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# Mutations of cytochrome *c* identified in patients with thrombocytopenia THC4 affect both apoptosis and cellular bioenergetics

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ABSTRACT

Inherited thrombocytopenias are heterogeneous diseases caused by at least 20 genes playing different role in the processes of megakaryopoiesis and platelet production. Some forms, such as thrombocytopenia 4 (THC4), are very rare and not well characterized. THC4 is an autosomal dominant mild thrombocytopenia described in only one large family from New Zealand and due to a mutation (G41S) of the somatic isoform of the cytochrome *c* (*CYCS*) gene. We report a novel *CYCS* mutation (Y48H) in patients from an Italian family. Similar to individuals carrying G41S, they have platelets of normal size and morphology, which are only partially reduced in number, but no prolonged bleeding episodes. In order to determine the pathogenetic consequences of Y48H, we studied the effects of the two *CYCS* mutations in yeast and mouse cellular models. In both cases, we found reduction of respiratory level and increased apoptotic rate, supporting the pathogenetic role of *CYCS* in thrombocytopenia.


## 1. Introduction

Inherited thrombocytopenias are a heterogeneous group of diseases due to mutations in at least 20 different genes [1]. Some of these forms are very rare being diagnosed only in a few patients worldwide. This is the case of thrombocytopenia 4 (THC4; OMIM 612004), an autosomal dominant form described in a single family from New Zealand [2]. In this family, the thrombocytopenia is caused by the G41S mutation of the somatic isoform of the cytochrome *c* (*CYCS*) gene, the only naturally occurring mutation identified in this gene.

Cytochrome *c* is an evolutionarily well-conserved small protein of the inner membrane of the mitochondrion. It contains a heme group that shuttles electrons from complex III to complex IV of the mitochondrial electron-transport chain. Moreover, cytochrome *c* is also involved in initiation of the intrinsic pathway of apoptosis. Upon its release to the cytoplasm, it leads to apoptosome formation, which in turn activates the caspase cascade [3].

Despite the ubiquitous expression of cytochrome *c*, the THC4 patients presented only with deregulated megakaryopoiesis, with ineffective platelet production due to ectopic platelet release within bone marrow [2]. Oxygen consumption was normal, suggesting that G41S does not affect electron transfer. It is instead stimulated apoptosome formation and caspase activation. However, it has recently been demonstrated that G41S alters the heme electronic structure of cytochrome *c* and increases its electron self-exchange rate, suggesting that the heme changes might be responsible for the proapoptotic effect of the mutation [4].

Although no *CYCS* mutation was previously revealed in 77 unrelated patients selected from a large series of individuals with inherited thrombocytopenias [5], routine screening led us to identify a second missense mutation of *CYCS*, whose pathogenetic role was investigated in mouse and yeast models.

## 2. Materials and methods

### 2.1. Patients

The proband (III-3; Fig. 1A) was a 3-year-old boy, who was referred to the Division of Pediatric Hematology/Oncology (University of Catania) because of thrombocytopenia revealed on a routine check-up. His

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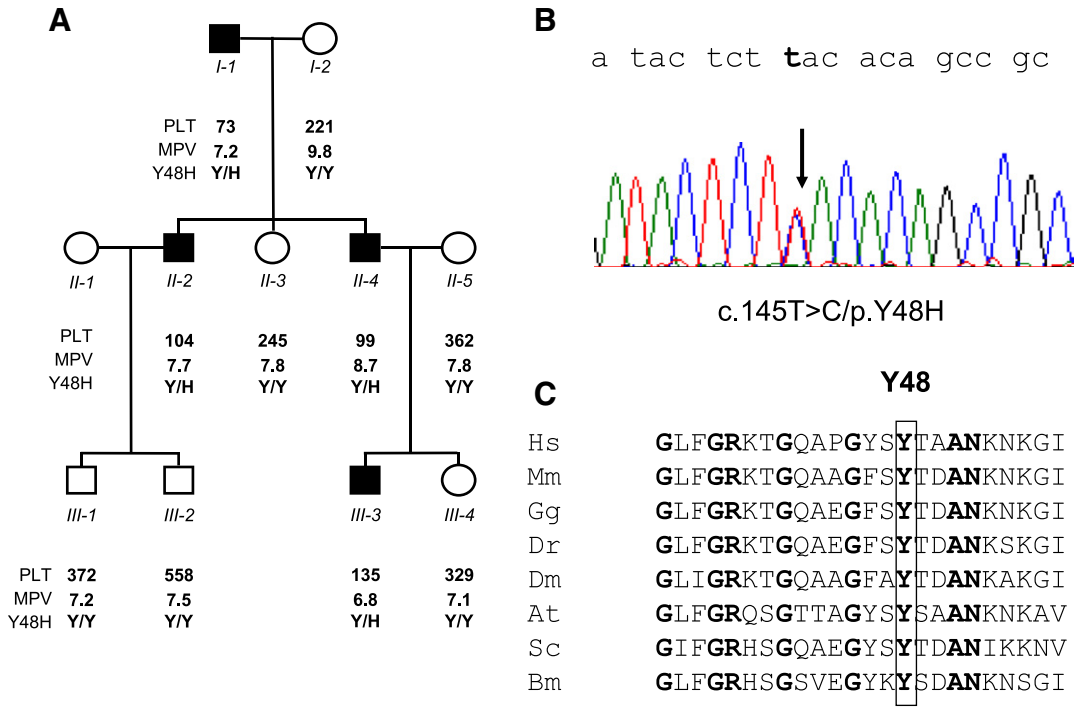


**Fig. 1.** Identification of theY48H mutation. (A) Proband's (III-3) family pedigree showing platelet number (PLT, reported in $\times 10^9$/L), mean platelet volume (MPV, reported in fL) and segregation of the p.Y48H mutation. To conform to mutation nomenclature of literature, in this paper we refer to the amino acid after the first methionine of cytochrome *c* (NP_061820.1) as residue +1. (B) Direct sequencing of PCR product with the overlapping peaks of the heterozygous c.145T>C mutation. (C) Alignment of cytochrome *c* in different species, showing conservation of tyrosine 48. Hs, *Homo sapiens* (NP_061820.1); Mm, *Mus musculus* (NP_031834.1); Gg, *Gallus gallus* (NP_001072946.1); Dr, *Danio rerio* (NP_001002068.1); Dm, *Drosophila melanogaster* (NP_477176.1); At, *Arabidopsis thaliana* (NP_173697.1); Sc, *Saccharomyces cerevisiae* (NP_012582.1); Bm; *Brucella melitensis* (NP_542046.1). Amino acids conserved during evolution are in bold.

platelet count was 135 × $10^9$/L, with mean platelet volume (MPV) of 6.8 fl (normal value 6.5–11). He had no personal or familial history of bleeding though his father also had thrombocytopenia. The investigation was extended to all family members, who gave written informed consent to the investigation according to the Declaration of Helsinki. All the other forms of inherited thrombocytopenia were excluded according to the diagnostic algorithm proposed by the Italian Platelet Study Group and subsequently updated [6,7].

### 2.2. Mutational screening of CYCS

The *CYCS* gene was screened for mutations using genomic DNA extracted from peripheral blood. Mutation analysis was performed by polymerase chain reaction (PCR) using oligonucleotides 5′-AGTGGC TAGAGTGGTCATTC-3′ and 5′-TTAACCACAAGCCAGTCTTAG-3′. PCR was carried out in a total volume of 25 μL with 80 ng of genomic DNA, 1 μL of each primer 10 μM, 2 μL of $MgCl_2$ 25 mM, 2 μL of dNTPs 2.5 mM, 1.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, USA) and 2.5 μL of the corresponding 10× PCR buffer. After an initial denaturation step at 95 °C for 12 min, amplification was performed for 30 cycles (denaturation at 95 °C for 40 s, annealing at 60 °C for 45 s and extension at 72 °C for 1 min). PCR products were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Read Reaction kit and ABI 1310 Genetic Analyzer (Applied Biosystems, USA).

### 2.3. Cloning of yeast and murine cDNA

The yeast cytochrome *c* gene (*CYC1*; NM_001181706.1) and its 5′ and 3′ flanking regions were amplified using genomic DNA of strain BY4741 as template and primers 5′-CCCCAAGCTTCGTGTGAGACGACA TCGTCG-3′ and 5′-CCCCGGATCCGGACCTAGACTTCAGGTTGTC-3′. The PCR product was cloned into the pFL38 centromeric plasmid [8]. The $CYC1^{Y53H}$ and $CYC1^{G46S}$ mutant alleles, which correspond to the human $CYCS^{Y48H}$ and $CYCS^{G41S}$ alleles, respectively, were produced by site-directed mutagenesis using QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using modified primers 5′-GGTCAAGCTGAAGG GTATTCG**CAC**ACAGATGCCAATATCAAGAA and 5′-TTCTTGATATTGGCAT CTGT**GTG**CGAATACCCTTCAGCTTGACC-3′ for $CYC1^{Y53H}$ or 5′-GGTATCTT TGGCAGACACTCT**AGT**CAAGCTGAAGGGTATTCG-3′ and 5′-CGAATACC CTTCAGCTTG**ACT**AGAGTGTCTGCCAAAGATACC-3′ for $CYC1^{G46S}$ (base changes are in bold). Both wild-type and mutagenized inserts were sequence-verified on both strands.

The murine cytochrome *c* (*Cycs*; NM_007808.4) coding region was amplified from cDNA using primers 5′-GGGACGTCTGTCTTCGAGTC-3′ and 5′-AAGCCATGAGACATTTGTTTTG-3′ with Platinum PCR SuperMix (Invitrogen, USA) and cloned into pCR8/GW/TOPO-TA (Invitrogen, USA). The G41S and Y48H mutations were introduced using the QuikChange II Site Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to manufacturer's protocol with primers 5′-TGTTCGGGCGGAAG ACA**A**GCCAGGCTG-3′ and 5′-CAGCCTGGC**T**TGTCTTCCGCCCGAACA-3′ for $Cycs^{G41S}$, and 5′-GGCCAGGCTGCTGGATTCTCT**C**ACACAGATGCC-3′ and 5′-GGCATCTGTGT**G**AGAGAATCCAGCAGCCTGGCC-3′ for $Cycs^{Y48H}$ (base changes are in bold). Plasmids were sequenced using the BigDyeCycle Reaction System v3.1 kit (Applied Biosystems, USA) and the ABI PRISM 3100 automated sequencer (Applied Biosystems, USA). The sequences of the wild-type and mutant *Cycs* were subcloned into the retroviral transducing vector pMSCV via homologous recombination, using the Gateway LR Clonase II Enzyme Mix Kit (Invitrogen, USA), according to manufacturer's protocol.

### 2.4. Yeast and murine cell culture, transformation and transduction

*Saccharomyces cerevisiae* strain BY4741 (*MATa*; *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0) and its isogenic strain *CYC1::kanMX4* (Δ*cyc1*) were

used. Cells were cultured in the YNB medium [0.67% yeast nitrogen base without amino acids (ForMedium™, United Kingdom)] supplemented with 1 g/L of drop-out powder containing all amino acids and bases except those required for plasmid maintenance [9]. Various carbon sources were added at 2% (w/v) (Carlo Erba Reagents, Italy). Media were solidified with 20 g/L agar (ForMedium™, United Kingdom). For respiration and cytochrome absorption spectra cells were grown to late-log phase in the YNB medium supplemented with 0.6% glucose. Transformation of yeast strain was obtained by the lithium acetate method [10]. Restriction-enzyme digestions, *Escherichia coli* transformation and plasmid extractions were performed using standard methods [11].

Mouse lung fibroblasts, where both somatic and testis cytochrome *c* isoforms were deleted ($Cycs^{-/-}/Cyct^{-/-}$ MLF) and mouse embryonic fibroblasts (MEF), were cultured as previously described [12]. Retroviral vectors were generated by co-transfecting the HEK293T cell line with pMSCV containing the wild-type or mutant *Cycs* sequences and with the helper pIK plasmid. The supernatants collected after 24 h or 48 h were used to transduce $Cycs^{-/-}/Cyct^{-/-}$ MLFs, which were then grown in medium supplemented with hygromycin (200 μg/mL), or wild-type MEFs.

### 2.5. Respiration measurements and cytochrome spectra

In yeast, oxygen uptake was measured at 30 °C using a Clark-type oxygen electrode in a 1 ml stirred chamber containing 1 ml of air-saturated respiration buffer (0.1 M phthalate–KOH, pH 5.0) and 10 mM glucose (Oxygraph System, Hansatech Instruments, England). The reaction was initiated with the addition of 20 mg of wet weight of cells, as previously described [13]. Differential spectra between reduced and oxidized cells of a suspension of cells at 60 mg/ml (wet weight) were recorded at room temperature, using a Cary 300 Scan spectrophotometer (Varian, Palo Alto, CA, USA).

Murine cells were grown under standard conditions. The culture medium was changed on the day before the measurements. Cells were collected by trypsinization and centrifugation, and resuspended at $7.5 \times 10^6$ cells per ml in standard medium (0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 1 mg/ml BSA, 10 mM $KH_2PO_4$, pH 7.4). Samples (0.3 ml) were transferred into a micro water-jacketed cell, magnetically stirred at 37 °C, and oxygen utilization was measured with a Clark oxygen electrode (Hansatech Instruments, UK). The reaction was inhibited with KCN (700 μM).

### 2.6. Cell lysates and immunoblotting

Whole-cell lysates of murine cells ($10^6$) were prepared in CHAPSO buffer (1% CHAPSO, 100 mM KCl, 50 mM HEPES pH 7.5, 1 mM EGTA) supplemented with complete protease-inhibitor mixture (Sigma-Aldrich, USA). Twenty micrograms of total cell lysate was separated by SDS-PAGE using the NuPAGE 4%–12% Bis Tris Gel (Invitrogen, USA), blotted on a polyvinyl difluoride membrane, probed with a 1:10,000 antibody against cytochrome *c* (BD Pharmingen, USA) and a 1:10,000 secondary HRP-conjugated antimouse antibody (Santa Cruz, USA), and analyzed by enhanced chemiluminescence (Amersham Pharmacia Biosciences, UK). As loading control, the membrane was probed with mouse anti-actin antibody (Sigma-Aldrich, USA).

### 2.7. Cell death

Cell death of murine cells was analyzed as previously reported [14]. Briefly, $3.5 \times 10^4$ transduced cells were treated with 2 μM staurosporine (STS) for 5 h. Cells were stained with propidium iodide (PI) and annexin-V-FITC (Prodotti Gianni, Italy) and cell death was measured by flow cytometry as the percentage annexin-V-positive/PI-negative cells.

## 3. Results

### 3.1. Identification of the Y48Y mutation

Mutational screening of the *CYCS* gene revealed a heterozygous substitution (c.145T>C; p.Y48H or $CYCS^{Y48H}$, LOVD database: http://databases.lovd.nl/shared/genes/CYC) in the proband of the family (Fig. 1). The c.145T>C substitution was not reported in the SNP or in he 1000 Genome databases (http://www.ncbi.nlm.nih.gov/SNP; http://www.1000genomes.org). As for the G41S mutation ($CYCS^{G41S}$) [1], amino acid alignments showed a perfect conservation of tyrosine 48 among cytochrome *c* orthologs (Fig. 1C), suggesting that the residue exerts a fundamental role in the structure and function of the protein.

Family studies showed that individuals I-1, II-2 and II-4 were also thrombocytopenic and carried the mutation (Fig. 1). In the affected individuals, platelet counts ranged from 73 to $135 \times 10^9$/L, suggesting complete penetrance for thrombocytopenia. In all the patients, MPV was within normal limits, and examination of peripheral blood smears showed that platelets had normal size and morphology. The proband, as well as the other three affected relatives, had no hematological or extra-hematological defects associated with thrombocytopenia.

### 3.2. $CYC1^{Y53H}$ partially complements growth defect of yeast Δcyc1 strain

As an initial attempt to understand whether the Y48H mutation of the human *CYCS* gene has any pathogenic role, we performed complementation studies in a *S. cerevisiae* strain carrying a deletion of *CYC1*, the yeast ortholog of human *CYCS*. The Δ*cyc1* mutant displays an OXPHOS (Oxidative Phosphorilation) negative phenotype characterized by failure to grow in media containing obligatory aerobic compounds as the only carbon sources [15]. The Δ*cyc1* strain was transformed with either wild-type *CYC1*, or mutant alleles (s$CYC1^{Y53H}$ and $CYC1^{G46S}$ corresponding equivalent to the human $CYCS^{Y48H}$ and $CYCS^{G41S}$, respectively), and as well as with the empty vector, and the oxidative growth was evaluated by spot assay analysis.

When the growth on non-fermentable carbon sources was evaluated at 28 °C, the OXPHOS negative phenotype of Δ*cyc1* could be rescued by expressing both mutant alleles. However, under temperature-induced stress conditions, i.e. at 37 °C [16,17], the oxidative growth of both mutants strains was significantly reduced when compared with that of the strain expressing the wild-type allele (Fig. 2), indicating that both mutations are not neutral change in yeast.

### 3.3. Defective respiratory activity in both yeast and mammalian cells expressing Y48H

The primary function of cytochrome *c* is to shuttle electron between complex III and IV of the mitochondrial respiratory chain. Since tyrosine 48 is located in close proximity to the heme redox center, amino acid substitutions at this residue could interfere with the mitochondrial electron flow. When the respiratory activity was measured in yeast strains expressing the $CYC1^{Y53H}$and $CYC1^{G46S}$alleles incubated at 37 °C, we observed a reduction in oxygen consumption of about 40% as compared to that observed in cells expressing the wild-type gene (Fig. 3A). Moreover, in order to assess the structural integrity of the mitochondrial respiratory chain complexes, the absorption spectrum of cytochromes was measured. Both the Y53H and G46S mutations were associated with specific reduction of about 30% in the cytochrome *c* content whereas that of cytochrome *b* did not change (Fig. 3B,C). These results corroborated, at least in yeast, the pathogenetic role of the human Y48H mutation.

In order to confirm the respiratory defect in mammalian cells, we reproduced the Y48H ($Cycs^{Y48H}$) and G41S ($Cycs^{G41S}$) mutations in the murine cDNA for their expression in immortalized mouse lung fibroblasts, where both somatic and testis cytochrome *c* isoforms were deleted ($Cycs^{-/-}/Cyct^{-/-}$ MLF) [12]. Cytochrome *c* expression level was

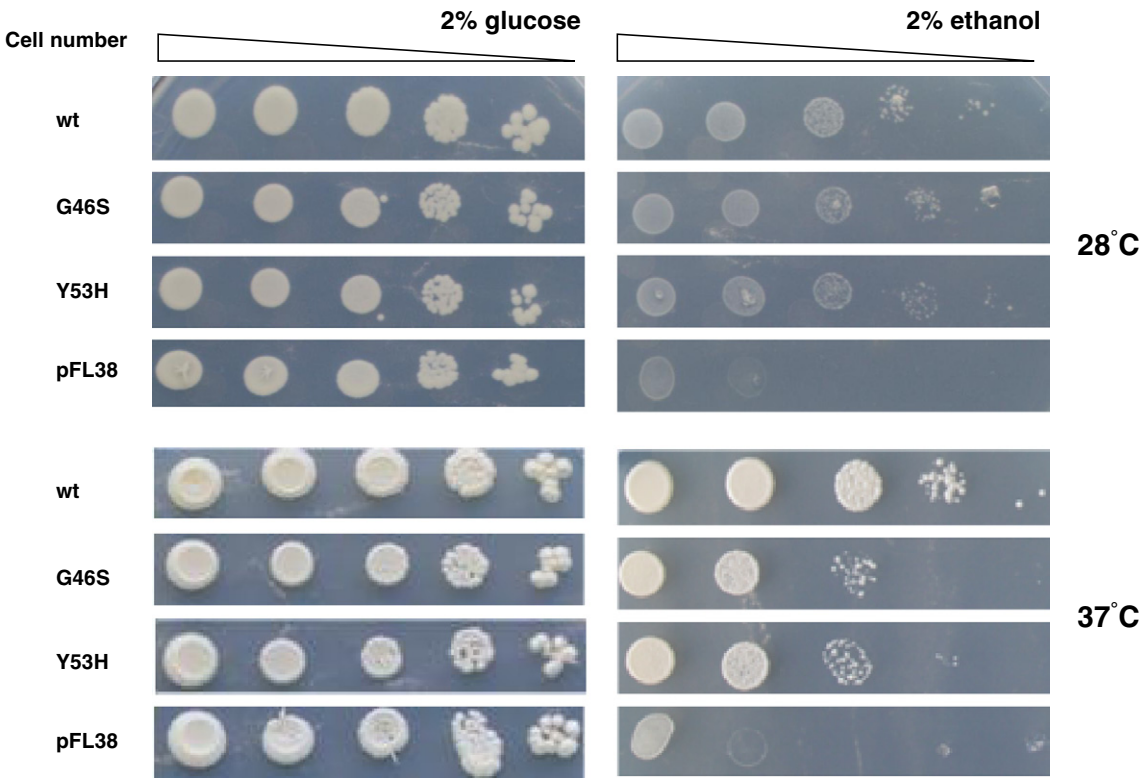


**Fig. 2.** Complementation studies of the *CYCS* mutations in yeast: oxidative growth phenotype. The BY4741 Δ*cyc1* strain was transformed with the pFL38 plasmid carrying the wild-type *CYC1*, the mutant alleles or the empty vector. Equal amounts of serial dilutions ($10^5$, $10^4$, $10^3$, $10^2$, and $10^1$ cells) of cells from exponentially grown cultures were spotted onto YNB plates supplemented with either 2% glucose or 2% ethanol. The growth was scored after 3 days of incubation at 28 °C or 37 °C.

similar for the three constructs, indicating that both mutant proteins are relatively stable (Fig. 4A). Since their expression levels were higher in the cells transduced with packaging supernatants collected at 48 h than at 24 h from virus generation, we used the former cells for the experiments in this paper. First of all, we measured oxygen consumption in $Cycs^{-/-}/Cyct^{-/-}$ MLFs transduced with the $Cycs^{Y48H}$ and $Cycs^{G41S}$ mutants. Consistent with data from yeast, intact cell coupled with endogenous respiration decreased at approximately 30% with the $Cycs^{Y48H}$ mutant (Fig. 4B). The decrement was even more evident with G41S. The effect was less significant in the wild-type MEFs, given the presence of the normal cytochrome *c* alleles.

### 3.4. The Y48H mutant has increased proapoptotic activity

In addition to its role in the mitochondrial electron transport chain, cytochrome *c* is active in the intrinsic apoptotic pathway. Indeed, the first G41S mutation identified in cytochrome *c* was shown to increase the apoptotic activity [2]. Therefore, we determined whether both mutants influenced the apoptotic response to staurosporine (STS), an intrinsic stimulus that requires the mitochondrial apoptotic pathway to induce cell death [18]. In $Cycs^{-/-}/Cyct^{-/-}$ MLFs, $Cycs^{Y48H}$ and $Cycs^{G41S}$ enhanced both basal and STS-induced apoptosis, compared to the wild-type cDNA (Fig. 5A). This effect was also visible (although to a lesser extent) when the mutant constructs were expressed in wild-type MEFs (Fig. 5B).

## 4. Discussion

Cytochrome *c* is a relatively well-characterized protein involved in multiple essential cellular pathways such as electron transport for mitochondrial ATP production and intrinsic apoptosis. Although its function has been largely studied in different models, no natural occurring mutations have been identified with the only exception of the G41S substitution, which was found in patients belonging to a large family affected with a mild form of autosomal dominant thrombocytopenia [2]. During our routine mutational screening of *CYCS* in patients with familial thrombocytopenia, we detected a novel variant (Y48H) that segregates in a small Italian family with four affected individuals. They all presented with a very mild thrombocytopenia with platelet size in the normal range and without any additional features associated, a phenotype comparable to that of patients carrying the G41S substitution [2].

Consistent with data previously published [2], we found an increased apoptotic activity when mouse cells were transfected with the $Cycs^{Y48H}$ and $Cycs^{G41S}$ mutants without or after an apoptotic stimulus. Megakaryocytes produce platelets by the extension of long and branching cytoplasmic processes, designated proplatelets, which are protruded into the lumen of marrow sinusoids to be released into the bloodstream. Proplatelets subsequently mature to platelets in the circulation. The apoptotic pathway regulates the timing of this process, as it is involved in triggering proplatelet extension when mature megakaryocytes approach marrow sinusoids [19]. Thus, it was hypothesized that the proapoptotic G41S mutant induces a premature proplatelet release within the bone marrow, rather than in the bloodstream, resulting in ineffective platelet production. Consistently, signs of premature proplatelet formation were observed in megakaryocytes from two patients with the G41S mutation [2]. Since Y48H showed a proapoptotic effect similar to that of G41S, it is reasonable to hypothesize that it induces thrombocytopenia through a similar mechanism. Unfortunately, samples from our patients were not available to confirm this hypothesis.

Tyrosine 48 is spatially close to glycine 41 and packs against the loop whose structure is defined by the presence of the glycine. Therefore, it is possible that their similar phenotypic consequences come from a similar effect on the protein folding. For both mutations, however, the molecular mechanisms leading to alteration of respiration and apoptosis remain obscure. As reported [4], G41S alters the heme electronic structure of cytochrome *c* and increases electron self exchange rate. In order

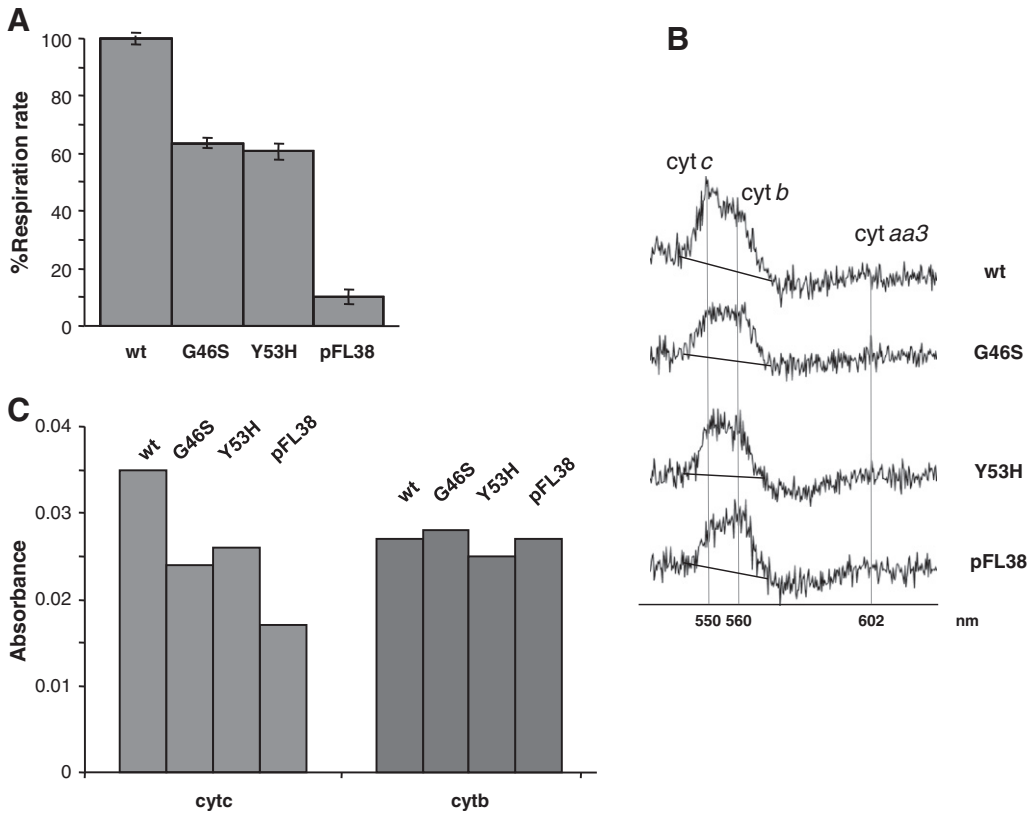


**Fig. 3.** Oxygen consumption rates and cytochrome spectra in yeast. (A) Respiration was measured in cells grown in YNB supplemented with 0.6% glucose at 37 °C. The values the *cyc1* mutants are expressed as percentage of the respiration obtained in cells expressing the wild-type *CYC1* gene, where the respiratory rate was 15 nmol $min^{-1}$ $mg^{-1}$. Values are the mean of three independent experiments. (B) Peaks at 550, 560 and 602 nm (vertical bars) correspond to cytochromes c (cytc), b (cytb) and aa3 (cytaa3), respectively. (C) The cytochrome content is expressed as the difference of absorbance value of each peak relative to the baseline of the corresponding spectrum. For each strain a suspension of cells at 60 mg/ml (wet weight) was utilized.

to explain the pro-apoptotic activity of the mutant protein, it was hypothesized that an increased electron transfer would accelerate generation of the oxidized form of cytochrome *c*, promoting apoptosis. However, the partial defect in respiration we found in both yeast and murine cells would argue against this hypothesis. Other hypotheses, including a mobilization of the mutant proteins from mitochondria into the cytosol, still need to be explored. These future studies should lead to a better understanding of the molecular mechanisms leading to thrombocytopenia.

## 5. Conclusions

In conclusion, we identified the second naturally occurring mutation of the CYCS gene in a family affected with a mild form of

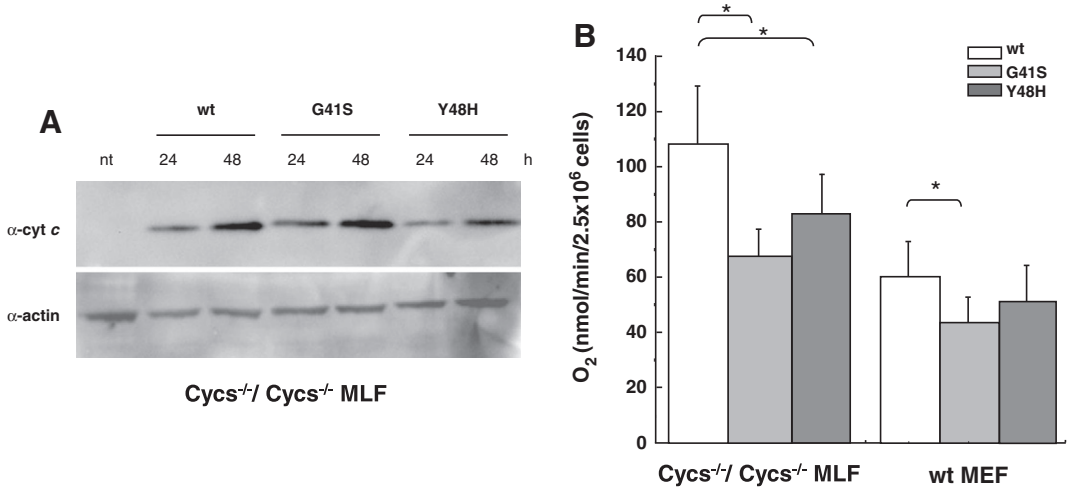


**Fig. 4.** Both G41S and Y48H mutations associated with mild decrease of endogenous respiration in mouse cells. (A) Protein (20 μg) expression level of the wild-type and mutant ($Cycs^{Y48H}$ and $Cycs^{G41S}$) forms of the *Cycs* cDNA in $Cycs^{-/-}/Cyct^{-/-}$ mouse lung fibroblasts (MLF) transduced with cell packaging supernatants collected at 24 h and 28 h from virus generation, as detected by SDS-PAGE and immunoblotted using antibodies against cytochrome *c* (cyt *c*) and α-actin. (B) Oxygen consumption rates in $Cycs^{-/-}/Cyct^{-/-}$ MLF or wild-type mouse embryonic fibroblasts (MEF) transduced with the wild-type or the mutant forms of the *Cycs* cDNA. nt, non transduced cells; * = $p < 0.05$.

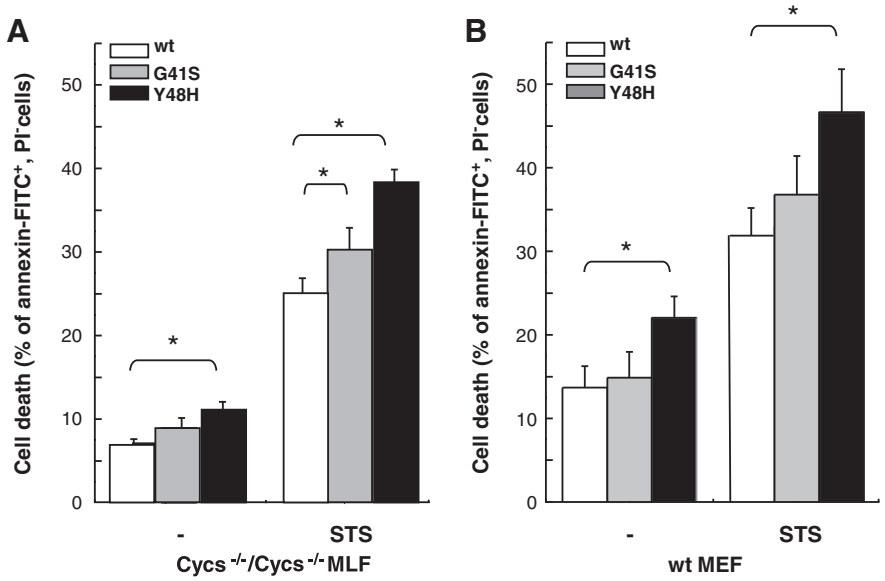


**Fig. 5.** The Y48H mutation enhances staurosporine (STS)-induced apoptosis. Cell death in (A) *Cycs*$^{-/-}$/*Cyct*$^{-/-}$ mouse lung fibroblasts (MLF) or (B) wild-type c mouse embryonic fibroblasts (MEF) transduced with the wild-type and mutant forms of the *Cycs* cDNA before and after treatment with STS (2 μM) for 5 h. * = $p < 0.05$.

thrombocytopenia. Both mutations are associated with a defective apoptosis and cellular bioenergetics. Further investigations are needed to understand how these defects compromise megakaryopoiesis and platelet production.

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