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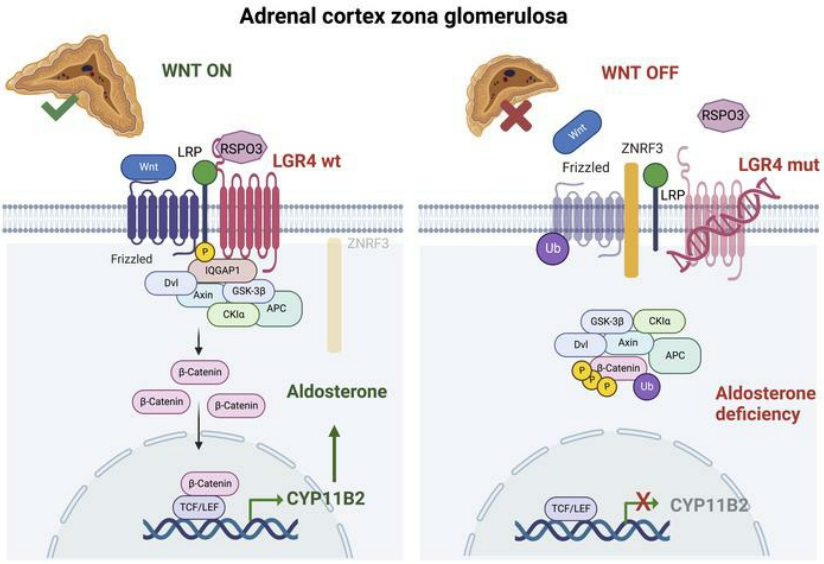
Graphical abstract



Loss of LGR4/GPR48 causes severe neonatal salt-wasting due to disrupted WNT signaling altering adrenal zonation

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Conflict-of-interest statement

The authors have declared that no conflict of interest exists.

ABSTRACT (199)

Disorders of isolated mineralocorticoid deficiency causing potentially life-threatening salt-wasting crisis early in life have been associated with gene variants of aldosterone biosynthesis or resistance, but in some patients no such variants are found. WNT/β-catenin signaling is crucial for differentiation and maintenance of the aldosterone producing adrenal zona glomerulosa (zG). We describe a highly consanguineous family with multiple perinatal deaths or infants presenting at birth with failure to thrive, severe salt-wasting crises associated with isolated hypoaldosteronism, nail anomalies, short stature, and deafness. Whole exome sequencing revealed a homozygous splice variant in the R-SPONDIN receptor *LGR4* gene (c.618-1G>C) regulating WNT signaling. The resulting transcripts affected protein function and stability, and resulted in loss of Wnt/β-catenin signaling *in vitro*. The impact of *LGR4* inactivation was analyzed by adrenal cortex specific ablation of *Lgr4*, using $Lgr4^{Flox/Flox}$ mated with *Sf1:Cre* mice. Inactivation of *Lgr4* within the adrenal cortex in the mouse model caused decreased WNT signaling, aberrant zonation with deficient zG and reduced aldosterone production. Thus, human *LGR4* mutations establish a direct link between *LGR4* inactivation and decreased canonical WNT signaling with abnormal zG differentiation and endocrine function. Therefore, variants in WNT signaling and its regulators should systematically be considered in familial hyperreninemic hypoaldosteronism.

Key words:

adrenal cortex, human adrenal cortex zonation, zona glomerulosa, mineralocorticoid deficiency**,** aldosterone, WNT/β-catenin signaling, *LGR4/GPR 48*, familial hyperreninemic hypoaldosteronism

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 as physiologic immaturity of the renal tubular system in the first year of life is contributing to impaired ability to regulate water and sodium homeostasis (2-4), while beyond the neonatal period a higher sodium intake in the diet regulated by salt appetite centrally may compensate for MC deficiency (5).

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 (6). 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 angiotensinogen (*AGT*), 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 (7-10). 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 (11). 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 (12). WNT4 and R-SPONDIN3 (RSPO3) are tissue specific expressed and thus important drivers of WNT activation and zG differentiation, through stabilization of β-catenin, which stimulates expression of CYP11B2 and angiotensin II receptor AGTR1 in the human adrenal cortex (13). Consequently, mouse models with *Ctnnb1*, *Wnt4*, or *Rspo3* deficiency have reduced zG differentiation(12, 14-16). 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 (17, 18). 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.

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. 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 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.

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/RNF43 (19). RSPOs bind to the first LRR and LRR3-LRR9 (20). 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 (ZNRF3, 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.

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 assay (21-26). 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-SPONDINS (21-27). 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 Lgr4cKO mice. RTqPCR showed a reduction in *Lgr4* mRNA, confirming efficient deletion of the floxed allele in Lgr4cKO 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 Lgr4cKO 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 Lgr4cKO 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 (12, 15).

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

Consistent with observations in our patient, zG differentiation anomalies in Lgr4cKO 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 Lgr4cKO mice was of primary adrenal origin. This was further supported by the almost complete extinction of CYP11B2 protein expression in the Lgr4cKO 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) (11), 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 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 (19, 28). LGR4 potentiates canonical WNT signaling, through inhibition of the ZNRF3/RNF43-mediated degradation of Frizzled receptors, after binding to R-SPONDINS (19). 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 activity (29). 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 (30). More recently, 3 rare heterozygous missense variants in *LGR4* were associated with delayed puberty, resulting from alterations in the development of hypothalamic GnRH neurons (31). In line with these findings, heterozygous family members as well as our index patient had delayed pubertal onset and low bone mineral density (Suppl Appendix Extended Case Report, Figure S4 and Suppl Table S1 and S3).

Loss of LGR4 is also potentially implicated in the rare aniridia-genitourinary anomalies-mental retardation (AGR) syndrome, where a heterozygous, contiguous gene deletion of the 11p13-14 region had been identified comprising the *LGR4* gene. 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 (32). 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 Lgr4cKO 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 (33, 34). 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.

METHODS

Genomic sequencing

We sequenced the exome of the affected child, her parents, and her two unaffected brothers. Details are provided in Supplementary Appendix. Next-generation sequencing data have been deposited in the European Genome-phenome Archive (EGA), which is hosted by the EBI and the CRG, under accession number EGAS00001006808 (https://ega-archive.org).

Bioinformatic and laboratory studies

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.

Statistics

Results are presented as means +/- SEM. The D'agostino and Pearson normality test demonstrated the absence of normality of the data. Therefore, statistical analyses between two or several groups were performed using Mann-Whitney or Kruskal-Wallis, respectively, using GraphPad Prism 9. A *P* value below 0.05 was considered statistically significant. **P*<0.05; ***P*<0.01; ****P*<0.001, *****P<0.00001*.

Study Approval

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

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Figure Legends

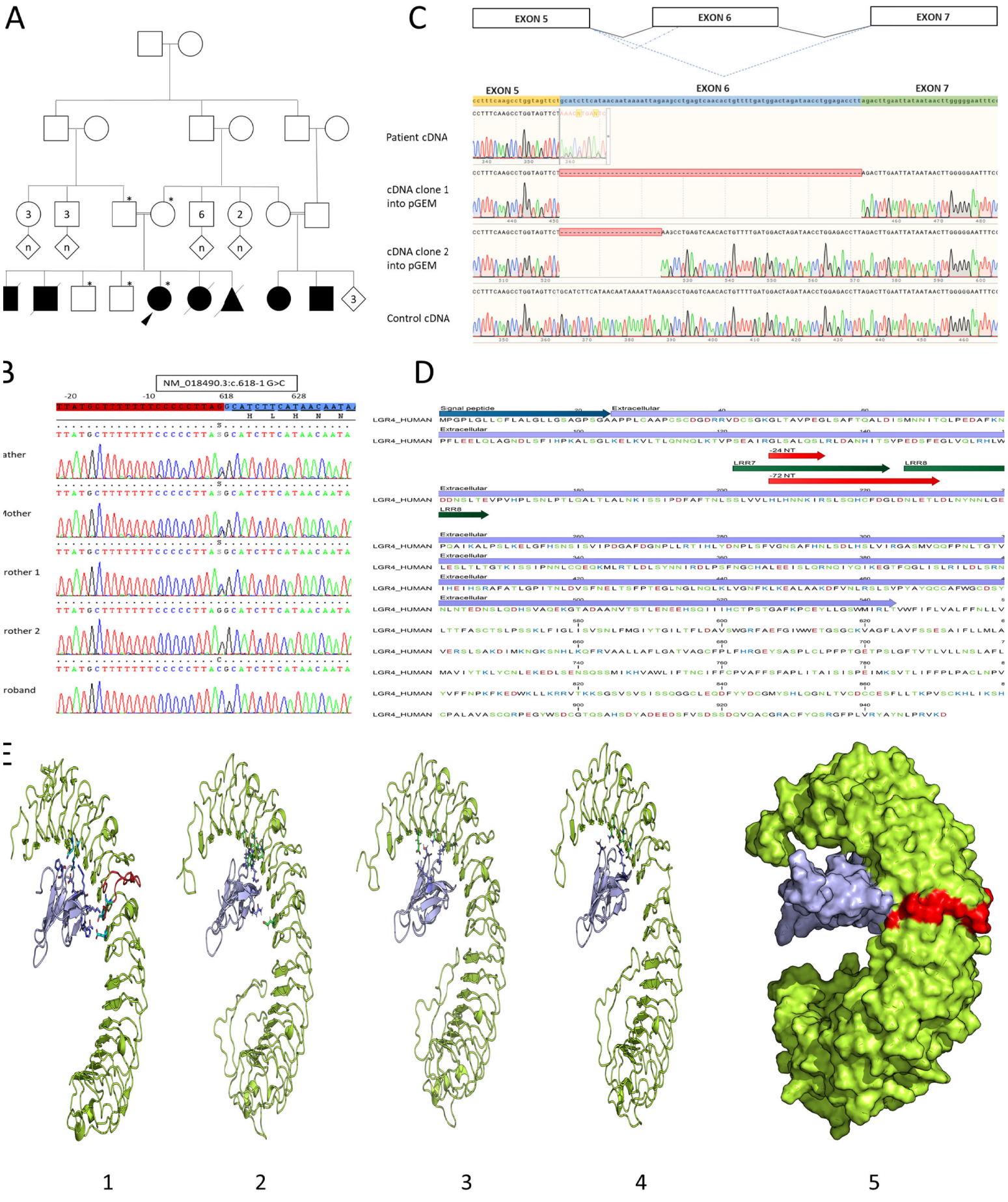
Figure 1. Genetic and structural characterization of a novel human *LGR4* mutation identified in a highly consanguineous family. A. Family pedigree showing first-degree consanguinity and multiple affected individuals. Squares, circles, and diamonds indicate male, female, unknown sex family members, respectively. 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

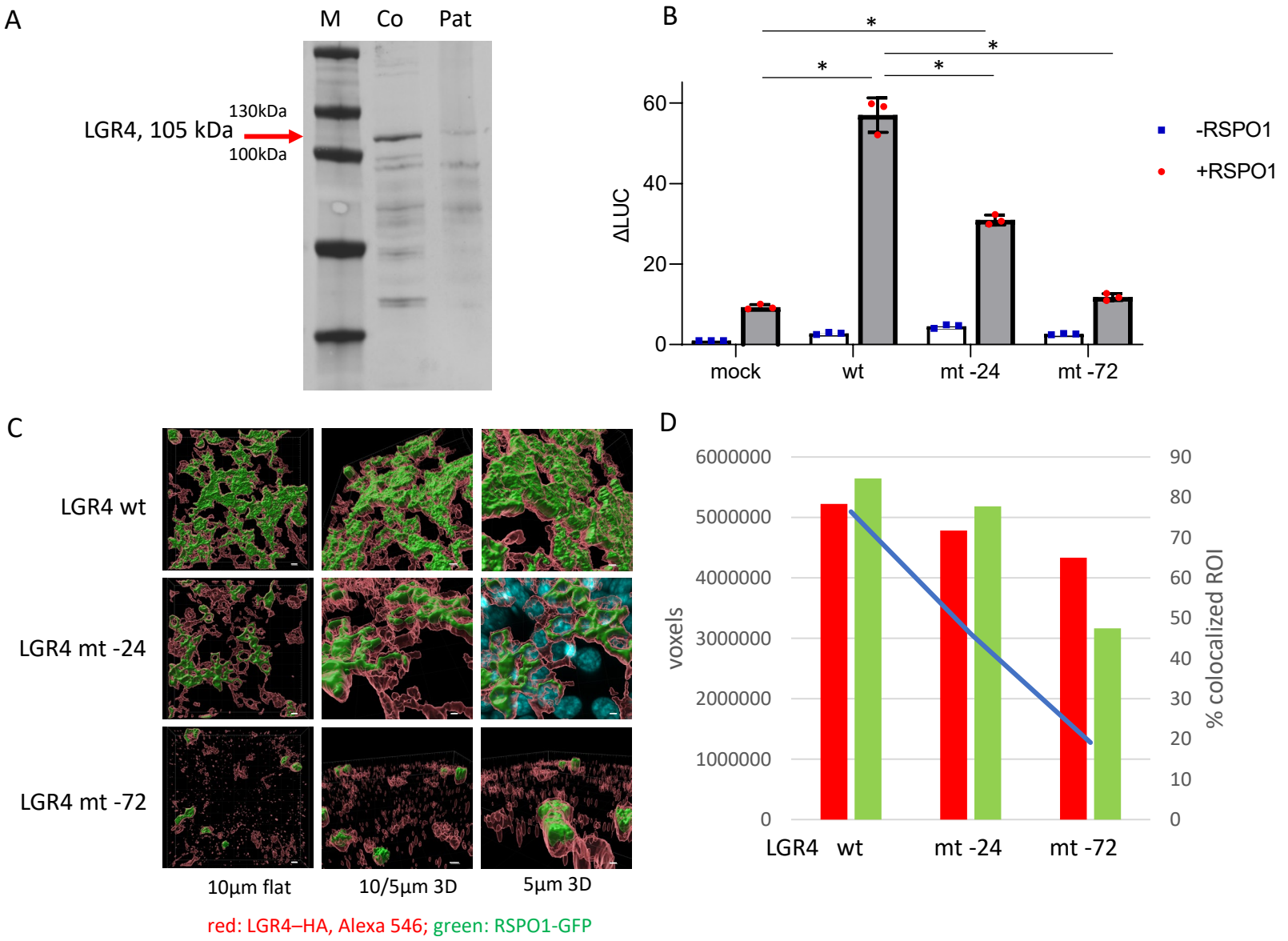
from the patient with missing 8 or 24 amino acids in LGR4. Several critical hydrogen bonding residues in LGR4 are missing due to mutations causing weaker interaction and binding of RSPO3 to mutant LGR4 proteins. 5) A surface view of the LGR4-RSPO3 complex, showing the close interaction points and the amino acids coded by exon 6 are shown in red.

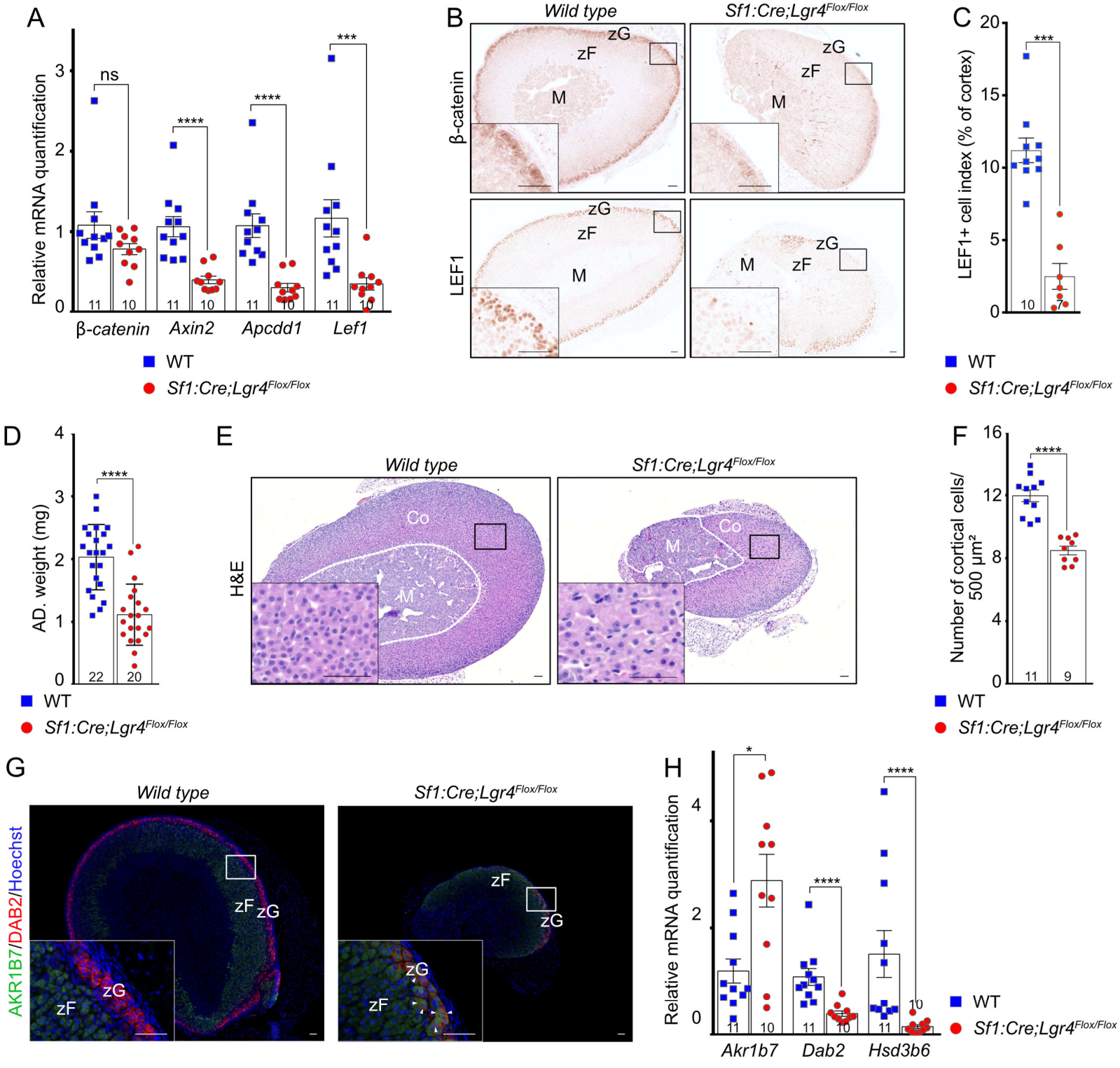
Figure 2. Protein expression and functional testing of the two LGR4 variants on Wnt/β-catenine signaling. Human fibroblasts and HEK cells were used. **A**. Western blot analysis for LGR4 protein expression in patient and control fibroblasts. A representative blot of 3 independent experiments is shown. 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. Student's t-test, * $P<0.01$. **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 analzyed. Representative pictures of confocal analysis at magnifications 40x are shown for wild-type and variants of LGR4. Scale bars show 5 and 10 µm, respectively. Quantification of colocalized LGR4 and RSPO1 was performed by Imaris (Bitplane AG, Zürich, Switzerland).

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 Lgr4cKO adrenals. F. Number of cortical cells per 500 μm^2 of the cortex. G. Co-immunostaining for *Akr1b7* and *Dab2* in wild type and Lgr4cKO 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 Lgr4cKO female mice. zF: zona fasciculata, zG: zona glomerulosa, M: medulla, Co : cortex. Scale bars, 50 µm. Bars represent the mean expression ± SEM. Numbers of individual samples analysed are indicated within the bars. Statistical analyses in panels A, C, D, F & H were conducted using Mann-Whitney tests in GraphPad Prism 9. ns, not significant *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

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 Lgr4cKO female mice. C. Immunohistochemical detection of CYP11B2 (scale bars 50 µm). D. Number of CYP11B2-poitive 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 Lgr4cKO female mice. Bars represent the mean expression ± SEM. Numbers of individual samples analysed are indicated within the bars. Statistical analyses in panels A, B, D, E, F & G were conducted using Mann-Whitney tests in GraphPad Prism 9. ns, not significant *P<0.05, **P<0.01.


A
C
EXON 5
EXON 6
EXON 7
Patient cDNA
cDNA clone 1 into pGEM
cDNA clone 2 into pGEM
Control cDNA
B
NM_018490.3:c.618-1 G>C
ather
lother
rother 1
rother 2
roband
D
Signal peptide
Extracellular
-24 NT
-72 NT
LRR7
LRR8
LRR8
LGR4_HUMAN
E
1
2
3
4
5


A
M
Co
Pat
130kDa
LGR4, 105 kDa
100kDa
B
*
*
*
*
60
40
20
0
ΔLUC
mock
wt
mt -24
mt -72
-RSPO1
+RSPO1
C
LGR4 wt
LGR4 mt -24
LGR4 mt -72
10µm flat
10/5µm 3D
5µm 3D
red: LGR4–HA, Alexa 546; green: RSPO1-GFP
D
6000000
5000000
4000000
3000000
2000000
1000000
0
voxels
90
80
70
60
50
40
30
20
10
0
% colocalized ROI
LGR4 wt
mt -24
mt -72


A
Relative mRNA quantification
ns
****
****
***
β-catenin
Axin2
Apcdd1
Lef1
WT
Sf1:Cre;Lgr4Flox/Flox
B
Wild type
Sf1:Cre;Lgr4Flox/Flox
β-catenin
LEF1
zG
zF
M
C
LEF1+ cell index (% of cortex)
***
D
AD. weight (mg)
****
E
Wild type
Sf1:Cre;Lgr4Flox/Flox
H&E
Co
M
F
Number of cortical cells/ 500 µm²
****
G
Wild type
Sf1:Cre;Lgr4Flox/Flox
AKR1B7/DAB2/Hoechst
zF
zG
H
Relative mRNA quantification
*
****
****
Akr1b7
Dab2
Hsd3b6

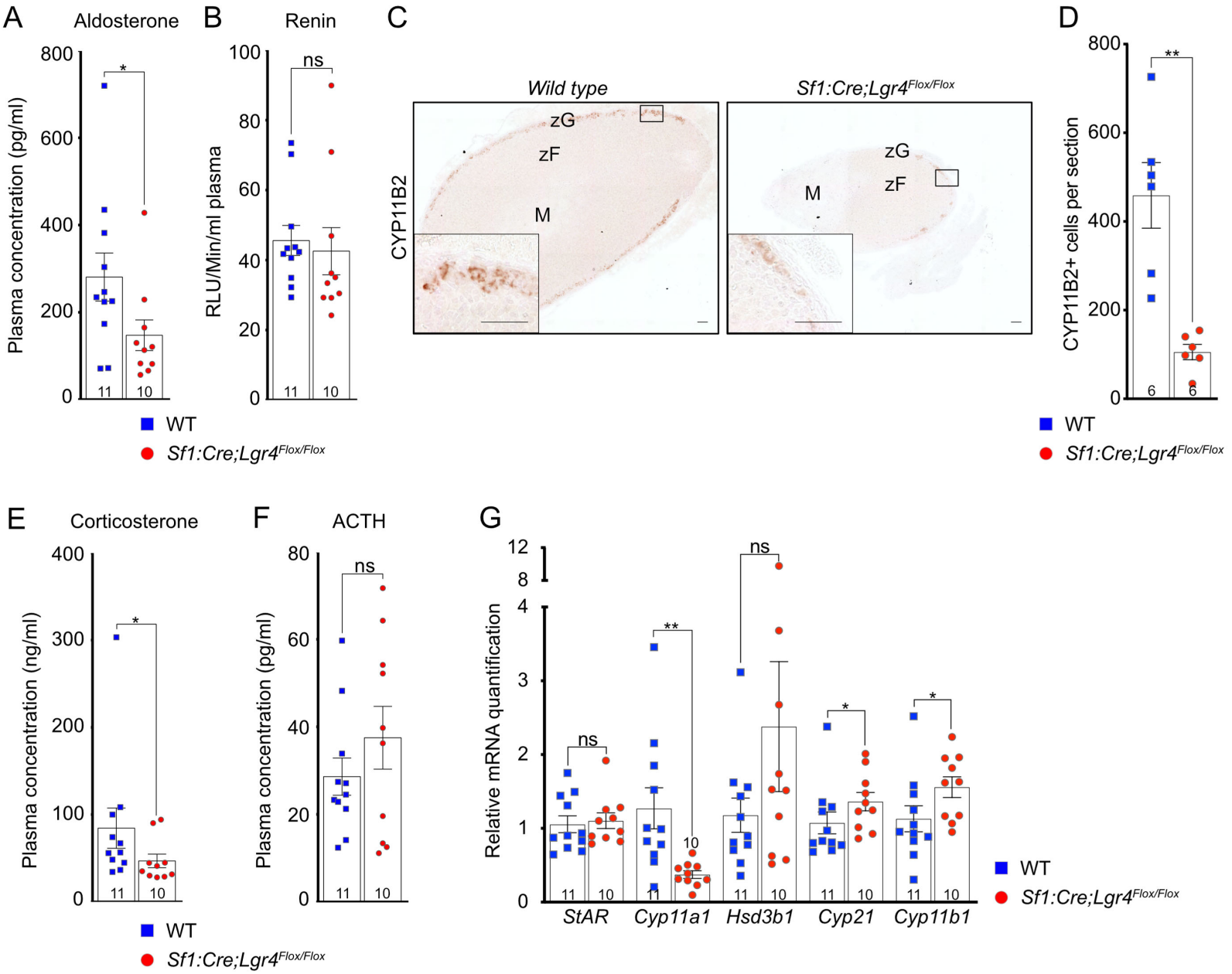

A
Aldosterone
Plasma concentration (pg/ml)
800
600
400
200
0
*
11
10
B
Renin
RLU/Min/ml plasma
100
80
60
40
20
0
ns
11
10
C
Wild type
Sf1:Cre;Lgr4Flox/Flox
CYP11B2
zG
zF
M
D
CYP11B2+ cells per section
800
600
400
200
0
**
6
6
WT
Sf1:Cre;Lgr4Flox/Flox
E
Corticosterone
Plasma concentration (ng/ml)
400
300
200
100
0
*
11
10
F
ACTH
Plasma concentration (pg/ml)
80
60
40
20
0
ns
11
10
G
Relative mRNA quantification
12
8
4
3
2
1
0
ns
**
*
*
10
StAR
Cyp11a1
Hsd3b1
Cyp21
Cyp11b1
WT
Sf1:Cre;Lgr4Flox/Flox

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/m2*					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/m2/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