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Identification of a novel leptin receptor (*LEPR*) variant and proof of functional relevance directing treatment decisions in patients with morbid obesity

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ABSTRACT

Background: Deficiency in the leptin-leptin receptor (LEPR) axis leads to severe, and potentially treatable, obesity in humans. To guide clinical decision-making, the functional relevance of variants in the *LEPR* gene needs to be carefully investigated.
Cases and methods: We characterized the functional impact of *LEPR* variants identified in two patients with severe early-onset obesity (1: compound heterozygous for the novel variant p.Tyr411del and p.Trp664Arg; 2: heterozygous for p.Arg612His) by investigating leptin-mediated signaling, leptin binding, receptor expression on cell surfaces, and receptor dimerization and activation for either wild-type and/or mutant LEPR.
Results: Leptin-induced STAT3-phosphorylation was blunted the novel p.Tyr411del or the p.Trp664Arg variant and mildly reduced with the p.Arg612His variant. Computational structure prediction suggested impaired leptin binding for all three LEPR variants. Experimentally, reduced leptin binding of all mutant proteins was due to diminished LEPR expression on the cell surface, with the p.Trp664Arg mutations being the most affected. Considering the heterozygosity in our patients, we assessed the heterodimerization capacity with the wild-type LEPR, which was retained for the p.Tyr411del and p.Arg612His variants. Finally, mimicking (compound) heterozygosity, we confirmed abolished STAT3-phosphorylation for the variant combination [p.Tyr411del + p.Trp664Arg] as found in patient 1, whereas it was retained for [p.Arg612His + wilde type] as found in patient 2.
Conclusions: The novel p.Tyr411del mutation causes complete loss of function alone (and combined with p.Trp664Arg) and is likely the cause for the early onset obesity, qualifying the patient for pharmacologic treatment. Heterozygosity for the p.Arg612His variant, however, appears unlikely to be solely responsible for the phenotype.
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1. Introduction

Leptin, produced by the adipose tissue, and its receptor (LEPR), expressed in the hypothalamus, are critical components in the central regulation of energy homeostasis and satiety [1–3]. Deficiency of these functional circuits can hence lead to severe and early onset obesity due to hyperphagia, as has been shown in rodent models [4,5] and also in human patients [6–8]. Even though monogenic obesity is rare in humans, access to modern techniques such as next generation sequencing identified a number of variants in the *LEPR* gene [8]. Nevertheless, their functional relevance needs to be carefully investigated.

Abbreviations: AT_1R, angiotensin II receptor type 1; BRET, Bioluminescence resonance energy transfer; CRH2, cytokine receptor homology 2 domain; ERK1/2, extracellular-signal regulated kinases 1/2; eYFP, enhanced Yellow Fluorescent Protein; FNIII, fibronectin III domain; IGD, immunoglobulin like domain; LEPR, leptin receptor; MAF, minor allele frequency; NTD, N-terminal domain; PI, propidium iodide; R612H, LEPR-p.Arg612His; SEM, standard error of the mean; (p)STAT3, (phosphorylated) signal transducer and activator of transcription 3; WT, wild-type LEPR; Y411del, LEPR-p.Tyr411del; W664R, LEPR-p.Trp664Arg.

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When leptin binds to its receptor in the hypothalamus, secretion of orexigenic neurotransmitters is inhibited and simultaneously anorexigenic hormones like alpha- melanocyte-stimulating hormone acting on the melanocortin 4 receptor (MC4R) are increasing, overall resulting in reduced energy intake [9]. The LEPR is a class I cytokine receptor and is expressed as a dimer on the cell surface. On the molecular level, ligand binding to the receptor's cytokine receptor homology 2 domain (CRH2) [10] induces a conformational change resulting in cross-phosphorylation and activation of the associated Janus kinase 2, which subsequently phosphorylates and thereby activates signal transducer and activator of transcription 3 (STAT3) [11]. Phosphorylated STAT3 dimerizes, translocates to the cell nucleus [12] and regulates the expression of target genes [13–15].

Variants in the *LEPR* gene were shown to cause impaired STAT3 phosphorylation [7], diminished cell surface expression and leptin binding [16], or protein truncation due to a splice-site-mutation leading to a receptor protein lacking the transmembrane and intracellular domain [6].

Clinically, all these patients show severe early-onset obesity [6–8,17–19] and most of them were described being hyperphagic from early childhood onwards [6–8,18,19]. In all families studied so far heterozygous individuals did not share the severe obese and hyperphagic phenotype of the homozygous or compound heterozygous LEPR-deficient patients [6–8,17,19].

In this study we performed a clinical characterization of two new patients harboring combinations of novel and known variants in the *LEPR* gene. Based on the comprehensive functional characterization of the variants regarding loss or retained LEPR function, suggestions for qualification for treatment with the MC4R agonist setmelanotide could be made.

2. Material and methods

2.1. Genetic analysis

Trio exome sequencing in patient 1 was performed after enriching approx. 37 Mb of coding sequences using the Illumina Nextera Rapid Capture Exome Kit. In patient 2, trio exome sequencing on an Illumina system was performed after enriching approx. 60 Mb of coding sequences using the Agilent SureSelect All Human Version 6. The bioinformatic analysis was performed by the software of Varvis (Limbus, Rostock, Germany). Identified, annotated variants were reported based on minor allele frequencies, impact on protein, segregation, relevance of the gene, as well as with the support of general in silico information such as conservation and prediction tools as described previously [20,21].

2.2. Plasmids

The wild-type construct pcDNA3 myc-LEPR was kindly provided by I. Sadaf Farooqi [7]. We inserted the respective variants (c.1231_1233 for p.Tyr411del, c.1835G > A for p.Arg612His and c.1990 T > A for p. Trp664Arg) into the wild-type pcDNA3-myc-LEPR plasmid via QuikChange-II-XL-Site-Directed-Mutagenesis-Kit (Agilent Technologies). Mutagenesis primers were obtained from biomers.net GmbH (Ulm, Germany). Furthermore, the above mentioned myc-tagged LEPR constructs were fused at the C-terminus with enhanced Yellow Fluorescent Protein (eYFP). For this purpose, the coding sequences of human LEPR and LEPR mutants were amplified by PCR using Phusion polymerase (Thermo Fisher Scientific, Waltham, MA), introducing a 5′ *Sal*I restriction site and a 3′ BamHI restriction site replacing the stop codon. The HA-hY_1R-eYFP-pVitro2 plasmid [22] was used as expression vector upon amplification of the eYFP-containing backbone (without HA-hY_1R coding sequence). After PCR product purification and restriction with *Sal*I and BamHI, the receptor DNA was ligated into the eukaryotic expression vector. To generate the wild-type leptin receptor as fusion protein with a C-terminal Nluc luciferase, the cDNA coding for the luciferase was amplified from the pNL1.3[secNluc] plasmid generously provided by Anette Kaiser (University of Leipzig, Institute of Biochemistry, Leipzig, Germany), using a 5′ primer introducing a BamHI restriction site, and a 3′ primer introducing a stop codon and *Xba*I restriction site. After fragment purification and restriction with BamHI and XbaI, the PCR product was ligated into the LEPR-eYFP-pVitro2 vector cut with the same enzymes. All of the constructs were verified by Sanger sequencing (Core Unit DNA-technologies, Faculty of Medicine, University of Leipzig). pEYFP-N1 (Clontech Laboratories, Inc., now Takara Bio Inc., Kusatsu, Japan) served as control and was kindly provided by Max Holzer (Paul Flechsig Institute of Brain Research, University of Leipzig, Germany).

2.3. Reagents and antibodies

Antibodies detecting myc-tag (9B11) (#2276), total STAT3 (D3Z2G) (#12640) and phospho- phospho-Tyr705 STAT3 (#9131) as well as horseradish peroxidase conjugated secondary goat anti-rabbit IgG (#7074) and horse anti-mouse IgG (#7076) antibodies were obtained from Cell Signaling Technology (Leiden, The Netherlands). Antibodies against β–actin (ab822J) were from Abcam (Cambridge, United Kingdom). Human LEPR APC-conjugated antibody (FAB867A) was obtained from R&D Systems (Minneapolis, MN, USA). Human recombinant leptin was purchased from Calbiochem (Merck Bioscience, Darmstadt, Germany). The Nluc substrate furimazine was purchased from Promega (Madison, WI, USA). Saponin was obtained from Sigma Aldrich (St. Louis, MO, USA). FuGENE HD Transfection Reagent was supplied by Promega (Madison, WI, USA). Lipofectamine® 2000 Transfection Reagent was obtained from Invitrogen (Carlsbad, CA, USA). MetafectenePro was purchased from Biontex (Munich, Germany). The nuclear stain Hoechst33342 was obtained from Sigma Aldrich (St. Louis, MO, USA).

2.4. Cell culture and transfection

For immunoblot analyses, flow cytometry and LEPR binding assays HEK293 cells were cultured in a humidified atmosphere at 37 °C and 5% CO_2 in DMEM/F12 (Gibco by Life Technologies) supplemented with 10% FCS and 1% penicillin/streptomycin. Cells were seeded into 6-well plates (500,000 cells/well) and were transfected 24 h later with the respective vectors using 2 μg of plasmid DNA and 8 μl FuGENE HD Transfection Reagent.

For live cell fluorescence microscopy and saturation BRET-assays HEK293 cells were cultured in DMEM/HAM's F12 (Lonza, Basel, Schweiz) supplemented with 15% FCS. For live cell fluorescence microscopy, cells were grown on poly-D-lysine-coated μ-slide 8 well slides (Ibidi, Martinsried, Germany) to 70–80% confluence. Transfection was performed with 800 ng plasmid DNA encoding the LEPR-eYFP constructs and Lipofectamine® 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol for 1 h at 37 °C. For saturation BRET-assays, HEK293 cells were seeded in six-well plates (1×10^6 cells/well) and transfected when 70–80% confluence was reached. 500 ng of BRET donor (LEPR-Nluc) and a gradient of eYFP-tagged LEPR constructs (BRET acceptors, 0-4000 ng) were co-transfected. As control, pVitro2-AT_1R-eYFP was used as BRET acceptor. The total DNA amount of 4500 ng per well was balanced by empty pcDNA3 vector. 3 μl MetafectenePro were used for each microgram DNA according to the manufacturer's protocol.

2.5. Protein isolation and western blot analysis

Stimulation of transfected cells with leptin and protein isolation was performed as described previously [7]. Equivalent amounts of protein were resolved by 4–12% SERVAGel™ TG PRiME™ Vertical Tris-Glycine Gel (SERVA, Heidelberg, Germany) by SDS-PAA gel electrophoresis,

blotted onto PVDF membrane (Amersham Hyperbond P0.45, GE Healthcare Life Sciences) and then incubated with primary antibodies direct against the myc-tag of the LEPR constructs, β–actin, phosphorylated or total STAT3. Analysis of densitometry was done using ImageJ 1.51 Software (NIH, USA). Statistical analysis was done using one-way ANOVA and Dunnett's Multiple Comparison Test via GraphPad Prism 5 (GraphPad Software, San Diego, CA).

2.6. Live cell fluorescence microscopy

24 h post-transfection, cells were starved for 20 min in Opti-MEM® reduced serum medium (Gibco by Life Technologies, Carlsbad, CA, USA) containing 2.5 μg/ml Hoechst33342 (Sigma) for nuclear staining. Membrane localization was examined on a Zeiss Axio Observer.Z1 using an ApoTome.2 Imaging System with an AxioCamMRm camera and an incubation chamber. To detect the fluorescence signals a C-Apochromat 63×/1.20 W objective was combined with a 46HE filter set (excitation: 488–512 nm; emission: 520–550 nm) for the YFP fluorophore and a 49 filter set (excitation: 335–383 nm; emission: 420–470 nm) to detect nuclear staining. The Zeiss ZEN2 software was used for image processing.

2.7. Analysis of cell surface LEPR expression with flow cytometry

48 h post-transfection, cells were detached using 5 mM EDTA solved in PBS, centrifuged at 200 xg and resuspended in PBS containing 0.5% BSA. Afterwards, 500,000 cells were incubated with 10 μl of APC-conjugated anti-human LEPR antibody for 30 min at room temperature in the dark. Then, cells were washed 3 times with staining buffer (PBS with 0.5% BSA and 0.1% Sodium Acide) and centrifuged through a cell sieve. Flow cytometry analysis was performed immediately with a BD LSR II (Core Unit fluorescence-technologies, Medical Faculty, University of Leipzig), following staining of dead cells with 1 μg/ml propidium iodide (PI). Forward/side scatter light was excited with a 488 nm laser and fluorescence signals of PI, YFP and APC were detected using the appropriate excitation laser wave lengths (355 nm, 488 nm, 633 nm) and emission filter sets (680 long pass, 515–545 nm, 656–676 nm). After gating for single cells (through the forward-scatter-height/forward-scatter- area-blot) and viable cells (through the propidium iodide/sideward-scatter-area blot), only cells expressing the eYFP-tagged LEPR were selected for further analyses, whereas cells without transfection served as negative controls.

2.8. Bioluminescence resonance energy transfer (BRET)-assay to investigate LEPR dimerization

24 h post-transfection, HEK293 cells were re-seeded into poly-D-lysine-coated white and black μclear-bottom 96-well plates (Greiner Bio-One, Kremsmünster, Austria) using phenol red-free DMEM/Ham's F12 (Gibco), supplemented with 15% FCS at 150,000 cells/well. BRET measurements were performed the next day at 37 °C using a plate reader (Tecan Spark; Tecan Group, Männedorf, Schweiz). To determine the BRET acceptor expression, the YFP fluorescence intensity was assessed in black 96-well plates (excitation/emission filter: 482(20)/544(25) nm). For measuring the BRET ratio, the medium was replaced with 100 μL BRET buffer (25 mM HEPES in Hanks' balanced salt solution adjusted to pH 7.4). Prior to addition of the luciferase substrate, the cells were incubated for 10 min with 0.01% saponin. The Nluc substrate furimazine was added at 2.1 μM 5 min before the BRET measurement. BRET fluorescence at 505-590 nm and luminescence at 410-430 nm were assessed with a delay of 1000 ms. The BRET acceptor/donor ratio (x-axis) was determined from the YFP fluorescence by direct excitation (black 96-well-plates) divided by the measured luminescence (white 96-well-plates). The netBRET value was determined by subtracting the BRET signals of cells transfected with only BRET donor. Nonlinear regression analysis was performed with GraphPad Prism (GraphPad Software, version 5.03, San Diego, CA). BRET ratios are presented in milliBRET units (1 milliBRET unit equals the value of the netBRET multiplied by 1000).

2.9. LEPR binding studies

Supernatants of HEK293 cells transfected with plasmids encoding eYFP-tagged LEPR fusion proteins or respective controls were collected 48 h post-transfection and concentrated 10-fold by lyophilization and resuspension in PBS. Leptin binding to the wild-type (WT) or mutated soluble form of the LEPR was assessed by ligand-immunofunctional assays as previously described [23]. For comparison, concentrations of total soluble LEPR in supernatants were quantified in the same samples by ELISA (Human LeptinR ELISA Kit, #DOBR00, R&D Systems) according to the manufacturer.

2.10. Biocomputational modelling of LEPR variants

RaptorX [24] is a computational protein structure prediction webserver based on the threading technique [25]. The RaptorX webservice was used to predict structures for the individual domains of the extracellular portion of *LEPR* where the mutations of interest are located. These models of the individual domains were further analyzed with Rosetta [26]. To estimate the structural impact of the p.Tyr411del variant on the given structure, we deleted the loop downstream of position 411 (residues 412–419) and used Rosetta's Generalized Kinematic Loop Closure algorithm [27] to create a new loop shortened by one residue. The effect on the thermodynamic stability was estimated with the Rosetta total energy.

The mutation of variant p.Arg612His was introduced in the CRHII domain using Rosetta. The structure of the leptin protein was obtained from the Protein Data Bank (PDB-ID 1AX8). It was docked [28] to the binding pocket of the mutated model as well as the wild type model for comparison. The best model was selected by tightest interaction energy from a pool of 300 models. The side chain packing of variants was assessed by averaging ten Rosetta packstat runs, which estimates remaining voids within a protein core. The electrostatic surface potential was calculated and visualized with Pymol.

3. Results

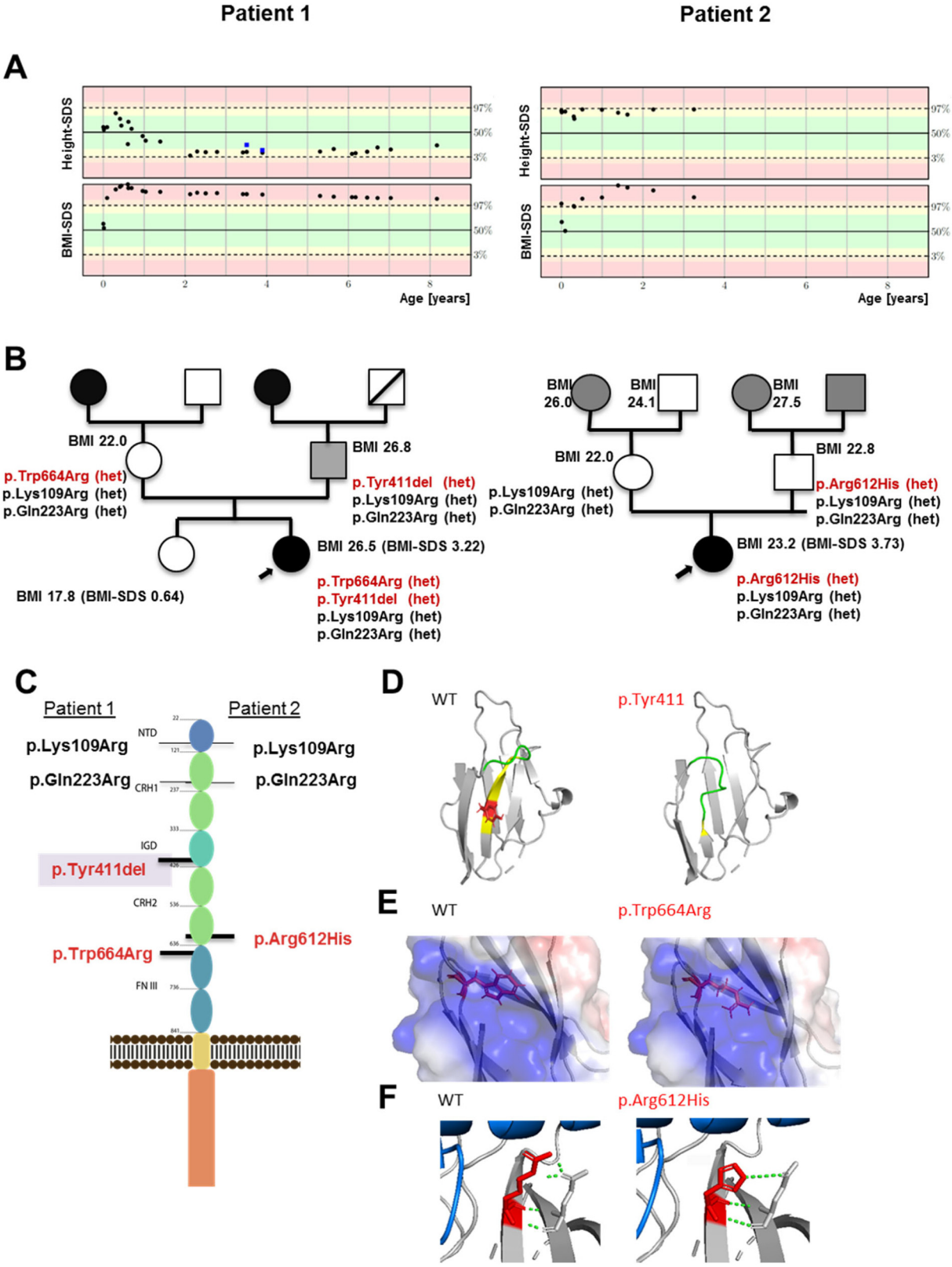
3.1. Clinical characteristics of patients

Patient 1: We followed a now 9 year old girl since the age of 2.5 years, when she first presented at our institution for assessment of severe obesity (BMI 29.7 kg/m^2, 5.15 SDS) and short stature (height of 85.5 cm, −1.52 SDS). She had been born eutrophic (3560 g birth weight, 52 cm birth length at 40 gestational weeks) from non-obese healthy parents of Caucasian origin. Parents reported insatiable hunger and constant crying for food from the age of 1 month onwards resulting in excessive weight gain (body weight 11 kg at 3 months of age) and consecutively upward crossing of BMI percentiles in the first months of life. BMI-SDS remained severely elevated despite tight eating restrictions by the parents (Fig. 1).

She had mild developmental delay of gross motoric function (assessed by the “Münchner funktionelle Entwicklungsdiagnostik”), likely to be secondary due to her body dimensions; otherwise clinical exam was unremarkable including cortisol levels and MRI brain scan. Of note, no increased frequency of infections was reported. Due to suspected hypothyroidism (thyrotropin 4.42 mU/l, free thyroxine 8.62 pmol/l), she had been treated with levothyroxine. As complications of obesity she developed genua valga, moderate obstructive sleep apnea (leading to an adenotonsillectomy) and metabolically presented with dyslipidemia (elevated total cholesterol and triglycerides) and hyperinsulinemia (peak insulin 1616 pmol/l in an oral glucose tolerance test). Circulating leptin levels of 24.9 ng/ml were within the reference

range adjusted to gender, pubertal stage and BMI (12.8–61.4 ng/mL at Tanner stage 1, BMI 26.5 kg/m^2).

Patient 2 was referred to our institution at the age of 1.6 years for evaluation of severe obesity (BMI of 23.2 kg/m^2, 3.73 SDS). She was born large for gestational age at 41 + 3 gestational weeks (4560 g birth weight, 55 cm birth length) to a Caucasian mother and Turkish father, both non-obese. Her parents reported hyperphagia, describing her daughter to be often thirsty, especially at night. At the age of six months,



her BMI-SDS crossed the 97th percentile and continued to increase ever since, while her height paralleled stably the 97th percentile (Fig. 1). There were no perinatal complications (including absence of maternal gestational diabetes), except for prolonged hyperbilirubinemia during her first 12 weeks of life. Clinical exam was unremarkable with normal psychological, cognitive and motoric development and no increased susceptibility for infections. Circulating leptin levels (10.8 ng/ml) were within the reference range adjusted to gender, pubertal stage and BMI (6.03–29.0 ng/ml at Tanner stage 1, BMI 23.2 kg/m^2).

For both patients, guardians gave written consent for genetic analyses and also permission for scientific workup and publication.

3.2. Identification of variants in the LEPR gene and biocomputational modelling of potential functional relevance

Based on the clinical presentation we suspected monogenic obesity and initiated genetic analyses by whole exome trio sequencing. In patient 1 we identified the novel, yet undescribed p.Tyr411del variant in compound heterozygosity with p.Trp664Arg in the *LEPR* gene (Fig. 1C). Based on GnomAD, this variant is absent in over 125,000 individuals. The position tyrosine at position 411 is conserved across species and the variant is absent in the general population. The p.Tyr411del variant is within the immunoglobulin-like domain (IGD) of the receptor binding region and important for leptin binding and receptor activation [8]. Computational structural modelling of the p.Tyr411del variant suggests disruption of the IGD β-sandwich tertiary structure (Fig. 1D). The predicted thermodynamic stability is worsened significantly by 120 energy units in Rosetta. The effect of this mutation is, therefore, predicted to be functionally deleterious.

The position tryptophan 664 is also highly conserved and the variant is extremely rare in the general population (minor allele frequency MAF = 0.000028, i.e. 7 heterozygous persons out of 124,789 persons, based on GnomAD, state of 01.04.2020). It is located in the fibronectin type III domain (FNIII), which is involved in leptin receptor activation [29]. The computational model of the p.Trp664Arg showed reduced side chain packing density of approximately 12% (Fig. 1E). Combined with the substantially different amino acid properties of Trp (hydrophobic) and Arg (hydrophilic), we suspect impaired formation of the FNIII domain.

A compound-heterozygote state of the two variants might thus significantly impact leptin binding as well as signal transduction.

Patient 2 was heterozygous for the missense variant (p.Arg612His) in the *LEPR* gene (Fig. 1C). The position arginine 612 is highly conserved, however, this variant is more frequent in the general population (MAF of 0.00035, i.e. 100 heterozygous persons out of 141,423 persons, no homozygous carriers). Since the position is part of the binding region, the p.Arg612His variant is predicted to reduce, but not completely abolish, the binding affinity for leptin (Fig. 1F). This mutation compromises a hydrogen bonding network in the interface of leptin and its receptor, as has also been suggested by a previous study [30].

In addition, both patients carry two frequent polymorphisms (p.Lys109Arg (MAF = 0.2959), p.Gln223Arg (MAF = 0.5089)) without an obvious structure-function relationship regarding the leptin/LEPR signaling axis [31,32]. No other genetic variants with potential relevance for the phenotype have been identified in exome sequencing.

3.3. Effect of LEPR variants on signal transduction

We tested the effect of the identified *LEPR* variants on LEPR signaling by analysing the leptin-dependent phosphorylation of STAT3, an important downstream effector of LEPR, in transiently transfected HEK293 cells expressing either wild-type or variant LEPR. We detected an almost complete loss of function for p.Tyr411del and p.Trp664Arg, while cells expressing the p.Arg612His variant showed reduced but retained STAT3 phosphorylation (Fig. 2A).

3.4. Effect of LEPR variants on cell surface expression and leptin binding

To analyse the potential cause for the compromised pSTAT3 response after leptin stimulation, we investigated cell membrane localization of the *LEPR* variants C-terminally fused to eYFP by fluorescence microscopy and FACS analyses.

For the wild-type construct we detected a distinct portion of cell membrane-residing LEPR in addition to vesicular YFP fluorescence. For p.Tyr411del and p.Arg612His we also detected cell membrane localization, but to a reduced extent, while p.Trp664Arg mutant was completely trapped intracellularly and thus, absent from the cellular membrane (Fig. 2B).

To confirm and quantify the microscopic studies by an alternative approach, we performed flow cytometry of transfected HEK293 cells using an antibody targeting the extracellular LEPR domain. The membrane localization of LEPR variants p.Tyr411del and p.Arg612His was reduced compared to wild-type LEPR, while the variant p.Trp664Arg seemed to be absent from the membrane as indicated by a signal intensity comparable to control cells. Equivalent levels of total LEPR expression levels were verified by similar intensities of the YFP-fluorescence signals (Fig. 2C).

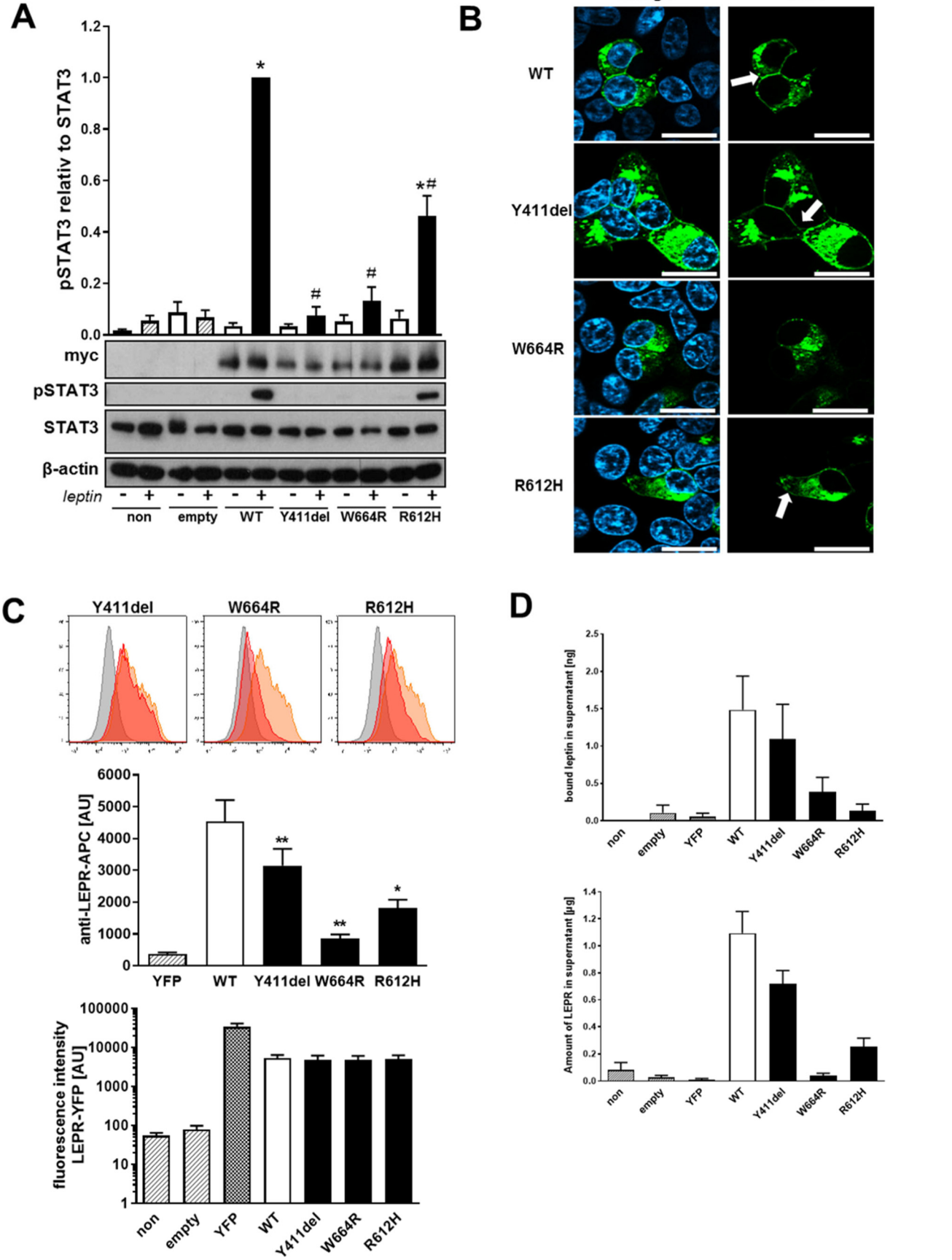
We finally assessed leptin binding to soluble LEPR variants in the supernatant of transfected HEK293cells. We found a similar pattern of reduced leptin binding to variants p.Tyr411del and p.Arg612His and almost complete loss of binding to p.Trp664Arg variant that were, however, due to reduced amount of soluble LEPR probably because of reduced cell surface expression (Fig. 2D).

3.5. Capability for dimerization and signaling of heterozygous states

Considering that the signaling capacity of LEPR is dependent on dimerization at the cell surface and that the patients were (compound) heterozygous, we further assessed the capacity for dimerization and signaling in a mimicked heterozygous state.

We investigated the capability for dimerization by saturation bioluminescence resonance energy transfer (BRET) assays in transiently transfected HEK293. The wild-type LEPR receptor was C-terminally fused to the nanoluciferase Nluc, the BRET donor; eYFP-tagged

Fig. 1. Clinical characterization of index patients. (A) Values of height-SDS (equivalent to percentiles) (upper panel) and BMI-SDS (lower panel) are depicted from birth to current age as dots for both patients. Dotted lines at 3rd and 97th percentile indicate normal age- and sex specific ranges. Both patients crossed the age- and sex-specific 97th BMI-percentile within their first year of life. (B) Analysis of pedigrees illustrates, that no severe obesity occurred among parents and siblings of both index patients (arrows). Squares represent male family members and circles female family members; open symbols represent unaffected family members, gray symbols family members with overweight and black symbols family members with obesity. Next to each symbol BMI is given in kg/m^2 for parents and additionally BMI-SDS in brackets. Genotypes are given for patients and parents (het = heterozygous). (C) Schematic overview of localization of genetic variants in LEPR and predicted structure-function relationship. (D) Computational modelling of p.Tyr411del in the IGD of LEPR showing tyrosine 411 (red) residues in a beta sheet (yellow) which is connected to its antiparallel strand via a loop (green). Corresponding computational modelling of the deletion variant shows a disruption of the tertiary structure and a shortened beta sheet (yellow) compared to the wild type model. (E) The model of the FNIII domain (gray) with residue 664 (red). The side chain of the hydrophobic wild type protein is part of the hydrophobic core. The side chain of variant p.Trp664Arg has a different shape and polarity which disrupts the ability to fold and reduces the predicted packing density. As a result, the electrostatic surface potential is altered and appears more positively (fraction of blue surface) compared to the wildtype potentially important for binding. (F) The model of the CRH II region of LEPR (gray) docked to its ligand (blue). The variant p.Arg612His exhibits less side chain polar interactions (green) with side chains of neighboring residues compared to the wild-type, ultimately reducing the binding affinity to the ligand. Abbreviations: N-terminal domain (NTD); cytokine receptor homology (CRH); immunoglobulin like domain (IGD); fibronectin III domain (FNIII). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)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YFP
WT
Y411del
W664R
R612H
fluorescence intensity LEPR-YFP [AU]
D
bound leptin in supernatant [ng]
Amount of LEPR in supernatant [µg]

LEPR wild-type and variants were used as BRET acceptors. Homodimerization of wild-type LEPR was used as positive control (Fig. 3A). To validate signal specificity, the angiotensin II receptor type 1 (AT_1R) was used as negative control as no specific interaction between these two membrane proteins is expected (Fig. 3B). LEPR homodimerization showed signal saturation with increasing amounts of acceptor expression, indicating a constitutive interaction in the absence of ligand. With pre-incubation of saponin, which permeabilises cellular membranes and has been used in BRET assays to characterize LEPR dimerization [27], the BRET ratio increased for this interaction. In contrast, the LEPR/AT_1R BRET does not reach saturation and is insensitive to saponin incubation, displaying weak BRET signals owing to a bystander effect. A netBRET signal saturation similar to the wild-type homodimerization was observable for both LEPR mutants Tyr411del (Fig. 3C) and p.Arg612His (Fig. 3D) indicating retained protein-protein interaction capability necessary for downstream STAT3 phosphorylation.

Finally, we addressed the functional relevance of the (compound) heterozygosity in our patients, which we mimicked by simultaneously transfecting HEK293 cells with the LEPR variants. We found retained signaling capacity if the wild-type LEPR was co-transfected with each of the variants p.Tyr411del, p.Trp664Arg or p.Arg612His (Fig. 3E). However, in cells expressing [p.Tyr411del + p.Trp664Arg] in combination, leptin induced STAT3 phosphorylation was absent.

Hence, the newly identified p.Tyr411del variant in the LEPR leads to complete loss of function in the homozygous and compound heterozygous state [p.Tyr411del + p.Trp664Arg]. This genotype is, thus, highly likely to explain the phenotype of patient 1. Even with partly or completely compromised LEPR signaling capacity due to genetic variants in the homozygous state, heterozygosity with wild-type LEPR is possible showing retained LEPR signaling capacity, as seen in patient 2.

4. Discussion

Driven by the clinical presentation in two young girls, we suspected a monogenic cause for obesity and identified a novel, along with known, genetic variants in the *LEPR* gene in these two patients. Nevertheless, it is crucial to demonstrate the functional relevance of the variants to claim them to be causative for the phenotype, particularly as they occurred in the (compound) heterozygous state.

This is particularly relevant for the newly identified variant p.Tyr411del. It is located in the IGD suggestive to impair leptin binding to the receptor. The other variant identified in patient 1, p.Trp664Arg, is presented in the membrane-proximal fibronectin type III (FNIII) domain. While it was not clear, whether both domains are involved in leptin-binding [10,33], IGD and FNIII are essential for activation of LEPR since lack of the domains leads to complete loss of signaling [10,33].

Previous attempts to characterize the binding pocket of leptin at the leptin receptor indicated that p.Tyr411 is critical for the ligand-receptor-interaction. Mutation of Tyr to Ala at this position in the receptor decreases agonist-mediated signaling, whereas a double alanine replacement of p.Tyr411 and p.Phe500 abolishes it completely [34]. Our finding that p.Tyr411del reaches the cell membrane, but shows almost complete loss of second messenger generation is in accordance with these findings.

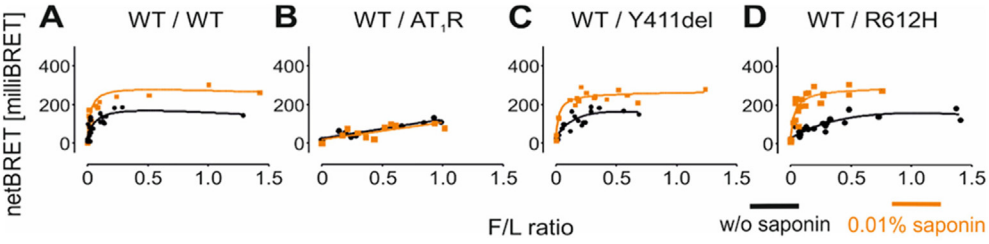
The variant p.Trp664Arg has been previously described as a homozygous mutation of the *LEPR* gene in an 8-year old daughter of first-degree cousins of Norwegian origin [7]. It causes severe loss of function as shown by blunted phosphorylation of STAT3 after leptin stimulation [7,16] or activation of a STAT3-dependent luciferase reporter construct [16] and also further upstream absent activation of extracellular-signal regulated kinases 1/2 (ERK1/2) [16].

The variant p.Arg612His identified in our second patient was first described in compound heterozygous expression along with a 1-bp deletion in codon 15 in a 5-year old girl of a non-consanguineous family from the United Kingdom [7]. Similar to p.Trp664Arg, p.Arg612His was associated with reduced leptin-induced phosphorylation of STAT3 as well as of ERK1/2 [7,16]. Hence, our findings of absent and significantly reduced signaling capacity for p.Trp664Arg and p.Arg612His variants, respectively, is consistent with previous findings.

This abrogation of leptin-mediated induction of the critical signaling cascades due to the variants in LEPR can be secondary to several upstream defects affecting cell membrane localization, dimerization of the receptor, or leptin binding.

The protein hormone leptin activates its cognate receptor by binding to extracellularly exposed domains. We determined membrane localization of the mutant receptors compared to the wild-type LEPR by fluorescence microscopy and flow cytometry. For both setups, the LEPR constructs were fused to an autofluorescent eYFP moiety at the distal C-terminus, allowing for the tracking of the protein in live cells, without interfering with protein synthesis, oligomerization and ligand binding [10,35]. By fluorescence microscopy we saw some intracellular retention for wild-type as well as for the mutant LEPR constructs, which has similarly been demonstrated previously with only 5–25% of total wild-type LEPR reaching the cell surface [36]. The variants LEPR p.Tyr441del and LEPR p.Arg612His also localize to the plasma membrane, although apparently to a lesser extent. However, for the LEPR p.Trp664Arg construct, no membrane expression was detectable by fluorescence microscopy, indicating a complete intracellular retention of the protein. Slightly reduced, although not completely abrogated cell surface expression of LEPR was previously shown for the p.Trp664Arg (to 82%) and p.Arg612His (84%) variants [16]. To further confirm the localization of the LEPR mutants, we conducted flow cytometry analysis by immunostaining of LEPR expressing HEK293 cells confirming significantly decreased cell membrane localization for p.Tyr411del and p.

Fig. 2. Effect of genetic variants on LEPR function. (A) Blunted leptin induced STAT3 phosphorylation (pSTAT3) in HEK293 cells expressing p.Tyr411del or p.Trp664Arg LEPR variants and reduced signal with p.Arg612His compared to wild-type receptor. Statistical analysis was performed using one-way ANOVA and Dunnett's Multiple Comparison Test. $^{*}p < 0.05$ compared to leptin-stimulated HEK293 cells expressing empty vector; $\#p < 0.05$ compared to leptin-stimulated cells expressing wild-type LEPR. (B) Membrane localization of LEPR variants in live HEK293 cells transiently transfected with fluorescent wild-type LEPR-eYFP, LEPR-p.Tyr411del-eYFP, LEPR-p.Arg612His-eYFP or LEPR-p.Trp664Arg-eYFP. All LEPR variants show high intracellular protein retention (accumulation of green vesicular structures). Membrane localization was distinct for wild-type LEPR, and less pronounced for the p.Tyr411del and p.Arg612His mutants (indicated by white arrows, eYFP-channel, right). The mutant LEPR-p.Trp664Arg-eYFP showed no observable signal at the cell membrane. Cell nuclei staining with Hoechst33342 (blue, merge picture, left); scale bar: 20 μm. (C) Cell surface expression of LEPR variants by flow cytometry analysis in HEK293 cells transfected with LEPR-eYFP fusion proteins as in (B) and LEPR staining with allophycocyanin (APC)-conjugated antibody targeting the extracellular domain (anti-LEPR-APC). Fluorescence intensities of the LEPR variants (red area) ranged between the intensity of WT receptor (orange area) and non-transfected cells (gray area) (upper panel). LEPR variants showed a reduced mean APC fluorescence intensity and thus a reduced cell surface expression (compared to WT LEPR), particularly the p.Trp664Arg mutant (fluorescence levels similar to control cells (YFP) (middle panel)). The mean YFP-fluorescence intensities (control for protein expression of the LEPR constructs) did not show significant differences indicating similar protein expression levels. Means with SD of 4 independent experiments are depicted. Statistical significance was determined using one-way ANOVA with a Tukey post-hoc-test, $^{**}p < 0.01$, $^{***}p < 0.001$. (D) Leptin binding (upper panel) and amount of soluble LEPR (lower panel) were assessed in supernatants of HEK293 cells transiently transfected with the LEPR-eYFP fusion constructs. Not transfected cells (non) and cells transfected with empty vector (empty) served as controls. The LEPR variants p.Trp664Arg and p.Arg612His showed significantly reduced leptin binding, while there was no significant effect for the p.Tyr411del variant. The amounts of the soluble LEPR variants p.Trp664Arg and p.Arg612His were strongly reduced in the supernatant, which was less pronounced for the p.Tyr411del variant. Data are presented as mean with SEM of 7 independent experiments. Statistical significance was determined using one-way ANOVA with a Tukey post-hoc-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Abbreviations: wild-type LEPR-eYFP (WT), LEPR-p.Tyr411del-eYFP (Y411del), LEPR-p.Arg612His-eYFP (R612H), LEPR-p.Trp664Arg-eYFP (W664R). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



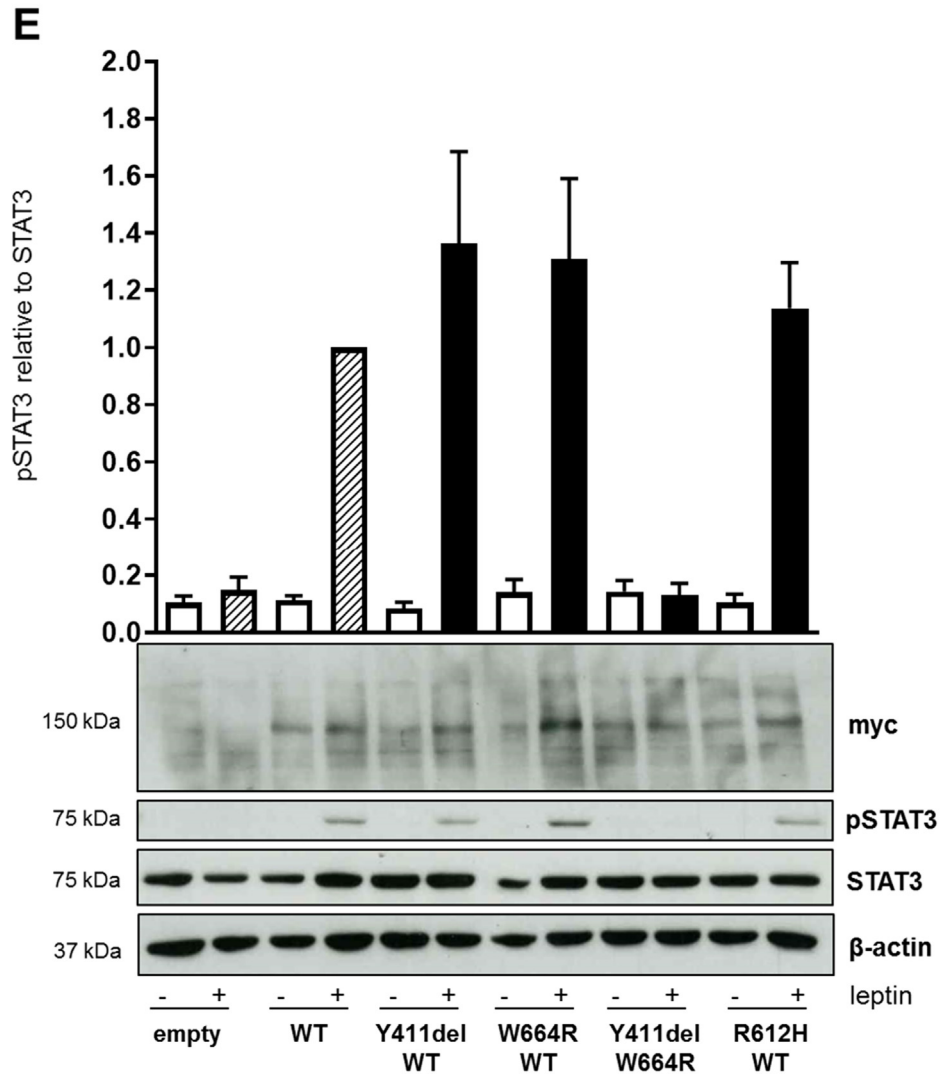


Fig. 3. LEPR function with constructs mimicking heterozygosity. (A) LEPR homo-/heterodimerization investigated by saturation BRET assays in HEK293 cells transiently transfected with wild-type LEPR, C-terminally fused to Nluc (BRET donor, WT) and either with LEPR-eYFP (A), AT_1R-eYFP (B), LEPR p.Tyr411del-eYFP (C) or LEPR p.Arg612His-eYFP (D) as BRET acceptors. Homodimerization of LEPR-Nluc/LEPR-eYFP was used as positive control. BRET signals for this interaction reach saturation in presence (black) and absence of saponin (orange). Heterodimerization of LEPR-Nluc/AT_1R-eYFP, used as negative control, does not reach saturation (linear correlation). Heterodimerization with wild-type LEPR was observed for both LEPR p.Tyr411del and LEPR p.Arg612His, similar to wild-type LEPR. For transfection, donor DNA was kept constant whereas acceptor DNA was added in increasing amounts (total DNA was kept constant). Saturation data are shown for n ≥ 2 independent experiments, performed in at least quadruplicates. One-site total binding (GraphPad Prism5) was used for fitting saturation BRET data. BRET ratios are presented in milliBRET (1 milliBRET unit equals netBRET value multiplied by 1000). (E) Effect of combined expression of distinct LEPR constructs on leptin stimulated STAT3 phosphorylation achieved by co-transfection of p.Tyr411del, p.Trp664Arg, p.Arg612His and wild-type (WT) as indicated. LEPR expression was confirmed by detection of the receptor (LEPR) or the myc-tag (myc). Detection of β-actin was used as loading control. Not transfected HEK293 cells (non) and cells transfected with empty pcDNA3 vectors (empty) served as controls. Densitometric analysis of each lane was performed in five independent Western Blots. Data are represented as mean (SEM). Statistical analysis was performed using one-way ANOVA and Dunnett's Multiple Comparison Test. $^*p < 0.05$ compared to leptin-stimulated HEK293 expressing empty vector; $\#p < 0.05$ compared to leptin-stimulated cells expressing wild-type LEPR.

Arg612His and particularly for p.Trp664Arg. Thus, the absence from the membrane explains the abolished leptin-mediated STAT3 phosphorylation for the p.Trp664Arg variant, as this LEPR variant is not accessible to extracellular agonist interaction. Thus, the reduced leptin binding for p.Tyr411del and p.Arg612His is at least in part due to impaired expression of the receptor mutants at the cell membrane.

Furthermore, dimerization of the LEPR at the cell membrane is crucial for the subsequent downstream signaling [10]. When trying to explain the genotype-phenotype association for our two patients, the (compound) heterozygous state needs to be considered. Therefore, we assessed the capability for dimerization of the variants with the wild-type partner applying a BRET-based setup. The LEPR wild-type served

as BRET donor and the YFP-tagged LEPR proteins acted as BRET acceptors. We first verified a constitutive dimerization of the long LEPR isoform as described previously [37]. Both tested variants of LEPR that are expressed on the cell surface did show a saturable BRET effect comparable to the wild-type LEPR homodimer, hence indicating the dimerization capacity was retained for p.Tyr441del and for p.Arg612His.

Finally, we mimicked the (compound) heterozygous state of our patients by simultaneous expression of the variants with wild-type and/or compound heterozygous partner with STAT3 phosphorylation as readout. Both variants in the LEPR found in the first patient, the novel p.Tyr441del as well as the p.Trp664Arg, showed inducible STAT3 phosphorylation in combination with wild-type receptor expression, even though both had blunted response when expressed homozygously. However, the combination of both variants without expression of wild-type receptor led to complete loss of signaling, hence further underlining that the combination of these two variants is deleterious for LEPR function and hence suggestive for being causative for the phenotype.

With this functional evidence and considering that a defect in LEPR function in the hypothalamus could be overcome by treatment with MC4R agonists that act down-stream of LEPR [18] this patient qualified for treatment with MC4R agonist setmelanotide within the frame of a clinical trial (EudraCT Number 2017–002005-36). Being enrolled at the age of 8 years, the girl lost 8 kg of body weight over a 1-year treatment period and dual-energy X-ray absorptiometry indicated preferential loss of fat mass in favor of muscle mass. The BMI decreased from 30.8 to 22.4 kg/m^2, hence from the range of extreme obesity to the range of overweight (1.74 SDS). Waist and hip circumference were 10 and 7 cm lower compared to pre-treatment, respectively. With 9 years of age, the patient developed first pubertal signs, mainly pubarche. According to her parents, she still experienced bouts of insatiable appetite and very conscious control of food intake by her parents and herself was still mandatory. To what extend this remaining hyperphagic drive was due to fixed behavioral patterns as opposed to neuroendocrine loss of control cannot be disentangled and accompanying psychological counselling should be considered for patients also under pharmacologic therapy. Furthermore, she experienced tanning of the skin and darkening of naevi as known side effects.

On the contrary, signaling capacity of the variant p.Arg612His found in patient 2 was only partially reduced in homozygous and well-preserved in combined expression with wild-type LEPR. Hence, our functional in vitro results did not imply that heterozygosity for p.Arg612His in LEPR was sufficient to explain the phenotype in this patient. Furthermore, the girl's father being heterozygous for the same *LEPR* variant did not suffer from hyperphagia and obesity (Fig. 1B) and this was similarly reported previously for heterozygous patients [7]. In line with those findings, current publications suggest that the classical "monogenic obesity phenotype" of early-onset severe obesity with hyperphagia is very unlikely to be caused by a heterozygous *LEPR* mutation alone [8,38]. For those reasons, patient 2 was not enrolled in a treatment trial with setmelanotide. Nevertheless, some milder effect of functionally relevant *LEPR* variants even in a heterozygous state cannot entirely be excluded. Intermediate phenotypes have been described for heterozygous *LEPR* variants in rodent models [39] and human [40]. Furthermore, heterozygous subjects have a higher body fat content than those with two wild-type LEPR alleles [7]. As a result, clinical trials on treatment response to setmelanotide are currently expanded to heterozygous genotypes [41,42]. Finally, there might still be the chance of a second hit in the index patient, although we were not able to detect any further mutations in commonly obesity-associated genes by whole exome sequencing data.

Of note, both patients showed serum leptin concentrations within the expected gender, pubertal stage and BMI-adjusted range, indicating that hyperleptinemia is not a suitable marker to detect leptin resistance due to *LEPR* mutations. Only one family with a *LEPR* mutation within the transmembrane region has been described so far with extensively elevated leptin concentrations, which were due to the high percentage of leptin bound by elevated amounts of soluble LEPR [43]. In line with other studies, *LEPR* mutations not affecting the transmembrane region, as found in here, do not lead to elevated serum leptin concentrations [7].

The strength of this study is its translational approach with a comprehensive experimental workup providing evidence for the relevance of the identified variants in patient 1 on both a cellular and clinical level. This directed the treatment decision and was proven to be successful by treatment response. A limitation remains as to what extent our experimental system of cotransfection of wild-type and variant LEPR reflects the biological situation in vivo and hence some uncertainty on the etiology for the phenotype of patient 2 remains.

5. Conclusions

In summary, we identified the novel p.Tyr411del variant in the LEPR in a young girl with severe early onset obesity leading to complete loss of function in the homozygous and compound heterozygous state [p.Tyr411del + p.Trp664Arg]. This genotype is, thus, highly likely to explain the phenotype of our patient. Even with partly or completely compromised LEPR signaling capacity due to genetic variants in the homozygous state, retained LEPR signaling capacity is possible in heterozygous states.

With the current advents of advanced genetic testing and new treatment options for monogenic (hypothalamic) obesity, patients with suggestive phenotypes should be subjected to genetic testing, and suitability of patients for pharmacologic treatment should then be based on experimentally proven functional relevance of the identified variants and/or results from clinical trials.

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CRediT authorship contribution statement

Franziska Voigtmann: methodology, formal analysis, investigation, writing – original draft, writing – review & editing, visualization; Philipp Wolf: methodology, formal analysis, investigation, writing – original draft, writing – review & editing, visualization; Kathrin Landgraf: methodology, investigation, writing – original draft, writing – review & editing, visualization, supervision; Samuel Schmitz: investigation, visualization, writing – review & editing; Robert Stein: methodology, investigation, writing – original draft, writing – review & editing; Jürgen Kratzsch: methodology, investigation, writing – review & editing; Rami Abou Jamra: investigation; writing – review & editing; Jens Meiler: methodology, supervision, writing – review & editing; Annette G. Beck-Sickinger: Writing – review & editing, supervision, funding acquisition; Wieland Kiess: investigation; writing – review & editing, funding acquisition; Antje Körner: conceptualization, investigation, writing original + review & editing, visualization, supervision, project administration, funding acquisition.

Declaration of competing interest

No conflict of interest.

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