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**Intercellular transfer of CD44 from bone marrow stroma
to chronic myeloid leukemia cells
as a driver of niche-mediated imatinib resistance**

PhD thesis

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ABSTRACT

Intercellular communication, while essential for normal organism function, is also exploited by cancer cells to interact with both malignant and non-malignant cells, thereby promoting tumor growth and adaptation to changing environmental conditions. In myeloid leukemias, including chronic myeloid leukemia (CML), leukemic cells interact directly and indirectly with various types of cells in the bone marrow niche. This contact is essential for the immune evasion, development of resistance, and the persistence of leukemic stem cells (LSC). Among the diverse modes of cell-cell communication, tunneling nanotubes (TNTs) have emerged as a distinct, unconventional type of direct intercellular communication mechanism. TNTs enable the transfer of a wide range of cargo, including mitochondria, vesicles, or proteins, between distant cells. However, despite significant advances in research on TNTs, substantial knowledge gaps persist, limiting our full understanding of these structures, their role in cancer progression and resistance, as well as their potential clinical application.

Our previous studies have shown that TNTs are exploited by bone marrow stromal cells (BMSCs) and CML cells to transfer cellular vesicles and proteins from stroma to leukemia cells. This results in the increased resistance of recipient cells to the imatinib drug.

Here, we identified CD44 as one of the stromal proteins transferred to leukemia. The general aim of this work was to examine the role of the stroma-derived CD44 protein in CML recipient cells. First, the stroma-to-leukemia CD44 transfer was confirmed using confocal microscopy and flow cytometry. The results showed that the transfer strictly depends on the direct cell-cell contact between stromal and leukemia cells. Moreover, it is mediated by tunneling nanotubes formed between connected cells. The RNA-seq analysis of CML cells that received and did not receive stromal CD44 revealed that CD44 transfer is linked to increased expression of genes related to cell adhesion, extracellular matrix remodeling, and cell migration. This was accompanied by increased activity of matrix metalloproteinases and higher invasiveness of these cells. Notably, in leukemic recipient cells, the endogenous CD44 was also upregulated at both mRNA and protein levels. Stroma-driven

upregulation of CD44 in CML cells was associated with the activation of the pro-survival ERK/AP-1 pathway and stroma-mediated protection from tyrosine kinase inhibitors. *In vitro*, pharmacological targeting of the interaction between CD44 and its main ligand, hyaluronan, disrupted stroma-driven protection and sensitized CML cells to imatinib.

An additional aim of this study was to investigate the ultrastructure of TNTs, which are the primary mediators of CD44 transfer. Focused ion beam scanning electron microscopy (FIB-SEM) confirmed the presence of TNTs in the stroma-leukemia co-cultures. However, TNTs identified by confocal microscopy using the current standard criteria do not consistently meet them when examined at higher resolution, using volume electron microscopy. Therefore, it was proposed here that current approaches should be re-evaluated. Furthermore, the presented data contradicted the theory that TNTs are completely open structures, indicating the existence of a specific regulatory system controlling cargo transfer via TNTs.

In summary, the studies presented in this dissertation provide new insights into stroma-driven resistance associated with horizontal CD44 protein transfer mediated by TNTs, leading to transcriptional reprogramming of leukemia acceptor cells toward a more invasive and resistant phenotype. Additionally, these findings deepen the understanding of TNTs' ultrastructure and critically evaluate the criteria and methodologies currently employed for their identification.

STRESZCZENIE

Komunikacja międzykomórkowa, kluczowa dla prawidłowego funkcjonowania organizmu, jest również wykorzystywana przez komórki nowotworowe do interakcji z komórkami nowotworowymi i prawidłowymi komórkami mikrośrodowiska. Sprzyja ona wzrostowi komórek nowotworowych i ich adaptacji do zmieniających się warunków. W białaczkach mieloidalnych, w tym w przewlekłej białaczce szpikowej (ang. chronic myeloid leukemia, CML), komórki białaczkowe oddziałują w sposób bezpośredni i pośredni z komórkami w niszy szpiku kostnego. Kontakt ten jest kluczowy dla unikania odpowiedzi ze strony układu odpornościowego, rozwoju oporności na leczenie oraz przetrwania białaczkowych komórek macierzystych (ang. leukemic stem cells, LSCs). Wśród różnych form komunikacji międzykomórkowej, nanotunele (ang. tunneling nanotubes, TNTs) reprezentują niekonwencjonalny mechanizm bezpośredniej komunikacji międzykomórkowej. TNTs umożliwiają transfer różnego rodzaju materiału, w tym mitochondriów, pęcherzyków i białek, pomiędzy oddalonymi od siebie komórkami. Pomimo znaczących postępów w badaniach nad TNTs, wciąż istnieją istotne luki w wiedzy, które ograniczają pełne zrozumienie działania tych struktur, ich roli w rozwoju nowotworów i oporności na leczenie, a także potencjalnego zastosowania klinicznego.

Nasze wcześniejsze badania wykazały, że TNTs powstają między komórkami podścieliska szpiku kostnego (komórki stromalne) (ang. bone marrow stromal cells, BMSCs) i CML, oraz pośredniczą w transporcie pęcherzyków komórkowych i białek z komórek podścieliska do komórek białaczkowych. Efektem jest zwiększona oporność komórek-biorców na lek imatinib.

W niniejszej pracy zidentyfikowano CD44 jako jedno z białek stromalnych transferowanych bezpośrednio z komórek stromy do komórek białaczki. Celem pracy było zbadanie roli stromalnego CD44 w białaczkowych komórkach-biorcach. Transfer CD44 potwierdzono przy użyciu mikroskopii konfokalnej i cytometrii przepływowej i wykazano, że wymaga on bezpośredniego kontaktu komórek stromalnych i białaczkowych oraz jest zależny od TNTs. Analiza RNA-seq wykazała, że transfer CD44 wiąże się ze zwiększoną ekspresją genów związanych z adhezją

komórkową, przebudową macierzy zewnątrzkomórkowej i migracją. Towarzystwo temu zwiększenie aktywności metaloproteinaz macierzy oraz większa inwazyjność tych komórek. Co istotne, w komórkach-biorcach zaobserwowano również wzrost endogennego CD44, zarówno na poziomie mRNA, jak i białka. Zależna od stromy zwiększona ekspresja CD44 w komórkach CML była związana z aktywacją prożyciowego szlaku ERK/AP-1 i ochroną przed inhibitorami kinaz tyrozynowych. W warunkach *in vitro* farmakologiczne zahamowanie interakcji między CD44 a jego głównym ligandem, hialuronianem, zaburzało ochronę zależną od stromy i uwrażliwiało komórki CML na imatinib.

Dodatkowym celem pracy było zbadanie ultrastruktury TNTs, które są głównymi mediatorami transferu CD44. Skaningowa mikroskopia elektronowa z wiązką jonów skupionych (ang. focus ion beam scanning electron microscopy, FIB-SEM) potwierdziła obecność TNTs w hodowlach ko-kultur stromy i białaczki. Zauważono, że TNTs zidentyfikowane mikroskopią konfokalną nie zawsze spełniały standardowo stosowane kryteria w analizie przy użyciu FIB-SEM. Zaproponowano konieczność ponownej oceny obecnie stosowanych podejść metodologicznych i związanych z nimi kryteriów do oceny tych struktur. Ponadto przedstawione dane kwestionują teorię, zgodnie z którą TNTs są całkowicie otwartymi strukturami, wskazując na istnienie specyficznego systemu regulującego transfer poprzez TNTs.

Podsumowując, przedstawione w niniejszej rozprawie badania dostarczają nowych informacji na temat oporności zależnej od stromy, związanej z bezpośrednim transferem białek przez TNTs, który prowadzi do transkrypcyjnego przeprogramowania komórek CML w kierunku bardziej inwazyjnego i opornego fenotypu. Dodatkowo wyniki pogłębiają zrozumienie ultrastruktury TNTs oraz oceniają kryteria i metodologie obecnie stosowane do ich identyfikacji.

ABBREVIATIONS

ABBREVIATION	DESCRIPTION
3D	Three-dimensional
7-AAD	7-Aminoactinomycin D
ADAM	A disintegrin and metalloproteinase
AF	Alexa Fluor
AKT	Protein kinase B
ALL	Acute lymphoblastic leukemia
Allo-HSCT	Allogeneic hematopoietic stem cell transplantation
AML	Acute myeloid leukemia
AP-1	Activating protein-1
APC	Allophycocyanin
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BCR-ABL1	Breakpoint cluster region protein and Abelson murine leukemia viral oncogene homolog 1
BIM	Bisindolylmaleimide
BMM	Bone marrow microenvironment
BMP	Bone morphogenetic protein
BMSCs	Bone marrow stromal cells
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
CAM-DR	Cell adhesion-mediated drug resistance
CD	Cluster of differentiation
Cdc42	Cell division control protein 42 homolog
cDNA	Complementary DNA
CIRE	CD44-ICD response element
CLEM	Correlative Light and Electron Microscopy
CML	Chronic myeloid leukemia
CSC(s)	Cancer stem cell(s)
DAPI	4',6-diamidino-2-phenylindole
DEG	Differentially expressed gene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECAR	Extracellular acidification rate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ERM	Ezrin, radixin, moesin
EV(s)	Extracellular vesicle(s)
F-actin	Filamentous actin
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	Food and Drug Administration

FITC	Fluorescein isothiocyanate
FIB-SEM	Focused ion beam-scanning electron microscopy
FLT3	FMS-like tyrosine kinase 3
FSC-A	Forward scatter area
FSC-H	Forward scatter height
FSC-W	Forward scatter width
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
(g)MFI	(geometric) mean/median fluorescence intensity
HA	Hyaluronic acid
HIF-1α	Hypoxia-inducible factor 1-alpha
HIF-2α	Hypoxia-inducible factor 2-alpha
HSC(s)	Hematopoietic stem cell(s)
ICAM	Intercellular cell adhesion molecule
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Mediuma
JAK	Janus kinase
LSCs	Leukemia stem cel(s)
MAPK	Mitogen-Activated Protein Kinase
MMP(s)	Metalloproteinase(s)
mRNA	messenger RNA
MSC	Mesenchymal stem cell
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
Myo	Myosin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear localization sequence
OCR	Oxygen consumption rate
PBMC(s)	Peripheral blood mononuclear cell(s)
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PFA	Paraformaldehyde
PI	Propidium iodide
PI3K	Phosphatidylinositol 3 kinase
PKC	Protein kinase C
PLL	Poly-L-lysine
PMA	Phorbol 12-myristate 13-acetate
Rab27a	Ras-related protein Rab-27A
Rac1	Rac Family Small GTPase 1
RhoA	Ras Homolog Family Member A
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute 1640 media
RUNX2	Runt-related transcription factor 2
SCF	Stem cell factor
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM	Scanning electron microscopy
shRNA	short hairpin RNA
SILAC	Stable isotope labeling by amino acids in cell culture
SSC-A	Side scatter area
SSC-H	Side scatter height
SSC-W	Side scatter width
STAT	Signal transducer and activator of transcription
TBS	Tris buffered saline
TBS-T	Tris buffered saline Tween-20
TEM	Transmission electron microscopy
TEMED	N,N,N',N'-tetramethylethane-1,2-diamine
TKI(s)	Tyrosine kinase inhibitor(s)
TNT(s)	Tunneling nanotube(s)
TPA	12-O-Tetradecanoylphorbol-13-acetate
TPO	Thrombopoietin
TRE	TPA-responsive element
WGA	Wheat germ agglutinin
Wnt	Wingless-related integration site
WT	Wild-type

1 INTRODUCTION

1.1 Chronic myeloid leukemia

1.1.1 Epidemiology and clinical overview

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm with an annual incidence of approximately 2 cases per 100 000 individuals, accounting for about 15% of newly diagnosed adult leukemia cases¹. In general, the CML can occur at any age, but the incidence increases with age, with a median age at diagnosis around 50-60 years. Only 2-3% of diagnoses occur under 20 years. CML is more common in men than in women, with a male-to-female incidence ratio of approximately 1.35². Exposure to high doses of ionizing radiation is the only well-established environmental risk factor associated with the development of CML. No familial or genetic predisposition to the disease has been identified. The introduction of imatinib in 2000 significantly reduced mortality from 10-20% to 1-2% and transformed CML from a fatal leukemia into a controllable chronic disease for a significant group of patients, improving their quality of life^{1,3}.

Chronic myeloid leukemia progresses through three main stages that differ in clinical presentation, blast cell percentage, and prognosis. CML typically begins in a chronic phase (CP-CML), which represents the earliest and most frequently diagnosed stage of the disease. This phase is characterized by preserved myeloid differentiation and a low blast count (<10%). The chronic phase, in which most patients (90-95%) are diagnosed, is often asymptomatic, while others present with nonspecific symptoms such as fatigue, weight loss, night sweats, and splenomegaly. Starting treatment at this stage yields the best therapeutic outcomes⁴. The absence of effective treatment leads to progression to accelerated phase (AP-CML), defined by 10-19% blasts in the bone marrow and increasing disease aggressiveness, and, ultimately, to blast crisis phase (BC-CML)⁵. The blast crisis phase is the most aggressive stage of the CML, resembling acute myeloid leukemia (AML). BC-CML is defined by $\geq 20\%$ blasts in the bone marrow and/or blood and is characterized by a complete loss of differentiation capacity and a poor prognosis^{1,6}. Disease progression is frequently associated with the acquisition of additional mutations.

The most frequently observed abnormalities include trisomy 8, an additional Philadelphia chromosome (+Ph), isochromosome 17q [i(17q)], and trisomy 19. The presence of additional abnormalities is associated with poorer overall survival^{7,8}.

1.1.2 Molecular pathogenesis

In the 1960s, Peter Nowell and David Hungerford identified the Philadelphia chromosome (Ph), a characteristic cytogenetic abnormality in CML patients^{9,10}. In 1972, Janet Rowley demonstrated that the Philadelphia chromosome results from a reciprocal translocation between chromosomes 9 and 22¹¹. Later, in the 1980s, the *BCR::ABL1* fusion gene was identified as the molecular driver of CML pathogenesis. Briefly, the Abelson murine leukemia (*ABL1*) gene from chromosome 9 is translocated to chromosome 22, where it fuses with the breakpoint cluster region (*BCR*) gene, forming the *BCR::ABL1* fusion gene (**Figure 1.1.1**)^{12,13}.

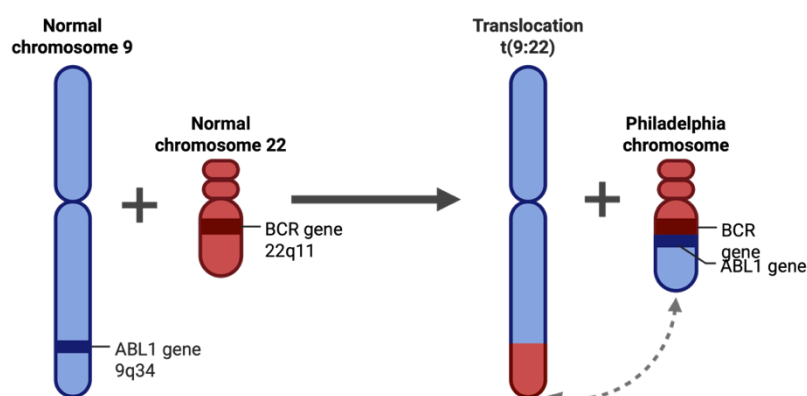


Figure 1.1.1 Schematic representation of the t(9;22)(q34;q11) reciprocal translocation leading to the formation of the Philadelphia chromosome and the *BCR::ABL1* fusion gene. Created with BioRender.com.

BCR::ABL1 mutation results in the formation of a tyrosine kinase of the same name¹⁴. The *BCR::ABL1* fusion protein contains multiple functional domains, including a tyrosine kinase domain with an ATP-binding site⁴. Most tyrosine kinase inhibitors (TKIs), such as imatinib, act by competitively binding to the ATP-binding site, thereby preventing phosphorylation of downstream effector proteins (**Figure 1.1.2**)¹⁵. Constitutive activity of the *BCR::ABL1* protein drives aberrant signaling through multiple pathways, promoting uncontrolled proliferation

(via RAS/MAPK), inhibition of apoptosis (PI3K/AKT) and enhanced cell growth and survival (via JAK/STAT, MYC, JUN)^{15–20}.

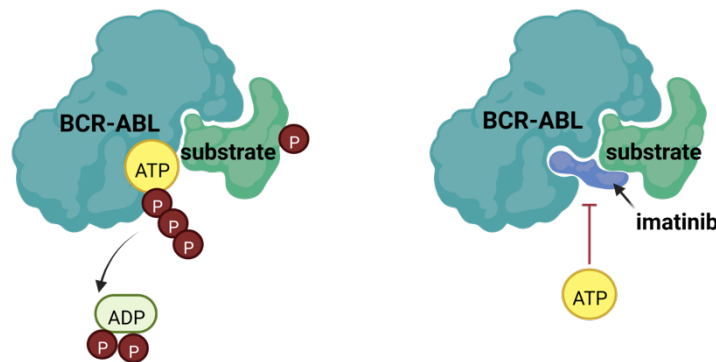


Figure 1.1.2 Schematic structure of the BCR::ABL1 fusion protein and mechanism of inhibition by imatinib. Created with BioRender.com.

1.1.3 Therapeutic strategies

In the pre-tyrosine kinase inhibitor era, treatment options for CML were limited and primarily aimed at controlling leukocytosis²¹. Initially, patients diagnosed with CML were treated with conventional chemotherapeutics, such as busulfan and hydroxyurea, to reduce white blood cell counts, but this did not prevent progression to blast crisis²². The introduction of interferon- α , either alone or in combination with cytarabine, became the standard therapy, inducing cytogenetic responses in a subset of patients and improving patients' survival²³. However, treatment with interferon- α was linked to high toxicity²¹. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) was the only curative approach, but it was associated with substantial treatment-related morbidity and mortality²⁴. Today, allo-HSCT is reserved for patients with treatment-resistance²⁵.

The introduction of tyrosine kinase inhibitors at the beginning of the 21st century revolutionized the treatment of CML, leading to a dramatic improvement in patient outcomes and survival^{5,8}. The mechanism of action of currently available TKIs relies on the blocking of the ATP-binding site in the BCR::ABL1 tyrosine kinase, preventing phosphorylation of downstream targets and inhibition of oncogenic signaling^{3,5}. Imatinib (Gleevec, STI571) was the first-generation TKI, which revolutionized the therapeutic approach to CML^{3,5}. Second-generation TKIs, such as dasatinib²⁶, nilotinib, and bosutinib²⁷, are characterized by substantially higher

affinity to the BCR::ABL1 kinase, allowing effective inhibition at lower concentrations and are effective against most BCR::ABL1 kinase domain mutations associated with imatinib resistance, excluding the T315L mutation²⁸. To overcome this mutation, resistant to both I- and II-generation TKIs, ponatinib was developed. It was specifically designed to bind the kinase domain even in the presence of the T315L mutation^{29,30}.

Recently, a novel class of drugs was developed, which act through the STAMP (Specifically Targeting the ABL Myristoyl Pocket) mechanism^{31,32}. Asciminib represents this class of agents and was approved for clinical use by the FDA in 2021⁵. The mechanism of action is based on binding to the myristoyl pocket of BCR::ABL1 instead of the ATP-binding side, which is targeted by older generations of drugs. In the native ABL1 kinase, the myristoyl pocket mediates autoinhibition, which is lost upon formation of the BCR::ABL1 protein. Asciminib mimics the endogenous myristoyl ligand and restores the inactive conformation of the kinase, effectively locking BCR::ABL1 in an “OFF” state³². Currently, asciminib is indicated as a third-line therapy for patients in the chronic phase who failed or are intolerant to at least two prior TKIs¹⁷. It is also effective in patients harboring the T315L mutation³³. In 2024, asciminib was also approved for use in adults with newly diagnosed CP-CML as first-line therapy³⁴.

Despite the high efficacy of tyrosine kinase inhibitors, the resistance in CML remains a significant problem. Approximately 5-10% of patients experience treatment failure associated with resistance and an increased risk of disease progression⁸.

1.1.4 CML resistance

1.1.4.1 Accumulation of secondary mutations

The best-characterized mechanism of CML resistance development involves BCR::ABL1-dependent alterations, including point mutations, as well as gene amplification leading to increased oncogene dosage^{8,35}. Leukemic cells accumulate additional cytogenetic and molecular abnormalities, thereby making conventional

inhibitors insufficient for effective disease control. These factors are the main reasons why the accelerated and blast phases of CML remain so difficult to treat⁵.

1.1.4.2 Leukemic stem cells

A major challenge in CML eradication is the persistence of leukemic stem cells (LSCs). LSCs are considered a key driver of relapse after TKI discontinuation, which occurs in approximately 50-60% of patients³⁶. Even when BCR::ABL1 transcripts become undetectable in peripheral blood, residual LSCs within the bone marrow niche can persist in a quiescent state (G0 cell cycle phase), insensitive to TKIs, and subsequently re-enter the cell cycle, reinitiating leukemic proliferation³⁷⁻³⁹. Moreover, LSCs may survive despite complete inhibition of BCR::ABL1 by relying on alternative pro-survival signaling pathways⁵. The identification of LSCs is challenging, as they share many features with normal hematopoietic stem cells (HSCs). Both types of stem cells are considered Lin⁻CD34⁺CD38⁻ population found in the bone marrow³⁸. Among the most promising currently identified markers for LSCs in CML are CD26^{40,41}, IL1RAP (interleukin 1 receptor accessory protein)⁴², and CD93⁴³.

Current strategies targeting CML leukemic stem cells focus on combination therapies, disruption of their interaction with the bone marrow niche, metabolic targeting, and immunotherapeutic approaches. For example, it was reported that BCL-2 inhibition in combination with TKIs enhances apoptosis of quiescent LSCs^{44,45}. Inhibition of the JAK/STAT pathway with ruxolitinib has also shown promising results in combination with TKIs⁴⁶. On the other hand, disruption of LSC-BMM interaction *via* inhibitors of E-selectin or CXCR4 mobilized LSCs from protective niches, thereby increasing their sensitivity to therapy⁴⁷⁻⁴⁹. Additionally, metabolic targeting of oxidative phosphorylation (OXPHOS), on which LSCs strongly depend, has emerged as a potential therapeutic approach^{50,51}.

1.1.5 Bone marrow microenvironment in leukemia

Bone marrow microenvironment is a specific niche, playing a fundamental role in the development of chronic myeloid leukemia and other hematologic malignancies. Krause and Scadden, in their review from 2015, refer to the bone marrow niche as a “hostel for hostile”, highlighting the role of bone marrow and

leukemia interactions in the protection of malignant cells⁵². It is a highly complex and dynamic niche, composed of diverse hematopoietic and non-hematopoietic cell types, interacting with each other and creating specialized conditions that promote leukemic cell survival. The bone marrow microenvironment consists of several distinct micro-niches. The endosteal niche is located near the bone surface (endosteum), includes mainly osteoblasts and osteoclasts, and maintains HSC quiescence and long-term protection. Importantly, osteoblasts produce essential secreted factors such as osteopontin, SDF-1, and SCF⁵³. This niche is more hypoxic in contrast to the perivascular niche, consisting mainly of endothelial cells lining blood vessels and regulating the trafficking of cells between the bone marrow and bloodstream, pericytes, and populations of mesenchymal stromal cells (MSCs). The perivascular niche promotes cell proliferation and differentiation⁵².

1.1.5.1 Bone marrow is a protective “sanctuary” for LSCs

The bone marrow microenvironment (BMM) plays a crucial role as a protective “sanctuary” for leukemic stem cells, protecting them from the TKIs and enabling their persistence⁵⁴. This support is mediated through a complex network of physical interactions, biochemical signaling, and metabolic adaptations^{55,56}. Firstly, the bone marrow niche is a hypoxic microenvironment, which stabilizes HIF-1 α . This induces a form of metabolic dormancy in LSCs⁵⁷ and may suppress BCR::ABL1 expression, thereby reducing the availability of the molecular target for TKIs⁵⁸. Additionally, bone marrow stromal cells and other components of the microenvironment secrete numerous factors that can compensate for LSCs’ dependence on the BCR::ABL1 oncoprotein, allowing their survival despite its therapeutic inhibition^{5,55,59}. For example, SDF-1 (CXCL12), predominantly secreted by MSCs, binds to CXCR4 on LSCs, which is essential for their homing to protective niches and maintenance of quiescence¹⁶. Conversely, leukemic stem cells can downregulate CXCL12 levels within the niche, impairing the function of normal HSCs⁶⁰. Another factor produced by neighboring cells – SCF, binds to the c-Kit receptor (CD117) on LSCs, supporting their survival and maintenance^{61,62}. In addition to the signaling axes described above, the bone marrow niche provides numerous other supportive factors that collectively promote LSCs’ survival, maintenance, and therapy resistance. TGF- β maintains their

quiescence via TGF- β /FOXO signaling, and its inhibition has been shown to increase sensitivity to TKIs⁶³. Inflammatory cytokines, such as IL-6⁶⁴ and IL-1 β , produced by MSCs and macrophages, modulate progenitor cells' fate and promote leukemogenesis³⁷.

1.1.5.2 The role of cell adhesion in the leukemic BMM

Adhesion interactions within the bone marrow microenvironment play a fundamental role in CML progression. In particular, LSCs exploit these interactions to anchor within niche compartments and enter a quiescent cell. The E-selectin/CD44 axis represents a key mechanism in the maintenance of the LSC pool within the bone marrow⁵⁴. E-selectin expressed on endothelial cells interacts with CD44 on the surface of LSCs, which is essential for their homing and retention within the bone marrow niche^{48,65–67}. Notably, CD44 expression increases with disease progression and is associated with poorer prognosis and enhanced therapeutic resistance⁶⁶. Another axis mediating adhesion of leukemia cells to stroma is VLA-4/VCAM-1. Engagement of VLA-4 integrin with its ligand VCAM-1 activates pro-survival signaling pathways in leukemic cells, including NF- κ B, directly protecting them from chemotherapy- or TKI-induced apoptosis^{68,69}. This interaction is considered a key determinant of minimal residual disease (MRD), which serves as a reservoir for disease relapse⁷⁰. In CML, cell adhesion-mediated drug resistance (CAM-DR) represents a major mechanism by which interactions with the bone marrow microenvironment actively protect leukemia cells against TKIs. Damiano and colleagues demonstrated that adhesion of K562 cells to components of the ECM protects these cells from apoptosis induced by BCR::ABL1 inhibition and γ -irradiation. They suggest that CAM-DR was induced by adhesion to fibronectin mediated by VLA-5 integrin⁷¹. Another study showed that adhesion of CML to stromal cells induces imatinib resistance through the activation of specific signaling pathways, particularly ERK and bone morphogenetic protein (BMP) signaling. Importantly, pharmacological inhibition of these pathways sensitized chemoprotected cells to treatment, highlighting their role in CAM-DR. Additionally, they observed that adherent cells exhibited reduced levels of mitochondrial ROS and upregulation of anti-apoptotic proteins, such as cIAP2, promoting cell survival⁷².

Puissant *et al.* demonstrated that continuous treatment of CML cells with imatinib selects for a subpopulation of adherent, imatinib-resistant cells displaying an epithelial-mesenchymal transition (EMT) phenotype⁷³. Cells characterized by high expression of CD44 and low CD24 were associated with enhanced aggressiveness, migratory capacity, and invasiveness. The authors identified integrin $\alpha_v\beta_3$ as a key mediator acting through activation of the FAK/Akt and ERK1/2 signaling pathways, enabling cell survival despite BCR::ABL1 inhibition⁷³.

Importantly, adhesion also enables direct intercellular exchange of material between leukemia cells and their surrounding microenvironmental cells. Connexin 43 (Cx43) forms gap junctions between leukemic stem cells and MSCs, enabling the transfer of ions (e.g., Ca^{2+}) and signaling molecules. Surprisingly, in CML, Cx43 expression is often reduced, and restoration of this intercellular communication induced by MSCs can promote leukemic cell apoptosis in response to imatinib⁷⁴. Additionally, it has been shown that miR-130b-3p-containing exosomes can regulate Cx43-dependent communication between MSCs and leukemic cells by attenuating gap junctional coupling, thereby contributing to the development of drug resistance and subclonal evolution⁷⁵. On the other hand, in AML, gap junction-mediated interactions with MSCs promoted chemoresistance in leukemic cells. Disruption of gap junctions using carbenoxolone impaired the protective niche effect, induced metabolic stress and apoptosis, and enhanced sensitivity to cytarabine both *in vitro* and *in vivo*⁷⁶. Collectively, gap junctions play a dual role in leukemia, exhibiting both pro-leukemic and leukemia-suppressive functions. However, they represent an important component of intercellular communication within the leukemia microenvironment.

Adhesion is also essential for the formation and stabilization of tunneling nanotubes (TNTs) and TNT-mediated intercellular transfer. At baseline, CML cells form few or no tunneling nanotubes between themselves, which is thought to reflect their reduced adhesive capacity³⁸. Interestingly, therapeutic intervention with TKIs, as well as IFN α can enhance cellular adhesion and promote TNT formation between CML cells. This increased intercellular connectivity is considered a pro-survival adaptation, enabling the exchange of organelles (e.g., mitochondria)⁷⁷. CML cells can

communicate via TNTs also with cells of the bone marrow niche⁷⁸. A detailed overview of tunneling nanotubes and their role in intercellular communication in leukemia and other biological contexts is provided in the following section.

1.2 Tunneling nanotubes (TNTs)

Tunneling nanotubes (TNTs) represent a unique form of direct, long-distance cell-to-cell communication, fundamentally challenging the classical perception of cells as discrete and isolated entities, introducing the concept of super-cellularity. They are thin, actin-rich, membranous channels connecting distant cells, enabling transfer of various cargo between connected cells, such as cellular vesicles, nucleic acids, organelles, and pathogens⁷⁹.

The history of tunneling nanotubes is relatively short, with its origins dating back to the beginning of the 21st century. As is frequently the case with major scientific breakthroughs, it began unexpectedly through a chance observation. A PhD student of Hans-Herman Gerdes from EMBL in Heidelberg, Amin Rustom, observed characteristic thin structures connecting neighboring rat pheochromocytoma PC12 cells during routine cell passaging. Initially, he mistook them for scratches on the cell culture dish. However, a few years later, these observations were published in *Science*, becoming one of the most important discoveries in the field of intercellular communication⁸⁰. The *mysterious lines* were named tunneling nanotubes, reflecting both their nanoscale diameter (approximately 50-200 nm) and their capacity to facilitate within the extracellular matrix. At the same time, structures of similar morphology were described in the immune system cells by the group of Professor Daniel Davis from Imperial College London⁸¹. Already at that point, both groups provided evidence that these newly discovered structures could function as *highways* for the transport of various types of cellular cargo^{80,81}.

1.2.1 Structural features of TNTs

Tunneling nanotubes are defined as thin, relatively straight membrane protrusions. Reported diameter range from 50 to 700 nm (with an average of 200 nm). Their length can vary from tens to several hundred micrometers, most commonly between 20 and 100 μm . One of the most important features of TNTs, enabling them

to be distinguished from other filopodia-like protrusions, is that they do not touch the substratum, hanging freely between connected cells in the extracellular space⁸². Two main categories of TNTs can be distinguished regarding their diameter:

- thin TNTs with a diameter below 700 nm, usually shorter (up to 20 μ m length), containing only F-actin, highly dynamic, fragile, and short-lived (less than 1 hour);
- thick TNTs with a diameter up to 700 nm and a length reaching several hundred micrometers, containing both F-actin and microtubules, more stable (lifetimes reaching several hours), supporting the transport of large cargo, such as mitochondria.

Depending on the source literature, these two categories have been designated as TNT-1 and TNT-2 accordingly, or *vice versa*. The inconsistencies in the nomenclature can still be found in the literature. However, there is general agreement concerning the criteria used to classify the two groups⁸³⁻⁸⁵.

F-actin is an essential structural component of all types of TNTs. It is arranged into highly organized, parallel bundles along the longitudinal axis of the nanotube. The actin filaments within TNTs remain continuous with the actin cortex of the connected cells^{86,87}. Microtubules are found primarily in thick TNTs, increasing their mechanical resistance and enabling transport of larger organelles via motor proteins (kinesins and dyneins)⁷⁹. Some of the cell types (e.g., epithelial cells, glial cells) form TNTs also containing intermediate filaments^{80,88}.

1.2.1.1 Distinguishing TNTs from other cellular protrusions

A major challenge in TNT research is the reliable discrimination of TNTs from other protrusions with similar morphology. The structures most mistaken for TNTs include filopodia, cytonemes, and intercellular bridges.

Filopodia are most frequently mistaken for TNTs, particularly in low-resolution microscopy. Both structures are thin, actin-based protrusions. The principal criterion distinguishing TNTs from filopodia in light microscopy is that filopodia typically adhere to the substratum, whereas TNTs remain suspended in the extracellular space^{79,89}. Cytonemes, a specialized form of filopodia, represent an even greater challenge, as their tips contact neighboring cells, enabling the transfer of

morphogens or signal transmission via ligand-receptor interactions^{89,90}. However, all filopodia and cytonemes, in contrast to TNTs, are closed-ended structures⁹¹. *Bona fide* TNTs are characterized by open-ended termini, establishing membrane and cytoplasmic continuity between cells. However, the question of whether TNTs are always open- or closed-ended remains a matter of debate in the TNT field^{92–95}. Some observations suggest the existence of TNTs containing gap junction-like structures, without full cytoplasmic mixing^{78,96–98}. Consequently, the correct identification of these structures is further complicated.

Intercellular bridges (cytokinetic bridges) are transient, membranous tubes formed following incomplete cytokinesis. Like TNTs, they connect two cells and enable cytoplasmic continuity. The structure allowing researchers to distinguish them from TNTs is the presence of a characteristic midbody structure^{77,99}, composed of densely packed microtubules derived from the mitotic spindle, typically visible as a central thickening along the bridge. Intercellular bridges are also generally positive for the CEP55 marker, whereas TNTs are CEP55-negative¹⁰⁰.

The distinguishing criteria are summarized in **Table 1-1**.

Table 1-1 Comparison of the intercellular membrane protrusions

Feature	Tunneling nanotubes	Filopodia	Cytonemes	Intercellular bridges
Contact with the substratum	No	Yes	Usually yes	Usually yes
End structure	Open (<i>bona fide</i> TNTs), sometimes closed	Closed	Closed	Open
Cytoskeleton	F-actin (thin TNTs), F-actin + microtubules (thick TNTs)	F-actin	F-actin	F-actin + microtubules
Transfer of cargo	Yes	No	Morphogens	Yes
Formation mechanism	Filopodial interplay, cell dislodgement	Outgrowth from the cell edge	Targeted outgrowth towards a signaling source	Incomplete cytokinesis
Typical length	Up to 100 – 300 μm	Usually short (up to 10 μm)	Up to 700 μm	Variable, up to 350 μm

1.2.1.2 Current knowledge of TNTs ultrastructure

Studies of TNT structure have evolved from simple microscopic observation to advanced nanoscale ultrastructural analyses, enabling more precise discrimination between TNTs and other cellular protrusions. Although ultrastructural studies are still limited, they have provided several important insights into tunneling nanotubes morphology.

Some of the most important observations were made by Sartori-Rupp *et al.*, who, using a combination of cryo-correlative light and electron microscopy (cryo-CLEM) and cryo-electron tomography (Cryo-ET), demonstrated that tunneling nanotubes appearing as single filaments in fluorescence microscopy often consist of bundles of multiple individual nanotubes (iTNTs)¹⁰¹. Their studies were performed on two neuronal cell models (mouse catecholaminergic CAD cells and human

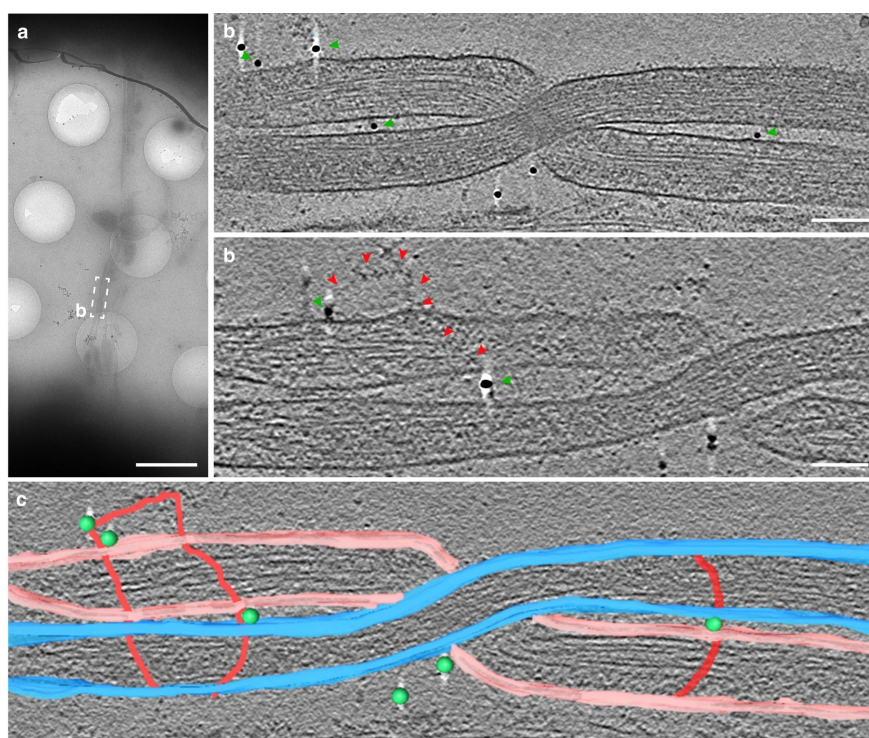


Figure 1.2.1 Ultrastructural analysis of N-cadherin in iTNTs. Original figure from Sartori-Rupp *et al.* (2019), reproduced under the Creative Commons Attribution 4.0 International License (CC BY 4.0). “**a** Low-magnification electron micrograph displaying N-Cadherin immunogold-labeled CAD cells connected by iTNTs. (**b-top**) White dashed rectangle in **a** correspond to two high-magnification non-consecutive cryo-tomogram slices (25 nm in thickness) shown in **b-top** and **b-bottom** (...) Green arrowheads in **b** and spheres in **c** indicate 10 nm gold particles attached to a polyclonal secondary antibody that binds to an N-Cadherin primary antibody. Red arrows indicate a long thin thread filament surrounding iTNTs anchored to the membrane of iTNTs by N-Cadherin molecules (green arrows). **c** Segmentation rendering of the tomogram described in **b**; N-Cadherin (green beads), thin thread surrounding iTNTs (green arrowheads). (...) Scale bars: **a** 2 μ m, **b** 100 nm”

neuroblastoma SH-SY5Y cells). They showed that iTNT bundles are stabilized by specific adhesion proteins, such as N-cadherin (validated by immunogold labeling)¹⁰¹.

Importantly, studies conducted by the group of Prof. Piwocka also demonstrated that TNTs connecting HS-5 stromal cells can form bundle-like structures composed of individual tunneling nanotubes, similar to those previously reported in neurons⁷⁸. These observations were made using CLEM and electron tomography, enabling three-dimensional reconstruction of their ultrastructure.

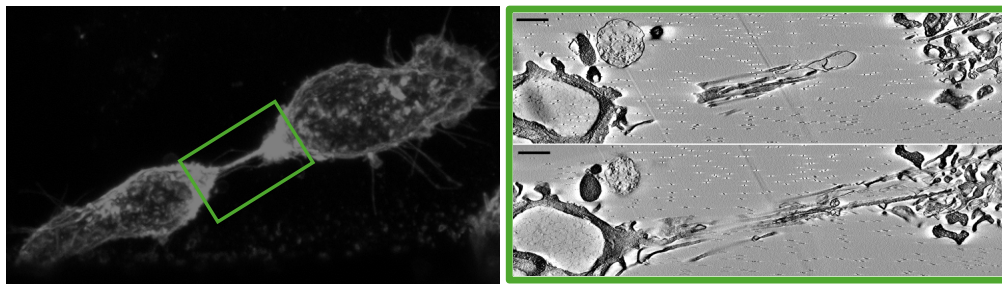


Figure 1.2.2 Bunches of single TNTs connecting HS-5 cells. Adapted from Kolba *et al.* (2019), used under the Creative Commons Attribution 4.0 International License (CC BY 4.0). “Fluorescence image of one thick TNT (left) and tomography of ROI (green frame) showing the bunches of single TNTs. Scale bar = 2 μ m (left image), 500 nm (right images).”

Electron microscopy has significantly contributed to studies on the open- and closed-ended nature of TNTs. Early studies on TNTs established the concept of open-ended, cytoplasmically continuous intercellular channels. TNT research pioneers, Rustom and Gerdes, using transmission electron microscopy (TEM), SEM, serial sectioning, and correlative 3D imaging approaches, demonstrated a seamless continuity between TNTs and the plasma membrane^{80,102}. On the other hand, later ultrastructural studies challenged this model. Sowinski *et al.* applied serial-section transmission electron microscopy (ssTEM) and identified closed-ended nanotubes in T cells. These TNTs were characterized by a junctional barrier at the interface with the recipient cell and lacked full cytoplasmic continuity⁹⁸. In subsequent studies, Thayanithy *et al.* suggested the presence of bundled filamentous structures within nanotubes connecting MSTO mesothelioma cells, based on scanning electron microscopy^{103,104}.

Currently, most reports support the coexistence of both types of TNTs. Analysis using focused ion beam – scanning electron microscopy (FIB-SEM)

performed by Sartori-Rupp *et al.* documented the existence of both open-ended and closed-ended TNTs in neuronal cells¹⁰¹. Closed-ended TNTs were further classified in the literature review written by Cervantes and Zurzolo as resting ends (lying on the recipient cell membrane) and invaginated ends (in which the terminal TNT structure was closed and deeply embedded within the recipient cell body)⁹². The authors highlighted that the possibility of closed structures representing pre-TNT fusion events cannot be excluded¹⁰¹. Our previous work on TNTs in the chronic myeloid leukemia microenvironment has led to similar conclusions. Both types, open- and closed-ended TNTs were reported in the Kolba *et al.*, where we used CLEM together with electron tomography and 3D reconstruction to analyze the ultrastructure of TNTs.

1.2.2 Mechanisms of TNTs formation

1.2.2.1 TNTs biogenesis

Currently, there are two main mechanisms proposed for TNTs formation. The first of these is the actin-driven protrusion mechanism, otherwise known as *filopodial interplay*. In this model, one cell actively extends a filopodia-like protrusion that elongates until it reaches the membrane of another cell. Following this, membrane fusion occurs at the tip of the protrusion, resulting in the formation of a continuous cytoplasmic channel (**Figure 1.2.3 A**). A specific variation of this process is the double-filopodial bridge model in which two filopodia originating from neighboring cells transform into TNT through helical deformation and the accumulation of

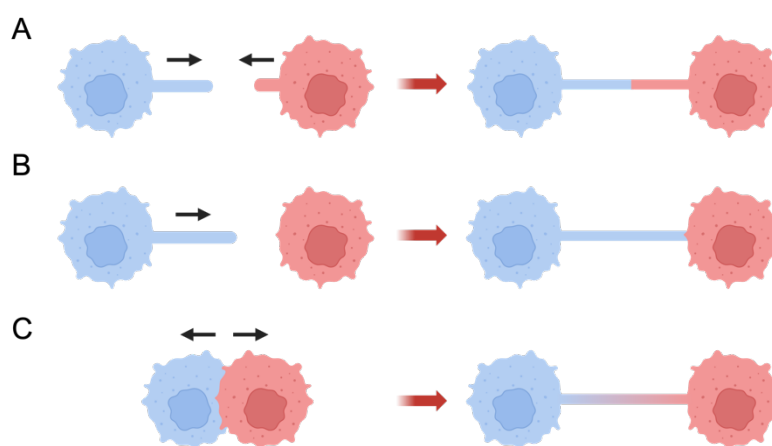


Figure 1.2.3 Mechanisms of TNTs formation - schematic representation. (A-B) Filopodia interplay mechanism **(C)** Cell dislodgement mechanism. Created with BioRender.com.

mechanical tension¹⁰⁵ (**Figure 1.2.3 B**). Filopodia-based models are typical for less mobile cells, such as neurons or epithelial cells^{106,107}. Another mechanism is *cell dislodgement*. Two cells, initially in direct cell-cell contact, begin to migrate away from one another. As they separate, a thin membrane structure is stretched between them and subsequently develops into a stable nanotube (**Figure 1.2.3 C**). This mechanism has been predominantly observed in immune system cells like NK cells or lymphocytes^{81,102}. Previously, it was also observed by our group in co-cultures of chronic myeloid leukemia cells and bone marrow stromal cells⁷⁸. Notably, these two mechanisms are not mutually exclusive and may operate simultaneously within the same cell population¹⁰².

1.2.2.2 Molecular basis of TNTs formation

The formation of TNTs is linked to the dynamic polymerization of globular actin (G-actin) into filamentous actin (F-actin). Like in filopodia, members of the Rho GTPase family, Cdc42 and Rac1, together with their effector molecules WASP and WAVE2, play a crucial role in initiating the cytoskeleton rearrangements required for tunneling nanotube formation. Inhibition of Cdc42 and/or Rac1 reduced the number of TNTs formed between macrophages¹⁰⁸.

M-Sec (also known as TNFAIP2) is considered one of the best-characterized inducers of TNT formation, particularly in cells of myeloid origin. In cooperation with ERp29, which stabilizes M-Sec and protects it from degradation, it promotes actin remodeling and membrane expansion required for TNT biogenesis. However, accumulating evidence suggests that M-Sec is not a universal regulator of TNT formation^{78,109,110}.

On the other hand, myosin X (Myo10) was reported to regulate the formation of TNTs^{106,111}. In neuronal cells, overexpression of Myo10 increased TNT formation, whereas its knockdown significantly impaired tunneling nanotube development¹⁰⁶. Mechanistically, Myo10 acts downstream of Cdc42 and promotes TNT formation independently of VASP¹⁰⁹.

However, universal regulators of TNTs formation and specific molecular markers distinguishing these structures have not been identified.

1.2.3 TNT-mediated transport: focus on leukemias

A key functional property of tunneling nanotubes is their ability to mediate intercellular cargo transport between connected cells, often involving relatively large cellular components, such as mitochondria. TNTs are often described as *intercellular highways* enabling direct transfer of cargo, from individual ions to entire organelles and pathogens (viruses, prions)¹¹².

Mitochondria

Among the wide range of cargo transported *via* TNTs, the transfer of mitochondria is the most extensively studied¹¹³. The first evidence that mitochondrial transfer can restore aerobic respiration in cells with damaged mtDNA was provided by Spees *et al.* in the studies on mesenchymal stem cells (MSCs)¹¹⁴. Mitochondrial transfer *via* TNTs is increasingly recognized as a key contributor to cancer progression, also in leukemias. Transferred mitochondria support malignant cell survival and therapy resistance, allowing for metabolic adaptation to the challenging bone marrow microenvironment, and contribute to cellular rescue from chemotherapy-induced stress¹¹⁵. In AML, mitochondrial transfer occurs predominantly from bone marrow stromal cells (BMSCs) to leukemic blasts. It was shown that AML cells exploit NADPH oxidase 2 (NOX2) to induce TNTs formation and initiate mitochondrial transfer from the BMSCs. AML cells that accepted stromal mitochondria exhibited increased metabolic fitness and proliferative capacity¹¹⁶. Another study showed that mitochondrial transfer can act as a rescue mechanism for leukemic cells undergoing drug treatment. Cytarabine (AraC) increased oxidative stress within the bone marrow microenvironment, which enhanced mitochondrial transfer from BMSCs to AML cells. As a result, a subpopulation of leukemia-initiating cells (LICs) that acquire mitochondria during chemotherapy exhibited increased survival and therapy resistance¹¹⁷. Similar observations were also made in other leukemias, such as acute lymphoblastic leukemia (ALL)¹¹⁸. On the other hand, in T-cell acute lymphoblastic leukemia (T-ALL), a distinct directionality of mitochondrial transfer was observed. In response to chemotherapy-induced stress, Jurkat cells actively exported large numbers of mitochondria to stromal cells, while acquiring only a limited amount in return. This transfer was dependent on the adhesion

molecule ICAM-1, and disruption of the ICAM-1 molecule impaired stromal protection of leukemic cells and sensitized T-ALL to chemotherapy¹¹⁹.

It is worth noting that neuronal systems represent the best-characterized context for TNT-mediated mitochondrial transfer and have provided key insights into its underlying transport mechanisms¹²⁰. Key players in TNT-mediated mitochondrial transport are molecular motors, such as myosin Va and myosin X in thinner TNTs^{106,121}. Other studies report increased expression of Miro1 on the outer mitochondrial membrane of donor cells¹²². Recently, TNT-mediated mitochondrial transfer has also been demonstrated *in vivo*. Using a zebrafish embryo model, Korenkova and colleagues showed that embryonic gastrula cells can exchange mitochondria through open-ended TNT-like structures, providing evidence that functional TNT-mediated cargo transfer occurs under physiological conditions and is not merely an *in vitro* culture artifact¹⁰⁰.

Proteins

There are relatively few reports on TNT-mediated protein transfer in leukemia cells, but several studies have described this phenomenon. For example, it has been shown that CD133 protein, a marker of cancer stem cells (CSCs), is transferred between primary human CD34⁺ hematopoietic progenitor cells and KG1a AML cell line¹²³. On the other hand, ALL cells have been shown to transfer the transmembrane protein ICAM1 to mesenchymal stromal cells. The authors suggest that this transfer may provide the anchoring interactions required for further formation and maintenance of TNTs formed between those cells¹²⁴. Our previous study⁷⁸ enriched the limited knowledge regarding TNT-mediated protein transfer in leukemia. In the leukemia-stroma co-culture model, the combination of cellular vesicles labeling and trans-SILAC followed by mass spectrometry, enabled identification of over 600 stroma-derived proteins in K562 acceptor cells. Among these, almost 300 proteins were found to be transported within stromal vesicles. Functional enrichment analysis revealed that these proteins belonged to distinct biological categories, including proteins involved in RNA synthesis and processing, such as translation initiation factors or spliceosomal proteins. In addition, TNT-transferred cargo was enriched in

proteins associated with cytoprotection. Observed vesicle and protein transfer resulted in the protection of acceptor cells against imatinib-induced apoptosis⁷⁸.

1.3 CD44

CD44 (synonyms: CDw44, homing cell adhesion molecule (H-CAM), phagocytic glycoprotein 1 (Pgp1), extracellular matrix receptor III (ECM-III), Hermes-1 antigen) is a class I transmembrane glycoprotein. It plays important roles in the various cellular processes, including cell adhesion, migration, and interactions between cells and the microenvironment.

1.3.1 Gene and protein structure

In humans, the CD44 gene is located on chromosome 11p13. It is comprised of 20 exons, where the first five (1-5) and exons 16-20 are constant, encoding the shortest form of CD44 called CD44 standard isoform (CD44s, 85-95 kDa)¹²⁵. In humans, exon 6 (exon v1) is absent, and exon 19 is usually spliced out¹²⁵. The remaining nine exons (v2-v10) can undergo alternative splicing and be combined with the exons of the CD44s, resulting in the formation of a plethora of CD44 variant isoforms (CD44v) (**Figure 1.3.1**)¹²⁶.

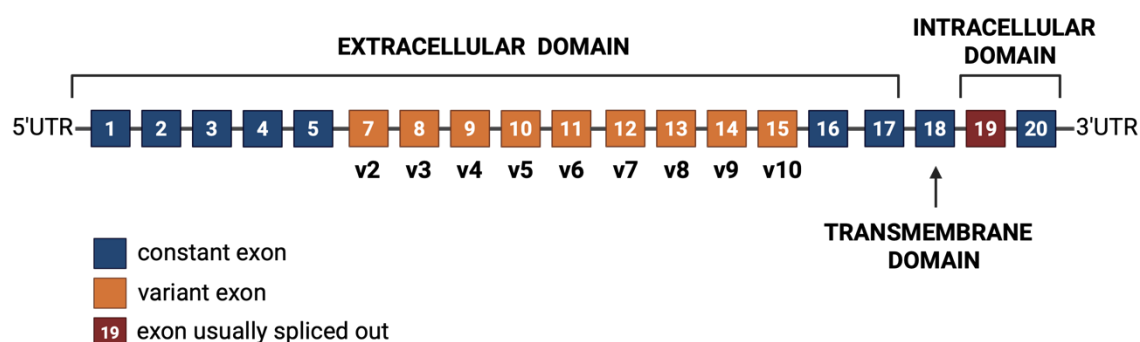


Figure 1.3.1 The genomic organization of the human CD44 gene. Created with BioRender.com.

CD44s is broadly expressed across tissues, including the central nervous system¹²⁷, hematopoietic cells, and lung¹²⁸, whereas the expression of CD44 variant isoforms is rather cell type-specific, with important roles in cancer^{129,130}. CD44 v8-v10 (CD44 epithelial isoform, CD44E) is predominantly found on epithelial cells and keratinocytes¹³¹. CD44v6 is one of the most extensively studied isoforms in cancer; its expression was linked to poor prognosis in several cancers, including colon¹³²,

bladder¹³³ , and breast cancer¹³⁴. In leukemias, especially AML, CD44v6 has been considered a potential target for CAR T-cell therapy, as FLT3 and DNMT3A mutations were linked to higher expression of this isoform in leukemic cells^{135,136}. Nevertheless, this isoform is also expressed in normal tissues such as the skin¹³⁷.

All CD44 protein isoforms comprise three main domains: extracellular (ECD), transmembrane, and intracellular domain (ICD). The extracellular domain encoded by exons 1 to 17 consists of an N-terminal ligand-binding region, and, in the alternatively spliced variants, a variable region generated by the inclusion of v2-v10 variant exons. The ECD structure is stabilized by disulfide bridges formed between pairs of cystine residues (**Figure 1.3.2**)¹³⁸. The ligand-binding region is mainly involved in the binding of hyaluronic acid (HA) and components of the extracellular matrix (ECM). ECD can undergo numerous post-translational modifications, involving N-glycosylation, O-glycosylation and glycosaminoglycanation^{139,140}.

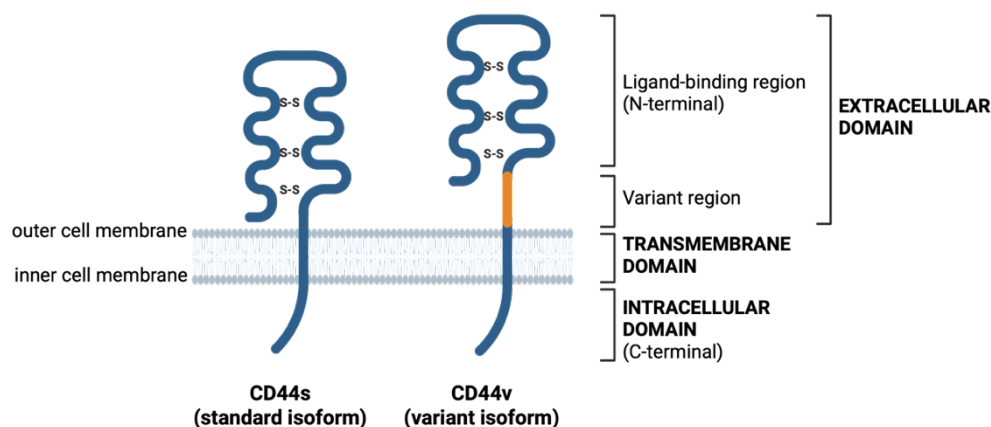


Figure 1.3.2 Simplified scheme of CD44s and CD44v protein structure.
Created with BioRender.com.

Post-translational modifications and the occurrence of multiple splice variants contribute to the high heterogeneity in CD44 molecular weight (85-250 kDa) and protein complexity. The hydrophobic transmembrane domain coded by exon 18 stabilizes CD44 in the lipid bilayer and is involved in CD44 dimerization and clustering, as well as the first step of ICD cleavage essential for further signaling events¹⁴¹. The intracellular domain serves as a signaling hub that links the CD44 protein to the cytoskeleton and plays a crucial role in intracellular signal transduction¹⁴². ICD can be phosphorylated, especially at Serine 325, which is

important for interaction with proteins connecting CD44 with the cytoskeleton¹⁴³. For instance, the C-terminal region of CD44-ICD interacts indirectly with actin filaments through the binding to proteins such as ezrin, radixin and moesin (ERM), thereby regulating cytoskeletal organization, cell adhesion, and migration¹⁴⁴. Additionally, CD44-ICD can be cleaved and translocated to the cell nucleus, where it co-regulates gene transcription¹⁴².

1.3.2 CD44 cleavage and its role in transcription regulation

The CD44 cleavage is a multi-step process, requiring the involvement of multiple enzymes. It is essential for CD44-mediated signaling and adhesive functions. First, the extracellular domain is released from the cell surface (often referred to as CD44 shedding) through the cleavage by metalloproteases from the MMP and ADAM families (e.g., MT1-MMP, ADAM10, ADAM17)^{145,146} at the membrane-proximal region of the extracellular domain (**Figure 1.3.3**). ECD shedding has been shown to be regulated mainly by protein kinase C (PKC) activity and Ca^{2+} influx^{147–149}, several studies also indicate the involvement of IL-1 β ¹⁵⁰. Therefore, extracellular domain cleavage promotes cell migration on hyaluronan substrates through the detachment of the cell from the ECM. CD44 shedding produces a soluble form of CD44, commonly referred to as sCD44 (usually ~25 kDa). It was shown that high levels of sCD44 in the serum are associated with poor cancer prognosis^{151,152}, also in leukemias¹⁵³. CD44 shedding is followed by the presenilin-dependent γ -secretase cleavage and generation of released ICD fragments (~15 kDa)¹⁵⁴.

Importantly, CD44-ICD contains a nuclear localization sequence (NLS), which facilitates CD44 nuclear translocation through the nuclear pore complex¹⁵⁵. In the nucleus, ICD can interact with transcription factors, co-regulating gene expression. CD44 has been reported to cooperate with RUNX2¹⁵⁶, regulating the expression of both *RUNX2* and, consequently, RUNX2-mediated transcription of *MMP9*. Miletto-González and colleagues performed ChIP analysis of the nuclear extracts from breast cancer and ovarian cancer cell lines and confirmed that CD44-ICD interacts physically with RUNX2 in the nucleus. Both proteins form a stable complex at the *MMP9* promoter, resulting in the robust activation of the *MMP9* transcription. For the first time, they showed that CD44-ICD binds to its consensus sequence

CCTGCG (named as *CD44-ICD response element*: CIRE). CIRE can be found not only near the promoter region of *MMP9* but is also present in many HIF-1 α -regulated genes. However, the authors propose that this group of genes can be regulated by CD44-ICD independently of HIF-1 α , under normoxic conditions¹⁵⁷. On the other hand, in hypoxia, CD44-ICD has been shown to stabilize HIF-2 α , enhancing the expression of HIF target genes¹⁵⁸. CD44-ICD can also bind to 12-O-tetradecanoylphorbol 13-acetate (TPA)-response elements (TRE) and regulate the expression of genes containing it. One of them is *CD44* itself, as it was reported by Okamoto *et al.* that CD44-ICD can regulate its own expression as a positive feedback loop¹⁵⁹. Additionally, by interacting with CREB, CD44-ICD regulates the expression of cyclin D1¹⁶⁰ and genes associated with glucose metabolism, including *PFKFB4*¹⁶¹, in each case promoting cancer progression. Additionally, CD44-ICD activity was also associated with overexpression of stemness factors, such as Nanog, Sox2 and Oct-4¹⁶². Importantly, not only ICD, but also the full-length CD44 was reported to regulate gene expression^{155,163,164}.

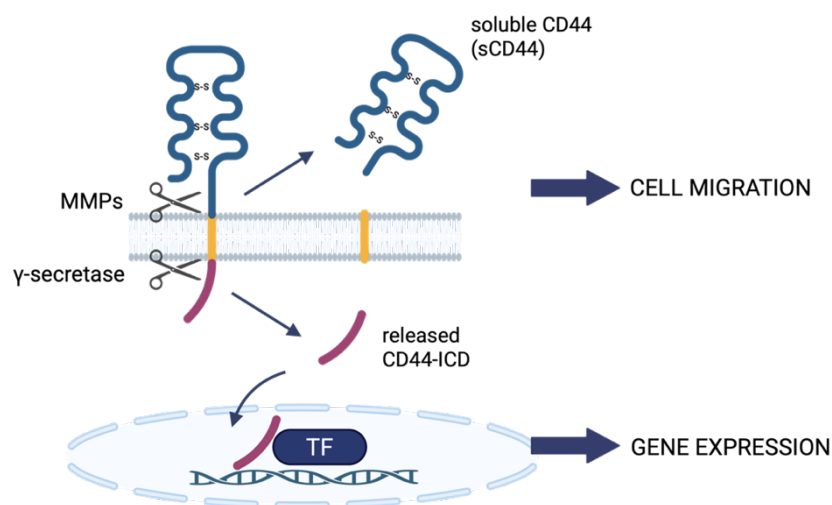


Figure 1.3.3 Schematic representation of CD44 cleavage. Created with BioRender.com.

1.3.3 CD44 ligands and CD44-mediated signaling in cancer

1.3.3.1 Hyaluronic acid

The main ligand for CD44 is hyaluronic acid (HA), a linear polysaccharide from the glycosaminoglycan family. HA is abundantly present in the extracellular matrix, and its interaction with CD44 is the best characterized, regulating multiple cellular

processes, such as migration, adhesion, and cancer progression¹⁶⁵. Importantly, binding sites for HA are localized in the N-terminal region of the extracellular domain, therefore all splicing forms of CD44 can bind HA. However, the ability to bind HA is strictly linked to CD44 density¹⁶⁶. Some CD44v isoforms were shown to have higher affinity to HA than CD44s, which was linked to their effective clustering modulated by N- and O-linked glycosylation¹⁶⁷. HA levels are elevated in many cancers, with stromal accumulation correlating with poor prognosis¹⁶⁸. What is important, the molecular weight of hyaluronic acid determines the functional outcome of its interaction with CD44. HA is classified into high-molecular-weight (HMW HA, > 1 MDa) and low-molecular-weight (LMW HA, < 50 kDa), binding of each results in distinct, often opposing biological effects. The HMW HA is present in normal tissues, and its binding typically promotes anti-proliferative, anti-angiogenic, and anti-inflammatory effects¹⁶⁹, while binding of LMW HA, frequently detected in malignant tumors, results in pro-inflammatory and pro-migratory outcomes^{170–172}.

The interaction between CD44 and hyaluronan results in the formation of a signaling platform linking ECM signals with intracellular pathways, controlling cell growth, survival, and migration. This interaction drives activation of pro-survival pathways, including Ras/MAPK/ERK and PI3K/AKT^{173,174}. The binding of LMW HA can lead to the cleavage of CD44-ICD, its translocation to the nucleus, and increased transcription of genes involved in cancer cells' migration and invasiveness¹⁴². CD44-HA interaction was reported to play a key role in the multiple myeloma progression, where stroma-derived HA and IL-6 secretion enhanced CD44 expression, Rac1 signaling, and cytoskeletal remodeling in cancer cells, promoting their adhesion and migration¹⁷⁵. Breast cancer cells can acquire resistance to PI3K α inhibitors through increased CD44-HA interaction which activates the Src-ERK-ezrin signaling cascade that bypasses PI3K α inhibition by sustaining AKT/mTOR activity¹⁷⁶. In chronic myeloid leukemia, imatinib treatment reduced both surface CD44 and HA levels in monocultured K562 cells. Exogenous HA attenuated the anti-proliferative effects of imatinib, increasing AKT phosphorylation and preventing imatinib-induced senescence¹⁷⁷.

Considering the increased expression of CD44 in many cancer types and cancer stem cells (CSC), the hyaluronic acid and its interaction with CD44 are used as a target for drug delivery systems¹⁷⁸. HA-coated nanoparticles can deliver drugs such as doxorubicin and paclitaxel directly to CD44-overexpressing cells, thereby enhancing efficacy and helping to overcome drug resistance¹⁷⁹. In cancer, also inhibition of the interaction between CD44 and HA is considered a promising therapeutic strategy, inhibiting CD44-HA mediated signaling and cancer invasiveness^{179,180}.

1.3.3.2 Osteopontin

Osteopontin (OPN) is a negatively charged protein acting both as a structural component and a cytokine. The binding of OPN by CD44 regulates cancer progression and metastasis. Osteopontin can increase cell-surface CD44 expression (both standard and alternative splicing forms) in various cell types¹⁸¹. CD44-OPN interaction has been shown to promote tumorigenesis and invasion, especially in H-Ras-transformed cells, *via* Rac signaling¹⁸². In gliomas, OPN and CD44 are co-expressed in perivascular niches, driving stemness and resistance *via* CD44-mediated signaling¹⁸³. In colorectal cancer, the presence of CD44 in cancer cells was linked to increased secretion of OPN by macrophages. Enhanced binding of OPN by CD44 resulted in increased clonogenicity of cancer cells as a result of CD44-dependent activation of c-Jun-NH2-kinase signaling¹⁸⁴.

1.3.3.3 Other interactions

Interestingly, alternative splicing variants expand the repertoire of available ligands for CD44. For instance, CD44v6 can interact with hepatocyte growth factor (HGF), the ligand for c-Met. CD44v6 is a c-Met co-receptor presenting HGF to c-Met, leading to its activation^{185,186}. A similar mechanism is observed when CD44v6 regulates the activation of another receptor tyrosine kinase, VEGFR-2, by promoting the binding of vascular endothelial growth factor (VEGF)¹⁸⁷. On the other hand, CD44v3 contains a heparan sulfate-binding site and is capable of binding other growth factors, such as the fibroblast growth factor (FGF) and the epidermal growth factor (EGF)¹⁸⁸.

1.3.4 CD44 in leukemias

In leukemias, CD44 plays a key role in the establishment of leukemic niches, cell survival, and resistance to pro-apoptotic signals¹⁸⁹. Crucially, CD44 is essential for the homing of leukemic stem cells to the bone marrow niche¹⁹⁰. In AML, binding of HA to CD44 triggers “inside-out” activation of the VLA-4 integrin. This process, mediated by receptor clustering on the cell surface, markedly enhances the adhesion of AML blasts to bone marrow stromal cells *via* VCAM-1, thereby promoting their retention within a protective niche¹⁹¹. Additionally, in CML, stem cells expressing BCR::ABL1 are highly dependent on CD44 for homing and engraftment, and its loss significantly impairs their ability to initiate disease in murine models^{66,192–194}. High expression of CD44 facilitates adhesion of T-cell acute lymphoblastic leukemia (T-ALL) cells to E-selectin within adipocyte-rich niches, promoting their survival and resistance to chemotherapy⁶⁷. CD44 is crucial not only for LSCs’ homing but is also an active regulator of their phenotype and function. In AML, cooperation between CD44 and CXCR4 receptor induces the expression of key stem cell transcription factors, including Sox2, Oct4, and Nanog¹⁹⁵. Moreover, the RNA-binding protein MBNL3 promotes alternative splicing of the CD44v3 isoform, which is required for AML stem cell self-renewal^{126,196}.

In leukemic cells, CD44 activates signaling pathways that protect cells from death induced by drugs. For instance, in CLL, CD44 stimulation by its ligands increases the level of the anti-apoptotic protein MCL-1 *via* the ERK and AKT pathways. Conversely, CD44 blockade with blocking antibodies induces apoptosis, particularly in aggressive ZAP-70⁺ cases¹⁹⁷. In AML, CD44-dependent adhesion activates the AKT, MAPK, and mTOR pathways, directly reducing chemotherapy-induced apoptosis (by doxorubicin or venetoclax)¹⁹¹. In IDH-mutant AML cells, CD44 is important for metabolic reprogramming that enhances NADPH production, which is required for the generation of the oncometabolite R-2-hydroxyglutarate (R-2HG). CD44 blockade synergizes with IDH inhibitors in the elimination of these cells¹⁹⁸. Additionally, imatinib-resistant CML cells often exhibit CD44 overexpression. Prolonged exposure to this TKI can lead to the selection of a CD44^{high}CD24^{low} cell population displaying stem cell-like properties and an epithelial-mesenchymal transition (EMT)-like

phenotype, which promotes invasiveness and survival^{192,199,200}. Li and colleagues showed that in CML cells, the AF1q protein promotes imatinib resistance by directly binding to the *CD44* promoter and upregulating the expression of CD44v6 and CD44v10 splicing variants. Moreover, CD44 blockade with the A3D8 antibody attenuated this AF1q-induced resistance²⁰¹. In another study, contact between CML cells and MSCs induced β -catenin and CD44 expression, which directly contributed to TKI resistance in the CML blast crisis phase²⁰². On the other hand, CD44 knockdown in K562 cells inhibited proliferation and induced G0/G1 cell cycle arrest through destabilization of the Wnt/ β -catenin signaling pathway and alterations in p21 and cyclin D1 levels²⁰³. It was also shown that CD44 stimulation by HA protects CML cells from the cytostatic effect of vincristine²⁰⁴. Anti-CD44 antibodies (e.g. IM7) can induce apoptosis in CML stem cells by downregulating c-MYC and BCL-2 expression and reducing NF- κ B activity¹⁹².

In summary, the role of CD44 in leukemias, including CML, is multifaceted. This protein contributes to several critical aspects of leukemia progression, ranging from bone marrow niche engraftment to the development of resistance to therapeutic agents.

2 AIMS

Bone marrow stromal cells (BMSCs) represent one of the key components of the bone marrow niche, supporting the survival and therapy resistance of chronic myeloid leukemia (CML) cells in several ways. One of the most poorly understood mechanisms of stroma-leukemia interactions is the communication through tunneling nanotubes (TNTs). Previous studies conducted at the Laboratory of Cytometry, Nencki Institute of Experimental Biology PAS have demonstrated that BMSCs transfer cellular vesicles to CML cells *via* tunneling nanotubes, leading to the increased resistance to imatinib-induced apoptosis in acceptor cells. Proteomic analysis of vesicle-accepting and non-accepting cells revealed that functional groups of proteins potentially involved in stroma-mediated resistance of leukemia are transferred together with stromal vesicles to leukemia cells⁷⁸. Although these studies have provided important insights into stroma-driven protection and resistance of leukemia, it remains unclear how specific transferred proteins remodel recipient leukemic cells and influence their resistance. We hypothesized that transfer *via* TNTs might be involved in the stroma-dependent upregulation of CD44, the protein with well-documented cancer-promoting properties. Furthermore, significant gaps remain in the understanding of TNTs, particularly regarding their precise ultrastructural organization and reliable discrimination from other similar structures.

Therefore, the general objective of this work was to evaluate the role of TNT-mediated transfer in the stroma-dependent CD44 upregulation in leukemia cells, and subsequently, the functional effects of CD44 uptake in the context of stroma-driven protection of leukemia. An additional goal was to expand the current knowledge on the ultrastructure of TNTs.

The specific aims were as follows:

1. To comprehensively characterize the 3D ultrastructure of TNTs formed between BMSCs and CML cells and critically assess the limitations of standard fluorescence microscopy techniques.
2. To identify CD44 among the proteins transferred from stroma to leukemia *via* TNTs and experimentally verify the TNT-mediated transfer.
3. To investigate how the CD44 protein transfer influences the transcriptomic profile of leukemic recipient cells.
4. To assess how upregulation of the CD44 protein in leukemia cells affects their key cellular pro-leukemic functions.
5. To validate the stroma-to-leukemia CD44 transfer as an element of the protection against the effects of tyrosine kinase inhibitors and a potential therapeutic target.

3 MATERIALS AND METHODS

3.1 Cell culture

3.1.1 Cell lines

3.1.1.1 K562 cell line

The K562 cell line (ATCC, #CCL-243) was used as a model for studying human chronic myeloid leukemia. This cell line is the first human, immortalized myeloid leukemia cell line. It was established in 1970 from the cells obtained from the 53-year-old woman with CML diagnosis in the blast crisis phase, after 4 years of chemotherapy²⁰⁵. K562 cells are non-adherent, round-shaped, characterized by the presence of the Philadelphia chromosome (*bcr::abl1* fusion) and BCR::ABL1 oncoprotein. However, there are other genetic abnormalities found in these cells²⁰⁶. Despite this, the K562 cell line is commonly used for *in vitro* CML research.

K562 imatinib-resistant (IR) and parental (PAR) cells

K562 Parental (PAR) and K562 imatinib-resistant (IR) cells were obtained from Prof. Laurie L. Parker from the Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, USA. Briefly, K562 cells were cultured in the presence of increasing concentrations of imatinib (for IR cells) for 90 days, which was followed by a constant concentration of 1 μ M imatinib. K562 PAR cells were cultured in the absence of the drug for the same period²⁰⁷.

3.1.1.2 LAMA-84 cell line

The LAMA-84 cell line (DSMZ, #ACC168) was used as another model of CML. It was derived in 1987 from a 29-year-old patient (woman) with the blast crisis of chronic myeloid leukemia²⁰⁸. Most cells in the culture display a round morphology and grow in suspension. Some cells are loosely adherent to the substratum, with spindle-like morphology. LAMA-84 cells are characterized by the presence of the Philadelphia chromosome and BCR::ABL1 oncoprotein. This cell line serves as another valuable model to study CML.

3.1.1.3 HS-5 cell line

The HS-5 cell line (ATCC, #CRL-11882) was used as a model of bone marrow stromal cells. It was established in 1995 from the bone marrow stroma of the healthy 30-year-old male donor. HS-5 cell line represents one of the 27 clones obtained by immortalizing isolated cells through their transduction with replication-defective recombinant retrovirus containing human papilloma virus-16 E6/E7 genes²⁰⁹. These cells are adherent and have fibroblast-like morphology. HS-5 cell line supports hematopoiesis without exogenous factors and mimics primary MSCs' immunophenotype, gene expression, and secretome²¹⁰. It is widely used to study interactions between leukemia and bone marrow stroma²¹¹.

3.1.1.4 HS-27A cell line

The HS-27A cell line (ATCC, #CRL-2946) was used as another model of BMSCs and was derived from the same patient as HS-5 cells as a subclone of HS-27²⁰⁹. These cells have an epithelioid morphology and are flattened with a polygonal shape. HS-27A secretes drastically lower levels of growth factors (e.g. IL-6, IL-8, G-CSF, GM-CSF)²⁰⁹ and exosomes²¹² compared to HS-5 cell line; however, they express high levels of glycoproteins (V-CAM1, ICAM-1)^{209,213}. This cell line exhibits less similarities to MSCs than HS-5 and does not support hematopoiesis; however, it supports the formation of “cobblestone” areas for CD34⁺CD38^{lo} cells^{209,214}.

3.1.2 Primary CD34⁺ CML cells

Patients' samples were obtained from the Department of Hematology, Transplantation and Internal Medicine, Medical University of Warsaw, following informed written consent, according to the Declaration of Helsinki and the guidelines for good clinical practice. All protocols were approved by the local Ethical Committee (Ethical and Bioethical Committee UKSW, Approval No. KEiB-19/2017, Approval No. WAW2/059/2019 and WAW2/51/2016). Peripheral blood mononuclear cells (PMBCs) were isolated from chronic myeloid leukemia patients' blood samples by density gradient centrifugation. Next, CD34⁺ cells were separated using EasySep human CD34⁺ selection cocktail (StemCell Technologies, #17856). The CD34⁺ population was subsequently assessed by flow cytometry, confirming purity of 98-99%. Following isolation, samples were cryopreserved and stored in liquid nitrogen

(-196°C) until use. The clinical characteristics of patients' samples used in this study are presented in **Table 3-1**. The predominance of CP-CML samples allowed the investigation of studied processes in the context of BCR::ABL1-driven disease, before the accumulation of secondary mutations commonly observed during blast crisis progression.

Table 3-1 CML Patients' samples characteristics

ID	Age	Sex	CML stage	Identified mutations (NGS)	% of blasts in the blood
P181	55	M	CML-CP	Primarily resistance ABL1 E255K mutation	1%
P371	30	M	CML-CP	No NGS diagnostics	8% IG (immature cells)
P372	51	F	CML-CP	No NGS diagnostics	81G/L leukocytosis
P356	52	F	CML-BC	MSH2 and WT1 mutations	27%

3.1.3 Cell culture conditions

In most of the experiments, cells were cultured under standard conditions at 37°C, 95% humidity, 5% CO₂, and 21% O₂ in the CO₂ HERAcell 150i (Thermo Fisher Scientific) incubator. Unless otherwise specified, cells were centrifuged at 200 × g for 5 min. All cell lines were routinely tested for Mycoplasma contamination.

3.1.3.1 Leukemia cell lines

Leukemia cell lines (K562 and LAMA-84) were grown in suspension, in the IMDM medium (Biowest, #L0191) for K562 and RPMI-1640 (Biowest, #L0490) for LAMA-84, both supplemented with 10% heat inactivated FBS (Gibco, #10500064), 100 µg/mL streptomycin, and 100 U/mL penicillin (Biowest, #392-0406). RPMI-1640 was additionally supplemented with 2 mM L-glutamine (Biowest, #X0550). K562-IR cells were cultured in the presence of 1 µM imatinib (MedChemExpress, #HY-15463). Cell density and viability were checked every 2 days following cell passage. The density was maintained within the range of 0.1-1 × 10⁶ cells/mL.

3.1.3.2 Stromal cell lines

Bone marrow stroma cell lines (HS-5 and HS-27A) were grown in cell culture-treated flasks to allow proper adherence. Cells were cultured in the IMDM medium supplemented with 10% FBS, 100 µg/mL streptomycin and 100 U/mL penicillin. The cell culture confluency and morphology were checked under the light microscope

every 2 days. Cells were passaged when they reached 80-90% confluence. Cells' detachment with trypsin 0.25% - EDTA 0.02% solution (Biowest, #L0932) was followed by neutralization with complete cell culture medium and reseeding into new culture flasks at the desired density. Cells were used for experiments up to passage 12, as higher passages exhibited signs of cellular senescence.

3.1.3.3 Primary CD34⁺ CML cells

After thawing, cells were cultured in the StemPro™-34 SFM (1X) (Gibco, #10639011) supplemented with 2 mM L-glutamine, 100 µg/mL streptomycin and 100 U/mL penicillin and cytokines as follows (**Table 3-2**):

Table 3-2 Cytokines used in the medium for primary CD34⁺ CML cells

Cytokine	Final concentration	Manufacturer	Identifier
Recombinant Human SCF	50 ng/mL	Gibco	#300-07
Recombinant Human TPO	50 ng/mL	Gibco	#300-18
Recombinant Human Flt3-Ligand	50 ng/mL	Gibco	#300-19
Recombinant Human IL-3	10 ng/mL	Gibco	#200-03
Recombinant Human IL-6	5 ng/mL	Gibco	#200-06
Recombinant Human GM-CSF	10 ng/mL	Gibco	#300-03

Cells were cultured for 2 days after thawing to achieve sufficient cell numbers. Cell density was maintained within the range of $0.1-1 \times 10^6$ cells/mL. Just before setting up the experiments, the CD34⁺ population was evaluated by flow cytometry to assess differentiation status. Only cultures containing more than 92% blasts (CD34⁺ cells) were selected for subsequent applications.

3.1.3.4 General stroma-leukemia co-culture setup

Leukemia monocultures and stroma-leukemia co-cultures were prepared for experiments involving subsequent flow cytometry or molecular biology analyses.

Briefly, stromal cells (HS-5 or HS-27A) were seeded at a density of 2.5×10^4 cells/cm² for 24h and 48 h co-cultures, and 1.6×10^4 cells/cm² for 72 h co-cultures in IMDM. For co-cultures designated for flow cytometry-based experiments and cell sorting, CML cells were labeled with eBioscience™ Cell Proliferation Dye eFluor™ 450 according to the manufacturer's instructions and cultured overnight to eliminate excess dye. This enabled flow cytometric discrimination between leukemic and stromal cell populations. Primary CML CD34⁺

cells were not labeled with tracking dyes, as they could be identified later in the co-culture, based on CD45 and CD34 expression. The following day, CML cells were counted, and cell suspensions were prepared in fresh cell culture medium at a density of 1×10^5 cells/mL for 24 h and 48 h co-cultures, and 0.8×10^5 cells/mL for 72 h co-cultures. Old medium from HS-5 cells was discarded and replaced with leukemia cells suspension at the previously established in our Laboratory stroma:leukemia 1:1 ratio. In parallel, leukemia monocultures were established at the same cell densities. The drugs were added at the time of co-culture setup. For Transwell controls, stromal cells were seeded in 1 mL of medium in the bottom chamber and leukemia cells in 1 mL on the upper surface of the insert, with a pore size of $1 \mu\text{m}$, 2×10^6 pores/cm² (Greiner Bio-One, #665610).

3.1.4 Routine assessment of cell viability and concentration

Cell viability and concentration were assessed routinely using the Muse Cell Analyzer (Merck) and the Muse Count and Viability Reagents (Merck, #LUMIMCH600103). 20 μL of cell suspension was added to 380 μL of the reagent, mixed, and incubated at room temperature (RT) for 2 min, followed by direct analysis.

3.2 Generation of genetically modified cell lines

3.2.1 Generation of cells with CD44 knockdown

To establish HS-5 and LAMA-84 cell lines with silenced CD44 expression, cells were transduced with MISSION® shRNA Lentiviral Transduction Particles carrying a pLKO.1_puro vector (Sigma-Aldrich, #SHCLNV). To ensure efficient gene silencing, five different shRNA clones targeting *CD44* were validated (listed in **Table 3-3**). As a negative control (shNEG), cells were transduced with MISSION® TRC2 pLKO.5-puro non-mammalian shRNA Control Transduction Particles (#SHC202V). The multiplicity of infection (MOI=10) was used based on prior optimization established in our Laboratory for these cell lines. After 48 hours of incubation with lentiviral particles, cells were selected with puromycin for 14 days (0.5 $\mu\text{g/mL}$ for HS-5 and 1 $\mu\text{g/mL}$ for LAMA-84). Cells were examined under the light microscope to assess their morphology, and their viability was routinely evaluated as described in **3.1.4**.

Immunoblotting and RT-qPCR were used to evaluate the knockdown efficiency of CD44. Cells transduced with the most effective Clone 5 were used in all experiments.

Table 3-3 Mission® shRNA lentiviral particles used for CD44 silencing

Target	Clone	TRC number	Source	Target region	Identifier
Non-targeting	-	-	Sigma-Aldrich	-	#SHC202V
CD44	1	TRCN0000057567		CDS	#SHCLNV
	2	TRCN0000289308		CDS	
	3	TRCN0000289233		CDS	
	4	TRCN0000296191		CDS	
	5	TRCN0000308110		3'UTR	

3.2.2 Generation of cells with CD44-GFP overexpression

To overexpress CD44 in the fusion with EGFP in HS-5 stromal cells, cells were transduced with custom-made, ready-to-use lentiviral particles: pLV[Exp]-EF1A>{CD44s(fl)}/3xGGGGS/EGFP; vector ID: VB240617-1475qbv (**Figure 3.2.1**). Expression vectors and lentiviral particles were constructed and packaged by VectorBuilder.

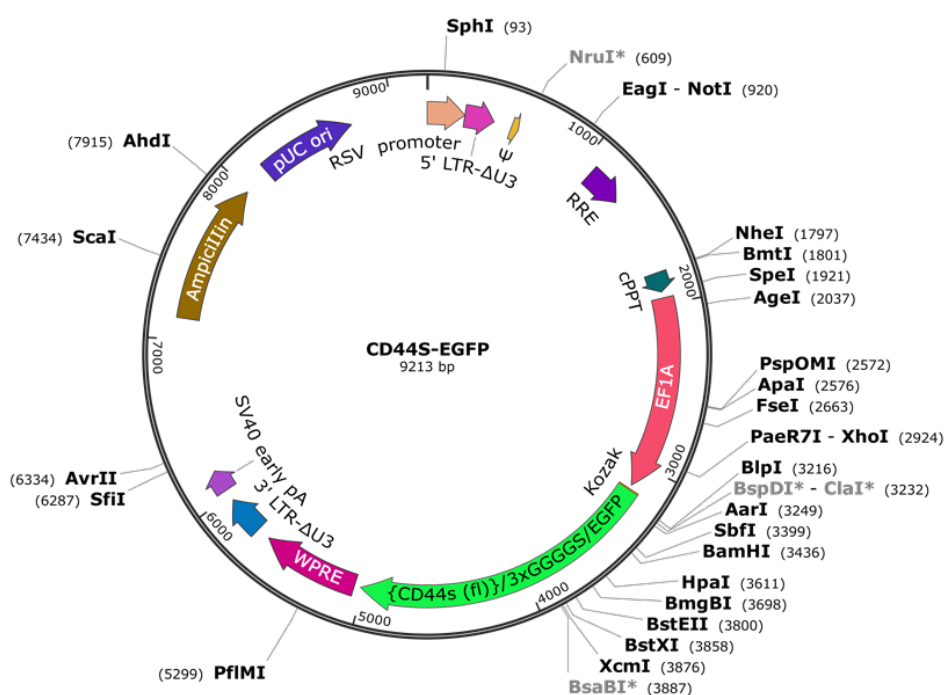


Figure 3.2.1 Schematic map of the lentiviral construct used for CD44-GFP overexpression in HS-5 cells. CD44 is expressed in-frame with GFP under the EF1A promoter, a 3xGGGGS linker was used between CD44 and GFP to allow proper folding and function.

The HS-5 cells were transduced with lentiviral particles (MOI=10) supplemented with 8 µg/mL polybrene (VectorBuilder) for 24 hours. Next, medium was replaced with standard culture medium, and cells were cultured for an additional 7 days, after which a homogenous GFP-positive population was sorted under sterile conditions by FACS using BD FACS Aria IIu cell sorter (85 micron nozzle, with sorting speed not exceeding 6000 events/second), according to the gating strategy shown in **Figure 3.2.2**. Sorted cells were expanded, cryopreserved and used for subsequent experiments.

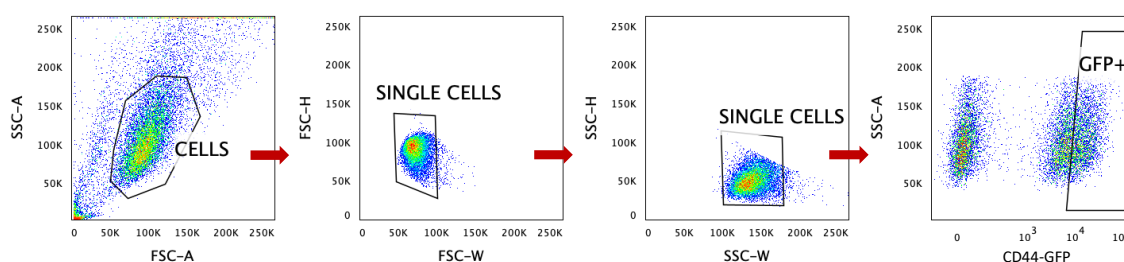


Figure 3.2.2 Sorting of HS-5 CD44-GFP cells. Representative gating strategy used to sort a homogenous GFP-positive population of HS-5 CD44-GFP cells.

3.3 Cell treatment with drugs

3.3.1 Tyrosine kinase inhibitors

Imatinib (MedChemExpress, #HY-15463), other names: STI-571, Gleevec™ is a first-generation tyrosine kinase inhibitor, commonly used in the treatment of CML. A 10 mM stock solution in DMSO was aliquoted and stored at -20°C or -80°C (for periods longer than 1 month). The stock was diluted 100× in cell culture medium and added to the cell culture to obtain a final concentration of 0.5, 1, 1.5 µM. These concentrations are routinely used in the Laboratory of Cytometry. The concentration and incubation time depended on the cell type and experimental design.

Nilotinib (MedChemExpress, #HY-10159) and **dasatinib** (MedChemExpress, #HY-10181) are second-generation TKIs routinely used in the treatment of Philadelphia chromosome-positive CML patients. 10 mM stock solutions in DMSO were aliquoted and stored at -20°C or -80°C (for periods longer than 1 month). The stocks were diluted 10 000× in cell culture medium and added to the cell culture to

obtain final concentration of 5, 10, 25, 50 nM for nilotinib and 1, 2.5, 5 and 10 nM for dasatinib. Cells were incubated with the drugs for 24 hours.

3.3.2 CD44-HA interaction inhibitor 2

CD44-HA interaction inhibitor 2 (MedChemExpress, #HY-149897) inhibits the interaction between hyaluronic acid (HA) and CD44¹⁸⁰. A 10 mM stock solution in DMSO was aliquoted and stored at -20°C or -80°C (for periods longer than 1 month). The stock was diluted 100× in cell culture medium and added to the cell culture to obtain a final concentration of 0.5 or 1 μM. The concentration and incubation time were dependent on the cell type and experimental design.

3.3.3 Phorbol 12-myristate 13-acetate (PMA)

Phorbol 12-myristate 13-acetate (PMA) (MedChemExpress, #HY-18739) is a phorbol ester commonly used as an activator of PKC and its downstream signaling. A 10 mM stock solution in DMSO was aliquoted and stored at -80°C. The stock was diluted 10 000× in cell culture medium and added to the cell culture to obtain a final concentration of 5 nM. Cells were incubated for overnight or 24 hours.

3.3.4 Bisindolylmaleimide I (BIM)

Bisindolylmaleimide I (BIM) (MedChemExpress, #HY-13867) is a selective, reversible PKC inhibitor showing selectivity for PKC α , β 1, β 2, γ , δ , and ϵ isozymes.²¹⁵ It was shown to inhibit glycogen synthase kinase 3 (GSK3) as well. A 1 mM stock solution in DMSO was aliquoted and stored at -20°C or -80°C (for periods longer than 1 month). The stock was diluted 10× in cell culture medium and added to the cell culture to obtain final concentration of 2.5 μM. The concentration was selected based on preliminary dose-response experiments.

3.3.5 RSL3

RSL3 (RAS-selective lethal 3) ((1S,3R)-RSL3) (MedChemExpress, #HY-100218A) is a potent, small-molecule glutathione peroxidase 4 (GPX4) inhibitor, which is a key enzyme responsible for maintaining redox balance in cells²¹⁶. RSL3 is commonly used to induce ferroptosis in *in vitro* studies²¹⁷. A 10 mM stock solution in DMSO was aliquoted and stored at -20°C or -80°C (for periods longer than 1 month).

The stock was diluted 1000× in cell culture medium and added to the cell culture to obtain final concentration of 150 nM. The concentration was selected based on preliminary dose-response experiments evaluating lipid peroxidation by BODIPY 581/591 C11 staining.

3.3.6 Ferrostatin-1

Ferrostatin-1 (MedChemExpress, #HY-100579) is an ethyl ester, a potent inhibitor of ferroptosis. This inhibitor acts as a lipid peroxyl radical scavenger, preventing oxidative membrane damage. Ferrostatin-1 protects cells from iron-dependent oxidative stress and eventual ferroptotic cell death by scavenging lipid peroxyl radicals and reducing oxidative membrane damage^{217–219}. A 10 mM stock solution in DMSO was aliquoted and stored at -20°C or -80°C (for periods longer than 1 month). The stock was diluted 100× in cell culture medium and added to the cell culture to obtain a final concentration of 1 µM. The concentration was selected based on preliminary dose-response rescue experiments evaluating the ability of ferrostatin-1 to reverse RSL3-induced lipid peroxidation measured by BODIPY 581/591 C11 staining.

3.3.7 GW4869

GW4869 (Tocris, #6741) is an inhibitor of neutral sphingomyelinase (nSMase2), one of the most commonly used inhibitors of exosome biogenesis²²⁰. The compound was dissolved in DMSO to obtain a 2 mM stock solution, which was aliquoted and stored at -20°C or -80°C (for periods longer than 1 month). The stock was diluted 100× in cell culture medium and added to the cell culture to obtain a final concentration of 100 nM. The concentration was previously optimized in our Laboratory²²¹.

3.3.8 Nexinhib20

Nexinhib20 (Tocris, #6089/5) is an inhibitor of Rab27a and neutrophil exocytosis²²². Rab27a is involved in the regulation of exosome secretion by mediating the docking of multivesicular bodies to the plasma membrane²²³. Consequently, Nexinhib20 is often used alongside GW4869 to inhibit exosome secretion in cell culture. The compound was dissolved in DMSO to obtain a 100 mM stock solution,

which was aliquoted and stored at -20°C or -80°C (for periods longer than 1 month). The stock was diluted 100× in cell culture medium and added to the cell culture to obtain a final concentration of 250 nM. The concentration was previously optimized in our Laboratory²²¹.

3.4 Cellular functional assays

3.4.1 Apoptosis

Apoptosis was assessed to evaluate the effect of the applied treatments. Cells were treated for 48 or 72 hours with drugs. After that, 2×10^5 cells were collected per tube. This was followed by centrifugation at $200 \times g$ for 5 min at room temperature and two washes with PBS. Then, cell pellets were resuspended in 100 μ L of the Annexin V Binding Buffer (BD Pharmingen, #556454) (1:10 dilution in distilled water), 5 μ L of Annexin V-FITC (BD Pharmingen, #556419) or Annexin V-PE (BD Pharmingen, #556421), and 5 μ L of 7-AAD (BD Pharmingen, #559925). Cells were incubated at RT in the dark for 15 min. Next, 300 μ L of diluted Annexin V Binding Buffer was added, and samples were analyzed using a BD LSR Fortessa flow cytometer (Becton-Dickinson). If required, immunophenotyping (e.g., anti-CD44 staining) was performed prior to apoptosis staining.

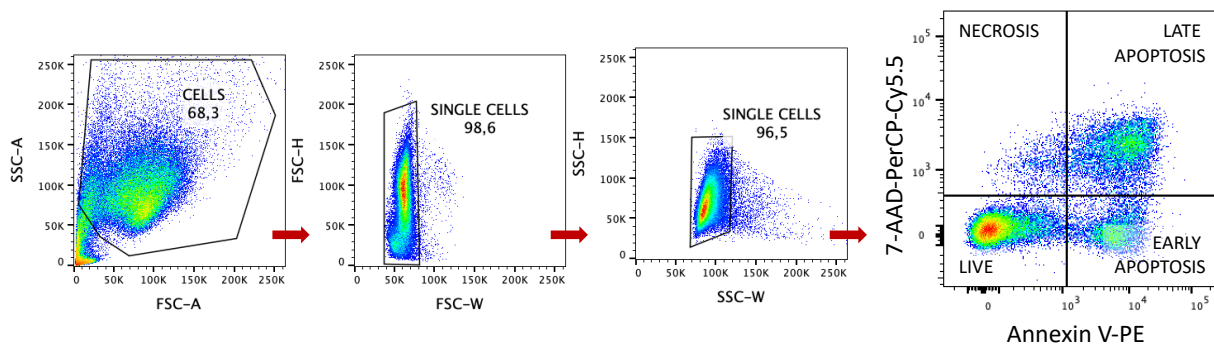


Figure 3.4.1 Representative gating strategy to analyze apoptosis using Annexin V and 7-AAD. Quadrants were defined as follows: LIVE (Annexin V⁻ 7-AAD⁻), EARLY APOPTOSIS (Annexin V⁺ 7-AAD⁻), LATE APOPTOSIS (Annexin V⁺ 7-AAD⁺), NECROSIS (Annexin V⁻ 7-AAD⁺).

After exclusion of cellular debris and doublets, live, early apoptotic, late apoptotic, and necrotic cells were identified using appropriate gates. The gating strategy and representative dot plot of apoptosis detection are shown in **Figure 3.4.1**.

The percentage of apoptotic cells was calculated as the sum of early and late apoptotic populations.

3.4.2 Cell cycle

To evaluate cell cycle distribution in HS-5 cells, 2×10^6 cells were seeded per T75 flask and cultured for 72 h. Next, cells were collected by trypsinization and centrifuged ($200 \times g$, 5 min, 4°C), washed twice with cold PBS and fixed in ice-cold 70% ethanol overnight at -20°C . Then, cells were centrifuged ($300 \times g$, 5 min, 4°C), washed twice with cold PBS and resuspended in 0.5 mL of extraction buffer (0.192 M Na_2HPO_4 and 4 mM citric acid) diluted 1:1 in PBS, followed by incubation for 5 min at RT. After centrifugation, cells were resuspended in 0.5 mL of staining solution containing 3.8 mM sodium citrate, 25 μg propidium iodide (Sigma-Aldrich, #P4170), 0.25 mg RNase A (Sigma-Aldrich, #4875), and PBS to the final volume and incubated for 30 min at RT in the dark prior to flow cytometry analysis on BD LSR Fortessa. Data was analyzed in ModFit LT software.

3.4.3 Cell proliferation

To measure cellular proliferation, cells were labelled with Cell Proliferation Dye eFluor450 (Invitrogen, #65-0842-90) according to the manufacturer's instructions. Next, cells were seeded at a density optimized for the culture of each cell line, and fluorescence was analyzed using flow cytometry (BD LSR Fortessa cytometer) on the day of labeling (0 h) and every 24 h for the subsequent two to three days. For quantification, the geometric mean fluorescence intensity (gMFI) was extracted and normalized to the value at 0 h.

3.4.4 Colony formation assay

To evaluate the long-term clonogenic potential of cells following treatment, a colony formation (clonogenic) assay was performed. K562 or CD34⁺ human primary CML cells were seeded in T25 flasks (K562) or 96-well plates (primary cells) in mono- and co-cultures with HS-5 cells, treated with imatinib, CD44-HA Interaction inhibitor 2, or both, for 72 hours. Then, cells were counted, and 500 cells (K562) or 2000 cells (primary cells) were seeded in gridded 35 mm dishes (Sarstedt, #83.3900.002), in 1 mL of Methocult. Methocult H4230 (StemCell Technologies, #04230) was used

for K562 cells, whereas Methocult H4434 (StemCell Technologies, #04434) was used for CD34⁺ primary CML cells. After 6 (K562 cells) or 12 days (CD34⁺ primary CML cells), all colonies present across the entire surface of each dish were manually counted under the inverted microscope.

3.4.5 Migration and invasion assays

In vitro migration and invasion assays were employed to evaluate the motility and extracellular matrix-penetrating capacity of leukemia CD44⁺ and CD44⁻ cells. For invasion assay, 24-well Corning® Fluoroblok™ Inserts with 8 µm pore size (Corning, #351152) were coated with Matrigel (Corning, #356234) according to the manufacturer's instructions. First, the coating solution was prepared by mixing 200 µg of Matrigel with 1 mL of coating buffer (0.01 M Tris pH 8.0, 0.7% NaCl). Next, 100 µL of coating solution was added at the top of each insert and was left for 3 h at 37°C. For the migration assay, inserts were not coated with Matrigel. After cell sorting and 3 h of resting, leukemic CD44⁺ and CD44⁻ cells were labeled with Green CMFDA (MedChemExpress). Then, cells were counted and suspended in culture media without FBS at a density of 2×10^5 cells/mL. 500 µL of each cell suspension (1×10^5 cells) was transferred on the top of the insert. On the bottom of the inserts, 750 µL of culture media containing 20% FBS was placed. After 48 h, cells present under the inserts were visualized with a Zeiss Axiovert microscope using a 20× objective (Zeiss, Germany) in a random 10 fields. The number of migrated cells was quantified with ImageJ.

3.5 Flow cytometry-based analyses

3.5.1 Antibodies and dyes used for flow cytometry

Antibodies and dyes used for flow cytometry analyses are listed in **Table 3-4**. For all flow cytometry experiments, antibody titration was performed to determine optimal concentrations. Fluorescent dyes used in the experiments involving flow cytometry are listed in **Table 3-5**.

Table 3-4 Antibodies used for flow cytometry staining

Antigen	Fluorochrome	Amount/test (volume/1 mln of cells)	Antibody (origin, clonality, clone)	Manufacturer	Catalog No
CD34	eFluor450	5 µL	mouse, monoclonal, 4H11	Invitrogen	48-0349-42
CD34	PE	5 µL	mouse, monoclonal, 581	Invitrogen	CD34-581-04
CD34	APC	10 µL	mouse, monoclonal, 581	BD Pharmingen	555824
CD44	PE	0.625 µL	rat, monoclonal, IM7	Biologend	103008
CD44	APC	0.32 µL	rat, monoclonal, IM7	Biologend	103012
CD44	PerCP-Cy5.5	5 µL	mouse, monoclonal, G44-26	BD Pharmingen	560531
CD45	APC	7.5 µL	mouse, monoclonal, HI30	BD Pharmingen	555485
CD45	Pacific Orange	5 µL	mouse, monoclonal, HI30	Invitrogen	MHCD4530
P-STAT3	Alexa Fluor 647	5 µL	mouse, monoclonal, 13A3-1	Biologend	651008
P-c-Jun	Alexa Fluor 647	5 µL	mouse, monoclonal, A19012A/R/C	Biologend	605555
P-c-Fos	Alexa Fluor 647	5 µL	mouse, monoclonal, A22009C	Biologend	631257
P-RelA (p65)	PE	20 µL	mouse, monoclonal, K10-895.12.50	BD Phosflow	558423

Table 3-5 List of dyes used in flow cytometry experiments

Fluorescent dye	Amount/test (volume) or Concentration	Manufacturer	Catalog No
7-AAD	5 µL	BD Pharmingen	559925
Annexin V FITC	5 µL	BD Pharmingen	556419
Annexin V PE	5 µL	BD Pharmingen	556421
BODIPY 581/591 C11	2 µM	Invitrogen	D3861
Cell Proliferation Dye eFluor™450	10 µM	Invitrogen	65-0842-90
CMFDA Green	2.5 µM	MedChemExpress	HY-126561
Fixable Viability Dye eFluor™ 455UV	1 µL	Invitrogen	65-0868-14
Fixable Viability Dye eFluor™ 506	1 µL	Invitrogen	65-0866-18
LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, for 633 or 635 nm excitation	0.4 µL	Invitrogen	L34976
Propidium iodide (PI)	25 µg	Sigma-Aldrich	P4170
Vybrant™ DiD Cell-Labeling Solution	1.5 µL per 1 mL	Invitrogen	V22887

3.5.2 Analysis of surface and phosphorylated protein expression

3.5.2.1 Staining of cell surface markers

For the assessment of surface protein expression by flow cytometry, cells were stained with fluorochrome-conjugated antibodies. First, cells were collected and centrifuged ($200 \times g$, 5 min, 4°C). It was followed by two washes in cold PBS. Up to 3×10^6 cells were used in a single staining sample (1 test). Cells were stained with an antibody cocktail prepared in FACS buffer (2% FBS in PBS), in a total volume of 100 μL , for 30 minutes, at 4°C . A suitable viability dye was included in the antibody cocktail to allow discrimination of dead cells (**Table 3-5**). After incubation, cells were washed twice, resuspended in cold PBS, and analyzed using the BD LSR Fortessa cytometer.

3.5.2.2 Intracellular staining of phosphorylated proteins

To analyze the levels of phosphorylated proteins by flow cytometry, first, surface proteins were stained as described in **3.5.2.1**. For viability, Fixable Viability Dyes were used (**Table 3-5**). After incubation with surface markers and one wash with cold PBS, cell pellets were resuspended in 250 μL of BD Cytofix Fixation Buffer (BD, #554655) and incubated for 15 minutes at 37°C . Next, cells were washed once with PBS ($400 \times g$, 5 min, 4°C), resuspended in 800 μL of ice-cold BD Phosflow Perm Buffer III (BD, #558050) and incubated on ice, in the dark for 30 minutes. This was followed by centrifugation and two washes with FACS buffer. Cells were then resuspended in the antibody cocktail prepared in FACS buffer and incubated for 30 minutes at room temperature. After incubation and two washes with FACS buffer, cell pellets were resuspended in PBS and analyzed using the BD LSR Fortessa cytometer.

3.5.3 Cargo transfer assays

3.5.3.1 Analysis of cellular vesicle transfer

Day 0

Stromal cells (HS-5 or HS-27A) were seeded at a density of 3.5×10^4 cells/cm² in IMDM. Leukemia cells were seeded at a density of 4×10^5 cells/mL.

Day 1

Stromal cells were covered with 1.5 μ M Vybrant™ DiD Cell-Labeling Solution (diluted in the culture medium) and incubated at 37°C, 5% CO₂ for 20 min. After this, the labeling solution was discarded, and cells were washed twice with PBS, followed by trypsinization. Next, cells were centrifuged, counted, and seeded into the final culture vessel at a density of 2.5×10^4 cells/cm² in IMDM and cultured overnight to allow cell attachment and efflux of excess dye.

To track leukemia in co-culture, leukemic cells were labeled with eBioscience™ Cell Proliferation Dye eFluor™ 450 according to the manufacturer's instructions and cultured overnight to eliminate excess dye. Primary CML CD34⁺ cells were not labeled with tracking dyes, as they could be identified later in co-culture based on CD45 and CD34 expression.

Day 2

Labeled leukemic cells were counted, and cell suspensions were prepared in fresh cell culture medium at a density of 1×10^5 cells/mL. Old medium from HS-5 cells was discarded and replaced with leukemia cells suspension at the previously established in our Laboratory stroma:leukemia 1:1 ratio. In parallel, monocultures of leukemia cells were seeded (at a density of 1×10^5 cells/mL). Drugs were added to the cell cultures, and cells were cultured for the next 24 hours.

Day 3

Cells were collected without trypsin, washed twice with PBS, and, if needed, the CD44 protein was stained with fluorochrome-conjugated antibodies. Samples

were analyzed using BD LSR Fortessa cytometer. The gating strategy used for DiD transfer analysis, combined with anti-CD44 staining, was presented in **Figure 3.5.1**.

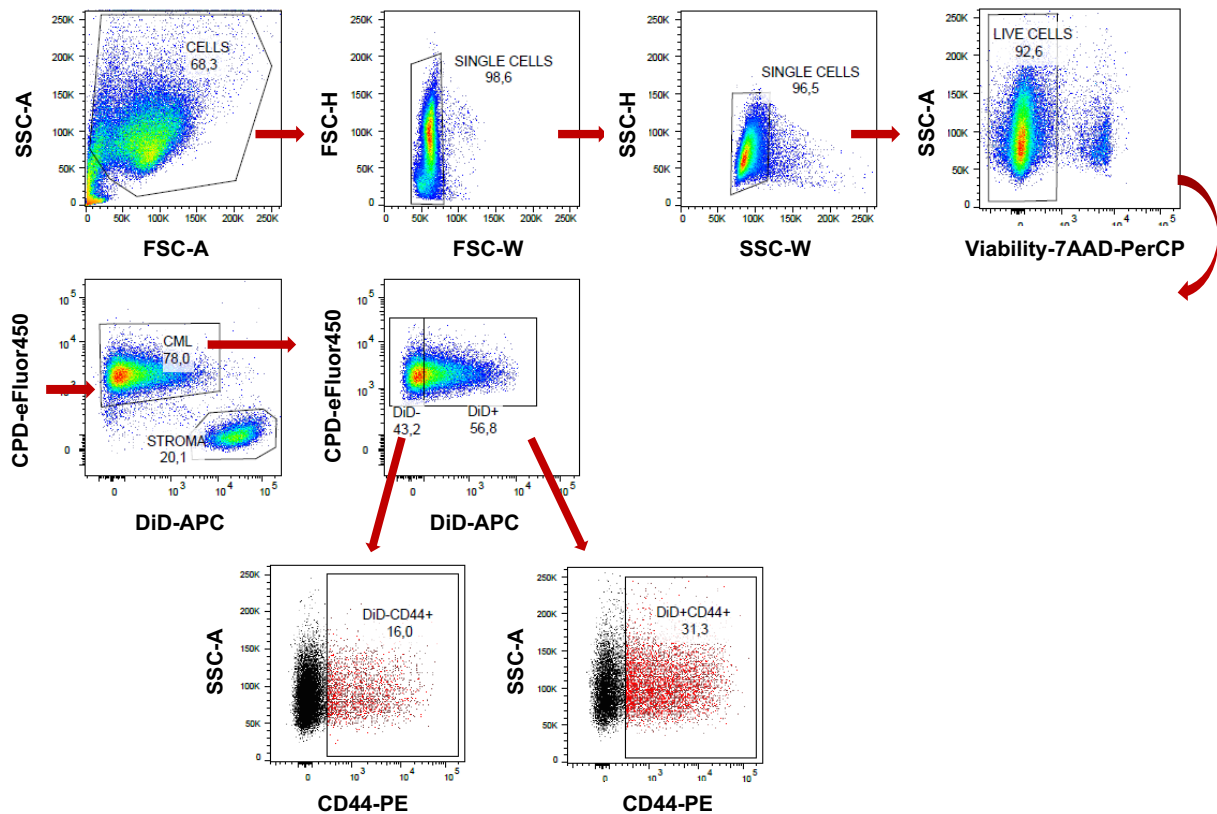


Figure 3.5.1 Analysis of vesicle uptake by leukemia cells combined with surface staining. Representative gating strategy to analyze the uptake of DiD-labeled stromal vesicles by leukemia cells. The percentage of live leukemia DiD⁺ cells was analyzed and counted. In identified DiD⁻ and DiD⁺ leukemia populations, CD44 expression was analyzed.

3.5.3.2 Analysis of CD44-GFP transfer

Day 1

HS-5 CD44-GFP stromal cells were trypsinized, counted, and seeded into the final culture vessel at a density of 2.5×10^4 cells/cm² in IMDM and cultured overnight to allow cell attachment. The staining strategy used to track leukemia cells was performed as described in **Section 3.5.3.1**.

Day 2

Mono- and co-cultures were established as described in **Section 3.5.3.1**. For Transwell controls, stromal cells were seeded in 1 mL of medium in the bottom chamber and leukemia cells in 1 mL on the upper surface of the insert, with a pore

size of 1 μm , 2×10^6 pores/ cm^2 (Greiner Bio-One, #665610). For conditioned medium controls, leukemic cells were cultured alone in a medium harvested from a 24-hour cell culture of HS-5 CD44-GFP cells.

Day 3

Cells were collected without trypsin, washed twice with PBS, and, if needed, surface proteins were stained with fluorochrome-conjugated antibodies. Samples were analyzed using the BD LSR Fortessa cytometer. The gating strategy used for CD44-GFP transfer was presented in **Figure 3.5.2**.

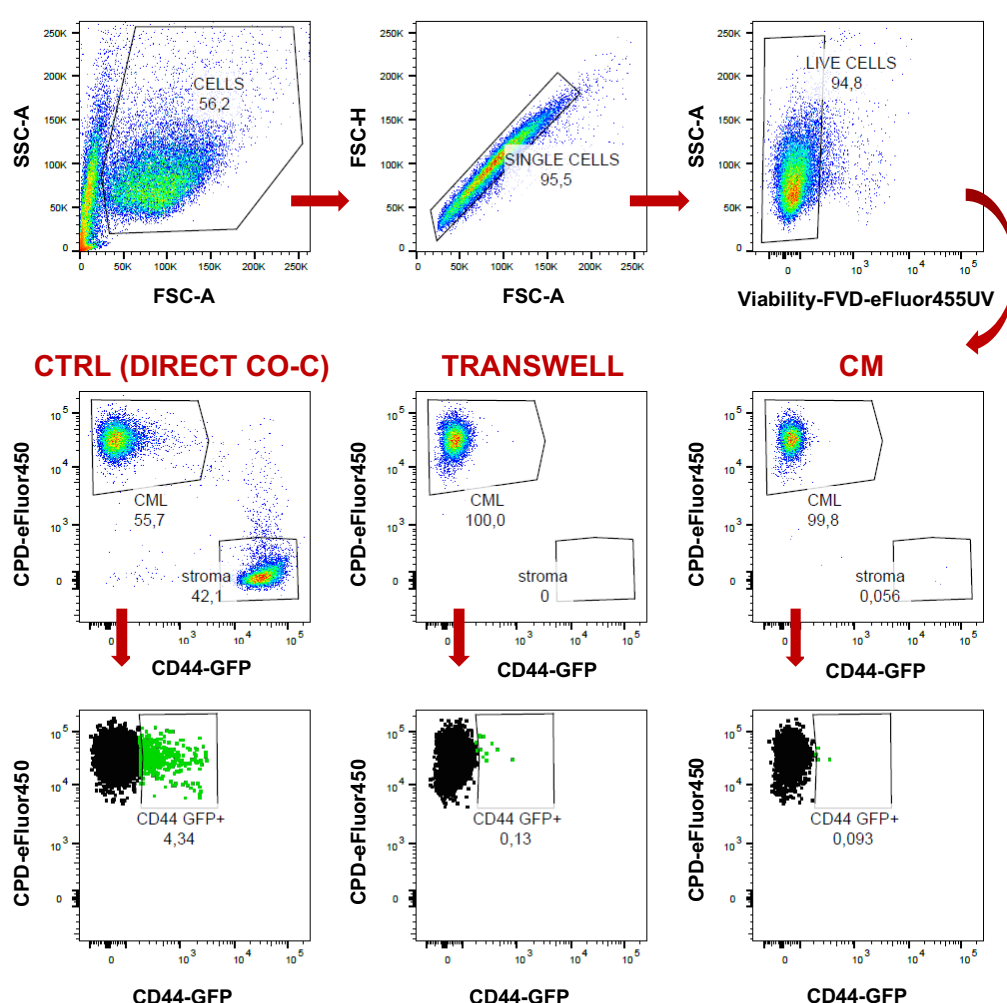


Figure 3.5.2 Analysis of the uptake of stromal CD44-GFP by leukemia cells. Representative gating strategy to analyze the uptake of stromal CD44-GFP by leukemia cells. The percentage of live leukemia GFP⁺ cells was analyzed and counted. CTRL – cells co-cultured directly, Transwell – cells co-cultured physically separated with cell culture insert, CM – leukemic cells cultured in conditioned media obtained from stromal cells.

3.5.4 Flow cytometry-based adhesion assay

To measure adhesion of CML cells to the stroma, fluorescently labeled CML cells were seeded onto HS-5 stromal cells in a 1:1 ratio. After 24 hours, non-adherent cells were removed, wells were gently washed with PBS, and the remaining cells were trypsinized, collected, and 30 000 events per tube were recorded using the BD LSR Fortessa cytometer. Results are presented as the CML:stromal cells ratio.

3.5.5 Flow cytometric analysis of protein synthesis

Global protein synthesis in leukemia cells was assessed using L-homopropargylglycine (HPG), a noncanonical amino acid analog of methionine that is incorporated into newly synthesized proteins during translation.

Cells were collected into FACS tubes (Corning, #352008) from 24 h mono- and co-cultures of K562 and HS-5 cells, established as described in **Section 3.1.3.4**. Cells were centrifuged ($200 \times g$, 5 min, RT), washed in PBS, and resuspended in 200 μ L of the methionine-free RPMI 1640 medium (Sigma, #R7513) supplemented as described in **Table 3-6**.

Table 3-6 RPMI 1640 methionine-free medium composition

Reagent	Final concentration	Manufacturer	Catalog No
FBS	10% (v/v)	Gibco	#10500064
L-glutamine	2 mM	Biowest	#X0550
Penicilin Streptomycin	100 μ g/mL 100 U/mL	Biowest	#392-0406
L-cysteine dihydrochloride	65 μ g/mL	MedChemExpress	#HY-W009203
Glucose	25 mM (4.5 g/L)	Biowest	#P5030
Sodium pyruvate	1 mM (0.088 g/L)	Gibco	#11360039

After 45 min incubation (37°C , 5% CO_2), cells were centrifuged ($200 \times g$, 5 min, RT) and resuspended in 200 μ L of medium described in **Table 3-6**, supplemented with 100 μ M L-homopropargylglycine (HPG) (MedChemExpress, #HY-140345). It was followed by 30 min incubation (37°C , 5% CO_2) and one wash in PBS. After centrifugation ($200 \times g$, 5 min, RT), cell pellets were resuspended in the 100 μ L of FACS buffer (2% FBS in PBS) containing LIVE/DEAD Fixable Near-IR stain (0.4 μ L) and anti-CD44-PE antibody (0.625 μ L) and incubated for 30 min in RT. 1 mL of FACS buffer was added to each tube and cells were centrifuged ($200 \times g$, 5 min, RT). Then, cells

were fixed in 2% PFA (MP Biomedicals, #199983) in PBS for 15 min at RT, followed by a wash in 1 mL of FACS buffer (300 × g, 5 min, RT) and permeabilized in 0.1% saponin (Calbiochem, #558255) in FACS buffer (15 min, RT). After washing in FACS buffer and centrifugation, cell pellets were resuspended in 200 µL of Click Reaction MIX (copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction mix) prepared in 100 mM Tris-HCl (pH = 7.4) as described in **Table 3-7**.

Table 3-7 Click Reaction MIX composition

Reagent	Final concentration	Manufacturer	Catalog No
CuSO ₄ • 5H ₂ O	0.5 mM	Sigma-Aldrich	#209198
THPTA Tris(3-hydroxypropyltriazolylmethyl)amine)	2 mM	MedChemExpress	#HY-W021042
(+)-Sodium L-ascorbate	10 mM	Sigma-Aldrich	#11140
Alexa Fluor 647 Azide Plus	0.5 µM	VectorLabs	#CCT-1482

After 30 min incubation at RT, 1 mL of FACS buffer was added to the tubes, followed by centrifugation (300 × g, 5 min, RT). After one more wash in FACS buffer, cells were centrifuged, resuspended in PBS, and analyzed using the BD LSRFortessa cytometer.

3.5.6 Lipid peroxidation analysis using BODIPY 581/591 C11

Lipid peroxidation is the ROS-driven oxidative damage of membrane lipids, particularly affecting phospholipid and polyunsaturated fatty acids. This leads to the formation of lipid peroxides, membrane disruption, and cell death, especially ferroptosis. To measure lipid peroxidation, the BODIPY 581/591 C11 probe was used. BODIPY 581/591 C11 enables detection of lipid peroxidation based on a fluorescence shift from ~590 nm (reduced state) to ~510 nm (oxidized state).

All centrifugation steps were performed under the following conditions: 200 × g, 5 min, room temperature. Cells were collected into FACS tubes, and 1 mL PBS was added to each tube as an initial washing step. Samples were centrifuged and washed once again with 1 mL PBS. After the final centrifugation, cell pellets were resuspended in 1 mL of a 2 µM BODIPY C11 working solution prepared in warm, serum-free RPMI medium. After vortex, samples were incubated for 30 minutes at 37°C in a humidified atmosphere containing 5% CO₂. Following incubation, 2 mL of PBS was added to each sample, and cells were centrifuged. Next, cell pellets were

resuspended in 100 μ L PBS containing LIVE/DEAD Fixable Near-IR stain (0.4 μ L/test) and incubated at RT for 15 minutes. It was followed by two washes with PBS. After final centrifugation, cells were resuspended in PBS and analyzed using the BD LSR Fortessa cytometer. The level of lipid peroxidation was determined as the ratio of geometric mean fluorescence intensity (gMFI) of the oxidized (FITC) signal to the reduced (PE) signal. If needed, to assess the effect of cell culture treatments, the obtained values were normalized to the untreated control condition.

3.5.7 Data acquisition and analysis

Unless otherwise specified, all samples for flow cytometry were collected using the BD LSR Fortessa cytometer (BD Biosciences), equipped with the 355 nm, 405 nm, 488 nm, and 635 nm lasers. For data analysis, FlowJo software (v. 10.10) (BD Biosciences) was used.

3.6 Fluorescence-activated cell sorting (FACS) of co-cultures

Fluorescence-activated cell sorting (FACS) was used to purify specific cell populations from co-cultures of stroma and leukemia for downstream analyses. After co-culture, cells were collected, centrifuged, and washed twice with PBS. For sorting K562 CD44⁺ and CD44⁻ cells, cells were stained with anti-CD44-PE antibody and incubated for 30 min at RT. This was followed by two washes with PBS. For sorting K562 DiD⁺ and DiD⁻ cells or K562 CD44-GFP⁺ and CD44-GFP⁻ cells, no additional staining procedures were performed. Cell pellets were resuspended in sterile 2% FBS in PBS, and cell suspensions were filtered through a 35 μ m sterile nylon filter (Falcon, #352235) into 5 mL polystyrene round-bottom tubes ("FACS tubes"). Cells were sorted into 15 mL tubes pre-coated with 2% FBS in PBS.

For samples intended for RNA or protein isolation, all procedures (including cell sorting) were performed at 4°C. Cell sorting of samples designated for further cell culture, invasion and migration assays, and Seahorse analysis was carried out at room temperature.

Most cell sorting experiments were performed at the Laboratory of Cytometry, Nencki Institute, using a BD FACS Aria IIu cell sorter (BD Biosciences). 85-micron nozzle was used, with sorting speed not exceeding 6000 events/second. An exception

was cell sorting performed for Seahorse metabolic analysis, for which a CytoFLEX SRT cell sorter (Beckman Coulter) was used (performed at the Laboratory of Immunology, University of Modena and Reggio Emilia).

3.7 Light microscopy

3.7.1 Immunocytochemistry

Cells were plated on collagen-I-coated coverslips, fixed with 4% paraformaldehyde, permeabilized with 0.1 Triton X-100, blocked with 2% BSA + 10% Normal Goat Serum, and incubated with antibodies. For actin staining, Phalloidin-eFluor570 was used (Invitrogen, #41-6559-05), and for nuclear staining - DAPI (Sigma-Aldrich, #D9542). For enhancing the GFP-fusion proteins signal, ChromoTek GFP-Booster Alexa Fluor® 488 was used (Proteintech, #CL488-66002). Images were acquired with a SP8 Leica microscope with a 63x objective and analyzed using ImageJ or Imaris software.

3.7.2 Confocal microscopy

3.7.2.1 Live cell microscopy and TNTs quantification

To perform live-cell imaging or quantify the number of TNTs formed in the co-cultures of stroma and leukemia, stromal cells were seeded at a density of 2.25×10^4 cells/cm² on collagen-I-coated Nunc™ Lab-Tek™ Chambered Coverglass (Thermo Scientific, #155411PK) and cultured overnight to allow proper adhesion. After this, leukemia cells were labeled with Proliferation Dye eFluor450 and gently added to the stromal cells at a 1:1 ratio for 24 hours. Co-cultures for live imaging were maintained in a total volume of 200 µL of IMDM, no phenol red medium (Gibco, #21056023) supplemented with 10% FBS and 1% penicillin-streptomycin. Immediately prior to the imaging, Wheat Germ Agglutinin conjugated to AF488 or AF647 (Invitrogen, #W11261 or #W32466) was used to label plasma membranes at a final concentration of 7.5 µg/mL. Images were acquired using the SP8 Leica microscope with a 63x objective (Leica).

TNTs quantification

For TNTs quantification, at least 10 fields of view (155 $\mu\text{m} \times 155 \mu\text{m}$) were imaged, with Z-stacks covering the entire volume of the cells. The data were analyzed manually with ImageJ software (Cell Counter plugin). For each field of view, calculations were performed as follows. First, numbers of leukemic (**a**) and stromal cells (**b**) were calculated, followed by quantification of three types of TNTs:

- heterotypic stroma-leukemia (**x**),
- homotypic leukemia-leukemia (**y**)
- homotypic stroma-stroma (**z**).

The average number of TNTs per 100 cells was quantified in a cell type- and TNT type-specific manner.

Homotypic TNTs

To estimate the average number of homotypic CML-CML TNTs per 100 CML cells, only homotypic CML-CML TNTs (**y**) were included in the analysis. However, each homotypic TNT connects two CML cells and was therefore weighted by a factor of two. Thus, the number of homotypic CML-CML TNTs per 100 CML cells was calculated as:

$$\text{Homotypic TNTs per 100 CML cells} = \frac{2y}{a} \times 100$$

The number of homotypic stroma-stroma TNTs per 100 stromal cells was calculated analogously:

$$\text{Homotypic TNTs per 100 stromal cells} = \frac{2z}{b} \times 100$$

Heterotypic TNTs

To estimate the average number of heterotypic stroma-leukemia TNTs per 100 CML cells, only heterotypic CML-stroma TNTs (**x**) were included in the analysis. The number of heterotypic TNTs per 100 CML cells was calculated as:

$$\text{Heterotypic TNTs per 100 CML cells} = \frac{x}{a} \times 100$$

The number of heterotypic stroma-stroma TNTs per 100 stromal cells was calculated analogously:

$$\text{Heterotypic TNTs per 100 stromal cells} = \frac{x}{b} \times 100$$

3.8 Electron microscopy

3.8.1 Focus Ion Beam – Scanning Electron Microscopy (FIB-SEM)

FIB-SEM experiments were performed in collaboration with Wioleta Dudka, PhD, Paolo Ronchi, PhD, and Yannick Schwab, PhD at the Electron Microscopy Core Facility, EMBL Heidelberg. The listed contributors were responsible for sample preparation and imaging. The author of the present dissertation was responsible for data analysis, quantification, and visualization.

3.8.1.1 Sample preparation

For co-culture, HS-5 cells and K562 cells were seeded in a 1:1 ratio, with a total of 2×10^5 cells, onto a poly-L-lysine-coated glass-bottom 35 mm μ -Dish (cell culture dish with a 500 μ m grid, Ibidi, #81168). One of the cell lines always expressed GFP to enable discrimination between the two populations. After 24 hours, plasma membranes were stained with wheat germ agglutinin coupled to Alexa Fluor 647. Cells were fixed gently by adding paraformaldehyde (PFA) to a final concentration of 4%. Samples were imaged using confocal microscopy (Zeiss LSM 880 Axio Observer or Abberior Instruments STED) to localize tunneling nanotubes on the gridded dish. After recording the imaging data and noting the regions of interest, the samples were prepared for FIB-SEM analysis.

Cells were then fixed with a 1:1 mixture of 2% osmium tetroxide and 3% potassium ferrocyanide using electron microscopy (EM) microwave processing (7×2 min). Samples were subsequently rinsed with cacodylate buffer and incubated for 10 min in 1% thiocarbohydrazide, followed by additional rinsing in cacodylate buffer. Cells were then post-fixed with 1% osmium tetroxide using EM microwave processing (7×2 min) and rinsed with distilled water. Next, samples were stained with 1% uranyl acetate using EM microwave processing (7×1 min), followed by rinsing with distilled water. Dehydration was performed through a graded ethanol

series (25%, 50%, 75%, 90%, 95%, 100%) with each step processed for 40 sec using the EM microwave. Samples were then infiltrated with Durcupan resin through increasing resin concentrations (25%, 50%, 75% and 100%) and polymerized at 90°C for 48 hours. After polymerization, the glass substrate was detached by consecutive immersion in boiling water and liquid nitrogen. The resin block was subsequently mounted onto an electron microscopy pin for further processing.

3.8.1.2 Imaging

FIB-SEM image acquisition was performed using FIB-SEM Crossbeam 550 microscope (Zeiss), at 8×8×8 nm voxel size.

3.8.1.3 Data analysis

The alignment of FIB-SEM image stacks was performed in Fiji (ImageJ) using the TrakEM plugin²²⁴. The images were then cropped, and the contrast was inverted. Segmentation of tunneling nanotubes and filopodia was performed in WEBKNOSSOS software²²⁵ (<https://webknossos.org/>), using Volume Annotation Tools (AI-based Quick Select Tool with manual correction using Brush and Eraser tools). For visualization, volume annotations were exported from the WEBKNOSSOS and imported into Amira software (Thermo Fisher Scientific, v. 2020.1) and aligned with the original volume FIB-SEM dataset. Figures were prepared in Amira. Measurements of structures were performed on segmented structures in WEBKNOSSOS, using the Skeleton tool.

3.9 Metabolic flux analysis

To assess differences in the metabolic profiles of K562 CD44⁺ and K562 CD44⁻ cells, Seahorse extracellular flux analysis was performed. 48-hour co-cultures of HS-5 and K562 cells were followed by anti-CD44 staining (CD44-PE antibody) and cell sorting (FACS) of K562 CD44⁺ and K562 CD44⁻ cells. After sorting, cells were rested overnight in the full IMDM media. Next, cells were counted, centrifuged (200 × g, 5 min, RT) and plated on poly-L-lysine (Sigma-Aldrich, #P8920) coated Seahorse XF plate in the XF DMEM Medium (Agilent Technologies, #103575), 1 × 10⁵ cells/well. Next, the plate with cells was centrifuged to attach the cells and incubated at 37°C, 0% CO₂, atmospheric O₂, for 40 minutes. For metabolic flux

analysis, the Seahorse XF Cell Mito Stress Kit (Agilent Technologies, #103015-100) was used, according to the manufacturer's instructions. The oxygen consumption rate (OCR) was measured in the presence of respiration modulators: oligomycin (1.5 μ M), FCCP (1 μ M), antimycin A/rotenone (0.5 μ M) with the Agilent XFe96 Seahorse analyzer using the recommended Seahorse XF Cell MitoStress Test analysis. Simultaneously, extracellular acidification rate (ECAR) was monitored to evaluate the properties of the glycolytic activity.

3.10 Gene expression analysis

3.10.1 RNA extraction

To isolate total RNA, $0.5 - 3 \times 10^6$ cells were centrifuged ($200 \times g$, 5 min, 4°C), washed twice with cold PBS, transferred to nuclease-free tubes, and resuspended in 1 mL of TRI Reagent (Zymoresearch, #R-2050-1). It was followed by further isolation with Total RNA Mini kit (A&A Biotechnology, #031-100), according to the manufacturer's instructions. Total RNA was eluted with 50-100 μ L of nuclease-free water (Invitrogen, #10977035) and stored at -80°C . The concentration and quality of isolated RNA were assessed using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific).

3.10.2 Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

3.10.2.1 Reverse Transcription PCR (RT-PCR)

1 μ g of total RNA was incubated with nuclease-free water and mix of oligo(dt)₁₈ and random hexamer primers (**Table 3-8, STEP 1**) and incubated for 5 minutes at 70°C . Next, samples were cooled down in ice for 1 minute and mixed with 11 μ L of the mix described in the **STEP 2 (Table 3-8)**. Then, reverse transcription PCR (RT-PCR) was performed on the MiniAmp™ Thermal Cycler (Applied Biosystems), with reaction conditions summarized in **Table 3-9**. cDNA samples were stored at -20°C .

Table 3-8 Reverse transcription reaction mix

REVERSE TRANSCRIPTION REACTION MIX (total volume: 25 μ L)					
	REAGENT	FINAL CONCENTRATION	ADDED VOLUME	MANUFACTURER	IDENTIFIER
STEP 1.	RNA	1 μ g	variable	-	-
	Oligo(dT)18 primer (0.5 μ g/ μ L; 100 μ M)	2 μ M (250 ng)	0.5 μ L	Thermo Fisher	SO132
	Rand. Hex. primer (0.2 μ g/ μ L; 100 μ M)	5.2 μ M (250 ng)	1.3 μ L	Thermo Fisher	SO142
	RNase-free water	-	To final volume 14 μ L	Invitrogen	10977035
STEP 2.	dNTP mix 100 mM (25 mM each)	4 mM (1 mM each)	1 μ L	Blirt	RP65
	Reverse transcriptase M-MLV (200 U/ μ L)	4 U/ μ L (100 U)	0.5 μ L	Promega	M1705
	M-MLV RT Buffer (5X)	1X	5 μ L	Promega	M1705
	RIBOPROTECT Hu (40 U/ μ L)	0.8 U/ μ L (20 U)	0.5 μ L	Blirt	RT36
	RNase-free water	-	4 μ L	Invitrogen	10977035

Table 3-9 RT-PCR reaction conditions

Step	Temperature [$^{\circ}$ C]	Time [min]
Primer annealing	37	60
Reverse transcription	95	5
Enzyme inactivation	4	∞

3.10.2.2 Quantitative PCR (qPCR)

The quantitative PCR was performed with the use of SensiFAST SYBR Green Hi-ROX kit (Meridian Bioscience, #BIO-92020) in the thermal cycler StepOnePlusTM (Applied Biosystems). Primers used in the study, listed **Table 3-10** were synthesized by Genomed S.A. and resuspended in 10 mM Tris pH 7.5. Different primer concentrations (125 nM, 250 nM, 500 nM) were tested to determine the optimal conditions. Primer specificity was verified by melting curve analysis and electrophoretic separation of qPCR products on a 3% agarose gel (3% agarose (Sigma-Aldrich, #A9539) in TAE buffer: 40 mM Tris-HCl, 1 mM EDTA, 20 mM acetic

acid) supplemented with SYBR Safe DNA Gel Stain (Invitrogen, #S33102). The concentration, which resulted in a single band on the agarose gel and a single peak in the melting curve analysis was selected. Master mix provided per well consisted of 5 μ L SensiFAST SYBR Green, 250 or 500 nM of each primer (forward and reverse), and nuclease-free water to a final volume of 8 μ L. cDNA was diluted 1:4, and 2 μ L was added to each well of the 96-well plate, resulting in a total reaction volume of 10 μ L. Additionally, no-template control (NTC, 2 μ L of water instead of cDNA) and no-reverse transcription controls (2 μ L of RNA used in RT-PCR instead of cDNA) were included. The plate was sealed with adhesive film, centrifuged, and subjected to qPCR under the conditions described in **Table 3-11**. Relative differences between samples were calculated using the comparative $\Delta\Delta C_t$ method. The amplified product was normalized to the endogenous expression of 18S rRNA or β -actin.

Table 3-10 Primer sequences used for RT-qPCRs

TARGET	SEQUENCE
<i>18S rRNA</i>	Forward 5'- GCGCGCTAGAGGTGAAATTC
	Reverse 5'- TTGGCAAATGCTTTCGCTC
<i>β-actin</i>	Forward 5'- GTGAAGGTGACAGCAGTCGGTT
	Reverse 5'- GAAGTGGGGTGGCTTTTAGGAT
<i>CD44s</i>	Forward 5'- TCGACTGTTGACTGCAATGC
	Reverse 5'- AAGGTGGAGCAAACACAACC
<i>CD44v2</i>	Forward 5'- ATCACCGACAGCACAGACAGAAT
	Reverse 5'- AACCATGAAAACCAATCCCAGG
<i>CD44v3</i>	Forward 5'- TACGTCTTCAAATACCATCTCAGCA
	Reverse 5'- AATCTTCATCATCATCAATGCCTG
<i>CD44v4</i>	Forward 5'- AACCACACCACGGGCTTTTG
	Reverse 5'- TCCTTGTGGTTGTCTGAAGTAGCA
<i>CD44v5</i>	Forward 5'- TGCTTATGAAGGAACTGGAAC
	Reverse 5'- TGTGCTTGTAGAATGTGGGGT
<i>CD44v6</i>	Forward 5'- CCAGGCAACTCCTAGTAGTACAACG
	Reverse 5'- CGAATGGGAGTCTTCTTTGGGT
<i>CD44v7</i>	Forward 5'- GCCTCAGCTCATACCAGCCATC
	Reverse 5'- TCCTTCTCCTGCTTGATGACCT
<i>CD44v8</i>	Forward 5'- TGGACTCCAGTCATAGTATAACGC
	Reverse 5'- GGTCTGTCTGTCCAAATC
<i>CD44v9</i>	Forward 5'- AGCAGAGTAATTCTCAGAGC
	Reverse 5'- TGATGTCAGAGTAGAAGTTGTT
<i>CD44v10</i>	Forward 5'- CCTCTCATTACCCACACACG
	Reverse 5'- CAGTAACTCCAAAGGACCCA
<i>SLC2A1 (GLUT1)</i>	Forward 5'-TTGCAGGCTTCTCCAAGTGGAC
	Reverse 5'-CAGAACCAGGAGCACAGTGAAG
<i>MMP2</i>	Forward 5'- TGGCAAGTACGGCTTCTGTC
	Reverse 5'- TTCTTGTGCGGGTCGTAGTC
<i>MMP3</i>	Forward 5'- CAGTTTGCTCAGCCTATCCA
	Reverse 5'- TCACATCTTTTCGAGGTCGT
<i>MMP9</i>	Forward 5'- GGGACGCAGACATCGTCATC
	Reverse 5'- TCGTCATCGTCGAAATGGGC
<i>SERPINE1</i>	Forward 5'- ACCGCAACGTGGTTTTCTCA
	Reverse 5'- TTGAATCCCATAGCTGCTTGAAT
<i>ITBG2</i>	Forward 5'- TTCGGGTCCTTCGTGGACA
	Reverse 5'- ACTGGTTGGAGTTGTTGGTCA
<i>GPX4</i>	Forward 5'- GAGGCAAGACCGAAGTAAACTAC
	Reverse 5'- CCGAACTGGTTACACGGGAA

Table 3-11 qPCR reaction conditions

Cycle	Temperature [°C]	Time [min:sec]	Number of cycles
Initial denaturation	95	00:20	1x
Amplification	95	00:03	40x
	60	00:30	
Melt curve stage	95	00:15	1x
	60	01:00	
	95	00:15	

3.10.3 RNA sequencing (RNA-seq)

RNA sequencing was performed by the Laboratory of Sequencing, Nencki Institute. Aleksandra Cabaj, PhD (Laboratory of Sequencing, Nencki Institute of Experimental Biology PAS) conducted the primary bioinformatic analyses of the generated data.

3.10.3.1 RNA-seq library preparation

The quality and integrity of total RNA were assessed with Agilent 2100 Bioanalyzer using an RNA 6000 Nano Kit (Agilent Technologies, #5067-1511). In total, strand-specific polyA-enriched RNA libraries were prepared using the KAPA Stranded mRNA Sample Preparation Kit according to the manufacturer's protocol (Roche, #07962207001). Briefly, mRNA molecules were enriched from 500 ng of total RNA using poly-T oligo-attached magnetic beads. The obtained mRNA was fragmented, and the first-strand cDNA was synthesized using a reverse transcriptase. Second cDNA synthesis was performed to generate double-stranded cDNA (dsDNA). Adenosines were added to the 3' ends of dsDNA, and adapters were ligated (adapters from NEB). Following the adapter ligation, uracil in a loop structure of the adapter was digested by the USER enzyme (NEB, #M5505S). Adapters containing DNA fragments were amplified by PCR using NEB starters. Library evaluation was done with Agilent 2100 Bioanalyzer using the Agilent DNA High Sensitivity chip (Agilent Technologies, #5067-4626). Mean library size was 300bp. Libraries were quantified using Quantus fluorometer and QuantiFluor ONE double-stranded DNA System (Promega, #E4871). Libraries were paired-end sequenced (2x151bp) on NovaSeq 6000 (Illumina).

3.10.3.2 RNA-seq data processing and analysis

Three independent biological replicates were analysed for each condition. Quality control of reads was performed using FastQC software (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) (v. 0.11.9). Adapter and quality trimming were performed using Cutadapt (v. 3.4)²²⁶ and TrimGalore (<https://www.trimgalore.com>) (v. 0.6.7) software, respectively. The TrimGalore quality parameter was set to 25. Alignment of reads to the human reference genome (GRCh38) was performed using the STAR aligner (v. 2.7.9a)²²⁷ with default settings. The GTF annotation file used was obtained from the Ensembl 105 release²²⁸. Duplicate reads were marked using Picard MarkDuplicates (<https://broadinstitute.github.io/picard/>) (v. 2.27.4-SNAPSHOT). Final quality control was collated with MultiQC (v. 1.13)²²⁹ from RSeQC (v. 3.0.1)²³⁰ and the tools described above. Reads were summarized and counted by featureCounts (v. 2.0.0)²³¹ on paired-end reads, with only primary alignments and reversely stranded reads counted. The minimum mapping quality score required for a read to be counted was set to 3.

3.10.3.3 Differential gene expression analysis

The differential and functional analyses were performed in the R environment (v. 4.1.3). The differential analysis was performed using DESeq2 (v. 1.34) with default parameters²³². If the sum of counts for a certain feature was less than 10 in all samples, this feature was discarded from the analysis. FDR-corrected P-value < 0.05 was considered significant. Plots were generated using ggplot2 (v. 3.5.1). GO Biological Process enrichment and transcription factor enrichment analyses were performed using ShinyGO (v. 0.77)²³³.

3.11 Analysis of publicly available transcriptomic datasets

Analyses of publicly available transcriptomic datasets were performed in collaboration with Dawid Stępnik from the Laboratory of Cytometry, Nencki Institute.

Expression analysis of the *CD44* gene in CML was performed using a microarray dataset available in the open database Gene Expression Omnibus (GEO;

GSE4170²³⁴, and RNA-sequencing data from GSE144119²³⁵. In the GSE4170 dataset, samples belonging to the two experimental groups corresponding to different responses to imatinib in chronic myeloid leukemia were analyzed: effective response (EFFECTIVE, n = 96) and treatment failure (FAIL, n = 15). The data were obtained as a normalized gene expression matrix directly from GEO using the GEOquery R package (version 2.78.0). The dataset had been previously preprocessed and normalized by the original authors. Samples annotated as “cd34” or “unknown” were excluded from further analysis. Differential expression between groups was assessed using Student’s t-test. For GSE144119, raw count data were downloaded and transformed to log2 counts per million (log2-CPM) using the edgeR R package (version 4.8.2). This dataset includes healthy donors (HD, n = 17) and CML patients (CML, n = 70). Differential expression between groups was assessed using Student’s t-test. All analyses were conducted in R (version 4.5.0).

3.12 Protein analysis

3.12.1 Western blot

3.12.1.1 Total protein isolation

2-10 × 10⁶ cells were centrifuged (200 × g, 5 min, 4°C) and washed twice with cold PBS. Cell pellets were resuspended in Sample Lysis Buffer (50 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, deionized water) preheated to 95°C. A ratio of 300 µL of the buffer per 6 × 10⁶ cells was maintained. Samples were incubated for 8 minutes at 95°C. Next, the solutions were passed five times through a syringe and incubated for another 3 minutes at 95°C. Protein lysates were stored at -20°C.

3.12.1.2 Isolation of nuclear and cytoplasmic protein fractions

Sorted cells were centrifuged (200 × g, 5 min, 4°C) to remove excess PBS and FACS sheath fluid. Then, cytoplasmic and nuclear protein fractions were isolated using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, #78833) according to the manufacturer’s instructions. Samples were stored at -80°C.

3.12.1.3 Protein concentration measurement

Protein concentration in the obtained cell lysates was assessed using the Bradford assay²³⁶. Briefly, 2 μ L of cell lysate was added to 998 μ L of Bradford Protein Assay reagent (Bio-Rad, #500-0006) diluted 25 $\times$ in deionized water. The diluted reagent served as a blank control. Absorbance was measured at 595 nm using a spectrophotometer (Eppendorf). Each sample was measured in triplicate, and protein concentration was calculated based on a standard calibration curve generated using a series of bovine serum albumin (BSA) (Sigma-Aldrich, #A3059) dilutions.

3.12.1.4 Immunoblotting

Protein lysates were mixed with 5 $\times$ SDS Sample-loading Buffer (final concentration: 62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue) supplemented with 250 mM DTT and heated for 5 minutes at 95°C. Depending on the molecular weight of the target proteins, polyacrylamide gels of 8-15% acrylamide were prepared. Resolving gels contained 0.375 M Tris-HCl pH 8.8, 0.1% SDS, 0.1% APS, and TEMED. Stacking gel (5% acrylamide) was prepared using Tris-HCl pH 6.8. Equal amounts of protein (minimum 15 μ g) were loaded per lane of the polyacrylamide gel. Electrophoresis was performed in running buffer (25 mM Tris pH 8.3, 192 mM glycine, 0.1% SDS, deionized water) using Mini-PROTEAN Tetra System (Bio-Rad). Electrophoresis was conducted at a constant voltage of 60 V for the stacking gel, and 100 V for the resolving gel.

Proteins separated by SDS-PAGE were transferred onto nitrocellulose membranes (GE Healthcare Life Sciences, #10600002) in a transfer buffer (25 mM Tris pH 8.3, 192 mM glycine, 0.05% SDS, 20% methanol). Transfer was performed at 4°C for 90 min at a constant 300 mA using a Mini-Trans-Blot Electrophoretic Transfer Cell (Bio-Rad).

Following transfer, membranes were briefly washed in TBST buffer (20 mM Tris pH 7.6, 136 mM NaCl, 0.1% Tween-20) and blocked in a blocking solution (either 5% BSA or 5% non-fat milk) for 1 hour at RT. Membranes were then incubated overnight at 4°C, with primary antibodies diluted in the appropriate blocking solution. Antibody details and incubation conditions are listed in **Table 3-12**. The following day,

membranes were washed three times for 10 minutes in TBST buffer and incubated for 1 hour at RT, with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000 dilution, goat anti-mouse #P0447 or goat anti-rabbit #P0448, both from Dako). Membranes were washed three times in TBST and developed using Super Signal West Pico ECL (Thermo Fisher Scientific, #34580) or Clarity Max Western ECL Substrate (Bio-Rad, #1705062). Western blot images were captured using a Chemidoc MP chemiluminescent imaging system (Bio-Rad).

Table 3-12 List of primary antibodies used for western blotting

TARGET	HOST	DILUTION	BUFFER	MANUFACTURER	IDENTIFIER
CD44 (C-terminus)	rabbit	1:2000	5% BSA	Abcam	#ab157107
CD44	rabbit	1:1000	5% milk	Proteintech	#15675-1-AP
ERK1/2	rabbit	1:2000	5% BSA	Cell Signaling	#4695
P-ERK1/2 (Thr202/Tyr204)	rabbit	1:1000	5% BSA	Cell Signaling	#4370
GFP (AF488 conjugated)	rabbit	1:5000	5% milk	Proteintech	#CL488-66002
GPX4	rabbit	1:1000	5% BSA	Abcam	#ab125066
HIF-1 α	rabbit	1:1000	5% BSA	Abcam	#ab179483
I κ B α	rabbit	1:1000	5% BSA	Cell Signaling	#9242
JNK	rabbit	1:1000	5% BSA	Cell Signaling	#9252
P-JNK (Thr183/Tyr185)	rabbit	1:1000	5% BSA	Cell Signaling	#9251
Lamin B1	rabbit	1:1000	5% milk	Abcam	#ab16048
STAT3	mouse	1:4000	5% BSA	Cell Signaling	#9139
P-STAT3 (Tyr705)	rabbit	1:1000	5% BSA	Cell Signaling	#9131
α -tubulin	mouse	1:5000	5% milk	Merck	#CP06

3.12.1.5 Densitometric analysis

Densitometric analysis was performed in Fiji ImageJ software, using the Analyse/Gels functionality.

3.12.2 Gelatin zymography

Gelatin zymography was performed to evaluate the enzymatic activity of MMPs (MMP2 and MMP9) in conditioned media obtained from the culture of leukemic CD44⁺ and CD44⁻ cells. First, K562 CD44⁺ and CD44⁻ cells were FACS-sorted from the co-culture with HS-5 cells and further cultured for 48 hours in Opti-MEM Reduced Serum Medium (Gibco, #11058021) at a density of 2×10^5 cells/mL. Next, conditioned media (CM) were collected *via* centrifugation ($1500 \times g$, 5 min, 4°C). Subsequently, CM were concentrated by adding 200 μ L of each sample to 1 mL of ice-cold 96%

ethanol and incubating at -20°C overnight. Then, samples were centrifuged (15 000 × g, 15 min, 4°C), ethanol was removed, cell pellets were dissolved in loading buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, H₂O), and loaded on 8% acrylamide gel containing 2 mg/mL gelatin (Sigma-Aldrich, #48723). As negative control, OptiMEM Reduced Serum medium was used, and for a positive control, IMDM containing 10% FBS was used. After SDS-PAGE separation, gels were rinsed twice with renaturation buffer (2.5% Triton X-100), 30 minutes each, at 37°C, with shaking. This was followed by an initial 10-minute incubation at 37°C in developing buffer (50 mM Tris-HCl pH=7.5, 10 mM CaCl₂, 1 μM ZnCl₂, 1% Triton X-100, 0.02% NaN₃). Next, the developing buffer was replaced with a fresh one, and gels were incubated at 37°C for the next three days with gentle agitation. Finally, gels were stained with staining solution (0.5% Coomassie Brilliant Blue R-250, 40% methanol, 10% acetic acid, water), and rinsed with deionized water or destaining solution (40% methanol, 10% acetic acid, water) until MMPs activity-related clear bands were visible. Gels were imaged using the Chemidoc MP chemiluminescent imaging system (Bio-Rad).

3.12.3 Multiplex analysis of soluble factors

The concentration of soluble factors in conditioned media (CM) obtained from cell cultures was analyzed using the bead-based quantitative LEGENDplex assay. To collect conditioned media from mono- and co-cultures of stromal and leukemic cells, unstained cells were seeded as described in **Section 3.1.3.4** and cultured for 48 hours. For the analysis of soluble factors concentration in the media obtained from sorted leukemic CD44-GFP⁺ and CD44-GFP⁻ cells (acceptors and non-acceptors of stromal CD44-GFP, respectively), cells were sorted as described in **Section 3.6** and further cultured for 48 hours in IMDM medium at a density of 2 × 10⁵ cells/mL for 48 hours. Then, cells were centrifuged at 400 × rcf at 4°C for 10 min and CM were collected, portioned, and stored at -80°C. CM were analyzed using the Human Essential Immune Response Panel (Biolegend, #740930) and Human Adhesion Molecule Panel (Biolegend, #740946) according to the manufacturer's instructions. Samples were analyzed using Cytex Aurora cytometer (Cytex Biosciences).

3.13 Statistical analysis

RNA-seq data and publicly available transcriptomic datasets were analyzed in R, as described in the respective Materials and methods sections. The remaining data obtained in this doctoral thesis were analyzed (visualized and statistically processed) using GraphPad Prism (v10) software. Statistical tests applied in each experiment are specified in the figure legends, unless no statistical analysis was performed. Briefly, statistical analyses of differences between two groups were performed using Student's t-test or the Mann-Whitney U test. For multiple group comparisons, one-way or two-way analysis of variance (ANOVA) was applied, followed by Tukey's or Šídák's post hoc tests. The number of biological replicates (n) is also indicated in the figure legends. Data are presented as mean $\pm$ standard deviation (SD). Differences were considered statistically significant if p value < 0.05. Levels of statistical significance were marked on graphs with asterisks (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

3.14 Data availability

RNA sequencing data generated in this study have been deposited in the NCBI Gene Expression Omnibus (GEO) database under accession number GSE327896. SILAC MS proteomics data reanalyzed in this study are available in the ProteomeXchange Consortium *via* the PRIDE [<https://www.ebi.ac.uk/pride>] partner repository under accession number PXD013504. Publicly available CML gene expression datasets were retrieved from the Gene Expression Omnibus [<https://www.ncbi.nlm.nih.gov/geo>] (GEO, accession numbers: GSE144119, GSE4170) and analyzed in this study.

3.15 Illustrations

Graphical illustrations in the figures were generated with BioRender.com (Nencki Institute license, user: Laura Turos-Korgul).

4 RESULTS

4.1 Ultrastructural analysis of tunneling nanotubes formed between stromal and leukemia cells

Tunneling nanotubes have been intensively studied as they represent a relatively novel mode of direct cell-to-cell communication, highly relevant to cancer progression. The significant role of TNTs in cancer, particularly in chronic myeloid leukemia, was also shown by us before⁷⁸.

Despite years of research, many aspects of TNTs remain poorly understood or unclear. There are still no specific regulators or inhibitors of TNTs, and the detailed structure and processes of their formation have yet to be fully elucidated. The field of tunneling nanotubes is characterized by heterogeneous definitions and the lack of unified terminology^{89,93,109}. One of the key challenges is to correctly distinguish TNTs from similar structures such as filopodia or gap junctions.

Significant limitations are restricting our ability to precisely characterize tunneling nanotubes. They are fragile and highly dynamic structures that are difficult to capture and analyze. Imaging is particularly challenging in co-cultures combining adherent and suspension cells, such as those used in our study. Microscopy is typically conducted on live cells or on gently fixed specimens maintained in fluid conditions. A comprehensive characterization of TNTs in stroma-leukemia co-cultures using mainly confocal microscopy was performed earlier by our group and served as the basis for a separate doctoral dissertation. Here, we decided to perform a deeper study and complement those results with detailed ultrastructural information obtained using the state-of-the-art focused ion beam scanning electron microscopy (FIB-SEM) imaging and 3D reconstruction of the obtained data. Analyses were performed in collaboration with Wioleta Dudka, PhD, Paolo Ronchi, PhD and Yannick Schwab, PhD at Electron Microscopy Core Facility, EMBL (Heidelberg, Germany). Three of the highest-quality datasets were selected for analysis.

Specific aims of this part of the study were:

1. to describe the ultrastructure of the entire volume of TNT formed between stromal and leukemic cells
2. to validate, using electron microscopy resolution, whether structures identified by confocal microscopy as TNTs fulfill the currently accepted criteria for their characterization
3. to assess the presence of open- and closed-ended TNTs in co-cultures of stromal and leukemic cells

4.1.1 Comparison of conventional confocal microscopy and FIB-SEM approach in the TNT imaging and identification

To image tunneling nanotubes formed between stromal and leukemia cells, HS-5 bone marrow stromal cells were co-cultured with K562 chronic myeloid leukemia cells for 24 hours on a cell culture dish with a grid. Cells were stained with wheat germ agglutinin coupled to Alexa Fluor 647 for plasma membrane visualization. To distinguish stroma and leukemia cells, one of the cell lines in co-culture always constitutively expressed GFP. Selected regions of interest were imaged, and their positions on the grid were documented for subsequent electron microscopy analysis.

Confocal microscopy revealed a classical structure of TNT connections formed between cells grown in co-culture. Both substrate-adherent (considered as filopodia) and non-adherent structures (classified as TNTs, indicated by arrows in **Figure 4.1.1**) were visible between connected cells (**Figure 4.1.1**). Confocal images were performed using conventional confocal microscopy (**Figure 4.1.1 A-B**) or high-resolution, stimulated emission depletion (STED) microscopy (**Figure 4.1.1 C**). As expected, high-resolution STED imaging revealed more connections, compared to traditional confocal microscopy (**Figure 4.1.1 C**), including even very thin structures.

TNTs were randomly selected for further FIB-SEM imaging without prior morphological preselection. However, structures exhibiting features characteristic of post-mitotic intercellular bridges, containing prominent membrane-rich bulges resembling midbodies, were excluded from the analysis.

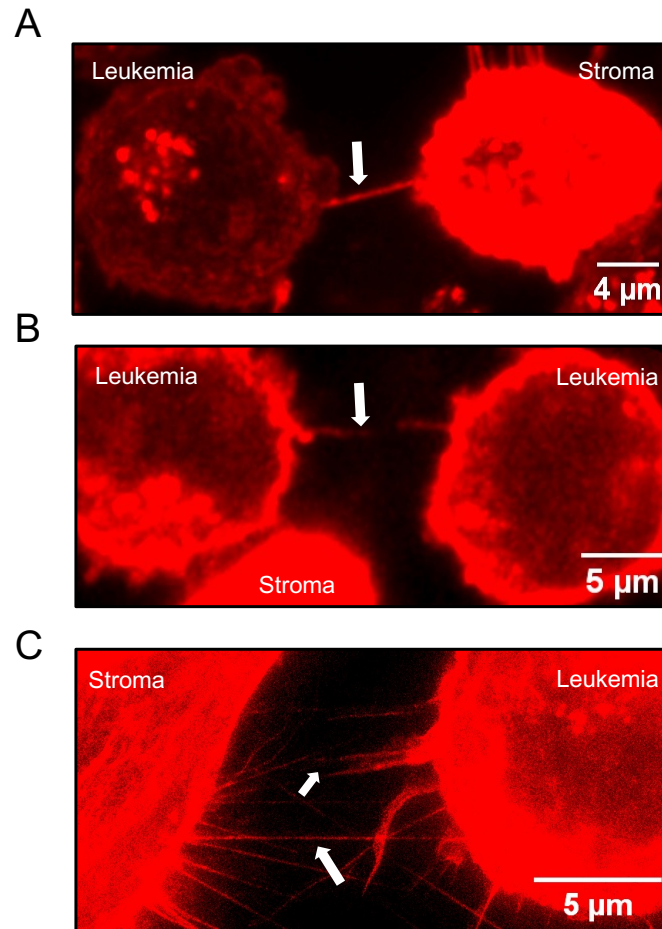


Figure 4.1.1 TNTs observed in stroma-leukemia co-cultures using confocal microscopy. (A) TNT connecting HS-5 (stroma) and K562 (leukemia). (B) TNTs emerging from two K562 cells. (C) TNTs connecting HS-5 and K562 cell. Red – plasma membranes stained with WGA-AF647. The GFP signal is not shown due to low signal intensity and high background levels. Structures identified as TNTs are indicated by arrows.

Next, the samples were further fixed and prepared for electron microscopy, as described in Materials and methods, **3.8.1** and FIB-SEM imaging was performed, followed by data analysis including TNTs and filopodia segmentation and 3D visualization in Amira software.

The most clearly visible structures observed in confocal datasets were successfully identified in the FIB-SEM volume reconstruction (**Figure 4.1.2 A-C, marked with arrows**). This suggests that sample preparation for FIB-SEM imaging did not disrupt the overall structural integrity. Additionally, FIB-SEM volume reconstruction revealed many additional structures not resolved in confocal imaging. It also showed a high level of structural detail not accessible by fluorescence microscopy. The three highest-quality FIB-SEM datasets were analyzed and

visualized in 3D reconstructions. Individual datasets, together with the specific structures identified within them, will be discussed separately.

Structure was classified as TNT if it was connected with another cell and did not touch the substrate at any point. Otherwise, it was classified as a filopodium. TNTs emerging from leukemic cells were color-coded in blue (in cases where multiple TNTs originated from a single site, additional colors included turquoise, green and yellow). TNTs originating from stromal cells were colored in red. Filopodia were colored in magenta. All identified TNTs were segmented, while only selected filopodia were included in the segmentation for quantitative and comparative analyses.

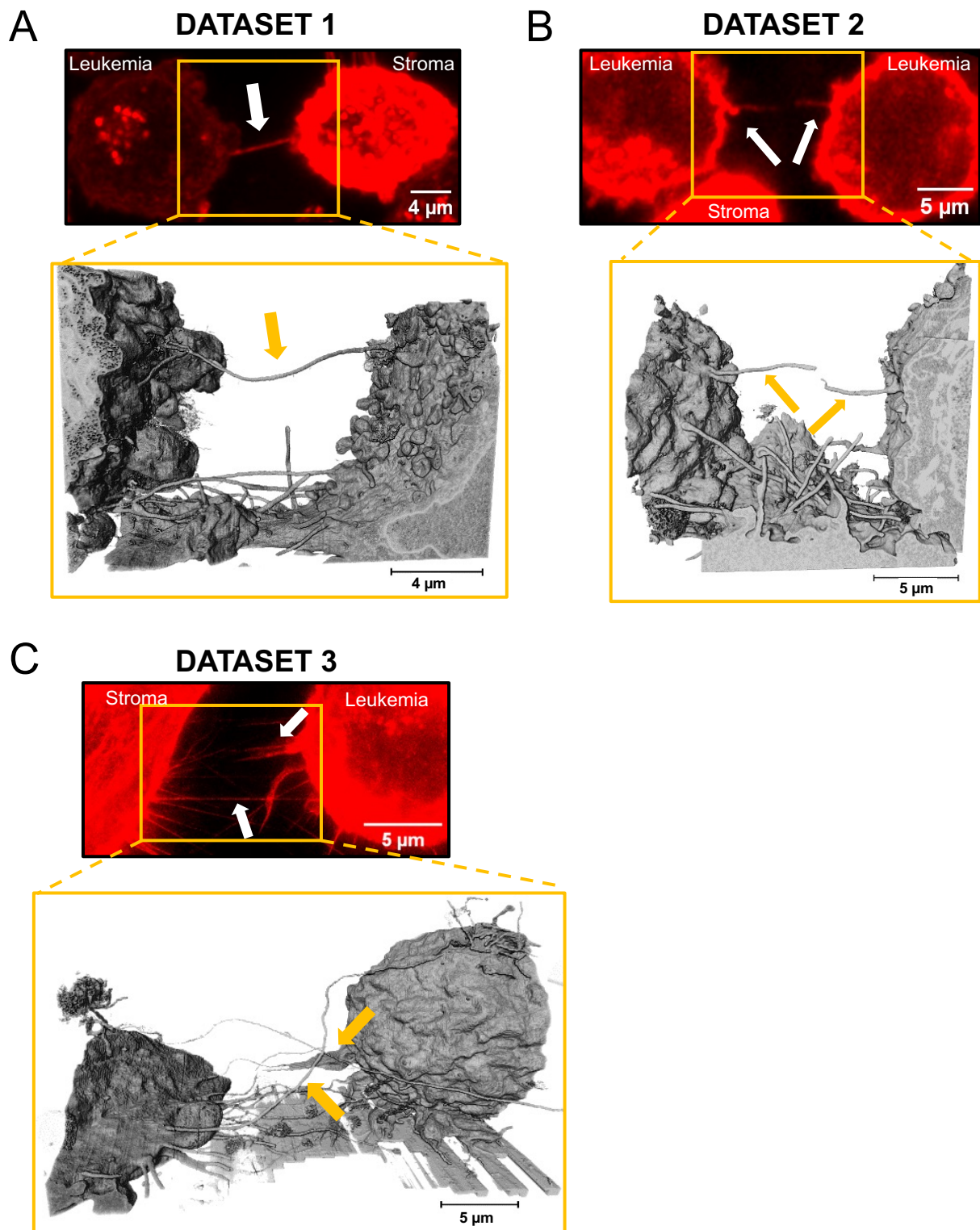


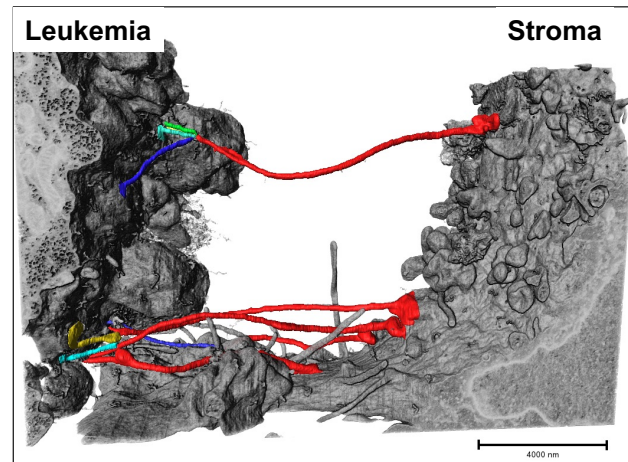
Figure 4.1.2 Confocal images and corresponding volume EM reconstructions. (A-C) Arrows indicate representative structures visible in confocal imaging and the corresponding regions in the 3D volume EM reconstruction. Scale bars are indicated on the images.

DATASET 1

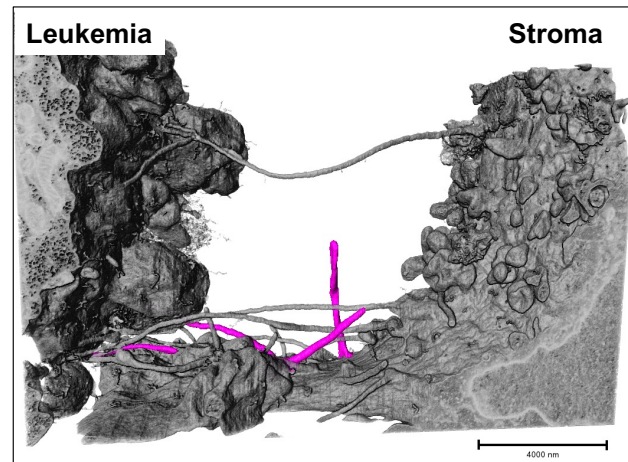
The first dataset (DATASET 1) depicts two cells, stromal (HS-5) and leukemic (K562) cell. This was the highest-quality dataset, enabling optimal image alignment and, consequently, the most reliable 3D reconstruction. In this Dataset, 12 structures connecting stroma and leukemia were classified as TNTs (**Figure 4.1.3 A**). 4 structures identified as filopodia were segmented (**Figure 4.1.3 B**).

Interestingly, the structure shown in (**Figure 4.1.3 C**) was clearly visible also in the confocal data (**Figure 4.1.2 A**). It is located at a considerable distance from the substrate along its entire length ($\pm 8 \mu\text{m}$) and connects two cells, fulfilling the fundamental criteria for classification as TNT, commonly used in experiments, where TNT structures are quantified using confocal microscopy. However, the 3D reconstruction of the volume EM data clearly identified five TNTs forming this connection. Four of these originate from leukemia cell, forming a characteristic TNT-bundle meeting at the junction with a long TNT emerging from a stromal cell. Additionally, volume EM analysis allowed the classification of seven other structures as TNTs, which are located relatively close to the substrate but do not contact it at any point. Connection shown in **Figure 4.1.3 D** resembles the filopodia-interplay type of TNT, where one TNT originates from leukemia cell and the other from stromal cell. However, the distance between the substratum and TNT emerging from the leukemic cell is only $1 \mu\text{m}$; therefore, its final classification remains debatable. The remaining identified TNTs either connect directly to the leukemic cell body (**Figure 4.1.3 E,G**) or to other TNTs emerging from the leukemic cell (**Figure 4.1.3 F**).

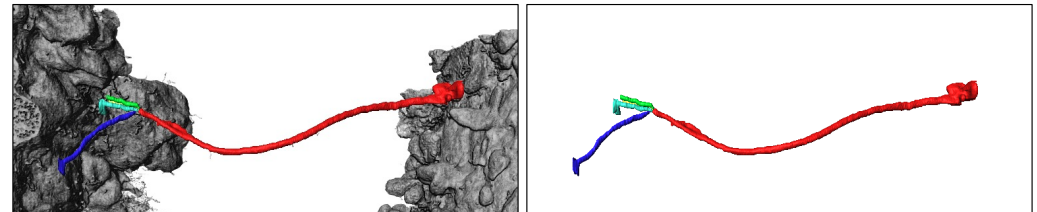
A



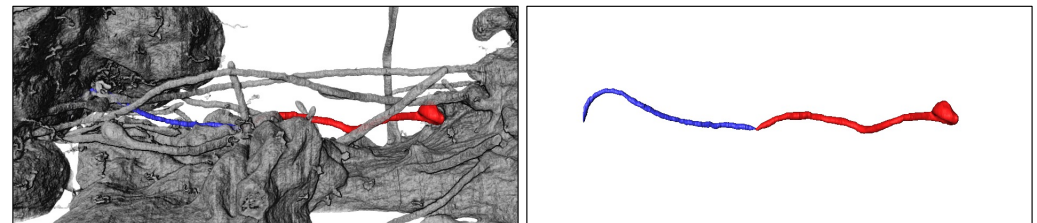
B



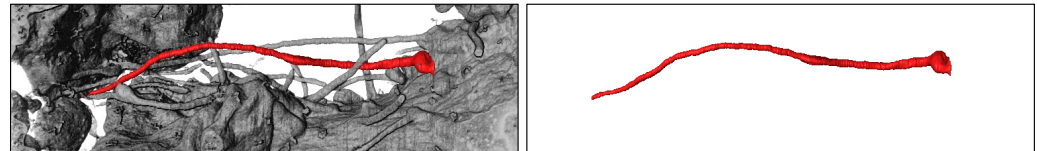
C



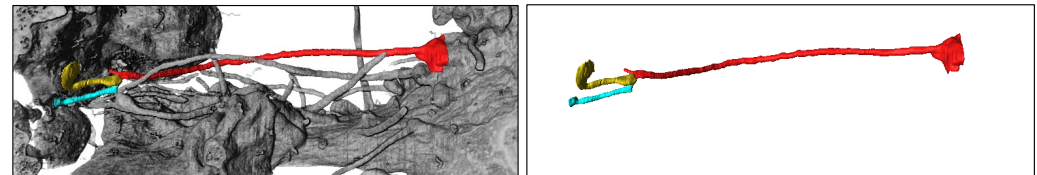
D



E



F



G

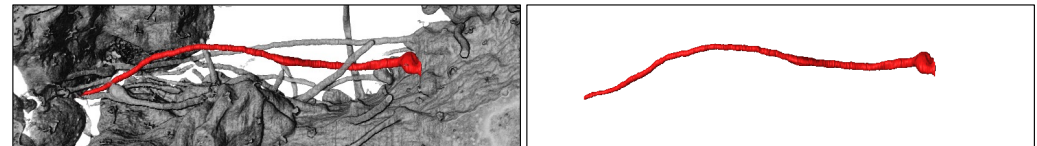


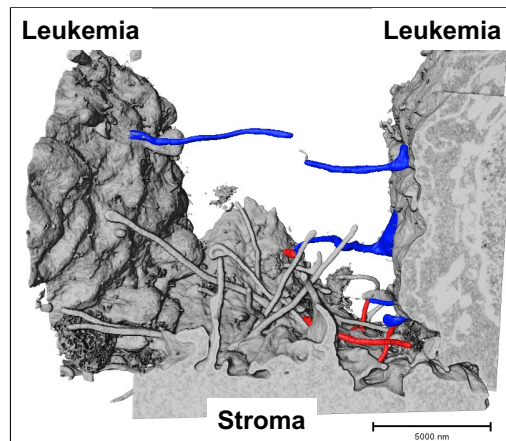
Figure 4.1.3 3D reconstruction generated from the FIB-SEM serial sections: DATASET 1. **(A)** 3D rendering of the FIB-SEM volume data with the segmentation aligned. Segmentation of the TNTs connecting HS-5 and K562 cell identified in this dataset. Scale bar = 4 μm . **(B)** 3D rendering of the FIB-SEM volume data with the segmentation aligned. Segmentation of the selected filopodia identified in this dataset. Scale bar = 4 μm . **(C-G) Left:** Zoomed-in view of the 3D reconstructions of the selected TNTs identified in the dataset. Data segmentation aligned. **Right:** Segmentation of the selected TNTs.

DATASET 2

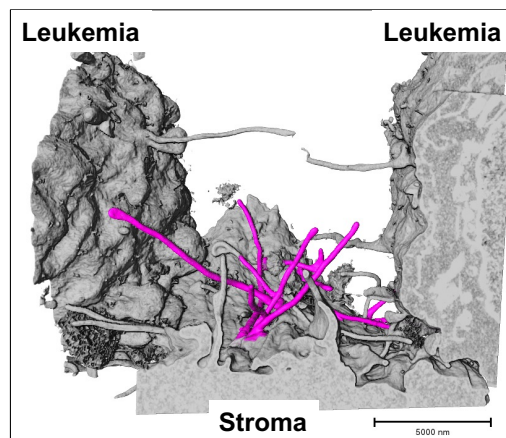
The DATASET 2 depicts two leukemic (K562) cells and a fragment of a stromal (HS-5) cell. In this Dataset, 7 structures were classified as TNTs (**Figure 4.1.4 A**); however, 2 of them did not fulfill the strict continuity criterion and may also be considered as filopodia (**Figure 4.1.4 C**). 10 structures identified as filopodia were segmented (**Figure 4.1.4 B**).

As mentioned above, the most difficult to classify structures are those shown in **Figure 4.1.4 C**. These structures were also visible in the confocal imaging data (**Figure 4.1.2 B**). As they do not contact neighboring cells, they would likely be classified as filopodia. However, due to the close proximity between the tips of both protrusions (~962 nm) and the characteristic structure at the tip of one of them (indicated by an arrow), suggesting a state immediately after rupture, it cannot be fully excluded that they were not TNTs (**Figure 4.1.4 C**). Other TNTs clearly met both classification criteria. In two cases, TNT emerging from one cell (**Figure 4.1.4 D** - from leukemia, **Figure 4.1.4 E** - from stroma) was connected to a characteristic bulge belonging to the soma of the other cell. On the other hand, the connection between TNTs presented in **Figure 4.1.4 F** resembled the formation of TNT driven by filopodia interplay. Interestingly, structures were oriented at a large angle relative to each other (**Figure 4.1.4 F**).

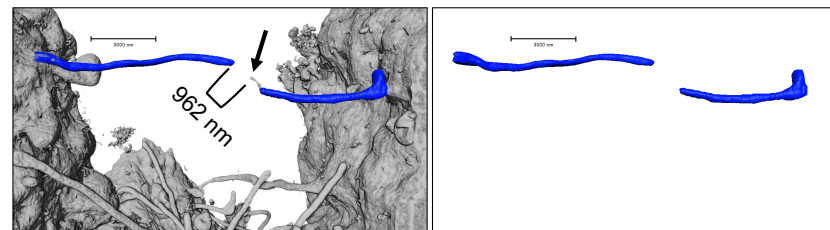
A



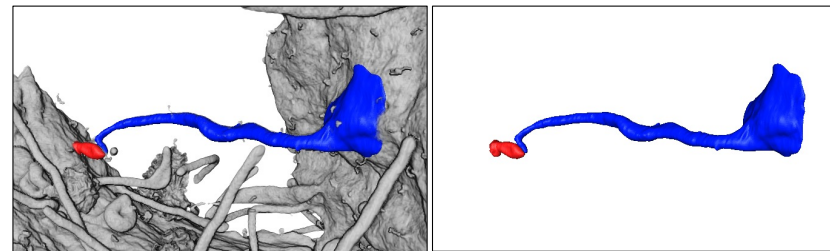
B



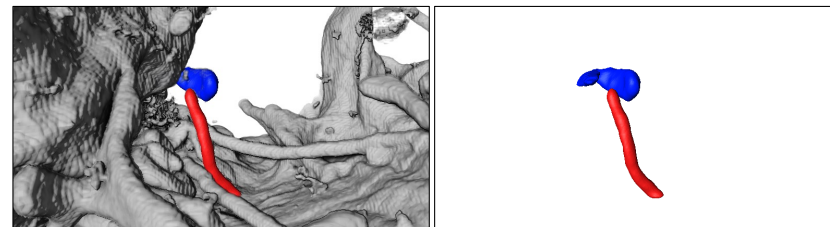
C



D



E



F

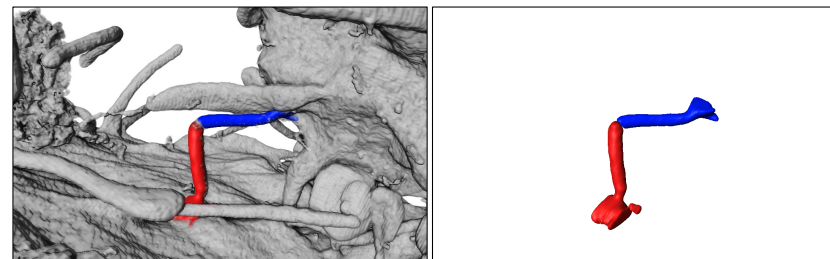


Figure 4.1.4 3D reconstruction generated from the FIB-SEM serial sections: DATASET 2. **(A)** 3D rendering of the FIB-SEM volume data with the segmentation aligned. Segmentation of the TNTs connecting HS-5 and K562 cell or interconnecting K562 cells identified in this dataset. Scale bar = 5 μm . **(B)** 3D rendering of the FIB-SEM volume data with the segmentation aligned. Segmentation of the selected filopodia identified in this dataset. Scale bar = 5 μm . **(C-F) Left:** Zoomed-in view of the 3D reconstructions of the selected TNTs identified in the dataset. Data segmentation aligned. **Right:** Segmentation of the selected TNTs.

DATASET 3

Owing to variability in FIB-SEM slice alignment, the DATASET 3 was processed into two separate subsets. The following description covers only the more complete subset. This subset of the DATASET 3 depicts one leukemic (K562) and one stromal (HS-5) cell. 4 structures connecting stroma and leukemia were classified as TNTs and 9 structures identified as filopodia were also segmented (**Figure 4.1.5 A**). The second part of the DATASET 3 (not shown) comprised fragments of two K562 cells, with 1 TNT and 6 segmented filopodia. Measurements of all segmented structures from both parts of the DATASET 3 were included in the statistical analyses presented in the subsequent sections of this chapter.

Initially, based on STED imaging, structures classified as TNTs did not fulfill the defining criteria, as no direct physical connection between the cells could be observed. Only FIB-SEM analysis at electron microscopy resolution revealed that two TNTs, positioned on the distinctive “platform” emerging from the K562 (leukemic) cell established contacts with their respective partner TNTs emerging from the HS-5 (stromal) cell (**Figure 4.1.5 B**). On the other hand, STED imaging revealed long, thin protrusions extending from the stromal cell toward the leukemia cell while remaining suspended above the substrate (**Figure 4.1.1 C-D**). Based on this, these types of structures would have been classified as TNTs. However, electron microscopy-scale analysis revealed that the tips of the protrusions did not, in fact, contact the target cell, thereby lacking the structural requirements for cargo transfer. Two representative cases were described below.

In the first case (**Figure 4.1.5 C**), an exceptionally long protrusion (26.5 μm) reached the leukemic cell without contacting the substrate at any point along its length. However, the terminal end of the structure did not contact the leukemic cell. Instead, the protrusion was “lying” on the leukemic cell surface, while its distal end continued into the extracellular space (**Figure 4.1.5 C**).

In the second case, a relatively long protrusion (15.5 μm) was observed to remain at an approximately constant distance of $\sim 2 \mu\text{m}$ from the substratum along its almost entire length. However, it was observed that at its tip, located beneath the

leukemic cell, it did not reach the target cell membrane, but the substratum (**Figure 4.1.5 D-E**).

Collectively, FIB-SEM analysis confirmed the existence of leukemia-stroma direct connections, including TNTs; however, it enabled a more critical evaluation of structures formed in the stroma-leukemia co-cultures. The results suggest that structures identified as TNTs using confocal microscopy may not always meet the appropriate criteria applied to TNTs and should be classified as filopodia. Conversely, structures that appear to be clear filopodia may represent TNT structures. Moreover, a single TNT identified by the fluorescent microscopy may in fact be built of a bunch of closely located thin TNTs.

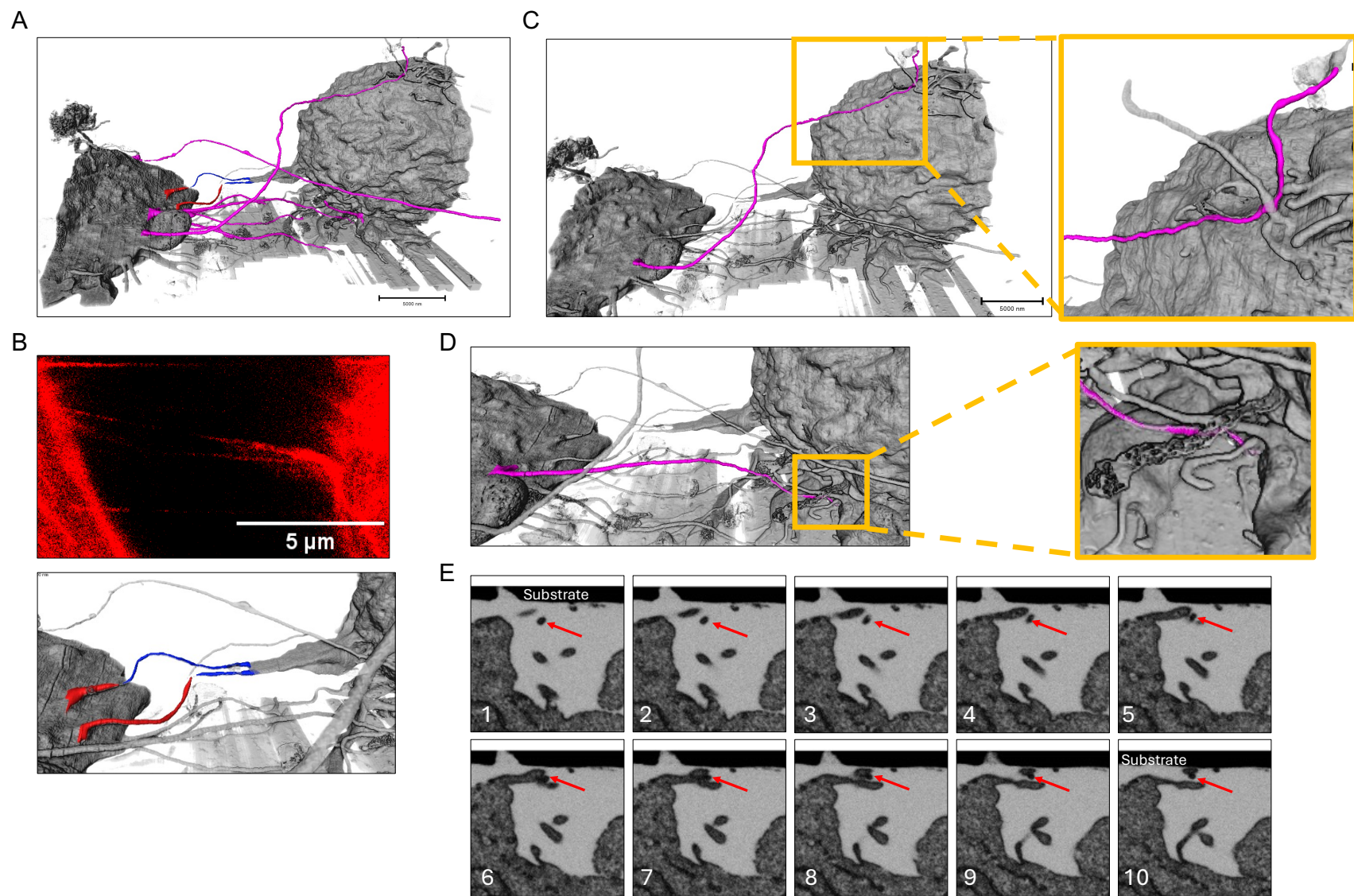


Figure 4.1.5 3D reconstruction generated from the FIB-SEM serial sections: DATASET 3.

(A) 3D rendering of the FIB-SEM volume data with the segmentation aligned. Segmentation of the TNTs connecting HS-5 and K562 cell and selected filopodia identified in this dataset. Scale bar = 5 μm . **(B) Up:** Zoomed-in view of the TNTs connecting HS-5 and K562 cell, confocal microscopy. Red – cell membranes stained with WGA AF647. **Down:** Zoomed-in view of the 3D reconstruction of the volume EM data presenting this connection with segmentation aligned. **(C)** 3D reconstruction of the volume EM data. Segmentation presents the example of the filopodium visible in confocal microscopy as TNT. **(D)** 3D rendering of the FIB-SEM volume data. Segmentation presents the example of the filopodium visible in confocal microscopy as TNT. **(E)** 3D rendering of the FIB-SEM volume data. Segmentation presents the example of the filopodium initially visible in confocal microscopy as TNT. **(F)** FIB-SEM slices presenting the distal end (red arrow) of the filopodium presented in (E), showing its connection with the substrate.

4.1.2 Verification of TNTs structural openness by FIB-SEM imaging

One of the most debated aspects of TNT's structure characterization concerns its open-ended or closed-ended state. Therefore, taking advantage of the full-volume imaging provided by FIB-SEM, we sought to examine this issue in our co-cultures of stroma and leukemia.

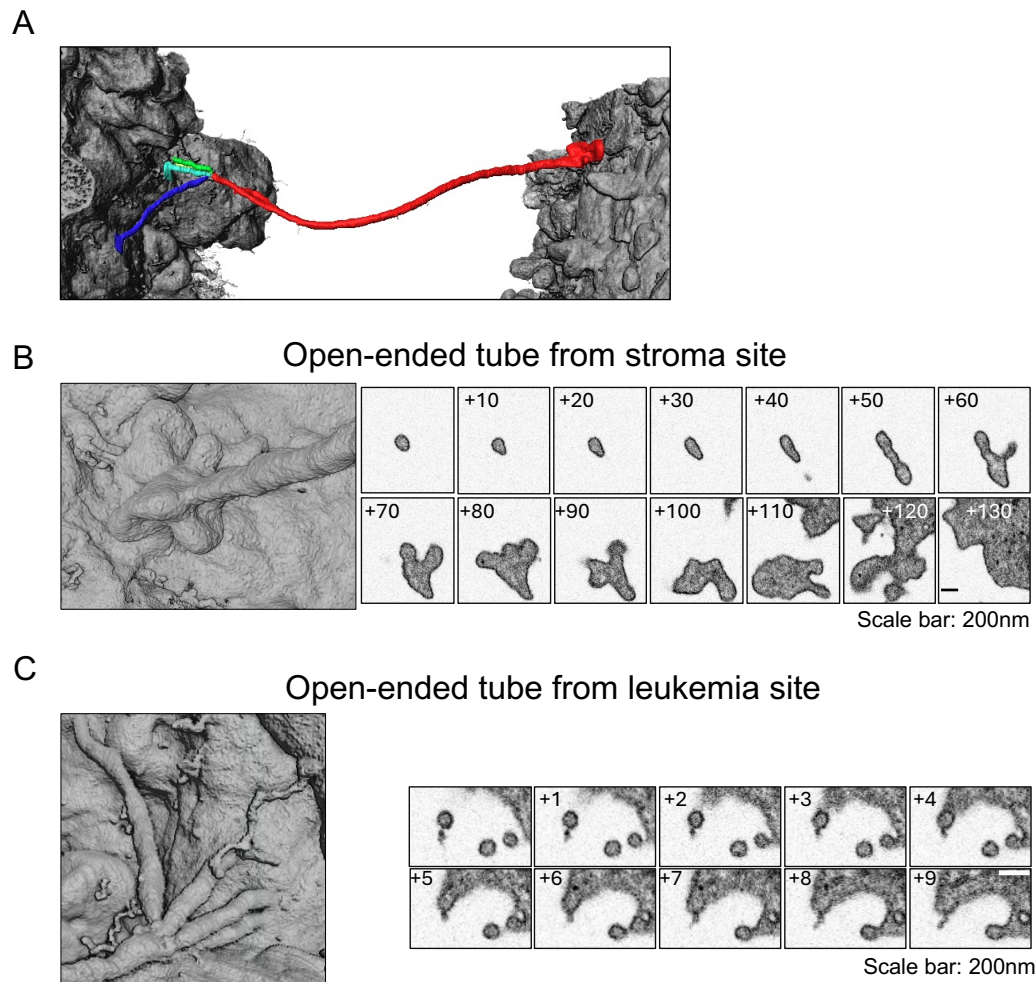


Figure 4.1.6 Representative open-ended TNTs at the site of emergence from the cell. **(A)** 3D reconstruction generated from the FIB-SEM serial sections. Segmented representative TNTs connecting HS-5 and K562 cell are displayed as surface-rendered objects aligned on the volume data. **(B) Left:** Zoomed-in view of the 3D reconstruction focusing on the site of TNT emergence from stromal cell. **Right:** Sequential FIB-SEM sections showing the open-ended TNT at the site of its emergence from stromal cell. **(C) Left:** Zoomed-in view of the 3D reconstruction focusing on the site of TNTs emergence from leukemia cell. **Right:** Sequential FIB-SEM sections showing the open-ended TNTs at the site of its emergence from leukemia cell.

Across all analyzed datasets, no TNT open along its entire length was identified. Although TNTs were open-ended toward the cell bodies of the cells from which they originated (**Figure 4.1.6**), their distal ends were closed. In cases where

TNT emerging from one cell connected with TNT/TNTs from another cell, all structures were clearly closed at the junction site (**Figure 4.1.7**).

Only a representative TNT structure and corresponding FIB-SEM slices are presented; however, similar observations were made in all analyzed TNTs.

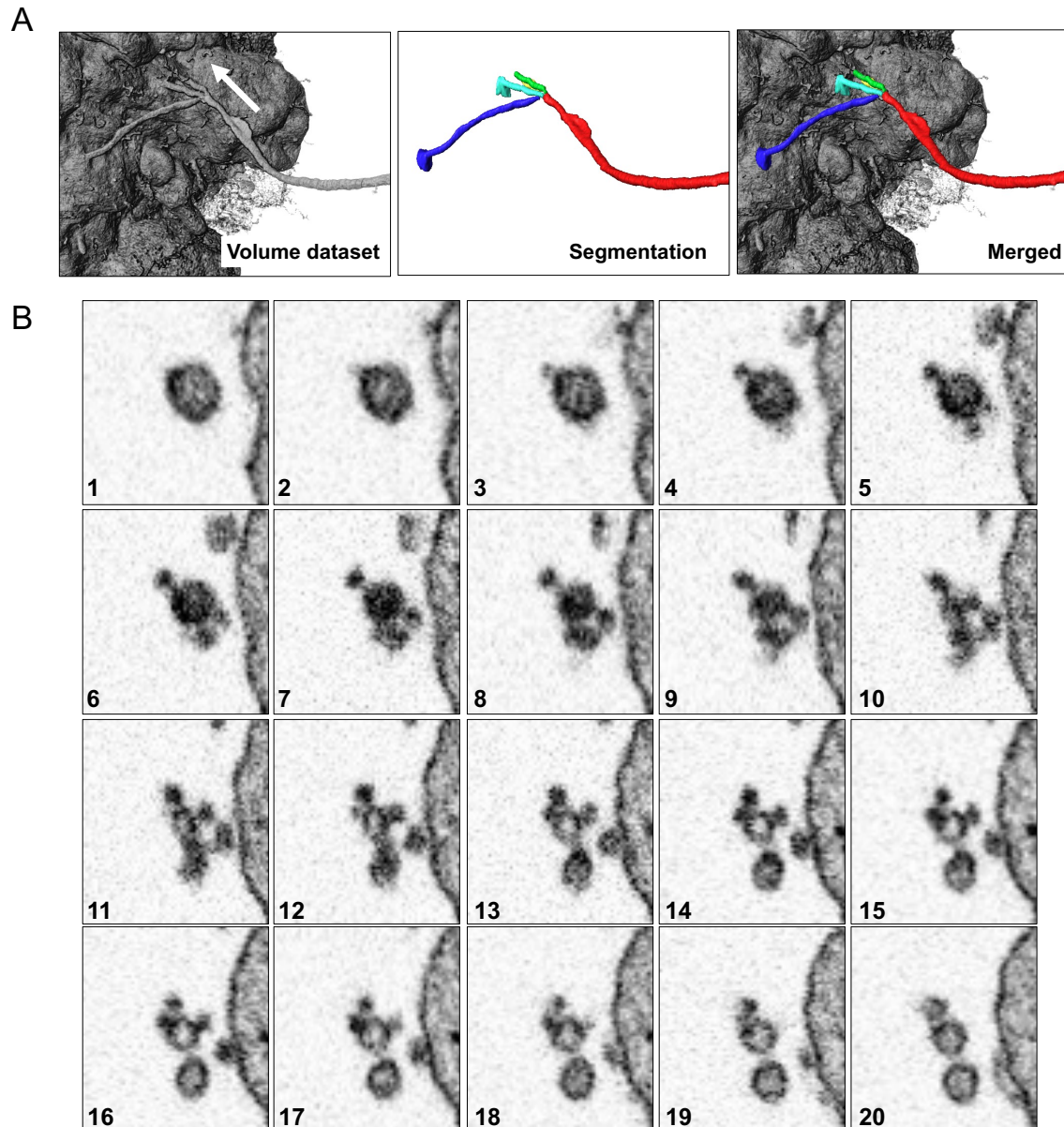


Figure 4.1.7 Representative junction of multiple TNTs visible as one TNT in lower resolution. **(A)** 3D reconstruction generated from the FIB-SEM serial sections. Segmented representative TNTs connecting HS-5 and K562 cell are displayed as surface-rendered objects aligned on the volume data. The white arrow indicates the direction of the slices presented in B. **(B)** Sequential FIB-SEM sections showing the closed distal ends of TNTs from stromal and leukemia cells, at the junction where all structures meet.

4.1.3 Quantitative analysis of TNTs and filopodia using FIB-SEM data

Based on segmentations performed on FIB-SEM sections, quantitative measurements of TNTs and filopodia were performed. The following parameters were measured:

- the distance of the protrusion from the substratum;
- the total length;
- the diameter;
- the volume.

The distance of the protrusion from the substratum was measured at three positions: at its emergence from the cell (START), at mid-length (MID) and at the terminal end (END) using the Skeleton tool in WEBKNOSSES (**Figure 4.1.8 A**).

The results revealed that TNTs were located at a mean distance of $4.4 \pm 2.7 \mu\text{m}$ from the substratum. Mean distances from the substratum remained similar across three

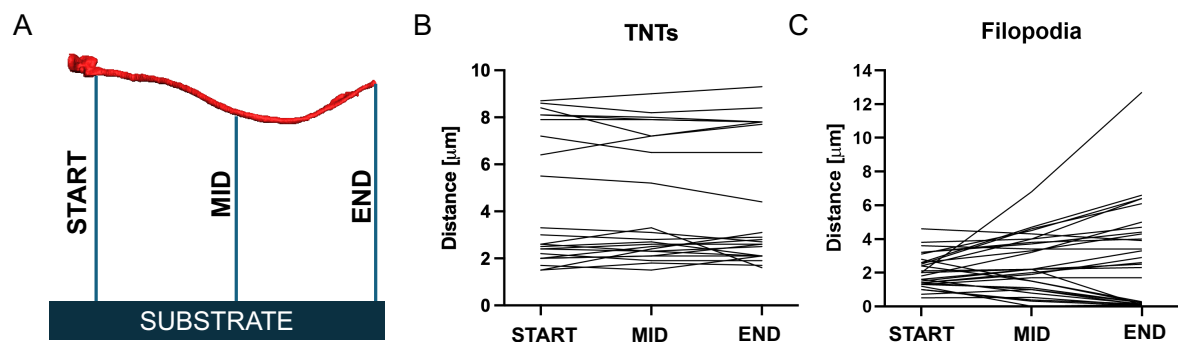


Figure 4.1.8 The distance from the substratum measured for TNTs and filopodia. (A) Schematic representation of the measurement sites (START – emerging site; MID – midpoint; END – distal end). **(B-C)** Distance from the substratum measured at three positions for TNTs **(B)** and for filopodia **(C)**.

measurement points: $4.4 \pm 2.8 \mu\text{m}$ at the proximal region (START), $4.4 \pm 2.6 \mu\text{m}$ at mid-length (MID) and $4.4 \pm 2.7 \mu\text{m}$ at the distal region (END). Only a subset of identified TNTs was located at a height greater than $4 \mu\text{m}$ above the substratum. As expected, filopodia were located closer to the substratum, with a mean distance of $2.5 \pm 2 \mu\text{m}$.

At the proximal region, the mean distance from the substratum was $2.1 \pm 1.0 \mu\text{m}$, at mid-length (MID) $2.5 \pm 1.7 \mu\text{m}$ and $2.9 \pm 3.0 \mu\text{m}$ at the distal length (END).

The diameter of the structures was measured at the same three positions as the protrusion-substratum distance using the Skeleton tool in WEBKNOSSES. In the 3D reconstructions of the FIB-SEM datasets, characteristic bulges were often observed at the sites of TNT emergence from the cell (**Figure 4.1.9 A**). Consequently, diameter measurements performed at this measure point were the highest, both for TNTs (**Figure 4.1.9 B**) and filopodia (**Figure 4.1.9 C**). In all TNTs, the diameter decreased toward the distal end (**Figure 4.1.9 B**), and a similar pattern was observed in filopodia (**Figure 4.1.9 C**).

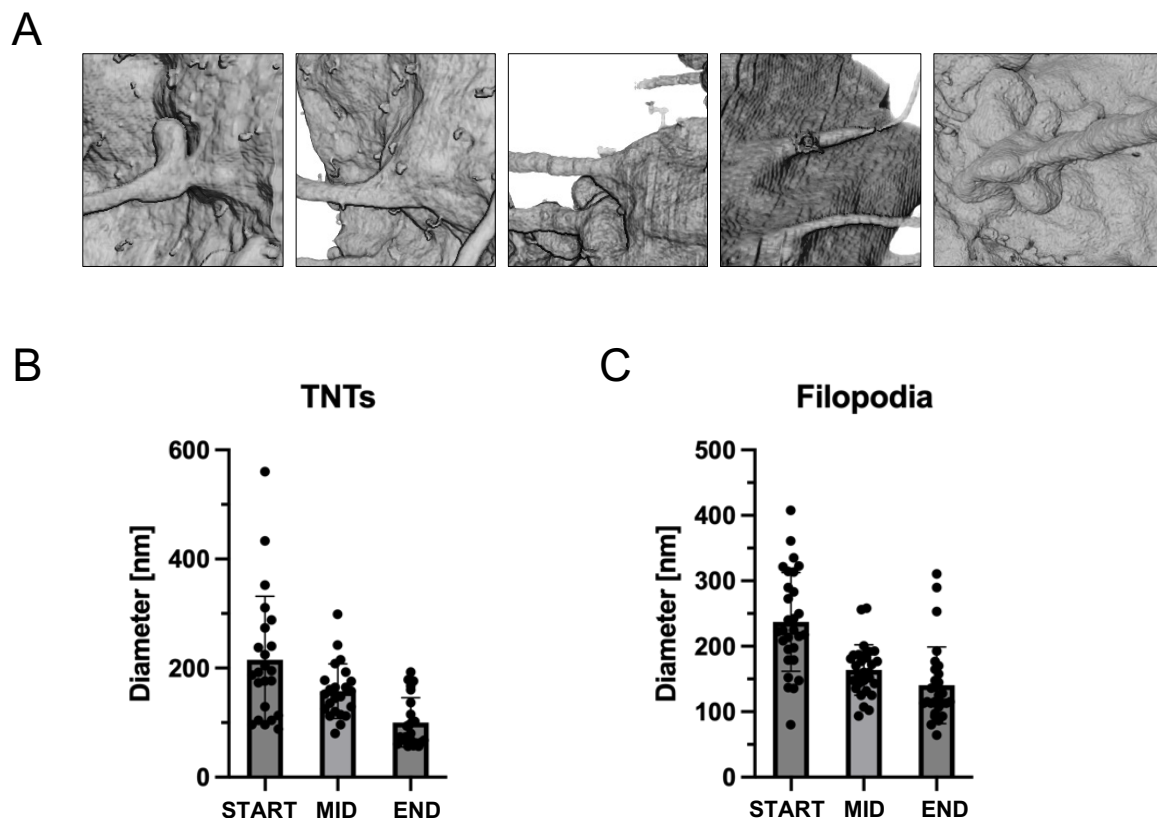


Figure 4.1.9 Diameter of the segmented structures. (A) 3D reconstructions from the FIB-SEM volume data of the bulges present at the TNTs' emerging sites. **(B-C)** Diameter of the TNTs **(B)** and filopodia **(C)** measured at three positions.

The total length of the protrusions was measured as the distance between the point of emergence from the cell and the distal end of the structure using the Skeleton tool in WEBKNOSSES. The mean total length of TNTs was shorter compared to the total length of filopodia, measuring $3.7 \pm 2.7 \mu\text{m}$ and $6.4 \pm 6.0 \mu\text{m}$, respectively.

The volume of segmented TNTs and filopodia was calculated in WEBKNOSS, based on the performed segmentation. The mean volume of TNTs was $0.10 \pm 0.09 \mu\text{m}^3$, similar to the mean volume of segmented filopodia $0.16 \pm 0.11 \mu\text{m}^3$.

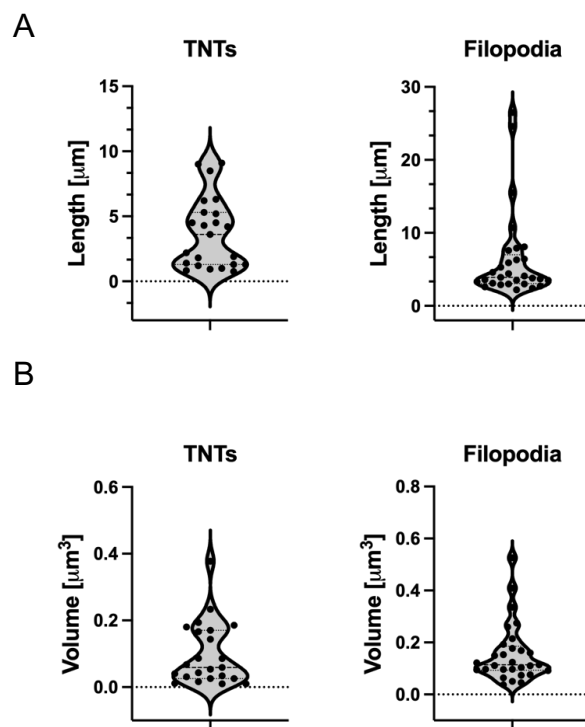


Figure 4.1.10 Total length and volume of the segmented structures. (A) The length of the segmented TNTs (left) and filopodia (right) measured from the START to END point. **(B)** The volume of the segmented TNTs (left) and filopodia (right).

Altogether, the data described in this chapter allowed us to address several questions concerning the ultrastructure of tunneling nanotubes. Importantly, FIB-SEM analysis confirmed the existence of TNTs formed between two cell types: stromal and leukemia cells. The obtained data also contradict the hypothesis that TNTs are fully opened structures, suggesting the presence of a specific regulation system controlling TNT openness and cargo transfer *via* TNTs. Lastly, the TNTs identified using confocal microscopy with the currently used criteria do not always meet them in the higher resolution applied in the volume EM.

4.2 Intercellular protein transfer *via* TNTs: focus on CD44 and its role in recipient cells

It was previously shown by us that HS-5 bone marrow stromal cells exploit tunneling nanotubes to connect with K562 chronic myeloid leukemia cells and transfer cellular vesicles and stromal proteins to CML⁷⁸. We reported that K562 cells, which received stromal vesicles specifically labeled with DiD fluorescent dye (leukemic recipients of stromal vesicles are referred to as K562 DiD⁺), are less sensitive to imatinib drug compared to cells that did not receive vesicles from stromal cells (K562 DiD⁻). The protein composition of transferred stromal vesicles was analyzed using a trans-SILAC MS approach. Briefly, donor cells' (HS-5, stroma) proteome was labeled with heavy isotopologues of amino acids, which allowed to identify stromal proteins in acceptor leukemia cells after co-culture. Then, HS-5 cells' vesicles were labeled with DiD dye, which was followed by co-culture with fluorescently tracked K562 cells. Vesicle acceptors (K562 DiD⁺) and non-acceptors (K562 DiD⁻) were sorted by FACS, and “heavy” proteins were identified in acceptor cells by mass spectrometry and bioinformatic analysis⁷⁸ (**Figure 4.2.1**).

The above phenomenon of TNT-mediated exchange of vesicles together with proteins was described before in another doctoral dissertation from Prof. Katarzyna Piwocka group and published⁷⁸. Here, these data were briefly discussed as they have made a ground for the biological concept of proteome transfer from the stroma to leukemia and provided a context for studies described in this dissertation.

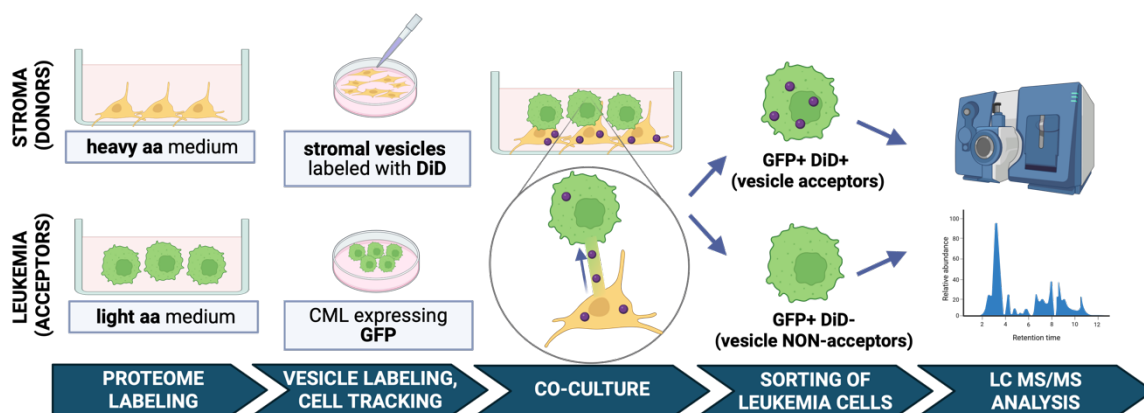


Figure 4.2.1 Overview of the experimental workflow used by Kolba *et al.* (2019). Created by the author of this dissertation using BioRender.com.

Additionally, our preliminary data showed that co-culture of leukemia cells together with stroma leads to increased levels of CD44 protein in leukemic cells (**Figure 4.2.2**). Therefore, we hypothesized that the stromal cells may directly transfer CD44 protein *via* TNTs and membrane vesicles, and by this regulate CD44 levels in leukemia. To address this, here we deeply investigated CD44 direct transfer, biological effects, and the role in stroma-dependent leukemia protection.

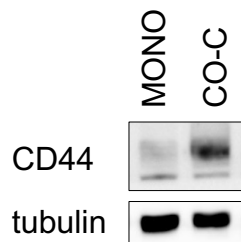


Figure 4.2.2 Representative immunoblot showing CD44 levels in K562 cells. K562 were cultured for 48 hours in monocultures (MONO) or in co-culture with HS-5 stromal cells (CO-C). Tubulin was used as a loading control.

4.2.1 CD44 is transferred together with vesicles from stromal to leukemic cells

First, the already existing open proteome database, which we created in our previous study⁷⁸, was reanalyzed (ProteomeXchange Consortium *via* the PRIDE [https://www.ebi.ac.uk/pride] partner repository, the dataset identifier: PXD013504). The trans-SILAC analysis revealed 294 proteins exclusively transferred from stroma to leukemia along with stromal vesicles. The identified proteins were primarily involved in the regulation of mRNA splicing, RNA localization, and translation. However, considering the importance of cell adhesion in the stroma-mediated resistance of leukemia, we focused our attention on CD44 protein (**Figure 4.2.3 A**). Heavy CD44 peptides (of stromal origin) were identified in K562 DiD⁺ cells, as shown by the normalized heavy-to light (H/L) ratio for CD44 detected in K562 cells. No heavy signal was detected in the population of K562 DiD⁻ cells (H/L=0), whereas the mean ratio for K562 DiD⁺ cells was 5.281 (**Figure 4.2.3 B**). This indicated that stromal CD44 is identified in leukemic cells that also received stromal vesicles.

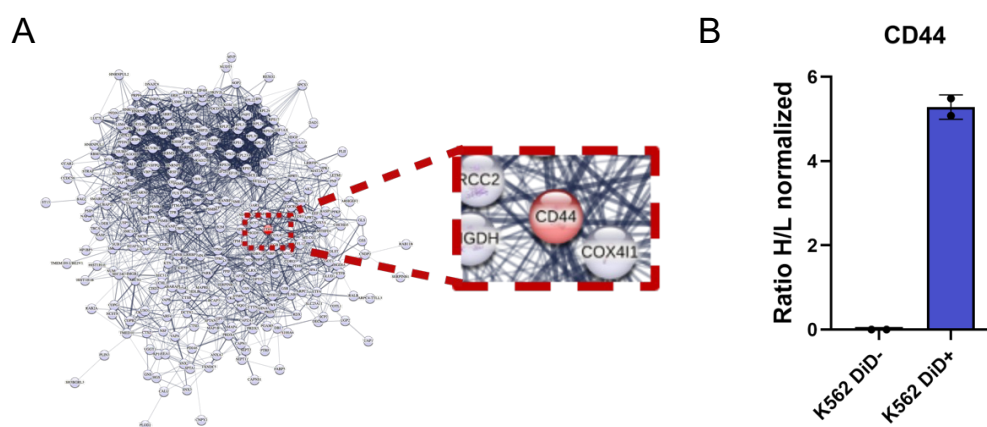


Figure 4.2.3 CD44 is transferred together with stromal vesicles to leukemia. (A) Schematic zoom-in of the STRING network showing CD44 as protein of interest, selected from all stroma-origin proteins identified in K562 cells. (B) Normalized heavy-to-light (H/L) ratio for CD44 detected in leukemic K562 cells by trans-SILAC mass spectrometry. The ratio shows the relative abundance of heavy-labeled (stroma-derived) versus light-labeled (endogenous) CD44 protein (n=2).

To strengthen the evidence for the involvement of stromal vesicles in the transfer of CD44 protein to leukemia, the levels of CD44 in K562 DiD⁺ and K562 DiD⁻ cells were compared using flow cytometry. Vesicles in HS-5 cells were labeled with DiD, followed by a 24-hour co-culture with fluorescently tracked K562 cells and anti-CD44 staining (described in the Materials and methods: **3.5.3.1**). Flow cytometry analysis revealed that K562 DiD⁺ population contains almost twice more CD44⁺ cells than K562 DiD⁻ population (**Figure 4.2.4 A**), indicating CD44 may be transferred along with stromal vesicles. To exclude the possibility that secretion and uptake of stromal exosomes is responsible for the CD44 transfer from stroma to leukemia, two inhibitors of exosome: GW4869 (exosome biogenesis) and Nexinhib20 (exosome secretion) were tested in co-cultures of HS-5 and K562 cells. Exosome inhibitors did not alter CD44 levels in K562 cells after co-culture with HS-5 stromal cells (**Figure 4.2.4 B**), nor did they impair transfer of DiD-labeled cellular vesicles *via* TNTs (**Figure 4.2.4 C**). This indicates that observed transfer is independent of exosome-mediated pathways and confirm contribution of contact-dependent TNT-associated vesicular transport.

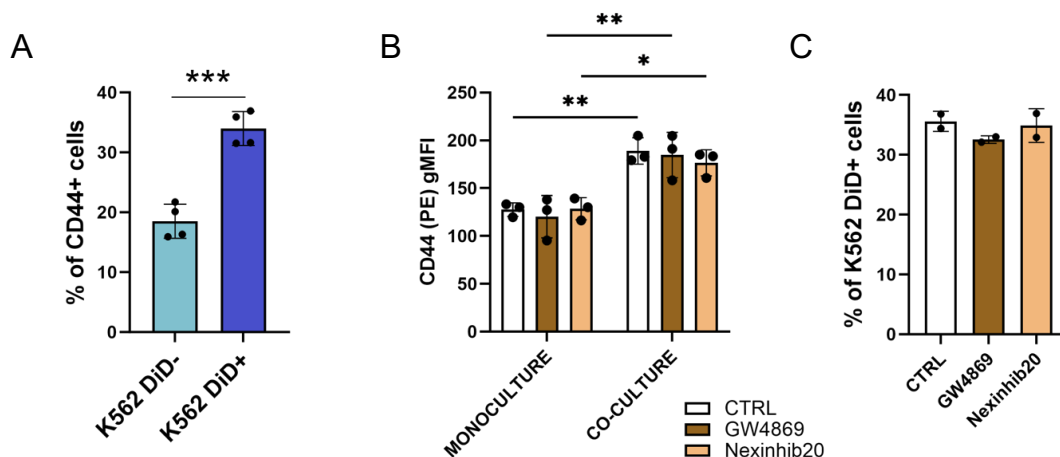


Figure 4.2.4 Co-transfer of stromal vesicles and CD44 protein to leukemia. (A) Enrichment of CD44 in K562 subpopulations of stromal vesicle recipients (K562 DiD⁺) and non-recipients (K562 DiD⁻). The percentage of K562 CD44⁺ cells is shown (n=4). (B) CD44 protein levels in viable K562 cells after 48 hours of monoculture and co-culture with HS-5 stromal cells, untreated or treated with exosome inhibitors: GW4869 (100 nM) or Nexinhib20 (250 nM). Data expressed as geometric mean fluorescence intensity – gMFI (n=3). (C) Uptake of DiD-labeled stromal vesicles by K562 cells after 24 h of co-culture with HS-5, untreated or treated with exosome inhibitors: GW4869 (100 nM) or Nexinhib20 (250 nM). The percentage of K562 DiD⁺ cells was shown (n=2). Data expressed as the mean ± SD, unpaired Student's t-test (A) or 2-way ANOVA with Tukey's posttest (B,C) were used to calculate statistical significance.

To elucidate CD44 importance in chronic myeloid leukemia, two independent, open GEO datasets with transcriptomic data obtained from analyses of CML patients' samples were reanalyzed. Both datasets revealed a significant clinical relevance of CD44 in CML, related to therapeutic resistance and progression. RNA-seq data from the GSE144119 dataset²³⁵ consisted of peripheral blood-derived samples, showed significantly upregulated *CD44* expression in newly diagnosed chronic-phase CML samples (CML) compared to samples from healthy donors (HD) (**Figure 4.2.5 A**). Moreover, analysis of the independent GSE4170 dataset²³⁴ revealed increased expression of *CD44* in the bone marrow aspirates obtained from patients for whom therapy with imatinib was ineffective (FAIL) compared to those who responded (EFFECTIVE) (**Figure 4.2.5 B**).

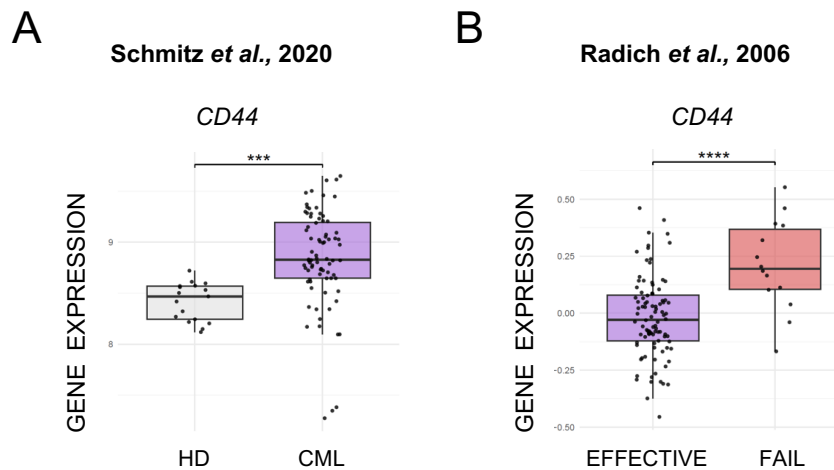


Figure 4.2.5 *CD44* expression in CML patients' samples. (A) *CD44* expression in samples from healthy donors (HD) and newly diagnosed chronic phase CML patients (CML), prepared based on RNA-seq data from the GSE144119 dataset. (B) *CD44* expression in CML samples obtained from CML patients for whom therapy with imatinib was ineffective (FAIL) or effective (EFFECTIVE), prepared based on the microarray dataset GSE4170. Data expressed as the mean $\pm$ SD, unpaired Student's t-test with Welch's correction was used to calculate statistical significance.

Considering its role in cell-cell adhesion, critical for stroma-driven protection of leukemia, and its clinical relevance in cancer and CML, the CD44 protein was selected as a vesicle-transferred protein candidate for further investigation in this study.

To further validate the transfer of stromal CD44 to leukemia cells, an *in vitro* co-culture model was used, in which HS-5 stromal cells stably expressing CD44-GFP (Figure 4.2.6) were co-cultured with fluorescently tracked leukemia cells (Figure 4.2.7).

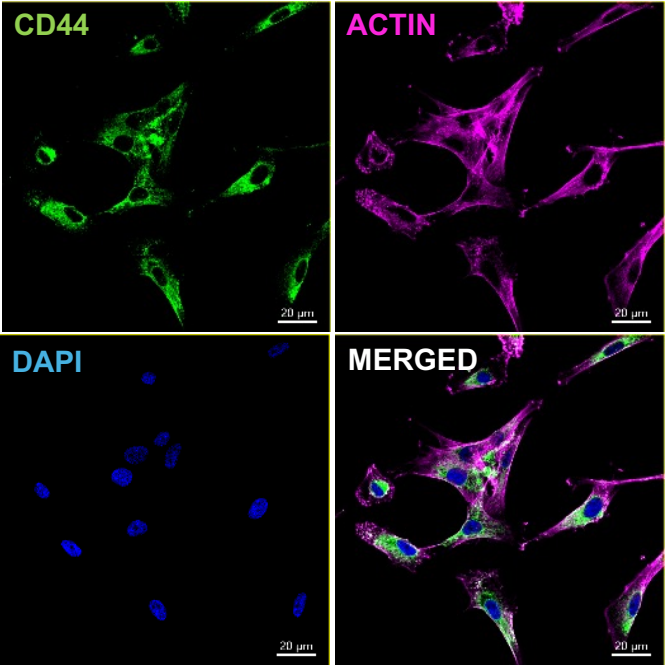


Figure 4.2.6 Representative immunofluorescence image of HS-5 cells expressing the CD44-GFP protein (green). Phalloidin-eFluor570 (magenta) was used to label actin and DAPI to visualize cell nuclei (blue). Scale bar: 20 µm.

Cells were co-cultured directly, indirectly – physically separated by a cell culture insert (Transwell control) or leukemia cells were grown as monocultures, in conditioned media obtained from HS-5 CD44-GFP culture (CM control) (Figure 4.2.7). Simultaneously, leukemia monocultures were maintained as additional controls. After 24 hours of co-cultures, stromal CD44-GFP was detected in around 4% of leukemia cells (Figure 4.2.8 A). Importantly, CD44 uptake

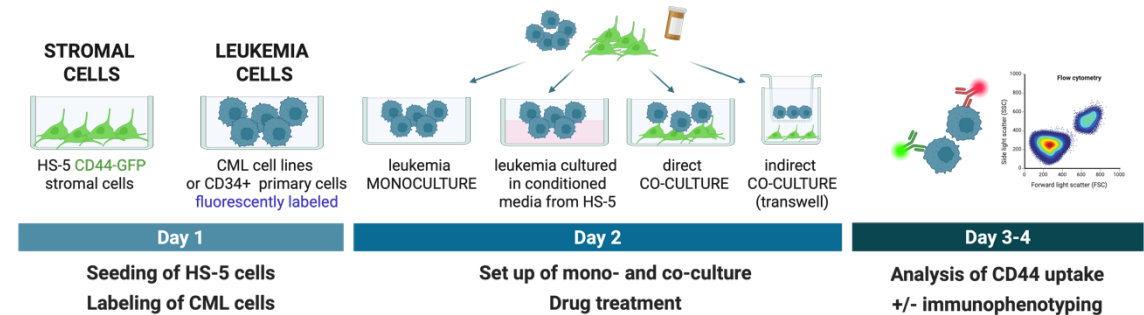


Figure 4.2.7 Experimental scheme for CD44-GFP uptake analysis. Created with BioRender.com.

by leukemia cells was strictly dependent on cell-cell contact, as no K562 GFP⁺ cells were detected in Transwell and CM control co-cultures.

To determine whether CD44 uptake depends on the intrinsic CD44 abundance in leukemia cells, we used additional cell lines characterized by different basal expression of CD44: LAMA-84 cells, imatinib-resistant K562 cells (K562 IR) and their parental counterparts (K562 PAR) (**Figure 4.2.8 B**). After co-culture with HS-5 CD44-GFP cells, stromal CD44 was present in all tested CML cell lines, regardless of their CD44 endogenous levels (**Figure 4.2.8 C**). Notably, these findings were further validated using primary patient-derived CD34⁺ CML cells (**Figure 4.2.8 D**).

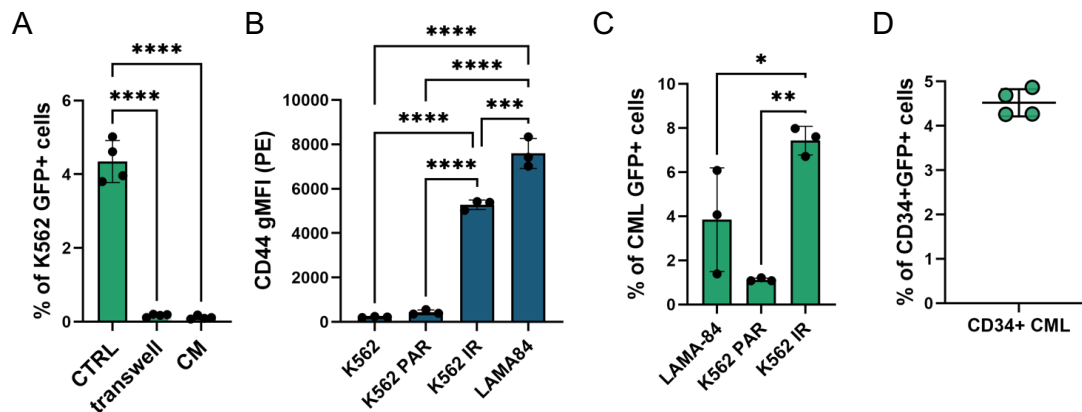


Figure 4.2.8 Uptake of stromal CD44-GFP by leukemia cells. (A) Uptake of stromal CD44-GFP by K562 cells after 24 h of co-culture. K562 cells were co-cultured with HS-5 CD44-GFP directly (CTRL), indirectly via cell culture insert (Transwell) or as monocultures in conditioned media obtained from HS-5 CD44-GFP cells. Shown as the percentage of K562 GFP⁺ cells (n=4). (B) Endogenous CD44 levels in monocultured K562 WT, Parental K562 (PAR), imatinib-resistant K562 (IR) and LAMA-84 cells. Measured with flow cytometry, shown as gMFI (n=3). (C) Uptake of stromal CD44-GFP by LAMA-84, K562 PAR and K562 IR cells after 24 h of co-culture with HS-5 CD44-GFP cells. Shown as the percentage of GFP⁺ leukemic cells (n=3). (D) Uptake of stromal CD44-GFP by primary CD34⁺ CML cells after 24 h co-culture with HS-5 CD44-GFP. Shown as the percentage of CD34⁺GFP⁺ cells (n=4). Data expressed as the mean $\pm$ SD, one-way (B) or 2-way ANOVA with Tukey's posttest (A,C) were used to calculate statistical significance.

To further confirm that stroma-derived vesicles serve as carriers of CD44, cellular vesicles were labeled in HS-5 CD44-GFP cells with DiD. Next, cells were co-cultured with fluorescently labeled K562 cells for 24 hours, after which the proportions of CD44-GFP⁺ cells in the K562 DiD⁺ and K562 DiD⁻ cells were compared using flow cytometry. Analysis revealed that K562 cells, which received vesicles from stroma (K562 DiD⁺), comprised a significantly greater proportion of CD44-GFP⁺ cells than K562, which did not accept stromal vesicles (K562 DiD⁻) (**Figure 4.2.9**).

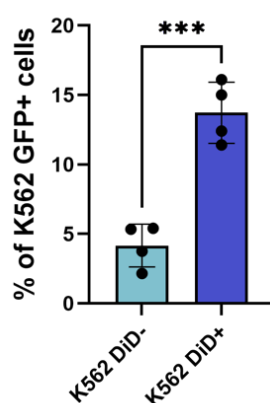


Figure 4.2.9 Co-uptake of stromal vesicles (DiD-labeled) and CD44-GFP by K562 cells. The percentage of K562 GFP⁺ cells in the populations of K562 DiD⁻ and K562 DiD⁺ cells is shown (n=4). Data expressed as the mean $\pm$ SD, unpaired Student's t-test was used to calculate statistical significance.

Based on these observations, we proposed that CD44 transfer from stroma to leukemia is mediated by TNT-dependent vesicle transport. Phorbol 12-myristate 13-acetate (PMA) is a well-documented protein kinase C (PKC) activator²³⁷, which was shown to promote TNT formation^{238,239}. Thus, PMA was used to induce TNT formation between stroma and leukemia to serve as a positive control. Overnight incubation with 5 nM PMA resulted in a marked increase in transfer of both stromal vesicles (**Figure 4.2.10 A**) and CD44-GFP (**Figure 4.2.10 B**) from stroma to leukemia cells. It was associated with increased adhesion of leukemia cells to stroma (**Figure 4.2.10 C**).

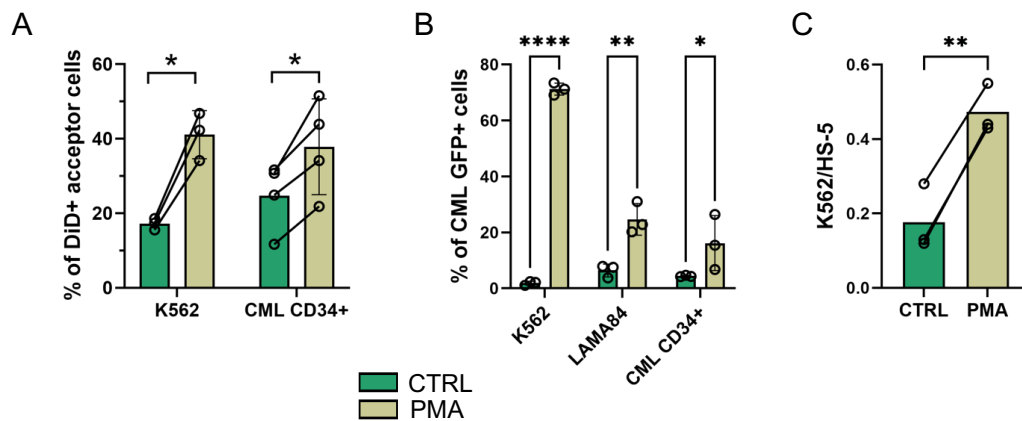


Figure 4.2.10 Effect of PKC activation with PMA on cargo transfer and cell adhesion. (A) Uptake of DiD-labeled stromal vesicles by K562 and primary CD34⁺ CML cells after 24 h of co-culture with HS-5, with or without PMA. The percentage of leukemic DiD⁺ cells was shown (n=4). (B) Uptake of stromal CD44-GFP by K562, LAMA-84 and primary CD34⁺ CML cells after 24 h co-culture with HS-5 CD44-GFP, with or without PMA. The percentage of leukemic GFP⁺ cells is shown (n=3). (C) Adhesion of K562 cells to HS-5 after 24 h co-culture, with or without PMA. The ratio of K562 to HS-5 cells is shown (n=3). Data expressed as the mean $\pm$ SD, 2-way ANOVA with Tukey's posttest (B) or unpaired Student's t-test (A, C) were used to calculate statistical significance.

Conversely, when PKC was inhibited with the specific, pan-PKC inhibitor bisindolylmaleimide I (BIM), both vesicle (**Figure 4.2.11 A**) and CD44-GFP transfer (**Figure 4.2.11 B**) were decreased.

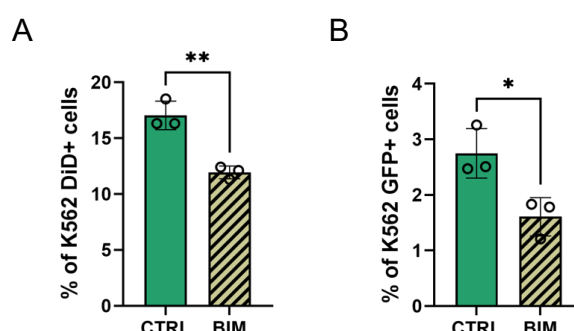


Figure 4.2.11 Effect of PKC inhibition with BIM I on cargo transfer. (A) Uptake of DiD-labeled stromal vesicles by K562 cells after 24 h of co-culture with HS-5, with or without BIM I (BIM). The percentage of K562 DiD⁺ cells was shown (n=3). (B) Uptake of stromal CD44-GFP by K562 cells after 24 h co-culture with HS-5 CD44-GFP, with or without BIM I. The percentage of K562 GFP⁺ cells is shown (n=3). Data expressed as the mean $\pm$ SD, unpaired Student's t-test was used to calculate statistical significance.

To visualize CD44 transfer, K562 cells labeled with Proliferation Dye eFluor450 (blue) were co-cultured with HS-5 CD44-GFP (green). This was followed by labeling cell membranes with WGA conjugated to Alexa Fluor 647 (red) and live cell imaging using confocal microscopy. The stromal CD44 was present not only within tunneling nanotubes connecting stromal and leukemic cells (**Figure 4.2.12, marked with an asterisks**), but also localized on the cell membranes of K562 acceptor cells (**Figure 4.2.12 A**) and was present inside K562 cells (**Figure 4.2.12 B**).

Taken together, CD44 is transferred from stromal to leukemic cells through direct, cell-cell contact. The results indicate that TNTs are involved in this process.

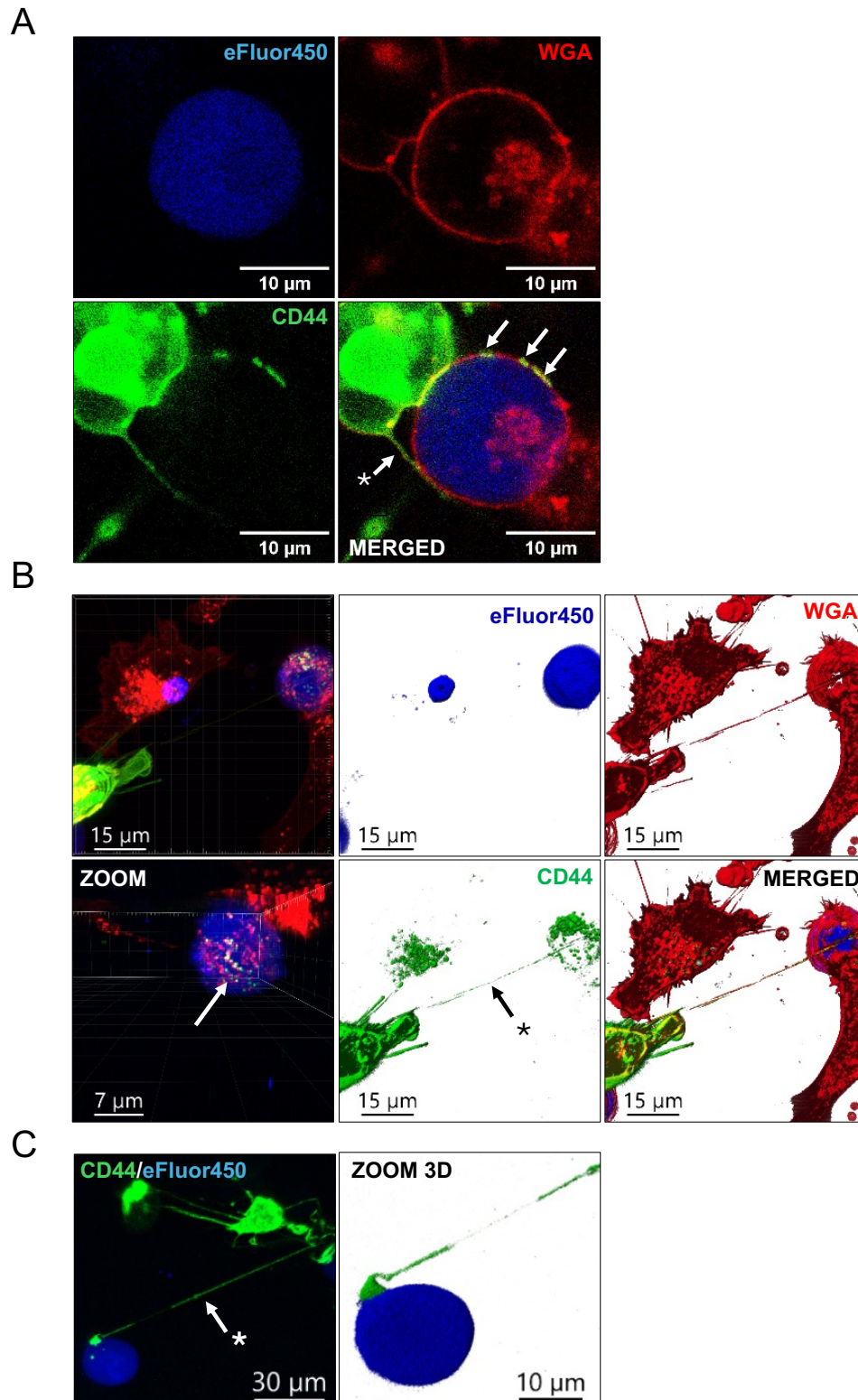


Figure 4.2.12 Representative images showing the localization of stromal CD44-GFP in K562 acceptor cells. Stromal CD44 was present on the leukemic cell membranes (**A**), inside leukemic cells (**B**) and localized within tunneling nanotubes formed between HS-5 and K562 cells (**A-C**, arrows with asterisks). (**C**) Left: image from live-cell confocal microscopy showing the presence of stromal CD44-GFP in the TNT formed between stroma and leukemia. Right: Zoomed-in 3D reconstruction. Green – stromal CD44-GFP, red – cell membranes labeled with WGA Alexa Fluor 647, blue – K562 cells labeled with Proliferation Dye eFluor450.

Interestingly, CD44-rich regions in stromal cell membranes at contact sites with leukemia cells were also observed (**Figure 4.2.13**). Our previously published data showed that formation of tunneling nanotubes between stromal and leukemic cells is a result of cell-cell contact followed by cell dislodgement⁷⁸, emphasizing the importance of adhesion in this process. Considering that CD44 is a cell adhesion molecule that was abundantly localized to TNTs formed between HS-5 and K562 cells, it was hypothesized that stromal CD44 is not only transferred *via* TNTs but may also play a role in their formation.

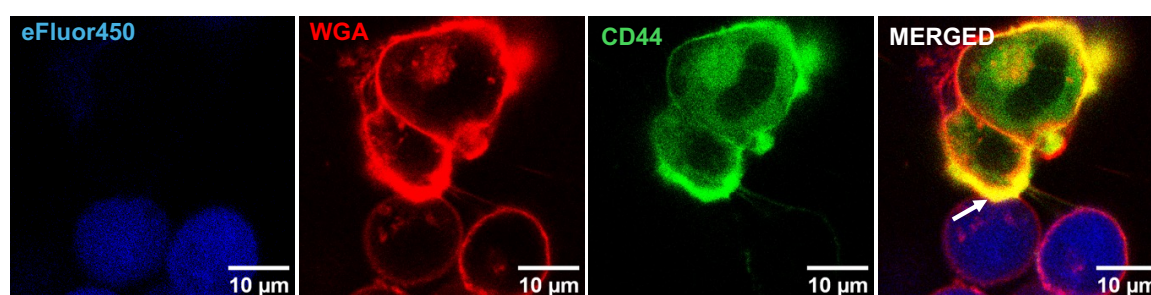


Figure 4.2.13 Representative image showing CD44-rich regions in stroma-leukemia contact sites. Green – stromal CD44-GFP, red – cell membranes labeled with WGA Alexa Fluor 647, blue – K562 cells labeled with Proliferation Dye eFluor450.

To investigate the role of CD44 in TNT formation, HS-5 cells with CD44 knockdown were established using shRNA (Materials and methods: **3.2.1**). Western blot and RT-qPCR analyses of silenced HS-5 clones revealed that Clone 5 achieved

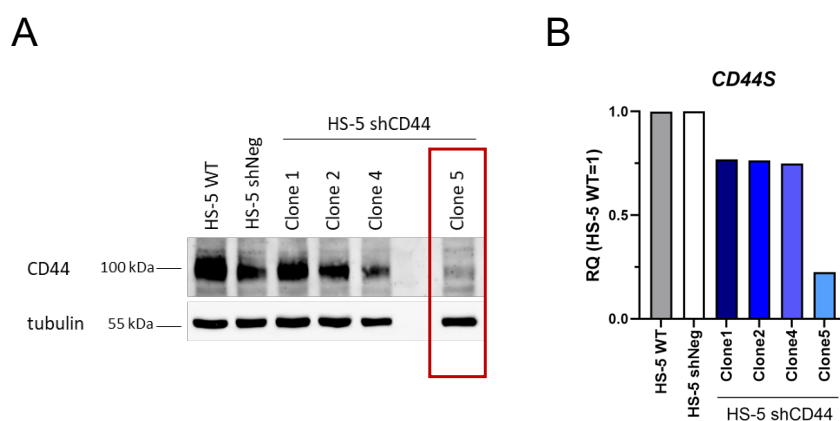


Figure 4.2.14 Validation of CD44 knockdown in HS-5 cells. **(A)** Western blot analysis showing CD44 protein levels in HS-5 cells. HS-5 WT – untreated cells, HS-5 shNeg – cells transduced with non-targeting shRNA, HS-5 shCD44 – cells transduced with shRNA targeting CD44 (different Clones 1-5). Red frame indicates the Clone 5 selected for downstream experiments. α -tubulin was used as a loading control. **(B)** RT-qPCR analysis of mRNA *CD44* levels in the cells described in **(A)**. 18S rRNA was used as endogenous control.

the highest level of *CD44* silencing in the HS-5 cells. Therefore, Clone 5 was selected for further downstream analyses (**Figure 4.2.14**). Co-culture of CML cells with HS-5 shCD44 stromal cells resulted in a significantly lower number of tunneling nanotubes formed between stroma and leukemia (referred to as heterotypic TNTs) (**Figure 4.2.15, left in A and B**) when compared to co-cultures with HS-5 shNEG. This effect was observed for both CML cell lines (K562 and LAMA-84 cells) grown in co-cultures and remained significant when normalized either to the number of CML cells or to the number of stromal cells. Additionally, CD44 knockdown in stromal cells did not decreased number of homotypic TNTs (formed between stromal cells themselves) (**Figure 4.2.15, right in A and B**). A detailed description of TNT quantification is provided in Materials and methods, **Section 3.7.2.1**.

What is important, the effects described above were not due to altered cellular functions in HS-5 shCD44 cells, as their cell cycle (**Figure 4.2.15 C**) and proliferation (**Figure 4.2.15 D**) remained unchanged.

To sum up, the presence of CD44 in stromal cells is crucial not only for its transfer to leukemia but also for the TNT formation between stromal and leukemic cells.

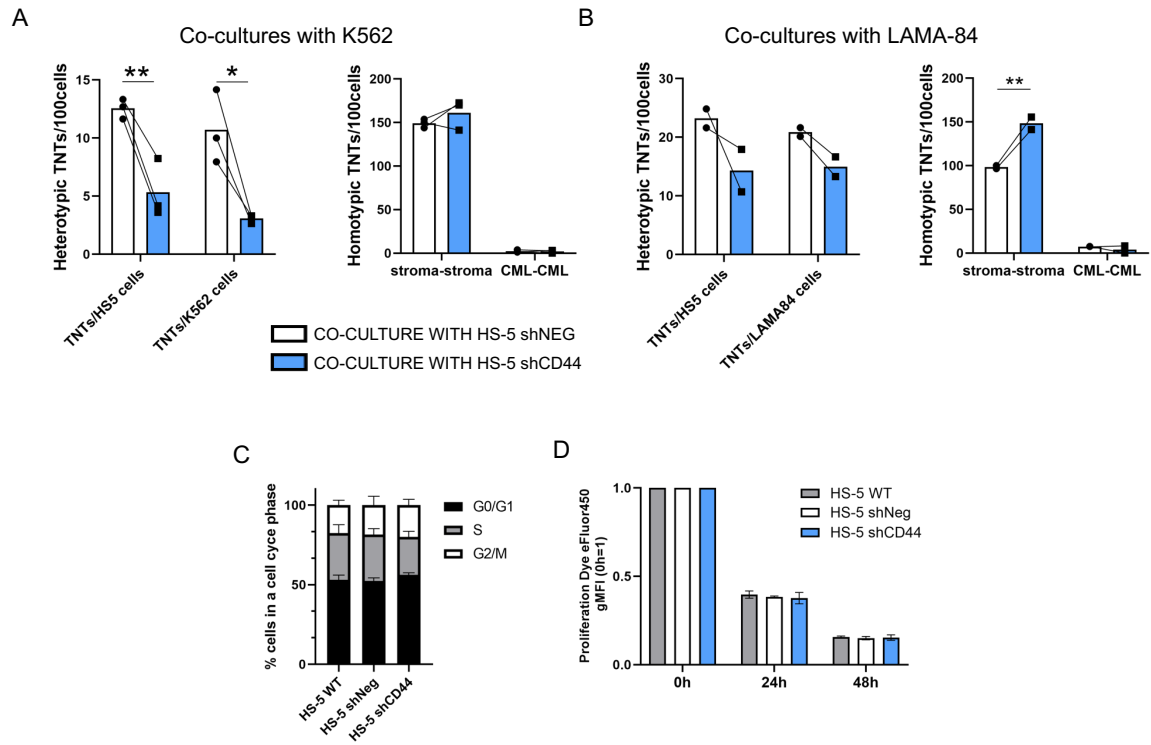


Figure 4.2.15 The influence of CD44 knockdown in stromal cells on TNT formation. (A) Average numbers of TNTs per 100 cells quantified in co-cultures of K562 cells with HS-5 shNEG or HS-5 shCD44 cells after 24 hours of co-culture. Left: number of heterotypic TNTs/100 cells calculated per 100 CML cells and per 100 stromal cells, right: number of homotypic TNTs interconnecting either stromal or CML cells, calculated per 100 stromal and per 100 CML cells, respectively. (B) Average numbers of TNTs per 100 cells quantified in co-cultures of LAMA-84 cells with HS-5 shNEG and HS-5 shCD44 cells after 24 hours of co-culture. Left: number of heterotypic TNTs/100 cells calculated per 100 CML cells and per 100 stromal cells, right: number of homotypic TNTs interconnecting either stromal or CML cells, calculated per 100 stromal and per 100 CML cells, respectively. (C) Cell cycle distribution measured by propidium iodide staining and flow cytometry in HS-5, HS-5 shNEG and HS-5 shCD44 cells. Results shown as stacked bars representing the percentage of cells in G0/G1, S and G2/M phases. (D) Proliferation of HS-5, HS-5 shNEG and HS-5 shCD44 cells measured with Cell Proliferation Dye eFluor450 at 0, 24 and 48 hours after labeling. Data normalized to 0 h (gMFI for 0 h = 1). Data expressed as the mean $\pm$ SD, 2-way ANOVA with Tukey's posttest was used to calculate statistical significance (A-D).

4.2.2 Recipients of stromal CD44 demonstrate altered transcription profiles

It has been reported that CD44 can be translocated to the cell nucleus, where it cooperates with transcription factors and remodels gene expression. Both the full-length CD44 protein and cleaved intracellular domain (ICD) can be involved^{155,163}. Therefore, the potential transcriptional role of transferred CD44 in CML acceptor cells was investigated. The CD44-GFP construct used to generate HS-5 CD44-GFP cells encoded CD44 fused to GFP at its C-terminus (**Figure 4.2.16 A**). This allowed us to track the localization of both full-length stromal CD44 and released CD44 ICD in leukemia cells.

K562 cells were co-cultured for 24 hours with HS-5 CD44-GFP cells. This was followed by FACS sorting of K562 cells and isolation of nuclear and cytoplasmic protein fractions. Western blot analysis revealed that co-culture with HS-5 CD44-GFP cells resulted in increased nuclear localization of CD44 in leukemia cells when compared to monocultured K562 (**Figure 4.2.16 B, upper panel**). Moreover, most of the nuclear-localized CD44 was of stromal origin as shown by anti-GFP immunodetection in nuclear and cytoplasmic protein fractions

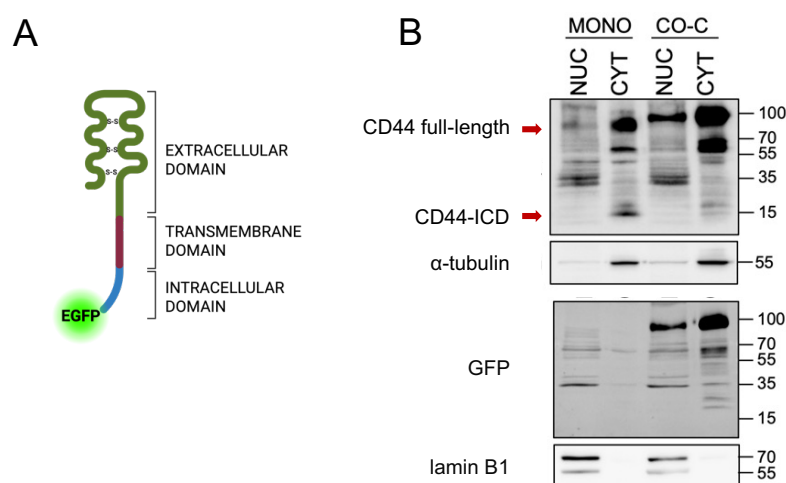


Figure 4.2.16 Nuclear localization of stroma-derived CD44 in leukemia cells upon co-culture. (A) Simplified scheme of the CD44 protein with EGFP fused to the protein C-terminus. (B) Immunoblot showing the cellular distribution of total CD44 and stromal CD44-GFP in K562 cells monocultured or sorted from co-cultures with HS-5 CD44-GFP cells. Total CD44 was detected using anti-CD44 antibody, and stromal CD44-GFP was detected with anti-GFP-AF488 antibody (upper and lower panel, respectively). The loading and contamination controls for cytoplasmic and nuclear fractions were α -tubulin and lamin B1, respectively.

(**Figure 4.2.16 B, lower panel**). Interestingly, no cleaved CD44-ICD and only the full-length CD44 was detected in the nuclear fraction of co-cultured K562 cells.

Considering the nuclear localization of stromal CD44 in leukemia cells after co-culture and its reported role in the co-regulation of gene transcription, it was further investigated whether CD44 uptake by leukemia cells influences gene expression in acceptor cells. To do this, co-cultures of K562 and HS-5 CD44-GFP cells, together with K562 monocultures, were established for 24 hours. Subsequently, K562 CD44-GFP⁺ (leukemia cells that accepted stromal CD44) and K562 CD44-GFP⁻ cells (non-acceptors of stromal CD44) were sorted by FACS. After this, RNA was isolated from sorted cell populations and from monocultured K562 cells, followed by bulk RNA-seq analysis (**Figure 4.2.17 A**). RNA-seq analysis of sorted K562 cells that received (CD44-GFP⁺) or did not receive (CD44-GFP⁻) stromal CD44, together with monocultured K562 cells, revealed distinct gene expression profiles across all analyzed populations, as shown by principal component analysis (PCA) (**Figure 4.2.17 B**). Venn analysis revealed a subset of 465 commonly upregulated genes in both CD44-GFP⁺ and CD44-GFP⁻ leukemia cells sorted from co-culture compared to leukemia monoculture, indicating a shared core of transcriptomic alterations not dependent on stromal CD44 transfer to leukemia, but shaped by other co-culture-derived factors (**Figure 4.2.17 C, left**). However, 791 genes were uniquely upregulated in CD44-GFP⁺ cells (leukemic cells that received stromal CD44) relative to monoculture, whereas only 60 genes were exclusively upregulated in CD44-GFP⁻ cells. 118 genes were specifically upregulated in CD44-GFP⁺ in comparison with CD44-GFP⁻ cells (**Figure 4.2.17 C left, bolded**). Only a few genes were downregulated when comparing recipients of stromal CD44 to non-recipients, pointing to a CD44-dependent trend toward transcriptional activation (**Figure 4.2.17 C, right**). The volcano plot illustrated the distribution of differentially expressed genes (DEGs) between K562 CD44-GFP⁺ (acceptors) and K562 CD44-GFP⁻ cells (non-acceptors). DEGs were identified using thresholds of $\log_2\text{FC} > 0.5$ for upregulated genes and $\log_2\text{FC} < -0.5$ for downregulated genes, with an adjusted p-value < 0.05 , displayed as $-\log_{10}(\text{p-adjusted})$ (**Figure 4.2.17 D**). The full list of DEGs is listed in **Table 9-1**.

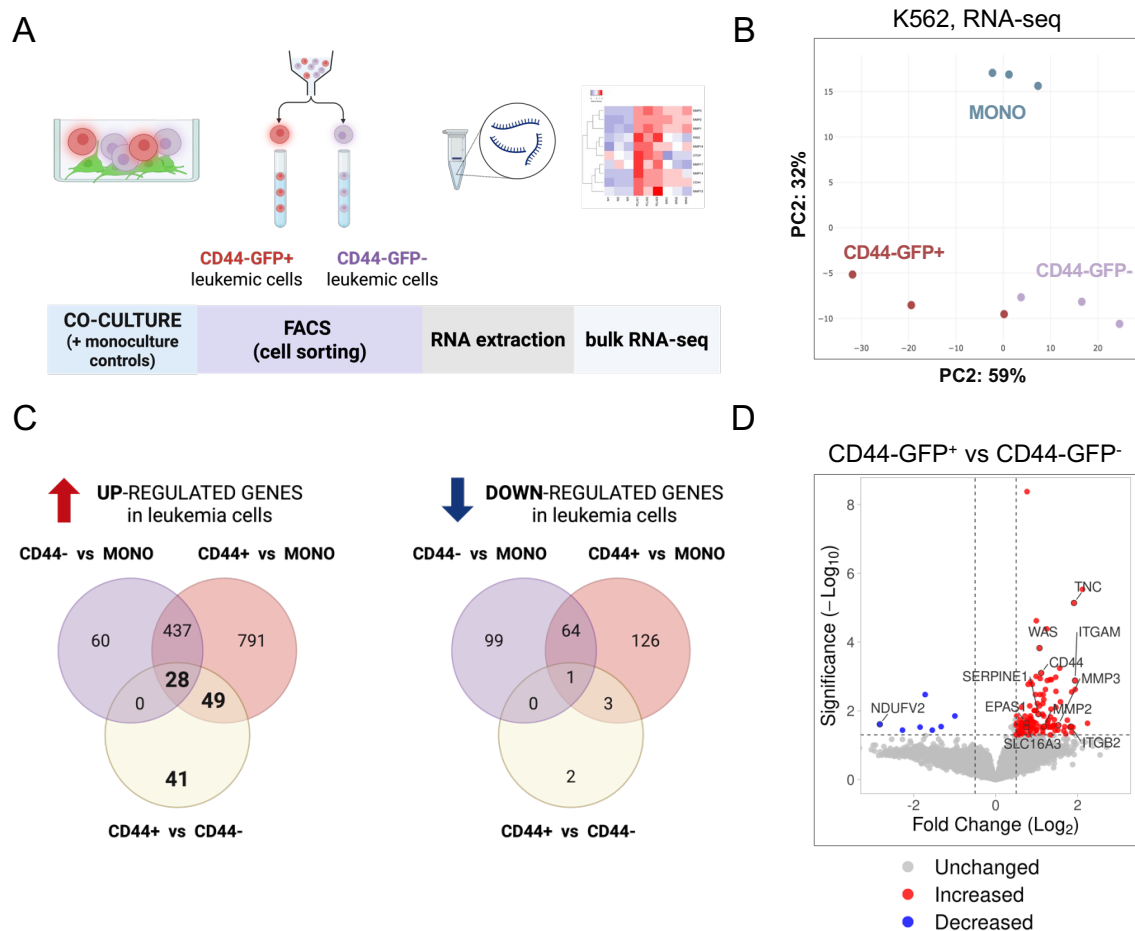
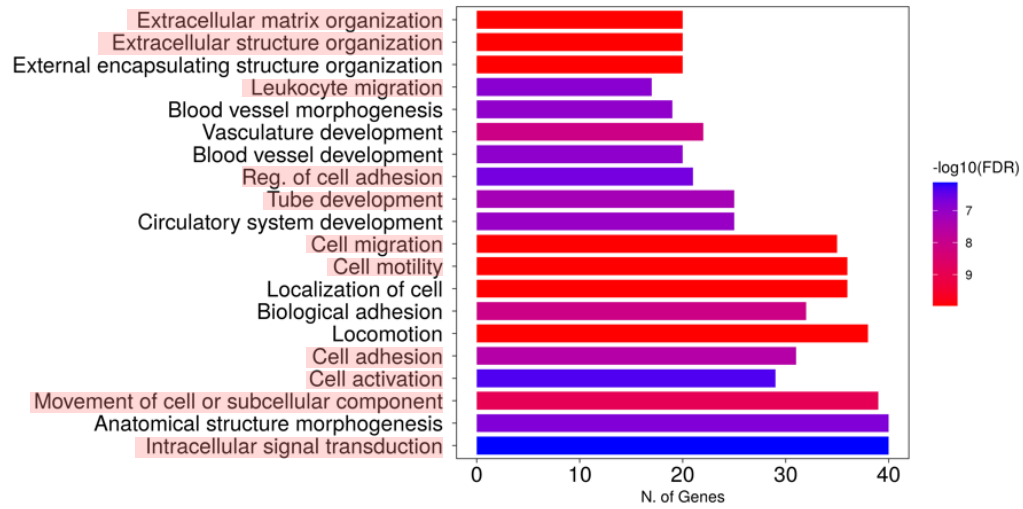


Figure 4.2.17 Transcriptomic changes in K562 cells after co-culture with HS-5 CD44-GFP cells. **(A)** Experimental scheme for bulk RNA-seq analysis. Created with BioRender.com **(B)** A principal component analysis (PCA) of bulk RNA-seq datasets from monocultured K562 (“MONO”), K562 acceptors of stromal CD44 (“CD44-GFP⁺”) and K562 non-acceptors of stromal CD44 (“CD44-GFP⁻”). **(C)** Venn diagrams showing shared and unique upregulated (left) and downregulated (right) genes among the three conditions: monoculture (MONO), stromal CD44-acceptors (CD44-GFP⁺; described here as CD44⁺) and non-acceptors (CD44-GFP⁻; described here as CD44⁻). Genes were considered significantly up- or downregulated if $|\log_2FC| > 0.5$, adj. $p \leq 0.05$. **(D)** Volcano plot illustrating differential gene expression between stromal CD44 acceptors (CD44-GFP⁺) and non-acceptors (CD44-GFP⁻) (adj. p -value < 0.05).

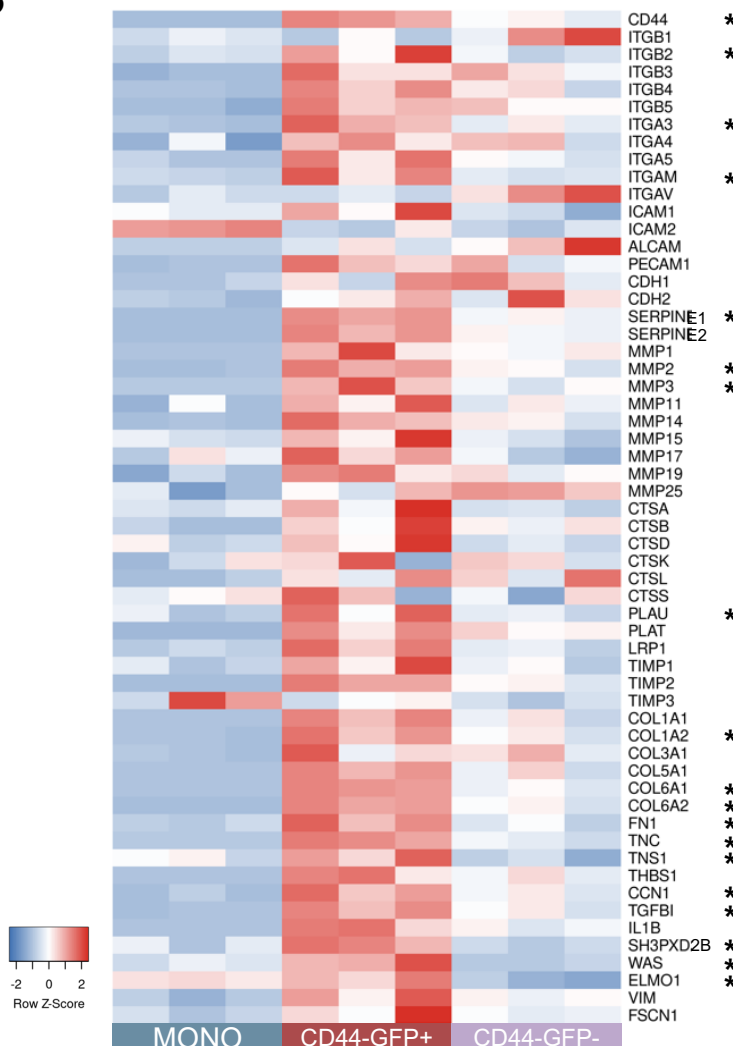
Next, functional enrichment analysis was performed against the *Homo sapiens* genome background. It revealed that genes upregulated following CD44 transfer in leukemia cells were significantly enriched (FDR < 0.05) in GO Biological Process terms related to: cell adhesion [GO:0030155; GO:0022610; GO:0007155], migration [GO:0050900; GO:0016477], and extracellular matrix organization [GO:0030198; GO:0043062] (**Figure 4.2.18 A**). Following GO Biological Process enrichment analysis, a heatmap was generated showing representative genes involved in enriched processes.

A

BIOLOGICAL PROCESS (GENE ONTOLOGY) ENRICHMENT



B



C

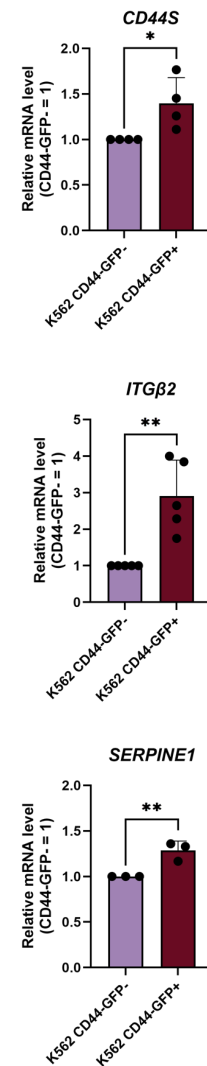


Figure 4.2.18 Upregulation of genes related to ECM remodeling in leukemic acceptors of stromal CD44. **(A)** GO Biological Process enrichment analysis showing overrepresented terms among upregulated genes in K562 cells which received stromal CD44 (CD44-GFP⁺). Analysis was performed using ShinyGO (v. 0.77). The color gradient represents $-\log_{10}(\text{FDR})$ values. **(B)** Heatmap showing expression of genes related to adhesion, migration and ECM remodeling across monocultured K562 (MONO), K562 acceptors of stromal CD44 (CD44-GFP⁺) and non-acceptors (CD44-GFP⁻). Expression values were log2-transformed ($\log_2(\text{FPKM})$) and standardized to Z-scores. Asterisks (*) indicate genes significantly altered (adjusted $p < 0.05$) when comparing K562 CD44-GFP⁺ and K562 CD44-GFP⁻ cells, while others are included for completeness and interpretative context. **(C)** RT-qPCR validation of RNAseq results showing relative expression of *CD44S*, *ITGB2*, *CD44S* in CD44 acceptors (K562 CD44-GFP⁺) versus non-acceptors (K562 CD44-GFP⁻) (n=3). Expression was normalized to CD44-GFP⁻ (set as 1), with actin as the endogenous control. Data expressed as the mean $\pm$ SD, unpaired Student's t-test.

The heatmap displays Z-score-normalized expression across the analyzed groups. Genes significantly upregulated in the K562 CD44-GFP⁺ cells relative to CD44-GFP⁻ cells are indicated by asterisks. The analysis further confirmed increased expression of genes related to adhesion (*CD44*, *ITGB2*, *ITGAM*), multiple proteases (e.g., *MMP2*, *MMP3*, *PLAU*), collagens, and genes involved in cytoskeleton regulation and mechanosensory signaling, like *ELMO1*, *WAS*, *VIM*, or *FSCN1* in acceptors of stromal CD44 compared to non-acceptors (**Figure 4.2.18 B**). Selected DEGs involved in cell adhesion and extracellular matrix organization were validated by qPCR (**Figure 4.2.18 C**).

Collectively, these data demonstrate that stromal CD44 is transported to the nuclei of leukemic recipient cells in its full-length form. This is accompanied by changes in the transcriptional profile of leukemic cells that received stromal CD44, characterized by upregulation of genes related to cell adhesion, migration, and extracellular matrix remodeling in these cells.

4.2.3 Stromal cells promote a positive feedback loop that enhances endogenous *CD44* expression in leukemia cells and prevents imatinib-induced loss of *CD44* protein

Importantly, RNA-seq analysis, confirmed by qPCR, revealed that *CD44* itself was among the most significantly upregulated genes in K562 cells, which accepted *CD44*-GFP from stroma (**Figure 4.2.17 D, Figure 4.2.18 C**). Furthermore, RT-qPCR analysis of RNA samples from sorted K562 DiD⁺ (acceptors of stromal vesicles) and K562 DiD⁻ (non-acceptors of stromal vesicles) cells revealed strongly increased upregulation of *CD44* standard (*CD44S*) and splicing variants compared to monocultured K562 (**Figure 4.2.19**). Importantly, a greater upregulation was detected in acceptors of stromal vesicles (K562 DiD⁺), in which *CD44* protein transfer had been previously observed (**Figure 4.2.3 and Figure 4.2.9**).

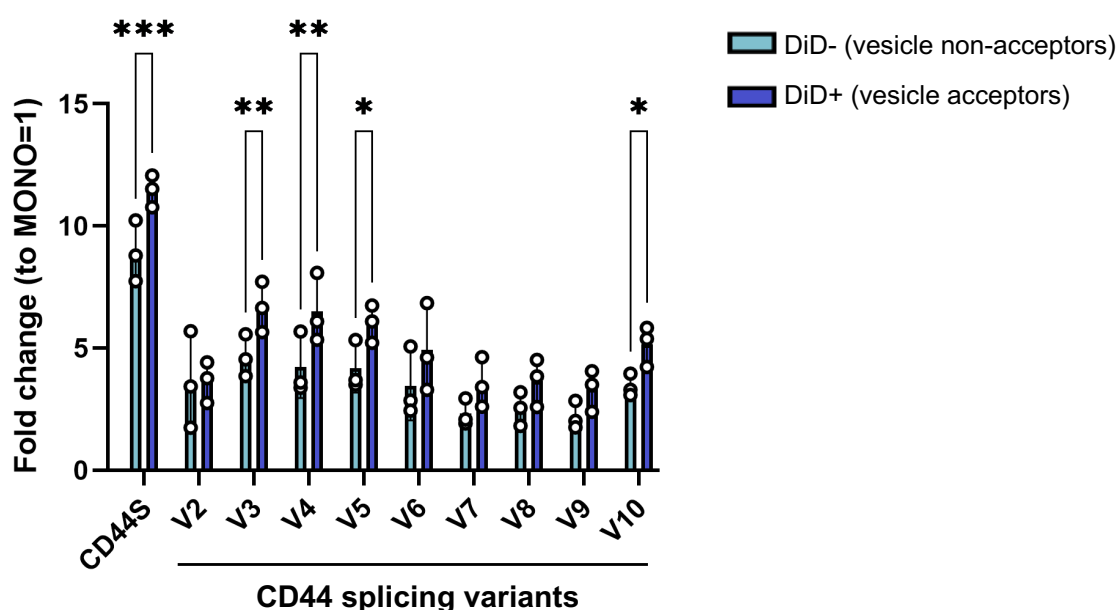


Figure 4.2.19 *CD44* mRNA levels in stromal vesicle acceptors and non-acceptors. Relative expression of *CD44S* and *CD44* splicing variants measured by RT-qPCR in K562 cells. Cells were cultured either in monoculture or co-culture with DiD-labeled HS-5 stromal cells, followed by FACS sorting of K562 DiD⁺ and DiD⁻ populations. Expression was normalized to monoculture samples, which was set to 1 (MONO=1). β -actin served as an endogenous control. Data expressed as the mean $\pm$ SD, 2-way ANOVA with Šídák correction was used to calculate statistical significance (n=3).

These findings indicate a possible feedback loop in which contact-dependent *CD44* transfer regulates its endogenous expression in leukemia cells.

To investigate whether direct cell-cell contact is required for the upregulation of endogenous *CD44* gene transcription in leukemia, K562 and HS-5 cells were co-cultured directly or in the Transwell system. This was followed by isolation of RNA from leukemia cells and assessment of *CD44* mRNA levels using RT-qPCR. The results revealed that direct co-culture with HS-5 stromal cells substantially increased *CD44* expression in leukemia cells, whereas physical separation using the Transwell system significantly attenuated stroma-mediated increase in *CD44* expression (**Figure 4.2.20 A**). Similar results were obtained in co-cultures of HS-5 cells with patient-derived CD34⁺ CML cells, confirming that interaction with stromal cells promotes upregulation of endogenous *CD44* expression in leukemia cells (**Figure 4.2.20 B**). Notably, the co-culture of leukemia with HS-5 cells with silenced *CD44* expression (HS-5 shCD44) did not result in a *CD44* increase in leukemic cells. This confirmed the critical role of stroma-derived *CD44* in the regulation of endogenous *CD44* transcription in leukemia cells (**Figure 4.2.20 C**).

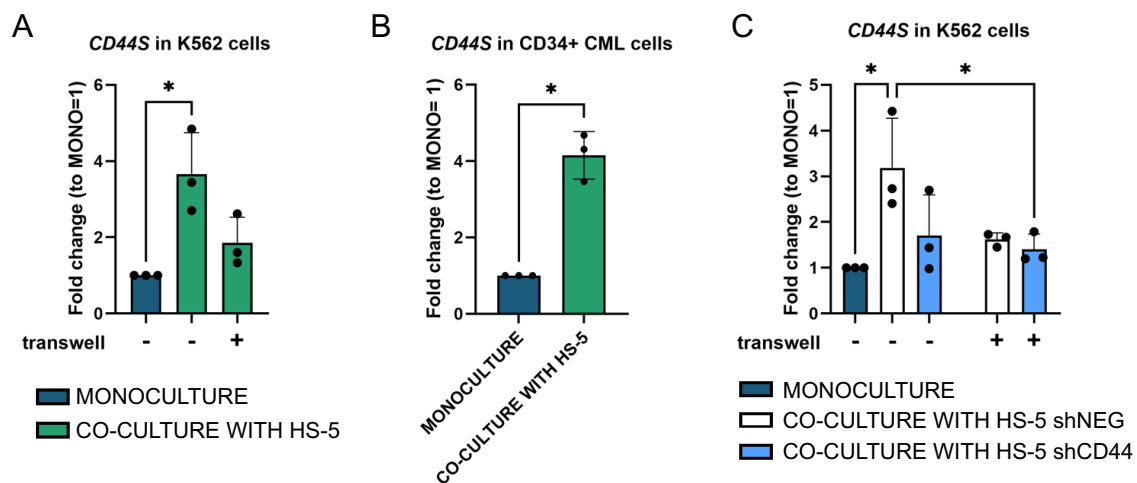


Figure 4.2.20 *CD44S* mRNA levels in leukemia cells co-cultured with stroma. (A) Relative expression of *CD44S* measured by RT-qPCR in K562 cells derived from monoculture and co-culture with HS-5 stromal cells. Co-cultures were performed either in direct contact or indirectly, separated by an insert (Transwell) (n=3). (B) Relative expression of *CD44S* measured by RT-qPCR in primary CD34⁺ CML cells grown in monoculture or in co-culture with HS-5 cells (n=3). (C) Relative expression of *CD44S* measured by RT-qPCR in K562 cells grown in monoculture or in co-culture with HS-5 shNEG and HS-5 shCD44 cells, either in direct contact or indirectly, separated by an insert (Transwell) (n=3). Data expressed as the mean $\pm$ SD, one-way ANOVA with Tukey's posttest (A, C) and paired Student's t-test were used to calculate statistical significance.

Importantly, stroma-induced increase in *CD44* mRNA levels corresponded to higher CD44 protein levels in leukemia cells. Anti-CD44 staining and flow cytometry analysis revealed a significant increase in the geometric mean fluorescence (gMFI) in the K562 cells co-cultured with HS-5 compared to K562 cultured alone, in monoculture. It was reported by others that treatment of monocultured K562 cells with imatinib leads to a decrease in CD44 protein levels¹⁷⁷. It was also shown in monocultures of K562 and LAMA-84 cells analyzed in this study. However, in co-cultures, stromal cells partially prevented CD44 protein decrease, suggesting a novel stromal protective mechanism (**Figure 4.2.21 A-B**). Similarly to mRNA levels, stroma-mediated CD44 protein upregulation in leukemia was strongly dependent on direct cell-cell contact, as shown by co-cultures in the Transwell system (**Figure 4.2.21 A**).

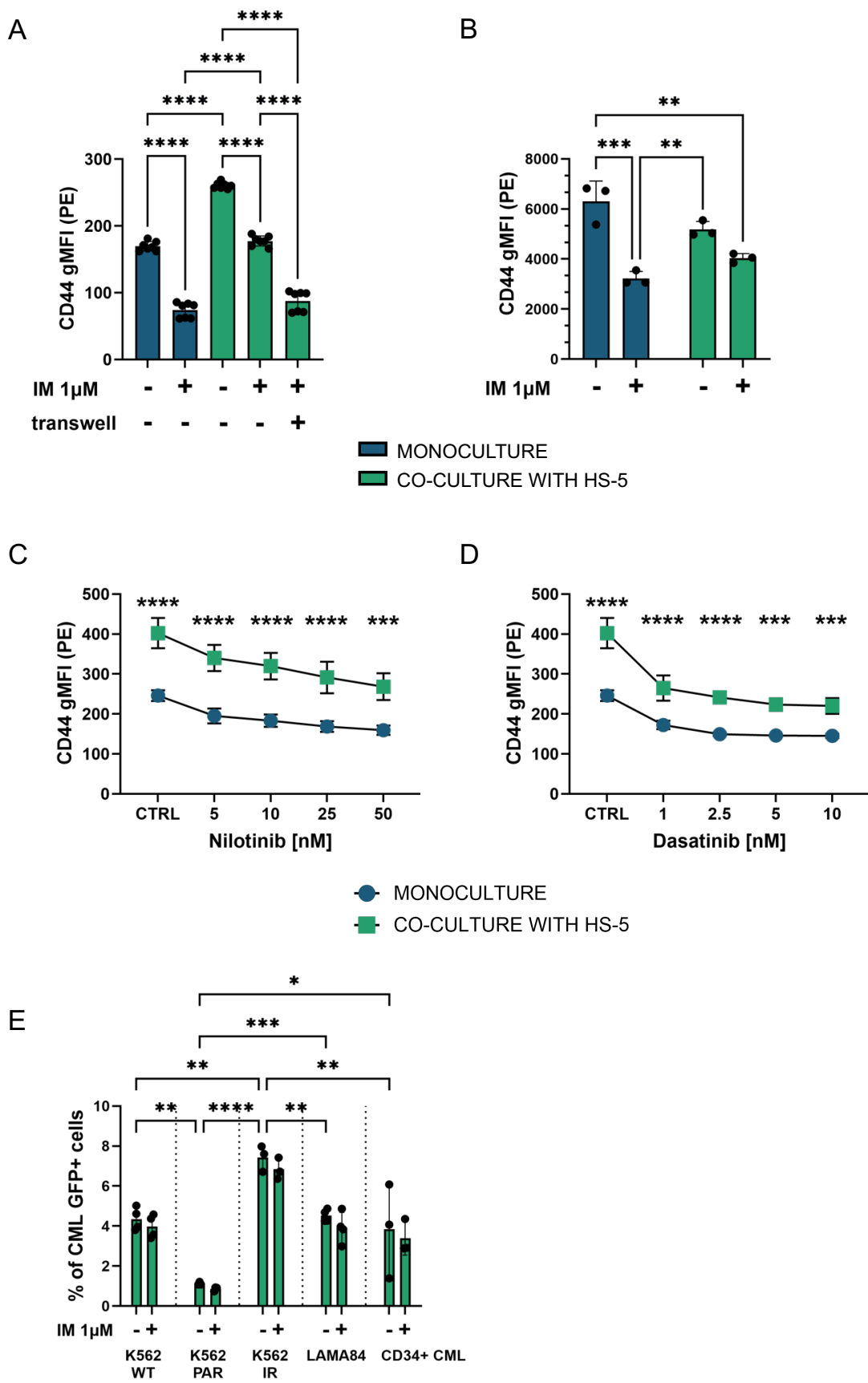


Figure 4.2.21 CD44 protein levels in leukemia cells after treatment with imatinib. **(A)** CD44 protein levels in viable K562 cells after 48 hours of monoculture and co-culture with HS-5 stromal cells, untreated or treated with 1 μ M imatinib. Leukemia and stromal cells were co-cultured directly or indirectly in the Transwell system (separated with inserts). Data expressed as geometric mean fluorescence intensity – gMFI (n=7). **(B)** CD44 levels (gMFI) in viable LAMA-84 cells after 48 hours of monoculture and co-culture with HS-5 stromal cells, untreated or treated with 0.5 μ M imatinib (n=3). **(C-D)** CD44 levels (gMFI) in viable K562 cells after 48 hours of monoculture or co-culture with HS-5 stromal cells, untreated (CTRL) or treated with increasing doses of nilotinib (left) and dasatinib (right) (n=3). **(E)** Uptake of stromal CD44-GFP by K562 WT, K562 PAR (Parental) and K562 IR (imatinib-resistant), LAMA-84 and CD34⁺ CML cells after 24 hours of co-culture with HS-5 CD44-GFP, untreated or treated with 1 μ M imatinib (n=3). Data expressed as the mean $\pm$ SD, one-way ANOVA with Tukey's posttest (A, B) and unpaired Student's t-test (C, D) and 2-way ANOVA (E) were used to calculate statistical significance.

Importantly, a similar protective effect was observed in cultures treated with second-generation TKIs, nilotinib and dasatinib (**Figure 4.2.21 C-D**). To investigate whether transfer of stromal CD44-GFP is still observed after inhibition of BCR-ABL1, co-cultures of HS-5 CD44-GFP and CML cells were treated with 1 μ M imatinib for 24 hours, followed by flow cytometric analysis of CD44-GFP uptake. In the presence of imatinib, CD44-GFP uptake remained at a level comparable to untreated co-cultures in all tested leukemia cell lines (both imatinib-sensitive K562 WT, K562 PAR and LAMA-84 cells, as well as imatinib-resistant K562 IR cells) and primary CD34⁺ CML cells, indicating that CD44 transfer from stroma is not dependent on BCR-ABL1 activity (**Figure 4.2.21 E**).

To exclude the possibility that the observed transfer and upregulation of CD44 in leukemia cells is driven by general, non-specific physical contact with neighboring cells and/or the general stress response caused by co-culture conditions, another stromal cell line, HS-27A, was applied. Unlike HS-5 cells, HS-27A cells do not protect CML cells from imatinib-induced apoptosis, as shown in **Figure 4.2.22 A**. Additionally, these cells transferred significantly less cellular vesicles to leukemia cells (**Figure 4.2.22 B**).

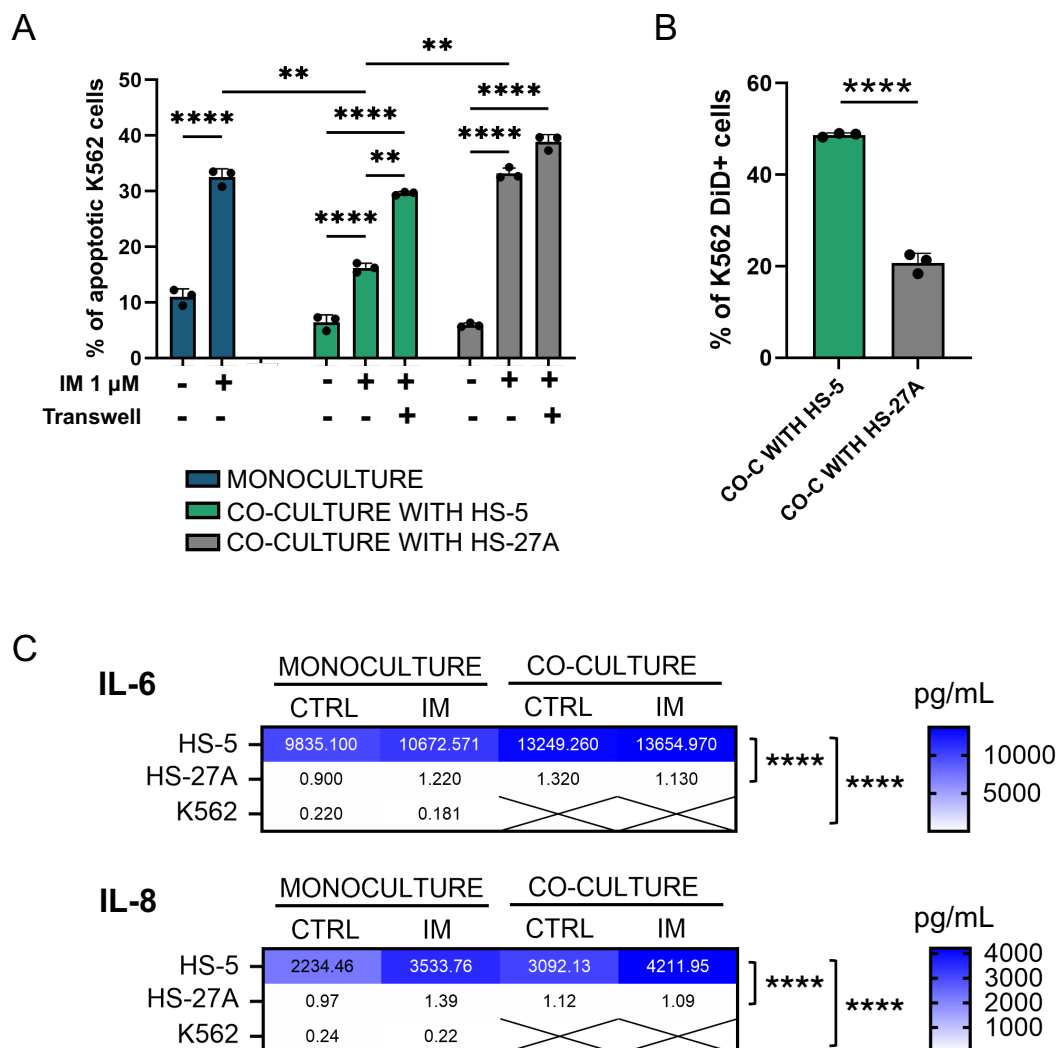


Figure 4.2.22 HS-27A cells as another model of bone marrow stroma. (A) Apoptosis levels measured in K562 cells after 48 h of monoculture or co-culture with stroma: HS-5 or HS-27A cells, untreated or in the presence of 1 μ M imatinib. Co-cultures were carried out either in direct contact or in a Transwell system (insert with 1 μ m pores). Graph bars represent the percentage of annexin V-positive K562 cells (n=3). (B) The percentage of K562 DiD⁺ cells (acceptors of stromal vesicles) after co-culture with DiD-labeled HS-5 or HS-27A stromal cells (n=3). (C) Secretion of IL-6 and IL-8 (in pg/mL) by monocultured leukemic and stromal cells or in co-cultures of leukemic and stromal cells, untreated or in the presence of 1 μ M imatinib (n=3). Data expressed as the mean $\pm$ SD, 2-way ANOVA with Tukey's posttest (A, C) and unpaired Student's t-test (B) were used to calculate statistical significance.

According to the literature, HS-27A cells secrete substantially less cytokines than HS-5²⁰⁹. LEGENDplex analysis of supernatants from leukemia and stroma monocultures, as well as co-cultures of stroma and leukemia confirmed these observations; both in control conditions (CTRL) and in the presence of 1 μ M imatinib, HS-27A cells produced minimal levels of IL-6 and IL-8 (**Figure 4.2.22 C**).

Remarkably, both stromal cell lines exhibited similar levels of CD44 (**Figure 4.2.23 A**); however, unlike HS-5 cells, HS-27A did not upregulate CD44 in leukemia cells (**Figure 4.2.23 B**). It was demonstrated (using Transwell and conditioned medium controls) that HS-5-mediated CD44 transfer and leukemic CD44 upregulation depend on direct cell-cell contact rather than soluble factors (**Figure 4.2.8, Figure 4.2.21**). However, given the numerous reports of a positive feedback loop between IL-6 and CD44 expression^{240,241}, exogenous recombinant IL-6 was added to the co-cultures of K562 and HS-27A cells to assess whether it would induce CD44 upregulation comparable to that observed in co-cultures with HS-5 stromal cells. Both lower (0.5 μ g/mL) and higher (1 μ g/mL) doses of IL-6 had no effect on CD44 levels in leukemia cells co-cultured with HS-27A, indicating that IL-6 secretion by HS-5 is not responsible for CD44 upregulation in leukemia cells (**Figure 4.2.23 C**). Interestingly, the lack of upregulation of CD44 and protection from imatinib-induced apoptosis observed in co-cultures with HS-27A were accompanied by reduced attachment of leukemia cells to the stroma, highlighting the importance of adhesion in these processes (**Figure 4.2.23 D**).

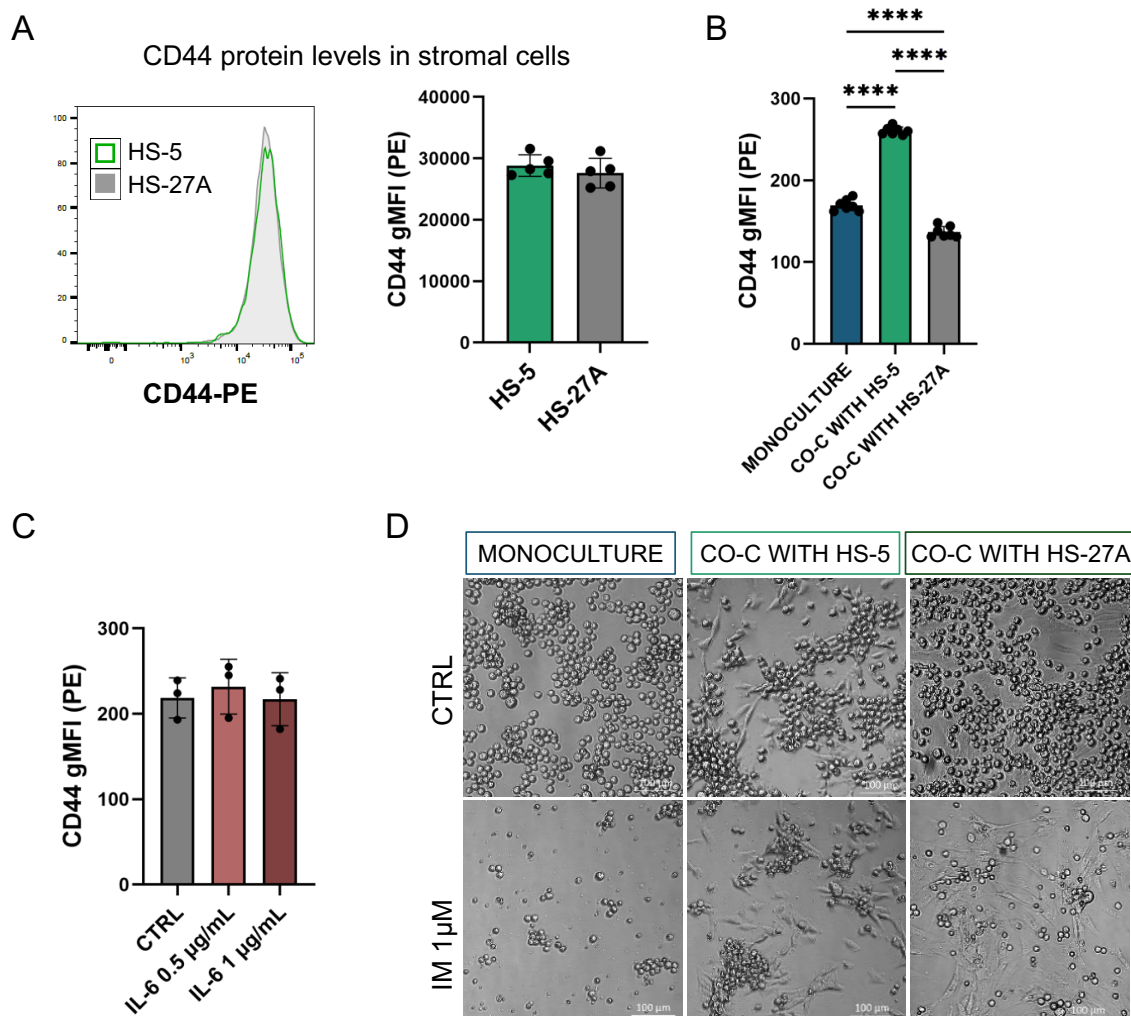


Figure 4.2.23 Co-culture with HS-27A cells did not result in CD44 upregulation in leukemia. **(A)** Left: Representative histograms from flow cytometry analyses presenting CD44 expression in HS-5 and HS-27A stromal cells. Right: CD44 levels (gMFI) in viable HS-5 and HS-27A cells (n=5). **(B)** CD44 protein levels in viable K562 cells after 48 hours of monoculture and co-culture with HS-5 or HS-27A stromal cells. Data expressed as geometric mean fluorescence intensity – gMFI (n=7). **(C)** CD44 protein levels in viable K562 cells after 24 hours of co-culture with HS-27A stromal cells, untreated (CTRL) or treated with low (0.5 µg/mL) and high (1 µg/mL) doses of IL-6. Data expressed as geometric mean fluorescence intensity – gMFI (n=3). **(D)** Representative bright-field images of K562 monocultures and K562 co-cultures with HS-5 or HS-27A stromal cells. Cultures were untreated (CTRL) or treated with 1 µM imatinib for 48 hours. Scale bars: 100 µm. Data expressed as the mean $\pm$ SD, unpaired Student's t-test (A) or One-way ANOVA with Tukey's posttest (B, C) and were used to calculate statistical significance.

4.2.4 Stroma-mediated CD44 upregulation is associated with ERK1/2-AP-1 and HIF-1 α signaling axes

To elucidate mechanisms underlying the transcriptional and phenotypic changes associated with CD44 uptake by leukemia cells, the first candidate transcription factors (TFs) for genes upregulated in CD44-GFP acceptors compared to non-acceptors were identified. For this purpose, the upregulated gene dataset was analyzed using ShinyGO combined with the TRRUST database. Among the predicted transcription factors, SP1, RELA, NFKB1, ETS1, HIF1A and JUN corresponded to the highest number of significantly induced genes and were selected for further downstream analyses (Figure 4.2.24).

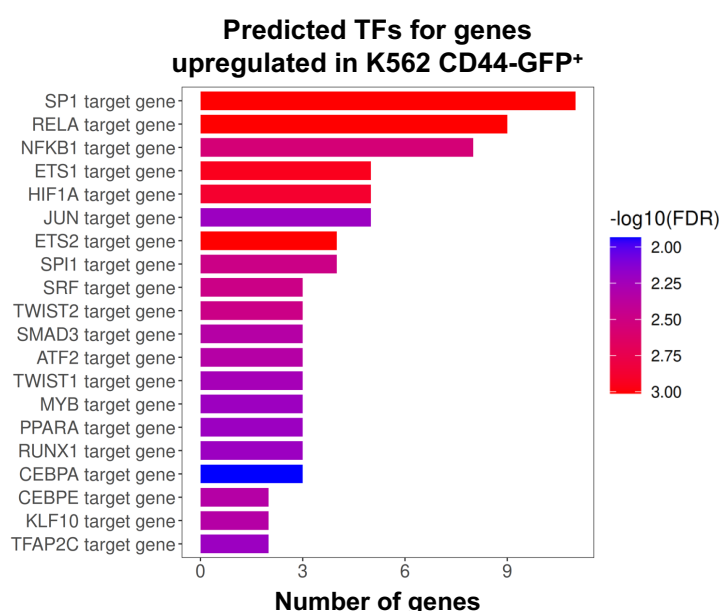


Figure 4.2.24 Transcription factor enrichment analysis of the upregulated genes in K562 CD44-GFP⁺ versus K562 CD44-GFP⁻ cells using ShinyGO (v. 0.77) and the TRRUST database. Bars indicate the number of genes regulated by the identified TF.

To assess the relevance of the selected TFs in the context of CML and our observations so far, we assessed their co-expression with the *CD44* gene using a publicly available RNA-seq dataset (GSE144119) derived from CML patients' samples. *CD44* was selected as a representative gene of interest from the initial pool of genes upregulated in CD44-GFP⁺ cells. Spearman correlation analysis showed significant positive correlations between *CD44* and *JUN*, *RELA*, *NFKB1*, *NFKB2* and *HIF1A*, but not *SP1* and *ETS*. Significant positive correlation with *CD44* was also

shown between other members of the AP-1 complex, like *JUND* and *FOS* (Figure 4.2.25). TFs with positively correlated expression were selected for further investigation.

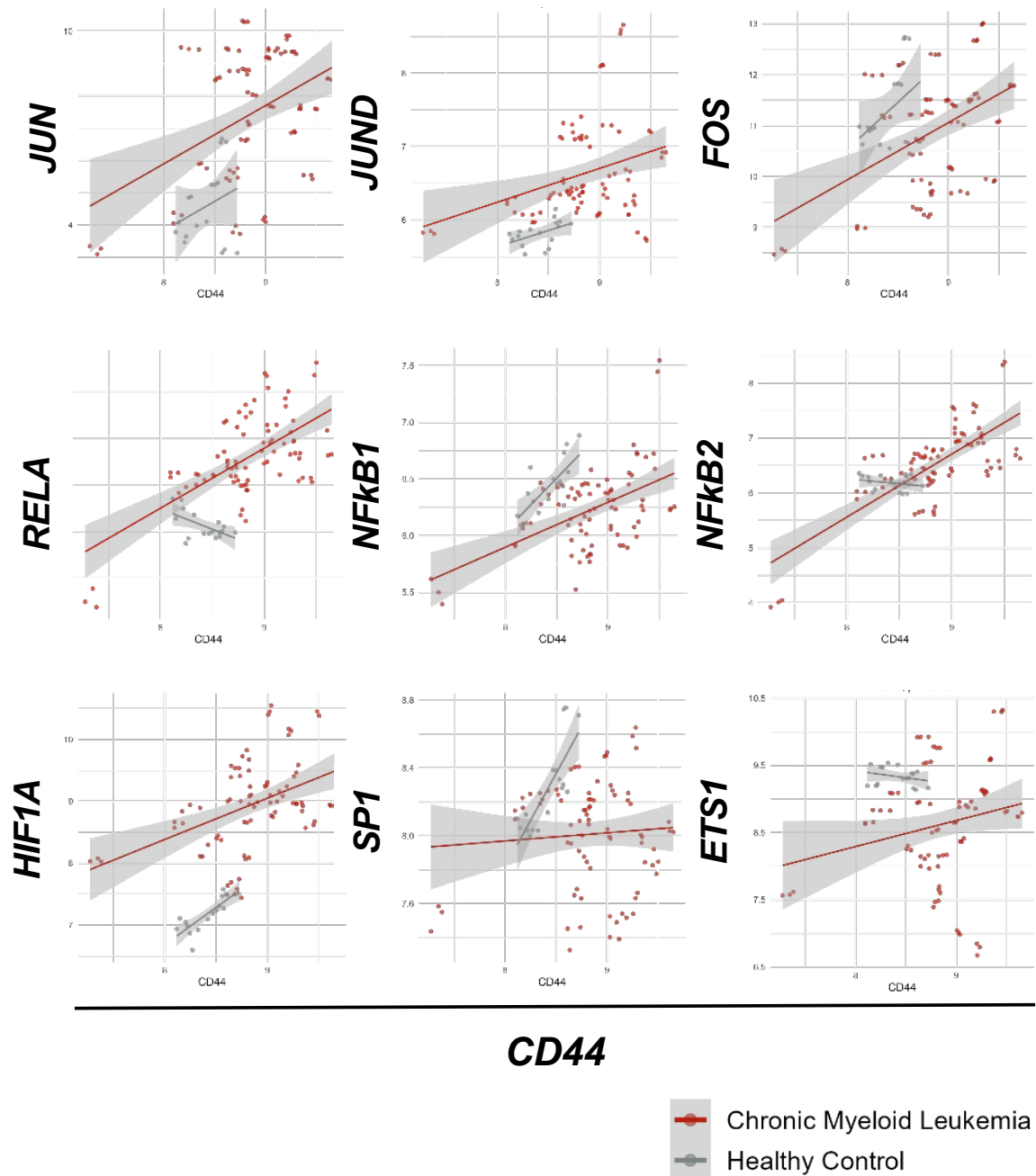


Figure 4.2.25 Spearman correlation analysis between *CD44* expression and selected TFs, based on data obtained from the RNA-seq dataset (GSE144119) derived from CML patients' samples. Spearman's rank correlation analysis was used to estimate the correlation coefficient. A significant positive correlation was observed for *CD44* and: *JUN* ($\rho=0.463$, $p=2.43 \times 10^{-6}$), *JUND* ($\rho=0.503$, $p=2.32 \times 10^{-7}$), *FOS* ($\rho=0.247$; $p=0.015$), *RELA* ($\rho=0.627$, $p<2 \times 10^{-16}$), *NFKB1* ($\rho=0.195$, $p=0.0559$), *NFKB2* ($\rho=0.663$, $p<2 \times 10^{-16}$), *HIF1A* ($\rho=0.612$; $p<2 \times 10^{-16}$), but not *SP1* ($\rho=-0.167$, $p=0.102$) and *ETS1* ($\rho=-0.051$, $p=0.618$).

To validate functional activity of selected TFs in K562 CD44⁺ cells, phospho-flow cytometry was performed for c-Jun, c-Fos, and RelA, as representative TFs of the AP-1 and canonical NF- κ B signaling pathways, respectively. HIF-1 α was not included in the phospho-flow analyses, as its activity is primarily regulated through protein stabilization rather than phosphorylation. After co-culture with HS-5 cells, phospho-c-Jun (Ser73) and phospho-c-Fos (Ser32) were upregulated in K562 CD44⁺ cells when compared to K562 CD44⁻ cells (**Figure 4.2.26 B-C**), whereas P-RelA levels were comparable between both populations (**Figure 4.2.26 A**).

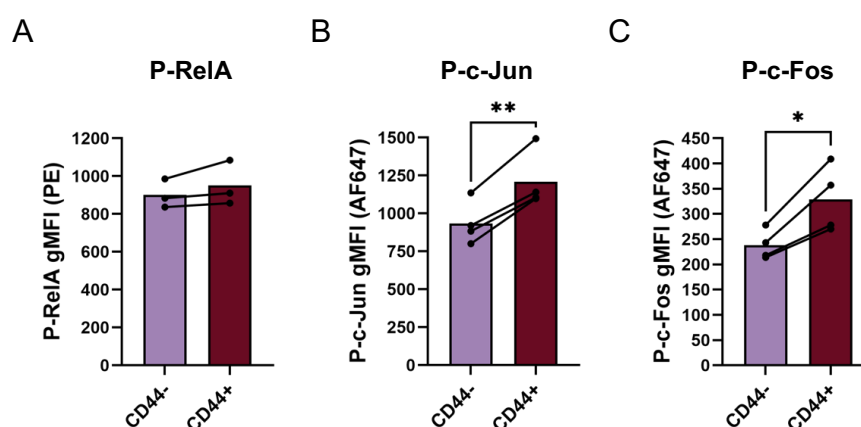


Figure 4.2.26 Phospho-flow analysis. (A-C) Bar plots showing gMFI of phosphorylated RelA (NF- κ B), c-Jun and c-Fos measured by flow cytometry in K562 CD44⁺ and K562 CD44⁻ cells, after co-culture with HS-5 stromal cells. Data shown as the mean of each group. Lines connect paired measurements, paired Student's t-test was used to calculate statistical significance.

To complement these data and to assess whether upstream signaling pathways for c-Jun, c-Fos and RelA depend on the presence of stroma and stroma-mediated CD44 transfer, the levels of P-ERK1/2, P-JNK, HIF-1 α , and I κ B α were compared by Western blot in K562 cells grown in monoculture, co-culture with HS-5, or non-protective HS-27A stromal cells, both under control conditions and after imatinib treatment. While co-culture with HS-5 cells strongly increased the phosphorylation of ERK1/2 (Thr202/Tyr204), which was not reduced by imatinib, phosphorylation in monoculture was markedly decreased. Conversely, co-culture with non-protective HS-27A stromal cells, which do not increase CD44 expression in leukemia cells, did not increase the phosphorylation of ERK1/2 and did not protect against the imatinib-mediated decrease in ERK1/2 phosphorylation (**Figure 4.2.27 A-B**). Interestingly, HIF-1 α levels were also increased in K562 cells

after co-culture with HS-5 but not HS-27A stromal cells and further increased in response to imatinib, confirming the activation of the adaptive protective response (**Figure 4.2.27 A,C**). The level of I κ B α remained unchanged regardless of the culture conditions (**Figure 4.2.27 A**). Additionally, no alterations in P-JNK (Thr183/Tyr185) levels were detected, suggesting that AP-1 activation is associated with an ERK-dependent mechanism rather than with JNK-mediated JUN phosphorylation (**Figure 4.2.27 A**). Taken together, the observed increase in c-Jun and c-Fos phosphorylation in K562 CD44⁺ leukemia cells, together with the analysis of signaling pathways, suggests that the activation of the mitogenic and pro-survival ERK/c-Jun/c-Fos signaling axes together with the HIF-1 α adaptative pathway may contribute to transcriptional regulation in CD44-GFP⁺ acceptor leukemia cells.

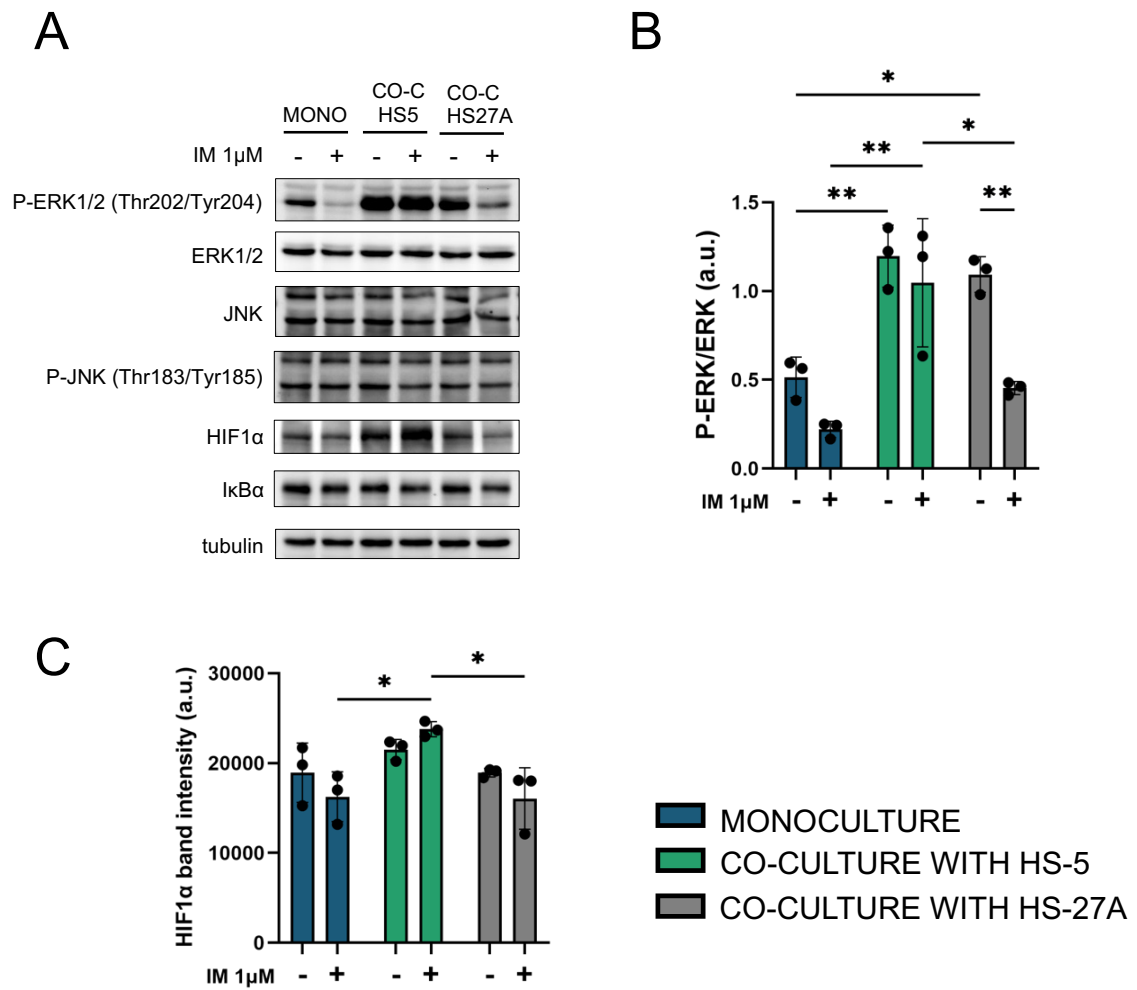


Figure 4.2.27 Western blot analysis of upstream signaling pathways upon co-culture with protective and non-protective stromal cells. (A) Representative immunoblots showing the expression of P-ERK1/2 (Thr202/Tyr204), ERK1/2, P-JNK (Thr183/Tyr185), JNK, HIF-1 α and I κ B α in K562 cells grown in monoculture and co-culture with HS-5 or HS-27A stromal cells, with or without 1 μ M imatinib for 24 hours. Tubulin was used as a loading control. (B-C) Densitometric quantification of phosphorylated ERK1/2 relative to total ERK1/2 levels (B) and HIF-1 α levels (C) are shown (n=3). Data shown as the mean $\pm$ SD, 2-way ANOVA (B,C) was performed.

4.2.5 Stroma-mediated upregulation of CD44 in leukemia cells increases their invasive and migratory properties

The results of RNA-seq suggested that the stroma-driven CD44 transfer and increase in leukemic CD44 expression are associated with the upregulation of genes promoting the invasive and migratory properties of acceptor cells. Metalloproteinases such as *MMP2*, *MMP3*, and *MMP9* play key roles in the extracellular matrix remodeling. qPCR analysis confirmed elevated mRNA levels of these MMPs in K562 cells which received CD44-GFP from stromal cells (Figure 4.2.28 A). An even greater increase was observed in the total K562 CD44⁺ population (compared to CD44⁻) sorted from the co-culture with HS-5 cells (Figure 4.2.28 B).

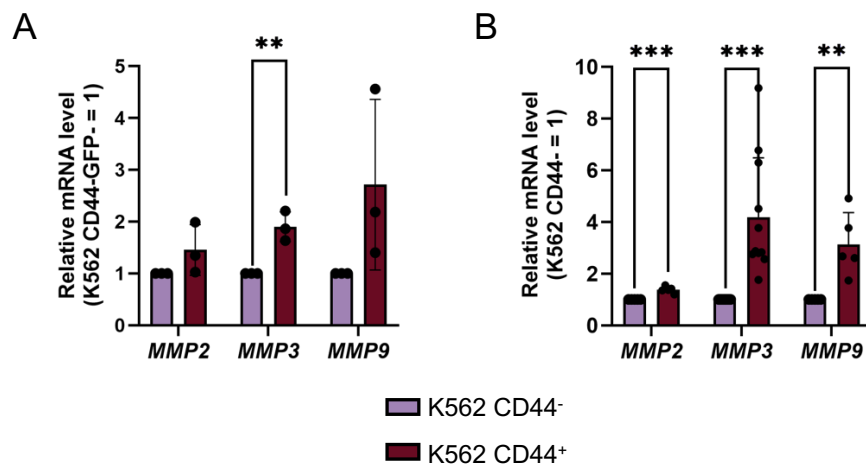


Figure 4.2.28 MMPs mRNA levels in K562 cells. (A) Relative mRNA expression of *MMP2*, *MMP3* and *MMP9* in K562 CD44-GFP⁺ and CD44-GFP⁻ cells sorted from co-culture with HS-5 CD44-GFP cells. Expression was normalized to CD44-GFP⁻ (set as 1), with actin as the endogenous control (n=3). (B) Relative mRNA expression of *MMP2*, *MMP3* and *MMP9* in K562 CD44⁺ and CD44⁻ cells sorted from co-culture with HS-5 cells. Expression was normalized to CD44⁻ (set as 1), with actin as the endogenous control (n=4). Data expressed as the mean $\pm$ SD. Unpaired Student's t-test.

Next, to evaluate the functional impact of stroma-driven CD44 upregulation in leukemia, co-cultures of K562 and HS-5 cells were established, followed by CD44 staining and sorting of K562 CD44⁺ and CD44⁻ cells. *In vitro* invasion and migration assays using insert-based systems with an FBS gradient (Figure 4.2.29 A) revealed that K562 CD44⁺ cells exhibited significantly greater invasive and migratory potential than cells with non-detectable CD44 (Figure 4.2.29 B-C). Importantly, the increased mRNA levels of *MMP2* and *MMP9* in K562 CD44⁺ cells translated into increased

gelatinolytic activity of MMP2 and MMP9, as shown by gelatin zymography of conditioned media obtained from sorted K562 CD44⁺ and CD44⁻ cells (**Figure 4.2.29 D**). Elevated levels of soluble CD44 (sCD44) have been shown to promote invasiveness by weakening cell-ECM adhesion, thereby facilitating cell detachment, migration and metastasis^{128,242,243}. LEGENDplex analysis of conditioned media obtained from sorted K562 and LAMA-84 cells, which received or did not receive stromal CD44 (CD44-GFP⁺ and CD44-GFP⁻, respectively), demonstrated increased levels of the soluble form of CD44 produced by acceptors of stromal CD44 (**Figure 4.2.29 E**). Interestingly, increased IL-8 was observed in acceptors of stromal CD44, and based on previous literature reports^{244,245}, it was considered a potential contributor to the increased invasiveness of these cells (**Figure 4.2.29 F**). IL-8 can influence cancer cells' invasiveness *via* activation of several pro-survival pathways, including PI3K/Akt/mTOR²⁴⁶, Ras/Raf/MAPK (ERK1/2)²⁴⁷, and one of the key pro-invasive pathways, JAK/STAT3^{246,248,249}. As demonstrated in the previous figure, ERK1/2 activation in leukemia cells was observed following co-culture with protective HS-5 cells, but not with non-protective HS-27A (**Figure 4.2.27 A-B**). The phosphorylation status of STAT3, a major regulator of invasiveness, was also assessed and found to be elevated in co-cultures with protective HS-5 stromal cells relative to monoculture conditions and co-culture with the non-protective HS-27A cells (**Figure 4.2.29 G**).

Overall, these results indicate that stroma-driven increase in CD44 level in CML cells is associated with their increased invasiveness, accompanied by enhanced CD44 shedding, MMPs expression and activity, as well as activation of pro-invasive pathways.

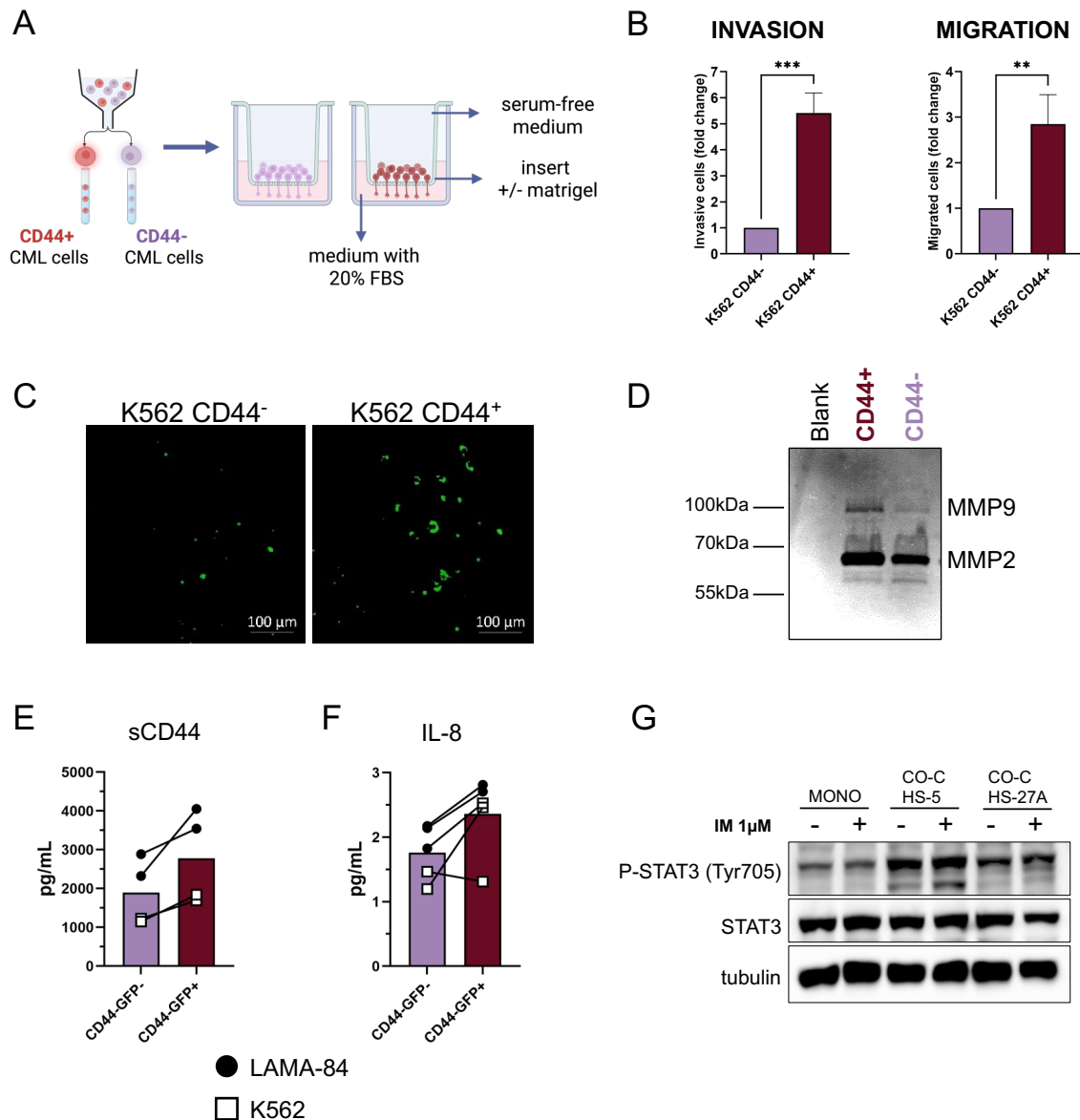
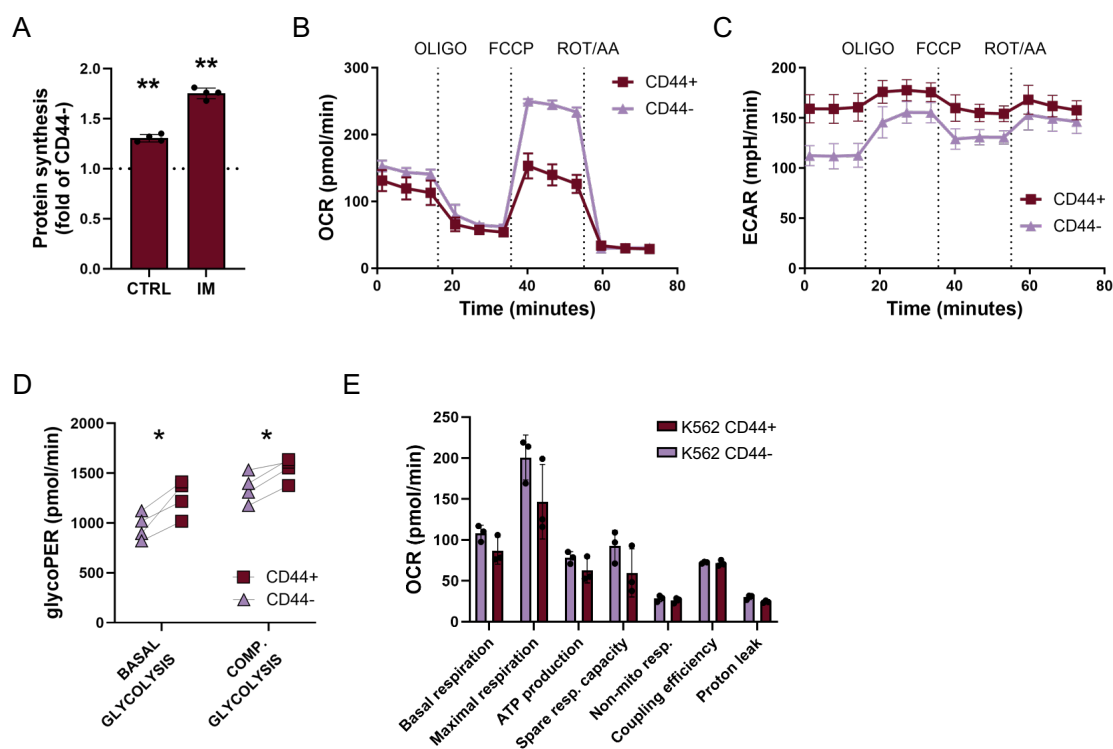


Figure 4.2.29 CD44 upregulation is associated with increased invasiveness of CML cells. (A) Experimental scheme for *in vitro* migration and invasion assays. Created with BioRender.com. (B) Number of invaded (left) and migratory (right) cells per one field, normalized to K562 CD44⁻ cells (n=3). (C) Representative images of invaded K562 CD44⁻ and CD44⁺ cells from light microscopy. (D) Zymogram showing MMP2 and MMP9 activity in conditioned media obtained from sorted and cultured K562 CD44⁺ and CD44⁻ cells. (E-F) The levels of soluble CD44 (sCD44) and IL-8 in the conditioned media obtained from sorted acceptors (CD44-GFP⁺) and non-acceptors (CD44-GFP⁻) of stromal CD44, expressed in pg/mL (n=4). (G) Representative immunoblots showing the expression of P-STAT3 (Tyr705) and STAT3 in K562 cells grown in mono- and co-culture with HS-5 or HS-27A stromal cells, with or without 1 μ M imatinib for 24 hours. Tubulin was used as a loading control. Data expressed as the mean $\pm$ SD. Unpaired Student's t-test (B,D-F).

4.2.6 Stroma-mediated CD44 upregulation results in metabolic changes in leukemia cells

The pro-invasive and pro-migratory phenotype often correlates with metabolic alterations in cancer cells. Click chemistry-based translation assay performed on K562 cells co-cultured with HS-5 stromal cells, showed that K562 CD44⁺ cells display higher protein synthesis rates compared with K562 CD44⁻ cells, particularly upon treatment with imatinib (**Figure 4.2.30 A**). Since increased translation levels are often associated with metabolic activity of the cells^{250,251}, Seahorse analysis was performed to compare metabolic profiles of leukemia CD44⁺ and CD44⁻ cells. After co-culture with HS-5 cells, CD44 protein was stained with fluorochrome-conjugated antibody followed by sorting of K562 CD44⁺ and K562 CD44⁻ cells using FACS.



After overnight rest following sorting, cells were analyzed using the Seahorse Mito Stress Test. The results revealed that K562 CD44⁺ cells displayed lower OCR (Oxygen Consumption Rate) and higher ECAR (Extracellular Acidification Rate) compared with CD44⁻ cells, indicating a shift toward a more glycolytic phenotype (**Figure 4.2.30 B-C**). Consistently, glycoPER (Glycolytic Proton Efflux Rate) calculations further showed increased basal and compensatory glycolysis in CD44⁺ cells relative to CD44⁻ cells (**Figure 4.2.30 D**). Although K562 CD44⁻ cells exhibited higher ATP production, basal and maximal respiration or spare respiratory capacity, these differences did not reach statistical significance (**Figure 4.2.30 E**).

Interestingly, metabolic shift towards glycolysis in K562 CD44⁺ cells was not linked to increased glucose uptake by these cells. Although GLUT1, the main glucose transporter in cancer cells, showed slightly elevated mRNA expression in leukemia cells with stroma-induced CD44 upregulation (**Figure 4.2.31 A**), no corresponding difference was observed at the protein level (**Figure 4.2.31 B**). An interesting observation was the substantial increase in GLUT1 protein levels following treatment with imatinib, in both cell subpopulations (**Figure 4.2.31 B**). Additionally, K562 CD44⁺ cells did not exhibit increased glucose uptake, as assessed by uptake of fluorescent D-glucose analog – 2-NBDG. Likewise, no global effect of stroma on glucose uptake in the total leukemia cell population was observed (**Figure 4.2.31 C**).

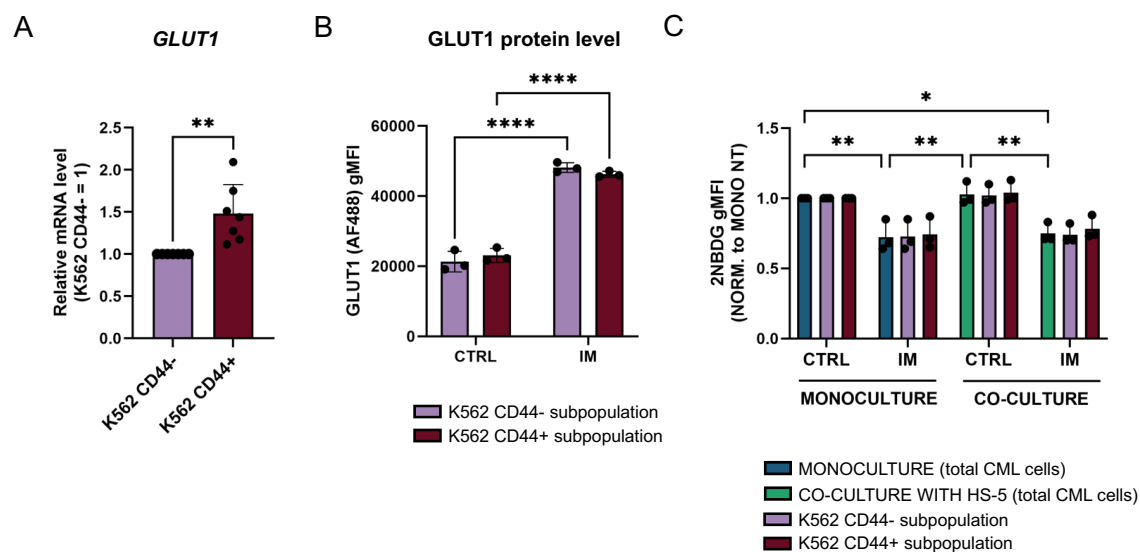


Figure 4.2.31 Glucose uptake by K562 cells. (A) Relative mRNA expression of *GLUT1* in K562 CD44⁺ and CD44⁻ cells sorted from co-culture with HS-5 cells. Expression was normalized to K562 CD44⁻ cells (set as 1), with actin as the endogenous control (n=7). (B) GLUT1 protein levels in viable K562 CD44⁺ and CD44⁻ cells after 48 hours of co-culture with HS-5 stromal cells, untreated (CTRL) or treated with 1 μ M imatinib (IM). Data expressed as geometric mean fluorescence intensity – gMFI (n=3). (C) 2-NBDG uptake in K562 cells monocultured or co-cultured with HS-5 cells, untreated (CTRL) or in the presence of 1 μ M imatinib (IM). Data are presented as gMFI values normalized to untreated monoculture (MONOCULTURE CTRL = 1) (n=3). Data expressed as the mean $\pm$ SD. Unpaired Student's t-test (A) and 2-way ANOVA with Tukey's posttest were used (B, C).

Collectively, these results suggest that upregulation of CD44 in leukemia cells leads to a more glycolytic phenotype accompanied by increased translational activity, as reflected by elevated protein production. These metabolic alterations, together with enhanced translational activity, may reflect adaptation to high energy and anabolic demands.

4.2.7 Stroma-driven CD44 upregulation rescues the ferroptosis-associated phenotype of CD44-deficient leukemia cells

An intriguing, recently discovered property of the CD44 protein is its ability to confer resistance to ferroptosis in malignant cells^{252–254}. So far, its described role has mainly been associated with enhancing SLC7A11 (xCT) stability, thereby promoting oxidative stress resistance in cancer cells^{252,255}.

To investigate whether CD44 modulates ferroptosis susceptibility in CML cells in the context of stromal support and imatinib sensitivity, a stepwise assessment of ferroptosis regulation was performed. For these studies, CD44 was silenced in LAMA-84 cells (**Figure 4.2.32 A**), characterized by high endogenous CD44 expression (**Figure 4.2.8 B**). First, basal lipid peroxidation was measured using BODIPY 581/591 C11, as an early indicator of altered ferroptosis sensitivity. Next, the expression of the key anti-ferroptotic regulator GPX4 was analyzed in CML cells. Finally, functional sensitivity to ferroptosis was evaluated upon RSL3-induced GPX4 inhibition, using lipid peroxidation and cell viability assays in the presence or absence of the ferroptosis inhibitor ferrostatin-1.

To monitor lipid peroxidation in LAMA-84 cells, the fluorescent probe BODIPY 581/591 C11 was used. This lipid-sensitive probe undergoes a shift in fluorescence emission upon lipid oxidation (from red to green) and therefore enables the detection of oxidized membrane lipids. Staining of LAMA-84 shNEG and shCD44 cells grown in monocultures and co-cultures with HS-5 cells revealed increased lipid peroxidation in LAMA-84 shCD44 cells compared to LAMA-84 shNEG cells in the presence of imatinib (**Figure 4.2.32 B-C**). Notably, this was associated with increased sensitivity of LAMA-84 cells with CD44 knockdown (LAMA-84 shCD44) to imatinib-induced apoptosis (**Figure 4.2.32 D**). What is important, co-culture with HS-5 stromal cells reduced both lipid peroxidation and apoptosis levels in imatinib-treated cells, in LAMA-84 shNEG and LAMA-84 shCD44 cells (**Figure 4.2.32 B-D**).

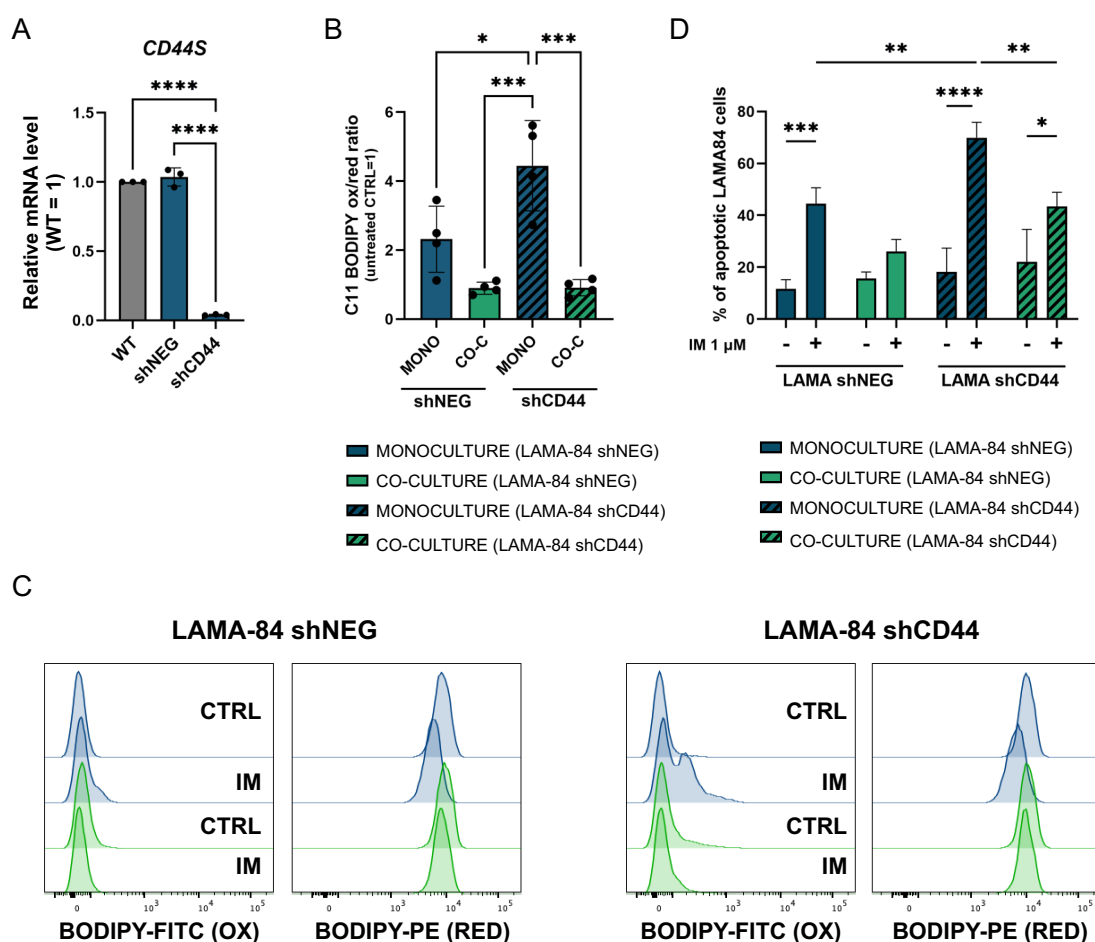


Figure 4.2.32 Increased vulnerability of LAMA-84 shCD44 cells to imatinib and lipid peroxidation. **(A)** RT-qPCR analysis of mRNA *CD44S* levels in the LAMA-84 cells. WT – untreated cells, shNEG – cells transduced with non-targeting shRNA, shCD44 – cells transduced with shRNA targeting CD44 (clone 5). Actin was used as endogenous control (n=3). **(B)** Lipid peroxidation measured using BODIPY C11 probe and flow cytometry. Oxidation index was calculated as the FITC/PE (oxidized/reduced) gMFI ratio and normalized to untreated controls within each group (n=4). **(C)** Representative flow cytometry histograms of BODIPY C11 staining in the FITC (oxidized) and PE (reduced) channels, shown for untreated and treated with 0.5 μ M imatinib LAMA-84 cells (left: shNEG, right: shCD44), cultured in monoculture (blue) or in co-culture (green) with HS-5 stromal cells for 24 h. **(D)** Apoptosis levels measured in LAMA-84 cells after 48 h of monoculture or co-culture with HS-5 cells, untreated or in the presence of 0.5 μ M imatinib. Graph bars represent the percentage of annexin V-positive LAMA-84 cells (n=3). Data expressed as the mean $\pm$ SD. Unpaired paired Student's t-test (A) or 2-way ANOVA with Tukey's posttest were used (B,D).

GPX4 protein is considered one of the central regulators preventing ferroptotic cell death²¹⁶. Notably, LAMA-84 cells with CD44 knockdown (shCD44) showed significantly reduced GPX4 mRNA and protein levels compared to LAMA-84 shNEG cells (**Figure 4.2.33 A-B**). This decrease was partially restored upon co-culture with HS-5 stromal cells, resulting in increased GPX4 expression in LAMA-84 shCD44 cells. However, this effect was attenuated when CD44 was also knocked down in stromal cells, suggesting that stromal CD44 contributes to the maintenance of GPX4 protein

levels in leukemia cells (**Figure 4.2.33 B**). Next, RSL3, a well-established GPX4 inhibitor used to induce ferroptosis, triggered a stronger increase in lipid peroxidation in LAMA-84 shCD44 cells compared with LAMA-84 shNEG cells, indicating increased sensitivity to ferroptosis of cells with CD44 knockdown. Importantly, the RSL3-induced effect was fully reversed by co-treatment with ferrostatin-1 (Fer1), a ferroptosis inhibitor (**Figure 4.2.33 C**).

Furthermore, treatment with ferrostatin-1 partially rescued LAMA-84 shCD44 cells viability in the presence of imatinib. This suggests that ferroptosis-related mechanisms may partially contribute to imatinib-induced cell death in CD44-deficient leukemia cells (**Figure 4.2.33 D**).

These findings suggest that CD44 protein and stroma-mediated CD44 upregulation may be involved in protection of leukemia cells from lipid peroxidation-associated stress. Loss of CD44 sensitizes cells to oxidative lipid damage and increases vulnerability to imatinib, while HS-5 stromal cells partially rescue this phenotype through the upregulation of GPX4.

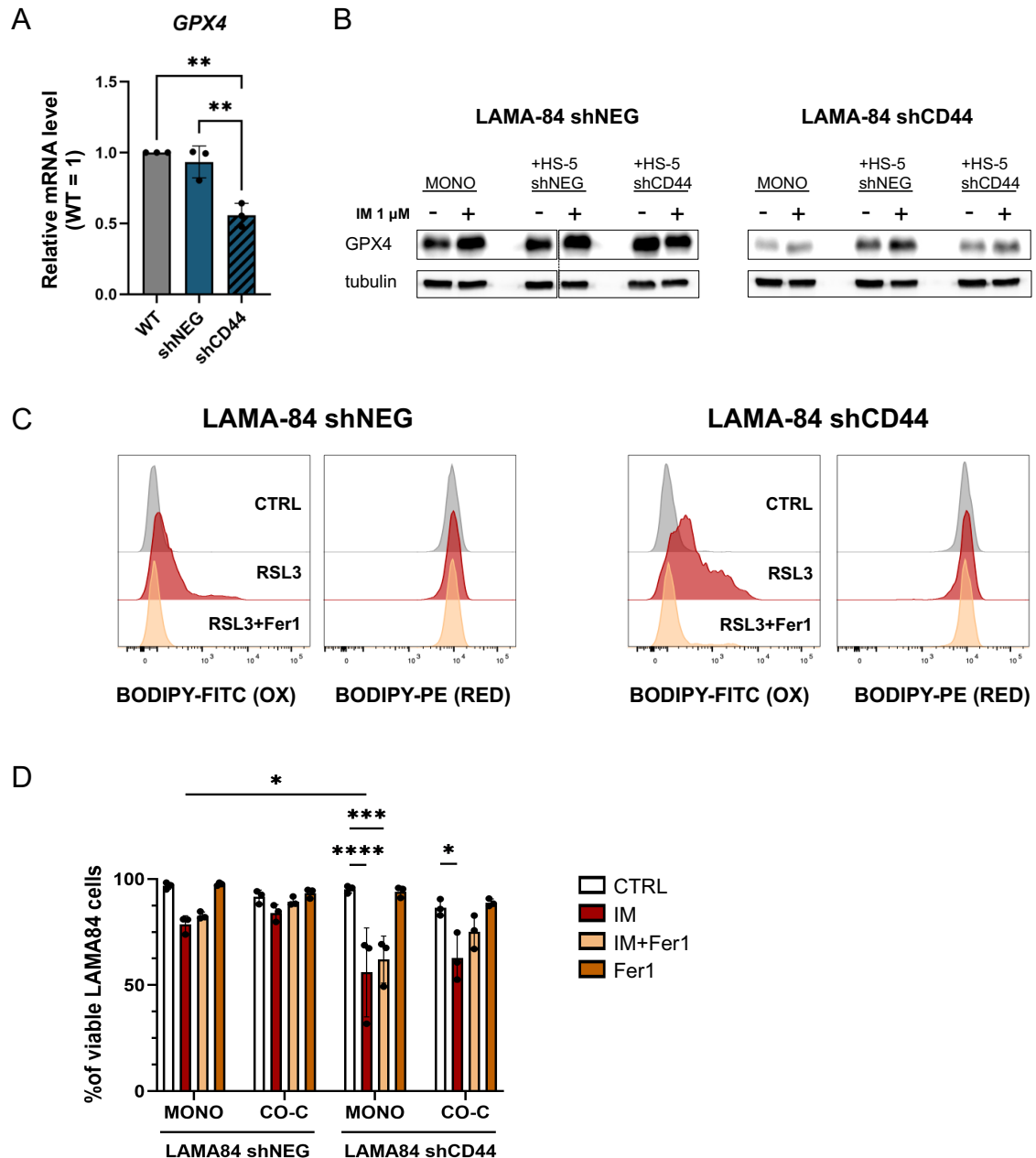


Figure 4.2.33 Reduced GPX4 expression in CD44-knockdown cells is partially restored by stromal cells. (A) Relative mRNA expression of *GPX4* in LAMA-84 (WT, shNEG and shCD44). Expression was normalized to levels in LAMA-84 WT (set as 1), with actin as the endogenous control (n=3). (B) Representative immunoblots of GPX4 protein expression in LAMA-84 shNEG (left panel) and LAMA-84 shCD44 cells (right panel), grown in monoculture or co-culture with HS-5 stromal cells (HS-5 shNEG or shCD44), untreated or treated with 0.5 μ M imatinib for 24 hours. (C) Representative flow cytometry histograms of BODIPY C11 staining in the FITC (oxidized) and PE (reduced) channels, shown for LAMA-84 shNEG (left) and shCD44 (right) cells grown in monocultures, untreated (CTRL) and treated with 100 nM RSL3, or 100 nM RSL3 with 1 μ M ferrostatin-1 (Fer1). (D) Cell viability analysis of LAMA-84 (shNEG and shCD44) cultured alone (MONO) or with HS-5 stromal cells (CO-C), treated with 0.5 μ M imatinib (IM) and/or ferrostatin-1 (Fer1). Bars represent percentage of viable cells (LIVE/DEAD Ner-IR-negative). Data expressed as the mean $\pm$ SD. Unpaired paired Student's t-test (A) or 2-way ANOVA with Tukey's posttest were used (D).

4.2.8 Targeting CD44-dependent downstream signaling enhances the anti-leukemia effect of imatinib under BMM conditions

The above findings indicate that the stroma-mediated protection of leukemia cells from imatinib is linked to the transfer of cellular vesicles and stromal CD44 to leukemia cells. The interaction between CD44 and its main ligand, hyaluronic acid initiates downstream signaling cascades, including ERK/AP-1, which regulate cell survival, proliferation, and other cell functions, such as invasion and migration^{256,257}. To obtain direct evidence that CD44-driven signaling mediates resistance to imatinib, the interaction between CD44 and HA was pharmacologically attenuated by CD44-HA inhibitor 2 (further referred to as CD44-HAi)¹⁸⁰.

Fluorescently tracked K562 cells were grown in monocultures or co-cultures with HS-5 cells and treated with imatinib, CD44-HAi, or both drugs for 48 hours, after which apoptosis levels were assessed (**Figure 4.2.34 A**). Although CD44-HAi alone had no cytotoxic effect, its combination with imatinib increased apoptotic cell death compared with imatinib alone, even when co-cultured with HS-5 cells, indicating the attenuation of stroma-driven resistance (**Figure 4.2.34 A**). Consistently, CD44-HAi alone did not affect colony formation by K562 or primary CD34⁺ CML cells; however, its combination with imatinib significantly enhanced the inhibitory effect of imatinib alone (**Figure 4.2.34 B-C**).

Overall, these findings suggest the potential for targeting the CD44-HA interaction to enhance TKI efficacy in CML and overcome stroma-mediated protection.

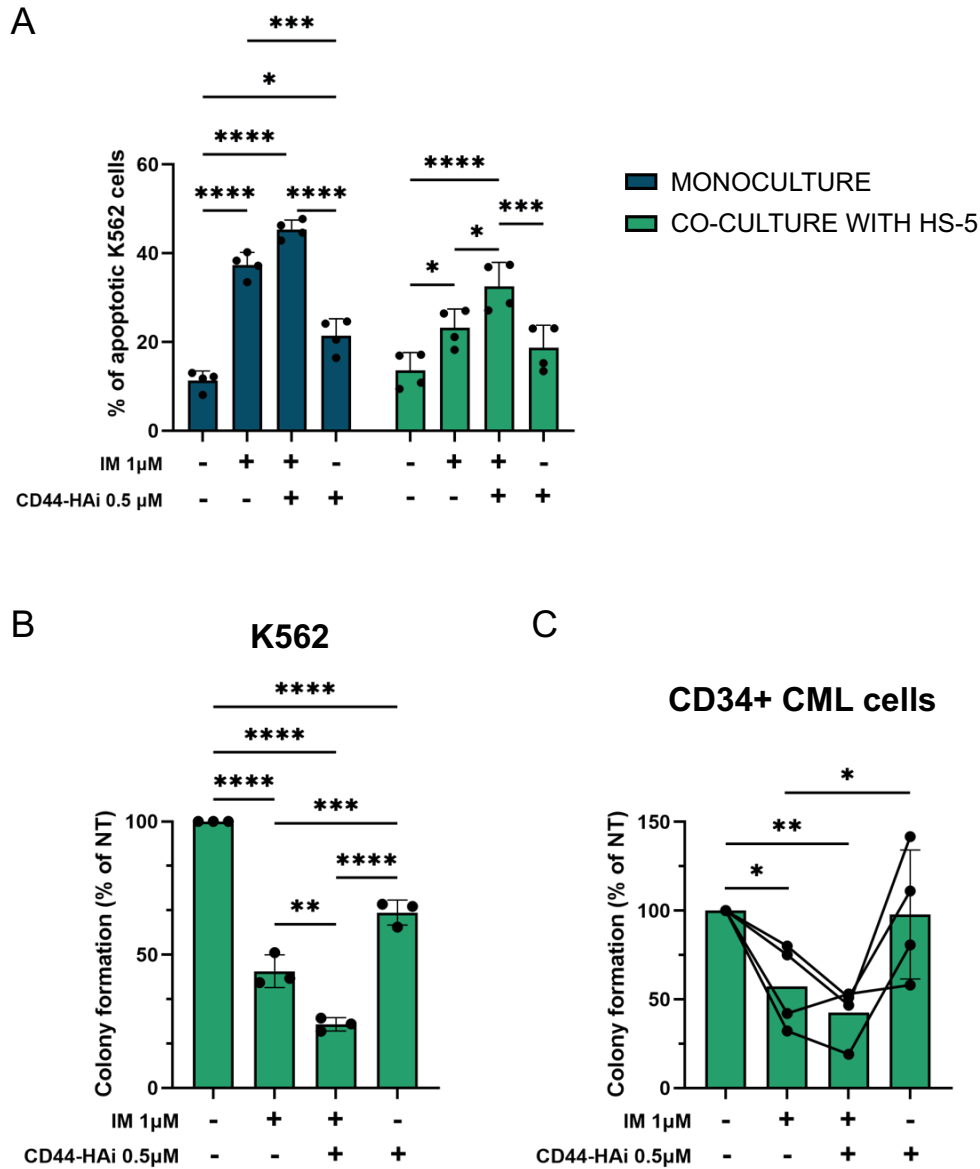


Figure 4.2.34 The effects of targeting CD44-dependent downstream signaling on the sensitivity to imatinib in leukemia. (A) Apoptosis level measured in K562 cells after 48 hours of culture in mono- and co-culture with stromal HS-5 cells, untreated or treated with 1 µM imatinib, 0.5 µM CD44-HAi, or both. Graph bars represent the percentage of annexin V-positive K562 cells (n=4). **(B)** Clonogenic assay showing survival and clonogenic capacity of K562 cells after co-culture with HS-5 in the presence of 1 µM imatinib, 0.5 µM CD44-HAi, or both. Data are expressed as the percentage of colonies relative to untreated controls (n=3). **(C)** Clonogenic assay showing survival and clonogenic capacity of CD34+ CML cells after co-culture with HS-5 cells in the presence of 1.5 µM imatinib, 0.5 µM CD44-HAi or both. Data are expressed as the percentage of colonies relative to untreated controls (n=4). Data expressed as the mean $\pm$ SD. 2-way ANOVA (A), One-way ANOVA with Tukey's posttest (B), paired Student's t-test (C) were used.

5 DISCUSSION

It is known that interactions within the bone marrow microenvironment, together with intrinsic oncogenic signaling, are essential for leukemia cell survival and therapeutic failure in chronic myeloid leukemia (CML). Tunneling nanotubes (TNTs) represent one of the mechanisms of direct cell-cell communication utilized by bone marrow stromal cells (BMSCs) and CML cells to transfer vesicles and proteins. However, despite extensive research on TNTs, substantial gaps in knowledge remain, particularly concerning their structure and proper distinction from other types of cellular protrusions. Moreover, the functional role of specific proteins transferred from BMSCs to CML cells *via* TNTs and direct cell-cell contact remains poorly understood.

The first part of the results of the following dissertation describes the entire volume of the TNTs formed between BMSCs and CML cells using FIB-SEM ultrastructural data. Analysis of the TNTs' entire volume allows for novel observations that can help to revise the current approach to the classification of tunneling nanotubes. The results indicate potential inaccuracies in the standard approach to TNTs identification using confocal microscopy.

The second part focuses on the transfer of stromal CD44 to leukemia cells. Firstly, the transfer of stromal CD44 to leukemia is verified. The obtained data indicate that CD44 is transferred along stromal vesicles *via* direct cell-to-cell contact, including TNTs. The stroma-driven transfer of CD44 results in its nuclear localization in recipient cells and induces transcriptomic changes in these cells. Acceptors of stromal CD44 are characterized by increased expression of genes involved in extracellular matrix remodeling, migration, and *CD44* itself, as a positive feedback loop. The upregulation of CD44 in leukemia at both the mRNA and protein levels results in the activation of the pro-leukemic ERK/AP-1 pathway, increased capacity for extracellular matrix remodeling, invasion, and protection against lipid peroxidation. Targeting the interaction of CD44 and its main ligand, hyaluronic acid, re-sensitizes leukemia cells to imatinib when co-cultured with protective BMSCs. The following aspects will be discussed in detail below.

5.1 Challenges in reliable TNTs identification

Although research on tunneling nanotubes has expanded significantly since their discovery in the early 2000's, numerous important knowledge gaps remain. A major challenge in the field is the lack of specific molecular markers of TNTs, as no protein or lipid has been shown to be uniquely restricted to TNTs and absent from other membrane protrusions or cytoskeleton-based structures, such as filopodia^{85,86,92}. Accordingly, no specific inhibitors of the TNT formation are known⁸⁸. The discovery of potential inhibitors relies on ongoing studies of the molecular regulators of TNT biogenesis. The validation of a protein as a potential TNT regulator typically involves modulation of its expression through gene silencing or overexpression, followed by confocal imaging and quantitative analysis of TNT formation^{78,88,258–261}. Therefore, accurate identification of TNTs and their discrimination from similar protrusions is crucial to avoid misleading interpretation. In this type of screening experiments, there are two main criteria the protrusions must meet to be considered as TNTs:

- it must connect two distant cells;
- it must not be attached to the substrate along its entire length⁸⁵.

5.1.1 The limitations of confocal microscopy in the proper TNT identification

Here, using a 2D co-culture system of CML cells and BMSCs, we applied the Focused Ion Beam Scanning Electron Microscopy (FIB-SEM) for visualization of the whole volume of protrusions formed by stromal and leukemia cells and compared this with the observations obtained from confocal microscopy. Notably, to the best of our knowledge, this is the first whole volume ultrastructural analysis of TNTs formed between two distinct types of cells, one of which is adherent (stroma, HS-5) and the other is suspension (leukemia, K562). Most of the existing studies using electron microscopy (SEM, TEM) have been conducted in adherent cell systems, in which cells of the same type are interconnected with TNTs (homotypic TNTs)^{92,101,262}. Co-cultures of adherent and suspension cells represent a significant challenge as TNTs are extremely fragile structures; however, careful and gentle sample preparation enables their successful analysis.

TNTs targeted earlier with confocal microscopy imaging, clearly observed at a significant height above the substrate, were also easily identified in the 3D reconstructions generated from FIB-SEM volume data (**Figure 4.1.2**). Surprisingly, volume EM analysis revealed many additional, similar protrusions connecting the cells, located just above the substratum, without touching it (**Figure 4.1.3 D-G, Figure 4.1.4 D-F, Figure 4.1.8 B**). Using a conventional approach in TNTs counting, based on the inspection of confocal z-stacks, these structures would likely have been classified as filopodia. On the other hand, in **Figure 4.1.5**, we presented two examples of protrusions that would have been classified as TNTs based on the currently accepted criteria, as they connect two distant cells and remain detached from the substrate along their length. Only FIB-SEM ultrastructural analysis revealed that the ends of these structures did not actually contact the target cells and should be considered as filopodia, not TNTs (**Figure 4.1.5 C-E**). In addition, some of the TNTs identified by the confocal imaging as one structure were, in fact, the bunches of thin TNTs formed close to each other (**Figure 4.1.3 C**).

These issues may be particularly relevant in studies where the TNT count is used as a direct measure of the influence of specific treatments or genetic manipulations. The proper identification is also crucial for experiments using TNTs microdissection and their proteome analysis, as performed by Gousset *et al.* The authors applied Laser Capture Microdissection to isolate various types of protrusions from CAD neuronal cells, including TNTs, filopodia, axons, and dendrites. Suspension above the substrate was a key criterion for the TNT identification²⁶³. In this type of study, potential contamination from the filopodia fraction would highly influence the results and the search for the potential TNT regulators or markers. Collectively, it may be worth considering defining more precise guidelines regarding distance from the substrate, including structures positioned closer to the substrate.

Another important aspect is the absence of standardized criteria for the unmistakable identification of TNTs and their discrimination from related structures in the complex tissue environments *in vivo*^{79,86,89}. Without a specific marker, it is extremely challenging to distinguish TNTs and other protrusions due to the dense packing of tissues and the fragile nature of TNTs. The criterion “not touching the

substratum” is difficult to apply to these conditions. However, the number of reports is increasing, together with the development of advanced imaging techniques. The identification relies mainly on morphological criteria and, importantly, evidence of cargo transfer. Using this approach, TNTs have been identified, for example, in the zebrafish embryos¹⁰⁰, mouse cerebral cortex²⁶⁴, and mouse embryonic hearts²⁶⁵, although in the last-mentioned study, the authors cautiously describe them as TNT-like structures²⁶⁵.

To sum up, until specific TNT markers/regulators are identified, confocal microscopy will remain the primary method for TNT identification. Volume EM is relatively expensive, time-consuming, and not easily accessible to be routinely employed in screening studies searching for TNT regulators or their potential inhibitors. However, FIB-SEM should be considered for critical validation experiments to avoid false conclusions.

5.2 Open- or closed-ended TNT: the need to examine the entire length

Whether TNTs exhibit open or closed ends remains one of the most actively debated issues in the TNT field. So far, both types of TNT termini have been detected, using both the FIB-SEM approach¹⁰¹ and more classical transmission electron microscopy (TEM)-based methods⁹⁴. Our ultrastructural analysis using FIB-SEM, performed with isotropic 8 nm resolution (8 nm in the x-, y-, and z-axes), was sufficient to examine the TNTs’ openness. We decided to visualize not only the sites of emergence from the cells, but also the entire length of connections formed between cells. Using this approach, we sought to verify the TNT openness throughout their entire length, which had not been demonstrated previously.

Our results confirmed the open-ended character of the TNTs’ emerging sites, as in the example shown in **Figure 4.1.6 B-C**. However, in all identified TNTs across the three analyzed datasets, none of the TNTs has shown a clear, open passable state. Imaging the volume of the entire length of the TNT, in confocal microscopy visible as one protrusion, revealed that in fact two or more TNTs are connecting at one junction point (**Figure 4.1.3 C, D, F; Figure 4.1.4 D-F; Figure 4.1.5 B**). At junction points, the diameters of the TNTs decrease significantly, and the “lumen” is no longer

detectable. On the FIB-SEM slices, the joining part is visible as a black point spanned over several sections (8 nm each). Volume EM performed only at the emerging sites would lead to false conclusions that these structures are open, hollow cylinders.

It is important to mention that TNT formation is a highly dynamic process. F-actin in the TNTs can undergo rapid polymerization and depolymerization, resulting in TNT formation times ranging from 30 seconds^{88,107} to several minutes⁸⁰. Additionally, TNTs are unstable and transient structures. Their lifetime varies between a few minutes^{266,267} and several hours^{268–270}. Meanwhile, our analysis is based on fixed-cell snapshots captured at different stages of the TNT formation process and its existence. Consequently, each dataset most likely represents distinct maturation stages occurring for different TNTs connecting cells. Furthermore, TNTs are exceptionally fragile and prone to damage. Their stability is limited by physical stress, photolability, and temperature fluctuations²⁷¹. All the factors mentioned above may lead to problems with the assessment of the TNTs' open or closed state. Again, this may represent an additional challenge for accurate TNT identification. An example of this kind of uncertainty is represented by protrusions shown in **Figure 4.1.4 C**. Their proximity and significant distance from the substratum suggest a “caught” filopodia, just before they fused into TNT (so-called “filopodia interplay” TNT formation mechanism). Therefore, taking the data together, it might be speculated that although the TNTs are open at the starting point, their opening at the distal end might be a very dynamic and strictly regulating process. However, this needs further studies to be performed.

It should also be noted that FIB-SEM may be associated with possible artifacts generated during sample preparation, ion beam milling, and long-term acquisition. The shrinkage of TNTs at certain points is also possible. These artifacts should be carefully considered when interpreting delicate structures, such as TNTs.

The exchange of cargo between cells connected by TNTs appears to be tightly regulated, specifically for the type of material being transferred. It is known that transport along cytoskeletal filaments (F-actin and microtubules) is mediated by specific motor proteins. However, this transfer may also be controlled by functional gating mechanisms. These “gates” could be located either at the sites where TNTs

emerge from the cell body or within the TNT structure. It has been proposed before that gap junctions can play a role as gating elements in the TNTs. Remarkably, some TNTs have been reported to contain gap junction components^{97,272,273}, including connexin-43, which is a primary gap junction protein²⁷². However, canonical gap junctions would be incompatible with the transfer of larger cargo, such as vesicles or mitochondria, due to their approximately 1.2 kDa size exclusion limit²⁷². Moreover, our ultrastructural data of the whole volume of TNTs obtained from FIB-SEM imaging clearly suggests the presence of a gating mechanism, visible as a dense membranous junction site, which presumably plays an important role in cargo transfer. However, the precise mechanisms determining whether cargo is permitted to pass this gate or redirected back remain unclear. It has been observed before that cargo transported *via* TNTs either reversed its direction of movement or displayed back-and-forth movements. Recently, Bénard and colleagues using advanced live-cell imaging (FLIM-STED) showed transient backtracking events in mitochondrial transport along thick TNTs²⁷⁴. They also observed mitochondrial rapid “round trips” along the TNT²⁷⁴. Similar observations have also been reported by other groups, suggesting that cargo transport within TNTs may undergo regulated movement patterns, such as intermittent “stop-and-go” and bidirectional “to-and-fro” motion^{275–277}. This supports the hypothesis of the existence of a certain type of gating mechanism, not only at the end of the TNT, but also along its length.

Together, FIB-SEM represents a state-of-the-art technique for whole-volume ultrastructural analysis. It enables precise identification of the protrusions and understanding of their morphology. Using volume EM followed by data segmentation and 3D reconstruction, we were able to identify as TNTs the protrusions that would not have been classified as TNTs using confocal microscopy alone. And conversely, we excluded structures that appeared to be typical TNTs. By imaging the entire length, we were able to observe that TNTs, even if open-ended at emerging sites, are probably closed, with some kind of gate mechanism, along their length. However, advanced volume EM techniques, including FIB-SEM, are still evolving, and maintaining software stability during extended imaging sessions (2-3 days) remains a significant technical limitation, which we also experienced in the present study.

They are also less accessible than fluorescence microscopy and time-consuming. Nevertheless, it should be strongly considered for the validation of key findings regarding tunneling nanotubes' potential regulators and TNTs' morphology.

5.3 CD44 is transferred from bone marrow stromal cells to chronic myeloid leukemia *via* direct cell-cell contact and TNTs

It was reported before that intercellular transfer of membrane proteins can be mediated by tunneling nanotubes¹¹². Molecules such as MHC I²⁷⁸, CD86²⁷⁹, CD133¹²³ have been shown to be transported between cells through this mechanism. The results presented in this dissertation reveal a previously unrecognized mechanism of stromal support for chronic myeloid leukemia, in which bone marrow stromal cells utilize direct cell-cell contact and tunneling nanotubes to transfer CD44 to leukemia cells.

Our findings indicate that transfer of stromal CD44 to leukemia cells is strongly dependent on direct cell-cell contact between stromal and leukemic cells, as shown in experiments with the Transwell system (**Figure 4.2.8 A**). However, accumulating evidence suggests that CD44 may be transferred between cells *via* secreted exosomes^{280–283}, promoting cancer progression, enhancing migratory and invasive phenotypes. For instance, CD44-enriched exosomes derived from doxorubicin-resistant breast cancer cells increased CD44 levels in doxorubicin-sensitive cells, leading to their increased resistance to the drug²⁸¹. In ovarian cancer, exosomes released by highly invasive cells transfer CD44 to less aggressive cells, directly enhancing their migratory and invasive capabilities²⁸². This led us to include additional controls with exosome inhibitors, such as GW4869 and Nexinhib20, in co-cultures of HS-5 and K562 cells. Nevertheless, the presence of exosome inhibitors did not affect stroma-driven upregulation of CD44 in leukemia, excluding this mechanism of transfer in stroma-leukemia co-cultures and further supporting contact-dependent transfer mechanisms. Importantly, exosome inhibitors did not affect the overall production of cellular vesicles and their transfer *via* direct cell-cell contact and TNTs (**Figure 4.2.4 B-C**).

Despite a significant enrichment of CD44-GFP⁺ cells within the population of leukemia cells, which received stromal vesicles (DiD⁺ cells), the presence of stromal CD44-GFP was not restricted to the vesicle acceptors, as a small proportion of DiD⁻ cells (~4%, compared to ~14% in the DiD⁺ population) was also positive for CD44-GFP (**Figure 4.2.9**). This suggests that CD44 may also be transferred to leukemia through contact-dependent, but non-vesicular mechanisms. Importantly, it was reported before that the whole membrane “patches” together with membrane-anchored proteins can be transferred *via* TNTs between cells^{258,279}. For example, it was shown that H-Ras protein from B cells can integrate in this way into the recipient T cell, preserving its original orientation and composition. Together with H-Ras, the B-cell marker, CD86, was also present in transferred membrane fragments²⁷⁹. Similar “patches” containing stromal CD44-GFP were also observed by us in the leukemia cells after co-culture with HS-5 CD44-GFP cells (**Figure 4.2.12 A**).

Stromal CD44 was clearly present along the TNTs connecting stromal and leukemic cells (**Figure 4.2.12**). However, without an available selective inhibitor of TNTs formation and/or activity, it is not possible to evaluate the precise contribution of TNT-mediated transport compared to other contact-dependent mechanisms in the transfer of a specific type of cargo. Also in this study, other mechanisms of protein transfer dependent on direct cell contact cannot be excluded. For instance, trogocytosis is a process of direct exchange of fragments of the plasma membrane along with the cytosol and surface proteins between cells in direct physical contact²⁸⁴. It is known that proteins’ transmembrane domains play a crucial role in the regulation of trogocytosis, taking part in the selection of proteins that can be transferred between cells. Elevated levels of CD44 on the activated T cells stabilize the immunological synapse, thereby facilitating trogocytosis²⁸⁵. However, direct CD44 transfer between cells *via* trogocytosis was reported before as inefficient. This was likely due to the short and stiff transmembrane domain of this protein^{286,287}.

5.4 CD44 – a possible important player in the TNTs formation?

Interestingly, we observed a significant decrease in the number of TNTs formed between stroma and leukemia when CD44 was knocked down in HS-5 stromal cells (**Figure 4.2.15 A-B, left graphs**). Actin polymerization is an essential

process for TNTs formation and their function^{88,288}. In the cell-dislodgement mechanism of TNTs formation, both actin polymerization and cell adhesion are essential²⁸⁹. CD44, as an adhesion molecule²⁹⁰ that can transduce external signals to cytoskeleton reorganization^{130,291,292}, may be an important factor in both processes. The interaction between CD44 and its main ligand, hyaluronan, leads to the binding of the ERM proteins (ezrin, radixin, moesin) and ankyrin by its intracellular domain (ICD). This leads to the cytoskeletal reorganization^{127,293}. ECM signals can also trigger the CD44-mediated signaling cascades and activation of small Rho GTPases, such as RhoA, Rad1 and Cdc42¹⁷³, reported previously to be involved in the formation of the TNTs¹⁰⁸. Interestingly, in neuronal cells, CD44 plays a key role in the dendritic spine's elongation. Through its interaction with serotonin receptor (5-HT7R), CD44 present on the dendritic spines is cleaved by MMP9, followed by detachment of CD44 extracellular domain and activation of Cdc42. This results in the elongation of dendritic spines^{294–296}. Cdc42 was reported to be involved in the TNTs elongation as well²⁹⁷. Thus, its knockdown in stromal cells may have resulted in a decreased number of TNTs connecting stromal and leukemia cells. On the other hand, it is intriguing that CD44 knockdown in stroma did not affect the number of homotypic (connecting stromal cells) TNTs (**Figure 4.2.15 A-B, right graphs**).

These findings should be considered preliminary, and the role of CD44 in TNTs formation requires further investigation. In particular, it will be important to determine whether additional CD44 knockout in leukemia cells would lead to comparable or greater effects. It should also be noted that quantification of TNTs was performed according to current standards, using confocal z-stacks. As discussed in previous sections of this work, this approach may introduce bias and requires further validation.

5.5 Stromal CD44 as a potential co-regulator of gene expression in recipient cells

As mentioned above, stromal CD44 is transferred to leukemia *via* direct cell-cell contact. An important question we sought to address is whether the protein remains functional in leukemia recipient cells and, if so, what role it plays.

Importantly, it was shown before that proteins transferred *via* TNTs remain functionally active in the recipient cells. For example, in the study of Guescini *et al.*, G protein-coupled receptors (A2a and D2) could still transduce signals in recipient cells, as was demonstrated by increased cAMP levels following the stimulation²⁹⁸.

Remarkably, the subcellular localization of stromal CD44 in leukemia recipients included the cell nucleus (**Figure 4.2.16 B**). CD44, mainly its intracellular domain (ICD), was reported to be involved in the regulation of transcription of some genes¹⁴². This led us to hypothesize that stromal CD44 protein may be involved in the co-regulation of transcription in leukemia cells. Previous reports on the involvement of CD44 in the transcriptional co-regulation have been limited to endogenous CD44 and translocation of its intracellular domain to the nucleus. This study highlights two novel aspects of this topic. The first of these findings is supported by some evidence in the literature. Most studies report the translocation of the cleaved ICD to the nucleus^{156–158,161,162,299}; however, we, along with two other independent groups, observed the translocation of the full-length CD44 form to the nucleus (**Figure 4.2.16**)^{155,163}. This suggests that the full form of the protein may also be responsible for transcriptome reprogramming, with our study being the first to report this in leukemia. Moreover, we discovered and demonstrated for the first time that CD44 derived from neighboring cells can be transferred to other cell types, followed by translocation to the nucleus of recipients, and is associated with their transcriptional reprogramming.

5.5.1 Uptake of stromal CD44 by leukemia cells results in their altered transcriptome profile

In leukemia cells that received stromal CD44, we observed the upregulation of genes related to adhesion, migration, and ECM remodeling (**Figure 4.2.18**). The upregulated genes included matrix metalloproteinases, which is consistent with the previous reports showing that CD44 (ICD) co-regulates the transcription of *MMP2*, *MMP9*, *MMP14*^{156,157,299}. The available data regarding transcription factors interacting with CD44 in the nucleus are still limited. Additionally, most reports have focused on the CD44 intracellular domain. One of the key partners of CD44-ICD are RUNX2^{156,157}, CREB¹⁶¹, Nanog, Oct4 and Sox2³⁰⁰ and HIF-2 α ¹⁵⁸. Even less is known about the nuclear

functions of the full-length CD44. Lee and colleagues, for the first time, showed that uncleaved CD44 is translocated to the nucleus¹⁵⁵. They presented that CD44 associates with STAT3 and p300 in the nucleus, binding directly to the cyclin D1 promoter, promoting its expression and enhancing cancer cells' proliferation. Janiszewska *et al.* identified a specific mechanism of CD44 nuclear import, demonstrating that full-length CD44 is translocated into the nucleus *via* a transportin-1-dependent manner. However, this study did not provide any details regarding the nuclear interaction partners of the full-length CD44. The authors only demonstrated that the presence of CD44 in the nucleus stimulates global RNA synthesis, as measured by BrdU incorporation¹⁶³. In another study, authors suggest that in liver cancer stem cells, the full-length CD44 is transported to the nucleus by importin β . Strikingly, it was suggested that full-length CD44 binds the promoter regions of *KLF2*, *KLF5*, and *ESRRB* directly, acting as the transcription factor¹⁶⁴.

Given the limited knowledge in this area, we aimed to identify potential TF partners interacting with full-length CD44 by analyzing publicly available databases (**Figure 4.2.24**). Among the transcription factors predicted to regulate transcription of genes upregulated in the acceptors of the stromal CD44-GFP, only NF- κ B³⁰¹ was reported previously to indirectly cooperate with CD44 to regulate gene transcription. However, CD44 may regulate gene transcription *via* TRE (TPA-response element), which are classical binding sites for the AP-1 (c-Jun/c-Fos) transcription factor complex^{142,159,302}, suggesting this cooperation. Additionally, even though CD44 does not interact with HIF-1 α , it can induce the expression of HIF-1 α -target genes, potentially bypassing its presence and activity¹⁵⁷. In this context, the observed increase in HIF-1 α protein levels in leukemia cells after co-culture with the protective stroma is intriguing (**Figure 4.2.27**). Firstly, if its activity is indeed “dispensable” in the presence of increased CD44, the biological role of its upregulation after stroma-mediated CD44 transfer remains unclear. Additionally, its upregulation is surprising given that experiments were performed under normoxic conditions. However, other researchers have shown that HIF-1 α can also be regulated by oxygen-independent mechanisms³⁰³, involving STAT3^{304–306} and ERK1/2, which can phosphorylate HIF-1 α , leading to its stabilization^{307,308}. Notably, in leukemia cells co-cultured with protective

HS-5 stromal cells, both ERK1/2 (**Figure 4.2.27**) and STAT3 (**Figure 4.2.29 G**) were activated. Additionally, c-Jun can stabilize HIF-1 α to protect it from degradation³⁰⁹ and cooperate with it in the regulation of gene transcription^{310,311}. This may explain its increase under co-culture with HS-5 and CD44 transfer. Moreover, additional regulation of transcription of genes upregulated in CD44 acceptors by HIF-1 α should not be excluded.

Additionally, correlation analysis between *CD44* expression and expression of *JUN*, *FOS* and *HIF1A* supported their potential involvement in *CD44* upregulation visible in leukemic acceptors of stromal CD44 (**Figure 4.2.25**). These findings, together with the increased expression of HIF-1 α protein, activation of the ERK1/2 pathway (**Figure 4.2.27**) and its downstream targets, c-Jun and c-Fos in leukemia cells after co-culture with HS-5 cells and CD44 transfer (**Figure 4.2.26**), can point to the AP-1 complex as the main CD44 partners in the gene transcription regulation. However, while our results are primarily descriptive in this context, they provide a useful framework for further validation, for example, by ChiP-seq, to confirm AP-1 and CD44 potential co-regulatory activity.

Interestingly, beyond MMPs, one of the top upregulated genes identified in leukemic acceptors of stromal CD44 was *SERPINE1* (**Figure 4.2.18 C**). Recently, it was shown that expression of *SERPINE1* in CD34⁺ CML cells is substantially increased when compared to CD34⁺ cells in healthy donors' samples. Moreover, it functions as a pro-survival factor in CML LSCs, suppressing caspase-9-mediated apoptosis, which directly contributes to imatinib resistance³¹². It was also shown that *SERPINE1* contributes to the retention of LCSs within the protective bone marrow niche³¹³. Literature points to the role of stromal TGF- β in the *SERPINE1* transcription. Stromal TGF- β binds to receptors on CML LSCs and induces Smad3 phosphorylation, leading to its translocation to the nucleus and *SERPINE1* transcription promotion³¹⁴. Considering the important role of direct cell-cell interactions of CML LSCs with BMSCs and our results, presenting the increased expression of pro-leukemic *SERPINE1* in acceptors of stromal CD44, we suggest that the potential role of CD44 in the regulation of *SERPINE1* transcription should be investigated.

5.5.2 The expression of the endogenous *CD44* is increased in leukemic acceptor cells

One of the upregulated genes in leukemic acceptors of stromal *CD44* was *CD44* itself (**Figure 4.2.18 C**). This indicates the existence of a positive feedback loop regulating the *CD44* expression in these cells. The ability of *CD44* to regulate its own expression has been documented previously; however, it was dependent on the *CD44*-ICD activity and involved *CD44* originating from the same cell¹⁵⁹. Here, we suggest that stromal *CD44* can induce expression of the endogenous *CD44* in leukemia acceptor cells. The experiments in which K562 cells were co-cultured with HS-5 BMSCs with silenced *CD44* expression confirmed that stromal *CD44* is necessary to upregulate endogenous *CD44* expression in leukemia. Another essential factor is direct cell-cell contact between the stroma and leukemia. Although there was a slight increase in *CD44* expression in leukemia cells co-cultured with stroma *via* cell culture insert (Transwell) (**Figure 4.2.20 A**), at a similar level to that observed in the co-cultures with HS-5 sh*CD44* cells (**Figure 4.2.20 C**), this effect did not reach statistical significance. However, the potential role of stroma-derived secreted factors and/or the role of endogenous leukemic *CD44* in this process should be considered.

Importantly, the increased mRNA levels of *CD44* in leukemia after co-culture with stroma corresponded to the increased protein levels. Clinically, *CD44* is often implicated as a factor corresponding to a poor prognosis^{189,315} and has been proposed for monitoring the therapeutic response and disease progression¹⁵³. In leukemia, an increase in *CD44* expression was observed during disease progression³¹⁶; however, the stroma-driven upregulation has not been extensively studied. Interestingly, we observed that stromal cells partially protected CML cells from the imatinib-induced *CD44* downregulation, clearly visible in leukemia monocultures (**Figure 4.2.21**). What is important, *CD44* protein levels were increased only in the co-cultures with stromal cells possessing protective properties for CML cells (**Figure 4.2.23**). To note, we showed that only HS-5 cells protect CML cells from imatinib-induced apoptosis, and the direct contact between cells was crucial for stroma-mediated protection (**Figure 4.2.22 A**). This excludes the possibility that the

absence of certain cytokines (e.g., IL-6, IL-8) secreted by HS-5 cells but not HS-27A²¹⁰ is responsible for this difference. Additionally, HS-27A cells transferred significantly fewer cellular vesicles to leukemia cells. We reported before⁷⁸ that cell contact-dependent, TNT-mediated transfer of cellular vesicles from HS-5 to K562 cells leads to protection from imatinib-induced apoptosis in CML acceptor cells. Here, we showed that CD44 is transferred together with vesicles from HS-5 cells to leukemia cells (**Figure 4.2.3**). Therefore, we suggest that reduced vesicle and CD44 transfer may be one of the reasons for the lack of HS-27A-driven protection. We hypothesize that the selective increase in CD44 levels observed only in co-cultures with stromal cells possessing protective abilities (HS-5) indicates that CD44 transfer is not a generic feature of intercellular contact but rather a property linked to the stroma-mediated protection of leukemia cells.

Interestingly, it is known that CD44 can influence gene expression also indirectly, by activation of various signaling pathways. For example, binding of CD44 to HA induces the formation of STAT3-Nanog complex, resulting in STAT3-dependent expression of multidrug resistance gene 1 (*MDR1*)³¹⁷. CD44-HA interaction also increased the expression and activity of the acetyltransferase p300, leading to the acetylation and activation of NF- κ B and β -catenin, inducing the expression of pro-survival genes, including Bcl-xL³⁰¹. Moreover, c-Jun and CD44 have been proposed to form a functional regulatory loop, where AP-1 induces CD44 expression and activated CD44, in turn, enhances c-Jun activity, promoting tumor growth^{182,256}.

We suggest that stroma-mediated upregulation of CD44 protein in leukemia cells results in increased total levels of CD44 protein in leukemia cells. This stroma-driven upregulation leads to enhanced activation of the ERK/c-Jun/c-Fos axis in CD44⁺ leukemia cells. Both stromal CD44 present in the leukemia nucleus and endogenous leukemic protein can influence the expression of pro-invasive genes. Additionally, their increased expression may be driven by enhanced pro-survival signaling pathways mentioned above. Altogether, these findings point to a complex network of CD44 transfer, the upregulation of endogenous leukemic CD44, activation of ERK/c-Jun/c-Fos signaling pathways, and increased expression of other genes

involved in adhesion, migration, and ECM remodeling, leading to increased invasiveness and resistance of leukemia cells.

5.6 Stroma-mediated CD44 upregulation in leukemia cells leads to functional consequences

5.6.1 Influence on the invasiveness of leukemia cells

As mentioned above, leukemic acceptors of stromal CD44 expressed higher levels of genes related to adhesion, migration, and ECM remodeling. Notably, leukemic cells in which co-culture with stroma triggered a positive feedback loop leading to sustained CD44 expression displayed a more invasive and migratory phenotype, as demonstrated by functional assays (**Figure 4.2.29 B**). In leukemic acceptors of stromal CD44, we observed not only higher expression and activation of MMPs, but also elevated levels of soluble CD44 and IL-8 (**Figure 4.2.29 E-F**). Both are strongly linked to the enhanced invasive potential of cancer cells. Additionally, in K562 CD44-GFP⁺ cells, the IL8 (*CXCL8*) was also upregulated at the transcript level, compared to non-acceptors; however, this increase did not reach statistical significance. Moreover, co-culture of leukemia cells with stroma induced activation of key signaling pathways, as evidenced by increased phosphorylation of STAT3 and ERK1/2, as well as AP-1 component, and upregulation of HIF-1 α . These changes suggest the establishment of a complex, interconnected signaling network that drives the acquisition of a more invasive phenotype of leukemia cells.

In B-cell chronic lymphocytic leukemia (CLL) and other malignancies, elevated serum IL-8 levels have been identified as a prognostic marker³¹⁸. It may act as an autocrine growth factor, with cancer cells producing it themselves to stimulate their own proliferation and escape host regulatory control mechanisms²⁴⁴. IL-8 activates major signaling cascades, such as PI3K/AKT/mTOR and Ras/Raf/MEK/ERK. Key TFs such as HIF-1 α , NF- κ B, AP-1, and STAT proteins integrate signals from these pathways to drive the expression of genes controlling cell survival, proliferation, and resistance to apoptosis^{244,319}. Interestingly, IL-8 induces phosphorylation of STAT3, promoting its nuclear translocation, which may lead to activation of Snail. This can further enhance IL-8 production, establishing a positive

feedback loop that drives tumor progression²⁴⁹. Since both CD44 and IL8 can activate STAT3^{155,246,320}, which may in turn regulate the expression of both factors, we propose that they may mutually reinforce each other's expression through a STAT3-dependent positive feedback loop in leukemia cells co-cultured with protective stromal cells.

Interestingly, one of the upregulated genes in the CD44 acceptors was *PLAU*, coding a serine protease uPA (**Figure 4.2.18 B**), that promotes tumor cell invasion by converting plasminogen into plasmin, initiating ECM degradation. In breast cancer, it has been shown that hyaluronan-induced CD44 signaling strongly enhances the expression of *PLAU*. CD44 knockdown markedly reduced *PLAU* transcript and protein levels, resulting in impaired invasive capacity of the cells³²¹. We suggest that *PLAU* may serve as one of the downstream proteolytic effectors of CD44 signaling.

Interestingly, CD44 can act as a multifunctional docking platform for MMPs, concentrating proteolytic activity at the cell surface. CD44 serves as a principal receptor for proMMP9³²² and forms functional complexes with MT1-MMP³²³. CD44-associated MMPs may be partially protected from inhibition by tissue inhibitors of metalloproteinases (TIMPs), creating a cell surface-restricted proteolytic niche with enhanced enzymatic activity. The high local concentration of MMPs within CD44 clusters further promotes their autoactivation, thereby amplifying invasive processes³²². However, the CD44 platform not only anchors enzymes at the cell surface but also participates in signal transduction. The interaction of MMP9 with CD44 induces ERK phosphorylation, promoting cell survival and migration³²⁴. This may indicate that transfer-dependent upregulation of CD44 in leukemia acceptor cells not only modulates the expression of genes associated with invasiveness but may also exert functional effects through interactions with MMPs. This could further explain the increased activity of MMP2 and MMP9 in conditioned media obtained from leukemic CD44 acceptors, as well as the elevation in ERK phosphorylation in leukemia cells co-cultured with protective stromal cells only.

Overall, these observations indicate that multiple linked processes, resulting from the transfer of stromal CD44 to leukemia, likely work together to increase leukemia cells' invasiveness. Nevertheless, precise dissection of the relative

contribution of each pathway will require further investigation using targeted pharmacological inhibition and functional validation approaches.

5.6.2 Increased glycolytic activity upon stroma-mediated CD44 upregulation in leukemia

Our results indicated that after co-culture with stroma, K562 CD44⁺ cells exhibit a metabolic shift toward glycolysis relative to K562 CD44⁻ cells (**Figure 4.2.30**). However, this shift was not accompanied by increased glucose uptake or changes in GLUT1 protein levels (**Figure 4.2.31**). It has been shown that CD44 contributes to the metabolic shift toward glycolysis through direct interactions with key metabolic enzymes, regulation of gene expression, and activation of multiple signaling pathways³²⁵. For instance, CD44 has been shown to interact directly with the M2 isoform of pyruvate kinase (PKM2) and promote its tyrosine phosphorylation. This modification suppresses PKM2 enzymatic activity, thereby promoting metabolic reprogramming toward aerobic glycolysis³²⁶. It was also reported that CD44 can influence the balance of the lactate dehydrogenase (LDH) isoenzymes, increasing the LDHA to LDHB ratio and favoring lactate-producing glycolysis, whereas CD44 silencing reduces this ratio and shifts cellular metabolism toward mitochondrial oxidative phosphorylation (OXPHOS)^{325,327}. An important aspect of CD44-mediated metabolic regulation is also its transcriptional co-regulatory activity. CD44-ICD has been shown to promote the expression of *PFKB4*, a key regulator of glycolysis that enhances glucose utilization and lactate production, as demonstrated in breast and prostate cancer models¹⁶¹. It was also reported that CD44 is required to maintain glucose uptake and the high levels of glucose transporter GLUT1 at the cell surface³²⁶.

In our model, the glycolytic shift observed in Seahorse assay performed on K562 CD44⁺ and CD44⁻ cells was likely not mediated at the level of glucose transport, as both populations of cells exhibited comparable uptake of a glucose analog 2-NBDG and had similar GLUT1 protein levels. Its potential transcriptional co-regulatory activity also does not appear to play a major role in this context, as we did not observe upregulation of metabolism-related genes in leukemic recipients of stromal CD44, with the notable exception of the lactate transporter *SLC16A3 (MCT4)* (**Table 9-1**) and a slight increase in *GLUT1* levels in the total K562 CD44⁺ population

from co-cultures with stroma (**Figure 4.2.31 A**). These findings suggest that the observed metabolic phenotype is driven by other mechanisms, possibly through direct or indirect interactions of CD44 with enzymes involved in metabolic regulation.

5.6.3 Impact on lipid peroxidation and ferroptosis sensitivity

Ferroptosis has emerged as one of the most intensively investigated areas in cell biology and cancer research. Although ferroptotic cell death has been observed in earlier studies, the term “ferroptosis” was formally introduced by Dixon and colleagues in 2012 to describe a distinct, iron-dependent form of regulated cell death²¹⁸. One of the principal mechanisms by which CD44 can protect cells from ferroptosis involves the stabilization of the cystine transporter xCT (SLCA11) at the plasma membrane. This increases cystine uptake and supports glutathione-dependent protection against lipid peroxidation and ferroptosis²⁵². These findings were subsequently confirmed and further investigated by Liu *et al.*²⁵⁵ and Thanne and colleagues, who identified the CD44v8-10 variant as a key regulator stabilizing xCT³²⁸.

Here, we demonstrated that knockdown of CD44 in LAMA-84 cells with intrinsically high expression of this protein sensitized them to lipid peroxidation induced by RSL3 or imatinib (**Figure 4.2.32**) and was accompanied by a marked reduction in the GPX4 levels (**Figure 4.2.33**). We showed that co-culture with HS-5 cells, which prevents the imatinib-induced decrease in leukemia CD44 levels, partially restores GPX4 levels in CML cells. Although the role of CD44 in regulating GPX4 remains poorly understood, available evidence suggests a potential link between CD44 variant expression and GPX4 expression. A newly published study analyzing patient-derived lung cancer samples revealed a strong positive correlation between expression of CD44v6 or CD44v9 and GPX4 mRNA and protein levels²⁵⁴.

We propose that CD44 transfer from BMSCs, together with the induction of endogenous CD44 expression in leukemia, protects against imatinib and ferroptosis inducers by enhancing GPX4 expression. It cannot be excluded that CD44 stabilizes xCT in leukemia cells; however, this was not investigated in the present study. Future work should also address whether specific isoforms of CD44 contribute to the observed effects.

5.7 CD44 as a potential therapeutic target in CML: limitations and future perspectives

The therapeutic potential of CD44 in cancer is broad and includes direct receptor targeting, advanced drug delivery systems, and immunotherapeutic approaches²⁹³. CD44 is considered a particularly attractive target due to its role as a marker of cancer stem cells and its involvement in cell survival, therapy resistance, and metastasis. However, its clinical exploitation is challenging as CD44 is also widely expressed in normal tissues, raising concerns about specificity and off-target effects¹⁸⁹.

In this study, we demonstrated that targeting CD44-hyaluronan interaction sensitizes CML cells to imatinib (**Figure 4.2.34**). Importantly, the use of an interaction inhibitor partially overcame stroma-mediated resistance to imatinib. Targeting HA and its interaction with CD44 has previously been explored in CML monocultures, where HA and CD44 were produced exclusively by leukemic cells¹⁷⁷. Strategies included the use of HA oligomers (o-HA), which bind CD44 without activating it, and inhibition of hyaluronan synthesis using 4-methylumbelliferone (4-MU). These interventions enhanced the response to imatinib *via* inhibition of PI3K/Akt signaling pathway and promoting senescence in CML cells¹⁷⁷. Similar studies on glioblastoma also showed that 4-MU significantly reduced the migratory capacity of tumor cells, which represents a critical determinant of glioblastoma aggressiveness³²⁹. However, CD44-HA interactions play fundamental roles in physiological processes, which represent one of the major challenges in designing safe anticancer therapies. For instance, this interaction is crucial for lymphocyte homing to lymph nodes and for the trafficking of HSCs to the bone marrow³³⁰. Importantly, CD44-HA interaction is also essential for maintaining the blood-brain-barrier structural stability and serves as a regulatory mechanism controlling the entry of immune and repair cells into the central nervous system^{127,331,332}. Despite these challenges, ongoing research is exploring strategies for selective cancer targeting. A key opportunity lies in differences in receptor activation states. In healthy cells, CD44 typically exhibits a low-affinity, inactive conformation, whereas in tumor cells it is often constitutively activated. Nanotechnology approaches, such as hyaluronan-coated nanoparticles,

exploit this difference by preferentially binding activated CD44 on cancer cells while sparing normal tissues. This strategy exploits CD44-HA interactions differently, using them as a gateway for more selective delivery of chemotherapeutic agents rather than a direct therapeutic target^{178,333,334}.

Another important approach involves antibody-based targeting of CD44. Monoclonal antibody therapies directed against CD44 represent a promising strategy for eradicating LSCs and overcoming therapy resistance by disrupting interactions between the tumor and the bone marrow niche¹⁹¹. Importantly, combined CD44 blockade with IM7 antibody and dasatinib treatment impaired the self-renewal capacity of leukemic stem cells in CML¹⁹². In the context of AML, A3D8 is one of the best-characterized antibodies used for targeting CD44. It binds to the standard form of CD44 on the cell surface and induces leukemic cell differentiation and apoptosis^{335,336}. However, as discussed above, a major limitation of antibody-based CD44 targeting is its expression in healthy tissues. A potential strategy to improve specificity is the use of alternatively spliced CD44 variants, which are generally less abundant in normal cells but frequently enriched in cancer²⁹⁶. Nevertheless, this approach is not without challenges, as illustrated by bivatuzumab (targeting CD44v6), whose clinical development was discontinued due to severe cutaneous toxicity, since keratinocytes also express CD44v6 abundantly^{337,338}.

Despite these limitations, CD44 remains an attractive therapeutic target due to its cell-surface localization, which facilitates drug accessibility. Therefore, systematic profiling of CD44 variant expression in healthy versus malignant cells may help refine targeting strategies. Additionally, combination therapies may further improve their efficacy. It should nevertheless be noted that CD44 blockade using antibodies may not always be a purely inhibitory process. The CD44 receptor engagement itself may trigger intracellular signaling, alter cytoskeletal stability, or promote CD44 trafficking to the nucleus, where it can function as a transcriptional co-activator. These potential effects should therefore be carefully considered when interpreting CD44-targeting strategies.

6 SUMMARY AND CONCLUSIONS

Physical interactions between leukemic cells and components of the bone marrow niche, such as stromal cells, create a protective microenvironment allowing leukemia cells to escape drug-induced effects. Tunneling nanotubes (TNTs) represent one of the mechanisms contributing to this phenomenon through the intercellular transfer of proteins and organelles. However, many aspects regarding TNTs' structure and their proper identification remain unresolved. Furthermore, the biological roles of specific proteins transported *via* TNTs are still largely unknown. Therefore, this study aimed to improve the understanding of TNTs' ultrastructure and the differences between TNTs and other cellular protrusions. Using state-of-the-art FIB-SEM methodology, we sought to critically evaluate current approaches to identification TNTs using conventional fluorescence microscopy. Then, we focused on the cell-contact-dependent, TNT-mediated transfer of stromal CD44 to chronic myeloid leukemia cells and assessed its role in the acceptor leukemic cells. Finally, a potential CD44-targeted therapeutic approach in combination with a conventional tyrosine kinase inhibitor was tested in the stroma-leukemia co-cultures.

The main outcomes of these studies are summarized below:

1. Not all TNTs formed between stromal and leukemia cells, fulfilling the currently established criteria, are visible using traditional confocal microscopy imaging. Some TNTs identified by confocal microscopy do not consistently meet the same criteria when examined at higher resolution using volume EM.
2. Although TNTs are open-ended at the donor-cell interface, visible gating structures are present along their length.
3. Bone marrow stromal cells (BMSCs) transfer CD44 protein to chronic myeloid leukemia cells *via* direct cell contact and TNTs; stromal CD44 is localized to leukemic cell membranes and the nuclei of leukemic recipient cells.
4. The transfer of stromal CD44 to leukemia cells results in the upregulation of genes related to adhesion, migration, and extracellular matrix remodeling in recipient cells. This leads to increased capacity for extracellular matrix remodeling and invasion.

5. One of the upregulated genes in leukemia cells that received stromal CD44 is *CD44* itself, leading to the upregulation of endogenous CD44 in leukemia, both on mRNA and protein levels.
6. CD44 transfer leads to activation of pro-survival signaling pathways, such as ERK/AP-1.
7. The presence of CD44 in leukemia cells is crucial for GPX4 expression and, consequently, may contribute to protection against lipid peroxidation and ferroptosis. The transfer of stromal CD44 to leukemia cells provides protection against these effects by restoring leukemic CD44 levels reduced by imatinib treatment.
8. Targeting of the CD44-HA interaction re-sensitizes leukemia cells to imatinib in the co-culture with BMSCs (bone marrow-mimicking conditions).

A summary of the findings described in points 3-8 is provided in **Figure 5.7.1**.

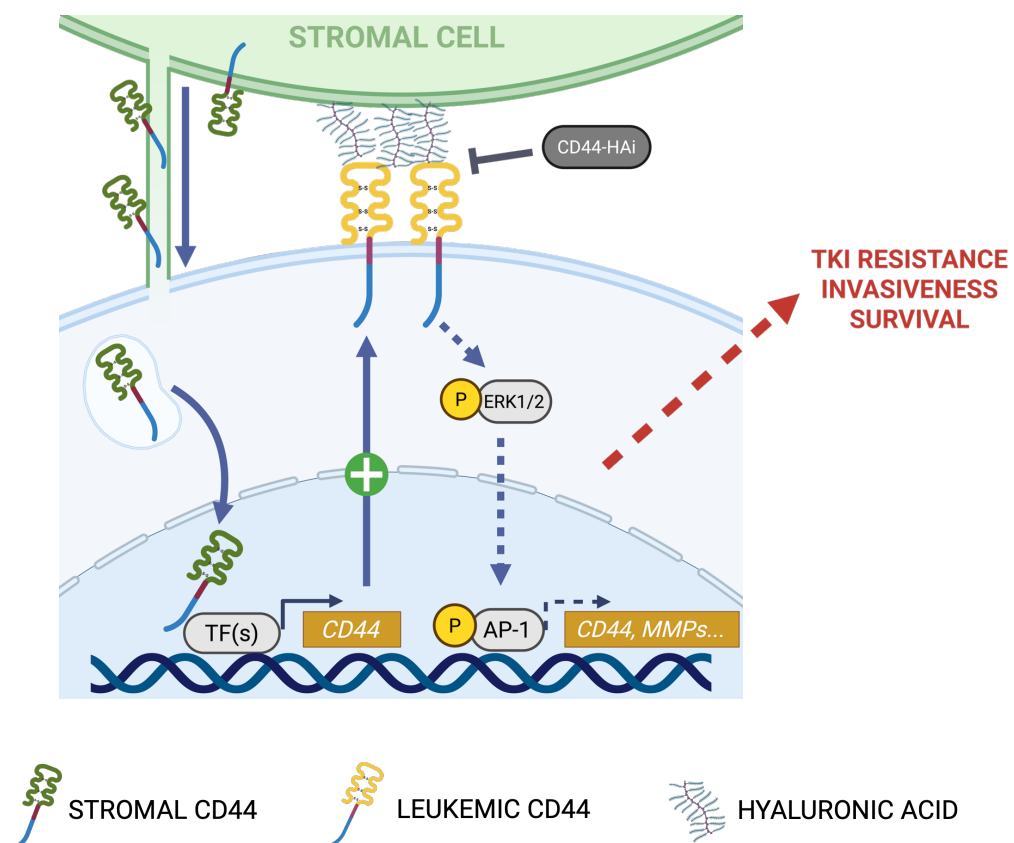


Figure 5.7.1 The proposed model of the stroma-mediated upregulation of CD44 in leukemia and its downstream effects. Created with BioRender.com.

Conclusions

Altogether, the presented data confirm that TNTs are formed between stromal and leukemia cells and play an important role in the stroma-driven protection, leading to their resistance to drug treatment. Our findings indicate the need for more rigorous verification of structures identified as TNTs, potentially using volume EM approaches. This is particularly relevant in studies aimed at identifying potential TNT regulators. Moreover, these results indicate a new regulatory loop driven by the transfer of CD44 from stromal to leukemia cells, in which stromal CD44 promotes the expression of endogenous CD44 in leukemia. This leads to transcriptomic changes and the activation of pro-survival signaling in CD44-acceptor cells, resulting in increased invasiveness and protection from imatinib. Pharmacological targeting of the CD44-hyaluronan axis can improve therapeutic outcomes in leukemic patients treated with TKIs, whose response is limited in the BMM because of stroma-mediated resistance. This might be a translational implication for the development of novel combined therapeutic strategies. Importantly, presented data, even if strictly related to the CML microenvironment, might have broader implications also for the other leukemias and cross-talk with the bone marrow niche.

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9 SUPPLEMENTARY INFORMATION

9.1 List of upregulated and downregulated genes

Table 9-1 List of differentially expressed genes in K562 CD44-GFP⁺ cells compared with K562 CD44-GFP⁻ cells.

List of the upregulated genes: K562 CD44-GFP⁺ (CD44 RECIPIENTS) vs K562 CD44-GFP⁻ (CD44 NON-RECIPIENTS)					
LAPTM5	TNS1	ELMO1	VSIR	OSGIN1	MYO1F
CDCA8	GNAI2	COL1A2	SNCG	ITGAM	RASGRP4
KIRREL1	LRRC15	WAS	PRF1	PLK1	CD22
PLXNA2	TRANK1	DPYSL2	CKAP4	VPS9D1	C3
CCN1	DAG1	FGFR1	LRP1	MVP	SIGLEC6
MXRA8	PPM1M	RHOBTB2	ADGRD1	APOBR	ANGPTL4
VWA1	GPX1	TNC	PTPN6	HASPIN	TYROBP
FNDC10	MGLL	SPTAN1	SELPLG	FKBP10	HOMER3
SH3BGRL3	TGFB1	SEMA4D	ADCY6	ITGA3	ARHGEF18
ACOT11	BASP1	INCENP	RIN3	TRAPPC1	SIGLEC10
PIK3CD	SH3PXD2B	TSPAN18	TNFAIP2	AURKB	LIF
PIK3C2B	LCP2	C11orf21	DLK1	LIMD2	INPP5J
EPAS1	SLC29A1	CD248	GPR132	SLC16A3	IL17REL
ADD2	GMPR	LGALS12	CSPG4	PHLPP1	COL6A2
INPP5D	TTYH3	SPI1	RPP25	FAM110A	SLC37A1
FEV	SERPINE1	MMP3	ANPEP	SMOX	ITGB2
PID1	SSPOP	CD44	MMP2	RGS19	COL6A1
FN1	SRRM3	PWWP2B	MT2A	SIRPA	DOP1B
OTOF	CLIP2	PLAU	RABEP2	EPB41L1	
EMILIN1	CHST12	DUSP5	CORO1A	GNA15	
List of the downregulated genes: K562 CD44-GFP⁺ (CD44 RECIPIENTS) vs K562 CD44-GFP⁻ (CD44 NON-RECIPIENTS)					
SCIN	CCT6P3	CA1	LINC02193	NDUFV2	RAD21L1

9.2 Publication record of the PhD Candidate

Most of the results presented in this doctoral dissertation have been published in:

1. **Turos-Korgul L**, Stepnik D, Zieminska A, Cabaj A, Kominek A, Ostrowska J, Kasak N, Krop A, Swatler J, De Biasi S, Cossarizza A, Stoklosa T, Piwocka K. (2026). Intercellular transfer of stromal CD44 drives transcriptional reprogramming to promote bone marrow-mediated resistance in chronic myeloid leukemia. *ResearchSquare* doi:10.21203/rs.3.rs-9440495/v1. **PREPRINT* (in revision)**

Other publications by the author of this dissertation:

2. Chroscicki P#, Kasak N#, Dymkowska D, **Turos-Korgul L**, Cysewski D, Chumak V, Stepnik D, Kusio-Kobialka M, Kominek A, Lebedzinska-Arciszewska M, Krop A, Szczepanowska J, Wieckowski M, Stoklosa T, Zablocki K, Piwocka K. (2025). Stroma-driven horizontal transfer of TCA-related proteins mediates metabolic plasticity and imatinib resistance in chronic myeloid leukemia. *Cell Communication and Signaling* 24(1):7.*
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The manuscript concerning TNT ultrastructure is in preparation.

- authors equally contributed

*** - publications related to the content of this dissertation**