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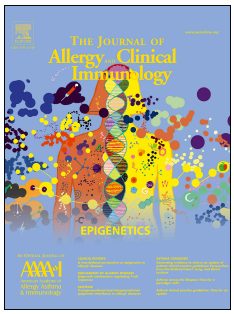

EROS mutations: decreased NADPH oxidase function and chronic granulomatous disease

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Full Title: : EROS mutations: decreased NADPH oxidase function and chronic granulomatous disease

Running Title: *EROS* mutations cause chronic granulomatous disease

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Conflict of interest statement

The authors have no conflicts of interest to declare.

Capsule Summary

We demonstrate for the first time that EROS (CYBC1/C17ORF62) regulates abundance of the gp91phox-p22phox heterodimer of the phagocyte NADPH oxidase in human cells and that EROS mutations are a novel cause of chronic granulomatous disease.

Key Words

EROS,

C17ORF62

CYBC1

Chronic granulomatous disease,

Nox2

gp91*phox*

Abbreviations

CGD – Chronic Granulomatous Disease

EROS – Essential for Reactive Oxygen Species

HLH – haemophagocytic lymphohistiocytosis

NADPH - Nicotinamide adenine dinucleotide phosphate

NBT Nitro blue tetrazolium chloride

DHR – Dihydrorhodamine

PBMC - Peripheral Blood Mononuclear Cell

Short Summary (for the Editor only)

The phagocyte respiratory burst is mediated by the phagocyte NADPH oxidase, a multi-protein subunit complex that facilitates production of reactive oxygen species and which is essential for host defence. Monogenic deficiency of individual subunits leads to chronic granulomatous disease (CGD), which is characterized by an inability to make reactive oxygen species, leading to severe opportunistic infections and auto-inflammation. However, not all cases of CGD are due to mutations in previously identified subunits. We recently showed that Eros, a novel and highly conserved ER-resident transmembrane protein, is essential for the phagocyte respiratory burst in mice because it is required for expression of gp91*phox*-p22*phox* heterodimer, which are the membrane bound components of the phagocyte NADPH oxidase. Eros has a human orthologue, *CYBC1*/*EROS*. We now show that the function of *CYBC1/EROS* is conserved in human cells and describe a case of CGD secondary to a homozygous *CYBC1/EROS* mutation that abolishes EROS protein expression. This work demonstrates the fundamental importance of *CYBC1/EROS* in human immunity and describes a novel cause of CGD.

To the editor:

The multi-subunit phagocyte NADPH oxidase generates reactive oxygen species and is crucial for host defence (1). Deficiencies in individual subunits (gp91*phox*, p22*phox*, p47*phox*, p67*phox* and p40*phox*) cause chronic granulomatous disease (CGD) but some patients with CGD do not have mutations in these genes (2). We recently found that Eros (3), a hitherto undescribed protein, is essential for the generation of reactive oxygen species because it is necessary for protein (but not mRNA) expression of the gp91*phox*-p22*phox* heterodimer, which is almost absent in *Eros*-deficient mice. *Eros*-/- animals succumb quickly following infection with *Salmonella* Typhimurium or *Listeria Monocytogenes*. *Eros* is highly

conserved and has a human orthologue CYBC1 (alias *C17ORF62*), hereafter referred to as *CYBC1* gene and EROS protein). We asked whether the gene fulfilled the same function in humans. We performed CRISPR-mediated deletion of *CYBC1/*EROS in PLB-985 cells, (**Fig. S1A**) and identified two clones with 8bp and 1bp deletions respectively (**Fig. S1B and C**). Neither clone expressed EROS protein (**Fig. 1A**) or detectable gp91*phox* (**Fig. 1B**). p22*phox* expression was also much lower in both *EROS*-deficient clones than in control cells (**Fig. 1C**). We verified the lack of surface gp91*phox* expression by flow cytometry (**Fig. 1D**). Both *CYBC1/*EROS-deficient clones had a severely impaired respiratory burst (**Fig. 1E**). In addition, *CYBC1/*EROS-deficient clones differentiated towards a neutrophil phenotype also demonstrated an impaired respiratory burst (**data not shown**). As expected, re-introduction of *CYBC1/*EROS using a lentiviral vector restored gp91*phox* expression to *CYBC1/*EROS-deficient clones **(Figure 1F**) and oxidase activity as measured by Nitro blue tetrazolium chloride (NBT) test (**Figure 1G**) and DIOGENES assay (data not shown).

We then identified a patient with a homozygous *CYBC1/*EROS mutation in a resource paper that details a thousand Saudi Arabian families with genetic disease(4). He presented with fever, splenomegaly, lymphadenopathy and short stature, but no immuno-phenotyping was detailed at that time. His full clinical history is as follows. He is a Saudi Arabian boy, born in 2007, the son of parents in a consanguineous marriage. He has three healthy older sisters. At 2 months of age, he developed a localized abscess following BCG vaccination. He was then relatively well until 8 years of age but was noted to be of short stature and experienced recurrent pulmonary infections and tonsillitis/pharyngitis despite tonsillectomy.

In August 2015, he became unwell with a febrile illness and an abnormal dihydrorhodamine (DHR) test was noted (**Fig. 2A,B**). He has a severely impaired DHR in response to both PMA

and zymosan. He was also profoundly lymphopenic. He subsequently developed an acute episode of hemolytic anemia in November 2015 and again in January 2017. During 2015, his fevers, infection, lymphopenia and elevated inflammatory markers met criteria for a diagnosis of haemophagocytic lymphohistiocytosis (HLH). He had no mutations previously implicated in HLH pathogenesis. He was treated with cyclosporine and steroids and was transferred to Boston Children's Hospital (BCH) in December 2016 for consideration of bone marrow transplantation. The DHR test was repeated and rhodamine fluoresence was again virtually absent. Further assessment demonstrated granulomatous inflammation in his lungs, and discrete granulomata in his bone marrow, with no evidence of infection. Following his open lung biopsy at BCH, he developed hemolytic anemia and required Intensive Care. He recovered with steroids, however on weaning of this therapy he developed recurrent pleural effusions. Due to his autoimmunity, features of lymphopenia with granulomata, and pleural effusions/hemolytic anemia he was started on sirolimus and he remains in this therapy. His steroids have been weaned to 4mg daily. While reasonably well clinically, he developed diminished anti-pneumococcal antibody responses, worsening lymphopenia, and declining immunoglobulin levels. He therefore underwent a myeloablative bone marrow transplant and has recovered well.

Whole exome sequencing demonstrated that the patient had a homozygous (c.127 A to G, NM_001033046) mutation in *CYBC1/*EROS. His sisters were all heterozygous for this mutation, confirmed by Sanger sequencing (**Fig. 2C**). Based on analysis of other family members and the likely important role of *CYBC1/*EROS in immunity, this mutation was identified as the most likely cause of the patient's disease. The mutation was not present in 10,000 whole genomes from the United Kingdom National Institute for Health Research BioResource - Rare Disease cohort (which includes 1000 patients with primary

immunodeficiency), nor in gnomAD. The variant was also absent in 3,300 ethnically matched exomes. It is, therefore, not seen in seen across 110,579 individuals with sequence data coverage across this position. There were no deleterious mutations in known NADPH oxidase subunits.

Splice site prediction algorithms including Mutation Tasting (**www.mutationtaster.org**) and Human Splicing Finder (**http://www.umd.be/HSF3/**) predict that the variant both disrupts an exonic splice enhancer (ESE) and creates an exonic splice silencer (ESS) which is likely to lead to a retained intron. This intron has 4 in-frame stop codons. It is therefore likely that translation would cease downstream of exon 4. Even if splicing is not disrupted, PolyPhen (**http://genetics.bwh.harvard.edu/pph2/**) predicts that the D43N mutation that would occur in the translated protein is also damaging.

We therefore performed western blot analysis on anti-CD3-CD28-CD2 expanded T cells from the patient, his sister and a healthy control, as well as primary T cells (either pre or post polyclonal stimulation) and peripheral blood mononuclear cells from healthy volunteers. The CRISPR targeted clones described above were used as positive and negative controls respectively. The patient had undetectable levels of EROS protein compared with cells from the healthy control or the primary T cells/PBMC, while his heterozygous sister had intermediate levels (**Fig. 2D**).

This work demonstrates that the function of the novel transmembrane protein, Eros, is conserved in humans. It also represents the first description of an immunodeficiency syndrome secondary to mutations in *CYBC1*/EROS. The severity of the disease seen in this patient underlines the importance of human EROS is for normal immunity. The patient has a

clinical history that is compatible with a diagnosis of autosomal recessive CGD, in that he had both infectious and auto-inflammatory manifestations, together with histo-pathological evidence of granuloma formation in the context of an impaired DHR response. While recurrent infections, BCG-it is (2, 5), granulomatous inflammation and HLH (6) are all recognised sequelae of CGD, this patient some unusual features such as autoimmune haemolytic anaemia. This is uncommon in CGD and may represent an effect of EROS-deficiency that is independent of its effects on the NAPDH oxidase.

The high sequence similarity between mouse and human EROS and the loss of gp91*phox* expression and the phagocyte respiratory burst that accompanies its absence suggests that EROS plays an almost identical role in human and murine immunity. In summary, we have shown that the function of EROS is fully conserved between human and mouse, and that homozygous mutations in EROS underlie a novel sixth cause of chronic granulomatous disease.

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Author Contributions

DCT designed and performed experiments and wrote the manuscript. LC analysed patient samples. **A Schejtman** designed guide RNAs, performed CRISPR-mediated deletion of EROS in PLB-985 cells and NBT assays. EC prepared plasmids for transfection experiments and cultured cells. LD performed western blots and assisted with culture of the PLB-985 cells. AB assisted with western blots and ROS assays. JL advised on and performed electroporation of PLB-985 cells. SC and A.Speak advised on cloning strategies and oversaw plasmid preparation. AT and GS oversaw CRISPR-mediated deletion experiments and advised on other experiments. FSA co-ordinated the sequencing of the patient and provided advice on experiments. HA and HAM treated and diagnosed the patient in Saudi Arabia. TC oversaw the clinical care of the patient in Boston and contributed to writing the manuscript. KGCS oversaw experiments in the project and wrote the manuscript.

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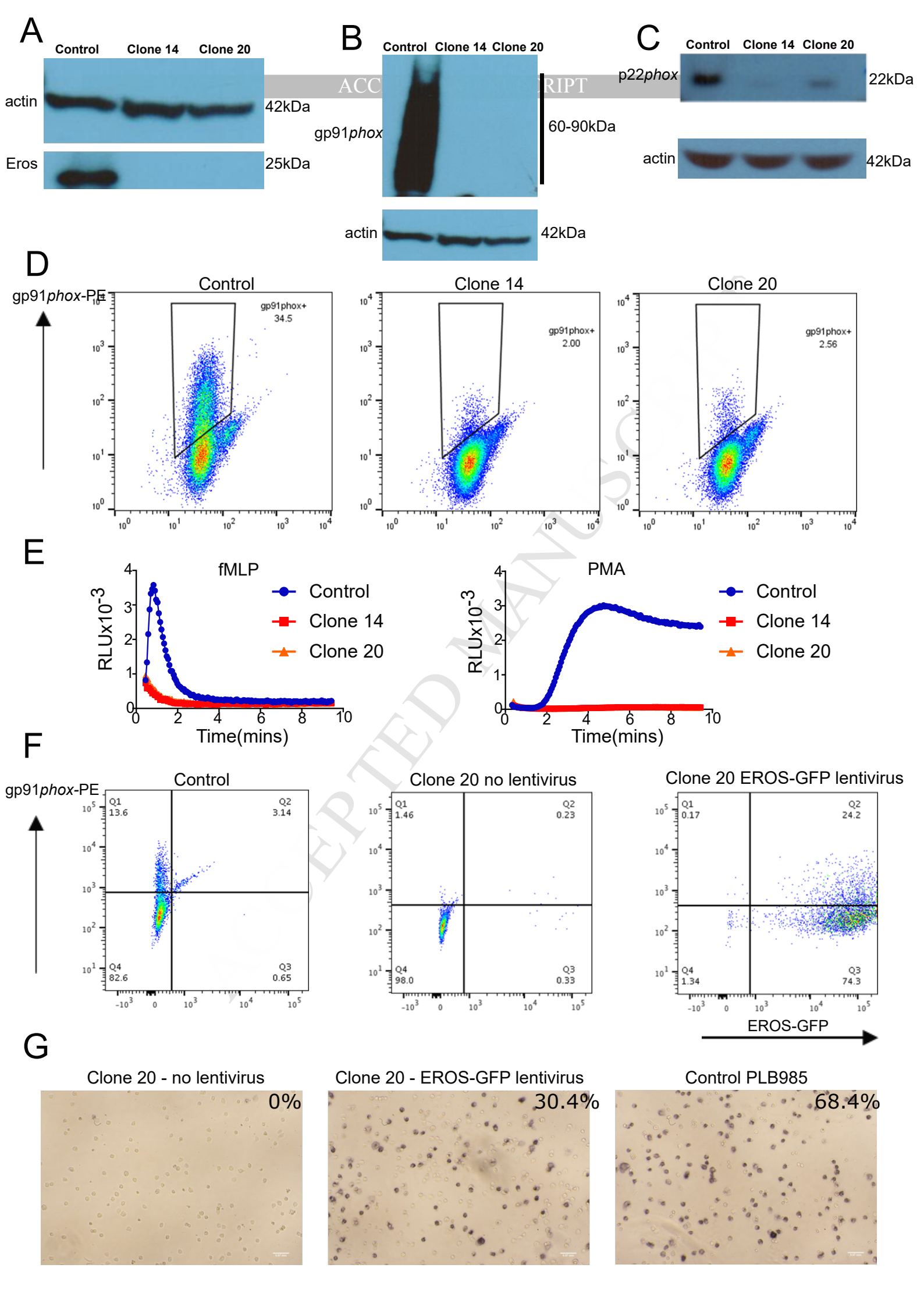
Figure 1: EROS function is conserved in humans. (**A-C**) Western blot of (**A**) EROS (**B**) gp91*phox* **(C)** p22*phox*, (**D**) surface gp91*phox* expression and **(E)** phagocyte respiratory burst in CRISPR targeted PLB-985 clones **(F)** surface expression of gp91*phox* in CRISPR targeted cells +/- overexpression of EROS-GFP. **(G)** NBT reduction following reconstitution with EROS-GFP lentiviral vector. Representative of 3 independent experiments.

Figure 2: **Homozygous *EROS* mutation has deleterious effects. (A, B)**: respiratory burst in patient and healthy control neutrophils. **(C)** homozygous c.127 A to G mutation **(D)** EROS protein expression in CD3/CD2/CD28 expanded PBMC from patient, sister and a healthy control, control CD4+ T cells pre and post CD3/CD2/CD28 or PBMC from a further healthy control. Representative of 2-3 independent experiments**.**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22kDa
actin
42kDa
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gp91phox+
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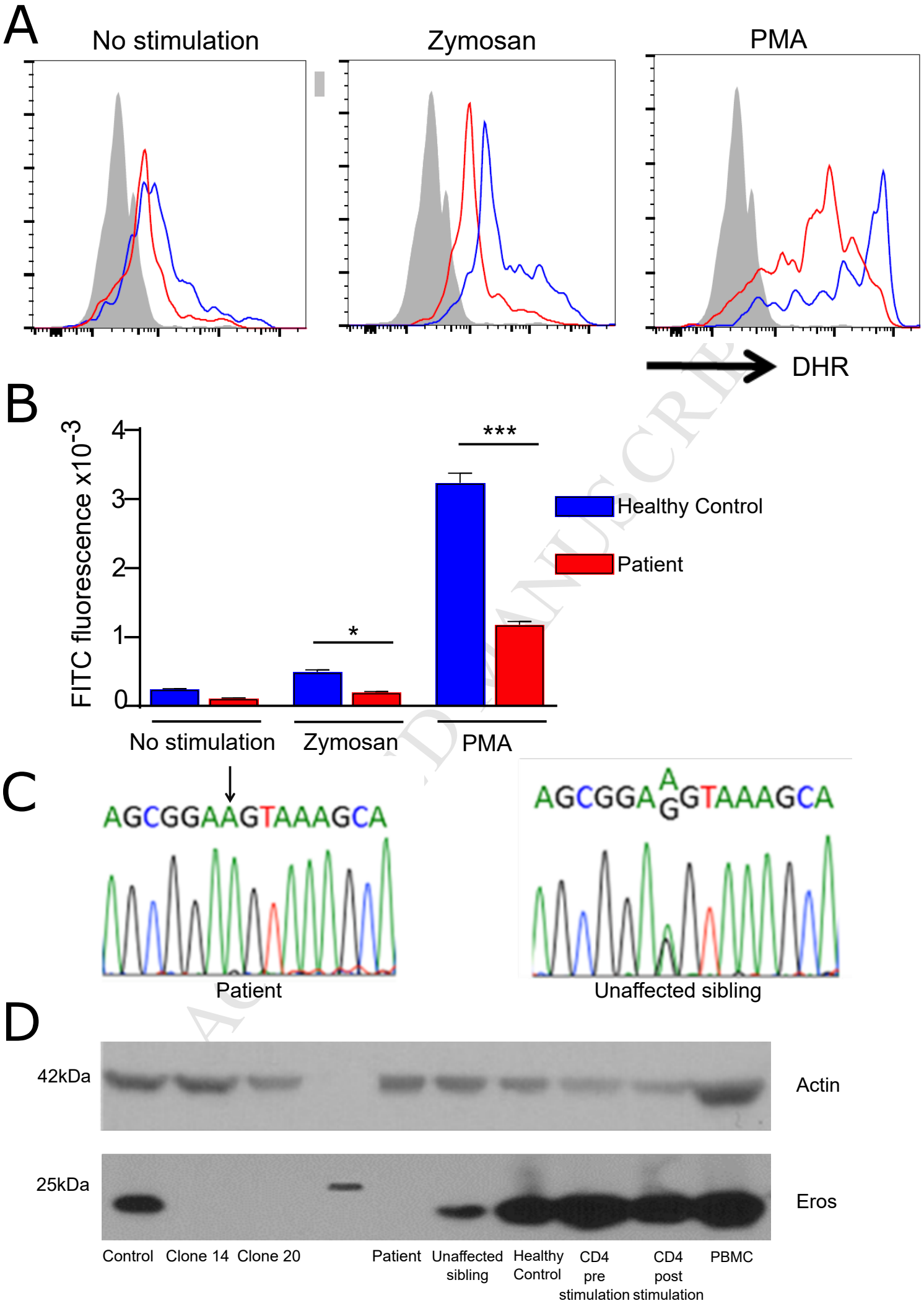
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Patient
No stimulation
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PMA
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Unaffected sibling
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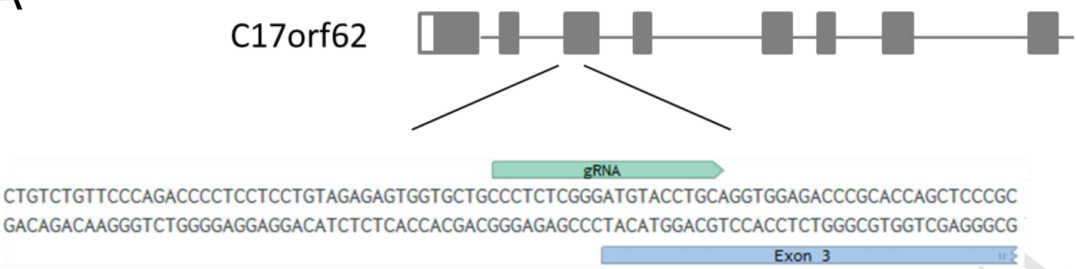
Actin
25kDa
Eros
Control
Clone 14
Clone 20
Patient
Unaffected sibling
Healthy Control
CD4 pre stimulation
CD4 post stimulation
PBMC

Legend for supplementary figure

Figure S1: **Targeting of EROS for deletion by CRISPR-Cas9 (A)** Schematic representation of the human *CYBC1* genomic locus and of guide RNA targeting exon 3 **(B, C)** Sequencing analysis from two clones derived from PLB985 cells that had been electroporated with Cas9:gRNA ribonucleoprotein complex, showing deletion of 8bp in clone 14 and deletion of 1 bp in clone 20.

A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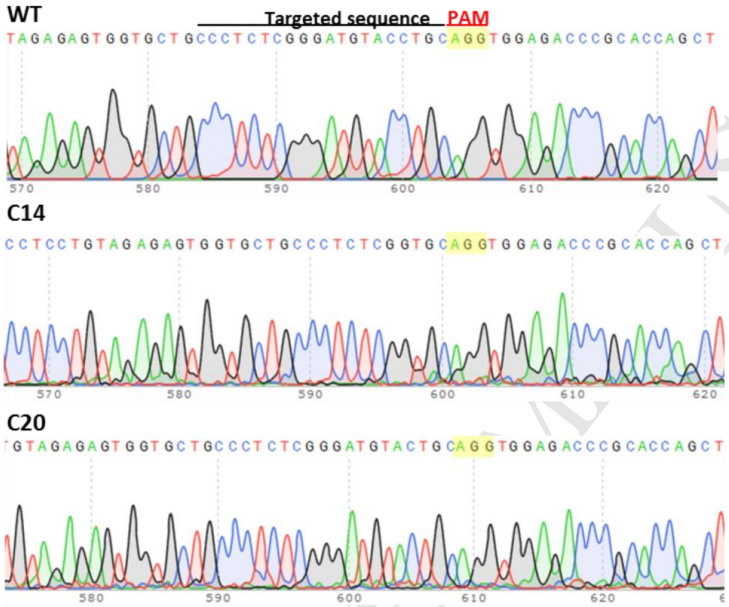
Exon 3

B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610
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C


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