The Human-Specific BOLA2 Duplication Modifies Iron Homeostasis and Anemia Predisposition in Chromosome 16p11.2 Autism Individuals

The human-specific *BOLA2* duplication modifies iron homeostasis and anemia predisposition in chromosome 16p11.2 autism patients

or

BOLA2 copy number modifies iron homeostasis and anemia predisposition in chromosome 16p11.2 autism patients

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Abstract

Human-specific duplications under positive selection at chromosome 16p11.2 lead to recurrent pathogenic 600 kb BP4-BP5 copy-number variations that are one of the most common genetic causes of autism. These duplications are copy-number polymorphic and include 3 to 8 copies of *BOLA2*, which is involved in the maturation of iron-sulfur cytosolic proteins. To investigate the potential advantage provided by the rapid expansion of *BOLA2*, we assessed hematological traits and incidence of anemia in 379,385 controls, 89 deletion, and 56 duplication chromosome 16p11.2 BP4-BP5 carriers of the UK Biobank (UKB), because such individuals have lost or gained copies of *BOLA2*. We found that the 16p11.2 deletion is strongly associated with anemia (18/89 carriers, 20%, *P*=4e-7, OR=5), particularly iron-deficiency anemia. Upon stratification by *BOLA2* copy number, we found an association between low *BOLA2* dosage and anemia (*P*=2e-3). In particular, 7 out of 15 (47%) individuals with three copies required iron supplementation and/or were anemic. In parallel, we analyzed hematological traits in mice carrying the 16p11.2 orthologous deletion or duplication, as well as, more specifically, *Bola2*$^{+/-}$ and *Bola2*$^{-/-}$ mice. The 16p11.2 deletion mouse model and *Bola2* haploinsufficient and deficient mice showed iron-deficiency anemia with lower blood iron and smaller red blood cells. *Bola2* deficient mice showed higher blood zinc protoporphyrin level.

Our results indicate that *BOLA2* participates in iron homeostasis. These data highlight a potential adaptive role of *BOLA2* expansion in improving human iron metabolism and the importance of monitoring *BOLA2* copy number in patients with chromosome 16p11.2 rearrangements.

Introduction

The human 16p11.2 chromosomal region is a hotspot of recurrent pathogenic copy number variation (CNV) with different size, breakpoints, and gene content. Most breakpoints map within homologous segmental duplication clusters consistent with non-allelic homologous recombination [1]. Among these CNVs, 600 kbp deletions and duplications with BP4-BP5 breakpoints are among the most frequent genetic causes of neurodevelopmental and psychiatric disorders [2-6]. They are also associated in a dosage dependent manner with head circumference, BMI, age at menarche, and the size of brain structures associated with reward, language, and social cognition [5, 7-11] (Mannik et al., unpublished). BP4-BP5 (breakpoint) rearrangements are mediated by non-allelic recombination of *Homo sapiens sapiens*-specific duplications that appeared at the beginning of the modern human lineage, ~282 thousand years ago [12]. These duplications rapidly spread and are nearly fixed in humans, suggesting the existence of a possible evolutionary advantage associated with this novel genetic structure that outweighs the accompanying chromosomal instability [12].

The human-specific duplications contain three genes — *BOLA2*, *SLX1,* and *SULT1A* — that have a single-copy ortholog in the mouse genome (**Figure 1A**). Whereas *BOLA2* and *SLX1* have copies only in the BP4-BP5 flanking repeats, *SULT1A* has also copies within the neighboring BP2. The BP4-BP5 duplicons also harbor copies of the primate gene family *NPIP* [13], the *BOLA2-SMG1* and *SLX1-SULT1A* fusion transcripts, and the *SMG1P* and LOC388242 pseudogenes. These 102 kbp long duplications are copy number variant in the genomes of contemporary humans but are single copy in archaic genomes for which DNA sequence data are available. They are located at both ends of the single-copy region and range from 3 to 8 diploid copies [12] (**Figure 1A**).

In this work, we questioned the potential adaptive role of the human-specific duplications at 16p11.2 BP4 and BP5. To this aim, we focused our study on the possible benefit associated with an increased dosage of *BOLA2*, as this is the only gene in the segment with convincing evidence of being duplicated specifically in *Homo sapiens* [12]. As *BOLA2* copy number positively correlates with both RNA expression and protein level in humans, and *BOLA2* expression in stem and lymphoblastoid cells is higher in human than in other large-bodied apes [12, 14], we reasoned that a possible advantage of *BOLA2* expansion derives from the increased expression level and protein abundance in some human cells.

BOLA2 encodes a protein that physically interacts with GLRX3 (glutaredoxin 3) to form a [2Fe-2S]-bridged heterotrimeric complex [15]. This complex participates in the maturation of cytosolic iron-sulfur proteins, transferring [2Fe-2S] clusters to CIAPIN1, also named anamorsin, an essential component of the cytosolic iron-sulfur assembly (CIA) system [16-18]. In yeast, besides the maturation of cytosolic iron-sulfur proteins, the [2Fe-2S]-bridged heterodimeric complex formed by the BolA-like protein Fra2 and the glutaredoxins Grx3/Grx4, respectively orthologs of BOLA2 and GLRX3, has essential roles in intracellular iron signaling and regulation [19, 20]. GLRX3 and CIAPIN1 are two molecules necessary for erythropoiesis, as the knockdown of *glrx3* in zebrafish impairs the synthesis of heme and maturation of hemoglobin [21] and *Ciapin1*-deficient mouse embryos are anemic [22].

We investigated phenotypes associated with *BOLA2* copy number variation in humans illuminating the contribution of this polymorphism in human phenotypic variation. We found that reduced *BOLA2* dosage associates with anemia in both humans and mice, suggesting a model where human-specific increases in *BOLA2* dosage might contribute to more efficient organismal iron homeostasis.

Results

16p11.2 deletion carriers have less copies of the *BOLA2-SLX1-SULT1A* segment

We had previously fully sequenced haplotypes of the 16p11.2 BP4-BP5 interval [12]. They differ in the number of copies and position, *i.e.* within the BP4 or BP5 low copy repeats, of a 102 kbp *BOLA2-SLX1-SULT1A* segment. In particular, H3, the most common haplotype, has one copy of the segment at BP4 (with the paralogs *BOLA2B*, *SLX1B,* and *SULT1A4*) and two copies at BP5 (with the paralogs *BOLA2A*, *SLX1A,* and *SULT1A3*) (**Figure 1A**). As alleles with 16p11.2 BP4-BP5 CNVs are generated through non-allelic homologous recombination between paralogous copies of this fragment [12], we expect that deletion and duplication alleles would have, respectively, less and more copies of it, together with one and three copies of the intervening single-copy genes from *SPN* to *CORO1A* (**Figure 2A**). To confirm this prediction, we quantified the number of copies of *BOLA2* in 16p11.2 BP4-BP5 deletion carriers collected by the European 16p11.2 consortium (n = 67) and the Simons Variation in Individuals Project (SVIP, n = 63) as described in [12].

Whereas European individuals from the 1000 Genomes Project and Human Genome Diversity Project (n = 635) have a mode of six copies of *BOLA2*, 16p11.2 deletion carriers showed a left-shifted distribution with a mode of four copies confirming reduced *BOLA2* copy number (**Figure 2B**). Consistently, expression of *BOLA2* is positively associated with its dosage in cells of 16p11.2 CNV carriers [23]. We identified nine and eight 16p11.2 deletion carriers with three *BOLA2* copies in the 16p11.2 European and SVIP cohorts (13%), respectively, compared to 0.2% (1 out of 635) in control individuals. Of note, we did identify neither in the general population nor in 16p11.2 deletion carriers, individuals with only two copies, which is the ancestral copy number state as both great apes and archaic hominin genomes have one *BOLA2* copy per haploid genome [12].

Chromosome 16p11.2 microdeletion is associated with anemia and mild hematological defects

Since 16p11.2 BP4-BP5 CNVs affect the dosage of the copy-number polymorphic *BOLA2*, *SLX1,* and *SULT1A* genes, it is possible that some associated phenotypes are due to the dosage change of one or combination of these genes. Because *BOLA2* binding partners have been implicated in hematological function, we evaluated hematological traits and the incidence of anemia in 16p11.2 BP4-BP5 CNV carriers *versus* control individuals.

We examined CNVs in 488,366 individuals from the UKB and identified 89 European unrelated individuals (35 females and 54 males) carrying the 16p11.2 BP4-BP5 deletion and 56 (32 females and 24 males) carrying the duplication. We compared 18 different hematological parameters to those of 379,385 control individuals with linear model and t-test combining and separating the genders (**Supplementary Table 1**).

Gene dosage at 16p11.2 was negatively correlated with platelet count (PLTc, β = -0.685, *P* = 1e-15) and platelet crit, a measure of total platelet mass (PLTcrit, Beta = -0.535, *P* = 4e-10), and positively associated with platelet volume (MPV, Beta = 0.444, *P* = 2e-7) (**Figure 3A**). Deletion carriers showed anisocytosis (higher red cell distribution width, RDW, Beta = -0.48, *P* = 2e-8) and higher mean reticulocyte volume in males (MRV, *P* = 4e-4) (**Figure 3A**), but all other red blood cell traits were in the normal range.

We then tested a possible association between 16p11.2 gene dosage and incidence of anemia using both main and secondary diagnoses collected for UKB participants. We found that 16p11.2 deletion is associated with a higher incidence of anemia (Fisher's exact *P* = 4e-

7, OR = 4.8) with 18 out of 89 carriers referred as anemic (20%) compared to 5% of control individuals (**Figure 3B**). Of these, four were females and 14 were males, with no significant difference in the gender ratio compared to all 16p11.2 deletion carriers identified (Fisher's exact *P* = 0.2). Out of the eleven cases for which the type of anemia was specified, nine individuals received the diagnosis of iron deficiency anemia, one of vitamin B12 deficiency anemia, and one of autoimmune hemolytic anemia. We next assessed the incidence of iron deficiency anemia in 16p11.2 deletion carriers *versus* control individuals (D50 category only), and confirmed the higher prevalence in the former group (six cases, Fisher's exact *P* = 4e-3). In contrast with deletion, 16p11.2 duplication carriers showed no association with anemia (Fisher's exact *P* = 0.8).

Reduced *BOLA2* copy number more strongly associates with anemia in 16p11.2 deletion carriers

Candidate genes for anemia in the 16p11.2 region are *BOLA2* and *ALDOA* [17]. *ALDOA* mutations cause an autosomal recessive form of hemolytic anemia [24], differently from 16p11.2 deletion that is associated mostly with iron deficiency anemia. We thus interrogated the possible involvement of *BOLA2* and hypothesized that *BOLA2* copy number variation might contribute to the incomplete penetrance of anemia with the lowest copy number possibly conferring a higher risk. To challenge this hypothesis, we estimated *BOLA2* copy number and collected information regarding the diagnosis of anemia from 82 families with chromosome 16p11.2 deletion. This includes 63 deletion probands from the SVIP cohort (**Supplementary Table 5**) and 25 deletion carriers belonging to 19 families from the European 16p11.2 cohort (**Supplementary Table 6**).

Six out of 63 deletion carriers from the SVIP cohort were anemic (~10%). We found a striking and significant difference in the incidence of anemia between deletion carriers with three copies of *BOLA2* (4 out of 8, 50%) and those with more than three copies (2 out of 55) (Fisher's exact *P* = 1.6e-3). In the European cohort, 5 out of 19 (~26%) unrelated 16p11.2 deletion carriers were anemic or iron deficient, a value similar to the incidence estimated in the UKB. The anemia prevalence increased with decreasing *BOLA2* copy number, with 3 anemic individuals out of 7 with three copies (~40%), 3 out of 11 with four copies (~30%), and zero out of 7 with five copies (one-sided logistic regression *P* = 0.1, considering only

probands when the information was available for more than one family member). Joining the two cohorts, the occurrence of anemia among deletion carriers with three *BOLA2* copies was significantly higher than among those with more than three copies (Fisher's exact *P* = 2e-3, OR = 9), together with an inverse relation between incidence of anemia and *BOLA2* copy number (one-side logistic regression *P* = 4e-3) (**Figure 3B**).

Mouse models of 16p11.2 deletion show lower blood iron, smaller red blood cell, lower corpuscular hemoglobin, and anisocytosis

To validate our human results, we measured heparin-plasma iron level and 44 hematological parameters in mouse models that carry a deletion (Del/+) or duplication (Dup/+) of the *Sult1a1-Spn* region syntenic to the human 16p11.2 BP4-BP5 locus [25]. In these models, the rearranged region includes 26 genes that are in single copy in both the human and mouse genomes together with the mouse single-copy *Sult1a1*, *Slx1b*, and *Bola2* genes whose orthologs map to the human-specific duplicated cassette. In contrast to other animal models of the 16p11.2 BP4-BP5 CNV [26, 27], these animals recapitulate the CNV both of the human single-copy genes and the copy-number variant *BOLA2*, *SLX1,* and *SULT1A* genes (**Figure 1A**). In particular, Del/+ mice allow the study of the effects of reduced *Bola2* dosage in the context of the whole 16p11.2 BP4-BP5 rearrangement.

We analyzed Del/+ and Dup/+ male and female mice, as well as their respective wild-type littermates at 7, 15, 29, and 50 weeks. An exception was made for 7-week-old females due to their low body weight and for which we only measured iron level. In parallel, we recorded the body weight from 5 to 53 weeks of age (**Supplementary Figure 1**). As reported in [25], Del/+ male and female mice were underweight. The weight of Dup/+ males was not different from that of their wild-type littermates, whereas Dup/+ females were overweight. Del/+ mice showed significantly lower plasma iron level at all ages and both genders. We observed no significant difference between Dup/+ mice and wild-type littermates although we note a trend towards higher iron level in the former (**Figure 1B, Supplementary Table 2**). Upon inclusion of both genotype and weight in the linear model, probably because of the already known effect of the genotype on the weight in Del/+ mice, neither variable was significantly associated with the iron level. We note that as some male mice had to be separated because of aggressive behavior, their husbandry status affected the plasma iron,

with male mice housed in single cages showing a trend toward higher iron levels (P = 0.06) (**Supplementary Table 2**).

We assessed differences in hematological parameters between genotypes and are describing here those that differ significantly (P < 0.05) in at least two time points. Del/+ males show lower mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red cell hemoglobin content (CH), mean corpuscular hemoglobin concentration (MCHCr), and hemoglobin content of reticulocytes (CHr), and higher RDW, consistent with a mild hypochromic, microcytic, iron-deficiency anemia (**Figure 1C**, **Supplementary Table 3**). Female mice show a similar trend. No hematological parameter differs significantly between Dup/+ mice and wild-type littermates in more than one time-replicate, in both females and males, except for a lower percentage of basophils in Dup/+ females (**Figure 1C**, **Supplementary Table 4**).

Bola2 haploinsufficient and deficient mice have lower blood iron, smaller red blood cells, higher zinc protoporhyrin, and higher mean platelet mass

We assessed the role of *Bola2* in organismal iron metabolism and levels of blood parameters, analyzing mouse models with heterozygous or homozygous ablation of *Bola2*. This knockout mouse line was engineered by the International Mouse Phenotyping Consortium (IMPC) [28]. They routinely measure an extensive series of parameters and evaluate if those are significantly different from non-littermate wild-type mice [29]. For the *Bola2* knockout line, they reported that *Bola2*$^{-/-}$ mice have decreased circulating iron level and mean corpuscular volume of red blood cells (see http://www.mousephenotype.org/).

To control for genetic background and environmental variability, we assessed hematological and iron-related parameters in *Bola2*$^{-/-}$ (homozygous mutants, ko/ko), *Bola2*$^{+/-}$ (heterozygous mutants, ko/+), and *Bola2*$^{+/+}$ mice (wild-type littermates, +/+). We created three different cohorts: i) a Bola2^{tm1} European cohort; ii) a Bola2^{tm1} US cohort; and iii) a Bola2^{tm1} US cohort where we excised the LacZ and neomycin cassette to eliminate potential position effects.

We sequenced the genome of a male *Bola2* knockout mouse where the neomycin cassette had been excised. We generated ~12-fold sequence coverage using the long-read single-molecule real-time (SMRT) sequencing platform and mapped and assembled long reads

mapping to the mouse *Bola2* locus. All sequence reads (10/10) confirmed the presence of the deletion (**Supplementary Figure 2**). Local assembly confirmed the deletion of 563 bp of genomic sequence, replaced with a 216 bp insertion, which includes one copy of the FRT recognition site. We blindly tested livers from homozygous and heterozygous mice for protein abundance by Western blot and confirmed, respectively, reduced level and absence of BOLA2 (**Supplementary Figure 3**).

To create the Bola2^{tm1} European cohort, we set 25 ko/+ x ko/+ crosses that generated 21 +/+, 54 ko/+, and 26 ko/ko mice born on the same day from 17 crosses, suggesting no prenatal lethality (**Figure 4A**). Ko/+ and ko/ko mice showed no weight alteration compared to wild-type littermates between 5 to 29 weeks of age or in liver weight at 31 weeks (**Figure 4B and Supplementary Figure 4**). We tested for a possible association between *Bola2* dosage and plasma iron level at three different ages, 8, 17, and 22 weeks, in both genders. We observe significant and positive association in females at 8 weeks (Beta = 3, *P* = 2e-3) and males at 22 weeks (Beta = 4.3, *P* = 6e-2) (**Figure 4C, Supplementary Table 7**). When adjusting for weight and, only for males, for weight and/or husbandry condition (single versus shared cage), the association of iron level with gene dosage remained. Of note, animals isolated to a single cage show significantly higher plasma iron levels, as observed for the larger 16p11.2 deletion mouse. As a result, we constructed two linear models to estimate the effect of the number of *Bola2* copies on the iron level, assessing mice in single and shared cages separately. The effect of the genotype on the iron level was stronger and significant only in male mice being in shared cages. However, the slopes of these two correlations do not differ significantly (*P* = 0.15).

Bola2 dosage affects red blood cells, reticulocytes, and platelets (**Figure 4D, Supplementary Table 8**). Specifically, the MCV (Beta = 0.7, P_{males} = 2e-2, $P_{females}$ = 4e-4 at 17 weeks), MCH (Beta = 0.3, P_{males} = 2e-4 at 22 weeks, $P_{females}$ = 2e-5 at 17 weeks), and CH (Beta = 0.2, P_{males} = 4e-2, $P_{females}$ = 8e-5 at 17 weeks) decrease in *Bola2*$^{+/-}$ and *Bola2*$^{-/-}$ mice, in both genders. This shows that *Bola2* haploinsufficiency and deficiency are associated with microcytosis and reduced hemoglobin in the blood. Immature red blood cells show the same features, *i.e.* their size and hemoglobin content are positively associated with *Bola2* copy number. We observe that some platelet traits show differences—the PCDW (Platelet Component Distribution Width, Beta = 1.4, P_{males} = 5e-2, $P_{females}$ = 8e-2) is positively associated with

Bola2 copy number, while the MPC (Mean Platelet Component concentration, $Beta_{males}$ = -8, P_{males} = 7e-3) and MPM (Mean Platelet Mass, Beta = -0.02, P_{males} = 3e-2, $P_{females}$ = 3e-3) are negatively associated.

To generate the US cohorts, heterozygous $Bola2^{tm1}$ mice on a pure C57BL/6N background were intercrossed. $Bola2^{tm1}$ animals with the LacZ and neo cassettes excised were backcrossed to C57Bl/6J for two generations and resulting heterozygous animals were intercrossed. We assessed the complete blood count and performed additional tests (**Supplementary Tables 9-12**). We replicated the mild microcytosis and found higher zinc protoporhyrin (ZPP) levels in the blood of $Bola2^{-/-}$ mice (**Figure 4E**). $Bola2^{-/-}$ showed no defective iron storage and ineffective erythropoiesis as shown by normal spleen to body weight ratio and spleen and liver iron levels that were similar to control mice. Peripheral blood had also no overt morphological defect.

In summary, these data show that *Bola2* haploinsufficiency and deficiency cause mild hypochromic, microcytic, iron-deficiency anemia and suggest that *BOLA2* is the causative gene for the iron-deficiency anemia phenotype in 16p11.2 deletion carriers.

Methods

Genotyping *BOLA2* copy number and statistical analysis in clinical cohorts

We estimated the *BOLA2A* and *BOLA2B* copy number of 16p11.2 BP4-BP5 deletion carriers with a molecular inversion probe (MIP) assay and probes mapping within the *BOLA2* gene as described in [12]. We collected iron metabolism-related (serum iron, ferritin, transferrin, coefficient of saturation of iron in transferrin) and hematological parameters of these individuals and/or information about diagnosis of anemia. Within the European cohort, a diagnosis of anemia was established if the individual had low blood iron level or low hemoglobin. Within the Simons VIP cohort, a diagnosis of anemia was established if the individual was identified as having anemia per caregiver or self-report on the standardized medical history interview and confirmed through medical records review. We used Fisher's exact test [30] to assess the statistical significance of the difference in anemia prevalence between individuals with low and high number of *BOLA2* copies.

CNV calling and association statistical analysis in UKB population cohort

UKB [31] is a volunteer-based general population biobank of the United Kingdom. Half a million participants were recruited through National Health Service patient registers and were aged 40-69 years at the time of recruitment (2006-2010). Participants have consented to provide personal and health-related information, biological samples, and to have their DNA tested. The UKB governing Research Ethics Committee has approved a generic Research Tissue Bank approval to the UKB, which covers the research using this resource.

SNP-genotyping in the UKB was performed using the UK BiLEVE and UKB Affymetrix Axiom platforms. Produced Log R ratio (LRR) and B Allele Frequency (BAF) values were formatted for CNV calling with Hidden Markov Model-based software PennCNV v1.0.4 [32]. We retained samples that passed the post-processing quality control parameters. To minimize the number of false positive findings, detected CNVs were stratified using a quality score (QS) [33]. Only validated (QS>|0.8|) carriers were included in downstream analyses. We considered "16p11.2 BP4-BP5 CNV carriers" those that had a deletion or duplication starting in the interval 29.4-29.8 Mb and ending 30.05-30.4 Mb (hg19). We restricted the analysis to those participants who declared themselves as white British.

We searched for evidence of association between gene dosage at 16p11.2 BP4-BP5 (deletion carriers, control individuals, and duplication carriers) and hematological traits relative to red blood cells, reticulocytes (immature red blood cells), and platelets, using linear model in the statistical package R [30]. We also ran pairwise comparisons of deletion carriers *versus* controls and duplication carriers *versus* controls using t-test. In linear models, only additive effects for each copy (del=-1, controls=0, dup=1) were considered, and age, age^2, sex, the first 40 principal components from the genetic analysis, acquisition route, acquisition time, device ID, and freeze-thaw cycles were included as covariates. Trait measures were normalized by quantile transformation, prior to the analyses.

To assess the prevalence of anemia, we analyzed hospital discharge diagnoses coded following the International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10) together with self-declared illnesses. This information is available under the data-field 41202 and 41204 (respectively main and secondary diagnoses) and data-field 20002 of self-reported non-cancer medical conditions. We

considered as anemic the individuals with ICD-10 codes D50, D51, D52, D53, D55, D58, D59, D60, D61, and D64, and self-reported non-cancer illness codes 1328, 1330, 1331, 1332, and 1446, as in [34]. We thus excluded from the counts individuals with anemia of known genetic origin (thalassemia and sickle-cell disease). A participant was coded as anemic if he/she had a diagnosis or self-reported the condition. We also used information under data-field 6179 (mineral and other dietary supplements) to assess the treatment with iron supplementation. We tested the difference in anemia prevalence between 16p11.2 deletion and duplication carriers versus controls using the Fisher's exact test.

Mouse models

We used the 16p11.2$^{Del/+}$ (Del/+) and 16p11.2$^{Dup/+}$ (Dup/+) mouse models [25] and the C57BL/6N-Bola2$^{tm1(KOMP)Wtsi/Nju}$ line (Bola2^{tm1}) produced by the Knockout Mouse Project and International Mouse Phenotyping Consortium. Bola2^{tm1} animals were acquired from the Nanjing Biomedical Research Institute of Nanjing University.

For the Del/+, Dup/+ and Bola2^{tm1} European cohort, all mice were born and housed in the Animal Facility of the Center for Integrative Genomics. We first removed the *agouti* allele from the Bola2^{tm1} line through backcrossing with C57BL/6N mice from Charles River. The animals were maintained on Kliba 3436 diet (250 ppm iron). All procedures were performed in accordance with protocols approved by the veterinary cantonal authority.

For the Bola2^{tm1} US cohort, 129S4/SvJaeSor-*Gt(ROSA)26Sor$^{tm1(FLP1)Dym}$*/J were acquired from the Jackson Laboratory and backcrossed (N>20) to the C57BL/6J background. The lacZ and neo cassettes in the Bola2^{tm1} animals were excised by breeding to FLP1 mice and then backcrossed to C57Bl/6J for two generations. Resulting heterozygous animals were intercrossed to generate a cohort for analysis. All genetically modified mice were born and housed in the barrier facility at Children's Hospital Boston. The animals were maintained on Prolab RMH 3000 diet (380 ppm iron; LabDiet). 8 and 15-week-old females were analyzed in studies of transgenic animals. All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee.

Both animal facilities employ a constant dark-night light cycle and all mice were provided both water and food ad libitum.

Blood and tissue iron analysis

For the Del/+, Dup/+ and Bola2^{tm1} European cohort, whole blood was collected from the tail vein in EDTA or heparin tubes (Sarstedt) for hematological and plasma iron measurements, respectively. These were executed by the Centre de PhénoGénomique, EPFL, Lausanne. The low body weight of 7-week-old Del/+ females did not allow collecting blood for hematological measurements because of limits imposed by the veterinary office.

For the Bola2^{tm1} US cohorts, whole blood for complete blood counts was collected from the retro-orbital sinus into EDTA-coated microtainer tubes (Becton Dickinson) from animals anesthetized with ketamine/xylazine (100-120 mg/kg ketamine and 10 mg/kg xylazine) in sterile saline. Samples were analyzed on an Avida 120 analyzer (Bayer) in the Boston Children's Hospital Department of Laboratory Medicine Clinical Core Laboratories. Whole blood for other purposes was collected by retro-orbital bleeding into serum separator tubes (Becton Dickinson), and serum was prepared according to the manufacturer's instructions. Serum iron values were determined with the Serum Iron/TIBC kit (Pointe Scientific) according to the manufacturer's instructions. Liver and spleen tissues were collected and tissue non-heme iron concentrations were determined as described previously [35]. ZPP values in whole blood were analyzed on a ProtoFluor-Z Hematofluorometer (Helena Laboratories) according manufacturer's instructions.

Statistical analyses (t-test and linear model) were performed using the R software environment [30].

Single-molecule real-time (SMRT) sequencing of *Bola2*$^{-/-}$ mouse genome

Genomic DNA was isolated from the buffy coat layer of the blood of a male null mouse [36]. We prepared one DNA fragment library (40-160 kbp inserts) with no additional shearing. After SMRTbell preparation, the library was size-selected with the BluePippin™ system (Sage Science) at a minimum fragment length cutoff of 40 kbp. Final library average size was 90 kbp. Single-molecule, real-time (SMRT) sequence data were generated using the PacBio Sequel instrument with Sequel Binding and Internal Ctrl Kit 3.0, Sequel Sequencing Kit 3.0, MagBead cleanup, Diffusion loading, and acquisition times of 10-hour movies. A total of three SMRT Cell 1M v3 cells were processed yielding 11.5-fold (ROI/3.2 G) or 11.9-fold (raw/3.2G) whole-genome sequence (WGS) data. The average subread length was 22.2 kbp

with a median subread length of 14.1 kbp and N50 subread length of 35.9 kbp. Raw reads were mapped back to the mm10 mouse reference genome with minimap2 [37] and inspected manually at the *Bola2* locus and genome-wide with PBSV (Pacific Biosciences). 10/10 reads mapping to the *Bola2* locus reveals the presence of deletion of exons 2-3 of the gene and a local assembly of those reads with the Canu assembler [38] confirms the deletion of 563 bp of genomic sequence, replaced with a 216 bp insertion which includes one copy of the FRT recognition site.

Discussion

The deciphering of how human-specific genetic changes determine unique human features has mostly focused on brain cortex expansion and higher cognitive functions. However, other genetic and physiological peculiarities might have allowed the survival of our species in contrast with the extinction of all other hominin species. This work sheds light on the possible benefit of increased dosage of *BOLA2* in humans, with six copies per genome (the most common copy number) compared to two copies present in other hominins, primates, and mammals [12]. Duplications containing *BOLA2* map to the 16p11.2 locus and generate chromosomal instability associated with neurodevelopmental and psychiatric disorders. *BOLA2* duplications are thus an example of genetic trade-off as their potential gain comes with the cost of predisposition to pathogenic rearrangements [12].

We assessed phenotypes associated with *BOLA2* copy number and those that are more prevalent in individuals carrying the minimum number of copies of *BOLA2*. We found that, as recently reported [34], 16p11.2 deletion is associated with anemia (~20% of carriers), particularly iron deficiency anemia, and that ~50% of deletion carriers with three *BOLA2* copies are anemic, suggesting a causative role of lower *BOLA2* dosage. We validated these results in mouse models of 16p11.2 deletion, encompassing the *BOLA2* ortholog, and *Bola2* haploinsufficiency and deficiency. All models presented iron deficiency and a mild form of microcytic and hypochromic anemia. Among Del/+ mice, only males had microcytosis, lower corpuscular hemoglobin, and anisocytosis. Transcriptome profiling of brain tissue shows that Del/+ females but not males have increased expression of *Glrx3*, *Glrx5*, *Bola1,* and *Ciapin1*, thus possibly compensating the decreased *Bola2* expression through increased expression of BOLA2 binding partner (GLRX3), mitochondrial counterparts (GLRX5 and

BOLA1), or pathway partner (CIAPIN1) (De Nittis *et al*., unpublished). Finally, *Bola2* knockout mice showed mild microcytosis, higher ZPP level, and lower plasma iron, although the last phenotype was not observed in all longitudinal measurements possibly due to compensatory mechanisms. These data suggest that *BOLA2* is a driver of iron deficiency anemia, however it is still possible that other genes in the 16p11.2 single-copy region or its flanking human-specific duplicon contribute to this phenotype, as the number of *BOLA2* copies coincides with the number of copies of the entire 102 kbp segment.

We found some discrepancies in hematological parameters between human and mouse. Both Del/+ male and *Bola2*$^{-/-}$ and *Bola2*$^{+/-}$ mice had significantly lower MCV and MCH compared to wild-type mice. We did not find the same features in red blood cells of 16p11.2 deletion carriers possibly due to an unreported iron supplementation therapy for anemia. In fact, we found no difference in dietary iron supplementation between 16p11.2 deletion carriers and controls from the UKB (data-field 6179, comparing iron versus "none of the above", Fisher's exact P = 0.6). We note that this information might be partial as it was collected by touchscreen questionnaire and 16p11.2 deletion is associated with loss of FSIQ points [5]. Platelet traits were overtly modified in human and mouse, however humans showed negative effect of gene dosage on PLTc and PLTcrit and positive on platelet volume, whereas Del/+ male, *Bola2*$^{-/-}$ and *Bola2*$^{+/-}$ mice showed higher platelet mass. These platelet modifications could be due to lower dosage of *BOLA2* and/or other 16p gene/s or be secondary to the iron deficiency, as serum iron is inversely related to PLTc and PLTcrit in iron deficiency anemia [39-41]. Human *versus* mouse discrepancy further confirms the fundamental divergence in gene expression between human and mouse hematopoiesis and the limitations of the mouse system to model human hematopoiesis [42, 43].

Overall these data suggest that the increased *BOLA2* dosage in humans improved our iron metabolism. Iron is an essential micronutrient and serves as a cofactor for numerous enzymes, including those involved in energy metabolism, synthesis of DNA and proteins, and a range of other biochemical functions in cells. It is important in brain development, kidney function, immune responses, and growth and is essential for oxygen transport and delivery as component of hemoglobin and myoglobin [44]. If overall iron deficiency decreases relative fitness, iron-deficient individuals are protected against infections and

have more chances to survive epidemics of infectious diseases with high mortality rates [45].

Veterinary hematological data for chimpanzee, bonobo, gorilla, and orangutan [46] (**Supplementary Table 13**) show that hemoglobin and iron levels in the blood do not differ between humans and great apes, suggesting that humans need higher BOLA2 levels to guarantee the same level of iron supply to the organism. Menstrual blood loss is much larger in human than in great apes. Different hypothesis have been proposed to explain this difference: elimination of pathogens from the reproductive tract; economy of energy as it is more costly to maintain the endometrium than to grow a new one; elimination of defective embryos; and adaptation to increased amounts of easily absorbable iron from meat [45]. It is possible that the increased copy number of *BOLA2* is necessary to cope with the increased menstrual iron loss, although males and females were equally affected by anemia due to low *BOLA2* dosage in 16p11.2 deletion carriers.

Higher BOLA2 levels might have been important in the past, for example during the transition from a red meat hunter-gatherer Paleolithic diet to the iron-reduced cereal grain Neolithic diet. This shift likely resulted in an increased incidence of iron deficiency anemia, especially in women of reproductive age, and selection could have favored individuals carrying additional *BOLA2* copies. Iron deficiency is still the most common micronutrient deficiency in the world and the most common cause of anemia [47], suggesting the difficulty to precisely tune iron metabolism in humans.

Finally, the effects of *BOLA2* copy number polymorphism on the phenotypic spectrum and incomplete penetrance of phenotypes of 16p11.2 deletion is a striking example of the contribution of genes in the flanking low-copy repeats to the traits associated with a genomic disorder, pointing out the evolutionary and medical relevance of this work.

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16p11.2 Consortium members

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Figure legends

Figure 1. **A)** Gene content of the human most common haplotype of the 16p11.2 BP4-BP5 region (*top*) and comparison with the syntenic single-copy region rearranged in 16p11.2 CNV mouse models [25] (*bottom*). Human single-copy genes are grey-shaded; the *BOLA2-SLX1-SULT1A* human duplicon is green-shaded; the human-specific *NPIP* duplicon is orange-shaded. At BP4 the *BOLA2B*, *SLX1B,* and *SULT1A4* paralogs map; at BP5 two copies of the *BOLA2A*, *SLX1A*, and *SULT1A3* map. **B)** Plasma iron level in 16p11.2$^{Del/+}$ and 16p11.2$^{Dup/+}$ mice and their wild-type littermates at different ages (f: female, m: male, w: weeks). C) Red blood cell traits in 16p11.2$^{Del/+}$ and 16p11.2$^{Dup/+}$ mice and their wild-type littermates at the age of 29 weeks. These traits are significantly different in at least two longitudinal measurements (**Supplementary Tables 3 and 4**). *P* <= 0.1 are shown. (MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, CH: red cell hemoglobin content, RDW: red cell distribution width, MCHCr: mean corpuscular hemoglobin concentration reticulocytes, CHr: red cell hemoglobin content reticulocytes).

Figure 2. A) Two models of non-allelic homologous recombination mechanism between copies of the *BOLA2-SLX1-SULT1A-NPIP* segment at BP4 and BP5 are presented. The BP4-BP5 structure corresponds to the most common H3 haplotype. For example, sequence misalignments could occur between one BP4 copy and either the distal (top panel) or proximal (bottom panel) BP5 copies. The resulting alleles with a BP4-BP5 duplication and deletion have, respectively, more and less copies of the *BOLA2*, *SLX1*, *SULT1A,* and *NPIP* genes (right panels). Numbers count the number of copies of the *BOLA2-SLX1-SULT1A* green segment in each haplotype. The color code is specified in Figure 1 legend. **B)** *BOLA2* copy number in 635 European individuals from the 1000 Genomes Project and Human Genome Diversity Project (Ctrl), 67 16p11.2 BP4-BP5 deletion carriers from the European 16p11.2 Consortium, and 63 deletion carriers from the SVIP Cohort.

Figure 3. A) Hematological parameters in 16p11.2 BP4-BP5 CNV carriers of the UK Biobank. Red blood cell distribution width (RDW), mean reticulocyte volume (MRV), platelet count (PLTc), mean platelet volume (MPV), and platelet crit (PLTcrit) in 16p11.2 deletion and

duplication carriers and control individuals of the UK Biobank. **B)** Prevalence of anemia in 379,385 control individuals and 89 16p11.2 deletion carriers of the UK Biobank (*top*) and 88 16p11.2 deletion carriers of the SVIP and European cohorts stratified by *BOLA2* copy number (*bottom*).

Figure 4. The Bola2^{tm1} knockout mouse line. A) Mouse mating strategy of the European Bola2^{tm1} line and gender and genotype ratios observed. **B)** Longitudinal body weight profiles. Note: the x-axis scale is not continuous. **C)** Plasma iron level in *Bola2*$^{+/-}$ and *Bola2*$^{-/-}$ mice and wild-type littermates (f: female, m: male, w: weeks). Red dots represent mice housed in single cages. **D)** Red blood cell (HGB: hemoglobin, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, CH: red cell hemoglobin content), reticulocyte (MCVr: mean corpuscular volume reticulocytes, MCHCr: mean corpuscular hemoglobin concentration reticulocytes, CHr: red cell hemoglobin content reticulocytes), and platelet (MPC: mean platelet component concentration, PCDW: platelet component distribution width, MPM: mean platelet mass) parameters of *Bola2*$^{+/-}$ and *Bola2*$^{-/-}$ mice and wild-type littermates at 17 weeks of age. Beta with $P <= 0.1$ are shown. **E)** Blood zinc protoporphyrin (ZPP) level of *Bola2*$^{-/-}$ mice and wild-type littermates at 8 weeks of age.

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