



HAL
open science

Loss of human LGR4/GPR48 causes a novel syndromic form of severe neonatal saltwasting due to disrupted WNT signaling altering adrenal zonation

Cécily Lucas, Kay-Sara Sauter, Michael Steigert, Delphine Mallet, James Wilmoth, Julie Olabe, Ingrid Plotton, Yves Morel, Daniel Aeberli, Franca Wagner, et al.

► To cite this version:

Cécily Lucas, Kay-Sara Sauter, Michael Steigert, Delphine Mallet, James Wilmoth, et al.. Loss of human LGR4/GPR48 causes a novel syndromic form of severe neonatal saltwasting due to disrupted WNT signaling altering adrenal zonation. *Journal of Clinical Investigation*, In press. hal-03868988

HAL Id: hal-03868988

<https://cnrs.hal.science/hal-03868988>

Submitted on 24 Nov 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Loss of human LGR4/GPR48 causes a novel syndromic form of severe neonatal salt-wasting due to disrupted WNT signaling altering adrenal zonation

Cécily Lucas^{1,2,3*}PhD, Kay-Sara Sauter^{4,5*}MSc, Michael Steigert^{6*}MD, Delphine Mallet^{1,7}PhD, James Wilmouth³MS, Julie Olabe³MD, Ingrid Plotton^{1,2,7}PhD, Yves Morel^{1,2,7}PhD, Daniel Aeberli⁸MD, Franca Wagner⁹MD, Hans Clevers¹⁰PhD, Amit V. Pandey^{4,5}PhD, Pierre Val^{3#}PhD, Florence Roucher-Boulez^{1,2,3#}PharmD, PhD, Christa E. Flück^{4,5#} MD

* shared first/ # last authors for equal contribution

¹ Laboratoire de Biochimie et Biologie Moléculaire, UM Pathologies Endocriniennes, Groupement Hospitalier Est, Hospices Civils de Lyon, Bron, France

² Univ Lyon, Université Claude Bernard Lyon 1, Lyon, France

³ Université Clermont Auvergne, CNRS, Inserm, GReD, F-63000 Clermont-Ferrand, France

⁴ Division of Pediatric Endocrinology, Diabetology and Metabolism, Department of Pediatrics, Inselspital, Bern University Hospital, University of Bern, 3010 Bern, Switzerland

⁵ Department of Biomedical Research, University of Bern, 3010 Bern, Switzerland

⁶ Department of Pediatrics, Cantonal Hospital Graubunden, 7000 Chur, Switzerland

⁷ Centre de Référence Maladies Rares du Développement Génital : du Fœtus à l'Adulte, Filière Maladies Rares Endocriniennes, Bron, France

⁸ Department of Rheumatology and Clinical Immunology/Allergology, Inselspital, Bern University Hospital, University of Bern, 3010 Bern, Switzerland

⁹ University Institute of Diagnostic and Interventional Neuroradiology, Bern University Hospital, Inselspital, University of Bern, 3010 Bern, Switzerland

¹⁰ Oncode Institute, Hubrecht Institute, Royal Netherlands Academy of Arts and Sciences and University Medical Centre Utrecht, 3584 CT Utrecht, The Netherlands

Corresponding Author:

Christa E. Flück

University Children's Hospital Bern

Freiburgstrasse 65 / C845

3010 Bern

Switzerland

Phone: +41 31 6320499

christa.flueck@dbmr.unibe.ch

ABSTRACT

BACKGROUND

Isolated mineralocorticoid deficiency causing potentially life-threatening salt-wasting has been associated with gene variants of aldosterone biosynthesis or resistance, but in some patients no variant is found. WNT/ β -catenin signaling is crucial for differentiation and maintenance of the aldosterone producing adrenal zona glomerulosa (zG).

METHODS

Whole exome sequencing in a girl presenting with adrenal crisis and born into a consanguineous family with multiple perinatal deaths identified a splice variant in the WNT signaling regulator *LGR4*. The variant was characterized by structural analysis, protein expression, binding studies, and confocal microscopy. *LGR4* inactivation was also analyzed by adrenal cortex specific ablation of *Lgr4* in mice.

FINDINGS

The proband presented with failure to thrive, salt-wasting crises associated with isolated hypoaldosteronism, nail anomalies, short stature, and deafness. We identified a homozygous splice variant in the R-SPONDIN receptor *LGR4* gene (c.618-1G>C). The resulting transcripts affected protein function and stability and resulted in loss of Wnt/ β -catenin signaling. Inactivation of *Lgr4* within the adrenal cortex in the mouse model caused decreased WNT signaling, aberrant zonation and reduced aldosterone production.

INTERPRETATION

LGR4 mutations establish a direct link between *LGR4* inactivation and decreased canonical WNT signaling with abnormal zG differentiation and endocrine function. Thus genetic variants in WNT signaling and its regulators should systematically be considered in familial hyperreninemic hypoaldosteronism.

FUNDING

Ligue Nationale Contre le Cancer 2021-2025 and ANR-21-CE14-0044-ADREMAC to PV. RECORDATI Rare Diseases 2020 of the French Society of Endocrinology and the Young Researchers 2021 grant by the Lyon University Hospital to FRB. SNF grant ID 320030_146127 to CEF.

Key words: mineralocorticoid deficiency, aldosterone synthesis, salt wasting, adrenal zona glomerulosa, WNT/ β -catenin signaling, LGR4/GPR48

RESEARCH IN CONTEXT

Evidence before this study

Human disorders of isolated mineralocorticoid deficiency with elevated renin are either caused by genetic defects of aldosterone synthesis (*CYP11B2* variants) and aldosterone resistance, or remain genetically unsolved. Mineralocorticoids are produced in the zona glomerulosa of the adrenal cortex, which is controlled in its differentiation by WNT signaling. WNT4 and R-SPONDIN3 (RSPO3) are important drivers of WNT activation and zG differentiation, through stabilization of β -catenin, which stimulates expression of CYP11B2 and angiotensin II receptor AGTR1. Mice with *Ctnnb1*, *Wnt4*, or *Rspo3* deficiency have reduced zG differentiation. However, mutations in the WNT signaling pathway in relation with mineralocorticoid deficiency, were not found in patients.

Added value of this study

Here, we show that homozygous human splice variants of *LGR4* (the R-SPONDIN receptor) are associated with potentially lethal neonatal salt wasting in the context of a syndrome with isolated aldosterone deficiency, short stature, deafness, and nail anomalies. We further show that consistent with our patient's phenotype, conditional *Lgr4* knockout in mice leads to aberrant adrenal cortex zonation and reduced aldosterone production. Our study demonstrates the central role of Wnt/ β -catenin signaling for proper adrenal cortex formation and function in humans.

Implications of all the available evidence

Our data suggest that *LGR4* variants and potential variants in other genes involved in the complex network of LGR4-Wnt/ β -catenin signaling should be considered in patients presenting with a salt-wasting crisis at birth, especially when manifesting with other syndromic features. Conversely, patients with known genetic alterations in WNT signalling should be carefully monitored for adrenal functions.

INTRODUCTION

Disorders of isolated mineralocorticoid (MC) deficiency are potentially life-threatening ¹. So far, they have been described in humans with primary defects in aldosterone biosynthesis or with MC resistance due to failure of aldosterone action. Patients mostly manifest in neonatal life with a salt-wasting crisis, e.g. dehydration, vomiting, and failure to thrive, due to high potassium, low sodium, metabolic acidosis, and high renin. The disorder becomes usually less severe with age. In humans, aldosterone is the principal MC produced in the zona glomerulosa (zG) of the adrenal cortex where the *CYP11B2* gene for aldosterone synthesis is expressed ². Aldosterone synthesis regulated by the renin-angiotensin-aldosterone (RAA) feedback loop, controls salt and water homeostasis and blood pressure.

Isolated hypoaldosteronism is mostly associated with autosomal recessive variants of the *CYP11B2* gene, catalyzing aldosterone synthesis. However, a subset of typical cases of aldosterone deficiency, grouped under Familial Hyperreninemic Hypoaldosteronism (FHHA2) remains genetically unsolved ¹. Mutations in genes that regulate aldosterone biosynthesis, downstream of renin, including genes encoding angiotensin (I), the angiotensin-converting enzyme (*ACE*) or the angiotensin II receptor *AGTR1* are associated with arterial hypotension in mice. In humans, these mutations are associated with renal tubular dysgenesis ³⁻⁶. However, they have not been linked with hypoaldosteronism. Other potential candidates comprise genes involved in the development and differentiation of the adrenal cortex.

Adrenal cortex physiology relies on functional zonation, essential for the production of aldosterone by the outer zG and glucocorticoids by the inner zona fasciculata (zF). The cortex undergoes constant cell renewal during postnatal life ⁷. This involves recruitment of subcapsular progenitor cells to zG fate and subsequent conversion to zF identity. This differentiation occurs in a centripetal manner, under the control of the WNT signaling pathway in zG and the PKA pathway in zF ⁸. WNT4 and R-SPONDIN3 (*RSPO3*) are important drivers of WNT activation and zG differentiation, through stabilization of β -catenin, which stimulates expression of *CYP11B2* and

angiotensin II receptor AGTR1⁹. Consequently, mouse models with *Ctnnb1*, *Wnt4*, or *Rspo3* deficiency have reduced zG differentiation^{8,10-12}. Conversely, constitutive WNT pathway activation resulting from activating *CTNNB1* mutations or downregulation of negative WNT regulators is associated with the development of aldosterone-producing adenomas^{13,14}. Despite the central role of canonical WNT signaling in zG differentiation, mutations in this pathway have not been associated with hypoaldosteronism in humans so far. Here, we identified a loss of function splice variant of the R-SPONDIN receptor coding *LGR4* gene (c.618-1G>C) in a girl born into a highly consanguineous family with a history of multiple perinatal deaths. The proband presented with failure to thrive, severe salt-wasting crises associated with isolated hypoaldosteronism, nail anomalies, short stature, and deafness. Our *in silico*, *in vitro* and *in vivo* studies establish a causal link between *LGR4* inactivation, decreased canonical WNT signaling, abnormal zG differentiation and endocrine function. This suggests that anomalies in WNT signaling pathway regulators should systematically be evaluated in familial hyperreninemic hypoaldosteronism.

METHODS

Patients

The index patient was referred at the age of 17 years for adrenal insufficiency, short stature, deafness, developmental delay, and dysplastic nails (Figure 1 and Suppl Figure S1). She was born at term and manifested within days, with failure to thrive and signs of an adrenal salt-wasting crisis. Corticosteroid treatment was successfully installed without further investigations. Parents are first-degree cousins from Syria. The presence of the same phenotype in 3 siblings deceased in the neonatal period, and two cousins (with consanguineous parents) was indicative of an autosomal recessive disorder. Laboratory workup revealed normal cortisol response to ACTH stimulation, but hyperreninemic hypoaldosteronism (Table 1). Thus the diagnosis was revised to isolated mineralocorticoid deficiency and therapy continued with fludrocortisone, while genetic workup was initiated. Additional important findings were short stature, microcephaly, structural

Table 1. Patient characteristics and laboratory findings at initial presentation and during 4 years follow-up

Age	years		17	17 1/3	17 1/3	18	20	21	21
Height	cm			141		142	142	142	
Weight	kg			33.5		36.1	34.7	36.4	
BMI	kg/m ²					17.9	17.2	18.05	
Blood pressure	mmHg			103/74		113/76	127/85	116/80	
Pubertal stage	Tanner			4-5		5	5	5	
Bone age	years GP			16-17		adult			
Hydrocortisone	mg/m ² /d		23.9	24h off treatment	0	0	0	0	
Florinef	ug/d		150	24h off treatment	0	100	100	24h off treatment	
		Normal range basal	basal	basal	ACTH stimulated	basal	basal	basal	ACTH stimulated
Na	mmol/l			136		138	137	142	
K				4.2		4.5	3.8	3.7	
Cl				106		108	106	110	
Creat	umol/l			79		72	69	64	
ACTH	ng/l	7.2-63.3	9.3	47.8		8	6.1	44.3	
Renin	ng/l (*mU/L)	1.7-23.9 (*4.4-46)	38	133		*187	20.8	9.1	
Corticosterone	nmol/l	1.69-63.8						1.08	9.91
Aldosterone	pmol/l	87-662	41	nd		49.2		nd	nd
17OHProg	nmol/l	0.24-6.84	4.2	16.6	20.2	3	1.5	8.26	15.7
Progesterone	nmol/l			20.5	15.6	3		42.1	
Cortisol	nmol/l	133-537		400	564	288.7	205	206	303
DHEA-S	umol/l	1.77-9.99	3.7	4.35	4.55	2.3	2.4	3.79	3.29
DHEA	nmol/l	1.7-38.3		8.2	9			7.93	11.3
Androstendione	nmol/l	1.06-7.72	8.5	13	15.9	3.4	3.3	6.89	7.45
Testosteron	nmol/l	0.31-2.29						1.33	1.54
E2	pmol/l	45-854	151	903			475	1264	
LH	U/l		11.8	17.7			12.2	5.7	
FSH	U/l		4.7	3.2			4.6	1.3	
AMH	pmol/l	7.14-57.1		13.3				22.4	
IGF-1	ng/ml		170	205			207		
IGF-BP3	mg/l		4.26	5.16			3.5		
iPTH	pg/ml	15-65						29.3	
25OHVitD3	nmol/l	50-135						60	
Osteocalcin	ng/ml	11-43						34.3	
b-Cross-Laps	pg/ml	<573						357	
totP1NP	ng/ml	15.1-58.6						84.3	
FGF23	pg/ml	10-50						50.7	

Footnotes: nd, not detected; numbers in bold mark findings outside the normative range

brain anomalies, and mental disability; deafness with functional but without structural anomalies of the cochlea and hearing nerve; low bone mineral density, and small kidneys with cortical microlesions (Suppl Figures S1-4 and Table S1). Pubertal development was late with menarche at 16 years.

Of note, two cousins with the same clinical phenotype (one female and one male) are alive under steroid replacement therapy in Syria but are not available for investigations. A detailed description of the clinical findings is given in the Supplementary Appendix.

Laboratory studies

Studies in humans or on human material were conducted in accordance with Swissethics (KEK Bern ID 04/07). Written informed consent was obtained from all subjects. Animal experiments were approved by the Auvergne ethics committee (CEMEAA) APAFIS #39127.

We sequenced the exome of the affected child, her parents, and her two unaffected brothers (details in Supplementary Appendix 1). Primary fibroblasts of skin biopsies from the proband and healthy controls permitted *LGR4* transcript analysis and studies of protein expression. The putative impact of the specific *LGR4* variants was analyzed *in silico* using the three-dimensional structure of the human *LGR4* extracellular domain in complex with a part of R-SPONDIN (PDB # 4KT1). The function of identified *LGR4* variants was analyzed *in vitro*, using the TOP-Flash WNT signaling luciferase reporter assay in the presence or absence of RSPO1. Localization of mutant *LGR4* and interaction with RSPO1 was investigated by confocal microscopy in HEK293 cells expressing HA-tagged *LGR4* and GFP-tagged RSPO1. The impact of *LGR4* inactivation *in vivo* was analyzed by adrenal cortex specific ablation of *Lgr4*, using *Lgr4*^{Flox/Flox} mice mated with *Sf1:Cre* mice. Full experimental details are provided in the Supplementary Appendix.

Role of the funding sources

Authors confirm that the study sponsors played no role in study design, data collection, analysis, and interpretation of data; they were also not involved in the writing of the report and in the decision to submit the paper for publication.

RESULTS

In a highly consanguineous family from Syria, newborns were found to suffer from salt-wasting crises soon after birth due to isolated aldosterone deficiency. In addition, they revealed a common syndromic phenotype of nail dysplasia, deafness, growth restriction, and mental disability (Figure 1A).

Identification of a human LGR4 variant

After exclusion of *CYP11B2* mutations, filtering of the exome sequences identified a homozygous mutation in the proband *LGR4* gene: NM_018490:c.618-1G>C. Both parents and one brother were heterozygous for the same *LGR4* mutation but showed no overt phenotype (Figure 1B; Suppl Table S2). The variant affects a splicing acceptor site, predicted to result in exon 6 skipping, and deletion r.618_689del or p.(His207_Leu230del). mRNA transcript analysis of patient's fibroblasts confirmed exon 6 skipping, but also identified a second transcript with an alternative acceptor site within exon 6, leading to a shorter deletion of -24 bp, r.618_641del or p.(His207_Arg214) (Figure 1C).

In silico analysis of LGR4 variants for prediction of pathogenicity

Leucine-rich repeat-containing G-protein-coupled receptors (LGR) are characterized by Leucine-Rich Repeats (LRRs) that provide the rigid structure of their large extracellular domain. LGR4 is a receptor for R-SPONDINS (RSPOs). The binding of RSPOs to LGR4 stimulates Wnt/ β -catenin signaling pathway by inhibiting the E3-ubiquitin ligases ZNRF3/RNF43¹⁵. RSPOs bind to the first

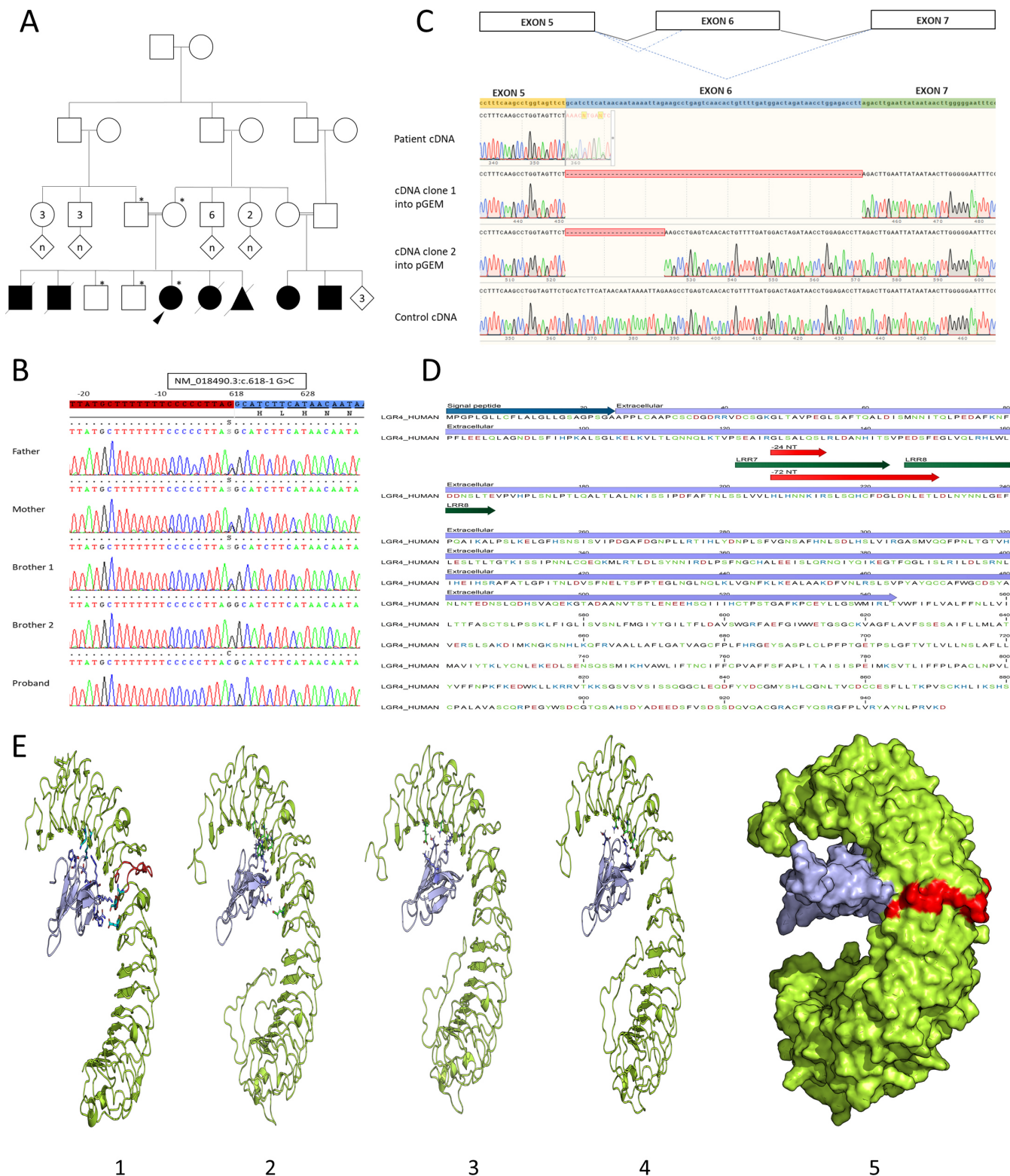


Figure 1. Genetic and structural characterization of a novel human *LGR4* mutation identified in a highly consanguineous family. A. Family pedigree showing first-degree consanguinity and multiple affected individuals. Squares, circles, and diamonds indicate male, female, unknown sex family members, respectively. Triangle indicates a miscarriage. Black symbols represent affected individuals and clear symbols unaffected individuals. Numbers in the symbol indicate multiple individuals. The black arrow indicates the index patient. Asterisk indicates individuals for whom DNA was sequenced. B. Partial chromatograms showing the identified *LGR4* mutation at NM_018490.5:c.618-1 G>C. The reference sequences of intron 5 and exon 6 are highlighted in red and blue, respectively. The proband's parents and one of the brothers are heterozygous, one brother revealed the wild-type sequence, while the proband is homozygous. C. *LGR4* mRNA analysis from fibroblast tissue of the proband. The reference sequences of exons 5, 6, and 7 are highlighted in yellow, blue and green, respectively. The first track represents the patient mRNA after reverse transcription indicating the presence of two transcripts. The two transcripts were separated by cloning and sequenced; results are shown in the two-middle tracks. The bottom track represents the sequencing of the cDNA of control fibroblasts. The scheme above indicates normal splicing in dark lines and the impact of the mutation on the splicing in blue dotted lines. D. Amino acid sequence of human *LGR4* and showing the extracellular domain of *LGR4* that binds to RSPO proteins. Amino acids deleted by mutations found in the patient are located in LRR7 (-8 AA) and LRR7/8 (-24) AA coded by exon 6 of *LGR4*. E. Structural analysis of *LGR4* and its interaction with RSPO proteins. From left to right: 1) Structure of human *LGR4* extracellular domain in complex with part of RSPO1 (PDB 4KT1). Amino acids coded by exon 6 are depicted in red. 2) Complex of human *LGR4* with human RSPO3. The RSPO3 shares high structural similarity to RSPO1 and binds to *LGR4* in similar manner, interacting with LRR7 and 8 of *LGR4*. 3 and 4) models of *LGR4* from the patient with missing 8 or 24 amino acids in *LGR4*. Several critical hydrogen bonding residues in *LGR4* are missing due to mutations causing weaker interaction and binding of RSPO3 to mutant *LGR4* proteins. 5) A surface view of the *LGR4*-RSPO3 complex, showing the close interaction points and the amino acids coded by exon 6 are shown in red.

LRR and LRR3-LRR9¹⁶. The amino acid deletions caused by the loss of 8 and 24 amino acids in LGR4 are located in LRR7 and 8, in the extracellular domain of LGR4 (Figure 1D). A protein sequence alignment of LGR4 across species revealed that sections of LGR4 that comprise LRR7 and LRR8 are highly conserved (Suppl Figure S5). Regions of RSPOs that interact with LGR4 are conserved among RSPOs isoforms (Suppl Figure S6) and across species (Suppl Figure S7). Mutations in the patient resulted in the deletion of parts of the LGR4 protein in its extracellular domain within LRR7 (for -8AA variant) and LRR7 and LRR8 (for -24AA variant). Structural analysis of contacts between LGR4 and RSPO1/RSPO3 showed that multiple contact points between the two proteins in the complex are located in LRR7 and LRR8 (Val204, His207, Asn226, Thr229, Tyr234 and Glu252) (Suppl Figure S8). A loss of multiple contact points due to the deletions would result in significantly weaker interaction between LGR4 and RSPOs and impact the overall structures of complexes involving other interaction partners (ZNR3, RNF43, UBB, UBC etc). In addition, the deletions were predicted to result in loss of protein stability and decreased half-life, which could alter LGR4 protein expression levels in the patients. Together with weaker complex formation, lower protein levels should result in an overall loss of interaction of the -8AA and -24AA variants of LGR4 found in patients, with the -24AA variant predicted to have a higher impact.

Analysis of LGR4 protein expression in human fibroblasts

Western blot analysis showed that patient fibroblasts expressed LGR4 protein minimally compared to controls (Figure 2A), but the expression was also low in control fibroblasts from healthy individuals. Unfortunately, biomaterial from heterozygote family members was not available.

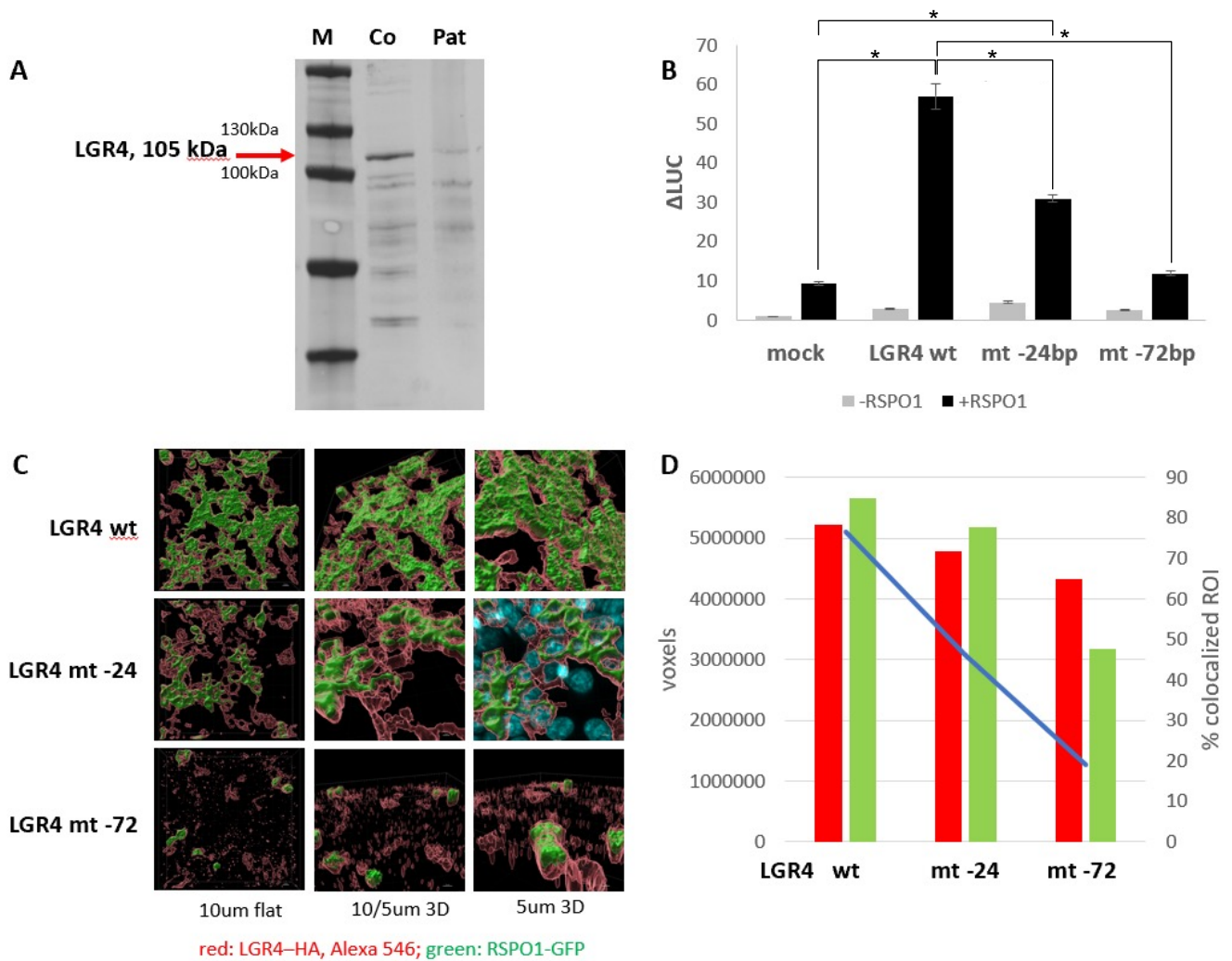


Figure 2. Protein expression and functional testing of the two LGR4 variants on Wnt/ β -catenine signaling. Human fibroblasts and HEK cells were used. A. Western blot analysis for LGR4 protein expression in patient and control fibroblasts. A representative blot of 3 independent experiments is shown. B-actin was used as a loading control. The molecular weight (kDa) of a protein standard is given. B. RSPO1 activated, LGR4 mediated Wnt signaling in HEK293 cells. Cells were transfected with wild-type (WT) or mutant LGR4 mt -24 and mt -72 plasmids (including a mock control) and reporter vectors TOP-Flash and Renilla. Signaling was stimulated by RSPO1 and assessed by the Dual-Luciferase assay (Promega). Results are expressed as relative LUC activities (RLU). Mean and SD of 3 independent experiments is shown. * $P < 0.01$. C, D. Interaction of RSPO1 with membrane-localized wild-type and variant LGR4. HEK293 cells were transfected with HA-tagged LGR4 plasmids (pcDNA3 LGR4wt, mt-24bp, mt-72bp) and incubated with conditioned RSPO1-GFP SN medium (previously produced in HEK cells transfected with pSpark- RSPO1-GFP). Cells were fixed with *Carnoy's* solution. Staining was with first antibody anti HA-Tag (green), second antibody anti-mouse Alexa 594 (red). Immunofluorescent microscopy was used to detect the cellular distribution of the tagged proteins as well as their colocalization (Zeiss LSM 710). Three independent experiments were analyzed. C. Representative pictures of confocal analysis at magnifications 40x are shown for wild-type and variants of LGR4. Scale bars show 5 and 10 μ m, respectively. D. Quantification of colocalized LGR4 and RSPO1 was performed by Imaris (Bitplane AG, Zürich, Switzerland).

Functional analysis of LGR4 variants in cell models

To assess the function of identified LGR4 variants on RSPO1 activated Wnt signaling, we used an established TOP-Flash luciferase reporter assay¹⁷⁻²². When using fibroblasts, RSPO1 stimulation of endogenous LGR4 – Wnt/ β -catenin signaling was not strong enough for luciferase readout. Therefore, we performed the studies in HEK293T cells that were transiently transfected with wild-type and variants of LGR4. While the basal activity of the Wnt/ β -catenin signaling was low and did not differ between wild-type and LGR4 variants, RSPO1 activated signaling was increased 6.1-fold with WT-LGR4 ($p < 0.0001$, Figure 2B). By contrast, the LGR4 nt-24 variant increased the Wnt/ β -catenin signaling only 3.3.-fold ($p < 0.0001$), and the LGR4 nt-72 variant completely failed to activate Wnt/ β -catenin signaling (Figure 2B). Thus, compared to WT, the LGR4 nt-72 showed loss of function, while the LGR4 variant nt -24 had 54% activity. In line with these results, loss of interaction by deletion of 24 amino acids in the nt-72 variant was predicted to have a higher impact on the binding as well as protein stability (Suppl Figure S8), which explains the very low level of LGR4 protein detected in western blots of patient fibroblasts.

Analysis of localization of LGR4 and binding to RSPO1 in HEK293 cells

LGR4 localizes to the cell membrane and reveals its signal-transducing functionality upon binding to R-SPONDINS¹⁷⁻²³. To assess localization and RSPO1 binding characteristics of wild-type and mutant LGR4 proteins, we expressed HA-tagged LGR4 in HEK293 cells and studied its binding to GFP-tagged RSPO1 by confocal microscopy. As depicted in Figure 2C, LGR4 localized to the cell surface. nt-24-LGR4 was expressed at a lower level than WT-LGR4, and cells carrying the nt-72-LGR4 expressed the lowest level of LGR4 (Figure 2D), in line with LGR4 protein expression in patient fibroblasts (Figure 2A). RSPO1 binding with LGR4 protein was also altered with nt-24-LGR4 compared to wild-type, and almost absent with nt-72 (Figure 2 C,D). Altogether, these *in vitro* data show that the mutant LGR4 proteins identified in the proband are deficient in their ability to bind R-SPONDINS and stimulate downstream WNT signaling.

***Lgr4* ablation results in disrupted *Wnt/β-catenin* signaling pathway in mice**

To study the role of LGR4 in adrenal function *in vivo*, we conditionally inactivated *Lgr4* within steroidogenic cells in the adrenal cortex of *Lgr4*cKO mice. RTqPCR showed a reduction in *Lgr4* mRNA, confirming efficient deletion of the floxed allele in *Lgr4*cKO mice (Suppl Figure S9A). Consistent with our *in vitro* data, conditional inactivation of *Lgr4* resulted in a significant decrease in expression of WNT target genes in the adrenals of *Lgr4*cKO mice (*Apcdd1*, *Axin2*, and *Lef1*) (Figure 3A) with decreased accumulation of both β-Catenin and LEF1 proteins in the presumptive zG of mutant mice, where they normally accumulate in control mice (Figure 3B-C).

***Lgr4* ablation causes adrenal hypoplasia and aberrant zonal differentiation**

The reduced canonical WNT signaling in *Lgr4*cKO mice was associated with decreased adrenal weight at 5 weeks (Figure 3D), massive cortical thinning, off-center localization of the adrenal medulla, steroidogenic cell cytomegaly, and a significant decrease in cortical cells numbers (Figure 3E-F). Interestingly, adrenal cortex thinning was not associated with decreased proliferation or increased apoptosis, suggesting that it relied on altered development/maintenance of the gland (Suppl Figure S9B-C). Analysis of adrenal cortex differentiation by immunohistochemistry showed a marked decrease in the number of cells expressing the zG marker DAB2 and expansion of the expression domain of zF marker AKR1B7 up to the capsule, where the zG normally resides (Figure 3G). *Lgr4* deficiency also resulted in the accumulation of cells with both zG (DAB2+) and zF (AKR1B7+) identity that were not found in control mice (Figure 3G, arrowheads), demonstrating a marked impairment of adrenal cortex differentiation. Aberrant cortical differentiation was further confirmed by RTqPCR showing increased *Akr1b7* (zF) and decreased expression of the zG markers *Dab2* and *Hsd3b6* (Figure 3H), consistent with previous data showing decreased zG differentiation and expansion of zF in mice with decreased adrenal WNT signaling^{8,11}.

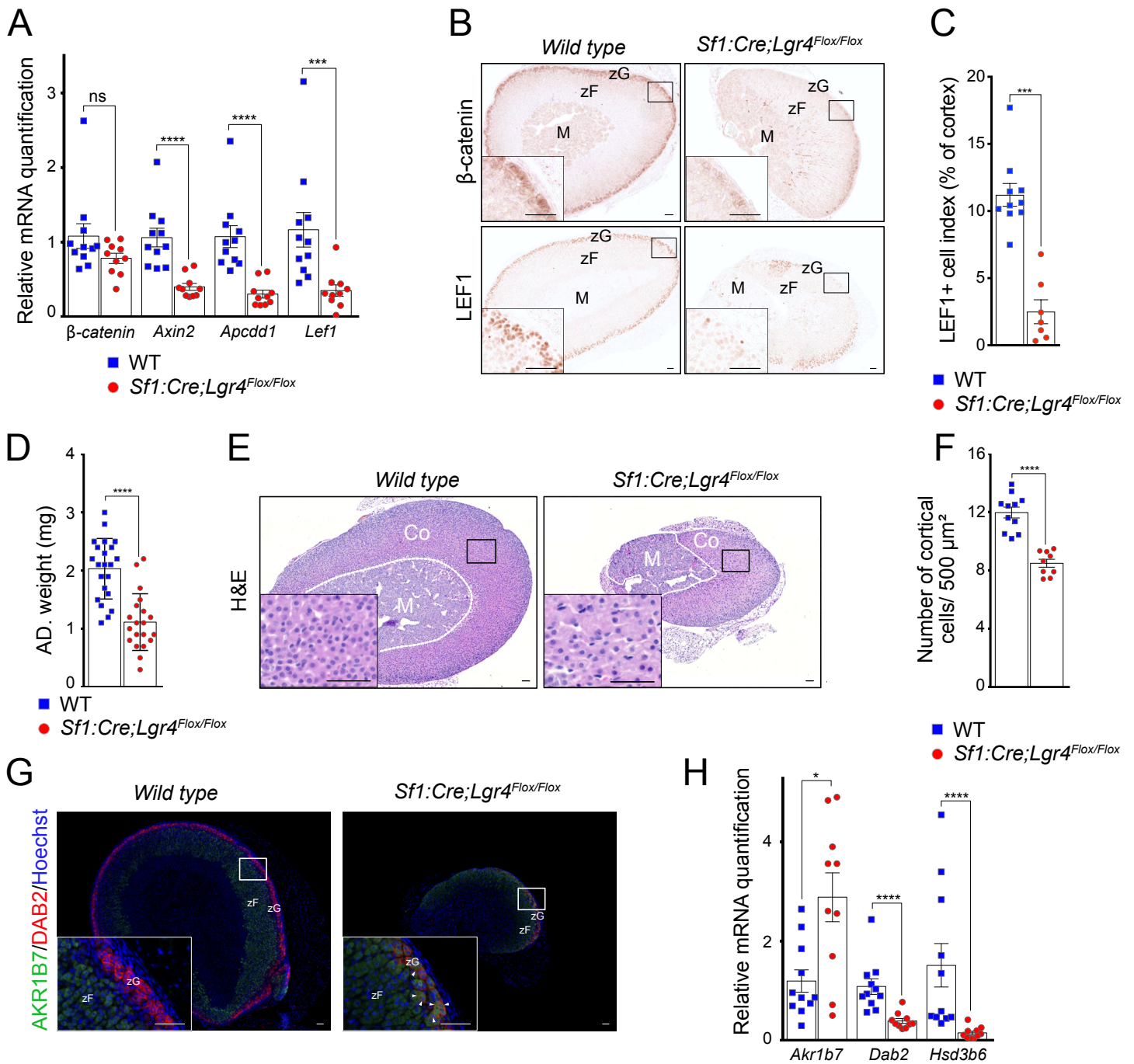


Figure 3. *Lgr4* ablation disrupts Wnt/ β -catenin signaling pathway resulting in adrenal hypoplasia and aberrant zonal differentiation. A. RT-qPCR analysis of mRNA encoding Wnt/ β -catenin signaling pathway associated genes. B. Immunohistochemical detection of β -catenin and Lef1. C. LEF1-positive cells index defined as the percentage of LEF1+ cells over the total number of cortical cells. D. Adrenal weight. E. Hematoxylin and Eosin staining of wild type and *Lgr4*cKO adrenals. F. Number of cortical cells per 500 μm^2 of the cortex. G. Co-immunostaining for *Akr1b7* and *Dab2* in wild type and *Lgr4*cKO adrenals. H. RT-qPCR analysis of mRNA encoding zone-specific markers (*Akr1b7*, *Dab2* and *Hsd3b6*). All analyses were conducted in 5 weeks wild-type and *Lgr4*cKO female mice. zF: zona fasciculata, zG: zona glomerulosa, M: medulla, Co: cortex. Scale bars, 50 μm . Bars represent the mean expression \pm SEM. ns, not significant * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

***Lgr4* ablation inhibits zG zonation resulting in primary hypoaldosteronism**

Consistent with observations in our patient, zG differentiation anomalies in *Lgr4*cKO mice resulted in a significant decrease in plasma aldosterone (Figure 4A) and an increase in hematocrit, suggestive of dehydration (Figure S9C). The observation of normal plasma renin activity (Figure 4B) suggested that hypoaldosteronism in *Lgr4*cKO mice was of primary adrenal origin. This was further supported by the almost complete extinction of CYP11B2 protein expression in the *Lgr4*cKO zG (Figure 4C-D). Interestingly, plasma corticosterone concentration was significantly decreased (Figure 4E), which was associated with a significant decrease in *Cyp11a1* expression, which is essential for the first step of both aldosterone and corticosterone synthesis (Figure 4G). However, there was a concomitant increase in *Cyp21* and *Cyp11b1* expression, which was not associated with altered plasma ACTH concentration (Figure 4F-G). This may reflect the aberrant expansion of zF at the expense of zG, and hence an increased ratio of zF to zG cells, rather than a direct effect of ACTH on steroidogenic gene expression.

Altogether, these data show that *Lgr4* inactivation is sufficient to significantly reduce WNT signaling in the adrenal cortex, which results in early-onset primary hypoaldosteronism.

DISCUSSION

Although adrenal insufficiency with MC and GC deficiencies has been reported for several complex syndromes where genetic variants lead to structural and/or functional defects of the adrenals and other organ systems (e.g. IMAGE, MIRAGE syndromes)⁷, an inherited syndrome with isolated MC deficiency at birth, has not been described so far. In this study, we identified a novel syndromic form of severe neonatal salt-wasting in a highly consanguineous family. In the index patient, isolated mineralocorticoid deficiency was diagnosed and treated successfully with mineralocorticoid replacement therapy, while cortisol production remained normal in the first two decades of life. Associated defects included nail anomalies, hearing loss, short stature, and

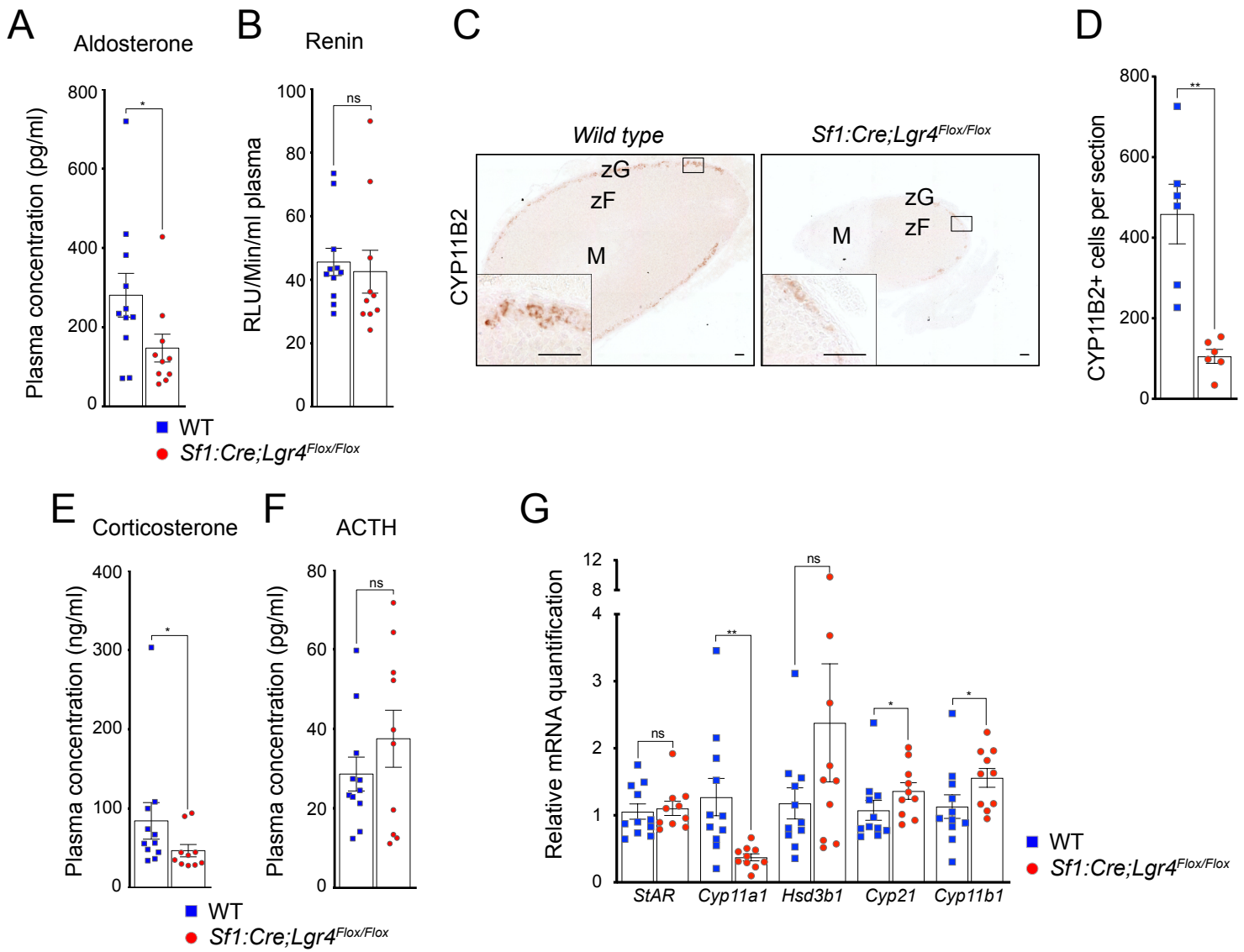


Figure 4. *Lgr4* ablation inhibits zG differentiation, resulting in primary hypoaldosteronism. A. Aldosterone plasma concentration and B. Renin activity in 5 weeks wild type and *Lgr4*cKO female mice. C. Immunohistochemical detection of CYP11B2 (scale bars 50 μ m). D. Number of CYP11B2-positive cells per adrenal section. E. Corticosterone and F. Plasma ACTH concentration. G. RT-qPCR analysis of mRNA encoding steroidogenesis-related genes. All analyses were conducted in 5 weeks wild-type and *Lgr4*cKO female mice. Bars represent the mean expression \pm SEM. ns, not significant * $P < 0.05$, ** $P < 0.01$.

mental disability in the index patient and both affected cousins (Figure 1A). We were aided by the consanguinity in this family to reveal the underlying genetic cause, involving a homozygous splice site variation in the *LGR4* gene (ch11p14.1) producing two shorter splice variants. LGR4, also named GPR48, is a leucine-rich repeat-containing G-protein coupled receptor, widely expressed in multiple tissues from early embryogenesis to adulthood^{15,24}. LGR4 potentiates canonical WNT signaling, through inhibition of the ZNRF3/RNF43-mediated degradation of Frizzled receptors, after binding to R-SPONDINS¹⁵. Consistent with this, our *in vitro* studies showed that the two aberrant *LGR4* transcripts found in the proband, coded for proteins with significantly reduced activity on WNT/ β catenin signaling *in vitro*. We further showed that genetic inactivation of *Lgr4* within steroidogenic cells of the adrenal cortex of transgenic mice resulted in decreased canonical WNT signaling, deficient zG differentiation, and reduced aldosterone production. Even though previous reports had shown adrenal dysgenesis in patients with inactivating mutations of WNT4 in the context of SERKAL syndrome, profound developmental defects resulted in embryonic lethality, precluding evaluation of adrenal differentiation and endocrine activity²⁵. Therefore, to the best of our knowledge, our study is the first to demonstrate a key role of LGR4 and more broadly of deficient canonical WNT signaling in adrenal differentiation and zG hypofunction in patients.

Beyond primary hypoaldosteronism, the index case also presented with a spectrum of defects including nail anomalies, hearing loss, and short stature, which were also associated with *in utero* death of presumably affected siblings. Our model of conditional *Lgr4* ablation within steroidogenic cells did not allow evaluation of LGR4 in these phenomena. However, studies of LGR4 variants in patients and of whole-body *Lgr4* knockout mice demonstrated the association between LGR4 alterations and fetal/ perinatal death, short stature, deafness, and dysplastic nails (Supplementary Table S3). This strongly suggests that the broad defects observed in our proband are the result of the identified LGR4 mutation. So far only individuals who carry heterozygous LGR4 variants

were described. Heterozygous human *LGR4* variants are associated with low bone mineral density, electrolyte imbalance, reduced testosterone production, and increased risk of cancers of the biliary system and skin ²⁶. More recently, 3 rare heterozygous missense variants in *LGR4* were associated with delayed puberty, resulting from alterations in the development of hypothalamic GnRH neurons ²⁷. Loss of *LGR4* is also potentially implicated in the rare aniridia-genitourinary anomalies-mental retardation (AGR) syndrome, where a contiguous gene deletion of the 11p13-14 region has been identified comprising the *LGR4* gene²⁸. Similar to the phenotype of AGR syndrome, whole-body deletion of *Lgr4* in mouse led to aniridia, polycystic kidney disease, genitourinary anomalies, and mental retardation. Although there were no reports of adrenal dysfunction in these cases, our results suggest that patients presenting with homozygous *LGR4*-associated genetic variations should be carefully evaluated for adrenal function.

Whereas *Lgr4*cKO mice show defects in both aldosterone and corticosterone secretion as early as 5 weeks, our index case presented with isolated mineralocorticoid deficiency in the first two decades of life. This phenotypic discrepancy could be accounted for by the residual activity of mutant *LGR4* proteins in our patient, compared with the complete inactivation of *LGR4* in the adrenal cortex of our transgenic model. However, ACTH stimulation testing of our patient at 21 years, showed subclinical glucocorticoid deficiency. This suggests that the endocrine phenotype may progress towards full-fledged adrenal deficiency over time. Lineage tracing studies in mice have shown that adrenal cortex cell renewal requires initial differentiation of progenitors into zG cells that subsequently differentiate into zF cells ^{29,30}. It is thus tempting to speculate that aberrant zG differentiation in our patient hampered cortical cell renewal, resulting in progressive exhaustion of the zF, associated with progressive glucocorticoid insufficiency. This warrants careful monitoring of patients initially presenting with primary hypoaldosteronism, without *CYP11B2* inactivating mutations.

In conclusion, we describe the first patients harboring biallelic *LGR4* variants and offer the mechanistic explanation for their life-threatening salt loss at birth, due to primary adrenal hypoaldosteronism. Our study confirms the important role of Wnt/ β -catenin signaling for proper adrenal cortex zG and zF formation and function. Thus *LGR4* variants and potential variants in other genes involved in the complex network of LGR4-Wnt/ β -catenin signaling should be considered in patients presenting with a salt-wasting crisis at birth, especially when manifesting with other syndromic features.

Contributors

C.L. - Performed experiments on mice biomaterials. Analyzed data. Created Figures. Contribution to manuscript writing

K.S.S. – Performed experiments on human biomaterials. Analyzed data. Created Figures. Contribution to manuscript writing

M.S. – Clinical workup of the patient. Contribution to manuscript writing and reviewing;

D.M. – Performed genetic test and analysis. Contribution to manuscript writing.

A.V.P. – Bioinformatic analyses and predictions. Created Figures. Contribution to manuscript writing and reviewing

J.W. - Performed experiments on mice biomaterials.

J.O. - Performed experiments on mice biomaterials.

I.P. – Performed hormonal tests.

Y.M. - Performed genetic test and analysis.

D.A. – Clinical workup of patient (bone phenotype), manuscript review.

F.W. - Clinical workup of patient (brain, cochlea phenotype); manuscript review.

H.C. – Mouse model.

P.V. - Study PI. Overall design, organization, data analysis and interpretation. Manuscript writing.

F.R.B. - Study PI. Overall design, organization, data analysis and interpretation. Manuscript writing.

C.E.F. – Study PI. Overall design, organization, data analysis and interpretation. Manuscript writing; corresponding author.

Declaration of interests

The authors declare no conflict of interest

Acknowledgments

We thank the patient and family for participating in the study. We acknowledge the help of Christelle Damon-Soubeyrand and the Anipath histopathology facility (iGReD), Clermont-Ferrand, for mouse tissue analyses. We also thank Khirredine Ouchen, Sandrine Plantade and Philippe Mazuel for animal care.

Data sharing

All data and materials presented in the manuscript will be made available upon written request to the corresponding author.

Funding

Pierre Val is supported by a program grant (Labellisation) by the “Ligue Nationale Contre le Cancer” 2021-2025. Florence Roucher-Boulez is supported by the Endocrinology Research Grant RECORDATI Rare Diseases of the French Society of Endocrinology (SFE) 2020 and the Young Researchers 2021 grant by the Lyon University Hospital (HCL). This work has also been supported by the Swiss National Science Foundation grant ID 320030_146127 to Christa E. Flück.

References

1. Miller WL, Fluck CE, Breault DT, Feldman BJ. Adrenal cortex and its disorders. In: Sperling MA, editor. *Sperling - Pediatric Endocrinology*; Elsevier; 2020.
2. Miller WL, Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev.* 2011; **32**(1): 81-151.
3. Krege JH, John SW, Langenbach LL, Hodgins JB, Hagaman JR, Bachman ES, et al. Male-female differences in fertility and blood pressure in ACE-deficient mice. *Nature.* 1995; **375**(6527): 146-8.
4. Tanimoto K, Sugiyama F, Goto Y, Ishida J, Takimoto E, Yagami K, et al. Angiotensinogen-deficient mice with hypotension. *J Biol Chem.* 1994; **269**(50): 31334-7.
5. Sugaya T, Nishimatsu S, Tanimoto K, Takimoto E, Yamagishi T, Imamura K, et al. Angiotensin II type 1a receptor-deficient mice with hypotension and hyperreninemia. *J Biol Chem.* 1995; **270**(32): 18719-22.
6. Gribouval O, Gonzales M, Neuhaus T, Aziza J, Bieth E, Laurent N, et al. Mutations in genes in the renin-angiotensin system are associated with autosomal recessive renal tubular dysgenesis. *Nature genetics.* 2005; **37**(9): 964-8.
7. Pignatti E, Fluck CE. Adrenal cortex development and related disorders leading to adrenal insufficiency. *Molecular and cellular endocrinology.* 2021; **527**: 111206.
8. Drelon C, Berthon A, Sahut-Barnola I, Mathieu M, Dumontet T, Rodriguez S, et al. PKA inhibits WNT signalling in adrenal cortex zonation and prevents malignant tumour development. *Nat Commun.* 2016; **7**: 12751.
9. Berthon A, Drelon C, Ragazzon B, Boulkroun S, Tissier F, Amar L, et al. WNT/beta-catenin signalling is activated in aldosterone-producing adenomas and controls aldosterone production. *Human molecular genetics.* 2014; **23**(4): 889-905.
10. Heikkila M, Peltoketo H, Leppaluoto J, Ilves M, Vuolteenaho O, Vainio S. Wnt-4 deficiency alters mouse adrenal cortex function, reducing aldosterone production. *Endocrinology.* 2002; **143**(11): 4358-65.
11. Vidal V, Sacco S, Rocha AS, da Silva F, Panzolini C, Dumontet T, et al. The adrenal capsule is a signaling center controlling cell renewal and zonation through Rspo3. *Genes & development.* 2016; **30**(12): 1389-94.
12. Leng S, Pignatti E, Khetani RS, Shah MS, Xu S, Miao J, et al. beta-Catenin and FGFR2 regulate postnatal rosette-based adrenocortical morphogenesis. *Nat Commun.* 2020; **11**(1): 1680.
13. Boulkroun S, Samson-Couterie B, Golib-Dzib JF, Amar L, Plouin PF, Sibony M, et al. Aldosterone-producing adenoma formation in the adrenal cortex involves expression of stem/progenitor cell markers. *Endocrinology.* 2011; **152**(12): 4753-63.
14. Berthon A, Sahut-Barnola I, Lambert-Langlais S, de Jossineau C, Damon-Soubeyrand C, Louiset E, et al. Constitutive beta-catenin activation induces adrenal hyperplasia and promotes adrenal cancer development. *Human molecular genetics.* 2010; **19**(8): 1561-76.
15. Ordaz-Ramos A, Rosales-Gallegos VH, Melendez-Zajgla J, Maldonado V, Vazquez-Santillan K. The Role of LGR4 (GPR48) in Normal and Cancer Processes. *Int J Mol Sci.* 2021; **22**(9).
16. Petrie EJ, Lagaida S, Sethi A, Bathgate RA, Gooley PR. In a Class of Their Own - RXFP1 and RXFP2 are Unique Members of the LGR Family. *Front Endocrinol (Lausanne).* 2015; **6**: 137.
17. Carmon KS, Gong X, Lin Q, Thomas A, Liu Q. R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/beta-catenin signaling. *Proceedings of the National Academy of Sciences of the United States of America.* 2011; **108**(28): 11452-7.

18. Glinka A, Dolde C, Kirsch N, Huang YL, Kazanskaya O, Ingelfinger D, et al. LGR4 and LGR5 are R-spondin receptors mediating Wnt/beta-catenin and Wnt/PCP signalling. *EMBO Rep.* 2011; **12**(10): 1055-61.
19. Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, et al. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC^{-/-} colon carcinoma. *Science.* 1997; **275**(5307): 1784-7.
20. Ruffner H, Sprunger J, Charlat O, Leighton-Davies J, Grosshans B, Salathe A, et al. R-Spondin potentiates Wnt/beta-catenin signaling through orphan receptors LGR4 and LGR5. *PLoS One.* 2012; **7**(7): e40976.
21. Tomaselli S, Megiorni F, Lin L, Mazzilli MC, Gerrelli D, Majore S, et al. Human RSP01/R-spondin1 is expressed during early ovary development and augments beta-catenin signaling. *PLoS One.* 2011; **6**(1): e16366.
22. de Lau W, Barker N, Low TY, Koo BK, Li VS, Teunissen H, et al. Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. *Nature.* 2011; **476**(7360): 293-7.
23. Kudo M, Chen T, Nakabayashi K, Hsu SY, Hsueh AJ. The nematode leucine-rich repeat-containing, G protein-coupled receptor (LGR) protein homologous to vertebrate gonadotropin and thyrotropin receptors is constitutively active in mammalian cells. *Molecular endocrinology.* 2000; **14**(2): 272-84.
24. Yi J, Xiong W, Gong X, Bellister S, Ellis LM, Liu Q. Analysis of LGR4 receptor distribution in human and mouse tissues. *PLoS One.* 2013; **8**(10): e78144.
25. Mandel H, Shemer R, Borochoy ZU, Okopnik M, Knopf C, Indelman M, et al. SERKAL syndrome: an autosomal-recessive disorder caused by a loss-of-function mutation in WNT4. *American journal of human genetics.* 2008; **82**(1): 39-47.
26. Stykarsdottir U, Thorleifsson G, Sulem P, Gudbjartsson DF, Sigurdsson A, Jonasdottir A, et al. Nonsense mutation in the LGR4 gene is associated with several human diseases and other traits. *Nature.* 2013; **497**(7450): 517-20.
27. Mancini A, Howard SR, Marelli F, Cabrera CP, Barnes MR, Sternberg MJ, et al. LGR4 deficiency results in delayed puberty through impaired Wnt/beta-catenin signaling. *JCI Insight.* 2020; **5**(11).
28. Yi T, Weng J, Siwko S, Luo J, Li D, Liu M. LGR4/GPR48 inactivation leads to aniridia-genitourinary anomalies-mental retardation syndrome defects. *The Journal of biological chemistry.* 2014; **289**(13): 8767-80.
29. Freedman BD, Kempna PB, Carlone DL, Shah M, Guagliardo NA, Barrett PQ, et al. Adrenocortical zonation results from lineage conversion of differentiated zona glomerulosa cells. *Dev Cell.* 2013; **26**(6): 666-73.
30. King P, Paul A, Laufer E. Shh signaling regulates adrenocortical development and identifies progenitors of steroidogenic lineages. *Proceedings of the National Academy of Sciences of the United States of America.* 2009; **106**(50): 21185-90.