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Brief Report

A new overgrowth syndrome is due to mutations in *RNF125*

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ABSTRACT

Overgrowth syndromes (OGS) are a group of disorders in which all parameters of growth and physical development are above the mean for age and sex. We evaluated a series of 270 families from the Spanish Overgrowth Syndrome Registry with no known overgrowth syndrome. We identified one *de novo* deletion and three missense mutations in *RNF125* in six patients from 4 families with overgrowth, macrocephaly, intellectual disability, mild hydrocephaly, hypoglycaemia and inflammatory diseases resembling Sjögren syndrome. *RNF125* encodes an E3 ubiquitin ligase and is a novel gene of OGS. Our studies of the RNF125 pathway point to upregulation of RIG-I-IPS1-MDA5 and/or disruption of the PI3K-AKT and interferon signaling pathways as the putative final effectors.

Key words: Overgrowth, macrocephaly, autoimmune disorder, intellectual disability, RNF125

Generalized overgrowth syndromes (OGS) include the classic overgrowth disorders in which all of most parameters of growth and physical development are above the mean for age and sex (Lapunzina, 2005; Lapunzina, et al., 2002). Partial, localized, or regional OGS are those disorders in which excessive growth is confined to one or a few regions of the body. Macrocephaly is usually part of the clinical features observed in many OGS such as the Sotos (MIM# 117550), Weaver (MIM# 277590), Simpson–Golabi–Behmel (MIM# 312870), Bannayan-Riley-Rubalcava (MIM# 153480), Primrose (MIM# 259050) (Cordeddu, et al., 2014) and the 17q11.2 deletion (van Asperen syndrome; MIM# 613675) (van Asperen, et al., 1998) as well as in hemimegalencephaly-capillary malformation (MIM# 602501) and CLAPO association (MIM# 613089) (Lopez-Gutierrez and Lapunzina, 2008). In 2007, Douglas & col. identified mutations in *RNF135* (an ubiquitin E3 ligase also known as Riplet mapping in the 17q11.2 deletion interval) in six families with a phenotype similar to the van Asperen syndrome but without neurofibromata (Douglas, et al., 2007). The authors demonstrated that haploinsufficiency of a RING finger gene was responsible for this overgrowth syndrome. All patients

had macrocephaly (ranging from +2 to +4.1 SD), most were tall and heavy and some of them had relatives with mutations and similar clinical features. This clinical entity shares many clinical features with the van Asperen syndrome and has been recently renamed as macrocephaly-macrosomia-facial dysmorphism syndrome (MMFD; MIM# 614192).

Ubiquitin-proteasome degradation is one of the major post-transcriptional processes regulating the levels and function of proteins. The ubiquitin pathway includes the activity of at least three different enzymes: an ubiquitin-activating enzyme or E1, a conjugating enzyme or E2, and a ligase enzyme or E3. More than 600 E3 ubiquitin ligases are expressed in the human genome, allowing for the specificity of the ubiquitination system. However, the implication of the ubiquitin-proteasome pathway in OGS is not well known. E3 ubiquitin-ligases are involved, among others, in the phosphoinositide 3-kinase (PI3K) pathway. PI3K enzymes generate 3-phosphorylated phosphoinositides that act as second messengers downstream of numerous cellular receptors implicated in the regulation of cell growth, proliferation, survival, differentiation, and cytoskeletal changes. One of the best characterized targets of PI3K lipid products are the protein kinases AKT (v-AKT murine thymoma viral oncogene). The PI3K-AKT pathway has been implicated in the pathophysiology of several OGS such as the Proteus (MIM# 176920), hemihyperplasia-hypoglycemia (240900), CLOVES (MIM# 612918) and hemimegalencephaly-capillary malformation (MIM# 602501) syndromes due to mutations in *AKT1, AKT2, AKT3, PI3KCA* and/or *PI3KR2* (Lindhurst, et al., 2012; Lindhurst, et al., 2011; Riviere, et al., 2012). In this paper we report six patients from four families with overgrowth, macrocephaly and other findings, in whom we have found mutations in another RING finger gene, *RNF125* which encodes an E3 ligase. Similarly to RNF135, RNF125 is upstream of RIG-I (Retinoic acid-inducible gene) and MDA5 (melanoma differentiation-associated gene 5), two proteins implicated in a variety of biological pathways including the interferons and the PI3K-AKT signalling pathways.

We selected a subset of 270 patients out of a total of 1632 individuals registered at the Spanish Overgrowth Syndrome Registry with non-syndromic overgrowth (as defined by Neri in 2001) (Cohen, et al., 2001). We included all individuals with OGS who were negative for *NSD1, EZH2, GPC3* and 11p methylation defects. We performed in all of them karyotypes and in most of them array CGH or SNP arrays (Supp. Materials and Methods). On SNP-array screening, one patient (patient 1, Figure 1A and C, Supp. Table S1) showed a *de novo* 9 Mb deletion at chromosome 18 (chr18:24,658,770-34,038,769) encompassing candidate genes, including *RNF125* and *RNF138* and many other RefSeq genes (Supp. Table S2). This finding led us to redirect our investigations in the subset, by screening *RNF125* and *RNF138* in all 270 individuals using multiplex ligation probe amplification (MLPA), high resolution melting (HRM), Sanger sequencing and pyrosequencing (Supp. Materials and Methods).

Three variants were identified in *RNF125* (c.336G>A [p.M112I], c.488C>T [p.S163L] and c.520C>T [p.R174C] (Figure 1D and E) which were predicted, by *in silico* analysis, to be pathogenic (Supp. Table S3). In one of the patients the mutation was *de novo* and in the other two patients the mutations were also observed in their affected mothers (patients 4 and 6, respectively) (Supp. Table S1, Figure 1A and F). Pyrosequencing of *RNF125* in patients 2-6 confirmed that heterozygous point mutations were 1:1 in dosage and that there was no evidence of mosaicism (Figure 1E). No pathogenic mutation in *RNF138* was found in any of the 270 patients. Clinical features and a summary of the studies performed in the four patients are listed in Supp. Table S1. The three missense mutations in *RNF125* were absent in 600 chromosomes from Spanish healthy controls and in 350 exomes of Spanish controls. We also explored the 1000 genome database (http://browser.1000genomes.org) and the Exome Variant Server (http://evs.gs.washington.edu/EVS/). None of the three variants were reported in the 1000 genomes. In the EVS we found that c.336G>A (p.M112I) was absent, while the other two variants were reported in 1/13,005 chromosomes (0.0077 %, Supp. Table S3). Predictions using the

Polyphen2, Mut Pred and Mutation Taster software suggest that all three changes are pathogenic or damaging (Supp. Table S3). Furthermore, methionine 112 and serine 163 residues are both highly conserved in mammals while arginine 174 is conserved in almost all but a few mammals (Supp. Figure S1). Protein modelling using Phyre2 predicted moderate to severe changes in the mutant proteins (Supp. Figure S2).

To test other possible genetic or epigenetic alterations we performed whole expression and whole methylation analysis (Supp. Figures S3 and S4). We retrospectively also applied whole exome sequencing of DNA samples from blood of patients 2 and 5, to exclude the presence of other pathogenic overgrowth-causing mutations (Supp. Materials and Methods). After filtering for common variants, known polymorphisms and comparison with Spanish databases of exomes, bioinformatic analysis demonstrated that they shared variants in *RNF125* and seven other genes (Supp. Table S4). Segregation analysis in two affected mothers strongly suggested *RNF125* as the unique candidate gene (Figure 1F). In all patients we also applied whole-genome expression arrays and subsequently RT-qPCR confirmation in the three main pathways, RIG-I-IPS1, PI3K-AKT and interferon signalling pathways, in which RNF125 participates. Clear differences were observed between patients and controls. The mRNA levels of *RNF125* were low in all patients (Figure 2A) compared to controls while *RNF135* levels were similar in the two groups (data not shown). In the RIG I-IPS1 pathway, some transcripts (*RIG-I, LGP2, MDA5, and IPS1)* were up-regulated in patients compared to age and sex-matched controls (Figure 2A). Other genes downstream of *RNF125* showed a non-homogeneous behaviour: *DUBA, TRAF3* and *TRAF6* were down-regulated, *IRF3 and TRADD* were up-regulated and *TRAF2* was equal to controls (Supp. Figure S4A). Similar results were obtained after the analysis of genes implicated in the PI3K-AKT pathway. Levels of *mTOR*, *PIK3R2*, *BTK*, *ILK, MYD88, PAK1, EIF4G1, HSPB1, PTEN* and *TLR4* (Figure 2A and Supp. Figure S4B) were up-regulated in patients compared to controls whilst *ATK2, AKT3, EIF4B, EIF4E, PIK3CA, PIK3R1* and *RPS6KB1* were down-regulated (Supp. Figure S4B). Finally, analysis of 15 genes of the interferon pathway showed

that only two genes, *IFNA8* and *IFNA14* were down-regulated (Supp. Figure S4C), whilst the remaining 13 genes showed no differences in their expression profile pattern between patients and controls. These results confirm that mutations in *RNF125* lead to transcriptional down-regulation of the gene and that haploinsufficiency of *RNF125* leads to up-regulation of *RIG-I, IPS1* and *MDA5* and misregulation of at least three main pathways (RIG-I-IPS1, PI3K-AKT and Interferon). We tested whether expression of RIG-I protein is dysregulated in patients with *RNF125* mutations and found that basal RIG-I levels were not significantly different in B-lymphocytes cells of patients 1 and 2 compared to controls (data not shown). However, after treatment with poly (I:C), which acts as a double strand RNA-activator of the RIG-I signaling pathway, RIG-I induction levels were doubled in patients compared to controls (Figure 2B-C). In order to demonstrate that RNF125 haploinsufficiency slows RIG-I degradation, we monitored the degradation kinetics of RIG-I after blocking translation with cycloheximide, a standard approach used to assay proteasome-mediated degradation. When protein synthesis was blocked, the turn-over of endogenous RIG-I was slower in patient cells than in controls (Figure 2D). After 12 hours post-cycloheximide treatment, patient-derived cells retained ~50% of the amount of RIG-I whilst RIG-I was almost fully degraded in control cells (Figure 2D-E).

Overgrowth, macrocephaly and intellectual disability are observed in a relatively small number of disorders (Lapunzina, 2005). We here report six individuals from four families with mutations in *RNF125* and macrocephaly, overgrowth, enlarged ventricles, a variable degree of intellectual disability and in some, hypoglycaemia and recurrent inflammatory disease resembling Sjögren syndrome. Two patients inherited the mutations from their affected mothers and in the other two the mutations were *de novo*. The p.M112I and p.R174C mutations cosegregated with the phenotypes of their affected mothers. *In silico* analysis of the amino acid changes in RNF125 indicates that they occurred in protein domains with important biological functions: p.M112I and p.R174C directly affect the first (C2HC) and third (C2H2) zinc finger domains of the protein, respectively (Supp. Figure S1). The p.S163L mutation is located between the second and the third

(C2H2) zinc finger domains, thus potentially affecting the intramolecular folding and the interactions among these zinc fingers (Giannini, et al., 2008). The introduction of a cysteine instead of an arginine at the third zinc finger disrupts the balance of the finger domain from C2H2 to C3H2 and potentially may also impair its function. In addition, *in silico* 3D analysis of the mutant proteins also suggest that the mutations may potentially alter the tertiary structure (Supp. Figure S2), thus, affecting their functions. Finally, and very importantly, expression studies confirmed that all analyzed patients showed decreased *RNF125* RNA levels.

All our patients with RNF125 mutations that resulted in down-regulation of *RNF125* were shown to have up-regulation of *RIG-I, MDA5* and *IPS1* (Figure 2A). RNF125 functions as a negative regulator of RIG-I and MDA5 signaling and a positive T cell regulator (Arimoto, et al., 2007). Dysregulation of RIG-I has been implicated in several infectious diseases and disorders of the immune system (Giannini, et al., 2008). Further, gain of function mutations of *IFIH1* (*MDA5*) have been recently described in patients with Aicardi-Goutieres syndrome (MIM# 225750) featuring CNS anomalies and autoinflammatory diseases, as observed in our patients (Rice, et al., 2014). In accordance with our findings, Rice et al. (Rice, et al., 2014) proposed that mutations in *IFIH1* (*MDA5)* cause a gain of function of the protein leading to an increase in its binding with the RNA in the innate immune response. Similarly, haploinsufficiency of *RNF125* (which is upstream in the same pathway and regulates both RIG-1 and MDA5) (Supp. Figure S5) would lead to an increase of MDA5 levels resulting in a gain of function of it and potentially dysregulating the immune response. In the interferon pathway both *IFNA8* and *IFNA14* were also down-regulated. Transcriptional up-regulation and increased protein levels of RIG-I and/or IPS1 have been reported to play a role in the pathophysiology of autoinflammation (Britto, et al., 2013). Interestingly, some patients reported herein suffered from recurrent inflammation with a chronic pattern (Supp. Table S1). Patient 2 had several episodes of Raynaud phenomena, dry conjunctivitis and recurrent aftous stomatitis and patient 4 suffered from continuous episodes of keratitis, conjunctivitis, limbitis, serous otitis and

pneumonia findings that are usually observed in the Sjögren syndrome (MIM# 270150), a common autoimmune disease. We observed transcriptional upregulation of RIG-I and IPS1 at the RNA and protein level in our patients. Three E3 ubiquitin ligases, RNF125, RNF135 and TRIM25 target RIG-I for ubiquitination (Arimoto, et al., 2007; Gack, et al., 2007; Oshiumi, et al., 2010) allowing RIG-I, and the three RIG I-like receptors, namely MDA5 [*IFIH1*], LGP2 (or DEXH box polypeptide 58 [*DHX58*], (MIM# 608588) and IPS1 to interact in a very complex cascade of downstream activations (Supp. Figure S5). IPS1 is activated by RIG-I and MDA5 which in turn both interacts with RNF125, RNF135 and TRIM25 (Oshiumi, et al., 2010) (Supp. Figure S5). The interaction between RIG-I and IPS1 is induced by TRIM25-mediated Lys63-linked polyubiquitination at Lys 172 of the RIG-I CARDs (caspase activation and recruitment domains) (Gack, et al., 2007). Previous studies demonstrated that RNF125 binds IPS1 as well as MDA-5 and was also ubiquitinately conjugated (Arimoto, et al., 2007) confirming that RNF125 has activity against RIG-I, MDA5, LGP2 and IPS1. These findings were also confirmed by the fact that the concentrations of MDA5 and IPS1 were reduced in cells overexpressing RNF125 ectopically and by a suppression of conjugation of ubiquitin to these proteins was observed by treatment with siRNA specific to RNF125 (Arimoto, et al., 2007).

Shared features in our patients were overgrowth, macrocephaly with prominent and large forehead and some degree of ID; however some other features were not observed in all individuals (Supp. Table S1). Our patients have phenotypic similarities with individuals with van Asperen syndrome (chromosome 17q11.2 deletion; MIM# 613675) and MMFD (MIM# 614192) due to mutations in *RNF135* (Douglas, et al., 2007). Although *RNF135* haploinsufficiency either to deletion or mutations leads to disease in humans, knockout of the mouse homolog, *rnf135*, does not cause any apparent developmental defects but rather severely reduced type I interferon, and abrogates the activation of RIG-I and RIG-I C-terminal domain polyubiquitination (Oshiumi, et al., 2010). In addition, *rig-i* deficient mice are usually embryonic lethal, but interestingly, a few mice were born alive and died within 3 weeks after birth showing growth retardation (the reverse phenotype of

overgrowth) (Kato, et al., 2005). Thus, mutations in *RNF125* and *RNF135* could lead to the same biological effect: haploinsufficiency of *RNF125* would prevent RIG-I binding thus, impairing IPS1 function while haploinsufficiency of *RNF135* would prevent activation of RIG-I and IPS1; similarly (Supp. Figure S5). Despite not knowing the precise mechanism through which RNF125 and RNF135 haploinsufficiency results in the dysregulation of the RIG-I-IPS1 pathway, we have clearly demonstrated that the patients with RNF125 mutations result in dysregulation of the two main activators of this pathway.

We have also observed transcriptional activation of some of the phosphatases of the PI3K pathway in patients with *RNF125* haploinsufficiency. There is considerable experimental evidence for the dysregulation of E3 ligases, PI3K signaling abnormalities and altered growth. Abnormal E3 ligases and growth retardation is reported in Angelman (MIM# 105830), Johanson-Blizzard (MIM# 243800) and Kaufman oculocerebrofacial (MIM# 615057) syndromes, among others. Additionally, some conditions with overgrowth have E3 ligases dysregulation. Firstly, synaptic overgrowth was observed in the absence of the E3 ubiquitin ligase "*highwire*" together with an increased PI3K signaling in motor neurons (Mozer and Sandstrom, 2012). Secondly, a Ring Finger E3 responsible of Ecdysone receptor (a steroid hormone) degradation in *Drosophila melanogaster* acts at high concentrations as a developmental timer and at basal levels contributes to the inhibition of tissue growth (Gradilla, et al., 2011). Thirdly, another E3 ligase complex associated to akt (SCF^{slimb}) inhibits neuroblast overgrowth in *drosophila* brains (Li, et al., 2014). Fourthly, the Big Brother (*bb*) gene, encoding an E3 ubiquitin ligase, represents a central regulator of organ size and growth in *Arabidopsis*. Plants lacking *bb* activity form larger organs; whilst conversely, plants expressing higher levels of *bb* produce smaller organs, indicating that *bb* acts as a negative regulator for organ size (Breuninger and Lenhard, 2012). RNF125 has a 46% (20/45) positive identity at the zinc finger domains with bb (amino acids 197-241). Exact amino acid identity between RNF125 and BB occurs at the eight conserved cysteines and histidines of the zinc finger domains. Fifthly, individuals with

haploinsufficiency of *NSD1* (a gene whose protein has several zinc fingers) develop Sotos syndrome (MIM# 117550), an overgrowth/macrocephaly disorder. Thus, haploinsufficiency of E3 ubiquitin ligases in plants (*Arabidopsis*) and *Drosophila melanogaster* leads to overgrowth and in humans, haploinsufficiency of *RNF135* (Douglas, et al., 2007) and now in this study, *RNF125*, leads to overgrowth, macrocephaly and intellectual disability. Finally, firm evidence of the implication of the PI3K-AKT pathways in overgrowth-macrocephaly syndromes and links among the PI3K and the RIG-I/interferon pathway have recently been communicated. Mutations in *PIK3CA*, *PIK3R2* and *AKT3* are observed in the megalencephaly-capillary malformation (Riviere, et al., 2012), in *AKT1* in Proteus syndrome (Lindhurst, et al., 2011) and in *AKT2* in hemihypertrophy-hypoglycemia (Lindhurst, et al., 2012). In addition, PI3K has been defined as one of the main factors activated through RIG-I mediated anti-pathogen response to enhance expression of type I interferons. Other features observed in some of our patients were intellectual disability, mild hydrocephaly and hypoglycaemia. Intellectual disability and hydrocephaly are also observed in patients with *AKT3*, *PI3KR2* and *PI3KCA* mutations whilst, hypoglycaemia has been observed in individuals with *AKT2* mutations (Lindhurst, et al., 2012; Lindhurst, et al., 2011; Riviere, et al., 2012). Intellectual disability has been also described in patients with mutations in other RING finger genes such as *RNF133* and *RNF148* (Bonora., et al., 2012).

In summary, we have identified six affected individuals from four OGS families with mutations in *RNF125*. We have demonstrated that mutations in *RNF125* result in a loss of function of *RNF125* and dysregulation of the RIG-I-IPS1, PI3K-AKT and interferon pathways. The exact mechanism/s by which haploinsufficiency of *RNF125* results in overgrowth, macrocephaly and in some patients intellectual disability, hypoglycaemia and Sjögren-like syndrome remains elusive. Though the complex pathways linking E3 ubiquitin ligases, RING fingers, CARD/CARD-like proteins and the RIG I-IPS1, PI3K-AKT and interferon pathways need further investigations, this paper

demonstrates the connections among these proteins and their implication in a novel disorder of growth, development and autoimmunity.

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Figure 1. A: Facial phenotypes of the patients 1, 2, 5 and 6. Correlation among the individuals and their position in the pedigree is in brackets. Note macrocephaly and large forehead in all patients and in some of them, a broad nose and synophrys. Patient 1 also presents a large mouth and a squared face, patient 2 has fleshy lips and upturned nose and patients 5 and 6 non-curved eyebrows and deep-set eyes. **B:** MRI of patient 2. Arrow indicates the anomaly of the Galenous' vein; note mild hydrocephalus and septum cavum pellucidum (white arrow). **C:** SNP-array of patient 1; the deleted region is shaded. The upper panel represents the log2 ratio score (dosage) and the lower panel shows the haplotype plot showing loss of heterozygosity (LOH) in the deleted region. The deletion coordinates are in hg19. **D:** Comparison of the mutant and wild type chromatagrams showing the *RNF125* mutations in the three probands (RefSeq NM_017831.3; data submitted to http://www.lovd.nl/RNF125). **E:** Pyrosequencing confirmation of each mutation showing a 1:1 ratio. F: Pedigrees of the four families showing cosegregation of the mutation with the phenotype in families 3 and 4. The pedigrees also include: genotype (+ =wt; M=mutant); y: (years), OFC (occipitofrontal circumference); H (Height) and mutation detected for each case. NR: Not Recorded.

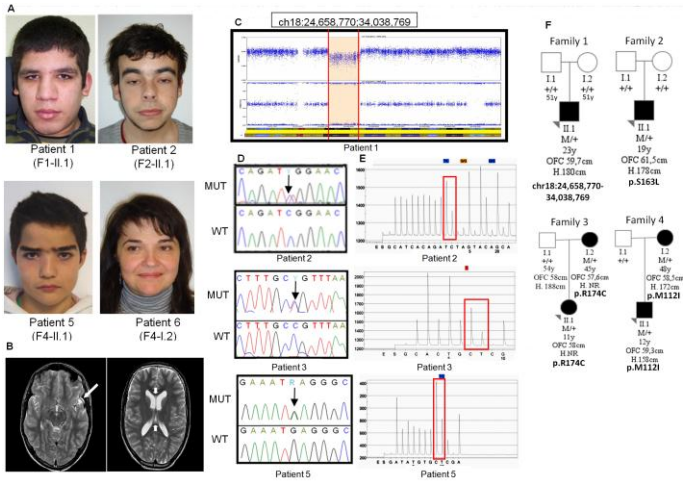

Figure 2. Functional analysis of RNF125. **A:** Expression arrays showing dysregulation of the RIG-I and PI3K-AKT pathways. Boxplot of mRNA expression of different genes included in the RIG-I and PI3K-AKT signalling pathways: *RNF125*; *RIG-I* (*DDX58*); *IPS-1*; *MTOR*; *PIK3R2*; *BTK*; *ILK* and *LGP2*. Case= average of normalized mRNA levels of patients (P1: Patient 1; P2: Patient 2; P5: Patient 5) Control= average of normalized mRNA levels of controls. For each patient one age and sex matched control were use. The colour range indicates the lowest (blue) to highest (red) expression. **B-E:** Western blots analysis of mutations in RNF125. RIG-I degradation is impaired in *RNF125* haploinsufficiency derived cells. **B:** RIG-I levels were examined by Western blot of untreated cells from each patients (1 and 2) or the corresponding controls (C) and compared with cells treated with 100µg/ml poly (I:C) for 16 hours. **C:** Densitometry was undertaken for three independent experiments, as in Figure 2B. The percentage of RIG-I induction was plotted in a graph, after values were corrected for actin expression. Patient cells showed a major percentage of induction compared to control cells. **D:** Patients' cells (patients 1, 2, 5 and 6) were treated with poly (I:C) followed by addition of cycloheximide (CHX). Cells were harvested at the indicated times after addition of CHX and RIG-I levels analyzed by Western blot. **E:** Degradation kinetic plots of for patients 1, 2, 5 and 6 versus controls showed a significance difference between the kinetic of the patients and controls.