LETTERS

Nck adaptor proteins link nephrin to the actin cytoskeleton of kidney podocytes

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The glomerular filtration barrier in the kidney is formed in part by a specialized intercellular junction known as the slit diaphragm, which connects adjacent actin-based foot processes of kidney epithelial cells (podocytes)[1]. Mutations affecting a number of slit diaphragm proteins, including nephrin (encoded by *NPHS1*)[2], lead to renal disease owing to disruption of the filtration barrier and rearrangement of the actin cytoskeleton[3], although the molecular basis for this is unclear. Here we show that nephrin selectively binds the Src homology 2 (SH2)/SH3 domain-containing Nck adaptor proteins[4], which in turn control the podocyte cytoskeleton *in vivo*. The cytoplasmic tail of nephrin has multiple YDxV sites that form preferred binding motifs for the Nck SH2 domain once phosphorylated by Src-family kinases. We show that this Nck–nephrin interaction is required for nephrin-dependent actin reorganization. Selective deletion of Nck from podocytes of transgenic mice results in defects in the formation of foot processes and in congenital nephrotic syndrome. Together, these findings identify a physiological signalling pathway in which nephrin is linked through phosphotyrosine-based interactions to Nck adaptors, and thus to the underlying actin cytoskeleton in podocytes. Simple and widely expressed SH2/SH3 adaptor proteins can therefore direct the formation of a specialized cellular morphology *in vivo*.

Nephrin is a 180-kDa transmembrane protein of the immunoglobulin superfamily that functions as an adhesion molecule and a structural component of the slit diaphragm[2,5]. Nephrin also serves as a signalling scaffold by recruiting proteins to the cytoplasmic face of podocyte foot processes[6], including CD2AP and podocin, which are necessary for the maintenance of normal podocyte structure[7,8], as well as IQGAP1, MAGIs, spectrins and α-actinin[9]. Consistent with a role in signalling, nephrin is tyrosine-phosphorylated and associates with Src-family kinases *in vivo*[10,11], which phosphorylate the cytoplasmic tail of nephrin upon clustering *in vitro*[12]. Genetic evidence supports a role for Src-family kinases in the maintenance of glomerular function, as mice deficient in Fyn or Fyn and Yes develop foot process effacement (loss) and proteinuria[10,13]. However, the physiological significance of phosphotyrosine-based nephrin signalling at the slit diaphragm, and its connection to the cytoskeleton, remain poorly understood.

The cytoplasmic region of nephrin has a series of conserved tyrosine-based motifs[14] (Fig. 1a), which upon phosphorylation can serve as binding sites for the SH2 domains of cytoplasmic targets such as phosphatidylinositol-3-OH kinase[15] and Fyn[10]. Notably, three tyrosine residues in the intracellular regions of human and mouse nephrin lie within potential binding sites for the SH2 domain of Nck adaptor proteins[14]. Nck1 (also Nckα) and Nck2 (also Nckβ or Grb4) comprise a family of adaptor proteins (collectively 'Nck') with three SH3 domains and a carboxy-terminal SH2 domain[16] (Fig. 1b). We have previously found that the SH2 domain of Nck binds with high affinity to a phosphorylated motif in the cytoplasmic tail of the Tir protein of enteropathogenic *E. coli* with the core sequence YDEV (Fig. 1c)[17]. Previous analysis of the binding properties of the Nck1 SH2 domain using a soluble degenerate peptide library has yielded the consensus motif phospho- (p)Tyr-Asp-Glu-Pro>Asp>Val. We have now probed an oriented phosphopeptide array library with the Nck1 SH2 domain, and find a strong selection for Asp in the +1 position relative to pTyr and for Val at +3 (Fig. 1d). Consistent with the data from enteropathogenic *E. coli* Tir and the oriented phosphopeptide array library, two of the potential Nck SH2-binding sites in nephrin are YDEV motifs, and the third is YDQV. Nck interacts through its SH3 domains with various effector proteins that regulate cytoskeletal organization, including N-WASp, which stimulates the Arp2/3 complex to initiate actin polymerization, and the protein kinase Pak[4,18,19]. Recruitment of Nck to phosphorylated YDxV sites on nephrin could therefore directly control the cytoskeletal architecture of podocytes (Fig. 1b).

To determine whether the SH2 domain of Nck adaptors can associate with nephrin, an immobilized glutathione *S*-transferase (GST)–Nck1 SH2 fusion protein was incubated with lysates from nephrin-transfected HEK 293T cells. Transiently expressed nephrin was not tyrosine-phosphorylated at detectable levels. However, treatment of these cells with the tyrosine phosphatase inhibitor pervanadate or co-expression of active Src kinase induced tyrosine phosphorylation of nephrin and its association with the Nck1 SH2 domain (Fig. 2a). To pursue these findings in the context of full-length Nck, we next analysed the association of transfected human nephrin with endogenous Nck adaptors in HEK 293T cells. Tyrosine-phosphorylated nephrin selectively co-precipitated with both Nck1 and Nck2 (Fig. 2b and data not shown). We also examined whether the interaction between nephrin and Nck could be detected in glomeruli from adult rats. Nephrin from glomerular lysates was tyrosine phosphorylated, and bound *in vitro* to the Nck1 SH2 domain (Fig. 2c and data not shown). Furthermore, we observed co-immunoprecipitation of phosphorylated nephrin and Nck from glomeruli (Fig. 2c). These experiments indicate that Nck binds through its SH2 domain to tyrosine-phosphorylated nephrin both in transfected cells and *in vivo*.

To address whether the YDxV sites in nephrin are required for association with Nck proteins, we introduced single, double or triple

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tyrosine (Y) to phenylalanine (F) substitutions into full-length human nephrin, at amino acid positions 1176, 1193 and 1217. Transient expression of these mutants in HEK 293T cells with active Src indicated that nephrin tyrosine phosphorylation was decreased in the double and triple mutants, suggesting that these sites might be substrates for Src-family kinases in cells (Fig. 2d). Consistent with this possibility, each nephrin YDxV motif is preceded by a leucine or isoleucine residue, which are the preferred amino acids at the −1 position for phosphorylation by Src-family kinases. Indeed, an *in vitro* kinase assay confirmed that the YDxV motifs on nephrin were preferentially phosphorylated by Fyn and Src (Fig. 1e), as previously seen with rat nephrin[11]. We then used lysates from HEK 293T cells transfected with nephrin mutants in co-immunoprecipitation experiments. Mutant forms of human nephrin with single Y-to-F substitutions all associated with Nck (Fig. 2d). However, the interaction of Nck with nephrin was selectively reduced by combined Y1193F/Y1217F substitutions, and was virtually undetectable for the Y1176F/Y1217F nephrin mutant (Fig. 2d). No binding of Nck to nephrin was detected when all three YDxV tyrosines were mutated to phenylalanine (Y3F) (Fig. 2d). The involvement of multiple YDxV motifs in Nck binding was also observed in rat and mouse nephrin (data not shown). Furthermore, the SH2 domain of Nck1 selectively associated with phosphopeptides representing each of the YDxV motifs on nephrin (Fig. 1e), indicating that nephrin has multiple docking sites for Nck.

The *in vivo* interaction with nephrin implies that Nck might have an important function in the kidney slit diaphragm. Consistent with this possibility, both Nck1 and Nck2 are expressed in podocytes (Supplementary Fig. 1a, b, e). However, mice lacking either *Nck1* or *Nck2* are viable[20] and show no apparent renal defects (Supplementary Fig. 1c, d), suggesting that Nck1 and Nck2 might have overlapping functions. Indeed, mice homozygous for both *Nck1*- and *Nck2*-null alleles die at embryonic day 9.5 (ref. 20), precluding analysis of Nck function in podocytes of double-null animals. To circumvent this lethality, we used a conditional *loxP*-flanked (floxed) allele of *Nck2* (J. Fawcett, F.B. and T.P., unpublished results) to selectively ablate *Nck2* expression in podocytes of *Nck1*-null ($Nck1^{-/-}$) animals by expressing Cre recombinase under the control of the podocyte-specific podocin promoter (Supplementary Fig. 1f). Mice homozygous for both the *Nck1*-null allele and a *Nck2* floxed allele, and carrying one copy of a podocin-*Cre* transgene (podocin-$Cre^{+/-}$; $Nck1^{-/-}$, $Nck2^{fl/fl}$, hereafter designated *Nck* mutant mice) were born at the expected mendelian frequency. However, by four days of age, *Nck* mutant pups were smaller than their littermates, and this growth retardation became more apparent by three weeks of age (Supplementary Fig. 2a). Urinalysis from *Nck* mutant mice showed

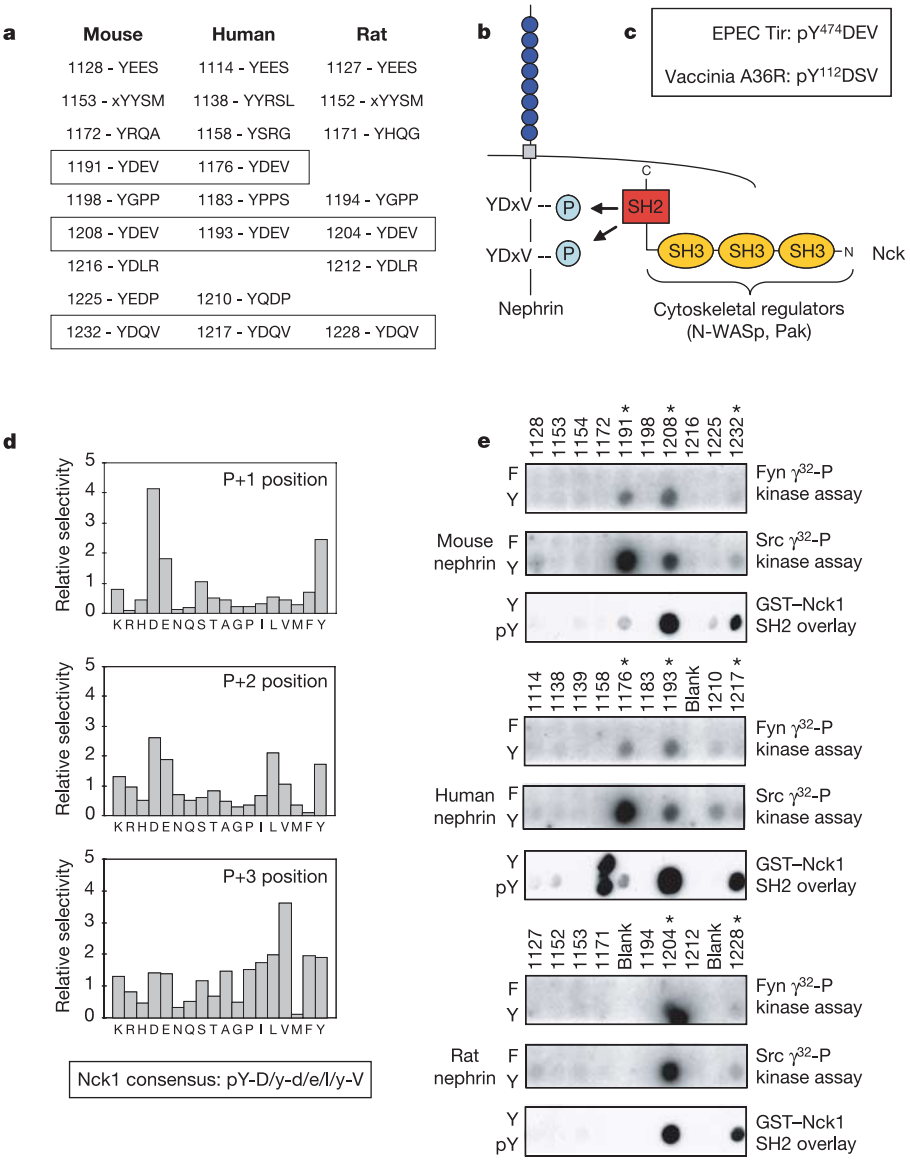

Figure 1 | The kidney slit diaphragm protein nephrin has multiple YDxV motifs that bind the Nck SH2 domain. **a**, Tyrosine residues and conserved YDxV motifs (boxed) in mouse, human and rat nephrin. Numbers indicate the amino acid position of the (first) tyrosine residue. **b**, Recruitment of Nck to phosphorylated YDxV sites on nephrin and downstream activation of N-WASp and Pak. **c**, Phosphorylated YDxV motifs shown to recruit Nck in enteropathogenic *E. coli* (EPEC) Tir and vaccinia A36R. **d**, Oriented phosphopeptide array libraries yield a consensus binding motif for the Nck1 SH2 domain of pY-D/y-d/e/l/y-V (residues under strong selection in upper case; residues under weak selection in lower case). **e**, Spot peptide arrays (centred around F, Y or phospho- (p)Y) were subjected to an *in vitro* kinase assay using recombinant Fyn or Src, or incubated with purified GST–Nck1 SH2 domain or GST alone. Binding to human Y1158 was also seen with GST alone (not shown). Asterisks indicate tyrosine residues within YDxV motifs.

nephrotic-range proteinuria, and SDS–polyacrylamide gel electrophoresis confirmed large amounts of albumin in the urine (Supplementary Fig. 2b). Histological examination of kidneys also revealed proteinaceous material within the tubules of mutant mice (Supplementary Fig. 2c, d). Most individual glomeruli remained relatively normal in the first few weeks of life in *Nck* mutant mice, as assessed by light microscopy (Fig. 3a–d). However, by 3.5 weeks of age, a range of glomerular defects were detected, including focal sclerosis (Fig. 3e). In some cases, much of the glomerulus had been replaced with sclerotic tissue, consistent with end-stage kidney disease (Fig. 3f). Expression of molecular markers of glomerular development (Wilm's tumour-1 (WT-1) and nephrin) in the glomeruli of *Nck* mutant mice was comparable to littermate controls at one week of age (Supplementary Fig. 3a–d) but was reduced by 3.5 weeks of age in a pattern consistent with segmental changes in podocyte distribution (Supplementary Fig. 3e–h), indicating a loss of podocytes.

Although the glomeruli in neonatal mutant mice did not seem morphologically abnormal by light microscopy examination (Fig. 3a–d), the presence of albuminuria indicated that the capillary filtration barrier might be damaged. This barrier normally consists of an inner fenestrated endothelium that is separated by a glomerular basement membrane from podocytes, with their characteristic foot processes (Fig. 3g). Electron micrographic examination of kidneys from four-day-old pups showed complete fusion of foot processes around the capillary loops of fully differentiated glomeruli in *Nck* mutant animals, compared to the regular appearance of these structures in control littermates (Fig. 3h, i). To determine whether foot processes can actually form in mutant mice or whether they develop and then degenerate, we performed similar ultrastructural analysis on kidney sections from embryos at day 16.5 to visualize podocytes that are beginning to differentiate and establish foot processes. In glomeruli from both mutant and control embryos, endothelial cell fenestrations were present and podocytes spread normally around the capillary loops (Fig. 3j, k). However, differentiated foot processes were notably absent at this stage of development in *Nck* mutant embryos compared to controls, consistent with the hypothesis that foot processes fail to form in *Nck* mutant animals.

The effacement of podocyte foot processes typically arises owing to perturbations in the actin cytoskeleton[3]. Nck adaptor proteins have been implicated in connecting phosphotyrosine signals to the actin cytoskeleton through SH3-mediated interactions with molecular effectors. Indeed, clustering of Nck SH3 domains at the plasma membrane is sufficient to induce localized actin polymerization[21]. We therefore investigated whether recruitment of Nck signalling complexes to the plasma membrane in response to nephrin clustering can stimulate reorganization of the actin cytoskeleton. To specifically activate intracellular nephrin signalling, we fused the intracellular (IC) domains of either wild-type (WT) nephrin or the Nck-binding mutant (Y3F) to the extracellular and transmembrane domains of the human immunoglobulin F_c receptors CD16 and CD7, respectively, to generate CD16/7–nephrin(WT)^{IC} and CD16/7–nephrin(Y3F)^{IC} chimaeras. These chimaeric proteins, which also included C-terminal green fluorescent protein (GFP), were expressed in murine embryonic fibroblasts (MEFs) lacking endogenous Nck1 and Nck2[20]. Clustering of these fusion proteins by treatment with anti-CD16 antibody resulted in markedly enhanced tyrosine phosphorylation of CD16/7–nephrin(WT)^{IC} and increased binding of a co-transfected Flag-tagged version of Nck2 or its target N-WASp (Fig. 4a and Supplementary Fig. 4). In contrast, neither nephrin

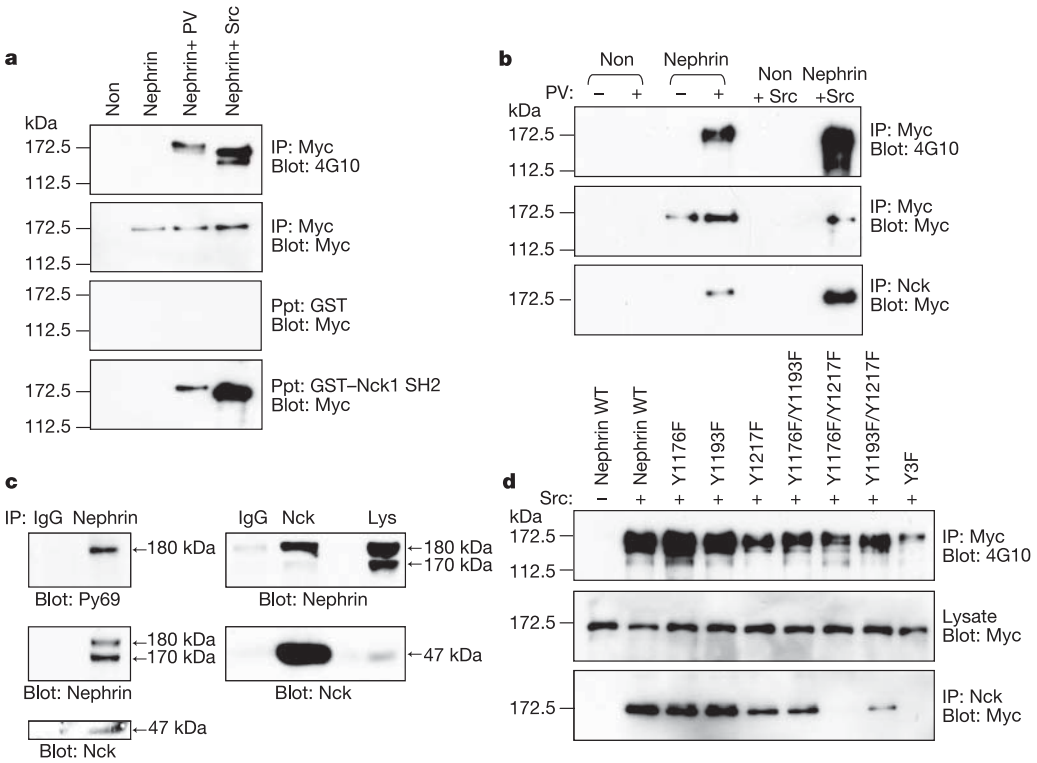

Figure 2 | Identification of a phosphotyrosine-dependent interaction between nephrin and the Nck SH2/SH3 adaptor. **a**, Myc-tagged nephrin is tyrosine-phosphorylated in HEK 293T cells stimulated with pervanadate (PV) or co-transfected with active Src, and can be precipitated (Ppt) by a GST–Nck1 SH2 domain. The 4G10 antibody is used to detect phosphotyrosine. Non, untransfected cells. **b**, Endogenous Nck associates exclusively with tyrosine-phosphorylated nephrin in parallel HEK 293T transfectants. **c**, In adult rat glomeruli, endogenous nephrin appears as a doublet of 180 and 170 kDa, and the upper band (180 kDa) is tyrosine-phosphorylated. Tyrosine-phosphorylated nephrin co-immunoprecipitates specifically with Nck (47 kDa) *in vivo*. Lys, total lysate. Py69 is another antibody that recognizes phosphotyrosine. **d**, Tyrosine-to-phenylalanine substitutions in nephrin variably reduce nephrin tyrosine phosphorylation and association with Nck. Interaction of nephrin with Nck is completely undetectable with the Y3F mutant. IP, immunoprecipitate; WT, wild type.

phosphorylation nor Nck2/N-WASp recruitment were detectably increased upon clustering of the CD16/7–nephrin(Y3F)^{IC} mutant (Fig. 4a and Supplementary Fig. 4). Clustering-induced tyrosine phosphorylation of nephrin seemed dependent on the action of Src-family kinases, as inhibitors specific for these kinases decreased this response, and phosphorylation was not observed in a MEF line lacking Src, Yes and Fyn (Supplementary Fig. 5). We confirmed by immunofluorescence microscopy that GFP aggregates corresponding to CD16/7–nephrin^{IC} proteins were the result of CD16 clustering by using a fluorescently labelled secondary antibody against CD16 in live cells (Fig. 4b).

To examine whether nephrin signalling influences actin organization, cells expressing CD16/7–nephrin^{IC} were fixed and stained with phalloidin to visualize F-actin after antibody-induced clustering. For this purpose, we used either MEFs lacking both *Nck1* and *Nck2*, or cells in which Nck2 expression had been restored, allowing us to test directly whether Nck adaptors are involved in coupling clustered nephrin to actin polymerization. In the absence of Nck, expression and clustering of CD16/7–nephrin(WT)^{IC} had no observable effect on actin organization (Fig. 4c, row 3). In contrast, co-expression of Nck2 in these same cells followed by clustering of CD16/7–nephrin(WT)^{IC} using an anti-CD16 antibody resulted in striking rearrangement of the actin cytoskeleton, notably in the form of polymerized actin 'comet tails' (Fig. 4c, row 4 and Fig. 4d). Before CD16 crosslinking, transfected Nck2 did not colocalize with CD16/7–nephrin(WT)^{IC} but remained diffusely distributed in a cytoplasmic pattern, as observed when Nck2 was expressed alone (Fig. 4c, rows 1 and 2). However, upon CD16 crosslinking, Nck2 became colocalized with clusters of CD16/7–nephrin(WT)^{IC}, which visibly capped many of the tails of polymerized actin induced by nephrin aggregation (the white 'heads' in Fig. 4c, row 4 and Fig. 4d). Notably, when the CD16/7–nephrin(Y3F)^{IC} mutant was co-expressed with Nck2 in *Nck*-deficient MEFs, Nck2 was not relocalized upon anti-CD16 treatment, nor was there any overt effect on actin polymerization, despite the fact that the Y3F mutant was aggregated upon crosslinking with anti-CD16 (Fig. 4c, row 5 and Fig. 4e). Similar results were obtained upon clustering of full-length nephrin (data not shown). Taken together, these results show that Nck adaptor proteins are required to link phosphorylated YDxV sites on activated nephrin to reorganization of the actin cytoskeleton.

Congenital foot process effacement due to loss of Nck in podocytes resembles that observed in mice deficient in nephrin[22,23], as well as other components of the slit diaphragm such as Neph1[24], FAT1[25] and α_3 integrin[26]. Although it is possible that Nck also acts downstream of other transmembrane podocyte proteins such as $\alpha_3\beta_1$ integrin, these molecules lack direct Nck-binding motifs. The results presented here argue that direct interaction of Nck with nephrin drives the elaboration of actin-based foot processes *in vivo*. The importance of actin remodelling in podocyte function is supported by genetic evidence in patients with adult-onset focal segmental glomerulosclerosis, who carry mutations in the actin filament crosslinking protein α-actinin-4 (*ACTN4*)[27]. Through interaction with α-actinin-4 and the Arp2/3 complex, the cytoplasmic adaptor CD2AP has also been proposed to anchor nephrin to the actin cytoskeleton[28]. Although mice lacking CD2AP or α-actinin-4 develop nephrotic syndrome, foot process formation is initiated in these mice[7,29], in contrast to *Nck*-deficient animals, suggesting that this complex might primarily be required for the maintenance of podocyte foot processes. The roles of Nck and

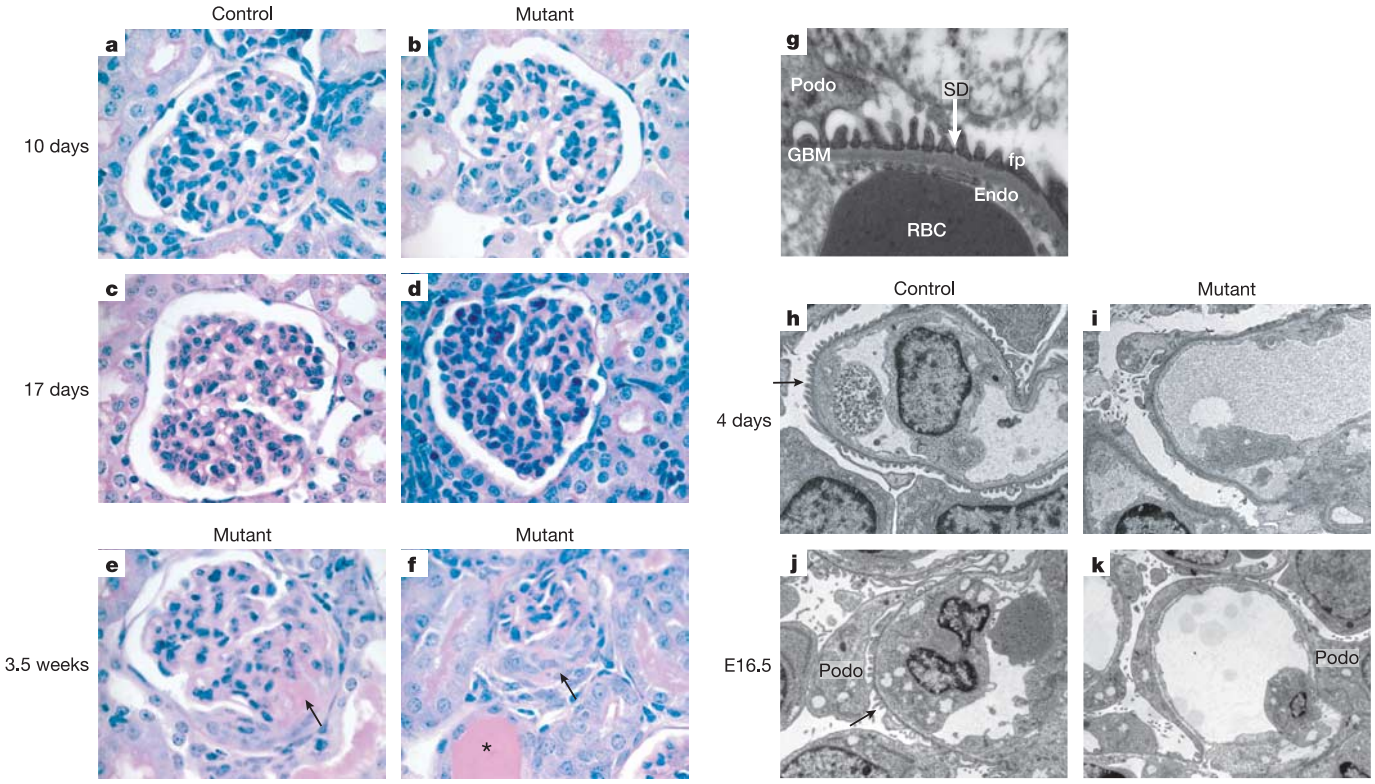

Figure 3 | Glomerulosclerosis and foot process fusion in mice lacking Nck expression in podocytes. **a**–**f**, Periodic Acid Schiff (PAS) staining of kidney sections reveals that *Nck* mutant glomeruli (**b**, **d**) are comparable to *Cre*$^{-/-}$ controls (**a**, **c**) at 10 and 17 days of age. However, after 3.5 weeks, both focal (**e**) and widespread (**f**) sclerosis (arrows) can be observed in mutant glomeruli. Asterisk indicates kidney tubule dilation and protein deposits. **g**, Electron micrograph of the normal glomerular filtration barrier. The slit diaphragm (SD) formed between adjacent foot processes (fp) is indicated. Endo, endothelium; GBM, glomerular basement membrane; Podo, podocytes; RBC, red blood cell. **h**, **i**, Distinct foot processes (arrow) normally seen at 4 days of age (**h**) cannot be distinguished in a comparable capillary loop from a *Nck* mutant (**i**), but are instead flattened, with an absence of slit diaphragms. **j**, **k**, At embryonic day (E)16.5, podocyte foot processes (arrow) form around a developing capillary loop in control littermates (**j**), but remain spread around the loop in *Nck* mutants (**k**).

CD2AP in signalling to the podocyte cytoskeleton are therefore likely to be distinct, and it will now be of interest to explore whether Nck expression is also required in the mature glomerular filtration barrier.

Genetic data support the function of nephrin as a signalling platform, as deletion of the cytoplasmic region of nephrin in patients with the Fin_{minor} mutation results in a similar disease phenotype to complete loss of nephrin[2]. This region of nephrin seems tailored to regulate the actin cytoskeleton via Nck through the presence of multiple YDxV motifs. Notably, such YDxV phosphorylation sites are also present in the bacterial EPEC Tir protein and the A36R vaccinia virus polypeptide (Fig. 1c), both of which become phosphorylated by cellular kinases, bind Nck and trigger actin polymerization through recruitment of effectors such as N-WASp and Arp2/3[17,30]. Our data indicate that these pathogens have mimicked the mechanism normally used by cell-surface proteins such as nephrin to reorganize the cytoskeleton, through the simple acquisition of YDxV sites. In addition to interacting with Nck, nephrin associates with several cytoplasmic proteins at the slit diaphragm[6], which may

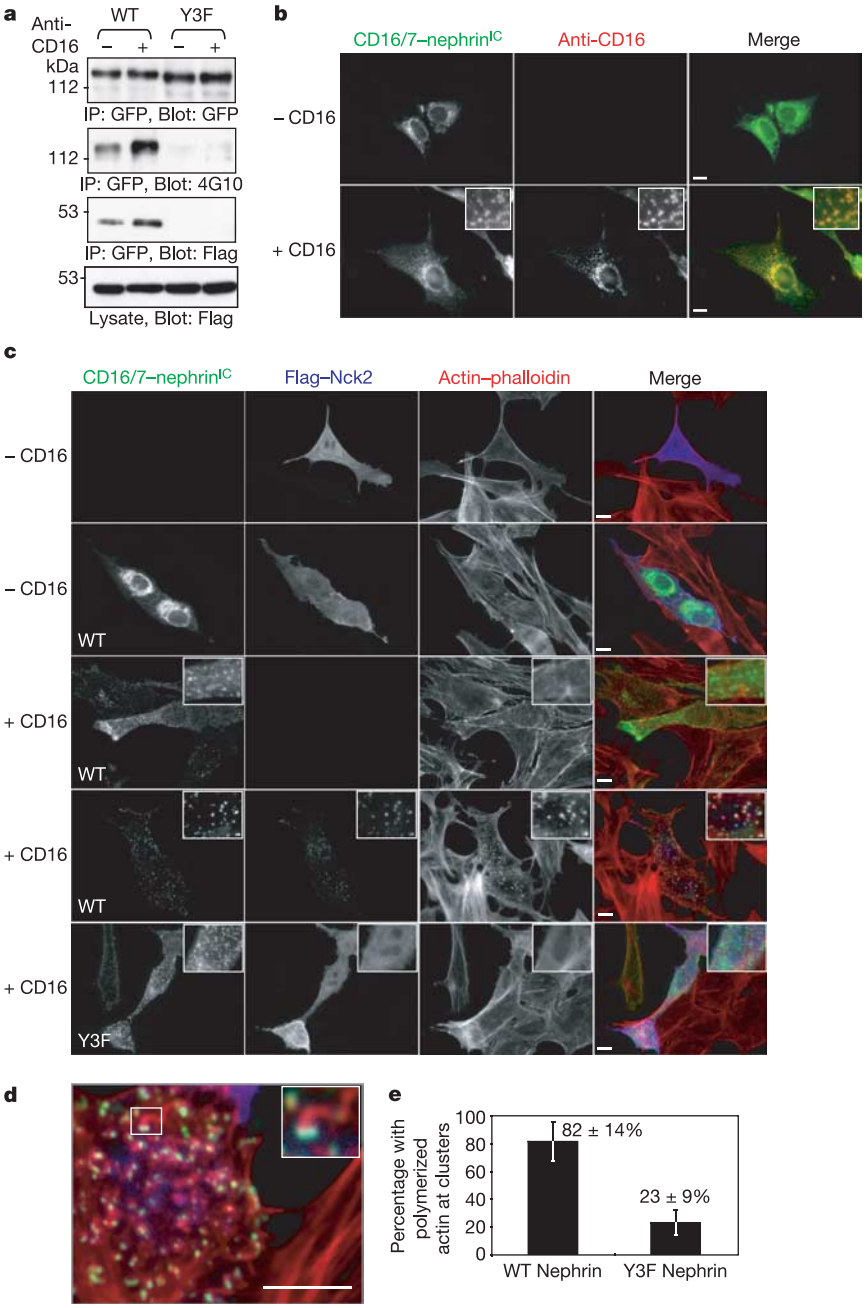

Figure 4 | Nck recruitment to phosphorylated nephrin facilitates localized actin reorganization. a, In *Nck1*$^{-/-}$, *Nck2*$^{-/-}$ MEFs expressing GFP-tagged CD16/7–nephrin^{IC} chimaeric proteins with Flag–Nck2, wild-type (WT) nephrin but not Y3F nephrin becomes tyrosine-phosphorylated and binds Nck2 after anti-CD16 treatment. **b**, Parallel transfectants were imaged using fluorescence microscopy to demonstrate co-localization of cell-surface CD16 (red) and intracellular GFP (green). **c**, Relocalization of Nck2 (blue) and comet tails of actin (red) can be seen in stimulated MEFs co-expressing both CD16/7–nephrin(WT)^{IC} (green) and Nck2 (row 4) but not in unstimulated (row 2) or singly transfected (rows 1, 3) cells, or in cells co-expressing CD16/7–nephrin(Y3F)^{IC} and Nck2 (row 5). The merged images in row 4 and panel **d** illustrate the overlap between CD16/7–nephrin(WT)^{IC} and Nck2, where the clusters appear as white 'heads' with red 'tails' of polymerized actin. Scale bars, 10 μm. **e**, Quantification of transfected *Nck*-null MEFs in rows 4 and 5 of **c**, showing polymerized actin at sites of nephrin clustering, expressed as a percentage of 100 cells counted at random in three independent experiments. Error bars show s.d.

modulate the cellular response to Nck signalling. Podocytes therefore present an intriguing system to explore how a widely expressed adaptor such as Nck interacts in a combinatorial fashion with more selectively expressed proteins to sculpt an elaborate actin-based cellular morphology *in vivo*.

METHODS

Plasmids. Myc-tagged human nephrin cDNA was provided by T. Huber. Tyrosine-to-phenylalanine substitutions were carried out using polymerase chain reaction (PCR)-based mutagenesis with Pfx Platinum polymerase (Invitrogen), and were confirmed by DNA sequencing. A plasmid containing the extracellular domain of CD16 and the transmembrane domain of CD7 was provided by G. Rivera and B. Mayer. The intracellular domain of human nephrin was PCR-amplified to generate an in-frame fusion with CD16/7 followed by a C-terminal EGFP epitope tag. Full-length human *NCK1* (NCBI accession number BC006403) and *NCK2* (BC000103) cDNAs (Open Biosystems) were PCR-amplified with a C-terminal Flag epitope tag and cloned into pcDNA3 (Invitrogen), or the SH2 domains of *NCK1* or *NCK2* were cloned into pGEX-4T-1 (Amersham Biosciences) to generate GST fusion proteins. Flag–N-WASp has been described previously[17].

Cell culture. All cell lines were grown in DMEM medium supplemented with 10% fetal bovine serum (Hyclone). Transient transfections were performed using polyethyleneimine for HEK 293T cells or Effectene reagent (Qiagen) for MEFs. Pervanadate (50 μM) stimulation of HEK 293T cells was performed for 10 min at 37 °C. MEFs were pre-treated with kinase inhibitors as indicated for 2 h before stimulation with anti-CD16. Protein blotting techniques and spot peptide arrays are described in Supplementary Information.

CD16 crosslinking and cell imaging. Antibody-mediated crosslinking of CD16/7 fusion proteins has been described previously[21]. Briefly, 1.5×10^5 MEFs were seeded onto glass coverslips and transfected the next day. After ~18 h, cells were incubated with 1 μg ml^{-1} mouse monoclonal anti-CD16 (Sigma) for 15 min at 37 °C, washed once with DMEM, incubated with 1 μg ml^{-1} fluorescence-conjugated secondary antibody for 15 min and fixed in 4% paraformaldehyde. Staining for Flag–Nck2 was performed using a polyclonal anti-Flag antibody (Sigma) and actin was visualized using fluorescently labelled phalloidin (Molecular Probes). For stimulation of transfected cells in preparation for phosphotyrosine analysis, anti-CD16 antibody was added at a dilution of 1:200 in culture medium for 10 min at 37 °C.

Generation of podocin-*Cre*$^{+/-}$; *Nck1*$^{-/-}$, *Nck2*$^{fl/fl}$ mutant mice. Mice carrying a floxed *Nck2* allele (*Nck2*$^{fl/fl}$) in which exon 1 is flanked by *loxP* sites to allow Cre-mediated excision (J. Fawcett, F.B. and T.P., unpublished results) and a *Nck1*$^{-/-}$ allele[20] were crossed to transgenic mice expressing Cre recombinase under the control of the podocyte-specific podocin (*NPHS2*) promoter (podocin-*Cre*). Cre-positive heterozygous offspring were backcrossed to *Nck1*$^{-/-}$, *Nck2*$^{fl/fl}$ mice to obtain podocin-*Cre*$^{+/-}$; *Nck1*$^{-/-}$, *Nck2*$^{fl/fl}$ mutant mice. Generation of *Nck2*$^{-/-}$ mice used for *lacZ* expression analysis has been described elsewhere[20]. Animal husbandry was carried out in accordance with Canadian Council on Animal Care protocols.

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