CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS
AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

UNIDAD ZACATENCO
DEPARTMENTO DE BIOMEDICINA MOLECULAR

“Efecto de la inhibición de la fosforilación y desacetilación de cortactina en la migración transendotelial, homing e infiltración extramedular en leucemia linfoblástica aguda de células B”

TESIS

Que presenta

Karina Elizabeth Jiménez Camacho

Para obtener el grado de

MAESTRA EN CIENCIAS

EN LA ESPECIALIDAD DE

BIOMEDICINA MOLECULAR

Directores de Tesis:

Dr. Michael Schnoor

Dr. Rosana Pelayo

Ciudad de México **Agosto 2019**

RESEARCH CENTER AND ADVANCED STUDIES OF
THE NATIONAL POLYTECHNIC INSTITUTE

ZACATENCO UNIT
DEPARTMENT OF MOLECULAR BIOMEDICINE

"Effect of inhibition of phosphorylation and deacetylation of cortactin in transendothelial migration, homing and extramedullary infiltration in B-cell acute lymphoblastic leukemia"

THESIS

Presented by

Karina Elizabeth Jiménez Camacho

To obtain the degree of

MASTER IN SCIENCES

IN THE SPECIALTY OF

MOLECULAR BIOMEDICINE

Thesis Directors:

Dr. Michael Schnoor

Dr. Rosana Pelayo

Mexico City **August 2019**

AGRADECIMIENTOS

Al Consejo Nacional de Ciencia y Tecnología (CONACYT), por la beca otorgada durante el periodo 2017-2019.

Al Dr. Michael Schnoor por aceptarme en su equipo y por su guía y apoyo durante el desarrollo de este proyecto.

A mi comité: Dra. Rosana Pelayo, Dr. Vianney Ortiz, Dr. Leopoldo Santos y Dra. Elisa Dorantes, por su tiempo, observaciones y recomendaciones.

A mis queridos compañeros de laboratorio: próximo M. en C. Armando Montoya (por ser mi mejor amigo desde que nos conocimos en el curso propedéutico y ser mi siamés), M. en C. Ramón Castellanos (por enseñarme y ser mi principal guía en este proyecto), próxima Dra. Sandra Chánez , M. en C. Idaira Guerrero, M. en C. Karla Castro, Dra. Hilda Vargas, por su apoyo y por hacer del laboratorio un lugar tan agradable.

A quienes conocí en esta maestría, principalmente a: los próximos M. en C. Alejandra Montañez, Ramón Muñóz y Víctor Sosa; así como también a Claudia Hidalgo, por su gran amistad y apoyo en todo momento.

A mis mejores amigos de La Paz en CDMX: Geovanni Ruiz, Dayan Carrión y Marcos Quiroz, porque tenerlos cerca es lo mejor.

A mis mejores amigas de siempre: Karla García y Karla Lau, por su bonita amistad y apoyo incondicional aún en la distancia.

A mis abuelitas: Chuyita y Veva, a mi abuelito Cuate y a mi querida familia, porque siempre tienen las palabras de amor y de apoyo adecuadas para alentarme a seguir creciendo profesionalmente aunque no esté en casa.

A mi abuelito Capi en el cielo, porque sin duda su recuerdo me mantiene cerca de él y siguiendo su consejo de permanecer centrada y enfocada en mis objetivos profesionales.

To my loving and supportive boyfriend, Sanyog Dwivedi, for giving me peace when I needed the most, for his patience and understanding throughout the time we have been together.

DEDICATORIA

A mi mami, Rossy Camacho, porque a pesar de lo difícil que es no estar juntas, siempre me ha apoyado y alentado a seguir adelante en este camino de la investigación.

A mi papi, José Jiménez, porque cada día me brinda palabras de apoyo y me alienta a seguir haciendo lo que me gusta aunque tenga que hacer sacrificios.

Gracias infinitas por todo lo que hacen por mí.

Los amo para siempre.

INDEX

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ABP	Actin binding protein
AL	Acute leukemia
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AURKA	Oncogenic serine/threonine kinase Aurora A
B-ALL	B-precursor cell acute lymphoblastic leukemia
BCR	B-cell receptor
BBB	Blood-brain-barrier
BGG	Bovine γ-globulin
BM	Bone marrow
CAM-DR	Cell adhesion-mediated drug resistance
CAR	CXCL12-abundant reticular
CLL	Chronic lymphoid leukemia
CNS	Central nervous system
CSC	Cancer stem cells
CSF	Cerebral spinal fluid
Cttn	Cortactin
CXCL12	C-X-C motif chemokine 12 or SDF1
CXCR4	C-X-C chemokine receptor type 4 (CXCR-4)
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF-R	Epidermal growth factor receptor
ERK	Extracellular signal-regulated
EX-527	6-Chloro-2,3,4,9-tetrahydro-1H-Carbazole-1-carboxamide
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FGF-R	Fibroblast growth factor receptor

FISH	Fluorescence in situ Hybridization
GM-CSF	Granulocyte colony stimulating factor
GPCR	G-protein coupled receptor
HAT	Histone acetylases
HDAC6	Histone deacetylase 6
HDACS	Histone deacetylases
HIF1-α	Hypoxia-inducible factor 1α
HNSCC	Head and neck squamous cell carcinoma
HPC	Hematopoietic progenitor cells
HSC	Hematopoietic stem cells
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular Adhesion Molecule 1
IL	Interleukin
IP3	Phosphatidylinositol 3
JAK	Janus kinase
KD	Knock down
LFA-1	Lymphocyte function-associated antigen 1
LICs	Leukemia-initiating cells
MFI	Mean fluorescence intensity
MMP-9	Matrix metalloprotease-9
MRD	Minimal residual disease
MSC	Mesenchymal stromal cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NEDD9	Neural precursor cell expressed, developmentally downregulated 9
NFκB	Nuclear factor-kappa B
NPF	Nucleation promoting factor
NTA	N-terminal acidic
N-WASP	Neuronal-Wiskott–Aldrich Syndrome protein
p38MAPK	p38 mitogen-activated protein kinase
PAGE	Polyacrylamide gel electrophoresis

PAK1	p21 activated kinase 1
PCAF	P300/CBP-associated factor
PDGFR	Platelet-derived growth factor receptor
PFA	Paraformaldehyde
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PKD	Protein kinase D
PLC	Phospholipase C
PP2	4-Amino-3-(4-chlorophenyl)-1-(t-butyl)-1Hpyrazolo[3,4-d]pyrimidine,4-Amino-5-(4chloro-phenyl)-7-(t-butyl)pyrazolo[3,4d]pyrimidine
PSGL-1	P-selectin glycoprotein ligand-1
PST	Proline-serine-threonine
PTM	Post-translational modification
RTK	Receptor tyrosine kinases
S1P	Sphingosine-1-phosphate
SCF	Stromal cell factor
SDF1	Stromal derived-factor 1
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SH3	Src-homology-3
SIRT1	Sirtuin-1
STAT	Signal transducer and activator of transcription
SV	Splice variant
TEM	Transendothelial migration
THPO	Thrombopoietin
TNF	Tumor necrosis factor
VCAM-1	Vascular adhesion molecule-1
VE	Vascular endothelial
VEGF	Vascular endothelial growth factor
VLA-4	Very Late Antigen-4 or integrin $\alpha_4\beta_1$
WIP	WASP-interacting protein

1. RESUMEN

La leucemia linfoblástica aguda de células B (LLA-B) es el tipo de leucemia más común en niños. Las células B leucémicas tienen la capacidad de extravasarse e infiltrar la médula ósea y órganos como testículos y sistema nervioso central (SNC). Este proceso migratorio depende del remodelamiento del citoesqueleto de actina. Cortactina es una proteína de unión a actina que se acumula en lamelipodios e invadopodios y regula la migración transendotelial de neutrófilos. Cortactina se encuentra sobreexpresada en células B de pacientes con LLA-B y correlaciona significativamente con colonización de médula ósea, infiltración de órganos, resistencia al tratamiento y recaída. Cortactina es blanco de diversas modificaciones post-traduccionales como fosforilación y acetilación, siendo sustrato de la cinasa Src y de la desacetilasas SIRT1. La fosforilación de cortactina en Y421 favorece la formación de lamelipodios e invadopodios, mientras que la acetilación disminuye su afinidad por F-actina y la capacidad de migración celular. En este trabajo, investigamos si la inhibición farmacológica de Src y SIRT1 en células B leucémicas reduce la migración transendotelial y la colonización de la médula ósea. De manera importante, encontramos un nivel de fosforilación de cortactina (Y421) bajo en la línea celular REH en condición basal, el cual se incrementó después del estímulo con CXCL12. Bajo condiciones de cultivo libres de suero fetal bovino (SFB), se observó el mismo nivel de fosfo-cortactina comparado con condiciones con SFB. PP2 y EX-527 son inhibidores de la cinasa Src y la desacetilasa SIRT1, respectivamente, y no causan muerte celular significativa después de 24 horas de tratamiento. La inhibición de la cinasa Src con PP2 en las células leucémicas disminuyó los niveles de fosfo-cortactina y afectó su capacidad de transmigración y colonización de médula ósea, lo cual también se observó después de la inhibición de SIRT1 con EX-527 al incrementar la acetilación de cortactina. Estos hallazgos resaltan el papel importante de las modificaciones post-traduccionales en las células B leucémicas y podrían contribuir a un mejor entendimiento de los mecanismos de la enfermedad, siendo útil como un potencial blanco terapéutico.

1. ABSTRACT

B-cell acute lymphoblastic leukemia (B-ALL) is the most common leukemia in children. B-ALL cells have the ability to extravasate and infiltrate bone marrow and organs such as lungs, testis and central nervous system. Dynamic actin cytoskeletal remodeling is needed to accomplish transendothelial migration. Cortactin is an actin-binding protein which accumulates in lamellipodia and invadopodia and regulates neutrophil transendothelial migration during inflammation. Cortactin is overexpressed in B-cells of B-ALL patients, and significantly correlates with bone marrow colonization, organ infiltration, drug resistance and disease relapse. Cortactin is a target for several posttranslational modifications such as phosphorylation and acetylation, being a substrate of Src kinase, and the deacetylase SIRT1. Phosphorylation of cortactin at residue Y421 supports lamellipodia and invadopodia formation, whereas acetylation of cortactin diminishes its affinity for F-actin and decreases cell migration. In this work, we investigated whether pharmacological inhibition of Src and SIRT1 in leukemic B-cells reduce transendothelial migration and bone marrow colonization. Importantly, we found a low phosphorylation level of cortactin (Y421) in pre-B ALL cell line REH at basal condition, which was increased after CXCL12 stimulation. PP2 and EX-527 inhibit Src kinase and SIRT1 deacetylase, respectively, and did not cause significant cell death at 50 μM after 24 hours of treatment. Of note, Src kinase inhibition with PP2 in leukemic B cells reduced phospho-cortactin levels and impaired leukemic cell transmigration and BM organoid colonization, an effect that was also observed after SIRT1 inhibition with EX-527. Taken together, these findings highlight the important role of post-translational modifications in leukemic B cells, which may contribute to a better understanding of the mechanisms implicated in disease progression and may be useful as a potential therapeutic target for B-ALL patients. In the future, we will analyze in detail whether cortactin is indeed the responsible Src and SIRT1 target that causes the observed effects.

2. INTRODUCTION

2.1 Childhood leukemia statistics

Childhood cancer comprises only a small proportion of the global cancer burden, but 84% of all childhood cancer cases arise in the low-income and middle-income countries. In 2018, GLOBOCAN estimated that about 230,000 new cancer cases in children aged 0–14 years occurred in these countries, including Latin America (GLOBOCAN, 2018). Due to the lack of cancer registries, these estimates are often the only unbiased source of incidence and mortality data. Besides many childhood cancers are not diagnosed, particularly leukemias and brain tumors as there is few data available in low- and middle-income countries (Magrath et al., 2013).

Acute leukemias (AL), especially acute lymphoblastic leukemias (ALL), are the most common childhood cancers throughout the world, accounting for about 30% of all cases. All has been reported to have a very elevated incidence worldwide varying between 20-35 cases per million for children under the age of 15 years (Magrath et al., 2013; Pérez-Saldivar et al., 2011). Although ALL is mainly considered a pediatric leukemia, 20% of cases occur in adults of around 50 years of age (Paul, Kantarjian, & Jabbour, 2016).

For Mexico City, the frequency of ALL is among the highest in the world (Pérez-Saldivar et al., 2011). In public hospitals, the immunophenotypes of children were determined for 113 pediatric AL cases: 26.5% corresponded to pro-B, 19.5% to pre-B and 32% contained pro-B and pre-B differentiation stages. Nearly 9% of the cases were T-ALL and 13% myeloid-lineage leukemias (Balandrán et al., 2016). Thus, B-lineage leukemias dominate within the lymphoid leukemia groups in children (Cobaleda & Sánchez-García, 2009).

2.2 B-cell Acute Lymphoblastic Leukemia

B-cell acute lymphoblastic leukemia (B-ALL) is a clonal malignant disease characterized by an uncontrolled production of hematopoietic B-precursor cells (blasts) within the bone marrow (BM) resulting in the suppression of normal

hematopoiesis (Cobaleda & Sánchez-García, 2009; Terwilliger & Abdul-Hay, 2017; Vilchis-Ordoñez et al., 2015).

The etiology of ALL is unknown. Less than 5% of cases can be attributed to genetic syndromes such as Down syndrome, Klinefelter syndrome, and Fanconi anemia, among others. Additional predisposing factors comprise exposure to ionizing radiation, pesticides, certain solvents or viruses such as Epstein-Barr Virus and Human Immunodeficiency Virus (Paul et al., 2016; Terwilliger & Abdul-Hay, 2017). The fetal environment is thought to play a vital role in the development of pediatric ALL, with the hypothesis that as cells proliferate during fetal development, random alterations occur creating a preleukemic clone (M. Greaves, 2018; Paul et al., 2016). In a small fraction of cases, the postnatal acquisition of secondary genetic changes drives conversion to leukemia. Microbial exposures earlier in life are protective but, in their absence, later infections trigger the critical secondary mutations. Hence, the causal mechanism is suggested to be a multifactorial mix of infectious exposure, inherited or constitutive genetics and chance, with patterns or timing of common infection in early life identified as the critical component (M. Greaves, 2018).

In the majority of cases, B-ALL appears as a *de novo* malignancy in previously healthy individuals, involving a large variety of genetic alterations: chromosome abnormalities, such as hyperdiploidy or translocations, point mutations and deletions (Cobaleda & Sánchez-García, 2009; M. F. Greaves & Wiemels, 2003; Terwilliger & Abdul-Hay, 2017). These oncogenic conversions could interfere with the networks controlling B-cell differentiation. For example, B-cell blasts might lose responsiveness to external cues that regulate normal differentiation; could acquire the capacity for unlimited self-renewal; or may activate pathways that mimic a particular stage of its normal differentiation (Cobaleda & Sánchez-García, 2009; Pui, Robison, & Look, 2008).

2.2.1 Clinical manifestations and diagnosis of B-ALL

Most of the clinical manifestations of B-ALL reflect the accumulation of malignant, poorly differentiated lymphoid cells within the bone marrow, peripheral blood and, extramedullary sites. Patients can present a range of ailments such as “B symptoms” (fever, weight loss, night sweats), infection, easy bruising/bleeding, dyspnea, and fatigue due to low blood cell counts. Also, patients may have leukemic infiltration in the central nervous system (CNS), testis, and liver, among other organs (Loghavi, Kutok, & Jorgensen, 2015; Paul et al., 2016; Terwilliger & Abdul-Hay, 2017).

Diagnosis is established by the presence of 20% or more lymphoblasts in the bone marrow or peripheral blood. Evaluation for morphology, flow cytometry, immunophenotyping and cytogenetic testing is valuable both for confirming the diagnosis and risk stratification (Paul et al., 2016; Terwilliger & Abdul-Hay, 2017). Morphologically, lymphoblasts are mainly of small size, with central and round nuclei, and very condensed chromatin. Immunophenotyping by flow cytometry analysis represents the diagnostic gold standard, where the most important markers for diagnosis of B-ALL are CD19, CD20, CD22, CD24, CD34 and CD79a. The earliest B-lineage markers are CD19, CD22 (membrane and cytoplasm) and CD79a. A positive reaction for any two of these three markers, identifies pro-B ALL. Cases with additional identification of cytoplasmic heavy μ chain comprise the pre-B group, while the presence of surface immunoglobulin light chains defines mature B-ALL. Cytogenetics represents an important step, where FISH (Fluorescence in situ Hybridization) karyotyping can be helpful in the identification of recurrent translocations (Chiaretti, Zini, & Bassan, 2014). With this technique, DNA probes are attached to fluorescent reporter molecules which under fluorescence microscopy confirm the presence or absence of a particular chromosomal aberration (Ryan Bishop, 2010).

2.2.2 Treatment

Treatment of B-ALL typically consists of an induction phase, a consolidation phase, and maintenance therapy to eliminate residual disease, with CNS prophylaxis given at intervals throughout therapy (Paul et al., 2016; Pui et al., 2008; Terwilliger & Abdul-Hay, 2017). The goal of induction therapy is to achieve complete remission and to restore normal hematopoiesis, where drugs like vincristine, anthracyclines such as daunorubicin or doxorubicin and corticosteroids (eg, prednisone or dexamethasone) are used. Then, consolidation treatment with similar drugs is applied to eradicate drug-resistant residual leukemic cells, thus reducing the risk of relapse. Finally, the maintenance therapy consists of daily 6-mercaptopurine, weekly methotrexate, monthly vincristine, and monthly pulses of prednisone administered for a period of 2 to 3 years to prevent relapse and prolong remission (Paul et al., 2016; Pui et al., 2008).

2.2.3 Relapse

Although the majority of patients with ALL achieve complete remission, approximately 20% are refractory to induction therapy or suffer relapse (Terwilliger & Abdul-Hay, 2017). Most of relapses occur during treatment or within the first 2 years after treatment achievement, although can also occur after 10 years from diagnosis (Gaynon, 2005). In contrast to the improved outcome of patients with newly diagnosed ALL, little progress has been made in the treatment of relapsed ALL, as leukemic blasts at relapse are more drug resistant than blasts at initial diagnosis. Of note, standard regimens for relapsed ALL are still mostly based on different combinations of the same agents used in frontline therapy in various doses and schedules(Locatelli, Schrappe, Bernardo, & Rutella, 2012). Therefore, development of strategies for accelerating the evaluation and clinical development of novel agents is a priority.

Overall survival is influenced by the site of relapse and duration of first complete remission. Isolated BM relapse carries the worst prognosis, with isolated CNS, isolated testicular or other extramedullary relapse having a better prognosis, and combined

BM and extramedullary relapse being associated with an intermediate prognosis (Bailey, Lange, Rheingold, & Bunin, 2008).

Most of relapses occur in the BM, in an isolated form or combined with involvement of another site as CNS or testes. Relapse isolated in the CNS or testicular is much less frequently (Locatelli et al., 2012). Normally, leukemic cells are found within the interstitial space where methotrexate concentrations from treatment are lower than those in the serum. Leukemic cells may enter the CNS from the BM of the skull into the subarachnoid space via the bridging veins or the cerebrospinal fluid (CSF) via the choroid plexus, invade cerebral parenchyma via brain capillaries, or infiltrate the leptomeninges via bony lesions of the skull (Pui & Jeha, 2007).

Leukemia relapse represents the outgrowth of a clonal cell population not completely eliminated by treatment (Locatelli et al., 2012). By studying DNA copy-number alterations and by lesion-specific backtracking studies in 61 patients, the cells responsible for relapse in most patients were identified as ancestral to the primary leukemia cells; and that the relapse clones were usually present as minor subpopulations at first diagnosis. This population then expanded during treatment, acquiring additional genomic lesions and thus becoming more aggressive (Bhojwani & Pui, 2013; Mullighan et al., 2008). Additionally, a rare, long-term subpopulation exhibiting the characteristics of dormancy, drug resistance and leukemia-initiating properties has been identified recently (Ebinger et al., 2016). Upon removal from their *in vivo* microenvironment, this population lost dormancy and drug resistance, suggesting a reversible nature of these characteristics and an important role for the interaction between B-ALL cells and the protective niche in the bone marrow for their survival and proliferation (Ebinger et al., 2016; Pramanik, Sheng, Ichihara, Heisterkamp, & Mittelman, 2013). Furthermore, these leukemia-initiating cells (LICs) are able to self-renew and generate more LICs (Chiarini et al., 2016).

2.3 Bone marrow niches

The hematopoietic microenvironment is a network of cells (mesenchymal cells, osteoblasts, fibroblasts, adipocytes, macrophages, endothelial cells) and their products (extracellular matrix molecules, cytokines and chemokines) that form a highly organized three-dimensional scaffold within the bone marrow. Studies in mice have identified at least three types of nonhematopoietic cells as candidate hematopoietic stem cells (HSC) niches (Figure 1), including osteoblasts lining the bone surface (endosteal niche), endothelial cells (vascular niche) and a population of reticular cells (reticular niche) (Purizaca, Meza, & Pelayo, 2012).

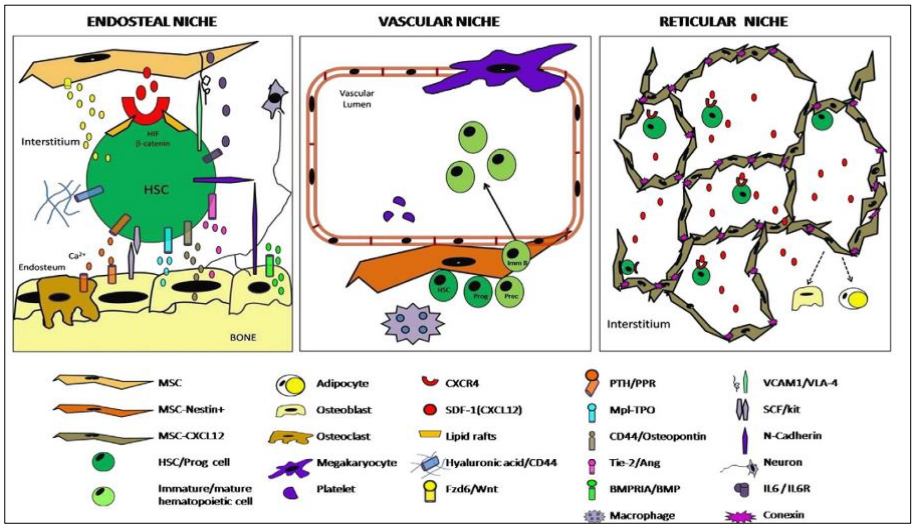

Figure 1. **Organization of bone marrow niches.** The endosteal niche is comprised of osteoblasts lining the bone surface, where microenvironment-HSC interactions promote maintenance of the HSC pool. The vascular niche involves endothelial cells expressing adhesion molecules to regulate HSC self-renewal, homing and mobility. The main component of the reticular niche is a population of reticular/mesenchymal cells, which provide the cues for HSC, lymphoid and erythroid progenitor cell proliferation and differentiation (Purizaca et al., 2012).

The endosteal niche is mostly conformed by osteoblasts within a hypoxic zone, and its role is sustaining the earliest events of hematopoiesis. Osteoblasts produce CXCL12, osteopontin and N-cadherin involved in HSC retention and maintenance. Signaling pathways induced by the niche, including CXCL12/CXCR4, Notch, and Wnt, are implicated in the regulation of cell cycling activity and self-renewal (Chiarini et al.,

2016; Purizaca et al., 2012). The vascular niche contains a network of blood vessels called vascular sinuses with sinusoidal endothelial cells as the major components and hematopoietic cells located in the extravascular spaces between the vascular sinuses. The high production of vascular endothelial growth factor (VEGF) and the expression of the adhesion molecules E-selectin, P-selectin, VCAM1, ICAM1, PECAM1 and vascular endothelial (VE)-cadherin by endothelial cells contribute to maintain HSC and hematopoietic progenitor cells (HPC) within this niche and regulate self-renewal, homing and mobility (Doan & Chute, 2012; Smith & Calvi, 2013; Suárez-Álvarez, López-Vázquez, & López-Larrea, 2012). In the reticular niche, nestin expressing cells and CAR (CXCL12-abundant reticular) cells are present (Sugiyama, Kohara, Noda, & Nagasawa, 2006). CAR cells are adipo-osteogenic progenitors that are required for HSC proliferation and for maintaining an undifferentiated state (Omatsu et al., 2010).

The bone marrow microenvironment plays an important role in the regulation of the growth, survival, and differentiation of normal and leukemic hematopoietic progenitors. It is thought that leukemic cells take over and destroy HSC-supportive microenvironments, changing the equilibrium from a state that supports steady-state hematopoiesis to one that instead leads to accelerated expansion of immature leukemic cells (Duarte, Hawkins, & Lo Celso, 2018). These competitions have been suggested to cause the exhaustion or migration of normal precursors, resulting in an almost total replacement by malignant cells. Hematopoietic precursors in childhood B-ALL have been shown to produce proinflammatory cytokines including TNFα, IL-1β, IL-12, and GM-CSF supporting the idea that malignant cells contribute to the pathogenesis by creating a pro-inflammatory microenvironment within the BM. This pro-inflammatory property is apparently mediated by the hyperactivation of NFκB and STAT3 pathways, maintaining the consecutive secretion of these proinflammatory cytokines (Balandrán et al., 2017a; Vilchis-Ordoñez et al., 2015), and inducing the disruption of the CXCR4/CXCL12 axis, crucial for maintenance of the proper hematopoietic cells niches (Figure 2) (Balandrán et al., 2017a).

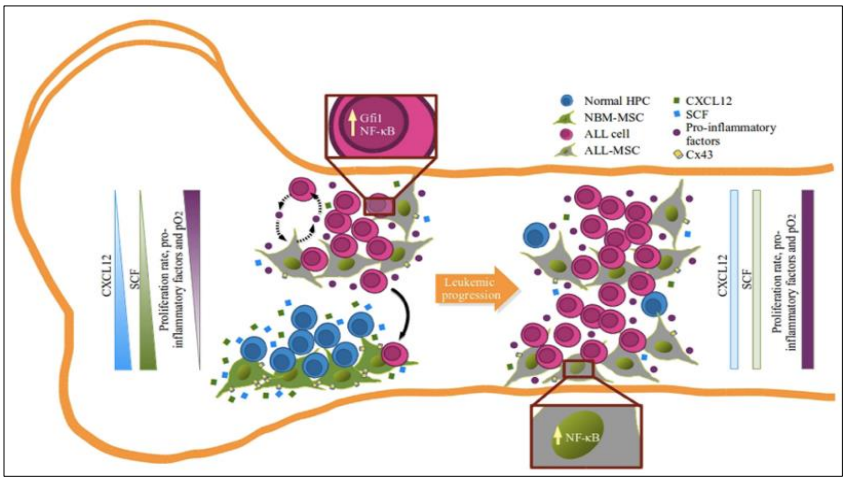

Figure 2. **Remodeling of the bone marrow microenvironment.** Hematopoietic niches supporting normal lymphopoiesis are formed by mesenchymal stromal cells (MSC) producing high levels of CXCL12 and SCF. During leukemogenesis, leukemic clones take over normal niches and induce reduction of CXCL12 expression in response to activation of MSC by pro-inflammatory cytokines that allow leukemic cell proliferation and suppression of normal lymphopoiesis (Balandrán et al., 2017a).

2.4 CXCL12/CXCR4 axis

Chemokines can induce distinct signaling pathways that mediate cell growth, transcriptional activation, as well as cell motility (Spiegel et al., 2008). The CXCL12/CXCR4 axis is involved in development and maintenance of healthy tissues and organs, however, it also appears to play an important role in hematopoietic disorders including ALL (Chiarini et al., 2016).

CXCL12 is a chemokine constitutively produced by many cell types, including immature osteoblasts, endothelial cells and stromal cells within the BM as well as by epithelial cells in other extramedullary tissues such as the lymph nodes, liver, spleen, thymus, lung, kidney and brain in mouse and human (Crazzolara et al., 2001; Müller et al., 2001). One of the primary physiologic receptors for CXCL12 is CXCR4, a G-protein coupled receptor (Nagasawa et al., 1996).

CXCL12 acts as a chemoattractant for a number of hematopoietic cell types expressing CXCR4 including normal $CD34^{+}$-hematopoietic progenitors, megakaryocytes, dendritic

cells, monocytes, and B and T lymphocytes. Moreover, it has been demonstrated that CXCR4 is expressed at high levels on all pre-B leukemic cells and related cell lines such as REH and Nalm6, being extremely responsive to CXCL12 (Chiarini et al., 2016; Jo, Rafii, Hamada, & Moore, 2000; Pramanik et al., 2013; Shen, Bendall, Gottlieb, & Bradstock, 2001).

In particular, CXCL12 causes multiple intracellular changes. After CXCL12 binding to CXCR4, various signaling pathways including Janus kinase/signal transducer and activator of transcription (JAK/ STAT), Src kinase, p38 mitogen-activated protein kinase (p38MAPK), MEK/ERK, phosphatidylinositol 3-kinase (PI3K)/Akt, and protein kinase C (PKC) are activated. These result in a plethora of responses such as increase in intracellular calcium, gene transcription, cell survival, proliferation, chemotaxis and cell migration (Chatterjee, Behnam Azad, & Nimmagadda, 2014; Cheng et al., 2017; Chiarini et al., 2016; Ganju et al., 1998; Perim, Amarante, Guembarovski, De Oliveira, & Watanabe, 2015a). Both HSC and B cell progenitors shared a dependence on PI3K signaling, while B-ALL leukemic cells demonstrated a minor involvement of this pathway with major signaling through p38 mitogen-activated protein kinase (p38MAPK). Yet, it needs to further investigated which pathways are the most important for migration and infiltration among the ALL subtypes (Perim, Amarante, Guembarovski, De Oliveira, & Watanabe, 2015b).

The majority of the signals that mediate interactions between leukemic cells and the BM niches, are similar to those of HSCs. However, leukemic cells are different from HSCs in their aberrant activation of key signaling pathways that control survival, proliferation, drug-resistance, and ability to invade and spread (Chiarini et al., 2016). High expression of CXCR4 by leukemic cells and hyperactivation of the CXCL12/CXCR4 axis is involved in leukemia progression, contributing to bone marrow infiltration, residence within the BM and disruption of normal hematopoiesis (Ayala, Dewar, Kieran, & Kalluri, 2009; Duarte et al., 2018).

Given the fact that CXCL12 is constitutively produced by stromal and CAR cells within the bone marrow, a chemoattractant gradient plays a role in the migration of circulating hematopoietic cells to the bone marrow during a process termed homing (Mohle et al., 1998). As CXCL12 is not only produced by stromal cells within the bone marrow, but also in extramedullary tissues, the CXCL12/CXCR4 axis stimulates integrin-mediated adhesion of circulating cells on the vascular endothelium, thus promoting tissue infiltration by leukemic blasts overexpressing CXCR4 (Chiarini et al., 2016; Crazzolara et al., 2001; Spiegel et al., 2008).

2.5 Leukemic cells infiltration

Since a primary role of lymphocytes is immune surveillance, it is not be surprising to find that leukemic cells migrating throughout the body and finding them in a variety of tissues (Pramanik et al., 2013). Although the mechanisms driving this phenomenon are not well understood, transendothelial migration as also executed by normal leukocytes is considered essential for leukemic cell infiltration of extramedullary organs (Gossai & Gordon, 2017; Pramanik et al., 2013).

B-ALL cells have the ability to infiltrate liver, kidney, lung, testes, spleen, lymph nodes and the central nervous system (CNS) (Crazzolara et al., 2001; Gossai & Gordon, 2017; Nies, Bodey, Thomas, Brecher, & Freireich, 1965; Spiegel et al., 2008). Despite bone marrow replacement being the major cause of leukemia symptoms, other important B-ALL syndromes result from extramedullary invasion, such as liver or spleen enlargement (Crazzolara et al., 2001). Moreover, the microenvironment of organs that are infiltrated by leukemic cells, plays a critical role in disease progression (Chiarini et al., 2016), as it is suggested that ALL cells migration into adipose tissue protects them against vincristine and daunorubicin (Pramanik et al., 2013).

2.6 Homing and transendothelial migration

Migration of hematopoietic cells from the blood back to the bone marrow niche is known as “homing”, which follows similar steps of leukocytes transendothelial migration of other leukocytes to sites of inflammation (Heazlewood, Oteiza, Cao, & Nilsson, 2014). Inflammation induces surface expression of endothelial adhesion molecules, actin remodeling, and activation of leukocyte integrins that enable leukocyte adhesion onto the endothelium lining the vascular wall and subsequent transendothelial migration (diapedesis). The sequence of adhesive interactions of leukocytes with endothelial cells is termed leukocyte extravasation cascade and involves a series of adhesive interactions including tethering, rolling, slow rolling, firm adhesion, crawling, and transmigratory cup formation on the apical endothelial surface (Figure 3) (Schnoor, Alcaide, Voisin, & Van Buul, 2015; Vestweber, 2015).

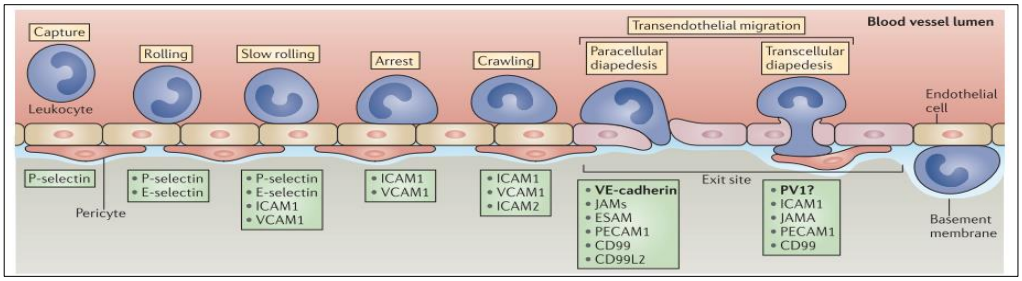

Figure 3. **Leukocyte transendothelial migration.** Cell adhesion receptors on endothelial cells mediate the different adhesive interactions of leukocytes with the luminal endothelial surface to accomplish transmigration through the endothelial barrier, which can occur via the paracellular or transcellular route (Vestweber, 2015).

For homing to the bone marrow (Figure 4), endothelial selectins (P- and E-selectin), as well as VCAM-1 are constitutively expressed by microvessels within the BM, and interactions with their HSPC-expressed counter-receptors, PSGL-1 (P-selectin glycoprotein ligand) and VLA-4 ($\alpha_4\beta_1$ integrin) support HSPC tethering, rolling and firm adhesion (Mazo, Massberg, & von Andrian, 2011). The interaction between CXCR4 expressed by HSPC and CXCL12 in the BM is a critical chemoattractant mechanism (Heazlewood et al., 2014), which induces activation of VLA-4 and LFA-1 to interact

with their ligands VCAM-1 and ICAM-1, respectively, thus leading to firm adhesion. CXCR4 induces downstream signaling, resulting in phospholipase C (PLC) activation, increased intracellular Ca^{2+}, and activation of small GTPases such as Rap1 and Rho-A driving actin remodeling required for cell adhesion and migration. Leukocytes then crawl on the endothelium until they find the ideal spot for transmigration (Nitzsche et al., 2017).

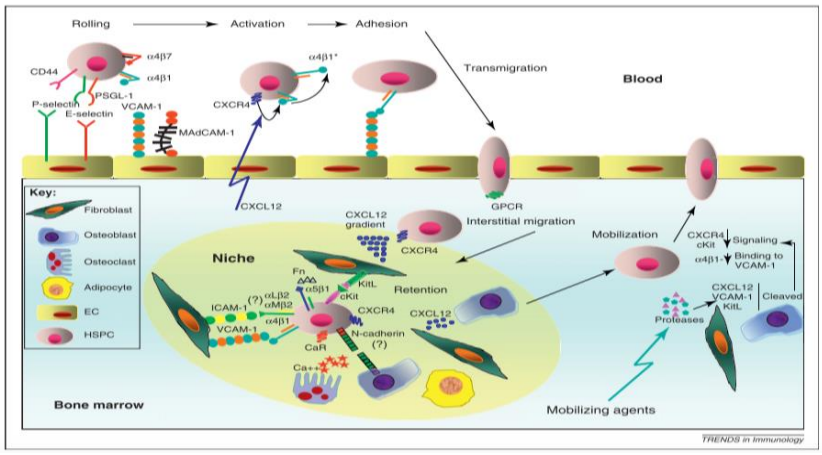

Figure 4. **HSPC homing to the bone marrow.** Endothelial cells express P- and E-selectin, which bind to PSGL-1 and CD44 on HSPC. Tethered cells then roll slowly by interacting with both endothelial selectins and the integrin $\alpha_4\beta_1$/VCAM-1 interactions. Both cell types are further activated by CXCL12 binding to CXCR4. The chemokine signal results in increased affinity for VCAM-1 mediating firm arrest followed by transmigration. Molecules involved in HSPCs retention within the bone marrow niche are also shown, such as $\alpha_4\beta_1$ with VCAM-1 and fibronectin, and β_2 integrins with ICAM-1, CXCR4 with CXCL12, and cKit with its ligand (KitL) (Mazo et al., 2011).

Diapedesis of any type of leukocyte can occur by crossing either endothelial cells contacts (paracellular) or the body of endothelial cells (transcellular). After crossing the endothelium, leukocytes additionally have to cross the pericyte layer and the basement membrane to conclude transmigration (Schnoor et al., 2015; Vestweber, 2015).

For all these steps of leukocyte transendothelial migration and homing, adhesive interactions and dynamic actin cytoskeletal remodeling in both transmigrating immune cells and endothelial cells are needed. Thus, actin-binding proteins (ABPs) such as cortactin play a central role in regulating the cellular movements involved during transmigration (Schnoor, 2015).

2.7 Cortactin: an Actin Binding Protein (ABP)

Human cortactin is encoded by the *cttn* gene within the chromosome region 11q13, a locus often amplified in various tumors that is associated with poor prognosis, invasion and metastasis (Cosen-Binker, 2006; Kirkbride, Sung, Sinha, & Weaver, 2011).

Cortactin is composed of around 550 amino acids and contains several key domains (Figure 5). The N-terminal half is responsible for coupling cortactin to structural elements of the cytoskeleton and includes the N-terminal acidic region (NTA), which harbors the DDW motif, the binding site for the Arp3 subunit of the Arp2/3 complex, followed by 6.5 tandem repeats of 37 amino acids responsible for F-actin binding. The C-terminal half is the regulatory segment of the protein, and is composed of an helical domain of unknown function, followed by a proline-serine-threonine-rich region (PST) and a Src homology 3 (SH3) domain, which mediates interactions with multiple actin cytoskeleton effectors or scaffolding proteins containing proline-rich SH3 interaction surfaces (Cosen-Binker, 2006; Schnoor, Stradal, & Rottner, 2017). All of these possible interactions indicate a diverse array of processes in which cortactin regulates cytoskeletal remodeling (Cosen-Binker, 2006; Lua & Low, 2005).

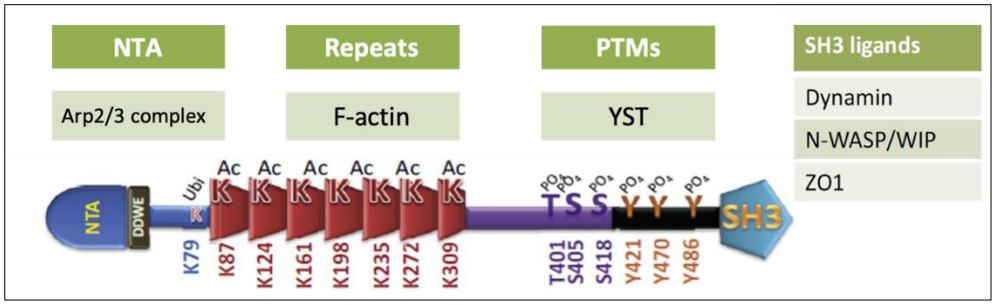

Figure 5. **Structural domains of cortactin.** At the N-terminus, the NTA domain interacts with and activates the Arp3 subunit of the Arp2/3 complex. Within the repeats region, the 4th repeat is essential for F-actin binding. At the C-terminus, the proline-rich domain contains phosphorylation sites for several kinases, and finally the SH3 domain, which mediates binding to many other proteins (Modified from Schnoor, Stradal, & Rottner, 2017).

Three cortactin isoforms exist with differently sized repeat regions, thus affecting its binding capacity to F-actin (Lua & Low, 2005). In addition to the WT isoform of 80 kDa, two major alternative splice variants have been identified: SV1-cortactin, lacking the

6th repeat (exon 11), and SV2-cortactin, lacking the 5th and 6th repeats (exon 10 and 11), generating proteins of 70 kDa and 60 kDa, respectively (van Rossum, Schuuring-Scholtes, van Buuren-van Seggelen, Kluin, & Schuuring, 2005).

Cortactin was first discovered as an 80/85 kDa substrate of Src kinase in the 1990s (Wu, Reynolds, Kanner, Vines, & Parsons, 1991). It was recognized as an ABP targeting actin structures in the cell cortex and as a molecule that links cytoskeletal organization with signal transduction (Cosen-Binker, 2006; Schnoor et al., 2017). Cortactin accumulates in actin-rich lamellipodia formed at the leading edge of migrating cells, and in invadopodia promoting the secretion of extracellular matrix (ECM)-degrading proteinases, crucial for tumor cell metastasis (Yin, Ma, & An, 2017). These structures are built by the actin branching activity of the Arp2/3 complex (Schnoor et al., 2017). F-actin is formed by reversible polymerization of globular (G)-actin providing the structural framework and mechanical force to induce morphological cell changes. Actin branching is driven by the Arp2/3 complex which is activated by nucleation promoting factors (NPFs), such as Neuronal-Wiskott–Aldrich Syndrome protein (N-WASP) that recruits G-actin monomer to the site of polymerization (Welch & Mullins, 2002). Cortactin acts as a type II NPF but is only a weak activator of Arp2/3. Instead, cortactin has recently been confirmed to rather stabilize newly formed branches (Cosen-Binker, 2006). The SH3 domain of cortactin may enhance actin nucleation via binding to the strong NPF WASP (Cosen-Binker, 2006; Yin et al., 2017). Thus cortactin associates with the “hot spots” of actin polymerization promoting nucleation, branching and stabilizing the newly formed branches (Cosen-Binker, 2006).

Cortactin also has an important role during transendothelial migration. In this process, endothelial cell ICAM-1 interacts with leukocyte integrins to facilitate leukocyte adhesion. ICAM-1 clustering and crosslinking on endothelial cells triggers Src phosphorylation of cortactin, which permits cortactin interaction with ICAM-1 engagement and cytoskeletal remodeling (Yang, Kowalski, Zhan, Thomas, & Luscinskas, 2006).

2.8 Post-translational modifications of cortactin

In response to signaling pathways including integrin- and cadherin-adhesion receptors and growth factor receptors, cortactin activity is modulated by several post-translational modifications (Kirkbride et al., 2011; MacGrath & Koleske, 2012).

The downstream kinases that phosphorylate cortactin include Src family kinases (Fer, Fyn, Syk and Src), tyrosine kinases (Abl and Arg), as well as serine/threonine kinases such as ERK, p21 activated kinase 1 (PAK1) and protein kinase D (PKD). Only some of the post-translational sites of cortactin have been associated with a specific function (Figure 6). Cortactin was first identified as a substrate of Src kinase, with the phosphorylation sites at tyrosines 421, 466, and 482 in mice (470 and 486 in human) within the proline-rich domain being main target residues. These modifications were originally proposed to activate the protein and induce cell migration (C. Huang, Liu, Haudenschild, & Zhan, 1998). Phosphorylation of these tyrosines is also accomplished by Abl family kinases, such as Abl or Arg, to induce dorsal membrane ruffling (Boyle, Michaud, Schweitzer, Predki, & Koleske, 2007).

S405 and S418 are phosphorylated by ERK to increase accessibility of the SH3 domain resulting in improved N-WASp binding to cortactin, which may account for increased cell motility and lamellipodial dynamics (Kirkbride et al., 2011). Likewise, phosphorylation of the same sites by PAK1 has been shown to increase N-WASp binding without affecting the Arp3- or actin-binding properties of cortactin (Kirkbride et al., 2011). PAK1 phosphorylates cortactin at S405 and S418 downstream of Rac1 and Cdc42, which is required for cortactin localization at the cell edge where it increases the association of cortactin with N-WASP (Grassart et al., 2010). PKD phosphorylates cortactin at S298, which increases cell migration due to activation of the Arp2/3 complex (Eiseler, Hausser, De Kimpe, Van Lint, & Pfizenmaier, 2010). Besides S298, PKD also phosphorylates S348 in invadopodia of breast cancer cells (De Kimpe et al., 2009).

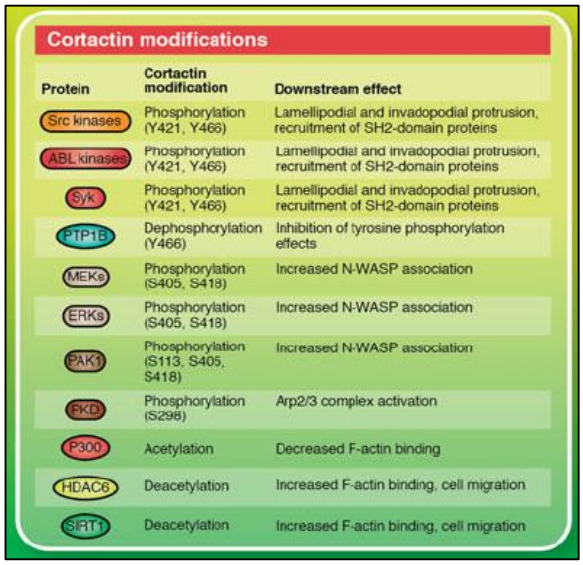

Figure 6. **Post-translational modifications of cortactin.** Some post-translational modifications have been associated with a specific downstream effect by altering cortactin interactions with other proteins (MacGrath & Koleske, 2012).

2.9 Acetylation and deacetylation of cortactin

In addition to phosphorylation, cortactin is also regulated through acetylation by histone acetyltransferases PCAF and p300, and deacetylation by histone deacetylase 6 (HDAC6) and sirtuin-1 (SIRT1) (Schnoor et al., 2017; Y. Zhang et al., 2009). Given that all the acetylation sites are within the tandem repeats region that regulates F-actin-binding and that acetylation neutralizes charged lysine residues, acetylation may diminish the affinity of cortactin to F-actin and decrease cell migration capability (Kaluza et al., 2011; Kirkbride et al., 2011; MacGrath & Koleske, 2012; Nakane et al., 2012; Schnoor et al., 2017; X. Zhang et al., 2007; Y. Zhang et al., 2009). Furthermore, only acetylated cortactin has been observed in the nucleus raising the question whether acetylated cortactin regulates transcription or other nuclear functions (Ito et al., 2015; Motonishi et al., 2015).

Reversible protein acetylation on the ε-amino group of a lysine residue is a key post-translational modification (PTM) which neutralizes the positive charge of this amino acid modifying protein function. This PTM is catalyzed by histone acetylases (HATs) and deacetylases (HDACs) (Choudhary et al., 2009; Parbin et al., 2014).

HDACs can be classified into four classes. Class I, II and IV include classical HDACs, which are Zinc-dependent amidohydrolases located in both the nucleus and the cytoplasm. Class III HDACs belong to the recently discovered sirtuin family (homologous to the yeast Sir2 (Silent information regulator 2) family of proteins), being NAD^+-dependent enzymes localized in the nucleus (Haberland, Montgomery, & Olson, 2011).

Several studies have revealed that reversible acetylation occurs in a large number of non-histone proteins, regardless of their cellular localizations, causing direct effects on multiple physiological processes including proliferation, apoptosis, metabolism and cell migration (Choudhary et al., 2009; Hofmann et al., 2011).
In tumorigenesis, the acetylation status at the whole proteome level is impaired by dysregulated deacetylases, which have a special participation in cancer initiation and progression (Li & Seto, 2016; Parbin et al., 2014).

Moreover, HDAC inhibitors have been shown to be effective therapeutic anticancer agents, including in leukemias. These are natural or synthetical products, which possess different structural characteristics, including aliphatic acids, cyclic peptides, benzamides and hydroximates (Eckschlager, Plch, Stiborova, & Hrabeta, 2017; Hyun-Jung & Suk-Chul, 2011; Xu, Parmigiani, & Marks, 2007). EX-527 (6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide) is a SIRT1 inhibitor with ∼100-fold more selectivity and affinity over other members of the sirtuin family (figure 7). This is a non-competitive inhibitor which occupies the nicotinamide site and a neighboring pocket to stabilize a closed conformation of SIRT1 that prevents product release (Gertz et al., 2013).

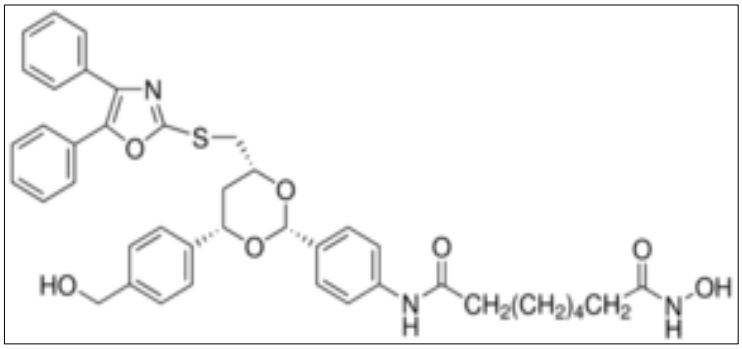

Figure 7. **EX-527, a SIRT1 inhibitor.**

Tubacin (Figure 8) is a molecule that selectively inhibits HDAC6 activity by binding to its catalytic domain impairing the interaction with tubulin, one of its major substrates. This inhibitor causes accumulation of acetylated α-tubulin, without affecting acetylation of histones, stability of microtubules and cell cycle progression (Haggarty, Koeller, Wong, Grozinger, & Schreiber, 2003).

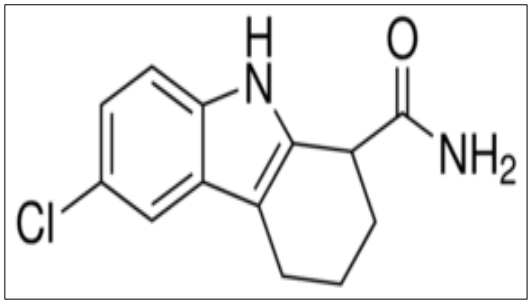

Figure 8. **Tubacin, a HDAC6 inhibitor.**

Cortactin was identified as a HDAC6 substrate by Zhang and collaborators, who reported that overexpression of HDAC6 resulted in reduced levels of acetylated cortactin. When cells expressed a 9KQ mutant of cortactin within the repeat region, cortactin was not capable of translocating to the cell periphery in response to growth factor stimulation. Moreover, F-actin binding was impaired and cortactin remained cytoplasmic, suggesting that deacetylation of cortactin may be required for its translocation to the cell periphery. Additionally, migration assays with ovarian cancer cell lines expressing different levels of acetylated cortactin showed that high levels of

HDAC6 and hypoacetylated cortactin led to faster migration when compared to those cell lines that expressed lower levels of HDAC6 with hyperacetylated cortactin (X. Zhang et al., 2007).

NEDD9 (neural precursor cell expressed, developmentally downregulated 9) is a scaffolding protein that activates AURKA (oncogenic serine/threonine kinase Aurora A), which phosphorylates HDAC6 to enhance its activity. The knockdown of NEDD9 or AURKA results in higher levels of acetylated cortactin and a reduction in its interaction with F-actin at the leading edge of migrating cells, an effect that was also observed after HDAC6 inhibition with Tubacin. Importantly, NEDD9 and AURKA colocalize with cortactin at the leading edge of breast cancer cells suggesting that NEDD9 is an upstream regulator of cortactin function, which acts through the AURKA-dependent activation of HDAC6 (Kozyreva et al., 2014).

Of note, SIRT1 levels are elevated in breast and ovarian cancer cells, correlating inversely with the levels of acetylated cortactin. When inhibiting SIRT1 activity with EX-527 in ovarian cancer cells, the amount of acetylated cortactin was increased. Likewise, by mutating nine acetylable lysines to glutamine in the repeat region of cortactin to mimic its acetylated status, it was shown by transwell assays that these mutations substantially reduced migration, whereas non acetylated cortactin promoted cell migration (Y. Zhang et al., 2009).

SIRT1 deacetylases cortactin in the nucleus and facilitates deacetylated cortactin localization to the cytoplasm. Recently, it was shown that after treating podocytes with EX-527, cortactin was dissociated from actin fibers, and WB analyses revealed that the ratio of acetylated cortactin to total cortactin was increased in a dose-dependent manner. Additionally, in the nuclear extract, acetylated cortactin levels were increased, whereas it was undetectable in the cytoplasmic extract. These data suggest that deacetylation activity of SIRT1 is important for cortactin function and localization (Motonishi et al., 2015).

2.10 Src mediated cortactin phosphorylation

Src is a non-receptor tyrosine kinase which associates with the inner surface of cell membranes. The basic function of Src is the transmission of external signals into the cell by phosphorylating tyrosine residues on substrates mainly downstream of receptor tyrosine kinases (RTK), G-protein coupled receptors and integrins (Guarino, 2010; Martin, 2001). All these type of surface receptors can induce simultaneous activation of different signal transduction cascades including Ras/MAPK, PI3K/Akt, and STAT3 pathways, thus achieving several biological functions such as cell survival, proliferation, cytoskeleton reorganization and invasion (Guarino, 2010; Huveneers & Danen, 2009; Martin, 2001).

Src overexpression or hyperactivation has been identified as one of the hallmarks of several cancer types promoting increased cancer cell growth and survival, and cytoskeletal reorganization to facilitate cell migration and metastasis (Bild et al., 2006; Yeatman, 2004). Therefore, Src kinase is an important target for anticancer drugs, comprising leukemias. PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) is a selective inhibitor of Src family kinases (Bain et al., 2008) (Figure 9), which binds between the N- and C-termini of the Src kinase domain thus interfering with substrate binding (Karni et al., 2003).

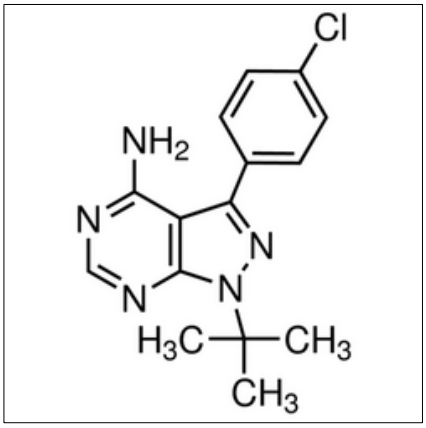

Figure 9. **PP2, a Src kinase inhibitor.**

Cortactin phosphorylated by Src (Wu et al., 1991) triggers cancer cell migration and cancer metastasis (C. Huang et al., 1998; J. Huang, Asawa, Takato, & Sakai, 2003;

Kirkbride et al., 2011).

It has also been reported that cortactin tyrosine phosphorylation by Src triggers the recruitment of the cytoplasmic protein Nck adaptor 1 (Nck1), linking cortactin with N-WASP and WIP thus increasing activation of the Arp2/3 complex and inducing lamellipodia protrusion and cell migration (Tehrani, Tomasevic, Weed, Sakowicz, & Cooper, 2007). Moreover, transmembrane integrins link the ECM to intracellular actin cytoskeleton. For example, at focal adhesions, clustered integrins recruit FAK and activate Src to phosphorylate cortactin and mediate association of F-actin with focal adhesions (Wang, Liu, & Liao, 2011).

Cortactin is recruited to and binds ICAM-1 in endothelial cells, becoming phosphorylated by Src kinase upon adhesion molecule engagement, thus regulating cytoskeletal remodeling and neutrophil transendothelial transmigration. After treating HUVEC with the Src kinase inhibitor PP2, tyrosine phosphorylation of cortactin was reduced, actin remodeling prevented, and neutrophil transmigration decreased (Yang, Kowalski, Yacono, et al., 2006).

Moreover, Luo and collaborators reported that CXCL12 activation of CXCR4 in HEK cells leads to cortactin translocation to the cell surface where is phosphorylated in a Src- and dynamin-dependent manner, thus regulating ERK activation and chemotaxis (Luo et al., 2006).

The importance of phospho-cortactin has additionally been confirmed in studies using a mutant form of cortactin with Tyr 421, 466, and 482 changed to Phe or Ala (3YF/3YA), which cannot be phosphorylated by Src. Cells expressing these mutants showed significantly impaired cell migration (C. Huang et al., 1998; Luo et al., 2006; Tehrani et al., 2007; Wang et al., 2011; Yang, Kowalski, Zhan, et al., 2006) .

Considering all these evidence about post-translational modifications of cortactin playing an important role in transmigration, it would be intriguing to determine how PTM's of cortactin influence transmigration potential of leukemic cells.

2.11 Cortactin in leukemia

Recently, cortactin was found to be overexpressed in B-cells from patients with chronic lymphoblastic leukemia (CLL), where most of the patients overexpressed the wild-type isoform of 80/85 kDa, which is absent in normal B-cells, and some overexpressed the splice variant 1 (SV1) of 70/75 kDa, which can be detected to some degree in normal B-cells (Gattazzo et al., 2014).

Overexpression of cortactin also correlates with markers of poor prognosis, such as ZAP70 and secretion of MMP-9 (Gattazzo et al., 2014). Moreover, depletion of cortactin in leukemic B cells reduced MMP-9 secretion, besides the migratory capacity of the cells. In CLL B-cells from patients, cortactin was highly and constituvely phosphorylated in Y421. After treating leukemic cells with PP2, phosphorylation of cortactin was decreased, as well as MMP-9 secretion and cell chemotaxis, suggesting that post-translational modifications of cortactin are critical for its functions in leukemia (Martini et al., 2017).

Assessing cortactin expression in the ALL pre-B cell line REH, our group detected the expression of only the SV2 cortactin variant of 60 kDa. Of note, clinical data of patients with relapse to bone marrow showed a positive correlation with high cortactin expression. In REH cells, high cortactin levels also correlated with CXCL12-dependent transendothelial migration, colonization of bone marrow organoids and organ infiltration in a xenotransplantation model. Importantly, cortactin-depleted REH cells showed reduced capacity to infiltrate organs. These data strongly suggest that cortactin may trigger invasion of peripheral organs by B-ALL cells (Velázquez-Avila et al., 2018). However, it remains unknown whether cortactin is post-translationally modified in this context.

Given the above-mentioned regulations of cortactin functions by post-translational modifications, it is tempting to speculate that different PTM also play an important role for the aggressiveness of B-ALL cells.

3. JUSTIFICATION

Cortactin is overexpressed in leukemic B cells and triggers transendothelial migration, extramedullary infiltration and bone marrow colonization. Post-translational modifications of cortactin regulate its functions, however, it remains to be explored whether these modifications modulate the aggressiveness of B-ALL cells. Thus, investigating whether pharmacological compounds inhibiting kinases and deacetylases that target cortactin functions can reduce transendothelial migration of leukemic cells and bone marrow colonization may reveal cortactin as a potential new therapeutic target for high risk B-ALL patients to prevent disease relapse.

4. HYPOTHESIS

Inhibition of Src and SIRT1 in leukemic B cells reduce transendothelial migration, organ infiltration and bone marrow colonization.

5. GENERAL OBJECTIVE

To investigate cortactin phosphorylation and acetylation in leukemic B cells and the relevance for transendothelial migration and bone marrow colonization.

6. PARTICULAR OBJECTIVES

1) To analyze phosphorylation of cortactin in leukemic B cells with and without CXCL12 stimulation.
2) To analyze the effect of enzyme inhibitors on leukemic B cells viability.
3) To test whether inhibition of Src or SIRT1 in leukemic B cells reduces their potential for transendothelial migration and bone marrow colonization.

7. MATERIAL AND METHODS

7.1 MATERIAL

7.1.1 Culture medium

Medium	**Company**
RPMI 1640	Sigma. Ref R4130-10X1L
Minimum Essential Medium Eagle	Sigma. Ref M4655-1L
Endothelial cell medium ECM	ScienCell. Ref 1001

7.1.2 Antibodies

Antibody	**Company**
Anti-human CD45-Phycoerytrin	Biolegend. Ref 304058
Anti-cortactin Alexa Fluor® 488 conjugated, clone 289H10	Kindly provided by Dr. Klemens Rottner, TU Braunshweig, Germany
Anti-phosphocortactin (Y421)	Cell Signaling Technology. Ref 4569
Anti-γ tubulin	ThermoFisher. Ref MA1-850
Alexa Fluor® 488 goat anti-rabbit	Invitrogen, ThermoFisher. Ref A-11034
Alexa Fluor® 488 goat anti-mouse	Invitrogen, ThermoFisher. Ref A-11001
TruStain fcX™ (anti-human)	Biolegend. Ref 422302
m- igGk BP-HRP	Santa Cruz. Ref SC-516102
Mouse anti-rabbit igG-HRP	Santa Cruz. Ref SC-2357

7.1.3 Kits

Kits	**Company**
DC™ Protein assay	Biorad. Ref 5000112
SuperSignal WestPico	ThermoFisher. Ref 34087
SuperSignal WestFemto	ThermoFisher. Ref 34095
MTT Cell Proliferation Assay	ATCC. Ref 30-1010K

7.1.4 Reagents

Chemical	**Company**
Recombinant Human SDF-1α (CXCL12)	Preprotech. Ref 300-28A
Fetal Bovine Serum	Biowest. Ref S1810
Antibiotic antimycotic 100X	Corning. Ref 30-004-Cl
Ficoll-Paque™ PLUS	GE Healthcare. Ref 17-1440-03
Tween 20	Sigma. Ref P1379-500ML
Triton™ X-100	Sigma. Ref T9284-500ML
Bovine Serum Albumin	Sigma. Ref A2153-100G
Trypsine-EDTA 0.25%	Sigma. Ref T4049-500ML
TrypLE™ Express	Gibco. Ref 12604-013
Complete protease inhibitor cocktail	Roche. Ref 11697498001

Nitrocellulose membrane, pore 0.45 μm	Biorad. Ref 1620115
$Na_2HPO_4 \bullet 7H_20$	J.T. Baker. Ref 3824-01
$KH_2PO_4 \bullet 7H_20$	Macron. Ref 7088-04
NaCl	J.T. Baker. Ref 3624-05
Nonidet™ P-40	Sigma. Ref 21-3277 SAJ
KCl	J.T. Baker. Ref 3040-01
NaF	Sigma. Ref S7902
Na_3VO_4	Sigma. Ref S6508
Tris base	J.T. Baker. Ref 4109-06
Glycine	J.T. Baker. Ref 4059-06
Glycerol	Sigma. Ref G6279-500ML
Ammonium persulfate	Biorad. Ref 161-0700
30% Acrylamide/bis solution	Biorad. Ref 161-0153
Sodium dodecyl sulfate (SDS)	Biorad. Ref 1610302
TEMED	Biorad. Ref 161-0801
Ponceau S	Merck-Millipore. Ref 159270025
β-mercaptoethanol	Sigma. Ref M3148-25ML
Saponin	Sigma. Ref B2261

7.1.5 Buffers

PBS 1X	138 mM NaCl 3 mM KCl 8.1 mM Na_2HPO_4 1.5 mM KH_2PO_4 pH 7.4
PBS-EDTA	PBS 1X 5 mM EDTA
RIPA lysis buffer	50 mM Tris-HCl pH 7.4 150 mM NaCl 2 mM EDTA 1% NP-40 0.1% SDS
SDS-PAGE buffer	25 mM Tris 192 mM Glycine 0.1% SDS pH 8.3
Transfer buffer	20% Methanol 25 mM Tris 192 mM Glycine 0.1% SDS pH 8.3
TBS 1X buffer	150 mM NaCl 10 mM Tris pH 8.0
TBS-T	TBS 1X 0.1% Tween20
Blocking buffer	TBS-T 5% BSA

7.2 METHODS

7.2.1 Cell culture

The B-ALL cell line REH (Pre-B leukemic cells, with the translocation 12:21 producing the *TEL/ETV6-AML1* fusion gene, from peripheral blood of a relapse case, ATCC CRL-8286) (Matsuo & Drexler, 1998) was cultured in RPMI 1640 (Sigma) medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic.

MS-5 stromal cells (ATCC CRL-1 me1882) and OP9 stromal cells (ATCC CRL-2749) were cultured in MEM medium (Sigma) supplemented with 10% FBS and 1% antibiotic/antimycotic.

7.2.2 Isolation and propagation of HUVEC

To obtain human umbilical vein endothelial cells (HUVEC), umbilical cords of at least 25 cm were washed with water to remove excess of blood. Under sterile conditions, in the upper end of the cord vein a canule with a syringe containing 20 mL PBS and 1% antibiotic solution was inserted and all content was flushed. The syringe was removed and ends were sealed with hemostatic clamps. 10 mL 0.25% trypsin-EDTA were added into the vein using a syringe and the cord was incubated at 37°C for 10 min in a water bath. Every 2 min the cord was gently massaged to facilitate the digestive process. Subsequently, the clamp of the lower end was removed and the content was recovered into a falcon tube and centrifuged at 1500 rpm for 5 min. Finally, the cell pellet was resuspended in ECM medium (ScienCell) and plated in a 60 mm cell bind dish (CellBind, Corning).

To propagate and maintain HUVEC, the medium was aspirated and cells were washed with PBS three times. Then 1 mL of 0.25% trypsin-EDTA was added and cells were incubated at 37°C for 5 min. 3 mL of serum-containing medium was added for trypsin inactivation, and the cell suspension was plated in three fresh 60 mm dishes.

7.2.3 Cell viability assay

REH cells were seeded at $3x10^4$ cells/well in a 96-well plate. Cells were treated with different concentrations of either PP2, EX-527 or Tubacin (50, 100 and 150 μM). Drugs were dissolved in DMSO and diluted to the final concentrations in RPMI medium. Control cells were treated with DMSO only. Following culture for 24 h with PP2, 10 μL/well MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg/mL) was added and incubated for 4 h at 37°C. Subsequently, to solubilize crystals 100 μL of acidic propanol (50 mL of Triton X-100, 4 mL of HCl, 446 mL of isopropanol) was added to each well and stirred continuously at room temperature and darkness for 3 h. The absorbance of plates was determined at 560 nm using a spectrophotometer (Infinite F500 TECAN). The dose-response curve was used to calculate the concentration of inhibitor resulting in 50% inhibition of cell viability (IC_{50}).

7.2.4 Cell culture treatment

REH cells were treated for 20 min with the Src inhibitor PP2 or the SIRT1 inhibitor EX-527 at 10, 20 and 50 μM. After 30 min incubation time at 37°C, cells were washed with PBS two times and were used in the functional assays described below.

7.2.5 Propidium iodide staining

$2x10^5$ REH cells were incubated with PP2 and EX-527 as described above. Subsequently, 2 μL of propidium iodide were added. Control cells were fixed with 4% paraformaldehyde (PFA) and permeabilized using Permwash (PBS+1% FBS, 0.1% saponin) and used as positive for cell death. Cells were analyzed using a CytoFLEX flow cytometer and FlowJo v10.0 software.

7.2.6 Flow cytometry

Phospho-cortactin (Y421) levels were evaluated after permeabilization and fixation of cells with 150 μL 4% PFA for 20 min at 4°C followed by washing with 1 mL, blocked with anti-FcRγ antibody (1:1000 dilution) for 15 min at room temperature, and washed again with 1mL Permwash. Then cells were incubated for 30 min at room temperature with polyclonal anti-phospho-cortactin (Y421) antibody (1:100 dilution; Cell Signaling Technology #4569) and washed with 1 mL Permwash. Cells were incubated with a secondary goat anti-rabbit Alexa 488-labelled antibody (1:1000) for 25 min. Finally, cells were analyzed using a CytoFLEX flow cytometer and FlowJo v10.0 software.

7.2.7 Western blotting

Cell lysis

Cells were lysed using RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS) supplemented with 1 mM Na_3VO_4, 5X Complete protease inhibitor cocktail and 2X Phosstop phosphatase inhibitor, followed by three pulses of sonication (10 seconds, 40% amplitude). Then, lysates were centrifuged at 14000 rpm for 10 min at 4°C. The supernatants were collected in a new 1.5 Eppendorf tube and protein concentration was quantified using the DC protein assay. After quantification, the supernatants were mixed with 5X Laemmli buffer to a final 1X concentration, boiled 5 min at 95°C and stored at -80°C until further use.

Protein quantification

The commercial kit DC Assay Method (BioRad, CA, USA) was used according to the manufacturer's instructions to quantify protein concentrations in cell lysates. Briefly, samples, diluent and reagents where prepared as follows: diluent was prepared by mixing the lysis buffer in deionized water in a 1:50 dilution; Reagent A' (RA') was prepared by diluting 1:50 Reagent S in reagent A, both provided with the kit. Samples were prepared by mixing 1 μL of the lysates with 19 μL of diluent. Then, the standard

curve with Bovine γ-globulin (BGG) was pipetted in a 96-well plate, as indicated in Table 1. Standard curve and samples were prepared in triplicates.

Table 1. **DC Assay Method standard curve reaction.**

BGG standard curve					
1	**2**	**3**	**4**	**← Reaction order**	
Diluent	**BGG stock (1 mg/mL)**	**Reagent A'**	**Reagent B**	**Final volume**	**Final concentration**
20 μL	0 μL	10 μL	80 μL	100 μL	0 μg/μL
19 μL	1 μL	10μL	80 μL	100 μL	1 μg/μL
18 μL	2 μL	10 μL	80 μL	100 μL	2 μg/μL
17 μL	3 μL	10 μL	80 μL	100 μL	3 μg/μL
16 μL	4 μL	10 μL	80 μL	100 μL	4 μg/μL
15 μL	5 μL	10 μL	80 μL	100 μL	5 μg/μL
14 μL	6 μL	10 μL	80 μL	100 μL	6 μg/μL
13 μL	7 μL	10 μL	80 μL	100 μL	7 μg/μL
12 μL	8 μL	10 μL	80 μL	100 μL	8 μg/μL

Afterwards, the plate was incubated in the dark for 15 min with gentle agitation. Then, absorbance was measured at 720nm using a 96-well plate spectrophotometer (Tecan, Männedorf, Switzerland). To obtain protein concentration, the BGG standard curve was analyzed by linear regression and absorbances of samples were extrapolated using Graph Pad Prism 5 software.

SDS-PAGE and Blotting

Equal protein amounts were separated by 8% SDS-PAGE for 2 hours at 100 V, transferred to nitrocellulose membranes (Millipore, 0.45 μm) for 1.5 hours at 220 mA, and blocked with 5% BSA in TBS with 0.1% Tween20 for 1 hour at room temperature. Membranes were incubated with primary anti-phospho-cortactin (Y421) antibody (1:1000 dilution; Cell Signaling Technology #4569), anti-cortactin (1:2000 dilution, GK-18 clone), or anti-γ tubulin (1:4000 dilution, loading control) at 4°C over night. Then membranes were washed three times with TBS containing 0.1% Tween20 and incubated with species-specific peroxidase-conjugated secondary antibody (1:10,000 dilution, Santa Cruz) for 2 hours at room temperature. Membranes were washed three times with TBS-T.

Imaging and quantification

Bands were revealed with SuperSignal® WestFemto chemiluminescent substrates. Chemiluminescence signals were recorded on a Chemidoc imaging system using Image Lab software (BioRad, California, USA). Band intensities were quantified using Image J software. Data were reported as relative pixel intensity obtained by dividing the pixel intensity of cortactin by the pixel intensity of tubulin of each sample, and normalizing phospho-cortactin with total cortactin.

7.2.8 Filter-based transendothelial migration assay

$8x10^4$ HUVEC were grown to confluence on 5 μm pore transwell filters (Corning) pretreated with attachment factor (Gibco, Thermo Scientific) for 30 min at 37°C. As a control to monitor monolayer formation, HUVEC were seeded in 96 well plates at the same density. Once the monolayer was formed (usually 48 h), $1.5x10^4$ REH cells untreated or previously treated with either PP2 or EX-527 at 10, 20 and 50 μM and washed twice with PBS were resuspended in 200 μL RPMI medium and placed on top of the endothelial monolayer. 100 ng/mL of CXCL12 (Peprotech) as chemoattractant

in 500 µl of fresh medium was added to the bottom well. Cells were allowed to transmigrate for 4 hours. Transmigrated cells from the bottom chamber were collected and counted using a Neubauer chamber.

7.2.9 *In vitro* spheroid colonization assay

OP9 or MS5 stromal cells were cultured in DMEM (Sigma) medium supplemented with 10% FBS and 1% antibiotic/antimycotic. To form spheroids in non-adhesion wells, a suspension of $2.5x10^4$ stromal cells were seeded in 96 well plates filled with 100 µL 1% agarose to avoid adhesion to the plastic surface and incubated for 48 hours at 37°C, 5% CO_2 thus allowing for cell aggregation and formation of spheroids.

$2.5x10^4$ cells previously treated with either PP2 or EX-527 at 10, 20 and 50 µM and washed twice with PBS were co-cultured with a spheroid for 4 hours. After this period, hematopoietic cells outside of spheroids were collected from supernatants, and spheroids with hematopoietic cells inside were collected separately after disaggregation of spheroids. To this end, spheroids were extensively washed with PBS-5mM EDTA and enzymatic digestion was performed using TrypLE (Thermo Scientific) by vigorous pipetting several times. The cell suspensions of inner and outer cells were stained using an anti-CD45 antibody (hematopoietic marker), and were analyzed by flow cytometry as described above.

7.2.10 Statistics

Graph Pad Prism 5 software was used to perform statistical data analysis. Differences between two groups were analyzed by Student's T-test, considering values of $p<0.05$ as statistically significant.

8. RESULTS

8.1 Determination of phospho-cortactin (Y421) levels in the pre-B ALL cell line REH

First, considering the importance of CXCL12/CXCR4 axis in leukemic B cells where Src kinase among other signaling pathways are activated, we analyzed Src-mediated cortactin phosphorylation at residue Y421 in the pre-B leukemic cell line REH. Using western blot, we found low phosphorylation levels of cortactin under basal conditions, which were increased after CXCL12 stimulation **(Figure 10A)**. Additionally, the same observation was corroborated by flow cytometry using the same specific phospho-cortactin antibody used in WB **(Figure 10B)**. Statistical analysis of our data did not show a significant difference on phospho-cortactin levels after CXCL12 stimulation, yet we can clearly observe a tendency towards an increase. Martini and colleagues previously reported constitutive phosphorylated cortactin levels at Y421 in CLL cells from patients, that were not changed under CXCL12 stimulation (Martini et al., 2017). Also, for the moment we do not have control samples to analyze cortactin phosphorylation in normal B cells. It has been reported that in B lymphocytes purified from healthy subjects cortactin presents a very low level of phosphorylation at Y421, which also increases after CXCL12 treatment (Martini et al., 2017).

REH cell culture was supplemented with fetal bovine serum, mainly because of its high levels of growth stimulatory factors that allow for proper cell growth in culture (Zheng et al., 2006). Activated growth factor receptors such as platelet derived growth factor receptor (PDGF-R), epidermal growth factor receptor (EGF-R) and fibroblast growth factor receptor (FGF-R), which are expressed on REH cells, trigger signal transduction inside the cells including Src activation to promote pleiotropic effects such as actin cytoskeletal rearrangements (Alvarez, Kantarjian, & Cortes, 2006; Biscardi, Ishizawar, Silva, & Parsons, 2000; Bjorge, Jakymiw, & Fujita, 2000). Thus, we analyzed whether the constitutive phosphorylation of cortactin at Y421 observed under basal conditions due to serum-derived growth factors that are known to target cortactin (Mezi, Todi, Orsi, Angeloni, & Mancini, 2012). By western blot, we observed that in serum-starved

condition for 16 h, REH cells showed similar levels of phospho-cortactin compared to REH cells cultured with fetal bovine serum **(Figure 11)**. Although growth factors found in FBS may contribute to the activation of Src kinase in REH cells, there might exist other stimuli or mutations in this kinase that triggers its activation and effect over cortactin. Further experiments are needed to determine what triggers the basal phosphorylation levels.

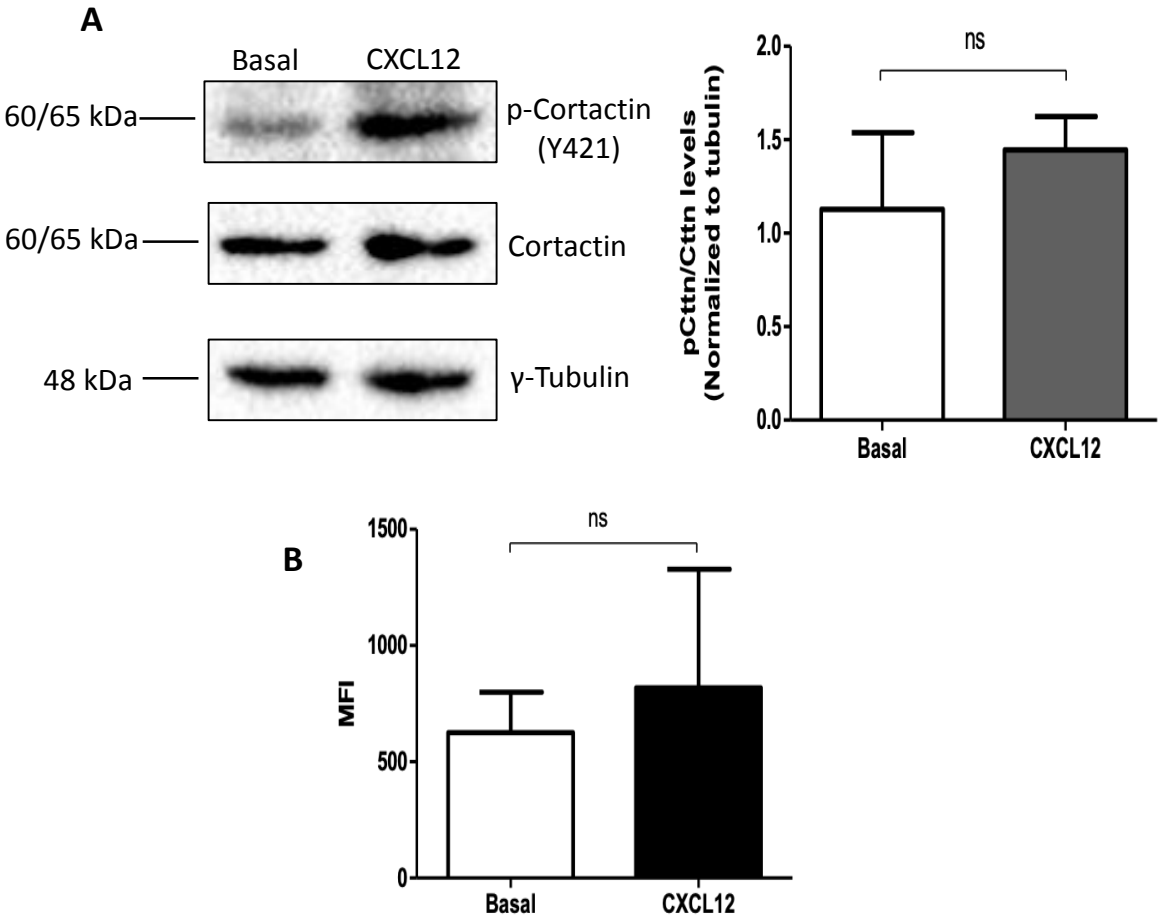

Figure 10. Phospho-cortactin (Y421) levels in basal and CXCL12 stimulated REH cells. A) Phosphorylation of cortactin was determined by Western blot using lysates from REH cells under basal conditions and after CXCL12 (100 ng/mL) stimulation for 20 minutes. Total cortactin served to normalize phosphorylation levels; and γ-Tubulin served as loading control. A representative blot of 3 independent experiments is depicted. Densitometric analysis was performed using ImageJ and Graph Pad Prism 5 software. Statistical significance was determined using paired Student's t-test. Data are represented as mean relative pixel intensity ± SD. n=3. B) Flow cytometry analysis was performed using a phospho-cortactin antibody (Y421). Each staining was performed independently three times. Data are displayed as mean fluorescence intensities (MFI) ± SD. FlowJo X software was used to analyze data. ns: not significant. n=3.

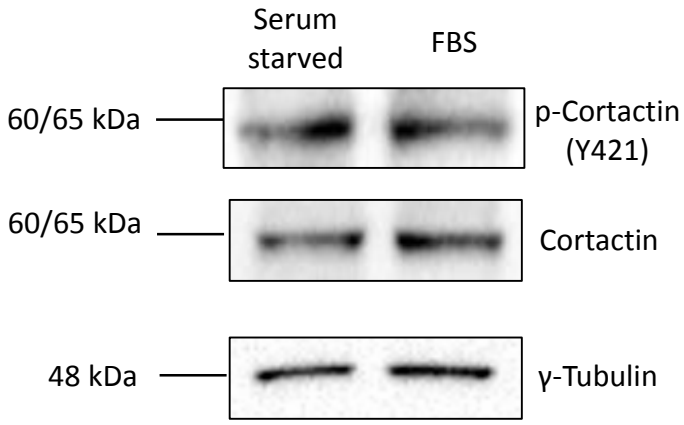

Figure 11. Phospho-cortactin (Y421) levels in serum starved REH cells. Phosphorylated cortactin levels were determined by Western blot using lysates of REH cells in serum-starved conditions for 16 h (0% FBS); and cultured with 10% FBS. Total cortactin served to normalize phosphorylation levels, and γ-Tubulin as loading control. n=2.

8.2 PP2, EX-527 and Tubacin do affect cell viability of REH cells only at high concentrations

A MTT assay to assess cell viability was performed to determine the IC_{50} value (maximal inhibitory concentration) of each of the pharmacological compounds that inhibit enzymes targeting cortactin. As this was the first time the inhibitors PP2, EX-527 and Tubacin were tested in the pre-B ALL cell line REH, we wanted to determine the most suitable concentrations to treat the cells before performing functional assays. The IC_{50} is the concentration of inhibitor to produce a 50% inhibition of cell viability. After 24 hours, the IC_{50} value of PP2 (Src kinase inhibitor) was 190.82 μM; the IC_{50} value of EX-527 (SIRT1 inhibitor) was 190.94 μM and the IC_{50} value of Tubacin (HDAC6 inhibitor) was 41.39 μM. All three compounds affected cell viability of REH cells in a dose-dependent manner with tubacin having the strongest cytotoxic effect **(Figure 12)**.

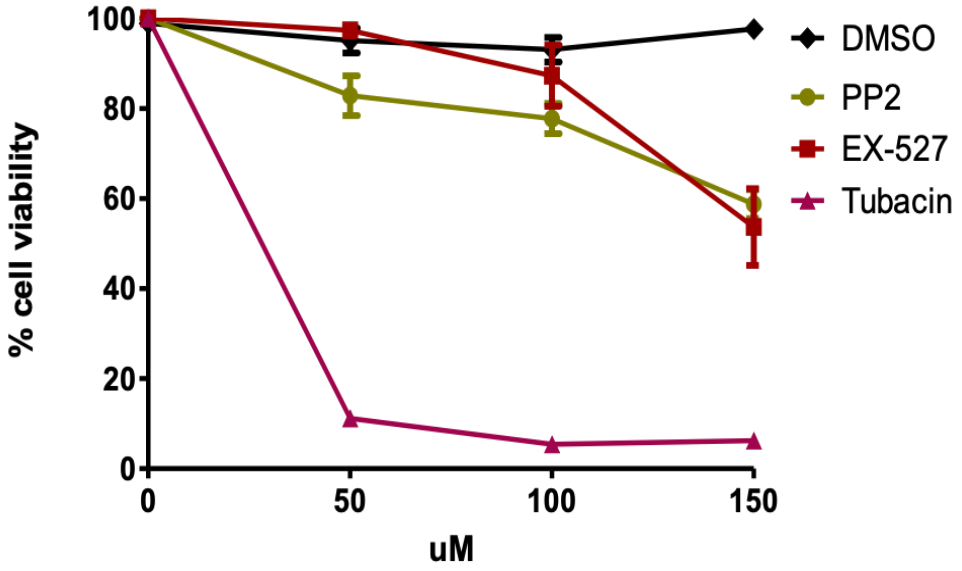

Figure 12. Cell viability as assessed by MTT assay. $3x10^4$ REH cells were plated into 96-well plates and then incubated with different concentrations of PP2, EX-527 and Tubacin (50, 100 and 150 µM) for 24 h. Control cells were treated with DMSO. Data are means ± SD of four independent experiments.

8.3 PP2 and EX-527 treatment do not cause REH cell death at low concentrations

As this was the first time that PP2 and EX-527 pharmacological inhibitors were tested in the REH cell line, we wanted to ensure that the concentrations of these inhibitors that have been used in the literature for functional assays in different cells would not cause significant cell death. We analyzed necrosis using flow cytometry analysis of propidium iodide (PI) uptake. After subsequent addition of propidium iodide to cells following incubations with PP2 and EX-527 for 20 and 30 minutes, respectively, we did not observe significant cell death **(Figure 13)**. Propidium iodide binds to DNA of those cells that have a permeable plasma membrane, a hallmark of cell death (Cummings & Schnellmann, 2004). Therefore, the concentrations of the pharmacological inhibitors and incubation times selected for further experiments are appropriate to apply them in functional experiments.

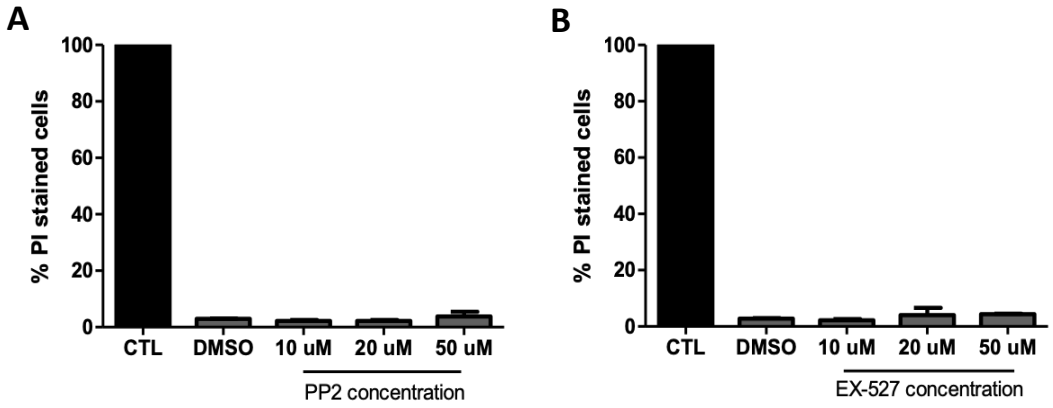

Figure 13. **Percentage of PI positive cells after treatment with either PP2 or EX-527.** A) $2x10^5$ REH cells were incubated for 20 minutes with 10, 20 and 50 μM PP2; or DMSO as control. B) $2x10^5$ REH cells were incubated for 30 minutes with 10, 20, 50 μM EX-527; or DMSO as control. Fixed and permeabilized cells were used as positive control in both experiments. After treatment, propidium iodide (PI) was added and cells were analyzed by flow cytometry. n=2.

8.4 The src inhibitor PP2 reduces phospho-cortactin levels in REH cells

Cortactin was discovered originally as a Src kinase substrate, being phosphorylated primarily at Y421 (Wu et al., 1991). Most studies report that high levels of Y421 phosphorylation correlate with enhanced cell migration (Kirkbride et al., 2011). Therefore, it is likely that Y421 phosphorylation in B cells confers enhanced migratory capabilities towards the chemoattractant CXCL12. First, we wanted to know whether the observed increased phosphorylation levels in response to CXCL12 are indeed src-dependent. We treated pre-B leukemic REH cells with CXCL12, a potent chemoattractant, and PP2, a selective inhibitor of Src family kinases (Bain et al., 2008). Using flow cytometry, we found that phospho-cortactin (Y421) levels were significantly decreased after treating the cells with 20 and 50 μM PP2 **(Figure 14)**, suggesting that PP2 can prevent cortactin activation and enhanced cell migration. These findings encouraged us to evaluate whether src-mediated cortactin phosphorylation is important for leukemic B cells to achieve transendothelial migration and bone marrow colonization.

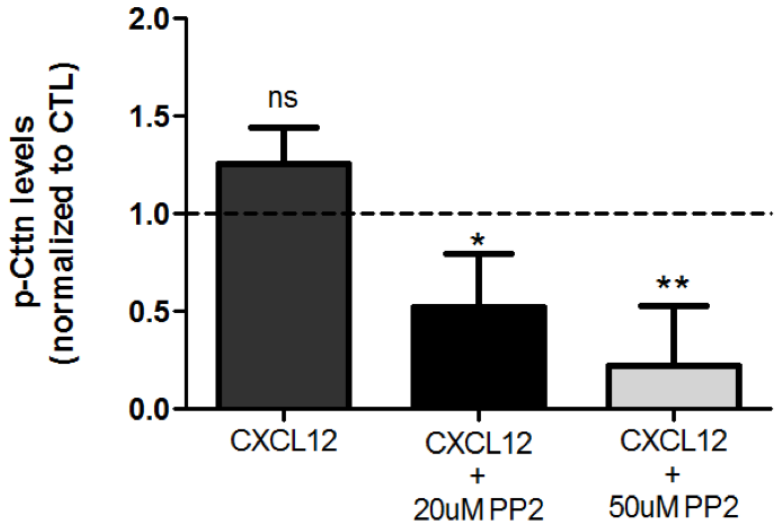

Figure 14**. Phospho-cortactin (Y421) levels are reduced after PP2 treatment.** 2x10^5 REH cells were incubated with CXCL12 (100 ng/mL) and 20 or 50μM PP2 in parallel for 20 minutes . Phospho-cortactin (Y421) expression was analyzed by flow cytometry. Data are represented as fold MFI increase ± SD normalized to basal (CXCL12) or DMSO-treated (CXCL12+PP2) conditions (set to 1, dotted line). Unpaired Student's t-test was performed to determine statistical significance. n=3. ns: not significant, *p<0.05, **p<0.005.

8.5 Src kinase inhibition impairs transendothelial migration and bone marrow colonization of REH cells

CXCL12 is a chemoattractant for normal and leukemic B cells (Chiarini et al., 2016; Jo et al., 2000; Pramanik et al., 2013; Shen et al., 2001). When CXCL12 binds to its receptor CXCR4, Src kinase among other signaling pathways are activated (Chatterjee et al., 2014; Cheng et al., 2017; Chiarini et al., 2016; Ganju et al., 1998; Perim et al., 2015a). Cortactin is recruited to the plasma membrane where it gets phosphorylated by Src at Y421 (Luo et al., 2006) to trigger cell migration (J. Huang, Asawa, Takato, & Sakai, 2003; Kirkbride et al., 2011). For this reason, we treated REH cells with 10, 20 and 50 μM PP2, and evaluated their capacity to transmigrate across HUVEC monolayers towards a CXCL12 gradient. It is important to mention that only REH cells were treated prior to the transmigration assay, and that endothelial cells, which also express cortactin and gets phosphorylated in a src-dependent manner, were never in

contact with Src inhibitor. Thus, possible effects can strictly be attributed to Src inhibition in REH cells. We found that the percentage of REH cells which were able to transmigrate was dramatically reduced **(Figure 15)**, suggesting that Src activation and likely also src-mediated cortactin phosphorylation (Y421) are important events for efficient REH cells transmigration.

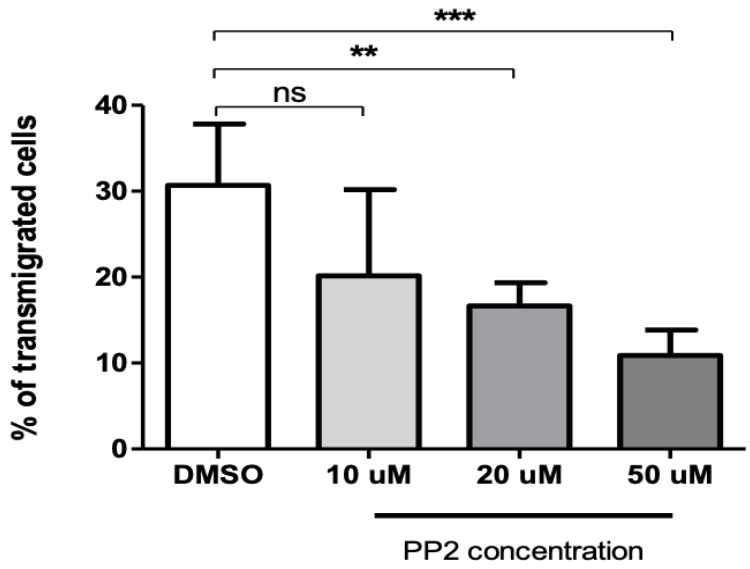

Figure 15. **The percentage of transmigrated REH cells is reduced after Src inhibition.** $1.5x10^4$ REH cells treated for 20 min with different concentrations of PP2 were washed with PBS and placed in the upper chamber of transwell filters containing HUVEC monolayers. Transmigrated cells towards a CXCL12 gradient (100 ng/mL) were recovered from the bottom chamber after 4 hours. Control cells were treated with DMSO. Data are represented as mean % of transmigration of total REH cells applied ± SD. Unpaired Student's t-test was performed to determine statistical significance. n=6. ns: not significant, **p<0.005, ***p<0.0005.

The bone marrow microenvironment is important for supporting the growth and survival of normal and leukemic cells. Leukemic cells take over and change the equilibrium leading to their expansion (Duarte et al., 2018). CXCL12 is a chemokine constituvely produced by bone marrow stromal cells to regulate the homing of circulating normal and leukemic cells to the bone marrow (Mohle et al., 1998). Thus, we wanted to know whether src activation in REH cells is critical to accomplish colonization of spheroids of stromal cells resembling BM (Balandrán et al., 2017b). After treating REH cells with 10, 20 and 50 μM PP2 and co-culture them with spheroids

for 4 hours, the number of cells inside the spheroids was analyzed by flow cytometry. We found that inhibition of Src showed a tendency towards reducing the percentage of leukemic B cells able to colonize the spheroid **(Figure 16)**. However, these experiments still need to be repeated to corroborate this observation.

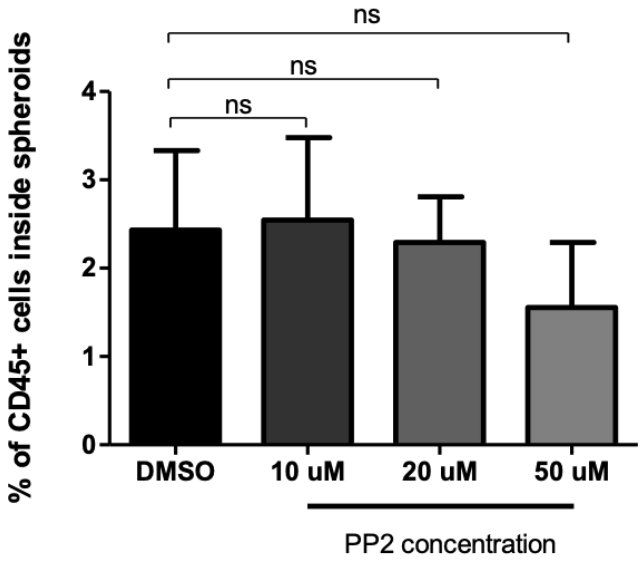

Figure 16. **Percentage of CD45$^+$ cells inside MS5 spheroids after PP2 treatment.** 2.5x10^4 REH cells were treated with 10, 20 and 50 μM PP2 for 20 minutes and washed with PBS. Co-cultures of treated REH cells with MS5 stromal cells were incubated for 4 hours. Then, spheroids were recovered, washed and disaggregated to obtain single cell suspensions. The number of CD45+ cells inside the spheroids was analyzed by flow cytometry. Control cells were treated with DMSO. Data are represented as % of CD45$^+$ cells of singlets ± SD. Unpaired Student's t-test was performed to determine statistical significance. n=6. ns: not significant.

8.6 SIRT1 inhibition with EX-527 diminishes transendothelial migration and bone marrow colonization of REH cells

SIRT1 is a histone deacetylase that deacetylases cortactin on lysine residues within its tandem repeat region to regulate cortactin affinity for F-actin. Acetylation in this region reduces the affinity for F-actin, whereas deacetylation favors affinity for F-actin and increases cell migration (Kaluza et al., 2011; Kirkbride et al., 2011; MacGrath & Koleske, 2012; Nakane et al., 2012; Schnoor et al., 2017; X. Zhang et al., 2007; Y. Zhang et al., 2009). Consequently, this prompted us to investigate whether inhibition of

SIRT1 would affect transendothelial migration of REH cells that overexpress cortactin. We treated REH cells with 10, 20 and 50 μM of the SIRT1 inhibitor EX-527 for 30 min, and evaluated transendothelial migration across HUVEC monolayers towards a CXCL12 gradient. Similar as with PP2 treatment, we found that inhibition of SIRT1 significantly reduced the percentage of transmigrated REH cells **(Figure 17)**, suggesting that active SIRT1 and likely SIRT-1 mediated cortactin deacetylation is critical for transmigration of leukemic B cells.

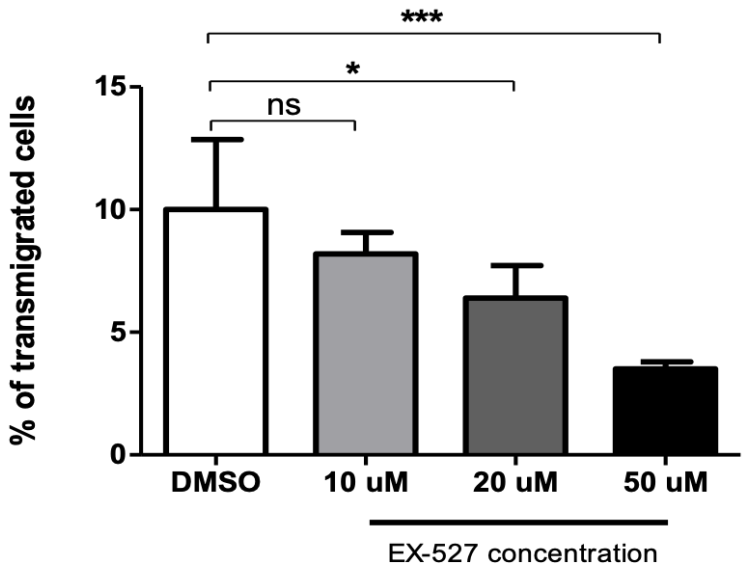

Figure 17. **The percentage of transmigrated REH cells is reduced after SIRT1 inhibition.** $1.5x10^4$ REH cells treated for 30 min with different concentrations of EX-527 were washed with PBS and placed in the upper chamber of transwell filters containing HUVEC monolayers. Transmigrated cells towards a CXCL12 gradient (100 ng/mL) were recovered from the bottom chamber after 4 hours. Control cells were treated with DMSO. Data are represented as mean % of transmigration of total REH cells applied ± SD. Unpaired Student's t-test was performed to determine statistical significance. n=6. ns: not significant, *p<0.05, ***p<0.0005.

Additionally, we also evaluated whether SIRT1 inhibition would impair bone marrow colonization. Treatment of REH cells with 10, 20 and 50 μM EX-527 showed a tendency towards diminishing the percentage of cells able to colonize the BM stromal cells spheroid **(Figure 18)**. Given that ovarian cancer cells showed increased amount of acetylated cortactin after inhibition of SIRT1 with EX-527, and that cell migration

was reduced in this context (Y. Zhang et al., 2009), it will be important to validate in future experiments the acetylation status of cortactin in leukemic B cells to allow a correlation of this status with the impaired transmigration of REH cells.

In summary, these data show for the first time that both src and SIRT1 activation in REH cells are important for transendothelial migration and bone marrow colonization.

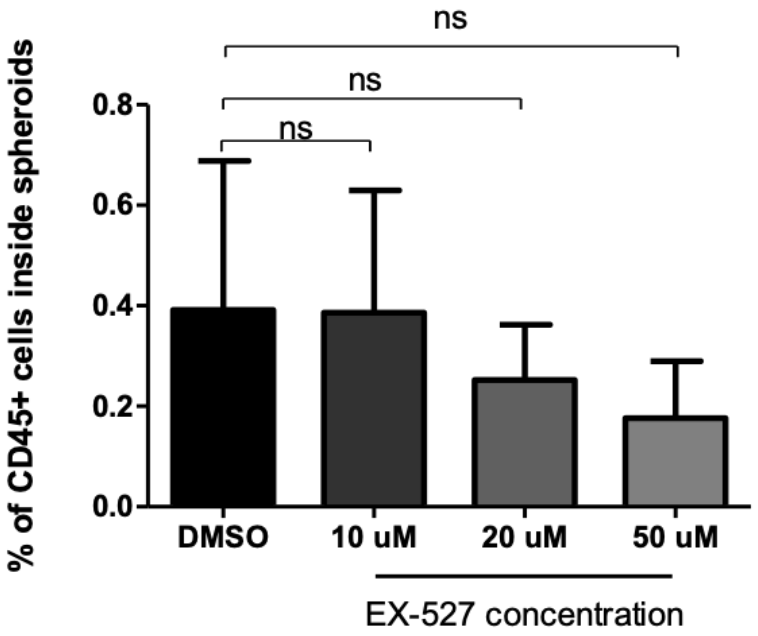

Figure 18. **Percentage of CD45$^+$ cells inside OP9 spheroids after EX-527 treatment.** 2.5x10^4 REH cells were treated with 10, 20 and 50 μM EX-527 for 30 minutes and washed with PBS. Co-cultures with OP9 stromal cells were incubated for 4 hours. Then, spheroids were recovered, washed and disaggregated to obtain single cell suspensions. Number of CD45$^+$ cells inside the spheroids was analyzed by flow cytometry. Control cells were treated with DMSO. Data are represented as % of CD45$^+$ cells of singlets ± SD. Unpaired Student's t-test was performed to determine statistical significance. n=5. ns: not significant.

9. DISCUSSION

The actin binding protein cortactin is regulated by several post-translational modifications in response to growth factors, chemokines, and integrin activation, thus linking different signaling pathways with cytoskeletal reorganization. Src kinase and SIRT1 deacetylase target cortactin to modify its ability to interact with scaffold proteins and F-actin. Our current work suggests that pharmacological inhibition of src kinase and SIRT1 deacetylase significantly reduces transendothelial migration and bone marrow colonization capabilities of leukemic B cells.

In cancer, the phosphorylation and acetylation status of many proteins is altered by dysregulated kinases and deacetylases (Parbin et al., 2014) , which are becoming targets for leukemia treatment. For example, PP2 is a selective inhibitor of Src family kinases (Bain et al., 2008) which interferes with the binding of the substrate (Karni et al., 2003). Among HDACs inhibitors, EX-527 is a SIRT1 inhibitor that occupies the nicotinamide site and stabilizes a closed conformation preventing product release (Gertz et al., 2013). Src and SIRT1 are enzymes that are activated by chemokines such as the B cell chemoattractant CXCL12; and target cortactin. Consequently, we aimed to treat leukemic REH cells, overexpressing cortactin, with these inhibitors prior to functional assays.

CXCL12 is an essential chemokine which acts as a chemoattractant for many hematopoietic cells, including leukemic B cells (Chiarini et al., 2016). When CXCL12 binds to its receptor CXCR4, highly expressed in B-ALL cells (Shen et al., 2001), different signaling pathways such as Rho GTPases and Erk and Src kinase regulating actin dynamics are activated (Perim et al., 2015a). CXCR4 activation leads to cortactin recruitment to the cell membrane where it becomes phosphorylated by src at Y421 in HEK and HeLa cells (Luo et al., 2006). These events trigger cortactin association with other cytoskeletal proteins such as Nck1 to enhance activation of the Arp2/3 complex and cell migration (Tehrani et al., 2007). Thus, it will be important to determine whether these observations are also true for normal and leukemic B-cells.

Given the importance of src-mediated cortactin phosphorylation, we analyzed the status of cortactin phosphorylation at Y421 in the pre-B leukemic cell line REH. Under basal conditions, we found low phosphorylation levels of cortactin, which were increased after stimulation with CXCL12 (**Figure 10)**. This is partially in agreement with a recent study describing high constitutive phosphorylation levels of cortactin at Y421 in patient-derived CLL B cells. However, in this study they did not observe an increase after CXCL12 stimulation (Martini et al., 2017), suggesting that cortactin maintains a conformation with higher affinity for F-actin and cytoskeletal proteins. We thought that the observed basal phosphorylation of cortactin may correlate with the fact that REH cells were cultured with fetal bovine serum, which contains several growth factors such as epidermal growth factor (EGF). It is well known that EGF stimulation in breast cancer cells leads to cortactin phosphorylation by src at Y421 and translocation to focal adhesions, where cortactin then contributes to remodeling of F-actin networks and regulating cell motility (Mezi et al., 2012). However, after serum starvation, we did not observe a difference in cortactin phosphorylation levels when compared to REH cells cultured with FBS (**Figure 11**), suggesting that src kinase may be constituvely hyperactivated to maintain cortactin in a hyperphosphorylated (activated) state in this leukemic B cell line. It is known that there BCR-Abl-positive acute leukemias exist, which are characterized by hyperactivated src kinase (Nimmanapalli et al., 2002). Yet, there are no reports supporting src mutations or hyperactivation in REH cell line. Given that REH cells are BCR negative (Köhrer, Havranek, Seyfried, & Hurtz, 2016), it will be important to experimentally show src hyperactivation in REH cells by comparing with normal B cells. It will also be important to include normal B cells as control to compare cortactin phosphorylation levels in normal vs leukemic B cells. Unfortunately, we have so far not had access to such controls. Moreover, it will be critical to determine the levels of phospho-cortactin (Y421) in B-ALL cells from patients. Given that cortactin has previously be suggested as biomarker to identify B-ALL patients at high risk to suffer relapse (Velázquez-Avila

et al., 2018), correlating cortactin phosphorylation with clinical parameters could be a more sophisticated tool to even better stratify such high-risk patients.

Previous studies have shown a critical role for src-mediated phosphorylation of cortactin in endothelial cells. Endothelial F-actin, cortactin and ICAM-1 colocalize in ring-like structures surrounding transmigrating leukocytes, where tyrosine phosphorylation of cortactin by src kinase is required for remodeling of the actin cytoskeleton and ICAM-1 clustering, leading to leukocyte diapedesis through endothelial cell-cell junctions. Thus, src-phosphorylated cortactin is intimately involved in generation and maintenance of endothelial-leukocyte interactions during transmigration (Yang, Kowalski, Zhan, et al., 2006). However, whether phosphorylation of cortactin by src in leukemic B cells is required to facilitate transendothelial migration was hitherto unknown. It has been shown before by our group, that cortactin overexpression in REH cells was necessary for efficient transmigration. Therefore, we focused on investigating whether src kinase inhibition in leukemic cells influence their transmigration abilities *in vitro*. PP2 is a selective src family inhibitor, with an inhibition efficiency of around 90% for src (Bain et al., 2008). Of note, chemotaxis of CLL cells from patients towards CXCL12 was impaired after treating the cells with PP2 (Martini et al., 2017). Similarly, in the transendothelial migration assays we performed, after PP2 treatment of REH cells we observed a remarkable decrease on the percentage of cells that were able to transmigrate across HUVEC monolayers (**Figure 15**). Also, we confirmed that phospho-cortactin (Y421) levels were reduced after PP2 treatment (**Figure 14**), which strongly supports the importance of cortactin phosphorylation by src in the transmigration of leukemic B cells in response to the chemoattractant CXCL12. To ensure the used concentrations did not affect REH cells viability, we performed MTT assays revealing that the IC_{50} concentrations were much higher than the ones applied in functional assays (**Figure 12)**, corroborating that the observed effects are not due to induced cell death (**Figure 13)**.

Within the bone marrow microenvironment, stromal and endothelial cells constituvely produce CXCL12 to support migration, proliferation and differentiation of hematopoietic cells. Accordingly, the CXCL12/CXCR4 axis is also critical for leukemic cells which colonize and reside in the BM as they express high levels of CXCR4 and bind CXCL12, secreted by multiple BM stromal cells (Duarte et al., 2018). After performing colonization assays with spheroids of stromal cells as model resembling the bone marrow microenvironment (Balandrán et al., 2017b), we found that after src inhibition with PP2, REH cells reduced their ability to colonize the spheroids (**Figure 16**). These results are in agreement with our previous observation that cortactin overexpression in REH cells was required for efficient BM spheroid colonization. Our data now add that src activation is also required for this process, suggesting that src-mediated cortactin phosphorylation is a key factor triggering BM colonization by REH cells.

Besides phosphorylation, cortactin is also regulated by acetylation and deacetylation on lysine residues within its tandem repeat region. These post-translational modifications modulate cortactin affinity for F-actin (Kirkbride et al., 2011). When cortactin is acetylated by PCAF or p300, affinity for F-actin is diminished. By contrast, when cortactin is deacetylated by SIRT1 or HDAC6, affinity for F-actin is increased, thus favoring cell migration (X. Zhang et al., 2007; Y. Zhang et al., 2009). EX-527 is a selective SIRT1 inhibitor (Gertz et al., 2013). After we treated pre-B ALL REH cells with EX-527, we observed a significant reduction of the percentage of transmigrated cells (**Figure 17**), which could be due to reduced cortactin-mediated F-actin remodeling required for transmigration. Similarly, we observed a decrease of the percentage of cells that were able to colonize bone marrow spheroids (**Figure 18**), suggesting that maintaining cortactin in a deacetylated state may be important for leukemic B cells to achieve BM colonization. Again, MTT viability assays revealed that the IC_{50} concentration for EX-527 was much higher than the applied concentration, confirming that the observed effects were not due to induced cell death

In future experiments, we need to validate whether cortactin acetylation levels are increasing after the inhibition of its deacetylase SIRT1, using an acetyl-cortactin specific antibody as it was shown previously (Y. Zhang et al., 2009). Another study showed that only acetylated cortactin was able to translocate into the nucleus (Motonishi et al., 2015). Thus, it will be interesting to determine how cortactin localization is regulated in leukemic B cells before and after treatments with the pharmacological inhibitors. Of note, SIRT1 is a nuclear deacetylase, but it recently has been reported to be found also in the cytoplasm of cancer cells, in response to PI3K/Akt signaling (Byles et al., 2010). Given that the PI3K/Akt signaling pathway is activated in response to CXCL12 binding to its receptor CXCR4 (Chatterjee et al., 2014), it may trigger SIRT1 localization into the cytoplasm of leukemic B cells favoring cortactin deacetylation. Further experiments are needed to confirm this idea, such as assessing SIRT1 and cortactin localization within leukemic cells in basal condition, under CXCL12 stimulation and after treatment with pharmacological inhibitor.

Cortactin is not the only substrate of src and SIRT1. Hence, it is critical for us to validate whether using cortactin KD leukemic B cells treated with PP2 and EX-527 would, similarly to WT REH cells, also reduce transmigration and BM spheroids colonization. Additionally, our findings encourage us to evaluate whether cortactin mutants of acetylation or phosphorylation residues would equally show impaired transmigration, allowing us to confirm that src mediated phosphorylation and SIRT1 deacetylation of cortactin are critical modifications in leukemic B cells. Thus to specifically connect the data from our inhibition experiments to cortactin, we will pursue the above described approaches in the future to validate our findings. However, so far, our findings at least suggest the importance of cortactin post-translational modifications for leukemic B cell migratory processes.

Additionally, there is a report demonstrating that cortactin is phosphorylated by Erk in S405, which enhances cortactin binding and activation of N-WASP in leukemic cells. In these cells, when src phosphorylates cortactin previously phosphorylated by Erk, the ability of cortactin to activate N-WASP is inhibited (Martinez-Quiles, Ho, Kirschner,

Ramesh, & Geha, 2004), suggesting that cortactin is regulated by phosphorylation-dependent on/off signals. This is a key aspect of cortactin regulation, which remains to be studied in REH cells: how phosphorylation of one residue modulates the phosphorylation of others. Also, the relationship between phosphorylation and acetylation of cortactin, i.e. whether phosphorylation would promote its deacetylation to enhance cell migration or vice versa, will be important to test with different mutant forms of cortactin within the phosphorylation and acetylation residues. Testing a combination of src and SIRT1 inhibitors in functional assays may unravel an additive effect suggesting how post-translational modifications of cortactin may be regulated.

Taken together, the results obtained in this work suggest the importance of src and SIRT1 activation and cortactin post-translational modifications for migratory processes of leukemic B cells, as we observed impaired transmigration and BM colonization capabilities after Src and SIRT1 inhibition. Therefore, inhibition of these enzymes targeting cortactin may be a suitable therapeutic approach for treating B-ALL patients.

10. CONCLUSION

Pharmacological inhibition of Src kinase and SIRT1 deacetylase, enzymes which regulate cortactin functions, robustly reduce the abilities of REH cells to transmigrate and colonize BM spheroids suggesting that a phosphorylated and deacetylated cortactin is crucial to maintain REH cells aggressiveness. Post-translational modifications of cortactin may be useful as a potential new therapeutic target for high risk B-ALL patients to prevent organ infiltration and disease relapse.

11. PERSPECTIVES

1. To treat leukemic B cells with Tubacin (HDAC6 inhibitor) and evaluate their ability to transmigrate and colonize BM.
2. To generate a cortactin-KD in REH cells and treat the cells with inhibitors to evaluate the importance of cortactin as target of Src kinase and SIRT1 and HDAC6 deacetylases in transmigration.
3. To validate the acetylated status of cortactin after SIRT1 inhibition with EX-527.
4. To determine cortactin localization in leukemic cells before and after treatment with the pharmacological inhibitors.
5. To express mutant forms of cortactin within the phosphorylation and acetylation sites deleted to validate the importance of these post-translational modifications for leukemic B cell transmigration and BM colonization.
6. To analyze phosphorylated and acetylated status of cortactin in patient-derived B-ALL samples.

12. REFERENCES

Alvarez, R. H., Kantarjian, H. M., & Cortes, J. E. (2006). The role of Src in solid and hematologic malignancies: Development of new-generation src inhibitors. *Cancer*, *107*(8), 1918–1929. https://doi.org/10.1002/cncr.22215

Ayala, F., Dewar, R., Kieran, M., & Kalluri, R. (2009). Contribution of bone microenvironment to leukemogenesis and leukemia progression. *Leukemia*, *23*(12), 2233–2241. https://doi.org/10.1038/leu.2009.175

Bailey, L. C., Lange, B. J., Rheingold, S. R., & Bunin, N. J. (2008). Bone-marrow relapse in paediatric acute lymphoblastic leukaemia. *The Lancet Oncology*, *9*(9), 873–883. https://doi.org/10.1016/S1470-2045(08)70229-8

Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C. J., Mclauchlan, H., … Cohen, P. (2008). The selectivity of protein kinase inhibitors: a further update. *Biochemical Journal*, *408*(3), 297–315. https://doi.org/10.1042/bj20070797

Balandrán, J. C., Purizaca, J., Enciso, J., Dozal, D., Sandoval, A., Jiménez-Hernández, E., … Pelayo, R. (2017a). Pro-inflammatory-related loss of CXCL12 niche promotes acute lymphoblastic leukemic progression at the expense of normal lymphopoiesis. *Frontiers in Immunology*, *7*(JAN), 1–14. https://doi.org/10.3389/fimmu.2016.00666

Balandrán, J. C., Purizaca, J., Enciso, J., Dozal, D., Sandoval, A., Jiménez-Hernández, E., … Pelayo, R. (2017b). Pro-inflammatory-related loss of CXCL12 niche promotes acute lymphoblastic leukemic progression at the expense of normal lymphopoiesis. *Frontiers in Immunology*, *7*(JAN), 1–14. https://doi.org/10.3389/fimmu.2016.00666

Balandrán, J. C., Vadillo, E., Dozal, D., Reyes-López, A., Sandoval-Cabrera, A., Laffont-Ortiz, M. D., … Pelayo, R. (2016). Analysis of Normal Hematopoietic Stem and Progenitor Cell Contents in Childhood Acute Leukemia Bone Marrow. *Archives of Medical Research*, *47*(8), 629–643. https://doi.org/10.1016/j.arcmed.2016.12.004

Bhojwani, D., & Pui, C. H. (2013). Relapsed childhood acute lymphoblastic leukaemia. *The Lancet Oncology*, *14*(6), e205–e217. https://doi.org/10.1016/S1470-2045(12)70580-6

Bild, A. H., Yao, G., Chang, J. T., Wang, Q., Potti, A., Chasse, D., … Nevins, J. R. (2006). Oncogenic pathway signatures in human cancers as a guide to targeted therapies. *Nature*, *439*(7074), 353–357. https://doi.org/10.1038/nature04296

Biscardi, J. S., Ishizawar, R. C., Silva, C. M., & Parsons, S. J. (2000). Tyrosine kinase signalling in breast cancer. Epidermal growth factor receptor: Convergence point for signal integration and diversification. *Breast Cancer Research*, *2*(3), 184–190. https://doi.org/10.1186/bcr52

Bjorge, J. D., Jakymiw, A., & Fujita, D. J. (2000). Selected glimpses into the activation and function of Src kinase. *Oncogene*, *19*(49), 5620–5635. https://doi.org/10.1038/sj.onc.1203923

Boyle, S. N., Michaud, G. A., Schweitzer, B., Predki, P. F., & Koleske, A. J. (2007). A Critical Role for Cortactin Phosphorylation by Abl-Family Kinases in PDGF-Induced Dorsal-Wave Formation. *Current Biology*, *17*(5), 445–451. https://doi.org/10.1016/j.cub.2007.01.057

Byles, V., Chmilewski, L. K., Wang, J., Zhu, L., Forman, L. W., Faller, D. V., & Dai, Y. (2010). Aberrant cytoplasm localization and protein stability of SIRT1 is regulated by PI3K/IGF-1R signaling in human cancer cells. *International Journal of Biological Sciences*, *6*(6), 599–612. https://doi.org/10.7150/ijbs.6.599

Chatterjee, S., Behnam Azad, B., & Nimmagadda, S. (2014). *The intricate role of CXCR4 in cancer*. *Advances in Cancer Research* (1st ed., Vol. 124). Elsevier Inc. https://doi.org/10.1016/B978-0-12-411638-2.00002-1

Cheng, Y., Qu, J., Che, X., Xu, L., Song, N., Ma, Y., … Liu, Y. (2017). CXCL12/SDF-1α induces migration via SRC-mediated CXCR4-EGFR cross-talk in gastric cancer cells. *Oncology Letters*, *14*(2), 2103–2110. https://doi.org/10.3892/ol.2017.6389

Chiaretti, S., Zini, G., & Bassan, R. (2014). Diagnosis and subclassification of acute lymphoblastic leukemia. *Mediterranean Journal of Hematology and Infectious Diseases*, *6*(1). https://doi.org/10.4084/mjhid.2014.073

Chiarini, F., Lonetti, A., Evangelisti, C., Buontempo, F., Orsini, E., Evangelisti, C., … Martelli, A. M. (2016). Advances in understanding the acute lymphoblastic leukemia bone marrow microenvironment: From biology to therapeutic targeting. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1863*(3), 449–463. https://doi.org/10.1016/j.bbamcr.2015.08.015

Choudhary, C., Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., … Mann, M. (2009). Lysine Acetylation Targets Protein Complexes and Co-Regulates Major (suppli).pdf. *Science*, *325*(August), 834–840. https://doi.org/10.1126/science.1175371

Cobaleda, C., & Sánchez-García, I. (2009). B-cell acute lymphoblastic leukaemia: Towards understanding its cellular origin. *BioEssays*, *31*(6), 600–609. https://doi.org/10.1002/bies.200800234

Cosen-Binker, L. I. (2006). Cortactin: The Gray Eminence of the Cytoskeleton. *Physiology*, *21*(5), 352–361. https://doi.org/10.1152/physiol.00012.2006

Crazzolara, R., Kreczy, a, Mann, G., Heitger, a, Eibl, G., Fink, F. M., … Meister, B. (2001). High expression of the chemokine receptor CXCR4 predicts extramedullary organ infiltration in childhood acute lymphoblastic leukaemia. *British Journal of Haematology*, *115*(3), 545–553. https://doi.org/10.1046/j.1365-2141.2001.03164.x

Cummings, B. S., & Schnellmann, R. G. (2004). Measurement of Cell Death in Mammalian Cells. *Current Protocols in Pharmacology*, (1), 1–22. https://doi.org/10.1002/0471141755.ph1208s25

De Kimpe, L., Janssens, K., Derua, R., Armacki, M., Goicoechea, S., Otey, C., … Van Lint, J. (2009).

Characterization of cortactin as an in vivo protein kinase D substrate: Interdependence of sites and potentiation by Src. *Cellular Signalling*, *21*(2), 253–263. https://doi.org/10.1016/j.cellsig.2008.10.015

Doan, P. L., & Chute, J. P. (2012). The vascular niche: Home for normal and malignant hematopoietic stem cells. *Leukemia*, *26*(1), 54–62. https://doi.org/10.1038/leu.2011.236

Duarte, D., Hawkins, E. D., & Lo Celso, C. (2018). The interplay of leukemia cells and the bone marrow microenvironment. *Blood*, *131*(14), blood-2017-12-784132. https://doi.org/10.1182/blood-2017-12-784132

Ebinger, S., Özdemir, E. Z., Ziegenhain, C., Tiedt, S., Castro Alves, C., Grunert, M., … Jeremias, I. (2016). Characterization of Rare, Dormant, and Therapy-Resistant Cells in Acute Lymphoblastic Leukemia. *Cancer Cell*, *30*(6), 849–862. https://doi.org/10.1016/j.ccell.2016.11.002

Eckschlager, T., Plch, J., Stiborova, M., & Hrabeta, J. (2017). Histone Deacetylase Inhibitors as Anticancer Drugs. *International Journal of Molecular Sciences*, *18*(7), 1–25. https://doi.org/10.3390/ijms18071414

Eiseler, T., Hausser, A., De Kimpe, L., Van Lint, J., & Pfizenmaier, K. (2010). Protein kinase D controls actin polymerization and cell motility through phosphorylation of cortactin. *Journal of Biological Chemistry*, *285*(24), 18672–18683. https://doi.org/10.1074/jbc.M109.093880

Ganju, R. K., Brubaker, S. A., Meyer, J., Dutt, P., Yang, Y., Qin, S., … Groopman, J. E. (1998). The a-Chemokine, Stromal Cell-derived Factor-1a, Binds to the Transmembrane G-protein-coupled CXCR-4 Receptor and Activates Multiple Signal Transduction Pathways. *The Journal of Biological Chemistry*, *273*(36), 23169–23175.

Gattazzo, C., Martini, V., Frezzato, F., Trimarco, V., Tibaldi, E., Castelli, M., … Trentin, L. (2014). Cortactin, another player in the Lyn signaling pathway, is over-expressed and alternatively spliced in leukemic cells from patients with B-cell chronic lymphocytic leukemia. *Haematologica*, *99*(6), 1069–1077. https://doi.org/10.3324/haematol.2013.090183

Gaynon, P. S. (2005). Childhood acute lymphoblastic leukaemia and relapse. *British Journal of Haematology*, *131*(5), 579–587. https://doi.org/10.1111/j.1365-2141.2005.05773.x

Gertz, M., Fischer, F., Nguyen, G. T. T., Lakshminarasimhan, M., Schutkowski, M., Weyand, M., & Steegborn, C. (2013). Ex-527 inhibits Sirtuins by exploiting their unique NAD+-dependent deacetylation mechanism. *Proceedings of the National Academy of Sciences*, *110*(30), E2772–E2781. https://doi.org/10.1073/pnas.1303628110

GLOBOCAN. (2018). Global Cancer Observatory. Retrieved June 23, 2019, from https://gco.iarc.fr/

Gossai, N. P., & Gordon, P. M. (2017). The Role of the Central Nervous System Microenvironment in Pediatric Acute Lymphoblastic Leukemia. *Frontiers in Pediatrics*, *5*(April), 1–8. https://doi.org/10.3389/fped.2017.00090

Grassart, A., Meas-Yedid, V., Dufour, A., Olivo-Marin, J. C., Dautry-Varsat, A., & Sauvonnet, N. (2010). Pak1 phosphorylation enhances cortactin-N-WASP interaction in clathrin-caveolin-independent endocytosis. *Traffic*, *11*(8), 1079–1091. https://doi.org/10.1111/j.1600-0854.2010.01075.x

Greaves, M. (2018). A causal mechanism for childhood acute lymphoblastic leukaemia. *Nature Reviews Cancer*, 1. https://doi.org/10.1038/s41568-018-0015-6

Greaves, M. F., & Wiemels, J. (2003). Origins of chromosome translocations in childhood leukaemia. *Nature Reviews Cancer*, *3*, 639. Retrieved from http://dx.doi.org/10.1038/nrc1164

Guarino, M. (2010). Src signaling in cancer invasion. *Journal of Cellular Physiology*, *223*(1), 14–26. https://doi.org/10.1002/jcp.22011

Haberland, M., Montgomery, R. L., & Olson, E. N. (2011). Physiology : Implications for Disease and Therapy. *Nature Reviews. Genetics*, *10*(1), 32–42. https://doi.org/10.1038/nrg2485.The

Haggarty, S. J., Koeller, K. M., Wong, J. C., Grozinger, C. M., & Schreiber, S. L. (2003). Domain-selective small-molecule inhibitor of tubulin deacetylation. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(8), 4389–4394.

Heazlewood, S. Y., Oteiza, A., Cao, H., & Nilsson, S. K. (2014). Analyzing hematopoietic stem cell homing, lodgment, and engraftment to better understand the bone marrow niche. *Annals of the New York Academy of Sciences*, *1310*(1), 119–128. https://doi.org/10.1111/nyas.12329

Hofmann, F. B., Board, E., Beavo, J. A., Barrett, J. E., Ganten, D., Geppetti, P., … Rosenthal, W. (2011). *Histone Deacetylases: the Biology and Clinical Implication* (Vol. 206). https://doi.org/10.1007/978-3-642-21631-2

Huang, C., Liu, J., Haudenschild, C. C., & Zhan, X. (1998). The role of tyrosine phosphorylation of cortactin in the locomotion of endothelial cells. *Journal of Biological Chemistry*, *273*(40), 25770–25776. https://doi.org/10.1074/jbc.273.40.25770

Huang, J., Asawa, T., Takato, T., & Sakai, R. (2003). Cooperative Roles of Fyn and Cortactin in Cell Migration of Metastatic Murine Melanoma. *Journal of Biological Chemistry*, *278*(48), 48367–48376. https://doi.org/10.1074/jbc.M308213200

Huveneers, S., & Danen, E. H. J. (2009). Adhesion signaling - crosstalk between integrins, Src and Rho. *Journal of Cell Science*, *122*(8), 1059–1069. https://doi.org/10.1242/jcs.039446

Hyun-Jung, K., & Suk-Chul, B. (2011). Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs. *American Journal of Translational Research*, *3*(2), 116–179. https://doi.org/www.ajtr.org /ISSN:1943-8141/AJTR1012002

Ito, A., Shimazu, T., Maeda, S., Shah, A. A., Tsunoda, T., Iemura, S., … Yoshida, M. (2015). The subcellular localization and activity of cortactin is regulated by acetylation and interaction with Keap1. Ito A, Shimazu T, Maeda S, Shah AA, Tsunoda T, Iemura S, Natsume T, Suzuki T, Motohashi H, Yamamoto M, Yoshida M. The subcellular localization a. *Science Signaling*, *8*(404),

ra120. https://doi.org/10.1126/scisignal.aad0667

Jo, D. Y., Rafii, S., Hamada, T., & Moore, M. A. (2000). Chemotaxis of primitive hematopoietic cells in response to stromal cell-derived factor-1. *J Clin Invest*, *105*(1), 101–111. https://doi.org/10.1172/JCI7954

Kaluza, D., Kroll, J., Gesierich, S., Yao, T. P., Boon, R. A., Hergenreider, E., … Urbich, C. (2011). Class IIb HDAC6 regulates endothelial cell migration and angiogenesis by deacetylation of cortactin. *EMBO Journal*, *30*(20), 4142–4156. https://doi.org/10.1038/emboj.2011.298

Karni, R., Mizrachi, S., Reiss-Sklan, E., Gazit, A., Livnah, O., & Levitzki, A. (2003). The pp60c-Src inhibitor PP1 is non-competitive against ATP. *FEBS Letters*, *537*(1–3), 47–52. https://doi.org/10.1016/S0014-5793(03)00069-3

Kirkbride, K. C., Sung, B. H., Sinha, S., & Weaver, A. M. (2011). Cortactin: A multifunctional regulator of cellular invasiveness. *Cell Adhesion and Migration*, *5*(2), 187–198. https://doi.org/10.4161/cam.5.2.14773

Köhrer, S., Havranek, O., Seyfried, F., & Hurtz, C. (2016). Pre-BCR Signaling In Precursor B-cell Acute Lymphoblastic Leukemia regulates PI3K/AKT, FOXO1, and MYC, and can be targeted by SYK inhibition. *Leukemia*, *30*(6), 1246–1254. https://doi.org/10.1038/leu.2016.9.

Kozyreva, V. K., McLaughlin, S. L., Livengood, R. H., Calkins, R. A., Kelley, L. C., Rajulapati, A., … Pugacheva, E. N. (2014). NEDD9 Regulates Actin Dynamics through Cortactin Deacetylation in an AURKA/HDAC6-Dependent Manner. *Molecular Cancer Research*, *12*(5), 681–693. https://doi.org/10.1158/1541-7786.MCR-13-0654

Li, Y., & Seto, E. (2016). HDACs and HDAC Inhibitors in Cancer. *Cold Spring Harb Perspect Med*, *3*(6), 1–10.

Locatelli, F., Schrappe, M., Bernardo, M. E., & Rutella, S. (2012). How i treat relapsed childhood acute lymphoblastic leukemia. *Blood*, *120*(14), 2807–2816. https://doi.org/10.1182/blood-2012-02-265884

Loghavi, S., Kutok, J. L., & Jorgensen, J. L. (2015). B-acute lymphoblastic leukemia/lymphoblastic lymphoma. *American Journal of Clinical Pathology*, *144*(3), 393–410. https://doi.org/10.1309/AJCPAN7BH5DNYWZB

Lua, B. L., & Low, B. C. (2005). Cortactin phosphorylation as a switch for actin cytoskeletal network and cell dynamics control. *FEBS Letters*, *579*(3), 577–585. https://doi.org/10.1016/j.febslet.2004.12.055

Luo, C., Pan, H., Mines, M., Watson, K., Zhang, J., & Fan, G. H. (2006). CXCL12 induces tyrosine phosphorylation of cortactin, which plays a role in CXC chemokine receptor 4-mediated extracellular signal-regulated kinase activation and chemotaxis. *Journal of Biological Chemistry*, *281*(40), 30081–30093. https://doi.org/10.1074/jbc.M605837200

MacGrath, S. M., & Koleske, A. J. (2012). Cortactin in cell migration and cancer at a glance. *Journal of Cell Science*, *125*(7), 1621–1626. https://doi.org/10.1242/jcs.093781

Magrath, I., Steliarova-Foucher, E., Epelman, S., Ribeiro, R. C., Harif, M., Li, C. K., … Howard, S. C. (2013). Paediatric cancer in low-income and middle-income countries. *The Lancet Oncology*, *14*(3), e104–e116. https://doi.org/10.1016/S1470-2045(13)70008-1

Martin, G. S. (2001). The hunting of the Src. *Nature Reviews Molecular Cell Biology*, *2*(6), 467–475. https://doi.org/10.1038/35073094

Martinez-Quiles, N., Ho, H.-Y. H., Kirschner, M. W., Ramesh, N., & Geha, R. S. (2004). Erk/Src Phosphorylation of Cortactin Acts as a Switch On-Switch Off Mechanism That Controls Its Ability To Activate N-WASP. *Molecular and Cellular Biology*, *24*(12), 5269–5280. https://doi.org/10.1128/MCB.24.12.5269-5280.2004

Martini, V., Gattazzo, C., Frezzato, F., Trimarco, V., Pizzi, M., Chiodin, G., … Trentin, L. (2017). Cortactin, a Lyn substrate, is a checkpoint molecule at the intersection of BCR and CXCR4 signalling pathway in chronic lymphocytic leukaemia cells. *British Journal of Haematology*, *178*(1), 81–93. https://doi.org/10.1111/bjh.14642

Matsuo, Y., & Drexler, H. G. (1998). Establishment and characterization of human B cell precursor-leukemia cell lines. *Leukemia Research*, *22*(7), 567–579. https://doi.org/10.1016/S0145-2126(98)00050-2

Mazo, I. B., Massberg, S., & von Andrian, U. H. (2011). Hematopoietic stem and progenitor cell trafficking. *Trends in Immunology*, *32*(10), 493–503. https://doi.org/10.1016/j.it.2011.06.011

Mezi, S., Todi, L., Orsi, E., Angeloni, A., & Mancini, P. (2012). Involvement of the Src-cortactin pathway in migration induced by IGF-1 and EGF in human breast cancer cells. *International Journal of Oncology*, *41*(6), 2128–2138. https://doi.org/10.3892/ijo.2012.1642

Mohle, R., Bautz, F., Rafii, S., Moore, M. A., Brugger, W., & Kanz, L. (1998). The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. *Blood*, *91*(12), 4523–4530. https://doi.org/10.1182/blood.v99.8.2977

Motonishi, S., Nangaku, M., Wada, T., Ishimoto, Y., Ohse, T., Matsusaka, T., … Inagi, R. (2015). Sirtuin1 Maintains Actin Cytoskeleton by Deacetylation of Cortactin in Injured Podocytes. *J Am Soc Nephrol*, *26*, 1939–1959. https://doi.org/10.1681/ASN.2014030289

Müller, a, Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., … Zlotnik, a. (2001). Involvement of chemokine receptors in breast cancer metastasis. *Nature*, *410*(6824), 50–56. https://doi.org/10.1038/35065016

Mullighan, C. G., Phillips, L. A., Su, X., Ma, J., Miller, C. B., Shurtleff, S. A., & Downing, J. R. (2008). Genomic Analysis of the Clonal Origins of Relapsed Acute Lymphoblastic Leukemia. *Science*,

322(5906), 1377–1380. https://doi.org/10.1126/science.1164266

Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S. I., Kitamura, Y., … Kishimoto, T. (1996). Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature*. https://doi.org/10.1038/382635a0

Nakane, K., Fujita, Y., Terazawa, R., Atsumi, Y., Kato, T., Nozawa, Y., … Ito, M. (2012). Inhibition of cortactin and SIRT1 expression attenuates migration and invasion of prostate cancer DU145 cells. *International Journal of Urology*, *19*(1), 71–79. https://doi.org/10.1111/j.1442-2042.2011.02888.x

Nies, B., Bodey, G., Thomas, L., Brecher, G., & Freireich, E. (1965). The persistance of extramedullary leukemic infiltrates during bone marrow remission of acute leukemia. *Blood*, *26*(2).

Nimmanapalli, R., O'Bryan, E., Huang, M., Bali, P., Burnette, P. K., Loughran, T., … Bhalla, K. (2002). Molecular characterization and sensitivity of STI-571 (imatinib mesylate, Gleevec)-resistant, Bcr-Abl-positive, human acute leukemia cells to SRC kinase inhibitor PD180970 and 17-allylamino-17-demethoxygeldanamycin. *Cancer Research*, *62*(20), 5761–5769.

Omatsu, Y., Sugiyama, T., Kohara, H., Kondoh, G., Fujii, N., Kohno, K., & Nagasawa, T. (2010). The Essential Functions of Adipo-osteogenic Progenitors as the Hematopoietic Stem and Progenitor Cell Niche. *Immunity*, *33*(3), 387–399. https://doi.org/10.1016/j.immuni.2010.08.017

Parbin, S., Kar, S., Shilpi, A., Sengupta, D., Deb, M., Rath, S. K., & Patra, S. K. (2014). Histone Deacetylases: A Saga of Perturbed Acetylation Homeostasis in Cancer. *Journal of Histochemistry and Cytochemistry*, *62*(1), 11–33. https://doi.org/10.1369/0022155413506582

Paul, S., Kantarjian, H., & Jabbour, E. J. (2016). Adult Acute Lymphoblastic Leukemia. *Mayo Clinic Proceedings*, *91*(11), 1645–1666. https://doi.org/10.1016/j.mayocp.2016.09.010

Pérez-Saldivar, M. L., Fajardo-Guti?rrez, A., Bern?ldez-R?os, R., Mart?nez-Avalos, A., Medina-Sanson, A., Espinosa-Hern?ndez, L., … Mejia-Arangure, J. M. (2011). Childhood acute leukemias are frequent in Mexico City: Descriptive epidemiology. *BMC Cancer*, *11*. https://doi.org/10.1186/1471-2407-11-335

Perim, A. D. L., Amarante, M. K., Guembarovski, R. L., De Oliveira, C. E. C., & Watanabe, M. A. E. (2015a). CXCL12/CXCR4 axis in the pathogenesis of acute lymphoblastic leukemia (ALL): A possible therapeutic target. *Cellular and Molecular Life Sciences*, *72*(9), 1715–1723. https://doi.org/10.1007/s00018-014-1830-x

Perim, A. D. L., Amarante, M. K., Guembarovski, R. L., De Oliveira, C. E. C., & Watanabe, M. A. E. (2015b). CXCL12/CXCR4 axis in the pathogenesis of acute lymphoblastic leukemia (ALL): A possible therapeutic target. *Cellular and Molecular Life Sciences*, *72*(9), 1715–1723. https://doi.org/10.1007/s00018-014-1830-x

Pramanik, R., Sheng, X., Ichihara, B., Heisterkamp, N., & Mittelman, S. D. (2013). Adipose tissue

attracts and protects acute lymphoblastic leukemia cells from chemotherapy. *Leukemia Research*, *37*(5), 503–509. https://doi.org/10.1016/j.leukres.2012.12.013

Pui, C.-H., & Jeha, S. (2007). New therapeutic strategies for the treatment of acute lymphoblastic leukaemia. *Nature Reviews Drug Discovery*, *6*, 149. Retrieved from https://doi.org/10.1038/nrd2240

Pui, C.-H., Robison, L., & Look, T. (2008). Acute Lymphoblastic Leukaemia. *The Lancet Oncology*, *371*, 1030–1043. https://doi.org/10.1007/978-1-61779-074-4_8

Purizaca, J., Meza, I., & Pelayo, R. (2012). Early Lymphoid Development and Microenvironmental Cues in B-cell Acute Lymphoblastic Leukemia. *Archives of Medical Research*, *43*(2), 89–101. https://doi.org/10.1016/j.arcmed.2012.03.005

Ryan Bishop. (2010). Applications of fluorescencein situ hybridization (FISH) in detecting genetic aberrations of medical significance. *Bioscience Horizons*, *3*(1), 85–95.

Schnoor, M. (2015). Endothelial Actin-Binding Proteins and Actin Dynamics in Leukocyte Transendothelial Migration. *The Journal of Immunology*, *194*(8), 3535–3541. https://doi.org/10.4049/jimmunol.1403250

Schnoor, M., Alcaide, P., Voisin, M. B., & Van Buul, J. D. (2015). Crossing the Vascular Wall: Common and Unique Mechanisms Exploited by Different Leukocyte Subsets during Extravasation. *Mediators of Inflammation*, *2015*(Figure 1). https://doi.org/10.1155/2015/946509

Schnoor, M., Stradal, T. E., & Rottner, K. (2017). Cortactin: Cell Functions of A Multifaceted Actin-Binding Protein. *Trends in Cell Biology*, *28*(2), 79–98. https://doi.org/10.1016/j.tcb.2017.10.009

Shen, W., Bendall, L. J., Gottlieb, D. J., & Bradstock, K. F. (2001). The chemokine receptor CXCR4 enhances integrin-mediated in vitro adhesion and facilitates engraftment of leukemic precursor-B cells in the bone marrow. *Experimental Hematology*, *29*(12), 1439–1447. https://doi.org/10.1016/S0301-472X(01)00741-X

Smith, J. N. P., & Calvi, L. M. (2013). Concise review: Current concepts in bone marrow microenvironmental regulation of hematopoietic stem and progenitor cells. *Stem Cells (Dayton, Ohio)*, *31*(6), 1044–1050. https://doi.org/10.1002/stem.1370

Spiegel, A., Kollet, O., Peled, A., Abel, L., Nagler, A., Bielorai, B., … Lapidot, T. (2008). result of altered CXCR4 expression and signaling Unique SDF-1 – induced activation of human precursor-B ALL cells as a result of altered CXCR4 expression and signaling. *Blood*, *103*(8), 2900–2907. https://doi.org/10.1182/blood-2003-06-1891

Suárez-Álvarez, B., López-Vázquez, A., & López-Larrea, C. (2012). Mobilization and homing of hematopoietic stem cells. *Advances in Experimental Medicine and Biology*, *741*, 152–170. https://doi.org/10.1007/978-1-4614-2098-9_11

Sugiyama, T., Kohara, H., Noda, M., & Nagasawa, T. (2006). Maintenance of the Hematopoietic Stem

Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches. *Immunity*, *25*(6), 977–988. https://doi.org/10.1016/j.immuni.2006.10.016

Tehrani, S., Tomasevic, N., Weed, S., Sakowicz, R., & Cooper, J. A. (2007). Src phosphorylation of cortactin enhances actin assembly. *Proceedings of the National Academy of Sciences*, *104*(29), 11933–11938. https://doi.org/10.1073/pnas.0701077104

Terwilliger, T., & Abdul-Hay, M. (2017). Acute lymphoblastic leukemia: a comprehensive review and 2017 update. *Blood Cancer Journal*, *7*(6), e577. https://doi.org/10.1038/bcj.2017.53

van Rossum, A. G. S. H., Schuuring-Scholtes, E., van Buuren-van Seggelen, V., Kluin, P. M., & Schuuring, E. (2005). Comparative genome analysis of cortactin and HS1: The significance of the F-actin binding repeat domain. *BMC Genomics*, *6*, 1–14. https://doi.org/10.1186/1471-2164-6-15

Velázquez-Avila, M., Balandrán, J. C., Ramírez-Ramírez, D., Velázquez-Avila, M., Sandoval, A., Felipe-López, A., … Pelayo, R. (2018). High cortactin expression in B-cell acute lymphoblastic leukemia is associated with increased transendothelial migration and bone marrow relapse. *Leukemia*, *1*, 1337–1348. https://doi.org/10.1038/s41375-018-0333-4

Vestweber, D. (2015). How leukocytes cross the vascular endothelium. *Nature Reviews Immunology*, *15*(11), 692–704. https://doi.org/10.1038/nri3908

Vilchis-Ordoñez, A., Contreras-Quiroz, A., Vadillo, E., Dorantes-Acosta, E., Reyes-López, A., Quintela-Nuñez Del Prado, H. M., … Pelayo, R. (2015). Bone marrow cells in acute lymphoblastic leukemia create a proinflammatory microenvironment influencing normal hematopoietic differentiation fates. *BioMed Research International*, *2015*. https://doi.org/10.1155/2015/386165

Wang, W., Liu, Y., & Liao, K. (2011). Tyrosine phosphorylation of cortactin by the FAK-Src complex at focal adhesions regulates cell motility. *BMC Cell Biology*, *12*(1), 49. https://doi.org/10.1186/1471-2121-12-49

Welch, M. D., & Mullins, R. D. (2002). Cellular Control of Actin Nucleation. *Annual Review of Cell and Developmental Biology*, *18*(1), 247–288. https://doi.org/10.1146/annurev.cellbio.18.040202.112133

Wu, H., Reynolds, A. B., Kanner, S. B., Vines, R. R., & Parsons, J. T. (1991). Identification and Characterization of a Novel Cytoskeleton-Associated pp6Osrc Substrate, *11*(10), 5113–5124.

Xu, W. S., Parmigiani, R. B., & Marks, P. A. (2007). Histone deacetylase inhibitors: Molecular mechanisms of action. *Oncogene*, *26*(37), 5541–5552. https://doi.org/10.1038/sj.onc.1210620

Yang, L., Kowalski, J. R., Yacono, P., Bajmoczi, M., Shaw, S. K., Froio, R. M., … Luscinskas, F. W. (2006). Endothelial cell cortactin coordinates intercellular adhesion molecule-1 clustering and actin cytoskeleton remodeling during polymorphonuclear leukocyte adhesion and transmigration.

Journal of Immunology (Baltimore, Md. : 1950), *177*(9), 6440–6449. https://doi.org/10.4049/jimmunol.177.9.6440

Yang, L., Kowalski, J. R., Zhan, X., Thomas, S. M., & Luscinskas, F. W. (2006). Endothelial cell cortactin phosphorylation by Src contributes to polymorphonuclear leukocyte transmigration in vitro. *Circulation Research*, *98*(3), 394–402. https://doi.org/10.1161/01.RES.0000201958.59020.1a

Yeatman, T. J. (2004). A renaissance for SRC. *Nature Reviews Cancer*, *4*(6), 470–480. https://doi.org/10.1038/nrc1366

Yin, M., Ma, W., & An, L. (2017). Cortactin in cancer cell migration and invasion. *Oncotarget*, *8*(50), 88232–88243. https://doi.org/10.18632/oncotarget.21088

Zhang, X., Yuan, Z., Zhang, Y., Yong, S., Salas-Burgos, A., Koomen, J., … Seto, E. (2007). HDAC6 Modulates Cell Motility by Altering the Acetylation Level of Cortactin. *Molecular Cell*, *27*(2), 197–213. https://doi.org/10.1016/j.molcel.2007.05.033

Zhang, Y., Zhang, M., Dong, H., Yong, S., Li, X., Olashaw, N., … Zhang, X. (2009). Deacetylation of cortactin by SIRT1 promotes cell migration. *Oncogene*, *28*(3), 445–460. https://doi.org/10.1038/onc.2008.388

Zheng, X., Baker, H., Hancock, W. S., Fawaz, F., McCaman, M., & Pungor, E. (2006). Proteomic analysis for the assessment of different lots of fetal bovine serum as a raw material for cell culture. Part IV. Application of proteomics to the manufacture of biological drugs. *Biotechnology Progress*, *22*(5), 1294–1300. https://doi.org/10.1021/bp060121o