ORIGINAL RESEARCH

# Alterations of Signaling Pathways in Essential Thrombocythemia with Calreticulin Mutation

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**Purpose:** Though mutations of the calreticulin (*CALR*) gene have been identified in essential thrombocythemia patients, the detailed mechanisms for *CALR* mutations have not been completely clarified. Our study is aimed at characterizing alteration of protein expression in ET patients with mutated $CALR^{del52}$ and further recognizing possible involvement of signaling pathways associated with *CALR* mutations.

**Patients and Methods:** Protein pathway array was performed to analyze the expression levels of proteins involved in various signaling pathways in peripheral blood neutrophils from 18 ET patients with mutated $CALR^{del52}$, 20 ET patients with $JAK2^{V617F}$ mutation and 20 controls.

**Results:** We found 20 proteins differentially expressed in ET patients with mutated $CALR^{del52}$ compared with healthy controls. These proteins were associated with molecular mechanisms of cancer in ingenuity pathways analysis (IPA) network. We identified top ten canonical pathways which including apoptotic pathways and cellular cytokine pathways might participate in pathogenesis of ET with mutated $CALR^{del52}$. Additionally, there were 8 proteins found to be dysregulated differently between ET patients with mutated $CALR^{del52}$ and those with $JAK2^{V617F}$ mutation. These proteins might be related to the unique signaling pathways activated by $CALR^{del52}$ mutation which were different to JAK/STATs pathway by $JAK2^{V617F}$ mutation.

**Conclusion:** Our study demonstrated that numerous alterations of signaling proteins and pathways in ET patients with mutated $CALR^{del52}$. These findings could help to gain insights into the pathological mechanisms of ET.

**Keywords:** essential thrombocytosis, $CALR^{del52}$ mutation, protein pathway array, signaling proteins

## Introduction

As a subcategory of myeloproliferative neoplasms (MPNs), essential thrombocytosis (ET) is characterized by an elevated platelets number, enhanced megakaryocyte count in bone marrow and increased risk for vascular events such as thrombosis or bleeding.[1] More than 85% of patients with ET carry driver gene mutations, including mutation of janus kinase 2 gene ($JAK2^{V617F}$), thrombopoietin receptor (*MPL*) genes and calreticulin (*CALR*) genes.[2–8] It's reported that JAK2V617F is mutated in nearly 50–60% of ET,[2–4] and MPL mutation is detected in 5% of cases.[5,6] The $JAK2^{V617F}$ mutation causes constitutive, independent activation of downstream JAK/STAT signaling and eventually disruption of gene transcription.[2–4] Furthermore, $JAK2^{V617F}$ mutation alone has been shown to result in MPN phenotypes in vivo and in vitro models.[9,10] In 2013, mutated *CALR* genes

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were found in 50% of *JAK2/MPL* negative ET.[7,8] Recent datas have shown that mutated *CALR* directly binds MPL and activates its downstream JAK/STAT signaling.[11–13] However, the detailed mechanisms for *CALR*(*CALR*[del52]) mutation has not been fully unraveled.

In this study, we undertook a comprehensive screening of signaling proteins using Protein Pathway Array (PPA)[14] for ET patients with *CALR*[del52] mutation to characterize the dysregulation of signaling protein expression and explored the potential involvement of signaling pathways related to *CALR*[del52] mutation. PPA is a proteomic method of analyzing alterations in intracellular protein expression in multiple signaling pathways, including apoptosis, cell proliferation and differentiation, invasion, cell cycle regulation, DNA repair and angiogenesis.[14–16] Using this method, several signaling proteins differentially expressed between *CALR*[del52] and *JAK2*[V617F] mutated ET and healthy controls were identified.

## Materials and Methods
### Patients and Samples
Peripheral blood samples were collected from patients with newly diagnosed ET patients at Xuan Wu Hospital, Capital Medical University January 2010 and April 2011. Granulocytes were isolated using the method previously reported.[17] Eighteen ET patients with mutated *CALR*[del52] were included in this study. In addition, 20 *JAK2*[V617F] mutation ET patients were included for comparison. The diagnosis of ET was established according to 2008 revision of WHO classification of myeloid neoplasms and acute leukemia.[18] Control samples (n=20) were collected from sex and age matched healthy volunteers. The study was approved by the ethics committee of Xuan Wu Hospital. All participants signed an informed consent form.

### Protein Pathway Array
Proteins were extracted from neutrophils of each sample and then 300 μg extracted protein was loaded in one well across the entire width of the gel. Furthermore, the separated proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad, Hercules, USA), which was then blocked using blocking buffer (3% BSA in 1×TBST containing 20mM Tris-HCL,100Mm NaCL and 0.1%Tween-20) at room temperature for 1 h. Next, the membrane was clamped with a Western blotting manifold in order to isolate 20 channels across the membrane (Mini-PROTEAN II Multiscreen apparatus, Bio-Rad Laboratories, Inc.). Three or two antibodies were added to each channel and the first set of 35

antibodies were allowed to hybridize proteins at 4°C overnight as described previously.[15] The blot was hybridized for 45 min with horseradish peroxidase conjugated secondary antibodies at room temperature. Then the membranes were developed with chemiluminescence substrate, and signals were caught by the ChemiDoc XRS system (Bio-Rad Laboratories, Inc.). The same membrane was stripped off and then used to detect a second set of 33 proteins-specific antibodies as described above. Total 128 antibodies were used for each membrane (Table 1). Proteins quantification was analyzed using densitometric scanning (Quantity One software; Bio-Rad Laboratories, Inc.) and normalized by internal standards.

## Signaling Pathway and Network Analysis
Ingenuity Pathway Analysis (IPA) (http://www.ingenuity.com) was used for functional pathway and network analysis.[14] Differentially expressed proteins identified by PPA were input into the IPA software and mapped to their corresponding gene objects in the Ingenuity Knowledge Base. In canonical pathways analyses, IPA was performed to determine which functional pathways were significantly related to

**Table 1** List of Antibodies Included in the Protein Pathway Array

| |
|---|
| **Cell signaling:** Akt, Axin, β-catenin, Calretinin, cPKCα, Erk1/2, EGFR, Endoglin, ERβ, ERα, FGF-8, H-Ras, IL-1β, IL-3Rα, JAK2, NEP, PTEN, Rab 7, Raf-B, Rap1, SOCS1, SOCS3, Wnt-1, CREB, Flt-3/Flk-2, Her2, Hsp90, IGFBP5, Jagged1, K-Ras, KAI1, p38α/β, Maspin, nm23-H1/2/3, PERK, patched |
| **Cell cycle:** Cdk2, Cdk4, Cdk6, Cdc25C, Cyclin B1, Cyclin D1, Cyclin E, Cdc42, p27, 14-3-3β, Cdc2p34, Cdc25B, CHK1, MDM2, Aurora A/AIK |
| **Transcription factor:** Stat3, Stat5, eIF4B, WT1, PDEF, Pax-2, ASCL1, FKHR, HDAC1, SLUG, SRC-1, HES1, p63, TTF-1, Nothc4, E2F-1, Stat1, NFATc1, TCF-1, E2A, ATF-1, Nkx-3.1, HMG-1, |
| **Apoptosis/Autophagy:** Bax, BID, Bak, NFκB p50, Bcl-6, Bcl-xL, RIP, c-IAP2, p53, XIAP, NFκB p52, NFκB p65, TNFα, Survivin, c-Flip, BECN1, Bad, Bcl-2 |
| **Angionesis:** EPO, HIF-1α, HIF-2α, HIF-3α, TGF-β, VEGF |
| **DNA repair:** TDP1, TFIIH p89, ERCC1, PCNA |
| **Epithelial-to-mesenchymal transition/Adhesion:** E-cadherin, N-cadherin, Mesothelin, PSM, L-Selectin, Ep-CAM, ICAM-1, VCAM-1, Vimentin, OPN, E-Selectin, HCAM, CD33 |
| **Others:** Cytokeratin 5, Cytokeratin 18, FAH, FTα, uPA, Factor XIII B, uPAR, PSCA, Cytokeratin 19, Keratin 10, Glutamine Synthetase, DRG1, Eg5 |

**Notes**: Underlines indicate detectable expression in samples from ET patients or healthy controls.

the input gene set through a Fisher's exact test. Furthermore, IPA identified local networks that were remarkably enriched for the input genes by computational algorithms.

## Statistical Analysis

Significant Analysis of Microarray (SAM) tool (http://www-stat.stanford.edu/~tibs/SAM/) was adopted to identify the proteins with differential expression between different groups. Fisher's Exact Test and Student $t$-Test were applied to compare laboratory findings and categorical variables. Statistical analyses were conducted with the software SPSS version 17.0 (SPSS Inc, Chicago, IL, USA) software. The $q$-value and $p$-value of less than 0.05 were considered as statistically significant.

## Results

### Patient-s' Clinicohematological Characteristics

The clinicopathological characteristics of ET patients are summarized in Table 2. The median age of patients with $CALR^{del52}$ and $JAK2^{V617F}$ mutation was 48.4±14.1 years (range, 25 and 74), and 58.4±12.9 years (range, 34–78), respectively. $CALR^{del52}$ mutated patients were younger than JAK2$^{V617F}$ mutated ones (P=0.029), consistent with previous reports.[19] Compared with the JAK2$^{V617F}$ mutated group (40%), $CALR^{del52}$ mutation was found more frequent in males (56%). Patients with a $CALR^{del52}$ mutation had lower white blood cell count (median of $7.1×10^9$/L versus 10.1 $×10^9$/L; P=0.007), higher platelet counts (median of $883×10^9$/L versus

$694 ×10^9$/L; P=0.02), and appeared to have more frequent thrombocytosis above $1000 × 10^9$/L (44% vs 20%) ($P$ =0.164). Median hemoglobin value was 131 g/L and did not differ from $JAK2^{V617F}$ mutated group ($P$ = 0.073) (Table 2).

## Identification of Differentially Expressed Proteins in ET with $CALR^{del52}$ Mutation

Of 128 proteins examined, 71 proteins were detected in all 38 samples. We found 20 proteins dysregulated in ET patients with $CALR^{del52}$ mutation compared with those in healthy controls (q<0.1). Among them, 13 proteins including PTEN, Erk1/2, Rap1, Cdc42, Raf-B, Axin, eIF4B, c-IAP2, NFκB p50 Bcl-Xl, CyclinD1, TGF-β, and SRC-1 were overexpressed, while 7 proteins including TDP1, ERβ, E-cadherin, cPKCα, Cdc2, Survivin and p27 were downregulated. These proteins were associated with molecular mechanisms of cancer in IPA network (Figure 1).

## Signaling Pathways Altered in ET with $CALR^{del52}$ Mutation

To determine the functional pathways most affected by differentially expressed proteins, these 20 proteins were uploaded into IPA for functional annotation and pathway analysis. We identified top 10 canonical pathways which included Cyclins and cell cycle regulation, IL-12 signaling and production in Macrophages, HGF signaling, Protein kinase A signaling, ILK signaling, Regulation of the epithelial-mesenchymal transition pathway, PI3K-AKT signaling, IL-8 signaling, Apoptosis signaling and PTEN signaling (Figure 2).

**Table 2** Main Features of Patients with ET

| | No. of Patients with CALR<sup>del52</sup> (n=18) | No. of Patients with JAK2V617F (n=20) |
|---|---|---|
| Gender;n(%)<br>Male<br>Female | <br>10(56)<br>8(44) | <br>8(40)<br>12(60) |
| Age in years; medina (range) | 51(25–74) | 60(33–78) |
| WBC, ×10⁹/L;median(range)<br>  >11×10⁹/L;n(%) | 7.1(5.1–12.4)<br>2(11) | 10.1(5.78–15.68)<br>7(35) |
| Hemoglobin, g/L;median(range) | 131(117–164) | 140(122–154) |
| Platelet, ×10⁹/L;median(range)<br>  >1000×10⁹/L;n(%)<br>Splenomegaly at diagnosis;n(%) | 883(560–1793)<br>8(44)<br>5(28) | 694(461–971)<br>4(20)<br>7(35) |
| Vascular risk factors; n(%)* | 4(22) | 7(35) |
| History of thrombosis; n(%) | 3(17) | 7(35) |

**Notes**: *Vascular risk factors include Hypertension, Diabetes mellitus, and Hyperlipidemia.
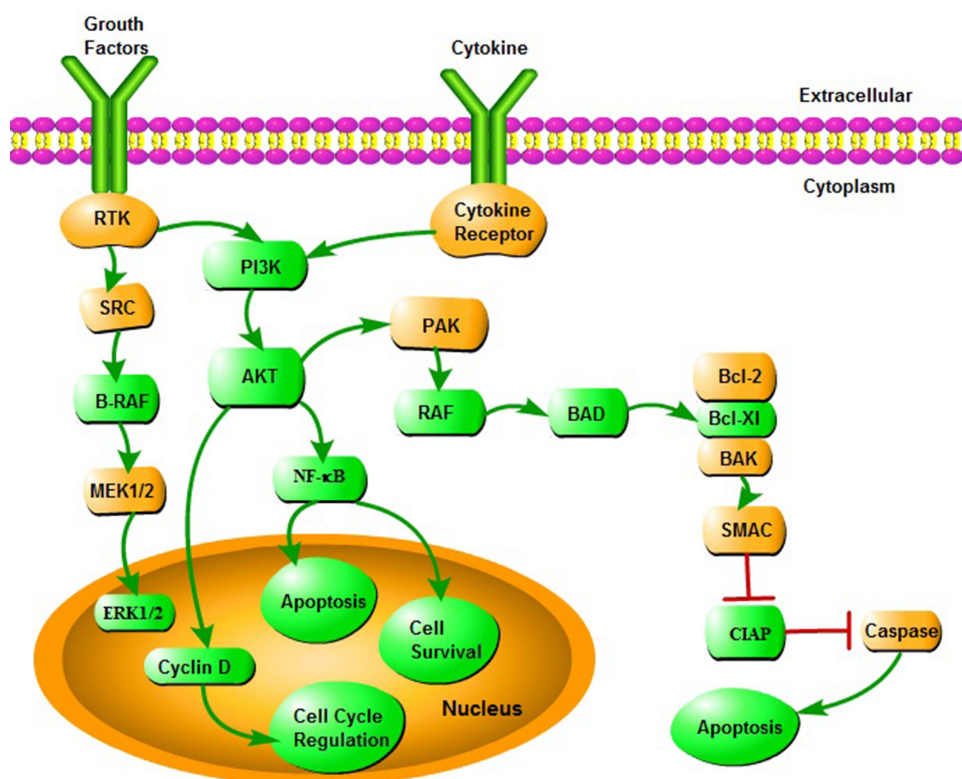
**Figure 1** The dysregulated proteins from the ET with CALR mutation highlighted in green within the current signaling pathway by IPA analysis.
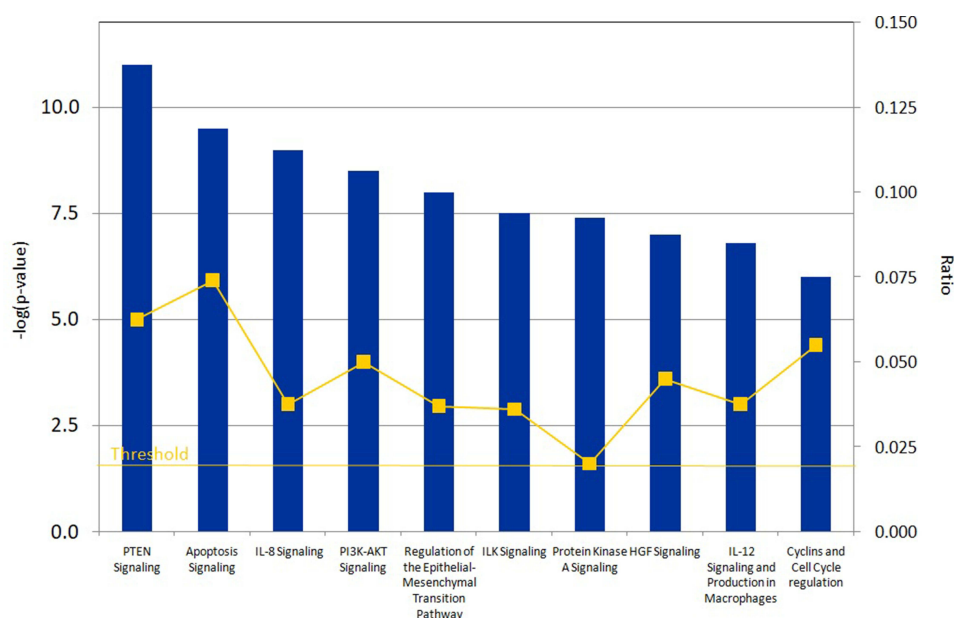


**Figure 2** The top canonical pathways affected by 20 dysregulated proteins in ET with CALR mutations.

## Signaling Network in ET with *CALR*^del52 Mutation

To further investigate the protein–protein interaction network in addition to the major regulators, IPA was conducted on the 20 proteins (genes) identified by PPA, and then the signal network was built (Figure 3). This complex network in *CALR*^del52 mutation appeared to affect many cellular functions, including differentiation of cells
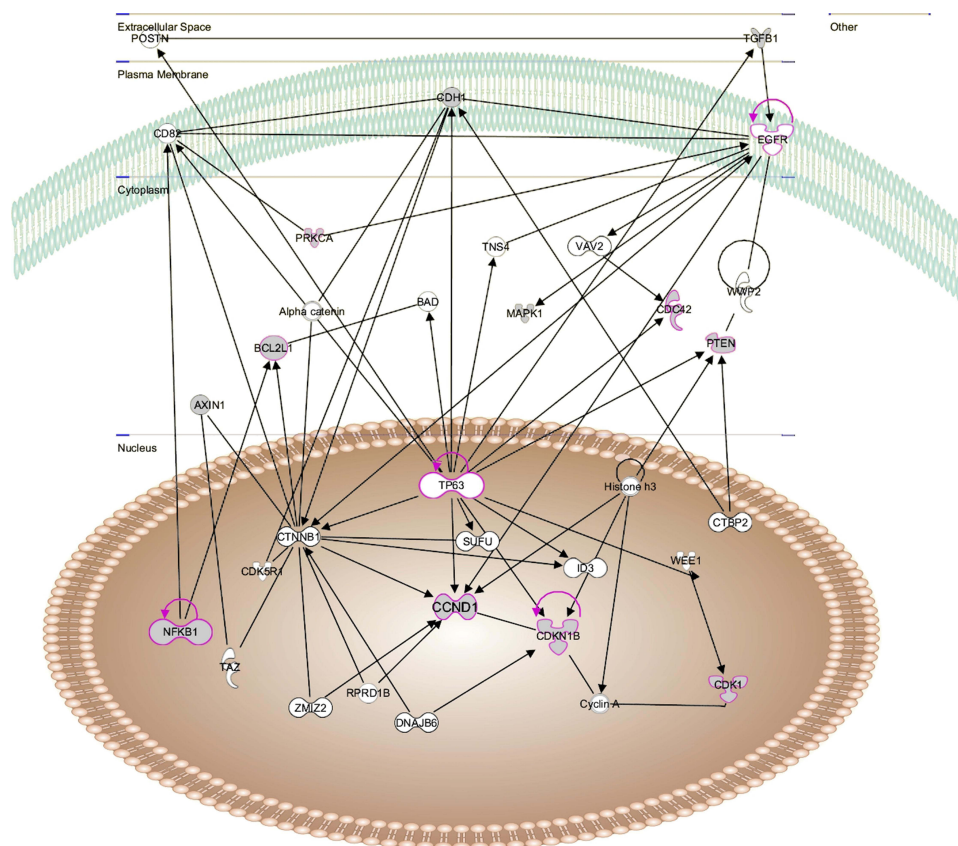
**Figure 3** Protein signaling network altered in ET with CALR mutations as determined by IPA. The dysregulated proteins shaded in gray. The network was shown graphically as nodes(proteins) and edges (the biological association between the nodes). The non-colored nodes were not evaluated in this study but identified by IPA as significant nodes involved in the network. The various shaped nodes represent the functional class of the proteins.

and cell cycle progression. Chronic inflammatory disorders were influenced as well. In this network analysis, core regulators included E-cadherin (*CDH1*), PTEN(*PTEN*), CyclinD1 (*CCND1*), NFκB (*NFKB1*), p27 (*CDKN1B*).

## Comparative Analysis of Protein Expression in ET with *CALR^del52* or *JAK2^V617F* Mutation

In order to determine the various changes in activated signaling pathways between ET patients with *CALR^del52* mutation and those with *JAK2^V617F* mutation, the protein expression levels in these 2 groups were compared and 8 proteins (CyclinD1, Stat5, HIF1α, IL-1β, TGF-β, p27, Erk1/2, ASCL) were found at different levels (q<0.05). These proteins might relate to the signaling pathways of activation by *CALR^del52* mutation, which were different from JAK/STAT signaling by *JAK2^V617F* mutation.

## Discussion

*CALR* mutations trigger JAK/STATs signaling dysregulation in ET, which is similar to *JAK2^V617F* mutation, however, *CALR* mutations were reported to be associated with distinct disease features.[19] We have also found that *CALR^del52* mutation status showed a tendency towards younger age and higher platelet counts. It suggests that additional signaling pathways might determine the disease phenotype, and participate in the development and propagation of the disease. Investigation of signaling pathways is of significance for further understanding the pathogenesis of ET with *CALR* mutations as well as for discovering effective therapeutic targets.

Having used large scale proteomic analysis, we succeeded in identifying numerous signaling proteins that were altered in ET with *CALR^del52* mutation. These 20 proteins involved cell signaling (Erk1/2, PTEN, Raf-B, Rap1, Axin, ERβ, TGF-β), cell cycle (Cdc42, Cdc2, CyclinD1, p27), apoptosis (Bcl-Xl, c-IAP2, NFκB p50, cPKCα, Survivin), transcription factor (eIF4B, SRC-1), DNA repair (TDP1) and adhesion

(E-cadherin). Among these proteins, a significant overexpression was identified for anti-apoptotic proteins (Bcl-Xl, c-IAP2), which might be associated with over-activation of signaling pathways against apoptosis.

Supporting this, the results of IPA analysis for these 20 differentially expressed proteins revealed that apoptosis signaling displayed a significant relationship with $CALR^{del52}$ mutated ET (Figure 2). It has been demonstrated that ET displayed dysregulation of apoptotic pathway.[20,21] Thus, the up-regulated anti-apoptotic proteins discovered in the current study could be involved in apoptotic defects and pathogenesis in $CALR^{del52}$ mutated ET.

Emerging evidence has shown that cytokine production was associated with clinical symptoms and contributed to BM fibrosis and disease progression and adverse prognosis in MPN.[22–24] Pourcelot et al showed that elevated levels of inflammatory cytokines such as IFNγ, IL8, IL4, VEGF, GM-CSF, PDGF, and MCP-1, occurred in ET.[25] In line with these reported results, the current study discovered two significant pathways associated with cellular cytokine pathways (IL-8 signaling, and IL-12 signaling pathways), suggesting a potential role of inflammatory process in the disease progression of ET with $CALR^{del52}$ mutation.

One of the other top pathways discovered in this study was ILK signaling. ILK has been shown to be involved in α-granule secretion of platelets and plays a main role in the regulation of platelet function. The ILK-deficient mice showed an increased bleeding time, and an obvious reduce in the stability of thrombi formed.[26,27] Further work will be required to explore whether ILK signaling plays a role in thrombosis of ET with $CALR^{del52}$ mutation. In addition, we discovered multiple other important signaling pathways which were reported to be involved in carcinogenesis and tumor progression, for example, PTEN signaling, HGF signaling, Regulation of the epithelial-mesenchymal transition pathway, cell cycle regulation (Figure 2). These signaling pathways might play a significant role in the pathogenesis and/or progression of ET with $CALR^{del52}$ mutation.

Furthermore, it is crucial to point out that the proteins identified in this study did not function independently but as a part of a complex signaling network. Therefore, signal networks were further built using IPA in our study to determine the interactions among these proteins (Figure 3). Out of these complicated networks, several core regulators in $CALR^{del52}$ mutated ET were identified. The finding of these core regulators with a higher degree demonstrated they had additional interactions with other molecules in the signaling network. For example, NF-kB

($NFKB1$) was not only upregulated in $CALR^{del52}$ mutated ET compared with control cells, but also served as the central node to establish the interaction network (Figure 3), which suggested this transcription factor played a central role in $CALR^{del52}$ mutation. Recently, Kleppe et al[28] reported that NF-kB signaling as a core pathway was constitutively active in MPN mouse models and NF-kB was a key regulator of inflammation involved in disease process. Taken together, these results underscore a role of NF-kB signaling in MPN pathogenesis, and NF-kB could be a potential therapeutic target for MPN.

Notably, in comparison of activated signaling pathways between $CALR^{del52}$ mutated and $JAK2^{V617F}$ mutated ET patients, 8 proteins were found to be differentially expressed which included CyclinD1, Stat5, Erk1/2, IL-1β, HIF1α, TGF-β, p27, ASCL. Increased phosphorylated STAT3 and STAT5 expression has been previously described in $JAK2$-mutated MPNs,[3,4,29] but STAT5 signaling patterns appeared to be distinct in $CALR$-mutated ET. MARIMO, a $CALR$-mutated cell line has been reported to possess markedly reduced levels of STAT5, pSTAT5, and constitutive activation of ERK1/2.[30,31] Furthermore, Lau et al[32] recently reported that STAT3 and STAT5 targeted genes were overexpression in granulocytes from ET patients with $JAK2$ mutations but not from those with $CALR$ mutations. Consistent with these data, we also observed decreased expression of STAT5 and increased expression of Erk1/2 in $CALR^{del52}$-mutated compared with in $JAK2^{V617F}$-mutated ET.

## Conclusion

In conclusion, our study reveals that a broad dysregulation of signaling proteins and distinct signaling patterns in ET patients with $CALR^{del52}$ mutations, including apoptotic proteins and pathways, which suggest ET with $CALR^{del52}$ mutation might possess unique signaling pathways involving disease pathogenesis and progression. Our findings are of importance in gaining insights into the pathological mechanisms of ET. Future investigations will be more focused on exploring the roles of these proteins in affecting tumor behavior, and hopefully unravel new therapeutic targets.

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## Disclosure

81400094), during the conduct of the study. The authors report no conflicts of interest in this work. The partial abstract of this paper was presented at the ASH Conference name "Alterations of Signaling Pathways in Essential Thrombocythemia with Calreticulin Mutation" as a poster presentation. The poster's abstract was published in "Poster Abstracts" in Blood Journal: https://doi.org/10.1182/blood.V128.22.4286.4286

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