from wild-type mice embryos were treated with CBE. We found increased levels of phospho-c-Abl (Fig. 1C), but total c-Abl protein levels were also significantly increased (Fig. 1D).

Furthermore, we found that phospho-c-Abl was increased in Hepa 1–6 cells (a hepatic cellular model) treated with CBE (Supplementary Fig. 2A), confirming that c-Abl is also activated in pharmacological GD models developed in other cell types.

We analyzed phospho-c-Abl levels in GD transgenic models using fibroblasts from four GD patients GM04394 (from GD type 1 patients) and GM00877, GM01260, and GM08760 (from GD type 2 patients). In all models, we found increased phospho-c-Abl levels compared to control fibroblasts (Fig. 1E). Moreover, we observed that the phospho-c-Abl signal localizes mainly in the nuclei of fibroblasts from GD patients (GM00877), a characteristic associated with cell damage dependent c-Abl activation [32,33] (Fig. 1F). Altogether, these results show that c-Abl is activated in pharmacological and genetic models of GD.

Next, we monitored c-Abl activation in a GD mouse model induced with CBE treatment [34]. We injected C57BL/6 mice every day with 50 mg/kg or 100 mg/kg of CBE, starting on postnatal day 8, for 21 days. As expected, both CBE doses decreased weight gain (Supplementary Fig. 3A) after 11 days, as described previously [35]. We consistently found an increased number of cells that were positive for the CD68 inflammation marker in the hippocampus and cortical cortex of the GD mouse model developed with both CBE doses (Supplementary Fig. 3B). However, with the 50 mg/kg dose, CD68 positive cells were only seen in layer V, while with the 100 mg/kg dose, CD68 positive cells were present in all layers of the cortex. This result indicates that the 100 mg/kg dose triggers a more severe GD phenotype, consistent with previously published results [36]. We then evaluated phospho-c-Abl levels in GD mice treated with 50 mg/kg of CBE which were increased in the liver as well as in the brain (Fig. 1G), while total c-Abl levels tended to increase only in the liver (Fig. 1G).

Then, we analyzed phospho-c-Abl levels in brain slices from 16-day-old transgenic GD mice. We observed a clear signal for phospho-c-Abl in layer V of the cortex (Fig. 1H), which is consistent with results showing that this area is one of the first to present damage: suggesting that this signaling pathway is relevant in GD.

Altogether, our results show that the tyrosine kinase c-Abl is active *in vitro* and *in vivo* in GD pharmacological and transgenic models.

2.2. RIPK3 activation by CBE is inhibited by c-Abl activation

After finding that c-Abl is activated in GD models, we evaluated its participation in RIPK3 activation. RIPK3 phosphorylation at threonine (Thr-231), serine-227 (Ser-227), and Ser-232 is indicative of activation: because these phosphorylated residues are particularly important for its interaction and activation of down-stream MLKL -the necroptosis effector [37]. First, we assessed whether c-Abl inhibition affects the phosphorylation of Thr-231 and Ser-232 residues of RIPK3 (phospho-RIPK3) in cells treated with CBE and the c-Abl inhibitor, Imatinib. As

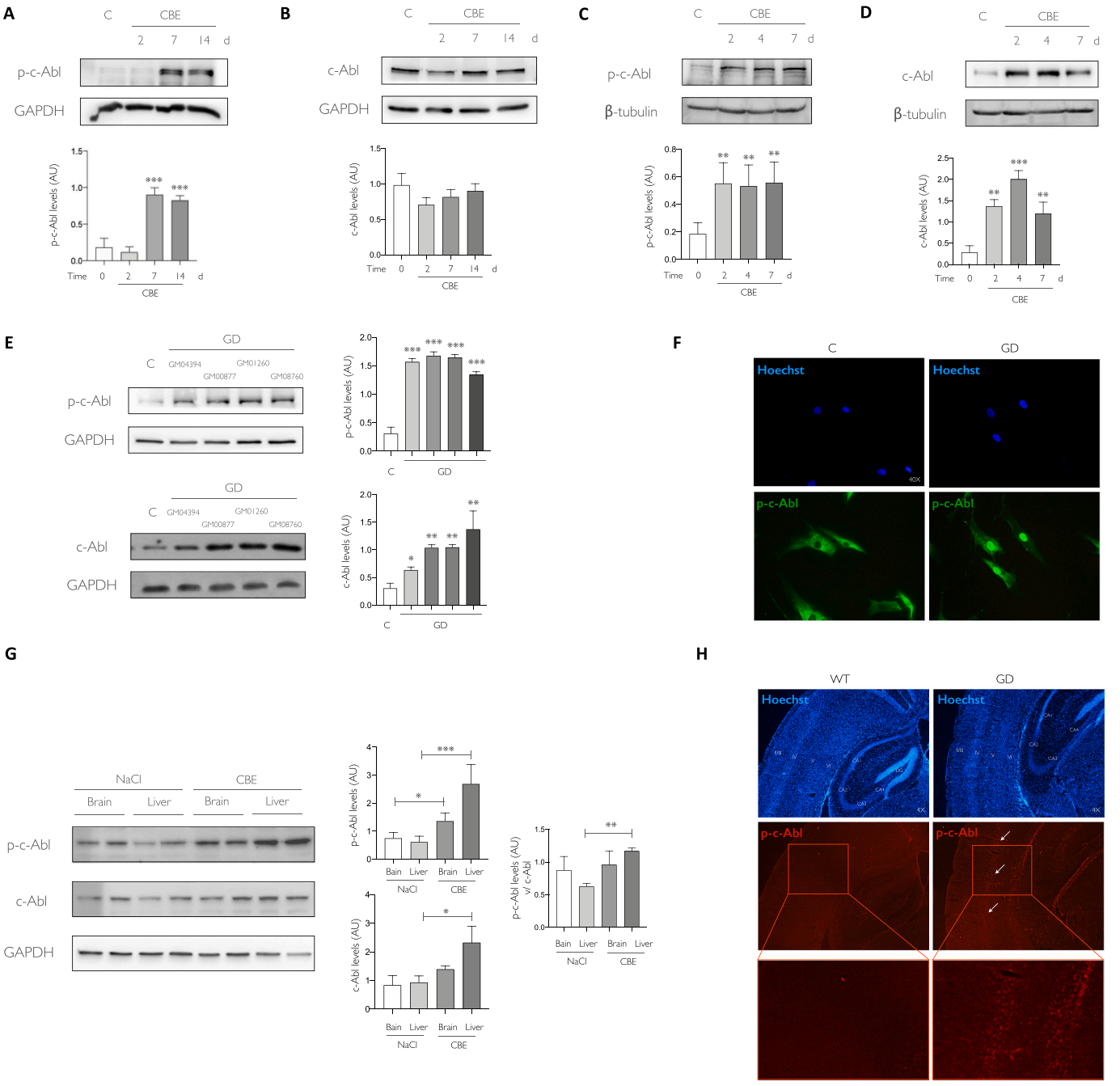

Fig. 1. c-Abl protein kinase is activated in different GD models. Phospho-c-Abl (phospho-c-Abl) (A) and c-Abl (B) levels of SHSY5Y cells treated with CBE (150 μM) for 2, 7, and 14 days. Phospho-c-Abl (phospho-c-Abl) (C) and c-Abl (D) levels in primary cultures of cortical neurons from wild-type mice embryos treated with CBE (150 μM) for 2, 4, and 7 days. (E) c-Abl and phospho-c-Abl (phospho-c-Abl) levels in different fibroblasts from GD patients. (F) Phospho-c-Abl (phospho-c-Abl) levels in fibroblasts from GD patients. (G) phospho-c-Abl (phospho-c-Abl) and c-Abl levels in brain and liver from 28-day old mice treated with vehicle (NaCl) or CBE (50 mg kg^{-1}) for 21 days. (H) Phospho-c-Abl (phospho-c-Abl) levels in slices of cerebral cortex from a 16-day old genetic GD mice model. Hippocampus regions and cortical layers are indicated.

expected, CBE treated SHSY5Y cells presented higher phospho-RIPK3 levels than controls (Fig. 2A). Co-treatment with the c-Abl inhibitor Imatinib (10 μM) prevented the CBE-induced increase in phospho-RIPK3 levels (Fig. 2A). Similar results were observed in Hepa 1–6 cells when treated with CBE and Imatinib. We found that CBE increased phospho-RIPK3 levels (Supplementary Fig. 2B) and co-treatment with Imatinib decreased these levels (Supplementary Fig. 2B).

These results suggest that activation of c-Abl by CBE treatment contributes to the signaling that triggers RIPK3 activation in GD models.

The high phospho-RIPK3 levels observed in fibroblasts from GD patients were decreased after treatment with Imatinib (10 μM) (Fig. 2B).

As a control for the inhibition of RIPK3 activation, we used a specific RIPK3 inhibitor (GSK′872). We observed that GSK˙872 also decreased phospho-c-Abl levels (Fig. 2A and B), suggesting that there is a cross-talk between these signaling pathways.

Altogether, our results show that the inhibition of c-Abl tyrosine kinase decreases the activation of RIPK3 in pharmacological and genetic models.

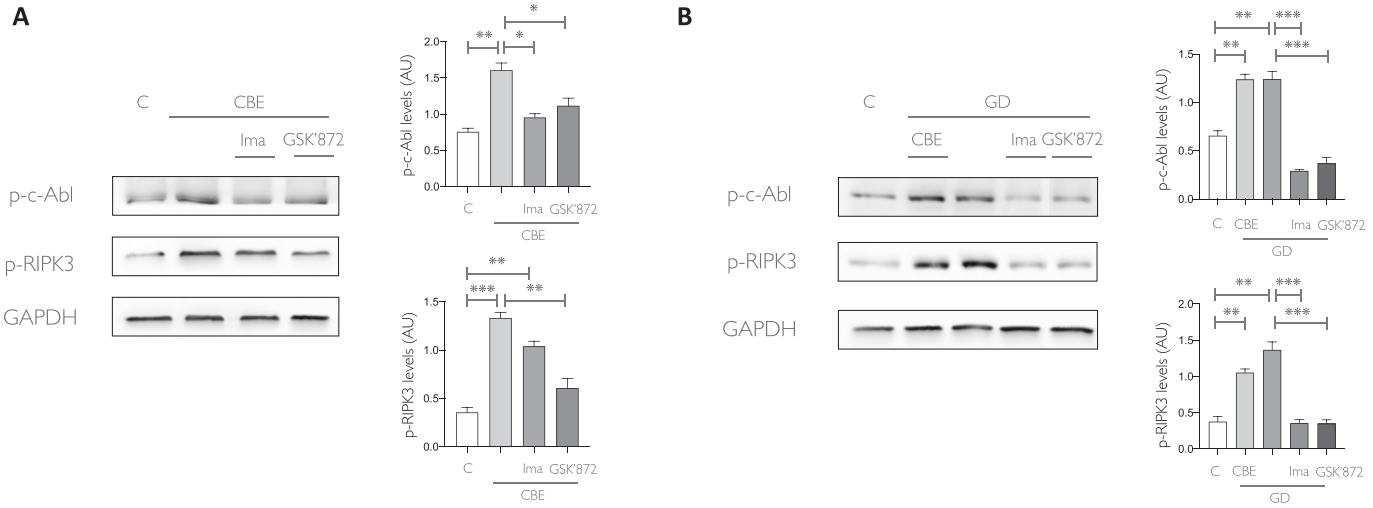

Fig. 2. c-Abl inhibition decreases RIPK3 signaling levels in different GD models. (A) Phospho-c-Abl (phospho-c-Abl) and phospho-RIPK3 (p-RIPK3) levels of SHSY5Y cells treated with CBE (150 μM) for 5 days and Imatinib (10 μM) or GSK′872 (1 μM) for 24 h. (B) Phospho-c-Abl (phospho-c-Abl) and phospho-RIPK3 (p-RIPK3) levels of fibroblasts from GD patients treated with CBE (150 μM) for 5 days or Imatinib (10 μM) or GSK′872 (1 μM) for 24 h.

2.3. c-Abl mediates RIPK3 tyrosine phosphorylation in GD cells

Our results indicate a functional interaction between c-Abl and RIPK3, therefore we tested a possible interaction in a GD model.

First, we treated SHSY5Y cells with CBE 150 μM for 5 days, and then we immunoprecipitated RIPK3 from whole-cell lysates from treated and untreated cells using an anti-RIPK3 antibody and evaluated c-Abl levels. Interestingly, we found that c-Abl-RIPK3 co-immunoprecipitated. Furthermore, we observed increased c-Abl immunoprecipitation after CBE treatment which decreased when using the c-Abl inhibitor Imatinib (Fig. 3A). As a control, we evaluated MLKL levels and found that Imatinib decreased the RIPK3-MLKL interaction (Fig. 3A).

So far, all the phosphorylations described in RIPK3 are at Ser and Thr residues, with no reports of Tyr phosphorylations. To assess whether c-Abl could phosphorylate RIPK3 we analyzed tyrosine phosphorylation of RIPK3 in CBE-treated SHSY5Y cells. Using an anti-phospho-tyrosine antibody we found that RIPK3 immunoprecipitated from whole-cell lysates of CBE treated cells is phosphorylated on tyrosine. Also, c-Abl was immunoprecipitated with tyr-phosphorylated RIPK3, and the phosphorylation of tyrosine induced by CBE was decreased when the cells were co-treated with Imatinib (10 μM) (Fig. 3B). These results suggest that c-Abl phosphorylates RIPK3 in this *in vitro* pharmacological GD

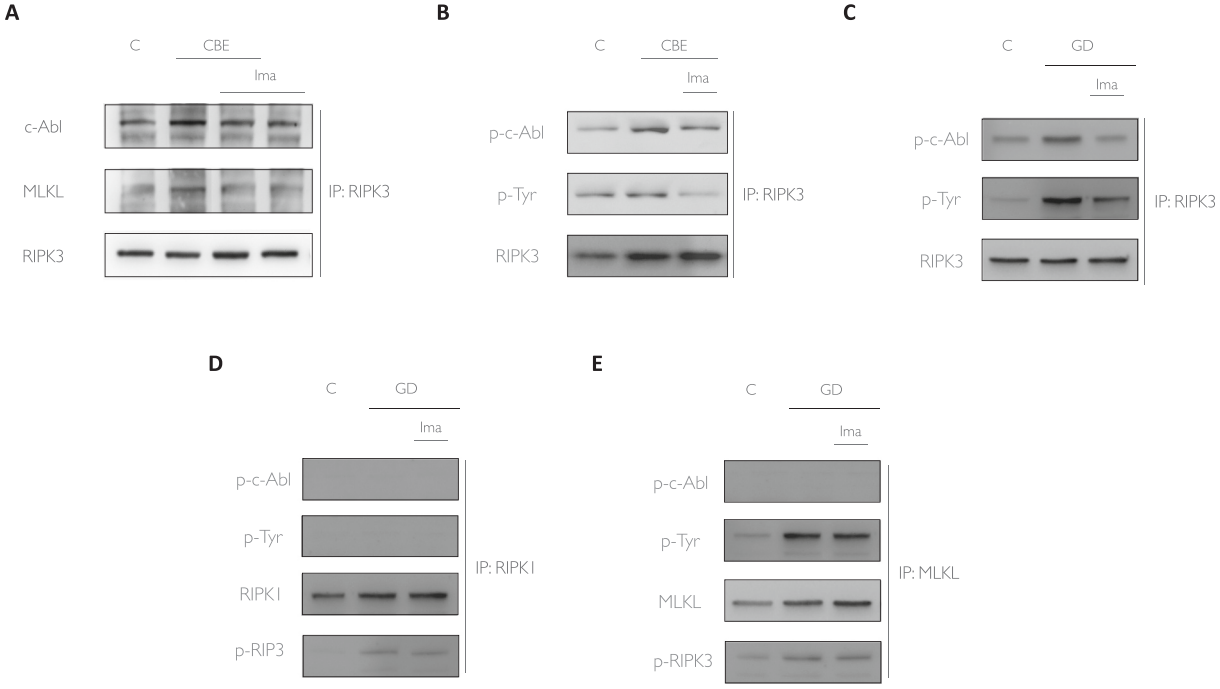

Fig. 3. RIPK3 is phosphorylated at tyrosine by c-Abl in a GD neuronal model and a GD patient's fibroblast. (A) Representative IP detection of RIPK3 with c-Abl and MLKL levels of SHSY5Y cells treated with CBE (150 μM) for 5 days and Imatinib (10 μM) for 24 h. (B) Representative IP detection of RIPK3 with phospho-c-Abl (phospho-c-Abl) and phospho-Tyr (p-Tyr) levels of SHSY5Y cells treated with CBE (150 μM) for 5 days or Imatinib (10 μM) for 24 h. Representative IP detection of RIPK3 (C), RIPK1 (D), and MLKL (E) with phospho-c-Abl (phospho-c-Abl) and phosphor-tyrosine (p-Tyr) levels of fibroblasts from GD patients treated with Imatinib (10 μM) for 24 h.

model.

RIPK3 is Tyr phosphorylated in untreated cells, although at lower levels than in CBE-treated cells. We suggest that RIPK3 is probably Tyr phosphorylated in normal conditions by c-Abl or other tyrosine kinases. The evidence accumulated in recent years indicates that c-Abl has several mechanisms that lead to its activation [38–40]. Indeed, there are numerous studies on physiological stimuli that act on c-Abl in different activation categories [39].

Remarkably, in the fibroblasts of GD patients, we found higher levels of RIPK3 phosphorylated at tyrosine than in control fibroblasts. We also found that c-Abl interacts with RIPK3, and its inhibition (using Imatinib) decreased tyrosine phosphorylation of RIPK3 (Fig. 3C). These results, obtained in pharmacological and genetic GD models, showed that RIPK3 is phosphorylated at tyrosine and that c-Abl could modulate necroptosis in GD (although it also promotes inflammatory engagement independent of necrosis).

GD fibroblasts also exhibit increased RIPK3 phosphorylation at Tyr. We propose that GD fibroblasts have constitutively active Tyr phosphorylation and that c-Abl or other tyrosine kinases might be participating.

Additionally, we did not detect tyrosine phosphorylation of RIPK1 in fibroblasts from GD patients or its interaction with c-Abl when we performed immunoprecipitation of RIPK1 from whole-cell lysates using an anti-RIPK1 antibody (Fig. 3D).

MLKL is phosphorylated by RIPK3 on Thr-357 and Ser-358 residues and these phosphorylation events are critical for MLKL activation [19,37]. However, phosphorylation on Tyr residues has not yet been described. To evaluate whether CBE induces tyrosine phosphorylation in MLKL, we performed immunoprecipitation of MLKL from GD cells using anti-MLKL antibodies and evaluated tyrosine phosphorylation levels (Fig. 3E). We observed an increase in the phosphotyrosine signal in MLKL in GD cells. However, c-Abl was not associated with MLKL and Imatinib did not significantly decrease the phosphotyrosine signal. Thus, MLKL could be phosphorylated on a tyrosine residue, that has not yet been described, by another tyrosine kinase (Fig. 3E).

To analyze which tyrosine on RIPK3 could be phosphorylated by c-Abl, we performed an in-silico analysis on RIPK3. We used the platforms netphos 2.0 to search for tyrosine consensus phosphorylation sites; and GPS3.0, to identify the tyrosine included in the c-Abl consensus phosphorylation site YX_5P with the highest phosphorylation probability [41]. The in-silico analysis identified Tyr 185 as the RIPK3 tyrosine with the highest probability of being phosphorylated by c-Abl (Score 26.237) (Supplementary Fig. 4). The other Tyr residues could be phosphorylated by other tyrosine kinases (Supplementary Fig. 4).

Our results show that c-Abl interacts with and can phophorylate RIPK3, possibly at Tyr-185.

2.4. c-Abl genetic ablation prevents RIPK3 activation and its downstream signaling in GD cells

Altogether our results show that c-Abl interacts with RIPK3 in a CBE-induced GD model and that a specific inhibitor of c-Abl decreases RIPK3 activation. RIPK3, RIPK1, and MLKL are indispensable for necroptosis but could also promote an inflammatory response in GD [11,19,24,37].

To assess the relevance of c-Abl signaling downstream of RIPK3, we determined whether genetic ablation of c-Abl prevents RIPK3 activation and downstream signaling in GD cells.

First, we followed the effects of c-Abl inhibition on RIPK1, which functions upstream of RIPK3. We analyzed the levels of the active (cleaved) RIPK1 since its cleavage by caspase-8 is a mechanism for dismantling death-inducing complexes [11]. We used SHSY5Y cells treated with 150 μM CBE for 5 days and 10 μM of Imatinib for 24 h and we observed that cleaved RIPK1 was significantly reduced by Imatinib (Fig. 4A). This observation was consistent with previous reports by us and others.

To confirm that the effect of Imatinib on phospho-RIPK3 levels is mediated by c-Abl inhibition, we used HeLa c-Abl knockout (KO) cells that we obtained using the CRISPR/Cas9 tool as detailed in Contreras et al. [42]. c-Abl KO HeLa cells were treated with 150 μM of CBE for 4 days and 10 μM of Imatinib for 24 h. Consistent with our previous results, we found that reduced c-Abl levels was associated with significantly decreased phospho-RIPK3 levels (Fig. 4B). It is important to mention that a residual band is observed in c-Abl knockout cells, since the antibody used probably recognizes another protein of the c-Abl family, such as Arg.

The interaction between RIPK3 and MLKL is important for inducing necrosome formation and consequently cell death [37,43–45]. Moreover, we observed that Imatinib decreases RIPK3-MLKL interaction in SHSY5Y cells treated with CBE 150 μM (Fig. 3A). Then, we analyzed the effect of genetic ablation of c-Abl on the downstream RIPK3 signaling protein, MLKL. We used primary cultures of cortical neurons from wild-type and c-Abl KO mice embryos treated with CBE 150 μM for 4 days and analyzed phospho-MLKL levels. Consistent with our previous results, we found decreased phospo-MLKL levels in c-Abl null neurons compared to WT neurons (Fig. 4C).

Altogether, our results show that c-Abl is active in GD and suggest that activation of the RIPK3 pathway may occur downstream of c-Abl. We propose that c-Abl could directly or indirectly phosphorylate RIPK3 in a new tyrosine residue, not previously described.

Since MLKL is phosphorylated by RIPK3, and all of the phosphorylation events are critical for necrotic cell death while also orchestrating necrosis-independent inflammation in GD [11,19,24], our results suggest that c-Abl could be involved in both processes.

3. Material and methods

3.1. Animals and brain tissues

$Gba^{flox/flox}$ mice were crossed with $Gba^{flox/+}$; nestin-Cre mice to generate $Gba^{flox/flox}$; nestin-Cre mice7 and $Gba^{flox/+}$; nestin-Cre mice, which served as healthy controls. Genotyping was performed by PCR using genomic DNA extracted from tails or embryonic brains. Only male mice were used in these studies. The colony was maintained in the experimental animal center of the Weizmann Institute of Science.

C57BL/6 mice (The Jackson Laboratory) were injected daily i.p. with 50 or 100 mg CBE per kg body weight or with PBS from 8 to 21 days of age. No animals were excluded from the study, the sample size was chosen based on the number required to validate statistical analyses, no randomization was used and the investigator was not blinded.

c-Abl KO mice were bred from $c\text{-}Abl^{loxp}$/$c\text{-}Abl^{loxp}$ and $Nestin\text{-}Cre^{+}$ was purchased from Jackson laboratory. The animal protocols used were reviewed and approved by the animal ethics board at our institution.

3.2. Antibodies

Rabbit anti-c-Abl (2862) and rabbit anti-RIPK1 (77565) were purchased from Cell Signaling Technology (Danvers, Massachusetts, USA). Hoechst 33342 (H3570) and Fluoromont-G with DAPI (00-4959-52) were purchased from Thermo Fischer Scientific (Waltham, Massachusetts, USA). Mouse anti-c-Abl (sc-23), mouse anti-GAPDH (sc-32233) were purchased from Santa Cruz Biotechnology (Dallas, United States of America). Rabbit anti-phospho-c-Abl (Tyr 412) (07-788) and GSK`872 (530389) were purchased from Sigma-Aldrich. Imatinib mesylate (13139) was purchased from Cayman Chemical Company (Ann Arbor, USA). Mouse anti-phospho-tyrosine antibody (4G10) and CBE (234599) were purchased from Merck Millipore. Rabbit anti-phospho-MLKL (Ser 358 and Ser 345) (ab187091 and ab196436), mouse anti-phospho-RIPK3 (Thr-231 and Ser-232) (ab20541), rabbit anti-phospho-RIPK3 (Ser-227) (ab209384), rabbit RIPK3 (ab62344) and rabbit anti-CD68 (ab213363) were purchased from Abcam.

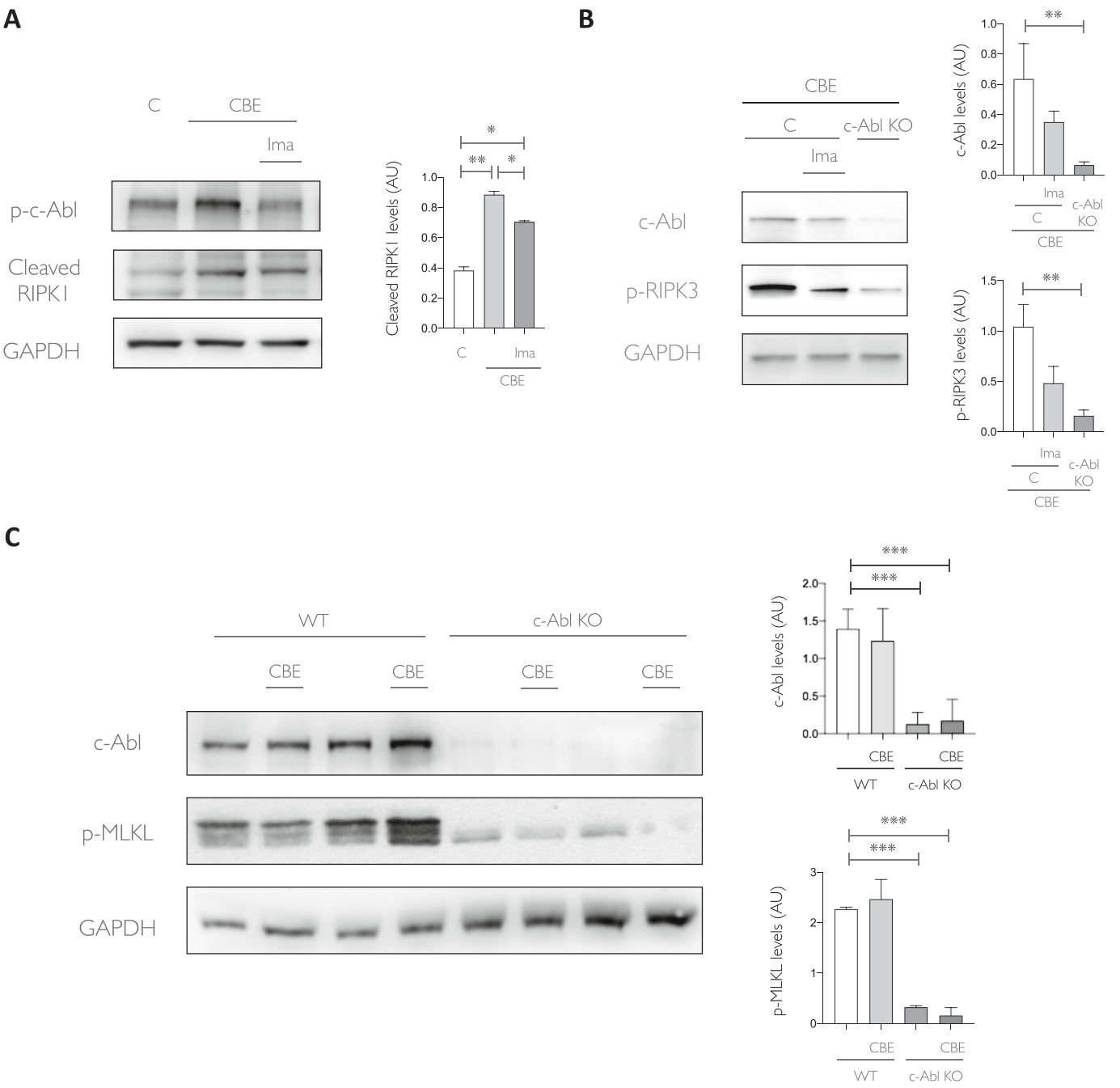

Fig. 4. c-Abl genetic ablation prevents RIPK3 activation and its downstream signaling in GD cells. (A) Phospho-c-Abl (phospho-c-Abl), c-Abl, and RIPK1 (Cleaved RIPK1) levels of SHSY5Y cells treated with CBE (150 μM) for 5 days and Imatinib (10 μM) for 24 h. (B) c-Abl and phospho-RIPK3 (p-RIPK3) levels of c-Abl KO HeLa cells treated with CBE (150 μM) for 4 days or Imatinib (10 μM) for 24 h. (C) c-Abl and phospho-MLKL (p-MLKL) levels of primary cultures of cortical neurons from wild-type and c-Abl KO mice embryos treated with CBE (150 μM) for 4 days.

3.3. Cell culture

SHSY5Y and Hepa 1–6 cells were obtained from ATCC (Virginia, USA). Human fibroblast GM05659, GM04393 (Leu444Pro), GM00877 (Leu444Pro), GM01260 (Pro415Arg) and GM08760 (Leu444Pro) were obtained from Coriell Institute (USA). All the 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.

3.4. Mice cortical neuron cultures

Cortical from c-Abl$^{floxo/floxo}$ Nestin Cre (c-Abl KO) and c-Abl$^{floxo/floxo}$ (wild-type) mice were removed on postnatal day 1 (the genotype of the animals (c-Abl$^{+/+}$ (WT) or c-Abl$^{-/-}$ (c-Abl KO) was determined 1 day later)) which were dissected in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS) and rinsed twice with HBSS. 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, supplemented with 10% horse serum (Invitrogen, Waltham, MA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. Dissociated hippocampal cells were seeded onto poly-Lysine coated coverslips. Cultures were maintained at 37 °C in 5% CO_2 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. On day 2, cultured neurons were treated with AraC 2 μM for 24 h; this method resulted in cultures highly enriched in neurons (approximately 5% glia).

3.5. Hela c-Abl KO cells

HeLa c-Abl KO cells were obtained using the CRISPR/Cas9 tool as detailed in Contreras et al. [42]. Hela cells were transfected with a plasmid containing all-in-one sgRNA clone for Human c-Abl gene, Target site: GTCTGAGTGAAGCCGCTCGT (Gene Copeia TM). After 48 h of transfection, cells were incubated with G418 0.5 mg/mL for 48 h. Then, cells were washed and incubated with G418 0.5 mg/mL for 2 weeks. We then selected colonies and incubated them in 96 well plates. Each clone was tested by western blot.

3.6. Imatinib, CBE, and GSK′872 treatments

All the cell lines were treated as indicated in the manuscript. Cells were treated with Imatinib 10 μM, CBE 150 μM, or GSK′872 1 μM.

3.7. Immunoprecipitations and RIPK3 tyrosine phosphorylation assay

The SHSY5Y cells were treated with CBE 150 μM for 5 days. Cells were lysed in immunoprecipitation buffer plus protease and phosphatase inhibitors. We used anti-RIPK3, anti-RIPK1, or anti-MLKL for the immunoprecipitations. RIPK3 tyrosine phosphorylation was evaluated with anti-RIPK3, anti-RIPK1, anti-MLKL, or anti-phospho-Tyr antibodies.

3.8. 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 20 μm thick sagittal sections using a cryostat (Leika) at −20 °C. Slices were permeabilized with 0.1% Triton X-100, blocked in 5% BSA or Gelatin in PBS, and incubated overnight with the antibody rabbit anti-p-c-Abl or anti-CD68 in 5% BSA in PBS. For Hoechst staining, tissues were treated with Hoechst after secondary antibody. The primary antibody was visualized with anti-rabbit Alexa-Fluor 555 or Alexa-Fluor 488.

3.9. Immunoblot analysis

Cells were lysed in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with cocktail of protease inhibitors (Roche): Na3VO4, NaF, PMSF and Aprotinina. The homogenates were maintained on ice for 30 min and then they were centrifuged at 10,000*g* for 10 min. The supernatant was recovered, and protein concentration was determined with the Pierce BCA protein assay kit (23225) purchased from Thermo Scientific (Waltham, United States of America). Proteins were resolved in SDS-PAGE, transferred to Nitrocellulose membranes (Thermo Scientific), and probed with primary antibodies against p-c-Abl, c-Abl, GAPDH, p-RIPK3, RIPK1, and p-MLKL. The reactions were followed by incubation with HRP labeled secondary antibodies and visualized using the ECL technique (Thermo Scientific).

3.10. GBA activity assay

GBA activity was assayed fluorometrically using artificial sugar substrates containing the fluorophore 4-methylumbelliferone (4-MU). For measuring β-glucocerebrosidase activity, samples were incubated in the presence or absence of 0.3 mM NB-DGJ for 30 min on ice before the assay. The substrate for GBA β-glucosidase activity was 4.5 mM 4-MU β-D-glucoside in 200 mM citrate/phosphate buffer, pH 5.2, 0.25% Triton X-100, 0.25% sodium taurocholate, 1.25 mM EDTA, and 4 mM 2-mercaptoethanol. GBA activity was defined as the NB-DGJ nonsensitive activity at pH 5.2. The digests (in triplicate) containing cell lysates in PBS with 0.1% Triton X-100 and artificial 4-MU substrate were incubated at 37 °C for 30 min. The reaction was stopped by adding cold 0.5 M Na_2CO_3, and the released fluorescent 4-MU was measured in a FLUOstar OPTIMA plate reader with excitation at 360 nm and emission at 460 nm. A standard curve of free 4-MU was used to calculate enzyme activity. Results were normalized to protein content.

3.11. Statistical analysis

Mean and SEM values and the number of experiments is indicated in each figure legend. One-way ANOVA tests were performed followed by Bonferroni post-test using the Prisma Software.

3.12. Statement of ethics

All protocols were approved and followed local guidance documents generated by the *ad hoc* committee of the Chilean Science and Technology Council (FONDECYT) and were approved by the Scientific Ethics Committee for the care of animals and the environment of Pontificia Universidad Católica de Chile #160803003. Protocols are in agreement with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals recommended by the Institute for Laboratory Animal Research in its Guide for Care and Use of Laboratory Animals.

4. Discussion

Here, we identified c-Abl as a new player in the signaling pathway involving RIPK3, which participates in cell damage and death in GD. RIPK3 has emerged as a new regulator of necroptosis which could also be involved in the inflammatory response, with important functions in GD induced by pathogenic mutations. Remarkably, RIPK3 deficiency improves the clinical course of GD in mice, with increased motor coordination and survival [11,24].

Our results strongly suggest that c-Abl activity is required for RIPK1, RIPK3, and MLKL activation. Moreover, our results show that c-Abl interacts with RIPK3 and that c-Abl could mediate phosphorylation in tyrosine residues of RIPK3, a phosphorylation status not previously described. Supporting a connection between RIPK3 and c-Abl, it was shown that Ponatinib, a c-Abl inhibitor, can inhibit RIPK3-mediated cell death in *in vitro* and *in vivo* models of inflammation [46]. c-Abl-dependent phosphorylation stabilizes many proteins such as p73 [47], c-Jun [48], ERα [49], C/EBPβ [50], and CDK5 [51], preventing their proteosomal degradation. We predict that a similar mechanism could be involved in the regulation of RIPK3 by c-Abl.

Using different models, we found that c-Abl kinase is active in GD cells. We observed that c-Abl is activated in *in vitro* as well as *in vivo* pharmacological GD models after CBE treatment for different periods. More importantly, we observed the activation of c-Abl in fibroblasts of GD patients and brain slices of GD mice.

Interestingly, c-Abl is initially active in layer V of the cerebral cortex and not in other layers and regions of the cortex. This result could explain the differential susceptibility of different areas of the brain that are associated with neurodegeneration in GD.

We have already shown the activation of c-Abl in other animal models of neurodegenerative diseases, including Alzheimer's and NPC. Interestingly, GD and NPC share several characteristics being both lysosomal lipid deposition disorders. NPC is characterized by the buildup of free cholesterol and glycosphingolipids while in GD there is an accumulation of GlcCer and to a lesser degree of glucosylsphingosine (GlcSph) in the endosomal-lysosomal system. The evidence suggests that lysosomal lipid accumulation and the dysregulation of lysosomal homeostasis are the major upstream stimuli that activate the c-Abl pathway [31]. These and other antecedents have led to the hypothesis that these diseases could share similar pathogenic pathways [52].

However, c-Abl activation in NPC and AD has been linked to the induction of apoptosis, whereas in GD necroptosis has been described as the main pathway that executes cell death. Considering the phenotypic characteristics of GD, we believe that dysregulation of lysosomal homeostasis activates c-Abl.

Gaucher patients show defective FAS-induced apoptosis and it has been proposed that this is due to the deterioration of some cell membrane lipids. This situation impairs the activation of caspases, although further studies are required to evaluate the putative role of membrane lipids as a trigger of such defect.

Although c-Abl was not previously linked to the RIPK3 pathway, it seemed interesting to evaluate a possible connection between both kinases. Indeed, it was known that the c-Abl inhibitor, Ponatinib, also inhibits RIPK3 [46]. The observation that activated c-Abl contributes to the increase in phospho-RIPK3 levels in GD reveals a new cell death pathway in which c-Abl activity could be participating.

The mechanisms that underlie cell fate choice between cell survival and cell death by apoptosis or necroptosis when cell homeostasis is seriously affected are being intensively investigated. Although both types of cell death can be initiated by death receptors and intracellular receptors, they are regulated differently and produce distinctive physiological consequences. Therefore, it is necessary to differentiate apoptosis from necroptosis. In response to inflammation, inhibitors of apoptosis, such as cIAP1, and cIAP2 elicit RIPK1 ubiquitination [53,54], and block necroptosis. Furthermore, the amplification of caspase-8-dependent apoptosis could cleave and decrease Ripk1/Ripk3 signals, preventing cell death [22,55]. Caspase-8 may even be the molecular switch that controls apoptosis and necroptosis preventing damage during embryonic development and adulthood [56]. RIPK3 is essential for necroptosis-like death but also governs whether the cell activates caspase-8 and dies by apoptosis [57]. Indeed, our data add additional information regarding the interplay of those two types of deaths and show data from a new player with a pivotal role. c-Abl could mediate both apoptosis and necroptosis, which are linked through multiple biochemical and functional associations. Undoubtedly, the most investigated mechanism of programmed cell death is caspase-mediated apoptosis, although increasing evidence indicates that necroptosis may trigger neuronal death in neurodegenerative disorders such as Gaucher disease [11,24], Huntington disease [58], and amyotrophic lateral sclerosis [59].

c-Abl contributes to the regulation of apoptosis and is activated in the nucleus upon DNA damage; where its signaling is required for the initiation of death by apoptosis. The proapoptotic function of c-Abl is partially mediated by its interaction with p73, and p53. Interestingly, our results show that c-Abl activates the RIPK3-dependent pathway and could play a role in triggering cell death.

It is tempting to speculate that background genetic differences between diseases, such as NPC and GD, could influence the different death responses involving c-Abl. Furthermore, the downstream proteins activated by c-Abl determines which one of the two types of deaths could be triggered.

Regardless of the exact pathological role mediated by c-Abl in GD, it is clear that inhibition of c-Abl may play an important role in RIPK3 signaling in this disease. It is necessary to find the stimulating mechanisms of apoptosis and necroptosis, which will be useful for understanding how the cell death pathway is chosen. Therefore, careful dissection of these pathways will provide better targets for therapeutic approaches in the future.

Our results clearly show that c-Abl is required for signaling *via* RIPK3. As mentioned, RIPK3 has emerged as a vital regulator of necroptosis-like death. Thr-231, Ser-227 (in human RIPK3), and Ser-232 (in mouse RIPK3) phosphorylation, as well as phosphorylation of Thr-357 and Ser-358 on MLKL, are indicative of necrosome formation and marks necroptosis triggering. The c-Abl inhibitor Imatinib, prevented the increase of RIPK3 phosphorylation induced by CBE in different GD models: results that allowed us to establish a connection between RIPK3 and c-Abl. In c-Abl KO HeLa cells treated with CBE the increase in RIPK3 phosphorylated at Ser-227 was not observed. Thus, c-Abl activated by CBE contributes to the increase in phospho-RIPK3 levels in GD.

When a RIPK3 inhibitor was used, there was also a decrease in c-Abl activation, which indicates that RIPK3 is also involved in c-Abl activation; suggesting a possible connection between the signaling pathways that have not yet been described.

Our results show that in c-Abl interacts with RIPK3 GD cells and decreases the levels of active phosphorylated RIPK3. As previously mentioned, c-Abl-dependent phosphorylation regulates the stability of several proteins such as p73 [47], HDAC [60], c-Jun [48], ERα [49], and C/EBPβ [50]. The identification of RIPK3 kinase as a critical mediator of cell death presents an exciting new opportunity for therapeutic developments for GD [11]. RIPK3 activation is modulated by caspase-mediated cleavage, ubiquitination, and phosphorylation. These post-translational modifications orchestrate the assembly of the necrosome. RIPK3 is tightly phosphorylated upon the induction of necrosis. However, only Ser and Thr phosphorylation but no tyrosine (Tyr) phosphorylation have been described. Phosphorylation sites of RIPK3 on Thr-231, Ser-227 (in human RIPK3), and Ser-232 (in mouse RIPK3) are induced by TNF alpha stimulation and are particularly important for its interaction with MLKL and its activation [19,37]. Although there are other phosphorylated Ser/Thr residues on RIPK3 [37], their roles in RIPK3 activation are not as clear. Interestingly, among RIPK3 post-translational modifications, tyrosine phosphorylation had not previously been described. We found that c-Abl could induce RIPK3 tyrosine phosphorylation, although our results do not rule out indirect phosphorylation by another protein. Tyrosine phosphorylation of RIPK3 by c-Abl appears to be especially relevant for necrosome development in cells exposed to CBE in a GD *in vitro* model.

RIPK1 and RIPK3 are key components of the necrosome, which mediates the activation of MLKL [61]. During TNF-initiated necroptosis, nuclear RIPK1 is ubiquitinated and RIPK3 phosphorylated, after which, MLKL is phosphorylated and oligomerized. These steps are key for neuronal necroptosis death in Type 2 GD.

The precise chain of events that destroys neurons in GD is still unclear. However, targeting c-Abl could be a strategy for the treatment of the disease.

Although our results do not show that inhibition of c-Abl prevents death, we know that c-Abl regulates apoptosis [62] and -as we have demonstrated in this work- it regulates one of the key elements of necroptosis. Therefore, our results support the idea that c-Abl kinase activity is an important hub in neuronal death.

Thus, c-Abl activation could efficiently regulate RIPK3 signaling and neuronal death in GD. c-Abl antagonists, already approved by the FDA, can be easily repurposed for the treatment of GD. Several c-Abl inhibitors are currently used for the treatment of chronic myeloid leukemia (CML) [63]. Also, c-Abl inhibitors have been used in different studies in patients with PD [64–67]. Among them, Imatinib, Nilotinib, and Bafetinib have been successfully used in pre-clinical models of PD [68,69]. However, selectivity, limited blood-brain barrier penetration, and toxicity are a concern. Imatinib, used in the current study, is a second-generation Bcr-Abl tyrosine kinase inhibitor (TKI) [70]. This is of particular interest in the context of cell death because the GD pathology is characterized by necroptosis. New drug targets are needed in the field as previous trials of potential therapies for LSD with neurological conditions such as NPC and GD have largely failed or demonstrated minimal efficacy. We recently reviewed the mechanism of c-Abl and its role in CNS diseases in general [71]. While c-Abl inhibitors are expected to also be effective for the treatment of peripheral human diseases, the possibility of developing highly specific c-Abl inhibitors that can cross the blood-brain barrier provides a unique opportunity for the treatment of LSDs diseases.

Finally, our results show that c-Abl signaling is a new upstream pathway that activates RIPK3 and that its inhibition could be an attractive therapeutic approach for the treatment of GD as well as other

lysosomal storage diseases.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2021.166089.

CRediT authorship contribution statement

M.J.Y., A.R.A., and S.Z. conceived the study. M.J.Y., A.D.K., A.R.A, and S.Z designed experiments. M.J.Y., F.C, and T.M. performed experiments. M.J.Y. performed quantitative analysis of the results. M.J.Y., A.D.K., A.H.F., A.R.A, and S.Z wrote the manuscript.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This article was funded by grants from CONICYT-Chile: Fondecyt 1161065 (ARA) and 1201668 (ARA), 1180337 (AK) and 1190334 (SZ), 3170710 (MJY) and FONDEF D10E1077; and from MSCA-RISE-2016-Lysomod-734825 (AK, AHF, and SZ).

References

[1] L. Ginzburg, Y. Kacher, A.H. Futerman, The pathogenesis of glycosphingolipid storage disorders, Semin. Cell Dev. Biol. 15 (4) (2004) 417–431.
[2] K.S. Hruska, M.E. LaMarca, C.R. Scott, E. Sidransky, Gaucher disease: mutation and polymorphism spectrum in the glucocerebrosidase gene (GBA), Hum. Mutat. 29 (5) (2008) 567–583.
[3] A.M. Vaccaro, M. Motta, M. Tatti, S. Scarpa, L. Masuelli, M. Bhat, M.T. Vanier, A. Tylki-Szymanska, R. Salvioli, Saposin C mutations in Gaucher disease patients resulting in lysosomal lipid accumulation, saposin C deficiency, but normal prosaposin processing and sorting, Hum. Mol. Genet. 19 (15) (2010) 2987–2997.
[4] G.A. Grabowski, Phenotype, diagnosis, and treatment of Gaucher's disease, Lancet 372 (9645) (2008) 1263–1271.
[5] J. Stirnemann, M. Vigan, D. Hamroun, D. Heraoui, L. Rossi-Semerano, M.G. Berger, C. Rose, F. Camou, C. de Roux-Serratrice, B. Grosbois, P. Kaminsky, A. Robert, C. Caillaud, R. Froissart, T. Levade, A. Masseau, C. Mignot, F. Sedel, D. Dobbelaere, M.T. Vanier, V. Valayanopoulos, O. Fain, B. Fantin, T.B. de Villemeur, F. Mentré, N. Belmatoug, The French Gaucher's disease registry: clinical characteristics, complications and treatment of 562 patients, Orphanet. J. Rare Dis. 7 (2012) 77.
[6] E. Sidransky, Gaucher disease: insights from a rare Mendelian disorder, Discov. Med. 14 (77) (2012) 273–281.
[7] T. Farfel-Becker, E.B. Vitner, S.N. Pressey, R. Eilam, J.D. Cooper, A.H. Futerman, Spatial and temporal correlation between neuron loss and neuroinflammation in a mouse model of neuronopathic Gaucher disease, Hum. Mol. Genet. 20 (7) (2011) 1375–1386.
[8] N. Conradi, M. Kyllerman, J.E. Mansson, A.K. Percy, L. Svennerholm, Late-infantile Gaucher disease in a child with myoclonus and bulbar signs: neuropathological and neurochemical findings, Acta Neuropathol. 82 (2) (1991) 152–157.
[9] K. Wong, E. Sidransky, A. Verma, T. Mixon, G.D. Sandberg, L.K. Wakefield, A. Morrison, A. Lwin, C. Colegial, J.M. Allman, R. Schiffmann, Neuropathology provides clues to the pathophysiology of Gaucher disease, Mol. Genet. Metab. 82 (3) (2004) 192–207.
[10] A.D. Klein, N.S. Ferreira, S. Ben-Dor, J. Duan, J. Hardy, T.M. Cox, A.H. Merrill Jr., A.H. Futerman, Identification of modifier genes in a mouse model of Gaucher disease, Cell Rep. 16 (10) (2016) 2546–2553.
[11] E.B. Vitner, R. Salomon, T. Farfel-Becker, A. Meshcheriakova, M. Ali, A.D. Klein, F. M. Platt, T.M. Cox, A.H. Futerman, RIPK3 as a potential therapeutic target for Gaucher's disease, Nat. Med. 20 (2) (2014) 204–208.
[12] M. Pasparakis, P. Vandenabeele, Necroptosis and its role in inflammation, Nature 517 (7534) (2015) 311–320.
[13] A. Degterev, J. Hitomi, M. Germscheid, I.L. Ch'en, O. Korkina, X. Teng, D. Abbott, G.D. Cuny, C. Yuan, G. Wagner, S.M. Hedrick, S.A. Gerber, A. Lugovskoy, J. Yuan, Identification of RIP1 kinase as a specific cellular target of necrostatins, Nat. Chem. Biol. 4 (5) (2008) 313–321.
[14] A. Degterev, Z. Huang, M. Boyce, Y. Li, P. Jagtap, N. Mizushima, G.D. Cuny, T. J. Mitchison, M.A. Moskowitz, J. Yuan, Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury, Nat. Chem. Biol. 1 (2) (2005) 112–119.
[15] D.W. Zhang, J. Shao, J. Lin, N. Zhang, B.J. Lu, S.C. Lin, M.Q. Dong, J. Han, RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis, Science 325 (5938) (2009) 332–336.
[16] Y.S. Cho, S. Challa, D. Moquin, R. Genga, T.D. Ray, M. Guildford, F.K. Chan, Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation, Cell 137 (6) (2009) 1112–1123.
[17] S. He, L. Wang, L. Miao, T. Wang, F. Du, L. Zhao, X. Wang, Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha, Cell 137 (6) (2009) 1100–1111.
[18] J. Wu, Z. Huang, J. Ren, Z. Zhang, P. He, Y. Li, J. Ma, W. Chen, Y. Zhang, X. Zhou, Z. Yang, S.-Q. Wu, L. Chen, J. Han, Mlkl knockout mice demonstrate the indispensable role of Mlkl in necroptosis, Cell Res. 23 (8) (2013) 994–1006.
[19] L. Sun, H. Wang, Z. Wang, S. He, S. Chen, D. Liao, L. Wang, J. Yan, W. Liu, X. Lei, X. Wang, Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase, Cell 148 (1–2) (2012) 213–227.
[20] X.N. Wu, Z.H. Yang, X.K. Wang, Y. Zhang, H. Wan, Y. Song, X. Chen, J. Shao, J. Han, Distinct roles of RIP1-RIP3 hetero- and RIP3-RIP3 homo-interaction in mediating necroptosis, Cell Death Differ. 21 (11) (2014) 1709–1720.
[21] W.D. Cook, D.M. Moujalled, T.J. Ralph, P. Lock, S.N. Young, J.M. Murphy, D. L. Vaux, RIPK1- and RIPK3-induced cell death mode is determined by target availability, Cell Death Differ. 21 (10) (2014) 1600–1612.
[22] W.J. Kaiser, H. Sridharan, C. Huang, P. Mandal, J.W. Upton, P.J. Gough, C. A. Sehon, R.W. Marquis, J. Bertin, E.S. Mocarski, Toll-like receptor 3-mediated necrosis via TRIF, RIP3, and MLKL, J. Biol. Chem. 288 (43) (2013) 31268–31279.
[23] L. Duprez, N. Takahashi, F. Van Hauwermeiren, B. Vandendriessche, V. Goossens, T. Vanden Berghe, W. Declercq, C. Libert, A. Cauwels, P. Vandenabeele, RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome, Immunity 35 (6) (2011) 908–918.
[24] L Galluzzi, O Kepp, FK Chan, G Kroemer, Necroptosis: Mechanisms and Relevance to Disease, Annu Rev Pathol 12 (2017) 103–130, https://doi.org/10.1146/annurev-pathol-052016-100247.
[25] P. Mandal, S.B. Berger, S. Pillay, K. Moriwaki, C. Huang, H. Guo, J.D. Lich, J. Finger, V. Kasparcova, B. Votta, M. Ouellette, B.W. King, D. Wisnoski, A. S. Lakdawala, M.P. DeMartino, L.N. Casillas, P.A. Haile, C.A. Sehon, R.W. Marquis, J. Upton, L.P. Daley-Bauer, L. Roback, N. Ramia, C.M. Dovey, J.E. Carette, F. K. Chan, J. Bertin, P.J. Gough, E.S. Mocarski, W.J. Kaiser, RIP3 induces apoptosis independent of pronecrotic kinase activity, Mol. Cell 56 (4) (2014) 481–495.
[26] X.S. Yang, T.L. Yi, S. Zhang, Z.W. Xu, Z.Q. Yu, H.T. Sun, C. Yang, Y. Tu, S.X. Cheng, Hypoxia-inducible factor-1 alpha is involved in RIP-induced necroptosis caused by in vitro and in vivo ischemic brain injury, Sci. Rep. 7 (1) (2017) 5818.
[27] A.R. Alvarez, P.C. Sandoval, N.R. Leal, P.U. Castro, K.S. Kosik, Activation of the neuronal c-Abl tyrosine kinase by amyloid-beta-peptide and reactive oxygen species, Neurobiol. Dis. 17 (2) (2004) 326–336.
[28] H.S. Ko, Y. Lee, J.H. Shin, S.S. Karuppagounder, B.S. Gadad, A.J. Koleske, O. Pletnikova, J.C. Troncoso, V.L. Dawson, T.M. Dawson, Phosphorylation by the c-Abl protein tyrosine kinase inhibits parkin's ubiquitination and protective function, Proc. Natl. Acad. Sci. U. S. A. 107 (38) (2010) 16691–16696.
[29] R. Katsumata, S. Ishigaki, M. Katsuno, K. Kawai, J. Sone, Z. Huang, H. Adachi, F. Tanaka, F. Urano, G. Sobue, c-Abl inhibition delays motor neuron degeneration in the G93A mouse, an animal model of amyotrophic lateral sclerosis, PLoS One 7 (9) (2012), e46185.
[30] A.R. Alvarez, A. Klein, J. Castro, G.I. Cancino, J. Amigo, M. Mosqueira, L. M. Vargas, L.F. Yévenes, F.C. Bronfman, S. Zanlungo, Imatinib therapy blocks cerebellar apoptosis and improves neurological symptoms in a mouse model of Niemann-Pick type C disease, FASEB J. 22 (10) (2008) 3617–3627.
[31] A. Klein, C. Maldonado, L.M. Vargas, M. Gonzalez, F. Robledo, K. Perez de Arce, F. J. Munoz, C. Hetz, A.R. Alvarez, S. Zanlungo, Oxidative stress activates the c-Abl/p73 proapoptotic pathway in Niemann-Pick type C neurons, Neurobiol. Dis. 41 (1) (2011) 209–218.
[32] S.T. Wen, P.K. Jackson, R.A. Van Etten, The cytostatic function of c-Abl is controlled by multiple nuclear localization signals and requires the p53 and Rb tumor suppressor gene products, EMBO J. 15 (7) (1996) 1583–1595.
[33] C.L. Sawyers, J. McLaughlin, A. Goga, M. Havlik, O. Witte, The nuclear tyrosine kinase c-Abl negatively regulates cell growth, Cell 77 (1) (1994) 121–131.
[34] J.N. Kanfer, G. Legler, J. Sullivan, S.S. Raghavan, R.A. Mumford, The Gaucher mouse, Biochem. Biophys. Res. Commun. 67 (1) (1975) 85–90.
[35] A. Vardi, H. Zigdon, A. Meshcheriakova, A.D. Klein, C. Yaacobi, R. Eilam, B. M. Kenwood, A.A. Rahim, G. Massaro, A.H. Merrill Jr., E.B. Vitner, A.H. Futerman, Delineating pathological pathways in a chemically induced mouse model of Gaucher disease, J. Pathol. 239 (4) (2016) 496–509.
[36] M.K. Pandey, G.A. Grabowski, Immunological cells and functions in Gaucher disease, Crit. Rev. Oncog. 18 (3) (2013) 197–220.
[37] W. Chen, Z. Zhou, L. Li, C.Q. Zhong, X. Zheng, X. Wu, Y. Zhang, H. Ma, D. Huang, W. Li, Z. Xia, J. Han, Diverse sequence determinants control human and mouse receptor interacting protein 3 (RIP3) and mixed lineage kinase domain-like (MLKL) interaction in necroptotic signaling, J. Biol. Chem. 288 (23) (2013) 16247–16261.
[38] J. Colicelli, ABL tyrosine kinases: evolution of function, regulation, and specificity, Sci. Signal. 3 (139) (2010), re6.
[39] O. Hantschel, G. Superti-Furga, Regulation of the c-Abl and Bcr-Abl tyrosine kinases, Nat. Rev. Mol. Cell Biol. 5 (1) (2004) 33–44.
[40] J.Y. Wang, The capable ABL: what is its biological function? Mol. Cell. Biol. 34 (7) (2014) 1188–1197.
[41] T.P. Cujec, P.F. Medeiros, P. Hammond, C. Rise, B.L. Kreider, Selection of v-abl tyrosine kinase substrate sequences from randomized peptide and cellular proteomic libraries using mRNA display, Chem. Biol. 9 (2) (2002) 253–264.
[42] P.S. Contreras, P.J. Tapia, L. González-Hódar, I. Peluso, C. Soldati, G. Napolitano, M. Matarese, M.L. Heras, C. Valls, A. Martinez, E. Balboa, J. Castro, N. Leal, F. M. Platt, A. Sobota, D. Winter, A.D. Klein, D.L. Medina, A. Ballabio, A.R. Alvarez, S. Zanlungo, c-Abl inhibition activates TFEB and promotes cellular clearance in a lysosomal disorder, iScience 23 (11) (2020), 101691.
[43] Z. Cai, S. Jitkaew, J. Zhao, H.C. Chiang, S. Choksi, J. Liu, Y. Ward, L.G. Wu, Z. G. Liu, Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis, Nat. Cell Biol. 16 (1) (2014) 55–65.
[44] Y. Dondelinger, W. Declercq, S. Montessuit, R. Roelandt, A. Goncalves, I. Bruggeman, P. Hulpiau, K. Weber, C.A. Sehon, R.W. Marquis, J. Bertin, P.

J. Gough, S. Savvides, J.C. Martinou, M.J. Bertrand, P. Vandenabeele, MLKL compromises plasma membrane integrity by binding to phosphatidylinositol phosphates, Cell Rep. 7 (4) (2014) 971–981.
[45] H. Wang, L. Sun, L. Su, J. Rizo, L. Liu, L.F. Wang, F.S. Wang, X. Wang, Mixed lineage kinase domain-like protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP3, Mol. Cell 54 (1) (2014) 133–146.
[46] M. Najjar, C. Suebsuwong, S.S. Ray, R.J. Thapa, J.L. Maki, S. Nogusa, S. Shah, D. Saleh, P.J. Gough, J. Bertin, J. Yuan, S. Balachandran, G.D. Cuny, A. Degterev, Structure guided design of potent and selective ponatinib-based hybrid inhibitors for RIPK1, Cell Rep. 10 (11) (2015) 1850–1860.
[47] K.K. Tsai, Z.M. Yuan, c-Abl stabilizes p73 by a phosphorylation-augmented interaction, Cancer Res. 63 (12) (2003) 3418–3424.
[48] B. Gao, S.M. Lee, D. Fang, The tyrosine kinase c-Abl protects c-Jun from ubiquitination-mediated degradation in T cells, J. Biol. Chem. 281 (40) (2006) 29711–29718.
[49] X. He, Z. Zheng, T. Song, C. Wei, H. Ma, Q. Ma, Y. Zhang, Y. Xu, W. Shi, Q. Ye, H. Zhong, c-Abl regulates estrogen receptor alpha transcription activity through its stabilization by phosphorylation, Oncogene 29 (15) (2010) 2238–2251.
[50] X. Li, X. Liu, G. Wang, X. Zhu, X. Qu, X. Li, Y. Yang, L. Peng, C. Li, P. Li, W. Huang, Q. Ma, C. Cao, Non-receptor tyrosine kinases c-Abl and Arg regulate the activity of C/EBPbeta, J. Mol. Biol. 391 (4) (2009) 729–743.
[51] G.I. Cancino, K. Perez de Arce, P.U. Castro, E.M. Toledo, R. von Bernhardi, A. R. Alvarez, c-Abl tyrosine kinase modulates tau pathology and Cdk5 phosphorylation in AD transgenic mice, Neurobiol. Aging 32 (7) (2011) 1249–1261.
[52] A.H. Futerman, J.L. Sussman, M. Horowitz, I. Silman, A. Zimran, New directions in the treatment of Gaucher disease, Trends Pharmacol. Sci. 25 (3) (2004) 147–151.
[53] M.J. Bertrand, S. Milutinovic, K.M. Dickson, W.C. Ho, A. Boudreault, J. Durkin, J. W. Gillard, J.B. Jaquith, S.J. Morris, P.A. Barker, cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination, Mol. Cell 30 (6) (2008) 689–700.
[54] M.A. O'Donnell, D. Legarda-Addison, P. Skountzos, W.C. Yeh, A.T. Ting, Ubiquitination of RIP1 regulates an NF-kappaB-independent cell-death switch in TNF signaling, Curr. Biol. 17 (5) (2007) 418–424.
[55] C.P. Dillon, A. Oberst, R. Weinlich, L.J. Janke, T.B. Kang, T. Ben-Moshe, T.W. Mak, D. Wallach, D.R. Green, Survival function of the FADD-CASPASE-8-cFLIP(L) complex, Cell Rep. 1 (5) (2012) 401–407.
[56] M. Fritsch, S.D. Günther, R. Schwarzer, M.C. Albert, F. Schorn, J.P. Werthenbach, L.M. Schiffmann, N. Stair, H. Stocks, J.M. Seeger, M. Lamkanfi, M. Krönke, M. Pasparakis, H. Kashkar, Caspase-8 is the molecular switch for apoptosis, necroptosis and pyroptosis, Nature 575 (7784) (2019) 683–687.
[57] K. Newton, D.L. Dugger, K.E. Wickliffe, N. Kapoor, M.C. de Almagro, D. Vucic, L. Komuves, R.E. Ferrando, D.M. French, J. Webster, M. Roose-Girma, S. Warming, V.M. Dixit, Activity of protein kinase RIPK3 determines whether cells die by necroptosis or apoptosis, Science 343 (6177) (2014) 1357–1360.
[58] S. Zhu, Y. Zhang, G. Bai, H. Li, Necrostatin-1 ameliorates symptoms in R6/2 transgenic mouse model of Huntington's disease, Cell Death Dis. 2 (1) (2011), e115.
[59] D.B. Re, V. Le Verche, C. Yu, M.W. Amoroso, K.A. Politi, S. Phani, B. Ikiz, L. Hoffmann, M. Koolen, T. Nagata, D. Papadimitriou, P. Nagy, H. Mitsumoto, S. Kariya, H. Wichterle, C.E. Henderson, S. Przedborski, Necroptosis drives motor neuron death in models of both sporadic and familial ALS, Neuron 81 (5) (2014) 1001–1008.
[60] M. Gonzalez-Zuñiga, P.S. Contreras, L.D. Estrada, D. Chamorro, A. Villagra, S. Zanlungo, E. Seto, A.R. Alvarez, c-Abl stabilizes HDAC2 levels by tyrosine phosphorylation repressing neuronal gene expression in Alzheimer's disease, Mol. Cell 56 (1) (2014) 163–173.
[61] K. Weber, R. Roelandt, I. Bruggeman, Y. Estornes, P. Vandenabeele, Nuclear RIPK3 and MLKL contribute to cytosolic necrosome formation and necroptosis, Commun. Biol. 1 (2018) 6.
[62] J.Y. Wang, Regulation of cell death by the Abl tyrosine kinase, Oncogene 19 (49) (2000) 5643–5650.
[63] D. Cilloni, G. Saglio, Molecular pathways: BCR-ABL, Clin. Cancer Res. 18 (4) (2012) 930–937.
[64] F. Pagan, M. Hebron, E.H. Valadez, Y. Torres-Yaghi, X. Huang, R.R. Mills, B. M. Wilmarth, H. Howard, C. Dunn, A. Carlson, A. Lawler, S.L. Rogers, R. A. Falconer, J. Ahn, Z. Li, C. Moussa, Nilotinib effects in Parkinson's disease and dementia with Lewy bodies, J. Parkinsons Dis. 6 (3) (2016) 503–517.
[65] F.L. Pagan, M.L. Hebron, B. Wilmarth, Y. Torres-Yaghi, A. Lawler, E.E. Mundel, N. Yusuf, N.J. Starr, J. Arellano, H.H. Howard, M. Peyton, S. Matar, X. Liu, A. J. Fowler, S.L. Schwartz, J. Ahn, C. Moussa, Pharmacokinetics and pharmacodynamics of a single dose Nilotinib in individuals with Parkinson's disease, Pharmacol. Res. Perspect. 7 (2) (2019), e00470.
[66] A. Elkouzi, V. Vedam-Mai, R.S. Eisinger, M.S. Okun, Emerging therapies in Parkinson disease - repurposed drugs and new approaches, Nat. Rev. Neurol. 15 (4) (2019) 204–223.
[67] T. Guttuso Jr., K.L. Andrzejewski, D.G. Lichter, J.K. Andersen, Targeting kinases in Parkinson's disease: a mechanism shared by LRRK2, neurotrophins, exenatide, urate, nilotinib and lithium, J. Neurol. Sci. 402 (2019) 121–130.
[68] S.S. Karuppagounder, S. Brahmachari, Y. Lee, V.L. Dawson, T.M. Dawson, H.S. Ko, The c-Abl inhibitor, nilotinib, protects dopaminergic neurons in a preclinical animal model of Parkinson's disease, Sci. Rep. 4 (2014) 4874.
[69] F.L. Pagan, M.L. Hebron, B. Wilmarth, Y. Torres-Yaghi, A. Lawler, E.E. Mundel, N. Yusuf, N.J. Starr, M. Anjum, J. Arellano, H.H. Howard, W. Shi, S. Mulki, T. Kurd-Misto, S. Matar, X. Liu, J. Ahn, C. Moussa, Nilotinib effects on safety, tolerability, and potential biomarkers in Parkinson disease: a phase 2 randomized clinical trial, JAMA Neurol. 77 (3) (2020) 309–317.
[70] M.E. Gorre, K. Ellwood-Yen, G. Chiosis, N. Rosen, C.L. Sawyers, BCR-ABL point mutants isolated from patients with imatinib mesylate-resistant chronic myeloid leukemia remain sensitive to inhibitors of the BCR-ABL chaperone heat shock protein 90, Blood 100 (8) (2002) 3041–3044.
[71] M.J. Yañez, T. Marín, E. Balboa, A.D. Klein, A.R. Alvarez, S. Zanlungo, Finding pathogenic commonalities between Niemann-Pick type C and other lysosomal storage disorders: opportunities for shared therapeutic interventions, Biochim. Biophys. Acta Mol. basis Dis. 165875 (2020).