Modulation of Clr-b Ligand for the Inhibitory NKR-P1B NK cell Receptor in Response to Transformation and Genotoxic Stress.

By

Jason Harris Fine

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Immunology
University of Toronto

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Abstract

Innate immunity is an important first-line defense against infectious disease and malignancies. Natural killer (NK) cells are large granular lymphocytes that are critical for the innate immune response. NK cells recognize target cells undergoing pathological alterations such as viral infection and transformation, through germline-encoded receptors. The recognition of targets undergoing genotoxic stress or transformation by NK cells has been limited to the upregulation of stimulatory NKG2DL and “missing-self” recognition of MHC-I. The ligand pair NKR-P1B:Clr-b participates in “missing-self” recognition of tumour and infected cells, but the role of this axis in sensing stressed cells remains unknown. This thesis focuses on the modulation of Clr-b by genotoxic stress and transformation.

In the first data chapter, we show that Clr-b is downregulated at the transcript and protein level upon genotoxic stress, in a time- and dose-dependent manner in tumour cells and fibroblasts. This loss occurs upon exposure to a variety of genotoxic agents with the exception of γ-irradiation and radiomimetics. The loss of surface Clr-b results in disinhibition of target cells using $^{51}$Cr-release assays and BWZ reporter assays.
In the next chapter, we investigate the mechanism(s) by which this phenotype occurs. Genotoxic stress-induced Clr-b downregulation is not modulated entirely by classical DNA damage response effectors including ATM, ATR, Chk1, Chk2 and p53. Furthermore, the loss of transcript can by uncoupled from the loss of surface protein following administration of proteasomal inhibitors. Lastly, inhibition of the autocatalytic hammerhead ribozyme harboured within the 3’UTR of the Clr-b transcript can abrogate genotoxic stress-induced Clr-b downregulation.

In the final chapter, we investigate the effect of oncogenic stress mediated through H-Ras$^\text{V12}$ on Clr-b expression in NIH3T3 fibroblasts. H-Ras$^\text{V12}$-mediated Clr-b downregulation occurs through the MEK-Erk pathway and can be inhibited through pharmacological inhibition of MEK1/2 or shRNA knockdown of Erk2. However, Erk2 alone is not sufficient to induce Clr-b downregulation and requires activation from upstream signals.

All together, our results explore the role of genotoxic stress and transformation on the modulation of Clr-b, ligand for the NKR-P1B. This work begins to shed light on the role of NKR-P1B:Clr-b system in “missing-self” recognition of target cells undergoing pathological alterations.
Dedications

This thesis is dedicated to everyone who supported me throughout my doctoral studies. Specifically, I would like to dedicate this work to my mom, dad, and grandparents, Jay & Hide Huang whose unconditional support brought me to where I am today. I would also like to dedicate this to my beautiful wife, Christa Fine. I couldn’t envision achieving this honour without you.
Acknowledgments

First off, I would like to take the opportunity to thank my supervisor Dr. James R. Carlyle for taking me into his lab and teaching me scientific techniques and the literary methods associated with academic writing. As his first graduate student, I received hands-on experience that helped me develop into the scientist I am today. You were very supportive through the years and together we went through all the ups and downs associated with promotions and starting a lab. I am especially thankful for the opportunity to attend multiple scientific meetings, both domestically and internationally. I am grateful for his nomination to receive the Richard Miller Award, an achievement he also received as a student. So, thank you. I wish you nothing but future success both professionally and personally.

I would like to thank my committee members Dr. Alberto Martin, Dr. Tania Watts and Dr. Cynthia Guidos for all of their help and guidance over the years.

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learned a great deal about what it takes to be a leader, as well as the enjoyment of a group-concerted effort. Immunology is truly the place to be!

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Christa, you have held my hand throughout my studies, and supported me unconditionally. You kept me laughing on the tough days, and comforted on the sad days. You have inspired me in life, and I can’t wait for our next adventure together.

Lastly, I would like to thank the chair, Dr. Michael Ratcliffe, the faculty members, graduate coordinator, graduate committee and fellow students in the Immunology department for their support and friendship. This has been an amazing journey and I have not only grown as a scientist, but also as an individual. Although bittersweet, as an era comes to an end, I now leave here with the confidence to tackle a new set of challenges.

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<th>Description</th>
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<tbody>
<tr>
<td>53BP1</td>
<td>Tumor suppressor p53 binding protein 1</td>
</tr>
<tr>
<td>ABI</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Ab1</td>
<td>Abelson</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>ATF2</td>
<td>Activating transcription factor 2</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>ATM and Rad3 related</td>
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<td>ATRIP</td>
<td>ATR-interacting protein.</td>
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<tr>
<td>B6</td>
<td>C57/Black 6</td>
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<tr>
<td>BARD1</td>
<td>BRCA1-associated RING domain protein 1</td>
</tr>
<tr>
<td>BASC</td>
<td>BRCA1-associated genome surveillance complex</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2 associated X protein</td>
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<tr>
<td>BD</td>
<td>Beckton Dickinson</td>
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<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>Bmi-1</td>
<td>B lymphoma Mo-MLV insertion region 1 homolog</td>
</tr>
<tr>
<td>BRCA1</td>
<td>breast cancer type 1 susceptibility protein</td>
</tr>
<tr>
<td>BRCA2</td>
<td>breast cancer type 2 susceptibility protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>C/EBP</td>
<td>CCAAT enhancer binding protein-beta</td>
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<td>C##</td>
<td>Cysteine-##</td>
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<tr>
<td>CA</td>
<td>Constitutive active</td>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<td>Cdc25</td>
<td>Cell division cycle 25</td>
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<td>CDKs</td>
<td>Cyclin Dependent Kinases</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
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<td>CDS</td>
<td>Coding sequence</td>
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<td>Chk1-s</td>
<td>Chk1 short form</td>
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<td>Chk2</td>
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<td>CIP</td>
<td>Calf intestinal phosphatase</td>
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<td>Clec</td>
<td>C-type lectin</td>
</tr>
<tr>
<td>Clr</td>
<td>C-type lectin related</td>
</tr>
<tr>
<td>Clr-b</td>
<td>C-type lectin related B</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
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<tr>
<td>CPRG</td>
<td>Chlorophenol-red-β-D- galactopyranoside</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
</tr>
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<td>Cs</td>
<td>Cesium</td>
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<td>Description</td>
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<td>-------------</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T-lymphocyte</td>
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<tr>
<td>DAP10</td>
<td>DNAX-activation protein 10</td>
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<td>DAP12</td>
<td>DNAX-activation protein 12</td>
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<td>DCs</td>
<td>Dendritic cells</td>
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<td>DDR</td>
<td>DNA damage response</td>
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<td>DLRA</td>
<td>Dual luciferase reporter assay</td>
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<td>DMEM-HG</td>
<td>Dulbecco's modified eagle medium-high glucose</td>
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<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<tr>
<td>DN</td>
<td>Dominant negative</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
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<tr>
<td>DNA-PKcs</td>
<td>DNA-dependent protein kinase catalytic subunit</td>
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<td>DNAM-1</td>
<td>DNAX accessory molecule-1</td>
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<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
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<tr>
<td>DSB</td>
<td>Double strand break</td>
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<tr>
<td>dTTP</td>
<td>Thymidine triphosphate</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ELK1</td>
<td>(ETS)-like transcription factor 1</td>
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<tr>
<td>Erk</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>ES</td>
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<tr>
<td>FA</td>
<td>Fanconi Anemia</td>
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<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<td>FANCD2</td>
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<tr>
<td>FANCL</td>
<td>Fanconi anemia, complementation group L</td>
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<td>FasL</td>
<td>Fas ligand</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FdUMP</td>
<td>fluorodeoxyuridine monophosphate</td>
</tr>
<tr>
<td>FdUTP</td>
<td>fluorodeoxyuridine triphosphate</td>
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<tr>
<td>FL</td>
<td>Full length</td>
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<tr>
<td>G3DPH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>GAP</td>
<td>GTPase-activating protein</td>
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<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>Guanosine diphosphate</td>
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<td>Guanine nucleotide exchange factor</td>
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<td>Green fluorescent protein</td>
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<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
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<td>Histone 2AX</td>
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<td>H2O2</td>
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<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
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<td>HDACi</td>
<td>Histone deacetylase inhibitors</td>
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<td>HHR</td>
<td>Hammerhead ribozyme</td>
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<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>hIgG</td>
<td>Human immunoglobulin G</td>
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<td>HLA-E</td>
<td>Human leukocyte antigen E</td>
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<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous recombination</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>hsp90</td>
<td>Heat shock protein 90</td>
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<td>Hypervariable region</td>
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<td>Interferon</td>
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<td>Internal ribosome entry site</td>
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<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
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<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibition motif</td>
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<td>JNK</td>
<td>C-Jun N-terminal kinase</td>
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<td>KIR</td>
<td>Killer-cell immunoglobulin-like receptor</td>
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<td>Killer cell lectin-like receptor subfamily G</td>
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<tr>
<td>KO</td>
<td>Knock out</td>
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<tr>
<td>LAK</td>
<td>Lymphokine activated killer</td>
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<td>LAT</td>
<td>Linker for T-cell activation</td>
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<td>LFA-1</td>
<td>Lymphocyte function-associated antigen 1</td>
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<tr>
<td>LLT1</td>
<td>Lectin-like transcript 1</td>
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<tr>
<td>LMP</td>
<td>Large multifunctional peptidase</td>
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<td>LN</td>
<td>Lymph node</td>
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<td>LTR</td>
<td>Long terminal repeat</td>
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<td>mA</td>
<td>Milliamps</td>
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<td>mAb</td>
<td>Monoclonal antibody</td>
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<td>MCS</td>
<td>Multiple cloning site</td>
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<td>MDC1</td>
<td>Mediator of DNA damage checkpoint protein 1</td>
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<td>Mdm2</td>
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<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
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<td>MEK</td>
<td>Mitogen-activate protein kinase</td>
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<td>MFI</td>
<td>Median fluorescence intensity</td>
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<td>MICA/MICB</td>
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<td>mLL-15</td>
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<tr>
<td>MILL</td>
<td>MHC class I-like located near the leukocyte receptor complex</td>
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<tr>
<td>min</td>
<td>minute</td>
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<tr>
<td>MNK</td>
<td>MAP kinase interacting serine/threonine kinase</td>
</tr>
<tr>
<td>MRN</td>
<td>Mre11/Rad50/NBS1</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MS-V</td>
<td>Murine sarcoma virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MSK</td>
<td>Mitogen and Stress-Activated Protein Kinase</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>Multi</td>
<td>Mouse UL16-binding protein-like transcript 1</td>
</tr>
<tr>
<td>MuLv</td>
<td>Murine leukemia virus</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NaN3</td>
<td>Sodium Azide</td>
</tr>
<tr>
<td>Nbs1</td>
<td>Nijmegen breakage syndrome gene</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear Factor-KappaB</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NKC</td>
<td>Natural killer cell gene complex</td>
</tr>
<tr>
<td>NKG2</td>
<td>Natural killer group 2</td>
</tr>
<tr>
<td>NKG2DL</td>
<td>Natural killer group 2 ligand</td>
</tr>
<tr>
<td>NKP</td>
<td>Natural killer cell precursor</td>
</tr>
<tr>
<td>NKp46</td>
<td>Natural killer cell p46-related protein</td>
</tr>
<tr>
<td>NKR-P1B</td>
<td>Natural killer cell receptor P1B</td>
</tr>
<tr>
<td>NKR-P1C</td>
<td>Natural killer cell receptor P1C</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization sequence</td>
</tr>
<tr>
<td>Ocil</td>
<td>Osteoclast inhibitory lectin</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>p21</td>
<td>Cyclin-dependent kinase inhibitor 1A</td>
</tr>
<tr>
<td>p38 MAPK</td>
<td>p38 mitogen-activated protein kinase</td>
</tr>
<tr>
<td>p53</td>
<td>protein 53</td>
</tr>
<tr>
<td>PAMPS</td>
<td>Pattern associated molecular patterns</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocyte</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>pDNA</td>
<td>Plasmid deoxyribonucleic acid</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIKK</td>
<td>Phosphatidylinositol 3-kinase-related kinase</td>
</tr>
<tr>
<td>PLB</td>
<td>Passive lysis Buffer</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonylfluoride</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>Qa-1</td>
<td>MHC class I b molecule Qa-1</td>
</tr>
<tr>
<td>Rad51</td>
<td>Rad51 homolog (S. cerevisiae)</td>
</tr>
<tr>
<td>Rae-1</td>
<td>Retinoic acid early inducible-1</td>
</tr>
<tr>
<td>RAET1</td>
<td>Retinoic acid early transcripts-1</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombinase activating gene 1</td>
</tr>
<tr>
<td>Ral</td>
<td>Ras-related protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RAP80</td>
<td>Receptor-associated protein 80</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>RBPJk</td>
<td>Recombination signal-binding protein 1 for J-Kappa</td>
</tr>
<tr>
<td>RFC</td>
<td>Replication factor C</td>
</tr>
<tr>
<td>rhIL-2</td>
<td>Recombinant human interleukin 2</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light unit</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNF</td>
<td>Ring-finger nuclear factor</td>
</tr>
<tr>
<td>RPA</td>
<td>Replication protein A</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RSK</td>
<td>Ribosomal s6 kinase</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>S/T</td>
<td>Serine/Threonine</td>
</tr>
<tr>
<td>S##</td>
<td>Serine-##</td>
</tr>
<tr>
<td>SA-APC</td>
<td>Streptavidin-allophycocyanin</td>
</tr>
<tr>
<td>SA-PE</td>
<td>Streptavidin-phycoerythin</td>
</tr>
<tr>
<td>Seid</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Shc</td>
<td>Src homology 2 domain containing</td>
</tr>
<tr>
<td>SHP-1</td>
<td>Src homology phosphatase-1</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>SLP-76</td>
<td>SH2 domain containing leukocyte protein of 76kDa</td>
</tr>
<tr>
<td>SMC1</td>
<td>Structural Maintenance of Chromosomes 1</td>
</tr>
<tr>
<td>SPL</td>
<td>Spleen</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>SUMO</td>
<td>Small ubiquitin-like modifier</td>
</tr>
<tr>
<td>Syk</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>T##</td>
<td>Threonine-##</td>
</tr>
<tr>
<td>TAP</td>
<td>Antigen peptide transporter 1</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-binding protein</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline tween-20</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>Tlk</td>
<td>Tousled like kinase</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TopBP1</td>
<td>DNA topoisomerase 2-binding protein 1</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>tRFP</td>
<td>Turbo red fluorescent protein</td>
</tr>
<tr>
<td>TSA</td>
<td>Trichostatin A</td>
</tr>
<tr>
<td>ULBP</td>
<td>UL16-binding proteins</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>Y##</td>
<td>Tyrosine-##</td>
</tr>
<tr>
<td>ZAP70</td>
<td>Zeta-chain-associated protein kinase 70</td>
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</tbody>
</table>
List of Publications


**Acknowledgements**

Chapter I

Introduction
1 Innate Immunity

Innate immunity constitutes an important first-line defense that functions as a barrier to infectious disease and malignancies. Innate effector cells respond rapidly and utilize the products of germline-encoded receptors to detect and eliminate pathogens and transformed host cells via a variety of mechanisms, including phagocytosis, complement-mediated opsonization and lysis, cell-mediated cytotoxicity, cytokine responses, and intrinsic and extrinsic pathways for induction of apoptosis. Target recognition is achieved through pattern recognition receptors that recognize foreign pathogen associated molecular patterns (PAMPs), or receptors that recognize self or induced-self molecules. Innate immune effector cells include neutrophils, eosinophils, macrophages, dendritic cells (DCs), and natural killer (NK) cells. The innate immune system plays an important role in the elimination of tumours through direct and indirect actions (stimulate adaptive immune response). Mice lacking some of these effectors, such as eosinophils and NK cells are more prone to developing tumours.

1.1 Natural Killer Cells

The origin of the initial NK cell discovery cannot be pinpointed to a specific research article, but rather of an era in which researchers were observing killing of tumour cell lines in vitro by splenocytes of healthy individuals. The previous belief was that tumour associated cytotoxicity arose specifically from T cells that had previously been sensitized to tumour associated antigens. Moreover, reactivity by normal individuals was blamed on technical problems potentially owing to the fact that the preparation of effector cells was not optimal. However, by the early 1970’s many researchers were publishing papers about NK cell activity observed in several species against allogeneic and syngeneic tumours, as well as Moloney Sarcoma Virus (MS-V) and Murine Leukemia Virus (MuLV) induced tumours.

NK cells are innate immune lymphocytes capable of recognizing and destroying transformed, infected, antibody-coated, transplanted, and “stressed” cells. Their effector functions can be classified into two important roles: (i) cell-mediated cytotoxicity via exocytosis of preformed granules containing perforin (a complement C9-related pore-forming protein) and granzymes (serine proteases that activate target cell caspases to induce apoptosis) or through
activation of intracellular caspases via TNF family ligand-receptor interactions (e.g. FasL:Fas, TRAIL:DR4/5;DCR1/2) \(^5\)\(^-\)\(^8\); and (ii) the early production of cytokines, most importantly IFN-\(\gamma\), which initiates and promotes T\(\text{H}1\) adaptive immune responses \(^2\)\(^-\)\(^9\). NK cells comprise between 2-10\% of total lymphocytes, depending on localization, and up to 18\% of PBLs in humans \(^10\).

1.1.1 NK Cell Development

Several cytokines are known to play a role in the commitment of an NK cell precursor (NKP) from a hematopoietic stem cell progenitor including stem cell factor (SCF), FLT-3 ligand, IL-7 and IL-15 \(^11\). The interaction of precursors with their environment is also important. In the bone marrow, stromal cells activated through lymphotoxin signaling are required for proper NK cell development in both function and absolute numbers \(^12\). Neutrophils are also required in the bone marrow and periphery for proper development and function. A lack of neutrophils causes the development of a hyperproliferative, hyporesponsive NK cell pool \(^13\).

NK cells can develop from at least two different anatomical sites, the bone marrow and the thymus. Bone marrow NK cell development follows at least 6 different stages, which mark the sequential acquisition of receptors targeting normal or altered self. The phenotype of the first NKP (NK precursor) committed to the NK cell lineage remains controversial. Generally speaking, the earliest NKP\(s\) express CD122 (IL2R\(\beta\)), which along with the common gamma chain (\(\gamma_c\,\text{CD132}\)) forms the IL-2/15 receptor. However, not all CD122\(^+\) Lin- cells become NK cells. NKG2D was also found to be expressed on a subset of CD122\(^+\) NKP\(s\) \(^11\). Recently, the transcription factor Id2, was found to more accurately identify Lin\(^-\)IL-7R\(\alpha^+\)CD122\(^+\) cells with NK cell potential, using an IRES-GFP knock-in in the Id2 3’UTR \(^14\). Concurrently, another group identified a CD122 low population (Lin\(^-\)c-kit\(^-\)Flk2\(^-\)CD27\(^+\)CD244\(^+\)IL-7R\(\alpha^+\)CD122\(^-\)) as capable of giving rise to NK1.1\(^+\)NKp46\(^+\) NK cells in RAG2\(^-\)IL2\(\gamma_c^+\) recipients \(^15\). Further work will be required to elucidate the identity of the earliest NK cell precursor, and whether the conventional belief that CD122 expression represents the first step of NK cell specific commitment. Sequential developmental events for the NKP involve the step-wise acquisition of receptors recognizing various forms of self. In most cases, the inhibitory isoform of a receptor family is acquired first before the stimulatory isoform \(^11\). During NK cell development, acquisition of NK cell receptors follows an orderly pattern, or a stochastic pattern, depending on
the receptor. During embryonic stem (ES) cell differentiation to NK cells, the CD94/NKG2 receptor family is acquired in the order of CD94, NKG2D, NKG2A, NKG2E, and NKG2C; at least at the transcript level, due to a lack of reagents to unambiguously discern the difference between different CD94/NKG2 heterodimers \(^{16}\). On the other hand, Ly49 receptor expression appears to follow a stochastic acquisition mechanism. Between 1-4 Ly49 genes can be expressed on an NK cell and a probabilistic bi-directional promoter determines the expression of each Ly49 gene. The Ly49G gene contains a second promoter upstream that is only active in immature NK cells. The transcription factors C/EBP and TBP in association with the NFκB p50 sub-unit bind overlapping sites on this promoter to favour either forward (coding transcript) or reverse (non-coding transcript) transcription. The decision made by the upstream promoter, which is not active on mature NK cells, dictates whether the Ly49 will be expressed on mature NK cells. Other Ly49 family members were also characterized to have similar elements as Ly49G preceding them \(^{17}\).

Thymic NK cells are derived from the thymus and do not represent a pool of peripheral NK cells that has populated the thymus, as demonstrated with thymus grafts into NK-deficient recipients \(^{11}\). They are found in the thymus, spleen, liver and lymph nodes, and have a greater cytokine secretion capacity and reduced cytolytic capabilities when compared to bone marrow derived NK cells \(^{11}\). Thymic NK cells possess a smaller receptor repertoire, and express lower levels of CD11b and CD43 compared to conventional splenic NK cells \(^{11}\). Unique to thymic NK cells is the expression of CD127 (the IL-7rα), which is dependent on GATA-3 activity \(^{18}\). It is believed that thymic NK cells share a precursor stage with T cells, and may represent T cell precursors that failed to differentiate beyond the DN2 stage based on \textit{in vitro} work that identified TCRγ rearrangements in fetal thymic and CD127\(^{+}\) LN NK cells \(^{19}\). However, more recent work assessed the impact of critical T cell developmental pathways in thymic NK cell development. It was found that \textit{in vivo}, RAG2 was expressed in a minority of thymic NK cells, and this finding coincided with the reduced expression of TCRγ \(^{19}\). Furthermore, bone marrow chimeras deficient for the transcription factor RBPJk which is essential for Notch signaling and T cell development, had no effect on thymic NK cell percentages or absolute numbers in the spleen \(^{20}\). These studies suggest that thymic NK cells develop independently of T cells and may not represent failed T cell precursors.
Although much work has been focused on discerning the origin and function of NK cells from these two compartments, the bone marrow and thymus are not the sole anatomical sites for the development of functionally specialized NK cells. NK cells found in the brain, joints, peritoneal cavity, pancreas, uterus, liver, and mucosal tissues have been characterized and are functionally or phenotypically different from conventional NK cells that circulate in the periphery\textsuperscript{21-23}. Thus, NK cells likely represent a heterogeneous group of cells that carry out specialized functions dependent on their anatomical distribution. How these cells get to these sites and the necessity for differential receptor expression requires further elucidation.

1.1.2 NK Cell Education/ Tolerance

During development, NK cells must be educated to be tolerant to self as they acquire receptors and functional activity. Two current models for NK cell tolerance are the Disarming model and the Licensing (Arming) model. In the Disarming model, NK cells enter into a state of hyporesponsiveness, similar to anergy of B and T cells when they do not encounter self-antigens, as a result of chronic stimulatory signals without engagement of a self-specific inhibitory receptor, such as the Ly49 family (receptor for MHC-I in mice). Hyporesponsive NK cells have a much reduced effector capacity compared to cells that have encountered MHC-I during development. The Disarming model can apply to either immature or mature NK cells, however the disarming of mature NK cells is only in theory. The Licensing model implies that an inhibitory receptor signal mediated through MHC-I:Ly49 interaction delivers a positive signal required for the functional maturation of an NK cell\textsuperscript{24}. Although an inhibitory receptor sending a stimulatory signal seems contradictory, another view is that interaction of an inhibitory receptor with an MHC-I allele is required for the functional maturation of NK cells. Thus, an NK cell that does not see any MHC-I alleles during development will become hyporesponsive\textsuperscript{25}. There is evidence to support both models, and in each, a hyporesponsive state is the result of a failure to encounter self-MHC-I\textsuperscript{24,25}. The missing-self responsiveness of an NK cell is increased/tuned depending on the number of functional interactions that occur with self MHC-I, earning the Rheostat model name\textsuperscript{26}. It has been shown that the hyporesponsive phenotype can be altered depending on the environment. Researchers used hyporesponsive splenic NK cells from a MHC-I deficient host and noted an increase in responsiveness to MHC-I when transferred into an MHC-I sufficient host. This responsiveness was notable on NK cells expressing Ly49C\textsuperscript{+}, Ly49I\textsuperscript{+},
and CD94/NKG2A\(^+\), as they are known to recognize the H-2\(^b\) host molecules\(^{27}\). Furthermore, a gain of function in responsiveness was demonstrated by transferring MHC-I deficient NK cells into a K\(^b\)D\(^b\)\(^−\)/ host expressing the H-2D\(^d\) transgene, which binds Ly49A. An increase in IFN-\(\gamma\) positive cells was observed in Ly49A\(^+\) versus Ly49A\(^−\) NK cells\(^{28}\). In these systems, the hyporesponsiveness of mature NK cells was altered with no significant effect on other cell surface receptors. However during development, the role of other receptor families such as the NKG2/CD94 family and NKR-P1 families, in generating functionally responsive cells has not been fully investigated, as these families, which also harbour inhibitory isoforms recognize self-molecules and are expressed earlier than the Ly49’s during mouse NK cell development. Recently, the stimulatory receptor NKp46, whose host endogenous ligand remains unknown, was implicated in the development of normal NK cell activity in mice. That is, mice lacking NKp46 display a hyperresponsive NK cell phenotype. The hyperresponsive phenotype is mediated by the transcription factor Helios, which is over-abundant in NKp46 knock out mice\(^{29}\). Thus, NK cell education likely requires a full complementation of both inhibitory and stimulatory receptors to develop the proper repertoire \textit{in vivo}.

### 1.1.3 Mature NK Cells

In general, murine NK cells can be distinguished as lacking canonical T cell markers such as TCR\(\beta\), CD3, and B cell markers (e.g. Ig), and by the presence of NK1.1 expression (PK136 antibody clone)\(^{30-32}\). The NK1.1 alloantigen can identify NK cells on CE, B6, NZB, C58, Ma/My, ST, SJL, FVB, and Swiss outbred mice, but not BALB/c, AKR, CBA, C3H, DBA, or 129 mice. The inability to bind NK cells from BALB/c mice is due to a non-synonymous amino acid substitution in the ectodomain of NKR-P1B and NKR-P1C that abolishes recognition of PK136\(^{33}\). The function of the NK1.1 alloantigen is further complicated by the fact that in different strains, PK136 binds either the stimulatory receptor NKR-P1C or inhibitory receptor NKR-P1B\(^{34,35}\). Subsets of mature NK cells can be differentiated through the markers CD11b and CD27. The most functionally immature CD11b\(^{dull}\)CD27\(^+\)cells, as determined through cell surface marker expression, are mainly found in the bone marrow and lymph nodes. The intermediate CD11b\(^+\)CD27\(^+\) cells are evenly distributed throughout the blood and tissues and the terminally differentiated CD11b\(^+\)CD27\(^{dull}\) cells tend to localize to the blood, lungs, spleen and liver\(^{36}\). These populations can also be divided based on CD94 expression, whereby in CD3\(^−\) NK1.1\(^+\)
cells, the CD94\textsuperscript{hi} population has more proliferative and cytotoxic capacity, as well as cytokine-induced IFN-\(\gamma\) production than the CD94\textsuperscript{lo} population. These two populations are evenly distributed in the CD11b\textsuperscript{dim}CD27\textsuperscript{+}, CD11b\textsuperscript{+}CD27\textsuperscript{+} and CD11b\textsuperscript{+}CD27\textsuperscript{dim} subsets\textsuperscript{37}.

In humans, NK cell subsets are distinguished through the markers CD56 and CD16. CD56\textsuperscript{dim}CD16\textsuperscript{+} comprise 90\% of the NK cell population in the blood and spleen. This subset has more cytotoxic capacity and produces more IFN-\(\gamma\) than the CD56\textsuperscript{bright}CD16\textsuperscript{−} population, which lacks perforin expression and is localized mainly to lymph nodes and tonsils. A functional intermediate population of CD56\textsuperscript{bright}CD16\textsuperscript{+} NK cells exists that was originally observed following hematopoietic stem cell transplantation and following TGF-\(\beta\) administration\textsuperscript{38-40}. These cells were the immature CD56\textsuperscript{bright}CD16\textsuperscript{−} population but had acquired the stimulatory receptors NKp46 and NKG2D\textsuperscript{40}. The CD56\textsuperscript{bright}CD16\textsuperscript{+} NK cells have comparable cytotoxic capacity to the more mature CD56\textsuperscript{dim}CD16\textsuperscript{+} cells and are enriched in elderly populations\textsuperscript{39}.

1.1.4 NK Cell Function

NK cell function is largely dictated by the inflammatory or cytokine milieu. Cytokines known to activate or stimulate proliferation of NK cells are IL-2, Type I IFN, IL-12, IL-18 and IL-15\textsuperscript{10}. Resting NK cells store transcripts for perforin and granzyme B, which are translated upon activation of the NK cell. This reduces the amount of time required for the effector function to be carried out\textsuperscript{41}. Activation through IL-12 can drive IFN-\(\gamma\) transcript production that is stored until activation by IL-2, at which point the transcripts are shuttled out of the nucleus and translated\textsuperscript{42}.

NK cells do not require prior sensitization, making them suitable for early defense, prior to the induction of adaptive immunity. Although their effector function or expansion is enhanced by reciprocal priming interactions with DCs, or interaction with CD8\textsuperscript{+} or CD4\textsuperscript{+} T cells, NK cells have the innate capacity to kill targets without priming. In contrast, in common inbred mouse systems there is a large portion of NK cells that are in a resting state. These NK cells express lower levels of key effector molecules perforin and granzyme B, which can be induced upon pathological insult, such as viral infection\textsuperscript{10}. NK cells can be primed through crosstalk with DCs, which enhances their cytolytic capacity\textsuperscript{43,44}. The trans-presentation of IL-15 by type I IFN primed DCs to NK cells is important for proper in vivo NK cell function following viral or
bacterial challenge. The Jagged2 (on DC)-Notch2 (on NK cell) interaction has also been implicated in activation of NK cells by increasing cytolytic anti-tumour responses. Conversely, a recent report using feral mice indicated that, compared to conventionally housed mice which have a much smaller pathogen load, the number of NK cells that were primed for effector function was greatly increased. This was evident through increased expression of stimulatory receptor NKp46, effector molecule granzyme B, and a greater capacity to secrete IFN-γ upon cytokine stimulation.

NK cells can also induce apoptosis in target cells in a perforin-independent manner through the interactions of TNF ligand-receptor families such as FasL:Fas, and TRAIL:DR4/5. NK cells express FasL and TRAIL which bind their corresponding receptor on target cells causing receptor trimerization, allowing for the recruitment of adaptor proteins to an intracellular death domain. Adaptor proteins recruit caspases, which are activated through autocleavage resulting in apoptosis of the target. Many TNF family ligands are expressed at low levels on NK cells, but can be induced following NK1.1 cross-linking, such as FasL. There is also differential use of TNF family ligands during NK cell development, such that immature human and mouse NK cells utilize TRAIL, but not FasL to recognize and destroy tumour targets.

1.1.5 NK Cell Memory

Akin to T cells, NK cell memory has been described in the context of cytokine induction and viral challenge. Naïve NK cells that were activated with IL-18, IL-12 and IL-15 to produce IFN-γ and transferred into Rag1-/- hosts initially stopped producing IFN-γ after the first week. However upon restimulation 7 days later with IL-12 and IL-15, or stimulatory receptor cross-linking, they were capable of mounting a greater IFN-γ response than transferred unactivated cells. In a mouse cytomegalovirus model, researchers demonstrated that NK cells with a virus specific receptor were capable of 100 and 1000 fold expansions in the spleen and liver following infection, respectively. These “memory”-like cells, contract following resolution of the infection and reside in the lymphoid and non-lymphoid organs. They were observed up to 70 days after infection and were capable of generating a more robust cytokine and degranulation response to plate bound antibody compared to naïve NK cells. Lastly, these memory NK cells, which harbour greater expression of KLRG1, CD43 and Ly6C, and decreased expression of CD27,
were capable of conferring increased resistance to viral infection in newborn mice when compared to naïve NK cells \(^{50}\). As this is a very new field, much work is left to help elucidate potential markers to distinguish this population in resting states, and determine whether it exists in humans.

1.1.6 Target Cell Recognition by NK cells

NK cells do not attack normal “self” cells but frequently recognize tumour cells and target them for destruction \(^{2}\). The lack of MHC-I expression on many tumour cell lines led to the suggestion that NK cells detect and respond to the absence of MHC-I self markers on target cells. This formed the basis of the “missing-self” hypothesis \(^{51}\): that is, NK cells possess inhibitory receptors specific for self MHC-I molecules, such that the loss of MHC-I (“missing self”) was sufficient to trigger NK cell cytotoxicity, due to the loss of normal inhibitory signals. This model was confirmed with the discovery of MHC-specific receptors on NK cells, encoded by the \(Ly49\) gene family in rodents and the \(KIR\) gene family in humans. It is now known that these receptors inhibit NK cell killing by recruitment of the SHP-1 phosphatase to their cytoplasmic ITIM motifs \(^{52}\).

Additionally, the “missing-self” model suggests that stimulatory receptors must also exist to direct granule exocytosis towards the target cells upon the loss of inhibitory stimuli. Some of these stimulatory receptors must recognize either constitutive self ligands (e.g., LFA-1 recognition of ICAM-1-bearing targets \(^{53}\)), or directly recognize “altered-self” or “induced-self” ligands that are upregulated on target cells. Thus, a more contemporary model proposes that NK cell function is regulated by a global balance or integrated sum of stimulatory and inhibitory receptor signals, which in turn are determined by target cell modulation of cognate cell surface ligands (Fig.1) \(^{9,54}\).
Figure 1. NK cell target recognition. NK recognition is mediated through a balance of both inhibitory and stimulatory signals. A normal cell, which expresses inhibitory ligands, such as MHC-I on the surface is protected from NK cytotoxicity. However following transformation or infection one of two things can happen. A missing-self target loses the expression of the inhibitory ligand thus favouring the balance of signals to the activating signal. In the case of an induced-self target, the upregulation of stimulatory ligands, such as NKG2DL in the presence of steady state inhibitory ligand is sufficient to favour the activating signal and cytotoxicity directed towards the target. It is important to note that missing-self, and induced-self targets are not mutually exclusive, and contribute overall to the sensitivity of a target to NK-mediated cytotoxicity.
In general, NK cell receptors belong to one of two main structural groups (Fig.2): (i) C-type lectin-like superfamily members, largely encoded by genes located within the NK cell gene complex (NKC; mouse chr.6, human chr.12); or (ii) Ig-like superfamily members, encoded by genes located within the leukocyte receptor complex (LRC; mouse chr.7, human chr.19). Rodent NK cell receptors specific for classical MHC-Ia ligands are C-type lectin-like (Ly49 family), while those in hominids are primarily Ig-like (KIR family) \(^{55,56}\). In addition, both the rodent and hominid genomes encode lectin-like NK cell receptors specific for ligands either distantly related or unrelated to MHC-I molecules. These include: (i) the CD94/NKG2 heterodimeric receptors for non-classical MHC-Ib molecules (e.g., Qa-1 in mice; HLA-E in humans \(^2\)); (ii) the NKG2D homodimeric receptor specific for induced-self ligands, largely oncofetal in nature (e.g., Rae-1, H-60, MULT-1 in mice; MICA/B, RAET-1, ULBP in humans) \(^2\); and the NKR-P1 homodimeric receptors for non-MHC lectin-like ligands (Ocil/C1r in rodents; LLT-1 in humans) \(^{57-64}\). NK cells display a plethora of other receptors that mediate self-nonself discrimination, such as NKp46, 2B4, CRACC, and DNAM-1 \(^{10,65,66}\).
Target recognition by NK cell is regulated by different NK cell receptor families

Figure 2. NK cell receptor repertoire. NK cells recognize their targets through a diverse array of cell surface receptors. There are both inhibitory and stimulatory receptors and the balance of signals dictates whether the target will be subject to NK-mediated cytotoxicity. There are receptor families that recognize classical MHC-I (Ly49 in mouse; KIR in human), Non-classical MHC-I such as Qa-1 in mouse and HLA-E in human (CD94/NKG2C,E in mouse and human), and distantly related MHC-I such as NKG2DL (NKG2D in mouse and human). Structurally, NK cell receptors are C-type lectin-like or part of the immunoglobulin superfamily. The NKR-P1 family consists of C-type lectin-like homodimeric receptors that recognize homodimeric C-type lectin-related ligands (Clr; CLEC2D). The CLEC2D genes are interspersed among the receptors facilitating co-inheritance of cognate ligand and receptor. To date, few NKR-P1 receptor ligand pairs are known. Some NKR-P1 receptors are capable of binding more than one Clr ligand, however in the case of NKR-P1B, the only known ligand is Clr-b.
1.1.7 NKG2D

NKG2D is a homodimeric type II transmembrane glycoprotein that is stimulatory in function. It is expressed mainly on NK cells, γδ T cells, and CD8+ T cells. Mouse and human NKG2D associate with the adaptor proteins DAP10 and DAP12 for proper surface expression and these different adaptor proteins engage different downstream pathways. In mice, two isoforms exist, which differ in the length of their cytoplasmic tail. The NKG2D long form associates with DAP10, whereas the short form is capable of associating with both DAP10 and DAP12. The differential use of DAP10 and DAP12 is reflective of different downstream signaling events as DAP10 activates PI3-K and DAP12 activates Syk and ZAP70 kinases. Engagement of NKG2D is sufficient to drive cell-mediated cytotoxicity as well as the secretion of cytokines such as IFN-γ, GM-CSF and TNF-α. Known ligands for NKG2D are comprised of 3 diverse groups in mice and humans. In humans, NKG2D (NKG2D ligands) belong to the MIC, and ULBP/RAET family. In mice the Rae-1 isoforms, Mult1, and H60 proteins have been characterized to bind NKG2D. The mouse NKG2DL are more evolutionarily similar to the human ULBP family. The MILL family discovered in rodents and marsupials appears to be most closely related to the MIC family in humans; however, they have lost their ability to bind NKG2D. NKG2D has been implicated in antiviral immunity and tumour clearance. NKG2D deficient mice are more susceptible to primary tumourigenesis and have an impaired ability to kill some tumours. Many tumours express NKG2DL and the discovery that some tumours could shed NKG2DL to avoid detection highlights the importance in targeting this receptor. In some viruses, the evolution of viral decoys that prevent NKG2DL surface expression through downregulation, retention or degradation also emphasizes the importance of this system.

1.1.8 The NKR-P1 Receptor Family

The NKR-P1 receptor family is composed of disulfide-linked homodimeric type II transmembrane C-type lectin-like receptors, conserved among rodents, birds, cattle, dogs, and hominids. They recognize Clr ligands, which are interspersed between the NKR-P1 genes. Their tight linkage likely facilitates coinheritance of receptor and cognate ligand genes. In contrast to the Ly49 system, the gene organization for the Nkr-p1-Clr family is well conserved between at least 3 mouse strains, B6, BALB/C, and 129, although significant allelic
polymorphisms do exist, such as that which abolishes NK1.1 reactivity in BALB mice. NKR-P1 receptors are expressed on NK cells, NKT cells, subsets of activated conventional T cells, and some myeloid lineages. Like most NK cell receptors, the NKR-P1 family is comprised of both stimulatory and inhibitory isoforms. NKR-P1 receptors have been implicated in recognition of both tumour cells and infected targets.

In mice, the NKR-P1A/C/F receptors are known to be stimulatory isoforms, NKR-P1E is an uncharacterized potential pseudogene, and NKR-P1B/D and NKR-P1G receptors are inhibitory in function. The NKR-P1F receptor appears to be expressed on most NK cells, at least in B6 mice, whereas NKR-P1B/D is expressed by NK cells, albeit in a variegated fashion. The NKR-P1C stimulatory receptor is expressed on NK cells, NK T cells, and activated T cells. Alleles of both the NKR-P1B and NKR-P1C isoforms confer the NK1.1 marker in selected mouse strains. Despite being the first NK cell receptor family identified, very few cognate receptor–ligand interactions were known until the last few years: the inhibitory NKR-P1B/D receptor interacts with Clr-b (C-type lectin-related-b; also known as Ocil, osteoclast inhibitory lectin), while the stimulatory NKR-P1F receptor interacts with Clr-g. Interestingly, the mouse and rat NKR-P1B:Clr-b systems appear to be orthologous, or at least homologous to the NKR-P1A:LLT1 system in humans, which has similarly been shown to inhibit NK cell function. Recent work that has expanded our knowledge of the Nkrp1-Clr system demonstrated that NKR-P1F from B6 or 129 strain mice was capable of interacting with not only Clr-g, but also Clr-d and Clr-c. Furthermore, the inhibitory NKR-P1G receptor was shown to interact with Clr-d, Clr-f and Clr-g. The ability of two Clr’s (Clr-d and Clr-g) to stimulate both inhibitory and stimulatory isoforms of a receptor family suggests that they may be under strong selective pressure, such that has been observed with viral decoys. However the functional significance for the dual recognition of inhibitory and stimulatory isoforms by endogenous host Clr’s is not known. Yet, in the case of NKR-P1B, regardless of the mouse strain, the only known ligand remains to be Clr-b.
1.1.9 NKR-P1B Receptor

NKR-P1B receptor cross-linking generates a localized inhibitory signal through its consensus ITIM motif (LxYxxL)\(^75\). Phosphorylation of the ITIM tyrosine residue is mediated by p56\(^\text{ck}\), which is recruited via a conserved cytoplasmic motif (CxCP) also found in the CD4 and CD8 co-receptor sequences\(^76\). Once phosphorylated, the ITIM recruits SHP-1 phosphatase, leading to de-phosphorylation of localized intracellular signaling proteins, including Syk, ZAP-70, PLC\(\gamma\)-1/2, LAT, SLP-76, Vav-1, Shc, Fc\(\varepsilon\)RI\(\gamma\), and (FcR\(\gamma\))\(^9,52,75\). These substrates include many of the signaling intermediates activated by stimulatory NKR-P1 receptors, which utilize the same CxCP motif to initiate phosphorylation of the ITAM-containing FcR\(\gamma\) adaptor protein and downstream Syk tyrosine kinase\(^77\). In general, equivalent inhibitory signals dominantly prevent co-localized stimulatory signals from inducing NK cell killing\(^35,75\).

1.1.10 Clr-b Regulation

Clr-b was first identified under the name Ocil, due to its ability to inhibit osteoclast formation from murine osteoblast spleen co-cultures. Expression of Ocil was found on osteoblasts, chondrocytes and extraskeletal tissues. The unknown receptor was expressed on macrophages/monocytes\(^78\). Ocil\(^{-/-}\) mice are viable and fertile, but adults exhibit mild osteopenia\(^79\). Not until the discovery that Clr-b was the cognate ligand for the NKR-P1B receptor was it determined that the Clr-b expression pattern actually resembles that of classical MHC-I: It is widely expressed on normal hematopoietic cells and is frequently downregulated on tumour cells\(^57\). However, Clr-b expression is \(\beta_2\)m-independent and unaffected by loss of MHC expression, making it the first documented MHC-independent missing-self ligand\(^57\). In addition to its demonstrated roles in normal self-tolerance and missing-self recognition of mouse tumour cells, Clr-b has recently been shown to play a role in missing-self detection of virus-infected cells in the rat\(^57,64\). Thus, Clr-b is downregulated under numerous pathological circumstances, by an unknown mechanism(s). Experimentally, Clr-b expression can be monitored, and NKR-P1B:Clr-b interactions can be blocked, using two mAb developed in our lab: 4A6 (\(\alpha\)-mouse-Clr-b) and R3A8 (\(\alpha\)-rat-Clr-b)\(^80\).
1.2 DNA Damage: Generalized Effect on Cell cycle and Apoptosis

DNA damage contributes to the evolution of cancer cells. Although epigenetic changes are contributing factors to cancer progression, underlying genetic changes leading to altered proteins can cause changes in cellular proliferation, differentiation, and death pathways of a cell. In turn, the cell activates tumour suppressors, such as p53, allowing the cell to attempt to repair the damage. Dysregulation of such tumour suppressors is a defining factor in many cancers.\(^\text{81}\).

Following DNA damage, a series of cellular responses can occur, such as cell cycle arrest, DNA repair, and activation of apoptotic pathways. In mammalian cells, DNA damage sensors, coupled with mediators engage the primary transducers of the DSB (double-strand break) repair signaling pathway such as RPA (Replication protein A; binds ssDNA), the 9-1-1 complex (Rad9-Rad1-Hus1), and the PI3-K-related (PIKK) proteins ATR and ATM.\(^\text{82,83}\).\(^\text{Fig.3}\).
Environmental insults that create single-stranded or double-stranded breaks, such as those generated by UV radiation and γ-irradiation, respectively, activate the DNA damage response (DDR). The DDR consists of proteins that detect lesions, transmit and amplify the DNA damage signal to downstream effectors such as p53, p21, and Cdc25 phosphatases. These proteins act on the cell cycle to halt progression at various checkpoints to allow the cell to attempt at repairing the damage. This includes the checkpoint prior to DNA synthesis (G1/S) and the checkpoint prior to mitosis (G2/M). The cell cycle is driven by cyclin-dependent kinases, with their catalytic cylin subunits. The expression and degradation of various cyclin partners is required to proceed through cell cycle.
Once activated, ATR (ATM and Rad3 related) and ATM (Ataxia-Telangiectasia Mutated) can phosphorylate numerous downstream targets, including the Chk1 (checkpoint kinase 1; primarily ATR target) and Chk2 (checkpoint kinase 2; primarily ATM target) checkpoint kinases, which are essential for cell cycle arrest before mitosis \(^{82-84}\). Although ATM and ATR respond to DSB, ATM responds to DSB during all phases of the cell cycle, whereas ATR acts primarily as a replication sensor (S-phase), responding to stalled replication forks (which generate SSB or DSB) \(^{85}\). Although there are distinct pathways that activate specific effectors (i.e. ATR-Chk1, ATM-Chk2), there is considerable overlap in the activation of downstream targets following ATR and ATM activation. This includes activation of the cdc25 phosphatases, which regulate cell cycle progression by acting on cyclin dependent kinases (CDKs) (Fig.3) \(^{83,86}\).

Cell cycle progression is controlled through the activity of catalytic CDKs, which are regulated through the activity of cyclins. Various CDK-cyclin complexes are activated during 4 different checkpoints in the cell cycle, namely G1, S, G2 (all part of interphase), and M (mitosis) \(^{87}\). The decision to undergo cell cycle arrest or apoptosis is largely governed by the p53 tumour suppressor protein. A primary target of p53 following DNA damage is p21, a CDK inhibitor. Through its inhibition of cyclin E-Cdk2 complexes, p53-activated p21 promotes cell cycle arrest in G1/S phase (Fig.3) \(^{83,88}\).

Following genotoxic stress, arrested cells that cannot repair their DNA will be committed to apoptosis. DNA damage-induced apoptosis is achieved primarily through p53, which acts as a transcriptional activator of genes encoding apoptotic effectors, including Bax, NOXA, and PUMA \(^{89}\). These pro-apoptotic proteins induce mitochondrial membrane permeabilization, resulting in the release of apoptogenic factors, including cytochrome c from the mitochondrial intermembrane space. Cytochrome C can act on downstream caspases resulting in the activation of caspase-activated DNase \(^{90,91}\).

### 1.2.1 DNA Damage Sensors and Mediators

There are numerous different DNA damage sensors within the cell, which serve to recruit different effectors to amplify and transmit the DNA damage signal depending on the type of lesion generated. These sensors work in conjunction with mediators to activate different aspects
of the DDR pathway. Important sensors and mediators of the DNA damage response include Rad17-RFC, the 9-1-1 complex, RPA, the MRN complex, H2AX, and the BRCT (BRCA1 C-terminus repeat) containing proteins, BRCA1, BRCA2, TopBP1 and MDC1 \(\text{(Fig.3)}\)\(^{82,83}\).

Following activating phosphorylation by ATM or ATR, depending on the nature of the genotoxic insult, the MRN complex and \(\gamma\)-H2AX (phosphorylated histone H2AX) act as mediators to amplify signals and aid in recruiting other mediators to the lesion \(^{92,93}\). MDC1 serves as a bridge between these protein complexes to recruit them to the same locations. The resulting complex can recruit more ATM and phosphorylate more downstream effectors \(^{83}\). The MRN complex is composed of three proteins: Mre11, Rad50 and Nbs1. Mre11 is a multifunctional protein that can interact with both Nbs1 and Rad50 independently. It has DNA nuclease, strand-dissociation, and strand annealing activities. These functions are modulated in part by ATP, Rad50, which stimulates Mre11 endonuclease and exonuclease activity, and Nbs1, which stimulates its endonuclease activity. In resting cells, Mre11 is distributed homogenously throughout the nucleus, and following genotoxic stress quickly forms nuclear foci, some of which are co-localized with \(\gamma\)-H2AX at the sites of damage \(\text{(Fig.3)}\). To date the function of the DDR-induced Mre11 foci is not quite known, however the general belief is that it concentrates DDR proteins at the sites of DSB \(^{92}\).

Histone variant H2AX is one of the earliest phosphorylated proteins following DNA damage. H2AX is phosphorylated at S-139 and the resulting \(\gamma\)-H2AX accumulates at the sites of DSB \(^{94}\). It can be phosphorylated by the PIKKs, ATM, ATR, and DNA-PK, and plays an important role in signal amplification; this is largely due to the recruitment of other DDR proteins at the sites of damage, including but not limited to MCPH1, 53BP1, BRCA1, RNF8/168, and the MRN complex. However the initial localization of these proteins to the sites of damage is not entirely dependent on \(\gamma\)-H2AX. Thus, \(\gamma\)-H2AX appears to participate in DDR, DNA repair, and checkpoint controls, but is not absolutely required \(^{94}\). This is evident in H2AX deficient cells, which have only minor defects in checkpoint signaling and DNA repair, and H2AX\(^{-/-}\) mice, which are viable, but have an increase in chromosomal abnormalities and radiosensitivity \(^{94,95}\).
During DNA repair and replication, ssDNA is formed as an intermediate. Subsequently, numerous RPA molecules coat the lesion. These complexes are directly recognized by ATRIP, which functions to localize ATR at the site of the DNA damage. However, for full activation of ATR, the 9-1-1 complex is required. The 9-1-1 complex is a heterotrimer that forms a ring-like structure. Following DNA damage, such as that caused by UV, IR, or replication inhibitors, the 9-1-1 complex gets loaded onto DNA by the Rad17-RFC complex. The Rad17-RFC complex has weak ATPase activity, which is enhanced when bound to ssDNA or primed DNA (dsDNA with ssDNA overhangs). Once bound to chromatin, the 9-1-1 complex facilitates the phosphorylation of downstream effectors such as ATR and ATM. MEFs or ES cells deficient in a single member of the 9-1-1 complex is sufficient to cause significant defects in Chk1 activation, a downstream kinase of ATR signaling. The actual method by which ATR is activated by the 9-1-1 complex following DNA damage has not been fully elucidated. Current insights suggest that the unique C-terminal tail of Rad9, which harbours at least 10 S/T residues for phosphorylation, may play a role. The phospho-tail of Rad9 is not required for 9-1-1 complex formation or loading onto chromatin, but harbours consensus PIKK phosphorylation sites. However, the contribution of the individual phosphorylation sites in ATR mediated Chk1 activation has not been fully elucidated. The BRCT repeat containing protein, TOPBP1, is recruited by the 9-1-1 complex through interactions with the phospho-tail of Rad9, and is an effective activator of the ATR-ATRIP kinase. Although in some systems, such as Xenopus, the Rad9 phospho-tail does not seem to be necessary to mediate TOPBP1 recruitment. Recently, a gene product was identified through a broad microscopy based assay using siRNA to screen and identify cells with inappropriate cell cycle entry after a large dose of IR. This protein, termed RHINO (Rad9,Rad1, Hus1 interacting nuclear orphan) was discovered to play a role in ATR-mediated activation of Chk1. More specifically, the 9-1-1 complex is required for the recruitment of RHINO. The 9-1-1 complex and TopBP1 are bound by RHINO independently, suggesting that RHINO may help bridge the gap between the 9-1-1 and TopBP1 to mediate optimal activation of ATR at the sites of DNA damage.

Important mediators of the DNA damage signal are the BRCT domain proteins 53BP1, BRCA1/2, an MDC1. These proteins facilitate the activation of downstream effectors such as Chk1, and Chk2. The BRCT domain is a ~100 amino acid tandem repeat that functions in protein-phospho-protein interactions. BRCA1 is an important protein in DDR, DNA repair,
and checkpoint activation as evidenced by increased cancer susceptibilities in humans with germline mutations in this protein. Mutations are commonly found in the C-terminal BRCT domain, or the N-terminal RING domain, which harbours E3 ubiquitin ligase activity. BRCA1 exists in different protein complexes depending on the downstream function. The BRCC complex, contains BRCA1, BRCA2, and Rad51 and appears to play a role in DNA repair, whereas the BASC complex, which contains many more proteins, including the MRN complex, mismatch repair proteins, DNA damage sensors, ATM, RFC and PCNA, functions in the sensing of DNA damage, and DNA replication. ATR and ATM can phosphorylate BRCA1 at both distinct and overlapping phosphorylation sites to influence variable downstream signaling. Serine-1423 is a residue that is important in both ATR and ATM activated pathways.

1.2.2 DNA Damage Transducers: PIKK family

The PI3-K like protein kinase family members (PIKK) ATM, ATR, ATX and DNA-PKcs transmit different DNA damage signals to the appropriate effector proteins through direct or indirect recognition of damaged DNA. Most of the PIKK family harbours serine/threonine kinase activity, and although they have domains similar to the PI3-K lipid kinase, they do not have any lipid kinase activity.

ATM is a protein kinase encoded by a gene that is mutated in humans with ataxia-telangiectasia. This condition is characterized by a notable cancer predisposition, genomic instability, immunodeficiency, cerebellar degeneration and clinical radiosensitivity. ATM responds to DSBs, such as those generated by ionizing radiation, and is recruited by the MRN complex; more specifically the Nbs1 C-terminus has been identified to contain an ATM interaction motif. Although it can still function in the absence of the MRN complex, activated ATM at the site of the MRN complex leads to more substrate recruitment and activation of downstream effectors such as Chk2 and p53. Oligomeric ATM exists as a dimer or multimer under steady state conditions and becomes autophosphorylated at S-1981 in humans following exposure to ionizing radiation, which leads to dissociation of the oligomers. The resulting ATM monomers can then act on numerous substrates. In mice, the equivalent phosphorylation site is at S-1987. Currently, there have been 5 phosphorylation sites identified to be functionally equivalent in humans and mice; S-367, -1893, -1981, -2996, and T-1885 in humans and S-367, -1987, -1999, -3006 and T-1891 in mice. However, the relative
importance of each site is unknown. Recently, a transgenic mouse was made in which 3 autophosphorylation sites were mutated to alanines: S-367, S-1893 and S-1981, all of which have been individually shown to be required for ATM activation and function. In this model, autophosphorylation at these residues was dispensable for DNA damage-induced ATM kinase activity, as measured through activation of downstream effectors such as p53, SMC1 and Chk2. However, this does not account for any other compensatory autophosphorylation sites, since several other sites have been noted. Other post-translational modifications of ATM, such as acetylation of K-3016 would also be intact in these mice.

ATR was discovered through a shared homology with ATM in the human genome database. However unlike ATM, knockout of ATR is embryonically lethal. It preferentially binds to DNA damaged by UV light, in contrast to the recruitment of ATM to DSB induced by ionizing radiation. The primary substrate that leads to ATR activation is ssDNA, which can result from DNA breaks, adducts, crosslinks, and stalled replication forks as a result of DNA polymerase inhibition. It is also activated in response to hypoxia, which does not activate ATM. ATR activation requires the accessory protein ATRIP for its recruitment and stabilization at DNA lesions. ATRIP has a C-terminal PIKK interaction motif. Without ATRIP, ATR fails to phosphorylate the downstream effector Chk1 at S-345 in response to UV light. TOPBP1 is also known to contribute to ATR-ATRIP kinase activity. Although ssDNA is a direct activator of ATR, dsDNA in the proximal area of the lesion also contributes to ATR activation due to the recruitment of the Rad17-RFC complex and the Rad9-Rad1-Hus1 (9-1-1) complex at the ssDNA:dsDNA junctions, both of which influence the downstream signaling through ATR. However, the recruitment of the Rad17-RFC complex and the 9-1-1 complex to the same sites as ATR-ATRIP-RPA occur independently of each other. The downstream substrates that are activated include, but are not limited to, p53 and BRCA1. With respect to the DSB response, ATR also appears to play a role in maintaining the phosphorylation status of some ATM substrates. Although many of the downstream effectors are shared between ATM and ATR, some appear to exhibit a preference, as exampled by Chk2 as the preferred substrate for ATM, and Chk1, the preferred substrate for ATR. Thus ATM and ATR have distinct, as well as redundant, or overlapping, functions that complement each other. Generally speaking, ATR acts primarily at the G2-M checkpoint, whereas ATM impacts the G1-S checkpoint.
ATX is the most recently discovered PIKK family member and appears to participate in a variety of responses, including the DNA damage response, and mRNA surveillance through the nonsense-mediated mRNA decay pathway, which helps remove mRNA species with premature stop codons. It has been shown to phosphorylate p53 in response to UV light and ionizing radiation\textsuperscript{105}.

DNA-PK is a component of the Non-Homologous End Joining pathway (NHEJ). DNA-PK consists of a catalytic subunit (DNA-PKcs) along with two accessory proteins Ku70 and Ku80. The Ku70:Ku80 heterodimer acts as a sensor for DSB and adheres to DNA ends, allowing recruitment of the catalytic subunit\textsuperscript{105}. It appears that the last 14 amino acids at the C-terminus of Ku80 harbour the actual PIKK interaction motif required for DNA-PKcs recruitment to DNA ends, even though such deletion did not impair Ku70:Ku80 heterodimer formation or DNA binding function\textsuperscript{104}. Deficiency in DNA-PK, and more specifically the catalytic subunit DNA-PKcs, is the culprit for severe combined immunodeficiency syndrome (SCID) in mice, dogs and horses. Hosts are immune-compromised due to a lack of T and B cells, which require the action of DNA-PK for production of primary receptor repertoires\textsuperscript{105,110}. Only recently has a missense mutation in DNA-PKcs been discovered in a T-B deficient (SCID) patient\textsuperscript{110}.

1.2.3 DNA Damage Transducers: Chk1 and Chk2

Following activation of the PIKK members ATM and ATR, the signal is amplified and transmitted to transducers that act on downstream effectors. Two important transducers of the DNA damage signal are Chk1 and Chk2 (Fig.3)\textsuperscript{82}.

Chk1 is mainly a cytosolic S/T kinase with some nuclear localization that acts directly downstream from ATR and has been well characterized in the context of the DNA damage checkpoint, more specifically the G2/M DDR\textsuperscript{82,111,112}. However it is also important during unperturbed S phase progression. During normal cell cycle, Chk1 protein expression is restricted to the S and G2 phases of cell cycle\textsuperscript{100,113}. Activation of Chk1 prevents premature mitotic entry, allowing for DNA to be properly replicated or repaired\textsuperscript{82,114}. Chk1 acts on numerous downstream targets, including proteins involved in cell cycle checkpoints, replication checkpoints, mitotic spindle checkpoints, DNA repair, and apoptosis (such as Cdc25A and Tlk Kinases)\textsuperscript{100,115}. Chk1 regulates the turnover of Cdc25A in a ubiquitin/proteasome-dependent
fashion, and in response to genotoxic stress, inhibits cyclin dependent kinases (CDKs), in turn leading to cell cycle arrest and/or a delay in G1/S or G2/M phases of cell cycle\textsuperscript{100}. Chk1 knockouts are embryonically lethal, which coincides with the fact that Chk1 is the preferred substrate of ATR, and ATR knockouts are also embryonically lethal\textsuperscript{82}. Phosphorylation by ATR at S-317 and S-345 increases the activity of Chk1, and requires the presence of the protein claspin. The actual recruitment of claspin to ssDNA requires ATR mediated phosphorylation of Rad17 as well as the Timeless/Tipin complexes bound to RPA\textsuperscript{93,115}. Recently, a truncated form of Chk1 (Chk-1s) was discovered, which was shown to be an endogenous repressor of Chk1 activation. Under steady state conditions, the N-terminally truncated protein associates with Chk1. Following DNA damage, such as that caused by camptothecin, a topoisomerase I inhibitor, Chk1 is phosphorylated at S-317 and S-345 which disrupts the Chk1-Chk-1s interaction, in turn allowing for normal Chk1 kinase activity, and finally leading to G2/M arrest. Under normal steady state conditions, forced overexpression of the Chk-1s variant results in premature mitotic entry, leading to mitotic catastrophe and cell death\textsuperscript{114}. Although, originally the ATM-Chk2 and ATR-Chk1 signaling arms were thought to be independent of each other, in recent years this view has been relaxed, due to numerous reports about crosstalk between ATR-Chk2 and ATM-Chk1 in the context of ionizing radiation, genotoxic stress, and other cellular stresses\textsuperscript{100,116}.

Chk2 is a nuclear S/T kinase, and although functionally similar to Chk1, it is structurally different\textsuperscript{117}. In contrast to Chk1, it is expressed through all phases of the cell cycle, but only activated in the presence of DNA damage, and contributes to the genomic integrity of the host in a conditional fashion\textsuperscript{100,117}. This is evident by the fact that Chk2 knockout mice are viable, likely compensated by Chk1 or other kinases, and have predominantly normal checkpoint responses. However, an increased resistance of Chk2\textsuperscript{−/−} mice to ionizing radiation and cellular defects in p53 function, especially in apoptosis have been observed\textsuperscript{100}. Following DNA damage, Chk2 activation occurs over several steps. The first step is the phosphorylation of T-68. This allows the N-terminal SQ/TQ-rich cluster on the Chk2 molecule to bind the FHA domain of another Chk2 molecule. The resulting dimer is required for full activation of Chk2. This is through trans-autophosphorylation of T-383 and T-387 in the T-loop of the kinase domain\textsuperscript{118}. In addition, oligomerization of Chk2 further increases its activity\textsuperscript{119}. Chk1 and Chk2 kinases exhibit redundancy as they share many substrates downstream such as the Cdc25 phosphatases and p53.
However, distinct functions of Chk2 have been uncovered over the years, including its role in regulating DNA repair by promoting the error-free homologous recombination repair pathway through its actions on BRCA1. In mouse cell lines, Chk2 is frequently lost due to point mutations, premature truncations, alternative splicing, and copy number variations, all of which reduce protein expression. Those that do retain high Chk2 activation, as measured by T-68 phosphorylation, are associated with p53 inactivation \textsuperscript{120}. In the human population, rare mutations to the Chk2 gene, CHEK2, have been discovered that are associated with an increase in susceptibility to develop cancer. The nature and location of the cancers has led to the consideration that Chk2 can prevent tumour progression, yet when dysregulated, Chk2 is a multi-organ tumour susceptibility gene. However, it must act in concert with other proteins because Chk2, itself does not directly predispose to cancer \textsuperscript{118}.

### 1.2.4 DNA Damage Effectors

Downstream from the DNA damage transducers are the DNA damage effectors such as p53 and Cdc25 phosphatases. They can be activated differentially, resulting in different downstream responses (Fig.3)\textsuperscript{83}.

The transcription factor p53 is activated upon genotoxic stress to transcriptionally activate genes that promote cell cycle arrest, senescence and apoptosis. There are also protein-protein interactions documented with p53 that enhance its role in stress responses. P53 plays an integral role in the DDR response, as germline mutations in TP53 result in increased risk in developing many cancers \textsuperscript{118,121}. A mutation at a single allele can lead to a loss in heterozygosity and eventual prevalence of the mutant allele in many tumours \textsuperscript{122}. In response to genotoxic stress numerous post-translational modifications can be made to modulate its function. This includes phosphorylation, acetylation, ubiquitylation, and methylation of specific residues. Under normal steady state conditions, Mdm2 is bound to p53, targeting it for ubiquitin-mediated degradation. Phosphorylation of p53 by DNA damage transducers prevents the association with Mdm2 allowing for function. Mdm4 is also a negative regulator of p53 that functions similarly to Mdm2. ATM and ATR are known to phosphorylate p53 at S-15 and T-18, and ATM-activated Chk2 phosphorylates p53 at S-20. Both Chk1 and Chk2 can phosphorylate p53 at similar and different residues \textsuperscript{118,121}. The nature and location of the phosphorylation or other post-translational modifications of p53 help dictate the resulting response. For example,
phosphorylation at S-46 or acetylation at K-320 and K-373 enables p53 to transcriptionally activate pro-apoptotic genes. In response to DNA damage, p53 induces the expression of p21, which mediates G1 cell cycle arrest through its inhibition of cyclin E-Cdk2 activity, in turn preventing cells from entering S phase. In the presence of DNA damage, p21 also binds to the Cdk4-Cyclin D complex, preventing the phosphorylation of Rb to initiate the transcription of S phase genes. Although p53 plays an important role in the DNA damage response prior to entry into S phase, it does not participate in the G2/M checkpoint, which prevents cells from undergoing mitosis in the presence of DNA damage.

The Cdc25 phosphatases, on the other hand, do participate in several DNA damage checkpoints, including the G2/M checkpoint. There are 3 mammalian phosphotyrosine-threonine phosphatase isoforms termed Cdc25A, Cdc25B, and Cdc25C, which dephosphorylate the Cdks, allowing for cell cycle progression. All three isoforms are involved in the G1-S and G2-M transitions through cell cycle; however, preference does exist. Cdc25A is primarily involved in activation of Cdk2-CyclinA or Cyclin E complexes within the G1/S phase, an Cdk1-CyclinB activation in G2/M phase. On the other hand Cdc25B and Cdc25C participate primarily in the entry into mitosis through their action on Cdk1-Cyclin B complexes, although some evidence supports their role in the entry to S phase as well. In response to diverse genotoxic stressors, ATM, ATR, Chk1 and Chk2 phosphorylate the Cdc25 phosphatase isoforms. Phosphorylation results in binding by 14-3-3 proteins, leading to cytoplasmic sequestration of Cdc25 isoforms from their target Cyclin/Cdk complexes, and subsequent degradation.

1.2.5 Classes of Genotoxic Agents

Many genotoxic agents employed are also antineoplastics used clinically, some of which can be classified based on the mode of action and the types of downstream repair pathways activated. This includes but is not limited to: i) ionizing radiation and radiomimetics, ii) alkylators (mono- or bifunctional), iii) antimetabolites, iv) topoisomerase inhibitors, and v) replication inhibitors. All of these agents act directly or indirectly to generate SSB and/or DSB that activate DDR pathways.

Ionizing radiation damages DNA bases and causes broadly distributed DSBs throughout the genome. Radiomimetics, such as bleomycin and phleomycin, are characterized as such due
to the similarities in the broad dispersion of the lesions generated. However, the actual mechanism of action is very different. Full activation of bleomycin requires ferrous iron as a cofactor and oxygen to create a free radical complex that oxidizes DNA bases preferentially at 5’-G(C/T)-3’ regions\textsuperscript{126}.

Alkylators such as cisplatin induce lesions by forming adducts with various substrates. Cisplatin is a platinum-based alkylating agent that generates nucleic acid (ssDNA, dsDNA, RNA) and protein adducts; These activate DNA damage proteins such as ATR, Chk1, Chk2 and p53, in turn leading to S and G2-phase cell-cycle arrest and apoptosis\textsuperscript{127,128}. In humans, the extracellular chloride ion concentration (~100mM) is high enough to keep cisplatin in an inactive state; however, once it enters the cell, where the chloride ion concentration drops to ~4mM, cisplatin becomes hydrated by water molecules, generating a charged species capable of preferential nucleophilic attack on N7 sites of purine bases\textsuperscript{127,129}. Uptake of cisplatin is mediated through the copper transporter Ctr1p in yeast, mouse and human cells, and can be enhanced in the presence of copper chelators\textsuperscript{130,131}.

Anti-metabolites such as 5-fluorouracil (5-FU) resemble nucleotides, precursors or cofactors involved in biosynthetic pathways that have an impact on the pool of available dNTPs\textsuperscript{125}. Inside cells, 5-FU is anabolized to FdUMP, which inhibits thymidylate synthase, resulting in the depletion of dTTP. If FdUMP is anabolized into FdUTP and incorporated into nascent transcripts, it can impair replication fork progression, due to base excision removal of the improper bases. If anabolized into FUTP, it can potentially incorporate into RNA, affecting function or stability of the transcript\textsuperscript{132}.

Camptothecin and etoposide are topoisomerase I and II inhibitors, respectively. The topoisomerases are enzymes that induce DNA breaks to prevent torsional strain that arises during replication\textsuperscript{125}. Topoisomerase I suppresses genomic instability by coordinating replication and transcription, specifically by preventing replication through active genes by inhibition of R-loop formation\textsuperscript{133}. During replication, topoisomerase I eases positive and negative supercoiling by causing a single stranded break in the DNA, allowing relaxing of the supercoiling, followed by religation of the broken DNA end. This is mediated through the cleavage complex, which is the catalytic intermediate of the topoisomerase bound to the DNA through a 5’tyrosyl adduct. It is
the cleavage complex that is the target of camptothecin, which is reversibly bound, generating both single and double-stranded breaks. Topoisomerase II is a homodimeric protein that plays an overall role in replication, chromosome segregation, and maintaining genomic stability. It generates DSB for which religation can be reversibly inhibited by topoisomerase II inhibitors such as etoposide. The resulting accumulation of DNA breaks activates the DDR. 

Aphidicolin is a DNA polymerase-α/β/ε inhibitor that activates the ATR checkpoint, promoting cell cycle arrest in G1/S phase by preventing the initiation or elongation during DNA replication. In *Xenopus* egg extracts, once the polymerase is inhibited, there is extensive unwinding of the DNA, generating a favourable ssDNA lesion for activation of ATR. UV-C induced DNA damage also causes nucleosome unwrapping and stalls DNA polymerases resulting in the activation of the ATR checkpoint.

Other genotoxic agents whose genotoxic effects haven’t been completely characterized include HDAC inhibitors (HDACi), such as Trichostatin A (TSA). HDACs are comprised of a family of 4 classes, by which the Type I-, II and IV are the most similar, whereas type III requires an NAD+ cofactor. Class I is nuclear, whereas Class II HDACs are both cytoplasmic and nuclear as well as more tissue restricted. HDACs play an important role in many processes, including mitosis and the DNA damage response through protein deacetylation. Although HDACi treatment does not generate DSB, it does stimulate the DDR. TSA is a type-I/II HDACi that prevents chromatin condensation, resulting in a broad but selective promotion of gene transcription. Anywhere from 2-10% of the transcriptionally active genes in the genome are affected through either upregulation or downregulation at equivalent ratios following treatment with HDACi. Moreover, the acetylation of a number of histone-independent proteins is augmented by HDAC inhibitors, including p53, TFIIE/F, hsp90, and p21^Waf1/Cip1^, the latter being associated with increased cell-cycle arrest.

### 1.2.6 Ubiquitin and the DNA Damage Response

Although originally identified as a tag that flagged proteins for degradation, ubiquitin plays a key role in the cell in various signaling processes, one of which is the DNA damage response. The ubiquitination process involves activating ubiquitin, transferring, and loading it onto a target protein through sequential steps involving E1, E2, and E3 ubiquitin ligases,
respectively. The common linkage involved in protein degradation is K-48; however, all 7 lysines (K-6, K-11, K-27, K-29, K-33, K-48 and K-63) in ubiquitin are capable of being either poly- or monoubiquinated, and some of these linkages are now known to mediate different cellular outcomes. For example, mono- or multiubiquitination of several lysine residues is involved in endocytosis, whereas K-63 polyubiquitin linkages, which have a more open structure than K-48 polyubiquitin linkages are involved in cell signaling.

Ubiquitin plays an important role in the DNA damage response. Interestingly, proteasomal inhibition alone, which depletes free ubiquitin, is sufficient to cause a reduction in the amount of phosphorylated DNA damage proteins such as ATM, 53BP1, Nbs1, BRCA1, and Rad51 in response to IR. At DSB foci, there is an accumulation of K-63 ubiquitin-linked proteins and complexes, and a lack of K-48 linked proteins. One such example is the DNA damage mediator, BRCA1, which is recruited to DSB sites in a K-63-dependent manner through the action of RAP80, a protein that contains many ubiquitin interaction motifs. Once activated by the PIKKs, ATR and ATM, the BRCA1-BARD1 heterodimer, with its E3-ligase activity deposits K-6 ubiquitin-linked chains on proteins at repairs sites. Deficiency in the E3-ligase activity of BRCA1-BARD1 dimer reduces the extent of Chk1 phosphorylation in response to ATR activation. However, the definitive role these ubiquitin chains have in modulating their downstream substrates is still being studied.

Another pathway that associates with BRCA1 in response to DNA damage is the Fanconi Anemia (FA) pathway proteins. The FA pathway is activated in response to DNA damage, more specifically DNA interstrand cross-links. Eight FA proteins form a complex, which monoubiquitinates FANCD2 through the subunit FANCL. Mutation in FANCD2 causes the human syndrome Fanconi’s Anemia, which is characterized by chromosomal aberrations that can lead to cancer due to defects in HR, translesional synthesis, and sensitivity to DNA cross-linking agents. Following ubiquitination, FANCD2 localizes to damaged chromatin and interacts with BRCA1/BRCA2, DNA repair enzymes, as well as other proteins. Proteasome inhibition, which is known to deplete the free ubiquitin pool over time, negatively affects the formation of ubiquitinated FANCD2 foci at the sites of DNA damage following ionizing radiation.
Post-translational modifications (PTM) such as ubiquitination have a notable effect in recruiting DDR proteins to the sites. But they are not the sole PTM responsible, as SUMOylation has also been reported to promote ubiquitination of proteins at the sites of DNA damage. Furthermore BRCA1 is targeted for SUMOylation and this modulates the E3 ligase activity of the BRCA1-BARD1 complex.

1.2.7 Cell Cycle and Genotoxic Stress

The mammalian cell cycle is regulated by cyclin-dependent kinases (CDK), which are heterodimeric serine/threonine kinases. CDK activity is regulated by regulatory subunits, termed cyclins. The ordered expression and destruction of cyclins enables the cell cycle to proceed in an uninterrupted fashion. In humans, there are currently 13 known CDKs and 25 Cyclins. Of these, only 5 CDKs (Cdk1, Cdk2, Cdk4, Cdk6, and Cdk7) and 10 cyclins (from the A-, B-, D-, and E-type cyclins) participate in the cell cycle. Following mitogenic stimulation, the D-type family of cyclins are induced, which bind and activate Cdk4 and Cdk6. The activated CDK complexes induce a partial inactivation of the pocket proteins, resulting in the expression of E-type cyclins that activate Cdk2. The pocket proteins include pRB, p107 and p130, and they are a family of proteins that bind and modulate E2F’s, a family of transcriptional repressors and activators. pRB is expressed in both proliferating and quiescent cells, whereas p107 is expressed mainly in proliferating cells, and p130 in arrested cells. Activated CDK/Cyclin E complexes trigger the full inactivation of pocket proteins, resulting in the expression of genes necessary for S phase. At this point, the cell has passed through the restriction point at the G1/S boundary and can proceed through cell cycle independent of a mitogenic stimulus. The S phase transition through G2 phase is driven by activated Cdk2/Cyclin A2 and Cdk1/Cyclin A complexes. Lastly, activated Cdk1/Cyclin B complexes are required to proceed through mitosis. Cdk7/Cyclin H complexes are also known to participate in the cell cycle through activation of Cdk1 and Cdk2 complexes. The biological inhibitors of CDK activity are the INK4A (p15, p16, p18, and p19) and CIP/KIP (p21, p27, and p57) families, through their inhibitory action on D-type and E-type cyclins, respectively.
The other mammalian cell CDKs are involved in processes such as neuronal development (Cdk5) and in the control of transcription (Cdk7, Cdk8, Cdk9, Cdk10, and Cdk11). These CDKs regulate RNA polymerase II activity, the formation of multi-subunit transcription factors, including those involved in RNA polymerase II elongation, and the RNA splicing machinery.

Pharmacological inhibition of CDK activity can be achieved with purine analogs such as roscovitine. Early work demonstrated that roscovitine was a potent inhibitor of Cdk1 and several types of Cdk2/Cyclin complexes in *Xenopus* oocytes, *Xenopus* embryos, and mouse cells, promoting arrest of cells in the G1/S and G2 phases. However, a study analyzing a larger panel of kinases established more targets for roscovitine, including Cdk3, Cdk5, Cdk7 and Cdk9. Roscovitine is also known to reduce Rb protein phosphorylation, and cause the depletion of many cyclin mRNAs such as D1, A and B1; it does this in part potentially through transcriptional inhibition by acting on Cdk7 and Cdk9. By depleting cyclin D1, roscovitine can act on Cdk4 by reducing the availability of the regulatory subunit. Thus, although roscovitine is characterized as a Cdk2 inhibitor, its effects in the cell and on the CDK family are more pleiotropic.

The DNA damage response and the cell cycle checkpoint pathways are interconnected and they share many similar endpoints. Furthermore, the CDK/Cyclin complexes enhance DNA repair and DNA damage sensing processes. During the G0/G1 phase, the NHEJ pathway predominates as the mode of DNA repair, whereas the high-fidelity HR pathway predominates in the S/G2 phase. In yeast, the loss of the S phase cyclins renders the cells more susceptible to genotoxic stimuli such as UV and IR. A more definitive link between the DDR and CDKs is the requirement for Cdk2/Cyclin A in the phosphorylation of ATRIP, which is required at ssDNA coated by RPA. Phosphorylation of ATRIP has been shown to be required for a proper G2/M checkpoint in response to IR and UV light. Furthermore, CDK-mediated phosphorylation of CtIP enhances the interaction between BRCA1 and the MRN complex. Lastly, CDK-mediated phosphorylation of Chk1 through Cdk1 and Cdk2 is required for a proper checkpoint response following exposure to ionizing radiation. The cyclins have also been implicated in the DDR. Cyclin E becomes stabilized in response to replication fork barriers induced by mitomycin C and UV, but not IR, resulting in a prolonged S phase. This DNA damage induced arrest is dependent on ATR. Cyclin D1 is highly expressed in many human cancers, and a proteomics analysis for
cyclin D1 partners identified several DNA repair proteins, including Rad51 and FANCD2. Cyclin D1 binds Rad51 in a BRCA2 dependent fashion in response to IR and appears to participate in HR.

1.2.8 Genotoxic Stress and NK cell recognition

Although many cell-intrinsic barriers exist to prevent malignant transformation of normal cells, there is a continually growing body of evidence to support the fact that the innate and adaptive immune systems can recognize transformed cells and target them for removal. In the case of the adaptive immune system, the presentation of tumour-associated antigens, or non-self antigens, can be recognized by B or T cells. In contrast, the innate immune system must rely on germline encoded receptors and ligands to differentiate self versus nonself. More specifically, while a number of NK cell receptor–ligand interactions have been discovered in the past few years, the underlying molecular basis behind the ability of NK cells to recognize “abnormal” or transformed target cells remains incompletely understood. The most well characterized interaction is that between MHC-I and Ly49 (mouse) or KIR (human) families. Tumour cells are known to escape recognition by the adaptive immune system by altering the antigen presentation machinery. The frequent loss of β2m, or TAP1/2 proteins results in the loss of MHC-I at the cell surface. In turn this will make the target cell more susceptible to NK cell killing, as the lack of MHC-I results in loss of engagement of the predominantly inhibitory Ly49, and KIR families. However, since NK cell cytolytic capacity is dictated through an integrated sum of inhibitory and stimulatory signals, at least one stimulatory ligand must be present to engage stimulatory receptors on the NK cell.

1.2.9 NKG2D-Ligands: Response in Healthy and Stressed Cells

Ligands for the stimulatory NKG2D receptor are known to be upregulated on virally infected, tumour, and stressed cells. NKG2DL are also known to be expressed at high levels on seemingly healthy cells. Although there are many theories to explain such a phenomenon, some studies suggest that NK cells are important in regulating the effector arm of the immune response. NK cells lyse regulatory T cells expanded in the context of Mycobacterium tuberculosis infection, which helps maintain a Th1 response necessary to clear such an infection. This is mediated in part by the upregulation of the NKG2DL, ULBP1. NKG2DL expression
is upregulated on cells that are rapidly proliferating, such as TCR/CD3-stimulated, CD28/PMA-induced, or PHA-induced CD4+ and CD8+ T cells. Furthermore, NKG2DL recognition by NK cells helps control T cell responses during viral infection, especially against viruses that have evolved ways to evade or alter adaptive effector responses, such as CMV. Additionally, perforin-deficient mice infected with LCMV exhibit greater lymphoproliferation of T cells compared to wild-type mice, suggesting a role for NK cells in the regulation of normal cells during effector processes. In contrast, NKG2DL recognition by NK cells can contribute to pathology in mice chronically infected with LCMV by impairing CD8+ T cell immunity. In fact, the viral dose (LCMV) may dictate the immunoregulatory role for NK cells, since only medium viral doses result in host death facilitated by NK cells, whereas low and high doses result in host survival through viral clearance and viral persistence, respectively.

### 1.2.10 DDR pathway and NK cell recognition

A common theme to the expression of NKG2DL on both healthy and stressed cells is the (ATR/ATM) DDR pathway. Activation of the ATR/ATM DDR pathways, through genotoxic stress, stalled replication forks, viral infection, T cell activation, or class switching in B cells has been shown to induce an upregulation of NKG2DL. The upregulation of NKG2DL has been observed using a diverse array of genotoxic stimuli, including γ-irradiation, 5-fluorouracil, aphidicolin, cisplatin, and UV-C. In this system, pharmacological inhibition of the ATM/ATR pathway blocked the induction of NKG2DL, and specific knockdown of ATR or Chk1 prevented aphidicolin-induced NKG2DL upregulation, whereas knockdown of ATM reduced constitutive NKG2DL expression. Furthermore, ligands for the DNAM-1 receptor, CD155 and CD112, are also upregulated upon activation of the ATM/ATR pathway. The DNAM-1 ligands, initially described as adhesion molecules, were shown to be induced on multiple myeloma cells following induction with the alkylating agent, melphalan, and the DNA intercalating agent doxorubicin, as well as the proteasome inhibitor, bortezimib. Pharmacological inhibition of the ATR/ATM pathway could block this effect, which was largely associated with cells arrested in the G2 phase of cell cycle. However, the modulation of inhibitory ligands during genotoxic stress remains undocumented to date.
1.3 Oncogenic transformation

Cellular senescence helps protect against tumour development, growth, and metastasis. When a cell escapes the checks and balances by activation of anti-apoptotic or pro-proliferative networks, the transformed cell becomes a prime candidate for tumour development \(^{164,165}\). Transformation is influenced through diverse factors and includes but is not limited to, anchorage-independent growth, genomic instability, CREB activation, epigenetic gene silencing, DNA damage, inactivation of cell cycle regulatory genes (e.g. p16INK4a, p53, pRB or p21CIP1/WAF1), NFκB-mediated inflammation, and overexpression of protooncogenes such as Ras, c-Myc, Abl tyrosine kinases, or Bmi-1 \(^{166-170}\). Two proteins of viral origin capable of transformation that are very well characterized are the HPV E6 and E7 proteins. These proteins are capable of inducing genomic instability by targeting Rb and p53, which modulates many processes within the cell, including cell cycle and cytokine secretion \(^{171}\). The Ras family was originally discovered as protooncogenes isolated from the Harvey and Kirsten Sarcoma Virus with transforming properties \(^{172,173}\). The human homologs were subsequently discovered and termed Ha-Ras (H-Ras) and Ki-Ras (K-Ras) \(^{174}\).

1.3.1 Ras Family

In humans and rodents, there are 3 Ras genes that functionally encode for four Ras proteins, H-Ras, N-Ras, K-Ras 4B, and the alternative splice variant, K-Ras 4A. The activity of Ras proteins are regulated by the actions of guanine nucleotide exchange factors (GEFs), and GTPase-activating proteins (GAPs), which activate and deactivate Ras, respectively, through the exchange of GDP and catalysis of GTP (Fig.4) \(^{170}\).
Figure 4. Generalized signaling through the Ras-MEK-Erk pathway. The Ras isoforms (H-, N-, K4A-, and K4B-) signal to downstream effectors which include the Raf kinases (A-, B- and C-). The Raf kinases are capable of hetero- and homodimerizing to affect downstream signaling differentially. However, the effect each dimer has individually on downstream signaling is not completely understood. Activated Raf kinases act on MEK 1 and MEK 2 kinases which are also capable of forming functional hetero- and homodimers. The activated MEK1/2 dimers act on their only known substrates, Erk1 and Erk2. Unlike the Raf and MEK1/2 kinases, Erk1 and Erk2 are only functional as homodimers. The activated homodimers are capable of acting on numerous downstream substrates through kinase and kinase-independent functions.
Following mitogenic stimulation, Ras transmits a proliferative signal downstream. In the presence of mutant forms of Ras the proliferative signal is constant and arrested cells can enter the cell cycle independent of mitogenic signals. The proliferative signals result in the induction of growth factor expression, growth factor receptor expression, integrins, and cell cycle transcription factors such as ELK1, ATF2, NFκB, and FOS. Cell cycle targets of these factors include cyclin D1 and cyclin G1. The signal generated by Ras can originate from the plasma membrane as well as intracellular compartments. The canonical downstream signaling pathway from Ras signaling is the activation of Raf proteins, MEK1/2, followed by ERK1/2. Other effectors of Ras include PI3-K, JNK Kinase, p38 MAPK and the Ral proteins. These proteins are implicated in the transformation capabilities of Ras; however, in the case of the Ral proteins, the downstream functions haven’t been fully elucidated.

Although a fair degree of homology exists between the ubiquitously expressed Ras proteins, it appears they have both specific and redundant roles in mammalian physiology. At the RNA level, the tissue distribution is similar and variable for all 3 isoforms. H-Ras RNA is more abundant in the skin, skeletal muscle and brain, whereas N-Ras RNA is more abundant in thymus and testis. K-Ras for the most part is expressed at comparable levels to N-Ras and H-Ras in various tissues such as the heart, lung, spleen and gut. Although comparable at the RNA level, K-Ras is the only gene required for mouse development, as H-Ras−/−, N-Ras−/− and H-Ras/N-Ras−/− knock out mouse models produced viable fertile offspring with no obvious defects. More specifically, the K-Ras 4B isoform is absolutely required, as K-Ras 4A was found to be dispensable for normal mouse development; although, a distinct function for K-Ras 4A in the differentiation of pluripotent embryonic stem cells with a tissue specific localization has been documented. With respect to function, Ras isoforms differ in their ability to activate downstream effectors such as PI3-K and C-Raf. K-Ras is more effective at recruiting and activating Raf-1 at the membrane, whereas H-Ras is more potent at activating PI3-K. The divergence in Ras function can be largely attributed to the C-terminus, which harbours the hypervariable region (HVR). The HVR, and more specifically the C-terminal CAAX motif is required for Ras to associate with the inner plasma membrane for efficient signaling. The CAAX motif is shared between the Ras isoforms, and thus the functional differences between them arises from post-translational modifications within the HVR. H-Ras harbours two
palmitoylation sites, and N-Ras, only one. K-Ras 4B does not contain a palmitoylation site, but instead a poly-basic domain comprised of 6 lysine residues. These sites may potentially alter the trafficking of the K-Ras isoforms. Evidence for this has come from quantitative EM analysis of the plasma membrane. Here, K-Ras is predominantly found associated with non-raft plasma membrane structures, whether it is loaded with GTP or GDP. On the other hand, H-Ras localizes to lipid rafts when bound to GDP, but is completely absent from rafts when bound to GTP \(^{175}\).

### 1.3.2 Oncogenic Ras

Mutations affecting GAP mediated hydrolysis of GTP promotes a state in which GTP is constitutively bound to Ras, resulting in persistent downstream signaling. In humans, activating mutations are found in as many as 20\% of tumours, with \(KRAS\) mutations in 85\% of them, \(NRAS\) mutations in \(\sim15\%\) and \(HRAS\) mutations in a very small fraction \(^{182}\). Generally speaking, \(KRAS\) mutations are found most frequently in colorectal, lung, and pancreatic carcinomas. \(HRAS\) mutations are found within skin, head and neck tumours as well as bladder cancers. \(NRAS\) is frequently mutated in liver, melanoma, and other hematopoietic malignancies \(^{170,183}\). Two common mutations that affect GTP hydrolysis directly or indirectly resulting in a constitutively active Ras are substitutions at Q-61, or G-12 and G-13, respectively. Mutations at Q-61 prevent GTP hydrolysis by impeding the interaction of a water molecule with the \(\gamma\)-phosphate of GTP. Mutations at the G-12 and G-13 residues attenuate GTP hydrolysis by preventing Vander Waals bond formation between Ras and the GAP resulting in steric hindrance of the Q61 residue \(^{170}\).

### 1.3.3 Raf Family

Immediately downstream in the Ras signaling cascade is the Raf family of serine/threonine kinases (Fig.4). In mammals, the Raf family consists of 3 isoforms, A-Raf, B-Raf and C-Raf. Activated Ras recruits Raf to the plasma membrane to signal to downstream kinases, the mitogen-activated protein kinases 1 and 2 (MEK1 and MEK2) \(^{182}\). Functionally, the Raf proteins are not subject to the redundancy that the Ras isoforms exhibit during development \(^{184}\). Structurally, the Raf proteins harbour 3 conserved regions, with numerous phosphorylation sites that are both shared and unique. At the transcript level, all three isoforms are ubiquitously expressed, however some tissue enrichment is observed, such as B-Raf in neuronal tissue and A-Raf in urogenital organs \(^{184}\). The Raf proteins function as homo- and heterodimers creating 6
possible activated complexes with different potential for downstream signaling. B-Raf-C-Raf heterodimers are thought to be more active than their respective homodimers, and C-Raf itself is more important in signaling to the downstream effectors MEK1 and MEK2. The differential function of the Raf proteins is highlighted by the fact that over-expressed kinase dead B-Raf
\[D594A\] is capable of activating C-Raf, which promotes aneuploidy in several mouse tissues. Furthermore, C-Raf but not B-Raf is required for the initiation of lung cancer by K-Ras\(G^{12D}\) in murine models. Genetic studies demonstrated that mice deficient in any of the isoforms do not survive. ARAF\(^{-/-}\) mice die shortly after birth due to gastrointestinal and neurological defects, whereas BRAF\(^{-/-}\) and RAF1\(^{-/-}\) mice die in utero between day 10.5-12.5 post-coitum, due to various defects. In humans, mutations that activate or hinder B-Raf function are fairly prevalent, especially in skin (melanomas) and thyroid tumours. The BRAF\(^{V600E}\) mutation has the highest incidence and results in an activated conformation. In contrast, RAF1 and ARAF mutations are extremely rare in human tumours. This may be due to the molecular events required to activate these kinases. B-Raf has a higher basal kinase activity, and recruitment to the plasma membrane by Ras through phosphorylation is sufficient to induce large changes in activity. Thus, single amino acid substitutions can have the potential to generate mutants with higher basal activity. The A-Raf and C-Raf kinases share conserved phosphorylation sites, and do not have a very strong basal activity. Thus, the generation of active isoforms would require a more complex set of mutations.

### 1.3.4 MEK Family

The Raf family of proteins signal downstream to the dual specificity kinases MEK1 and MEK2 (Fig.4). Mutations in these proteins are prevalent in some developmental disorders and human cancers. MEK1 and MEK2 are capable of phosphorylating their only known substrates to date, Erk1 and Erk2, at serine, threonine or tyrosine residues. Activation of MEK1/2 requires the phosphorylation of two serine residues that are in close proximity to each other: S-218, S-222 on MEK1 and S-222, S-226 on MEK2. Activated MEK1 and MEK2 are capable of homodimerizing, and have been recently shown to heterodimerize as well. Much like the other components of the Ras-Raf-MEK-Erk signaling cascade, MEK1/2 have both redundant and unique roles. The exact role the MEK1/2 proteins perform is largely dependent on the model being employed. In Hela cells, MEK1/2 are both required for proliferation.
Furthermore, in mouse and human epidermal cells, combined deletion of MEK1 and MEK2 was required to abolish the phosphorylation of Erk1/2, leading to apoptosis and ultimately impacting the skin barrier \(194\). In contrast, MEK1 knockdowns are embryonically lethal due to placental defects \(190\), whereas MEK2 knockdowns are viable and fertile with no apparent defects in the lymphoid subsets investigated, suggesting that MEK1 can compensate for the loss of MEK2 \(195\). In other systems using colon cancer cells, knockdown of MEK2 promotes proliferation of cells, with minimal effect on phospho-ERK activity, whereas knockdown of MEK1 induces G1/S growth arrest through sustained phospho-ERK activity through the induction of p21 \(196\). The unique functions of MEK1 and MEK2 can be further supported by the role that MEK1 has in MEK1/2 heterodimer function. The MEK1/2 heterodimer is stable within cells through negative feedback phosphorylation of T-292 on MEK1 by the Erk1/2 proteins. Mutations that prevent heterodimerization result in prolonged Erk activation through the actions of an unregulated MEK2 \(192\). Although the heterodimer has a novel function it supports current bodies of evidence that MEK2 is a more potent activator of ERK2 than MEK1 \(197\). Furthermore, the nature of the phosphorylation impacts the intracellular sorting of the Erk targets. MEK2 activated Erk2 tends to localize to the cytoplasm whereas MEK1 activated Erk2 accumulates in the nucleus \(198\). Interestingly, it appears the Raf family targets the MEKs differently. In HeLa cells, A-Raf is a more potent activator of MEK1 in response to EGF stimulation, whereas C-Raf activates both MEK1 and MEK2 \(199\).

### 1.3.5 ERK Family

Immediately downstream from the MEK1/2 proteins are the Erk1 and Erk2 kinases (Fig.4). They are ubiquitously expressed proteins involved in a diverse number of cellular processes such as proliferation, differentiation, transcriptional suppression, and chromatin remodeling \(200,201\). Erk1/2 are activated through dual phosphorylation of threonine and tyrosine on a conserved TEY motif \(202\). They share 83\% protein identity and have redundant and unique functions \(200,201\). Erk2 knockout mice are embryonically lethal due to placental defects and problems with mesoderm differentiation. In contrast, Erk1 knockout mice are viable, with only reported minor defects in T cell development and activation \(203\). Both Erk1 and Erk2 participate in the G1/S cell cycle transition following activation of Ras. This is achieved through the accumulation of D-type cyclins due to transcriptional and post-translational regulation. The
resulting phosphorylation of Rb family members allows for the expression of genes required for DNA replication. In NIH3T3 fibroblasts, Erk1 antagonizes Erk2 in oncogenic H-Ras^Q61L^-dependent proliferation, an effect that is not dependent on Erk1 kinase activity. RNAi knockdown of Erk2 alone, but not Erk1 prevents oncogenic Ras-mediated cell proliferation. Furthermore, Erk1 shuttles between the cytoplasm and the nucleus at a lower rate than Erk2 owing to an additional 20 amino acids of sequence in its N-terminus. This affects the ability of Erk1 to carry proliferative signals to the nucleus. In contrast, HeLa cells exhibited no difference in proliferation rate following silencing of either Erk1 or Erk2. In support of this is recent work using genetically-deficient MEFs in which Erk1/2 are determined to be functionally redundant with respect to induction of replicative senescence following G1 cell cycle arrest. However, these experiments were performed independent of oncogenic Ras. Thus, the duration and the magnitude of the Erk1/2 signal may potentially impact the downstream pro-proliferative response. In fact, studies have demonstrated that activation of Erk1/2 by constitutively active Ras or Raf is sufficient to induce the expression of Cdk inhibitors p16, p15, and p21, leading to senescence through cell cycle arrest in immortalized as well and non-immortalized cells. This senescent phenotype has been recapitulated in vivo in K-Ras^V12^-induced lung adenomas.

During resting states, most of the Erk1/2 pool is cytosolic and associated with the microtubule network and scaffold proteins. Activation results in phosphorylation of Erk1/2 leading to homodimerization and nuclear import. Erk1/2 can homodimerize with phosphorylated and unphosphorylated Erk1/2, although the functional significance of the hemiactive homodimer is unclear. Although Erk1/2 do not have a canonical nuclear localization sequence (NLS), importin7 was demonstrated to be required for nuclear shuttling. There are hundreds of known targets of Erk1/2, including but not limited to RSKs, MSKs, and the MNK kinase families as well as the immediate early gene, Elk1, the transcriptional suppressor, Erf1, and Raptor, a component of mTOR signaling. Erk1/2 phosphorylated Elk1 induces transcription of the gene C-fos, which is important in proliferation and differentiation. Erf1 is normally unphosphorylated and suppresses transcription in resting cells. Following phosphorylation by Erk1/2, Erf1 is exported out of the nucleus allowing for the transcription of genes, such as those involved in cell cycle progression. Erk1/2 also carry out cell cycle functions independent of their catalytic activity as demonstrated by transfecting kinase-dead Erk2^K52R^-K52R into Erk1/2 knockdown cells. The Erk2^K52R^-K52R mutation produces a
nonproductive binding mode for ATP, preventing proper catalysis \(^{211,212}\). Erk2 is also capable of directly repressing the transcription of some genes by binding to the G/CAAAG/C consensus sequence \(^{213}\).

The Ras-Raf-Mek-Erk pathway is frequently mutated, or subject to over-stimulation in cancers, due to its capacity to enhance proliferation. Since the MEK proteins are the only known activators of Erk1/2, they are a frequent target of pharmacological intervention to prevent the integrated signals from Ras and Raf proteins to reach the nucleus \(^{189}\). Two commonly used MEK1/2 inhibitors are the small molecule inhibitors, UO126 and PD98059. They are highly specific ATP-independent competitive inhibitors and prevent Erk1/2 phosphorylation by binding and locking MEK1/2 in a catalytically inactive conformation \(^{189}\). However, these inhibitors lacked the pharmaceutical properties to be used therapeutically. They are also not as specific as originally believed, since the Erk5/BMK1 pathway is also inhibited by PD98059 and UO126. This pathway is not inhibited \textit{in vitro} when using the MEK inhibitor, PD184352 at lower concentrations (below 10\(\mu\)M), which was the first of its kind to enter clinical trials. Current derivatives of PD184352 are being optimized to increase bioavailability and pharmacological effect \(^{189,214}\).

\subsection*{1.3.6 Ras signaling and the DDR.}

Increased proliferative signals from constitutively active Ras, such as that from H-Ras\(^{G12V}\), promote an increase in the amount of DNA replication origins and collapsed replication forks, which activates the DDR. Numerous senescence-associated DNA-damage foci that contain ATM, ATR, Nbs1, and H2AX DDR proteins are present upon by H-Ras\(^{G12V}\) transfection, but only when in the presence of active replication \(^{215}\). H-Ras\(^{G12V}\) is also known to cause cells to bypass the G2 DNA damage checkpoint through an accelerated G2/M transition, and induce mitotic spindle checkpoint defects. Thus oncogenic Ras participates in the generation of genomic instability \(^{170}\).

\subsection*{1.3.7 NK cell sensing of oncogenic transformation}

\textit{In vivo}, oncogenic transformation has been demonstrated in an E\(\mu\)-C-Myc mouse model in which NKG2D ligands are upregulated upon lymphoma development \(^{216}\), thus increasing susceptibility to NK cell killing. NKG2D ligands were also found to be increased on tumours
derived from transformed cells \(^{162}\). In mouse fibroblasts, oncogenic transformation mediated through Ras causes a loss of cell surface MHC-I, ligand for the inhibitory Ly49 receptors on NK cells \(^{217}\). This is due to loss of proteins important in MHC-I processing and presentation such as TAP1, TAP2, LMP2 and LMP7 \(^{170}\). NK cells are also capable of recognizing the BCR-ABL1 oncogene expressing primary bone precursors in an MHC-I dependent manner \(^{218}\). To date, whether oncogenic stress modulates other NK cell ligands, such as those that mediate MHC-independent missing-self recognition remains undocumented. Future work will help to elucidate the complement of NK cell receptor-ligand interactions required for recognition of targets undergoing oncogenic transformation.
1.4 Thesis goals

Natural killer (NK) cells are capable of targeting tumour cells lacking “self” markers. The receptor-ligand pair, NKR-P1B:Clr-b, has been shown to mediate “missing-self” recognition of tumour and infected cells. In contrast to the rationale for the selective loss of MHC-I on tumour cells (pressure to evade T cell-mediated immunity), the basis for the “missing-self” loss of Clr-b on tumour cells is unclear. Understanding how a programmed cell-autonomous response mediates Clr-b downregulation in real-time during genotoxic stress and transformation will complement our knowledge of the events that lead to innate recognition of transformed cells, how malignant NK cell-resistant tumours evolve, and how chemotherapeutic agents influence NK cell recognition of tumour cells.

Tumour cells possess dramatically reduced Clr-b expression relative to normal cells *ex vivo*. Events that lead to transformation may initiate an intrinsic programmed cellular response pathway resulting in loss of Clr-b at the cell surface. In addition, genotoxic and oncogenic stress have been shown to enhance NK cell susceptibility by upregulating stimulatory NKG2D and DNAM-1 ligands and downregulating MHC-I. Therefore, similar responses to DNA-damaging events may be sufficient to cause a decrease in function of Clr-b, resulting in enhanced NK cell susceptibility.

The goal of my thesis was to investigate the effects of genotoxic and oncogenic stress and the corresponding signaling pathways on the expression and function of Clr-b as a ligand for the inhibitory NKR-P1B NK cell receptor. This will aid in our understanding of the mechanism by which MHC-independent “missing-self” regulation of Clr-b is achieved in normal versus transformed cells. The long-term goal is to delineate the series of events that leads to loss of Clr-b on tumour cells.
Chapter II

Methods
2 Methods

2.1 Mice, Cell Lines, and Tissue Culture

C1498 and NIH3T3 cells were purchased from the ATCC and maintained in culture for less than 6 months from resuscitation. MNK-1 was generated in our lab from primary mouse fetal thymic NK cells, as described previously. Primary mouse embryonic fibroblasts (MEF) and IL-2 lymphokine-activated killer (LAK) cells were generated from B6 and CD-1 mice bred and maintained in our own facilities. BWZ.36 cells were received from Dr. Nilabh Shastri (University of California, Berkeley, USA). Human 293T cells, EL4, RMA, and RMA-S were obtained from Dr. David Raulet (University of California, Berkeley, USA). All cells were cultured in Dulbecco’s Modified Eagle’s Medium-High Glucose (DMEM-HG), with 2 mM glutamax (Invitrogen), 10 U/mL penicillin, 10 mg/mL streptomycin, 100 µg/mL gentamicin, 110 µg/mL sodium pyruvate, 50 µM 2-mercaptoethanol, 10 mM HEPES, supplemented with either 10% or 20% FBS.; MNK-1 cells were maintained in rhIL-2 (300-500 U/mL Proleukin; Novartis) 35. For most treatments, 2-5x10^5 cells were seeded in 2mL complete media in 6-well plates and dosed for 24h, with or without 1h pre-treatments with caffeine, MG132, or lactacystin. Pre-treatment with toyocamycin was three hours prior to addition of genotoxic agent.

2.2 Chemicals and Chemotherapeutic Agents.

Aphidicolin, bleomycin, cisplatin, camptothecin, etoposide, 5-fluorouracil, phleomycin, roscovitine, Scriptaid, trichostatin-A, tunicamycin, 5-aza-2’-deoxycytidine, caffeine, H_2O_2, α-amanitin, actinomycin-D, cycloheximide, parthenolide, pifithrin-α, SB203580, UO126, FR180204, SP600125 and PD98059 were purchased from Sigma-Aldrich. BIX02189 was purchased from Cedarlane Laboratories. CI-1040 was purchased from US biological. Ly294002, MG132 and lactacystin were purchased from Calbiochem. γ-irradiation was performed using a Gammacell-1000 Cs-source irradiator. Toyocamycin was purchased from Berry & Associates. IFN-α4 was provided by Dr. Eleanor Fish. UV-C irradiation was performed using a XL-1000 Spectrolinker. Chemicals were dissolved according to manufacturers instructions in DMSO, water, unsupplemented media or PBS.
2.3 Ras/Erk Experiments

Dominant negative vector sets for CREB, p53, IκBα, Raf and Ras were purchased from Clontech. Co-transfection of dominant negative vectors was performed with either pMaxGFP (Lonza) or CMV-GFP-NLS at a 10:1 ratio. The H-Ras wild type (WT), Constitutive active (CA), and Dominant-negative (DN) sequences were cloned into the pBud4.1CE vector that was modified to harbour eGFP (Clontech) in the BamHI site of the CMV driven MCS (Invitrogen). ERK2 reconstitution experiments were performed with H-Ras CA cloned into the pBud4.1CE eGFP vector (gift from Dr. Eleanor Fish).

2.4 PCR and Cloning

PCR amplifications for the purposes of cloning were performed using the relevant primers with the ExpandPLUS High Fidelity enzyme (Roche Applied Science, Laval, QC) (Table 1). PCR products were cut from gels and purified using a gel extraction kit (Invitrogen). The resulting PCR products were TA cloned directly into pcDNA3.1/V5/His/TOPO (Invitrogen), and confirmed through sequencing (Macrogen Inc., Seoul, South Korea or TCAG Sequencing Facility, Sick Kids Hospital, Toronto, Ontario, CAN).

2.5 GeneSOE ERK2

Wild type Erk2 was cloned from MMM1013-9201102 (Open Biosystems) based on NCBI accession number BC058258. Mutant Erk2 products were generated by GeneSOE using the ExpandPLUS High Fidelity enzyme (Roche) with the indicated primers (Table 1). The two step reaction was achieved by performing a primary amplification of the two mutated fragments [94°C, 120s; 94°C, 30s; 58°C, 30s; 72°C 90s] x 15 cycles, which were then gel purified. The purified products were used as template with the full-length Erk2 primers at the same amplification conditions to generate a full-length mutant, which was then cloned and sequenced. All mutant products required 2 fragments to be fused together with exception of Erk2<sup>L73P S151D</sup> which required 3 fragments to be fused together. WT Erk2 and mutant variants were cloned into the EF-1α driven MCS of the pBud4.1CE vector that was modified to harbour turboRFP or eGFP in the CMV driven MCS (Invitrogen).
2.6 Flow Cytometric Analysis.

All commercial mAbs were purchased from BD Pharmingen or eBioscience. Biotinylated anti-mouse-Clr-b monoclonal antibody (4A6 mAb) was generated previously. NKG2DL were visualized using NKG2D/hIgG fusion protein plus goat-anti-hIgG-PE (Jackson ImmunoResearch). MHC-I levels were analyzed using anti-K\textsubscript{b}D\textsuperscript{b} mAb or anti-K\textsuperscript{d} FITC (BD Pharmingen). Streptavidin-R-phycoerythrin (SA-PE) or streptavidin-allophycocyanin (SA-APC) were used as secondary reagents (Invitrogen). Briefly, cell suspensions were stained in 100 µl of staining buffer (HBSS, without phenol red or Mg\textsuperscript{2+}/Ca\textsuperscript{2+}, plus 0.5% BSA and 0.03% NaN\textsubscript{3}) for 25 minutes on ice for primary, washed in staining buffer, stained for 20 minutes with secondary reagent on ice, then washed again. Cells were then analyzed using a BD FACSCalibur flow cytometer and FlowJo software (TreeStar). All plots show cells gated for viable cells, as determined by live-cell gating by forward scatter, side scatter, and lack of propidium iodide uptake; viability for all experiments was greater than 80-90%. Untreated control stains show analysis of cells treated with solvent alone.

Experiments in Chapter V were acquired on a BD LSR II with a laser configuration of 405nm (Violet), 488nm (Blue), 545nm (Yellow-Green), and 633nm (Red). Live cell and doublet gating was determined through forward scatter by side scatter, area and width, and a lack of DAPI uptake. Cells were stained as above.

2.7 Cell Cycle Analysis

One million cells were stained with primary biotinylated antibody for 25 minutes on ice. The washed cells were spun down at 300g for 5min, and fixed with mixture of PBS:70% ethanol at a 1:4 ratio under gentle vortexing. The cells were incubated on ice for 60 minutes then washed with PBS, followed by a wash in staining buffer. The secondary antibody was then added for 20 minutes on ice. Cells were washed and resuspended.

2.8 BWZ Reporter Assay.

BWZ.36 reporter cells expressing CD3\textgreek{z}-fusion receptors were described previously. For cell-based assays, 10\textsuperscript{5} stimulator cells were treated for 24h, washed with PBS, then co-cultured overnight with 5x10\textsuperscript{4} BWZ reporter cells prior to analysis of β-galactosidase activities.
Activity was measured by adding 100µl of buffer containing 90 mg/L chlorophenol-red-β-D-galactopyranoside (CPRG) (Roche Applied Science), 9 mM MgCl$_2$, and 0.1% NP-40 (Sigma-Aldrich) in PBS. Plates were incubated at room temperature, and analyzed using a Varioskan microplate reader (Thermo Scientific, VWR) with a signal-background subtraction set at OD$_{595}$.

2.9 RT-PCR and Quantitative Real-Time PCR.

Total RNA was prepared using either Trizol, the PureLink RNA Mini Kit (Invitrogen), or the RNeasy Mini Kit (Qiagen). RNA was reverse-transcribed using the Super-Script III Kit and oligo-dT$_{12-18}$ primer (Invitrogen). PCR on resulting cDNA was performed using a PTC-240G Tetrad (Bio-Rad) for 35 cycles (Clr-b) or 28 cycles (G3PDH/GAPDH), as follows: 94°C, 30s; 53°C, 30s; 72°C, 60s with the relevant primers (Table 1).

Quantitative real-time PCR was performed using iTaq SYBR Green with ROX (Bio-Rad) on an ABI Prism 7000 (Applied Biosystems), as follows: 95°C, 5min; [95°C, 30s; 61°C, 60s x 37 cycles]; or performed with SsoFast EvaGreen (Bio-Rad) on a CFX96 (Bio-Rad) at: 98°C, 120s; [98°C, 2s; 61.5°C, 5s. x 40 cycles] with the relevant primers (Table 1) Products were confirmed by dissociation curve analysis.

2.10 $^{51}$Cr-Release Cytotoxicity Assays.

Lymphokine activate killer (LAK) effector cells were prepared from bone marrow (BM) cells and splenocytes grown in complete medium containing 1000-2000 U/mL rhIL-2 plus 15 ng/mL mIL-15 (Peprotech) for 5-6 days. Cytokines were replenished every 48 hours. Target C1498 cells were incubated with 100mCi of sodium chromate (Perkin-Elmer) for 1.5 hours at 37°C in 100µl FBS. Targets were washed by underlaying the suspension with 1ml of FBS, then washed 3 times with supplemented DMEM-HG and mixed with the effectors plated in 1:3 dilutions in a 96-well V bottom plate. If necessary, blocking was performed for 30 min at room temperature. The resulting mixture was briefly pelleted at 1000 RPM for 1 min. The effector:target mixture was allowed to incubate for 4 hours, at which point, 100µl of supernatant was aliquoted onto a solid scintillation LumaPlate-96 (Perkin Elmer) and dried down overnight. Supernatants from target cells alone or target cells plus 1% SDS yielded the spontaneous or
maximal release counts, respectively. Counts were read on a TopCount NXT Microplate Scintillation Counter (Packard Instrument Company). The counts obtained from the culture supernatant at different effector:target (E:T) ratios were used to determine the percent specific lysis, calculated as 100 x \(rac{[(\text{experimental CPM}) - (\text{spontaneous CPM})]}{[(\text{maximal CPM}) - (\text{spontaneous CPM})]}\).

2.11 Retroviral Transduction

The full-length Clr-b coding sequence was subcloned into pMSCV2.2-CMV-IRES-GFP. Retroviral shRNA constructs based upon the pMND-Banshee vector (which contains an LTR-driven GFP reporter gene) were previously characterized, validated and provided by Dr. Stephan Gasser (National University of Singapore)\(^{221}\). shRNA target sequences were Chk1, 5’-CAA CTT GCT GTG AAT AGA AT-3’; Chk2, 5’-GAA GTG GAG CCT TCG TAA A-3’; ATM, 5’-GAG GTG GCT CTT ATT CTA C-3’; scrambled control, 5’-AAC AAT CTG TTG CAG TGA TA-3’. Retroviral supernatants were generated in 293T cells and stable transductants of C1498 and NIH3T3 cells were isolated by “spinfection”, as previously described\(^{57}\). Stable transductants (GFP\(^{+}\)) were sorted after 48 hours, allowed to grow for 5 days, then mixed with untransduced cells in a 1:1 ratio in 6-well dishes, followed by treatment with the indicated chemicals. After 24 hours, the cells were harvested and analyzed by flow cytometry.

2.12 Lentiviral shRNA Knockdown of ERK Proteins

Proviral vectors to generate lentivirus harbouring shRNA were obtained from Drs. Jason Moffatt and Troy Ketela (University of Toronto). Lentivirus was produced by transfecting proviral vector with packaging vectors pCMV-VSV-G and pCMV-dR8.2 dvpr (obtained from Sam Kung (University of Manitoba)) at a ratio of 5:4:1 (Proviral vector: pCMV-dR8.2 dvpr: pCMV-VSV-G) into 293T cells. Supernatant was collected after 48 hours and NIH3T3 fibroblasts were infected with 80\(\mu\)l of viral supernatant in complete media containing 8\(\mu\)g/ml polybrene at room temperature for 30 minutes. Cells were plated and after 24 hours selected with 2.5 \(\mu\)g/ml Puromycin for 4 more days. Cells were divided into 3 fractions and either frozen down, made into lysates for Western Blot analysis or plated for transfection. The shRNA target sequences for Erk proteins were as follows: Erk2 (Mapk1) clone A3 GACATGGAGTTGGACGACTTA; Erk1
2.13 Western Blotting

Cells were pelleted at 1500RPM for 5min. Following aspiration, 75µl of RIPA Buffer + 2mM PMSF (Bioshop) + 1X Protease Inhibitor cocktail (Bioshop) was added to the pellet. Lysate was incubated on ice for 30 min then spun down at 12,000g for 10min at 4°C. The supernatant was then transferred to a new tube. Protein concentrations were determined by mixing diluted lysates with Bio-Rad Protein Assay Dye Reagent (Bio-Rad) and compared to a BSA standard curve run at the same time. OD$_{595nm}$ was read after incubating with Protein Assay Reagent for 5 minutes. Normalized protein amounts were mixed with 2X SDS (1X final) and boiled at 110°C for 5 minutes. Samples were stored at -20°C until needed. Lysates, kaleidoscope (Bio-Rad) and biotin ladder (Cell Signal) were loaded on 10% Mini-Protean TGX Pre Cast gels (Bio-Rad) in a Mini-Protean Tetra System with Running Buffer (w/1% SDS) at 0.4mA until a desired band migration was achieved. Blots were transferred for 65V at 90 minutes onto an Immobilon P$^{SQ}$ membrane (Millipore) that was pre-soaked in 100% methanol and washed in transfer buffer. The resulting blot was blocked with 5% Milk in TBS-T for 1 hour at room temperature or overnight at 4°C. The blot was then washed 3 times for 5 min. in TBS-T. Primary antibody was incubated overnight at 4°C on the blot in either 5% BSA TBS-T or 5% Milk in TBS-T depending on manufacturers guidelines. Erk1 (rabbit pAb), Erk2 (rabbit pAb), Erk1/2 (rabbit mAb), ERK5 (rabbit pAb) and β-Tubulin (rabbit mAb) were purchased from Cell Signal. The blot was then washed 3 times for 5 min in TBS-T. Secondary antibody was incubated for 1 hour at room temperature in 5% BSA TBS-T. Anti-rabbit HRP was purchased from Jackson Immunoresearch. Anti-biotin HRP was purchased from Cell Signal. After washing, the blot was incubated with Immobilon Western HRP chemiluminescent reagent (Millipore) and visualized on a ChemiGenius2 using GeneSnap and GeneTools software (Syngene, Frederick,MD, USA).
2.14 Cell Transfections and Nucleofection

Cells were transfected either with Effectene (Qiagen) following manufacturers protocol, with Lipofectamine 2000 (Invitrogen), at a modified ratio of 1µg pDNA to 2µl of reagent for NIH3T3 fibroblasts, or with PolyJet (FroggaBio), at a modified ratio of 1µg pDNA to 1.5µl of reagent. Media was changed after 24 hours and transfections were assessed after 24 or 48 hours. For experiments relating to H-Ras cell were transfected for 24 hours, then dosed with inhibitors or vehicle alone for another 24 hours. Flow cytometry was then performed.

Nucleofection was performed with 1 million cells. Following centrifugation and careful aspiration, 1µg pDNA was added to the cells. Then 100µl Nucleofector Reagent R (for NIH3T3) or Reagent V (for C1498) was added to the cells and mixed gently before adding the suspension into the electroporation cuvette. The mix was subjected to either the U-030 program for NIH3T3 fibroblasts, or the A-020 program for C1498 cells. Warmed media was gently added to the suspension to remove the cells, and the desired dilution of cells was made for plating. Cells were allowed to recover and begin gene transcription for 4 hours before any other manipulations were made.

2.15 Ribozyme Inhibition Experiments

pSIcheck2 vectors (Promega) harbouring the wild type, enzyme mutant and substrate mutant of Clr-b were kindly provided by Dr. Lucas Horan (Berkeley, California). Following nucleofection of pSIcheck2 vectors in C1498 and NIH3T3, cells were dosed with genotoxic agents following a 4 hour recovery period. Following 24 hours with chemical, cells were subjected to a Dual Luciferase Assay. Toyocamycin experiments with accompanied genotoxic agent were performed by pre-incubation of cells with toyocamycin for 3 hours prior to addition of genotoxic agent. Both chemicals were left in contact with the cells for the remainder of the experiment.
2.16 Dual Luciferase Assays

Nucleofected cells were plated in 24 well plates at the desired density. Cells were dosed for 24 hours, at which point they were lysed with 100µl 1x Passive Lysis Buffer (PLB). Plates were shaken with PLB for 25 min at room temperature, then 20 µl of lysate was pipetted into a white opaque 96 well plate. The plates were loaded into a Varioskan Plate Reader with two mechanical dispensers (Thermo Scientific, VWR). The pumps were primed with the Dual-Luciferase Substrates and the machine was programmed to dispense 100µl of Luciferase substrate, read for 10 seconds, then dispense 100µl of Stop-N-Glo Buffer followed by another 10 second read. The ratio of Luciferase/Renilla Relative Light Units (RLU) was then calculated.
**Table 1: Primer pairs utilized throughout the thesis.**

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<th>Gene Product</th>
<th>Forward Primer (5' → 3')</th>
<th>Reverse Primer (5' → 3')</th>
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Chapter III

Chemotherapy-induced genotoxic stress promotes sensitivity to NK cell
cytotoxicity by enabling missing-self recognition

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All assays were performed by J Fine. shRNA validation was performed by Dr. S. Gasser.
Additional independent experiments (biological replicates) contributed by P. Chen and A. Mesci

This work has been published in Cancer Research. 2010. Sep 15;70(18):7102-13.
3.1 Abstract

Natural killer (NK) cells can recognize and kill tumour cells lacking “self” markers, such as class I MHC, but the basis for this recognition is not completely understood. NKR-P1 receptors are members of the C-type lectin-related NK cell receptor superfamily that are conserved from rodents to humans. Identification of Clr ligands for the NKR-P1 receptors has facilitated functional analysis of MHC-independent target cell recognition by NK cells. One receptor-ligand pair, NKR-P1B:Clr-b, can mediate “missing-self” recognition of tumour and infected cells, but the role of this axis in sensing stressed cells remains unknown. Here, we show that Clr-b is rapidly downregulated on cells undergoing genotoxic and cellular stress at the level of both RNA and surface protein. Stress-mediated loss of Clr-b on leukemia cells enhanced cytotoxicity mediated by NKR-P1B+ NK cells. Notably, Clr-b downregulation was coordinated functionally with stress-mediated upregulation of NKG2D ligands (but not class I MHC). Our findings highlight a unique role for the MHC-independent NKR-P1B:Clr-b “missing-self” axis in recognition of stressed cells.

3.2 Introduction

Innate immunity constitutes an important front-line defense and barrier to infectious disease and malignancies [1] . NK cells are innate lymphocytes capable of recognizing and eliminating a wide variety of target cells, including transformed, infected, transplanted, antibody-coated, and stressed cells [2]. NK cell effector mechanisms include cell-mediated cytotoxicity mediated by perforin, granzymes, and cell-surface molecules (e.g., FasL, TRAIL), and rapid secretion of cytokines (e.g., IFN-γ, TNF-α, chemokines) [2]. While NK cells are tolerant to normal “self” cells, they frequently respond to abnormal cells undergoing pathological alterations. How NK cells mediate self-nonself discrimination at the molecular level remains the focus of intense research.

The frequent correlation between heightened NK cell cytotoxicity and a lack of MHC-I expression on tumour cell lines led to the hypothesis that NK cells sense the absence of “self” MHC-I markers on target cells. Termed “missing-self” recognition [5], this pathway is governed by inhibitory receptors specific for self MHC-I molecules that normally override stimulatory
NK–target cell interactions. In turn, the loss of MHC-I molecules upon malignant transformation or infection becomes sufficient to trigger NK cell cytotoxicity, via disinhibition of effector function \(^{52}\). However, this mechanism alone is insufficient to explain the complex outcomes that regulate NK–target cell interplay.

Indeed, other potent stimulatory NK cell receptors such as NKG2D recognize “induced-self” ligands dynamically upregulated during stress responses. Thus, a contemporary dual-recognition model proposes that NK cell function is regulated by a balance of stimulatory and inhibitory receptor signals, which in turn are determined by target cell modulation of numerous cognate ligands \(^{9,54}\). This more target-centric model highlights the need to understand how both NK cell receptors and their cognate ligands are modulated in real-time under pathological conditions, in order to fully appreciate complex NK cell recognition events \textit{in vivo}.

The underlying molecular basis behind the ability of NK cells to recognize “stressed” cells remains incompletely understood. Recent evidence indicates that cells exposed to chemotherapeutic agents, genotoxic stimuli, or stalled DNA replication cycles dynamically upregulate stimulatory NKG2DL \(^{221}\). This induced-self ligand modulation is mediated in part through the DNA damage response (DDR) pathway, in particular involving ATR, ATM, and Chk1 \(^{222}\). NKG2DL upregulation has been observed in response to diverse genotoxic stimuli (including aphidicolin, cisplatin, 5-fluorouracil, \(\gamma\)-irradiation, and ultraviolet (UV) radiation) \(^{162}\), as well as heat shock responses \(^{223}\), antigen-mediated activation of T cells \(^{161,224,225}\), and dysregulation of Dicer expression \(^{226}\). In addition, ligands for other stimulatory NK cell receptors, such as DNAM-1 (CD226), appear to be similarly upregulated \(^{163}\). However, the modulation of inhibitory NK cell ligands during stress responses remains undocumented to date.

In addition to MHC-I molecules, several MHC-independent inhibitory ligands have recently been characterized, including Clr-b (Clec2d), ligand for the NKR-P1B (Klrb2) receptor \(^{9,57,58,71,73,78}\). Importantly, the expression pattern of Clr-b closely resembles that of classical MHC-I antigens. Clr-b is widely expressed on normal hematopoietic and non-hematopoietic cells and is frequently lost on tumour cells \(^{57}\). Moreover, like MHC-I, Clr-b expression is rapidly downregulated following CMV infection \(^{64}\). Thus, Clr-b appears to be lost under numerous pathological circumstances, and may function as an inhibitory rheostat in the routine detection of
abnormal cells. While the mechanism(s) governing Clr-b regulation remain to be elucidated, events leading to transformation or infection appear to initiate a programmed cellular response pathway culminating in the loss of Clr-b at the cell surface \textsuperscript{64,71}. This intrinsic host response pathway differs from the extrinsic CTL-selected or immunoevasin-induced loss of MHC-I on tumour or infected cells, and thus may share common elements known to be involved in the DDR pathway responsible for NKG2DL upregulation.

In this report, we investigated the influence of cell stress on Clr-b expression. Various chemotherapeutic agents and inducers of genotoxic or physiologic stress were found to promote a rapid functional downregulation of Clr-b transcripts and cell surface protein. Notably, MHC-I cell surface molecules were not substantially altered by similar treatments (or actually increased). Loss of Clr-b rendered targets cells more susceptible to NK cell killing. Collectively, these results demonstrate that genotoxic stress promotes missing-self regulation of the NK cell inhibitory ligand, Clr-b, at both the RNA and surface protein levels.
3.3 Results

3.3.1 Rapid and dose-dependent loss of cell surface Clr-b in response to diverse genotoxic agents.

We have previously shown that Clr-b is broadly expressed on most normal cells, yet frequently lost on tumour cells; this renders transformed target cells more susceptible to NK cell cytotoxicity via NKR-P1B-mediated missing-self recognition. However, the underlying basis for the loss of Clr-b during transformation remains unclear. To gain better insight into the regulation of Clr-b expression, we investigated whether Clr-b levels are influenced by genotoxic stress, an initiating event in transformation. To this end, several Clr-b+ mouse cell lines were screened for their responses to various genotoxic agents, then evaluated for surface expression of Clr-b. For comparison, we also monitored expression of classical MHC-I molecules and NKG2DL.

As previously shown, C1498 cells (NKT-like acute leukemia), MNK-1 cells (IL-2-dependent thymic pre-NK line), NIH3T3 fibroblasts, and MEF cells express high levels of cell surface Clr-b. Strikingly, treatment of these cells with chemotherapeutic agents and chemicals known to induce genotoxic stress promoted a rapid downregulation of Clr-b surface protein (Fig.5; Fig.6; Fig.2; Fig.8A). Loss of Clr-b expression was a rapid and dynamic event, and the magnitude of downregulation was controlled in a dose- and time-dependent manner (Fig.5A).
Figure 5. Rapid and dose-dependent downregulation of Clr-b on C1498 leukemia cells in response to genotoxic and cellular stress. (A) C1498 cells were treated as indicated and analyzed by flow cytometry. Histograms show untreated cells (thick black line), treated cells (thin solid lines, with maximal treatment indicated by gray shading), or secondary reagent alone (dotted line). (B) C1498 cells were treated as indicated for 24h, then analyzed as in (A). Data are representative of 3 independent experiments.
Figure 6. Clr-b downregulation on 4 different cell lines in response to genotoxic and cellular stress. C1498, NIH3T3, MNK-1, and B6-strain MEF cells were treated as indicated in Fig. 5, and analyzed by flow cytometry. Cells were stained with anti-Clr-b antibody (4A6 clone), recombinant NKG2D human Fc chimera for NKG2DL, or anti-K{\textsuperscript{a}}D{\textsuperscript{b}} or anti-K{\textsuperscript{q}} for MHC I. Bar graphs indicate the fold up/down-regulation in median fluorescence intensity (MFI) levels of Clr-b, NKG2DL, and MHC I, relative to untreated control cells. Error bars indicate standard deviations of 3 experiments.
Figure 7. DMSO treatment of C1498 and NIH3T3 cells. C1498 leukemia cells and NIH3T3 fibroblasts were treated for 24 hours with DMSO or no treatment and analyzed by flow cytometry. Histograms show untreated cells (black line), treated cells (shaded histogram), or secondary reagent alone (dotted line). Data are representative of 3 independent experiments.
Figure 8

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Figure 8. Downregulation of Clr-b on various Clr-b+ cell lines in response to chemotherapeutic agents and inducers of genotoxic or cellular stress. Flow cytometric analysis of (A) Clr-b, (B) NKG2DL (NKG2D human Fc chimera), and (C) MHC-I (K<sup>Q</sup>D<sup>B</sup> or K<sup>S</sup>) expression on four Clr-b+ cell lines (C1498, MNK-1, NIH3T3, and B6-strain MEF) treated with various agents for 24h. The concentrations utilized were as follows: aphidicolin (16μM), cisplatin (15μM), camptothecin (40μM), etoposide (20μM), roscovitine (20μM), trichostatin-A (15nM; 150nM for C1498), Scriptaid (6μM), UV-C (40J/m²), γ-irradiation (27Gy), bleomycin (40μg/mL), 5-fluorouracil (200μM), tunicamycin (0.5μ g/mL), or H<sub>2</sub>O<sub>2</sub> (400μM). Histograms show treated cells (shaded area), untreated cells (solid line), or secondary reagent alone (SA-PE or goat-anti-hIgG-PE or isotype control mAb; dashed line). Data are representative of 3 independent experiments.
To investigate distinct modes of cellular stress, Clr-b modulation was evaluated in response to a variety of chemotherapeutic and genotoxic agents, as well as agents that induce alternative stress responses. This effect was not due to the solvent used for delivery as concentrations double the amount used in the assays did not result in a change in Clr-b expression (Fig.7). Several but not all genotoxic agents efficiently promoted Clr-b downregulation on 4 cell lines at pharmacological doses within 24h (Fig.6; Fig.8A). Interestingly, analogous treatments had similar effects on Clr-b expression, including: (i) camptothecin and etoposide (topoisomerase-I/II inhibitors); (ii) trichostatin-A and Scriptaid (histone deacetylase inhibitors); and (iii) γ-irradiation, bleomycin, and phleomycin (ionizing radiation and radiomimetic agents). This highlights a correlation between similar stress response pathways and the extent of Clr-b downregulation.

In contrast, NKG2DL were frequently upregulated under these conditions (Fig.6; Fig.8B), while MHC-I surface levels remained largely unaffected (or increased somewhat; (Fig.6;Fig.8C)). Collectively, these combined responses are expected to enhance the susceptibility of target cells to NK cell cytotoxicity. Since heterogeneity in responses existed among the cells tested, factors such as transformation state, cell type, or other cell-intrinsic properties may influence the degree or magnitude of Clr-b or NKG2DL modulation. Notably, Clr-b was strongly and consistently downregulated on several cell lines in response to treatment with aphidicolin (DNA replication inhibitor), cisplatin (DNA crosslinking agent), and roscovitine (CDK inhibitor), but not bleomycin (DNA-cleaving radiomimetic) (Fig.5;Fig.6;Fig.8A). Therefore, we tested whether the responses to these agents had functional consequences on NK cell recognition.

3.3.2 Genotoxic stress enhances susceptibility to NK cell cytotoxicity and diminishes NKR-P1B-mediated recognition of Clr-b ligand.

Loss of surface Clr-b on cells undergoing genotoxic stress would be expected to enhance NK cell cytotoxicity, as previously observed for tumour cells. To address this, treated target cells were analyzed in standard ⁵¹Cr-release cytotoxicity assays. C1498 targets were used because they represent a model acute leukemia cell line used broadly in cytotoxicity assays, they are one of few tumour lines that express high levels of Clr-b, and they modulate Clr-b but not other known NK cell ligands in response to several genotoxic agents. To further
control for receptor specificity, we took advantage of known NKR-P1B allelic polymorphisms between two mouse strains, CD-1 and B6, which have been previously shown to influence the magnitude of NKR-P1B:Clr-b-dependent inhibition in cytotoxicity assays: CD-1-strain LAK are strongly inhibited via the NKR-P1B\textsuperscript{CD-1} allele, while B6-strain LAK are only weakly inhibited via the NKR-P1B\textsuperscript{B6} allele (a.k.a., NKR-P1D\textsuperscript{33}), in response to target cells expressing Clr-b\textsuperscript{57,230}. Because the NKR-P1B receptor is known to exhibit variegated expression on NK cell subsets\textsuperscript{9,230}, the use of CD-1-strain LAK allowed us to monitor surface expression of the NKR-P1B\textsuperscript{CD-1} allele (via PK136 mAb\textsuperscript{9,33,35,57}), while mAb specific for the NKR-P1B\textsuperscript{B6} allele are not commercially available\textsuperscript{58,230}.

As shown in Fig.9A, both BM-derived and splenic LAK from CD-1 mice express high levels of NKR-P1B. Furthermore, C1498 targets treated with aphidicolin or cisplatin possess strongly reduced levels of surface Clr-b, while NKG2DL and MHC-I surface levels remain largely unaltered (Fig.9B; Fig.6; Fig.8B/C). In contrast, C1498 cells treated with roscovitine lose Clr-b and induce NKG2DL, while cells treated with bleomycin maintain Clr-b levels and moderately upregulate NKG2DL. As expected, treatment with genotoxic agents rendered C1498 targets more sensitive to cytotoxicity mediated by CD-1 strain LAK (Fig.9C). Moreover, target cytotoxicity was increased to a greater extent in response to aphidicolin, cisplatin, or roscovitine (which strongly downregulate Clr-b), compared to bleomycin (which does not promote loss of Clr-b). On the other hand, cytotoxicity mediated by B6-strain LAK was minimally affected by aphidicolin or cisplatin, while cytotoxicity was increased by roscovitine or bleomycin, consistent with the moderate increase in NKG2DL expression observed in the latter treatments. Importantly, known receptor polymorphisms affecting CD-1 versus B6 LAK function are limited to the NKR-P1B and Ly49 receptors, yet MHC-I levels do not change upon aphidicolin or cisplatin treatment. This rules out a role for loss of MHC-I ligands or induction of stimulatory ligands recognized by non-polymorphic NK cell receptors in contributing to the augmented cytotoxicity observed for CD-1-strain LAK. This strongly argues that enhanced cytotoxicity mediated by NKR-P1B\textsuperscript{+} LAK is due to loss of Clr-b expression on C1498 targets exposed to the genotoxic agents, aphidicolin and cisplatin.
Figure 9. Loss of NKR-P1B-mediated inhibition of NK cytotoxicity and Clr-b ligand function in response to genotoxic stress. (A) CD-1-strain BM and splenic LAK cultures were analyzed by flow cytometry for NKR-P1B expression (shaded histogram) versus secondary reagent alone (dotted line). (B) Flow cytometric analysis of Clr-b, NKG2DL, and MHC-I (Kb Db) expression on treated C1498 cells (shaded area), untreated cells (DMSO alone; solid line), or secondary reagent alone (dotted line). C1498 cells were treated overnight (24h) with aphidicolin (20µM), cisplatin (10µM), roscovinine (10µM), or bleomycin (40µg/mL). (C) Standard ⁵¹Cr-release cytotoxicity assay of CD-1 or B6 LAK cells versus C1498 target cells treated as in (B). Plots indicate mean of triplicate percent specific lysis values ± SEM for the indicated effector:target (E:T) ratios. (D) Left panel: BWZ– parental cells or BWZ.CD3c/NKR-P1B reporter cells were plated overnight in media alone (—), stimulated using PMA+ionomycin (P/I), or mixed with BWZ.Clr-b stimulator cells (Clr-b). Normalized OD₅₉₅₅₅₅ values are shown. Right Panel panel: C1498 stimulator cells were treated with the indicated concentrations of agents in situ, then incubated with BWZ.P1B reporter cells overnight. (E) Normalized OD₅₉₅₅₅₅ values for MNK-1, NIH3T3, and MEF are treated with aphidicolin (APH;16µM), bleomycin (BLM;40µg/mL), cisplatin (CIS;30µM), or roscovinine (ROS;20µM) or DMSO alone. Stimulator cells were treated with the indicated concentrations of agents in situ for 24h, then incubated with BWZ.P1B reporter cells overnight. Data are representative of 3 independent experiments.
To specifically address the loss of surface Clr-b ligand function in isolation, BWZ reporter assays were performed. Here, BWZ.36 reporter cells \(^{219}\) expressing a chimeric CD3ζ/NKR-P1B fusion-receptor (BWZ.CD3ζ/P1B cells \(^{57}\)) were used to assess NKR-P1B-dependent recognition of treated stimulator cells. As shown in Fig.9D, BWZ.CD3ζ/P1B reporter cells respond specifically to stimulator cells expressing Clr-b ligand, while BWZ– parental cells fail to respond. Next, C1498 stimulator cells were incubated in situ for 24h with various genotoxic agents in 96-well plates, followed by washing and overnight incubation with BWZ– or BWZ.CD3ζ/P1B reporter cells. Importantly, these results confirm that Clr-b ligand function is lost in a dose-dependent manner, yet only in response to genotoxic agents that downregulate Clr-b cell surface expression (i.e., aphidicolin, cisplatin, roscovitine, but not bleomycin; (Fig.9D; Fig.5; Fig.6; Fig.8A)). This loss of cognate ligand function was independently confirmed with the 3 other cell lines treated with the same 4 genotoxic agents (Fig.9E)
3.3.3 Genotoxic stress-mediated loss of Clr-b occurs at the transcript level.

It was previously shown that Clr-b is rapidly lost at both the transcript and surface protein levels in response to cytomegalovirus (CMV) infection. However, it is not known whether Clr-b downregulation following genotoxic stress occurs at the transcript level or whether the surface protein is being actively internalized. To address this, Clr-b transcripts were analyzed by semi-quantitative RT-PCR using primers spanning the entire coding sequence. As shown in Fig.10A, Clr-b transcripts are rapidly lost in C1498 cells undergoing genotoxic stress. Moreover, the loss of Clr-b transcripts following stress induction correlates temporally with the loss of Clr-b protein at the cell surface (Fig.10A; Fig.1; Fig.6; Fig.8A), similar to previous observations following CMV infection. Thus, like virus infection, genotoxic stress like that promoted by transformation directly regulates steady-state levels of endogenous Clr-b transcripts.

Figure 10

Figure 10. Genotoxic stress promotes a loss of endogenous Clr-b transcripts. Semi-quantitative RT-PCR analysis of Clr-b transcript expression (full-length coding sequence) in C1498 cells either untreated or treated with cisplatin (5 μM) as indicated (using 3-fold serial cDNA dilutions). Inverse ethidium bromide gel images (Left Panel) and quantitative signal intensities (Right Panel) (using Quantity One software) are shown.
3.4 Discussion

Clr-b (also known as Ocil/Clec2d \(^{71,73,78}\)) is a C-type lectin-like ligand for the inhibitory NKR-P1B receptor that functions in MHC-independent missing-self recognition by NK cells \(^{57,58}\). Although Clr-b is expressed on almost all nucleated hematopoietic cells, and many non-hematopoietic cells, it is frequently lost on tumour cells \(^{57}\) and rapidly downregulated during cytomegalovirus infection \(^{64}\). How these pathological processes regulate Clr-b expression is currently unknown, as is whether Clr-b surface expression is influenced on normal cells in response to stress. It has been previously shown that stimulatory ligands for the NKG2D and DNAM-1 immunoreceptors are upregulated by the DDR pathway following genotoxic stress, an effect that enhances the susceptibility of target cells to NK cell-mediated lysis \(^{162,231}\). Here, for the first time, we demonstrate that genotoxic stress promotes the functional downregulation of an inhibitory NK cell ligand, Clr-b, at the transcript and surface protein levels on mouse tumour cells and fibroblasts. In particular, several agents that induce genotoxic stress via diverse mechanisms were found to promote a rapid loss of Clr-b cell surface expression in a dose- and time-dependent manner. Consequently, “missing-self” Clr-b ligand downregulation on target cells undergoing genotoxic stress was found to functionally diminish NKR-P1B-mediated recognition and thereby enhance NK cell-mediated cytotoxicity. The ability of numerous distinct chemotherapeutic and genotoxic agents to promote Clr-b downregulation suggests that cell stress may initiate a conserved and programmed cellular response governing Clr-b expression. Thus, pathways governing the cellular response to transformation \(^{57,162}\), genotoxic stress (an initiating event in transformation), virus infection \(^{64,232,233}\), and other pathological states may share common features.

How Clr-b transcript and surface protein levels might be regulated requires further investigation. Activation of the DNA damage response has been demonstrated to be involved in modulating the expression of ligands for NKG2D and DNAM stimulatory receptors \(^{162,163}\). It is unknown whether the DDR pathway response influences the loss of Clr-b in response to genotoxic stress. Additionally, post-translational modification has been shown to be involved in the upregulation of NKG2DL in response to cellular stress \(^{223}\). Furthermore, activation of PI3-K is required for the induction of NKG2DL following MCMV infection. \(^{234}\) Another possible mechanism is via the direct control of Clr-b (Clec2d) promoter activity and the production of
nascent Clr-b transcripts. Lastly, a potential mechanism is through the post-transcriptional regulation of mRNA stability. This intriguing possibility was recently suggested by the finding that the rodent Clr-b mRNAs (i.e., the mouse Clec2d8 and rat Clec2d11 gene products\textsuperscript{64,71}) possess an embedded autocatalytic discontinuous hammerhead ribozyme sequence\textsuperscript{235}. Thus, cleavage of steady-state Clr-b transcripts by the internal ribozyme upstream of the polyadenylation site could promote the loss of mRNA stability or impair protein translation. Whether protein factors normally antagonize autocatalysis mediated by the ribozyme sequence, or whether co-factors are recruited to the cleavage site upon genotoxic stress require further testing. On the other hand, pre-existing Clr-b transcripts may also be regulated at the level of mRNA stability and/or protein translation by microRNA-mediated silencing mechanisms, such as that observed for NKG2DL expression\textsuperscript{236,237}. Thus, the DDR response, post-translational modification, Clec2d promoter activity, elements within the Clr-b untranslated regions, and endogenous or pathogen-encoded Clr-b-specific microRNAs are all relevant targets to warrant future studies on the “missing-self” control of Clr-b expression in response to pathological processes\textsuperscript{57,64,71}.

In conclusion, how NK cells recognize abnormal targets remains incompletely understood. Understanding how a programmed cellular response mediates Clr-b downregulation in real-time during genotoxic stress will enhance our knowledge of the events that lead to innate recognition of transformed and infected cells, how malignant NK cell-resistant tumour cells evolve, and how chemotherapeutic agents might influence NK cell recognition of normal and tumour cells. Future insight into this MHC-independent mode of “missing-self” ligand modulation will impact our views of NK cell-mediated recognition.
Chapter IV

Investigation into the mechanism(s) of genotoxic stress induced downregulation of Clr-b

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All assays were performed by J Fine. shRNA validation was performed by Dr. S. Gasser. pSIcheck2 vectors harbouring Clr-b UTR’s were provided by Dr. L. Horan. Additional independent experiments (biological replicates) contributed by P. Chen, T. Kirkham and A. Mesci. T. Kirkham for assistance with p53 genotyping and tissue culture treatments.

Figures 11, 12, 17 and 18 have been published in Cancer Research. 2010. Sep 15;70(18):7102-13. The remainder is unpublished.
4.1 Abstract

NK cells are lymphocytes of the innate immune system that target cells through the balance of stimulatory and inhibitory signals generated via cell surface receptors. The receptor-ligand pair, NKR-P1B:Clr-b, mediates “missing-self” recognition of tumour and infected cells in an MHC-independent manner. How the inhibitory ligand Clr-b is regulated in the context of transformation and infection remains unknown. Stimulatory ligands for the NKG2D and DNAM-1 (CD226) receptors have been shown to be regulated through the classical DDR pathway. Here we show that in response to genotoxic stress, the classical DDR pathway does not clearly affect Clr-b modulation. Additionally, targeting post-translational and post-transcriptional modulation of Clr-b through inhibition of select pathways prevents genotoxic stress-mediated loss. However, these pathways do not directly promote the loss of Clr-b in response to genotoxic stress. Altogether, multiple mechanisms may be involved in the regulation of Clr-b following genotoxic stress, ultimately leading to a loss of Clr-b transcript and cell surface protein.

4.2 Introduction

NK cells mediate self-nonself discrimination through the balance of stimulatory and inhibitory signals. The mechanism by which their germline-encoded ligands for receptors for self-nonself are regulated is a focus of intense research. Recent work demonstrates that the activation of the DDR pathway is involved in the upregulation of ligands for the NKG2D and DNAM-1 (CD226) receptors. More specifically, the ATM, ATR, and Chk1 S/T kinases are involved in this phenotype. Post-translational modifications, mediated through ubiquitylation are also involved in the induction of NKG2DL in response to UV and heat shock. In addition, PI3-K is involved in the upregulation of NKG2DL upon MCMV infection. We have shown that the NK cell inhibitory ligand, Clr-b, is functionally lost on tumour cells and fibroblasts in response to genotoxic stress. Whether the loss of Clr-b at the RNA and/or surface protein level is mediated through the DDR pathway, such as that observed with NKG2DL, is unknown. How other forms of regulation will impact Clr-b modulation in response to genotoxic stress such as post-translational modifications and post-transcriptional modifications are also poorly understood. The recent finding that the rodent Clr-b mRNAs possess an embedded
autocatalytic discontinuous hammerhead ribozyme sequence \(^{235}\), prompts the question as to whether cleavage of steady-state Clr-b transcripts is influenced by genotoxic stress to affect mRNA stability and/or impair protein translation.

In the previous chapter, various chemotherapeutic agents and inducers of genotoxic or physiologic stress were found to promote a rapid functional downregulation of Clr-b transcripts and cell surface protein on tumour cells and fibroblast, while other surface molecules, such as MHC-I proteins were not substantially altered by similar treatments. Extending previous work, NKG2DL upregulation was found to be blocked by both caffeine and shRNA-mediated silencing of the ATR-Chk1 pathway \(^{162}\). However, Clr-b downregulation was differentially affected by caffeine treatment and remained largely unaffected by independent shRNA-mediated knockdown of the ATR/Chk1 and ATM/Chk2 pathways. Downregulation also occurs independent of p53, a classical DDR pathway effector. This suggests that loss of Clr-b following genotoxic stress may occur independently of the classic DDR pathway. Interestingly, ectopic expression of Clr-b transcripts prevented stress-mediated loss of Clr-b surface protein. Moreover, pharmacologic inhibition of the ubiquitin-proteasome degradation pathway uncoupled the downregulation of Clr-b surface protein from the stress-mediated loss of endogenous Clr-b transcripts. Additionally, inhibition of the hammerhead ribozyme embedded in the 3’UTR increased expression of Clr-b on many of the tumour cells and fibroblasts tested, at the transcript and surface protein levels. Ribozyme blockade was also sufficient to prevent genotoxic stress-induced loss of Clr-b transcript and protein. However, results from reporter assays suggest that, although the ribozyme decreases the amount of transcript available when compared to enzymatic mutants, there is no alteration in the amount of ribozyme cleavage upon genotoxic stress induction. This implies that genotoxic stress does not directly affect the post-transcriptional regulation of Clr-b, and that ribozyme catalysis may occur constitutively. Collectively, these results suggest that loss of Clr-b following genotoxic stress may occur through multiple mechanisms that target the Clr-b transcript and protein, independently of the classical DDR pathway.
4.3 Results

4.3.1 Potential involvement of the ATM/ATR pathways in stress-mediated Clr-b downregulation.

Having established in Chapter III that Clr-b is functionally downregulated in various cell types in response to genotoxic and cellular stress, we sought to investigate the mechanism(s) regulating this response. Previous studies demonstrated a role for the DDR pathway in aphidicolin-induced NKG2DL upregulation on fibroblasts (via ATR/Chk1), as well as constitutive NKG2DL expression on tumour cells (via ATM)\textsuperscript{162,222}. In these studies, inhibition of the ATM/ATR pathways using caffeine abrogated NKG2DL upregulation on fibroblasts in response to aphidicolin treatment\textsuperscript{162}. Therefore, we investigated the involvement of this pathway in stress-mediated Clr-b downregulation. Interestingly, caffeine blocked both Clr-b downregulation and NKG2DL upregulation on NIH3T3 fibroblasts in response to aphidicolin treatment (Fig.11A,B). However, caffeine did not block aphidicolin-mediated loss of Clr-b on C1498 leukemia cells; rather, caffeine treatment alone promoted Clr-b downregulation, and the combination of caffeine and aphidicolin promoted a more striking loss of Clr-b. In agreement with previous work\textsuperscript{157}, both constitutive NKG2DL expression and aphidicolin-mediated NKG2DL upregulation were abrogated on both C1498 leukemia cells and NIH3T3 fibroblasts by caffeine (Fig.11A,B). These findings are consistent with differential effects observed previously: The ATR pathway seems to regulate aphidicolin-induced NKG2DL on fibroblasts, while the ATM pathway may regulate constitutive NKG2DL expression on some tumour cells\textsuperscript{162,222}. 
Figure 11. Differential effects of general ATR/ATM inhibition by caffeine on aphidicolin-mediated Clr-b downregulation on C1498 leukemia cells and NIH3T3 fibroblasts. (A,B) Flow cytometric analysis of Clr-b or NKG2DL expression on C1498 leukemia cells and NIH3T3 fibroblasts treated overnight with aphidicolin (16μM) in the absence, or presence of 1h caffeine pretreatment (2mM or 10mM). Histograms show staining with anti-Clr-b antibody or NKG2D:hIgG fusion protein (shaded area) versus secondary reagent alone (SA-PE) (dotted line). A vertical dashed line indicates the median fluorescence intensity (MFI) of untreated cells; numbers indicate MFI values of shaded histograms. Data are representative of 3 independent experiments.
Because caffeine exhibits pleiotropic effects independent of ATR/ATM inhibition\textsuperscript{238-241}, previously characterized shRNA vectors\textsuperscript{162} were utilized to specifically silence the ATM, ATR, Chk1, and Chk2 gene products. Clr-b modulation on C1498 and NIH3T3 cells was then reassessed. Extending previous findings\textsuperscript{162}, aphidicolin-induced NKG2DL upregulation on NIH3T3 fibroblasts could be blocked by shRNA-mediated knockdown of Chk1 or ATR, but not ATM or Chk2 (Fig.12A,B,C). In contrast, none of the shRNA constructs prevented aphidicolin-mediated Clr-b downregulation on either NIH3T3 fibroblasts or C1498 cells. In fact, ATR/Chk1 silencing (but not ATM/Chk2 silencing) modestly enhanced aphidicolin-mediated downregulation of Clr-b, and somewhat lowered constitutive Clr-b expression, on NIH3T3 fibroblasts (Fig.12A,B).
Figure 12. Differential effects of shRNA-mediated ATR/ATM and Chk1/Chk2 silencing on aphidicolin-mediated Clr-b downregulation on C1498 leukemia cells and NIH3T3 fibroblasts. (A,B) Flow cytometric analysis of Clr-b or NKG2DL expression on C1498 leukemia cells and NIH3T3 fibroblasts treated overnight with aphidicolin (16μM). Cells were first modified by retroviral shRNA-mediated knockdown of ATR, Chk1, ATM, or Chk2, or using a scrambled shRNA. Transduced cells were gated on GFP reporter expression. Histograms show untreated cells (solid line), treated cells (shaded area), secondary reagent alone (dotted line). Numbers in the upper right corner of the histogram indicate MFI of untreated cells, and the numbers below indicate MFI of aphidicolin-treated cells. (C) Bars indicate fold change in median fluorescence intensity including samples from A and B. Data are representative of at least 3 independent experiments. For NIH3T3 a two-tailed t-test of equal variance was performed where * = 0.001, and *=0.002.
Collectively, although redundancy in the DDR pathway cannot be excluded, the failure of shRNA-mediated silencing of these kinases to inhibit aphidicolin-mediated Clr-b downregulation (on both NIH3T3 and C1498 cells), suggests that loss of Clr-b in response to genotoxic stress may occur via a novel mechanism, independent of the ATR/ATM pathways. The lack of ATM involvement is further supported by the failure of γ-irradiation and the radiomimetic drugs, bleomycin and phleomycin, to promote Clr-b downregulation (Fig.5; Fig.6; Fig.8A). In addition, studies using p53−/− fibroblasts and inhibitors of p53 function (such as pifithrin-α) failed to reveal a clear involvement of p53, (a downstream effector of the ATM/ATR-mediated DDR signaling pathways) on genotoxic stress-mediated Clr-b downregulation (Fig.13; Fig.14). Nor was p21 involved, another downstream effector of DDR signaling (Fig.13). In turn, the partial ability of caffeine to abrogate aphidicolin-mediated Clr-b downregulation (on NIH3T3 but not C1498 cells), may be due to effects unrelated to the DDR pathway, such as cell cycle blockade, or inhibition of PI3-K, DNA-PK, PAK1, or other caffeine-sensitive kinases. In addition, the ability pifithrin-α to partially block genotoxic stress-mediated downregulation of some DNA damaging agents (on NIH3T3 but not C1498 cells), may be due to undocumented cellular effects. Recent reports indicate that in fibroblast lines such as NIH3T3, pifithrin-α, is quickly metabolized into pifithrin-β in culture medium and has cytotoxic effects with no effect on p53 transactivation or protein expression.
Figure 13.

Genotoxic stress promotes Clr-b downregulation on p53<sup>-/-</sup> and p21<sup>-/-</sup> fibroblasts. B6, p53<sup>-/-</sup> and p21<sup>-/-</sup> ear fibroblasts were treated overnight (24h) with aphidicolin (20μM), cisplatin (30μM), roscovitine (20μM), Trichostatin A (500nM) or bleomycin (40μg/mL) and analyzed by flow cytometry. Histograms show DMSO treated cells (thick black line), treated cells (shaded histogram), or secondary reagent alone (dotted line). Data are representative of 3 independent experiments.
**Figure 14.** Inhibition of p53 activation has partial effects in preventing genotoxic stress-mediated Clr-b downregulation. C1498 cells (top panel) and NIH3T3 fibroblasts (lower panel) were pre-treated with 30μM pifithrin-α (PFT-α) for 1hr followed by administration of either DMSO, aphidicolin (16μM), cisplatin (30μM), roscovitine (20μM), or Trichostatin A (500nM) for 24 hours and analyzed by flow cytometry. Histograms show DMSO treated cells (thick black line), treated cells (shaded histogram), or secondary reagent alone (dotted line). Data are representative of 3 independent experiments.
A downstream function of DDR signaling is cell cycle checkpoint activation. Thus, we investigated whether the Clr-b downregulation phenotype on cells exposed to genotoxic stress was associated with a specific stage of the cell cycle. To this end, C1498 cells were treated with the indicated genotoxic agents (Fig. 15) for 24 hours before cell cycle analysis was performed in conjunction with staining for surface Clr-b. Consequently, the Clr-b downregulation phenotype does not appear to be restricted to any stage of the cell cycle, since cells in G1, S and G2/M all downregulated Clr-b when exposed to aphidicolin, cisplatin, roscovitine and TSA (Fig. 15). Strikingly, γ-irradiation and the radiomimetic bleomycin, which do not induce Clr-b downregulation exhibited identical cell cycle phenotypes (Fig. 15).

Analogous to what has been observed for Clr-b mRNA, transcript-level regulation has been previously reported for at least some NKG2DL that are upregulated upon transformation, infection or genotoxic stress. Other NKG2DL (e.g., Mult1), were recently shown to be posttranslationally regulated in response to cell stress. Therefore, we examined protein-level regulation of Clr-b on cells exposed to genotoxic stress.
Figure 15. Cell cycle analysis of C1498 cells treated with various genotoxic agents. C1498 cells were treated for 24h with aphidicolin (16μM), cisplatin (15μM), roscovitine (10μM), trichostatin A (500nM), bleomycin (40μg/mL) or 27 Gy γ-irradiation and analyzed by flow cytometry. Cell cycle histograms (panel above the 3 histograms) were gated on G1, S and G2/M phases of cell cycle as determined through comparison to DMSO treated cells and Clr-b expression was compared (panel of 3 histograms). Histograms show DMSO treated cells cells (thick black line), treated cells (shaded histogram), or secondary reagent alone (dotted line). Data are representative of 3 independent experiments.
4.3.2 Regulation of cell surface Clr-b protein following genotoxic stress

Although Clr-b expression is regulated at the transcript level, it is still possible that Clr-b surface protein might also be posttranslationally internalized, either constitutively or following genotoxic stress. The Clr-b cytoplasmic tail contains a number of motifs that could impact constitutive and genotoxic stress-induced cell surface expression, including several lysine and cysteine residues that may serve as substrates for ubiquitination. Indeed, several viruses have evolved E3 ubiquitin ligases that posttranslationally regulate cell surface expression of MHC-I, B7 family members, ICAM-1, or NKG2DL. To investigate the role of putative posttranslational modification sites a truncation analysis was performed with the cytoplasmic tail to systematically remove each putative site. This analysis was complicated by the fact that expression of the full-length Clr-b coding sequence, ectopically overexpressed under the control of a heterologous CMV promoter (pMCIG vector) did not exhibit genotoxic stress-mediated loss of Clrb in C1498 cells. As shown in Fig.17, stable overexpression of Clr-b transcripts prevents downregulation of Clr-b surface protein mediated by diverse agents. Therefore, we could not assess the role of posttranslational modification sites, due to the inability of overexpressed Clr-b to behave like endogenous Clr-b in the context of genotoxic stress.
Figure 16. The cytoplasmic tail of Clr-b contains several potential sites for posttranslational modification. (A) The Clr-b cytoplasmic tail sequence was analyzed for putative posttranslational modification motifs using the ELM website (http://elm.eu.org/links.html). Notable sequences include consensus CK1 and CK2 phosphorylation motifs (arrows), several S/T phosphorylation sites (P circles), a consensus TRAF2-recruitment motif (SPQE), and several K and C residues for ubiquitination (chains of filled circles). The putative transmembrane (TM) domain is indicated at the C-terminus represented by gray shading; the ectodomain is not depicted. (B) A truncation analysis of the Clr-b cytoplasmic tail was performed by removing important residues through PCR amplification. The first amino acid of the forward primer is depicted by the arrowhead above the sequence.
Figure 17. Ectopic expression of Clr-b transcripts abrogates Clr-b downregulation following genotoxic stress. Flow cytometric analysis of endogenous or ectopically overexpressed Clr-b protein on C1498 cells following treatment as indicated. Retroviral C1498 transductants overexpressing a CMV promoter-driven full-length Clr-b coding sequence were gated according to IRES-GFP reporter gene expression. Histograms show Clr-b expression (shaded area) versus secondary reagent alone (dotted line); numbers indicate MFI values. A vertical dashed line indicates the median Clr-b expression on untreated cells. Data are representative of 3 independent experiments.
Some NKG2DL are posttranslationally regulated through ubiquitination following genotoxic and cellular stress\textsuperscript{223}. Thus to broadly address the potential role for post-translational modifications, such as that mediated through ubiquitination we employed pharmacological inhibition of the 26S proteasome via MG132. Interestingly, inhibition of the ubiquitin-proteasome degradation pathway via MG132 pre-treatment abrogated Clr-b downregulation following treatment of C1498 cells with diverse agents, including aphidicolin, cisplatin, roscovitine, and trichostatin-A, while MG132 treatment alone slightly (but consistently) increased steady-state Clr-b surface expression (Fig.18A). Furthermore, MG132 administration also blocked downregulation of surface Clr-b in response to generalized inhibitors of transcription, translation, and posttranslational processing, such as α-amanitin, actinomycin-D, cycloheximide, and tunicamycin (Fig.18B), the latter of which also promotes ER stress.

Because MG132 can exert pleiotropic effects independent of blocking ubiquitin-dependent proteasomal degradation, including an ability to inhibit calpains and cathepsins, another proteasomal inhibitor of greater specificity, lactacystin, was also utilized\textsuperscript{250}. As shown in Fig.18C, both MG132 and lactacystin prevented stress-mediated Clr-b downregulation in a dose-dependent manner. Similar results were observed using NIH3T3 fibroblasts (Fig.18E). Importantly, the administration of proteasomal inhibitors had no influence on the downregulation of Clr-b transcripts following administration of genotoxic agents or transcriptional inhibitors, as revealed by quantitative real-time RT-PCR (Fig.18D). This demonstrates that downregulation of Clr-b transcript can be uncoupled from a loss of Clr-b surface protein.
Figure 18. Inhibition of the ubiquitin-proteasome pathway uncouples stress-mediated Clr-b surface protein downregulation from loss of endogenous transcripts. (A) Flow cytometric analysis of Clr-b expression on C1498 cells either untreated or treated overnight in the presence or absence of 1h pretreatment with MG132 (2 μM). Histograms show Clr-b expression (shaded area) versus secondary reagent alone (dotted line) on cells treated with aphidicolin (16 μM), cisplatin (15 μM), roscovitine (5 μM), or trichostatin A (100 nM). A vertical dashed line indicates median Clr-b expression on untreated cells. (B) As in (A), except cells were treated with α-amanitin (30 μM), actinomycin D (5 nM), cycloheximide (40 μg/mL), or tunicamycin (0.5 μg/mL). (C) As in (A), except cells were treated with cisplatin (15 μM) with increasing concentrations of MG132 or lactacystin. Histograms show Clr-b expression at maximum inhibitor concentrations (shaded area), or decreasing concentrations (solid lines, as indicated), relative to median Clr-b expression on untreated cells (vertical dashed line), or secondary reagent alone (dotted line). (D) Real-time quantitative RT-PCR analysis of Clr-b expression on C1498 cells treated as in (A) with aphidicolin (16 μM), cisplatin (15 μM), trichostatin A (100 nM), α-amanitin (30 μM), or actinomycin D (5 nM). Relative ΔΔCt values were calculated using G3PDH as internal control, normalized to untreated samples. (E) Flow cytometric analysis of Clr-b expression on NIH3T3 fibroblasts either untreated or treated overnight in the presence or absence of 1h pretreatment with lactacystin (25 μM). Histograms show DMSO treatment (solid black line), Clr-b expression (shaded area) versus secondary reagent alone (dotted line) on cells treated with aphidicolin (16 μM), cisplatin (30 μM), roscovitine (20 μM) or trichostatin A (500 nM). Data are representative of 3 independent experiments.
Nonetheless, the MG132 and lactacystin data do not necessarily implicate the proteasome itself in direct degradation of Clr-b protein. Pharmacological inhibition of the proteasome leads to the accumulation of non-degraded polyubiquitinated aggregates and a rapid depletion of free ubiquitin levels, resulting in general impairment of ubiquitin-dependent processes.\textsuperscript{251} One such proteasome-independent process, the endolysosomal trafficking pathway, targets endocytosed monoubiquitinated proteins for recycling and/or lysosomal degradation.\textsuperscript{143} Interestingly, chloroquine, an inhibitor of endolysosomal acidification and autophagy,\textsuperscript{252,253} also impaired aphidicolin-, cisplatin- and TSA-mediated Clr-b downregulation (Fig.19) Collectively, these results suggest that ubiquitin-dependent processes play an important role in normal Clr-b cell surface turnover, while stress-mediated Clr-b downregulation is ultimately controlled at the transcript level. Therefore, any perturbation in Clr-b transcript levels may rapidly influence Clr-b surface expression.
Figure 19. Inhibition of the endolysosomal pathway partially abrogates genotoxic stress-mediated downregulation. Flow cytometric analysis of Clr-b expression on C1498 cells either untreated or treated overnight in the presence or absence of 1h pretreatment with chloroquine (50µM) followed by addition of indicated genotoxic agent. Histograms show Clr-b expression (shaded area) versus secondary reagent alone (dotted line) on cells treated with aphidicolin (16µM), cisplatin (15µM), or trichostatin A (100nM). Data are representative of 3 independent experiments.
4.3.3 Post-transcriptional regulation of Clr-b

As it has clearly been established that a loss of Clr-b transcript during infection and genotoxic stress correlates with a loss in cell surface protein we sought to investigate the contribution of the 3’ UTR of Clr-b. The rodent Clr-b mRNAs (i.e., the mouse Clec2d8 and rat Clec2d11 gene products) both possess an embedded autocatalytic discontinuous hammerhead ribozyme sequence, which is not found in the human orthologue LLT1. Cleavage of the ribozyme in vitro and in vivo has been demonstrated to cause a reduction in protein expression in a heterologous system.

The recent discovery of ribozyme inhibitors using a high-throughput screening of chemical libraries yielded the adenosine analog toyocamycin. Incorporation of toyocamycin into nascent RNA transcripts prevents ribozyme cleavage, and thus a stable transcript is generated that can be translated. Treating various mouse cell lines harbouring low to high levels of Clr-b with toyocamycin for 24 hours demonstrated a marked increase in Clr-b surface expression (Fig.20A), which is reflective of an increase in transcript levels (Fig.20B). In the case of BWZ and EL4, an increase in transcript is observed, which does not completely reflect what is observed at the cell surface (Fig.20B). Notably, treatment of Clr-b low cell lines with MG132 does not result in an increase in Clr-b expression (Fig.20C), which suggests that the protein is not actively being recycled from the surface in these cell lines, and that the observed Clr-blo phenotype is due to a lack of steady state transcripts, whether due to affects on transcript stability or production of nascent transcripts. Inhibition of ribozyme cleavage by toyocamycin also prevented aphidicolin- and cisplatin- mediated downregulation of surface Clr-b in C1498 and NIH3T3 cells (Fig.21A). This result is also reflective of an increase in transcripts, as assayed by real-time qRT-PCR (Fig.21B). Whether the ribozyme is directly modulated by genotoxic stress cannot be concluded from these experiments. However, increasing the amount of steady state transcripts by inhibiting ribozyme cleavage in vitro is sufficient to block genotoxic stress-mediated downregulation, and increase Clr-b expression on certain Clr-blo cell lines.
Figure 20

(A) Fibroblast and tumour cells expressing variable levels of Clr-b were treated with 0.1 nM toyocamycin for 24 hours and stained for Clr-b. Histograms show untreated cells (black line), treated cells (shaded histogram), or secondary reagent alone (dotted line). Data are representative of 3 independent experiments. (B) Fibroblast and tumour cells expressing variable levels of Clr-b were treated with 0.1 nM toyocamycin for 24 hours. RNA was extracted and qRT-PCR was performed. ΔΔCt values for Clr-b are normalized to GAPDH and are an average of 3 independent experiments. (C) Fibroblast and tumour cells expressing variable levels of Clr-b were treated with 1μM MG132 for 24 hours and stained for Clr-b. Histograms show untreated cells (black line), treated cells (shaded histogram), or secondary reagent alone (dotted line). Data are representative of 3 independent experiments.
Figure 21. Toyocamycin treatment prevents aphidicolin- and cisplatin-induced Clr-b downregulation on C1498 and NIH3T3 cells at the surface and transcript levels. (A) C1498 (Left Panel) and NIH3T3 fibroblasts (Right Panel) were treated with 0.1 nM toyocamycin (TOYO) for 3 hours prior to addition of 16μM aphidicolin (APH) or [15μM C1498] or [30μM NIH3T3] cisplatin (CIS) for 21 hours and stained for Clr-b. Histograms show untreated cells (black line), treated cells (shaded histogram), or secondary reagent alone (dotted line). Data are representative of 3 independent experiments. (B) C1498 (Left Panel) and NIH3T3 fibroblasts (Right Panel) were treated with 0.1 nM toyocamycin (TOYO) for 3 hours prior to addition of 16μM aphidicolin (APH) or [15μM C1498] or [30μM NIH3T3] cisplatin (CIS) for 21 hours. RNA was extracted and qRT-PCR was performed for Clr-b. Clr-b ΔΔCt values for C1498 are normalized to GAPDH, and NIH3T3 are normalized to GAPDH/TBP/28sRNA combination. Real time data is represented as an average of 3 independent experiments.
Although addition of a ribozyme inhibitor increases the amount of available steady-state transcripts we sought to study whether there is a change in the amount of ribozyme cleavage in response to genotoxic stress or whether this effect is constitutive. To achieve this, we utilized the pSlcheck2 (pSi2) vector system, which was modified to contain the Clr-b 3’UTR downstream of the Luciferase gene. The vector also contains a Renilla gene under the control of an SV40 promoter, which serves as an internal control (Fig.22A). The Luciferase/Renilla ratio for empty pSi2 vector, enzyme- and substrate- mutants (Fig.22B) were similar for NIH3T3 fibroblasts and C1498 cells. As has been previously observed, less luciferase protein production was observed when the WT Clr-b HHR was fused to the transcript (Fig.22C), compared to the empty vector and the HHR enzyme and substrate mutants. In the case of C1498 cells, the Luc/Ren ratios for the enzyme- and substrate- mutant UTRs were not as high as the empty vector alone, yet still much higher than wild type UTR (Fig.22C). These results reaffirm previous work, in that cleavage of the WT Clr-b HHR reduces the amount of transcript, ultimately decreasing the amount of reporter protein. When the HHR is mutated, there is no loss of transcript relative to empty pSlcheck2 vector, which does not harbour the Clr-b 3’UTR within the luciferase transcript.
Figure 22. Analysis of wild type (WT), enzyme mutant (ENZ) and substrate mutant (SUB) hammerhead ribozymes (HHR) using a luciferase-based reporter system. (A) Modified pSIcheck2 vector harbouring the Clr-b 3’UTR. (B) Alignment of the WT, ENZ and SUB HHR mutants with mutated bases indicated in green. (C) Luciferase to Renilla ratio of NIH3T3 fibroblasts (Left Panel) and C1498 cells (Right Panel) nucleofected with pSIcheck2 vectors for 24 hours before performing dual luciferase assay. Data are representative of 3 independent experiments.
To investigate the effect of genotoxic stress on the ribozyme, NIH3T3 and C1498 cells were nucleofected with the pSi2 vectors and after a 4-hour recovery period, dosed with genotoxic stressors, or IFN-α4 and toyocamycin for 24 hours. The lysates were subjected to a dual-luciferase reporter assay (DLRA) and the ratios were calculated. As would be expected, incorporation of toyocamycin into the WT HHR resulted in increased production of luciferase. This effect is not observed with the HHR substrate or enzyme mutants, which do not cleave, nor the empty vector (Fig.23A,B Right Panel). In the context of genotoxic stress, cisplatin, aphidicolin, or Trichostatin A (Fig.23A,B Right Panel) did not have a noticeable effect on the amount of luciferase produced, suggesting that the ribozyme is not cleaving more or less in the presence of genotoxic stress. This was the case for both C1498 cells and NIH3T3 fibroblasts, which were validated in the context of these genotoxic agents and transfection conditions through flow cytometry prior to the DLRA (Fig.23A,B Left Panel). IFN-α4, which causes an increase in Clr-b surface expression in many different cell types, did not have any noticeable effect on the amount of luciferase produced (Fig.23A,B Right Panel). Collectively, the data suggests that although inhibition of the Clr-b HHR can prevent genotoxic stress-mediated Clr-b downregulation, the HHR itself does not appear to be modulated by genotoxic stress.
Figure 23

A

Figure 23. Analysis of wild type (WT), enzyme mutant (ENZ) and substrate mutant (SUB) hammerhead ribozymes in a reporter system following cellular or genotoxic stress. (A) Modified pSIcheck2 vector harbouring the Clr-b 3′UTR, or unmanipulated vector was nucleofected into C1498 cells and were dosed after 4 hours. Flow cytometry (Left Panel) and dual luciferase assay (Right Panel) was performed on the cells after 22 hours incubation with chemical. (B) as in (A) but with NIH3T3 cells. Flow cytometry (Left Panel) and dual luciferase assay (right panel) was performed on the cells after 22 hours incubation with chemical. Chemical concentrations used were aphidicolin (16μM), cisplatin (15μM C1498; 30μM NIH3T3), interferon-α4 (5000U), trichostatin A (500nM) and toyocamycin (100nM). Normalized signal is calculated as Luciferase/Renilla signal divided by empty pSIcheck2 vector treatment, followed by normalizing to DMSO for each independent vector. Data is plotted as an average of 3 independent experiments. Histograms represent treatment (shaded), DMSO control (solid line), and secondary reagent alone (dashed line).
Discussion

Genotoxic stress activates numerous cellular pathways, resulting in the induction of cell-cycle arrest and/or apoptosis. Upregulation of ligands for the stimulatory NKG2D and DNAM-1 receptors have been shown to be mediated in part via the DDR pathway\textsuperscript{162,165}. Specifically, NKG2DL upregulation could be blocked through inhibition of the PI3K-like ATR and ATM kinases, both pharmacologically (i.e., by caffeine\textsuperscript{243}) and genetically (i.e., via shRNA-mediated knockdown\textsuperscript{162}). In contrast, Clr-b downregulation in response to genotoxic stress may occur independently of the ATR/ATM pathway, as pharmacological inhibition yielded conflicting results, depending on the cell lines employed. For example, the lymphoid cell line, C1498, was found to downregulate constitutive Clr-b expression in response to caffeine alone, and caffeine pre-treatment followed by aphidicolin treatment enhanced Clr-b downregulation. On the other hand, aphidicolin-mediated Clr-b downregulation of Clr-b on NIH3T3 fibroblasts was blocked by caffeine pre-treatment, suggesting the involvement of the ATR/ATM pathways or other PIKK family members. However, specific shRNA-mediated knockdown of ATM, ATR, Chk1, or Chk2 alone could not recapitulate this effect. This is despite the fact that shRNA-mediated knockdown of ATR/Chk1 prevented NKG2D upregulation on fibroblasts in response to aphidicolin, as reported previously\textsuperscript{162}. The potential discrepancies between these responses, with respect to Clr-b modulation, may be attributed to several distinct factors.

First of all, the pleiotropic effects of caffeine may influence ATM/ATR-independent pathways. For example, although 10mM caffeine did inhibit Clr-b downregulation on fibroblasts, no effect was observed using 2mM caffeine, which should be sufficient to inhibit ATM/ATR activity in vitro\textsuperscript{243}. Thus, blockade of Clr-b downregulation by caffeine could be attributable to a block in cell cycle\textsuperscript{239}, inhibition of PI3-K, DNA-PK, PAK1, or other effects\textsuperscript{238,240,241,244}. Secondly, the differential ability of caffeine alone to promote a loss of Clr-b constitutive expression on C1498 cells, yet not on NIH3T3 fibroblasts, may reflect the nature of the cells or the cell type. For example, transformed versus non-transformed or lymphoid versus fibroblast cell lines may be differentially sensitive to the effects of caffeine. While fibroblasts predominantly maintain cell-cycle checkpoint arrest in response to caffeine, transformed cells frequently bypass checkpoint delays prematurely when treated with caffeine\textsuperscript{255}. As a result,
caffeine treatment can differentially drive these cell populations through cell cycle checkpoint arrest following genotoxic stress.

The incomplete contribution of the ATM/ATR signaling cascade to Clr-b downregulation is further supported by observations using p53\(^{-/-}\) primary mouse adult ear fibroblasts. p53 is a downstream effector of the DDR signaling pathway through the ATM/ATR kinases, and dictates cell fate in response to DNA damage\(^82\). The genotoxic stress-mediated downregulation of Clr-b on primary p53\(^{-/-}\) ear fibroblasts was found to be comparable to, or greater than, that observed using wild-type fibroblasts over a 24 h period. Similar results were achieved with p21\(^{-/-}\) mouse ear fibroblasts, which is an important cell cycle mediator downstream of p53. In addition, studies using pifithrin-\(\alpha\) (a reversible inhibitor of p53-dependent transcriptional activation\(^{242}\)) failed to reveal any clear effect on genotoxic stress-mediated Clr-b downregulation (Fig.13;Fig.14). However, this inhibitor clearly influences other cellular processes independent of p53 function\(^{245}\). Collectively, the data suggests that stress-mediated loss of Clr-b is p21- and p53-independent. In addition, cell cycle analysis of C1498 cells suggests that genotoxic stress-mediated loss of Clr-b is not restricted to any particular stage of cell cycle.

Recently, it was shown that cell surface expression of the NKG2DL, Mult1, was upregulated independently of the ATR/ATM pathway in response to cell stress induced by heat shock and UV radiation\(^{223}\). In this report, surface expression of Mult1 was shown to be dependent upon inhibition of the ubiquitin-proteasome and endolysosomal degradation pathways responsible for retention of Mult1 protein within normal, non-stressed cells. These findings highlight a potential ubiquitin-dependent mechanism for Clr-b downregulation under stressed versus non-stressed conditions. Specifically, rapid constitutive Clr-b turnover may be regulated by a similar pathway, such that genotoxic stress-mediated downregulation of Clr-b transcripts may promote a subsequent and proportionate loss of Clr-b protein at the cell surface.

Notably, the cytoplasmic tail of Clr-b contains four lysine and two cysteine residues that may serve as potential targets of ubiquitination, several S/T target residues for phosphorylation, and other potential motifs for post-translational modification (Fig.16A). Because genotoxic stress-mediated downregulation of Clr-b was blocked using the 26S proteasome inhibitors, MG132 and lactacystin (Fig.18), internalization and proteasome-mediated degradation of
ubiquitinated Clr-b protein remains a possible mechanism for loss of surface ligand following the loss of transcripts. Nonetheless, the mechanism by which blockade of ubiquitination affects Clr-b surface levels remains unknown. The effect could be due to direct inhibition of Clr-b internalization and/or degradation, or due to indirect blockade of the degradation of other cellular intermediates. For example, MG132 is also known to inhibit cathepsins and calpains 250. Apoptotic pathway proteins, cell cycle kinases, DNA damage mediators, and DNA repair factors may also be directly modulated by the proteasome 145,256. Furthermore, proteasomal inhibition results in the depletion of free ubiquitin, due to the accumulation of aggregates of poly-ubiquitinated proteins that fail to undergo degradation 251,257. Proteasome inhibition may also elicit global changes in gene expression profiles 258. Alternatively, the blockade of Clr-b downregulation by chloroquine suggests that mono- or multi-ubiquitin-dependent internalization and endolysosomal trafficking may also play a role 143. Furthermore, chloroquine is capable of inhibiting autophagy 253, a process that also requires ubiquitin 259. Thus downregulation of Clr-b surface protein following genotoxic stress may occur through one of these pathways.

In any case, it is likely that steady-state turnover of Clr-b surface protein is mediated through ubiquitin-dependent internalization, while the loss of Clr-b transcripts plays a predominant role in the dynamics of Clr-b surface levels following genotoxic stress. This is exemplified by the observation that steady-state transcripts decrease following genotoxic stress, yet do not change upon blockade of ubiquitin-dependent processes. Further evidence in support of this view arises from the fact that inhibition of RNA polymerase II-mediated transcription via α-amanitin or actinomycin D is sufficient to induce a loss of Clr-b surface levels, an effect that is reversed upon addition of MG132. Finally, because overexpression of the full-length Clr-b coding sequence in C1498 cells is not influenced by genotoxic agents, the modulation of Clr-b in response to genotoxic stress likely occurs at the level of steady-state transcripts, which could occur through decreased promoter activity or increased mRNA degradation. These findings reveal a second level of Clr-b regulation by the ubiquitin-proteasome and endolysosomal degradation pathways. Thus, a high rate of constitutive Clr-b protein turnover likely facilitates coupling of the stress-induced loss of Clr-b transcripts with a loss of Clr-b ligand function.
Whether the transcripts are being degraded through canonical mRNA degradation pathways, through ribozyme mediated catalysis or through a decrease in promoter activity has to be further elucidated. Pharmacological inhibition of HHR cleavage increases expression of surface Clr-b on Clr-b<sup>lo</sup> cells and prevents genotoxic stress-induced loss of Clr-b on C1498 and NIH3T3 cells. This occurs at both the transcript and protein levels. In contrast, by utilizing a dual-luciferase reporter system, genotoxic stress does not appear to decrease the amount of protein made in the context of the wild type HHR. The HHR is still functional in the reporter assay, because inhibition of the WT HHR with toyocamycin does increase the amount of reporter protein. The discrepancies between the in vitro assays and the reporter system could be influenced by the sequences used. The vector harbouring the WT HHR does not contain the consensus Clec2d poly-adenylation signal sequences, which would be present in the endogenous transcript for Clr-b. Instead the pSIcheck2 vector utilizes a late SV40 poly-A signal sequence. Thus if there is a difference in the processing of the endogenous transcript and reporter system transcript, then they may not behave the same in the context of genotoxic stress. Lastly, other proteins may be required for proper folding of the ribozyme, as has been observed in known cofactor-dependent ribozyme systems, such as the GlmS ribozyme<sup>260</sup>. The reporter system also does not harbour the mClr-b 5’-UTR. Since transcripts require circularization before translation<sup>261</sup>, the conformation of the ribozyme lacking the endogenous poly-A tail and 5’UTR in the reporter system could be different than that in the endogenous state. However, since toyocamycin mediated inhibition of ribozyme cleavage does function, the current data suggests that the ribozyme is a component in the processing of the endogenous transcript and that HHR inhibition can increase the amount of transcript and protein, which may compensate for the loss of transcript that would normally occur following genotoxic stress.

Ribozymes are catalytically active RNA secondary structures that are capable of cleaving themselves in vitro and in vivo. In vitro work has demonstrated that the hammerhead ribozyme is the simplest and most common RNA structure under near-physiological conditions<sup>262</sup>. The hammerhead ribozyme is composed of three helices, which are termed Stems I, II, and III. At the centre of the helices is the catalytic core, which is largely invariant. Additional nucleotides on the stems form loops, which give the ribozyme the shape of a hammerhead<sup>263</sup>. Cofactors predominantly required for proper folding and activity include divalent cations, such as Mg<sup>2+</sup>, Ca<sup>2+</sup>, and Mn<sup>2+</sup><sup>264</sup>, although some studies implicate the involvement of proteins in enhancing the
ribozyme cleavage activity. An improvement in computational algorithms has allowed many researchers to find these catalytic RNAs across many phylogenies and species. Originally discovered in plant viruses, these catalytic RNAs can be found in Archaea, schistosomes, insects, rodents, marsupials, Laurasiatheria and humans. In humans, ribozymes appear to be predominantly associated with intronic regions. They are also found as tandemly repeated elements in the genomes of Bacteria, Chromalveolata, Plantae, and Metazoa kingdoms and have been proposed to be potentially new forms of retrotransposable elements. The Clr-b ribozyme appears to be conserved in the mouse (Clec2d8) and rat (Clec2d11) C-type lectin transcripts as well as in mammalian CLEC-2-like sequences in horse, elephant, cow, dog and platypus. The function of the ribozyme can be further complicated by work in which ribozymes were engineered to generate an active hammerhead endonuclease ribozyme capable of transcribing a ribozyme. Furthermore, ribozymes are being engineered for clinical use. It has been demonstrated that targeting a ribozyme to the 3’UTR of the survivin gene was sufficient to decrease expression of survivin in two prostate cancer cell lines tested. The cells, which became polyploid, underwent caspase-9-dependent apoptosis. In another study, a ribozyme was developed to target a tumour-specific variant of the EGF receptor termed EGFvII. This ribozyme was capable of reducing expression of EGFvII in breast cancer cell lines, with no effect on other EGF family members. The loss of the constitutively active EGFvII reduced the tumourigenicity of the breast cancer cells in athymic nude mice.

The complete role that the ribozyme plays in the stability of the Clr-b transcript has yet to be fully elucidated. In our hands, the ribozyme does not appear to be modulated by genotoxic stress, although its inhibition can prevent genotoxic stress-mediated loss of transcript and surface protein. Thus, further research to understand the programmed cellular response that mediates Clr-b downregulation in real-time during genotoxic stress will enhance our knowledge of the events that lead to innate recognition of transformed and infected cells. Future insight into this MHC-independent mode of “missing-self” recognition in response to pathological processes will influence our views of NK cell-mediated recognition.
Chapter V

Investigating the effect of oncogenic stress on Clr-b modulation in NIH3T3 fibroblasts

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All assays were performed by J Fine. shRNA validation was performed with Miho Tanaka. Additional independent experiments (biological replicates) were contributed by M. Tanaka, O. Aguilar, T. Kirkham and A. Mesci

This work is unpublished.
5.1 Abstract

Cellular transformation is an early event, which increases the likelihood of tumourigenesis. Through dysregulated anti-apoptotic or pro-proliferative networks, a cell can replicate uncontrollably. Oncogene-expressing cells are known to modulate ligands recognized by NK cell receptors including upregulated ligands for the stimulatory receptor NKG2D, and downregulated MHC-I proteins. The Ras family of protooncogenes is mutated in many human tumours. Whether MHC-independent inhibitory ligands are also modulated in this context is currently unknown. Here we show that Clr-b, the NKR-P1B ligand, is downregulated upon H-RasV12 transfection of NIH3T3 fibroblasts. This effect is mediated through the MEK-Erk pathway, and specifically requires Erk2. However, overexpression of wild type Erk2 alone is not sufficient, and requires activation through H-RasV12 to cause Clr-b downregulation. In addition, H-RasV12-mediated downregulation is facilitated through kinase-dependent and kinase-independent functions of Erk2, but requires homodimerization. Collectively, these results contribute to our knowledge of how NK cells recognize targets undergoing pathological alterations.

5.2 Introduction

Cells that undergo transformation through activation of anti-apoptotic, or pro-proliferative networks can influence tumour development. Various factors influence the ability of a cell to escape the normal checks and balances, such as loss of DNA damage and cell cycle regulatory proteins, and the activation of protooncogenes. Ras was originally discovered as a group of protooncogenes isolated from different strains of Sarcoma Virus172,173 with the subsequent discovery of the human homologs H-Ras and K-Ras174. This family of proteins drives pro-proliferative signals within the cell, and can lead to tumourigenesis when dysregulated170.

NK cells are known to mediate self-nonsel discrimination of transformed cells or tumour cells through various stimulatory (e.g. DNAM-1, Nkp30, NKG2D) and inhibitory receptors (e.g. Ly49, KIR (human), NKR-P1B, NKR-P1A (human), CD94/NKG2A)9,274. Thus, a lack of inhibitory ligand such as MHC-I, or upregulation of a stimulatory ligand such as NKG2DL, will
alter the balance of signals favouring killing of the target. In vivo, oncogenic transformation has been demonstrated in an Eµ-C-Myc mouse model in which NKG2D ligands are upregulated upon lymphoma development, thus increasing susceptibility to NK cell killing. NKG2D ligands were also increased on tumours derived from transformed cells. In fibroblasts, oncogenic transformation mediated through Ras caused a loss of cell surface MHC-I, ligand for the inhibitory Ly49 (KIR(h)) receptors on NK cells.

Clr-b, ligand for the NKR-P1B receptor is frequently lost on tumour cell lines, whereas ex vivo cells express high levels (Fig. 20A). This loss has been shown to increase target susceptibility to NK cell-mediated cytotoxicity. How transformation, an event that precedes immortalization of a cell, or tumourigenesis, influences Clr-b expression is currently unknown. To this end, we screened a panel of wild-type, dominant-negative, and constitutive-active protooncogenes and assessed their effect on Clr-b expression. Notably, Clr-b expression was markedly decreased on NIH3T3 fibroblasts expressing constitutively-active H-RasV12, and to a lesser extent with the constitutive-active form of human C-Raf. H-RasV12-mediated downregulation of Clr-b could be blocked with inhibitors of the MEK-Erk pathway, U0126 and CI-1040. More specifically, knockdown of Erk2, and not Erk1 or Erk5, largely prevents H-RasV12-mediated loss of Clr-b. However, the expression of Erk2 alone was not sufficient to cause Clr-b downregulation, but synergized with H-RasV12 in a conditional manner dependent upon the Erk2 TEY motif, which is required for Erk2 homodimerization and activation. This is indicative that a signal derived from Ras, either to activate Erk2, or through activation of other downstream signaling molecules, is required to induce the loss of surface Clr-b. Erk2 kinase activity and DNA-binding activity may also contribute to the loss of Clr-b but are not sufficient when evaluated alone. Collectively, these results suggest that loss of Clr-b following H-RasV12-induced oncogenic stress occurs through the MEK-Erk pathway, and is mediated by homodimerized Erk2 through kinase-dependent and kinase-independent functions. Overall, the ubiquitously expressed MHC-independent inhibitory ligand, Clr-b, is modulated by oncogenic H-Ras. Together, these findings contribute to our current knowledge of how NK cells recognize targets in healthy versus stressed states.
5.3 Results

5.3.1 Investigating the effects of protooncogenes on Clr-b expression in NIH3T3 fibroblasts.

In contrast to normal, healthy cells, which express high levels of Clr-b, this ligand is frequently lost on tumour cells, rendering transformed target cells more susceptible to NKR-P1B-mediated missing-self recognition. To gain further insight into the underlying basis for the loss of Clr-b during transformation, we screened a panel of protooncogenes in wild-type (WT), dominant-negative (DN), and constitutive-active (CA) forms, overexpressed in NIH3T3 fibroblasts (Fig.24).
Figure 24

<table>
<thead>
<tr>
<th>Vector</th>
<th>Expression-Level</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-Ras WT</td>
<td>Overexpression</td>
<td>Codon-optimized Human WT H-Ras</td>
</tr>
<tr>
<td>H-Ras N17 (DN)</td>
<td>Dominant-negative</td>
<td>Serine to Asparagine mutation at residue 17 - Blocks endogenous Ras expression.</td>
</tr>
<tr>
<td>H-Ras V12 (CA)</td>
<td>Constitutive-active</td>
<td>Glycine to Valine mutation at residue 12 - Loss of Ras-mediated GTPase activity, results in constitutively bound GTP</td>
</tr>
<tr>
<td>C-Raf WT</td>
<td>Overexpression</td>
<td>Human WT C-Raf</td>
</tr>
<tr>
<td>C-Raf S621A (DN)</td>
<td>Dominant-negative</td>
<td>Serine to Alanine mutation at residue 621 - Block phosphorylation site for activation of Raf</td>
</tr>
<tr>
<td>C-Raf CAAX (CA)</td>
<td>Constitutive-active</td>
<td>Encodes K-Ras carboxyl-terminal localization signals CAAX – Targets C-Raf to the plasma membrane independent of Ras activation</td>
</tr>
<tr>
<td>p53 WT</td>
<td>Overexpression</td>
<td>Human p53</td>
</tr>
<tr>
<td>p53mt135</td>
<td>Dominant-negative</td>
<td>G to A mutation at position 1016, leading to non-synonymous mutation that prevents interaction with p53 binding sites</td>
</tr>
<tr>
<td>CREB WT</td>
<td>Overexpression</td>
<td>Human CREB protein</td>
</tr>
<tr>
<td>CREB133</td>
<td>Dominant-negative</td>
<td>Human CREB protein harbouring a Serine to Alanine mutation at amino acid 133, corresponding to the mouse mutant CREB protein.</td>
</tr>
<tr>
<td>KCREB</td>
<td>Dominant-negative</td>
<td>Acts as dominant repressor by binding CREB to form inactive dimers that can no longer bind DNA elements to promote transcription</td>
</tr>
<tr>
<td>IxBα WT</td>
<td>Overexpression</td>
<td>Human IxBα protein</td>
</tr>
<tr>
<td>IxBμM</td>
<td>Dominant-negative</td>
<td>Harbours 2 Serine to Alanine mutations at residues 32 and 36 that cannot be phosphorylated, preventing dissociation from NFκB and resulting in a block in NFκB pathway signaling</td>
</tr>
</tbody>
</table>

Figure 24. Clontech vectors employed to test protooncogenic stress on Clr-b modulation. The vector sets included dominant-negative, wild-type and constitutive-active forms of signaling mediators involved in oncogenesis p53, C-Raf, H-Ras, CREB and IxBα.
Over-expression of the WT human C-Raf protein did not cause a significant loss in surface Clr-b expression; however, the CA C-Raf caused a noticeable loss (Fig.25). The human WT H-Ras molecule, which functions upstream of C-Raf signaling displayed a notable downregulation, and an even greater degree of Clr-b loss was observed following overexpression of CA H-Ras\(^{V12}\). The degree of downregulation induced by WT H-Ras appeared to be near equivalent to that of CA C-Raf, whereas a DN H-Ras\(^{N17}\) caused a slight upregulation of Clr-b (Fig.25). Although C-Raf functions directly downstream of H-Ras, the difference in phenotype as represented by the degree of downregulation in Clr-b, could arise from several factors. The human and mouse H-Ras are 100% identical at the protein level, whereas C-Raf is only 97.7% identical between mouse and human. Furthermore, redundancy exists within the different Ras isoforms, in contrast to the Raf isoforms, which are structurally different and capable of homo- and heterodimerizing. Thus, the downstream signals activated by Ras may activate more than one Raf isoform, whereas activation of C-Raf merely addresses one downstream effector of Ras signaling. CREB and p53 over-expression did not cause a significant change in Clr-b surface expression following a 24h transfection (Fig.25). Over-expression of the WT IκB\(\alpha\), which would inhibit NFκB in the absence of exogenous signals, caused a subtle decrease in surface Clr-b, whereas the dominant negative form, which would prevent NFκB activation, did not (Fig.25). However, in the latter, GFP expression from the co-transfection was markedly reduced in the presence of the DN IκB\(\alpha\) form making interpretation inconclusive.
Figure 25. Flow cytometric analysis of NIH3T3 fibroblasts transfected with dominant-negative (DN), wild type (WT) and constitutive active (CA) forms of known protooncogenes p53, C-Raf, H-Ras, CREB and IkBα. In the case of CREB, the constitutive active heading is actually a DN vector. NIH3T3 fibroblasts were co-transfected with the vector harbouring the protooncogene and GFP-NLS at a 10:1 ratio. Flow cytometry was performed 24 hours post-transfection. Histograms represent protooncogene as gated on the transfected cells (GFP high population; shaded), untransfected control (GFP low population; solid line), and secondary reagent alone (dashed line).
5.3.2  H-Ras\textsuperscript{V12} -mediated loss of Clr-b involves the MEK-Erk cascade

Based on the substantial effect CA H-Ras\textsuperscript{V12} had on Clr-b surface expression, we sought to further investigate the specific signaling arm(s) responsible for this phenotype, since Ras influences many downstream kinases, such as the MEK kinases, PI3-K, JNK, and p38 MAPK. To this end, several pathways downstream from H-Ras signaling were targeted through pharmacological inhibition, including NFκB, JNK kinase, p38 MAPK, MEK1/2, Erk1/2, MEK5 and PI3-K (Fig.26)\textsuperscript{170,176}. Inhibition of p38 MAPK and JNK kinase pathways had no effect on H-Ras\textsuperscript{V12}-mediated Clr-b downregulation using SB203580 and SP600125, respectively, nor did inhibition of PI3-K with LY294002, or NFκB with parthenolide. However, the non-competitive inhibitors of MEK1/2, UO126 and CI-1040 (PD184352), were capable of inhibiting H-Ras\textsuperscript{V12}-mediated Clr-b downregulation. CI-1040 had a partial effect at 1µM, which is slightly above the IC\textsubscript{50} for the MEK proteins, but a more pronounced effect at 20µM\textsuperscript{201,214}. The inhibitor for Erk1/2, FR180204\textsuperscript{276}, did not have a significant effect on H-Ras\textsuperscript{V12}-mediated Clr-b downregulation even though the Erk proteins are the only known targets of MEK1 an MEK2.

Although originally characterized for their ability to inhibit MEK1/2, UO126 and CI-1040 are also capable of inhibiting the MEK5-Erk5 pathway when used at higher concentrations, such as those employed in this study\textsuperscript{277}. In addition, both C-Raf and H-Ras modulate Erk5 by a mechanism distinct from activation of the traditional Erk1/2 pathway\textsuperscript{278}. Thus, we employed an inhibitor of Erk5 phosphorylation (BIX02189\textsuperscript{279}) to aid in deciphering whether the effect observed with UO126 and CI-1040 was due to inhibition of the MEK5-Erk5 pathway (Fig.26). Notably, BIX02189 caused an upregulation of Clr-b in the GFP\textsuperscript{+} fraction; however, this increase was also observed in the GFP\textsuperscript{−} (non-transfected) fraction. Thus, Erk5 inhibition does not cause a block in H-Ras\textsuperscript{V12}-mediated downregulation, but rather promotes a generalized increase in Clr-b surface expression, independent of Ras signaling.
Figure 26

Figure 26. Pharmacological inhibition of downstream Ras signalling following transfection with constitutive active H-Ras highlights the MEK-Erk pathway. NIH3T3 fibroblasts were transfected with pBud 4.1 eGFP harbouring H-RasV12 for 24 hours then dosed with the indicated inhibitors (target in brackets) for 24 hours. (A) Flow cytometry was performed 48 hours following the original transfection. Histograms represent chemical-treated GFP-negative population (solid line), chemical-treated GFP-positive population (shaded), and secondary reagent alone (dashed line). (B) The grey bars represent fold downregulation in the Clr-b median fluorescence intensity (MFI) of the GFP+ transfected population relative to the GFP- (untransfected) population for each treatment. The vertical dashed line is arbitrarily aligned to the approximate median of the GFP- DMSO treated population for reference purposes. Data are representative of 3 independent experiments.
5.3.3 H-Ras$^{V12}$-mediated loss of Clr-b requires ERK2, but not ERK1, or ERK5

A caveat to pharmacological inhibition is that many chemicals can have pleiotropic or undocumented effects across numerous different proteins/pathways. This is evident through the ability of CI-1040 and UO126 to inhibit MEK1, MEK2, and MEK5 signaling. To address the role of the MEK1/2-Erk1/2 pathway and the potential involvement of the MEK5-Erk5 pathway in H-Ras$^{V12}$-mediated loss of Clr-b, we knocked down Erk1, Erk2, or Erk5 in NIH3T3 fibroblasts using lentiviral shRNA, in comparison to a LacZ shRNA control (Fig.27). Cells were transiently transfected with H-Ras$^{V12}$ following five days of puromycin selection for the indicated shRNA after stable transduction, and cultured for 48 hours before assaying Clr-b expression. Lysates were made following selection and assayed by Western Blot for the protein of interest. We reproducibly achieved 80-100% knockdown for ERK1/2 and 50-70% for Erk5 (Fig.27A). Knockdown was specific, as knockdown of the related kinases, Erk2 and Erk1, did not have an effect on the opposite protein, respectively (Fig.27A). Erk2, but not Erk1, Erk5, or LacZ shRNA caused a notable block in Ras$^{V12}$-mediated downregulation of Clr-b (Fig.27B). The loss in Clr-b was quantified as a fold-change relative to the GFP$^{-}$ untransfected cells for each given knockdown (Fig.27C). Overall, the results implicate Erk2 as a central player in H-Ras$^{V12}$-mediated downregulation of Clr-b, and that although Erk1 is highly related (~87% amino acid identity), little or no redundancies exist downstream of MEK1/2 in this system.
Figure 27. Investigating the effects of constitutive active H-RasV12 following knockdown of Erk proteins on Clr-b regulation in NIH3T3 fibroblasts. NIH3T3 fibroblasts were transduced with lentivirus harbouring shRNA within the pLKo.1 vector for Erk1, Erk2, and Erk5. Following 5 days of selection in 2.5 μg/ml puromycin cells were harvested and; (A) Prepared lysates for western blot for Erk proteins to confirm the degree of knockdown compared to the β-tubulin reference gene. Relative expression normalized to NIH3T3 cells was calculated as \[
\frac{\text{shRNA}_{\text{tubulin}}}{\text{NIH3T3}_{\text{tubulin}}} \times \frac{\text{shRNA}_{\text{Erk}}}{\text{NIH3T3}_{\text{Erk}}}
\] (B) Flow cytometry was performed 48 hours following transfection with pBud 4.1 eGFP harbouring H-RasV12. Histograms represent untransfected cells (GFP negative population; solid line), and H-RasV12 expressing cells (GFP positive population; shaded). Data are representative of 3 independent experiments. (C) The grey bars represent fold downregulation in the Clr-b median fluorescence intensity (MFI) of H-RasV12 transfected GFP+ cells versus non-transfected GFP- cells. The average values and SEM for the Erk proteins are derived from 5 independent experiments whereas, the LacZ values are calculated from 3 independent experiments. Significance calculated as a one-tailed t-test with equal variance, where * p=0.001 ** p =0.0001 *** p=0.001.
5.3.4 Overexpression of WT Erk2 enhances H-Ras\textsuperscript{V12}-mediated Clr-b downregulation but is not sufficient in isolation

Since Erk2 appears to be necessary in causing the loss of surface Clr-b following H-Ras\textsuperscript{V12} transfection, we sought to investigate whether overexpression of Erk2 was sufficient to mediate this effect. Erk2, as well as several functional mutants were cloned into the pBud4.1CE eGFP vector and transfected for 48 hours before flow cytometry was performed. The K52R mutation results in a kinase-dead form of Erk2, that acts as a dominant-negative \textsuperscript{212}. The L73P/S151D mutations generate a CA form of Erk2 kinase activity \textsuperscript{280}. Erk2\textsuperscript{S321-7A} represents a mutant in which residues 321-327 are replaced with alanines, and lacks a site required for nuclear import and thus resides exclusively in the cytosol \textsuperscript{281}. Lastly, the T183A/Y185F mutant lacks the TEY motif that is required for activation of Erk2, thus preventing activating phosphorylation and homodimerization \textsuperscript{209}. Surprisingly, expression of WT Erk2 or any of the mutant forms did not cause a loss of Clr-b surface expression, as quantified through fold-change in MFI of the GFP\textsuperscript{+} fraction versus the GFP\textsuperscript{-} fraction as normalized to empty pBud4.1 GFP vector (Fig.28A).

Since overexpression of Erk2 alone was not sufficient to induce a loss in surface Clr-b, we wanted to determine whether the Erk2 wild-type and mutant proteins could modulate the H-Ras\textsuperscript{V12}-mediated loss of Clr-b. Erk2 coding sequences were cloned into the pBud4.1CE tRFP vector and H-Ras\textsuperscript{V12} was cloned into the pBud4.1CE eGFP vector. The two vectors were co-transfected at a 1:1 ratio into NIH3T3 fibroblasts for 48 hours before flow cytometry was performed (Fig.28B). The fold-change in MFI for Clr-b was compared between the GFP\textsuperscript{+}RFP\textsuperscript{+} population and the GFP\textsuperscript{-}RFP\textsuperscript{-} fraction and normalized to the control, pBud4.1CE eGFP H-Ras\textsuperscript{V12} co-transfected with empty pBud4.1CE tRFP. Thus, the phenotype depicted is the fold change normalized to H-Ras\textsuperscript{V12} alone. The Erk2 wild-type vector as well as the Erk2\textsuperscript{L73P/S151D}, and Erk2\textsuperscript{S321-7A} mutants all increased the degree of downregulation of Clr-b in the context of H-Ras\textsuperscript{V12}. The Erk2\textsuperscript{T183A/Y185F} mutant did not enhance the loss of Clr-b, but it did slightly antagonize H-Ras\textsuperscript{V12}-mediated Clr-b downregulation, although not significantly. Interestingly, both the kinase-dead Erk2\textsuperscript{K52R} and a mutant deficient in DNA-binding, Erk2\textsuperscript{K257A/R259A} showed a partial block in the degree of synergistic downregulation as compared to H-Ras\textsuperscript{V12} + Erk2\textsuperscript{WT}. 
This suggests that kinase-dependent and kinase-independent functions may be mediating the synergistic loss of Clr-b at the cell surface.
Figure 28

(A) TPNZ fibroblasts were transfected with pBud 4.1 eGFP harbouring wild type or mutant forms of Erk2 for 48 hours then analyzed by flow cytometry. The grey bars represent fold up or downregulation in the Clr-b median fluorescence intensity (MFI) between the transfected cells (GFP+) and untransfected cells (GFP-) for each given condition normalized to empty pBud 4.1 eGFP. Data are an average of 3 independent experiments with SEM.

(B) TPNZ fibroblasts were co-transfected with pBud 4.1 eGFP harbouring H-Ras\(^{V12}\) and pBud 4.1 tRFP harbouring wild type or mutant forms of Erk2 for 48 hours then analyzed by flow cytometry. The grey bars represent fold up or downregulation in the Clr-b MFI for the GFP/RFP positive co-transfected population versus the GFP/RFP negative untransfected population as normalized to pBud 4.1 H-Ras\(^{V12}\) + empty pBud 4.1 tRFP. Data are representative of at least 4 independent experiments. SEM is plotted and statistical comparison between Ras\(^{V12}\)+Erk2\(^{WT}\) and mutant vectors is based on a two-tail equal variance t-test where \(* = 0.0002, ** = 0.000001, and *** = 0.002.\)
5.4 Discussion

Oncogenic transformation is an early event in tumourigenesis. The early detection of cells that escape the checks and balances that precede these pathological alterations is important in host immunosurveillance. NK cells have been demonstrated to be important in self-nonself discrimination of cells expressing oncogenes such as C-Myc, which induce NKG2DL, or Ras, which lose surface MHC-I. Activation of the Ras family of protooncogenes have been implicated in as many as 20% of human tumours. Based on the rationale that the NKR-P1B ligand, Clr-b, is frequently lost on tumour cell lines, and that transformation is an early event in the immortalization of a cell, hypothetically leading to the evolution of a tumour, we sought to investigate the role of oncogenic stress on Clr-b modulation in NIH3T3 fibroblasts. After screening a small panel of protooncogenes, we found that H-Ras, and more specifically the CA form, H-RasV12, had the most pronounced effect on surface Clr-b expression. Thus, we continued the remainder of our investigation using the oncogenic CA H-RasV12.

H-RasV12-mediated loss of Clr-b appears to be dependent on the MEK1/2-Erk1/2 pathway, as pharmacological inhibition using CI-1040 and UO126 blocked this effect. Pharmacologically, we ruled out the direct involvement of other pathways such as PI3-K, JNK, NFκB, and p38 MAPK, although specificity and efficacy remain a concern. Aside from inhibition of MEK1 and MEK2, CI-1040 and UO126 can also act on the MEK5-Erk5 pathway, which can be activated by stress and/or mitogenic stimulation. However, experiments employing the inhibitor of Erk5 phosphorylation, BIX02189, demonstrated a generalized increase in Clr-b expression, regardless of CA H-RasV12 transfection state. Thus, this constitutive upregulation, as opposed to blocking downregulation, appears to be independent of H-RasV12 signaling. Nevertheless, it is interesting to speculate what role the MEK5-Erk5 pathway has in the steady-state expression of Clr-b, and that perhaps basal signaling moderates Clr-b expression.

As previously stated, the pharmacological inhibition of MEK1 and MEK2 was sufficient to prevent H-RasV12-mediated loss of Clr-b. However, inhibition of the only known downstream kinases Erk1 and Erk2 using FR180204 was not sufficient to prevent this phenotype. One potential explanation is that the inhibitor was originally characterized against human Erk1 and Erk2 and may not bind its mouse counterparts as efficiently. However, human Erk2 and Erk1
share ~99% and ~97% protein homology with mouse, respectively. Furthermore, the residues determined to be important for binding of FR180204 to Erk1 and Erk2, as determined through crystal structure analysis, are present in both mouse and human Erk1/2. A more likely explanation for this discrepancy can be clarified by the functions of the inhibitors themselves. FR180204 is a competitive inhibitor for ATP by binding the Erk1/2 ATP-binding pocket, and thus would prevent activated Erk dimers from phosphorylating downstream substrates. On the other hand, MEK inhibitors such as UO126 and CI-1040 are non-competitive inhibitors of MEK1 and MEK2 that prevent the phosphorylation, activation and formation of Erk homodimers. Thus, the Erk inhibitor would prevent the downstream kinase activity of Erk homodimers, whereas the MEK1/2 inhibitors would prevent the dimerization of Erk1/2, which in turn would prevent proper Erk1/2 activation and nuclear translocation. Indeed kinase independent functions of the Erk1/2 have been documented and theoretically would still be functional in the presence of the competitive Erk inhibitor.

Due to the broader substrate specificity of CI-1040 and UO126, we sought to further delineate which Erk proteins were responsible for H-RasV12-mediated loss of Clr-b. To this end, we performed specific knockdown of Erk1, Erk2, or Erk5, to address their potential involvement individually. H-RasV12-mediated downregulation of Clr-b was largely prevented in Erk2 knockdown cells. The requirement for Erk2 highlights a non-redundant role for the Erk proteins in Ras-MEK-Erk signaling. This contributes to a current body of evidence that supports the fact that Erk1 and Erk2 have unique functions, even though they harbour ~87% protein identity. In fact, in NIH3T3 fibroblasts, the knockdown of Erk1 drives a proliferative response due to a dysregulated Erk2. In contrast, the loss of Erk2 appears to slow cell cycle. This is in agreement with reports that Erk1 acts in a manner to attenuate Erk2 activity. In our hands, the loss of Erk1 or Erk5 did not render NIH3T3 resistant to H-RasV12-mediated Clr-b downregulation. Whether the loss of Erk2 alters the cell-cycle state following H-RasV12 transfection, and whether this directly correlates with the loss of surface Clr-b, is a topic area that requires further investigation. Data we have generated to date suggests that cells need to be cycling to evoke a loss of Clr-b expression. However, this has been difficult to clearly document using various different in vitro model systems when investigating the response to genotoxic stress. Nevertheless, if active replication is required, and the cells have become quiescent, or
senescent, following Erk2 knockdown, then H-Ras<sup>V12</sup> transfection would be expected to not have an effect on Clr-b surface levels.

Since the loss of Erk2 prevented H-Ras<sup>V12</sup>-mediated downregulation of Clr-b, we sought to determine if Erk2 was sufficient to trigger this effect. Remarkably, the over-expression of WT Erk2 alone was not sufficient to cause Clr-b downregulation in normal NIH3T3 fibroblasts. Nor did Erk2 mutants that target Erk2 kinase activity, phosphorylation-induced dimerization, or nuclear import. Unexpectedly, the Erk2<sup>L73P/S151D</sup> CA mutant did not have an effect. Although this result was unexpected, it could be potentially explained by the fact that the Erk2<sup>L73P/S151D</sup> mutation was characterized for its kinase activity on common Erk2 substrates RSK, Elk-1, and myelin basic protein<sup>280</sup>. Since there are hundreds of known and theoretical Erk2 substrates<sup>283</sup>, the specific requirements for activation of Erk2 to achieve Clr-b downregulation in the absence of H-Ras<sup>V12</sup>-driven signals remains unknown and the Erk2<sup>L73P/S151D</sup> mutation appears to be insufficient. Nevertheless, the Erk2<sup>L73P/S151D</sup> mutations do exhibit a greater degree of basal autophosphorylation than WT Erk2<sup>284</sup>. We would have to confirm in our hands whether the amounts of Erk2 phosphorylation following transfection of WT Erk2, or Erk2<sup>L73P/S151D</sup> alone and following H-Ras<sup>V12</sup> transfection, are different.

Co-transfection experiments demonstrated that although not sufficient on its own, Erk2 synergizes with the H-Ras<sup>V12</sup> signal to induce a greater degree of Clr-b downregulation compared to H-Ras<sup>V12</sup> alone. This was also the case with the nuclear-import deficient (Erk2<sup>S321/7A</sup>), and constitutive-active (Erk2<sup>L73P/S151D</sup>) mutants. The kinase-dead Erk2<sup>K52R</sup> showed a partial block in the synergistic downregulation. The fact that Erk2<sup>K52R</sup> only partially affects the synergistic downregulation of Clr-b suggests that the kinase activity of Erk2 is not absolutely sufficient for H-Ras<sup>V12</sup>-mediated Clr-b downregulation. This is also in agreement with pharmacological inhibition experiments with FR180204, which would target the phosphokinase functions of Erk1 and Erk2, but not the kinase-independent functions. Indeed, kinase-independent functions of Erk2 have been documented through the ability of kinase-deficient mutants to directly activate topoisomerase II, PARP-I (poly (ADP-ribose) polymerase I), and MKP-3 (MAPK phosphatase-3). Kinase deficient Erk2, as well as WT Erk2 are also capable of binding to DNA, through transcription factor complexes, and directly to G/CAAAG/C motifs within 90 base pairs of transcriptional start sites. The original identification of these DNA-binding motifs was performed
with human Erk2; however, the K-259 and R-261 residues that are important for DNA binding and promoter occupancy are also conserved within the mouse Erk2, at residues K-257 and R-259, respectively \(^{213,282}\). The Erk2\(^{K257A/R259A}\) mutant, which also showed a partial block in the synergistic downregulation, would have intact kinase activity, but lack the ability to bind DNA \(^{213}\). Thus, the synergistic loss of Clr-b may require both kinase-dependent, and kinase-independent (DNA-binding) functions of Erk2. The Erk2\(^{T183A/Y185F}\) mutant, which lacks the necessary residues in the TEY motif for activation and dimerization of Erk2, did not increase the amount of H-Ras\(^{V12}\)-mediated Clr-b downregulation compared to the WT Erk2, and displayed a phenotype similar to H-Ras\(^{V12}\) alone. The Erk2\(^{T183A/Y185F}\) mutant cannot form active homodimers, and can associate with endogenous Erk2 to form non-functional hemi-active dimers \(^{209}\). Thus, it is surprising that overexpressing this mutant is not sufficient to at least partially reduce the degree of H-Ras\(^{V12}\)-mediated downregulation, due to the formation of hemi-active dimers with endogenous Erk2. Performing these experiments with shRNA-mediated knockdown of Erk2, and reconstitution with Erk2 constructs that harbour silent mutations for the target shRNA are required to fully elucidate the role of Erk2 in this system.

The necessity for Erk2 to synergize with H-Ras\(^{V12}\) to mediate the loss of Clr-b suggests that other H-Ras\(^{V12}\) activated pathways are required for the proper activation of Erk2, or more simply, that activated MEK1 and MEK2 are required for proper activation of Erk2 to mediate Clr-b downregulation. This is logical due to the fact that Erk1 and Erk2 are the only known substrates for MEK1 and MEK2, and although Erk1 and Erk2 are capable of autophosphorylation, their full activation requires MEK1 and MEK2 \(^{285}\).

Collectively, these results are beginning to shed light on the some of the programmed cellular responses responsible for Clr-b downregulation in response to oncogenic stress and transformation. H-Ras\(^{V12}\)-mediated downregulation of Clr-b occurs through the MEK-Erk pathway and is largely dependent on Erk2. The loss of this inhibitory self-ligand would result in missing-self recognition by NK cells in response to oncogenic H-Ras-induced transformation. Together, these findings contribute to our current knowledge of innate recognition of transformed targets, and more specifically, how NK cells recognize targets in healthy versus diseased states.
CHAPTER VI

Concluding Remarks
6 Thesis Goals

6.1 Investigating the effects of genotoxic stress on Clr-b modulation

In chapter III of this thesis we have demonstrated that genotoxic stress promotes Clr-b ligand downregulation on tumour cells and fibroblasts in a time-dependent and dose-dependent manner. This loss occurs at both the RNA level and surface level. Functionally, this was demonstrated using a BWZ reporter assay as the cell surface ligand loss translated into reduced BWZ.NKR-P1B reporter cell stimulation. Genotoxic stress-induced loss of Clr-b on murine leukemia cells renders them more susceptible to NK cell-mediated cytotoxicity by NKR-P1B\textsuperscript{+} LAK cultures. This effect was less pronounced using B6 LAK cultures. However NKR-P1B\textsuperscript{B6}, also known as NKR-P1D, is stimulated by Clr-b to a lesser extent than NKR-P1B\textsuperscript{Swiss} using reporter systems \textsuperscript{57}, which suggests that the inhibitory effect of Clr-b on B6 LAK is not very strong.

Remarkably, Clr-b downregulation was coordinated functionally with stress-mediated upregulation of NKG2D ligands, in most cases. Any discrepancies may be reflective of the different types or cellular states of the cells used, as many cells acquire various mutations that affect the DDR and cell cycle as they become transformed or immortalized. Notably, levels of MHC-I which is lost on cells during transformation or viral infection, was not significantly altered by genotoxic stress, which argues that the genotoxic stress-mediated loss of Clr-b is independent of MHC-I regulation. Having different pathways to modulate different ligands and enhance NK cell susceptibility is important, as viruses and tumours are known to evolve to avoid innate recognition \textsuperscript{67}. Overall, our findings highlight a unique role for the MHC-independent NKR-P1B:Clr-b “missing-self” axis in recognition of stressed cells.

6.2 Investigating the pathways/mechanisms responsible for genotoxic stress-mediated Clr-b downregulation

In Chapter IV of this thesis, we have further investigated the phenomenon of genotoxic stress-induced downregulation of Clr-b in an attempt to delineate the pathway(s) and/or mechanism(s) involved in mediating this effect. Stimulatory ligands for the NKG2D and DNAM-1 (CD226) receptors have been shown to be regulated through the classical DDR
pathway. Pharmacological inhibition of ATM and ATR was sufficient to prevent DNA-damage induced NKG2DL and DNAM-1 ligand upregulation, and specific shRNA-mediated knockdown of ATR prevented aphidicolin-induced NKG2DL upregulation, while ATM knockdown decreased constitutive expression of NKG2DL. Here, we show that in response to genotoxic stress, components of the classical DDR pathway, mainly ATM, ATR, Chk1 and Chk2 do not affect Clr-b modulation, at least not individually. However, one cannot completely exclude the possibility of redundancy, which exists in the DDR pathway, to maintain the genotoxic stress-induced downregulation phenotype in the presence of single knockdowns. Yet, aphidicolin primarily activates ATR, due to replicative stress, and we were able to reproduce published data with respect to NKG2DL, as single knockdown of ATR was sufficient to prevent aphidicolin-induced NKG2DL upregulation. Thus, the inability of ATR alone to prevent aphidicolin-induced Clr-b downregulation is surprising, even if redundancies in this system do exist. However, recent evidence demonstrates that in mice, activation of the replication checkpoint following aphidicolin administration occurs independently of ATR and ATM. In addition, knockout fibroblasts for p53 and p21, two downstream effectors of ATR and ATM maintain the genotoxic stress induced downregulation phenotype.

In the cell systems employed, the general inhibition of ATR/ATM via caffeine appears to have opposing roles, as caffeine-treated fibroblasts do not lose Clr-b upon aphidicolin treatment, whereas caffeine-treated murine leukemia cells demonstrate a synergistic loss following aphidicolin treatment. This can potentially be explained by the fact that caffeine is capable of activating replication checkpoints independent of ATM and ATR. Furthermore, caffeine is capable of reversing chemically-induced replication arrest in transformed cells, yet, not in normal fibroblasts. Thus, the phenotype observed may be reflective of irreversible growth arrest in the case of NIH3T3 fibroblasts, and a bypass in the replication checkpoint in the case of C1498 cells. A caveat to all of these experiments is that the mutational status of the all of the DNA damage proteins in NIH3T3 and C1498 cells is not known. CHEK2 and p53 mutations have indeed previously been documented in other cell lines (e.g. NCI-60). However, our use of many different cell types, and low passage-number mouse embryonic and ear fibroblasts, demonstrates that the Clr-b downregulation phenotype is intact in many cell types.
The genotoxic stress-mediated loss of Clr-b appears to be independent of any particular stage of the cell cycle. However, the accumulation of cells in G2/M with bleomycin and γ-irradiation, which do not promote Clr-b downregulation, poses the question of whether active replication is required to cause Clr-b downregulation. Experiments performed early on in this thesis work supported this hypothesis, yet they were difficult to duplicate, and thus not included. In the end, the assays employed, such as CFSE dilution following genotoxic stress and cytokine deprivation of IL-2-dependent MNK-1 cells (because of loss of IL-2 dependency) in the context of genotoxic stress did not provide a clear answer to this question.

In this work, we demonstrated that targeting post-translational and post-transcriptional mechanisms could block genotoxic stress-induced downregulation of Clr-b. The post-translational mechanism targeted is hypothesized to involve the ubiquitin-proteasome pathway, which when inhibited by pre-treatment with MG132 or lactacystin prevents Clr-b downregulation in response to genotoxic agents. However, this pathway is not exclusively involved in mediating genotoxic stress-induced loss of Clr-b since there is no blockade in the loss of transcript when comparing genotoxic agent alone versus genotoxic agent coupled with proteasome inhibitor. Thus, transcript and protein level loss is uncoupled using proteasomal inhibitors. The ability of chloroquine to have partial effects suggests the possibility that Clr-b may be recycled through an endolysosomal pathway. The endolysosomal pathway utilizes ubiquitin, and thus would be inhibited by proteasomal inhibitors. Yet, attempts to delineate whether endocytic mechanisms play a role, such as clathrin or dynamin-mediated endocytosis, proved difficult to dissect as mixing inhibitors of these pathways along with genotoxic agents proved to be too toxic to the cells.

We also sought to target the post-transcriptional regulation of Clr-b, since we know that Clr-b transcript is lost upon exposure to genotoxic agents, and this results in the loss of cell surface Clr-b. We chose to investigate this through the embedded HHR within the 3’UTR of the Clr-b gene, clec2d. Under normal steady state conditions the HHR cleaves the transcript, which removes the poly-A signal from the mRNA, resulting in an unstable transcript that may ultimately be degraded \(^{235}\). Inhibition of the ribozyme through pre-treatment of cells with toyocamycin \(^{254}\) causes an increase in Clr-b transcripts and protein under normal conditions and prevents genotoxic stress-mediated loss of Clr-b. However, using reporter systems, it does not
appear that there is specific increase in the amount of cleaved reporter transcript following addition of the genotoxic agents. That has lead to the current conclusion that the ribozyme does not affect the genotoxic-stress mediated loss of Clr-b, although it may generally be responsible for normal turnover of transcript. Furthermore the inability of some Clr-b\textsuperscript{lo} cell lines to increase Clr-b expression upon exposure to toyocamycin suggests that the ribozyme is not the only means for regulating the abundance of the transcript. Yet to be fully examined is the promoter of Clr-b and how it is affected under genotoxic stress conditions.

6.3 Investigating the effect of oncogenic stress and the pathways involved in Clr-b modulation during transformation

In Chapter V of this thesis, we investigated the effect of oncogenic stress on Clr-b modulation. Screening several protooncogenes lead us to investigate the constitutively-active form of H-Ras, H-Ras\textsuperscript{V12}, as it had the most pronounced effect on Clr-b modulation. With the use of pharmacological inhibitors, we determined that the downregulation of Clr-b following transfection of NIH3T3 fibroblasts with H-Ras\textsuperscript{V12} occurs through the MEK-Erk pathway and not through other Ras-activated pathways. The MEK1/2 kinases activate Erk1/2 kinases, so we employed a lentiviral-shRNA system to specifically assess the role of the Erk proteins in mediating this effect. Erk2 is required in H-Ras\textsuperscript{V12}-mediated Clr-b downregulation, but it alone is not sufficient to mediate Clr-b loss. Remarkably, this was still the case when using a constitutive active form of Erk2, which is capable of autophosphorylation. Thus, a synergistic signal upstream from Erk2 is required for proper activation to mediate Clr-b downregulation. Whether the loss of cell surface protein in this model requires a loss of transcript has not yet been investigated. However, work in Chapters III, and IV, as well as previously published work using a viral model\textsuperscript{64}, supports the notion that the loss of Clr-b transcript may be required. Since Erk2 exists and performs functions in both the cytosol and nucleus, although primarily in the latter, it is interesting to speculate what function of Erk2 is actually mediating this effect. The kinase-defective mutant of Erk2 only partially synergized with H-Ras\textsuperscript{V12} to cause Clr-b downregulation when compared to Erk2\textsuperscript{WT}, which suggests that kinase-independent functions of Erk2 may also contribute to Clr-b downregulation. Overall, this may be the first instance documenting that oncogenic stress is involved in the regulation of the MHC-independent missing-self ligand,
Clr-b. Collectively, these results contribute to our knowledge of innate surveillance through NK cell recognition of target cells undergoing transformation.

### 6.4 Mechanism(s) of Clr-b downregulation and future directions

Although more than one mechanism may ultimately be involved in the downregulation of Clr-b in the context of genotoxic stress and transformation, a common theme seems to be emerging that, when cells undergo replicative stress, they lose Clr-b expression. This may explain why genotoxic agents such as aphidicolin, cisplatin and TSA, all of which cause replicative stress through stalled or collapsed replication forks $^{86,127,137}$, promote downregulation of Clr-b, whereas bleomycin and $\gamma$-irradiation, which do not cause Clr-b downregulation, generate broadly distributed dsDNA break lesions. Furthermore, H-Ras$^{V12}$ expression, which induces Clr-b downregulation, causes senescence via replicative stress through an increased abundance and collapse of replication forks $^{203}$.

Although cells in different stages of the cell cycle downregulated Clr-b when exposed to different genotoxic agents a distinction that was not made was whether the cells were actively dividing and at what stage they were within the G2/M checkpoint (Fig.15). Thus, a stain for Ki-67 $^{288}$ or phosphorylated-histone H3 (phosphorylated S10) $^{289}$ may more clearly mark proliferating and mitotic cells, respectively. Thus, cells exposed to radiomimetics or $\gamma$-irradiation may not actually be in mitosis or actively proliferating, even though they would be expected to be 4n after 24 hours (Fig.15). Furthermore, silencing Erk2 in NIH3T3 fibroblasts almost completely abrogates oncogenic Ras-dependent cell proliferation. As only Erk2 was capable of preventing H-Ras$^{V12}$-mediated Clr-b downregulation, it may be due to the fact that the cells are impaired in their ability to proliferate following transfection $^{205}$. Consequently, the loss of Clr-b from the surface in response to genotoxic or oncogenic stress may in fact be dependent on the proliferative state of the cells.

Through work in this thesis and previous work using viral systems $^{64}$, we know that the Clr-b transcript is lost along with cell surface protein. The initial discovery of the clec2D HHR $^{235}$ posed an intriguing and plausible mode of regulation of the transcript. Work done here suggests that active cleavage by the HHR within transcripts, which reduces surface protein and reporter protein expression, is not modulated (i.e. augmented) by genotoxic stress. Following
clec2d HHR cleavage, the substrate portion of the HHR remains with the coding sequence of the mRNA, and the enzyme portion is liberated\textsuperscript{235}. An interesting point to note is that the substrate for clec2d, shares homology with other Clec2 genes, which lack the enzyme portion. This makes trans-interactions between different Clec2 transcripts a possibility. Whether these interactions occur remains to be documented. Another possibility is that the HHR requires interaction with other proteins for proper folding and catalysis, or regulation, as has been observed with some other ribozymes\textsuperscript{263,265}. With the reporter system employed, it is possible that the HHR fused to the reporter gene transcript does not encounter its endogenous interacting partner(s). Although the HHR mode of post-transcriptional regulation may not be unique to genotoxic stress, it does not necessarily mean that Clr-b is not subject to post-transcriptional regulation, as HHR cleavage may be responsible for normal turnover, independent of DDR-mediated activation.

Lastly, the promoter has yet to be fully investigated in the context of genotoxic stress, transformation and viral infection. A reduction in transcription rate could be a simple explanation for genotoxic and oncogenic stress-mediated loss of Clr-b. This could be reflective of an increase in repressive transcription factors or a reduction in transcription factors that promote Clr-b transcription. Erk2 can bind directly to DNA to repress transcription at G/CAAAG/C sites clustered close together, normally within 100bp of the transcriptional start site of some genes\textsuperscript{213}. The Erk2\textsuperscript{K257A/R259A} mutant, whose mutations affect its DNA-binding capability\textsuperscript{213}, only partially synergized with H-Ras\textsuperscript{V12} to cause Clr-b downregulation equivalent to that of the kinase dead Erk2\textsuperscript{K52R}. Thus, the DNA-binding capability of Erk2 may be involved in H-Ras\textsuperscript{V12}-mediated Clr-b downregulation. A quick search of the genomic DNA for clec2d reveals a cluster of these sites ~250bp from the transcriptional start site. Whether Erk2 can bind these sites and influence H-Ras\textsuperscript{V12}-mediated Clr-b downregulation remains to be elucidated.

In conclusion, it appears that multiple mechanisms are responsible for Clr-b regulation. So, why would this ubiquitously expressed non-polymorphic ligand for an inhibitory NK cell receptor be under such tight regulation? The adaptation of certain viruses to counteract the loss of surface Clr-b\textsuperscript{64} highlights the NKR-P1B:Clr-b axis as an important contributor to NK cell-mediated target recognition. Future work will help to clarify how this monomorphic ligand serves as an early indicator of cells undergoing pathological alterations.
References


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Rabinovich, B. A. *et al.* Activated, but not resting, T cells can be recognized and killed by syngeneic NK cells. *J Immunol* 170, 3572-3576, (2003).


