

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

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INDIAN INSTITUTE OF TECHNOLOGY DELHI**

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MYELOPROLIFERATIVE NEOPLASM**

*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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## 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 ( $\Delta 52$ ) 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- $\beta$  signalling, to interact with CALR WT/ $\Delta 52$  /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 ( $\Delta 52$  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  $\Delta 52$  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  $\Delta 52$ . 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  $\Delta 52$  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), थ्रोम्बोपोइटिन 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 ( $\Delta 52$ ) 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। म्यूटेन्ट 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 म्यूटेन्ट्स (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 म्यूटेन्ट CALR की ऑन्कोजेनिक (कैंसर उत्प्रेरक) क्षमता TpoR और JAK-STAT पथ से परे भी हो सकती है। इसलिए, TpoR-स्वतंत्र रूप से CALR म्यूटेन्ट के ऑन्कोजेनिक गुणों का अध्ययन आवश्यक है। इसके अतिरिक्त, KDEL अनुक्रम के हटने से म्यूटेन्ट 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 म्यूटेन्ट CALR ने विभिन्न कोशिकीय स्थानों में नए इंटरैक्टिंग पार्टनर प्राप्त कर लिए होंगे। इस उद्देश्य से, हमने CALR WT (वाइल्ड टाइप) और म्यूटेन्ट टेल को क्लोन एवं एक्सप्रेस किया। बीड-बाउंड रिकॉम्बिनेन्ट CALR WT और म्यूटेन्ट टेल का उपयोग पुलडाउन एस्से में किया गया, जिसके बाद मास स्पेक्ट्रोमेट्री द्वारा इनके इंटरैक्टिंग पार्टनर्स की पहचान की गई। हमने CALR

म्यूटेन्ट 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 डीयूबिकिटिनेज़) को विशेष रूप से CALR WT और ins5 के साथ इंटरैक्ट करते पाया गया। हमने यह भी दर्शाया कि TGFBR3, जो TGF- $\beta$  सिग्नलिंग का को-रिसेप्टर है, CALR WT,  $\Delta$ 52 और ins5 टेल के साथ इंटरैक्ट करता है। यह तीसरा रिसेप्टर है जो CALR म्यूटेन्ट्स 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 म्यूटेन्ट्स दोनों के साथ इंटरैक्ट करते पाए गए। दिलचस्प रूप से, हमारे परिणामों से यह पता चला कि CALR म्यूटेन्ट्स ( $\Delta$ 52 और ins5) में लाइसोसोम की संख्या कम थी और mTOR का लाइसोसोम पर कोलोकलाइज़ेशन भी घटा हुआ था। हालांकि, दोनों म्यूटेन्ट्स के बीच लाइसोसोम वितरण में स्पष्ट अंतर पाया गया —

- CALR  $\Delta$ 52 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  $\Delta$ 52 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 म्यूटेन्ट 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  $\Delta$ 52, *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

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

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

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