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A mutation of human cytochrome *c* enhances the intrinsic apoptotic pathway but causes only thrombocytopenia

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We report the first identified mutation in the gene encoding human cytochrome *c* (*CYCS*). Glycine 41, invariant throughout eukaryotes, is substituted by serine in a family with autosomal dominant thrombocytopenia caused by dysregulated platelet formation. The mutation yields a cytochrome *c* variant with enhanced apoptotic activity *in vitro*. Notably, the family has no other phenotypic indication of abnormal apoptosis, implying that cytochrome *c* activity is not a critical regulator of most physiological apoptosis.

Apoptosis is crucial for normal development and tissue homeostasis. The intrinsic apoptotic pathway, involving release of cytochrome *c* from mitochondria, is essential for apoptosis occurring in response to cellular stress and damage[1]. Knock-in mice whose cytochrome *c* fails to activate the apoptotic downstream partner, Apaf-1, but retains normal electron transfer function have enlarged brains and abnormal lymphocyte homeostasis but minimal phenotypic abnormalities in other tissues[2]. Such results have led some workers to question whether mitochondrial cytochrome *c*–induced caspase activation is rate limiting for apoptosis[3]. Because these knock-in mice seldom survive, it has not yet been possible to evaluate the role of cytochrome *c* in apoptosis after birth.

The opportunity to examine the role of cytochrome *c* arose from the analysis of a family with mild autosomal dominant thrombocytopenia for which a six-generation pedigree was available (**Supplementary Fig. 1** online). The mean platelet count of 29 affected individuals in this family was 109 × 10^9/l (range 73–167, reference interval 150–430). Platelet morphology and volume were normal, as were other peripheral blood counts (**Supplementary Table 1** online). Clinical manifestations of thrombocytopenia were absent or mild. Affected individuals had normal longevity, fertility and fitness, with no evidence of neurodegenerative, muscular, eye or kidney disease or diabetes.

We established genetic linkage to a 1.12-Mb region on chromosome 7p15.2 (**Supplementary Table 2** online). Sequencing of 38 putative

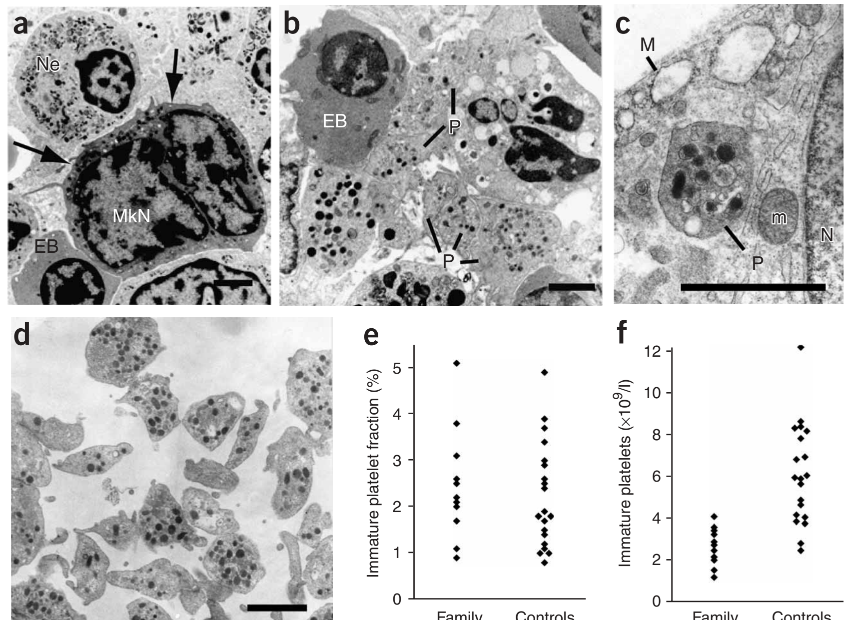

Figure 1 Defective platelet formation but normal platelet lifespan. (**a**) Electron micrograph of a naked megakaryocyte nucleus (MkN) with a thin rim of cytoplasm (arrows), adjacent to neutrophil (Ne) and erythroblast (EB). (**b**) Platelets (P) within the marrow space, indicating intramedullary release. (**c**) A phagocytosed platelet in a marrow macrophage (M), confirming intramedullary release (N, nucleus; m, mitochondrion). (**d**) Numerous platelets produced *in vitro* as early as day 6 of culture. Scale bars in **a**–**d**, 2 µm. (**e**) Percentage of immature platelets in 11 affected family members compared to 20 controls (mean ± s.d. = 2.5 ± 1.3% and 2.3 ± 1.1%, respectively). (**f**) Absolute number of immature platelets compared to controls (2.7 ± 0.9 and 6.1 ± 2.4 × 10^9/l, respectively, $P < 0.00001$).

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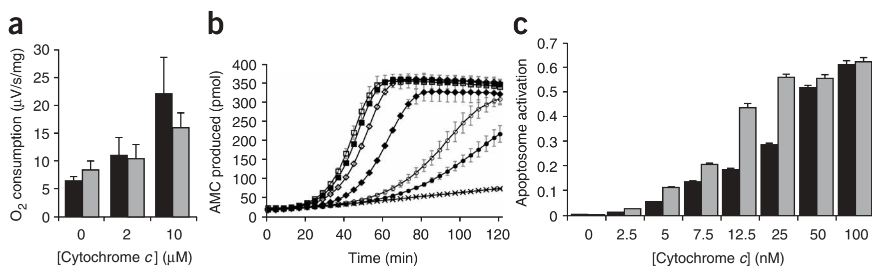

Figure 2 G41S variant of cytochrome *c* has enhanced proapoptotic activity but normal redox activity. (**a**) Relative oxygen consumption by mitoplasts depleted of endogenous cytochrome *c* in the presence of WT (black bars) or G41S (gray bars) cytochrome *c*. There was no significant difference at any concentration ($n = 6$ and values are mean ± s.d.). (**b**) U937 cell cytosol was incubated with 0 (×), 5 (circles), 25 (diamonds) or 100 (squares) nM of G41S (open symbols) or WT (filled symbols) cytochrome *c* in the presence of dATP and the caspase-3 substrate DEVD-AMC ($n = 3$ ± s.d.). (**c**) Apoptosome activation, derived from the rate of DEVD-AMC cleavage in **b**, by WT (black bars) and G41S (gray bars) cytochrome *c* over a range of cytochrome *c* concentrations.

exons in the region identified a single cosegregating unreported variant, a G-to-A transition at nucleotide 132 of exon 2 of the gene encoding cytochrome *c* (*CYCS*) that causes substitution of glycine by serine at residue 41 (G41S). Cytochrome *c* is a highly conserved protein, and glycine 41 is invariant among 113 eukaryotic species[4]. Allele-specific PCR confirmed that 26 affected family members were heterozygous for the mutation, which was not detected in 360 other individuals, including 27 unaffected family members, nor in 1,990 cDNA and EST sequences in NCBI databases. Mass spectrometric analysis of leukocyte cytochrome *c* gave a WT/G41S ratio of 2:1 in an affected heterozygote (**Supplementary Methods** online).

Platelets are formed when bone marrow megakaryocytes undergo a compartmentalized activation of the intrinsic apoptotic pathway involving cytochrome *c* release and caspase activation without fragmentation of nuclear DNA[5]. The resulting proplatelets protrude into the marrow sinusoids, forming mature platelets that circulate for 10 d before dying by an apoptotic process[6]. Thrombocytopenia can be caused by ineffective platelet formation or premature death in the circulation.

To determine whether early death of circulating platelets contributed to the thrombocytopenia, we measured the immature platelet fraction, a noninvasive measure to distinguish decreased production from increased destruction (**Fig. 1**)[6,7]. Peripheral blood from affected family members contained the same proportion of immature platelets as controls (**Fig. 1e**), indicating no reduction in platelet lifespan. However, the absolute number of immature platelets was lower, suggesting reduced production (**Fig. 1f**).

To examine platelet production, we assessed megakaryocyte development and platelet formation in affected family members. Electron microscopy (EM) of bone marrow from a 76-year-old showed intramedullary naked megakaryocyte nuclei and platelets (**Fig. 1a–c**). These abnormal observations[8] are indicative of dysregulated megakaryopoiesis with premature release of platelets into the marrow space rather than into sinusoids. This dysregulation was confirmed when blood-derived megakaryocyte stem cells from two family members were grown *in vitro* (**Supplementary Methods**). Unusually numerous platelets, confirmed by EM (**Fig. 1d**), were produced as early as day 6. Normally, $CD34^+$ cell–derived megakaryocytes produce visible platelets only after 10–12 d in culture[9]. Flow cytometry showed a tenfold increase in the ratio of platelet-like particles ($CD41^+$) to megakaryocytic cells at days 6 and 7 of culture (11 and 8.0 in a family member compared to 1.0 and 0.8 in control cultures, respectively). Such early and enhanced production of platelets is unusual and is consistent with the thrombocytopenia being caused by enhanced activation of the apoptotic machinery.

Cytochrome *c* is a multifunctional protein with roles in electron transport, antioxidant defenses and apoptosis. Thus, we examined the functional and structural properties of recombinant wild-type (WT) and G41S cytochrome *c*. An X-ray structure of reduced G41S cytochrome *c* at 2.7-Å resolution indicated that this mutation does not confer any large-scale structural alteration (**Supplementary Fig. 2** online). The redox potential of the G41S variant was 221.4 mV, similar to the 220.4 mV for WT cytochrome *c*, which is in agreement with previous reports[10]. As thrombocytopenia is not a feature of mitochondrial diseases, and members of the affected family do not have symptoms characteristic of a defect in mitochondrial oxidative phosphorylation[11], the mutation was predicted not to affect mitochondrial respiration. Indeed, there was no significant difference between WT and G41S cytochrome *c* in oxygen consumption rate (**Fig. 2a**) or in steady-state reduction of cytochrome *c* (**Supplementary Fig. 3** online). In addition, G41S cytochrome *c* had the same superoxide oxidase activity as WT cytochrome *c* (**Supplementary Fig. 3**).

Cytochrome *c* mediates apoptosis by interacting with monomeric Apaf-1, triggering a conformational change that enables apoptosome formation and caspase activation. Glycine 41 is close to lysine 39, a residue involved in the cytochrome *c*–Apaf-1 binding interface[12,13]. At low cytochrome *c* concentration (≤25 µM), the G41S variant was significantly more effective than the WT form at triggering cleavage of the caspase-3 substrate DEVD-AMC in a cell-free caspase activation assay (**Fig. 2b**,**c**). Combining equal amounts of the WT and G41S proteins gave an intermediate level of caspase 3 activity (**Supplementary Fig. 4** online). This is the first mutation identified that increases the apoptotic activity of cytochrome *c*. In contrast, all engineered mutations of cytochrome *c* either do not affect or decrease apoptotic activity[12–14].

It has been argued that, in many cell types, although cytochrome *c*–mediated caspase activation is necessary for rapid cell death, it is not a driving force[3]. Thus, the presence of a cytochrome *c* variant that enhances caspase activation would make cell death more efficient but would not necessarily cause a fundamental change in developmental or physiological outcome. The observations of premature platelet release *in vivo* and early platelet formation *in vitro* argue strongly that the increased caspase-activating activity of G41S cytochrome *c* is responsible for the familial thrombocytopenia. Furthermore, these observations suggest that platelet formation is particularly sensitive to changes in the activation of the intrinsic apoptotic pathway. This is not surprising because proplatelet formation must be restricted to a very narrow developmental stage and specific sinusoidal location[15]. The observation that the members of the affected family are otherwise healthy and long-lived implies that, at least in its heterozygous form, the presence of a more 'active' cytochrome *c* has little or no effect on the apoptotic outcome in most organs during development and adult life.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

I.M.M. and E.J.C. initiated the study, recruited and evaluated participants; A.J.H., M.W.L., F.M.d.S.T., M.S.A.C. and N.A.B. performed linkage studies, haplotype refinement and sequencing; M.P.S., E.M.C., S.F. and C.C. performed functional platelet and megakaryocyte studies and electron microscopy; P.L.C., R.J.W., E.C.L., A.L., S.M.K.D., G.H., S.M.W., R.D.F. and D.A.P. expressed the proteins and performed functional and structural studies; and I.M.M., E.C.L., E.M.C. and S.M.W. wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturegenetics/.

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