Molecular mechanism of MPN development by mutant calreticulin

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COI disclosure

Name of author: Norio Komatsu

I currently have, or I have had in the past two years, an affiliation or financial interest with business corporation(s):

(3) Others: No
WHO classification of MPN (2017)

Myeloproliferative neoplasms (MPN)
- Chronic myeloid leukemia (CML), BCR-ABL1+
- Chronic neutrophilic leukemia (CNL)
- Polycythemia vera (PV)
- Primary myelofibrosis (PMF)
  - prefibrotic/early stage
  - overt fibrotic stage
- Essential thrombocythemia (ET)
- Chronic eosinophilic leukemia, NOS
- MPN, unclassifiable

MPNs arise from the hematopoietic stem cell compartment

JAK2V617F, MPLW515x, CALR, BCR/ABL1
### WHO classification of MPN (2017)

**-Driver mutations in MPNs-**

<table>
<thead>
<tr>
<th>MPNs</th>
<th>Driver mutations</th>
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<tbody>
<tr>
<td>Chronic myelogenous leukemia</td>
<td><em>BCR-ABL1</em></td>
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<tr>
<td>Chronic neutrophilic leukemia</td>
<td><em>CSF3R</em> mutations</td>
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<tr>
<td>Polycythemia vera</td>
<td><em>JAK2V617F</em> • <em>JAK2exon12</em></td>
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<td>Essential thrombocythemia</td>
<td><em>JAK2V617F</em> • *MPLW515K/L • <em>CALRmut</em></td>
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<tr>
<td>Primary myelofibrosis (prefibrotic/overt)</td>
<td><em>JAK2V617F</em> • *MPLW515K/L • <em>CALRmut</em></td>
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<td>Chronic eosinophilic leukemia, NOS</td>
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<td>MPN, unclassifiable</td>
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</table>
Constitutive activation of cytokine receptors by mutant proteins in ET and PMF patients

**Normal**

- Normal cytokine receptors
- TPO
- MPL
- JAK2
- ERK1/2
- AKT
- STATs

**ET and PMF**

- JAK2 V617F (ca. 50%)
- MPL W515K/L (ca. 5%)
- CALRmut (ca. 25%)

Today’s talk

• What is calreticulin?

• How does mutant calreticulin contribute to MPN development?

• What is the molecular mechanism by which mutant calreticulin activates the thrombopoietin receptor MPL?
What is calreticulin (CALR)?
Calreticulin

1. was first identified as a **Ca\(^{2+}\)-binding protein** of the muscle sarcoplasmic reticulum in 1974

2. also known as calregulin, CRP55, CaBP3, calsequestrin-like protein, and endoplasmic reticulum resident protein 60 (ERp60)

3. is located in storage compartments associated with the **endoplasmic reticulum**

4. functions as a **molecular chaperone** to assist in the folding and subunit assembly of the majority of Asn-linked glycoproteins that pass through the endoplasmic reticulum

5. acts as an **important modulator of the regulation of gene transcription** by nuclear hormone receptors for glucocorticoid, androgen and retinoic acid

6. is engaged in **cellular invasion** and **metastasis** through the induction of cell migration
Structure and function of calreticulin (CALR)

CALR is a 417 amino acid, 46 kDa, **multi-functional protein** that primarily localizes within the lumen of the **endoplasmic reticulum**.
Somatic Mutations of Calreticulin in Myeloproliferative Neoplasms


the unique features of CALR mutations

Somatic CALR Mutations in Myeloproliferative Neoplasms with Nonmutated JAK2


Structure of calreticulin (CALR)

Endoplasmic reticulum (ER)-localized molecular chaperon.

Frameshift mutation is always +2 but not +1.

CALR mutations are gain of function mutations.
Mutant CALR gene is located in exon 9


ER-retention signal
mostly frequently observed forms

ET (n = 50)
PMF (n = 11)
How does mutant calreticulin contribute to MPN development?
Activation of the thrombopoietin receptor by mutant calreticulin in CALR-mutant myeloproliferative neoplasms

Marito Araki,1,∞ Yinjie Yang,2,∞ Nami Masubuchi,2,3 Yumi Hironaka,2 Hiraku Takei,2 Soji Morishita,1 Yoshihisa Mizukami,2,4 Shin Kan,2,5 Shuichi Shirane,2 Yoko Edahiro,2 Yoshitaka Sunami,2 Akimichi Ohsaka,1 and Norio Komatsu2


Calreticulin mutants in mice induce an MPL-dependent thrombocytosis with frequent progression to myelofibrosis

Caroline Marty,1,3 Christian Pecquet,4,5 Harini Nivarthi,6 Mira El-Khoury,1,3 Ilyas Chachoua,4,5 Micheline Tulliez,1,3 Jean-Luc Villeval,1,3 Hana Raslova,1,3 Robert Kralovics,6 Stefan N. Constantinescu,4,5 Isabelle Plo,1,3,∞ and William Vainchenker1,3,∞


Thrombopoietin receptor activation by myeloproliferative neoplasm associated calreticulin mutants

Ilyas Chachoua,1,2,∞ Christian Pecquet,1,2,∞ Mira El-Khoury,3,5,∞ Harini Nivarthi,6 Roxana-Irina Albu,1,2 Caroline Marty,3,5 Vitalina Gryshkova,1,2 Jean-Philippe Defour,1,2 Gaëlle Vertenoeil,1,2 Anna Ngo,7 Ann Koay,7 Hana Raslova,3,5 Pierre J. Courtoty,2 Meng Ling Choong,7 Isabelle Plo,3,5 William Vainchenker,3,5 Robert Kralovics,6 and Stefan N. Constantinescu1,2

UT-7 family

Komatsu N. Blood 87: 4552-4560, 1996
Komatsu N. Blood 89: 4021-4033, 1997
UT-7

established from the BM cells of a patient with M7 phenotypic characteristics of megakaryocytic lineage dependent on IL-3/GM-CSF/EPO for growth and survival

Ideal model for analysis of cytokine signal transduction

bone marrow  UT-7

UT-7 family

IL-3
GM-CSF
EPO

UT-7

GM-CSF

UT-7/GM

UT-7/EPO

EPO

Erythroid cells

GM-CSF

UT-7/TPO

TPO

Megakaryocytes

Komatsu N. Blood 87: 4552-4560, 1996
Komatsu N. Blood 89: 4021-4033, 1997
Functional TPO receptors are expressed in UT-7/TPO but **NOT** in UT-7/EPO cells.


Komatsu N. Blood 82: 456-64, 1993
Komatsu N. Blood 87: 4552-60, 1996
Komatsu N. Blood 89: 4021-33, 1997
CALR mutants induce autonomous growth of UT-7/TPO cells but **NOT** of UT-7/EPO cells

**UT-7/TPO**

- **TPO-free**
  - vector
  - CALR WT
  - CALR Del52
  - CALR Ins5

**UT-7/EPO**

- **EPO-free**
  - vector
  - CALR WT
  - CALR Del52
  - CALR Ins5

retrovirus vector system

Araki M, Yang Y and Komatsu N. Blood 2016: 127; 1307-16
MPL is required for the mutant CALR-dependent cell growth in UT-7/TPO cells

UT-7/TPO/CALR Del52 shRNA virus infection

0hr  24  48  72
Viability RNA Viability

MPL mRNA

MPL/GAPDH mRNA

Cell viability

Viability (%)

0  20  40  60  80  100

24hr  72hr

Non-target sh1 sh2 sh3

Non-target sh1 sh2 sh3

lentiviral vectors Araki M, Yang Y and Komatsu N. Blood 2016: 127; 1307-16
Mutant CALR protein binds to MPL

Do c-MPL and mutant CALR proteins physically interact?

Araki M, Yang Y and Komatsu N. Blood 2016: 127; 1307-16
Mutant CALR interacts with JAK2 via MPL

**co-IP assay**

Do JAK2 and mutant CALR proteins physically interact?

Araki M, Yang Y and Komatsu N. Blood 2016: 127; 1307-16
Mutant CALR activates the JAK2 pathway via MPL activation to drive oncogenic transformation

Robust phosphorylation of ERK1/2 and STAT5

Araki M, Yang Y and Komatsu N. Blood 2016: 127; 1307-16
Short summary

- Mutant CALR requires thrombopoietin receptor MPL for oncogenic transformation of hematopoietic cells.
- Mutant CALR interacts with JAK2 via MPL.
- Mutant CALR promotes cytokine-independent growth by activating the JAK2 pathway.
Which domain of mutant CALR binds to c-MPL?
Identification of MPL-binding domain of mutant CALR

**co-IP assay**

Araki M, Yang Y and Komatsu N. Blood 2016: 127; 1307-16
The mutant CALR-specific sequence is required for interaction with c-MPL

Araki M, Yang Y and Komatsu N. Blood 2016: 127; 1307-16
Identification of MPL binding domain in mutant CALR

Autonomous growth

- - + -

Binding activity of Ins5 to MPL

Araki M, Yang Y and Komatsu N. Blood 2016: 127; 1307-16
Identification of MPL binding domain in mutant CALR

N-domain of mutant CALR is the bona fide MPL binding domain.

Araki M, Yang Y and Komatsu N. Blood 2016: 127; 1307-16
What is the function of the mutant-specific C-terminal domain of mutant CALR?
Mutant-specific C terminal domain is required for mutant CALR to interact with MPL via the N-domain

When the P-domain is present and the mutant-specific C terminal domain is missing, all CALR mutants carrying the N-domain failed to interact with c-MPL

N-domain could bind to c-MPL in either the presence of the mutant specific C terminal domain or the absence of P-domain

Araki M, Yang Y and Komatsu N. Blood 2016: 127; 1307-16
A hypothetical model for mutant CALR-specific binding to MPL

P-domain functions as a blockade of binding of the N-domain to MPL in wild-type CALR, whereas this function of the P-domain is blocked by the mutant-specific C-terminal domain of mutant CALR.

Araki M, Yang Y and Komatsu N. Blood 2016: 127; 1307-16
What is the molecular mechanism by which mutant calreticulin activates thrombopoietin receptor MPL?
Mutant, but not wild type, CALR proteins may form homomultimeric complexes.

**Fractionation by density gradient centrifugation**

WT Del52 Ins5

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**UT-7/TPO**

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Intermolecular interactions occur among mutant CALR proteins

A co-IP assay on the extracts from HEK293T cells harboring Del52, Ins5, or wild type CALR, with either a V5 or a FLAG epitope tag.
Does the mutant specific C terminal domain mediate intermolecular interaction between mutant CALR?
Mutant-specific C terminal domain mediates intermolecular interaction between mutant CALR

Flag-tagged CALR Ins5ΔN/P (prey) interacts with V5-tagged CALR Ins5ΔN/P (bait) in vitro. *IgG light chain

Mutant-specific C terminal domain is required for the intermolecular interaction between mutant CALR proteins.

A series of truncated FLAG-tagged Ins5

WT

Flag-tagged CALR (prey)

V5-tagged CALR Ins5ΔN/P (bait)

Mutant-specific C terminal domain is required for intermolecular interaction between mutant CALR

CALR WT

CALR\textsuperscript{mut}

CALR\textsuperscript{mut}
Is mutant CALR multimerization essential for MPL activation?”
Scheme of competition assay

Competitor
(Lacking an MPL-binding domain)

intermolecular interactions within CALR molecules

Homomultimerization of mutant CALR is required for the MPL binding and activation

MPL-binding assay

STAT5 reporter assay

A new model for the constitutive activation of cytokine receptor signaling by a mutant chaperone

The mutant CALR serves as a fake ligand of MPL by forming a homo-multimeric complex and constitutively activates MPL and its downstream signaling molecules, leading to MPN development.

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