Novel etiology of hereditary erythroid disorders

Ph.D. Thesis

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Declaration/Prohlášení

Hereby I declare that I have written this work on my own under the supervision of Assoc. Prof. Vladimír Divoký, Ph.D., and that all used literature is cited and mentioned in references.

Tímto prohlašuji, že předloženou práci jsem napsala samostatně, pod vedením školitele doc. RNDr. Vladimíra Divokého, Ph.D. a s použitím citované literatury.

In Olomouc/V Olomouci

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Abstract/Abstrakt

Our study defines new etiology of two distinct congenital erythroid disorders: β-thalassemia and polycythemia and provides novel insights into phenotypic heterogeneity associated with the positional effect of *VHL* mutations.

β-thalassemia is a common hereditary hemoglobin disorder characterized by quantitative reduction of functional *β-globin* chains. Earlier, we have reported a novel etiology of β-thalassemia caused by insertion of an LINE-1 element into the intron-2 of the *β-globin* gene, leading to $\beta\text{-globin}_{L1+}$ allele. The exact mechanism how this intronic insertion of transposable element attenuates human *β-globin* gene expression was not known. Therefore, we tested several hypotheses which led to the elucidation of the molecular mechanism leading to the thalassemia phenotype due to the LINE-1 insertion.

Germline heterozygous *von Hippel-Lindau* (*VHL*) gene mutations underlie dominantly inherited familial VHL tumor syndrome comprised of a predisposition for different tumors. However, recessively inherited congenital polycythemia, exemplified by Chuvash polycythemia, has been associated with two separate homozygous 3' *VHL* gene mutations (R200W, H191D). We described and characterized a novel homozygous *VHL* mutation, in exon-2 (c.413C>T):P138L, which is associated in the affected homozygote with congenital polycythemia but not in her, or her heterozygous relatives, with cancer. We also reported a second polycythemic Croatian VHL^{H191D} homozygote and performed several biochemical and molecular tests to better define the phenotype.

Tato práce se zabývá studiem dvou různých typů vrozených poruch erytropoezy: β-talasémie a polycytémie a přináší nové informace k pochopení fenotypové heterogenity asociované s různou pozicí mutací ve *VHL* genu.

β-talasémie jsou vrozené chronické anémie, vznikající v důsledku snížení, nebo absence syntézy β-globinového polypeptidového řetězce. Úplně novou etiologií β-talasémie je inserce funkčního retrotransposonu LINE-1 do druhého intronu *β-globinového* genu. Přesný mechanismus, jakým LINE-1 element může modulovat expresi lidských genů, není znám. V našem případě jsme dokázali, že talasemický fenotyp je důsledek kombinace několika různých defektů na molekulární úrovni.

Vrozené heterozygotní mutace *von Hipple-Lindau (VHL)* genu jsou nejčastěji asociovány s VHL syndromem a různými druhy rakoviny. Zatímco recesivně dědičné homozygotní mutace na 3´konci *VHL* genu, v exonu 3 (R200W, H191D), jsou příčinou vzniku polycytémie, jejíž nejznámějším příkladem je tzv. Čuvašská polycytémie. U nově diagnostikované pacientky s vysokou hladinou hemoglobinu a erytropoetinu jsme objevili a popsali doposud nepublikovanou homozygotní mutaci v druhém exonu *VHL* genu (c.413C>T):P138L. Jde o první mutaci v druhém exonu *VHL* genu, která je asociovaná jen s polycytémií a nikoliv s nádorovým onemocněním. Podrobně jsme také popsali druhý případ výskytu VHL^{H191D} mutace v Chorvatsku.

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I Theoretical Background

1 Introduction

1.1 The erythropoiesis

Hematopoiesis is dynamic process, where the hematopoietic stem cells (HSC) give rise to all of the different mature blood cell types (myeloid and lymphoid lineage). HSCs are self-renewing and multipotent, i.e. have the potential to develop into all blood cells, but cannot develop to other cell types.

Traditionally, the hematopoietic process is divided into primitive and definitive hematopoiesis based on the developmental program and type of blood cells generated. During embryogenesis, the yolk sac derived hematopoiesis occurs in two distinct waves in the extraembryonic blood islands. The first wave produces primitive macrophages and primitive erythrocytes, thus providing the developing embryos with oxygen [1]. The second wave of the yolk sac hematopoiesis is a transient wave of definitive erythroid precursors that enter early embryonic circulation. The long-term definitive hematopoiesis produces multipotent blood cells from the hemogenic endothelium of the embryo that includes the aorta-gonad-mesonephros (AGM) region of the embryo; these cells also seed the yolk sac, and mainly fetal liver. After the birth, the site of adult hematopoiesis, where HSCs undergo differentiation to generate lineage-committed progenitors and self-renewal to maintain a constant supply of HSCs, is bone marrow. The shifting power during the developmental stages of hematopoiesis, also connected with hemoglobin switching, is thought to be regulated predominantly at the transcriptional level by a network of several transcription and chromatin remodeling factors.

Erythropoiesis is physiological process of the red blood cells (RBCs, erythrocytes) production. The first step in HSCs differentiation takes common multipotent CFU-GEMM progenitor (colony-forming unit granulocytic, erythroid, megakaryocyte, macrophage), followed by generation of committed erythroid progenitors: "early" BFU-E (burst forming unit – erythroid), and "later" CFU-E (colony forming unit – erythroid) [2]. Already committed erythroid progenitors develop into the morphologically distinguishable erythroid precursors – proerythroblasts and erythroblasts. The final stages of maturation are accompanied by hemoglobin synthesis and nucleus extradition - the reticulocytes and mature erythrocytes are produced. Erythropoiesis generates ~ 2×10^{11} new erythrocytes (1% of the total red cell mass) every day and the same amount is removed every day from the circulation [3]. The mature RBCs transport oxygen from the lungs to the rest of the body and then return carbon dioxide from the body to the lungs.

1.1.1 Morphology and composition of the erythrocyte

Normal RBCs have a diameter of 7.5 to 8.7 μm, average volume of 90 fl and a surface area approximately 136 μm^2 [3]. The normal resting shape of the erythrocyte is a flexible biconcave disc. The erythrocyte spends most of its circulatory life span (100- to 120- days) within capillary channels of the microcirculation. The erythrocyte's membrane has a unique capacity to "tank-tread" – rotate around the red cell contents. This arrangement transmits shocks from wall contact through the membrane to the viscous hemoglobin solution in the interior rather than concentrating the energy of contact in the membrane [3].

Hemoglobin (Hb) is a two-way respiratory carrier, enables RBCs delivering oxygen from the lungs to the tissues and facilitating the return transport of carbon dioxide [4]. The Hb synthesis in erythroid cells is dependent on three distinct processes: synthesis of globins, synthesis of heme and iron intake. All of these processes have to be tightly regulated and coordinated in order to prevent pathological conditions such as anemia, porphyria, hemochromatosis or polycythemia.

Normal mammalian Hb contains two pairs of unlike polypeptide chains: one chain of each pair is α or α-like (ζ) and the other is *β*, or *β*-like (*γ, δ* or *ε*). The α-chains of all human hemoglobins, except for some embryonic hemoglobins, are the same during all developmental stages. The non-α chains include the *β*-chain of normal adult hemoglobin (Hb A (α2*β*2)), the γ-chain of fetal hemoglobin (Hb F (α2*γ*2)), and the *δ*-chain of hemoglobin A2 (Hb A2 (α2*δ*2)), the minor component which accounts for 2.5% of the Hb of normal adults [3]. The globin gene switches occur during development: the embryonic to fetal globin switch (from ζ- to α-chain and *ε*- to *γ*-chain), which coincides with the transition from embryonic (yolk sac) to definitive (fetal liver) hematopoiesis; and the fetal to adult switch, which occurs at the perinatal period [5]. The α-*globin* gene cluster is located on chromosomes 16 and *β-globin* gene cluster on the short arm of chromosome 11.

Each globin subunit must form a stable linkage with heme situated on the external surface of the protein so that oxygen in the RBC cytosol can bind reversibly to the heme's iron atoms [6]. A heme (ferrous protoporphyrin IX) is a chemical compound which serves as a prosthetic group consisting of an iron ion (Fe^{2+}) situated in the center of a large heterocyclic organic ring called a porphyrin (four pyrrolic groups joined together by methine bridges). The erythroid heme biosynthesis pathway involves 8 different enzymes, when first and last three are mitochondrial and the intermediate four are cytosolic [7]. Approximately 85% of heme is synthesized to meet requirements for hemoglobin synthesis, the rest is synthesized in the liver, when is largely required for cytochromes P450 [8]. The iron metabolism and the regulation of heme synthesis are different in hemoglobin-synthesizing as compared with non-erythroid cells, but limiting factor is always the availability of iron.

Iron is an element essential for living organism, but can be also toxic due to its capacity to react with oxygen and catalyze the production of reactive oxygen species [9]. Much of the iron in the human cells occurs in heme form, when hemoglobin, which is 0.34% iron in weight, contains approximately 2 g of body iron in men and 1.5 g in women [3]. The heme iron is continuously recycled following phagocytosis and catabolism of senescent RBCs by the macrophages of reticuloendothelial system. Only a small part represents iron absorbed through the gastrointestinal tract, mostly through the duodenum. The amount of iron absorbed is tightly regulated according to body needs.

The central regulator of iron homeostasis is the hepatic antimicrobial peptide hepcidin. Ferroportin serves as the receptor for hepcidin and is destroyed when the complex is formed [3]. Ferroportin transports iron across basolateral membrane of enterocytes into blood

circulation. Ferroportin-associated enzyme hephaestin oxidizes the ferrous iron to the ferric form. Once ferric iron is released to the plasma by ferroportin, it is bound by transferrin, which after forming a complex with the transferrin receptor, transports the metal into erythroid cells. Once bound to the receptor, the transferrin-iron complex is internalized and iron is released inside the cells into an acidified vesicle. Within the vesicle, STEAP3 (STEAP family member) effects the reduction of ferric to ferrous iron and another protein DMT-1 (divalent metal transporter 1) induces the release of Fe^{2+} into the cytosol, where it is taken up by mitochondria for heme synthesis [3].

1.1.2 Regulation of erythropoiesis

Many hormones/cytokines, receptors and transcription factors control the development of RBCs from HSCs, but the primary controlling molecular hub is erythropoietin-receptor (EPOR) signaling pathway, with erythropoietin (EPO) as principal regulating hormone. Among the other growth factors, which positively stimulate erythropoiesis, are stem cell factor, interleukin-3, granulocyte macrophage colony-stimulating factor and trombopoietin. Each maturation stage requires for growth and survival different combinations of cytokines, but the first EPO-dependent stage of erythroid differentiation represents CFU-E. The key lineage-specific transcription factors are GATA-1 (GATA-binding factor 1), KLF-1 (erythroid Krüppel-like factor 1) or NF-E2 (nuclear factor - erythroid-derived 2), that are absolutely necessary for normal erythropoiesis and activate many erythroid specific genes, including those for globins and erythroid specific membrane proteins.

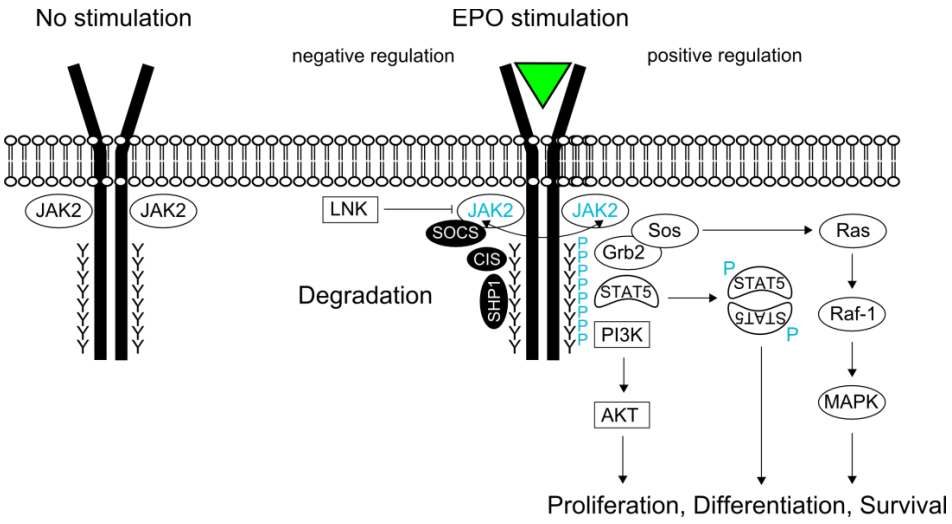

Figure 1. Schematic representation of EPO/EPOR signaling pathway. Conformational changes of the receptor after the EPO binding induce the transphosphorylation of associated JAK2 molecules and phosphorylation of tyrosine residues in the cytoplasmic tail of the receptor, which allows binding and phosphorylation of signaling molecules. Grb2/Sos complex is adaptor which mediates the cross-talk between EPO/EPOR and Ras/Raf/MAPK pathways. Negative regulators (e.g. SOCS, CIS) are transcriptionally induced by the activated JAK2. See text for details.

EPO is heavily glycosylated protein, which is produced primary in kidney, by peritubular interstitial fibroblast [1] in response to O_2 tension. EPO production is regulated almost exclusively by hypoxia at the transcription level (described in detail below, chapter 1.1.3). EPO is not stored but secreted immediately [2]. EPO binds to the cognate receptor (EPOR) of erythroid progenitor cells promoting the survival, proliferation and differentiation of these cells.

EPOR belongs to the hemopoietin/interferon super-family of receptors, which lack intrinsic catalytic function therefore has to be associated with signal-transducing proteins. EPO ligation on its receptor induces the conformational alteration of the pre-existing receptor dimers allowing associated tyrosine kinase Janus kinase 2 (JAK2) activation [3]. Activated JAK2 phosphorylates multiple key tyrosine residues in the EPOR cytoplasmic domain, thereby provides docking sites for downstream signaling molecules containing Src homology 2 domain. The signaling cascade initiated by EPO binding includes multiple pathways, e.g. the STATs, PI-3K/AKT, Ras/Raf/MAPK (**Figure 1**). Beside JAK family kinase (which is primary and critical), erythroid cells can facilitate cellular signaling also via Src family kinase members, e.g. Lyn [4], but its precise involvement in erythropoiesis is not fully elucidated. An important feature of EPOR signaling is its temporal activation of down-stream negative regulators, such as CIS (cytokine inducible SH2 protein) or SOCSs proteins (suppressors of cytokine signaling) and phosphatase SHP1 (Src homology region 2 domain-containing phosphatase-1), which represent the classical feedback loop [5]. In addition, LNK (SH2B adaptor protein 3) adaptor molecule, through its SH2 domain, negatively modulates EPOR signaling by attenuating JAK2 activation, and regulating EPO-mediated erythropoiesis [6].

1.1.3 Regulation of oxygen sensing

All nucleated cells in the body sense and respond to conditions of reduced oxygen availability (hypoxia). Under hypoxia, hypoxia-inducible factors (HIFs) regulate the expression of genes that mediate adaptive response. Within any given cell type, HIFs control the expression of hundred genes, including these that promote glucose uptake, facilitate glycolysis, inhibit the Krebs cycle, increase mitochondrial electron-transport chain efficiency, promote iron mobilization, stimulate angiogenesis, regulate apoptosis and increase the synthesis of EPO [1]. Oxygen depending-regulation of HIFs is depicted in the **Figure 2**.

HIF is a heterodimeric transcription factor that consists of an O_2-sensitive α-subunit and a constitutively expressed β-subunit, which is insensitive to changes in oxygen tension and is identical to aryl hydrocarbon receptor nuclear translocator (ARNT) [2]. Three HIF α-subunits are known, HIF-1α, HIF-2α and HIF-3α [3]. HIF-1 was first identified in human Hep3B hepatoma cells using DNA sequences that were derived from the 3'-hypoxia enhancer of the *EPO* gene [4]. HIF-1α has a ubiquitous pattern of expression in tissues, whereas HIF-2α is restricted to certain cell types including endothelial cells, cardiomyocytes, hepatocytes or glial cells [5]. Less is known about HIF-3α which in certain contexts have been shown to be inhibitory [6], whereas HIF-1 and HIF-2 heterodimers are transcriptional activators.

In well-oxygenated cells (normoxia), HIF-α-subunits are continuously synthesized and rapidly degraded. The key event regulating oxygen-sensitive turnover is, in case of HIF-1α, hydroxylation of Pro^{402} or Pro^{564} (Pro-OH), or both, by the prolyl hydroxylase domain protein 2 (PHD2), which is a dioxygenase that utilizes O_2 and α-ketoglutarate as substrates while generating CO_2 and succinate as by-products [7]. Hydroxylated HIF-1α interacts with

the von Hippel-Lindau (VHL) tumor-suppressor protein which recruits an ubiquitin E3 ligase. The polyubiquitination of HIF-1α flags the protein for degradation by the 26S proteasome. Factor inhibiting HIF-1 (FIH-1) also uses oxygen to hydroxylate HIF-1α on an asparagine residue 803 (Asn-OH) [7]. HIF-1α containing Asn-OH cannot be bound by the coactivator protein p300, thereby preventing HIF-1α from activating gene transcription [8].

Under hypoxic conditions PHD2 activity is reduced due to substrate limitation, inhibition of the catalytic center, or both [9]. Proline and asparagine hydroxylation reactions are inhibited, and HIF-α (i.e., either HIF-1α or HIF-2α) rapidly accumulates, dimerizes with HIF-1β, recruits p300, binds to hypoxia response elements, and activates the transcription by RNA polymerase II (Pol II) of hundreds of target genes [10]. Thus, HIF-α hydroxylation provides a mechanism for transducing changes in oxygen availability to the nucleus as changes to gene transcription.

Although *in vitro* approaches identified HIF-1 as the transcription factor responsible for the hypoxic induction of EPO, HIF-2 has now emerged as the main regulator of EPO production *in vivo* [11]. Perturbation of PHD-VHL-HIF pathway leads to the development of benign erythrocytosis/polycythemias that are associated with increased or inappropriately normal serum EPO levels (with respect to hemoglobin levels, described in detail below, chapter 1.2.2.3).

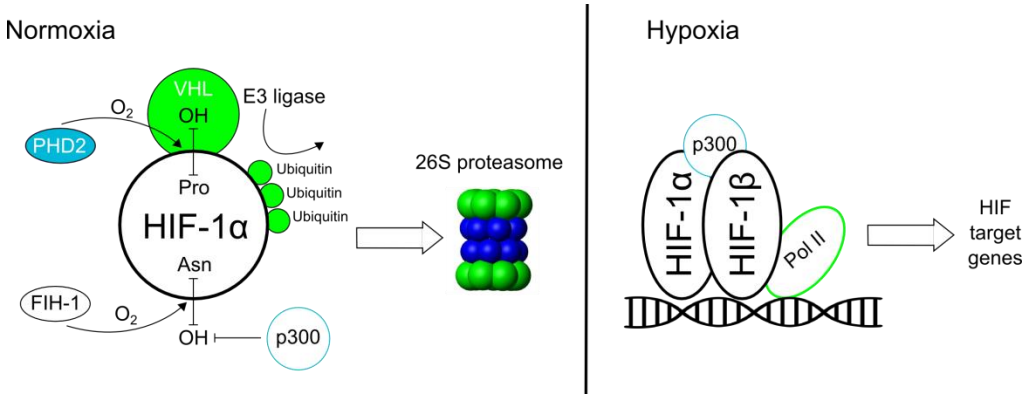

Figure 2. Oxygen sensing, gene expression and adaptive response to hypoxia [adapted from ref. 10]. (Left) Under normoxic conditions, PHD2 protein constitutively hydroxylates HIF-1α on specific prolyl residues, and FIH-1 constitutively hydroxylates HIF-1α on a specific asparaginyl residue. Hydroxylated HIF-1α subunit is recognized by VHL, a component of an E3 ubiquitin ligase complex and targeted for degradation by the proteasome. (Right) Under hypoxic conditions, HIF hydroxylases are inactivated allowing HIF-1α to heterodimerises with HIF-1β, binds DNA, recruits the p300 co-activator to form an active complex leading to transcription of downstream genes. See text for details.

1.2 Clinical manifestation and classification of erythroid disorders

Erythroid disorders are traditionally divided into two groups: (1) anemia and (2) polycythemia, which both can be caused by acquired or inherited genetic defects. In general,

anemias are characterized by a decrease and polycythemias by an increase of the red cell mass.

1.2.1 Anemia

There are several different ways of classifying anemia. Anemia is defined by the World Health Organization (WHO) as Hb < 120 g/l in women and Hb < 130 g/l in men. Hemoglobin concentration expresses the oxygen-carrying capacity of blood, which is in fact decreased in anemia. Based on determination of the red cell mass, anemia can be classified as relative or absolute. Relative anemia represents the states, where Hb concentration falls as the result of an increase in the plasma volume and red blood cell mass is not influenced. Classification of absolute anemias with decreased red cell mass is very difficult. In general, the anemias are caused by decreased production or increased destruction of red cells. Pathophysiological classification of anemias is listed in **Table 1**. Since we investigated the novel molecular mechanism of thalassemia, only pathophysiology of this hereditary disorder of globin synthesis will be further discussed.

In thalassemia, there are defects in the production of either the α-like (α-thalassemia) or in β-like (β-thalassemia) globin chains resulting in an imbalance between production of globin chains and deleterious effect of the globin subunits that are produced in excess. Less common forms of thalassemia include γ-, γδβ-, δ- and εγδβ- thalassemias [1]. There are 95 different mutations causing α-thalassemia described worldwide [2]. The majority of most common α-thalassemia determinants are due to deletions that remove some, or all, of the α-*globin* gene cluster. More than 200 β-thalassemia alleles have been described in the database of human hemoglobin variants, which involve mutations affecting any of the stages from transcription to RNA processing and translation of *β-globin* mRNA [3]. New etiology of β-thalassemia was described by us, when insertion of the full-length transposable element LINE-1 into the intron-2 of the *β-globin* gene caused severe reduction in *β-globin* mRNA production.

WHO calculations estimated that at least 5.2% of the world population (and over 7% of pregnant women) carry an affected Hb allele [4] and about 60 000 severely affected infants are born every year [5]. In addition, at least 20% of the world population suffers from α-thalassemia [4], which is more frequently and widely distributed than β-thalassemia. Epidemiological studies strongly suggest that in populations in which malaria is (or was) endemic, individuals with mild form of either α-thalassemia or β-thalassemia trait are protected against *Plasmodium falciparum* infection, which explains the high carrier frequency via natural selection.

The α-*globin* gene is duplicated (α1 and α2) on each copy of chromosome 16, so there are a total of four α-*globin* genes in a normal genotype (αα/αα). The clinical severity of α-thalassemia relates to the number of genes affected, when clinically significant types are Hb H disease and Hb Bart's hydrops fetalis [6]. There are three broad clinical phenotypes in patients with β-thalassemia: minor, intermedia and major. These phenotypes are associated with mutations that either reduce (β^+-thalassemia) or abolish (β^0-thalassemia) expression of *β-globin* gene. The only forms of treatment for thalassemic patients are regular blood transfusions, iron chelation therapy (to prevent iron overload) and splenectomy (in cases complicated by hypersplenism). In case of β-thalassemia, experimental approaches (pharmaceutical agents e.g. azacitidine and decitabine [7] or somatic gene therapy [8]) are used to increase expression of *γ-* or *β-globin* genes in order to restore the balance between globin chains production.

Table 1. Classification of Anemia [adapted from ref. 9]

A. RELATIVE

1. Macroglobulinemia
2. Pregnancy
3. Nutritional deficiency
4. Splenomegaly

B. ABSOLUTE

1. Anemia predominantly caused by decreased red cell production
   a) Disturbance of proliferation and differentiation of hematopoietic stem cells
      1. Aplastic anemia
      2. Anemia of leukemia and of myelodysplastic sydromes
   b) Disturbance of proliferation and differentiation of erythroid progenitors
      1. Pure red cell aplasia
      2. Anemia of chronic renal failure
      3. Anemia of endocrine disorders
      4. Congenital dyserythropoietic anemia
   c) Disturbance of DNA synthesis (megaloblastic anemia)
      1. Vitamin B_{12} deficiency
      2. Folic acid deficiency
      3. Acquired and congenital defects in purine and pyrimidine metabolism
   d) Disturbance of hemoglobin synthesis (hypochromic anemia)
      1. Iron deficiency
      2. Congenital atransferrinemia and idiopathic pulmonary hemosiderosis
      3. Thalassemia
   e) Disturbance of erythropoietic regulation
      1. Low oxygen affinity hemoglobinopathies
   f) Unknown or multiple mechanisms
2. Anemia caused predominantly by increased erythrocyte destruction or loss
   a) Intrinsic abnormality
      1. Membrane defect
         (a) Hereditary spherocytosis, elliptocytosis, and pyropoikilocytosis
         (b) Hereditary acanthocytosis and stomatocytosis
      2. Enzyme deficiency
         (a) Glucose-6-phosphate dehydrogenase deficiency
         (b) Pyruvate kinase and other enzyme deficiency
         (c) Porphyria
      3. Globin abnormality (hemoglobinopathy)
         (a) Sickle cell disease and related disorders
         (b) Unstable hemoglobins
      4. Paroxysmal nocturnal hemoglobinuria
   b) Extrinsic abnormality
      1. Mechanical
      2. Chemical or physical
      3. Infections
      4. Antibody-mediated
      5. Hyperactivity of the monocyte-macrophage system
      6. Blood loss

1.2.2 Polycythemia

Polycythemia (also known as erythrocytosis) is characterized by an increased red cell blood mass. Polycythemias can be primary or secondary. Primary polycythemias are caused by somatic or germline mutations leading to changes within the erythroid progenitors causing an augmented response to erythropoietin. Secondary polycythemias are caused by either an appropriate or inappropriate increase in the red cell mass as a result of augmented levels of erythropoietin. In some instances, exemplified by Chuvash polycythemia (CP) [1], the first known congenital disorder of hypoxia sensing, erythroid progenitors are in *in vitro* cultures hypersensitive to EPO; thus, Chuvash polycythemia shares features of both primary and secondary polycythemia. Classification of polycythemias is listed in **Table 2**.

Table 2. Classification of Polycythemia/Erythrocytosis [adapted from ref. 2]
A. RELATIVE (decreased plasma volume)
1. Dehydration
B. APPARENT (normal plasma and red cell volume)
1. Stress or smoker's erythrocytosis
C. ABSOLUTE (increased red cell volume)
1. Primary a) Polycythemia vera b) Primary familial and congenital polycythemia 2. Secondary a) Appropriate 1. Altitude 2. Cardiopulmonary disorder 3. Increased hemoglobin affinity for oxygen b) Inappropriate 1. Renal cysts and tumors 2. Hepatoma 3. Cerebellar hemangioblastoma 4. Essential 5. Augmented hypoxia-sensing

1.2.2.1 Primary polycythemia

Polycythemia vera (PV) and primary familial and congenital polycythemia (PFCP) are primary polycythemic disorders with erythroid progenitors hypersensitive to EPO. They are caused by somatic (PV) or germ-line (PFCP) mutations that are intrinsic to erythroid progenitors and result in an augmented response to EPO [2].

PV is an acquired clonal hematopoietic stem cell disease characterized by increased production of erythrocytes, granulocytes, platelets and largely polyclonal T lymphocytes [3]. PV erythroid progenitors exhibit disease-specific functional characteristic, i.e. erythropoietin independent colony-formation, a hallmark of PV [4]. Over 95% of PV patients carry a somatic *JAK2*^{V617F} gain-of-function mutation [5]. *JAK2*^{V617F} in a large proportion of the PV cases is homozygous due to acquired uniparental disomy on chromosome 9p [6]. Evidence suggests that *JAK2*^{V617F} is not the disease-initiating mutation and constitutes only part of the

clone [7,8]. Thus, the full mutation landscape and whether and how these alterations contribute to disease initiation and clonal evolution or myelofibrotic transformation are not fully deciphered.

PFCP is congenital disorder characterized by an autosomal-dominant mode of inheritance [9]. The distal cytoplasmic region of the EPOR, in association with SHP-1, is required for down-regulation of EPO-mediated activation of JAK2/STAT5 proteins. To date, ten mutations of the *EPOR* have been convincingly linked to PFCP [10]. All of these mutations result in truncation of the EPOR cytoplasmic carboxyl terminus, leading to loss of its negative regulatory domain and resulting in a gain-of-function of the EPOR.

1.2.2.2 Secondary polycythemia

Secondary polycythemia may be subdivided into appropriate and inappropriate, when the former responding normally to tissue hypoxia (i.e. high-altitude polycythemia and hemoglobins with increased affinity for oxygen) and the latter is stimulated by aberrant production of EPO.

Polycythemia is often considered a universal, uniform adaptation to hypoxia that would arise in all normal individuals, but high altitude population e.g. Tibetans, are genetically adapted to the environmental stress of high altitude, having normal Hb concentration both at sea level and at high altitude. Several haplotypes have undergone positive selection in Tibetans, including variations at or near the *EGLN* locus, which encodes PHD2. *PHD2*^{D4E} and *PHD2*^{C127S} alterations that originated on the same haplotype about ~6,000 years ago contribute to protection from polycythemia at high altitude, by abrogation of hypoxia-induced HIF-mediated augmentation of erythropoiesis [1].

1.2.2.3 Congenital disorders of hypoxia sensing

Chuvash polycythemia has autosomal-recessive trait and is the first recognized hereditary condition of augmented hypoxia-sensing [2]. The Chuvash people reside in the mid-Volga River region in Russia where CP affects hundreds of individuals (homozygous *VHL* c.598C>T; *VHL*^{R200W} mutations), making it the most common congenital polycythemia [3]. Outside of Chuvashia, CP has also been found sporadically in diverse ethnic and racial groups [4,5,6] and a high prevalence of this disorder was reported in the Italian island of Ischia [7]. *VHL*^{R200W} mutation impairs the interaction of VHL with α-subunit of HIF, thus reducing the rate of ubiquitin-mediated HIF destruction. As a result, the levels of the HIF-1 and HIF-2 heterodimers increase and lead to increased expression of target genes including *EPO*, *vascular endothelial growth factors* (*VEGF*), and *plasminogen activator inhibitor* (*PLA*) among others [3].

Clinically, CP patients are predisposed to develop thrombosis, bleeding, cerebrovascular events, and increased mortality independent of the increase in hematocrit [8]. Despite increased expression of HIF-1α and VEGF in the normoxic state, which has been proposed to be related with development of hemangioblastoma and renal cell carcinoma [9], CP patients do not display a predisposition to tumor formation [8]. In contrast, autosomal dominant mutations of the *VHL* gene cause VHL syndrome [9]. Heterozygotes for these dominant *VHL* mutations are at increased risk of developing hemangioblastomas, renal cell carcinoma, pheochromocytoma, pancreatic endocrine tumors, and endolymphatic sac tumors when they acquire a somatic mutation in the normal *VHL* allele in trans [10,11].

Some patients with VHL syndrome also develop acquired polycythemia due to EPO production by a tumor [9].

Other than *VHL*^{R200W} germline mutations also cause polycythemia. Some patients with congenital polycythemia have proven to be compound heterozygotes for the Chuvash mutation, a few cases of congenital polycythemia, known to have mutations of only one *VHL* allele were reported, but lacking an obvious pathophysiological explanation [12].

The mutation in PHD2 (*PHD2*^{P317R}) was identified in the family, in which heterozygotes for this mutation have mild or borderline polycythemia [13]. The P317R mutation affects a residue that is in very close vicinity of the catalytic domain and impairs binding to both HIF-1α and HIF-2α. Since then, five additional patients with unexplained polycythemia who are heterozygote carriers of different *PHD2* mutations have been reported [14]. Almost all patients with *PHD2*-associated polycythemia have normal EPO level. If the cause of the polycythemia is the haploinsufficiency or dominant negative effect remains unsolved.

Study of different families with polycythemia also revealed the presence of heterozygous missense mutation in the coding sequence of *HIF-2α*. Patients with *HIF-2α* mutations have typically elevated EPO [14]. There is heterogeneity in the functional defects associated with *HIF-2α* mutations but all the findings support a critical role of *HIF-2α* in controlling the expression of human EPO.

1.3 Mobile elements and human diseases

1.3.1 Mobile elements

Human genome is flooded with the repetitive sequences capable of moving to new locations, by process known as a 'transposition' (excision of the sequences from current genomic location and insertion into a new genomic site). Transposable elements (TEs; also known as "jumping genes") occupy almost half, 45% [1], of the human genome, making the TE content of our genome one of the highest among mammals, second only to the opossum genome with a reported TE content of 52% [2]. The actual contribution of repeats to mammalian genomes is significantly larger, as the older, ancestral repeats have diverged beyond the current recognition limit [3].

TEs can be separated, based on their mechanism of replication, into two major classes: DNA transposons and retrotransposons. DNA transposons, which move by a 'cut-and-paste' mechanism using an encoded transposase gene [4] are currently not mobile in the human genome, they were active during early primate evolution until ~37 million years ago (Myr) [5]. In contrast, retrotransposons duplicate through RNA intermediates that are reverse transcribed and inserted at new genomic locations, referred as 'copy-and-paste' mechanism [5]. Retroelements are subdivided into two major groups: those containing long-terminal repeats, LTR retroelements, and all others belong into the category of non-LTR retroelements [2]. LTR retroelements are endogenous retroviruses, which account for ~8% of the human genome but their activity is presently very limited, when the peak of accumulation was estimated ~25 Myr [6]. Non-LTR retrotransposons include autonomous and non-autonomous members. The autonomous long interspersed element-1 (LINE-1 or L1), and its non-autonomous partners, such as 'SINE-R, VNTR, and Alu' (SVA) and the short interspersed element (SINE), are the only mobile elements with clear evidence of current retrotranspositional activity in the human genome [2], as indicated by the more than

96 reported cases of *de novo* insertions that are responsible for genetic disorders and cancer [5,7,8]. SVA and SINEs lack the ability to retrotranspose, but can co-opt L1 machinery for their replication [9].

There are >500,000 L1 copies in the human genome as a result of their continued mobilization activity over the past 150 Myr [5]. Most L1s are inactive due to point mutations, rearrangements, or truncations with only a subset of estimated 80 - 100 elements [8], currently functional in any individual. A substantial fraction of the human genome, >30%, is derived directly or indirectly from L1 retrotransposon activity [8], which makes them the most successful TEs in the human genome by mass. Although L1 transcription and retrotransposition may occur in any cell type at the level of an individual, only events occurred during germ-cell development will be incorporated into the germline lineage and contribute to future generations [3]. However, a growing evidence has indicated that somatic retrotransposition in mammals not only occurs, but is likely to occur at a substantial frequency [10].

The ability of TE to cause diseases via the inactivation of genes by insertional mutagenesis together with the abuse of cellular mechanisms for their own propagation has long been characterized as "parasitic". But there are also several ways how L1 can generate new alleles. When L1 retrotransposes it can also co-transpose adjacent non-L1 sequences to its new integration site. In this process (designated as '3' transduction') the presumed L1 polyadenylation signal is bypassed in favor of a downstream endogenous polyadenylation signal, allowing downstream flanking host sequences to "come along for the ride" [11]. Similarly, '5' transduction' occurs when a cellular promoter, localized upstream to the donor L1, transcribes both 5' adjacent sequence and L1 which is then subjected to reverse transcription/integration [11]. Some of these L1-generated alleles have survived the test of time, and strongly suggest that L1 may create a platform of potential alleles that can be subjected to the forces of natural selection. Nowadays there is increasing tendency to assume, that TEs are cultivated in the genome for their beneficial possibilities, in particular, to drive genome evolution and alter gene expression [7,12] – key components of plasticity necessary for adaptation and survival.

1.3.2 The role of mobile elements in the pathogenesis of human diseases

The fact that many sporadic human diseases have sub-group of unknown etiology suggests a possibility that TE-associated DNA disruption plays crucial role at least in some of them. While the disruption of normal gene function by transposable elements upon integration into exonic regions is obvious, their post-insertional effects on gene expression have not received much attention [1]. Up to date, the known impact on the expression of host genes is via modification of the transcript quality or quantity [2], transcriptional interference [3], or by the control of pathways that affect the mRNA life-cycle [4]. Germline mutations caused by TE insertions have led to several human genetic disorders such as cystic fibrosis, retinitis pigmentosa, hemophilia etc. Recently, the new generation sequencing techniques allow identification of genome and transcriptome aberrations on the level of individuals and help to clarify the extent of the contribution of mobile elements to genetic instability in many human diseases, including different types of cancer.

Genetic instability is one of the key features associated with cancer. In contrast to normal cells, the majority of human cancers, and cancer-derived cell lines, support variable, but typically much higher endogenous full-length L1 mRNA expression [5]. The major mechanisms for silencing of TE's potentially harmful retrotransposing activity is DNA

methylation at the CpG site in the L1 promoter [6] and malignant tumor cells are generally featured by global hypomethylation, including L1 [7]. The hypomethylation of L1 has been reported in urothelial bladder carcinoma, malignant testicular tumors and prostatic adenocarcinoma [8]. The association of retroelement expression with genetic instability in cancer remains largely correlative [9,10], but the observations of augmented mutation rates in the majority of human cancers suggest, that retroelement expression and presumably activity could be one of the forces which accelerate tumor evolution [5].

Another negative fitness consequence arising from the proliferation of TE sequences within mammalian genomes involves the tendency to serve as sources of homology for non-allelic homologous recombinations (NAHR) [11]. Such misrouted recombination events involving TEs represents a viable mechanism for large-scale chromosomal translocation mutations [12]. A much larger fraction of TE-related diseases in humans results from recombination mutations than from insertional mutations [11]. NAHR events have been well recognizes as a major source of DNA damage that leads to either duplications deletion of the sequences between the two participating Alu elements [5]. Numerous examples of Alu/Alu NAHR contributing to cancer have been reported. For example, 23 out of 29 reported recombination events in the *BRCA1* gene, which has an unusually high density of Alu elements (41.5% of the gene), involve Alu elements [5]. Interestingly, Alu/Alu cancer-related recombination events do not occur only in germ-lines but also somatically. Among them are recurring duplication of the MYB locus that can contribute to T-cell acute lymphoblastic leukemia or the case of MLL (mixed lineage leukemia), where Alu mediated recombination generates tandem duplications of exons [13,14]. L1 contributes to the instability in human cancers also by creating double strand breaks (DSBs) [15]. DSBs are disruptive forms of DNA damage that are typically corrected prior to its proceeding through the cell cycle.

II Original Research

2 Aims of the Thesis

This study is focused on molecular pathophysiology of congenital erythroid disorders with novel etiologies. It was aimed to:

1. describe molecular mechanism, how the presence of active retrotransposon in the non-coding region of *β-globin* gene attenuates its expression and thus leads to β-thalassemia,

2. investigate the role of novel homozygous *VHL* mutation in the pathogenesis of polycythemia.

Secondary, it was aimed to:

3. characterize the clinical and biological differences between two known positionally close inherited homozygous *VHL* mutations causing polycythemia: Chuvash R200W and Croatian H191D.

II Original Research

3 Materials and Methods

The Materials and Methods section contains only detail information about experiments which were performed by me either at Department of Biology, Faculty of Medicine and Dentistry, Palacky University or at Division of Hematology, School of Medicine, University of Utah. All the other experiments, done by collaborators at different Institutions are presented only in brief and cited accordingly. Each chapter in this section is subdivided into paragraphs and each paragraph is labeled (on the right margin) with the abbreviation of the corresponding project, i.e. *L1* (see chapter 4.2), *VHL P138L* (see chapter 4.3) and *VHL H191D* (see chapter 4.4).

3.1 Patient samples

Peripheral blood from propositus and her mother were obtained by venipuncture. All the samples were obtained with approval of the Institutional Review Board (IRB) committee of Palacky University in Olomouc, Czech Republic. Informed consent for all subjects was provided according to the Declaration of Helsinki. *L1*

The propositus is a 15-year-old girl of Asian Indian extraction (Punjabi ethnicity), who has been known to be polycythemic from infancy. Her parents are hematologically normal and are of the same ethnicity but not known to be related. Peripheral blood of the propositus and her parents was obtained by venipuncture after obtaining a signed University of Utah's IRB informed consent. *VHL P138L*

Blood samples from 23 persons were collected from two different families, including two propositi with polycythemia and 21 relatives. Peripheral blood samples were collected in EDTA and/or ACD tubes. Written inform consent was obtained from all participants. The IRB of the University of Utah approved the study. *VHL H191D*

3.2 Mutation screening

Genomic DNA was isolated from whole peripheral blood using phenol-chloroform protocol [1]. To characterize the β-thalassemia mutation, genomic DNA was analyzed by restriction mapping using several restriction endonucleases and Southern blot hybridization (for details see chapter 9, Supplemental Materials and Methods). *L1*

Granulocyte and mononuclear cell fractions from peripheral blood were isolated according to previously published protocol [2]. Genomic DNA was isolated from granulocytes using Gentra-Puregene Kit (Qiagen, Germantown, MD), and *VHL* gene's *VHL P138L* *VHL H191D*

exons were amplified using Hot Star Master Mix (Qiagen). The sequences of primers and reaction conditions are listed in Supplements and Appendices. Sequencing was performed using the standard protocol and the same amplification primers.

3.3 Cell culture

Interspecific hybrids of the patients' Epstein Barr Virus (EBV) transformed lymphocytes and mouse erythroleukemia (MEL) cells were generated as described elsewhere [3]. From the positive cell clones were further derived two lines – one contained affected chromosome 11 (designated "MEL *HBB*$^{L1+}$") and the second with normal chromosome 11 (designated "MEL *HBB*^{wt}"). Hybrid mouse/human erythroleukemia cell lines were maintained in humidified atmosphere at 37°C, 5% CO_2 in high-glucose Iscove's Modified Dulbecco's Media (IMDM) with GlutaMAX (Invitrogen, NY, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin (both Invitrogen). *L1*

786-0 cell line (renal cell adenocarcinoma origin) was purchased from ATCC (CRL-1932™, Manassas, VA). Cells were maintained in humidified atmosphere at 37°C, 5% CO_2 in high-glucose Dulbecco's Modified Eagle's Medium (D-MEM) with GlutaMAX (Invitrogen, NY, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin (both Invitrogen). *VHL P138L*

3.4 Drugs and inhibitors

Hybrid human/mouse cells were treated by 1.8% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) for 72 h to induced differentiation.

For purpose of different experiments MEL cell lines were treated: 24h with emetine (0.5 μM, Sigma-Aldrich) for nonsense-mediated mRNA decay (NMD) inhibition; in various time points (0, 1, 2, 4, 8 and 12 hours) with actinomycin D (Act-D, 1 μM, Sigma-Aldrich) to block mRNA synthesis. *L1*

To test the DNA methylation pattern/reactivation of silenced gene expression the hybrid cells were treated with 5-aza-2'-deoxycytidine (DAC, 2 μM or 5 μM, Sigma-Aldrich) and/or trichostatin A (TSA, 100 nM, Sigma-Aldrich) for 24h simultaneously with DMSO or for 24h and 48h before the induction of differentiation by DMSO.

3.5 Nuclear Run-On Assay

Nuclei from hybrid MEL *HBB*$^{L1+}$and MEL *HBB*^{wt} cells were isolated with Nuclei EZ Prep Nuclei Isolation Kit (Sigma-Aldrich), according to the manufacturer's protocol. The total nuclei were split into two equal aliquots and 2x reaction buffer was added (containing 30mM Tris, pH 8; 2,5 mM $MgCl_2$; 150 mM KCl; 20% glycerol; 1mM DTT and 40 U RNasin (Promega, Madison, WI) and reaction was set as described [4]. *L1*

The *in vitro* transcription reaction was initiated with the addition of 0.5 mM of each ribonucleotide triphosphates (rATP, rCTP, rGTP, rUTP) (rNTP) (Invitrogen) and incubated for 40 minutes at 30°C. Second aliquot without added rNTPs was incubated at the same

conditions. The rate of transcription (normalized fold increase) was determined as the ratio of nuclear fraction primary *β-globin* transcript measured in exon-1 (primers *HBB* ex1 F and R) with added rNTPs to the same fraction basal level (without added rNTPs) after 40 minutes of *in vitro* transcription. *LDHA* was used as a reference gene for quantification. The sequences of primers and reaction conditions are listed in Supplements and Appendices. *L1*

3.6 RNA isolation and quantitative RT-PCR

3.6.1 Human *β-globin* transcripts quantification

Total RNA from MEL *HBB*$^{L1+}$and MEL *HBB*^{wt} cell lines was isolated with TRI reagent (Roche Applied Science) followed by TURBO DNA-free DNase I treatment (Ambion, Life Technologies, NY). For quantification of human *β-globin* mRNA by quantitative RT-PCR (qPCR) RNA was reverse-transcribed using a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). cDNA was quantified on Light Cycler 480 (Roche Applied Science). For quantification of aberrantly spliced variants and NMD activation assay, exon-3 primers (*HBB* ex3 F and R) and UPL (Universal Probe Library, Roche Applied Science) probe #56 were used. For mRNA stability assay and effect of AZA and/or TSA treatment, exon-2 primers (*HBB* ex2 F and R) were used for *β-globin* mRNA and primers HBG F a R for *γ-globin* mRNA quantification. Levels of human *β-globin* and *γ-globin* transcripts were normalized to human *LDHA* (primers h*LDHA* F and R and UPL probe #47) using efficiency corrected formula for relative expression ratio [5]. The sequences of primers and reaction conditions are listed in Supplements and Appendices. *L1*

3.6.2 Relative ratio of exon-3 to exon-2 of human *β-globin* primary transcript

Nuclei's RNA from MEL *HBB*$^{L1+}$ and MEL *HBB*^{wt} cell lines was isolated with TRI reagent (Roche Applied Science) followed by TURBO DNA-free DNase I treatment (Ambion, Life Technologies, NY). The *HBB* exon-3/exon-2 ratio of MEL *HBB*$^{L1+}$ native primary transcript from the nuclear fraction that was prepared as mentioned above was normalized to the exon-3/exon-2 ratio of the control MEL *HBB*^{wt} native primary nuclear transcript using formula $2^{-\Delta\Delta Ct}$. The method for calculating ΔΔCt was similar to the method used for the relative quantification and is as follows: $(Ct_{MEL\ HBBL1+\ ex2} - Ct_{MEL\ HBBwt\ ex2}) - (Ct_{MEL\ HBBL1+\ ex3} - Ct_{MEL\ HBBwt\ ex3})$. Exon-2 primers *HBB* ex 2 F and R, exon-3 primers *HBB* ex 3 F and R, and exon-3 UPL probe #56 were used for the determination of this ratio. The efficiency of both amplicons was measured using a dilution series standard curve and was very close to 2. The sequences of primers and reaction conditions are listed in Supplements and Appendices. *L1*

3.6.3 Effect of the *VHL* mutations on expression of HIFs' target genes

Total RNA was isolated from granulocytes using TRI reagent solution (Molecular Research Center, Cincinnati, OH) and then treated with DNA-free™ DNase Treatment & Removal Reagents (Ambion, Life Technologies, NY) to remove any contaminating DNA. *VHL P138L*

500 ng of DNA-free RNA was reverse-transcribed using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) according to manufacturer's instruction protocol. qPCR were performed with specific TaqMan® Gene Expression probes (Applied Biosystems, Carlsbad, *VHL H191D*

CA) for following genes: *ADM* (Hs00181605), *TFRC* (Hs00951083), *NDRG1* (Hs00608387), *PDK1* (Hs00176853), *SLC2A1* (Hs00892681), *VEGF* (Hs00900055), *BNIP3* (Hs00969291), *BNIP3L* (Hs00188949), *HK1* (Hs00175976). All samples were assayed in triplicates. Data were normalized to *HPRT* (4333768F) and *GAPDH* (4333764F) reference genes. The statistical significance of relative expression changes of target mRNA levels normalized to a reference genes was analyzed by the pair-wise fixed reallocation randomization test using the REST© 2009 software [6].

VHL P138L

VHL H191D

3.7 DNA methylation analysis

Bisulfite modification was performed on genomic DNA from MEL *HBB*$^{L1+}$ and MEL *HBB*^{wt} cells using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA). The promoter and enhancer region of *β-globin* gene and the *β-globin*$_{L1+}$ L1 promoter were amplified with semi-nested PCR. *β-globin* promoter primers were published elsewhere [7], enhancer region was amplified with *HBB*enh bis F and R primers for first round, then *HBB*enh bis F2 and R for semi-nested PCR. L1 promoter was amplified with L1 meth F and R primers for first round, followed by semi-nested round with primers L1 meth F2 and R. The purified products were subcloned into the pCR®2.1-TOPO® plasmid (Invitrogen) and at least 8 positive clones for each region were sequenced (in the case of *β-globin*$_{L1+}$ promoter and 3' enhancer 10 to 20 clones were sequenced). The sequences of primers and reaction conditions are listed in Supplements and Appendices.

L1

3.8 *In vitro* assay of the sensitivity of erythroid progenitors to EPO

3.8.1 *In vitro* assay in semisolid medium

In vitro sensitivity of erythroid progenitors to EPO was performed on mononuclear cells isolated from the peripheral blood using Histopaque (Sigma-Aldrich) density gradient centrifugation and plating (2.3×10^5/mL) to methylcellulose media (MethoCult® H4531; StemCell Technologies, Vancouver, BC) without addition of EPO or with addition of various concentrations of EPO (StemCell Technologies), ranging from 0.015 to 3.0 U/mL. Cell cultures were maintained in humidified atmosphere of 5% CO_2 at 37°C for 14 days. Erythroid burst-forming unit colonies (BFU-Es) were scored by standard morphologic criteria.

VHL P138L

VHL H191D

3.8.2 *In vitro* assay in liquid culture

Expansion of the progenitor cells from the mononuclear cell population was performed based on our published protocol [8]. Briefly, $1x10^6$ cells/mL were cultured in the StemSpan™ Serum-Free Expansion Medium (StemCell Technologies) containing different cytokine cocktail - day 1-7 (100 ng/mL of fetal liver tyrosine kinase 3 ligand, 100 ng/mL of thrombopoietin, and 100 ng/mL of stem cell factor), day 8-14 (50 ng/mL of stem cell factor, 50 ng/mL of insulin like growth factor-1, and 3 U/mL of EPO), day 15-21 (50 ng/mL of insulin like growth factor-1, and 3 U/mL of EPO). All cytokines were kind gift of Amgen (Thousand Oaks, CA).

VHL H191D

3.9 Functional analysis of VHL protein

VHL Human cDNA ORF Clone was obtained from OriGene (RC216151, Rockville, MD). Site-directed mutagenesis was performed using the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) and allele-specific oligonucleotides for VHL^{P138L} and VHL^{H191D}. The sequences of primers and reaction conditions are listed in Supplements and Appendices. Transfection of all mutated, wild type and empty plasmids (which was used as a negative control) was done using Lipofectamine 2000 reagent (Invitrogen).

Cells were selected 48 hours after transfection using 1 mg/mL G418 (Invitrogen) and cultured for 21 days. Resistant clones were isolated using 96-well limiting dilution. 30 single clones were picked up for each *VHL* construct and tested for expression of *VHL*(Myc-DDK) by TaqMan® Gene Expression assay on demand (Applied Biosystems).

VHL P138L

To determine the half-life of pVHL, 786-0 stable transfected clones were treated with 200 μM cycloheximide (Sigma-Aldrich) and cells were harvested at different time-points (0, 2, 4, 6, 8 and 10 hours) to ice cold RIPA buffer (Sigma-Aldrich) supplemented with following protease inhibitors: 1 μM DTT, 1 μM NAF, 10 μM β glycerophosphate, 10 μg/mL leupeptin, 2 mg/mL aprotinin, 0.1 μM Na_3VO_4 and 0.1 μM PMSF.

VHL H191D

Proteins were electrophoretically resolved on SDS-polyacrylamide gels and electroblotted onto Immobilon® PVDF mebranes (Millipore, Billerica, MA). Membranes were incubated with primary antibodies rabbit anti-human VHL (FL-181, Santa Cruz, Dallas, TX, 1:500) and rabbit anti-human actin (Sigma-Aldrich, 1:1000) at 4°C overnight, washed in PBS with 0.05% Tween 20, and incubated for 1h with the goat anti-rabbit horse radish peroxidase (HRP)-conjugated secondary antibody (Thermo Fisher, Waltham, MA). HRP activity was detected with ECL detection kit (Pierce, Rockford, IL, USA) on Blue Lite Autorad films (BioExpress, UT, USA). Quantitation of pVHL signals was performed using ImageJ software.

II Original Research

4 Results and Discussion

4.1 List of Publications and Meeting Abstracts

This thesis contains data that were presented in publications and on meetings listed below:

Publications

- **Lanikova L**, Kucerova J, Indrak K, Divoka M, Issa JP, Papayannopoulou T, Prchal JT, Divoky V. β-thalassemia due to intronic LINE-1 insertion in the *β-globin* gene: molecular mechanisms underlying reduced transcript of the *β-globin*$_{L1}$ allele. *Human Mutation*. 2013; doi: 10.1002/humu.22383/epub ahead of print.

- **Lanikova L**, Lorenzo F, Yang CH, Vankayalapati H, Drachtman R, Divoky V, Prchal JT. Novel homozygous *VHL* mutation in exon 2 is associated with congenital polycythemia but not with cancer. *Blood*. 2013;121(19):3918-24.

- **Piterkova L***, Tomasic Ljubas N*, Huff Ch, Bilic E, Yoon D, Miasnikova GY, Sergueeva AI, Niu X, Nekhai S, Gordeuk V, Prchal JT. Polycythemia due to Croatian homozygous *VHL* (571C>G:H191D) mutation has a different phenotype than Chuvash polycythemia (*VHL* 598C>T:R200W). *Haematologica*. 2013; 98(4):560-7.

Selected Meeting Abstracts

- **Lanikova L**, Prchal JT. *VHL* mutations associated with congenital polycythemia.

  - 6th Symposium on Advances in Molecular Hematology, XXVII. Olomoucké hematologické dny s mezinárodní účastí, May 12-14 (2013), Olomouc.

- **Piterkova L**, Lorenzo F, Vankayalapali H, Drachtman R, Prchal JT. Novel homozygous *VHL* mutation in exon 2 (413C>T:P138L) is associated with congenital polycythemia, elevated level of *RUNX1/AML1* transcript, but not with the cancer. *Blood*. Nov 2012; 120(21): 2081.

  - 54th Annual Meeting of American Society of Hematology, December 8-11 (2012), Atlanta (Georgia), USA.

- Divoka M, Mojzikova R, **Piterkova L**, Pospisilova P, Partschova M, Horvathova M, Pospisilova D, Laluhova Striezencova Z, Cermak J, Indrak K, Divoky V. Molecular Characterization of Hemoglobinopathies and Red Cell Enzymopathies in the Czech and Slovak Populations: An Update. *Blood*. Nov 2011; 118(21): 5307.

  - 53rd Annual Meeting of American Society of Hematology, December 10-13 (2011), Sand Diego (California), USA.

- Divoka M, Partschova M, Mojzikova R, **Piterkova L**, Horvathova M, Cermak J, Pospisilova D, Indrak K, Divoky V. Molecular characterization of beta-thalassemia and hemoglobin variants in the Czech and Slovak populations: An Update. *Haematologica*. 2011; 96(s2): 626.

  - 17th Annual Meeting of European Society of Hematology, June 9-12 (2011), London, United Kingdom.

- **Piterkova L**, Kucerova J, Indrak K, Divoky V. Decreased rate of *β-globin*$_{L1+}$ allele transcription due to intronic LINE-1 insertion in the *β-globin* gene is associated with *β-globin*$_{L1+}$ promoter and enhancer hypermethylation which is not reverted by decitabine. *Blood*. 2010; 116(21): 860.

  - 52nd Annual Meeting of American Society of Hematology, December 4-7 (2010), Orlando (Florida), USA.

- **Piterkova L**, Kucerova J, Indrak K, Divoky V. Intronic LINE-1 insertion in *β-globin* gene cause β-thalassemia due to aberrant splicing, nonsense-mediated decay and decreased rate of *β-globin*$_{L1+}$ allele transcription.

  - XXII. Biochemický zjazd, Martin, September 8-12 (2010), Martin, Slovak Republic.

- **Piterkova L**, Kucerova J, Indrak K, Divoky V. Intronic LINE-1 insertion in the *β-globin* gene causes β-thalassemia due to aberrant splicing, nonsense-mediated decay and decreased rate of *β-globin*$_{L1+}$ allele transcription. *Haematologica*. 2010; 95(s2): 417-418.

  - 16th Annual Meeting of European Society of Hematology, June 10-13 (2010), Barcelona, Spain.

- **Piterkova L**, Kucerova J, Indrak K, Divoky V. Negativní vliv retrotransposonu LINE-1 na lidský genom: nová molekulární příčina vzniku β-talasémie.

  - X. mezioborové setkání mladých vědeckých a výzkumných pracovníků, Sigma-Aldrich, May 25-28 (2010), Hotel Devět skal. **Best oral presentation award.**

- **Piterkova L**, Kucerova J, Indrak K, Divoky V. A novel etiology of β-thalassemia: Evidence for LINE-1 retrotransposon contribution to human disease.

  - Conference of Medical and Pharmaceutical Schools, November 19-21 (2009), Hradec Králové.

- **Piterkova L**, Kucerova J, Indrak K, Divoky. Molekulární mechanismus působení L1 elementu na expresi *β-globinového* genu: nová příčina vzniku β-talasémie.

  - Science conference of Ph.D. students, September 8-9 (2009), Faculty of Medicine and Dentistry, Palacky University, Olomouc. **2nd place in Best Oral Presentation Competition.**

4.2 Molecular mechanisms underlying reduced transcription of *β-globin* gene with intronic LINE-1 insertion leading to β^+-thalassemia phenotype

β-thalassemia is an inherited disorder of β-globin chain production. The known molecular mechanisms responsible for β-thalassemia include point mutations located in the *β-globin* gene or its promoter (the majority of the β-thalassemia mutations) and a small number of deletions removing either a part of the *β-globin* gene or its locus control region - LCR [1]. The point mutations underlying β-thalassemia are classified according to the mechanism by which they affect gene regulation. Mutation affecting transcription can involve either the conserved DNA sequences in the *β-globin* promoter (e.g. in the TATA or CCAAT boxes) or the stretch of 50 nucleotides in the 5' UTR. There are over 50 different mutations that affect splice junction dinucleotides (GT at 5' and AG at 3') which completely abolish or reduce the efficiency of normal splicing. Several mutations also activate cryptic splice sites, which disturb the normal splice sites during the processing of pre-mRNA. Other RNA processing mutants affect the polyadenylation signal (AATAAA) and the 3' UTR [2,3,4]. There are rare forms of β-thalassemia, which are associated with mutations independent of the *β-globin* complex, e.g. *GATA 1* [5] or *Xeroderma Pigmentosum D* genes [6].

We described a mother and daughter of Ukrainian descent with clinical presentation of β^+-thalassemia trait. Inexplicably their phenotypes were more severe than that of typical β^+-thalassemia heterozygotes. The propositus (25-year-old Caucasian female) had the hemoglobin concentration values 11 to 12 g/dl, MCV of 60-70 fl, and MCH of 19-20 pg. Red cell morphological abnormalities included hypochromic microcytosis, target cells, poikilocytes and 0.3 to 1.5% of reticulocytes. The HbA2 levels were elevated to 5.3% and the HbF levels were slightly just above 1%. No inclusion bodies of precipitated globin chains were detected in the patient's erythroid cells after brilliant cresyl blue staining. Hematological indices of the propositus' mother were comparable. No neurological or other systemic defects were found on both patients physical examination.

Analysis of the patient's *β-globin* genes revealed no obvious mutation except that each was heterozygous for an unexpected rearrangement detected by gene mapping and this finding provided the impetus for further studies. Hybridization of several restriction enzyme digests of propositus' DNA to an intron-2 (β-IVS-II) probe showed two abnormal fragments, one major and one minor, indicated unreported rearrangement involving an insertion into the *β-globin* gene.

The insertion (named $L1_{\beta\text{-thal}}$, **Figure 3**) occurred 98 bp 5' to the 3' end of β-IVS-II. The L1 was flanked by a 9-13 bp target site duplication, with the presumptive first 9 bases (T/AAAATAAAA) forming a consensus L1 endonuclease cleavage site [7]. Analysis of the inserted retrotransposon demonstrated its antisense orientation with respect to the *β-globin* gene. The inserted L1 was full length, displayed 99.5% homology with a consensus sequence of retrotranspositionally-active human L1s (33 nucleotide differences in 6.0 kb [8]), including only 6 bp differences in the 910 bp 5' UTR containing the L1 promoter (GenBank accession: AF149422.1). It had two open reading frames and an intact 3' poly (A) track.

The β^+-thalassemia nature of this mutation was investigated in reticulocytes and in interspecific hybrids of propositus' chromosome 11 and mouse erythroleukemia cells. Both mRNA and globin-chain data revealed that the L1 insertion leads to reduced production of *β-globin* transcript and peptide from the mutant locus, i.e. to β^+-thalassemia. $L1_{\beta\text{-thal}}$ led to a

dramatic reduction of *β-globin* transcripts produced by the affected allele, as assessed by RT-PCR using the propositus' reticulocyte RNA.

We were also able to identify abnormally spliced *β-globin* transcripts by RT-PCR; the sizes of these PCR products suggested the presence of rare messages with unusual combinations of *β-globin* exons with intronic and/or insertion-derived sequences. All aberrantly spliced *β-globin* transcripts contain premature translation termination codon, which makes them susceptible to nonsense-mediated mRNA decay (NMD). We proved that NMD participates in reduction of steady-state mRNA level from affected allele. The low mRNA amount could also result from other causes influencing *β-globin*$_{L1}$ mRNA stability.

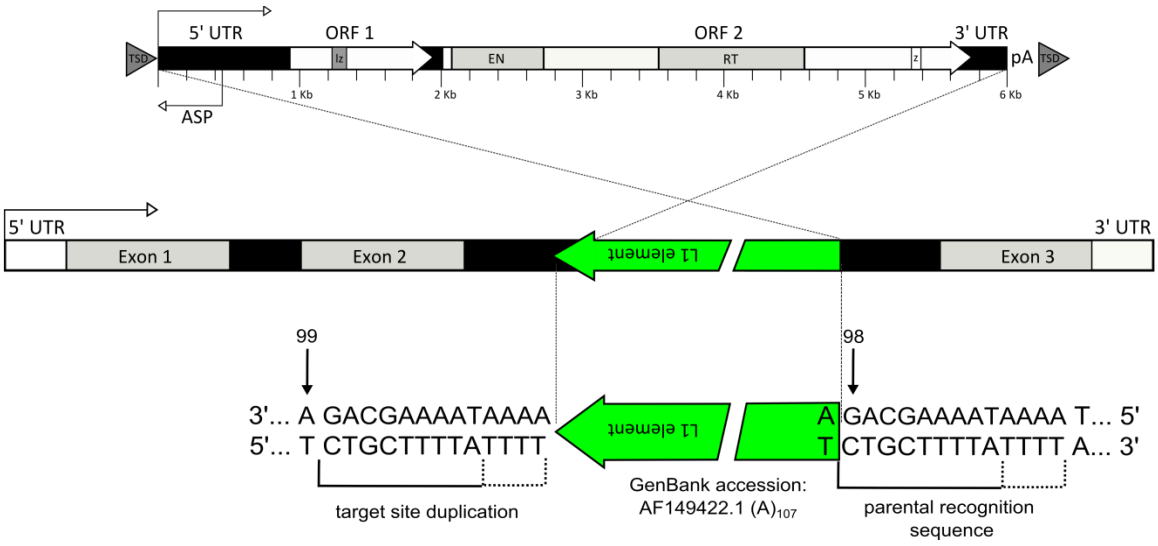

Figure 3. Schematic diagram of the *β-globin*$_{L1}$ allele. (middle) The three exons of *β-globin* gene are shown as grey rectangles, introns as black; the 6 kb insertion is depicted as a green rectangle; the β-IVS-II is divided into two segments of different sizes. (up) Structure of the human L1 retrotransposon [adapted from ref. 9]. The full-length human L1 retrotransposon is 6 kb, and contains: 1) a 910-bp 5' UTR region with bidirectional promoter activity; ASP (antisense promoter) 2) ORF1 region, which encodes a 40-kD a basic RNA-binding protein with a leucine zipper domain (lz); 3) ORF2 region, which encodes a 150-kDa protein with endonuclease (EN) and reverse transcriptase (RT) activities, and a conserved C-terminal zinc knuckle domain (z); and 4) a 3' UTR region dispensable for retrotransposition activity. The 3' UTR contains a functional polyadenylation signal, which occasionally may be bypassed in favor of a stronger downstream signal. L1 terminates with a poly(A) sequence (pA), and is flanked by 2-bp to 20-bp target site duplications (TSDs). (below) Schematic diagram of the L1$_{\beta\text{-thal}}$ insertion and *β-globin* gene breakpoints. The arrows above the nucleotide sequence show the residues at positions 98 and 99 bp 5' to the 3' end of β-IVS-II. The L1 insertion was flanked by a 9-13 bp target site duplication (the run of four As, shown by the hatched bracket, could be duplicated or could be a part of the L1-poly(A) tail).

Lower steady-state amount of mRNA produced by *β-globin*$_{L1}$ allele also resulted from reduced rate of transcription and decreased production of full-length *β-globin* transcripts. Hypermethylated promoter/enhancer sequences of *β-globin*$_{L1}$ allele indicated epigenetic

modification caused by the presence of L1. Treatment with demethylation agent did not lead to restoration of transcription. Histone deacetylase inhibitor partially reactivated the *β-globin*$_{L1}$ transcription in spite of permanent *β-globin*$_{L1}$ promoter CpG methylation suggesting that decreased rate of transcription from *β-globin*$_{L1}$ allele is associated with altered chromatin.

In conclusion, we report a combination of several molecular events leading to the β^{+}-thalassemia phenotype due to intronic LINE-1 insertion in the *β-globin* gene. Although similar effects of intronic L1 sequence on normal mRNA processing have been reported for other host genes (such as altered splicing, hybrid L1/host gene transcripts, truncation of host gene transcripts [reviewed by ref. 10]) we demonstrated these defective transcript-processing mechanisms in a combination with an L1-mediated epigenetic silencing of the host gene expression. Our data suggested that the main effect of intronic L1 insertion on the host gene expression is transcriptional repression due to regional spreading of methylation affecting both 5' promoter and 3' enhancer. However, our attempts to reactivate the silenced *β-globin*$_{L1}$ expression revealed important consequences: not a demethylation agent, but trichostatin, a potent histone deacetylase inhibitor, led to (partial) restoration of the host gene transcription suggesting that decreased rate of transcription from *β-globin*$_{L1}$ allele is associated also with altered chromatin caused either by *β-globin*$_{L1}$ promoter-enhancer displacement due to insertion and/or possibly by spreading of repressive marks induced by L1 to the neighboring *β-globin*$_{L1}$ gene promoter [11,12]. These results might have therapeutic implications in diseases caused by deleterious effects of retrotransposons on the expression of nearby genes.

4.3 Novel homozygous *VHL* mutation in exon 2 is associated with congenital polycythemia but not with cancer

Inherited mutations of the *VHL* gene cause VHL syndrome, an autosomal dominant disorder [13] that demonstrates marked phenotypic variability and age-dependent penetrance. Heterozygotes for such mutations are at increased risk of developing retinal and central nervous system haemangioblastomas, clear cell renal cell carcinoma, phaeochromocytoma, pancreatic islet tumors and endolymphatic sac tumors [14]. Tumors develop from cells that acquire a somatic mutation of the unaffected *VHL* gene in addition to the germ line mutation on the other allele (two hit model of cancerogenesis) [15]. In rare cases, affected patients may present with polycythemia, a paraneoplastic manifestation of VHL syndrome presumably due to inappropriate production of EPO by the tumor cells. In fact, production of EPO by the tumor has been demonstrated both in the case of hemangioblastoma and renal cell carcinoma; the polycythemia usually resolves after removal of the tumor [16].

There are two known homozygous *VHL* gene mutations causing polycythemia that are not associated with a VHL cancer syndrome - an R200W mutation endemic in Chuvashia causing the first recognized disorder of augmented hypoxia sensing in normoxia [17], and an H191D mutation of Croatian origin [18]. Both are located in the distal exon-3 of the *VHL* gene of its C-terminal domain. As different positions of loss-of-function mutations of *VHL* gene are associated with different type of cancers, it has been proposed that only C-terminal domain *VHL* mutations would cause polycythemia. However, we contradicted this notion. We report a novel homozygous variant of the *VHL* gene located in the middle of coding region in exon-2; c.413C>T:P138L. The propositus is a 15 year old Punjabi female with

congenital polycythemia; i.e. elevated EPO 40 mIU/mL, no JAK2 mutations, and hemoglobin 19-20 g/dl. Her parents are *VHL*^{P138L} heterozygotes and no VHL tumors are reported in the extended family, in contrast to the other *VHL* P138 residue (P138R [19], P138T [20]) mutations that have been reported in VHL syndrome and renal cancer.

pVHL is a negative regulator of hypoxia inducible transcription factors as it degrades α subunits of HIFs. It has been proposed, that an association between mutated pVHL and suppressor of cytokine signaling 1 leads to JAK2 up-regulation and enhanced erythropoiesis [21]. We show that the *VHL*^{P138L} mutation, which lies in the catalytic HIF-1α peptide ligand-binding region, perturbs pHIF-1α pVHL interaction due to a conformational effect on the W117 and S111 residues lying within 2.8 to 4.3 Å distance from the mutated L138. The effect of this single mutation on overall structure is a shift of 1.9 Å RMSD (root mean square deviation) from the wild-type complex structure (see **Figure 4**). The accumulation of HIFs and up-regulated transcription of downstream target genes including those for *glucose transporter-1* (*SLC2A1*) and *transferrin* (*TF*) were found in the propositus' granulocytes.

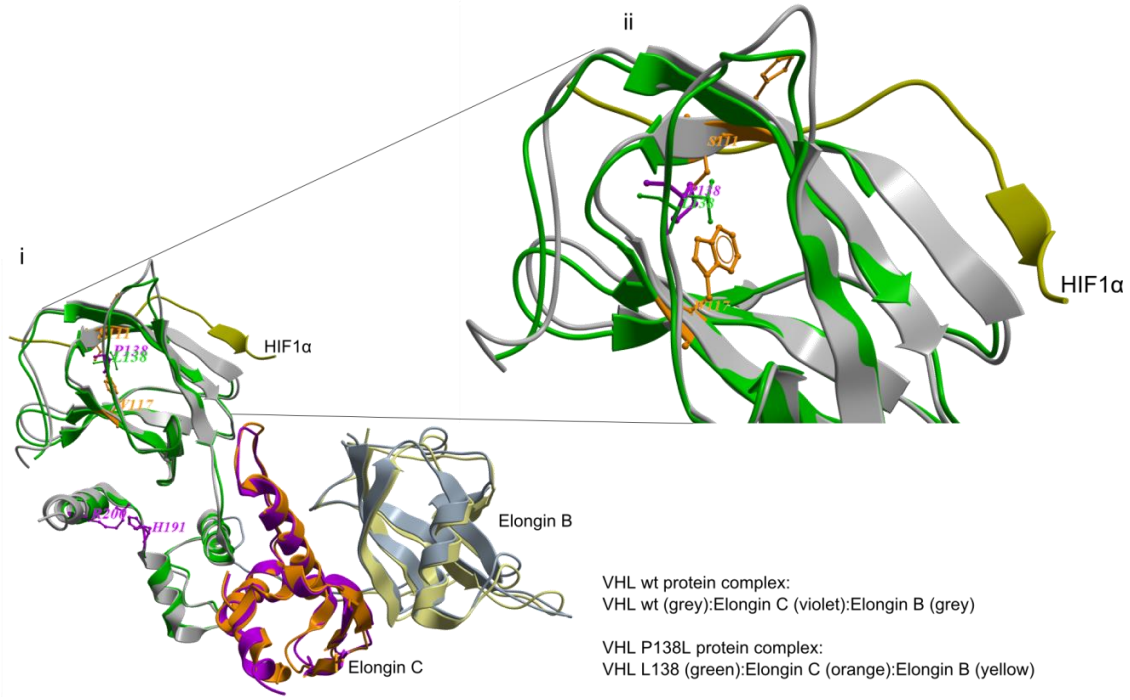

Figure 4. Molecular dynamics simulations study of pVHL-ElonginC-ElonginB complex and interaction with HIF-1α. (i) Superimposition of wild type (grey color) and mutated pVHL (green color) is shown. The wt P138 (in violet) and mutated L138 (in green) sites and the critical active site residues for the HIF-1α peptide (PDB:1LM8) binding region are depicted. The P138L mutations perturbs pHIF-1α interactions with pVHL due to the conformational effect on the W117 and S111 residues (shown in orange) at 2.8 to 4.3 Å distance from mutated L138. (ii) Detail superimposition of wild type and P138L pVHL in the interaction with HIF-1α.

We then analyzed the *VHL*^{P138L} propositus and her heterozygous parents' erythroid progenitors and found them to be hypersensitive to EPO (a feature of primary polycythemias without EPO independent colonies). Because of reports that *RUNX1/AML1* transcript levels are specifically upregulated in erythroid progenitors in polycythemia vera and allegedly responsible for PV EPO hypersensitivity [22,23], we analyzed erythroid *RUNX1/AML1* transcripts in the propositus and found them increased.

In conclusion, we demonstrated that a homozygous mutation in exon-2 of the *VHL* gene can also be associated with a polycythemic phenotype rather than VHL syndrome tumors. Further, we report that increased levels of *RUNX1/AML1* transcripts are not specific for PV but can be seen in other primary polycythemias. This report provides further evidence for the heterogeneity of clinical phenotypes of *VHL* mutations resulting in cancers or in augmented erythropoiesis. The molecular basis of these differences remains unclear. We can only speculate that the relatively small VHL peptide comprised of 213 codons yet encoded by a large >11,2 kb VHL gene may have multiple functions, possibly due to interactions with other modifying factors, that await future clarification. We submit that description of families with congenital disorders and unique phenotypes due to *VHL* mutations provides an attractive opportunity for structure-function relationship analyses of VHL protein and leads to an enhanced understanding of polycythemic disorders and diseases of hypoxia sensing.

4.4 Polycythemia due to Croatian homozygous *VHL* (c.571C>G:H191D) mutation has a different phenotype than Chuvash polycythemia (*VHL* c.598C>T:R200W)

The only known endemic polycythemia, Chuvash polycythemia, is a disorder of global augmented hypoxia sensing due to elevated hypoxia inducible transcription factors (HIF-1 and HIF-2). The underlying genetic defect is the homozygosity with respect to a C→T missense mutation in *VHL* gene, causing an arginine-to-tryptophan change at amino-acid residue 200 (Arg200Trp) [17]. The mutation has also non-erythroid consequences such as increased stroke and low blood pressure, pulmonary hypertension [24,25], and increased PIA [26], which have even greater impact on morbidity and mortality than the initially uncovered erythroid effect. The estimated time of origin of *VHL*^{R200W} mutation is prior to divergence of the human races approximately 14,000 - 62,000 years ago, suggesting that while those homozygous for this mutation have increased mortality and are selected against, more numerous heterozygotes must have a survival or reproductive advantage yet to be defined [27]. Beside Chuvash polycythemia, there is accumulating evidence of other congenital polycythemias characterized by *VHL* mutations [16,18,28].

In the study, we report second homozygous polycythemic patient for the *VHL* c.71C>G (H191D) germ-line mutation, a 5 year old Croatian girl from Herzegovina, a region located in the southern part of Bosnia contiguous to Dalmatia. Herzegovina is the counterpart to the Croatian region and is populated largely by people of Croatian ethnicity along the border. We set up to define the phenotype of this mutation in this and the previously reported homozygote [18] with particular emphasis on a critical comparison with CP. We also pursued a hypothesis that a putative survival advantage of H191D heterozygotes may account for the possible high prevalence of heterozygosity in Croatians and thus set up to

determine its approximate origin in evolution by determining haplotype sharing among affected individuals.

We analyzed the two homozygotes (p12 and p18, see **Figure 5**) by high-density genotyping to examine their relatedness and the *VHL* haplotype. We detected evidence of a significant relationship between the two individuals ($p=4x10^{-18}$), with a maximum likelihood estimate of 8th degree relatives (95% C.I. 6th degree - 11th degree) [29], exactly matching the known pedigree (see **Figure 5**). These individuals share a 16 cM haploid segment on chromosome 11 and a 24 cM haploid IBD (identity by descent) segment on chromosome 3 (see **Figure 6**). Within the segment on chromosome 3, a 15.6 cM autozygous segment is present in the female (p18), and a 1.6 cM autozygous segment is present in the male (p12). Both autozygous segments contain the *VHL* mutation, and the smaller segment is diploid IBD. Two other large autozygous segments are present in the male (15.5 cM on chromosome 15 and 10 cM on chromosome 4). The presence of autozygosity at the *VHL* mutation and elsewhere in the genome is a strong indication that all four of the parents of p12 and p18 are related, sharing a common ancestor between approximately 3 and 6 generations ago. This common ancestor is likely the founder of the VHL^{H191D} mutation. Since the analysis demonstrated a recent origin of this mutation and, thus, the frequency of this mutation among tested families cannot be used in arguments for or against any survival benefit or detriment.

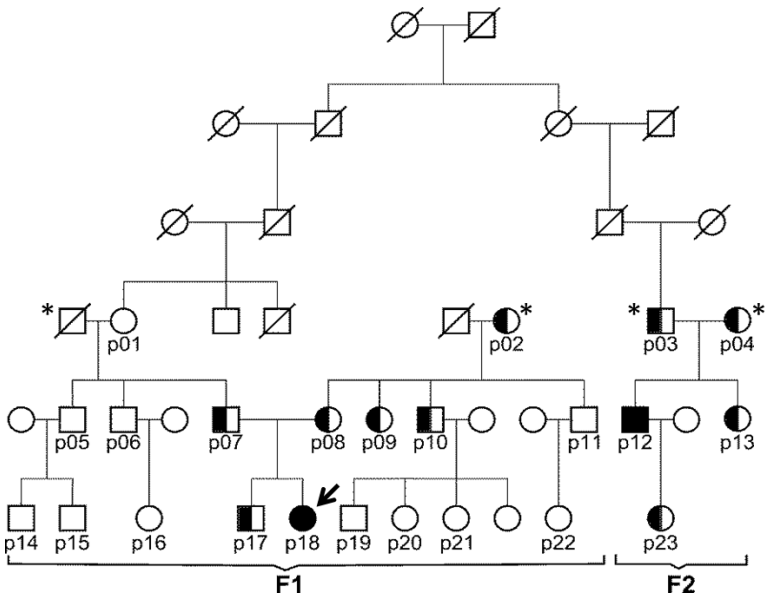

Figure 5. Pedigree of the *VHL* ^{H191D} families available for study with extended family history. Each family is denoted by a family number (F1, F2). The 'p' code (p01, p02 and so on) indicates individuals from whom DNA samples have been obtained. The arrow indicates new polycythemic patient. The *VHL* mutation of the paternal side of F1 must have been inherited from the husband of p01. The husband of p01 was not related to F2 according to the pedigree and the mutation did not segregate among the known relatives. Heterozygote individuals denoted by asterisk as well as the husband of p01 presumably shared the common ancestor, the founder of the mutation.

While previously was shown [17] that in CP, the erythroid progenitors are intrinsically hyperproliferative and the elevated EPO levels (a feature of secondary polycythemia) further contribute to augmented erythropoiesis. The *VHL*^{H191D} mutation differs from *VHL*^{R200W}, as the EPO levels seen in both *VHL*^{H191D} homozygotes appear to be higher than in CP, but more importantly, we have been unable to find evidence of a primary polycythemic functional defect in the native erythroid progenitors of this family. Therefore, we conclude that the *VHL*^{H191D} polycythemic phenotype is solely driven by EPO.

Our data provide additional evidence of as yet unexplained variations of phenotypes with different locations within the *VHL* gene, but clearly more work is needed to define the molecular basis of the array of phenotypic differences arising from changes in this rather small VHL peptide.

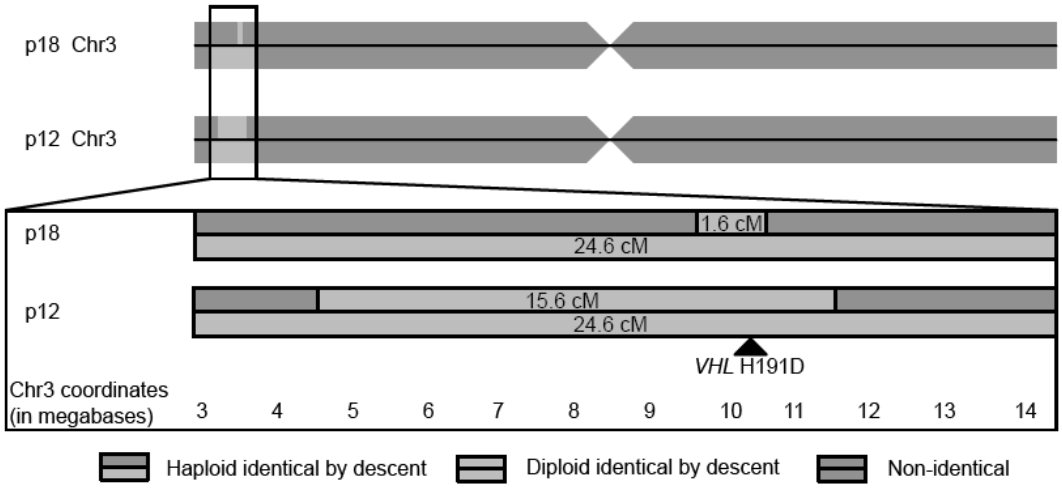

Figure 6. IBD and autozygosity on chromosome 3. The two individuals homozygous for *VHL*^{H191D} share a 24.6 cM haploid IBD segment, a 15.6 cM haploid IBD segment that is autozygous in the female, and a 1.6 cM diploid IBD segment that is autozygous in both individuals. All segments contain the *VHL* mutation. The presence of large autozygous IBD segments strongly suggests that all four copies of the *VHL* mutation originated from a recent founder.

II Original Research

5 Summary

In 1988, a novel molecular mechanism producing inactivation of gene expression and causing human disease, genomic insertion of a long interspersed nuclear elements (LINE-1, L1), was described [1]. The human genome contains about 5 x 10^5 copies of these retrotransposons [2]; most of which are randomly 5' truncated and inactive. However, there are 3000 - 4000 full-length L1 elements in the human genome and roughly 80 - 100 of them are capable of ongoing retrotranspositon [3]. De novo L1 insertions into genes such as *factor VIII* [1], *dystrophin* [4] or *retinitis pigmentosa* [5] demonstrated the mutagenic potential of active L1 elements in the genesis of human disease. Our group has described a family with β-thalassemia due to the insertion of a full-length L1 element into the *β-globin* gene and shown that this L1 element retained the capacity for high retrotransposition frequency. This was the first example of an intact, functional L1 causing human disease. We now demonstrated that this retrotransposon was inserted in the antisense orientation into the 3' end of intron-2 of the *β-globin* gene and led to a severe reduction of *β-globin* gene expression due to aberrant splicing, nonsense-mediated decay, decreased production of full-length transcripts and epigenetic transcriptional repression of $\beta\text{-}globin_{L1}$ allele. Although mutations in the human *globin* genes have been extensively studied for a long time, this kind of molecular etiology of thalassemia has not been previously described.

Studies on Chuvash polycythemia and other *VHL*-associated polycythemias are important because *VHL* was originally identified as a tumor-suppressor gene mutated in VHL syndrome, where tumors are initiated by biallelic *VHL* inactivation and are associated with abnormal activation of hypoxic gene response pathways [6]. Whereas, the majority of the VHL syndrome mutations abolish the ability of VHL to polyubiquitinate HIF-α, polycythemic *VHL* mutations lead to a more modest partial loss of activity [7]. Quantitative difference in the loss of activity could explain the variable phenotype among *VHL* mutations, but *VHL* gene may have also other functions, possibly due to interactions with other modifying factors, that can contribute to the onset of diseases and which await future clarification.

We described a new homozygous *VHL* exon-2 mutation, VHL^{P138L}, which is associated in the affected homozygote with congenital polycythemia but not in her, or her relatives, with cancer or other VHL syndrome tumors. This contrasts with reports of other heterozygous *VHL* mutations encoding the same amino acid residue (*VHL* P138R, P138T) that have been reported in VHL syndrome and renal cancer. We also show that this mutation is not only associated with elevated EPO levels but also with a hallmark of primary polycythemia, i.e. EPO hypersensitivity. We also provided evidence that polycythemia due to Croatian homozygous VHL^{H191D} mutation has a different phenotype than Chuvash polycythemia.

III Supplements and Appendices

6 Bibliography

1 Introduction

1.1 The erythropoiesis

1.1.1 Morphology and composition of the erythrocyte

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1.3 Mobile elements and human diseases

1.3.1 Mobile elements

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4 Results and Discussion

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5 Summary

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III Supplements and Appendices

7 Acronyms and Abbreviations

ACD	citrate-dextrose solution
Act-D	actinomycin D
ADM	adrenomedullin
ARNT	aryl hydrocarbon receptor nuclear translocator
Asn	asparagine
ATP	adenosine triphosphate
BFU-E	burst forming unit - erythroid
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3 like
BRCA1	breast cancer 1
cDNA	complementary deoxyribonucleic acid
CFU-E	colony forming unit - erythroid
CFU-GEMM	colony-forming unit granulocytic, erythroid, megakaryocyte, macrophage
CIS	cytokine inducible SH2 protein
CP	Chuvash polycythemia
CTP	cytosine triphosphate
DAC	decitabine: 2'-deoxy-5-azacytidine
D-MEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DMT-1	divalent metal transporter 1
DNA	deoxyribonucleic acid
DSBs	double strand breaks
DTT	dithiothreitol
EDTA	ethylene-diamine-tetraacetic acid
EBV	Epstein-Barr virus
EGLN	egl nine homolog 1
EPO	erythropoietin
EPOR	erythropoietin receptor
FIH-1	factor inhibiting hypoxia inducible factor 1
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GATA-1	GATA-binding factor 1
GTP	guanine triphosphate
Grb2	growth factor receptor-bound protein 2
Hb	hemoglobin
HBB	beta-globin
HBG	gamma-globin
HIF	hypoxia inducible factor
HK1	hexokinase 1
HPRT	hypoxanthine-guanine phosphoribosyl transferase
HSC	hematopoietic stem cell
IBD	identity by descent
IVS	intervening sequence
JAK2	Janus kinase 2

KCl	potassium chloride
KLF-1	erythroid Krüppel-like factor 1
L1/ LINE-1	long interspersed element-1
LCR	locus control regions
LDHA	lactate dehydrogenase A
LNK/SH2B	src homology 2 B adaptor protein 3
LTR	long terminal repeats
MAPK	mitogen-activated protein kinase
MCH	mean corpuscular hemoglobin
MCV	mean corpuscular volume
MEL	murine erythroleukemia cell line
MLL	mixed lineage leukemia
Myr	million year
NAF	sodium fluoride
NAHR	non-allelic homologous recombination
NDRG1	N-myc downstream regulated 1
NF-E2	nuclear factor - erythroid-derived 2
NMD	nonsense-mediated decay
NTPs	nucleotide triphosphate
ORF	open reading frame
PDK1	pyruvate dehydrogenase kinase 1
PFCP	primary familial and congenital polycythemia
PHD2	prolyl hydroxylase protein 2
PI-3K/AKT	phosphoinositide 3-kinase/protein kinase B
PIA	plasminogen activator inhibitor-1
Pol II	polymerase II
PMSF	phenylmethylsulfonyl fluoride
Pro	proline
PV	polycythemia vera
RBCs	red blood cells
REST	relative expression software tool
RMSD	root-mean-square deviation
RNA	ribonucleic acid
RT-PCR/PCR	reverse transcription polymerase chain reaction
RUNX1/AML	runt-related transcription factor 1/acute myeloid leukemia
SH2	src homology 2
SHP1	src homology region 2 domain-containing phosphatase-1
SINE-R	short interspersed elements ('R' indicating a sequence of retroviral origin)
SLC2A1	solute carrier family 2 (facilitated glucose transporter), member 1
SOCS	suppressor of cytokine signaling
Sos	son of sevenless
STATs/STAT 5	signal transducer and activator of transcription 5
STEAP3	6-transmembrane epithelial antigen of prostate family member 3
SVA	SINE-R VNTR Alu
TE	transposable element
TFRC	transferrin receptor protein
TSA	trichostatin
TSD	tandem side duplication
UPL	universal probe library
UTP	uracil triphosphate
UTR	untranslated region
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau gene/protein
VNTR	variable number tandem repeat

III Supplements and Appendices

8 Supplements

8.1 List of primers and PCR reaction conditions

8.1.1 List of primers

Primer name	Sequence
VHL exon 1 F	5' CGAAGACTACGGAGGTCGAC
VHL exon 1 R	5' GGCTTCAGACCGTGCTATCG
VHL exon 2 F	5' GTGTGGCTCTTTAACAACC
VHL exon 2 R	5' CTGTACTTACCACAACAACC
VHL exon 3 F	5' TCCTTGTACTGAGACCCTAG
VHL exon 3 R	5' AGCTGAGATGAAACAGTCTA
HBB ex1 F	5' TGAGGAGAAGTCTGCCGTT
HBB ex1 R	5' GGGCCTCACCACCAACTT
HBB ex2 F	5' CAAGGGCACCTTTGCCACA
HBB ex2 R	5' CCTGAAGTTCTCAGGATCCACG
HBB ex3 F	5' ACAAGTATCACTAAGCTCGCTTTCT
HBB ex3 R	5' TAGTTGGACTTAGGGAACAAAGG
hLDHA F	5' CTGTCATGGGTGGGTCCTT
hLDHA R	5' GCAACATTCATTCCACTCCA
HBG F	5' GGCAACCTGTCCTCTGCCTC
HBG R	5' GAAATGGATTGCCAAAACGG
*HBB*enh bis F	5' TTTGATTTTATTTAGTTTTTTTGTTTAGAG
*HBB*enh bis R	5' CCAACAAACCTCTAATCTCTTCCTA
*HBB*enh bis F2	5' TTTTAGTTGTTTTTTATGAATGTTTTT
L1 meth F	5' TTGAGTTAGGTGTGGGATATAGTTT
L1 meth F2	5' GTTTTTTTAGGTGAGGTAATGTTT
L1 meth R	5' ATATAAACATAATTAACAAAAAAACCTAAC
VHL P138L_FW	5' CTGAATTATTTGTGCTATCTCTCAATGTTGA
VHL P138L_RV	5' TCAACATTGAGAGATAGCACAAATAATTCAG
VHL H191D_FW	5' GAAGATCTGGAAGACGACCCAAATGTGCAGA
VHL H191D_RV	5' TCTGCACATTTGGGTCGTCTTCCAGATCTTC

8.1.2 PCR reaction conditions

Table 3. PCR conditions for *VHL* mutation screening			
Exon	1	2	3
Denaturation	95°C – 15 min	95°C – 15 min	95°C – 15 min
Cycles	35	35	35
Denaturation	95°C – 30 sec	95°C – 30 sec	95°C – 30 sec
Annealing	58°C – 30 sec	58°C – 10 sec	58°C – 10 sec
Elongation	72°C – 30 sec	72°C – 10 sec	72°C – 10 sec
Final Elongation	72°C – 7 min	72°C – 7 min	72°C – 7 min

Table 4. qPCR conditions for *β-globin* transcripts				
Gene	*hLDHA*	*HBB* ex1	*HBB* ex2	*HBB* ex3
Assay type	Taqman	SYBR	SYBR	Taqman
Denaturation	95°C – 5 min	95°C – 5 min	95°C – 5 min	95°C – 5 min
Cycles	45	45	45	45
Denaturation	95°C – 10 sec	95°C – 10 sec	95°C – 10 sec	95°C – 10 sec
Annealing	63°C – 30 sec	60°C – 10 sec	63°C – 10 sec	60°C – 30 sec
Elongation		72°C – 10 sec	72°C – 10 sec	
		Melting analysis	Melting analysis	
		95°C – 5 sec 55°C – 1 min	95°C – 5 sec 55°C – 1 min	
		continuous increase by 0.1°C/sec to 97°C	continuous increase by 0.1°C/sec to 97°C	

Table 5. PCR conditions for methylation analysis			
Locus	*β-globin* promoter	L1 promoter	*γ-globin* promoter
1st round			
Denaturation	95°C – 15 min	95°C – 15 min	95°C – 15 min
Cycles	35	35	35
Denaturation	95°C – 30 sec	95°C – 30 sec	95°C – 30 sec
Annealing	58°C – 30 sec	58°C – 10 sec	58°C – 10 sec
Elongation	68°C – 1 min	68°C – 1 min	68°C – 1 min
Final Elongation	68°C – 7 min	68°C – 7 min	68°C – 7 min
2nd			
- reaction conditions are the same as for 1st round except the cycles			
Cycles	25	25	25

Table 6. PCR conditions for site mutagenesis		
Mutation	*VHL*^{P138L}	*VHL*^{H191D}
Denaturation	95°C – 30 sec	95°C – 30 sec
Cycles	16	16
Denaturation	95°C – 30 sec	95°C – 30 sec
Annealing	55°C – 1 min	60°C – 1 min
Elongation	68°C – 6 min	68°C – 6 min

8.2 List of other Publications and Meetings Abstracts

Publications

- Ye Z, Liu CF, **Lanikova L**, Dowey SN, He C, Huang X, Brodsky RA, Spivak JL, Prchal JT, Cheng L. Differential sensitivity to JAK inhibitory drugs by isogenic human erythroblasts and hematopoietic progenitors generated from patient iPSCs. *Stem Cells*. 2013; accepted.

- Lorenzo FR, Yang CH, **Lanikova L**, Burtos L, Prchal JT. Novel Compound *VHL* Heterozygosity (*VHL* T124A/L188V) Associated With Congenital Polycythemia. *Br J Haematol*. 2013; doi: 10.1111/bjh.12431/epub ahead of print.

- Prchal JT, Swierczek SI, **Piterkova L**, Jelinek J, Parker CJ, Cairns B. Reliable assessment of X-chromosome inactivation via DNA or RNA based assays remains controversial. *Blood*; *e-Letter*, Jun 2012.

- Swierczek SI, **Piterkova L**, Jelinek J, Agarwal N, Hammoud S, Wilson A, Hickman K, Parker CHJ, Cairns B, Prchal JT. Methylation of AR locus does not always reflect X chromosome inactivation state. *Blood*. 2012; 119(13): e100 - e109.

Meeting Abstracts

- **Piterkova L***, Tian L*, Wang L, Ye Z, Cheng L, Wheeler DA, Hakonarson H, Prchal JT. Whole Genome Sequencing of Four CD34+-Derived iPSC Polycythemia Vera Clones From a Single Female. *Blood*. Nov 2012; 120(21): 705.

  - 54th Annual Meeting of American Society of Hematology, December 8-11 (2012), Atlanta (Georgia), USA.

- Wang L, Swierczek SI, **Piterkova L**, Hickman K, Wheeler DA, Prchal JT. Whole Exome Sequencing of Polycythemia Vera Reveals Novel Recurrent Somatic and Germline Variation. *Blood*. Nov 2012; 120(21): 1755.

  - 54th Annual Meeting of American Society of Hematology, December 8-11 (2012), Atlanta (Georgia), USA.

- Ye Z, **Piterkova L**, Liu C, Dowey S, Chou BK, Huang X, Spivak J, Swierczek S, Moliterno A, Prchal JT, Cheng L. Distinct Induced Pluripotent Stem Cell Clones with Somatic Mutations Prepared From PV Patients. *Blood*. Nov 2011; 118(21): 2826.

    - 53rd Annual Meeting of American Society of Hematology, December 10-13 (2011), San Diego (California), USA.

III Supplements and Appendices

9 Appendices

Original version of Publications

- **Lanikova L**, Kucerova J, Indrak K, Divoka M, Issa JP, Papayannopoulou T, Prchal JT, Divoky V. β-thalassemia due to intronic LINE-1 insertion in the *β-globin* gene: molecular mechanisms underlying reduced transcript of the *β-globin*$_{LI}$ allele. *Human Mutation*. 2013; doi: 10.1002/humu.22383/epub ahead of print.

- **Lanikova L**, Lorenzo F, Yang CH, Vankayalapati H, Drachtman R, Divoky V, Prchal JT. Novel homozygous *VHL* mutation in exon 2 is associated with congenital polycythemia but not with cancer. *Blood*. 2013;121(19):3918-24.

- **Piterkova L***, Tomasic Ljubas N*, Huff Ch, Bilic E, Yoon D, Miasnikova GY, Sergueeva AI, Niu X, Nekhai S, Gordeuk V, Prchal JT. Polycythemia due to Croatian homozygous *VHL* (571C>G:H191D) mutation has a different phenotype than Chuvash polycythemia (*VHL* 598C>T:R200W). *Haematologica*. 2013; 98(4):560-7.

BRIEF REPORT

Human Mutation

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β-Thalassemia Due to Intronic LINE-1 Insertion in the β-*Globin* Gene (*HBB*): Molecular Mechanisms Underlying Reduced Transcript Levels of the β-$Globin_{L1}$ Allele

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ABSTRACT: We describe the molecular etiology of β^+-thalassemia that is caused by the insertion of the full-length transposable element LINE-1 (L1) into the intron-2 of the *β-globin* gene (*HBB*). The transcript level of the affected *β-globin* gene was severely reduced. The remaining transcripts consisted of full-length, correctly processed *β-globin* mRNA and a minute amount of three aberrantly spliced transcripts with a decreased half-life due to activation of the nonsense-mediated decay pathway. The lower steady-state amount of mRNA produced by the β-$globin_{L1}$ allele also resulted from a reduced rate of transcription and decreased production of full-length *β-globin* primary transcripts. The promoter and enhancer sequences of the β-$globin_{L1}$ allele were hypermethylated; however, treatment with a demethylating agent did not restore the impaired transcription. A histone deacetylase inhibitor partially reactivated the β-$globin_{L1}$ transcription despite permanent β-$globin_{L1}$ promoter CpG methylation. This result indicates that the decreased rate of transcription from the β-$globin_{L1}$ allele is associated with an altered chromatin structure. Therefore, the molecular defect caused by intronic L1 insertion in the *β-globin* gene represents a novel etiology of β-thalassemia.

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KEY WORDS: β-thalassemia; *β-globin*; *HBB*; LINE-1; epigenetic repression

We have previously reported that the insertion of a full-length LINE-1 (L1) element (GenBank accession: AF149422.1) in the antisense orientation into the 3′-end of intron-2 of the *β-globin* gene (*HBB*; MIM #141900; GenBank accession: NM_000518.4) causes β^+-thalassemia in a mother and daughter of Ukrainian descent [Divoky et al., 1996]. The β-thalassemia trait in the propositus and her mother was diagnosed in the laboratory of the late Dr. Huisman (Augusta, GA) [Indrak et al., 1992]. We have shown that this L1 element retained the capacity for high-frequency retrotransposition in cultured cells [Kimberland et al., 1999]. Here, we analyzed the molecular mechanisms that are responsible for the severe reduction in *β-globin* gene expression in the presence of the L1 insertion and cause the β^+-thalassemia phenotype.

The β^+-thalassemia nature of this mutation was investigated in the reticulocytes from the patient and in the interspecific hybrids created from the fusion of transformed lymphocytes of the propositus with mouse erythroleukemia cells (MEL) containing either the affected or normal chromosome 11 [Papayannopoulou et al., 1986]. The cell line containing the mutant chromosome 11 from the propositus was designated as "MEL HBB^{L1+}," and the control cell line with a normal chromosome 11 was designated as "MEL HBB^{wt}." Blood samples from the propositus and her mother were obtained with the approval of the institutional review board committees of Palacky University in Olomouc and the Institute of Hematology and Blood Transfusion in Prague, Czech Republic. Informed consent was obtained as per the Declaration of Helsinki. The details of the experimental procedures and the sequences of the primers used for this study are found in the Supp. Methods and Supp. Tables.

The propositus (25-year-old Caucasian female) had microcytic anemia (Hb 11.3 g/dL, red blood cells 5.5×10^{12}/L, mean corpuscular volume 63 fL, and mean corpuscular hemoglobin 20 pg) with a normal reticulocyte count. Her HbA_2 was increased to 5.3% and the HbF was just above 1%. No inclusion bodies of precipitated globin chains were detected in her erythroid cells after brilliant cresyl blue staining. Hematological indices of the patient's mother were comparable; neither patient had neurological or other systemic abnormalities. Southern blot and sequencing analyses revealed heterozygosity for the full-length retrotransposon (L1, i.e., $L1_{\beta\text{-thal}}$) inserted in the antisense orientation into the 3′-end of intron-2 (β-IVS-II) of the *β-globin* gene (hereafter referred to as β-$globin_{L1}$; Supp. Fig. S1) deposited into the HBVar database (http://www.lovd.nl/HBB). The

Additional Supporting Information may be found in the online version of this article.

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insertion occurred at 98 bp 5′- to the 3′-end of β-IVS-II and was flanked by a 9–13-bp target site duplication [Divoky et al., 1996; Kimberland et al., 1999].

The *β-globin* transcripts from affected and unaffected *β-globin* alleles of the propositus were distinguishable by a silent codon 2 polymorphism [Antonarakis et al., 1985; Orkin et al., 1982]. This polymorphism allowed the quantification of the transcript amount and the determination of the β^+-thalassemia phenotype (Fig. 1A). The propositus was a codon 2 CAT/CAC heterozygote, whereas the mother of the propositus, who also carried the L1 insertion, was a codon CAC homozygote. Thus, the L1-containing allele was derived from framework 1 [Antonarakis et al., 1985] with a codon 2 CAC sequence that creates a recognition site for the *Bsi*HKAI enzyme.

To assess whether the β^+-thalassemia is due to abnormal splicing of the *β-globin* message, reticulocyte and MEL HBB^{L1+} mRNAs were analyzed by RT-PCR using several primers. Sequencing of the RT-PCR products revealed both correctly processed transcripts as well as three rare messages with unusual sequences produced by the $\beta\text{-}globin_{L1}$ allele (Fig. 1B). All of the aberrantly spliced *β-globin* transcripts contained a premature translation termination codon, which makes them susceptible to nonsense-mediated mRNA decay (NMD). To determine whether the NMD pathway degrades the $\beta\text{-}globin_{L1}$ mRNA and thus is responsible for the reduction in the steady-state mRNA level produced by the mutant allele, we cultivated the induced MEL hybrids cells overnight with the NMD inhibitor emetine [Yang et al., 2007]. Quantitative real-time PCR (qPCR) showed a 2.4-fold increase ($P < 0.05$ vs. control) in the accumulation of transcripts from the $\beta\text{-}globin_{L1}$ allele in the presence of emetine (Fig. 1C), whereas no increase in the amount of wild-type $\beta\text{-}globin_{L1}$ transcripts was observed under the same conditions. To further confirm that the low stability of the $\beta\text{-}globin_{L1}$ transcripts was due to NMD, transcription in the MEL HBB^{L1+} and MEL HBB^{wt} hybrids was blocked with actinomycin D in the presence and absence of emetine. Indeed, the decreased stability of the $\beta\text{-}globin_{L1}$ mRNA was mainly due to NMD. The half-life of the $\beta\text{-}globin_{L1}$ mRNA was restored almost to normal when emetine was used (Supp. Fig. S2).

In addition to a decrease in RNA stability, a decreased rate of transcription could be responsible for the reduced amount of $\beta\text{-}globin_{L1}$ mRNA. This possibility was examined using a nuclear run-on assay [Yang et al., 2007]. The increase in the *β-globin* nuclear fraction primary transcript level during 40 min of *in vitro* transcription in the presence or absence of ribonucleotide triphosphates (rNTPs, see Supp. Methods for experimental details) was measured. The data showed that the $\beta\text{-}globin_{L1}$ allele has a transcription rate that is reduced 4.7-fold versus the wild type (Fig. 1D).

Previous studies have demonstrated that L1 insertions disrupt the formation of the full-length transcript of the target gene by producing prematurely polyadenylated mRNA. These truncated transcripts form because of the use of the L1 polyadenylation signal(s). Additionally, the release of RNApol-II from the L1 sequence causes inefficient elongation [Han et al., 2004]. We measured the relative amount of the $\beta\text{-}globin_{L1}$ primary transcript using qPCR with primers flanking the insertion (Fig. 1E). The nuclear fraction RNA was collected from MEL HBB^{wt} and MEL HBB^{L1+} hybrids, and the relative amount of wild-type and $\beta\text{-}globin_{L1}$ primary transcripts was measured at exon-2 and exon-3 and expressed as the exon-3/exon-2 ratio normalized to the control as described [Pfaffl, 2001] (see Supp. Methods). The ratio was 0.805, which suggests that 19.5% of the MEL HBB^{L1+} primary transcript is not extended beyond the L1 insertion. The truncated transcripts could result from either premature polyadenylation or incomplete elongation.

Retrotransposons are considered to be epigenetic mediators of gene expression in mammalian cells [Whitelaw and Martin, 2001]. We examined whether the decreased rate of $\beta\text{-}globin_{L1}$ allele transcription is associated with the $\beta\text{-}globin_{L1}$ promoter and 3′-enhancer methylation that could be reversed by a demethylating agent. The locations of the CpG dinucleotides (CpGs) within the $\beta\text{-}globin_{L1}$ promoter and enhancer and also within the L1 promoter are depicted in Figure 2A. The results from bisulfite sequencing (Fig. 2B) show that the $\beta\text{-}globin_{L1}$ promoter sequence was hypermethylated (65% Me-CpG vs. 35% Me-CpG in the control MEL HBB^{wt} cell line). The 3′-enhancer sequence was fully methylated in comparison with the control sequences. Treatment of the induced hybrid MEL cells with decitabine (5-aza-2′-deoxycytidine, DAC, 2 μM) for 24 hr did not change the methylation status of the $\beta\text{-}globin_{L1}$ promoter, nor did it lead to an increase in expression of $\beta\text{-}globin_{L1}$ mRNA (Fig. 2B and C). Different treatment conditions (treatment time 48 hr or 5 μM DAC) gave similar results. Expression of the *γ-globin* gene, which was used as a treatment control, was increased after DAC exposure as expected [Mabaera et al., 2008] (Fig. 2C). The partial demethylation of the $\beta\text{-}globin_{L1}$ erythroid-specific 3′-enhancer sequence [Boehringer et al., 1987] (Fig. 2B) suggested that insensitivity to DAC under these assay conditions was a feature of the $\beta\text{-}globin_{L1}$ and L1 promoters. Even the more stringent DAC-treatment conditions [Qin et al., 2009] did not reactivate $\beta\text{-}globin_{L1}$ expression (data not shown).

The histone deacetylation of the surrounding chromatin was implicated in the inactivation of L1 host genes [Garcia-Perez et al., 2010]. To test whether transcriptional silencing of the $\beta\text{-}globin_{L1}$ allele is related to repressive chromatin modifications such as deacetylation, we treated induced MEL HBB^{L1+} cells with trichostatin A (TSA), a histone-deacetylase (HDAC) inhibitor (Fig. 2D). The TSA-induced reactivation of developmentally silenced *γ-globin* expression in the somatic cell hybrids was previously documented [Swank et al., 2003], and therefore, it was used as an internal treatment control (Fig. 2E). Although exposure of the induced MEL HBB^{L1+} cells to TSA (alone or in combination with DAC) did not change the densely methylated profile of the $\beta\text{-}globin_{L1}$ promoter sequence (Fig. 2D), this treatment partially restored $\beta\text{-}globin_{L1}$ expression (Fig. 2E). This result suggests that histone deacetylation is (in part) responsible for the $\beta\text{-}globin_{L1}$ silencing that could be relieved by TSA.

$L1_{\beta\text{-thal}}$ displays characteristics of an active retrotransposon [Kimberland et al., 1999]. Therefore, it is possible that transcripts originating from the $L1_{\beta\text{-thal}}$ promoter could interfere with the *β-globin* gene that is transcribed in the opposite orientation to this L1 [Whitelaw and Martin, 2001]. The *ε-globin* gene silencing by a transcribed Alu element provides precedence for in cis transcriptional interference by neighboring transcripts from the antisense strand in globin gene regulation [Wu et al., 1990]. Thus, we assayed for the presence of $L1_{\beta\text{-thal}}$-specific transcripts in patient mRNA that was isolated from harvested BFU-E colonies and in mRNA from the interspecific hybrids (see Supp. Methods and Supp. Tables for details). No $L1_{\beta\text{-thal}}$-specific transcripts could be detected in the BFU-Es from the patients. Several L1 transcripts were detected in the induced MEL HBB^{L1+} cells; however, their sequence analyses revealed other than $L1_{\beta\text{-thal}}$ origin (data not shown). Correspondingly, bisulfite sequencing revealed a highly methylated pattern along the $L1_{\beta\text{-thal}}$ promoter region (Fig. 2B and D), suggesting that the absence of endogenous L1 transcripts is a consequence of the $L1_{\beta\text{-thal}}$ promoter hypermethylation. We also investigated whether the $L1_{\beta\text{-thal}}$ insertion splits the *HBB* gene's transcript, that is, the "gene-breaking" model [Wheelan et al., 2005]. We identified neither upstream *HBB*-specific transcripts that terminate in the major antisense polyadenylation signal [Han et al., 2004] nor evidence for L1 antisense promoter-derived *HBB*-specific transcripts (see Supp. Methods and Supp. Tables for details).

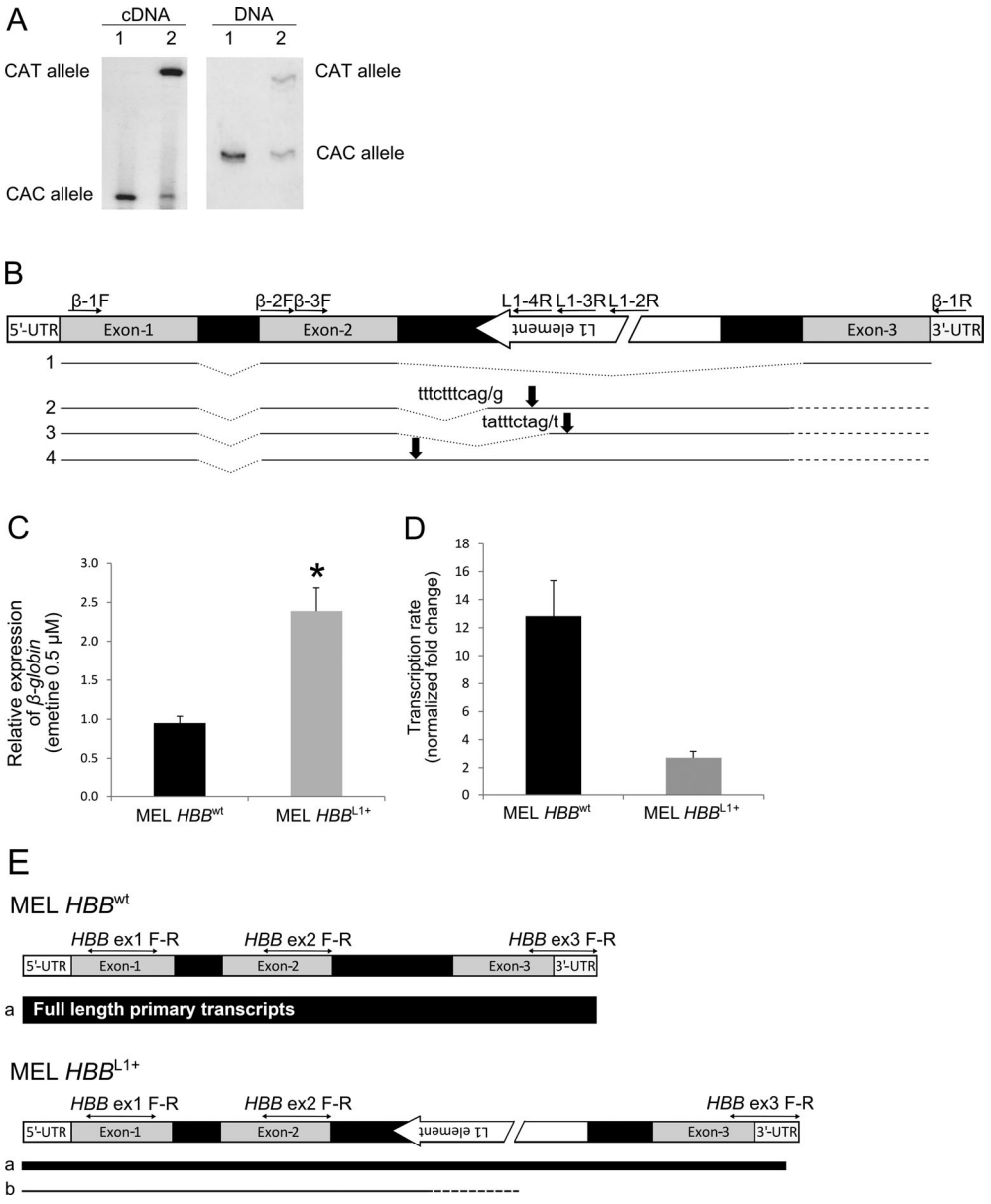

Figure 1. Analyses of transcription from the β-globin$_{L1}$ allele. **A:** Total reticulocyte RNA of the propositus (lane 2, codon 2 CAT/CAC polymorphism heterozygous) and the wild-type control (lane 1, codon 2 CAC homozygous) was subjected to RT-PCR, which amplified exons 1 and 2 of all existing mature β-*globin* transcripts. Aliquots of the labeled PCR products were digested with the *Bsi*HKAI enzyme, which cuts the CAC allele. The ratio of digested to undigested cDNA was 10%–15% to 90%–85% instead of the expected heterozygous ratio of 50% to 50%. Concurrent experiments performed with genomic DNA served as a control for digestion and quantification of PCR products. **B:** The structure of the β-*globin*$_{L1}$ gene is depicted at the top. Gray rectangles represent the β-*globin* exons, black rectangles indicate the β-*globin* introns, and the β-IVS-II is divided by L1 insertion (white rectangle not drawn to scale). The correctly spliced β-*globin* message (1) and three abnormally spliced β-*globin*$_{L1}$ transcripts (2–4) were detected by RT-PCR using three forward (β-1F, β-2F, and β-3F) and four reverse (β-1R, L1-2R, L1-3R, L1-4R) primers (primers are listed in Supp. Table S1, locations are delineated as arrows at the top). The solid lines indicate transcript sequences, the dotted lines are sequences eliminated by splicing, and the dashed lines are portions of transcripts that were not sequenced. One splicing variant resulted from activation of a cryptic acceptor site within β-IVS-II (tttctttcag/g), and the second variant resulted from activation of a cryptic acceptor site within the L1 sequence (tatttctag/g). The third aberrantly processed β-*globin* transcript displayed retention of the globin intron including the inserted L1 sequence. The arrows (⬇) denote stop codons in the aberrant β-*globin* reading frame. **C:** MEL *HBB*$^{L1+}$ and MEL *HBB*^{wt} hybrids were treated with an NMD inhibitor emetine (0.5 μM). The qPCR quantification showed no increase in wild-type β-*globin* mRNA in the MEL *HBB*^{wt} cell line versus a 2.4-fold increase (*, $P < 0.05$ vs. control) in β-*globin*$_{L1}$ mRNA in MEL *HBB*$^{L1+}$ cells. Data are the mean ± SD of three independent experiments. **D:** A PCR-based nuclear run-on assay revealed a 4.7-fold decrease in the β-*globin*$_{L1}$ allele transcription rate compared with the wild-type β-*globin* allele. To include all transcript variants originating at the β-*globin*$_{L1}$ promoter, exon-1 primers were used for quantification. The rate of transcription is displayed as the fold increase of primary β-*globin*/β-*globin*$_{L1}$ transcript over its basal level (not containing rNTPs) after 40 min of in vitro transcription. Data are the mean ± SD of two independent experiments. **E:** Schematic representation of the primary transcripts of the β-*globin* and β-*globin*$_{L1}$ alleles (full-length [a, solid line] and truncated [b, partially dotted line]). The thickness of the lines represents the overall relative amount of the produced primary transcript. According to the PCR-based nuclear run-on assay, the wild-type allele creates 4.7-fold more primary transcript than the mutant β-*globin*$_{L1}$ allele. Additionally, the nuclear RNA was evaluated by β-*globin* exon-2 and exon-3 quantification. The results indicated that a portion of the primary β-*globin*$_{L1}$ transcripts (19.5%) do not contain the sequences downstream of the L1 insertion. The details of the experimental procedures are described in the Supp. Methods.

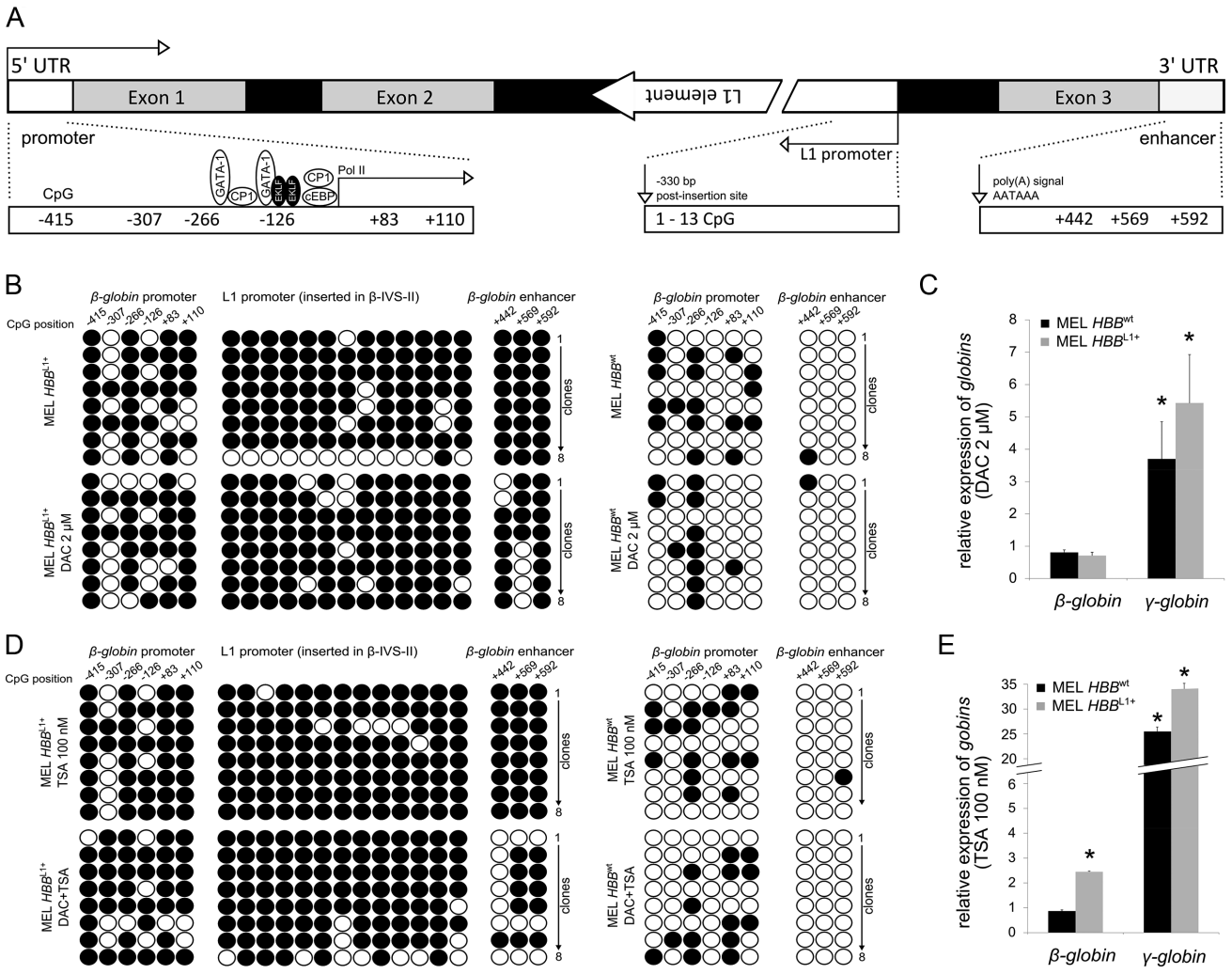

Figure 2. Analysis of epigenetic silencing of the β-*globin*$_{L1}$ allele. **A**: Diagram of the human β-*globin* gene promoter and enhancer regions showing the locations of binding sites for transcription factors, poly(A) signal, and CpG dinucleotides [adapted from Mabaera et al., 2007]. The position of the CpGs within the L1 promoter is also shown. **B:** Genomic DNA from MEL hybrid cells was isolated and bisulfite treated to determine the methylation status of the six CpGs between –415 and +110 bp (bases downstream and upstream of the transcription start site) on the promoter region, 13 CpGs on the promoter of the inserted L1 and three CpGs between +442 and +592 bp on the enhancer region (bases upstream of the poly(A) signal) of the mutated and normal β-*globin* alleles (plotted horizontally). Treatment of DMSO-induced MEL *HBB*$^{L1+}$ cells with a demethylating agent (DAC, 2 μM) did not change the methylation profile of the β-*globin*$_{L1}$ gene and the L1 promoters but did decrease the methylation of the enhancer. • represents methylated sequence, ○ represents unmethylated sequence. **C:** Consequently, DAC treatment did not increase the expression of β-*globin*$_{L1}$ mRNA, whereas the expression of the γ-*globin* gene (*HBG1*; MIM #142200, GenBank: NM_000559.2) was increased. Data are the mean ± SD of three independent experiments (*, $P < 0.05$ vs. untreated cells). For the effect of DAC and TSA treatment expressed as the γ- to β-*globin* mRNA ratio [Swank et al., 2003], see Supp. Figure S3. **D:** Treatment of induced hybrid MEL *HBB*$^{L1+}$ cells with the HDAC inhibitor TSA (100 nM) alone and in combination with the demethylating agent (DAC, 2 μM) did not change the hypermethylation profile of the β-*globin*$_{L1}$ promoter. However, the combined treatment partially reversed the methylation of the β-*globin*$_{L1}$ enhancer. • represents methylated sequence, ○ represents unmethylated sequence. **E:** Despite the permanent methylation pattern of the β-*globin*$_{L1}$ promoter, TSA partially reactivated expression from the mutant β-*globin*$_{L1}$ gene (see also Supp. Fig. S3) indicating that silencing is mediated by histone deacetylation. The data are the mean ± SD of three independent experiments (*, $P < 0.05$ vs. untreated cells). The details of the experimental procedures are described in the Supp. Methods.

In conclusion, we report a combination of molecular events leading to β^+–thalassemia caused by an intronic L1 insertion in the β-*globin* gene. In addition to the known effects of the intronic L1 sequence on mRNA processing [reviewed by Hancks and Kazazian, 2012], our data reveal that transcriptional repression of the host gene is due to regional spreading of methylation that affects both the 5′-promoter and the 3′-enhancer. Our attempts to reactivate the silenced β-*globin*$_{L1}$ expression revealed that the HDAC inhibitor TSA, but not the demethylating agent DAC, leads to a partial restoration of host gene transcription. This result indicates that the decreased rate of transcription from β-*globin*$_{L1}$ is associated with altered chromatin that is likely caused by the spreading of repressive marks induced by L1 to the neighboring β-*globin*$_{L1}$ gene promoter [Garcia-Perez et al., 2010; Montoya-Durango et al., 2009]. These results have therapeutic implications for diseases caused by the deleterious effects of retrotransposons.

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Disclosure Statement: The authors declare no conflicts of interest.

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SUPPLEMENTAL METHODS, TABLES AND FIGURES

Databases

The mutation has been deposited in the LOVD database and HBvar database:

http://lovd.bx.psu.edu/variants.php?select_db=HBB&action=view&view=0002122%2C0001811%2C0

http://globin.bx.psu.edu/cgi-bin/hbvar/query_vars3?mode=output&display_format=page&i=2892

Diagnosis of β-thalassemia

The HbA_2 levels were measured by cation exchange high-performance liquid chromatography (HPLC) and the HbF levels by alkali denaturation and HPLC (Kutlar, et al., 1991). Analysis of the *β-globin* genes of the patient revealed no obvious mutation except that they were heterozygous for an unexpected rearrangement that was detected by gene mapping (not shown).

RNA quantification by primer extension of RT-PCR products

Total reticulocyte RNA from the propositus and the control was subjected to RT-PCR with primers β-F and β-Ex2-R (**Supp. Table S1**). After 19 cycles of PCR, the amplified products were labeled with one cycle of primer extension using a [γ-^{32}P] ATP end-labeled primer β-Ex2-R (95°C, 2 min; 58°C, 2 min; 72°C, 8 min). Aliquots of the PCR products were then digested for 2 h at 65°C with *Bsi*HKAI (New England Biolabs, Beverly, MA), analyzed on an 8% non-denaturing polyacrylamide gel, and visualized by autoradiography. The intensities of the bands were quantified using a PhosphorImager (Molecular Dynamics).

Preparation of human/mouse cell hybrids

Interspecific hybrids of Epstein Barr Virus (EBV) transformed lymphocytes from the propositus and mouse erythroleukemia (MEL) cells were generated. The fusions were done in the presence of polyethylene glycol (PEG, 1500, 50 % w/v) as previously described (Papayannopoulou, et al, 1996). After a period of two to three weeks, clonal outgrowths were evident in many of the wells. Hybrid cells from the clonal outgrowths that contained human chromosome 11 were selected based on reactivity to antibody 53/6, which recognizes an antigen encoded by chromosome 11 (Yagi, et al., 1987). Two lines were derived from the positive cell clones: one containing the affected chromosome 11 (designated "MEL *HBB*$^{L1+}$") and one with the normal chromosome 11 (designated "MEL *HBB*^{wt}"). The hybrids were expanded and cultured in IMDM

supplemented with 10% bovine calf serum (Invitrogen, Life Technologies, NY) in a humidified incubator at 37°C and 5% CO_2 and were induced to differentiate by adding 1.8% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) for 72 h.

The hybrid human/mouse cells were treated under various conditions for different experiments as follows: for 24 h with emetine (0.5 μM, Sigma-Aldrich, St. Louis, MO) for nonsense-mediated mRNA decay (NMD) inhibition; for various times (1 - 12 h) with actinomycin D (Act-D, 1 μM, Sigma-Aldrich, St. Louis, MO) to block mRNA synthesis; and for 24 h simultaneously with DMSO or for 24 h and 48 h before the induction of differentiation by DMSO with 5-aza-2'-deoxycytidine (DAC, 2 μM or 5 μM, Sigma-Aldrich, St. Louis, MO) and/or trichostatin A (TSA, 100 nM, Sigma-Aldrich, St. Louis, MO) to test the DNA methylation pattern/reactivation of silenced gene expression.

Subcloning and sequencing of alternatively spliced transcripts

RNA was isolated from the reticulocytes from the propositus and from the *in vitro* cultured human/mouse cell hybrids. The *β-globin* transcripts were analyzed by RT-PCR and sequencing of the subcloned PCR products. The primer pairs used to amplify and sequence the transcripts were: β-1F and β-1R, for first round PCR; β-2F with L1-4R, β-3F with L1-4R, β-2F with L1-2R and β-3F with L1-3R for nested PCR. The sequences of the PCR primers are listed in **Supp. Table S1**.

Human *β-globin* transcript quantification by real-time qPCR

Total RNA from the MEL HBB^{L1+} and MEL HBB^{wt} cell lines was isolated with TRI reagent (Roche Applied Science) followed by TURBO DNA-free DNase I treatment (Ambion, Life Technologies, NY). For quantification of the human *β-globin* mRNA by qPCR, RNA was reverse-transcribed using a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). cDNA was quantified on a Light Cycler 480 (Roche Applied Science). For quantification of the aberrantly spliced variants and the NMD activation assay, exon-3 primers (*HBB* ex3 F and R) and UPL (Universal Probe Library, Roche Applied Science) probe #56 were used. For the mRNA stability assay and the effect of AZA and/or TSA treatment, exon-2 primers (*HBB* ex2 F and R) were used for *β-globin* mRNA and primers *HBG* F a R for *γ-globin* mRNA quantification. The levels of the human *β-globin* and *γ-globin* transcripts were normalized to

human *LDHA* (OMIM 150000, GenBank accession: NM_005566.3) (primers *LDHA* F and R and UPL probe #47) using an efficiency corrected formula for the relative expression ratio (Pfaffl, et al., 2001). The sequences of the primers and the reaction conditions are listed in **Supp. Tables S1 and S2**.

PCR-based Nuclear Run-On Assay

Nuclei from the hybrid MEL *HBB*$^{L1+}$ and MEL *HBB*^{wt} cells were isolated with the Nuclei EZ Prep Nuclei Isolation Kit (Sigma-Aldrich, St. Louis, MO), according to the manufacturer's protocol. The total nuclei were split into two equal aliquots and 2x reaction buffer was added (containing 30 mM Tris, pH 8; 2,5 mM $MgCl_2$; 150 mM KCl; 20% glycerol; 1 mM DTT and 40 U RNasin; Promega, Madison, WI), and the reaction was performed, as previously described (Yang, et al., 2007). The *in vitro* transcription reaction was initiated by the addition of 0.5 mM of each ribonucleotide triphosphate (rATP, rCTP, rGTP, rUTP) (rNTP) (Invitrogen, Life Technologies, NY) and incubated for 40 min at 30°C. The second aliquot, which did not contain rNTPs, was incubated under the same conditions. The rate of transcription (normalized fold increase) was determined as the ratio of the nuclear fraction primary *β-globin* transcript measured in exon-1 (primers *HBB* ex1 F and R) with added rNTPs to the same fraction at basal level (without added rNTPs) after 40 min of *in vitro* transcription. *LDHA* was used as a reference gene for quantification. The sequences of primers are listed in **Supp. Table S1**.

Relative ratio of exon-3 to exon-2 of the human *β-globin* primary transcript

The *HBB* exon-3/exon-2 ratio of MEL *HBB*$^{L1+}$ native primary transcript from the nuclear fraction that was prepared as mentioned above was normalized to the exon-3/exon-2 ratio of the control MEL *HBB*^{wt} native primary nuclear transcript using formula $2^{\Delta\Delta Ct}$. The method for calculating ΔΔCt was similar to the method used for the relative quantification and is as follows: $(Ct_{MEL\ HBBL1+\ ex2} - Ct_{MEL\ HBBwt\ ex2}) - (Ct_{MEL\ HBBL1+\ ex3} - Ct_{MEL\ HBBwt\ ex3})$. Exon-2 primers *HBB* ex 2 F and R, exon-3 primers *HBB* ex 3 F and R, and exon-3 UPL probe #56 were used for the determination of this ratio. The efficiency of both amplicons was measured using a dilution series standard curve and was very close to 2. The sequences of PCR primers are listed in **Supp. Table S1**.

DNA methylation analysis

Bisulfite modification was performed on the genomic DNA from MEL *HBB*$^{L1+}$ and MEL *HBB*^{wt} hybrids cells using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA). The promoter and enhancer regions of the *β-globin* gene and the L1$_{\beta\text{-thal}}$ promoter were amplified using semi-nested PCR. The *β-globin* promoter primers were published elsewhere (Mabaera, et al., 2007); the enhancer region was amplified with *HBB*enh bis F and R primers for the first round, and then *HBB*enh bis F2 and R were used for semi-nested PCR. The L1 promoter was amplified with L1 meth F and R primers for the first round, followed by primers L1 meth F2 and R for the semi-nested round. The purified products were subcloned, and at least 8 positive clones for each region were sequenced. In the case of the *β-globin$_{L1}$* promoter and 3' enhancer, 10 to 20 clones were sequenced. The sequences of the PCR primers are listed in **Supp. Table S1**.

L1-specific RT-PCR and sequencing

In vitro cultures of BFU-E colonies were performed as described (Horvathova, et al., 2012). Total RNA from reticulocytes and from harvested BFU-Es was treated twice with DNAse I (10 U/1 μg of RNA) at 37°C for 1 hour and then re-extracted using phenol-chloroform and precipitated with ethanol. Overall, 5 pmols of the L1-specific primer were annealed (in 45°C for 30 min) to 1 μg of total RNA (isolated from reticulocytes and from BFU-E colonies) in 1x hybridization buffer (1 M NaCl; 10 mM Tris-HCl pH 7.5; 1 mM EDTA; 0.2% SDS) and were extended with reverse transcriptase (Superscript II, Invitrogen, Life Technologies, NY) according to manufacturer's instructions. This cDNA template was amplified by semi-nested PCR using the same antisense primer as for reverse transcription and two sense primers (L1 F 1 for the first round and L1 F 2 for the second round). DNA sequencing was performed in both directions. The sequences of the primers are listed in **Supp. Table S1**.

Gene-breaking RT-PCR and sequencing

Total RNA from MEL *HBB*$^{L1+}$ and MEL *HBB*^{wt} cell lines was isolated with TRI reagent followed by TURBO DNA-free DNase I treatment. Transcripts terminating in L1 major antisense polyadenylation site were searched by using 0.12 μM L1-polyT chimera primer and reverse transcriptase (Superscript II, Invitrogen, Life Technologies, NY) (Wheelan, et al., 2005); the cDNA synthesis was performed with 3 μg total RNA. 10 μL of the cDNA synthesis solution

was used for PCR amplification with HotStarTaq Master Mix Kit (Qiagen, QIAGEN Valencia, CA) and 0.5 μM primers for the first round: NRO B1 F and MAPS L1 R and second round: NRO B2 F and MAPS L1 R. In searching for transcripts starting in the L1 antisense promoter GeneRacer™ Oligo dT primer was used (GeneRacer™ Kit, Invitrogen, Life Technologies, NY) and ASP L1 F primer (Mätlik, et al., 2006) for the first round, and ASP L1 F and HBB ex3 R primers for the second round. The PCR products (major bands) were purified on agarose gels, cloned by using a TOPO TA Cloning (GeneRacer™ Kit, Invitrogen, Life Technologies, NY) and sequenced. The sequences of the primers are listed in **Supp. Table S1**.

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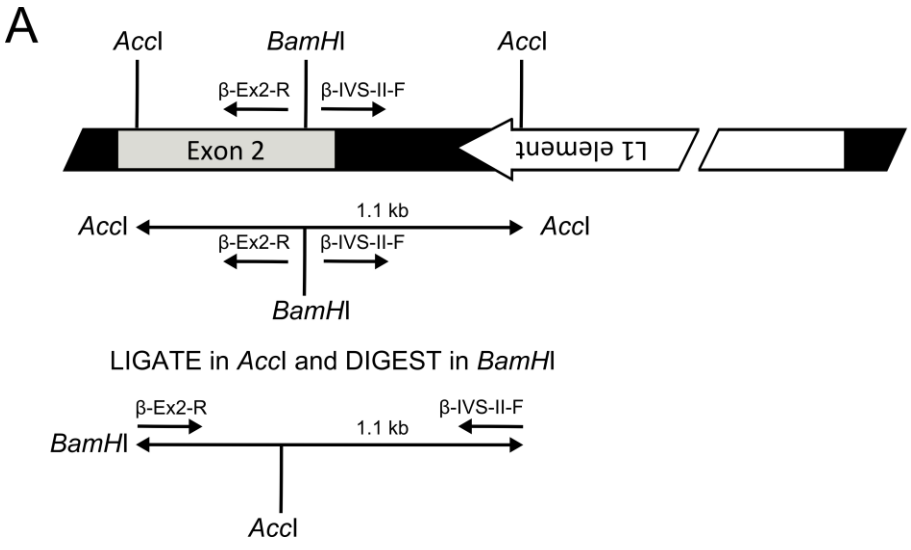
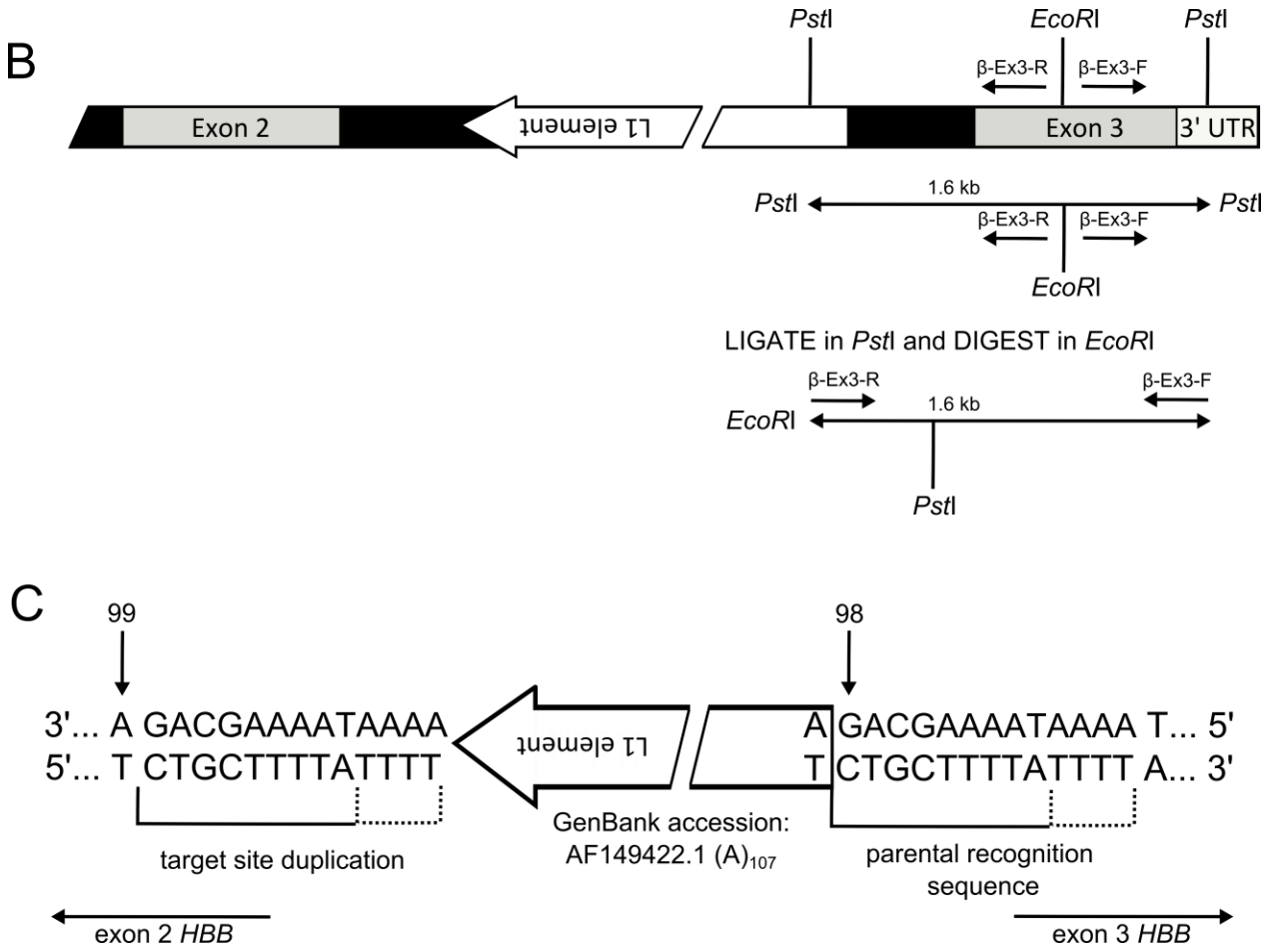
Supp. Figure S1. Schematic diagram of the L1$_{\beta\text{-thal}}$ insertion and *β-globin* gene breakpoints. (A and B) The inverse PCR strategy used to clone the 5' and 3' breakpoints. (A) An *Acc*I genomic fragment (1.1 kb) containing the 5' junction of the *β-globin* IVS-II with the insertion sequence was self-ligated in the *Acc*I site and then linearized in the *BamH*I site. Primers β-Ex2-R and *β*-IVS-II-F were used to amplify the junction sequence. (B) A *Pst*I genomic fragment (1.6 kb) containing the 3' junction of the insertion with *β-globin* IVS-II sequence was ligated in the *Pst*I site and then linearized in the *EcoR*I site. Primers β-Ex3-F and β-Ex3-R were used to amplify the linear fragments containing the junction. (C) Sequence of the breakpoints. The insertion occurred at 98 bp 5′ to the 3′ end of *β*-IVS-II. The arrows above the nucleotide sequence show the residues at positions 98 and 99 bp 5' to the 3' end of *β-globin* IVS-II. The L1 insertion was flanked by a 9-13 bp target site duplication (the run of four As, shown by the hatched bracket, could be duplicated or could be a part of the L1-poly(A) tail) (Kimberland, et al., 1999). The L1-poly(A) tail length is 107(A); the GenBank sequence database (accession AF149422.1) shows only 27(A).

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EcoRI
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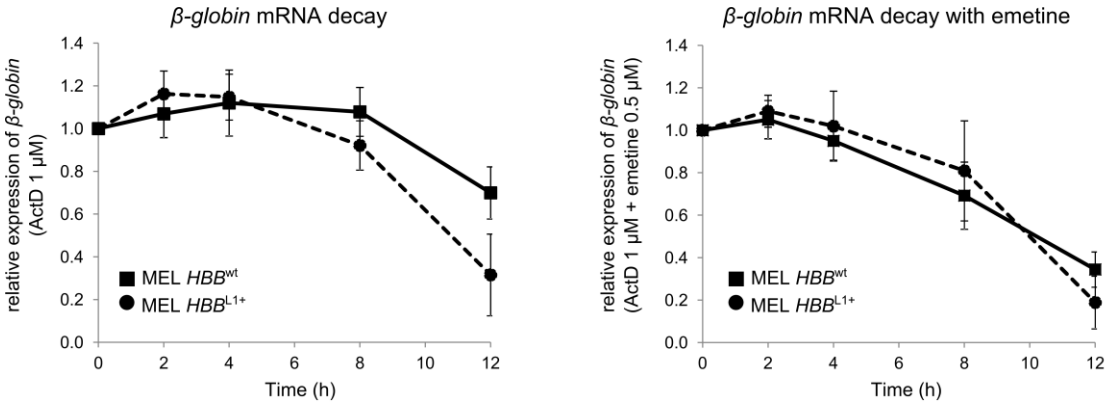
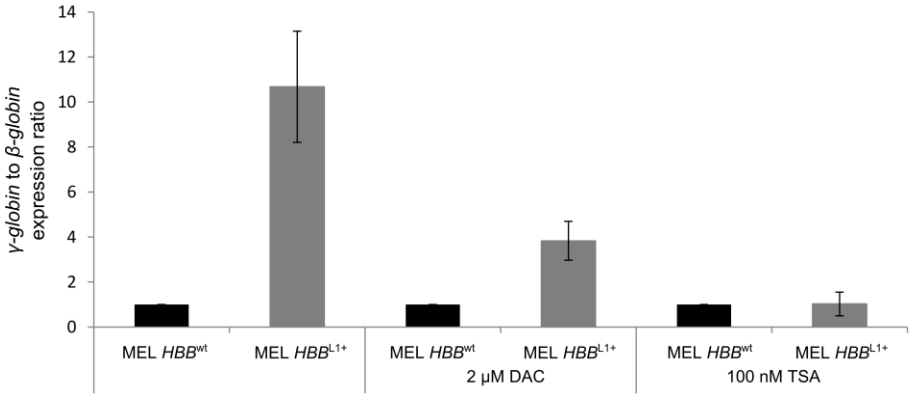
3'... A GACGAAAATAAAA
5'... T CTGCTTTTATTTT
L1 element
A GACGAAAATAAAA T... 5'
T CTGCTTTTATTTT A... 3'
target site duplication
GenBank accession:
AF149422.1 (A)107
parental recognition
sequence
exon 2 HBB
exon 3 HBB

Supp. Figure S2. The half-life of *β-globin* and *β-globin$_{L1}$* mRNA was determined after blockage of transcription for various times (1 – 12 h) with actinomycin D (ActD, 1 μM) in induced hybrid MEL cells. The half-life was calculated (Dölken, et al., 2008) from the relative amount of transcript present at each time point during ActD treatment. The half-life of the wild type *β-globin* mRNA in the control MEL *HBB*^{wt} hybrid cell line was ~11.71 h. The half-life of total mRNA from *β-globin$_{L1}$* allele, measured in MEL *HBB*$^{L1+}$ cells, was only ~4.31 h. To test whether the low stability of *β-globin$_{L1}$* transcripts is solely a consequence of NMD, we also measured the mRNA half-life in the presence of emetine (0.5 μM). The stability of the *β-globin$_{L1}$* mRNA was restored almost to normal levels when emetine was used. The data are the mean ± SD of three independent experiments.



Supp. Figure S3. The ratio of *γ*- to *β-globin* mRNA in hybrid MEL cells treated with DAC (2 μM) and TSA (100 nM) at day 3 of 1.8% DMSO-induced differentiation. The *γ*/*β-globin* transcript ratio of MEL *HBB*$^{L1+}$ cells was normalized to the *γ*/*β-globin* transcript ratio of control cells MEL *HBB*^{wt} using the formula $2^{\Delta\Delta Ct}$ where ΔΔCt was calculated as: $(Ct_\gamma - Ct_\beta)_{MEL\ HBBwt} - (Ct_\gamma - Ct_\beta)_{MEL\ HBBL1+}$. Data are the mean ± SD of three independent experiments. The increased *γ*/*β-globin* mRNA ratio in DMSO-induced and DAC-treated MEL *HBB*$^{L1+}$ cells is consistent with the activation of *γ-globin* in the presence of persistent suppression of *β-globin* expression due to the L1 insertion. TSA treatment resulted in an equal *γ*/*β-globin* transcript ratio in MEL *HBB*^{wt} and MEL *HBB*$^{L1+}$ cells.



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Supp. Table S1.

Table S1. List of primers used	
Primer name	**Sequence**
β-1F	5'TGAGGAGAAGTCTGCCGTTA
β-1R	5'CAGAATCCAGATGCTCAAGG
β-2F	5'GTGGTCTACCCTTGGACCC
β-3F	5'CTTTGGGGATCTGTCCACT
L1-2R	5'TTCCCTATTTAATAAATGGT
L1-3R	5'AATATCCAGAATCTACAATGAA
L1-4R	5'CCAAATGTCCAACAATGATAGTCTGGA
β-F	5'CATTTGCTTCTGACACAACT
β-Ex2-R	5'CCATAACAGCATCAGGAGTG
β-Ex3-F	5' TGCAGGCTGCCTATCAGAAA
β-Ex3-R	5' GCACACAGACCAGCACGTTG
β-IVS-II-F	5' GTGGAAGTCTCAGGATCGTT
HBB ex1 F	5' TGAGGAGAAGTCTGCCGTT
HBB ex1 R	5' GGGCCTCACCACCAACTT
HBB ex2 F	5' CAAGGGCACCTTTGCCACA
HBB ex2 R	5' CCTGAAGTTCTCAGGATCCACG
HBB ex3 F	5' ACAAGTATCACTAAGCTCGCTTTCT
HBB ex3 R	5' TAGTTGGACTTAGGGAACAAAGG
HBG F	5' GGCAACCTGTCCTCTGCCTC
HBG R	5' GAAATGGATTGCCAAAACGG
LDHA F	5' CTGTCATGGGTGGGTCCTT
LDHA R	5' GCAACATTCATTCCACTCCA
*HBB*enh bis F	5′TTTGATTTTATTTAGTTTTTTTGTTTAGAG
*HBB*enh bis R	5' CCAACAAACCTCTAATCTCTTCCTA
*HBB*enh bis F2	5' TTTTAGTTGTTTTTATGAATGTTTTT
L1 meth F	5' TTGAGTTAGGTGTGGGATATAGTTT
L1 meth F2	5'GTTTTTTAGGTGAGGTAATGTTT
L1 meth R	5' ATATAAACATAATTAACAAAAAAACCTAAC
L1-specific primer	5'TCAGTTTTAGGGTACATGTGC
L1 F 1	5'TGCTCATCATCACTGGCCAT
L1 F 2	5'AGTCAGGAAACAACAGGTGC
L1-polyT chimera primer	5' TTTTTTTTTTTTTTTTTTAAAGACA
NRO B1 F	5' ACGTGGATGAAGTTGGTGGT
MAPS L1 R	5' TGTGCACATGTACCCTAAAACTG
NRO B2 F	5' GCACGTGGATCCTGAGAAC
ASP L1 F	5' CTGCTGTGCTAGCAATCAGC

Supp. Table S2.

Table S2. Real-time PCR conditions				
Gene	*LDHA*	*HBB* ex1	*HBB* ex2	*HBB* ex3
Assay type	Taqman	SYBR	SYBR	Taqman
Denaturation	95 °C – 5 min	95 °C – 5 min	95 °C – 5 min	95 °C – 5 min
Cycles	45	45	45	45
Denaturation	95 °C – 10 sec	95 °C – 10 sec	95 °C – 10 sec	95 °C – 10 sec
Annealing	63 °C – 30 sec	60 °C – 10 sec	63 °C – 10 sec	60 °C – 30 sec
Elongation		72 °C – 10 sec	72 °C – 10 sec	
		Melting analysis	*Melting analysis*	
		95 °C – 5 sec 55 °C – 1 min	95 °C – 5 sec 55 °C – 1 min	
		continuous increase by 0.1 °C/sec to 97 °C	continuous increase by 0.1 °C/sec to 97 °C	

RED CELLS, IRON, AND ERYTHROPOIESIS

Novel homozygous *VHL* mutation in exon 2 is associated with congenital polycythemia but not with cancer

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Key Points

- We describe a novel homozygous mutation in exon 2 of the *VHL* gene causing congenital polycythemia.
- We demonstrate the *VHL*^{P138L} effect on the augmentation of erythropoiesis, along with structural and functional studies of this mutation.

Germline *von Hippel–Lindau* (*VHL*) gene mutations underlie dominantly inherited familial VHL tumor syndrome comprising a predisposition for renal cell carcinoma, pheochromocytoma/paraganglioma, cerebral hemangioblastoma, and endolymphatic sac tumors. However, recessively inherited congenital polycythemia, exemplified by Chuvash polycythemia, has been associated with 2 separate 3′ *VHL* gene mutations in exon 3. It was proposed that different positions of loss-of-function *VHL* mutations are associated with VHL syndrome cancer predisposition and only C-terminal domain-encoding *VHL* mutations would cause polycythemia. However, now we describe a new homozygous *VHL* exon 2 mutation of the *VHL* gene:(c.413C>T):P138L, which is associated in the affected homozygote with congenital polycythemia but not in her, or her-heterozygous relatives, with cancer or other VHL syndrome tumors. We show that *VHL*^{P138L} has perturbed interaction with hypoxia-inducible transcription factor (HIF)1α. Further, VHL^{P138L} protein has decreased stability in vitro. Similarly to what was reported in Chuvash polycythemia and some other instances of HIFs upregulation, *VHL*^{P138L} erythroid progenitors are hypersensitive to erythropoietin. Interestingly, the level of *RUNX1/AML1* and *NF-E2* transcripts that are specifically upregulated in acquired polycythemia vera were also upregulated in *VHL*^{P138L} granulocytes. (*Blood*. 2013;121(19):3918-3924)

Introduction

The von Hippel-Lindau (VHL) tumor suppressor gene encodes a multifunctional protein that interacts with diverse partners and promotes degradation of hypoxia-inducible transcription factors (HIFs) by facilitating their ubiquitinization and eventual proteasomal degradation. Germline dominantly inherited mutations in *VHL* predispose patients to highly vascularized malignant tumors, including renal cell carcinoma of the clear-cell type, hemangioblastoma, and pheochromocytoma/paraganglioma.[1,2] Less frequently, *VHL* mutations have been associated with benign tumors, including those of the inner ear (endolymphatic sac tumor), pancreas (pancreatic cysts, serous cystadenoma, and pancreatic neuroendocrine tumors), and testes (epididymal cystadenomas).[2] The incidence of VHL disease is thought to be about 1 in 36 000 births with an estimated de novo mutation rate of 4.4×10^{-6} gametes per generation.[3] VHL tumors generally develop after age of 12[4] and have more than 90% penetrance in the highest age classes (96% at 51–60 years of age, 99% at 61–70 years of age).[3]

Polycythemia (also known as erythrocytosis) is characterized by an increased red cell blood mass. Polycythemias can be primary or secondary. Primary polycythemias are caused by somatic or germline mutations leading to changes within the erythroid progenitors causing an augmented response to erythropoietin (EPO). Secondary polycythemias are caused by either an appropriate or inappropriate increase in the red cell mass as a result of augmented levels of EPO. In Chuvash polycythemia, the first known congenital disorder of hypoxia-sensing,[5] erythroid progenitors in in vitro cultures are hypersensitive to EPO; thus, Chuvash polycythemia shares features of both primary and secondary polycythemia.[6]

Because different positions of loss-of-function mutations of the *VHL* gene are associated with different type of cancers, it has been proposed that only C-terminal domain–encoding *VHL* mutations would cause polycythemia.[7] There are 2 known homozygous *VHL* gene mutations causing polycythemia that are not associated with any VHL syndrome malignant or benign tumors; these are the *VHL*^{R200W} mutation, constituting the disorder of augmented hypoxia-sensing in normoxia (ie, Chuvash polycythemia[5]) and the *VHL*^{H191D} mutation found in a Croatian subject.[8] Both are located in the distal portion of exon 3 of the *VHL* gene in its C-terminal domain. In addition, there are several reports of compound heterozygotes associated with congenital polycythemia, 3 in combination with *VHL*^{R200W} mutation, and others employing different exon 3 *VHL* missense

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Table 1. Homozygous and compound heterozygous *VHL* mutations in individuals with erythrocytosis/polycythemia reported in the literature

VHL genotype		Ethnicity	Notes
Compound heterozygous *VHL* genotype			
235 C→T/586 C→G	R79C/L188V	Caucasian[9]	
376 G→A/548 C→T	D126N/S183L	British[10]	
598 C→T/574 C→T	R200W/P192A	American (white)[11]	
598 C→T/562 C→G	R200W/L188V	American (white)[11]	
598 C→T/388 G→C	R200W/V130L	American (white)[8]	
Homozygous *VHL* genotype			
598 C→T/598 C→T	R200W/R200W	Chuvash,[5] Italian,[12] Danish,[11] German,[13] Turkish,[13] American (white)[11]	Frequent thrombotic complications
571 C→G/571 C→G	H191D/H191D	Croatian[11,27]	
413 C→T/413 C→T	P138L/P138L	Punjabi	

mutations (Table 1).[9-13] It has been suggested that the genomic configuration of the 3′ region of *VHL* exon 3 gene has a specific erythropoiesis-promoting effect independent of EPO by Janus Kinase-2 (JAK2) hyperactivation, because JAK2 is the crucial component of intracellular activation of EPO/EPO receptor signaling.[14] Compatible with this report, to date no congenital polycythemia associated with *VHL* homozygous or compound heterozygous mutations outside of *VHL* exon 3 have been described. We now challenge this premise by reporting a family with congenital polycythemia with a new homozygous *VHL* mutation in exon 2 along with structural and functional studies of the mutation (Figure 1).

Materials and methods

Patient samples

The propositus is a 15-year-old girl of Asian Indian extraction (Punjabi ethnicity), who has been known to be polycythemic from infancy. Her parents are hematologically normal and are of the same ethnicity but not known to be related. Peripheral blood of the propositus and her parents was obtained by venipuncture after obtaining a signed Institutional Review Board informed consent in accordance with the Declaration of Helsinki. This study received approval from Institutional Review Board protocol #17665; molecular biology of polycythemia and thrombocytosis. Granulocyte and mononuclear cell fractions were isolated according to a previously published protocol.[15]

Mutation screening

Genomic DNA was isolated from granulocytes using Gentra-Puregene Kit (Qiagen, Germantown, MD), and *VHL* gene was amplified using Hot Star Master Mix (Qiagen) and the following primers: VHL_exon1_F 5′CGAA GACTACGGAGGTC GAC; VHL_exon1_R 5′GGCTTCAGACCGTGC TATCG; VHL_exon2_F 5′GTGTGGCTCTTTAACAACC; VHL_exon2_R 5′CTGTACTTACCACAACAACC; VHL_exon3_F 5′TCCTTGTACTGA GACCCTAG; and VHL_exon3_R 5′AGCTGAGATGAAACAGTCTA. Sequencing was performed using the same amplification primers and protocol established by the University of Utah, Core DNA Sequencing Facility.

In vitro assay of erythroid progenitors' sensitivity to EPO

In vitro sensitivity of erythroid progenitors to EPO was performed on mononuclear cells isolated from the peripheral blood using Histopaque (Sigma-Aldrich, St. Louis, MO) density gradient centrifugation and plating (2.3×10^5/mL) to methylcellulose media (MethoCult H4531; StemCell Technologies, Vancouver, BC) with addition of various concentrations of EPO (StemCell Technologies), ranging from 0.015 to 3.0 U/mL. Cell cultures were maintained in humidified atmosphere of 5% CO_2 at 37°C for 14 days. Erythroid burst-forming unit colonies (BFU-Es) were scored by standard morphologic criteria. The number of BFU-Es grown in individual concentrations of EPO was expressed as a percentage of maximum vs the concentration of EPO. Maximum growth (100%) represents the highest colony number grown in culture. The assay was carried out on erythroid progenitors from a VHL^{P138L}-homozygous patient (n = 1), from patient with heterozygous gain-of-function $HIF2\alpha^{M535V}$ mutation (n = 1), and 8 healthy controls (n = 8). Results for healthy controls are pooled and T bars designate standard deviations.

Quantitative analysis of HIF target genes expression

RNA was isolated from the patient's granulocytes using TRI reagent and residual DNA was removed by DNA-free DNase Treatment & Removal Reagents (Ambion, Life Technologies, NY). Gene expression experiments were performed on a FastPCR 7500 instrument using TaqMan Gene Expression assays *TFRC* (Hs00951083), *SLC2A1* (Hs00892681), *HK1* (Hs00175976), *RUNX1* (Hs00231079), *NF-E2* (Hs00232351), and reference

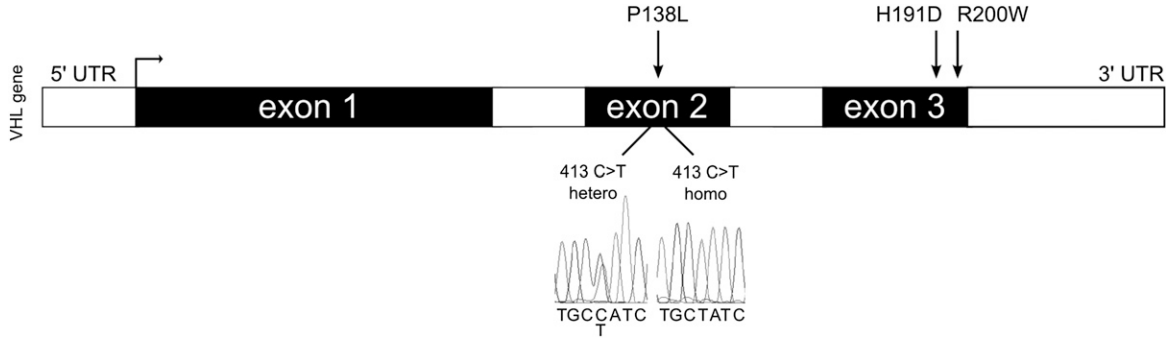


Figure 1. The schematic structure of the *VHL* gene. Sequencing of the second exon of the *VHL* gene revealed c.413C>T:P138L homozygous *VHL* mutation in the propositus, which was inherited from her parents, both VHL^{P138L} heterozygous. UTR, untranslated region.

Table 2. Characteristics of patients with *VHL* homozygous mutation (adjusted to age)

VHL mutation	Age, y	Sex	Hct, %	MCV, (fL)	EPO, (mIU/mL)	In vitro assay
VHL^{P138L}	15	Female	59.2	88.1	40	Hypersensitive
VHL^{H191D}	5	Female	53.1	63.1	201.6	Normal[27]
VHL^{R200W}	16	Male	53.4	81.2	31	Hypersensitive[27]

genes *HPRT* (4333768F) and *GAPDH* (4333764F). All samples were investigated in triplicate. The data represent the mean of 3 independent experiments; T bars designate SEM. The statistical significance of relative expression changes of target mRNA levels was analyzed using REST 2009 software.[16]

Cell culture, plasmids, and transfection

The 786-0 renal cell carcinoma cell line was purchased from ATCC (CRL-1932; Manassas, VA) and *VHL* human cDNA open reading frame clone was obtained from OriGene (RC216151; Rockville, MD). Site-directed mutagenesis was performed using the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) and allele-specific oligonucleotides for VHL^{P138L} and VHL^{H191D} mutations. Transfection of mutated and empty plasmids was done using Lipofectamine 2000 reagent (Invitrogen, Life Technologies). Cells were selected 48 hours after transfection using 1 mg/mL G418 (Invitrogen, Life Technologies) and cultured for 21 days. Resistant clones were isolated using 96-well limiting dilution. Thirty single clones were picked up for each *VHL* construct and tested for expression of *VHL*(Myc-DDK) by TaqMan Gene Expression assay on demand (Applied Biosystems, Carlsbad, CA).

Determination of pVHL stability

To determine the half-life of protein VHL (pVHL), 786-0 stable transfected clones (VHL^{wt}, VHL^{P138L}, and VHL^{H191D}) were treated with 200 μM cycloheximide (Sigma-Aldrich) and cells were harvested at different time points (0, 2, 4, 6, 8, and 10 hours). Western blot was performed with antibodies VHL (FL-181, Santa Cruz, 1:500) and actin (Sigma-Aldrich, 1:1000). Quantitation of pVHL signals was performed using densitometric analysis by ImageJ software according to the software manual.[17] The data represent the mean of 3 independent experiments; T bars designate standard deviations.

Structural and energetic modeling

Computational analysis (molecular graphics, structural manipulations, energy minimization, molecular docking, and molecular dynamics simulations) were carried out using Internal Coordinate Mechanics molecular modeling program (Molsoft, La Jolla, CA). Energy grids representing the active site (van der Waals, hydrogen bonding, electrostatics, and hydrophobic interactions) were calculated with 0.5 Å grid spacing, and protein ligand docking experiments were performed using the defined site map–binding pocket with the application of our docking workflow.[18]

Immunoprecipitation assay

HIF1α ubiquitination and pVHL binding was determined through immunoprecipitation assay as previously described.[19,20] Briefly, 786-0 cells were co-transfected with VHL plasmids (VHL^{wt}, VHL^{P138L}, VHL^{H191D}, and VHL^{R200W}) together with hemagglutinin (HA)-tagged HIF1α plasmid using Lipofectamine 2000 (Invitrogen; Life Technologies). Cells were lysed in either NP40 lysis buffer for pVHL binding or radio-immunoprecipitation assay lysis buffer supplemented with 1% sodium dodecyl sulfate for ubiquitination assay. Lysate was immunoprecipitated using Protein G Dynabeads co-immunoprecipitation kit (Invitrogen, Life Technologies) and anti-HA antibody (Covance, Gaithersburg, MD). Bound protein was eluted in loading buffer supplemented with 1% sodium dodecyl sulfate. Eluents were subsequently analyzed by western blot and quantitation of signals was performed using densitometric analysis by ImageJ software.

Results

VHL^{P138L} mutation

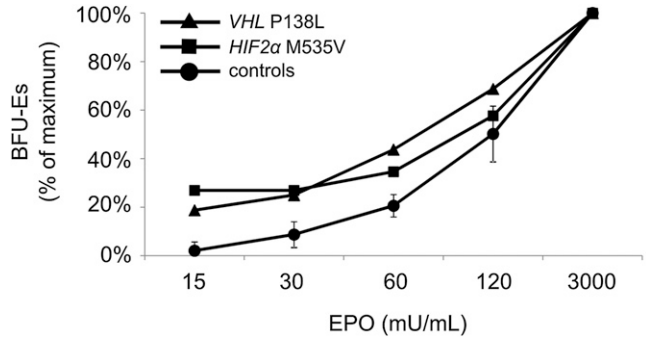
We report a novel homozygous variant of the *VHL* gene located in the middle of exon 2: c.413C>T, VHL^{P138L}. The propositus is a 15-year-old Punjabi female with congenital polycythemia (hemoglobin 19-20 g/dL), elevated EPO at 40 mIU/mL, no evidence of high-affinity hemoglobin mutations or 2,3-DPG deficiency as determined by a normal p50,[21] and absence of $JAK2^{V617F}$ or exon 12 *JAK2* mutations. Her parents were found to be VHL^{P138L} heterozygous. No VHL syndrome malignant or benign tumors have been encountered in the propositus' parents or in their extended family.

In vitro analysis of VHL^{P138L} native erythroid progenitors

We then analyzed the VHL^{P138L} propositus early erythroid progenitors in BFU-E assay and found them to be hypersensitive to EPO (a feature of primary polycythemias), as shown in Figure 2.

The effect of VHL^{P138L} mutation on HIFs signaling

The accumulation of transcripts of HIF-regulated target genes, including those for glucose transporter-1 (*SLC2A1*), transferrin receptor (*TFRC*), and hexokinase-1 (*HK1*), was measured in the



	No. of BFU-E colonies		
EPO (mU/mL)	*VHL* P138L	*HIF2α* M535V	controls
15	6	7	1 ± 1
30	8	7	2 ± 1
60	14	9	3 ± 1
120	22	15	7 ± 2
3000	32	26	14 ± 6

Figure 2. Response of BFU-E erythroid progenitors to EPO. EPO dose–response curves derived from the homozygous VHL^{P138L} patient (△), patient with heterozygous gain-of-function $HIF2\alpha^{M535V}$ mutation (□), and healthy controls (●; n = 8, T bars = SD). VHL^{P138L}-affected erythroid progenitors display hypersensitivity to low concentration of EPO (15–60 mU/mL). There was a relatively higher number of BFU-Es in comparison with healthy controls (number of colonies ± SD) in all analyzed EPO concentrations. The assays of VHL^{P138L} and $HIF2\alpha^{M535V}$ erythroid progenitors were not done concomitantly.

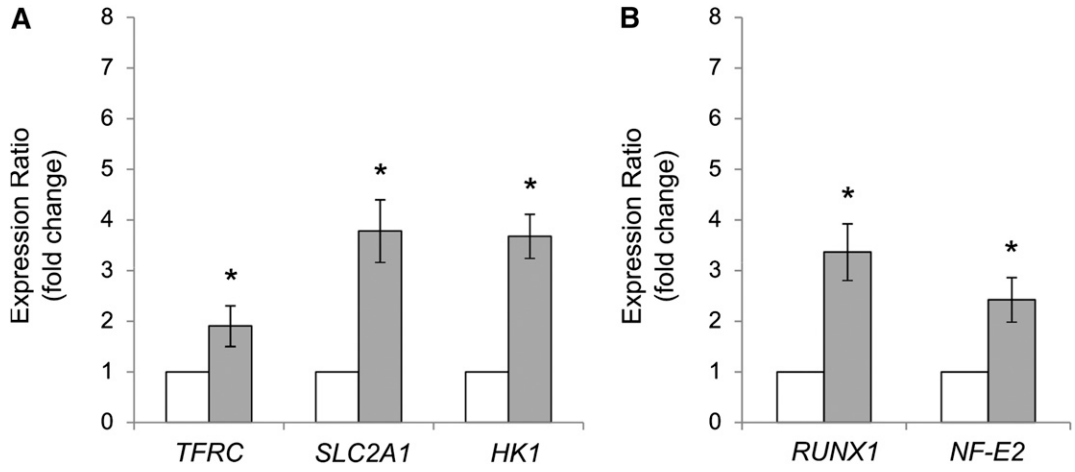


Figure 3. Relative expression of HIF target genes induced by hypoxia and *AML1/RUNX1* and *NF-E2* genes. (A) Expression of *TFRC, SLC2A1*, and *HK1* genes and (B) *AML1/RUNX1* and *NF-E2* genes was evaluated by quantitative polymerase chain reaction in granulocytes isolated from homozygous patient for VHL^{P138L} mutation (grey columns), and normal controls (n = 8, white columns). Data are normalized to *HPRT* and *GAPDH* reference genes. T bars = SEM. *$P < .01$.

propositus' granulocytes. As shown in Figure 3A, the transcripts of these HIF-regulated genes were significantly increased.

RUNX1/AML1 transcript levels in granulocytes

The upregulation of *RUNX1/AML1* transcript levels was reported in erythroid progenitors and granulocytes and claimed to be specific for an acquired polycythemic disorder (ie, polycythemia vera [PV]) and to be responsible for PV EPO hypersensitivity.[22,23] However, we considered the possibility that the increased transcripts of these genes may be a feature associated with other primary polycythemic states with increased EPO sensitivity.[8] We thus analyzed *RUNX1/AML1* and *NF-E2* transcripts in the propositus and found them to be increased (Figure 3B).

In vitro analysis of VHL^{P138L} effect in VHL-null 786-0 renal carcinoma cells

We analyzed the effect of homozygosity of VHL^{P138L} on pVHL stability. Renal carcinoma cells 786-0, which do not express a detectable pVHL, were stably transfected with plasmids expressing VHL^{wt}, VHL^{P138L}, and VHL^{H191D} proteins. Mutant and wild-type cell lines were treated with cycloheximide (CHX) for different lengths of time and pVHL levels were determined by western blot. We show a decreased stability of both mutated VHL peptides (VHL^{P138L} and VHL^{H191D}) in the transfected cells (Figure 4).

Molecular dynamics simulation study of VHL^{P138L} mutation effect

Molecular dynamics simulation showed that the VHL^{P138L} mutation, which lies in the catalytic HIF1α peptide ligand–binding region, perturbs HIF1α/pVHL interaction because of the conformational effect on the Trp117 and Ser111 residues (Figure 5A-B), both within 2.8 to 4.3 Å distance from the mutated Leu138. The effect of this single mutation on overall structure was calculated as a shift of 1.9 Å root mean square deviation from the wild-type complex structure. Our model also predicted that HIF1α binding energy would change from −34.86 kcal/mol to −29.84 kcal/mol, mainly from the loss of 2 ligand hydrogen-bonding interactions within the residues Ser111 and Arg107.

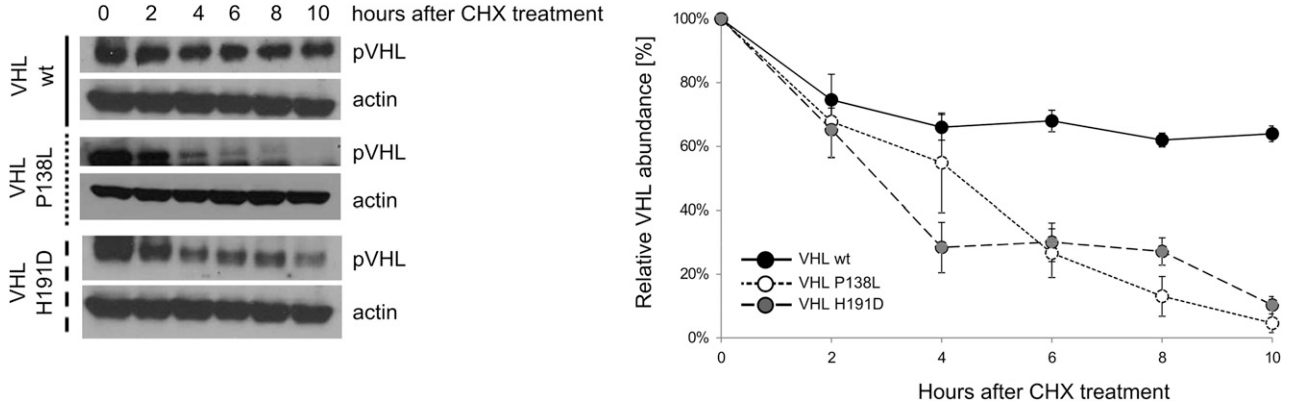


Figure 4. CHX assay measuring pVHL stability, showing mutant VHL proteins decreased half-life in vitro. The 786-0 cells were stably transfected with plasmids expressing VHL^{wt}, VHL^{P138L}, and VHL^{H191D} mutants. Clones were treated with cycloheximide (CHX, 200 μM) for 0, 2, 4, 6, 8, and 10 hours, and lysates were subjected to western blot as indicated. Actin was used as the loading control. (Right) The relative quantitation of pVHL.

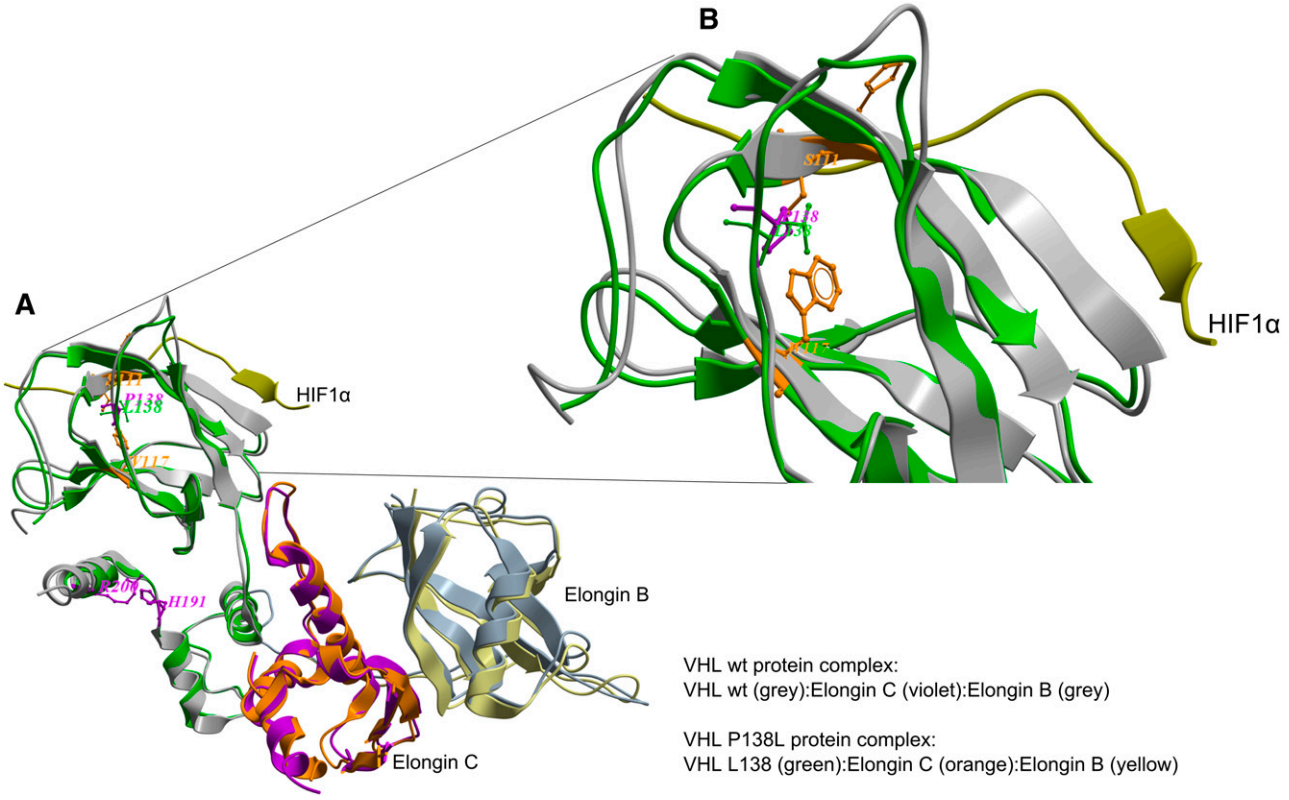


Figure 5. Molecular dynamics simulations study of pVHL–ElonginC–ElonginB complex and interaction with HIF1α. (A) Superimposition of wild-type (wt; grey) and mutated pVHL (green) is shown. The wt P138 (violet) and mutated L138 (green) sites and the critical active site residues for the HIF1α peptide (PDB:1LM8)-binding region are depicted. The P138L mutation perturbs pHIF1α interactions with pVHL because of the conformational effect on the W117 and S111 residues (orange) at 2.8 to 4.3 Å distance from mutated L138. (B) Detail superimposition of wt and P138L pVHL in the interaction with HIF1α.

Immunoprecipitation assay of HIF1α ubiquitination and pVHL binding

To determine the effect of the Pro138Leu substitution on pVHL function, we carried out an in vitro ubiquitination assay. The level of HIF1α ubiquitination was markedly decreased in cells expressing all 3 mutant proteins (P138L, H191D, and R200W), with the highest reduction associated with pVHL^{P138L} (Figure 6A). To further investigate the impact of the *VHL*^{P138L} mutation on HIF1α signaling, we analyzed the binding of mutated pVHL to the HIF1α peptide. Radioactively labeled VHL^{P138L} protein showed decreased affinity to HIF1α peptide (Figure 6B), with only 26.6% of the VHL protein bound. By comparison, the wild-type VHL protein (100%) and also proteins with other homozygous polycythemic mutations (H191D, R200W) have much stronger affinity for the HIF1α peptides, with 49.0% and 62.2% of the bound VHL protein, respectively. These data indicate that the P138L mutation specifically reduced the affinity of pVHL for HIF1α, resulting in a reduced rate of ubiquitination under nonhypoxic conditions.

Discussion

We describe a new homozygous *VHL* exon 2 mutation, *VHL*^{P138L}, which is associated in the affected homozygote with congenital polycythemia but not in her parents or her relatives, with cancer or other VHL syndrome tumors. This contrasts with reports of other heterozygous *VHL* mutations encoding the same amino acid

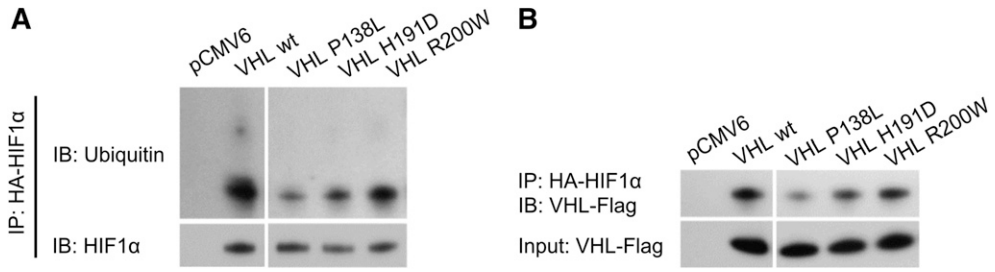


Figure 6. Immunoprecipitation assay of HIF1α ubiquitination and VHL binding. The 786-0 cells were cotransfected with plasmids expressing pVHL (pCMV6 denotes empty plasmid without VHL open reading frames) and HA-tagged HIF1α. Total protein was extracted from cells and HIF1α was precipitated with magnetic beads coupled with anti-HA antibody. pVHL binding and HIF1α ubiquitination was then tested by western blot. Samples were run on the same gel but were noncontiguous, as indicated by white spaces.

residue (*VHL* P138R, P138T) that have been reported in VHL syndrome and renal cancer.[24-26] We also show that this mutation is not only associated with elevated EPO levels but also with a hallmark of primary polycythemia (ie, EPO hypersensitivity).

This novel, polycythemia-associated germline homozygosity for the hypomorphic VHL^{P138L} allele, albeit present in a different domain of the VHL protein, has a similar erythropoiesis-stimulating effect to the Chuvash VHL^{R200W} mutation in regard to EPO levels and BFU-Es EPO hypersensitivity (Table 2). It has been suggested that these features are due to a conformational change of SOCS1's binding groove encoded by *VHL* gene's exon 3. SOCS1 is a negative regulator of JAK2, and it has been suggested that the impaired interaction of VHL^{R200W} and VHL^{H191D} with SOCS1 is a cause of EPO hypersensitivity.[9] However, this putative mechanism is contradicted by our data of EPO-hypersensitive VHL^{P138L} erythroid progenitors (Figure 2) and also by the fact that VHL^{H191D} native erythroid progenitors are not EPO hypersensitive,[27] even though VHL^{H191D} is located in the SOCS1's binding groove.[9] Further, our data reveal that some *HIF2α* gain-of-function mutation (c.1603A>G:M535V)[28] also have EPO-hypersensitive erythroid progenitors. We conclude that the molecular mechanisms of EPO-hypersensitive stimulation of erythroid proliferation (a feature of primary polycythemia) remain unexplained and await further studies.

We show that the loss of function of the VHL^{P138L} mutation (as well as VHL^{H191D}) is at least in part from decreased stability of VHL protein (Figure 4). Similar protein instability was previously shown for the Chuvash VHL^{R200W} mutation.[29] Further, our computer modeling of the $pVHL^{P138L}$ structure predicted significant interference with binding of the α subunit of the HIF1 peptide, which was confirmed by HIF1α ubiquitination and a pVHL-binding assay (Figure 6). These functional abnormalities would be expected to prolong the half-life of HIF1 or HIF2, essential transcriptional factors regulating hypoxia sensing. Indeed, this assumption is directly confirmed by the enhanced expression of HIF-regulated genes *SLC2A1*, *TFRC*, and *HK-1* (Figure 3A). These data suggest that the impaired stability, together with reduced affinity for the HIF α subunit of the polycythemic pVHL mutants, are common features resulting in the delayed ubiquitinization and degradation of HIFs.[30]

We also demonstrate that granulocytes from the VHL^{P138L} subject have increased transcripts of *RUNX1* and *NF-E2* genes (Figure 3B). It was reported that increased transcription of *NF-E2* is specific for the acquired primary polycythemic disorder, PV.[22,23] Our results contradict this assumption. The nonspecificity of increased transcripts of these genes in PV is further supported by our unpublished data in the study of *HIF2α* germline gain-of-function mutation. These data suggest that the activation of *RUNX1* and *NF-E2* genes is likely secondary to augmented erythropoiesis in polycythemia and not a candidate "driver" of PV augmented erythropoiesis. It remains to be shown if the increased transcripts of these genes in PV may be due to augmentation of the hypoxia sensing pathway, also possibly present in PV.

This report provides further evidence for the heterogeneity of clinical phenotypes of *VHL* mutations resulting in cancers[1] or in augmented erythropoiesis. The molecular basis of these differences remains unclear at this time. One can only speculate that the relatively small VHL peptide comprising 213 codons yet encoded by a large >11.2-kb *VHL* gene may have multiple functions, possibly from interactions with other modifying factors, which await future clarification. We submit that descriptions of families with congenital disorders and unique phenotypes resulting from *VHL* mutations provide an attractive opportunity for structure-function relationship analyses of VHL protein and lead to an enhanced understanding of polycythemic disorders and diseases of hypoxia sensing.

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Authorship

Contribution: L.L. designed the research, performed the research, analyzed data, and wrote the paper; F.L. performed the research and reviewed the paper; C.Y. performed the immunoprecipitation assay and wrote the paper; H.V. performed molecular dynamics simulations study and wrote the paper; R.D. recruited study subjects and reviewed the manuscript; V.D. analyzed data and reviewed the manuscript; and J.T.P. conceived the project, designed the study, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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The phenotype of polycythemia due to Croatian homozygous *VHL* (571C>G:H191D) mutation is different from that of Chuvash polycythemia (*VHL* 598C>T:R200W)

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ABSTRACT

Mutations of *VHL* (a negative regulator of hypoxia-inducible factors) have position-dependent distinct cancer phenotypes. Only two known inherited homozygous *VHL* mutations exist and they cause polycythemia: Chuvash R200W and Croatian H191D. We report a second polycythemic Croatian H191D homozygote distantly related to the first propositus. Three generations of both families were genotyped for analysis of shared ancestry. Biochemical and molecular tests were performed to better define their phenotypes, with an emphasis on a comparison with Chuvash polycythemia. The *VHL* H191D mutation did not segregate in the family defined by the known common ancestors of the two subjects, suggesting a high prevalence in Croatians, but haplotype analysis indicated an undocumented common ancestor ~six generations ago as the founder of this mutation. We show that erythropoietin levels in homozygous *VHL* H191D individuals are higher than in *VHL* R200W patients of similar ages, and their native erythroid progenitors, unlike Chuvash R200W, are not hypersensitive to erythropoietin. This observation contrasts with a report suggesting that polycythemia in *VHL* R200W and H191D homozygotes is due to the loss of JAK2 regulation from *VHL* R200W and H191D binding to SOCS1. In conclusion, our studies further define the hematologic phenotype of *VHL* H191D and provide additional evidence for phenotypic heterogeneity associated with the positional effects of *VHL* mutations.

Introduction

Von Hippel-Lindau (*VHL*) is a tumor-suppressor gene, mutations of which have long been recognized to predispose to renal cancer, pheochromocytoma and other cancers.[1] Notably, mutations clustering at different positions appear to favor certain tumor types.[2-4] VHL is a negative regulator of hypoxia inducible transcription factors (HIF). Two mutations located at the 3' portion of the *VHL* coding region cause polycythemia but not VHL syndrome cancers. The most frequent cause of congenital polycythemia is homozygosity for the hypomorphic *VHL* 598C>T (R200W) mutation leading to a reduced rate of ubiquitination of α subunits of HIF-1 and HIF-2, the principal mechanism underlying Chuvash polycythemia.[5] This disease is endemic in the Chuvash Autonomous Republic of the Russia Federation[6] and in the Italian island of Ischia,[7] and is sporadic worldwide; it is associated with decreased survival of homozygotes partly due to cerebral vascular events and systemic thrombosis. Other common manifestations are varicose veins and vertebral hemangiomas.[8] Haplotype analyses demonstrated that the mutation likely arose from a single founder 14,000 – 62,000 years ago, indicating a survival advantage of heterozygotes,[9] which may in part be due to protection from anemia.[10] In addition, compound heterozygosity for R200W and other *VHL* mutations has been reported in a few patients with congenital polycythemia.[11-14]

HIF are master transcription factors that determine cellular responses by oxygen-dependent destruction of α subunits. VHL is a substrate-recognition component of an E3 ubiquitin-protein ligase complex that, under normoxic conditions, ubiquitinates HIF1α and HIF2α and targets them for proteasomal degradation.[15] Disruption of the interaction between the α-subunits of HIF and VHL protein causes accumulation of HIF and altered transcription of downstream target genes including those for glucose transporter-1 (*SLC2A1*), vascular endothelial growth factor (*VEGF*), transferrin (*TF*) and erythropoietin (*EPO*).[5]

Eight years ago we described the first example of a homozygous germ-line *VHL* mutation other than the *VHL* R200W mutation, i.e. the 571C>G (H191D) mutation in a polycythemic boy from a region in the south of Croatia called Dalmatia.[16] This Croatian *VHL* mutation is positioned in the same structural region as the Chuvash polycythemia muta-

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tion that leads to a modest partial loss of VHL activity since the affected residue is distant from the functional VHL domain.[17]

A high level of erythropoietin, due to increased HIF, was assumed to be a major cause of polycythemia in patients with these *VHL* R200W and H191D mutations, which are hallmarks of secondary polycythemic disorders. However, Chuvash polycythemia R200W erythroid progenitors are also hypersensitive to erythropoietin,[5,18] a hallmark of primary polycythemic disorders, by an as of yet unknown underlying mechanism. Recently, Russell and colleagues hypothesized that the mutated VHL R200W and H191D regions bind more avidly to suppressor of cytokine signaling 1 (SOCS1), a potent negative regulator of erythropoiesis.[19] They proposed that this abnormal association between the VHL protein and SOCS1 hinders Janus kinase 2 (JAK2) degradation leading to JAK2 up-regulation, which can potentially explain the erythroid hypersensitivity to erythropoietin observed in Chuvash polycythemia, and they predicted that this erythroid hypersensitivity to erythropoietin would also be observed in the Croatian *VHL* H191D mutation.[19]

In this study, we report another homozygous patient for the *VHL* 571C>G (H191D) germ-line mutation, a 5-year old Croatian girl from Herzegovina, a region located in the southern part of Bosnia contiguous to Dalmatia. Herzegovina is the counterpart to the Croatian region and is populated largely by people of Croatian ethnicity along the border. We set out to define the phenotype of this mutation in this and the previously reported homozygote[16] with particular emphasis on a critical comparison with Chuvash polycythemia. We also pursued a hypothesis that a putative survival advantage of H191D heterozygotes may account for the possible high prevalence of heterozygosity in Croatians and thus set out to determine its approximate origin in evolution by determining haplotype sharing among affected individuals.

Design and Methods

Blood samples from 23 persons were collected from two different families, including two propositi with polycythemia and 21 relatives (Figure 1). Peripheral blood samples were collected into tubes containing EDTA and/or ACD. Written informed consent was obtained from all participants. The Institutional Review Board of the University of Utah approved the study.

Mutation analysis of the VHL gene

Genomic DNA was isolated from granulocytes using the TRI reagent (Molecular Research Center, Cincinnati, OH, USA). For genotyping, 100 ng of patients' genomic DNA was used. Polymerase chain reactions (PCR) were performed using the HotStar Taq Master Mix Kit (QIAGEN, Germantown, MD, USA) and the following primers: VHL F agttgttggcaaagcctctt, VHL R caaaagctgagatgaaacagtg. As the *MslI* endonuclease abolishes a restriction site of the *VHL* 571C>G mutation, the PCR product was then purified with a QIAquick PCR Purification Kit (QIAGEN) and subjected to restriction with *MslI* enzyme (New England Biolabs, Beverly, MA, USA) according to the manufacturers' instructions. Cleavage products were evaluated on 2% agarose gels. PCR-direct sequencing was performed to confirm the mutation screening, using a standard protocol and the same amplification primers.

Analysis of recent shared ancestry

The two individuals homozygous for *VHL* H191D were genotyped using the Illumina HumanOmni1-Quad BeadChip at the Children's Hospital of Philadelphia, Center for Applied Genomics. Beagle 3.2[20] was used to phase and impute missing genotypes, with the phase two release of 30 HapMap CEU trios as a reference.[21] GERMLINE 1.4.1[22] inferred the locations and extents of identity by descent (IBD) segments (parameters err_het = 2, err_hom = 1, and min_m = 1cM, with marker positions given on the HapMap r22 genetic map). ERSA 1.0 was applied to the GERMLINE output to test for evidence of recent shared ancestry.[23]

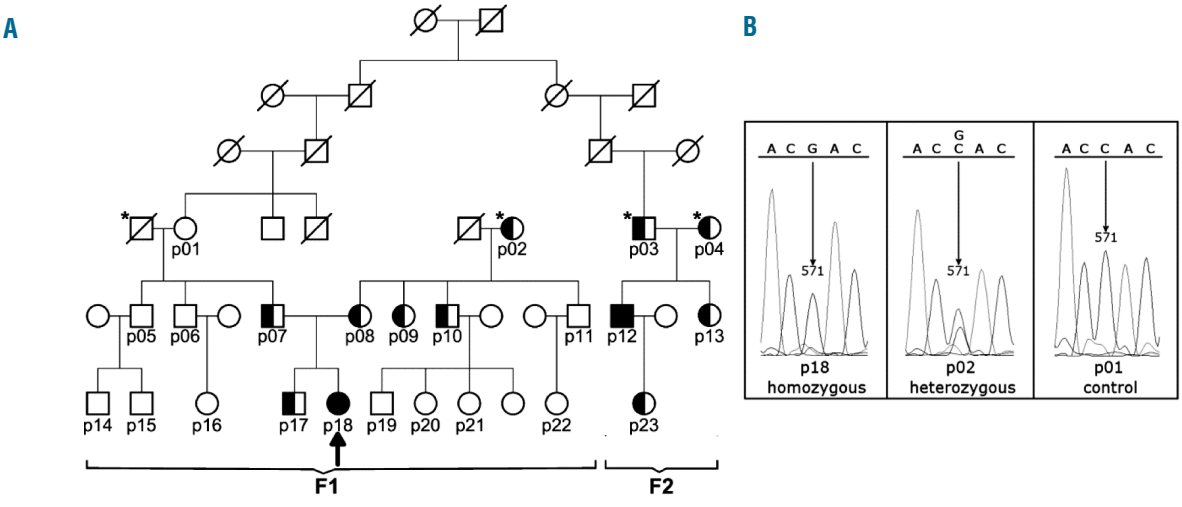

Figure 1. Pedigree with genotyping. (A) Pedigree of the family available for study with extended family history. Each family is denoted by a family number (F1, F2). The "p" code (p01, p02 and so on) indicates individuals from whom DNA samples have been obtained. The arrow indicates a new polycythemic patient. The *VHL* mutation of the paternal side of F1 must have been inherited from the husband of p01. The husband of p01 was not related to F2 according to the pedigree and the mutation did not segregate among the known relatives. Heterozygote individuals denoted by an asterisk as well as the husband of p01 presumably shared the common ancestor, the founder of the mutation. (B) Examples of sequences of the third exon of the *VHL* gene in an unaffected subject (p01), in a heterozygote subject (p02), and in a homozygote (p18) patient.

In vitro *assay of the sensitivity of erythroid progenitors to erythropoietin*

In vitro sensitivity of erythroid progenitors to erythropoietin was determined on mononuclear cells isolated from the peripheral blood using Histopaque (Sigma-Aldrich, St. Louis, MO, USA) density gradient centrifugation and plating (2.3×10^5/mL) on methylcellulose media (MethoCult® H4531; StemCell Technologies, Vancouver, BC, Canada) without addition of erythropoietin or with addition of various concentrations of erythropoietin (StemCell Technologies), ranging from 0.015 to 3.0 U/mL. Cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C for 14 days. Erythroid burst-forming unit colonies (BFU-E) were scored by standard morphological criteria. The assay was carried out on erythroid progenitors from one homozygous and three heterozygous *VHL* H191D individuals, two wild-type relatives and a polycythemia vera patient as a positive control. The protocol and interpretation of these assays were identical to those used for homozygous and heterozygous native progenitors with the Chuvash polycythemia *VHL* R200W mutation.[5]

In vitro *expansion of human erythroid progenitors in liquid culture*

The progenitor cells were expanded from the mononuclear cell population using our published protocol.[24] Briefly, 1×10^6 cells/mL were cultured in StemSpan™ Serum-Free Expansion Medium (StemCell Technologies) containing different cytokine cocktails from day 1-7 (100 ng/mL of fetal liver tyrosine kinase 3 ligand, 100 ng/mL of thrombopoietin, and 100 ng/mL of stem cell factor), day 8-14 (50 ng/mL of stem cell factor, 50 ng/mL of insulin-like growth factor-1, and 3 U/mL of erythropoietin), and day 15-21 (50 ng/mL of insulin-like growth factor-1, and 3 U/mL of erythropoietin). All cytokines were a kind gift from Amgen (Thousand Oaks, CA, USA).

Real-time polymerase chain reaction assay

Total RNA was isolated from granulocytes using TRI reagent solution (Molecular Research Center, Cincinnati, OH, USA) and then treated with DNA-free™ DNase Treatment & Removal Reagents (Ambion, Life Technologies, NY, USA) to remove any contaminating DNA. Five hundred nanograms of DNA-free RNA were reverse-transcribed using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Life Technologies, NY, USA) according to the manufacturer's instruction protocol. Quantitative PCR were performed with specific TaqMan® Gene Expression probes (Applied Biosystems, Carlsbad, CA, USA) for the following genes: *ADM* (Hs00181605), *TFRC* (Hs00951083), *NDRG1* (Hs00608387), PDK1 (Hs00176853), *SLC2A1* (Hs00892681), *VEGF* (Hs00900055), *BNIP3* (Hs00969291), *BNIP3L* (Hs00188949), and *HK1* (Hs00175976). All samples were assayed in triplicate. Data were normalized to *HPRT* (4333768F) and *GAPDH* (4333764F) reference genes. The statistical significance of relative expression changes of target mRNA levels normalized to a reference genes was analyzed by the pair-wise fixed reallocation randomization test using REST© 2009 software.[25]

Biochemical studies

The complete blood count was performed by an automated analyzer (Sysmex XT 2000i, Sysmex Corporation, Kobe, Hyogo, Japan). The concentration of erythropoietin in the serum was determined by an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Analyses were performed with Stata 10.1 (StatCorp., College Station, TX, USA).

Results

Patients' characteristics

The proposita is a 5-year old girl who was referred to the University Hospital of Zagreb due to failure to thrive at the age of 2 years. Routine blood analysis at the time of the current study revealed an erythrocyte count of $8.42\text{x}10^{12}$/L and hemoglobin concentration of 14.5 g/dL. She was iron deficient (ferritin 6.3 μmol/L) and her erythropoietin was increased (202 mIU/mL; normal range, 4-28). Her weight and height were below the 5th percentile for age and gender and psychomotor development was delayed, but she was otherwise normal. She experiences headaches about twice weekly, on average. Besides adequate hydration and daily acetylsalicylic acid (1 mg/kg), she receives no other treatment. Her parents were born in the same part of the country and deny being related.

The previously reported homozygous patient was diagnosed with polycythemia and elevated erythropoietin at the age of 12 years. He is now 26 years old and has occasional headaches and malaise, and tires easily. He is phlebotomized once or twice a month depending on symptoms and hematologic findings. He has been iron deficient because of the regular phlebotomies to reduce hemoglobin concentration.

We found no individuals among the heterozygotes who had evidence or a history of polycythemia. Neither homozygote had evidence of varicose veins which are common in young Chuvash polycythemia patients and virtually invariable after the age of 18 years.[8]

Analysis of the VHL *H191D mutation and mapping of the* VHL *locus*

Among 23 members (12 female, 11 male) of the families of the two propositi, we found 10 heterozygotes (6 female, 4 male; Figure 1) for the *VHL* H191D mutation. The father of the proposita is distantly related to the first *VHL* H191D homozygote polycythemic subject but genotyping revealed that the *VHL* mutation in family F1 must have come from the husband of p01 who was not related to family F2 (Figure 1). Thus, the *VHL* H191D mutation does not segregate among the known relatives between these two families, and no evidence suggests that the mutation originated from one of two known common ancestors depicted in Figure 1A.

We analyzed the two homozygotes (p12 and p18) by high-density genotyping to examine their relatedness and the *VHL* haplotype. We detected evidence of a significant relationship between the two individuals ($P=4\text{x}10^{-18}$), with a maximum likelihood estimate of 8th degree relatives (95% CI 6th degree - 11th degree)[23], exactly matching the known pedigree (Figure 1). These individuals share a 16 cM haploid segment on chromosome 11 and a 24 cM haploid IBD segment on chromosome 3 (identical copies of a gene segregating from a common ancestor within the defined pedigree, Figure 2). Within the segment on chromosome 3, a 15.6 cM autozygous segment is present in the female (p18), and a 1.6 cM autozygous segment is present in the male (p12). Both autozygous segments contain the *VHL* mutation, and the smaller segment is diploid IBD (Figure 2). Two other large autozygous segments are present in the male (15.5 cM on chromosome 15 and 10 cM on chromosome 4). The presence of autozygosity at the *VHL* mutation and elsewhere in the genome is a strong indication that all four of the parents of p12 and

p18 are related[23] and share a common ancestor. This common ancestor is likely the founder of the *VHL* H191D mutation. Because *VHL* H191D does not segregate among the known relatives of the two families, the evidence for a common founder indicates multiple undocumented relationships. The four carriers of this mutation in the generation of the grandparents are p02, p03, p04, and the husband of p01 (Figure 1). The size and number of autozygous (two alleles at a locus originate from a common ancestor by way of non-random mating) segments in p12 and p18 suggest that the four carriers in the grandparental generation are separated from the common founder by two to five generations (by 3-6 generations for propositi).

Response of erythroid progenitors to erythropoietin

We performed the erythroid progenitor colony (BFU-E) assay on peripheral blood mononuclear cells from one *VHL* H191D polycythemic patient, heterozygous subjects and two healthy controls. The growth of BFU-E of the affected individuals and mutation carriers, unlike those of

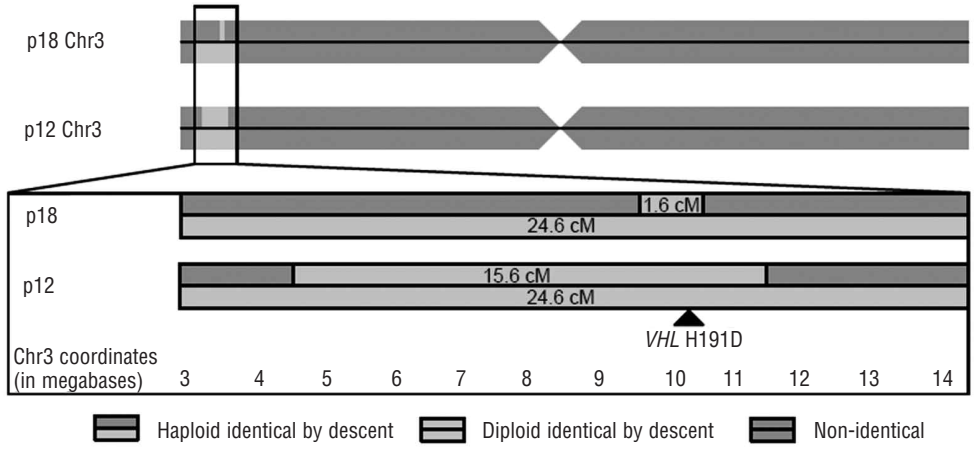

Figure 2. IBD (gene identity by descent) and autozygosity on chromosome 3. The two individuals homozygous for *VHL* H191D share a 24.6 cM haploid IBD segment, a 15.6 cM haploid IBD segment that is autozygous in the female, and a 1.6 cM diploid IBD segment that is autozygous in both individuals. All segments contain the *VHL* mutation. The presence of large autozygous IBD segments strongly suggests that all four copies of the *VHL* mutation originated from a recent founder.

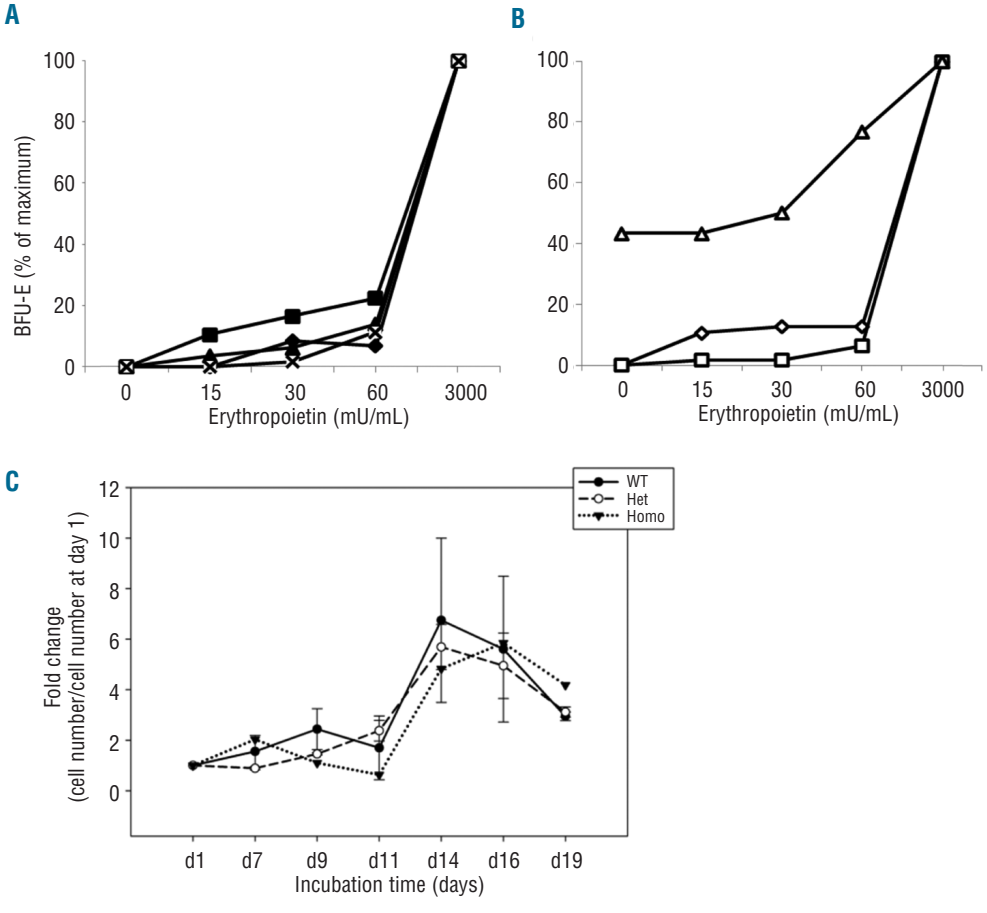

Figure 3. Sensitivity to EPO and *in vitro* proliferation of BFU-E erythroid progenitors. (A) Erythroid progenitor growth curves show the relative percentages of colonies, i.e. the percentage of colonies of each genotype, for a given concentration of erythropoietin, relative to that at the maximum concentration (3000 mU/mL). Normal response of BFU-E to erythropoietin was found in the patient homozygous for *VHL* H191D mutation (p12,×). Heterozygous individuals (p03, ◆), (p04, ■), (p08, ▲) also showed normal responses in the presence of a low concentration of erythropoietin. (B) Concomitantly tested healthy controls (◇), (□) and a patient with polycythemia vera used as a hypersensitive control (△). (C) *In vitro* expansion of cells from homozygous *VHL* H191D patient (p12), three heterozygous individuals (p03, p04 and p08) and healthy controls (n=2). The number of expanded cells was determined at each time point. Fold increase in the number of cells was determined from the initial total number of peripheral blood progenitors used for expansion. Data are presented as the mean and the whiskers represent SE intervals.

Chuvash polycythemia homozygotes, resembled the growth of normal controls (Figure 3A,B). The result of the second homozygous *VHL* H191D patient (p18) is not included in Figure 3A because only a limited amount of her peripheral blood progenitors was available, precluding the whole erythropoietin response curve. However, no BFU-E colonies were observed at low erythropoietin concentrations (15, 30 and 60 mU erythropoietin/mL). Concomitantly analyzed BFU-E from a patient with polycythemia vera were markedly hypersensitive to erythropoietin. Proliferation of *VHL* H191D erythroid progenitors in liquid cultures was also normal, when compared to that of healthy controls (Figure 3C).

Effect of the VHL *H191D mutation on expression of the target genes of hypoxia inducible factors 1 and/or 2*

We evaluated the expression level of several target genes of HIF-1 and/or HIF-2 (*ADM*, *TFRC*, *NDRG1*, *PDK1*, *SLC2A1*, *VEGF*, *BNIP3*, *BNIP3L* and *HK1*) in granulocytes from the two homozygous patients and eight normal controls (Figure 4). The homozygotes for *VHL* H191D mutation had increased expression of several HIF-1 and/or HIF-2 regulated genes (*TFRC*, *SLC2A1*, *VEGF*, *BNIP3* and *HK1*) and two genes (*ADM* and *BNIP3L*) were down-regulated. Changes of expression of HIF target genes are often tissue-specific and our analyses were limited to only available tissue to which the studied subjects consented.

Comparison of clinical variables and biological markers among VHL *genotypes*

The clinical characteristics of the study participants are summarized in Table 1 according to *VHL* phenotype. Comparing *VHL* H191D heterozygotes with subjects with wild-type *VHL*, it was found that the former had higher mean values of the mean corpuscular volume (P=0.030) and of serum ferritin concentration (P=0.007) and lower mean cell hemoglobin concentration (P=0.033). The limited number of homozygous samples precludes their statistical evaluation, and furthermore the clinical studies in the homozygotes are affected by the fact that one of them has been frequently phlebotomized. Consistent with the phlebotomy program of p12 and the iron deficiency of both homozygotes (p12, p18), mean corpuscular volume, mean cell hemoglobin, mean cell hemoglobin concentration and ferritin were decreased among the *VHL* H191D homozygotes compared to both the other two groups: *VHL* wild-type and *VHL* H191D heterozygotes. Nevertheless, the hematocrit and red blood cell count were increased among the *VHL* H191D homozygotes. The extremely low mean cell hemoglobin and mean cell hemoglobin concentration could explain the discrepancy between red blood cell volume hematocrit and hemoglobin concentration in addition to the high concentration of erythropoietin, which was increased despite the lack of anemia. Moreover, high erythropoietin is associated with plasma volume contraction[26] which can further elevate hematocrit. The erythropoietin concentrations of both *VHL* H191D homozygotes are higher than those of Chuvash polycythemia patients of similar age and with similar hemoglobin concentration (Table 1 and Figure 5). We also compared other hematologic parameters between *VHL* H191D homozygotes and heterozygotes and relevant Chuvash polycythemia counterparts (Table 1). The *VHL* R200W heterozygotes were older and had lower mean corpuscular volumes and ferritin levels compared to the *VHL* H191D heterozygotes.

Discussion

We describe a 5-year old Croatian girl with recessively inherited congenital polycythemia due to homozygosity for the *VHL* H191D mutation. We studied her extended family, as well as revisited and expanded the study of the originally described polycythemic patient with the same *VHL* H191D mutation[16] and also his extended family. This homozygous *VHL* mutation, along with Chuvash polycythemia *VHL* R200W, are the only *VHL* mutations thus far not predisposing to tumor development but rather causing polycythemia.

While we had previously shown[5] that in Chuvash poly-

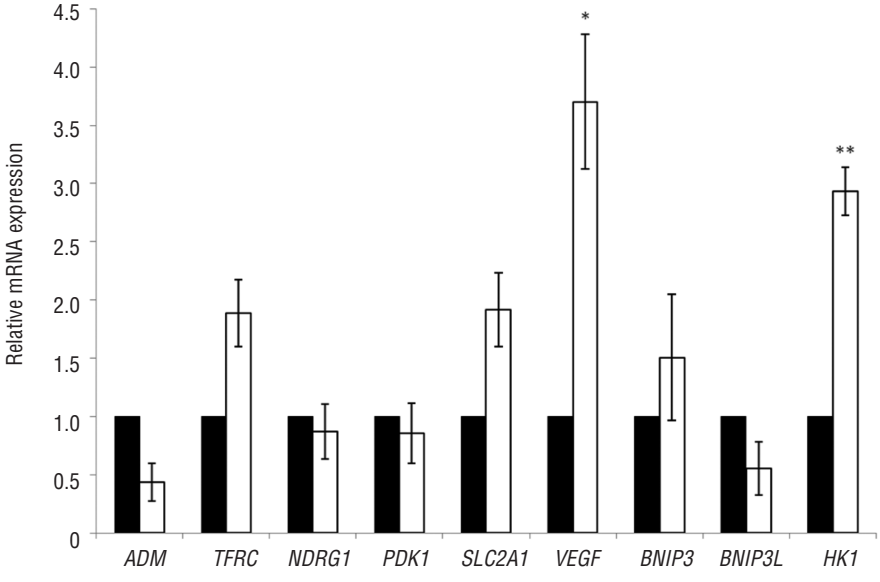

Figure 4. Expression of HIF-1 and HIF-2 target genes. Expression of ***ADM***, ***TFRC***, ***NDRG1***, ***PDK1***, ***SLC2A1***, ***VEGF***, ***BNIP3***, ***BNIP3L*** and ***HK1*** genes was evaluated by quantitative PCR in granulocytes isolated from two patients homozygous for ***VHL*** H191D mutation (p12, p18, white columns) and normal controls (n = 8, black columns). Data are normalized to ***HPRT*** and ***GAPDH*** reference genes. All samples were investigated in triplicate. Results pooled from three separate experiments are shown. The bars represent mean mRNA levels relative to the mean levels calculated for wild-type controls. The whiskers represent SE intervals. Significant mean value deviations from normal controls are presented as *(***P***<0.05) and **(***P***<0.01).

cythemia, the erythroid progenitors are intrinsically hyperproliferative (a feature of primary polycythemia), their average elevated erythropoietin levels (a feature of secondary polycythemia) further contribute to augmented erythropoiesis. No differences of BFU-E response of Chuvash heterozygotes have been detected in our multiple studies over the last decade. The *VHL* H191D mutation differs from *VHL* R200W, as the erythropoietin levels seen in both *VHL* H191D homozygotes appear to be higher than those in patients with Chuvash polycythemia, but more importantly, we have been unable to find evidence of a primary polycythemic functional defect in the native erythroid progenitors of this family. We can, therefore, conclude that the *VHL* H191D polycythemic phenotype is solely driven by erythropoietin.

There is a significant, non-erythroid phenotype in Chuvash polycythemia; specifically, there is an increased risk of thrombotic and hemorrhagic strokes, non-central nervous system thrombotic complications, pulmonary hypertension, and other abnormalities[8] which are associated with decreased survival of homozygotes and, thus, one would expect a negative survival pressure of the *VHL* R200W mutation. However, examination of the population effect of this mutation in a large number of individuals of Asian-Indian, Caucasian and Chuvash (population from Central Asia) origin suggested that this mutation arose from a single founder, possibly prior to diversification of the human races.[9] This implies that there is some, possibly subtle survival advantage for heterozygotes which allows this mutation not only to persist, but to increase. As shown in Figure 1, the *VHL* H191D mutation in two seemingly unrelated individuals led us to examine whether this mutation may be much more frequent in Croatians, perhaps exhibiting a similar survival advantage for heterozygotes for *VHL* H191D as that shown for *VHL* R200W. However, our haplotype analysis demonstrated a recent origin of this mutation and, thus, the frequency of this mutation cannot be used in arguments for or against any survival benefit or detriment.

The increased intrinsic sensitivity to erythropoietin of erythroid progenitors with the *VHL* R200W mutation has not yet been clarified. A recent paper[19] suggested that this is due to selective binding of the mutated VHL protein in the regions of *VHL* H191D and *VHL* R200W to an inhibitor of erythropoiesis (SOCS 1). However, this report erroneously quoted the *VHL* H191D mutation as a Chuvash polycythemia mutation and concluded that the same erythropoiesis-augmenting mechanism applies for both mutations. Clearly, this is not the case, as shown by our data demonstrating no intrinsic augmented erythroid proliferation with *VHL* H191D, unlike that present in erythroid progenitors bearing the *VHL* R200W mutation. By repeated testing of many Chuvash and non-Chuvash individuals homozygous for the *VHL* R200W mutation, we consistently showed that BFU-E from patients with Chuvash polycythemia are erythropoietin hypersensitive. Unfortunately, we did not have enough material, or consent from the patients with *VHL* H191D for repeated blood sampling, which precluded SOCS 1 analysis in their BFU-E.

We evaluated hematologic and iron data from our heterozygous and two homozygous patients and compared them to our previous findings in homozygous and heterozygous relatives of patients with Chuvash polycythemia. The *VHL* H191D heterozygotes had larger mean cell volumes and ferritin concentrations than the

Table 1. Comparison of clinical variables between individuals with *VHL* H191D or *VHL* R200W mutations.

	VHL wildtype, *VHL* H191D and *VHL* R200W heterozygotes[#]					*VHL* H191D and *VHL* R200W homozygotes[§]			
	VHL wildtype (n = 11)	*VHL* H191D heterozygote (n = 10)	*P*[$]	*VHL* R200W heterozygote (n = 34)[10]	*P*[&]	p18	*VHL* R200W homozygote comparison for p18 (n =1)	p12	*VHL* R200W homozygote comparisons for p12 (n = 20; results in range)
Age (years)	26±2	35±2	0.3	53±2	<0.001	5	16	26	25-56
N. of females	5 (45%)	6 (60%)	0.5	19 (56%)	NS	female	male	male	13 (65%)
HBF (%)	1.3±0.4	1.5±0.4	0.4	ND		0.5	ND	1.4	ND
Hemoglobin (g/dL)*	14.0±0.3	14.1±0.3	0.8	13.4±0.2	0.09	14.50	16.4	13.7	12.2-15.7
Hematocrit (%)*	44.6±1.0	46.1±1.0	0.3	ND		53.1	53.4	59.6	37.8-51.8
RBC*x10^6 (cells/μL)	4.94±0.08	4.78±0.08	0.2	ND		8.42	6.58	8.89	4.59-7.59
MCV (fL)**	89.9±1.8	96.8±1.9	0.030	88±0.8	<0.0001	63.1	81.2	67.1	59.6-93.3
MCH (pg)**	28.4±0.6	29.7±0.6	0.172	ND		17.2	24.9	15.4	18.3-32.7
MCHC (g/dL)**	31.5±0.2	30.6±0.3	0.033	ND		27.3	30.7	23.0	30.3-35.4
WBCx10^3 (cell/μL) **	5.6±0.52	6.46±0.55	0.3	7.6±0.29	0.1	6.4	5.96	4.1	3.5-8.8
Plateletsx10^3 (cell/μL) **	260±25	290±26	0.4	238±12	0.053	310	247	199	114-369
Ferritin (ng/mL)[£]*	39 (32-48)	112 (88-143)	0.007	62 (57-73)	0.005	6.3	11.9	2.3	0.4-20.0
Erythropoietin (mIU/mL)***	8.2 (6.1-10.7)	9.6 (7.2-26)	0.7	11 (10.8-11.2)	0.4	201.6	31	469	27-230

*[#]Results as mean ± SE or mean (range), unless otherwise indicated. [§]VHL R200W homozygote comparisons for either pediatric or adults subjects with hemoglobin concentration within 2 g/dL of the Croatian subjects. [$]Comparison of VHL wildtype and VHL H191D heterozygotes. [&]Comparison of VHL H191D and R200W heterozygotes. [£]Geometric mean and SE range. For analysis of ferritin, two outliers excluded. *Analysis by ANOVA with adjustment for pedigree and gender. ** Analysis by ANOVA with adjustment for pedigree. *** Analysis by ANOVA with adjustment for pedigree and hemoglobin; geometric mean and SE range. RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean cell hemoglobin; MCHC: mean cell hemoglobin concentration; WBC:white blood cells.*

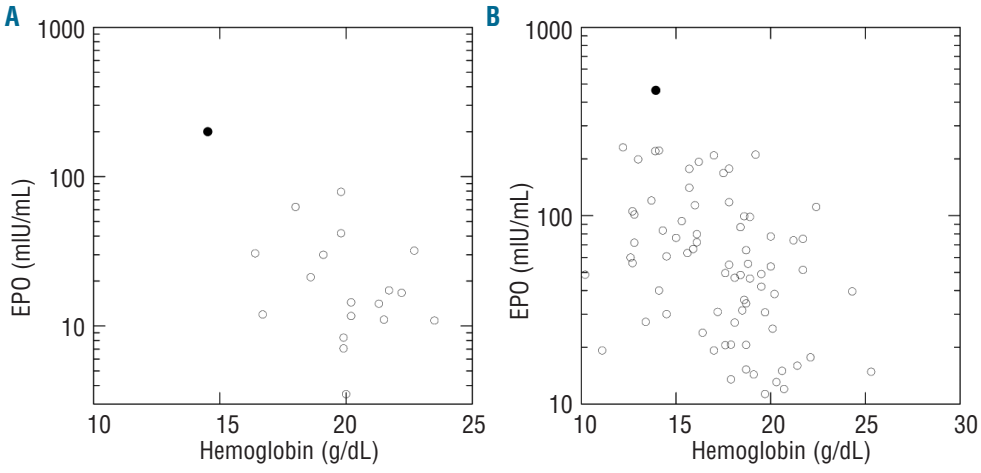

Figure 5. Serum EPO levels in VHL H191D Croatian patients, as compared to CP patients of similar age. The vertical axes represent logarithmic scale of serum EPO values in relation to hemoglobin concentration on horizontal axes. (A) Croatian p18 patient, and CP patients up to 20 years old (B) Croatian p12 patient, and CP patients at least 20 years old. EPO concentration is higher in Croatian (●) than in CP patients (○).

R200W heterozygotes but similar hemoglobin and erythropoietin concentrations.[10] The two *VHL* H191D homozygotes tended to have higher erythropoietin concentrations relative to the hemoglobin concentration compared to *VHL* R200W homozygotes.[8] In both types of polycythemia (Chuvash and Croatian) our data indicate an impaired interaction of VHL with hypoxia inducible factors, reducing the degradation rate of alpha subunits resulting in increased expression of downstream target genes including *SLC2A1*, *TFRC* and *VEGF*.

In summary, our data provide additional evidence of an as yet unexplained variations of phenotypes with different locations within the *VHL* gene. While *VHL* is a relatively large gene (12 kb) with three exons and two introns, the coding region consists of only 213 codons. The resultant very small protein has been well-studied as a cause of one of the first and most comprehensively investigated tumor predisposition syndromes. Germ-line mutations predicted to cause truncated protein and exon deletions are characteristic for the development of retinal and central nervous system hemangioblastomas and clear renal cell carcinoma, while germ-line missense mutations typically cause pheochromocytomas.[27] Thus, it was unexpected that the large number of patients with Chuvash polycythemia (bearing the *VHL* R200W mutation either in heterozygous or homozygous forms) have not been associated with any classical VHL syndrome tumors, but with polycythemia. We now provide some evidence that patients with the *VHL* H191D mutation, which is positionally close to the *VHL* R200W mutation, appear to have a similar, but apparently not identical, phenotype suggesting subtle functional differences of these two different *VHL* mutations. More work is needed to define the molecular basis of the array of phenotypic differences arising from changes in this rather small VHL peptide.

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Authorship and Disclosures

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