

THE ROLE OF CALRETICULIN IN CELL ADHESION

by

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B.Sc., Alzahra University, 2015

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Cell and Developmental Biology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

September 2019

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis entitled:

The role of calreticulin in cell adhesion

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Submitted by Foujan Pedari in partial fulfillment of the requirements

for the degree of Master of Science

in Cell and Developmental Biology

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## Abstract

Acute lymphoblastic leukemia (ALL) is the most commonly diagnosed childhood cancer in Canada. Even though childhood ALL has a high overall survival rate, therapeutic options remain limited for those experiencing relapse; therefore, understanding the causes for treatment failure is vital. Adhesion of leukemic cells to bone marrow and the extracellular matrix provides chemotherapy protection to ALL, leading to drug resistance. In this thesis, I investigated the involvement of an endoplasmic reticulum (ER) chaperone, calreticulin (CRT), in integrin-mediated cell adhesion. I show that integrin-mediated adhesion of Jurkat T-lymphoblasts is disrupted in CRT<sup>-/-</sup> cells, and CRT re-expression restores the wildtype phenotype.

To determine the requirement of cytosolic CRT for integrin-mediated cell adhesion, I used ERp57<sup>-/-</sup> cells, previously characterized to express no CRT in the extra-ER compartment. Interestingly, ERp57<sup>-/-</sup> cells have decreased adhesion compared to wildtype cells, indicating the involvement of cytosolic CRT in integrin-mediated cell adhesion.

To evaluate the requirement of LRP1 as a surface receptor for CRT during immunogenic cell death (ICD), I utilized CRISPR-Cas9 technology to generate LRP1<sup>-/-</sup> lymphoblasts. I show that drug-treated stimulation of surface CRT presentation is lost in LRP1<sup>-/-</sup> cells, a result consistent with LRP1 as a cis-acting receptor for CRT during ICD.

I utilized CRT<sup>-/-</sup> HEK cells expressing various CRT constructs that are localized in different cellular compartments. All constructs successfully rescued the adhesion defect of CRT<sup>-/-</sup> cells. Furthermore, I found that mutant variants of CRT that lack the C-terminal KDEL ER-retention motif were proteolytically truncated, and that the N-terminal truncated fragment can be detected on the cell surface.

## **Lay Summary**

Leukemia is a cancer caused by the excess production of immature white blood cells. One third of childhood cancers are leukemias. Despite advances in chemotherapy, relapses still occur and treatment options remain limited for relapsed patients. One of the relapse mechanisms is the survival of a few tumor cells that reside within the bone marrow and developing resistance to chemotherapy. Furthermore, survivors of childhood leukemia experience long-term health problems in adulthood. Therefore, new treatment options are needed to improve outcomes for leukemia patients. This study investigates the mechanism by which leukemia cells adhere to the bone marrow. I focused on the role of a protein called calreticulin in cell adhesion. In the absence of calreticulin, cells lose their ability to adhere to the bone marrow. These findings further our understanding of the mechanisms contributing to relapse and may be exploited for development of new therapeutic strategies to minimize or prevent relapse.

## Preface

The original hypothesis for this study, that calreticulin mediates chemoresistance by interacting with the  $\alpha 4$ -integrin cytoplasmic tail, was formulated by Dr. Chinten James Lim (CJL) and Dr. Chi Chao Liu (CCL). I expanded and formulated the final hypothesis under the supervision of CJL. All work was completed at the BC Children's Hospital Research Institute located in Vancouver, Canada.

Other than the exceptions listed below, I performed the planning and set-up of all the experiments, the data acquisition, the data analysis, the figure presentation, and the interpretation of the results.

## Chapter 3

HEK CRT<sup>-/-</sup> cell line was generated by CJL. ssGFP-CRT, ssGFP-CRT-KDELdel, ssGFP-CRT-Mutant careticulin constructs were generated by CCL.

## Chapter 4

The Jurkat-based CRT<sup>-/-</sup> and ERp57<sup>-/-</sup> cells used in this study was generated by CCL. The integrin  $\beta 1$ <sup>-/-</sup> cells used in this study was generated by Pascal Leclair (PL). The recombinant protein substrate GST-CS1 was made by PL.

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## List of Abbreviations

Akt	RAC serine/threonine-protein kinase, also known as PKB
ALL	acute lymphoblastic leukemia
ATP	adenosine triphosphate
B-ALL	B-cell acute lymphoblastic leukemia
bp	base pair
BSA	bovine serum albumin
CALR	gene encoding calreticulin
CAMDR	cell adhesion mediated drug resistance
CIB	calcium and integrin binding protein
CRISPR-Cas9	clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9
CRT	calreticulin
CS1	fibronectin connecting segment 1
DAMPs	damage-associated molecular patterns
Doxo	doxorubicin
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ERp57	ER protein of 57-kDa
ERK	Extracellular signal-regulated kinases
FAK	focal adhesion kinase
FBS	fetal bovine serum

Fluo-4-AM	Fluo-4-acetoxymethyl ester
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
gp96	heat shock protein gp96
GST	glutathione <i>S</i> -transferase
HMGB1	high mobility group box 1
HSP27	heat shock protein beta-1
HSP70	heat shock protein 70-kDa
HSP90	heat shock protein 90-kDa
ICD	immunogenic cell death
ILK	integrin linked kinase
IMDM	Iscove's Modified Dulbecco's Medium
IR-dye	infrared dyes
JAK	Janus kinase
<i>JAK2</i>	Janus kinase 2
JNK	(c-jun N-terminal kinase)
KDEL	ER retention sequence, Lysine-Aspartic Acid-Glutamic Acid-Leucine
LRP1	low-density lipoprotein receptor related protein 1
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase
MCSF	macrophage colony-stimulating factor
MDGI	mammary-derived growth inhibitor
MMEC	Myocardial Microvascular Endothelial Cells

MPL	myeloproliferative leukemia virus oncogene
MPNs	myeloproliferative neoplasms
Mss4	guanine nucleotide exchange factor MSS4
NES	nuclear export signal
NMR	nuclear magnetic resonance
Oxa	oxaliplatin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PKI	protein kinase inhibitor
PS	phosphatidylserine
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RUNX1	runt related transcription factor 1
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Src	proto-oncogene tyrosine-protein kinase Src
STAT	signal transducer and activator of transcription
T-ALL	T-cell acute lymphoblastic leukemia
tRNA	transfer ribonucleic acid
TSP1	thrombospondin 1

## **Acknowledgements**

I want to start by thanking my research supervisor, Dr. Chinten James Lim, for his mentorship and guidance throughout the duration of my Master of Science degree. He provided an excellent environment to grow and develop as a scientist. His mentorship has provided me with the skills to succeed in my future endeavors.

I thank my supervisory committee members: Dr. Calvin Roskelly, and Dr. Gregor Reid, who have spent their valuable time attending my committee meetings, offering constructive advice and evaluating my progress.

Thanks to Pascal Leclair and Chi Chao Liu for teaching me many of the experimental techniques and for helping me since day one.

Thanks to Lakshana Sreenivasan, Axel Chu and Li Ling for on-going technical and emotional support.

Thanks to the Michael Cuccione Foundation, who provided one year of graduate studentship support.

I would like to thank my boyfriend, Mojtaba Mansouri for his on-going emotional support throughout this degree who has enabled me to stay focused and motivated to complete this degree.

Special thanks are owed to my parents, Nori Pedari and Mina Javadzadehpour, and my brother Farbod who have supported me throughout my years of education. I am thankful to having a supportive family.

## **Dedication**

Dedicated in memory of my grandmother,

Mahin Saraydari

# **1. Introduction**

## **1.1 Acute Lymphoblastic Leukemia**

ALL symptoms are caused by the accumulation and overexpansion of immature lymphoblasts, which occupy bone marrow resources and lead to insufficient numbers of normal white cells, red cells and platelets<sup>1,2</sup>. Many symptoms of ALL are non-specific; therefore, they can be mistaken for other diseases<sup>1</sup>. Typically, these symptoms include signs of anemia such as general weakness, fatigue and pallor, and tachycardia. Since ALL patients have significantly low levels of normal white cells including neutrophils, they are at higher risk of getting infections. They are also more susceptible to bruising and bleeding due to thrombocytopenia<sup>1-3</sup>.

Three out of 100,000 people develop ALL worldwide with the highest incidence occurring in ages between 1 and 9 years old<sup>4,5</sup>. With 80% of ALL cases being pediatric, ALL is the most common childhood cancer, representing more than a quarter of all pediatric cancers.<sup>3</sup> Pediatric ALL can be classified into two subtypes based on the lineage of the originating hematopoietic cell: precursor B-cell ALL (B-ALL) or precursor T-cell ALL (T-ALL). Approximately 85% of pediatric ALL are B-ALLs while 15% are T-ALLs<sup>6</sup>. T-ALL patients usually have a worse prognosis than those with B-ALL; therefore, they require stronger treatment<sup>6</sup>. Multi-agent chemotherapy has improved the outcome of pediatric ALL and has led to an overall cure rate approaching 90%<sup>7</sup>. However, 10%-15% of children experience relapse after a transient remission, with the long-term overall survival rate for relapsed patients dropping to 15%-50%<sup>8</sup>. In addition, the survivors of pediatric ALL suffer from late-term secondary toxic effects of intense chemotherapy and struggle with health problems such as cardiovascular



disease, diabetes, and adiposity in adulthood<sup>9,10</sup>. Therefore, novel treatment options are needed that are both more effective and less toxic for ALL patients.

Relapse of pediatric ALL often occurs by expansion of treatment-resistant leukemic cells that were present in the bone marrow, thus the bone marrow microenvironment is thought to facilitate their survival<sup>11</sup>. Indeed, cell adhesion to matrix proteins found in the bone marrow is known to confer drug resistance<sup>11</sup>. The work outlined in this thesis aims to understand the molecular factors that govern T-ALL cell adhesion as a basis for understanding cell adhesion-mediated resistance to chemotherapy.

Survival differences between pediatric cancer types are well-studied. In epidemiologic survival analyses, males and females are normally grouped together<sup>12,13</sup>. However, male children diagnosed with leukemia continue to show poorer prognosis and worse survival than females<sup>12,13</sup>. The survival differences between sexes are most likely to be multifactorial and they vary based on sex differences in diagnosis delay, pharmacogenetics and cancer biology<sup>12,13</sup>.

## **1.2 Treatment of ALL**

Diagnosed patients are classified into high risk and standard risk groups based on their clinical and laboratory features. The intensity of the treatment varies among different subsets of children with ALL. Treatment of ALL is divided into three phases: remission induction, consolidation, and maintenance. The aim of the induction phase is to destroy as many leukemic cells as possible. High risk patients are put on a more intensive and toxic induction therapy regimen that includes a combination of anthracycline, usually doxorubicin or daunorubicin, which induce apoptosis by producing free radicals that damage cell membranes, proteins and lipids, glucocorticoids that arrest growth and induce apoptosis,

vincristine which inhibits formation of microtubules in mitotic spindle and arrests cell division, and L-asparaginase that halts cell division by inhibiting RNA and DNA synthesis<sup>14</sup>. In contrast, standard-risk patients undergo a modest therapy that does not include anthracyclines, in order to reduce unwanted toxicities<sup>4,15,16</sup>. By the end of remission induction, if patients have less than 5% of detectable lymphoid blasts, it is called complete remission (CR) and patients are then further classified into minimal residual disease (MRD) positive or negative. Patients with less than 0.01% lymphoid blasts (non-detectable) are classified as MRD negative, while those with 0.01%-5% lymphoid blasts are classified as MRD positive. Generally, end-of-induction MRD negative patients have excellent outcomes, while MRD positive patients have a poorer prognosis with increased likelihood of relapse<sup>17</sup>. When a complete remission is achieved, children undergo consolidation therapy, also known as the intensification phase, to eliminate the submicroscopic residual disease that may cause a relapse<sup>17,18</sup>. In this phase, the treatment is stronger and wider variety of drugs are used to prevent occurrence of drug resistance, including mercaptopurine, an inhibitor of nucleic acid synthesis, methotrexate which inhibits enzymes responsible for nucleotide synthesis, thioguanine blocks the utilization and synthesis of the purine nucleotides, cyclophosphamide which prevents DNA separation for synthesis or transcription, etoposide that induce apoptosis by halting DNA synthesis, and cytarabine that acts through direct DNA damage and incorporation into DNA<sup>14,16</sup>. An allogeneic bone marrow transplant is usually recommended to patients classified as very high risk at this phase<sup>17</sup>. Before the transplant, patients may receive either high dose chemotherapy or total body irradiation (TBI)<sup>17</sup>.

### 1.3 Hallmarks of Cancer

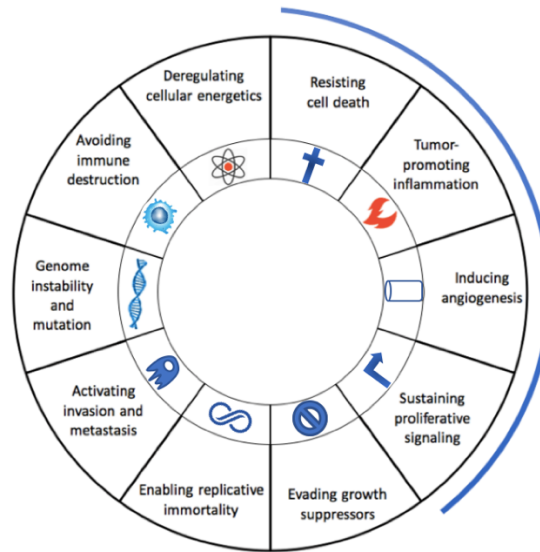
Hanahan and Weinberg's 2011 publication proposed ten hallmarks of cancers; *sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, genome instability and mutation, tumor-promoting inflammation, deregulating cellular energetics, and avoiding immune destruction* (Figure 1.1)<sup>19</sup>. Each of these hallmarks not only enables tumor growth and metastatic dissemination, but also provides the foundation required for the emergence of other hallmarks<sup>19</sup>.

Like other malignancies, T-ALL exhibits hallmark characteristics that include: activating mutations of NOTCH1 that are present in 60% of T-ALL<sup>20</sup>, constitutively active PI3K/AKT pathway that is mostly as a result of the PTEN inactivation<sup>21,22</sup> and less commonly due to gain-of-function mutations in PI3K subunits (~5% of T-ALL cases) or in AKT (~3% cases)<sup>22</sup>; mutations in IL7 receptor, JAK1, JAK3 and STAT5 which result in unusually active JAK/STAT pathway leading to uncontrolled cell proliferation<sup>23,24</sup>; and increased RAS/MAPK signaling via KRAS and NF1 mutations that promote disease progression<sup>25</sup>.

Also, mutations in tumor suppressor genes such as Lymphoid Enhancer Binding Factor 1 (LEF1), Wilms Tumor 1 (WT1), Runt Related Transcription Factor 1 (RUNX1) and GATA Binding Protein 3 (GATA3) contribute to the overall transcriptional deregulation of T-ALL<sup>26</sup>.

Another contributing factor that gives rise to multiple hallmarks of cancer is the tumor microenvironment<sup>19</sup>. Microenvironmental factors such as nutrient availability, chemokines, and growth factors and their receptors promote tumor growth by the activation of signal transduction pathways such as PI3K/AKT/mTOR signaling, AMPK signaling, and NFAT signaling. In leukemia, the stromal cells in the bone marrow provide anti-apoptotic and sustained proliferative

signaling. Therefore, understanding the interaction between cancer cells with their tumor microenvironment may facilitate development of more effective treatment options.



**Figure 1.1 Hallmarks of Cancer**

The ten hallmarks of cancer as adapted from Hanahan, D., and R.A. Weinberg. 2011. Hallmarks of cancer: the next generation. *Cell*. 144:646-674. Hallmarks marked in blue are considered as contributors to the tumor microenvironment.

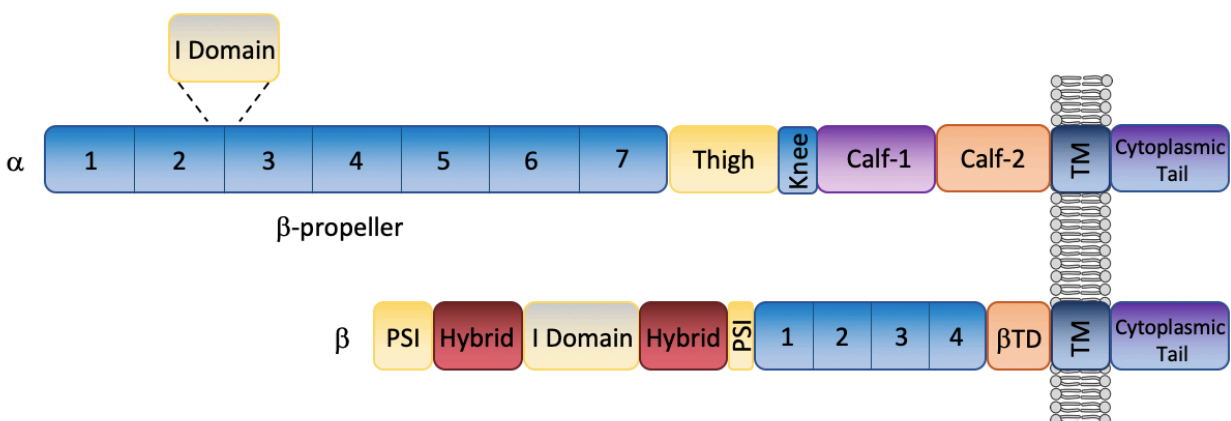
## 1.4 Integrins in Cancer

### 1.4.1 Integrin Structure and Signaling

Integrins are heterodimeric cell adhesion molecules that relay signals across the plasma membrane<sup>27-30</sup>. The  $\alpha$  and  $\beta$  subunits of integrins are non-covalently associated. There are 18  $\alpha$  and 8  $\beta$  subunits in vertebrates that can assemble into 24 different integrins specific for different ligands<sup>27,31</sup>. The  $\alpha$  subunit mainly defines the ligand specificity of the integrin<sup>30-34</sup>. Each subunit consists of a single membrane-spanning helix and a short unstructured cytoplasmic tail<sup>35-38</sup>. The extracellular subunits are composed of about 700 amino acids for  $\alpha$  and 1000 amino acids for  $\beta$

and form stalks with globular ligand-binding heads<sup>39</sup>. The ligand binding head consists of seven repeats of about 60 amino acids that fold into a seven-bladed  $\beta$  propeller structure<sup>40</sup>. Approximately half of the  $\alpha$  integrins and all  $\beta$  integrins have insertions, called the I-domain and I-like domain respectively, located within the  $\beta$  propeller, and directly participate in ligand binding<sup>41</sup>. I-domains contain a “metal ion-dependent adhesion site” (MIDAS) and I-like domains contain a similar metal-binding motif. Binding of a ligand to the integrin head alters the coordination of the metal ion and the shifts I-domain from a closed to an open conformation. This conformation change increases the affinity for ligand and strengthens adhesion<sup>41</sup>.

The cytoplasmic tails of integrins are less than 75 amino acids in length. There is a significant homology between the cytoplasmic tails of  $\beta$  integrins, while the cytoplasmic tails of  $\alpha$  integrins are highly diverse, except for the conserved GFFKR motif located next to the transmembrane domain. The GFFKR motif is essential for the association of  $\alpha$  and  $\beta$  integrin tails<sup>42</sup>. Several cytoskeletal and signaling proteins have been shown to bind  $\beta$  cytoplasmic tails and some proteins have been reported to interact with  $\alpha$  tails<sup>43</sup>.



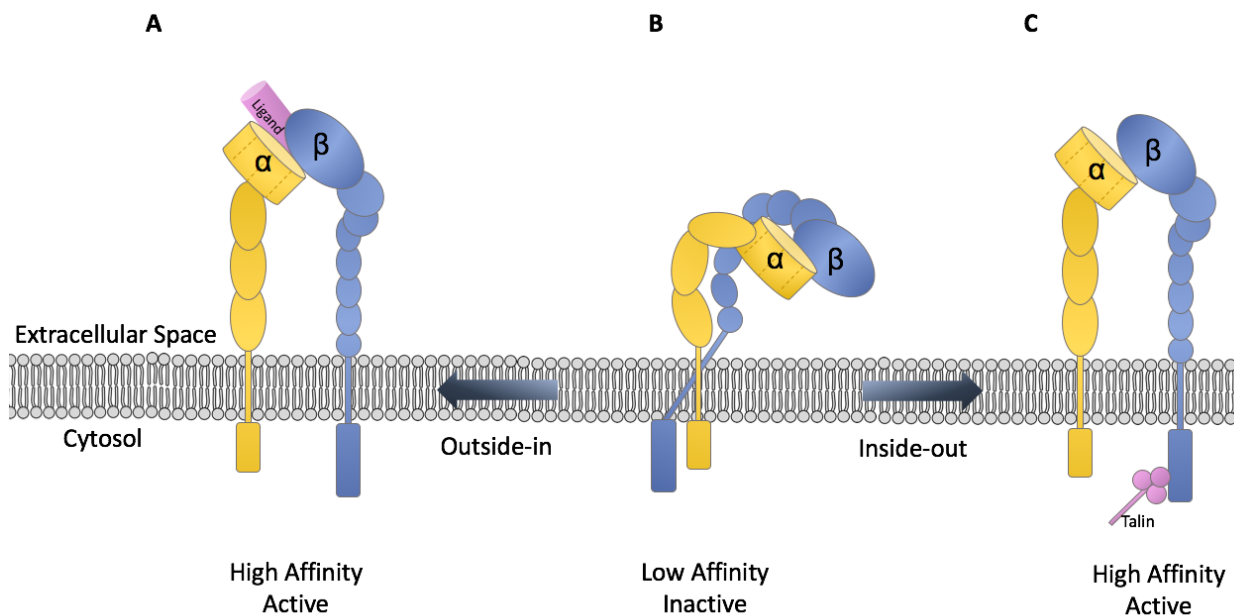
**Figure 1.2 Structures of Integrins**

Adopted from Zhang, K. and J. Chen, The regulation of integrin function by divalent cations. *Cell Adh Mig* 2012<sup>44</sup>. Integrins contain a short cytoplasmic tail, a transmembrane region, and a long extracellular domain.

Integrins act as bridges between the extracellular matrix (ECM) and the cytoskeleton, enabling cells to react appropriately to external signals by detecting changes in the environment<sup>32</sup>. Also, changes in the cytoskeleton and intracellular signaling can change integrins' affinity to ECM ligands such as fibronectin, collagen, laminin and vitronectin, which allows integrins to signal in both an inside-out and outside-in manner<sup>45</sup>. Integrin function depends on its activation status, which is related to its conformation. Inactive integrins are in a bent-closed conformation and can be activated to take on the high affinity ligand binding state by both the outside-in and inside-out signaling. During outside-in signaling, increased extracellular ligand concentration enhances the binding of integrins to these ligands and changes the conformation of the heterodimer from a bent to an extended conformation (Figure 1.3)<sup>46</sup>. This conformation change facilitates integrin binding to signaling proteins, leading to cell survival, proliferation and differentiation. During inside-out signaling, binding of an activator protein such as talin to the  $\beta$  integrin cytoplasmic tail leads to conformation shifts in the integrin dimer that result in higher ligand binding affinity, which promotes cell adhesion and migration<sup>47-51</sup>. The conformation change is also accompanied by snorkeling, piston-like insertion, of the entire GFFKR motif into the cytosol, and separation of the  $\alpha$  and  $\beta$  integrin transmembrane domains and the inner membrane clasp, which is a set of salt bridges on the cytoplasmic side of the transmembrane domain of the integrin  $\alpha$  and  $\beta$  subunits (Figure 1.4)<sup>45,52</sup>.

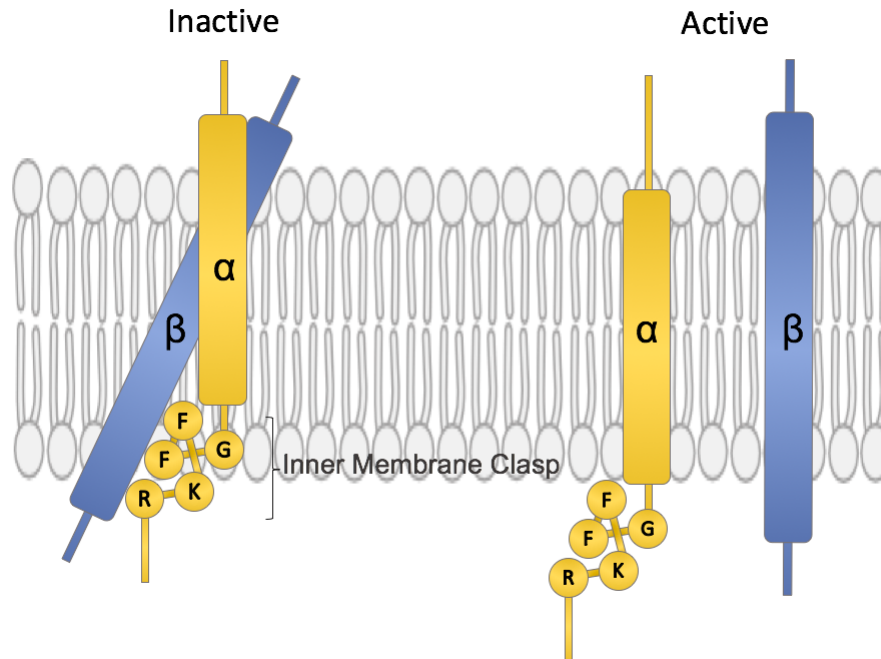
Some signaling proteins have been reported to interact specifically with the  $\alpha$  integrin tail via the GFFKR motif, including calreticulin (CRT), Sharpin, mammary-derived growth inhibitor

(MDGI), calcium and integrin-binding protein (CIB), and guanine nucleotide exchange factor MSS4 (Mss4). Dr. Shoukat Dedhar's laboratory first reported CRT as a GFFKR-binding protein in 1994<sup>53</sup>. CRT functions in several cellular mechanisms, such as regulation of calcium homeostasis, phagocytosis of apoptotic cells, and regulation of integrin-mediated adhesion<sup>54,55</sup>. Sharpin interaction with the GFFKR motif leads to inactivation of integrins and detachment of migrating cells<sup>56,57</sup>. Similarly, binding of MDGI to the  $\alpha$  integrin tail inhibits its activation<sup>56,58</sup>. Haataja *et al.* reported increased integrin-mediated cell adhesion upon binding of CIB to  $\alpha$  integrin<sup>59</sup>. Lastly, Mss4 regulates matrix metalloproteinase activation and fibronectin remodeling via interaction with GFFKR<sup>60</sup>.



**Figure 1.3 Model of integrin outside-in and inside-out signaling**

Adopted from C.C Liu thesis, 2016<sup>61</sup>. Inactive integrins (B) are in the bent-closed conformation. Integrins can be activated to take on the high-affinity ligand binding state by both (A) outside-in and (C) inside-out signaling of integrins.



**Figure 1.4 Structure of the integrin transmembrane domains**

Adopted from C.C.Liu thesis, 2016<sup>61</sup>. A schematic presentation of the integrin transmembrane domain in the inactive and active conformation. Activation of integrin leads to ‘Snorkeling’ of the  $\alpha$  integrin transmembrane domain, which shifts the GFFKR motif into the cytosolic space from its partially membrane-embedded inactivated form.

### 1.4.2 Integrin Contribution to Hallmarks of Cancer

In healthy cells, integrins regulate intracellular processes and enable multicellular life<sup>62</sup>. In cancer, they contribute to hallmarks of cancer, such as *uncontrolled and limitless proliferation, invasion of tumor cells, promotion of tumor angiogenesis, evasion of apoptosis and resistance to growth suppressors*.

The crosstalk between integrins and growth factor receptors gives rise to cancer cell proliferation<sup>63</sup>. For example, in ovarian cancer, binding of  $\alpha 5 \beta 1$  to fibronectin leads to phosphorylation and activation of c-Met, which further activates Src and FAK and promotes proliferation and invasion<sup>64</sup>. Also, binding of the oncogene MYC (v-myc avian



myelocytomatosis viral oncogene homolog) to the  $\alpha 1\beta 1$  promoter element enhances transcription of this integrin<sup>65</sup>. Moreover,  $\alpha v\beta 3$  and  $\alpha v\beta 5$  integrins are involved in tumorigenesis. In ovarian cancer, integrins  $\alpha v\beta 1$  and  $\alpha v\beta 3$  were shown to enhance proliferation via integrin-linked kinase (ILK) and blocking  $\alpha V$  was sufficient to arrest the cell cycle<sup>66</sup>.

To be able to invade, tumor cells have to acquire the ability to get rid of cell-cell contacts, cross the basal membrane, cross the stroma, enter the circulatory system, invade a new site, and colonize. The first step of cell invasion is epithelial-mesenchymal transition (EMT)<sup>67</sup>. The cytokine TGF- $\beta 1$  that induces EMT resides in the ECM in an inactive form. Inactive TGF- $\beta 1$  is bound to two latency-associated peptide (LAP) peptides and one of four latent TGF $\beta$  binding proteins (LTBP)<sup>68</sup>. Binding of  $\alpha v\beta 3$ ,  $\alpha v\beta 5$ ,  $\alpha v\beta 6$ ,  $\alpha v\beta 8$  and an unidentified  $\beta 1$  integrin to the tripeptide Arg-Gly-Asp (RGD)-motif of the LAP protein induces a conformational change and exposes TGF- $\beta 1$  to the adjacent cells<sup>68,69</sup>. Activated TGF- $\beta$  downregulates epithelial proteins like E-cadherin and upregulates mesenchymal proteins such as N-cadherin. It also enhances malignancy by upregulation of EGFR<sup>70</sup>. Also, tumor cells are able to migrate through ECM by modifying it. Overexpression of  $\alpha v\beta 6$  in ovarian cancer cells and squamous carcinoma cells resulted in increased expression of urokinase-type plasminogen activator (uPA), uPA-receptor (uPAR) and matrix metalloproteinases (MMP), such as MMP-2 and MMP-9<sup>71,72</sup>. The uPA-uPAR pathway activation leads to ECM degradation, tumor invasion, and angiogenesis<sup>73-75</sup>. Binding of uPAR to  $\beta 1$  and  $\beta 3$  integrins in tumor cells modulates their signaling<sup>76</sup>. Also, interaction of uPAR with the  $\alpha 5\beta 1$ -fibronectin complex mediates constitutive ERK1/2 activation<sup>77</sup>.

Another characteristic of cancer cells is their ability to proliferate indefinitely and evade the replicative limit of normal cells<sup>19</sup>. A possible mechanism that enables cancerous cells to replicate indefinitely is the expression of the DNA repair enzyme complex that induces

telomerase, an RNA-dependent DNA polymerase that maintains telomere length<sup>78,79</sup>. The telomerase reverse transcriptase subunit (hTERT), a regulator of telomerase activity, is downregulated upon  $\beta 1$  integrin mediated MMP-9 downregulation in glioblastoma cells<sup>80–82</sup>. Moreover, silencing hTERT via siRNA in cancer cell lines leads to downregulation of integrin  $\alpha V$  and leads to the inhibition of cell growth and proliferation<sup>83</sup>.

In the absence of functional blood vessels, tumors cannot exceed a diameter of 2 mm; therefore, they induce the formation of new vessels which facilitate tumor survival, tumor invasion, and metastasis<sup>84</sup>. The most important activators of angiogenesis are VEGF and FGF2 and their receptors, which promote mechanisms such as the remodeling of the ECM via MMPs, uPA, and migration and proliferation of endothelial cells to form new vessels<sup>85</sup>.  $\alpha v\beta 5$  regulates VEGF-mediated angiogenesis while  $\alpha v\beta 3$  and  $\alpha 5\beta 1$  regulate FGF-mediated angiogenesis<sup>86</sup>. Several integrins, including  $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$ ,  $\alpha 4\beta 1$ ,  $\alpha 5\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha 6\beta 4$ ,  $\alpha 9\beta 1$ ,  $\alpha v\beta 3$  and  $\alpha v\beta 5$ , are reported to regulate cell growth, survival, and migration during angiogenesis<sup>87</sup>.

Integrins are key players in anoikis, a phenomenon characterized by controlled cell death upon loss of cell-ECM attachment. Adhesion to ECM via integrins activates FAK-PI3K-AKT and FAK-MAP kinase pathways that prevent cell death<sup>88</sup>. In the absence of the ligand,  $\alpha 8\beta 1$  remains inactivated, thus the PI3K-AKT pathway is switched off<sup>89</sup>, and the integrin  $\beta 1$  and  $\beta 3$  subunits recruit and activate caspase-8, a protein involved in apoptosis, which leads to integrin-mediated cell death<sup>90</sup>. Cancer cells can avoid anoikis by downregulating caspase-8 via promoter methylation<sup>91</sup>.

Moreover, altered integrin expression can also regulate cell death. Upregulation of  $\alpha v\beta 3$  expression after ultraviolet B (UVB) exposure enables cell adhesion to the collagen-rich dermis, therefore enhancing melanoma cell adhesion and migration<sup>92</sup>. Also, hypoxia in brain capillaries

results in overexpression of  $\alpha 5\beta$  leading to active vascular remodeling and increased capillary density<sup>93</sup>.

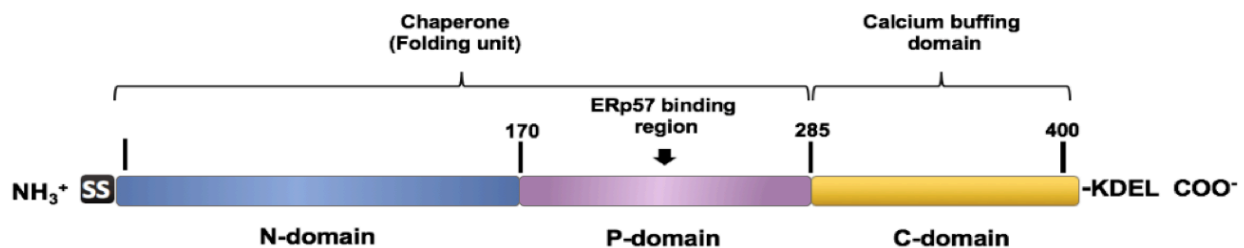
Cell adhesion to ECM enhances the drug resistance of tumor cells in hematological malignancies, a process known as cell adhesion-mediated drug resistance (CAMDR)<sup>94–96</sup>. Previously our lab demonstrated the involvement of CRT in integrin-dependent CAMDR<sup>97</sup>. In the next section, I will describe the many attributes of this ubiquitous protein with a diverse array of functions that is matched by its equally diverse subcellular and extracellular localization.

### **1.5 Calreticulin: One Protein, Many Functions**

CRT is a pleiotropic protein that normally resides in the lumen of the endoplasmic reticulum (ER)<sup>98</sup>. It has various functions in the ER, including regulation of calcium homeostasis and ensuring proper protein folding<sup>98,99</sup>. More recently, CRT gained special interest for its cytosolic and extracellular functions<sup>97,100,101</sup>.

CRT consists of three domains: the globular N-terminal domain, the proline-rich P domain and an acidic C-terminal domain<sup>102</sup>. The N-terminal domain (residues 1–180) is the most conserved domain among different species. It has no net charge and is involved in chaperone function and protein-protein interaction<sup>53,103</sup>. A cleavable signal sequence is located at the beginning of the N-domain, which guides the protein into the ER lumen following synthesis<sup>104,105</sup>. The P-domain (residues 181–280) is rich in the amino acid proline and contains a high affinity-low capacity binding site for calcium and a lectin-like chaperone site. The negatively charged C domain is rich in amino acids that give CRT a low-affinity ( $K_d \sim 2$  mM), high capacity ( $\sim 20$  mole calcium/mole CRT)  $\text{Ca}^{2+}$  binding activity<sup>98,106</sup>. This acidic domain terminates with the KDEL ER retrieval signal that binds to KDEL receptors in the Golgi apparatus, which recycle CRT back into the ER compartment (Figure 1.5)<sup>107,108</sup>.

Many studies have shown that CRT can also be detected in various cellular compartments other than the ER<sup>53,54,98,108,109</sup>. This multifaceted protein is implicated in many unexpected roles on the cell surface, in the cytosol, within the nucleus, and in the extracellular matrix. Also, a low level of CRT is detectable in human serum<sup>110</sup>.



**Figure 1.5 Structure of Calreticulin**

Adopted from Michalak, M., Groenendyk, J., Szabo, E., Gold, L. I. & Opas, M., Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum. *Biochem. J* 417, 651–666 (2009)<sup>102</sup>. Linear representation of CRT domains. The protein contains an N-terminal amino acid signal sequence (black box), N-domain (blue box), P-domain (purple box), C-domain (yellow box) and a C-terminal KDEL ER retrieval signal.

### 1.5.1 ER Functions of Calreticulin

ER is the main organelle for synthesis, folding, and transportation of secretory proteins. Molecular chaperones carry out these functions by facilitating correct protein folding and assembly. CRT, a lectin-like chaperone, is involved in quality control during synthesis of proteins such as surface receptors and transporters<sup>98,111–114</sup>. CRT in complex with calnexin (CNX) retains folding intermediates, misfolded glycoproteins, or partly assembled oligomers to

prevent their aggregation and assist their conformational maturation<sup>114–116</sup>. Moreover, CNX and CRT cooperate with ERp57, a protein disulfide isomerase, to correct disulfide bonding during the folding of glycoproteins<sup>117,118</sup>.

In addition to its chaperone function, CRT is one the most important  $\text{Ca}^{2+}$ -binding proteins in the ER and is considered an intracellular  $\text{Ca}^{2+}$  regulator since it contains two  $\text{Ca}^{2+}$ -binding sites within the P-domain (low capacity, high affinity), and an acidic C-domain (high capacity, low affinity)<sup>119–121</sup>. Since more than 50% of  $\text{Ca}^{2+}$  stored in the ER lumen is associated with CRT, higher levels of CRT may increase intracellular  $\text{Ca}^{2+}$  storage, while CRT-deficient cells have reduced capacity for  $\text{Ca}^{2+}$  storage in the ER lumen<sup>121–123</sup>. Studies on CRT-deficient mice showed defective cardiac development as a result of impaired  $\text{Ca}^{2+}$  homeostasis of CRT<sup>124,125</sup>.

### 1.5.2 Cytosolic and Nuclear Functions of Calreticulin

There is accumulating evidence for diverse roles of CRT localized in the cytosol and nucleus. A number of published studies have highlighted a calcium-dependent interaction between CRT and the GFFKR motif of the cytoplasmic tail of  $\alpha$  integrins<sup>126–128</sup>. The direct interaction appears to facilitate CRT-mediated cell adhesion and regulate surface CRT presentation<sup>97</sup>. Downregulation of CRT has been shown to decrease adhesive capacity and migration of cells through c-Src and calcium-calmodulin kinase II signaling<sup>52,128–132</sup>. Xu *et al.* reported that overexpression of cytosolic CRT in Myocardial Microvascular Endothelial Cells (MMEC) upregulates the expression of  $\alpha$  integrin and promotes interaction of CRT and phosphorylation of FAK, which then activates downstream pro-survival and pro-migration signal pathways<sup>52</sup>. CRT overexpression inhibits microwave radiation-induced MMEC injury by

decreasing cell apoptosis and lactate dehydrogenase (LDH) activity, enhancing cell migration capacity and preserving ultrastructure and cytoskeleton integrity<sup>52</sup>. Also, knock down of CRT leads to aggravated microwave-induced injury<sup>52</sup>.

Recent studies have identified functions for CRT in the nucleus. Here, CRT can act as a factor to regulate mRNA stability. CRT has been shown to bind the AU-rich region in 3'-UTR to destabilize type I angiotensin II receptor mRNA<sup>133</sup>. Totary-Jain *et al.* reported that CRT can also destabilize mRNA under high-glucose conditions by binding to a specific element in 3'-UTR of glucose transporter-1 mRNA<sup>134</sup>. Also, CRT has been identified as a receptor for nuclear protein export for glucocorticoid receptor (GR) and protein kinase inhibitor (PKI)<sup>135</sup>. The CRT-dependent export of GR occurs through the direct interaction of CRT with the KGFFKR sequence of GR<sup>135,136</sup>. However, a nuclear export signal (NES) is needed for export of PKI<sup>135,137</sup>.

### **1.5.3 Cell Surface Functions of Calreticulin**

Results from several laboratories have demonstrated that CRT on the surface of apoptotic tumor cells is recognized as the dominant pro-phagocytic signal and serves an important role for cancer immunity<sup>108,138–140</sup>. Drug treatment, in particular with anthracyclines, induces ER stress and leads to translocation of CRT to the cell surface, a key feature of immunogenic cell death<sup>141</sup>. Moreover, suppression of CRT in mice inhibits the anthracycline-induced phagocytosis by dendritic cells and curtails their immunogenicity<sup>139</sup>. It is the ratio of CRT (eat-me signal) to CD47 (don't eat-me signal) that sets the fate of the cell. During phagocytic clearance of dying cells, also known as efferocytosis, binding of CRT on the surface of a dying cell in *trans* to a LRP1 receptor expressed on macrophages, leads to engulfment of the apoptotic cell<sup>142</sup>.

Therefore, therapeutics that increase cell surface CRT exposure are a potential strategy to augment immune antitumor activity.

Landmark studies indicated that cell surface CRT on bovine aortic endothelial cells and fibroblasts interact with thrombospondin (TSP1), which then stimulates the association of CRT and LRP1 at the cell membrane, leading to focal adhesion disassembly that is essential for cell migratory process<sup>143–147</sup>. Formation of a CRT/TSP1/LRP1 complex activates signaling through PI3K-dependent FAK and ERK activation, resulting in cytoskeletal reorganization and loss of focal adhesions<sup>143,146,148,149</sup>. The molecular players involved in this pathway is different from that in efferocytosis, where the CRT/TSP1/LRP1 signaling complex acts in *cis* mode on the same cell. In addition, another study showed binding of TSP1 with the CRT/LRP1 complex confers resistance to anoikis by inhibiting apoptotic signaling via caspase 3 and PARP1<sup>150</sup>.

#### **1.5.4 Immunogenic Cell Death**

Immortality and the ability to avoid immunological surveillance are two important properties that allow tumor growth<sup>151</sup>. Thus, an effective therapeutic strategy is one that restores cancer cell susceptibility to death and enhances the immune recognition of these cells by leveraging immunogenic properties of cancer cell death<sup>152</sup>. Cancer cell death can be classified into immunogenic or non-immunogenic based on the initiating stimulus<sup>153</sup>. Apoptosis was initially considered to be immunologically silent, without any impact on the immune system, or tolerogenic, a phagocytic process invoking active immunosuppression against the initiating stimulus<sup>154,155</sup>. However, late apoptotic cells are rapidly recognized by phagocytic cells due to the presence of various “eat me” signals on their surface<sup>139</sup>. Recent studies indicate that some chemotherapeutic agents such as anthracyclines can induce an immunogenic type of

apoptosis<sup>139,156</sup>. Such drugs generate danger signaling through oxidative-ER stress<sup>157</sup>. Immunogenic apoptosis, aka immunogenic cell death (ICD), involves a composition change of the cell surface followed by the release of soluble mediators; therefore, it stimulates the immune system of the host and enhances immunological responses after the therapies<sup>158</sup>. These signals belong to a category called the damage-associated molecular patterns (DAMPs). The important DAMPs that collaboratively trigger immune responses include, but are not limited to, the exposure of CRT on the cell surface, and release of large amounts of HMGB1, ATP, and a number of heat shock proteins such as HSP27, HSP70 and HSP90<sup>153,159,160</sup>. During ICD, CRT translocate from the ER to the cell surface and act as “eat me” signals for phagocytes<sup>139,151</sup>, while ATP is released or secreted into the extracellular space by apoptotic cells and functions as “find me” signals<sup>161</sup>. Release of HMGB1 is required for the optimal presentation of tumor antigens to phagocytes<sup>162</sup>.

### **1.5.5 Translocation of Calreticulin from ER to Cell Surface**

Since CRT functions in multiple cellular locales, it needs to translocate from ER to its target locations, including cytoplasm and cell surface. In the ER, CRT and other chaperones are distinguished from other secretory proteins by a carboxyl terminus sequence of Lys-Asp-Glu-Leu (KDEL). This sequence retains these proteins in the ER, and the deletion of this sequence leads to the secretion of ER chaperones<sup>105,163</sup>. Chaperones bearing a KDEL motif can traffic protein between the ER, Golgi complex, intermediate ER-Golgi (ERGIC) complex, and Trans Golgi Network (TGN). These chaperones are returned to the ER via KDEL receptors expressed in the Golgi apparatus. However, many ER chaperones containing the KDEL motif can be detected outside of the ER and several mechanisms have been proposed for CRT translocation<sup>164–166</sup>.



Studies have shown that changes in the ER environment can influence the folding of ER proteins such as CRT, resulting in masking of the KDEL motif and release of CRT from ER<sup>167</sup>. The conformation change occurs in response to high concentrations of reactive oxygen species (ROS) that are produced by apoptotic cells in a high-stress environment<sup>167</sup>. Also, stress environments lead to changes in pH which can decrease the binding efficiency of the KDEL receptors and increase surface presentation of ER chaperones<sup>166</sup>. Panaretakis *et al.* have shown that a glycosylated form of CRT can translocate to the cell surface via Golgi anterograde pathway in multiple cell lines<sup>168</sup>.

Some studies suggest that CRT can translocate to the cell surface through retrotranslocation<sup>169</sup>. Usually, retrotranslocated proteins are marked as damaged and sent toward proteasomal degradation<sup>116,169</sup>. However, after retrotranslocation from the ER to the cytoplasm, CRT avoids ubiquitylation and degradation since it is not an efficient substrate for the proteasome<sup>169</sup>. The decrease in  $\text{Ca}^{2+}$  in the ER leads to translocation of CRT to the cytosol, where it can be modified by arginyl transferase. The attachment of an arginine residue to the N-terminal aspartic acid residue of the protein localizes CRT to cytoplasmic stress granules<sup>170</sup>. Other post-translational modification such as citrullination of CRT may also be important for its cell surface presentation<sup>170</sup>.

Previously, work from our lab using a T-lymphoblast cell line confirmed that CRT requires ERp57 for its translocation from the ER to the cytosol. Interestingly, ERp57 can also translocate to the cell surface in a manner that is independent of CRT<sup>171</sup>.

### **1.5.6 LRP1 as a Surface Receptor of Calreticulin**

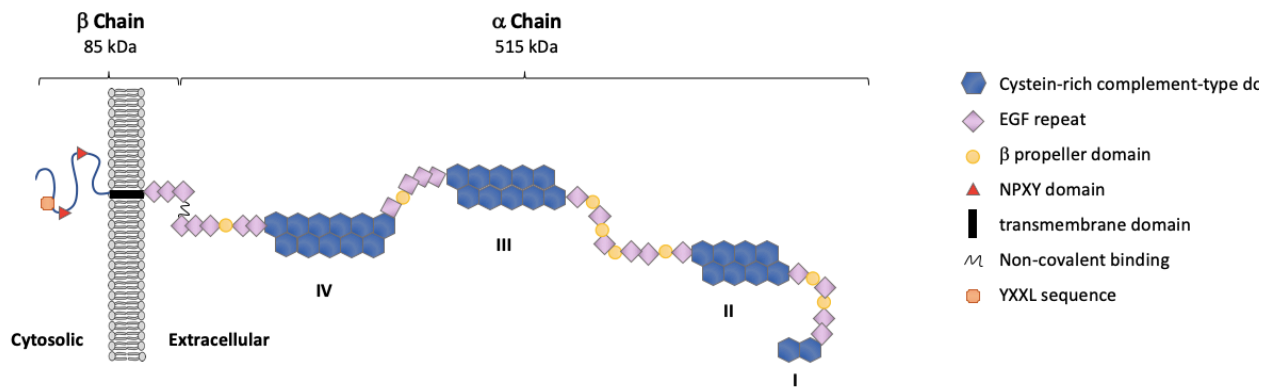
LDL receptor-related protein-1 (LRP1) is an endocytic and cell-signaling receptor, which has more than 100 ligands<sup>172,173</sup>. LRP1 was first described for its involvement in lipoprotein

metabolism<sup>174</sup> and later was shown to play diverse roles in various physiological and pathological processes, including degradation of proteases, activation of lysosomal enzymes, cellular entry of bacterial toxins and viruses, cholesterol homeostasis and cancer development<sup>173,175,176</sup>.

The protein consists of two non-covalently linked  $\alpha$  and  $\beta$  chains. The 515-kDa  $\alpha$  chain, entirely extracellular and responsible for most of the ligand-binding activity of the receptor, is connected to the cell surface by the 85-kDa  $\beta$  chain. The  $\beta$  chain has three domains: an ectodomain, a transmembrane domain, and an intracellular tail (Figure 1.6)<sup>177</sup>. The intracellular tail contains two NPXY motifs with the distal one overlapping an YXXL sequence. The NPXY is essential for recruiting molecular adaptors and signaling proteins, while YXXL is crucial to triggering endocytosis<sup>178</sup>. LRP1 limits ECM remodeling by mediating internalization and catabolism of several extracellular proteinases<sup>179,180</sup>.

The role of LRP1 in tumor progression is controversial. LRP1 was initially thought to prevent tumor aggressiveness because of its role in controlling extracellular proteolysis<sup>179</sup>. Also, studies on patients' samples confirmed the correlation between decreased expression of LRP1 and poor survival<sup>181–183</sup>. Other groups have reported that LRP1 supports invasion and survival of breast cancer and thyroid carcinoma<sup>184–187</sup>. Also LRP1 was shown to control the turnover of actin cytoskeleton and focal adhesion complexes in tumor cells<sup>184</sup>. In thyroid carcinoma cells, LRP1 ensures the proper distribution of paxillin and FAK in focal adhesions and optimizes cell adhesion and invasion by these cells by supporting ERK and inhibiting JNK pathways<sup>185</sup>. Moreover, Perrot *et al.* reported LRP1 as a key endocytic receptor for CD44, regulating the morphology of the tumor cell<sup>188</sup>.

LRP1 and CRT have been shown to interact in *cis* and in *trans* modes. The *cis* interaction of LRP1 and surface CRT was shown to stimulate focal adhesion disassembly by activating the PI3K and ERK pathways<sup>143</sup>. Also, there are numerous studies of *trans* interaction between CRT and LRP1 during efferocytosis<sup>138,139,142</sup>. However, little is known about the *cis* CRT-LRP1 interaction during ICD. Furthermore, no *cis*-acting receptor has been identified for surface CRT. Therefore, in this thesis I will investigate the requirement of LRP1 for surface presentation of CRT for cells undergoing ICD.



**Figure 1.6 Structure of mature low-density lipoprotein receptor-related protein-1 (LRP-1)**

Adopted from Emonard H, Théret L, Bennisroune AH, Dedieu S. Regulation of LRP-1 expression: Make the point. *Pathol Biol.* 2014;62(2):84-90. doi:10.1016/j.patbio.2014.02.002<sup>189</sup>. The transmembrane domain joins the extracellular heavy chain and intracellular light chain. The protein contains a short cytoplasmic tail and a long extracellular domain that consists of staggered arrangement of ligand-binding repeats, EGF-like repeats.

## 1.6 Somatic Calreticulin Mutation Associated with Myeloproliferative Neoplasms

Myeloproliferative neoplasms (MPNs) are a rare and heterogeneous group of hematological neoplasms with similar biology<sup>190</sup>. In MPNs, mutations originating within

hematopoietic stem cells lead to clonal amplification of certain myeloid progenitors, which ultimately results in excess production of mature and immature cells in either a single or multiple myeloid lineages. The classic MPNs include polycythemia vera, essential thrombocythemia, and primary myelofibrosis. MPNs start out as benign tumors, but they can transform into either myelodysplastic syndromes or acute myeloid leukemias<sup>191,192</sup>.

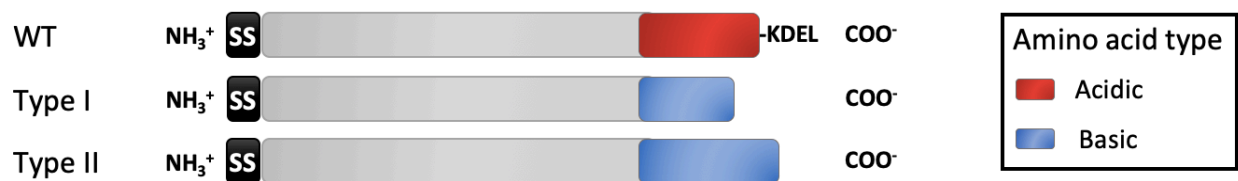
Genomic studies have identified multiple recurrent and mutually exclusive somatic mutations in myeloproliferative neoplasms, including *CRT*, Janus kinase 2 (*JAK2*), and thrombopoietin receptor (*MPL*) mutations. 70-84% of MPN patients who are both *JAK2* and *MPL* negative, carry somatic mutations of *CALR*, the gene encoding for *CRT*<sup>101,190</sup>. The *JAK2V617F* gain-of-function mutation can be found in around 95% of polycythemia vera patients, approximately 50% of essential thrombocythemia patients, and 65% of primary myelofibrosis patients<sup>193</sup>. Taken together, after *JAK2* mutation, somatic mutation of *CALR* is the most commonly occurring marker for MPNs<sup>101,190</sup>.

All somatic *CALR* mutations happen in exon 9; these insertion or deletion mutations cause a frameshift and result in a new C-terminal domain for the mutated *CRT* protein<sup>101,190</sup>. Type I and type II *CALR* variants are the most frequently occurring ones, resulting from a 52-base pair deletion and a 5-base pair insertion, respectively (Figure 1.7)<sup>190</sup>. The *CRT* proteins encoded by these two variants have an altered C-terminus; therefore, such protein lacks the KDEL ER retention motif and the acidic  $\text{Ca}^{2+}$  binding C-terminal domain is largely replaced by a polypeptide that is basic and hydrophobic. Importantly, a large proportion of negatively charged amino acids is replaced with positively charged amino acids, abolishing proper  $\text{Ca}^{2+}$ -binding<sup>194</sup>.

Interestingly, the MPN-associated activities of mutated *CRT* are related to the loss of the negatively charged C-terminus. Araki and his group suggested a mechanism by which mutated

CRT induces proliferation of myeloid cells leading to MPN. CRT facilitates the activation of the JAK/STAT signaling pathway downstream of myeloproliferative leukemia protein (MPL), aka thrombopoietin cytokine receptor<sup>195</sup>. Loss of the negatively charged tail results in interruption of the N-P domain interaction and exposure of the N-domain for interaction with the JAK2-MPL protein complex<sup>195</sup>. The complex of mutated CRT, JAK2, and MPL constitutively activates JAK2, downstream phosphorylation, and activation of STAT proteins in a manner independent of thrombopoietin as a cytokine<sup>195</sup>.

Additionally, Arshad *et al.* showed that the acidic C-terminus of CRT is required to recruit it to the peptide loading complex of MHC-I for peptide binding<sup>196</sup>. They found that MPN-related mutant CRTs are nonfunctional, as the decrease in surface MHC-I in CRT<sup>-/-</sup> cells was not reversed by expression of either MPN type I or type II mutant CRTs<sup>196</sup>.



**Figure 1.7 Somatic mutation of CRT**

Adopted from Imai, M., Araki, M. & Komatsu, N. Somatic mutations of calreticulin in myeloproliferative neoplasms. *Int. J. Hematol.* 105, 743–747 (2017)<sup>197</sup>. Protein structure models depicting the overall folding of WT CRT, Type I and Type II mutant. The C-terminus are color coded.

### 1.6.1 Enrichment of Mutated Calreticulin in the Nucleus and Extracellular Matrix

Studies have shown that MPN-mutated CRT is secreted and functions as a pseudo-cytokine<sup>195,198</sup>. Secreted mutant CRT has been shown to induce the proliferation of myeloid cells

by acting as a cell surface cytokine for MPL activation in an autocrine manner, leading to activation of JAK/STAT signaling pathway<sup>195,198</sup>. Araki *et al.* reported a model by which the CRT P-domain blocks the interaction of MPL with the N-domain; however, in mutant CRT the inhibitory function of the P-domain is lost, and CRT can interact with the extracellular domain of MPL, resulting in activation of downstream signaling pathways<sup>195</sup>.

Garbati *et al.* showed that HEK and HeLa cells expressing type I and type II mutant CRT have significantly reduced total CRT expression (intracellular CRT) compared to wildtype CRT<sup>199</sup>. These cells had similar *CALR* mRNA levels; however, mutant CRT was enriched in the nucleus and in conditioned media compared to wildtype. This phenotype is likely attributed to loss of the KDEL ER-retention motif<sup>199</sup>. In non-hematopoietic cells, expression of mutant CRT does not directly activate JAK/STAT signaling compared to *JAK2-V617F* expression. Interestingly, monocytes cultured in media conditioned by cells expressing mutated CRT produced more TNF- $\alpha$  compared to normal cells with or without treatment with a toll-like receptor agonist, suggesting a paracrine effect of cells expressing mutant CRT<sup>199</sup>.

## 1.7 Hypotheses and Aims

Adhesion of leukemic cells to components of the bone marrow through integrin cell adhesion receptors mediates drug resistance that may contribute to relapse of pediatric ALL. In addition, CRT has been shown to bind to cytosolic GFFKR motif of  $\alpha$  integrins, and cell adhesion stimuli increases this interaction. Therefore, I hypothesize that cytosolic CRT, via its interaction with the GFFKR motif of  $\alpha$  integrins, is a mediator of cell adhesion. The work outlined in this thesis aims to characterize the role of subcellular localized CRT in integrin-mediated cell adhesion. Such mechanistic insights improve our understanding of the molecular

underpinnings of tumour-stromal interactions that support tumour growth such that they may be targeted therapeutically for improved outcomes. A secondary aim of this thesis investigated the requirement of LRP1 as a receptor for cell surface CRT.

## **2. Materials and Methods**

### **2.1 Materials**

#### **2.1.1 Cells and Cell Culture**

The human T-ALL cell line, Jurkat clone E6-1, was obtained from American Type Culture Collection (ATCC). CRT<sup>-/-</sup> and ERp57<sup>-/-</sup> Jurkat-derivative cells were generated in the Lim laboratory by Dr. C-C Liu using CRISPR-Cas9 methodology<sup>97,171</sup>. All cells were cultured at 37°C, 5% CO<sub>2</sub> in complete RPMI (RPMI 1640 [R8758; Sigma- Aldrich] supplemented with 10% fetal bovine serum [FBS], penicillin-streptomycin and nonessential amino acids [Gibco, ThermoFisher Scientific]).

The HEK293 cell line was obtained from Dr. Christopher Maxwell (BCCHRI). The HEK-based CRT<sup>-/-</sup> cell line was generated by Dr. C-C Liu and Dr CJ Lim using CRISPR-Cas9 methodology. All cells were cultured at 37°C, 5% CO<sub>2</sub> in complete DMEM (Dulbecco's Modified Eagle's Medium - high glucose [D5796; Sigma-Aldrich] supplemented with 10% fetal bovine serum [FBS], penicillin-streptomycin and nonessential amino acids [Gibco, ThermoFisher Scientific]).

#### **2.1.2 CRISPR-Cas9 Generation of LRP1<sup>-/-</sup> Cells**

The LRP1<sup>-/-</sup> cells were generated by CRISPR-Cas9 methodology<sup>200</sup>. The guide RNA sequence targeting the first coding exon for LRP1 GGGGCCTCGTCAGATCCGTCTGG (reverse) was ligated into the Bbs1 site of pX458 (Addgene #48138). Jurkat cells were nucleoporated and clonally sorted into 96-well dishes. LRP1<sup>-/-</sup> clones were confirmed by sequencing of the targeted genomic loci. Clone hLRP1 2-5 was used to generate the data presented in this thesis.



### **2.1.3 Plasmids**

GFP-CRT is an N-terminal Green Fluorescent Protein (GFP) fusion to human CRT (amino acids 18-417)<sup>201</sup>. ssGFP-CRT, ssGFP-CRT-KDELdel, ssGFP-CRT-Mutant were generated by Dr. C-C Liu<sup>61</sup>. To reconstitute the ER-targeting function, the signal sequence (ss) of CRT (amino acids 1-17) was fused to the N-terminus of GFP-CRT, producing ssGFP-CRT. To generate ssGFP-CRT-KDELdel, essentially ssGFP-CRT without the C-terminal KDEL ER-retention sequence, a stop codon was inserted prior to the KDEL coding sequence. The ssGFP-CRT-mutant was subcloned using fragments generated by PCR of ssGFP-CRT that was ligated to double stranded oligonucleotides encoding the MPN-related type I mutant CRT.

### **2.1.4 Recombinant Proteins and Fibronectin**

Glutathione S-transferase (GST)-tagged proteins were purified from BL21 Escherichia coli lysates by affinity chromatography through glutathione sepharose (GE Healthcare) according to the manufacturer's instructions. GST-CS1 is GST fused to fibronectin CS1 region<sup>202</sup>. Fibronectin from human plasma was purified in house by affinity chromatography through gelatin sepharose (GE Healthcare).

### **2.1.5 Antibodies**

The following antibodies were used for flow cytometric labeling of cell surface proteins: CRT (D3E6) from Cell Signaling, GFP (FM264G) from BioLegend, and Goat Anti-Rabbit (DyLight 650) from Thermofisher.

Antibodies used for immunoblotting are: CRT (FMC75) from Enzo; CRT (ab2907), GFP(GF28R) from Abcam; CRT (D3E6), P44/42 MAPK (T202/Y204), phospho-p44/42 MAPK, phospho-Ser473 Akt (D9E), phospho-Thr308 Akt (C31E5E), Akt pan (40D4) from Cell Signaling; GAPDH (FF26A/F9) from BioLegend; and Goat Anti-Rabbit (Alexa Fluor Plus 800),

and Goat Anti-Mouse (Alexa Fluor Plus 800 from Invitrogen).

### **2.1.6 Services**

Flow cytometry work was conducted at the BC Children's Hospital Research Institute (BCCHRI) Flow Core Facility (BD FACS Aria and LSRFortessa) or at the Michael Cuccione Laboratories at BCCHRI (BD Accuri). Post-acquisition analysis was done using FlowJo (Tree Star).

## **2.2 Methods**

### **2.2.1 Cell Transfection**

HEK cell transfections with CRT constructs were performed using Lipofectamine 3000 reagent according to manufacturer's instructions. Cell transfections of CRISPR-Cas9 plasmids were performed using Opti-MEM (Gibco) by nucleofection using the program X-001 on the Amaxa Nucleofector. Routinely, transfection used  $2 \times 10^6$  cells in 100  $\mu$ L of Opti-MEM with 2500 ng of plasmids. Cells stably expressing the desired receptor levels were sorted. Cells with LRP1 knockout by CRISPR-Cas9 were cloned in single cell suspension and identified through flow cytometry and sequencing for the lack of LRP1.

### **2.2.2 Preparation of Adhesion Substrates**

Routinely, tissue culture dishes (Corning Costar) were incubated with 5  $\mu$ g/mL Fibronectin and 20  $\mu$ g/mL of GST- CS1, and bovine serum albumin (BSA) in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ , 8 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.4 (Sigma)) overnight at 4°C. Following 2 washes with PBS, the coated dishes were ready for

seeding with cells.

### **2.2.3 Cell Adhesion Assay**

Some adhesion assays were done with cells pre-labeled with CellTracker<sup>TM</sup> Green CMFDA (Invitrogen), according to manufacturer's instructions. Cells are seeded at  $1 \times 10^5$  cells/well in substrate-coated 96 well plates, and incubated at 37°C for 45 minutes. Fluorescence readings were acquired with an Enspire spectrophotometer before and after 3 washes with PBS. Background fluorescence from the incubation medium was subtracted from each reading and the percent adhesion was calculated as follows:  $100 \cdot (\text{Fluorescence after washes} / \text{Initial fluorescence})$ .

### **2.2.4 Western Blot Analysis**

Cell lysates were routinely prepared in PN-buffer (5 mM PIPES, 25 mM NaCl, 75 mM sucrose, 25 mM NaF, 20 mM  $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$ , 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 1% Triton X-100, complete protease inhibitors (Roche). For phospho-Akt analysis, Jurkat cells were serum-starved in 0.5% FBS/RPMI for 24 hours before plating on 6-well substrate-coated plates for up to 60 minutes. For phospho-ERK analysis, Jurkat cells were serum-starved in 0.5% FBS/RPMI for 24 hours before plating on 6-well substrate-coated for up to 60 minutes.

For Western blot analyses, SDS-PAGE separated proteins were transferred onto nitrocellulose membranes (BioRad) and incubated with primary and IR-dye conjugated secondary antibodies (Pierce and Rockland). Blots were imaged on an Odyssey Imaging System (LI-COR).

### **2.2.5 Stimulation of Cell Surface Calreticulin**

Cells were serum-starved in 0.5% FBS in RPMI for 24 hours prior to drug treatments.

Routinely,  $1 \times 10^5$  cells/mL in 0.5% FBS/RPMI were treated with or without 3  $\mu\text{g/mL}$  doxorubicin (Tocris Bioscience) for 4 hours, at 37° C. Cell surface CRT levels were measured by flow cytometry analysis of  $\alpha$  CRT antibody-labeled cells using 650nm excitation. Only the non-apoptotic population was gated for analysis using Geometric Mean calculation.

### **2.2.6 Statistical Analysis**

The unpaired 2 tail Student's t-test was used to calculate p-values. Error bars shown are the standard deviation values obtained from at least 3 treatment replicates conducted within an experiment. Each experiment is representative of similar results obtained in at least 2 independently conducted experiments.

### **3. Involvement of Calreticulin in Adhesion of HEK Cells**

#### **3.1 Overview and Rationale**

CRT is essential for integrin-mediated calcium signaling and cell adhesion.<sup>130</sup> Cytosolic CRT binds to the GFFKR sequence of the  $\alpha$  integrin tail in a  $\text{Ca}^{2+}$ -dependent manner<sup>128</sup> and this may enhance the adhesive potential of cells by stabilizing the integrin-ligand complex, activating the integrin-linked kinase, and enhancing the adhesion of cells for fibronectin<sup>52</sup>.

HEK293 is a cell line derived from human embryonic kidney and has been extensively used as an expression tool for recombinant proteins since it was generated over 25 years ago. They are one of the most common cell lines used for research, second only to HeLa cells. Their popularity is due to ease of growth and transfection. The biochemical machinery of HEK cells can carry out most of the post-translational folding and processing required to generate mature and fully functional protein and they are very efficient at protein production. Since our lab had successfully generated a HEK293-based CRT<sup>-/-</sup> cells, I utilized this cell line to stably re-express various wildtype and mutated forms of CRT to study the involvement of subcellular localized CRT in cell adhesion.

#### **3.2 Re-expression of Different CRT Constructs in HEK CRT<sup>-/-</sup> Cells**

To determine the source of CRT involved in adhesion, I used multiple CRT constructs tagged with green fluorescent protein (GFP) for visualization (Figure 3.1). GFP-CRT is a GFP-tagged CRT construct that lacks the N-terminal ER-targeting signal sequence found in full length CRT, therefore, GFP-CRT is expected to be expressed only in the extra-ER cytosolic compartment<sup>203</sup>. The ssGFP-CRT construct reproduces the 17-amino acid ER-targeting signal

sequence at the N-terminus that directs the synthesis of ssGFP-CRT into the ER lumen<sup>204</sup>. The ssGFP-CRT-KDELdel construct is similar to ssGFP-CRT except that the terminal KDEL motif is deleted (Figure 3.1). This protein cannot engage KDEL receptors that retrieve KDEL-containing proteins from the Golgi apparatus back to the ER, thus promoting its exit from the ER<sup>205</sup>. ssGFP-CRT-mutant type I reproduces the alternate C-terminal reading frame identified as the causative somatic mutation in patients with myeloproliferative neoplasms (MPN) (Figure 3.1)<sup>101,190</sup>. Importantly, CRT-mutant type I's alternate C-terminus also lacks the KDEL ER retention motif<sup>101,190</sup>. Garbati *et al.* showed that mutant CRT does not accumulate efficiently in cells and is enriched in extracellular space, inducing monocyte hyperreactivity to toll-like receptor agonists through a paracrine mechanism<sup>197</sup>.



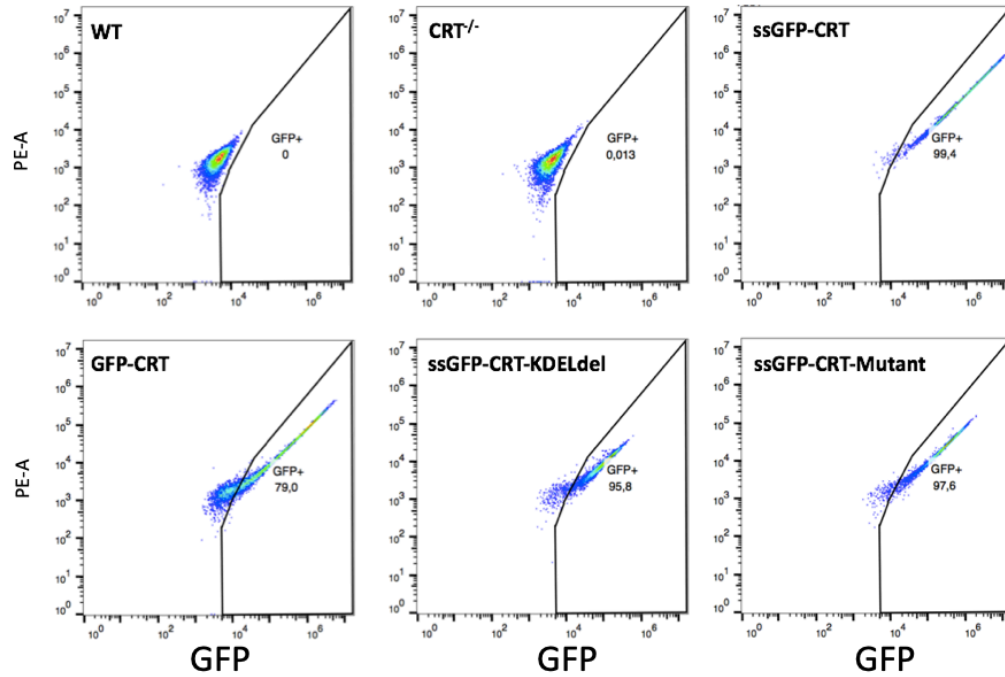
**Figure 3.1 Schematic of variant CRT constructs compared with the wildtype**

Adopted from C.C Liu thesis, 2016<sup>61</sup>. Wildtype CRT is shown with the N-terminal 17 amino acid signal sequence (ss) and the C-terminal KDEL ER-retention motif. GFP-CRT lacks the signal sequence and is targeted to the cytosol. ssGFP-CRT, ssGFP-CRT-KDELdel and ssGFP-CRT-mutant type I are ER-targeted due to the signal sequence. ssGFP-CRT-KDELdel and ssGFP-CRT-mutant type I do not contain the ER-retention motif. The yellow box shows the alternate C-terminal reading frame for CRT associated with myeloproliferative neoplasms (type I mutant). GFP: green fluorescent protein.

I transfected a clonal derivative of HEK CRT<sup>-/-</sup> cells with the various CRT constructs.

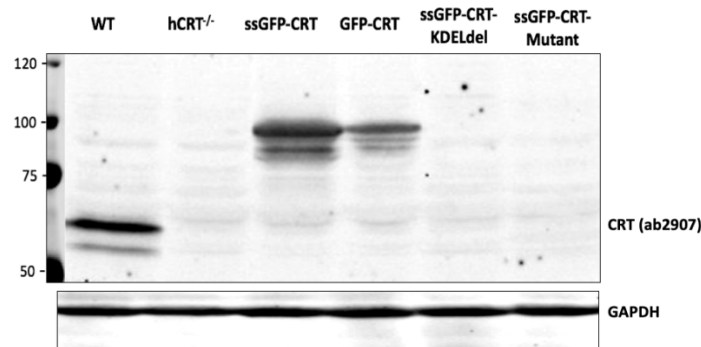
Cell lines were sorted for GFP positivity for 4 months until stable cell lines expressing GFP-tagged CRT proteins was established (Figure 3.2). However, western blot labeling for CRT using ab2907, a polyclonal antibody, did not detect CRT in lysates of cells expressing ssGFP-CRT-KDELdel and ssGFP-CRT-mutant (Figure 3.3). Subsequently, I used two different CRT antibodies, D3E6 and FMC75, both of which are monoclonal antibodies raised against residues near the N-terminus of human CRT protein. Both D3E6 and FMC75 were able to detect CRT in ssGFP-CRT-KDELdel and ssGFP-CRT-mutant cell lysates; however, the proteins were smaller than the expected molecular weight, suggesting they may have been post-translationally truncated (Figure 3.4 A, B). Using a GFP-specific antibody, I confirmed that the detected proteins contained the GFP moiety, suggesting they were N-terminal portions of GFP-CRT (Figure 3.4C). Rather surprisingly, the truncated forms of ssGFP-CRT-KDELdel and ssGFP-CRT-mutant were very different in molecular weights, suggesting the proteolytic modification events were not identical between the two proteins despite much of their N-terminal sequences being identical.

In addition, overexpression of CRT can be observed in ssGFP-CRT, GFP-CRT and ssGFP-CRT-Mutant. The levels of protein expression are slightly different using different CRT antibodies, D3E6 and FMC75. However, it is yet to be confirmed whether the cleaved C-terminal fragments of ssGFP-CRT-noKDEL and ssGFP-CRT-Mutant remain expressed in the cells, since the available commercial antibodies do not detect these domains.



**Figure 3.2 Example of flow cytometry gating scheme for analysis of GFP+ cells**

The healthy population is gated and it is analyzed for GFP fluorescence to distinguish between cells stably expressing GFP-tagged CRT (GFP<sup>+</sup>) and cells that do not (GFP<sup>-</sup>). X-axis is FL1 for GFP, while y-axis is FL2 for autofluorescence.

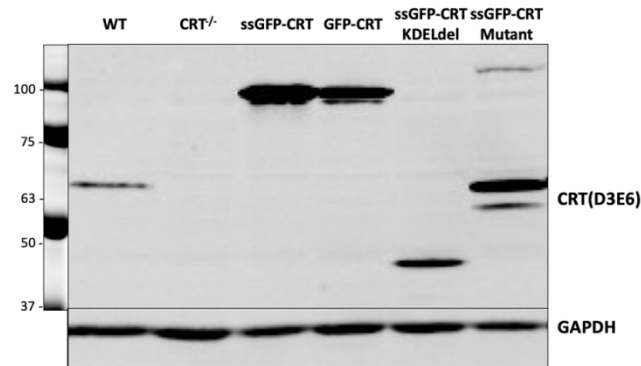


**Figure 3.3 Western blot detection of CRT expression in HEK cells using ab2907**

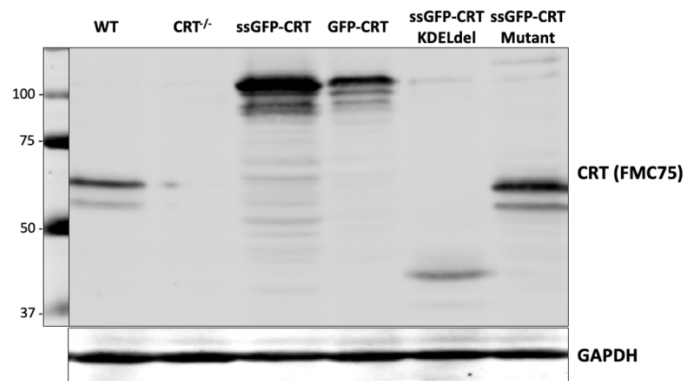
Whole cell lysates of WT, CRT<sup>-/-</sup>, ssGFP-CRT, GFP-CRT, ssGFP-CRT-KDELdel and ssGFP-CRT-mutant cells were assayed by Western blotting for expression of CRT (ab2907) and GAPDH. The results shown are representative of more than 3 experiments.



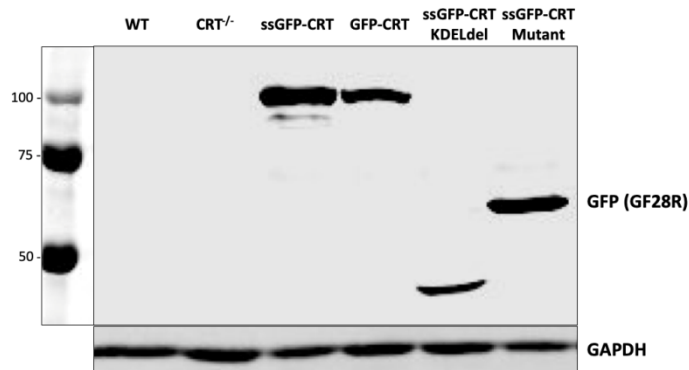
A



B



C



**Figure 3.4 Western blot detection of GFP-CRT expression in HEK cells using N-terminal targeted CRT antibodies and a GFP antibody**

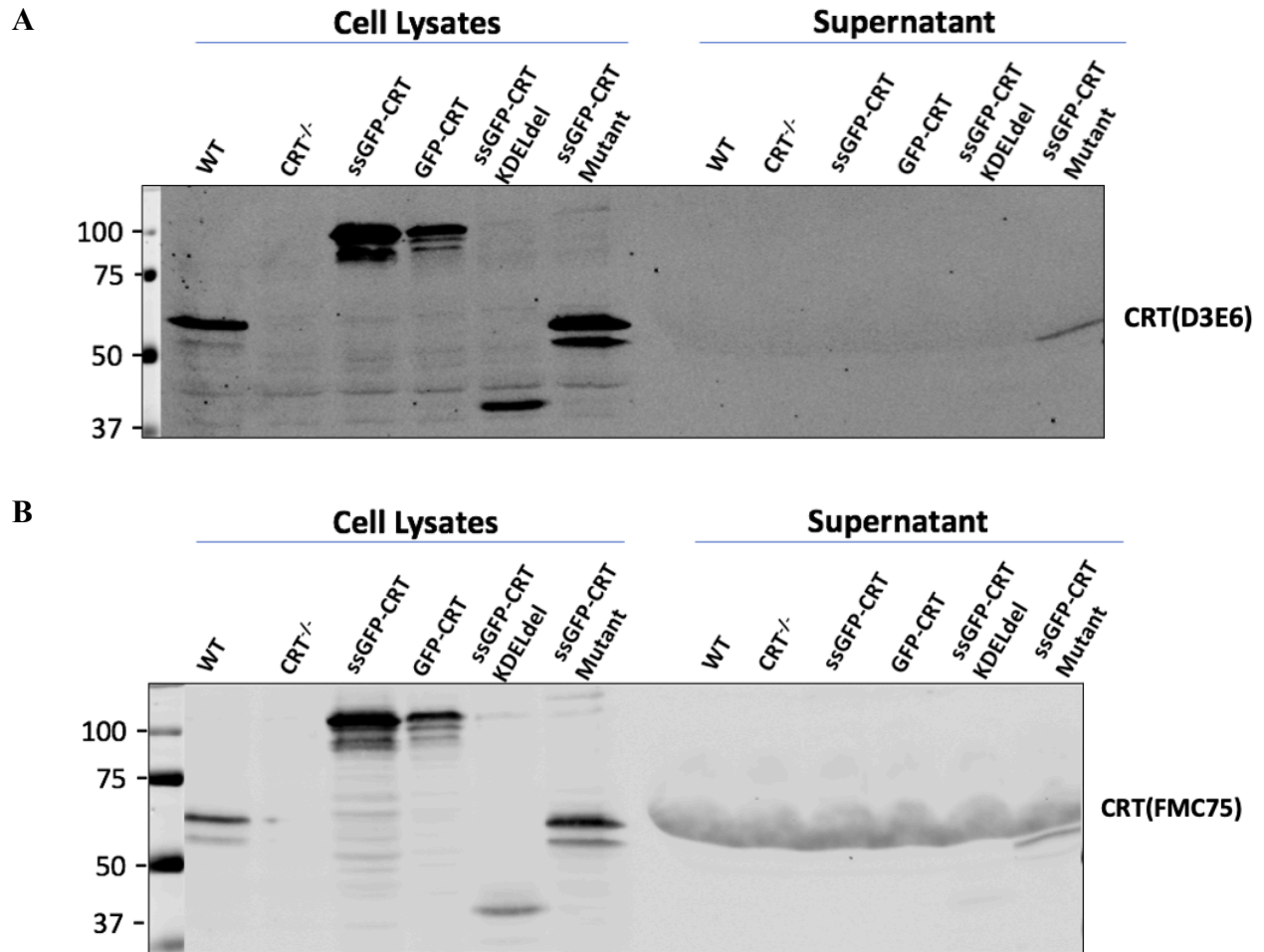
Comparison of total CRT expression in lysates of WT, CRT<sup>-/-</sup>, ssGFP-CRT, GFP-CRT ssGFP-CRT-KDELdel and ssGFP-CRT-mutant cells by western blotting (A) using CRT(D3E6) antibody, (B) using CRT(FMC75) antibody, and (C) using GFP(GF28R) antibody. Equal protein loading was assessed by immunoblotting for GAPDH. The results shown are representative of

more than 3 experiments.

### **3.3 Calreticulin Expressed Without the KDEL Motif Leads to its Secretion**

Previous studies have shown that CRT-Mutant type 1 is secreted out of the cells due to the novel C-terminus<sup>197,199</sup>. To see the fate of these proteins when expressed in HEK cells, I collected and immunoblotted cell-free supernatant for CRT using D3E6 and FMC75 antibodies. A secreted form of CRT was detectable in each of the supernatants of cells expressing ssGFP-CRT-KDELdel or ssGFP-CRT-mutant, but not of cells expressing ssGFP-CRT or GFP-CRT (Figure 3.5). The detected form of secreted CRT is identical in molecular weight to the truncated protein detected in the corresponding cell lysates.

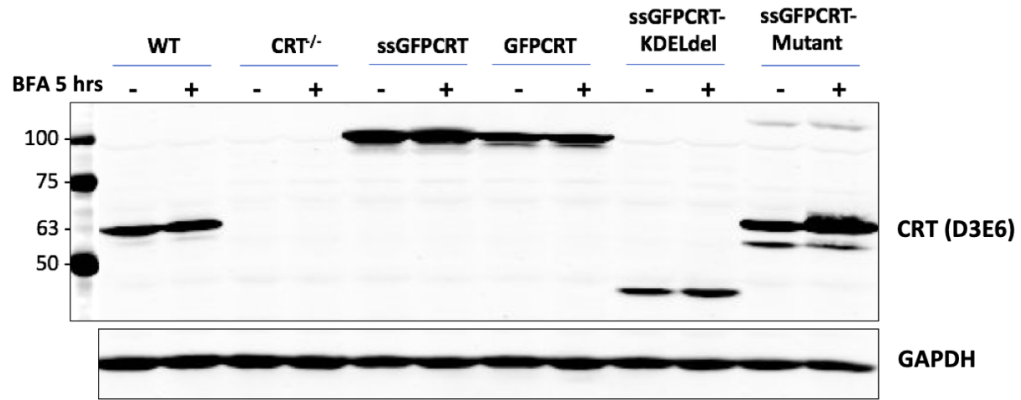
To see if their secretion could be prevented by blocking a protein secretory pathway, I treated cells with Brefeldin A (BFA), an inhibitor that interferes with anterograde transport from the endoplasmic reticulum to the Golgi. Treatment with 0.6 µg/ml BFA for 5 hours resulted in enrichment of Mutant-CRT and KDEL-CRT in the cell lysates (Figure 3.6A,C), with a corresponding decrease in CRT levels found in the cell-free supernatants (Figure 3.6B,D). When probed with a GFP antibody, I found that BFA-treated ssGFP-CRT-mutant cell lysates also showed increased retention of CRT, suggesting the secreted version is likely to have maintained its GFP tag (Figure 3.6E).



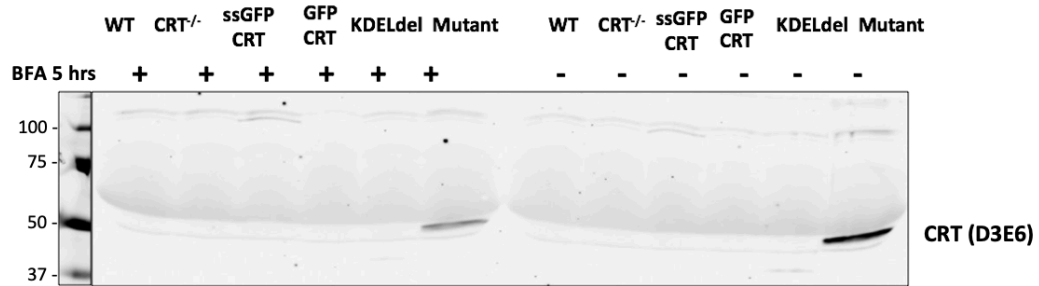
**Figure 3.5 Western blot detection of CRT in cell-free supernatant**

Comparison of total CRT expression in both cell lysates and supernatant of WT, CRT<sup>-/-</sup>, ssGFP-CRT, GFP-CRT ssGFP-CRT-KDELdel and ssGFP-CRT-mutant cells by Western blotting using (A) CRT (D3E6) antibody and (B) CRT (FMC75) antibody. The results shown are representative of more than 3 experiments.

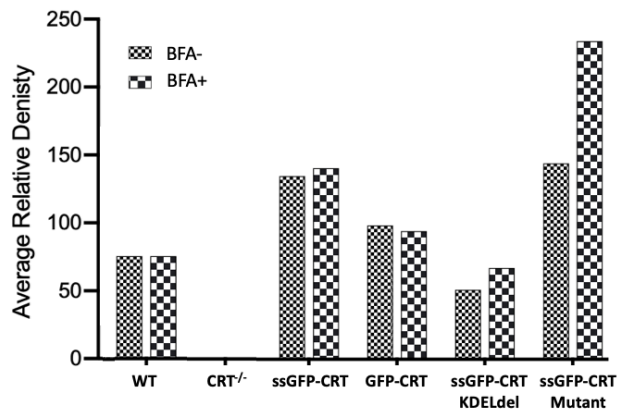
A



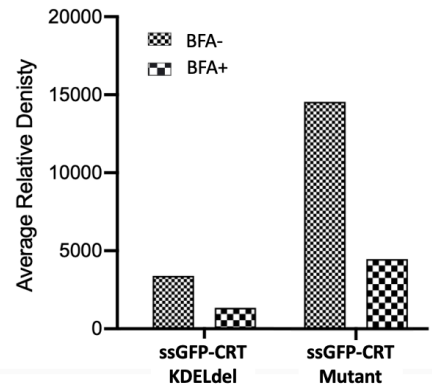
B



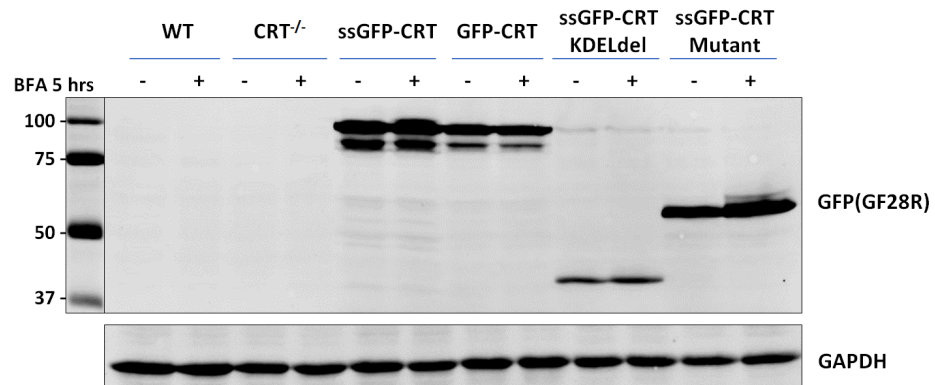
C



D



E

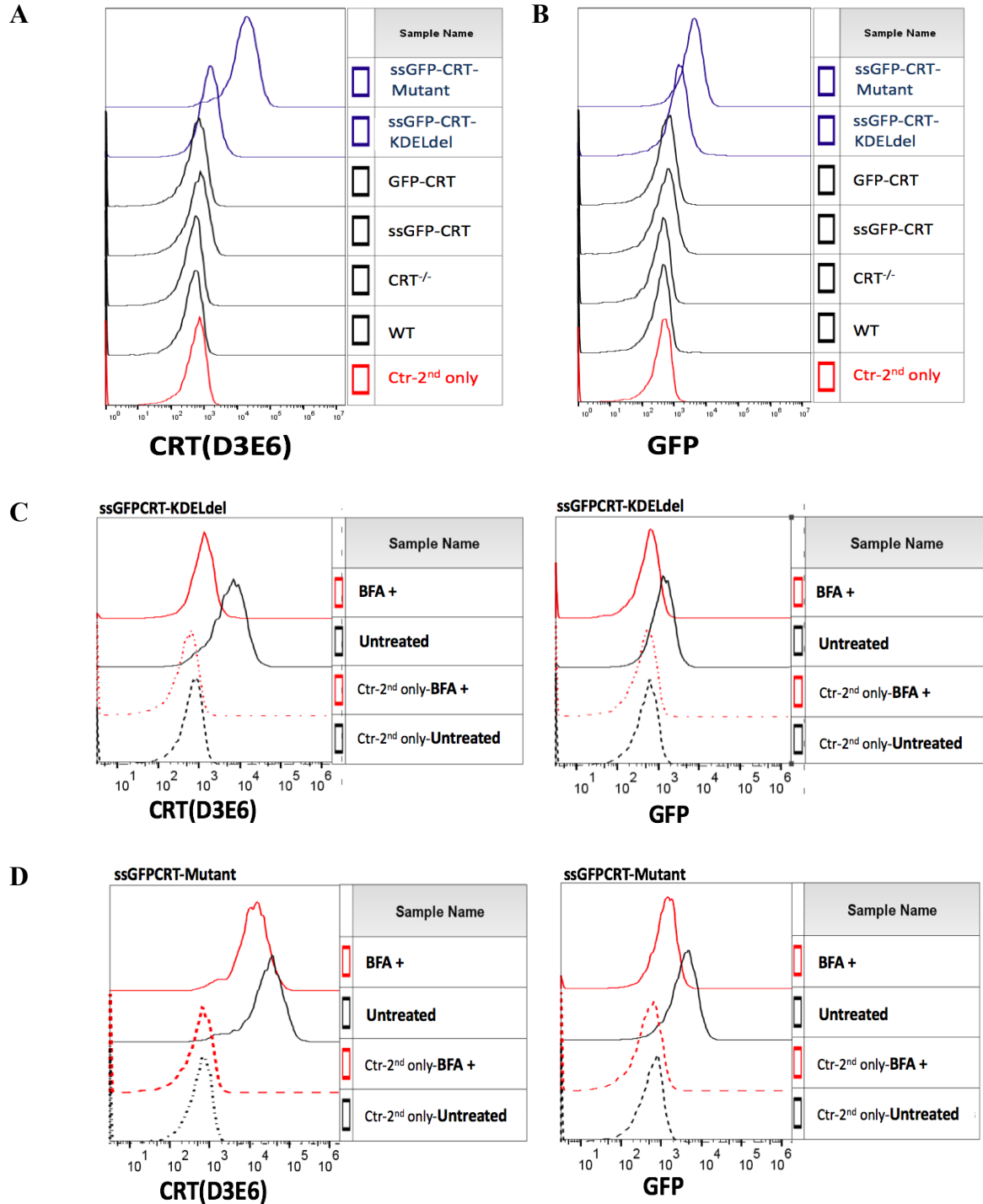


### **Figure 3.6 Effect of BFA treatment on localization of ssGFP-CRT-KDELdel and ssGFP-CRT-mutant proteins**

Comparison of (A) total CRT expression in lysates and (B) cell-free supernatants of WT, CRT<sup>-/-</sup>, ssGFP-CRT, GFP-CRT ssGFP-CRT-KDELdel and ssGFP-CRT-mutant cells by western blotting after treatment with 0.6 µg/ml BFA using CRT(D3E6) antibody. (C) Densitometry quantification of CRT signals of (C) figure 3.6A and (D) figure 3.6B (signals in figure 3.6C are normalized against GAPDH). (E) Comparison of total GFP-CRT expression in lysates using GFP (GF28R) antibody. Equal protein loading was assessed by immunoblotting for GAPDH.

### **3.4 Cell Surface Calreticulin**

Given that cell surface CRT could arise from a mechanism that involves CRT secretion, I assessed the HEK cell lines for surface CRT expression using flow cytometry. Rather surprisingly, I found that both the secreted forms, ssGFP-CRT-KDELdel and ssGFP-CRT-mutant, could be detected as a cell surface antigen (Figure 3.7A). By performing the same analysis using an antibody targeting GFP, I confirmed that the surface CRTs were GFP-tagged (Figure 3.7B). I also found that ssGFP-CRT-KDELdel and ssGFP-CRT-mutant cells treated with BFA exhibited reduced surface GFP-CRT when detected with either CRT or GFP antibodies (Figure 3.7C), indicating that a similar pathway involved in extracellular secretion is also involved in cell surface translocation. Taken together, my results indicated that calreticulin expressed without the KDEL ER-retention motif in HEK cells leads to its presentation as a cell surface antigen as well as its secretion to the extracellular space. In addition, these proteins were also subjected to proteolytic modification.



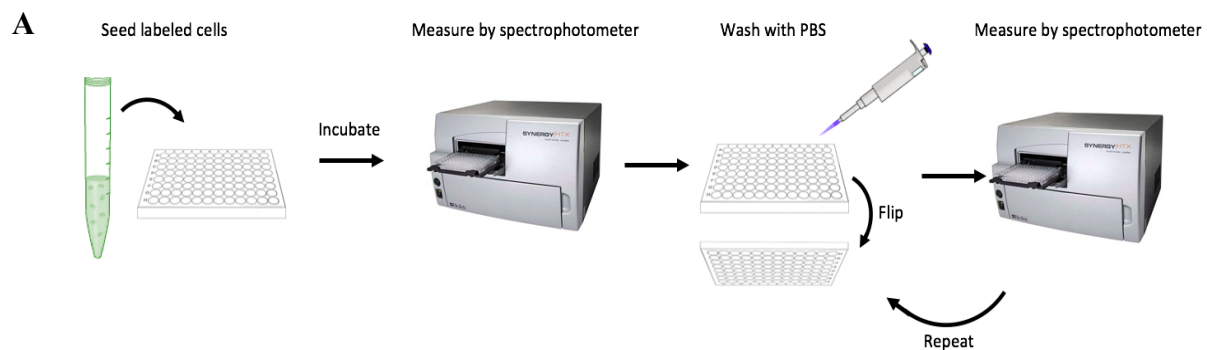
**Figure 3.7 Surface expression of ssGFP-CRT-KDELdel and ssGFP-CRT-mutant**

Flow cytometry was used to determine the cell surface expression of GFP-CRT in HEK cell lines using (A) CRT antibody D3E6 and (B) GFP antibody. (C) ssGFP-CRT-KDELdel and (D)

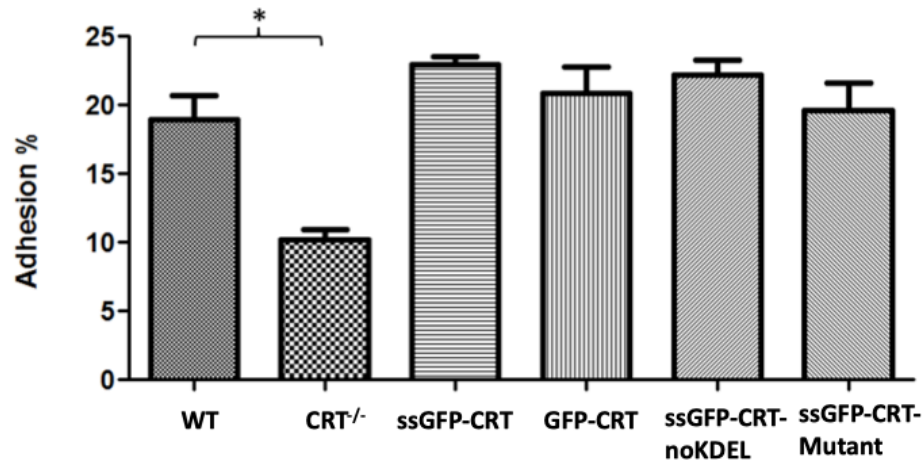
ssGFP-CRT-mutant cell lines were treated with and without 0.6  $\mu\text{g/ml}$  BFA for 5 hours, and surface CRT or GFP assessed. The data includes overlays with untreated (Black) and BFA treated (Red).

### 3.5 Involvement of Calreticulin in Adhesion of HEK Cells

To determine if CRT and its expression isoforms mediate cell adhesion, I conducted a static adhesion assay with WT, CRT<sup>-/-</sup>, ssGFP-CRT, GFP-CRT, ssGFP-CRT-KDELdel and ssGFP-CRT-mutant HEK cells. The cells were labeled using the fluorescent dye, CellTracker Green, and then seeded on tissue culture plates that were pre-coated with Fibronectin or BSA. BSA is used as a control substrate that will not engage integrins, while fibronectin can engage at least ten different integrins<sup>202,206</sup>. The plates were incubated at 37°C for 1 hour and the adhesion index was measured by spectrophotometer after serial washes with PBS (Figure 4.8A). CRT-deficient cells showed reduced adhesion to fibronectin while all ssGFP-CRT, GFP-CRT, ssGFP-CRT-KDELdel and ssGFP-CRT-mutant cells were able to rescue the CRT<sup>-/-</sup> adhesion defect to WT-like levels (Figure 4.8B).



**B**



**Figure 3.8 CRT-deficient cells have reduced adhesion to integrin substrate**

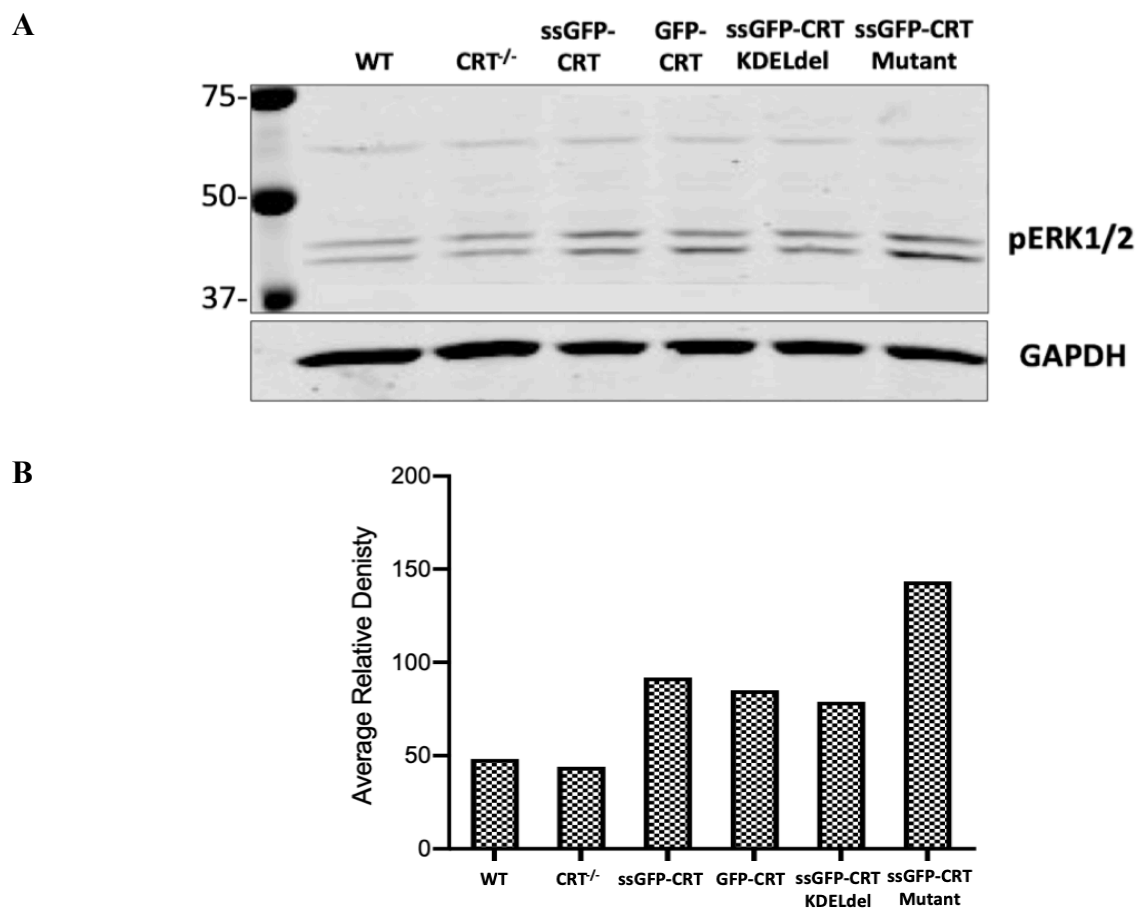
(A) Schematic of the adhesion assay procedure. Cells were labeled with either CellTracker<sup>TM</sup> Green, followed by seeding on dishes coated with fibronectin for 1 hour. Fluorescence readings were acquired with an Enspire spectrophotometer before and after 5 washes with PBS. (B) Background fluorescence from the incubation medium and cells was subtracted from each reading, and the percentage of total cells that adhered was calculated. The bars show the mean  $\pm$  S.D.;  $n=3$ ;  $*p<0.05$

### 3.6 Involvement of Calreticulin in MAPK ERK pathway

Outside-in activation of integrins leads to the activation of multiple intracellular signaling events via the recruitment of signaling proteins to integrin containing adhesion complexes. Some of the most important downstream signaling events that occur upon cell adhesion are phosphorylation of FAK<sup>207,208</sup>, Src-mediated tyrosine phosphorylation of adhesion proteins<sup>196</sup> and stimulation of the MAPK/ERK cascade<sup>209–212</sup>. A large body of evidence now indicates the involvement of ERK in adhesion-mediated signaling. Integrin engagement leads to activation of v-Src,<sup>201,202</sup> which then activates ERK<sup>209,210,212,213</sup>. Moreover, ERK activity has been implicated in ECM-dependent cell migration<sup>214,215</sup>.



To determine if CRT is involved in integrin mediated MAPK/ERK activation, I seeded cells on integrin substrate and performed Western blot analysis to detect phosphorylated ERK as an indicator of ERK activation. Phospho-ERK1/2 levels were not changed for CRT<sup>-/-</sup> cells seeded on fibronectin compared to WT. However, all the rescues had higher phospho-ERK1/2 levels compared to that of WT and CRT<sup>-/-</sup>, suggesting that overexpressing CRT may increase integrin mediated ERK phosphorylation (Figure 3.9).



**Figure 3.9 Effect of Calreticulin Overexpression on ERK phosphorylation**

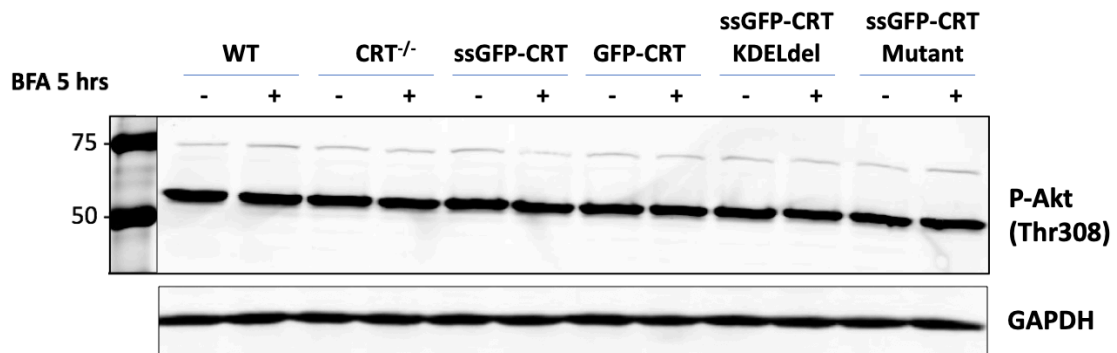
(A) WT, CRT<sup>-/-</sup>, ssGFP-CRT, GFP-CRT, ssGFP-CRT-KDELdel, ssGFP-CRT-Mutant cells were seeded on dishes coated with Fibronectin for 30 mins, and cell lysates were immunoblotted to detect phospho-ERK1/2 levels. Equal protein loading was assessed by immunoblotting for

GAPDH. (B) Densitometry quantification of pERK1/2 signals shown in A, signals are normalized over GAPDH.

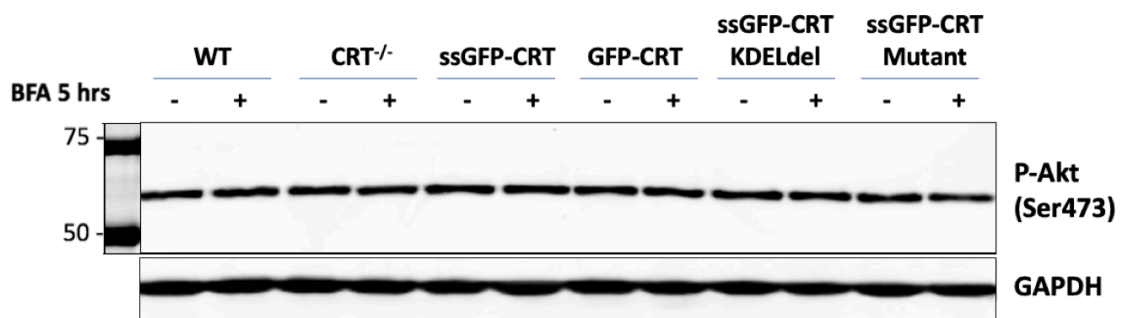
### 3.7 Involvement of Calreticulin in AKT pathway

Integrin-mediated cell adhesion can also mediate the activation of Akt<sup>216</sup>, which promotes cell survival and increased cell proliferation<sup>217</sup>. To assess whether cytosolic CRT is involved in integrin mediated Akt activation, HEK cells were seeded on an integrin substrate, fibronectin, and when applicable cells were treated with BFA to enrich for CRT in the cytosol and prevent its secretion. Western blot analysis was performed to detect phosphorylated Akt as an indicator of Akt activation. WT, CRT<sup>-/-</sup>, ssGFP-CRT, GFP-CRT, ssGFP-CRT-KDELdel, ssGFP-CRT-Mutant cells had comparable levels of both phospho(T308)-Akt and phospho(S473)-Akt, suggesting that CRT is not involved in adhesion-mediated Akt activation (Figure 3.10).

**A**



**B**



### **Figure 3.10 Cytosolic Calreticulin is not involved in integrin-mediated Akt activation**

WT, CRT<sup>-/-</sup>, ssGFP-CRT, GFP-CRT, ssGFP-CRT-KDELdel, ssGFP-CRT-Mutant cells were seeded on dishes coated with Fibronectin for 30 mins and when applicable, treated with 0.6 µg/ml BFA, and cell lysates were immunoblotted to detect (A) phospho(T308)-Akt and (B) phospho(S473)-Akt levels.

## **3.8 Discussion**

Cell adhesion is necessary for all aspects of cell growth, differentiation and migration. Cellular adhesion molecules (CAMs) are involved in several cellular functions such as cellular communication, embryogenesis, signal transduction, and apoptosis<sup>218</sup>. It has been shown that CRT interacts with the cytoplasmic tail of  $\alpha$  integrin and this interaction may influence integrin-mediated cell adhesion<sup>126,219</sup>. This chapter addresses the involvement of subcellular localized CRT in integrin-mediated cell adhesion in HEK cells. Using CRT<sup>-/-</sup> reconstituted with ssGFP-CRT, GFP-CRT, ssGFP-CRT-KDELdel, or ssGFP-CRT-Mutant, I confirmed the involvement of CRT in cell adhesion. All four cell lines rescued with the various constructs showed CRT expression and were able to rescue the adhesion defect observed in CRT<sup>-/-</sup> cells (Figure 3.8). This included GFP-CRT, a form predicted to be located in the cytosol in a manner that bypasses its transit through the ER. Interestingly, both ssGFP-CRT-KDELdel and ssGFP-CRT-Mutant were able to rescue the adhesion phenotype despite expressing CRT that appears to be truncated. It remains unclear if the rescued adhesion seen in these cells is mediated by the N-domain segment of CRT that I was able to detect, or by the remaining segment that might be expressed in the cell but not detectable using our antibodies (Figure 3.8 B). Using antibodies targeted to different domains of CRT, we can confirm if other pieces of ssGFP-CRT-noKDEL and ssGFP-CRT-Mutant are still presented in the cell<sup>220</sup>. It is important to note that all the rescues have

overexpression of CRT which may lead to higher levels of CRT in the cytosol, thus the adhesion index of these cells are slightly higher than that of the WT. In addition, I was not able to distinguish between adhesion of ssGFP-CRT and GFP-CRT as both exhibited similar adhesion to fibronectin. I suspect that this is due to overexpressed ssGFP-CRT resulting in their increased cytosolic accumulation when recycling KDEL receptors become saturated.

Studies have shown cell surface accumulation and secretion of mutant CRT<sup>195,198</sup>. I found that both ssGFP-CRT-KDELdel and ssGFP-CRT-Mutant resulted in CRT secretion. BFA treatment, which blocks the anterograde transport from the ER to the Golgi apparatus and leads to the collapse of Golgi stacks and accumulation of proteins in the ER<sup>221,222</sup>, enhanced the amount of ssGFP-CRT-Mutant and ssGFP-CRT-KDELdel in the whole cell lysates and prevented their secretion to the supernatant. I showed accumulation of cell surface CRT on ssGFP-CRT-KDELdel, ssGFP-CRT-Mutant, which could be reduced by blocking secretory pathways using BFA. This increase was detectable with either CRT or GFP antibodies, indicating that the translocation involved the fusion protein.

Surface CRT serves as the “eat me” signal and is essential for the clearance of apoptotic cells by the immune system mediated by phagocytes<sup>100,151,223</sup>. It is yet to be shown whether ssGFP-CRT-KDELdel and ssGFP-CRT-Mutant expressed on cell surface is sufficient to stimulate phagocytosis of these cells.

Several studies have established that integrin engagement is required for adhesion-mediated activation of ERK<sup>209–213,224,225</sup> downstream of v-Src activation<sup>226,227</sup>. I sought to determine whether cytosolic CRT is involved in activation of ERK signaling in HEK cells. Increased phosphorylation of ERK was seen in cells expressing ssGFP-CRT, GFP-CRT, ssGFP-CRT-KDELdel, and ssGFP-CRT-Mutant when compared to WT or CRT<sup>-/-</sup> cells, suggesting a

possible role for cytosolic CRT in ERK activation. In contrast, CRT over-expression did not affect AKT activity in these cells, suggesting the effect may not impact upon all classically activated pathways of integrin adhesion signaling.

## **4. Involvement of Calreticulin in Adhesion of Jurkat T-ALL Cells**

### **4.1 Overview and Rationale**

A critical factor in cancer recurrence is the development of chemotherapeutic resistant forms of the tumour<sup>49</sup>. Interaction between ALL cells and their microenvironment plays a critical role in leukemia cell survival and progression. Tumor cell adhesion to the extracellular matrix (ECM) via integrins contributes to minimal residual disease, patient relapse and development of chemoresistance<sup>49</sup>. Overexpression of several integrins in patient ALL has been shown to correlate with poor survival outcome<sup>228,229</sup>. Interaction between the cytosolic tail of  $\alpha$  integrins and CRT has been studied by several groups<sup>48,126,129,230</sup>. Work from our lab showed that integrin-mediated cell adhesion enhances the interaction between  $\alpha$  integrin and CRT, and that CRT may play a role in integrin-driven cell adhesion-mediated drug resistance (CAMDR)<sup>231</sup>. When plated to engage integrin substrates and treated with drug, CRT<sup>-/-</sup> cells experienced significantly higher apoptosis compared to WT cells<sup>61</sup>. Also, work described in chapter 3 combined with results from several groups suggests involvement of cytosolic CRT in cell adhesion. In this chapter, I sought to determine if CRT is involved in leukemic cell adhesion using Jurkat cells as a T-lymphoblast model. In addition, I also generated an LRP1<sup>-/-</sup> cell line to assess if LRP1 may function as a cis-acting receptor for cell surface CRT.

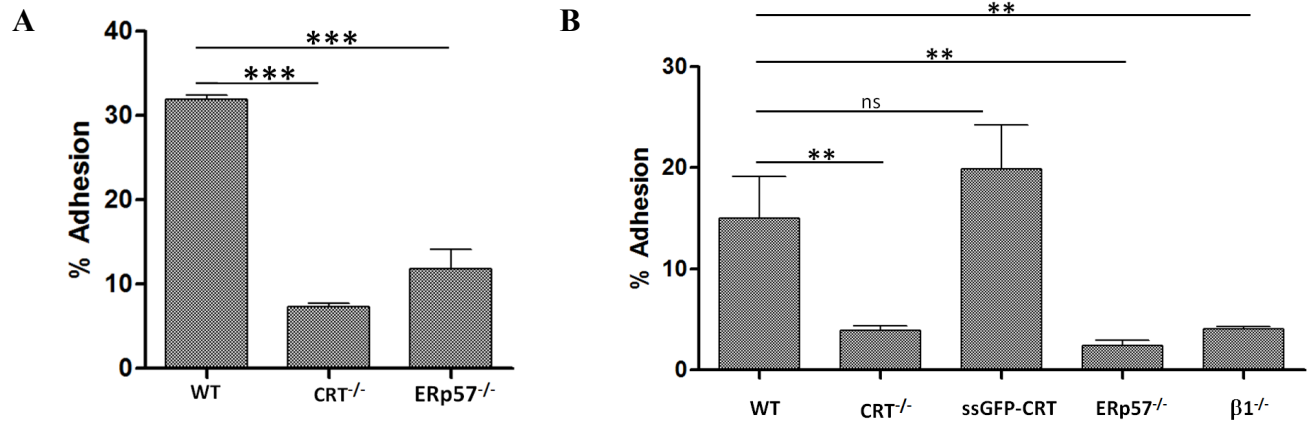
### **4.2 Knocking out Calreticulin Reduces Cell Adhesion of Jurkat T-lymphoblasts**

Jurkat is a T-lymphoblastic cell line established from the peripheral blood of a 14-year-old male with T-ALL<sup>232</sup>.

Jurkat cells are used as a model for T-ALL and a powerful genetic model to study various T cell functions. Our laboratory has a collection of Jurkat derivative cell lines that are deficient

in specific protein expression, including CRT, ERp57 and  $\beta$ 1-integrin. Therefore, the work reported in this chapter utilizes Jurkat lymphoblasts as a genetic and biochemical signaling model for the functional study of CRT in cell adhesion of T-ALL.

To determine if CRT is involved in cell adhesion, Jurkat cells were labeled using cell tracker green and seeded on tissue culture plates that were pre-coated with the integrin substrates fibronectin or GST-CS1 (glutathione S-transferase fusion with CS1). Fibronectin is used as a substrate able to engage multiple integrins, while CS1 is a polypeptide fragment derived from fibronectin that specifically engages integrins  $\alpha$ 4 $\beta$ 1<sup>202,206</sup>. The plates were incubated at 37°C for 45 minutes and the cell adhesion was measured by spectrophotometer after serial washes with PBS. Herein, WT refers to the parental Jurkat strain, CRT<sup>-/-</sup> refers to the calreticulin-null strain and ERp57<sup>-/-</sup> the ERp57-null strain. The calculated adhesion index shows that CRT<sup>-/-</sup> cells have reduced adhesion to both fibronectin and GST-CS1 compared to WT (Figure 4.1). Interestingly, ERp57<sup>-/-</sup> cells also have reduced adhesion to both adhesion substrates and it is comparable to that of the CRT<sup>-/-</sup> (Figure 4.1). We have shown earlier that ERp57<sup>-/-</sup> cells have depleted levels of CRT in the extra-ER cytosol<sup>171</sup>, suggesting the involvement of cytosolic CRT in cell adhesion. Re-expression of CRT (as ssGFP-CRT) in CRT<sup>-/-</sup> cells was able to rescue the adhesion defect observed in CRT<sup>-/-</sup> cells on GST-CS1.



**Figure 4.1 Cell adhesion assay of Jurkat derivative cells to integrin substrate**

(A) Labeled WT, hCRT<sup>-/-</sup> and ERp57<sup>-/-</sup> were seeded on plates coated with Fibronectin for 45 minutes. (B) Labeled WT, CRT<sup>-/-</sup>, ssGFP-CRT, ERp57<sup>-/-</sup>, β1<sup>-/-</sup> were seeded on plates coated with GST-CS1 for 45 minutes. Fluorescence readings were acquired with an Enspire spectrophotometer before and after 3 washes with PBS. Background fluorescence from the incubation medium was subtracted from each reading, and the percentage of total cells that was adhered was calculated. The bars show the mean S.D.; n=3; ns, p not significant; \*\**p*<0.01; \*\*\**p*<0.001.

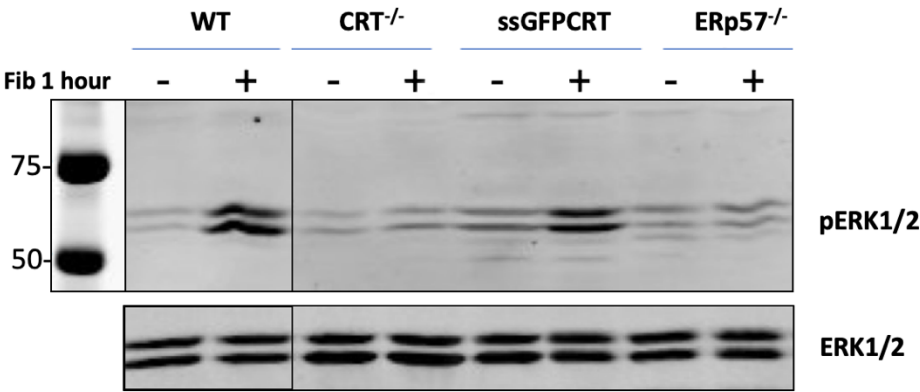
### 4.3 Involvement of Calreticulin in integrin/MAPK/ERK pathway in T-ALL

In order to study the involvement of CRT in integrin-mediated ERK activation, cells were serum-starved (0.05% FBS) overnight and then seeded on either fibronectin or BSA coatings for 1 hour. ERK activation was assessed by detection of phospho-ERK1/2 using western blot analysis. WT cells seeded on fibronectin showed increased levels of phospho-ERK1/2 when compared to cells seeded on BSA (Figure 4.2). In contrast, CRT<sup>-/-</sup> cells exhibited little change in phospho-ERK1/2 on fibronectin. Re-expression of CRT in CRT<sup>-/-</sup> cells as ssGFP-CRT was able to rescue fibronectin-mediated increase in phospho-ERK1/2, indicating the involvement of CRT in integrin-mediated ERK activation. Interestingly, phospho-ERK1/2 levels were not changed for

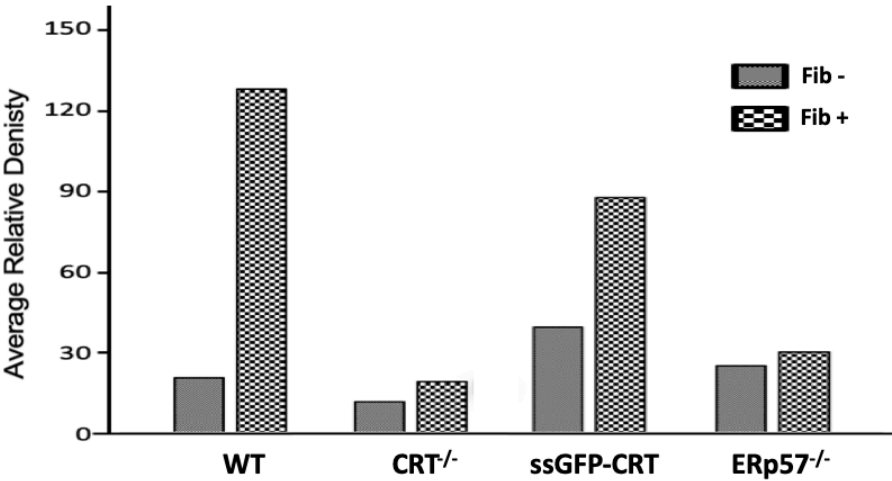


ERp57<sup>-/-</sup> cells (cytosolic CRT<sup>-/-</sup>) (Figure 4.2), suggesting the requirement of cytosolic CRT for activation of ERK downstream of integrin-mediated cell adhesion.

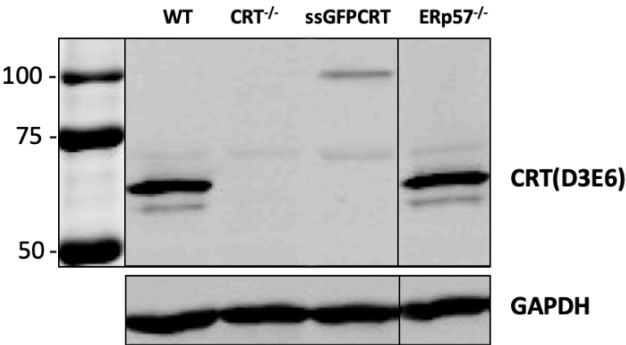
**A**



**B**



**C**



### Figure 4.2 Calreticulin is required for integrin-mediated ERK phosphorylation.

(A) WT, CRT<sup>-/-</sup>, ssGFP-CRT, ERp57<sup>-/-</sup> cells were seeded on dishes coated with fibronectin (Fib +) or BSA (Fib -) for 1 hour, and cell lysates were immunoblotted to detect phospho-ERK1/2 levels. (B) Densitometry quantification of pERK1/2 signals shown in A, signals are normalized over ERK1/2. (C) CRT expression in WT, CRT<sup>-/-</sup>, ssGFP-CRT, and ERp57<sup>-/-</sup>. Equal protein loading was assessed by immunoblotting for GAPDH. The black lines divide lanes from the same gel and blot that are non-adjacent.

### 4.4 Involvement of Calreticulin in AKT pathway

It has been reported that activation of Akt can be induced by integrin-mediated cell adhesion<sup>216</sup>, which promotes cell survival and increases cell proliferation<sup>217</sup>. To assess whether cytosolic CRT is involved in integrin-mediated Akt activation in leukemic cells, Jurkat WT, CRT<sup>-/-</sup>, ssCFPCRT and ERp57<sup>-/-</sup> cells were serum-starved (0.05% FBS) overnight and then were seeded on either fibronectin or BSA coatings for 30 minutes and western blot analysis was performed to detect phosphorylated Akt as an indicator of Akt activation. WT, CRT<sup>-/-</sup>, ssGFP-CRT, and ERp57<sup>-/-</sup> cells had comparable levels of phospho(T308)-Akt, suggesting that CRT is not involved in adhesion-mediated Akt activation (Figure 4.3).

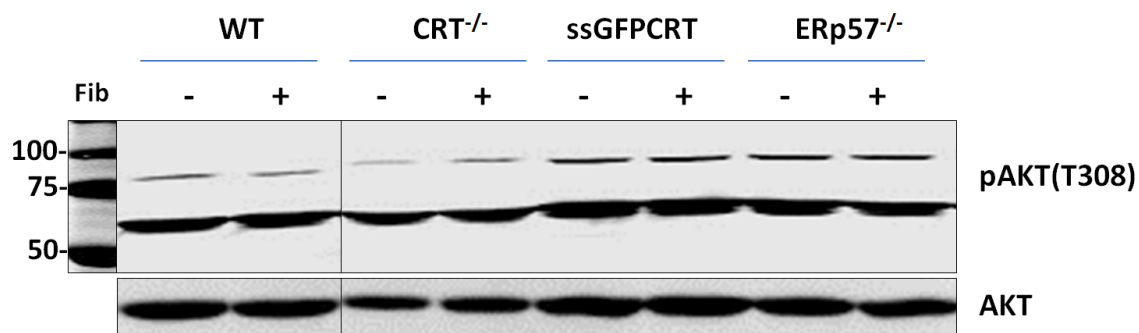
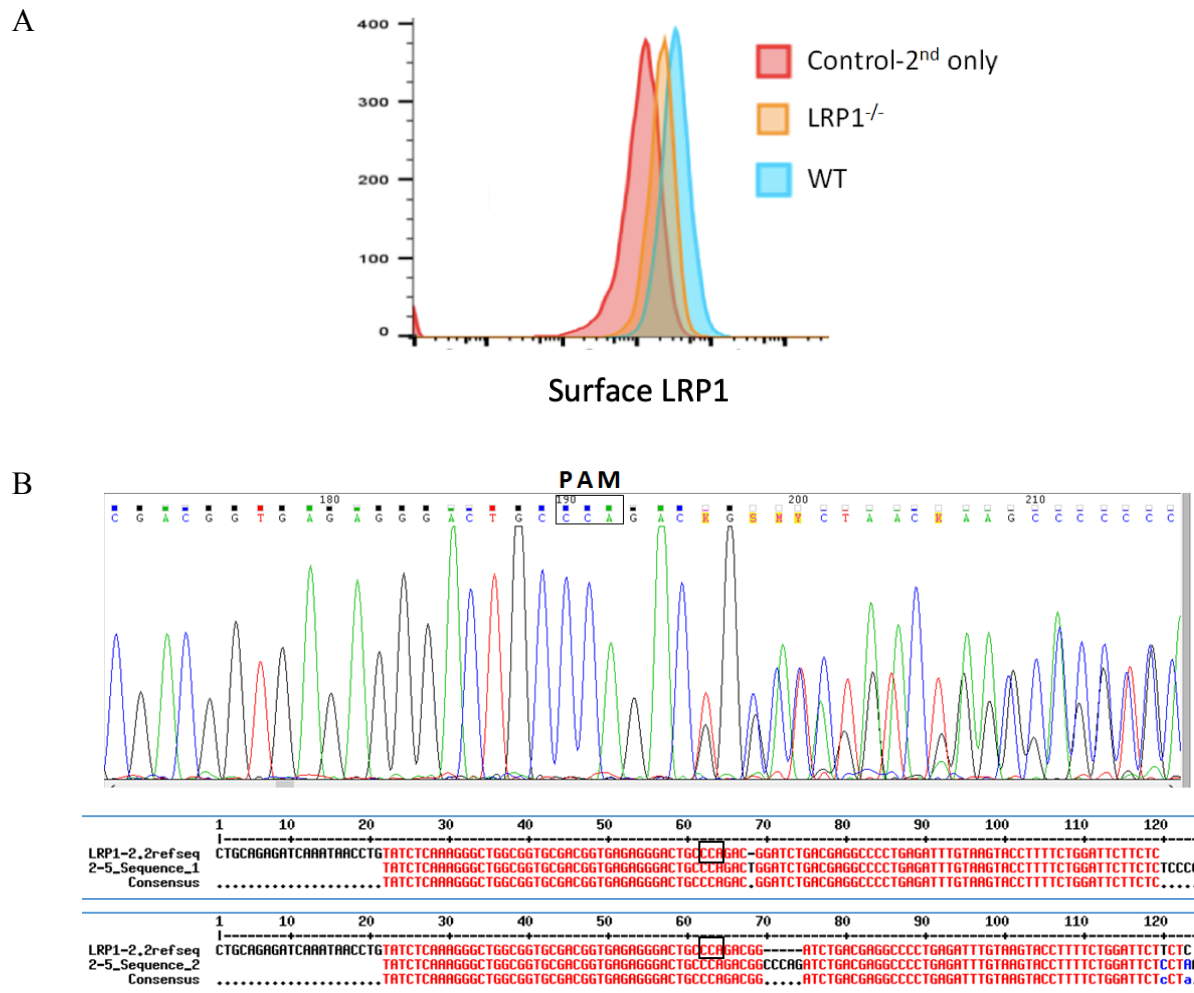


Figure 4.3 Calreticulin is not involved in integrin-mediated Akt activation

WT, CRT<sup>-/-</sup>, ssGFP-CRT, ERp57<sup>-/-</sup> cells and seeded on dishes coated with Fibronectin for 30 mins, and cell lysates were immunoblotted to detect (A) phospho(T308)-Akt. The cells were serum-starved to reduce constitutive activation of Akt known in Jurkat cells.

#### **4.5 LRP1, a receptor for cell surface CRT**

Professional phagocytes, including macrophages and dendritic cells, are known to utilize LRP1 as a *trans* receptor for CRT presented on the surface of cells undergoing immunogenic apoptosis to promote cell engulfment<sup>138</sup>. However, it is unknown what receptors, if any, mediate surface CRT presentation on the apoptotic tumour cell. To study whether cell surface CRT directly binds to LRP1 of apoptotic cells in *cis* mode, I utilized CRISPR-Cas9 technology to generate LRP1<sup>-/-</sup> lymphoblasts (Figure 4.4). Initially, I used flow cytometry to screen for clones with reduced LRP1 expression, however I was not able to get convincing loss of expression using this method. In addition, I was not successful in identifying an LRP1 antibody that works for western blotting. Hence, clones with reduced LRP1 expression as determined by flow was selected for sequencing analysis of the targeted genomic loci. Out of nine clones that was sequenced, one (clone 2-5) was confirmed with insertional mutations in both alleles, and each was predicted to result in early termination of LRP1 protein translation. Taken together, clone 2-5 is an LRP1<sup>-/-</sup> clonal derivative, although further confirmation by protein expression would be advisable. This could be done by western blotting, or by mass spectrometry.

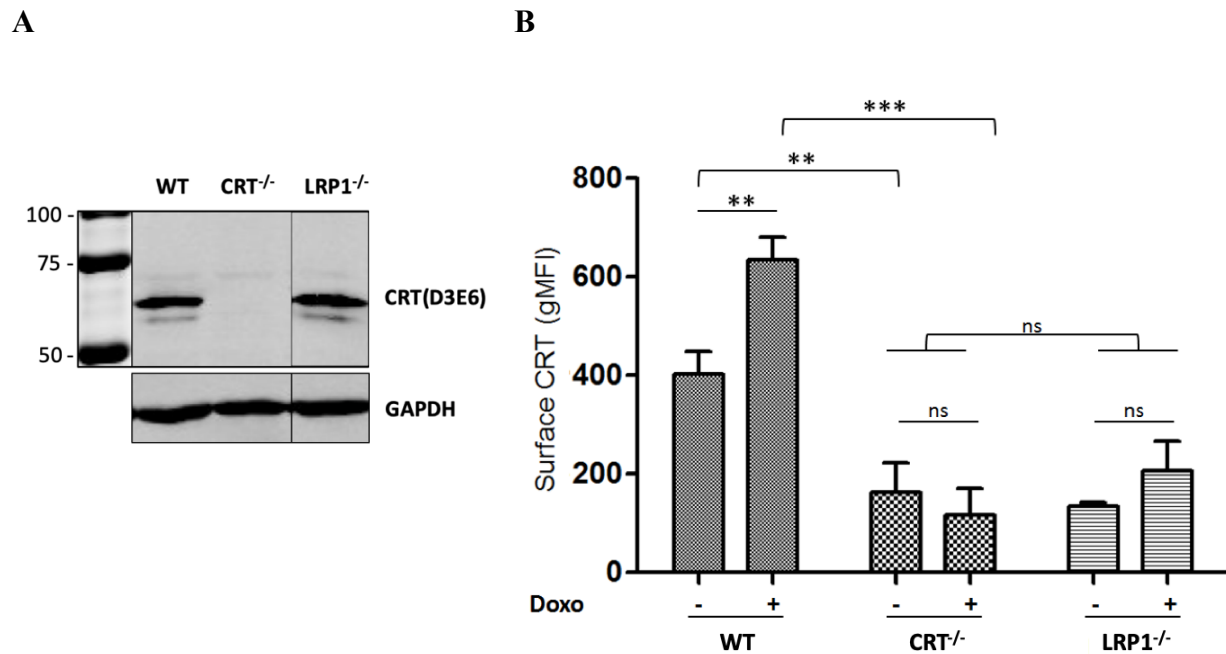


**Figure 4.4 CRISPR-Cas9 generation of LRP1<sup>-/-</sup> Jurkat cells**

(A) Flow cytometry analysis of fixed and permeabilized Jurkat WT and LRP1<sup>-/-</sup> samples for expression of LRP1. (B) Top: PCR amplicon sequencing of the CRISPR-Cas9 targeted genomic loci of Jurkat LRP1<sup>-/-</sup> clone (named hLRP1 2-5). The PAM motif is as indicated. Bottom: Sequence alignment analysis in comparison to the parental WT sequence showing each mutated alleles. *Allele 1*: Single nucleotide insertion occurs at 68 bp from the start codon. *Allele 2*: A five-nucleotide insertion occurs at 70 bp from the start codon.

First, I assessed and found that total CRT expression in LRP1<sup>-/-</sup> cells is comparable to that seen in the parental WT cells (Figure 4.5A), indicating that loss of LRP1 had little effect on CRT

stability. To assess if loss of LRP1 affects surface CRT presentation, I treated WT, CRT<sup>-/-</sup> and LRP1<sup>-/-</sup> cells with 3 µg/mL of doxorubicin for 4 hours to induce immunogenic cell death and assayed surface CRT expression using flow cytometry. In the absence of drug treatment, I found that LRP1<sup>-/-</sup> and CRT<sup>-/-</sup> cells had reduced surface CRT presented when compared to WT cells (Figure 4.5 B). In addition, I also found that LRP1<sup>-/-</sup> cells treated with the immunogenic cell death inducer, doxorubicin, failed to upregulate surface CRT levels in the manner that was observed in WT cells. Thus, the results obtained are consistent with an interpretation that LRP1 is a *cis*-acting receptor for CRT during ICD.



**Figure 4.5 Doxorubicin induced elevation of surface CRT is reduced in the absence of LRP1**

(A) Western immunoblot to detect CRT and GAPDH (loading control) in lysates of the cells as indicated. The black lines divide non-adjacent lanes that are from the same gel and blot. (B) WT, CRT<sup>-/-</sup>, and LRP1<sup>-/-</sup> cells were treated with 3 µg/mL of doxorubicin for 4 hours. Flow cytometry was used to measure the gMFI of surface CRT labeled with CRT antibody (D3E6). The bars show the mean S.D.; n=3; ns, p not significant; \*\**p*<0.01; \*\*\**p*<0.001.

## 4.6 Discussion

Acquired drug resistance is a significant problem in the treatment of T-ALL. It results in poor responsiveness to chemotherapy and leads to relapse. Adhesion to the extracellular matrix (ECM) via integrins has been extensively described as a key mediator of the survival and drug resistance of malignant cells of hematopoietic origin. CRT has been shown to be involved in CAMDR via interaction with cytosolic tail of  $\alpha$  integrins<sup>231</sup>. Furthermore, several studies reported the involvement of CRT in cell adhesion and the possibility of involvement of extra-ER, cytosolic form of CRT. Since cell adhesion was previously shown to increase CRT interaction with  $\alpha$  integrins via the juxtamembrane, cytosolic GFFKR motif<sup>126,231,233</sup>, the work described in this chapter sought to further our understanding of the involvement of CRT in integrin-mediated cell adhesion in a T-lymphoblast model and to determine the source of CRT involved in this process. The availability of ERp57<sup>-/-</sup> cells, which we had previously shown to express no detectable levels of the extra-ER cytosolic form of CRT (hence, it is a functionally null strain for cytosolic CRT, while still expressing ER-resident CRT)<sup>171</sup>, provided an opportunity to study the requirement of cytosolic CRT for cell adhesion. Using CRT<sup>-/-</sup>, ssGFP-CRT, and ERp57<sup>-/-</sup> (cytosolic CRT<sup>-/-</sup>) cells, I showed that T-lymphoblast adhesion to an integrin substrate was significantly reduced for cells lacking CRT (CRT<sup>-/-</sup>) and for cells lacking cytosolic CRT (ERp57<sup>-/-</sup>). Re-expression of ssGFP-CRT in CRT<sup>-/-</sup> cells, a form comparable to wildtype CRT in its targeted synthesis and major enrichment within the ER, was able to rescue the adhesion phenotype (Figure 4.1). Combined with the adhesion results observed in the HEK cell lines, this work provides supportive evidence that the cytosolic pool of CRT plays an important role in integrin-mediated cell adhesion.

Several studies have established that integrin engagement is required for adhesion-mediated activation of ERK<sup>197,198,203,204,211–213</sup> downstream of v-Src activation<sup>202,214</sup>. I sought to determine whether cytosolic CRT is involved in activation of ERK pathway in T-ALL. In the absence of CRT, levels of phospho-ERK1/2 did not increase for Jurkat T cells plated on an integrin substrate; this increase in phosphorylation was observed in WT and CRT<sup>-/-</sup> cells expressing ssGFP-CRT. Furthermore, phospho-ERK1/2 levels in ERp57<sup>-/-</sup> cells was comparable to that of CRT<sup>-/-</sup> cells, suggesting a cytosolic role for CRT in activating the ERK pathway. Further experiments will be needed in order to fully understand how ERK activation is mediated by CRT, including where within the MAPK cascade that requires the activity of cytosolic CRT. The levels for phospho(T308)-Akt remained constant, suggesting that Akt activity is CRT-independent in Jurkat T-ALL cells. Based on literature and previous results from our lab, I incubated the cells on fibronectin for 30 minutes; however, it is possible that other time points could reveal differences not evident at 30 minutes to better show the involvement of CRT in Akt signaling.

Next, I sought to determine the requirement for LRP1 as a receptor for cell surface CRT. Apoptotic cells are removed by phagocytosis upon recognition of the dying cells by professional phagocytes, and surface presentation of CRT is one of the important events during ICD which acts as a pro-phagocytic signal for macrophages<sup>223</sup>. Under normal physiological conditions, the bulk of CRT resides primarily within the ER lumen. Treatment of cells with an ICD-inducer, such as doxorubicin, induces the translocation of CRT from the ER to the cell surface<sup>223</sup>. Previous studies have established that surface CRT on apoptotic cells interacts in *trans* with LRP1 expressed on macrophages to mediate phagocytosis<sup>138,234</sup>. The work described in this chapter furthers our understanding of the relationship between surface CRT and LRP1 during

ICD. This work is the first to establish the requirement of LRP1 receptor for cell surface CRT presentation. By generating LRP1<sup>-/-</sup> T-lymphoblasts, I was able to investigate the requirement of LRP1 as a receptor for surface CRT during ICD. Treatment of WT, LRP1<sup>-/-</sup>, or CRT<sup>-/-</sup> cells with the ICD inducer, doxorubicin, resulted in increased surface CRT presented only in WT cells, but not in CRT<sup>-/-</sup> or LRP1<sup>-/-</sup> cells. Even though LRP1<sup>-/-</sup> cells had higher surface CRT compared to CRT<sup>-/-</sup> cells, it was not as high as seen in WT cells, suggesting there may be additional *cis*-acting receptors for surface CRT, in addition to LRP1. The fact that doxorubicin treatment did not increase surface CRT presented on LRP1<sup>-/-</sup> cells suggest that LRP1 may be the major receptor involved in ER to surface translocation of CRT.

I used doxorubicin as an ICD inducer drug; however, previously our lab established better induction using oxaliplatin, compared to that induced by doxorubicin. I was not able to use oxaliplatin due to solubility issues of the newer commercially available batches. Also, using doxorubicin prevented me from performing meaningful phagocytosis assays since this drug is highly fluorescent across a wide spectrum, imposing technical limitations on the ability to distinguish target and effector cells using the flow cytometer.



## 5. Conclusion and Discussion

### 5.1 Key Results

#### 5.1.1 Involvement of CRT in integrin mediated cell adhesion

Previously, our lab and others have published that integrin-mediated adhesion promotes binding of CRT to  $\alpha$  integrin tails in a manner requiring the GFFKR motif<sup>53,97,126,231,233</sup>. Also, our lab has described a regulatory role for  $\alpha$  integrin function in cell surface CRT presentation, suggesting that increased integrin activation leads to increased interaction of CRT with integrin, which results in reduced free cytosolic CRT able to translocate to the extracellular surface<sup>97,171</sup>. Adhesion of T-lymphoblasts to an integrin substrate leads to reduced surface CRT in cells treated with ICD inducers<sup>97</sup>.

In this study, I investigated the involvement of CRT in cell adhesion. The absence of CRT decreased adhesion of lymphoblasts to an adhesion substrate, while re-expression of CRT rescued the adhesion defect in these cells. The requirement of CRT for adhesion was also observed in HEK cells, suggesting the phenomenon may be widely applicable to multiple cell types. To investigate the source of CRT involved in this phenomenon, I used ERp57<sup>-/-</sup> cells, which we had shown to express little to no cytosolic CRT<sup>171</sup>. ERp57<sup>-/-</sup> cells showed reduced adhesion comparable to CRT<sup>-/-</sup> cells, suggesting that integrin-mediated cell adhesion is facilitated by cytosolic CRT (Figure 5.1).

#### 5.1.2 Involvement of CRT in integrin/MAPK/ERK pathway

Several studies have established the involvement of ERK in adhesion-mediated signaling downstream of v-Src activation<sup>210,211</sup>. Also, overexpression of CRT has been shown to

contribute to the development and progression of pancreatic cancer through ERK/MAPK signaling<sup>235</sup>. Furthermore, our lab previously demonstrated that integrin activation promotes binding of CRT to the  $\alpha$  integrin GFFKR motif<sup>97,231</sup>. My study describes the requirement of cytosolic CRT for activation of ERK downstream of integrin-mediated cell adhesion in a T-lymphoblast cell model (Figure 5.1). Comparing the parental WT, CRT<sup>-/-</sup>, ERp57<sup>-/-</sup> and ssGFPCRT rescued cells, I showed that cytosolic CRT plays a significant role in facilitating ERK phosphorylation downstream of integrin-mediated cell adhesion. CRT<sup>-/-</sup> and ERp57<sup>-/-</sup> cells are shown to have significantly lower ERK phosphorylation compared to parental wildtype and rescued cells when seeded on fibronectin. The interaction between CRT and MEK/ERK pathway may provide new ideas for applying new gene-targeted chemotherapy to ALL. However, the mechanism of this specific interaction in lymphoblasts is unknown and needs to be further investigated.

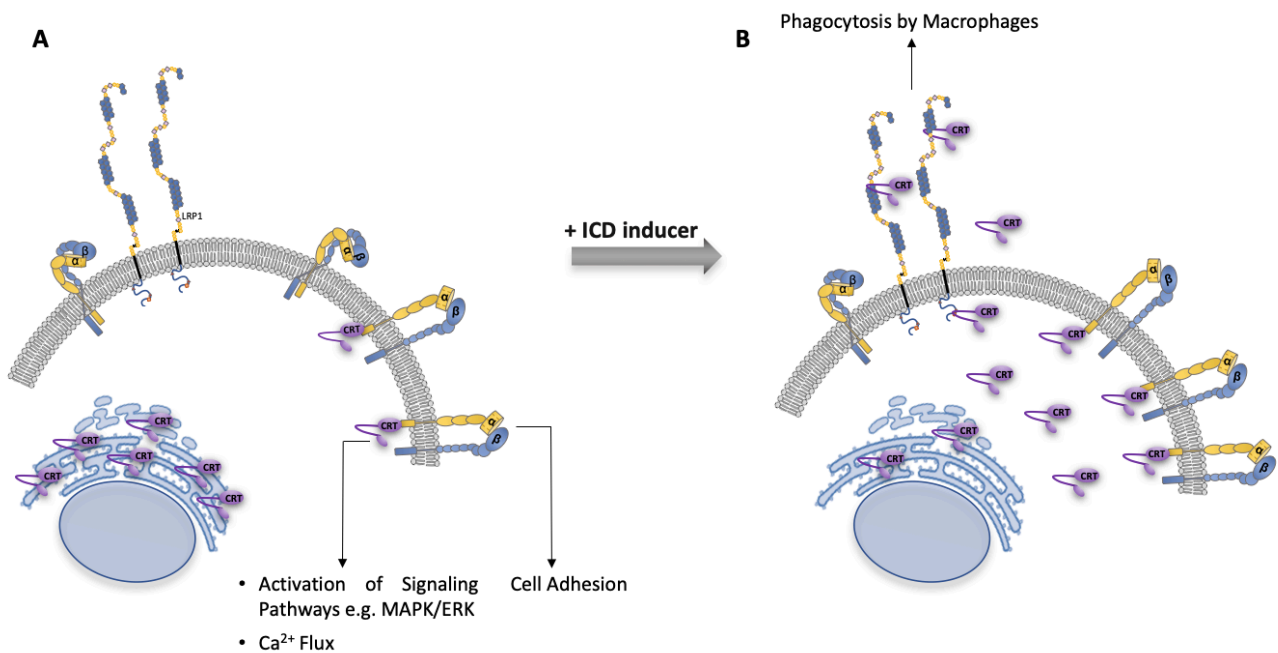
### **5.1.3 LRP1 a receptor for surface CRT**

Several studies have shown that during immunogenic cell death (ICD), cell surface CRT serves as a phagocytic signal for dendritic cells (DCs) that subsequently invoke cytotoxic T-cell activation and anti-tumor activity<sup>138,151,236</sup>. CRT is now regarded as one of the most potent danger-associated molecular patterns (DAMPs), with the surface CRT triggering restoration of homeostasis by immune stimulation<sup>138,151,236</sup>.

In this study, I used CRISPR-Cas9 technology to knock out LRP1 and to study the requirement of LRP1 as a surface receptor for CRT during ICD. I found that in the absence of LRP1, there is less surface CRT compared to WT; however, LRP1<sup>-/-</sup> cells had slightly higher

surface CRT compared to CRT<sup>-/-</sup>, indicating that LRP1 may not be the only receptor for CRT (Figure 5.1).

CRT can be a potential target for developing anticancer therapeutics and preventive strategies; therefore, understanding the mechanism by which CRT is presented on the cell surface and identifying its receptors can help the development of therapeutics which capitalize on the anti-tumour benefits of immunogenic cell death.



**Figure 5.1 A model depicting the involvement of CRT in cell adhesion, signaling and phagocytosis.**

(A) In healthy cells, CRT is primarily enriched within the lumen of the ER; however small amounts of CRT exist in the cytosol which can bind to GFFKR motif of activated integrins and promote cell adhesion and/or activate downstream signaling pathways such as MAPK/ERK. (B) When cells are treated with an ICD-inducer, the resultant ER stress promotes the release of ER-resident CRT into the extra-ER cytosolic space. Cytosolic CRT can translocate to the cell surface and bind to its receptor, LRP1. This binding presents CRT as a DAMP that promotes phagocytosis of the cell by professional phagocytes.

## 5.2 Concluding Remarks

The findings presented in this thesis indicate that CRT is required for integrin-mediated cell adhesion and that cytosolic CRT is involved in this process. Also, cytosolic CRT appears to have positive regulatory effect on ERK/MAPK pathway. Furthermore, I showed that LRP1 is a putative receptor for cell surface presentation of CRT during ICD.

Further elucidation of the exact mechanism by which CRT mediates cell adhesion is necessary to further our understanding of integrin function and the role of cell adhesion in T-ALL. It has been shown that T-cell adhesion promotes the elevation of intracellular  $\text{Ca}^{2+}$  and that CRT has a critical role in regulating  $\text{Ca}^{2+}$  flux<sup>237,61</sup>. It has yet to be shown whether CRT mediates cell adhesion by structural activation of integrins via binding of CRT to GFFKR motif, or if cell adhesion defects observed in these cells are mediated by  $\text{Ca}^{2+}$  flux regulated by CRT. We now have the complete model system to study the involvement of cytosolic CRT in  $\text{Ca}^{2+}$  flux by comparing the activities of Jurkat WT, CRT<sup>-/-</sup>, and ERp57<sup>-/-</sup> (cytosolic CRT null) cells under non-adherent basal, as well as under integrin-engaging, adherent conditions. Also,  $\text{Ca}^{2+}$  flux assays using CRT<sup>-/-</sup> HEK cells expressing various CRT constructs that are localized in different cellular compartments can further our understanding of the role of CRT in  $\text{Ca}^{2+}$  signaling and its effect on cell adhesion.

To date, no receptor has been proposed for surface CRT on apoptotic cells during ICD. My work has established that LRP1 is partially required for cell surface presentation of CRT on dying cells. Tarr *et al.* have shown that CRT binds directly to phosphatidylserine (PS) in a  $\text{Ca}^{2+}$ -dependent manner<sup>201</sup>. CRT was also shown to be associated with lipid rafts in association with ERp57<sup>238</sup>. However, whether CRT associated with lipid rafts can leave the cells through

dimerizing and clustering in rafts that bud from the cell is unknown. The mechanism by which CRT binds to LRP1 remains to be resolved. There are two possibilities for binding of CRT to its receptor in *cis* mode. First is the co-translocation of CRT and LRP1 from the ER to cell surface. Second, CRT is first released to extracellular space upon which CRT is recaptured by binding to LRP1 expressed on cell surface. Another question arising from my study is whether expression of LRP1 on apoptotic cells is required for the clearance of these cells by professional phagocytes.

Further investigation is needed to show if presentation of CRT is inside out, requiring intracellular activation signals, or outside in, requiring extracellular stimuli. This can be determined by comparing the surface CRT levels of Jurkat WT, CRT<sup>-/-</sup> and LRP1<sup>-/-</sup> cell lines incubated with exogenous CRT, with or without ICD induction. Under such conditions, CRT<sup>-/-</sup> cells are expected to have higher surface CRT levels when compared to LRP1<sup>-/-</sup> cells, a possible indication that LRP1 is sufficient to bind exogenously added CRT. Alternatively, surface CRT may only be presented in a manner requiring LRP1-CRT interaction originating in an intracellular, which would be revealed using ICD-inducing agents.

It is clear now that clinical success of conventional chemotherapy results in both tumor cell toxicity and engagement of the innate and adaptive immunity for recognition and further elimination of tumour cells that may arise in the future, minimizing the chances of relapse. Surface CRT is vital for the clearance of tumor cells by the immune system mediated by phagocytes<sup>100,151,223</sup>. Thus, cell physiological events that contribute to reduced surface CRT expression may lead to reduced antitumor responses resulting from ICD-based chemotherapy. Therefore, the findings in this thesis may enhance the effectiveness of chemotherapy using ICD inducers in a manner that engages the anti-tumour immune responses to provide lasting benefits to patients.

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