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Neurofilament Medium Polypeptide (NEFM), a marker for Zona Glomerulosa Cells in Human Adrenal, Inhibits Dopamine D1 Receptor Mediated Secretion of Aldosterone

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Abstract

Heterogeneity among APAs has been highlighted by the discovery of somatic mutations. *KCNJ5* mutations predominate in large zona fasciculata (ZF)-like APAs; mutations in *CACNA1D, ATP1A1, ATP2B3* and *CTNNB1* are more likely to be found in small zona glomerulosa (ZG)-like APAs. Microarray comparison of *KCNJ5* mutant versus wild-type APAs revealed significant differences in transcriptomes. *NEFM*, encoding a neurofilament subunit which is a dopamine D1 receptor (D1R) interacting protein, was 4-fold-upregulated in ZG-like vs. ZF-like APAs, and 14-fold more highly expressed in normal ZG vs. ZF. Immunohistochemistry confirmed selective expression of NEFM in ZG and in ZG-like APAs. Silencing *NEFM* in adrenocortical H295R cells increased basal aldosterone secretion and cell proliferation; silencing also amplified aldosterone stimulation by the D1R agonist, fenoldopam, and inhibition by the D1R antagonist, SCH23390. NEFM co-immunoprecipitated with D1R, and its expression was stimulated by fenoldopam. Immunohistochemistry for D1R was mainly intracellular in ZG-like APAs but membranous in ZF-like APAs. Aldosterone secretion in response to fenoldopam in primary cells from ZF-like APAs was higher than in cells from ZG-like APAs. Transfection of mutant KCNJ5 caused a large reduction in NEFM expression in H295R cells. We conclude that NEFM is a negative regulator of aldosterone production and cell proliferation, in part by facilitating D1R internalization from the plasma membrane. Down-regulation of NEFM in ZF-like APAs may contribute to a D1R/D2R imbalance underlying variable pharmacological responses to dopaminergic drugs among patients with APAs. Finally, taken together, our data point to the possibility that ZF-like APAs are in fact ZG in origin.
Key words: adrenal cortex, primary aldosteronism, aldosterone, dopamine, endocrine.
Introduction

Aldosterone producing adenomas (APAs) are responsible for about 5% of hypertension, and are probably its most common curable cause.\textsuperscript{1} The heterogeneity of these tumors has been highlighted by the landmark discovery of somatic mutations of \textit{KCNJ5}, followed by others in \textit{ATP2B3}, \textit{ATP1A1}, \textit{CACNA1D} and \textit{CTNNB1}\textsuperscript{2-5}. The genotype and histological features delineate two overlapping APA subtypes: the common, large \textit{KCNJ5} mutant APAs histology is usually similar to cortisol producing zona fasciculata (ZF); smaller APAs with other mutations are mainly composed of zona glomerulosa (ZG)-like compact, lipid-depleted cells.

The two subtypes present other phenotypical differences: for instance, elevated secretion from an APA of hybrid steroids 18-hydroxycortisol and 18-oxocortisol could be considered a fingerprint of \textit{KCNJ5} mutation\textsuperscript{6}. Further differences regarding the response to aldosterone secretagogues such as angiotensin II or metoclopramide have been previously reported\textsuperscript{7,8}.

Dopamine is one of the main regulators of aldosterone secretion\textsuperscript{9}. The five known dopamine receptors belong to the G-protein-coupled receptor (GPCR) superfamily and are classified into two major subgroups (D1- and D2-like) according to their structure and opposite action on cAMP production. Activation of the D1-like subfamily members (D1R and D5R) causes an increase of intracellular cAMP levels. The D2-like subfamily includes D2R, D3R, and D4R receptors; their transduction of signal causes a reduction of cAMP production\textsuperscript{10}.

In human adrenal, most attention has been paid to the D2R sub-type. This is expressed in
normal ZG and mediates dopamine inhibition of aldosterone response to ANG II (but not to ACTH or potassium)\textsuperscript{11,12} and tonic dopaminergic aldosterone suppression\textsuperscript{13}: indeed, antagonists such as metoclopramide, but not agonists, increase plasma aldosterone concentration in normal subjects and in APA patients. However, the response to metoclopramide is inversely proportional to the percentage of ZF-like cells in the APA, attributed to downregulation of D2R in these cells\textsuperscript{13-15}.

Expression of the D1R sub-type is well documented in the ZG of non-human species, as demonstrated by in situ hybridisation, IHC and binding studies\textsuperscript{16,17}. In normal human adrenal, D1R expression has been demonstrated by RT-PCR\textsuperscript{18}, but an autoradiographic study found only D2R (and D4R) specific binding\textsuperscript{19}. Among possible explanations is rapid internalisation of D1R after stimulation by dopamine, and failure to re-traffic to the plasma membrane.

We were therefore interested to find \textit{NEFM}, encoding neurofilament medium polypeptide, in human ZG. \textit{NEFM} is one of the four subunits (alongside NEFH, NEFL and peripherin or α-internexin) comprising neurofilaments (NF), the most abundant intermediate filaments in the nervous system. Besides its structural role in the neuronal cytoskeleton, \textit{NEFM} has specialised biological functions in the synaptic densities where it co-localises with D1R and regulates its agonist-induced desensitization.

\textit{NEFM} was the fourth most up-regulated gene in ZG vs. ZF in a microarray comparison of the transcriptome of ZF, ZG, and APAs of 21 patients\textsuperscript{20,21}. \textit{NEFM} is also a biomarker for the wild-type, ZG-like subtype of APAs; compared to these it was four-fold down-regulated in \textit{KCNJ5}
mutant, ZF-like APAs\textsuperscript{22}. We hypothesized that NEFM plays a role in regulating human zona glomerulosa endocrine function through a similar relationship with D1R as described in the nervous system.

**Methods**

*(Further details are provided in the online-only Data Supplement for each method).*

**Human subjects**

Human adrenal tissues from patients who underwent adrenalectomy after being diagnosed with unilateral APA or phaeocromocytoma were obtained from Cambridge University Hospitals’ Human Research Tissue Bank post-surgery at Addenbrooke’s Hospital, Cambridge, United Kingdom. All tissues were obtained with approval from the Cambridgeshire Research Ethics Committee with written informed consent prior to surgery. APAs and their paired adjacent normal adrenal were identified and macroscopically dissected by histopathologists and separated into three categories for processing: a) snap-frozen in liquid nitrogen and then stored at -70°C for immunohistochemistry or DNA extraction, or b) stored in RNA-later for RNA extraction or c) digested with collagenase for two hours and then placed in Dulbecco's Modified Eagle's Medium at 37 °C in 5% CO\textsubscript{2} for cell culture.

**qPCR Analysis of Gene Expression**

Reverse transcription was performed using the reverse transcriptase system (Promega) with a 1:1 mixture of random and oligo primers according to the manufacturer's instructions. mRNA expression of genes of interest was quantified using TaqMan probes (Applied
Biosystems), and CYP11B2 expression was quantified using custom-made probes as specified before.

**Western blotting**

Western blotting was conducted using total protein extracts. Proteins were separated by electrophoresis in 8-10% SDS-polyacrylamide gel and transferred to a PVDF membrane, immunoblotted with anti-NEFM (Sigma-Aldrich, UK; 1:500 dilution). Anti-GAPDH (#G8795, Sigma, UK; 1:10,000 dilution) was used as an internal control for protein abundance.

**Co-immunoprecipitation**

500 µg of whole-cell lysates protein with a final volume of 1 ml were pre-cleared by incubation with 10 µl of Protein A/G PLUS-Agarose (Santa Cruz, sc-2003) at 4 °C for 1 hour followed by centrifugation at 4 °C for 1 min at 4000 rpm. The supernatant was subjected to immunoprecipitation by the addition of 1 µg of mouse anti-D1R (Santa Cruz, sc-33660) antibodies and incubated overnight at 4 °C, followed by incubation with Protein A/G PLUS-Agarose for 2 hours. After wash and centrifugation, the samples were resolved by SDS-PAGE and subjected to Western blot analysis.

**Cell culture**

H295R cells were plated into 24-well plates (10^4 cells/well) in 0.5 mL of antibiotic-free medium. Vector controls, pCMV6-AC-GFP (Origene), and green fluorescent protein (GFP)-tagged KCNJ5 WT or mutants (Dell157 and T158A) were transfected using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s guidelines.

Silencing was performed using individual ON-TARGETplus NEFM small interfering RNA (siRNA) and non-targeting siRNA as control (Dharmacon; 10 nM). After 48 hours, H295R cells were serum deprived for 8 hours, and further drug treatments with dopamine only or
D1R agonist fenoldopam and antagonist SCH23390 or D2R antagonist metoclopramide were carried out with 24 hours incubation from this point. All drugs were diluted in media in presence of AngII (10^{-8} M), Dopamine (10^{-7} M) and vitamin C (0.1\%)^{23,24}. Supernatants for aldosterone concentration measurement were collected, and cells were harvested for protein assay or RNA extraction.

Primary adrenocortical cells were obtained from the adrenals surgically removed from patients with APA at Addenbrooke’s Hospital, Cambridge, UK. APAs were categorised as ZF-like or ZG-like, as previously described, and we used APAs with somatic mutation of either KCNJ5, or CACNA1D, respectively^{25}.

**NEFM shRNA**

In order to visualize cells at confocal microscopy, we transfected cells with shRNA plasmid expressing eGFP. pLVTH was a gift from Didier Trono (Addgene plasmid # 12262). ShRNA sequence targeting NEFM was cloned into pLVTH plasmid using QS site directed mutagenesis kit (NEB).

**Cell Proliferation Assays**

H295R cells were transfected with non-targeting control and NEFM siRNA and IncuCyte Kinetic Live Cell Imaging System (Essen BioScience) was used to measure live cell growth at 37°C and 5% CO₂ in 5 wells per treatment over 72 hours.

**Aldosterone and protein measurement**

Commercially available Homogenous Time Resolved Fluorescence Resonance Energy Transfer (HTR-FRET) assay from Cisbio Bioassays, France was used according to
manufacturer’s instructions. The aldosterone concentrations from H295R and primary cells were normalized to total cell protein, which was determined by performing the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, USA).

**Confocal Imaging**

H295R cells were cultured in supplemented media on sterilised cover-slips for 24-h. After treatment and proper incubations, cells were incubated with the 597-conjugated chicken anti-D1R antibody (a kind gift of Professor Felder and Professor Wang, 1:100 dilution) in 3% BSA-PBST. Confocal images were taken using Zeiss LSM510 Meta confocal microscope and analysed using Zen 2011 software.

**Immunohistochemistry**

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded sections (4 µm) using an automated immunostainer with cover tile technology (Bond-III system, Leica Biosystems). The commercial antibody anti-NEFM (Sigma, HPA022845), anti-D1R (Sigma, HPA013393), anti-KCNJ5 (Sigma, HPA017353), custom made anti-CYP11B1 (Severn Biotech Ltd.), and anti-CYP11B2 antibody (a kind gift from Dr. Celso E. Gomez-Sanchez) were used as the primary antibodies.

**Data Analysis**

Each experiment was performed with biological replicates and the averages were calculated. Results are expressed as mean values with SEM. Differences between two groups were analysed for statistical significance by t test and multiple groups were analysed by one- or two-way ANOVA followed by Tukey’s post hoc test. The significance level of P<0.05 was considered to indicate statistical significance. Statistical analysis was performed as indicated using the standard statistical software, Prism 6 (GraphPad Software, Inc).
Results

NEFM expression in normal zona glomerulosa and in KCNJ5 mutant and wild-type tumours

The antibodies used for IHC have been validated by either immunizing peptide blocking experiments or by WB following silencing (Supplementary Figure S1). NEFM protein expression in the normal adrenal glands adjacent to APA or phaeocromocytoma was highly selective for ZG vs. ZF (Figure 1, panel A). The staining shows two patterns: diffusely cytoplasmic (Figure 1, panel A, sub-panel ii)) or more localized in the perimembranous compartment (Figure 1, panel A, sub-panel iii)). Compared to other potential markers for ZG, CYP11B2 staining is more patchy than NEFM, and KCNJ5 is less selective because the expression extends into the outer ZF. (Supplementary Figure S2).

There was similar diffuse cytoplasmic staining for NEFM in ZG-like APAs. By contrast, NEFM staining was absent in normal ZF and ZF-like, KCNJ5 mutant APA (Figure 1, panel B).

NEFM regulates aldosterone secretion

Silencing of NEFM in H295R cells, which reduced NEFM mRNA by 51% compared with the non-targeting control and resulted in >80% decrease in protein levels (Supplementary Figure S1), increased aldosterone secretion by 38% (P<0.05; Figure 2); this was accompanied by 57% and 64% decrease in CYP11B2 and NR4A2 mRNA expression levels, respectively (P<0.05; Figure 2). Similar results were obtained when the gene silencing was performed by transfection of shRNA plasmid (Supplementary Figure S3).
NEFM regulates proliferation

*NEFM* silencing in H295R cells caused an increase in cell confluence as detected by Incucyte (42% at 72 hours for silencing NEFM vs. 29% for control, *P*< 0.0001, Supplementary Figure S4 panel A). The capability of forming colonies was consistently increased in H295R cells after *NEFM* silencing by shRNA. At 3 weeks after shRNA transfection, the number of colonies was higher in comparison to empty vector (*P*<0.05, Supplementary Figure S4 panel B).

NEFM prevents aldosterone response to D1R stimulation in H295R cells

The responses to dopamine are the net effect of adenylate cyclase stimulation and inhibition by, respectively, D1R and D2R. To test whether NEFM knock down affects the D1R-mediated aldosterone response of H295R cells we performed drug experiments with dopamine only or the D1R agonist fenoldopam and antagonist SCH23390.

The net response to dopamine alone was a reduction in aldosterone secretion, by 65±10%, whereas in si-NEFM transfected cells dopamine stimulated aldosterone secretion by 26±10% (*P*< 0.001, Figure 3, panel A).

In the experiments using selective D1R agonist and antagonist, non-significant trends in the control (non-silenced) cells were amplified by silencing of NEFM. The D1R antagonist SCH23390 inhibited aldosterone secretion by 41±4% in si-NEFM transfected cells (*P*<0.05), compared to 13±6% (NS) in control cells (Figure 3, Panel B). The D1R agonist fenoldopam increased aldosterone by 75±25% in si-NEFM transfected cells (*P*<0.05) compared to 24% in controls (Figure 3, panel C). In the presence of D2R blockade by metoclopramide, the net
response was an increase in aldosterone secretion, and there was no impact on this of si-NEFM transfection (Figure 3, panel D).

**Interaction between NEFM and D1R in primary adrenal tissue**

D1R expression in normal adrenal followed that of NEFM, appearing mainly in ZG (and medulla), and mainly cytoplasmic; weak staining of ZF was mainly membranous (Supplementary Figure S5). Similarly, ZG- and ZF-like APAs differed in their D1R protein staining pattern. In ZG-like APAs, higher NEFM protein expression was associated with mainly cytoplasmic D1R staining; in ZF-like APAs, which have lower or absent NEFM expression, D1R protein staining was mainly membranous (Figure 4, panel A).

We evaluated the aldosterone response to D1R agonist fenoldopam in primary cell cultures from the two sub-types of APA. Fenoldopam increased aldosterone secretion in primary cells from ZF-like APAs (low NEFM expression) by 2.3±0.3 fold vs. 1.3±0.1 fold in those from ZG-like APAs (high NEFM expression, P<0.05, Figure 4, panel B).

**Interactions between NEFM and D1R proteins in H295R cells**

We found that endogenous D1R protein was localised in the cytoplasm of H295R cells (Supplementary Figure S6). To further support the relationship between NEFM and D1R we performed a pull-down experiment and found that NEFM co-immunoprecipitates with D1R in H295R cells (Figure 4, panel C). NEFM knock-down by shRNA increased D1R localization at plasma membrane, as found at immunofluorescence microscopy in transfected H295R cells (Figure 4, panel D).
In addition, fenoldopam stimulated NEFM protein expression, consistent with a role in D1R desensitization and internalization in response to agonist (Supplementary Figure S7).

**KCNJ5 mutants affect NEFM expression**

The possibility that reduced NEFM expression in ZF-like APAs is consequent on the KCNJ5 mutation was addressed by expression of KCNJ5 mutants in H295R cells. Transfection with I157DEL or T158A reduced NEFM mRNA by, respectively, 40% and 60% in comparison to KCNJ5 wild-type (P<0.05, Figure 5 panel A), associated with a similar reduction at protein level (Figure 5 panel B).

**Discussion**

The comparison of ZG vs. ZF transcriptomes has revealed several unsuspected genes many-fold up-regulated in human ZG. NEFM was the fourth most up-regulated gene in ZG\(^21\). Moreover, its expression was ~4-fold higher in ZG-like vs. ZF-like APAs\(^22\). Our previous functional studies of two of the ZG-specific genes, LGR5 and DACH1, showed that they inhibit aldosterone secretion and proliferation. This surprising finding, together with their absence from up-regulated genes in rodent ZG\(^26\), and patchiness of aldosterone synthase in human adrenal, was interpreted as an adaptive mechanism to high salt intake in western diet\(^20,21\).

NEFM encodes one of the subunits forming the Neurofilament (NF), the type IV intermediate filament of mature neurons, one of the most abundant proteins in the nervous
system. NF subunits perform extra-cytoskeletal roles in the neuronal synapses as receptor-interacting proteins. For instance, NEFL interacts directly with one of the subunits of the N-methyl-d-aspartate (NMDA) receptor in the synapses of glutamatergic neurons\textsuperscript{27}. NEFM is a DR-interacting protein. It interacts directly with the cytoplasmic loop of D1R\textsuperscript{28}. In transgenic mice, the specific deletion of NEFM, but not of NEFL or NEFH, amplified the D1R-mediated motor responses to cocaine and caused the redistribution of postsynaptic D1R to the plasma membrane from the endocytic-recycling compartment\textsuperscript{29}. In brain and extra-neuronal cancer NEFM, NEFL and NEFH play a role as onco-suppressors, as their epigenetic silencing affects cell proliferation, and correlates with aggressiveness and prognosis\textsuperscript{30–33}.

Similar to other up-regulated genes in human ZG, we found that NEFM suppresses aldosterone secretion and cell proliferation. In addition, it reduced dopamine stimulation of D1R responses. We infer that, as in the synapse, NEFM in normal ZG and in ZG-like APAs binds to D1R, facilitating its desensitisation by internalisation, so attenuating adenylate cyclase activation and stimulation of aldosterone secretion. Consistent with this conclusion, we observed a different D1R staining pattern in ZF-like APAs, where the plasma membrane localisation is attributable to the down-regulation of NEFM. In support of this histological observation, \textit{in vitro} aldosterone response to the D1R agonist fenoldopam was larger in primary cells from ZF-like APAs than in those from ZG-like APAs.

The net effect of dopamine is usually to inhibit aldosterone secretion, this predominant action being mediated by D2R\textsuperscript{8}. Interestingly, the dopaminergic inhibition of aldosterone
secretion in APAs varies with their cellular composition, with the consequence that aldosterone secretion in response to the D2R antagonist metoclopramide is inversely correlated to the percentage of ZF-like cells. D2R was subsequently reported to be down-regulated in APAs with high ZF-like cell composition. This report was prior to recognition of KCNJ5 mutations in ZF-like APAs. Our results offer further insight to dopaminergic responses in this sub-type: not only is D2R down-regulated, but the dominant effect of dopamine is likely to be on their D1R, consequent on reduced NEFM expression.

Our study presents some limitations. Because the primary focus of our investigation was NEFM, we have concentrated on the D1R, in the regulation of which NEFM was previously implicated. The D2R function and expression in normal ZG and APAs and its correlation with the cellular composition relies mainly on previous literature. Our experiment using metoclopramide showed that D2R blockade un masks a net stimulatory effect of dopamine on aldosterone secretion, but future studies on the relationship between APA genotype and D2R function may be required. We used immunostaining, which is a semi-quantitative technique, rather than autoradiography, to show probable surface expression of D1R in ZF-like APAs and NEFM-silenced adrenocortical cells and our range of colours did not allow for use of a plasma membrane marker. However, D1R agonist and antagonist modulated aldosterone secretion in H295R cells and, more importantly, in primary cells from APAs, suggested that they express functional receptors.

One further finding needs comment, the reduction in CYP11B2 expression associated with the increased aldosterone secretion consequent on silencing of NEFM. We have noted
aldosterone secretion and synthesis to change in opposite directions in previous published experiments, and infer that this points to regulatory influences downstream of transcription, for instance on cholesterol uptake into the inner mitochondrial membrane via the steroidogenic acute regulatory (STAR) protein. Although in recent years the emphasis in pathways to aldosterone secretion has been via stimuli to CYP11B2 transcription, earlier literature showed that many effects, including postural changes and those of dopamine, occur within minutes – namely too fast for a transcriptional response.34–36

Finally, the recognition of NEFM as a selective marker of ZG-like APAs gave us the opportunity for testing whether the ZF-like phenotype might be a consequence of its commonest genotype, namely the KCNJ5 mutation, rather than from arising in ZF cells, where KCNJ5 expression is scarce. The disappearance of NEFM from KCNJ5 transfected cells would favour the first of these alternatives, and indeed explain why D1R remains on the surface of ZF-like APA cells. But clearly further experiments are required to settle this important issue.

In conclusion, we have found that NEFM contributes to the dopamine regulation of aldosterone secretion in human normal ZG. D1R is functionally expressed in ZG and ZG-like APAs but its interaction with NEFM facilitates internalization and desensitization. NEFM down-regulation in ZF-like APAs cells contributes to D1R localization on the plasma membrane and its activation by agonists.

**Perspectives**

APAs represent the most common surgically curable cause of hypertension. The somatic mutations recently discovered in APAs prompted investigation of transcriptome differences
between genotypes, and discovery that these vary in their resemblance to normal adrenocortical ZG and ZF. NEFM is selectively expressed in human ZG where its function is similar to the non-cytoskeletal role played in the neuronal synapses. NEFM expression differs between the two APA subtypes and its down-regulation in ZF-like APAs appears to increase their D1R responsiveness. The measurement of plasma aldosterone levels (in peripheral or adrenal veins) after D1R stimulation with fenoldopam could help to predict APA genotype.

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Disclosures

None.
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NOVELTY AND SIGNIFICANCE

What is new?

*NEFM* is shown to be a ZG-selective transcript that is not just a marker, but plays a functional role in desensitizing dopamine-mediate stimulation of aldosterone secretion. The different consequences of dopamine stimulation or blockade in ZF- and ZG-like APAs is the first demonstration of a functional consequence of their differing transcriptomes.

What is Relevant?

NEFM expression variability between the two APA subtypes represents an example of how differences in genotypes can have potential clinical consequences, such as variable responses to pharmacological agents used in diagnosis and/or therapy.

Summary

*NEFM* is a ZG-selective transcript in human adrenal which reduces aldosterone secretion directly and in response to dopamine. The two APA subtypes differ in *NEFM* expression, whose inverse correlation with D1R response appeared due to NEFM-mediated internalisation of D1R. The negative regulation of NEFM expression following transfection of mutant *KCNJ5* suggests that ZF-like properties may be a consequence of mutation, rather than tissue of origin.
Figure Legends

Figure 1. Immunohistochemistry (IHC) of NEFM in formalin-fixed paraffin-embedded human adrenal sections. 
A, IHC of NEFM in adjacent normal adrenal cortex. NEFM is highly expressed in zona glomerulosa (ZG) cells. The staining shows two patterns: diffusely cytoplasmic (panel A, sub-panel ii)) or more localized in the perimembranous compartment (panel A, sub-panel iii)).
B, IHC of NEFM in APAs. NEFM is more expressed in ZG-like than ZF-like APAs. i) and iv) scanned images. ii), iii), v), vi) insets of left panels at different magnifications.

Figure 2. Effect of NEFM on aldosterone production. Silencing of NEFM in H295R cells increased aldosterone production compared with control (non targeting). H295R cells were transfected with nontargeting siRNA or siNEFM for 48 h after which 24-h supernatant was collected for aldosterone measurement (n=12).

Figure 3. Effect of silencing NEFM on aldosterone response to dopamine and D1R and D2R drugs. Panel A. Silencing NEFM blunted dopamine inhibition on aldosterone secretion. Panel B and C. Silencing NEFM amplified aldosterone inhibition and stimulation by D1R antagonist SCH23390 and agonist fenoldopam, respectively. Panel D. D2R mediated aldosterone suppression was not affected as aldosterone response to its antagonist metoclopramide was not affected (n=16).

Figure 4: Interactions between NEFM and D1R proteins in primary adrenal tissue and H295R
Panel A: IHC for D1R of paraffin-embedded human adrenal sections including ZG-like (sub-panels i), ii, iii), and ZF-like APAs (sub-panels iv), v), vi). In ZG-like APA D1R is mainly localized in the cytoplasm. In ZF-like APAs D1R is mainly localized on the plasma membrane. i), iii) scanned images. ii), iv), zoomed insets of left panels.

Panel B: Aldosterone secretion in primary adrenal cells from ZG-like and ZF-like APAs (n = 12 each) in response to fenoldopam. Each concentration was replicated 4 times within each individual patient samples. The stimulation by fenoldopam at maximal dose (10⁻⁷ M) was bigger in KCNJ5 mutant, ZF-like APAs (P<0.05 at 10⁻⁷ M). Aldosterone results are relative to 0 M of treatment. Two-way ANOVA was used to calculate significance.

Panel C: Co-immunoprecipitation of D1R pulls down NEFM, confirming the interaction between NEFM and D1R in H295R. Monoclonal mouse anti-D1R (or PBS as negative control) was used to precipitate the protein complexes containing D1R, and the presence of NEFM protein in these complexes was subsequently examined by immunoblotting with a polyclonal rabbit anti-NEFM antibody.

Panel D: Immunofluorescence staining of D1R in H295R cells 48 hours after NEFM-shRNA transfection. GFP-positive transfected cells showed more perimembranous fluorescence staining for D1R (red).

Figure 5: Effect of the transfection of KCNJ5 mutations on NEFM mRNA and protein levels in H295R cells. In comparison to wild-type, KCNJ5¹⁵⁷⁰del and KCNJ5¹⁵⁸⁸Δ caused a decrease of NEFM mRNA level by 34% and 59%, respectively (panel 1 n=8, P<0.05). Similar results were obtained at protein level (panel 2).