DIFFERENTIAL SIGNALLING BY TYPE I AND TYPE II CALRETICULIN (CALR) MUTATIONS IN MYELOPROLIFERATIVE NEOPLASM

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FEBRUARY 2026

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by

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Submitted

in the fulfilment of the requirements of the degree of Doctor of Philosophy

to the

INDIAN INSTITUTE OF TECHNOLOGY DELHI

FEBRUARY 2026

“To my parents, and to the younger me who endured, believed, and became”

CERTIFICATE

This is to certify that the thesis entitled "**Differential signalling by type I and type II calreticulin (CALR) mutations in myeloproliferative neoplasms**" being submitted by **Ms. Saadia Naseer** to the **Kusuma School of Biological Sciences, Indian Institute of Technology Delhi** for the award of the degree of "**Doctor of Philosophy**" is a record of the bonafide research work carried out by her, prepared under my supervision, in conformity with the rules and regulations of the 'Indian Institute of Technology Delhi'. The research report and the results present in the thesis have not been submitted to any other University or Institute for the award of any other degree or diploma.

Date: 04/02/2026

Place: New Delhi

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ACKNOWLEDGEMENTS

According to the holy Quran, **'Verily, with hardship, comes ease.'** The completion of my Ph.D. work is not the end, rather a living testament of this promise. No difficulty presents itself without the blessings hidden inside. This journey had been incredibly humbling and culmination of scientific exploration, following the hunches, personal growth and invaluable support of numerous people becoming my blessing. I would like to take this moment to convey my heartfelt appreciation to all the people who have directly or indirectly played a role in bringing this work to fruition.

To begin with, I would like to express my sincerest gratitude to my supervisor, **Prof. Anita Roy**, for her intellectual support and mentorship playing a pivotal role throughout my research journey. Her willingness to let me to pursue leads had helped me garner independent thinking while upholding scholarly rigor. I am thankful for her to providing me this opportunity to work on this project as it aligned with my interest and providing me the niche to grow and explore my curious hunches. Her perceptive feedback, meticulous attention to detail and passionate commitment have predominantly responsible for the conceptual framework and forming the structure of this thesis. Her commitment to academic excellence and resilience in the challenging times has been deeply inspiring, traits that have shaped my own scholarly approach.

I express my profound gratitude to my SRC committee members (Prof. C.S. Dey, Prof. Tapan K. Chaudhari and Prof. Ritu Kulshretha) for their constant support and invaluable insights for this project during these years. Their constructive criticism and thoughtful observation have honed my analytical viewpoints and hence, led to enhanced quality and clarity of this work.

I would like to extend my sincere gratitude to all the labs of Kusuma department for paving the way for me as I joined in the new lab with hopeful eyes. The sharing culture of Kusuma has allowed me to perform my experiments smoothly and opened the windows of possibilities through enriching academic conversations. Special mention for the members of MBM lab (Ms. Khushboo, Ms. Khadija, Ms. Shayeri, Ms. Himanshi, Ms. Shabih and Prof. Manoj Menon for generously providing space and equipment in the challenging times. Special mention to AKP and MB lab for letting me use their cold chamber for my protein studies. I would like to thank Mr. Manthan for his guidance for protein expression, Mr. Ashish for electrocompetent cell preparation, Ms. Akanksha for confocal imaging, Mr. Saurabh and Ms. Mamta for flow

cytometry. Their selfless candour and expertise (and also patience which I tested enormously by making them wait endlessly for freeing up the Geldoc) have been pivotal in data acquisition phase of this Ph.D. journey.

I would like to express my heartfelt appreciation for current and former AR lab colleagues- Dr. Rishi, Mr. Saurabh, Ms. Shayeri, Ms. Aditi, Mr. Farmaan, Ms. Sweta, Mr. Louis, Mr. Dev, Mr. Kavan, Mr. Chetan, Ms. Malvika, Ms. Mrittika, Ms. Mamta, Mr. Abhiraj and Ms. Nithila for their constant support, encouragement and playing a crucial part in my Ph.D. journey. I would also like to acknowledge the support from the SMARt lab members, Ms. Debashruti, Ms. Ayushma, Ms. Nikita, Mr. Himanshu, Ms. Adrija and Ms. Anjali for being the best neighbouring lab.

This beautiful and fulfilling journey wouldn't be complete without mentioning my support system-my friends. Our interaction began in the classroom during our formative years and got enriched all through these years. I would forever be grateful the members of Pina Colada (Ms. Prachi and Ms. Gagan) and SIPS (Ms. Priyanka, Mr. Sidharth and Mr. Ishu) for all your encouragement, support and contribution in my life and the project itself. Their companionship and understanding provided the balance and positivity amidst the demanding academic life. Additionally, I wish to acknowledge my long-time friends- Ms. Shweta, Mr. Vikas and Ms. Kirti whose once in a year conversations have been my source of comfort. Their friendship remains steadfast despite of long distance and rare meetups but is deeply appreciated.

The source of my motivation and positivity lies with my family for their endless love, encouragement, and belief in my dreams. Their belief in my potential has been both a wellspring of strength and a constant reminder of my purpose. I owe them more than words can express.

In the end, nothing would have been possible without the divine guidance, strength and perseverance from the Almighty throughout my journey. Every accomplishment in this endeavour reflects that grace.

Saadia Naseer
04/02/2026

ABSTRACT

Myeloproliferative neoplasm (MPN) is the disorder of hematopoietic stem cells and progenitor cells with various mutations implicated in its pathogenesis, including Janus kinase 2 (JAK2), thrombopoietin receptor (TpoR), and calreticulin (CALR) mutations. MPN patients harbouring frameshift CALR mutations exhibit megakaryocyte hyperplasia, thrombocytosis, with or without marrow fibrosis. Two of the most prevalent mutations implicated in 80% of CALR-mutated patients are CALR Type I (Δ52) and CALR Type II (ins5) mutations. All the CALR mutations are characterised by a unique C-terminal tail rich in positively charged amino acids and loss of KDEL ER retrieval sequence located in the exon 9 tail of the CALR gene. Mutant CALR binds to the N117 residue in the extracellular domain of the TpoR to dimerise the receptor inducing ligand-independent activation of TpoR. This renders the JAK2/STAT1/3/5 pathway active, promoting megakaryopoiesis, leading to MPN. The CALR mutants are efficient in driving ligand-independent activation of TpoR. Despite this, the difference between the two CALR mutants lie in the prognosis and prevalence. The Type I CALR harbouring patients have higher myelofibrosis cases, whereas younger age at presentation is seen in Type II CALR patients. This difference in patient outcome cannot be explained by the ability of CALR Type I and Type II mutants to activate TpoR. Furthermore, CALR mutations are not restricted to the myeloid lineage but penetrate the lymphoid lineage. This highlights the possible oncogenic capabilities of mutant CALR beyond TpoR and JAK-STAT pathway. Therefore, studying the oncogenic properties of mutant CALR independent of TpoR is imperative. Moreover, KDEL deletion enables the mutant CALR to secrete from the ER and is reported to be found in the Golgi (cis and trans), vesicles, cell membrane, and even in the nucleus. Thus, we hypothesised that mutant CALR might have gained new interacting partners in the various compartments. Hence, we cloned and expressed the recombinant CALR WT and mutant tail. The bead-bound recombinant CALR WT and mutant tails were used as the bait to perform a pulldown followed by mass spectrometry to identify their interacting partners. We identified 21 pulldown partners of the CALR mutant tail, out of which 8 were shared with CALR WT tail. Among the shared pulldown partners of CALR WT and mutant tail, we report ARHGAP21 (a GTPase activating protein (GAP) for CDC42 activity) and USP9X, (a deubiquitinase) interacted with CALR WT and Ins5 exclusively. We showed TGFBR3, co-receptor for TGF-β signalling, to interact with CALR WT/Δ52 /ins5 tail. TGFBR3, is the third receptor to interact with CALR mutants apart from the already reported and characterized

receptors TpoR and Transferrin receptor. Moreover, VPS13C and BORC5, which are involved in the lysosome movement and distribution, were found to interact with CALR WT and mutants. Interestingly, our results suggested low lysosomal count resulting in low mTOR colocalization on the lysosomes in CALR mutants (Δ52 and ins5) compared to the CALR WT. However, the difference between the two CALR mutants was apparent in terms of lysosomal distribution inside the cells. On one hand, CALR Δ52 exhibited majorly perinuclear clustering of lysosomes, whereas CALR ins5 showed peripheral localization of the lysosomes, leading to robust activation of mTOR (S2481) by CALR ins5 and diminished mTOR (S2481) activity by CALR Δ52. This differential activation of mTOR (S2481) by the CALR mutants disappears when supplemented with pyruvate-rich medium. Therefore, CALR mutations differentially dictate the lysosomal dynamics impacting their number and distribution by modulating mTOR signalling. Furthermore, we also report downmodulation of basal mTORC2-Akt S473 axis mediated through c-JUN in CALR Type I mutant. Type II CALR mutations rendered robust activation of the mTORC2-Akt S473 axis. Our data supports that Type I CALR mutation compromised mTORC2 assembly which was rescued either by overexpression of mTORC2 assembly subunits RICTOR and mSIN1 or by c-JUN knockdown. We report that CALR Δ52 occupies the promoter and distal enhancers of *jun*, hence, directly regulating c-JUN expression through chromatin immunoprecipitation. Finally, we observed implications of downregulated mTORC2-Akt S473 axis in terms of reduced glucose uptake and ATP levels in cells expressing Type I CALR mutations. This was reversed by c-JUN knockdown. Hence, c-JUN mediated mTORC2-Akt S473 axis interplay was found to be responsible for diminished glucose metabolism in CALR Type I mutant. In contrast, CALR Type II mutations enhanced the glucose uptake in the cells by activating basal mTORC2-Akt S473 signalling, demarking a crucial difference between the two CALR mutations.

सारांश

मायलोप्रोलिफेरेटिव नियोप्लाज़्म (Myeloproliferative Neoplasm, MPN) एक ऐसी विकृति है जो हीमाटोपोएटिक स्टेम कोशिकाओं (Hematopoietic stem cells) और प्रोजेनिटर कोशिकाओं (progenitor cells) में उत्पन्न होती है, जिसमें कई प्रकार के म्यूटेशन इसके रोगजनन (pathogenesis) से जुड़े हुए हैं – जैसे Janus kinase 2 (JAK2), थ्रॉम्बोपोइटिन रिसेप्टर (TpoR), और कैलरेटिक्युलिन (CALR) म्यूटेशन। CALR फ्रेमशिफ्ट म्यूटेशन वाले MPN रोगियों में मेगाकारियोसाइट हाइपरप्लासिया, थ्रोम्बोसाइटोसिस, और कभी-कभी बोन मैरो फाइब्रोसिस देखने को मिलता है। CALR Type I (Δ52) और CALR Type II (ins5) दो प्रमुख म्यूटेशन हैं, जो लगभग 80% CALR-म्यूटेटेड रोगियों में पाए जाते हैं। सभी CALR म्यूटेशन की एक सामान्य विशेषता यह है कि इनमें एक पॉज़िटिव चार्ज वाली C-टर्मिनल टेल होती है और KDEL ER रिट्रीवल सीक्वेंस (जो CALR जीन के एक्सॉन 9 में स्थित होता है) का लोप हो जाता है। म्यूटेंट CALR, TpoR के बाह्य डोमेन (extracellular domain) के N117 अवशेष (residue) से बंधता है, जिससे रिसेप्टर का लिगैंड-स्वतंत्र डायमराइजेशन होता है। यह JAK2/STAT1/3/5 सिग्नलिंग पाथवे को सक्रिय कर देता है, जिससे मेगाकारियोपोएसिस बढ़ती है और परिणामस्वरूप MPN उत्पन्न होता है। हालाँकि दोनों CALR म्यूटेंट्स (Type I और Type II) TpoR को लिगैंड-स्वतंत्र रूप से सक्रिय करने में सक्षम हैं, लेकिन इनके बीच प्रग्नोसिस (रोग-पूर्वानुमान) और प्रचलन (prevalence) में अंतर पाया जाता है। Type I CALR म्यूटेशन वाले रोगियों में मायलोफाइब्रोसिस की घटनाएँ अधिक होती हैं, जबकि Type II CALR म्यूटेशन वाले रोगियों में रोग का प्रकट होना अपेक्षाकृत कम आयु में देखा जाता है। यह अंतर केवल TpoR सक्रियता से नहीं समझाया जा सकता। CALR म्यूटेशन केवल मायलोइड वंश (myeloid lineage) तक सीमित नहीं हैं, बल्कि ये लिम्फॉइड वंश (lymphoid lineage) में भी प्रवेश करते हैं। इससे संकेत मिलता है कि म्यूटेंट CALR की ऑन्कोजेनिक (कैंसर उत्प्रेरक) क्षमता TpoR और JAK-STAT पथ से परे भी हो सकती है। इसलिए, TpoR-स्वतंत्र रूप से CALR म्यूटेंट के ऑन्कोजेनिक गुणों का अध्ययन आवश्यक है। इसके अतिरिक्त, KDEL अनुक्रम के हटने से म्यूटेंट CALR का ER से स्राव (secretion) संभव हो जाता है, और इसे Golgi (cis एवं trans), वेसिकल्स, कोशिका झिल्ली, यहाँ तक कि नाभिक (nucleus) में भी पाया गया है। अतः यह परिकल्पना की गई कि म्यूटेंट CALR ने विभिन्न कोशिकीय स्थानों में नए इंटरैक्टिंग पार्टनर प्राप्त कर लिए होंगे। इस उद्देश्य से, हमने CALR WT (वाइल्ड टाइप) और म्यूटेंट टेल को क्लोन एवं एक्सप्रेस किया। बीड-बाउंड रिकॉम्बिनेंट CALR WT और म्यूटेंट टेल का उपयोग पुलडाउन एस्से में किया गया, जिसके बाद मास स्पेक्ट्रोमेट्री द्वारा इनके इंटरैक्टिंग पार्टनर्स की पहचान की गई।हमने CALR

म्यूटेंट टेल के 21 पुलडाउन पार्टनर्स पहचाने, जिनमें से 8 पार्टनर्स CALR WT टेल के साथ साझा थे। साझा पार्टनर्स में, ARHGAP21 (एक GTPase एक्टिवेटिंग प्रोटीन जो CDC42 गतिविधि को नियंत्रित करता है) और USP9X (एक डीयूबिक्विटिनेज़) को विशेष रूप से CALR WT और ins5 के साथ इंटरैक्ट करते पाया गया। हमने यह भी दर्शाया कि TGFBR3, जो TGF-β सिग्नलिंग का को-रिसेप्टर है, CALR WT, Δ52 और ins5 टेल के साथ इंटरैक्ट करता है। यह तीसरा रिसेप्टर है जो CALR म्यूटेंट्स से इंटरैक्ट करता है, पहले दो पहले से ज्ञात रिसेप्टर TpoR और Transferrin receptor हैं। इसके अलावा, VPS13C और BORC5, जो लाइसोसोम की गति और वितरण में शामिल हैं, CALR WT और म्यूटेंट्स दोनों के साथ इंटरैक्ट करते पाए गए। दिलचस्प रूप से, हमारे परिणामों से यह पता चला कि CALR म्यूटेंट्स (Δ52 और ins5) में लाइसोसोम की संख्या कम थी और mTOR का लाइसोसोम पर कोलोकलाइज़ेशन भी घटा हुआ था। हालांकि, दोनों म्यूटेंट्स के बीच लाइसोसोम वितरण में स्पष्ट अंतर पाया गया —

- CALR Δ52 में लाइसोसोम मुख्यतः परिन्यूक्लियर क्लस्टरिंग (नाभिक के पास समूहित) दिखे,
- जबकि CALR ins5 में लाइसोसोम पेरिफेरल (किनारों पर) स्थित थे, जिससे mTOR (S2481) की मजबूत सक्रियता हुई, जबकि CALR Δ52 में यह सक्रियता घटित रही।

यह अंतर पाइरुवेट-समृद्ध माध्यम देने पर समाप्त हो गया, जिससे यह निष्कर्ष निकला कि CALR म्यूटेशन mTOR सिग्नलिंग को प्रभावित करते हुए लाइसोसोम की संख्या और वितरण को नियंत्रित करते हैं। हमने यह भी पाया कि CALR Type I म्यूटेंट में c-JUN द्वारा मध्यस्थित mTORC2-Akt S473 अक्ष का डाउनमॉड्यूलेशन होता है, जबकि Type II CALR म्यूटेशन इस अक्ष को मजबूती से सक्रिय करते हैं। हमारे आंकड़े बताते हैं कि Type I CALR म्यूटेशन में mTORC2 कॉम्प्लेक्स का असेंबली बाधित होता है, जिसे या तो RICTOR और mSIN1 की अधिक अभिव्यक्ति या c-JUN नॉकडाउन द्वारा पुनर्स्थापित (rescue) किया जा सकता है। हमने दर्शाया कि CALR Δ52, *jun* जीन के प्रोमोटर और डिस्टल एन्हांसर क्षेत्रों से बंधता है और इस प्रकार सीधे c-JUN अभिव्यक्ति को नियंत्रित करता है। अंततः हमने पाया कि Type I CALR म्यूटेशन में mTORC2-Akt S473 अक्ष की कमी से ग्लूकोज़ अवशोषण और ATP स्तर में कमी आती है, जिसे c-JUN नॉकडाउन से पुनः बहाल किया जा सकता है। इसके विपरीत, Type II CALR म्यूटेशन mTORC2-Akt S473 सिग्नलिंग को सक्रिय करके ग्लूकोज़ अवशोषण बढ़ाते हैं, जिससे दोनों CALR म्यूटेशन के बीच एक महत्वपूर्ण जैविक अंतर स्थापित होता है।

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ABBREVIATIONS AND SYMBOLS

β	Beta
µ	Micron
µL	Microlitre
µg	Microgram
Δ	Deletion
°C	Degree Celsius
4EBP1	Eukaryotic translation initiation factor 4E-binding protein-1
AKGDH	α-ketoglutarate dehydrogenase
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ARHGAP21	Rho GTPase activating protein 21
ATP	Adenosine triphosphate
BORC	BLOC-1 Related Complex
CALR	Calreticulin
CLP	Common Lymphoid Progenitors
CMP	Common Myeloid Progenitor
DEPTOR	DEP domain-containing mTOR-interacting protein
EpoR	Erythropoietin receptor
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ERK	Extracellular signal-regulated kinase
ET	Essential thrombocythemia
FOXO	Forkhead box class O
G-CSF-R	Granulocyte colony-stimulating factor receptor
GM-CSF-R	Granulocyte macrophage colony-stimulating factor receptor
GSK3β	Glycogen synthase kinase 3 beta
GST	Glutathione S-Transferase
GTP	Guanosine-5'-triphosphate
HACBP	High-affinity calcium-binding protein
HSC	Hematopoietic stem cell
IDH	Isocitrate dehydrogenase
IL	Interleukin

JAK	Janus Kinase
JNK	c-Jun N-terminal kinases
LAMP1	Lysosomal-associated membrane protein 1
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
Mk	Megakayocyte
mLST8	mTOR-associated protein, LST8 homolog
MPN	Myeloproliferative neoplasms
mSIN1	Mammalian stress-activated MAP kinase-interacting protein-1
mTORC1	Mechanistic target of rapamycin complex 1
mTORC2	Mechanistic target of rapamycin complex 2
NDRG1	N-Myc Downstream Regulated 1
PDH	Pyruvate dehydrogenase
PDK1	Phosphoinositide-dependent kinase 1
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5 bisphosphate
PIP3	Phosphatidylinositol (3,4,5) trisphosphate
PMF	Primary myelofibrosis
PRAS40	40-kDa proline-rich Akt substrate
PV	Polycythemia vera
RAPTOR	Regulatory-associated protein of mTOR
RBC	Red blood cells
RHEB	Ras homolog enriched in brain
RICTOR	Rapamycin-insensitive companion of mammalian target of rapamycin
RTK	Receptor tyrosine kinase
RTK	Receptor tyrosine kinase
S6K1	Ribosomal protein S6 kinase beta-1
Ser	Serine
SGK	Serum and glucocorticoid-regulated kinase
SOCE	Store-operated calcium entry
STAT	Signal transducer and activator of transcription
TFE3	Transcription factor E3
TFEB	Transcription factor EB

TGFβ3	Transforming growth factor beta 3
Thr	Threonine
TPO	Thrombopoietin
TpoR	Thrombopoietin receptor
TSC	Tuberous sclerosis complex
ULK1	Unc-51 like autophagy activating kinase 1
UPR	Unfolded protein response
USP9X	Ubiquitin specific peptidase 9 X-linked
VPS13C	Vacuolar protein sorting 13 homolog C
WHO	World Health Organization
WT	Wildtype