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Disruption of the MAGI2-RapGEF2-Rap1 signaling axis contributes to podocyte dysfunction in congenital nephrotic syndrome caused by mutations in MAGI2

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Abstract:

The essential role of MAGI2 in podocytes is reflected by the severe glomerulosclerosis phenotypes of both MAGI2 knockout mice and of humans with congenital nephrotic syndrome (CNS) caused by mutations in MAGI2. Here, we show MAGI2 directly binds the Rap1 guanine nucleotide exchange factor, RapGEF2, and that this interaction is lost when expressing MAGI2 CNS variants. Co-expression of RapGEF2 with wild-type MAGI2, but not MAGI2 CNS variants, enhanced activation of the small GTPase Rap1, a central signaling node in podocytes. In mice, podocyte-specific RapGEF2 deletion resulted in spontaneous glomerulosclerosis, with qualitative glomerular features comparable to MAGI2 knockout mice. Knockdown of RapGEF2 or MAGI2 in human podocytes caused similar reductions in levels of Rap1 activation and Rap1-mediated downstream signaling. Furthermore, human podocytes expressing MAGI2 CNS variants show dramatically decreased actin polymerization and abnormal cellular morphology that is completely rescued by pharmacological activation of Rap1 via a non-MAGI2 dependent upstream pathway. Finally, immunostaining of kidney sections from CNS patients with MAGI2 mutations showed reduced podocyte Rap1-mediated signaling. We conclude that MAGI2-RapGEF2-Rap1 signaling is essential for normal podocyte function and that disruption of this pathway is an important cause of the renal phenotype induced by MAGI2 CNS mutations.

Keywords:

podocyte, focal segmental glomerulosclerosis, nephrotic syndrome

Introduction:

Membrane associated guanylate kinase 2 (MAGI2) belongs to the MAGUK family of scaffolding proteins. It is composed of five PDZ domains, two WW domains, a Src homology (SH3) domain, and a catalytically inactive guanylate kinase domain. These multiple protein-protein interacting domains predict dual functions as a molecular scaffold and signaling hub. The MAGI subfamily has three members, designated MAGI1, MAGI2, and MAGI3, which share identical protein domains and similar molecular structure. In kidney, MAGI2 is exclusively expressed in podocytes ¹, the terminally differentiated epithelial cells that line the outer aspect of the glomerular basement membrane and regulate the filtration of blood to form urine. Within the podocyte, it is specifically expressed at the slit diaphragm ^{1,2}, a specialized cell-cell junction that bridges adjacent interdigitating podocyte foot processes. All forms of human proteinuric kidney disease, regardless of specific etiology, result in podocyte injury with loss of normal foot process architecture, a pathological process known as effacement.

Multiple recent individual MAGI2 knockout mouse models all demonstrate the gene's essential roles in podocyte development and function ³⁻⁶. Two global knockout mouse models die within the first 24 hours after birth from anuric renal failure with absence of slit diaphragm formation ^{3,5}. The third global knockout model ⁴ and a podocyte-specific knockout ⁶ both develop severe focal segmental and global glomerulosclerosis with renal failure and early lethality. In humans as well, convincing disease-causing mutations in MAGI2 give rise to congenital nephrotic syndrome (CNS) ^{7,8}. Most cases present with severe nephrotic syndrome in the first few months of life. While these findings establish the critical role of MAGI2 in proper podocyte function in mice and humans, the underlying mechanisms are poorly understood.

MAGI proteins have previously been implicated as upstream activators of the small GTPase Rap1^{9,10}, which regulates multiple essential downstream signaling pathways in podocytes including activation of β 1 integrin¹¹. In fact, mice with reduced podocyte Rap1 expression develop severe glomerulosclerosis and early death from renal failure by 8 weeks of age¹¹. Rap1 cycles between an active GTP-bound form and an inactive GDP-bound form. The balance between active and inactive Rap1 is tightly regulated by numerous upstream factors including guanine nucleotide exchange factors (GEFs), which turn on signaling by catalyzing the exchange of GDP to GTP, and GTPase activating proteins (GAPs), which terminate signaling by hydrolyzing GTP to GDP¹². Rap1-specific GEFs include RapGEF1, RapGEF2 (PDZ-GEF1), RapGEF3 (EPAC1), RapGEF4 (EPAC2), RapGEF5, and RapGEF6 (PDZ-GEF2). RapGEF2 has been shown to bind MAGI proteins¹⁰, to have enriched expression in the mammalian glomerulus¹³, and to be important in maintenance of the glomerular filtration barrier in zebrafish¹⁴. With this background, we aimed to investigate the interaction between RapGEF2 and MAGI2 as potential upstream regulator of podocyte Rap1 activation.

Results:

MAGI2 variants that cause congenital nephrotic syndrome fail to interact with RapGEF2 and are unable to induce Rap1 activation

Two homozygous truncating point mutations in the human MAGI2 gene (pGly39* and pTyr746*) have been identified as directly causative of CNS⁷ (comparison of domain organizations, figure 1A). Both of the affected patients presented with severe CNS early in childhood⁷. Reciprocal co-immunoprecipitation experiments performed between FLAG-RapGEF2 and full-length GFP-MAGI2 show a robust protein:protein interaction (figure

1B). This protein interaction, however, is completely lost when substituting either of the GFP-tagged MAGI2 disease causing variants. GFP-Toca, used as a negative control, also failed to interact with FLAG-RapGEF2.

To look for a functional consequence to the loss of this interaction, we tested the ability of each to induce Rap1 activation (figure 2A). We utilized calcium switch as a means to induce cellular Rap1 activation using a previously established protocol^{9,10}. Confluent transfected 293T cells were exposed briefly to calcium chelation to disrupt intercellular contacts (EGTA washout), after which full calcium containing medium was restored (calcium switch). Consistent with published data, we found a general increase in levels of Rap1 activation in all transfectants after calcium switch (figure 2A). However, this effect was dramatically enhanced in 293T cells expressing both RapGEF2 and full-length MAGI2, but was not present in cells expressing both RapGEF2 and either of the MAGI2 mutant variants. Previous work had also implicated MAGI1 as a regulator of podocyte Rap1 activation¹⁰. However, using an identical calcium switch system, the combined expression of MAGI1 and RapGEF2 did not augment Rap1 activation beyond baseline (figure 2B). Overall, these findings demonstrate that the interaction of MAGI2, but not disease causing MAGI2 variants, with RapGEF2 functions as a potent stimulus for Rap1 activation.

Podocyte-specific RapGEF2 KO mice develop FSGS with comparable qualitative glomerular features compared to MAGI2 KO mice

We next performed detailed immunofluorescence studies to localize RapGEF2 protein within wild-type kidney (figure 3A). Unlike MAGI2, RapGEF2 is robustly expressed in both the glomerular and tubular compartments. Within the glomerulus, it is

strongly expressed in podocytes. Its cellular localization within the podocyte is complex, with expression predominantly in the cytoplasm, where it co-localizes with podocyte cytoplasmic marker nestin.

We hypothesized that if RapGEF2 function was mediated by its interaction with MAGI2, then RapGEF2 knockout mice, similar to MAGI2 deficient mice, would demonstrate severe podocyte dysfunction. Because, global RapGEF2 knockout mice are early embryonic lethal ¹⁵, we bred a conditional floxed RapGEF2 knockout mouse line ¹⁵ to a transgenic mouse line that expresses cre recombinase under podocyte-specific promoter podocin ¹⁶. This allowed the generation of podocyte-specific knockout of RapGEF2. RapGEF2 immunostaining verifies loss of glomerular, but preservation of tubular, RapGEF2 expression in these mice (figure 3B).

Podocyte-specific RapGEF2 KO mice develop overt proteinuria at between 6 and 16 weeks of age (figure 4A) with progressive glomerulosclerosis and severe proteinuria by 20 weeks of age (figure 4B). The renal phenotype is comparable to that described for podocyte-specific MAGI2 KO ⁶ and global MAGI2 KO mice ⁴, although onset of proteinuria for the RapGEF2 model is later and glomerulosclerosis scores overall less severe (figure 4C). Renal histological analysis reveals similar qualitative glomerular features between RapGEF2 and MAGI2 models that include focal segmental and global glomerulosclerosis, mesangial expansion, podocyte loss, and glomerular epithelial cell proliferation with pseudocrescent formation (figure 4D). Electron microscopic features are similar between the two models: diffuse severe foot process effacement with basal actin matting, simplification of overall podocyte cellular shape, loss of normal primary and secondary process architecture, and cytoplasmic vacuolization (figure 4E). Podocyte loss is a major driving force for development of glomerulosclerosis ¹⁷. To quantify podocyte

depletion in our model, we calculated the number of WT1-positive cells per glomerular tuft area (figure 4F). Consistent with the severity of their pathologic findings, podocyte loss was greater in MAGI2 KO mice than in RapGEF2 KO mice, but both models showed substantially fewer podocytes compared to control mice.

RapGEF2 or MAGI2 knockdown in podocytes similarly diminish Rap1-GTP activation and signaling

We hypothesized that podocyte dysfunction in both the RapGEF2 and MAGI2 KO mouse models was caused by similar reductions in podocyte Rap1 activation and downstream Rap1 signaling pathway activation. To investigate this possibility, we transduced a shRNA expression plasmid that targeted either RapGEF2 or MAGI2 RNA into a conditionally immortalized human podocyte cell line¹⁸. To eliminate the possibility of non-specific off target effects, we utilized two different hairpins per gene, each with a distinct RNA target sequence. In this way, we generated two distinct RapGEF2 knockdown and two distinct MAGI2 knockdown podocyte cell lines. By western blotting, we confirmed severely reduced RapGEF2 or MAGI2 protein expression in each of the knockdown podocyte cell lines compared to control podocytes that were transduced with a scrambled shRNA expression plasmid (figure 5A).

In the setting of calcium switch, RapGEF2 and MAGI2 knockdown podocytes showed similar reductions in the level of Rap1 activation (figure 5A) as well as dramatically reduced levels of essential Rap1 mediated downstream signals including β 1 integrin activation and Erk phosphorylation (figure 5B). To detect levels of activated β 1 integrin, native PAGE electrophoresis (non-reducing and non-denaturing sample buffer) was utilized; under these conditions, the monoclonal antibody 12G10 specifically detects

the activated form of $\beta 1$ integrin. Next, we studied cellular migration rates, which are substantially reduced in podocytes that have diminished cellular Rap1 activation ¹¹, a reliable phenotype observed in podocyte-specific knockdown of other genes known to cause nephrotic syndrome ^{7,19,20}. Again, RapGEF2 and MAGI2 knockdown podocyte cell lines behaved similarly, each showing dramatic reductions in migratory rates (figure 5C). Diminished cellular Rap1 activation was also previously reported to enhance podocyte susceptibility to apoptosis ¹¹. By Annexin PI staining, we found comparable high rates of podocyte apoptosis under basal condition for both RapGEF2 and MAGI2 knockdown podocytes compared to control cells (figure 6A, 6B). Taken together, these data demonstrate that loss of either RapGEF2 or MAGI2 in podocytes causes disruption to Rap1 activation and downstream signaling.

Human podocytes that express MAGI2 CNS causing variants are phenotypically rescued by pharmacological activation of Rap1

Here, we generated individual human podocyte cell lines that express FLAG-tagged wild type MAGI2 and each of the FLAG-tagged CNS MAGI2 human mutant variants (pGly39* and pTyr746*). This was accomplished by transduction of a lentiviral expression plasmid into human podocytes under permissive conditions (vector backbone pSF-Lenti), followed by selection with puromycin. Robust expression of wild type and mutant versions of MAGI2 in transduced podocytes is shown by immunofluorescence using an anti-FLAG antibody as compared to control vector-alone transduced podocytes (figure 7A). Interestingly, while wild type MAGI2 strongly localized to sites of intercellular contact, both of the MAG2 mutant proteins were completely mislocalized,

showing robust accumulation in cytoplasmic vesicles and almost no expression at intercellular contacts. Control podocytes lacked any immunoreactivity.

Differentiated podocytes that expressed either of the human MAGI2 disease causing variants, demonstrated severe abnormalities of cellular morphology, including an elongated cellular shape and dramatically diminished actin polymerization (figure 7B, C). Vector-alone and wild type MAGI2 transduced podocyte lines each demonstrated normal morphology and overall actin content. Podocytes expressing either of the MAGI2 disease causing mutations show diminished basal Rap1 activation compared to podocytes expressing wild type MAGI2 (figure 7D). To show that inactivation of the MAGI2-RapGEF2-Rap1 signal is the cause of podocyte dysfunction induced by disease causing MAGI2 mutations, rescue experiments were performed using pharmacological activation of Rap1 via a non-MAGI2 dependent upstream pathway. Individual podocyte cell lines were split from the same flask prior to moving to 37°C, and then maintained under non-permissive conditions for seven days, either with or without the potent Rap1 activating drug 8-pCPT-2'-O-Me-cAMP (10µM)²¹. In the presence of the drug, levels of activated Rap1 were dramatically increased in the mutant MAGI2 expressing podocyte cell lines to levels similar to wild type MAGI2 expressing cells (figure 7D). Impressively, mutant MAGI2 transduced podocytes were completely rescued by pharmacological Rap1 activation including normalization of cellular shape and robust levels of actin polymerization (figure 7B, C). These findings suggest that inactivation of RapGEF2-Rap1 signaling is an important cause of the renal phenotype induced by MAGI2 CNS mutations.

Immunostaining of kidney sections from CNS patients with MAGI2 mutations is consistent with reduced glomerular Rap1GTPase mediated signaling

To corroborate our findings in humans, we performed immunohistochemistry on kidney sections from patients with CNS due to mutations in MAGI2⁸. Because direct staining for activated Rap1 is not feasible, we investigated activation of the Rap1-mediated downstream signal Erk1/2 (figure 8A, 8B). While phosphorylated Erk1/2 is highly enriched in podocytes of control kidney, it is essentially undetectable in podocytes from patients with CNS caused by MAGI2 mutations. Levels of Erk1/2 phosphorylation were only moderately reduced in podocytes from idiopathic FSGS patients compared to controls, suggesting that such dramatic loss of podocyte Erk1/2 phosphorylation cannot be generalized to all forms of podocyte injury. Importantly, total Erk1/2 was ubiquitously expressed in all samples, including substantially in podocytes, suggesting that podocyte loss is not the main explanation for our observed reductions in phosphorylated Erk1/2 staining. Overall, these findings are consistent with reduced levels of podocyte Rap1 activation in CNS patients harboring mutations in MAGI2.

Discussion:

In the current work, we propose a novel mechanism of podocyte injury in patients with CNS caused by mutations in MAGI2 (figure 9). In normal podocytes under physiological conditions, MAGI2 interacts directly with RapGEF2, and this interaction is required to sustain adequate levels of Rap1 activation and Rap1-mediated downstream signaling. In patients with MAGI2 mutations, however, the interaction between MAGI2 and RapGEF2 is lost, causing inability of this complex to activate Rap1. Over time, the resulting inadequate levels of podocyte Rap1 activation as well as Rap1-mediated downstream signaling induce podocyte injury and mediate FSGS. We conclude that under

the basal condition, the interaction between MAGI2 and RapGEF2 is a critical upstream signal for Rap1 activation in podocytes.

The FSGS phenotypes of global and podocyte-specific MAGI2 KO mice ^{4,6} are more severe than for podocyte-specific RapGEF2 KO mice. This difference is reflected in a more completely penetrant phenotype, earlier age of onset of proteinuria, more severe pathological findings including higher glomerulosclerosis, and earlier lethality. There are several potential explanations for this finding. First, it is likely that MAGI2 has important functions beyond its interaction with RapGEF2. MAGI2 protein contains multiple protein interacting domains (PDZ and WW) and mediates numerous protein-protein interactions essential for proper podocyte function ^{2,22,23}. For example, MAGI2 directly interacts with nephrin and forms part of the intricate cytoplasmic protein complex at the slit diaphragm ^{2,24}. Two of the three described global MAGI2 knockout mouse models have demonstrated alterations in nephrin expression and localization, suggesting that MAGI2 may have a role in nephrin stability and/or signaling ^{4,5}. Another proposed function of MAGI2, via its direct interaction with dendrin, is as regulator of Hippo signaling ^{3,25}, which has recently been shown to be critical to podocyte function ²⁶. In podocyte-specific MAGI2 knockout mice, loss of MAGI2 induced dendrin translocation to the nucleus, alterations in the Hippo regulator YAP, and podocyte apoptosis ⁶. Beyond these known interactions with nephrin and dendrin, MAGI2 may mediate other yet uncharacterized protein interactions governing podocyte maintenance. A second potential explanation for the milder phenotype of RapGEF2 KO mice is redundancy in the network of proteins that regulate Rap1 function. RapGEF2 loss of function, under the appropriate conditions, might be compensated by upregulation of other Rap1 activators including other Rap1-specific GEFs. A suitable candidate activator is RapGEF6, which is structurally similar to RapGEF2 ²⁷. Ongoing

studies will look at whether RapGEF6 is expressed in podocytes and if so, whether it can also activate Rap1 by a direct interaction with MAGI2.

We acknowledge that because several upstream signaling cascades can induce Erk1/2 phosphorylation, measuring its staining intensity as a secondary read-out for levels of Rap1 activation has limitations. However, our available starting material from MAGI2 CNS patients was limited to paraffin embedded slides only. Because there is no technically feasible way to stain directly for levels of Rap1 activation on such sections, our best option was to assay for a major downstream Rap1-mediated signal as a surrogate read-out. We selected to use immunostaining for Erk1/2 phosphorylation because it is a major downstream target of Rap1 that can be reliably detected on paraffin embedded material by a well-characterized commercially available antibody and is also relatively podocyte-specific in kidney. To minimize the possibility of a secondary effect, we analyzed levels of podocyte Erk1/2 phosphorylation in multiple cases of idiopathic FSGS and compared them to MAGI2 CNS. Levels of podocyte Erk1/2 phosphorylation were uniformly lower in MAGI2 CNS compared to idiopathic FSGS, suggesting the effect to be specific and could not be generalized to all forms of podocyte injury. Overall, these findings are consistent with our observations in MAGI2 knockdown cultured podocytes, where it was technically possible to demonstrate that diminished Erk1/2 phosphorylation was downstream of reductions in Rap1 activation.

MAGI2 expression is one of the most dramatically downregulated of all podocyte-expressed genes in human proteinuric kidney diseases including diabetic nephropathy and idiopathic FSGS (⁵, nephroseq database). Levels of podocyte activated Rap1 are likewise reduced in the same conditions ¹¹. Further studies will be required to determine whether reduced expression of MAGI2 is a cause of diminished podocyte Rap1 in these diseases.

What is clear, however, is that the activation state of podocyte Rap1 is tightly regulated both under the basal condition and in the setting of podocyte injury. Importantly, several of the regulatory proteins located upstream of Rap1, including MAGI2 but not RapGEF2, are mainly podocyte-specific. An innovative therapeutic approach would be to pharmacologically target such podocyte-specific regulatory proteins. This targeted approach would increase levels of activated Rap1 specifically in podocytes and thereby avoid potentially undesirable effects in other cell types.

Methods:

Mice

Podocin-cre transgenic mice ¹⁶, floxed RapGEF2 conditional knockout mice ¹⁵, and global MAGI2 knockout mice ⁴ were previously described. All mice were genotyped according to their previously described protocols.

Human kidney samples

We analyzed paraffin embedded kidney sections (one biopsy, one nephrectomy) from two patients with CNS caused by MAGI2 mutations ⁸. Paraffin embedded deidentified human kidney sections from archived human biopsies were provided by Vivette D'Agati with appropriate IRB approval (five separate cases). All biopsies were clinically indicated and carried a confirmed pathological diagnosis of idiopathic FSGS. Paraffin-embedded normal human kidney sections were obtained from nephrectomy samples from the Department of Pathology at Icahn School of Medicine. In all cases, only remaining tissue not required for diagnostic purposes was utilized.

Antibodies

The following antibodies were used: Rap1 (ThermoFisher Scientific, 16120), total Erk1/2 (Cell Signaling Technology, 137F5), phospho-Erk1/2 (Cell Signaling Technology (Thr202/Tyr204) D13.14.4E), activated β 1 integrin (Millipore, clone 12G10), total β 1 integrin (BD Biosciences, clone 18), MAGI2 (Sigma Aldrich, M2441), RapGEF2 (Sigma Aldrich, clone 1E8, WB), RapGEF2 (Lifespan Biosciences, LS-B12608, IF), WT1 (Abcam, CAN-R9 (IHC)-56-2), nestin (BD Biosciences, 556309), FLAG (Sigma Aldrich, M2), GFP (Clontech, 632377).

Plasmids

Expression plasmids for GFP-MAGI2 wild type, GFP-MAGI2 pGly39*, and GFP-MAGI2 pTyr746* were previously described ⁷. The expression plasmids for FLAG-RapGEF2 ²⁸ and myc-MAGI1 ⁹ have been described previously. The lentiviral expression plasmid also encoding puromycin resistance, pSF-Lenti, was purchased from Sigma-Millipore. FLAG-MAGI2 wild type, FLAG-MAGI2 pGly39*, and FLAG-MAGI2 pTyr746* were synthesized and cloned into pSF-Lenti by GenScript Biotech Corporation.

Histopathology and immunohistochemistry

All kidneys were perfused in vivo with 4% PFA. For histological analysis, kidneys were left in PFA overnight and then embedded in paraffin. Sections were cut at 2 μ m and stained with PAS. For IF, kidneys were fixed in PFA for 4 hours, transferred to 18% sucrose overnight, and then flash-frozen in OCT medium. For electron microscopy, samples were cut into 1-mm cubes and fixed overnight in 2.5% glutaraldehyde before being epoxy-embedded using standard techniques (JEOL 1011 electron microscope).

Podocyte cell culture

A conditionally immortalized human podocyte cell line was propagated and differentiated as has been described previously¹⁸.

Lentiviral infection and production

All shRNA plasmids were purchased from Origene, already cloned into the pGFP-C-shLenti backbone: RapGEF2 shRNA plasmids (TL316875), MAGI2 shRNA plasmids (TL311600), and scrambled shRNA control (TR30021). Hairpin sequences specific for human RapGEF2 are as follows: CAATGTCAGTGAGGCGAGAACTCTGTGCT (#1) and TGGTCAGTCTCAAGATGACAGCATAGTAG (#2). Hairpin sequences specific for human MAGI2 are as follows: GAACCTGAGCCATACAGAAGTAGTGGATA (#1) and TCTACTTCATTGACCATAACACAAAGACA (#2). FLAG-tagged wild type and mutant MAGI2 lentiviral expression plasmids were synthesized and cloned into pSF-Lenti by GenScript Biotech Corporation. All lentiviral preparation and infections were performed as previously described²⁹. Infections were done at the permissive temperature in conditionally immortalized human podocytes, and then stable cell lines were established by selection with puromycin at a concentration of 1-2 ug/ml. Knockdown and control transduced cell lines were grown at 37°C for at least 1 week prior to use in experiments.

Reciprocal co-immunoprecipitations

Plates of 293T cells were individually transfected with FLAG-RapGEF2, GFP-MAGI2 wild type, GFP-MAGI2 pGly39*, and GFP-MAGI2 pTry746*. Reciprocal co-immunoprecipitations were performed using antibodies to each protein's tag (either GFP-

agarose (Allele Biotechnology) or Anti-FLAG M2 affinity gel (Sigma Aldrich). Total cellular lysates were harvested in RIPA buffer, combined at equal concentrations, and then incubated overnight with either the GFP-agarose or FLAG gel. Beads were washed with RIPA buffer x 3 and then boiled. Western blotting was performed on resulting eluates and on input lysates.

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Rap1-GTP pull downs

Relative levels of active Rap1 were assessed by pull-downs using a GST-tagged fusion protein, corresponding to amino acids 788–884 of the human RalGDS-RAP-binding domain bound to glutathione-agarose. Experiments were performed using the Active Rap1 Pull-down and Detection Kit, (ThermoFisher Scientific, 16120).

Calcium switch

Confluent cells were serum-deprived overnight in medium containing 1% serum. To chelate extracellular calcium, cells were exposed to 1 mM EGTA (293T cells) or 2 mM EGTA (podocytes) for 2 minutes (293T cells) or 10 minutes (podocytes). Cells were washed twice with PBS and then returned to full serum calcium-containing medium for the indicated time.

Apoptosis Assay

Staining was performed with Annexin V APC (eBioscience) and 7-AAD (eBioscience). Samples were collected using a FACSCanto II (BD) flow cytometer and analyzed using Cytobank.

Migration Assays

Differentiated podocytes were plated to complete confluence on collagen type I-coated 6-well plates. A scratch was created using a 200- μ l sterile pipette, and loosely adherent cells were removed by washing. At specific time intervals, images were obtained at several fixed locations along the scratch. Percent wound closure was calculated using ImageJ processing program.

Quantification of immunostaining

Immunostained images with a final magnification of 400X were obtained. The level of immunostaining was measured using ImageJ software. First, acquired images were converted to 8-bit grayscale. Glomerular regions were selected for measurement of area and integrated intensity. Background intensity was measured by selecting three distinct areas that showed no staining. The corrected optical density was calculated as previously described³⁰.

Calculation of podocyte number

The number of WT1-positive cells per glomerular tuft was determined for approximately 20 glomeruli per animal (n=3 mice per group, total of 60 glomeruli per group). Glomeruli were selected at random. Glomerular area was calculated using ImageJ software.

Pharmacological Rap1 Activation

Podocytes were split into two flasks at time of thermoshift to 37°C and allowed to differentiate for one day. At this point, we added the potent Rap1 activating drug, 8-pCPT-2'-O-Me-cAMP (from Sigma Aldrich) at a concentration of 10 μ M to one flask and vehicle

only to the other. Cells were allowed to continue to differentiate under these conditions for an additional seven days prior to performing experiments. The drug 8-pCPT-2'-O-Me-cAMP activates Rap1 through EPAC²¹, a pathway independent of MAGI2 mediated Rap1 activation.

Statistics

All experiments were repeated at least three or more times. Bar graphs represent combined results from all experiments. All data is represented as mean \pm SEM. Statistical significance was determined by utilizing the 2-tailed Student's *t* test. A *P* value of less than 0.05 was considered significant. Prism software was utilized for comparison between groups.

Study Approval

All mouse studies were approved by the IACUC at Icahn School of Medicine at Mount Sinai in accordance with NIH guidelines.

Disclosure:

The authors have declared that no conflict of interest exists.

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Author contributions:

BZ, AC, JL, JY, JW, VD, and LK performed the experiments, data acquisition, and analysis. BZ, JL, SA, AB, MM, CS, SH, KC, MS, JH, FH, VD, WP, and LK provided materials, conceptualized, and designed the studies. All authors participated in the drafting and critical appraisal of the manuscript.

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Figure Legends:

Figure 1. MAGI2, but not variants that cause congenital nephrotic syndrome, interacts with RapGEF2. (A) Schematic representation of protein domain organization of wild type MAGI2 and two of its disease-causing variants, Gly39* and Tyr746*. Each variant carries a homozygous truncating point mutation. Image was generated using the Simple Modular Architecture Research Tool (SMART) web resource and then modified. (B) Reciprocal co-immunoprecipitations between FLAG-tagged RapGEF2 and GFP-tagged MAGI2 was performed using antibodies to their tags. FLAG-RapGEF2 and the indicated GFP-tagged MAGI2 protein lysates were mixed at equal concentration and incubated overnight with FLAG (upper blot) or GFP (lower blot) antibodies bound to agarose. A robust interaction is present between RapGEF2 and wild type MAGI2, but is completely lost for MAGI2 mutant variants or for the negative control, GFP-Toca. Predicted molecular weights for each protein are as follows: GFP-MAGI2 WT, 185 kDa; GFP-MAGI2 Gly39*, 37 kDa; GFP-MAGI2 Tyr746*, 110 kDa; GFP-Toca, 93 kDa; FLAG-RapGEF2, 167 kDa. **, non-specific band.

Figure 2. Combined expression of RapGEF2 and MAGI2, but not disease causing MAGI2 variants, induce Rap1 activation. (A) Confluent transfected 293T cells were exposed briefly to calcium chelation followed by restoration of full calcium-containing medium (calcium switch). Cellular lysates were collected both before and after the calcium switch and assayed for levels of active Rap1. While there was a generalized increase in Rap1 activation in all samples after calcium switch, this response was dramatically augmented in cells that expressed both RapGEF2 and MAGI2, but not in cells expressing RapGEF2 and disease causing MAGI2 alleles. 293T cells were transfected with the following expressions plasmids: lanes 1 and 6: GFP-MAGI2 WT, lanes 2 and 7: FLAG-RapGEF2, lanes 3 and 8: GFP-MAGI2 WT and FLAG-RapGEF2, lanes 4 and 9: GFP-MAGI2 Gly39* and FLAG-RapGEF2, and lanes 5 and 10: GFP-MAGI2 Tyr746* and FLAG-RapGEF2. Lower panel shows the western blot densitometric quantification comparing relative levels of Rap1 activation between groups. Ratio of active to total Rap1 was calculated for three individual experiments and then normalized. * $P < .025$, ** $P < .035$, *** $P < .03$, **** $P < .04$. Arrow indicates non-specific blotting artifact over 9th lane, which was excluded from densitometric analysis. (B) Levels of Rap1 activation were also not augmented in cells expressing RapGEF2 and MAGI1. 293T cells were transfected with the following plasmids: lane 1: GFP-MAGI2 WT and FLAG-RapGEF2, lane 2: GFP-MAGI2 Gly39* and FLAG-RapGEF2, lane 3: GFP-MAGI2 Tyr 746* and RapGEF2 and lane 4: GFP-MAGI1 and FLAG-RapGEF2. Densitometric quantification of active to total

Rap1 was calculated for three separate experiments and then normalized. * $P < .02$, ** $P < .045$, *** $P < .025$.

Figure 3. Glomerular RapGEF2 expression is lost in podocyte-specific RapGEF2 KO mice. (A) Immunostaining for RapGEF2 on kidney sections of control mice demonstrates widespread expression in both tubules and podocytes. Its podocyte expression is mainly cytoplasmic, where it co-localizes with the podocyte cytoplasmic marker nestin. Scale bar low power 50 μm ; high power 10 μm . (B) In podocyte-specific RapGEF2 KO mice, glomerular RapGEF2 expression is absent, but tubular expression preserved. Scale bar low power 50 μm ; high power 10 μm .

Figure 4. Podocyte-specific RapGEF2 KO mice develop FSGS with comparable qualitative glomerular features compared to MAGI2 KO mice. (A) Survival curve showing age of onset of proteinuria. Urine protein:creatinine ratios were measured weekly with the onset of proteinuria defined by a protein:creatinine ratio of greater than 400 $\mu\text{g}/\text{mg}$ for two consecutive weeks. (B) Calculated protein:creatinine ratios for RapGEF2 KO mice ($n=12$, at age 20 weeks or at time of sacrifice) and global MAGI2 KO mice ($n=4$, age 5 weeks). Both models develop severe proteinuria. (C) For each mouse with proteinuria, the percentage of glomeruli demonstrating glomerulosclerosis on PAS staining was calculated. (D) Comparative pathologic analysis of proteinuric RapGEF2 KO mice (age 12 weeks) with MAGI2 KO mice (age 5 weeks). Both models demonstrate focal segmental and global glomerulosclerosis, mesangial expansion, and parietal cell proliferation with pseudocrescent formation. Original magnification for control: 200X upper, 600X lower. Original magnification for RapGEF2 and MAGI2 KO: 200X upper left; 400X upper right and middle left; 600X middle right and lower left and right. Arrow shows parietal cell proliferation with pseudocrescent. (E) Electron microscopy reveals comparable qualitative features between proteinuric podocyte-specific RapGEF2 KO and MAGI2 KO mice. Both show diffuse foot process effacement, actin cytoskeletal matting parallel to the GBM, and simplification and loss of primary and secondary processes. Scale bar low power 5 μm , high power 2.5 μm . Arrow shows actin cytoskeletal matting present in both models. (F) Podocyte loss was evident in both MAGI2 and proteinuric RapGEF2 KO mice. The number of WT-1 positive cells per glomerular tuft area was determined for 20 glomeruli per mouse. $n=3$ mice per group (* $P < .0001$, ** $P < .03$, *** $P < .03$).

Figure 5. RapGEF2 or MAGI2 knockdown in podocytes similarly diminish Rap1 activation and Rap1 mediated downstream signaling. (A) Human cultured podocytes transduced with shRNA expression plasmid targeting RapGEF2 or MAGI2 mRNA demonstrate diminished RapGEF2 or MAGI2 protein expression, respectively, compared to podocytes expressing a scrambled shRNA. Two distinct shRNA plasmids were used for both RapGEF2 and for MAGI2, each with a unique mRNA target sequence. Levels of Rap1 activation induced by calcium switch were dramatically reduced in all four knockdown cell lines compared to control cells. Ratio of active to total Rap1 was calculated for three individual experiments and then normalized (for control cells versus RapGEF2 #1 and #2: * $P < .03$, ** $P < .04$; for control cells versus MAGI2 #1 and #2: * $P < .01$ for both). α , β , and γ indicate major MAGI2 splice isoforms. The asterisk represents a non-specific band. (B) Western blotting of podocytes transduced as indicated was performed after calcium switch. Levels of $\beta 1$ integrin activation and Erk1/2 phosphorylation (both

major Rap1 downstream signals) were substantially diminished. Densitometric ratios of active to total $\beta 1$ integrin was calculated for three individual experiments and then normalized. * $P < .02$, ** $P < .01$, *** $P < .001$. Quantification combined the band intensities of the two isoforms of active $\beta 1$ integrin (shown by arrows) and compared it to the combined intensities of the bands for total $\beta 1$ integrin. Densitometric quantification for phospho to total Erk1/2 is also shown. * $P < .03$, ** $P < .02$, *** $P < .01$. (C) RapGEF2 and MAGI2 knockdown cells migrated more slowly than control cells. The percent wound closure was quantified at fixed locations along the scratch. * $P < .0001$, ** $P < .002$ (each versus controls), no significant difference between RapGEF2 and MAGI2.

Figure 6. RapGEF2 or MAGI2 knockdown in podocytes results in similarly high rates of apoptosis. (A) Differentiated RapGEF2 and MAGI2 knockdown podocytes were cultured for 48 hours under basal conditions, after which adherent and non-adherent cells were collected, combined, and then assayed for Annexin V and 7-AAD by FACS analysis. Both RAPGEF2 and MAGI2 knockdown podocytes show enhanced levels of apoptosis compared to controls. Experiments were repeated a total of three times using newly grown cells each time. (B) The ratio of Annexin V positive cells relative to controls was calculated for each experiment and results averaged. * $P = .0005$ (RapGEF2 versus control), ** $P = .0002$ (MAGI2 versus control), no significant difference between RapGEF2 and MAGI2.

Figure 7. Podocytes expressing MAGI2 CNS variants are rescued by pharmacological activation of Rap1 (A) Human podocytes were transduced with lentiviral expression plasmids carrying full length FLAG-MAGI2, FLAG-MAGI2 pTyr746*, FLAG-MAGI2 pGly 39*, or with lentiviral vector alone (control). Each podocyte line was fully differentiated, and then immunofluorescence was performed using an anti-FLAG antibody. Wild type FLAG-MAGI2 robustly accumulated at sites of intercellular contacts. In both of the CNS causing MAGI2 variants, however, FLAG-MAGI2 was dramatically mislocalized demonstrating punctate protein accumulation throughout the cytoplasm. Control vector transduced podocytes showed no immunoreactivity. Scale bar 20 μ m. (B) Each podocyte cell line was differentiated for seven days either with or without the potent Rap1 activator, 8-pCPT-2'-O-Me-cAMP (8-pCPT) and then stained for actin using phalloidin. While vector-alone and wild type MAGI2 transduced podocyte lines each demonstrated normal morphology and robust actin polymerization, podocytes that expressed either of the human MAGI2 disease causing variants demonstrated severe abnormalities of cellular morphology and dramatically diminished actin content (top row). However, when cultured in the presence of 8-pCPT, the MAGI2 mutant phenotype was completely rescued with normalization of cell shape and restoration of robust actin polymerization (lower row). Scale bar 50 μ m. (C) Quantification of actin staining is shown. Five hundred phalloidin stained cells were assessed for each podocyte line by a blinded observer. Cells were considered positive when actin filaments were robust throughout the entire cell. ** $P < .0001$ (each versus control or MAGI2); NS, not significant. (D) Podocyte lines were differentiated with either vehicle or 8-pCPT and then protein was harvested for active Rap1 pull down assays or western blotting. Levels of active Rap1 were substantially reduced in podocytes expressing mutant MAGI2 variants, but in the presence of 8-pCPT were restored to levels similar to podocytes expressing wild type MAGI2. Densitometric

quantification of active to total Rap1 was calculated for three separate experiments and then normalized. * $P < .02$, ** $P < .01$; NS, not significant.

Figure 8. Immunohistochemistry of kidney from congenital nephrotic syndrome patients caused by MAGI2 mutations is consistent with reduced podocyte Rap1 signaling. (A) Immunostaining for the Rap1 downstream effector phospho-Erk1/2 was performed on paraffin embedded human kidney sections. Representative images are shown. Scale bar low power 100 μ m; high power 20 μ m (B) Quantification of glomerular staining intensity was calculated and is shown as a fold change relative to MAGI2 CNS. Podocyte phospho-Erk1/2 staining is severely reduced in MAGI2 CNS patients compared to controls, but is only intermediately reduced in idiopathic FSGS patients. Intensity of total Erk1/2 staining is identical between the groups. Total number of glomeruli analyzed: $n=60$ control (20 glomeruli from each of three individual patients), $n=72$ idiopathic FSGS (12 glomeruli from each of six individual patients), $n=50$ MAGI2 CNS (two individual patients, 32 glomeruli from nephrectomy sample, 18 glomeruli from biopsy sample) (* $P < .001$, ** $P < .004$, ns is no significant difference).

Figure 9. The interaction between MAGI2 and RapGEF2 is a critical upstream signal for Rap1 activation in podocytes. In normal podocytes, the interaction of RapGEF2 with MAGI2 is required to maintain sufficient levels of activated Rap1 and essential Rap1 downstream signals (left panel). In patients with CNS caused by mutations in MAGI2, however, the interaction between RapGEF2 and MAGI2 is lost and cannot induce Rap1 activation (right panel). This causes a reduction of critical Rap1-mediated downstream signals and over time induces podocyte injury and ultimately FSGS.