BBA - Molecular Basis of Disease 1865 (2019) 1041–1066

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

TRPC channels: Regulation, dysregulation and contributions to chronic kidney disease

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ARTICLE INFO

Keywords:
Chronic kidney disease
TRPC6
TRPC5
Fibrosis
Glomerulosclerosis
Nephrotic syndrome

ABSTRACT

Mutations in the gene encoding canonical transient receptor potential-6 (TRPC6) channels result in severe nephrotic syndromes that typically lead to end-stage renal disease. Many but not all of these mutations result in a gain in the function of the resulting channel protein. Since those observations were first made, substantial work has supported the hypothesis that TRPC6 channels can also contribute to progression of acquired (non-genetic) glomerular diseases, including primary and secondary FSGS, glomerulosclerosis during autoimmune glomerulonephritis, and possibly in type-1 diabetes. Their regulation has been extensively studied, especially in podocytes, but also in mesangial cells and other cell types present in the kidney. More recent evidence has implicated TRPC6 in renal fibrosis and tubulointerstitial disease caused by urinary obstruction. Consequently TRPC6 is being extensively investigated as a target for drug discovery. Other TRPC family members are present in kidney. TRPC6 can form a functional heteromultimer with TRPC3, and it has been suggested that TRPC5 may also play a role in glomerular disease progression, although the evidence on this is contradictory. Here we review literature on the expression and regulation of TRPC6, TRPC3 and TRPC5 in various cell types of the vertebrate kidney, the evidence that these channels are dysregulated in disease models, and research showing that knockout or pharmacological inhibition of these channels can reduce the severity of kidney disease. We also summarize several areas that remain controversial, and some of the large gaps of knowledge concerning the fundamental role of these proteins in regulation of renal function.

1. Introduction

The gradual loss of renal function, more typically referred to as chronic kidney disease (CKD), describes a large class of primary and secondary conditions that impair the ability of the kidney to filter wastes, regulate salt and electrolyte balances, recapture essential metabolites and cofactors, and to regulate production of red blood cells. Once renal function has essentially ceased, a stage known as end-stage renal disease (ESRD), dialysis or kidney transplantation is necessary for life. However, even modest declines in renal function are associated with significant increases in morbidity and mortality, often from cardiovascular diseases [1,2]. It is now estimated that between 13 and

Abbreviations: Ang II, angiotensin II; AT1R, type 1 angiotensin receptor; BK_{Ca}, large-conductance Ca^{2+}-activated K^+ channel, also known as KCa1.1; BKP2, [*N*-{4-[3,5-*bis*(trifluoromethyl)-1*H*-pyrazol-1-yl]phenyl}-4-methyl-1,2,3-thiadiazole-5-carboxamide]; BUN, blood urea nitrogen; Ca_V, dihydropyridine-sensitive voltage-activated Ca^{2+} channel; CKD, chronic kidney disease; CN, calcineurin; D609, *O*-(octahydro-4,7-methano-1*H*-inden-5-yl) carbonopotassium dithioate; DAG, diacylglycerol; ESRD, end-stage renal disease; FSGS, focal and segmental glomerulosclerosis; GBM, glomerular basement membrane; GFR, glomerular filtration rate; GPCR, G protein-coupled receptor; GTP-βS, guanosine 5′-*O*-(3-thiotriphosphate); 20-HETE, 20-hydroxyeicosatetraenoic acid; IP3R, inositol (3,4,5) triphosphate receptor; LPS, lipopolysaccharide; LysoPC, lysophosphatidylcholine; ML-204, 4-methyl-2-(piperidin-1-yl) quinoline; NFAT, nuclear factor of activated T-cells; NFκB, nuclear factor kappa B; Nox2, NAHPH oxidase-2; NTS, nephrotoxic serum; PAS, periodic acid-Schiff's stain; PAN, puromycin amino nucleoside; PHB, prohibitin domain; PLA, phospholipase A; PLC, phospholipase C; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; P2XR, type 2 ionotropic purinergic receptor; P2YR, type 2 metabotropic purine nucleotide receptor; ROS, reactive oxygen species; SAR-7334, 4-[[(1*R*,2*R*)-2-[(3*R*)-3-amino-1-piperidinyl]-2,3-dihydro-1*H*-inden-1-yl]oxy]-3-chlorobenzonitrile dihydrochloride; SGLT2, Na^+-dependent glucose transporter-2; SliM, short linear motif; STZ, streptozotocin; suPAR, soluble urokinase receptor; TGFβ1, transforming growth factor-β1; TNF, tumor necrosis factor; TRP, transient receptor potential; TRPA1, ankyrin-type transient receptor potential-1 channel; TRPC3, canonical transient receptor potential-3 channel; TRPC5, canonical transient receptor potential-5 channel; TRPC6, canonical transient receptor potential-6 channel; UUO, unilateral ureteral obstruction

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https://doi.org/10.1016/j.bbadis.2019.04.001
Received 23 February 2018; Received in revised form 20 December 2018; Accepted 6 January 2019
Available online 04 April 2019
0925-4439/ © 2019 Published by Elsevier B.V.

16% of the population of the United States suffers from some degree of CKD, and the burden of ESRD is greater in certain racial and ethnic subsets of the population [3].

The majority of CKD is the consequence of systemic pathologies, such as diabetes mellitus, lupus erythematosus, hypertension, or HIV disease, which drive renal failure, typically accompanied by dysfunction of other organ systems. However some other forms of CKD, for example primary focal and segmental glomerulosclerosis (FSGS), polycystic kidney disease, some forms of glomerulonephritis, and some obstructive conditions that block urine outflow, emerge from pathological processes that are initially apparent within the kidney itself. Many of these are autoimmune disorders. It is further possible to distinguish between diseases in which the renal manifestations begin within glomeruli, as opposed to diseases which originate in tubules, the interstitium, or the renal vasculature. Regardless of the nature of the primary insult, inflammation and fibrosis throughout the kidney are hallmark features of most forms of CKD. Diseases that originate in glomeruli, if sustained, eventually affect other parts of the kidney, and vice versa. Reduced renal function in and of itself is sufficient to cause deleterious systemic changes, including hypertension, atherosclerosis, dyslipidemias, anemia, metabolic acidosis, muscle wasting, and increased susceptibility to infection and bone fracture [4].

There is currently an unmet need for effective therapies to treat many forms of CKD. The mainstays of therapy, especially for glomerular diseases, remain inhibition of the renin-angiotensin system, aggressive immunosuppression, and treatment of dyslipidemias and other metabolic and systemic issues that arise as a result of impaired renal function [5,6]. In many cases these approaches ultimately fail. For example, primary FSGS that does not respond to treatment with glucocorticoids often progresses inexorably to ESRD [7,8], and then recurs in as many as 40% of patients who receive a kidney transplant [9]. Consequently there is a strong motivation to identify new therapeutic targets for the various forms of CKD [10]. Ideally, these therapeutic targets should be amenable to modulation by small molecules, should reduce or reverse the progression of CKD without compromising the function of other physiological systems, and should be more specific than the broadly acting immunosuppressive agents currently employed.

Here we will review the status of one class of potential therapeutic targets known as canonical transient receptor (TRPC) channels, a group of proteins classified within a larger superfamily of distantly related so-called transient receptor potential (TRP) channels. TRP superfamily members outside of the TRPC family are beyond the scope of this review. Among the members of the TRPC family, TRPC6 has received the most attention but we will also have reason to discuss TRPC3 and TRPC5. A major advantage of ion channels as therapeutic targets is that the active molecules are often accessibly located on the cell surface, and the nature of their pores and regulatory domains make them highly amenable to inhibition by small molecules. In addition, many channels, including TRPC channels, are components of complex pathways, which means that targets up- or down-stream of a channel can be identified by studies of their physiological regulation, and may also be potential targets for therapeutic agents.

2. TRPC6 mutations in familial FSGS

In 2005, Michelle Winn and her colleagues identified a mutation in the *TRPC6* gene in a large extended family in which many members had a highly aggressive hereditary form of FSGS that appeared to be transmitted with an autosomal dominant mode of inheritance [11]. The disease in this family occurred with an adult onset, mostly in the third or fourth decade. ESRD occurred 4–20 years after the initial presentation, and the disease did not recur after transplantation [12]. This mutation (TRPC6-P112Q) exhibited a gain of function compared to wild-type TRPC6 when these channels were characterized in a heterologous expression system (HEK-293 cells). Biochemical data suggested that TRPC6-P112Q exhibited increased steady-state expression on the cell surface, indicating some change in trafficking into or out of the plasma membrane. Shortly after this initial report, several other TRPC6 mutations were identified in families with autosomal dominant forms of hereditary FSGS [13]. Again, most of these mutations exhibited a gain of function when the encoded channels were characterized in HEK-293 cells and activated by co-expressed G protein coupled receptors (GPCRs). The biophysical processes that lead to gains of function in those mutations were not established. It was also observed that TRPC6 channels are expressed in podocytes, and it was of particular interest that they were present in foot processes, within or close to the membrane domains containing the filtration slit diaphragm [11,13,14]. However a subset of TRPC6 also appears to be present in the podocyte cell body and along major processes, possibly in contact with the subpodocyte space [15].

Since those initial observations, many other TRPC6 mutations have been identified in patients with FSGS [16–20]. Most have been shown to cause a gain in function, and one in particular, TRPC6-M132T, was notable for both the magnitude of the gain of function seen in heterologous expression systems and the childhood onset of FSGS in the patient in which the mutation was identified [18]. Other mutations have been identified in children more recently [19]. However, while most TRPC6 mutations have been shown to exhibit a gain of function, this is not universally the case. For example, at least five mutations associated with FSGS, including TRPC6-G757D, result in a loss of channel function, and indeed, TRPC6-G757D acts as a dominant-negative [21]. Assuming that those mutants do not produce pathology via a process unrelated to ion permeation, such as endoplasmic reticulum stress, this suggests that human renal function requires at least a baseline level of TRPC6 function, whereas chronic excessive activation of TRPC6 results in glomerular disease. For reasons that are not known, most of the inactive or dominant-negative forms were associated with childhood disease onset.

Mutations in TRPC6 channels may be associated with changes in their interactions with other essential proteins, especially within podocyte foot processes. (Foot processes are highly differentiated structures that are relevant to FSGS, and will be discussed further below.) Therefore assays carried out in heterologous expression systems must be interpreted with caution, especially if no change in function is observed, because those systems do not endogenously express several of the important regulatory proteins found in podocyte foot processes. It was observed early on that TRPC6 interacts with other essential slit diaphragm, proteins, for example podocin and nephrin [13,14]. As will be discussed in detail further below, the interaction with podocin is functionally significant for TRPC6 gating, and the direct interaction occurs between cytosolic C-terminal domains of both proteins [22]. The interaction between TRPC6 and the slit diaphragm adhesion/organizing protein nephrin regulates the trafficking of TRPC6 and other associated ion channels to the podocyte cell surface [23,24]. Moreover, TRPC6 mutations suppress the interactions between heterologously expressed TRPC6 and slit diaphragm proteins, including nephrin [24] and podocin (unpublished observations from our laboratory). The interactions between TRPC6 and other proteins also raise the possibility that in addition, to regulating Ca^{2+} influx, TRPC6 could have a simple scaffolding function at the slit diaphragm.

3. General structural aspects of TRPC channels

TRPC is a subgroup of a larger superfamily of distantly related cation channels known as the TRP family. The TRPC group is most closely related to *Drosophila* transient receptor potential (TRP) channels, which were the first members of this superfamily to be identified. The TRPC family of vertebrates is encoded by seven different genes (Fig. 1), although *Trpc2* has become a pseudogene in humans. Among the remaining channels, TRPC3/6/7 and TRPC4/5 appear to cluster separately on the basis of both primary sequence, functional characteristics, and probably the extent to which they can form functional

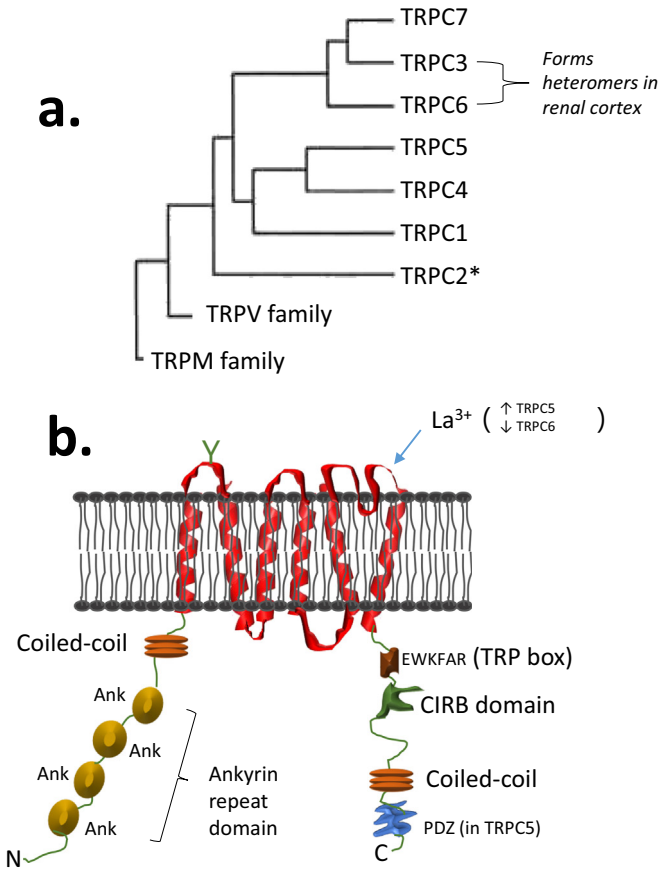

Fig. 1. General structural aspects of TRPC channels. (a) Relationships between various TRPC channels and two outgroups. TRPC6 and TRPC3 can form functional heteromers, and we have detected these heteromers in kidney by biochemical methods. However, heteromultimerization is not obligatory and there are probably renal cells where TRPC6 and TRPC3 act independently [96]. TRPC6 and TRPC5 do not form heteromultimers in kidney. (b) General structural features and important motifs in a single subunit of TRPC channels. Functional channels are comprised of four such subunits. Many of the domains identified in this schematic underlie functionally significant interactions with other proteins. TRPC channels typically have relatively high affinity La^{3+}-binding domains near the pore. Micromolar concentrations of La^{3+} acting at those sites cause inhibition of TRPC6 and enhanced activation of TRPC5 [29]. Lanthanides at millimolar concentrations are always inhibitory.

heteromultimers (Fig. 1a). A schematic diagram depicting the most extensively characterized domains of TRPC channels is shown in Fig. 1b. As with many other cation channels and K^+ channels, all TRPC channels subunits have six membrane-spanning alpha-helical domains, with N- and C-termini facing the cytosol. A series of three or four ankyrin-like motifs near the N-terminus together give rise to the ankyrin-repeat domain present in all TRPC channels. These are also found in other TRP channels, although the number of ankyrin domains may differ quite dramatically (for example as many as eighteen ankyrin motifs are found in TRPA1). Coiled-coil domains are found in both the N- and C-terminal cytosolic regions of TRPC channels. The C-terminal region has a so-called TRP box that contains the sequence EWKFAR, which defines members of the TRPC family, although highly homologous TRP box domains with slightly different sequences are found in other TRP channels [25]. This is followed by a site that allows Ca^{2+}-dependent binding of calmodulin and the IP3 receptor (IP3R), known as the CIRB domain [26]. It is likely that interactions between those proteins and TRPC channels are competitive, which raises the possibility that at least some TRPC members may function as store-operated channels [26]. However, the CIRB domain may play a more important role in regulating trafficking of TRPC channels, especially members of the TRPC3/6/7 group, to the cell surface [27]. A second coiled-coil domain is found near the C-terminus, which could mediate a variety of protein-interactions. TRPC5 channels have a PDZ domain near the C-terminus, which has been recently shown to interact with proteins that regulating the gating of the channel [28]. TRPC channels have high affinity binding sites for trivalent cations such as La^{3+} near the mouth of the pore. Micromolar La^{3+} inhibits TRPC6 channels but enhances activation of TRPC5, a feature that has proved to be quite useful experimentally [29].

Functional TRPC channels are tetramers, and different members of this group can form heterotetramers, although the precise stoichiometry of endogenously expressed TRPC channels is not usually known. We have observed that TRC6 can co-immunoprecipitate with TRPC3 but not with TRPC5 in extracts of renal cortex. We will return to this further below. A major advance in the field occurred recently when several groups published structures of TRPC3 and TRPC6 channels obtained from cryo-electron microscopy [30–33]. The resolution of these images ranged from 2.2 to 4.4 Å, and they revealed extensive interactions between N-terminal ankyrin repeats and C-terminal coiled-coil domains in assembled tetramers [30]. There also appears to be allosteric coupling between C-terminal domains and transmembrane domains that modulates channel gating [33].

4. Regulation of TRPC6 channels in podocytes

Podocytes are terminally differentiated cells attached to the surface of the glomerular basement membrane (GBM), where they interact with each other to form the final and most selective components of the glomerular filtration apparatus. As with astrocytes, podocytes are highly polarized cells that extend processes that control the permeability of an underlying capillary [34]. The pathology of FSGS is thought to begin as a result of podocyte dysfunction through death, detachment, or changes in the ultrastructure of the foot processes and filtration slits [35–37]. Glomerulosclerosis is the result of a complex sequence of events that stem from this initial insult, a key event being the formation of an adhesion between Bowman's capsule and stressed podocytes or denuded portions of the glomerular basement membrane [38]. One of the most important ultrastructural features of podocytes is the slit diaphragm, a specialized but delicate junction between adjacent interconnecting podocyte foot processes. The slit diaphragm forms a fine filter that allows sieving of molecules based on their size and shape [39,40]. The cytoplasmic components of the slit diaphragm also appear to function as a signaling hub in podocytes [41]. Podocyte stress results in changes in the organization of foot processes and loss of slit diaphragms [42]. If the stress is sustained, the number of podocytes in affected glomeruli is eventually reduced, and a loss of around 25% of podocytes usually results in glomerulosclerosis and eventual loss of function in the entire nephron [43].

Podocyte TRPC6 channels are found within the slit diaphragm domains of foot processes, and they reside in complexes with several other essential components of the glomerular filter [13,14,22,23,44]. TRPC6 channels have also been observed along major processes and in the cell body [15]. The TRPC6 channels in these compartments have distinct functions for the cell and appear to exist within distinct macromolecular complexes. For example, podocin, a functionally significant TRPC6-interacting protein, is expressed in foot processes but not on the cell body surface [45], whereas TRPC6 is found on the surface of both cell compartments [15]. TRPC6 co-immunoprecipitates with TRPC3, at least in podocyte cell lines, and it is likely that at least some functional TRPC6 channels are heteromeric (as in most other tissues where these channels are found). Formation of such heteromers has been shown to have functional consequences in other systems. For example, TRPC6/TRPC3 heteromers have enhanced mechanosensitivity compared to homomers of either subtype [46]. Hereafter we will refer to TRPC6 channels in various renal cells with the understanding that many of the

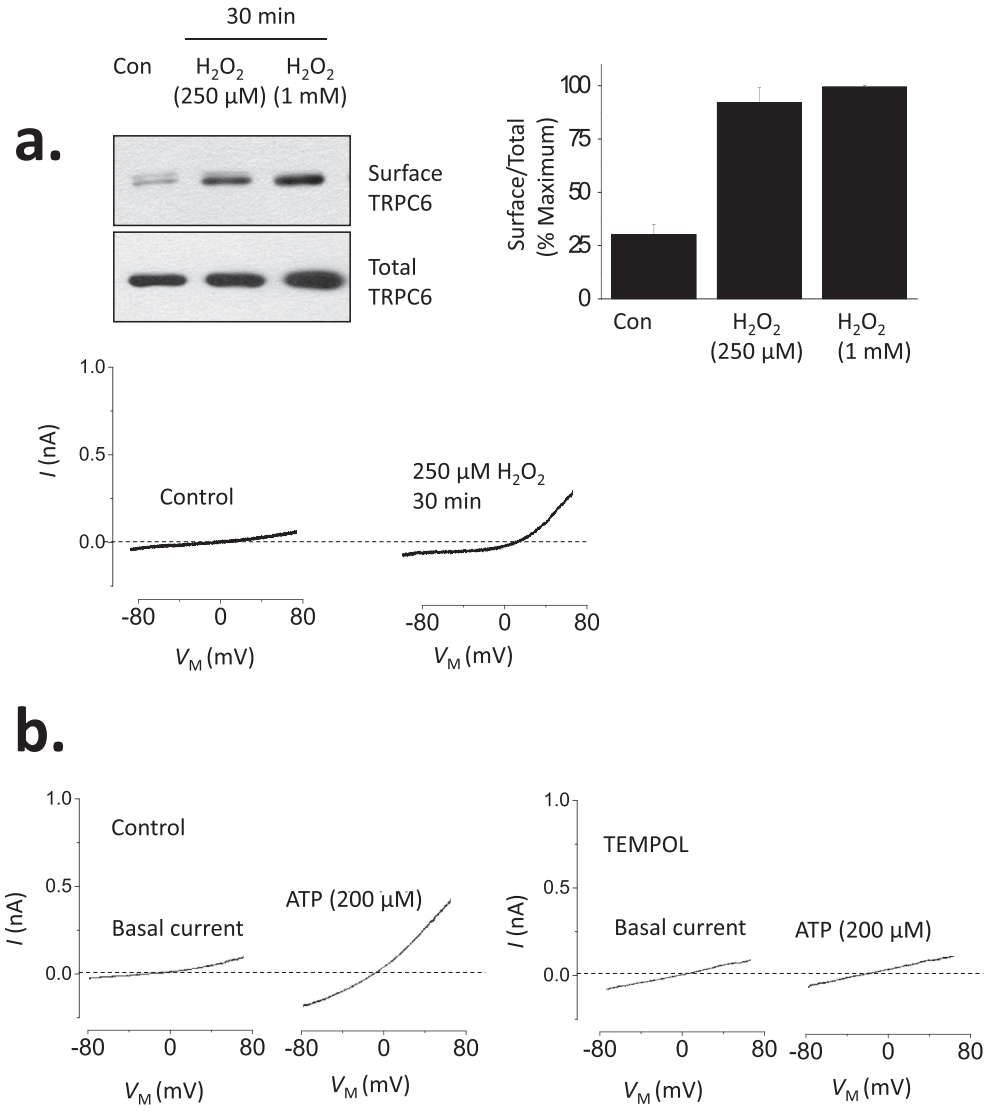

Fig. 2. TRPC6 channels are redox-sensitive proteins. In this figure we show examples of oxidative modulation of endogenous TPC6 channels in cultured mouse podocytes. (a) Application of H_2O_2 to podocytes for 30 min is sufficient to markedly increase the abundance of TRPC6 protein on the cell surface and to increase an outwardly rectifying current. (b) Activation of TRPC6 in podocytes in response to ATP, an effect mediated through P2Y receptors, does not occur in cells pretreated with TEMPOL, a membrane permeable quencher of ROS. Many other physiological and pathophysiological modulators of TRPC6 act on TRPC6 through ROS that is often generated by NADPH oxidases.

endogenously expressed channels are probably heteromeric. TRPC6 subunits in podocytes seem to be essential to detect functional currents, based on analyses in knockout animals and using siRNA knock-down. It is also worth noting that podocyte TRPC6 interacts with other ion channel proteins in podocytes, such as KCa1.1 [47], which will be discussed further below.

The most extensively studied mode of TRPC6 activation in podocytes occurs as a consequence of activation of GPCRs, especially by angiotensin II (Ang II) acting through losartan-sensitive AT1 receptors [48,49]. In whole-cell recordings from podocytes attached to isolated glomeruli, or in immortalized MPC-5 podocyte cell lines, Ang II at nanomolar concentrations activates an outwardly-rectifying cationic current that reverses very close to 0 mV in physiological conditions or when K^+ ions in internal or external solutions are replaced with Cs^+. As an aside we note that the detailed rectification properties of evoked currents are not useful to ascertain whether a particular macroscopic current is mediated by TRPC6, and the complex dual rectification properties sometimes seen with heterologously expressed TRPC6 is almost never seen in recordings from podocytes [50]. Ang II-evoked TRPC6 currents are completely blocked in the presence of micromolar concentrations of La^{3+} [48], a feature that distinguishes them from TRPC5, which is enhanced by micromolar La^{3+} [29,51]. Ang II-evoked cation currents in podocytes are reduced or eliminated by TRPC6 knockdown [48]. They are also blocked by inhibitors of PLC signaling cascades [48]. ATP and other agonists of P2Y receptors (e.g. UTP and UDP) produce a virtually identical response in cultured podocytes as well as in podocytes still attached to GBM in acutely isolated glomeruli [52]. As an aside, we have never seen responses mediated by P2X receptors in podocytes in acutely isolated glomeruli or in immortalized podocytes of low passage number that robustly express essential podocyte markers. The effects of ATP on podocyte TRPC6 are blocked by suramin, a relatively non-selective inhibitor of P2Y receptors. The responses to various nucleotides suggest that $P2Y_1$, $P2Y_2$, and $P2Y_6$ receptors are positively coupled to TRPC6 channels in podocytes [52]. The responses to ATP and Ang II in podocytes are eliminated by introducing guanosine 5′-*O*-(2-thiodiphosphate) (GDP-βS) into the cell cytosol, which indicates that G protein cascades are required for ATP-evoked activation of TRPC6 [48,52]. ATP-evoked cation currents were also blocked by the PLC inhibitor D609 [52].

Modulation of podocyte TRPC6 channels by GPCRs has also been characterized in single-channel recordings [49,51]. In cell-attached patch recordings Ang II was observed to activate a homogeneous population of TRPC6 channels with a unitary slope conductance of 22 pS with physiological external Na^+ and Ca^{2+} concentrations. The current-voltage relationship of TRPC6 channels in this recoding configuration is essentially linear, in contrast to what is more typically seen in whole-cell recordings [49]. This is discussed in more detail elsewhere [50]. Crucially, ion channel activation by Ang II was not detected in

podocytes examined in glomeruli acutely isolated from TRPC6 knockout mice [49].

A landmark study in 1999 established the canonical activation pathway for TRPC6 channels by which certain diacylglycerols (DAG) produced during GPCR-mediated transduction cascades evoke an increase in the open-probability (P_O) of cell surface TRPC6 channels [53]. Importantly, those responses were observed even in excised membrane patches, indicating that at least part of the effect of DAG is membrane-delimited and represents actions directly on or close to the TRPC6 proteins. Membrane-permeable DAG analogs such as 1-oleoyl-*sn*-glycerol (OAG) increase TRPC6-mediated cationic currents in podocytes, consistent with pharmacological analyses of the responses to Ang II and ATP described above (especially inhibition by GDP-βS and D609) [44,54]. However, there is evidence that GPCR signaling stimulates exocytotic insertion of TRPC6 channels into the plasma membrane, which can occur as little as 30 s following onset GPCR signaling, and at very low agonist concentrations [55]. Consistent with this, OAG stimulates a marked increase in the abundance of TRPC6 proteins on the podocyte cell surface [54]. Therefore it is likely that modulation by GPCRs entails two distinct processes; mobilization of channels to the surface accompanied by an increase in the open-probability (P_O) of TRPC6 channels already at the cell surface. Very similar effects are seen in podocytes treated with the arachidonic acid metabolite 20-hydroxyeicosatetraenoic acid (20-HETE) [56], as is also seen in heterologous expression systems [57].

A large body of evidence now indicates a signaling role for reactive oxygen species (ROS) during normal modulation of podocyte TRPC6 channels (and also in dysregulated responses that will be described further below). Simply exposing podocytes to H_2O_2 is sufficient to mobilize TRPC6 channels to the surface of podocytes and increase rectifying cationic currents, which occurs in minutes (Fig. 2a). In addition, activation of podocyte TRPC6 by Ang II and by ATP is blocked by agents that quench ROS (Fig. 2b) and requires activation of the NADPH oxidase Nox2 [48,52]. ATP and Ang II also increase the generation of ROS in podocytes. Importantly, increased ROS generation is also seen after application of OAG [44].

In cultured podocytes the catalytic subunit of Nox2 ($gp91^{phox}$) forms a ternary complex containing TRPC6 and podocin, and TRPC6-Nox2 interactions are not detected in podocytes following podocin knockdown, suggesting that podocin plays a role as a bridge or scaffold [44]. It is likely that these complexes normally form within sterol-rich membrane domains that are organized by podocin, which occurs as a higher order oligomer [58,59]. Interactions with podocin may also connect TRPC6 to the underlying cytoskeleton through interactions with CD2-associated protein (CD2AP) [58]. This arrangement may serve in part to allow relatively high concentrations of ROS to be generated within the immediate vicinity of the channel molecules, without large changes in the bulk cytosolic ROS concentration further away from the channels. Consistent with this, DAG analogs such as OAG stimulate formation of active Nox2 complexes on the cell surface containing the regulatory subunit $p47^{phox}$, leading to increased surface expression of TRPC6. Importantly, that effect was blocked by agents that quench ROS, by agents that inhibit Nox2 (which includes inhibitors of Rac1), and by procedures that result in disorganization of lipid rafts, including podocin knockdown [44]. The role of podocin and Nox2 in activation and mobilization of podocyte TRPC6 during GPCR cascades is summarized schematically in Fig. 3.

The molecular and biophysical mechanisms whereby ROS lead to increased surface expression of TRPC6 are not yet known. However TRPA1 channels show a similar sensitivity to ROS [60], which appear to cause TRPA1 activation in part through oxidation of cysteine residues within ankyrin-repeats in the cytosolic amino-terminus [61]. TRPC6 has similar ankyrin-repeats in its amino terminus, albeit substantially fewer of them than TRPA1 [62]. Indeed, redox regulation seems to a feature of many members of the TRP superfamily [63]. In the case of TRPC6 it would be useful to understand the mechanisms of redox regulation in more detail, and to examine a possible role for reactive nitrogen species, which can S-nitrosylate various TRP channels, including endothelial TRPC5 channels [64].

Podocyte TRPC6 channels can be activated by a second mechanism that appears in this cell type to be entirely distinct from processes that occur during GPCR signaling, namely activation by mechanical stimuli [22,65,66]. TRPC6 becomes active in response to a variety of mechanical stimuli in podocytes [22]. While it is not a physiological stimulus for podocytes, the most reproducible and experimentally straightforward mechanical stimulus is a stretch of the membrane evoked by briefly placing cells into a hypoosmotic (70%) external solution, which causes cell swelling. In podocytes these responses are fully reversible for membrane stretches of up to 5 min in duration. A limitation of this method is that one cannot exclude the possibility that hypoosmotic swelling results in rapid changes in cytoskeletal structures that in turn alter intrinsic membrane tension. In any case, stretch-evoked cationic currents in podocytes are clearly mediated by TRPC6 since they are abolished by TRPC6 knockdown and by the highly selective TRPC6 inhibitor SAR-7334 [22,65]. An example of such an experiment is shown in Fig. 4, in this case from podocytes in acutely isolated rat glomeruli. Stretch-evoked cation currents in podocytes are also blocked by micromolar concentrations of external La^{3+}, as well as by GsMTx4, a spider toxin that blocks mechanically-evoked activation of a wide range of channels [22]. Mechanical activation of podocyte TRPC6 appears to be a completely distinct activation mode from those mediated through GPCRs because it persists in the presence of ROS quenchers, as well as in cells internally perfused with GDP-βS, or in cells treated with PLC or PLA inhibitors [22]. By contrast, activation of podocyte TRPC6 channels by OAG or by 20-HETE is blocked by ROS quenchers [44,56]. Therefore, mechanical activation of TRPC6 in podocytes cannot be attributed to stretch-evoked activation of GPCRs, phospholipases, or NADPH oxidases, although there is evidence that this can occur in other cell types [67]. In addition, there is at least one TRPC6 mutation that eliminates mechanical activation of TRPC6 while allowing activation by OAG or by GPCRs [68]. We favor the hypothesis that TRPC6 channels are directly mechanosensitive. However, mechanosensitivity may be dependent on interacting proteins and the biophysics of the local membrane, which will vary from cell to cell, and even in different compartments of the same cell. Bear in mind that the mechanics of the cell surface will depend in part on interactions with underlying cytoskeleton, which can affect the basal tension or curvature of the plasma membrane. For these reasons, TRPC6 channels in podocyte foot processes and in the cell body may not show identical behavior.

Podocin plays a complex role in modulating responses to both mechanical and chemical stimuli in podocytes (Figs. 3, 5). Huber and coworkers [14] originally predicted that podocin would be required for mechanoactivation of TRPC6. This was based on the role of the *C. elegans* MEC2, a podocin ortholog, in mechanotransduction in sensory neurons. While that was a perfectly reasonable conjecture, it turns out that podocin plays a role that is more complex than what is known for *C. elegans* MEC2. Thus, TRPC6 activation through GPCRs or by OAG is reduced or eliminated following podocin knockdown, whereas mechanical activation of TRPC6 is greatly enhanced (as much as 5- to 20-fold) [22]. In other words, one of the normal physiological roles of podocin may be to suppress mechanical activation of TRPC6 in podocytes, which could be especially important in foot processes that are subjected to constant movements of the glomerular capillary [69] and to ongoing fluid shear forces [37,70]. As with other members of the stomatin-like protein family, podocin has a cholesterol-binding prohibitin (PHB) domain, also known as the stomatin/prohibitin/flotillin/HflKC domain [71]. It is possible that podocin holds TRPC6 channels within sterol-rich membrane domains that might be relatively resistant to at least certain types of shape perturbations caused by mechanical stimuli, given the overall structure of the foot process and its underlying cytoskeleton. This hypothesis for podocin modulation of mechanical

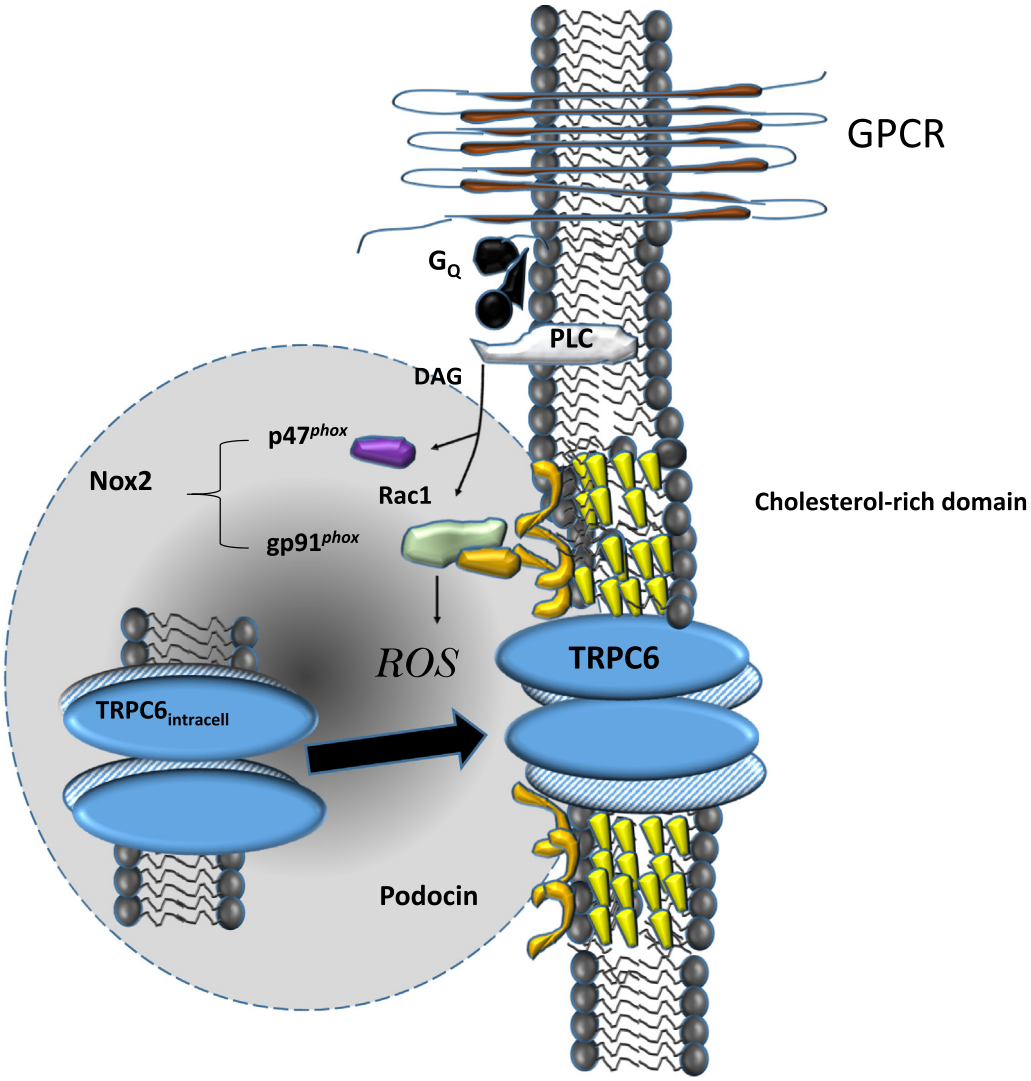

Fig. 3. Schematic diagram showing mechanisms of podocyte TRPC6 activation following activation of GPCRs, including P2YRs and AT1Rs. A portion of the action of these modulators entails mobilization of TRPC6 to the cell surface, which can occur very quickly. It is likely that diacylglycerol (DAG) produced in this cascade causes direct or membrane-delimited actions to cause activation of TRPC6 channels already at the cell surface, as originally described by [53]. In podocytes, an essential component of GPCR pathways to TRPC6 is ROS-dependent, with ROS generated by the NADPH oxidase Nox2, and in some cases Nox4. Nox2 becomes active upon Rac1 activation caused by as yet unknown pathways downstream of DAG. In podocytes a ternary complex between Nox2, podocin and TRPC6 can be detected. The interactions of Nox2 and TRPC6 with podocin tether the complex to sterol-rich domains surrounding the slit diaphragm in podocyte foot processes, and Nox2 and TRPC6 do not interact in the absence of podocin. Consequently TRPC6 in foot processes is normally maintained within the region of ROS concentration surrounding Nox2, and loss of podocin disrupts transduction from GPCRs to TRPC6. Additional details and evidence for these pathways are in [44].

activation of podocyte TRPC6 is shown schematically in Fig. 5b. This model is clearly simplistic since it illustrates only one direction of mechanical force. In addition, it ignores contributions from the cytoskeleton or from cell-cell adhesions. Indeed, in our original report we noted that stretch-evoked TRPC6 activation in podocytes is enhanced after disruption of F-actin with cytochalasin-D [22]. Podocin, in addition to having a cholesterol-binding motif, is known to interact with the cytoskeleton through interactions with CD2AP [58], and therefore its effects to suppress stretch activation of TRPC6 could entail multiple biophysical mechanisms.

Regardless of the biochemical and biophysical mechanisms of its actions, it is clear that podocin modulates the *dominant mode* of TRPC6 activation; specifically it promotes activation by DAG and ROS and hence by GPCRs, but reduces sensitivity to mechanical perturbation, which in podocytes does not depend on GPCRs. We believe that mechanical activation of TRPC6 is best thought of as a pathophysiological process, at least in foot processes, and may occur primarily in cells that already have reduced podocin content. Podocin is not expressed in other cell types except for the testis [72], and this type of modulation of TRPC6 may therefore be a unique regulatory feature in podocyte foot processes. However it is possible that other PHB-domain proteins such as stomatin and stomatin-like proteins [73] play a similar role in modulating TRPC family channels in other tissues. PHB-domain proteins can certainly regulate gating of other types of channels. We have already noted that in *C. elegans* a podocin ortholog known as MEC-2 is required for mechanical activation of a degenerin-type cation channel that is closely related to the ENaC channels of the distal nephron [74]. Stomatin in the form of a higher-order complex inhibits proton-gated currents mediated by acid-sensing ion channels in mice [75,76]. It is particularly interesting that stomatin-like protein 3 enhances mechanical activation of Piezo1 channels evoked by molecular scale membrane displacements [77]. Thus multiple PHB-domain proteins regulate cell mechanosensitivity, as well as the dominant activation mode of multimodal channels. It has been proposed that at least part of these effects occur through regulation of resting membrane tension [78]. Whether a PHB-domain protein favors one activation mode or another probably depends on details of cytoskeleton, membrane and channel structure that vary from one system to the next.

Protein phosphorylation also plays an important role in modulation of TRPC6 channels. In podocytes, TRPC6 channels can be detected in complexes containing Src family tyrosine kinases, which phosphorylate TRPC6 on multiple residues and thereby enhance its expression on the cell surface [24,66]. Among these, residue Y284 may be especially important, as it is necessary for TRPC6 to traffic to the cell surface, and phosphorylation at this residue also regulates interactions with various components of the slit diaphragm [24]. Moreover, Src activation in podocytes occurs in response to increased ROS [66]. Co-localization of Src to the vicinity of TRPC6 may entail interactions with nephrin [24].

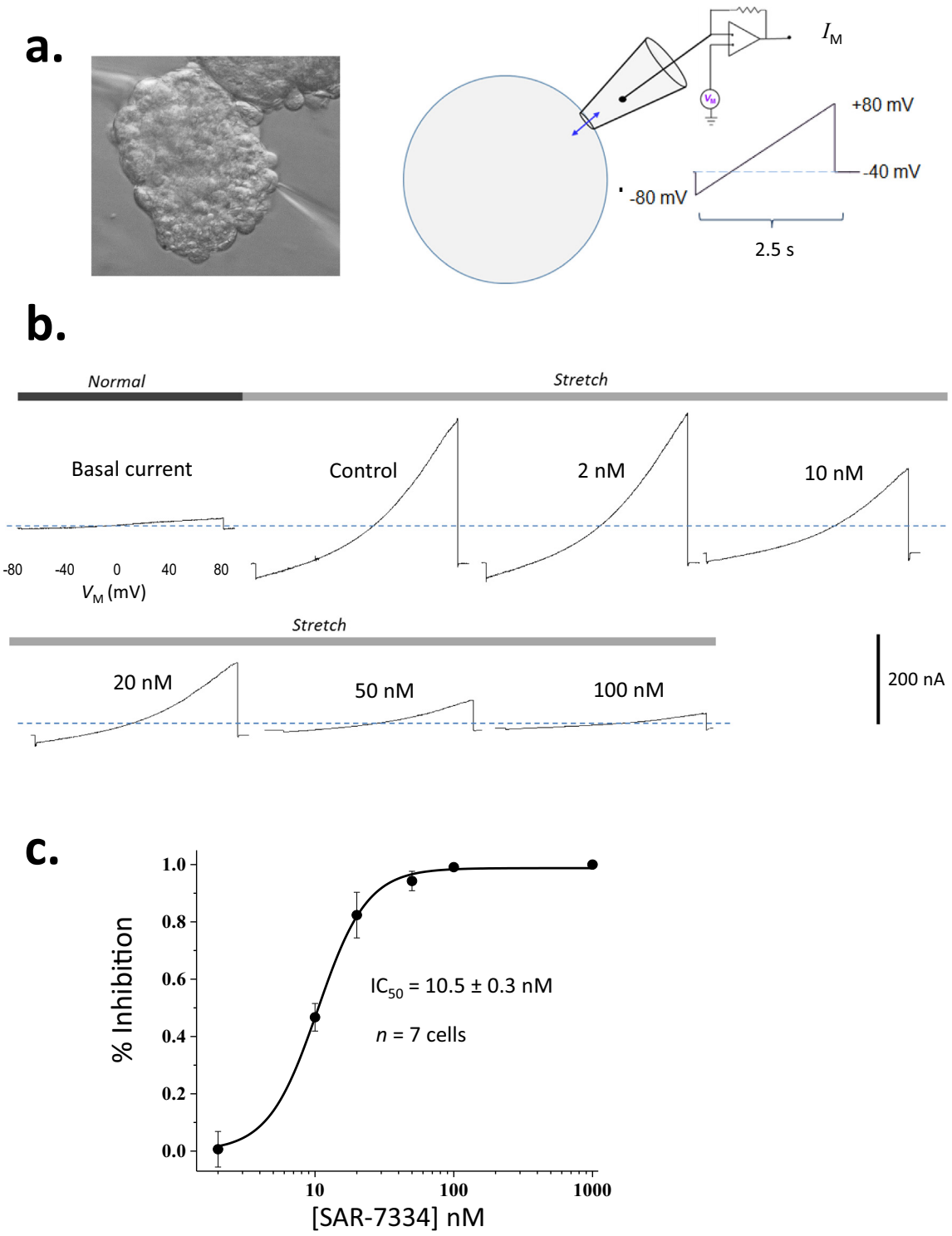

Fig. 4. Mechanical activation of TRPC6 in podocytes measured by whole-cell recordings from cells in acutely-isolated de-capsulated rat glomeruli. (a) Recording arrangement for typical experiment, with a whole-cell recording electrode on a podocyte located on the outer surface of an isolated glomerulus. Ramp voltage commands typically used to study TRPC channels are shown to the right. (b) Hypoosmotic stretch of the plasma membrane as described in [22] results in activation of an outwardly rectifying cation current that is blocked by the selective TRPC6 inhibitor SAR-7334 at low nanomolar concentrations. (c) Concentration response relationship for this response, which can be compared to those reported for cloned TRPC6 channels [224].

5. Regulation of TRPC6 channels in mesangial cells

Mesangial cells have several complex functions, and appear to be heterogeneous [79]. Many are found within glomeruli and form the inner wall of multiple capillary loops [80] and are connected by a dense matrix to the GBM and to glomerular endothelial cells. A subpopulation of extraglomerular mesangial cells, also known as Lacis cells, is located between the afferent and efferent arterioles at the vascular pole of the glomerulus, and form part of the juxtaglomerular apparatus. Mesangial cells, at least in cell culture, exhibit many features typically seen in smooth muscle, and it is possible to detect contractions of those cells mediated in part through activation of dihydropyridine-sensitive

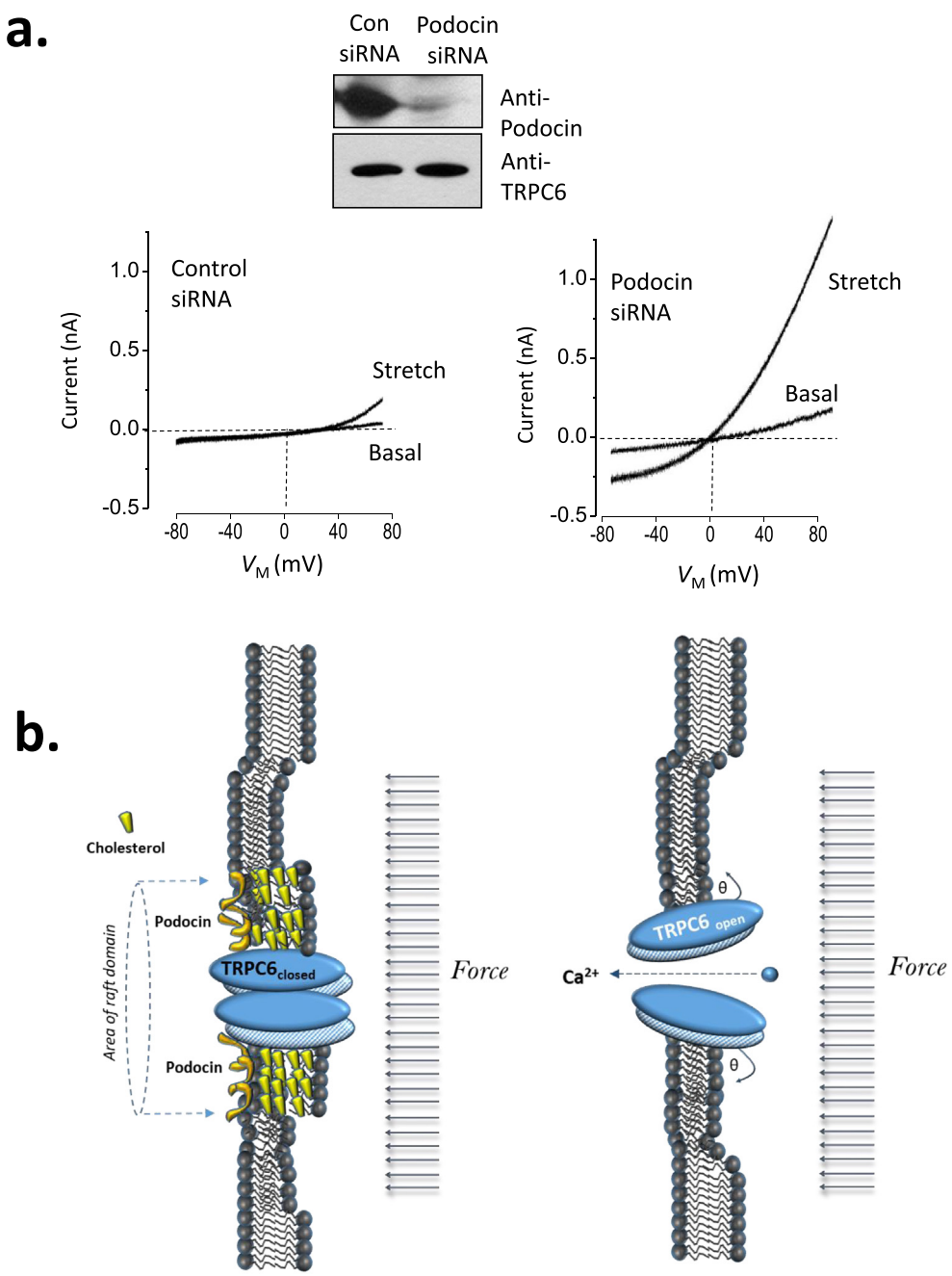

Fig. 5. Podocin suppresses TRPC6 activation in response to mechanical stimuli in cultured podocytes. Consequently, stretch-evoked currents are substantially larger on average in podocytes following podocin knockdown. Additional details are in [22]. (b) Schematic diagram showing possible role of podocin, which binds to TRPC6 at their respective C-terminals. Podocin also has a cholesterol-binding PHB domain that interacts with sterol-rich domains in plasma membranes (left). Sterol-rich domains are thought to have mechanical properties distinct from other regions of the plasma membrane owing to differences in lipid and protein composition. It is possible that sterol-rich domains in podocytes are relatively resistant to mechanical perturbation. In the absence of podocin (right), more TRPC6 channels will be found outside of sterol-rich domains. In this model, perturbation in those areas causes transmembrane alpha helices of TRPC6 move or tilt in order to maintain stable interactions between hydrophobic side chains and the fatty acid acyl chains in the core of the membrane. Cytoskeletal elements not shown here may also play an important role in regulating plasma membrane mechanics and stretch-activation of TRPC6 [22].

voltage-activated Ca^{2+} channels (Ca_v channels) [81,82]. Mesangial cells secrete a dense matrix that can bind and store growth factors when the matrix is hydrolyzed, and are able to secrete a wide range of growth factors, vasoactive agents, chemokines, and matrix metalloproteinases [83,84]. Under certain conditions they become hypertrophic and proliferate, resulting in excess production of matrix and excess secretion of soluble signals that affect the functions of other glomerular cell types [85].

Mesangial cells are reported to express multiple TRPC channels including TRCPC1, 3, 4 and 6 [86]. As with podocytes, there is biochemical evidence that endogenously expressed mesangial cell TRPC6 channels contain some TRPC3 subunits [87]. Functional studies have shown that Ang II is able to evoke activation of cationic currents in mesangial cells with properties expected for TRPC6. These currents are reduced or eliminated following TRPC6 knockdown [88]. Mesangial cell TRPC6 channels also become active in response to calcium sensing receptors [87] and glucagon [89]. There is evidence supporting a pathway from cyclic AMP to MEK and Erk1/2 that ultimately leads to TRPC6 activation in mesangial cells [89]. In mesangial cells, TRPC6 activation leads to changes in the cytoskeleton [90] and stimulation of proliferation [87,91]. It is also likely that TRPC6 can provide a depolarizing stimulus that can drive activation of Ca_v channels, which is supported by the observations that TRPC6 is required for mesangial cell contraction in response to Ang II [92] and reduced contractility in mesangial cells in TRPC6 knockout mice [93]. Because mesangial cells express Ca_v channels, contraction may not depend directly on Ca^{2+}-permeation through TRPC6 per se. In those cells it may be sufficient for TRPC6 to simply induce depolarization, thereby causing Ca_v channels to become active and to drive the majority of Ca^{2+} influx. Bear in mind that depolarization will occur under physiological ionic conditions even if TRPC6 channels are only transporting monovalent cations.

TRPC6 channels of mesangial cells are inhibited by ROS and by stimuli that generate ROS, at least if elevated ROS is maintained over periods of several hours. This is different from what is seen in

podocytes, and it was originally noted by examining responses to elevated external glucose [88], which we will discuss in more detail further below. This was also seen in response to H_2O_2 [92]. These effects were associated with reduced expression of mesangial cell TRPC6 channels and their transcripts over periods of hours to days, and they were blocked by pretreatment with ROS quenching agents [88,92]. The inhibitory effect of ROS on mesangial cells requires protein kinase C activation, and these factors appear to act through nuclear factor κB (NFκB) to suppress TRPC6 at a transcriptional level [94]. To our knowledge the role of ROS in TRPC6 modulation over shorter time scales in mesangial cells has not been studied, and this remains an important unresolved question.

The fact that at least some stimuli produce opposing effects on TRPC6 in mesangial cells and podocytes (e.g. glucose and ROS) is consistent with an admittedly speculative model in which expansile forces within a glomerular capillary under certain conditions are absorbed by suppression of mesangial cell contraction that is accompanied by TRPC6-driven changes in podocyte cytoskeleton that prevent their detachment while maintaining the integrity of the slit diaphragm. Through such a mechanism, sustained expansile forces that might result in podocyte detachment could be directed away from the outer walls of the glomerular capillary.

6. TRPC6 channels in other renal cell types

There are reports of TRPC6 channels in other renal compartments. An immunohistochemical study found evidence for TRPC3 and TRPC6 co-localization with aquaporin-2, suggesting their expression in principle cells of the collecting duct. These channels are also expressed in M1 and IMCD-3 cells, which are derived from the collecting duct [95–97]. There is evidence that TRPC6 and TRPC3 traffic independently in the collecting duct, which implies that they have distinct functions and may not heteromerize in those cells [96]. It has been proposed that TRPC6 could play a role in driving flow-mediated production of endothelin-1 in cortical collecting ducts [98]. Moreover, tubular cells appear to lose TRPC6 in response to ischemic injuries, and this effect is reversed following infusion of erythropoietin [97].

There is pharmacological and functional evidence suggesting that TRPC6 and/or TRPC3 channels contribute to Ang II- and norepinephrine-evoked Ca^{2+} responses in afferent arterioles [99,100] and could therefore play a role in regulation of renal blood flow. As with mesangial cells, the primary role of TRPC channels in these cells may be to function as receptor-operated channels that provide sufficient depolarization to activate dihydropyridine-sensitive Ca_V channels, which then drive the majority of Ca^{2+} influx required for contraction. If these are mechanosensitive, they might also play a role in autoregulatory myogenic responses in those vessels.

TRPC6 channels are often expressed in phagocytic cells [101] and fibroblasts [102] and play an important role in the functions of these cells. Dendritic cells and macrophages form a complex network within the kidney [103], and therefore TRPC6 could contribute to regulation of innate immunity and fibrosis in the kidney. We have already noted that a subpopulation of mesangial cells is also phagocytic. A possible role for TRPC6 in triggering renal fibrosis will be discussed further below.

7. TRPC6 dysregulation in acquired forms of glomerular disease

TRPC6 came to the attention of experimental nephrologists owing to studies of inherited nephrotic syndromes. However familial forms of FSGS are quite rare, and mutations in *TRPC6* comprise a relatively small subset of these [104]. For example, mutations in the *NPHS2* gene, which encodes podocin, are much more frequently detected, especially in FSGS with childhood onset [105,106]. The question nevertheless arises as to whether TRPC6 dysregulation can be discerned in patients with the more common non-familial or “acquired” forms of glomerular disease. This first evidence for this appeared in 2007, when Jochen Reiser and his colleagues reported increased expression of wild-type TRPC6 channels and transcripts in glomeruli of patients with various forms of CKD. These workers observed increased *TRPC6* mRNA and anti-TRPC6 staining in glomeruli of patients with primary FSGS, minimal change disease, and membranous glomerulonephritis, compared to patients who did not have glomerular disease [107]. This has since been confirmed for primary FSGS [108]. Increased glomerular TRPC6 was also seen in passive Heymann nephritis in rats, an animal model of human membranous nephropathy, as well as in acute and chronic puromycin aminonucleoside (PAN) nephrosis, a rat model for secondary forms of FSGS [107,109]. More recently, increases in renal cortical TRPC6 have been described in the nephrotoxic serum (NTS) model of rapidly progressing autoimmune crescentic glomerulonephritis [110]. In this regard, there is also an increase in renal cortical TRPC3 in chronic PAN nephrosis and in the NTS model in rats [109,110].

Given that mutations in TRPC6 are associated with familial forms of FSGS, it is important to address if wild-type TRPC6 channels are dysregulated in the presence of disease factors driving primary (non-familial) forms of FSGS. To test this, our group cultured immortalized mouse podocytes with serum and plasma samples collected from patients with primary FSGS whose disease recurred after transplantation [65]. The recurrence of FSGS after transplantation has long been considered evidence for circulating factors, most likely associated with innate immune responses that drive pathological processes in podocytes [111–114]. We observed that the majority of the relatively small number of samples that we were able to test increased the abundance of TRPC6 channels on the podocyte cell surface after exposures of 6–24 h [65]. An example of this is shown in Fig. 6a. With most but not all samples, increased surface expression of TRPC6 was accompanied by a concurrent loss of podocin expression. Knocking down podocin by itself does not alter surface abundance of TRPC6 in cultured podocytes, so it seems likely that effects on TRPC6 and podocin represent pathways arranged in parallel rather than in series. Moreover, these plasma and serum samples induced a marked increase in the activation of TRPC6 channels by membrane stretch (Fig. 6b), a point that we will return to further below. In a few cases we noted that the effect of the plasma and serum samples was greater with samples taken when patients were in a relapse of their disease, whereas samples taken during temporary remissions after plasma exchange or plasmapheresis were less active [65]. Finally, the effects of these patient samples on TRPC6 were mimicked by putative circulating glomerular “permeability factors” implicated in primary FSGS, including the soluble urokinase and plasminogen activator receptor (suPAR) and tumor necrosis factor (TNF) [65,66]. It is interesting that only suPAR also evoked a concurrent loss of podocin, whereas TNF increased surface TRPC6 but had no effect on podocin.

A role for suPAR as a permeability factor driving primary and recurrent FSGS was initially suggested in 2011 [115] and Kronbichler et al. [116] have written a well-balanced review of the literature that appeared over the next five years. Different groups have used different methods used for measurement of suPAR, and suPAR measurements have been made from urine or serum. Interpretation of results depends to some extent on whether changes in circulating suPAR are affected by disease-related reductions in GFR. More recent studies indicate that circulating suPAR levels prior to reductions in GFR strongly predict future declines in renal function and independently associate with incident kidney disease [117,118]. These data provide a strong motivation for studies of suPAR in CKD. There are also extensive older data that have received less attention supporting a role for TNF in primary and recurrent FSGS. These include case reports showing efficacy of TNF inhibitors [119,120] as well as increased TNF production from monocytes from patients with primary nephrotic syndrome [121] and increased TNF in serum and urine of FSGS patients [122]. It may be relevant that most of the reports of TNF in FSGS come from pediatric patients.

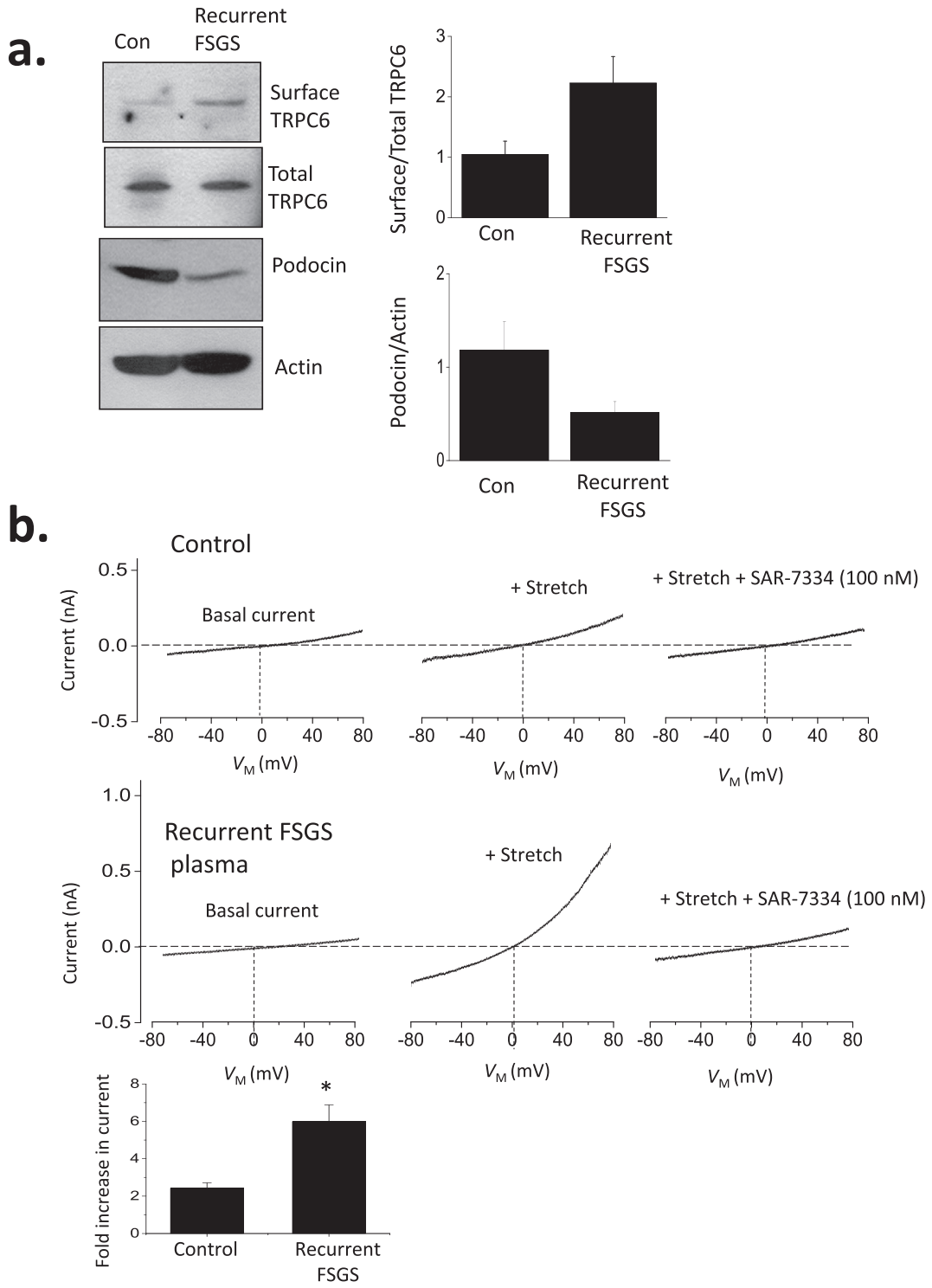

Fig. 6. Exposing podocytes to serum or plasma samples from patients with recurrent forms of FSGS alters the distribution and properties of TRPC6 channels. (a) Exposing mouse podocytes to plasma from a patient with recurrent FSGS for 24 h resulted in increased abundance of TRPC6 on the cell surface, and also resulted in a loss of podocin. Loss of podocin increases cumulatively over a period of 6–24 h. (b) Whole-cell recordings from cultured mouse podocytes exposed for 24 h to plasma from the same patient. Note markedly increased response to hypoosmotic membrane stretch, which was blocked by SAR-7334, a selective inhibitor of TRPC6 channels. Bar graph shows summary of several repetitions of this experiment. Additional details and other examples can be found in [65]. Most but not all recurrent FSGS samples that we have examined result in a loss of podocin, while nearly all increase the abundance of TRPC6 on the cell surface.

We have observed that TNF and suPAR produce greater than additive effects in cultured mouse podocytes on several responses, including increasing abundance of TRPC6 on the cell surface and loss of podocin [65]. The latter was seen even though TNF by itself does not result in a loss of podocin at any concentration that we have tested. Moreover, antibodies against either suPAR or TNF partially neutralized the activity in a recurrent FSGS plasma sample, but complete neutralization of the activity could only be produced by simultaneous application of both antibodies [65]. Those observations imply that there may not be a single circulating factor driving primary FSGS; rather

there may be a milieu of circulating factors that produce interacting effects to ultimately destabilize podocytes [123], in part via dysregulation of TRPC6 and, in most cases, through loss of podocin [65]. Indeed, even suPAR occurs in multiple forms, including a newly described dimeric form [124].

Of course, the loss of podocin may have impacts on other components of the slit diaphragm as well. We must emphasize that TNF and suPAR are not the only factors proposed to play such a role [123] and primary FSGS patients are likely to be heterogeneous (as suggested by our data on the effects of patient serum and plasma samples on podocin abundance). A "multiple circulating factor" model is also consistent with the common observation that relapse of nephrotic syndromes can be triggered by minor infections, especially of the upper respiratory tract [125,126], which result in production of a host of circulating immunomodulatory factors. Moreover, if multiple factors act additively or synergistically to drive overall pathology, statistical correlations between disease and any one of these circulating factors may be quite difficult to obtain across a wide range of patients (which was at one point a critique of the suPAR hypothesis). In other words, it is possible that a single circulating factor could contribute significantly to disease progression, and could be targeted for therapeutics, without being a useful biomarker. Moreover, circulating factors could drive increased production of local factors in glomeruli that are capable of modulating TRPC6 [127,128].

The effects of suPAR, and also the effects of FSGS patient plasma and serum samples, appear to require activation of αvβ3-integrin, which is known to function as a receptor for suPAR [115]. Specifically the effects of suPAR and FSGS plasma on podocin and TRPC6 are blocked by cilengitide, an agent that stabilizes the inactive configuration of αv-containing integrins [129]. In addition, severe glomerular disease in mice over-expressing suPAR is reduced by crossing those animals with mice bearing deficiency mutations in β3-integrins [124]. In addition, mice with deficiencies in the inducible costimulator ligand (ICOSL), an endogenous inhibitor of αvβ3-integrin, had increased susceptibility to proteinuria [130]. TNF has its own family of receptors. Interestingly, it is known that TNF can activate cascades that result in inside-out activation of various integrins [131] including β3-containing integrins [120]. Soluble syndecan-4, another protein that can activate β3-containing integrins, also upregulates podocyte TRPC6 channels [127]. However, neither TNF nor syndecan-4, applied by themselves, affect podocin abundance, suggesting that transduction cascades leading to podocin and TRPC6 differ in some ways, and are biased depending on what ligand activates the integrin, and/or the mechanism whereby the integrin is activated. One possibility is that other co-receptors affect the transduction evoked by a subset of integrin agonists. Modulation of podocyte TRPC6 by suPAR leads to αvβ3-integrin activation, which in turn causes activation of Rac1 and Nox2, leading to increased ROS generation. In endothelial cells, integrins can modulate Rac1 through Gα13 actions on radixin, a protein found at junctions between the cytoskeleton and the plasma membrane [132]. Radixin occurs in a complex with podocalyxin in foot processes [133] and therefore a similar pathway may exist in podocytes. Increased ROS in podocytes leads to activation of Src kinases, which in turn can catalyze phosphorylation of TRPC6 and modulation of its function [24,66]. Finally we note that FSGS patient plasma samples and suPAR are both able to induce expression of β3-integrin in podocytes, which could provide for a positive feedback loop in which pathogenic circulating factors can upregulate their own receptors [65].

Similar functional changes in podocyte TRPC6 channels (especially an increase in activation by mechanical stimuli) occur in chronic PAN nephrosis, an animal model of secondary FSGS (Fig. 7). This model differs from primary FSGS in that there is no circulating factor driving the disease. Rather, PAN is taken up selectively by podocytes, and its metabolism in cytosolic compartments leads to acute toxicity resulting in loss of a certain number of podocytes, the number of which depends on dose and frequency of administration. If 25–50% of podocytes are lost in response to the initial insult, the animals exhibit a profound nephrosis but appear to recover [43]. Over the course of several weeks, albuminuria returns, at which point FSGS lesions can be detected. Plasma from PAN-treated animals has no effect on podocytes, in contrast to samples that we examine from patients with primary or recurrent FSGS (unpublished observations). We have made whole-cell recordings of podocytes in glomeruli isolated acutely from rats with chronic PAN nephrosis as well as saline-treated healthy controls. Podocytes in nephrotic animals exhibited markedly increased activation of TRPC6 in response to hypoosmotic stretch stimuli (Fig. 7a, c) and markedly attenuated responses to OAG (Fig. 7b, c). Analysis of isolated glomeruli also revealed diminished podocin abundance in chronic PAN nephrosis [109]. This suggests some common features in primary and secondary FSGS, especially enhanced TRPC6 activation by mechanical stimuli that one expects to be constantly present in vivo. By contrast, during the acute phase of PAN nephrosis there is modestly increased activation of TRPC currents in response to either ATP or OAG. There is also a discernible increase in stretch activation, but less than is seen in the chronic phase of PAN nephrosis (Fig. 7d). In other words, the switch in preferred activation mode is not seen in acute PAN nephrosis.

8. Protective effects of TRPC6 inactivation or suppression in animal models of kidney disease

If TRPC6 plays a role in driving progression of podocyte diseases, animals that do not express TRPC6, or with diminished TRPC6, should be protected in glomerular disease models. On the other hand, animals that over-express the protein should be more susceptible to disease processes. These predictions have been tested in several ways. An early study examined TRPC6 knockout mice and littermate controls following sustained (28-day) infusion with Ang II using subcutaneous osmotic pumps [134]. It bears noting that TRPC6 channels comprise an essential component of Ca^{2+} responses and cationic currents evoked by Ang II in podocytes [48,49]. TRPC6 knockout and wild-type mice developed comparable increases in blood pressure in response to Ang II, and mice of both genotypes exhibited modest increases in urine albumin excretion by the second week of Ang II treatment. Albuminuria was significantly decreased on weeks 2 and 3 in the TRPC6 knockout group compared to wild-type controls. However, this protective effect was no longer seen at 4 weeks. In this model, glomerulosclerosis and other pathological changes in glomeruli are very subtle and only a small percentage of glomeruli (< 1%) are affected, even when examined after 28 days of continuous Ang II infusion.

Overexpression of wildtype or mutant forms of TRPC6 selectively in mouse podocytes also resulted in albuminuria, modest glomerulosclerosis, and foot process effacement, although it is curious that wild-type forms of TRPC6 and mutant forms that cause FSGS in humans were equally effective [135]. A similar outcome was observed in a study of mice induced to express a constitutively active mutation of Gq selectively in podocytes [136]. Recall that Gq is an essential component of the pathway from Ang II to TRPC6. Animals over-expressing this transgene exhibited glomerulosclerosis in response to PAN (which does not normally produce glomerular disease in mice) and they exhibited more severe diabetic nephropathy when crossed with Akita mice [136]. Animals with podocyte-specific over-expression of NFAT also exhibited albuminuria accompanied by increased TRPC6 expression in glomeruli [137]. In this regard, TRPC6 upregulates its own expression in podocytes through an NFAT-dependent positive feedback loop whereby TRPC6 activation can over time upregulate its own expression [137,138]. These studies highlight a limitation of mouse models; specifically mice tend to be resistant to experimental procedures that produce quite severe glomerulosclerosis in other species.

More robust evidence suggesting a role for TRPC6 in glomerulosclerosis has been obtained in rats, in which there are several models that produce more severe disease phenotypes that more closely model renal pathology seen in humans. For example, vitamin D [139] as well

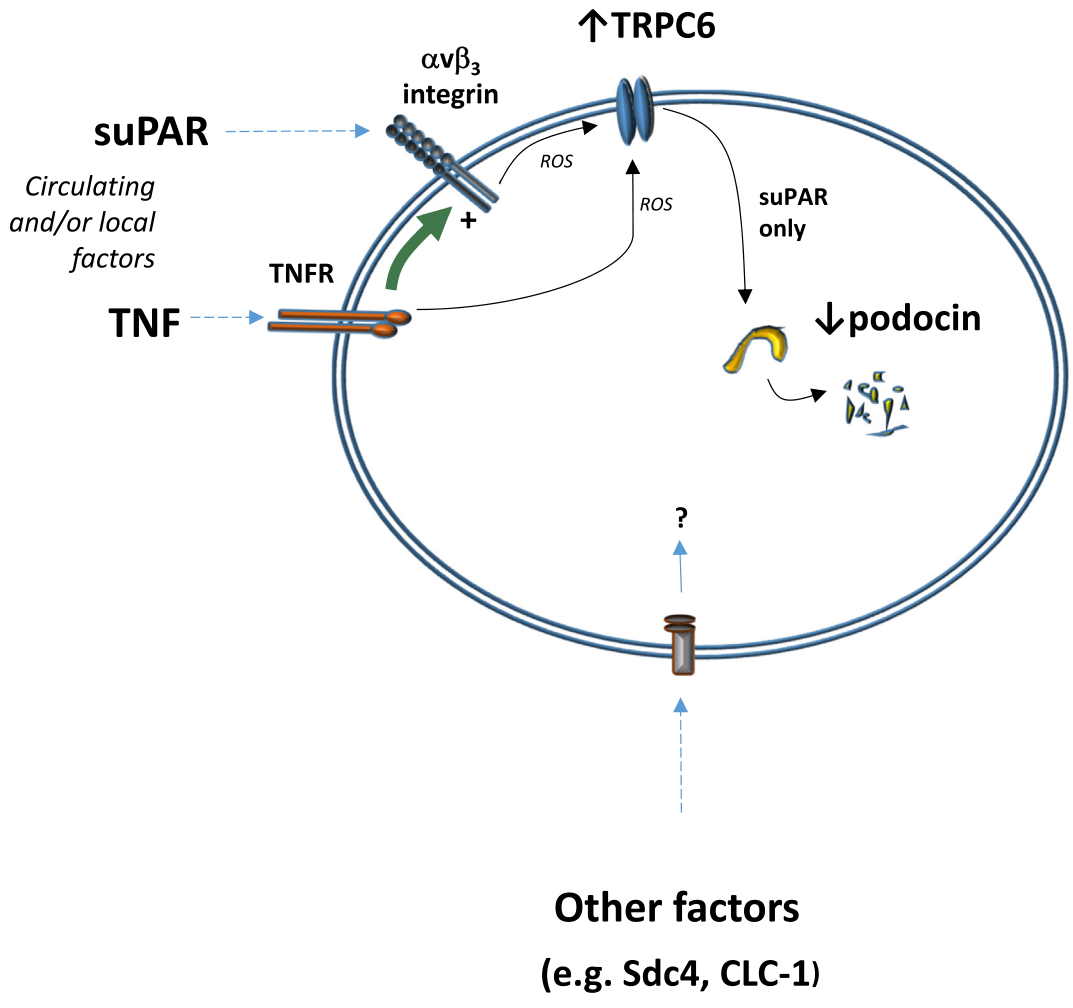

Fig. 7. Schematic showing regulation of podocyte TRPC6 channels by multiple factors found in the circulation. suPAR binds directly to αVβ3-integrin resulting in increased surface expression of TRPC6 by mechanisms that entail increased ROS generation and Src signaling [65,66]. TNF also increases TRPC6 abundance on the cell surface, possibly as a result of pathways leading to inside-out activation of αVβ3-integrin. Exposure to suPAR, but not TNF, leads to loss of podocin. Other circulating factors, such as Sdc4, cardiotrophin-like cytokine 1, and several others may produce similar effects.

as sildenafil or pioglitazone [140,141] reduce expression of TRPC6 in glomeruli and reduce glomerulosclerosis evoked by doxorubicin or hyperglycemia. Interpretation of these studies is limited by their correlative nature, as all of those treatments will affect many other cellular processes other than the expression of TRPC6. Nevertheless they support the hypothesis that excessive TRPC6 activity contributes to glomerular disease.

More recently our group used CRISPR/Cas9 methods to generate Sprague-Dawley rats with a deletion in Exon 2 of the *Trpc6* gene, which encodes a portion of the ankyrin-repeat domain required for assembly of functional channels [109]. This is not a pure knockout per se. Instead, the 239-bp deletion introduces a premature stop codon in Exon 2 and the animals remove the entire exon through a splicing process that occurs after transcription, a phenomenon that is now well documented to occur in numerous other genes [142,143]. No functional TRPC6 channels can be detected using whole-cell recordings in glomerular cells from the *Trpc6*$^{del/del}$ rats and only trace amounts of the truncated TRPC6 protein can be detected by immunoblot of renal cortex [109]. However, robust TRPC6 currents and immunochemically detectable TRPC6 are seen in wild-type Trpc6$^{wt/wt}$ littermates. The disease seen in wildtype *Trpc6*$^{wt/wt}$ rats 60 days into the chronic PAN model is severe, with very high urine albumin excretion, severe dyslipidemias, and markedly increased blood urea nitrogen and reduced creatinine clearance (Fig. 9). In addition, glomerulosclerosis was seen in a large majority of glomeruli, accompanied by a number of completely collapsed glomeruli, severe foot process effacement and GBM thickening, as well as tubular atrophy, tubular hyalinization, and marked interstitial inflammation and fibrosis [109]. *Trpc6*$^{del/del}$ rats exhibited significantly less severe kidney disease in every parameter measured. At 60 days after PAN injection the protection was not complete, with most of the measured parameters being approximately half as severe in *Trpc6*$^{del/del}$ rats [109]. In contrast to the chronic model, *Trpc6*$^{del/del}$ rats were not protected during the acute phase of PAN nephrosis (measured 9 days after PAN treatment) and the severity of albuminuria in rats examined at 60 days after PAN treatment was not correlated with the severity of albuminuria seen at 9 days (Fig. 9). In other words, the protective effect seen in chronic PAN nephrosis was not due to some systematic genotype-dependent difference in the severity of the initial PAN-evoked injury. Some parameters of kidney function in chronic PAN nephrosis in *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats are shown in Fig. 9c.

Trpc6$^{wt/wt}$ rats during chronic PAN nephrosis have increased expression of TRPC6 and TRPC3 in renal cortex compared to vehicle-treated control rats, but there was no change in the abundance of TRPC5 [109]. As noted earlier, stretch-evoked currents through TRPC6 in podocytes are enhanced in wild-type rats during chronic PAN nephrosis, whereas response to diacylglycerol analogs is decreased. TRPC3 abundance is increased in glomeruli of *Trpc6*$^{del/del}$ rats (even in vehicle-treated controls), which is reminiscent of earlier studies in vascular smooth muscle of TRPC6 knockout mice [144]. Therefore, it is possible that renal protection would be more complete if TRPC3 was

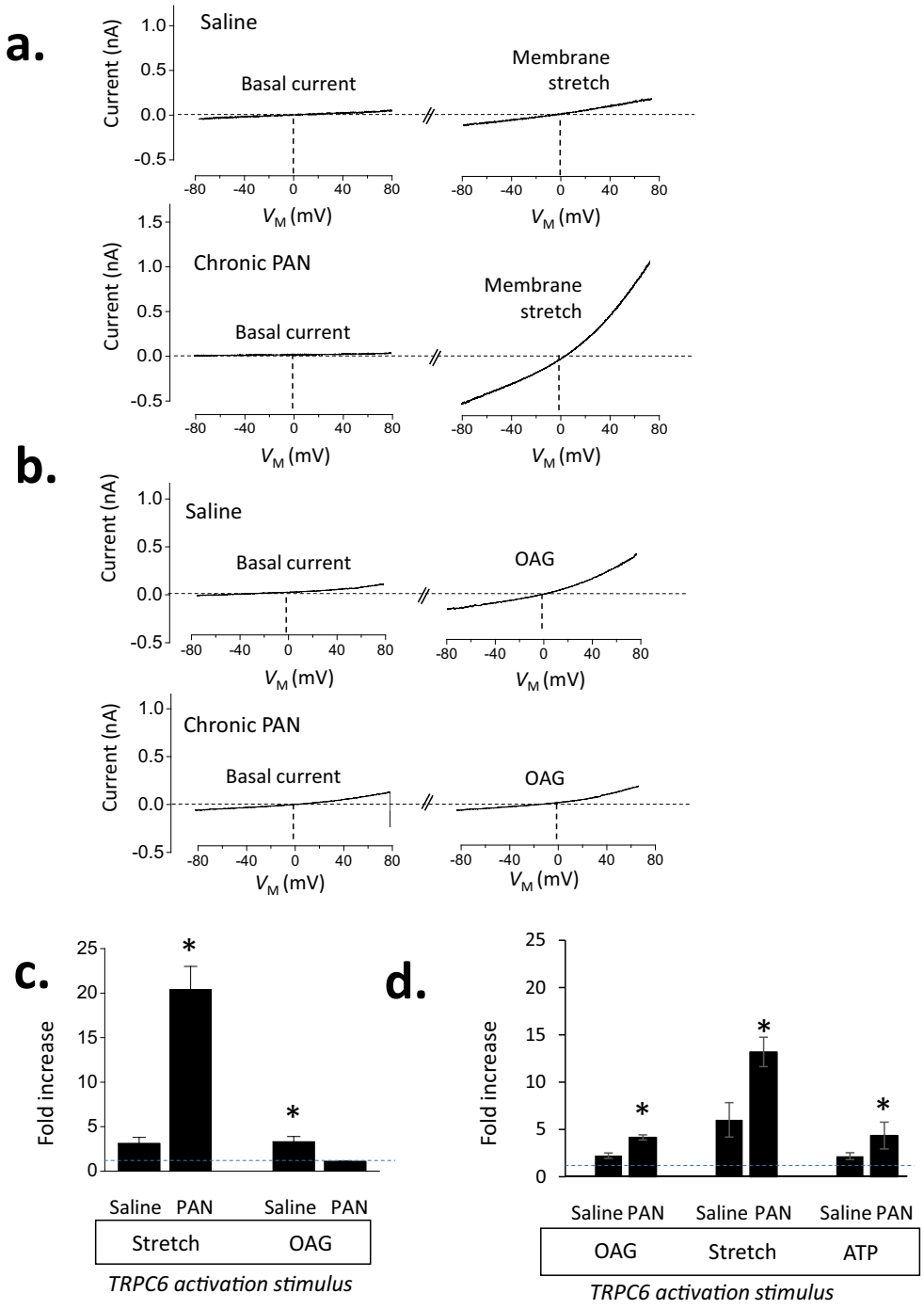

Fig. 8. Example of dysregulation of podocyte TRPC6 channels in an animal model of FSGS. In these experiments the puromycin aminonucleoside (PAN) nephrosis models of glomerular disease were induced as described elsewhere [43,109]. Control animals were treated with saline. Whole-cell recordings were from podocytes in isolated glomeruli, 60 days after the first PAN injection, using methods diagramed in Fig. 4. This is the chronic phase of PAN nephrosis, a stage when marked glomerulosclerosis can be detected by light microscopy. (a) Whole-cell recordings showing responses to membrane stretch 60 days after initial PAN injection. Currents evoked by membrane stretch are much larger in glomeruli taken from a rat with chronic PAN nephrosis compared to a saline-treated control animal. (b) Responses to OAG, a membrane-permeable DAG analog, are nearly eliminated in a rat podocyte during chronic PAN nephrosis, whereas OAG consistently obtained in cells from saline-treated control animals. (c) Summary of responses to stretch and OAG in podocytes from saline- or PAN-treated animals. During chronic PAN nephrosis there was a switch in the preferred mode of TRPC gating in favor of mechanical activation. (d) During the acute phase of PAN nephrosis, 9 days after animals were injected with PAN, there were modest and comparable fold-increases in cationic currents in response to OAG, membrane stretch, and 200 μM ATP. Thus, during acute PAN nephrosis there was no change in the dominant gating mode, but TRPC6 currents were larger on average, regardless of activation stimulus. Urine albumin excretion is markedly elevated at both time points, although it is more severe during the acute phase, as outlined in the next figure.

also inhibited or inactivated, but as yet rat strains do not exist that allow for testing of this hypothesis. A limitation of any studies on the *Trpc6*$^{del/del}$ rats that we developed is that the inactivation is global, and therefore we cannot state that the extent to which protective effects reflect actions on podocytes. Indeed, there are reasons to suspect that some effects of TRPC6 inactivation reflect immunosuppression or inhibition of fibrosis pathways [145]. We will return to this further below.

From a pathophysiological perspective, there are some similarities between diabetic nephropathy and secondary FSGS. For example, increases in glomerular capillary pressure are hallmark features of both diseases. Elevated glucose upregulates podocyte TRPC6 channels, acutely through increases in ROS [146,147], and more chronically through Ang II- and NFAT-dependent upregulation of TRPC6 expression [148]. There are a number of studies that correlate TRPC6 expression levels to the severity of diabetic nephropathy in various models and following various treatments [139,141,148–151]. More direct studies using TRPC6 knockout animals are less clear cut. The authors of a recent study suggest that TRPC6 knockout may have a slight protective effect in Dahl salt-sensitive rats made diabetic by streptozotocin (STZ), but it is important to note that they did not see statistically significant reductions in blood glucose, albuminuria or improvements in glomerular filtration rates in the TRPC6 knockout animals [152]. As with humans [153], increases in albumin excretion are a very early marker of diabetes in rats [154], and therefore a failure to see an effect on this parameter is a surprising result if protection actually occurred. Histological and morphological data were not quantified, blood pressures were not reported, and urine nephrin levels were measured with an

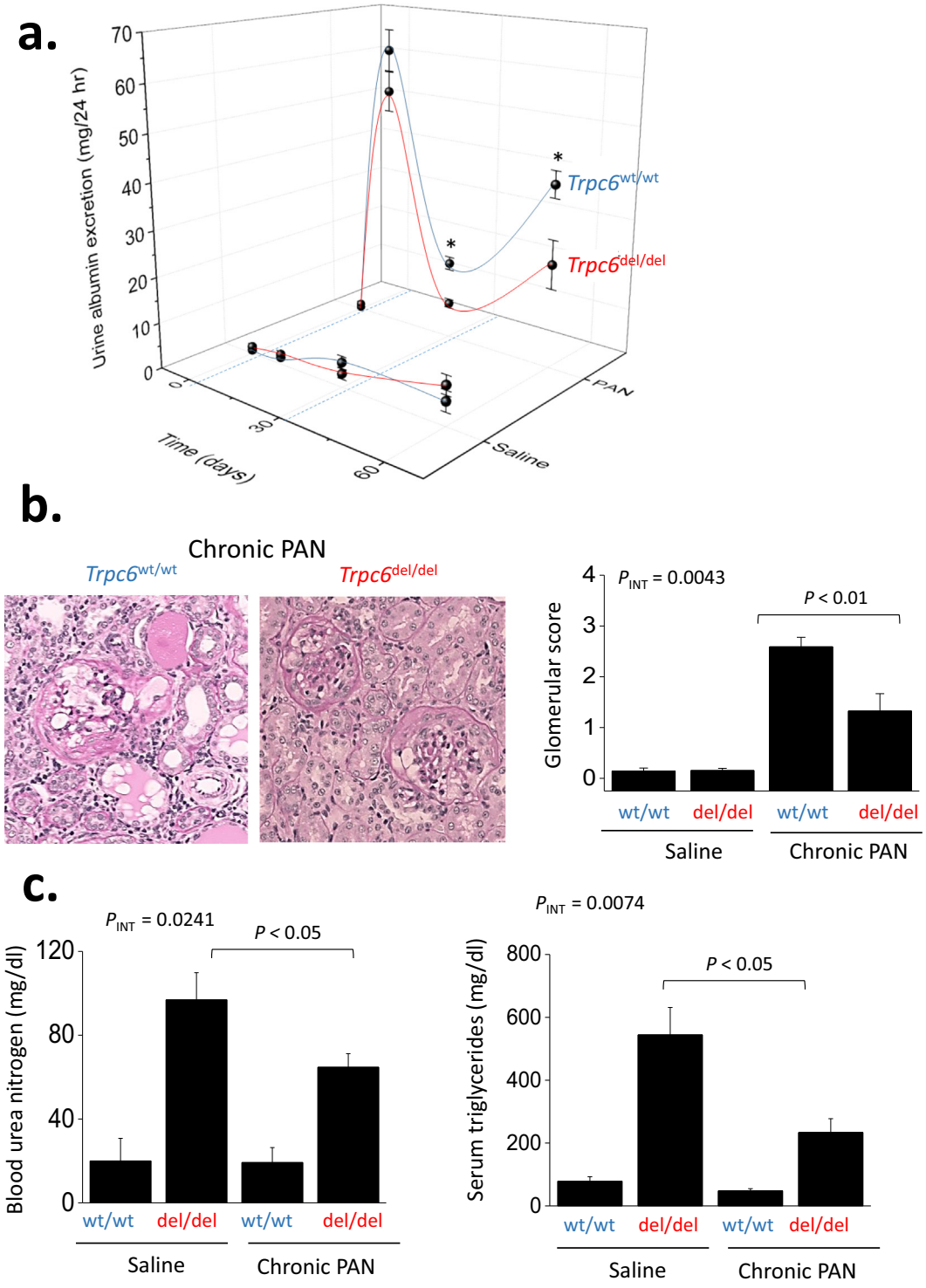

Fig. 9. Effects of TRPC6 inactivation in Sprague-Dawley rats on severity of kidney disease during chronic phase of PAN nephrosis. TRPC6 was inactivated using CRISPR/Cas9 methods described in detail in [109], which resulted in a *Trpc6*^{del} allele. The *Trpc6* transcripts and TRPC6 subunits in homozygous *Trpc6*$^{del/del}$ rats lack Exon 2, which encodes a substantial portion of the essential Ankyrin-repeat domain. (a) Urine albumin excretion in wild-type rats (*Trpc6*$^{wt/wt}$) and *Trpc6*$^{del/del}$ littermates before and at different times after injection of PAN, and in saline-treated control rats of both genotypes [109]. Renal structure and function appeared completely normal in saline-treated *Trpc6*$^{del/del}$ rats. There was severe albuminuria around 9 days after PAN injection (acute PAN nephrosis), which at this stage was equally severe in *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats. Albuminuria subsided but remerged by 30 days into the disease, becoming even more severe by 60 days. There was significantly less albuminuria in *Trpc6*$^{del/del}$ rats at both time points in the chronic phase, although protection was only partial at 60 days. (b) Glomerulosclerosis was severe in chronic PAN nephrosis in *Trpc6*$^{wt/wt}$ rats. Histology on the left is periodic acid-Schiff's staining of PAN-treated animals 60 days after the first of two PAN injections [109]. Glomerulosclerosis was still detectable but was markedly less severe in *Trpc6*$^{del/del}$ rats. The bar graph shows semi-quantitative analysis of glomerulosclerosis by an observer blind to treatment groups. There was a highly significant effect of *Trpc6* Exon 2 deletion on severity of disease based on two-way ANOVA analysis. (c) *Trpc6*$^{del/del}$ rats also preserved more renal function as measured by blood urea nitrogen, and dyslipidemias were less severe.

antibody that appears to be non-specific [152]. A more recent study has addressed a possible role for TRPC6 in diabetic nephropathy by examining *Trpc6* knockout in the Akita mouse model of type I diabetes [155]. These workers observed a protective effect of *Trpc6* knockout in this model at 12 and 16 weeks of age. However, much as was seen with Ang II infusions, this effect was transient, and by 20 weeks of age there was no longer a protective effect seen in kidneys of *Trpc6* knockout Akita mice, and indeed TRPC6 knockout at that point exacerbated the injury. These authors also reported that *Trpc6* knockout mice develop insulin resistance accompanied by increased expression of p38 and cyclooxygenase pathways which could exacerbate nephropathy, and possibly due to loss of insulin receptor substrate-2 (IRS2). In other words, the protective effects seen early in the Akita model are lost in part because *Trpc6* knockout animals are no longer able to respond effectively to residual amounts of insulin being secreted [155]. It is not known if the effect of TRPC6 knockout on insulin responsivity is species- or strain-specific. In preliminary experiments using STZ-evoked diabetes we have seen no protective effects at any time after STZ injection in the same line of *Trpc6*$^{del/del}$ rats in which we saw robust protection from PAN nephrosis. While this might appear to contradict the study by Spires et al. [152], a significant difference is that our rats were generated on a different genetic background, and it is certainly possible that TRPC6 channels contribute more to the progression of diabetic nephropathy on a Dahl salt-sensitive strain. In any case, the protection seen by Spires et al. [152] was subtle and they observed no improvement in albuminuria or GFR; rather they reported improvements in glomerular ultrastructure (although quantitative data on this were not presented). Therefore our results to date are in agreement with most of those reported for the Dahl S strain.

It is possible that coordinated inhibition of multiple TRPC channel variants would be more effective than inactivation of TRPC6 alone. This has actually been addressed in triple-knockout mice in which multiple diacylglycerol-responsive TRPC channels (TRPC3, TRPC6, and TRPC7) were eliminated [156]. The authors report reduced urine albumin excretion, glomerular hypertrophy and podocyte loss in STZ-evoked diabetes compared to wild-type controls. Therefore it is possible that blockade of multiple TRPC channels would be more effective than a selective inhibition of TRPC6, although it is also worth noting that the triple knockout animals have markedly reduced body weight compared to WT mice, and this effect exacerbates the reductions in body weight that are a normal feature of STZ-evoked diabetes in mice [156]. Disease processes in diabetes are more complex than those occurring in PAN nephrosis, as elevated glucose produces highly pleiotropic effects in a vast number of different cell types, whereas renal effects in PAN nephrosis, and probably in primary FSGS, stem initially from dysfunction or loss of a single cell type. From this perspective it is perhaps not surprising that TRPC6 inactivation is less effective in diabetes models. It is also important to remember that different species may respond differently to channel inactivation in an otherwise similar disease state.

TRPC6 channels may play a normal role in normal responses of glomeruli to changes in blood glucose and other metabolites, as well as to hormones that respond to blood glucose. Thus, insulin increases the surface expression of TRPC6 channels in podocytes [54,157,158]. This effect is seen within minutes of insulin exposure [54], and it may be part of a mechanism to adapt glomerular filtration mechanisms to acute changes in glomerular capillary pressure which occur whenever blood glucose increases as a result of sodium-dependent reuptake of glucose in the proximal tubule via SGLT2 [159–161]. One expects that TRPC6 activation would return to normal after blood glucose returns to basal levels and during normal physiology in healthy animals the expectation is that TRPC6 activation is phasic rather than sustained. We have already noted that mesangial cell TRPC6 is upregulated by glucagon [89], which again suggests that TRPC6 channels of mesangial cells and podocytes will respond in opposite ways to elevated blood glucose, which may serve to protect components attached to the outer walls of glomerular capillaries.

9. Role of TRPC6 channels in non-glomerular and autoimmune models of renal fibrosis

TRPC6 is involved in functionally significant Ca^{2+} responses in macrophages [101], neutrophils [162] and other cell types involved in innate immunity. TRPC6 is also reported to drive trans-differentiation of fibroblasts into myofibroblasts [102], and to modulate endothelial permeability [163] and the translocation of immune cells across endothelial layers of the microcirculation [164]. Therefore, it is interesting that a recent report described a partial protective effect of TRPC6 knockout in the development of renal fibrosis following unilateral ureteral obstruction (UUO) in mice [145]. These authors observed reduced renal expression of numerous fibrosis markers (collagen-1, α-smooth muscle actin, vimentin, matrix metalloproteinases 2 and 9, and TGFβ1) in TRPC6 knockout mice compared to controls, and an anti-fibrotic effect was also seen in quantitative analyses of histology. These authors observed a similar protective effect following administration of the pan-TRPC inhibitor BTP2 (which also blocks Orai-coupled channels). Moreover, the UUO procedure resulted in upregulation of renal *Trpc3* and *Trpc6* transcripts. Importantly, however, they observed that TRPC3/6 double-knockout mice did not exhibit significantly greater protection following UUO than mice in which only TRPC6 was knocked out [145]. The authors further presented evidence that protective effects of soluble klotho in UUO are mediated by suppression of renal TRPC6, and that soluble klotho does not produce additional kidney protection once TRPC6 is knocked out [145]. Soluble klotho may reduce cardiac fibrosis by a similar mechanism [165] and also appears to reduce TRPC6 abundance in cultured podocytes [166].

Given the marked protective effects of TRPC6 knockout in this model, the question arises as to whether TRPC6 knockout or inactivation would always reduce renal fibrosis. For example, we observed reduced tubulointerstitial fibrosis in *Trpc6*$^{del/del}$ rats subjected to chronic PAN nephrosis, in which the initial insult at least is confined to glomeruli [109]. By contrast, tubulointerstitial fibrosis was equally severe in *Trpc6*$^{del/del}$ rats subjected to the NTS model of auto-immune glomerulonephritis, even though there was less glomerulosclerosis in rats with inactivated TRPC6 [110]. These results suggest that the anti-fibrotic effects of TRPC6 inactivation are model-dependent and/or species- or even strain-dependent. The reasons why TRPC6 inactivation produces different results in these models are not as yet understood. It could depend on how much of the kidney is involved in the initial insult (primarily podocytes in the case of PAN nephrosis, primarily the distal nephron in obstructive models). By contrast, autoimmune processes almost immediately induce effects outside of the site of deposition of the triggering antigens, with consequences that persist thereafter [167]. Only examination of the effects of TRPC6 knockout or inactivation in additional animal models will allow the role of these channels in fibrotic processes to be more fully elucidated. It would be of considerable interest to examine if TRPC6 regulates release of pro-inflammatory cytokines from distal tubules and glomerular cells, and indeed the extent to which TRPC6 knockout affects a range of immune responses in mice and rats.

10. Short-term versus long-term activation of TRPC6 and its normal role in renal physiology

While a substantial body of work has examined the regulation of TRPC6 in dissociated cells and isolated preparations, and its dysregulation in various circumstances, almost nothing is known about the normal role of TRPC6 channels in the overall regulation of renal physiology. One of the few studies to address this in animal models showed that TRPC6 knockout mice have an increased GFR and lower serum creatinine, possibly owing to reduced mesangial cell tone [93]. In an earlier study showing acute mobilization of podocyte TRPC6 channels in response to insulin, we suggested that this effect could play a role in regulating glomerular permeability or podocyte cytoskeleton so as to

protect glomeruli in the face of acute changes pressure that would normally occur in response to a glucose load [54]. Recall that elevated blood glucose causes an increase in glomerular capillary pressure as a result of its sodium-dependent uptake into the proximal tubule [160,161]. This has been suggested to explain hyperfiltration seen in early stages of diabetes [159]. We suggested several years ago that insulin-dependent mobilization of TRPC6 in podocytes [54] could play a role to maintain glomerular integrity in the face of what would normally be a relatively transient hyperfiltration. There is evidence that TRPC6 plays a role in regulating glucose uptake into podocytes themselves [168]. The primary point to be made here is that in full health, stimuli that have been shown to induce TRPC6 activation would normally be relatively transient, with actions that persist over time scales of minutes to hours. Presumably activation of TRPC6 over this time frame does not overload the ability of cells to handle increased Ca^{2+} influx, and indeed TRPC6 activation in this context might be protective. Indeed, there is evidence that TRPC6 knockout mice are more susceptible to podocyte damage in response to acute infusions of complement [169]. Nevertheless, it seems clear that sustained hyper-activation of TRPC6 ultimately drives loss of podocytes resulting in glomerulosclerosis and renal fibrosis. This was apparent even with the earliest studies of autosomal dominant TRPC6 mutations, which in most cases lead to disease with an adult onset [11,13], and only the most severe gain-of-function mutations are seen to produce FSGS with a childhood onset [18]. In fact, an even earlier onset childhood FSGS occurred with a dominant-negative TRPC6 mutation [21]. In this regard, based on the effects of podocin knockdown on mechanical activation of TRPC6 [22], one would expect a greater gain of TRPC6 function following mutations in *NPHS2* than is actually seen with the majority of TRPC6 mutations reported to date. This effect would be severe and unrelenting, as mechanical stimuli in functional glomeruli will always be present. The early FSGS seen with a dominant-negative mutation in TRPC6 [21] certainly suggests that TRPC6 plays some essential role in glomerular function, at least in humans. Given that, it is actually curious that TRPC6 knockout mice and rats do not show any obviously deleterious renal phenotype (e.g. glomerulosclerosis or proteinuria). The presence of functional TRPC3 in TRPC6 knockout mice and rats, and the fact that TRPC3 can be upregulated in response to TRPC6 inactivation [109,144] may obscure this role in most of the experimental designs employed to date, especially in TRPC6 knockout animals where TRPC3 is still present or even upregulated. The fact remains that TRPC6 presumably has a normal role in podocytes, mesangial cells, and other renal cell types, and it will be important in the future to design experiments to address this question. Establishing the precise physiological roles of TRPC6 channels in renal function will ultimately require development of cell type-specific knockout models. The fact that TRPC6 is expressed in a wide range of tissues and cell types currently restricts interpretations of data obtained from global and constitutive knockouts.

11. Intracellular pathways downstream of TRPC6 activation

In order to understand the consequences of TRPC6 dysregulation, it is important to understand the pathways downstream of its activation, both in normal conditions and during kidney disease. These are not fully understood and will clearly depend on the cell type examined. To date, the best documented pathways downstream of TRPC6 entail activation of calcineurin (CN), a ubiquitously expressed 57–59 kD serine-threonine phosphatase [170]. CN is nearly ubiquitously expressed and becomes catalytically active upon its interaction with Ca^{2+}-calmodulin, although CN also binds Ca^{2+} independently [171]. CN participates in a host of signal transduction cascades. The most extensively studied pathway leads to activation of a family of transcription factors known as nuclear factor of activated T-cells (NFAT). The various NFAT proteins (NFATc1–4 and the more divergent NFAT5) were initially described as part of a transcriptional regulatory complex induced by signals onto activated T-cells that result in PLC-dependent increases in intracellular free Ca^{2+} and activation of calmodulin and CN [172]. Active CN dephosphorylates cytosolic NFAT, thereby triggering its translocation to the nucleus. Once inside the nucleus, NFAT is again phosphorylated by various nuclear kinases, whereupon it forms large complexes with other transcriptional regulatory proteins [173]. This pathway is responsible, at least in part, for profound immunosuppression seen in response to the CN inhibitors cyclosporine and tacrolimus, which provided the initial motivation for using these agents in nephrotic syndromes such as primary FSGS. This immunosuppressive effect may certainly contribute to their efficacy in this context. However, TRPC6 activation also leads to CN activation in podocytes. Moreover, TRPC6 channels themselves are regulated at a transcriptional level by CN-NFAT pathways [137,138], which provides the basis for a positive feedback loop that could in time cause constitutive upregulation of TRPC6 [137]. Because of the highly polarized structure of podocytes, and because of diffusional barriers, Ca^{2+} influx that alters podocyte transcription probably occurs within or close to the cell body (where TRPC6 channels are present, as noted previously).

The TRPC6-dependent pathways in podocyte foot processes are almost certainly somewhat different from those that occur in the cell body. TRPC6 channels in both compartments appear to be coupled to CN. However, in foot processes, active CN causes de-phosphorylation of synaptopodin, which then allows for its proteolysis by cathepsin L [174]. Synaptopodin is a cytoskeletal regulatory protein found in podocyte foot processes that plays a role to stabilize those structures in the face of stimuli or forces that would otherwise lead to their effacement [174,175]. Consequently, sustained activation of TRPC6 and CN would tend to promote foot process destabilization. This pathway may contribute to the protective effects of drugs such as cyclosporine A or tacrolimus noted in case reports of patients with familial nephrotic syndromes [176,177]. In other words, the efficacy of these agents may not be due to immunosuppression, rather they may stabilize foot processes and/or prevent podocyte detachment [174]. Synaptopodin tends to suppress surface expression of TRPC6, at least in cultured in podocytes [178]. Therefore, as with the transcriptional dynamics in the cell body, sustained activation of TRPC6 may act to increase the availability of TRPC6, giving rise to a positive feedback loop eventually giving rise to podocyte disease.

The possible range of pathways downstream of TRPC6 can be appreciated from the number of proteins already confirmed to be substrates for CN, which includes cytoskeletal proteins, mitogen-activated protein kinases, proteins involved in the cell cycle and apoptosis, scaffolding proteins, ion channels known to be expressed in podocytes, and several transcription factors in addition to NFAT proteins [179,180]. In fact, > 50 substrates for CN have been confirmed, and it is likely that this is only a fraction of those that are likely to exist [179]. CN does not act on specific substrate motifs adjacent to phosphorylated serine or threonine residues, rather it recognizes proteins containing so-called short linear motifs (SLiMs) that are conserved within the NFAT family but which are present in many other proteins. Structural analysis of SLiMs has allowed for elucidation of a large CN interaction network that includes proteins that regulate cell junctions, cell cycle, transcription, ion transport, and vesicle trafficking [181]. Therefore, it is likely that only a small portion of the possible consequences of TRPC6 activation in the kidney and elsewhere have been identified.

CN is just one of a wide range of potentially Ca^{2+}-dependent signals in renal cells. In principle nearly any Ca^{2+}- or calmodulin-dependent protein localized within the accessible diffusional radius surrounding TRPC6 channels could represent a downstream pathway for TRPC6. A recent study has suggested that calpain-1 contributes to sustained podocyte injury and structural destabilization following TRPC6 activation [182]. TRPC6 can also increase apoptosis in cultured podocytes through activation of caspases [183]. This pathway has also been reported in mesangial cells [184]. TRPC6 can also contribute to autophagy induced by various stimuli [185]. There is also evidence that TRPC6 activation leads to activation of RhoA [51], although the mechanism whereby this

occurs is not known.

We have already mentioned that TRPC6 itself can be considered a downstream target of Ca^{2+} influx through TRPC6 and closely associated proteins. This can result in both negative- and positive-feedback effects. From studies of heterologously expressed TRPC6, it has long been known that the effects of Ca^{2+} influx on TRPC6 activation exhibit a bell-shaped relationship, with increased TRPC6 activation seen at lower levels of Ca^{2+} influx, and suppression or inactivation of current seen under conditions that allow for greater Ca^{2+} influx [186]. In this regard, TRPC6 and other TRPC channels have binding sites for Ca^{2+}-calmodulin in cytosolic-facing domains at the C-terminus [187] and possibly also in the N-terminus [188]. The site near the C-terminus of TRPC channels, known as the CIRB region, has been extensively characterized, and overlaps with a binding site for the inositol (1,4,5)-triphosphate receptor [189]. This domain also interacts with other Ca^{2+}-binding proteins such as S100A1 [190]. Direct binding of Ca^{2+}-calmodulin tends to suppress TRPC6 activation [188,191] and this effect may regulate translocation of TRPC6 to the surface of endothelial cells [192]. On the other hand, phosphorylation of TRPC6 by Ca^{2+}-calmodulin-dependent protein kinase II can enhance TRPC6 activation [193]. Any or all of these interactions could in principle be altered in renal cells in response to circulating factors, locally generated stimuli, or as a result of mutations in TRPC6. However these processes have not been investigated for endogenously expressed TRPC channels in renal cells.

While not as extensively discussed, it is important to note that permeation of Ca^{2+} through TRPC6 channels exhibits a complex dependency on the transmembrane electric field; more specifically while Ca^{2+} can permeate the TRPC6 channel, it also blocks the pore in a voltage-dependent manner [194]. As a result of the interaction between Ca^{2+} and the pore, as the membrane potential approaches 0 mV, TRPC6 increasingly functions as a monovalent cation channel. This is not simply due to reduced driving force for Ca^{2+} influx with membrane depolarization, and it is not a consequence of voltage-dependent conformational gating changes such as are seen in Ca_V channels. Rather, this is an intrinsic property of the pore itself that arises from the interactions of permeant ions with residues lining the inside of the pore as they permeate the channel [194]. An important consequence is that because TRPC6 activation results in cell depolarization, its own activation will tend to reduce Ca^{2+} influx unless there is a mechanism in place to reduce or prevent depolarization [50,194]. In cells that express voltage-dependent Ca_V channels (e.g. cardiac muscle, vascular smooth muscle and mesangial cells) TRPC6 activation can trigger membrane depolarization sufficient for Ca_V channels to become active. This will occur even if TRPC6 is functioning as a pure monovalent cation channel (since it is only the depolarization that matters, and not what ions are carrying the current). However, podocytes and many other non-excitable cell types do not express functional Ca_V channels, which makes the question of how TRPC6 can trigger receptor-evoked Ca^{2+} influx problematic given the biophysics of ion permeation through these channels.

In podocytes we have observed that TRPC6 co-immunoprecipitates with large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca} channels) encoded by the *KCa1.1* gene, also known as Slo1 channels [47]. BK_{Ca} channels are also present in human podocytes [195]. This interaction allows Ca^{2+} influx through TRPC6 to cause BK_{Ca} activation, which by reducing the tendency for depolarization, could allow TRPC6 to function as an efficient and sustained source of Ca^{2+} influx. These dynamics might explain why insulin increases the surface expression of both channels in podocytes [54,157,158,196,197]. In addition, podocytes also express a sodium-calcium exchange protein, which could also allow for increased Ca^{2+} influx following TRPC6 activation, even if the channel is primarily conducting Na^+ [198].

12. TRPC5 channels, podocyte cell biology, and kidney disease

TRPC6 is not the only TRPC channel expressed in glomeruli [51,54]. It has been reported that inhibition of TRPC5 channels by knockout or by administration of high systemic doses (20 mg/kg) of a small molecule inhibitor of TRPC5 (ML-204) reduced albuminuria and rescued the podocyte cytoskeleton in mice challenged with lipopolysaccharide (LPS) or protamine sulfate [199]. That study also noted rapid activation of TRPC5 channels in cultured podocytes following application of LPS (100 μg/ml), and also in response to muscarinic cholinergic agonists. Both of those responses were blocked by 10 μM ML-204 in vitro. More recently, this group has developed a novel TRPC5 inhibitor known as AC1903 that they argue has greater specificity than ML-204. They have reported that AC1903 reduces albuminuria in transgenic rats that overexpress the human angiotensin type 1 receptor (AT1R) selectively in podocytes [200]. The AT1R rats normally develop severe albuminuria starting at 8–15 weeks. Albuminuria increases progressively with time, although creatinine clearance, a surrogate measure for GFR, is not reduced until 32 weeks of age [201]. The AT1R rats also exhibit pseudocysts within glomeruli by 15 weeks of age, with adhesions between capillary tufts and parietal cells appearing somewhat later, and with evidence of tubulointerstitial disease appearing by 11 months of age [201]. Importantly, because the transgenic AT1R is limited to podocytes, these animals do not develop systemic hypertension. AC9103 delivered twice daily by intraperitoneal (i.p.) injection at 50 mg/kg for a period of just seven days reduced albuminuria and decreased the appearance of pseudocysts in AT1R rats. Quite remarkably, this treatment protocol was reported to be effective in AT1R rats that already had advanced glomerular disease. If confirmed, this would seem to imply some role for TRPC5 in podocyte regeneration since glomerulosclerosis generally occurs as a result of a loss of podocytes. AC9103 treatment also reduced unitary channel activity attributed to TRPC5 in excised inside-out patches in glomeruli isolated from these animals. These workers also reported a modest reduction in proteinuria and podocyte loss in Dahl S rats treated with AC1903 starting at the same time that animals were given high NaCl intake to induce systemic hypertension [200]. These data suggest that TRPC5 inhibitors could represent a useful and novel class of therapeutic agents for glomerular disease.

However, the role and significance of TRPC5 is controversial. For example, over-expression of either a wild-type TRPC5 or a dominant-negative TRPC5 pore mutant (TRPC5-DN) in C57BL/6 mice did not result in albuminuria, foot process effacement, or glomerulosclerosis at up to 8 months of age [202]. Moreover, global overexpression of TRPC5 or TRPC5-DN did not affect albuminuria evoked by LPS [202], in contrast to what was seen following ML-204 treatment in mice, or in TRPC5 knockout mice [199]. Ratiometrically measured Ca^{2+} responses in podocytes cultured from animals over-expressing wildtype TRPC5 were increased compared to podocytes from wild-type controls, suggesting that some of the TRPC5 channels over-expressed in podocytes of these animals were functional, even though no pathology ensued [202]. In addition, treatment with Englerin-A, an agent that causes activation of TRPC5 [203,204], did not evoke albuminuria in wild-type mice or in mice over-expressing either TRPC5 or TRPC5-DN [202]. In those experiments mice received two i.p. injections of Englerin A (3 mg/kg. by i.p. route) separated by 24 h before examining urine albumin excretion. Englerin A causes a reduction in mouse locomotor behavior that lasts for about 1 h after a single i.p. injection at 2 mg/kg [205]. It is not known if that reflects difficulties in crossing the blood-brain barrier or overall pharmacokinetics, but it is possible that Englerin A simply did not activate TRPC5 long enough to induce adverse renal effects.

In the most recent study, LPS-evoked albuminuria was not altered by treatment with the TRPC5 inhibitor ML-204 [202]. Mice were given three intraperitoneal applications at 2 mg/kg, a markedly lower dose than was used in the earlier study by Schaldecker et al. [199]. A recent publication examining the effects of ML-204 in mouse models of arthritis and joint inflammation found that the ML-204 was effective by multiple criteria at a dose of 2 mg/kg i.p., with no adverse effects seen with that protocol [206]. Consequently, it is possible that the ten-fold

higher in vivo doses of ML-204 used by Schaldecker et al. [199] produced effects independent of TRPC5. In this regard, ML-204 and AC9103 were not tested in TRPC5 knockout animals to rule out off-target effects, which would be a particular concern given the doses of ML-204 used by Schaldecker et al. [199]. In our hands ML-204 can block stretch-evoked activation of TRPC6 in podocytes at sub-micromolar concentrations (unpublished data). On the other hand, Schaldecker et al. observed that ML-204 does not block TRPC6 activation in excised patches activated by a diacylglycerol analog [199].

TRPC5 was readily detected by immunochemical methods but changes in TRPC5 abundance in renal cortex were not observed in rats during chronic PAN nephrosis [109] or in autoimmune glomerulonephritis [110] although there was increased abundance of TRPC6 and TRPC3 in both of those disease models. While TRPC5 can certainly be detected in kidney, TRPC5 expression in human kidney biopsy samples has not been presented. This is in contrast to TRPC6, which is expressed at higher levels in glomeruli of patients with FSGS [107,108]. When considering ion channels, the number of functional and active proteins on the cell surface is often more relevant than total abundance or transcript levels. However, changes in total abundance will occur when a given channel is regulated by transcriptional positive feedback loops such as the ones that are well established for podocyte TRPC6 [137].

The first functional characterization of TRPC5 channels in podocytes was based on characterization of Ang II-evoked channels in single-channel recordings from immortalized mouse podocytes overexpressing the AT1R [51]. Much of the electrophysiological evidence for expression of functional TRPC5 channels in podocytes ex vivo has been in glomeruli from rats with podocyte-selective over-expression of AT1R [200]. Moreover, functional TRPC5 channels detected in single-channel recordings appeared to be more numerous in AT1R transgenic rats with established glomerular disease, and were considerably sparser at the time of disease onset. Functional TRPC5 channels were nearly undetectable in wild-type control animals [200]. That observation is consistent with an earlier study using single-channel recordings from podocytes in glomeruli isolated from wildtype mice, which found no evidence for TRPC5 activation in response to Ang II, and which noted abolition of Ang II-evoked cation current in TRPC6 knockout mice [49]. A similar conclusion was reached based on whole-cell recordings from cultured mouse podocytes following TRPC6 knockdown with siRNA in which cationic currents were activated by either Ang II [48] or ATP [52], although those later studies must be considered lest definitive since siRNA cannot produce as complete of an inhibition as a gene knockout. It is possible that over-expression of AT1Rs in podocytes in vivo or in vitro allows for “promiscuous” activation pathways that do not occur if these receptors are present at normal endogenous levels of expression. In support of the TRPC5 concept, we observe that TRPC5 is readily detectable by biochemical analyses of rat glomeruli and in mouse podocyte cell lines. Moreover plasma samples from at least one patient with recurrent FSGS produced a large increase in stretch-evoked current in cultured podocytes, and a component of this current persisted in the presence of micromolar La^{3+} [65]. This is relevant because TRPC6 and TRPC5 show opposite responses La^{3+} at this concentration, and TRPC5 should be increased under those conditions, whereas TRPC6 should be completely inhibited [29,51]. Thus, it is possible that the residual current seen in the presence of La^{3+} in those recordings reflects enhanced mobilization of TRPC5 induced by the FSGS plasma sample. Given the relatively small number of patient samples that we have tested in this way, it is possible that a subpopulation of patients have circulating factors that would more specifically up-regulate TRPC5.

One factor that potentially confounds interpretation of available studies is the nature of the disease models used to date, and in particular their time course. LPS produces an acute and reversible albuminuria in rats and mice. In our hands TRPC6 inactivation in rats has no effect on LPS-evoked nephrosis in rats (unpublished observations), reminiscent of what we observe in acute PAN nephrosis, and in marked contrast to chronic PAN nephrosis [109] or autoimmune glomerulonephritis [110]. In our view, it is not clear that any acute manipulation (e.g. acute PAN, protamine sulfate or LPS) is a realistic model for a human nephrotic syndrome, except perhaps to demonstrate that if one kills podocytes, or alters their cytoskeleton sufficiently, glomerular dysfunction ensues very quickly. Therefore, whether or not TRPC5 inhibition or knockout is effective in acute disease models, which as noted above is not universally agreed upon, is not particularly informative. Data obtained from chronically enhanced Ang II signaling would seem to be more informative [200] but it is still puzzling that chronic over-expression of TRPC5 does not produce a renal phenotype, even after administration of TRPC5 activating agents, and given evidence that the over-expressed channels are functional [202].

In spite of arguments to the contrary [200] the role of TRPC6 and TRPC5 in kidney disease should not be regarded as an “either-or” choice. TRPC6 and TRPC5 do not form functional heteromultimers and it is not possible to co-immunoprecipitate these proteins from kidney. (TRPC5 can form heteromers with TRPC4 whereas TRPC6 can form heteromers with TRPC3.) However, TRPC6 stimulates translocation of TRPC5 to the surface of endothelial cells and mobilization of TRPC5 by lysophosphatidylcholine (lysoPC) requires TRPC6 [207,208]. As an aside, we have observed that knockdown of TRPC5 reduces the surface expression of TRPC6 in cultured podocytes, and vice versa (Fig. 10), similar to the earlier reports on endothelial cells. We do not know if this would occur with podocytes in vivo, but the possibility needs to be considered, especially since it is reported that TRPC5 promotes activation of Rac1 [51]. TRPC5 channels, like TRPC6, are reported to be mechanosensitive, or at least components of mechanotransduction cascades [209,210] and they are activated in response to oxidative stress [211]. In heterologous expression systems TRPC5 activation is increased during agonist-evoked increases in intracellular Ca^{2+} and calmodulin [212,213], and Ca^{2+}-calmodulin-dependent light chain kinase can increase trafficking of TRPC5 to the cell surface [214]. Increases in Ca^{2+}-calmodulin certainly occur following TRPC6 activation in podocytes. Therefore it is quite possible that TRPC6 and TRPC5 in podocytes are mobilized to the cell surface and activated in a coordinated fashion by at least some of the same stimuli, as has been demonstrated directly in endothelial cells [208]. If so, it is possible that the protective effects of TRPC5 inhibition reported to date [199,200] could at least in part reflect suppression of TRPC6 through this type of indirect pathway. One of the mysteries surrounding TRPC5 is what, if any, are the normal physiological stimuli that cause those channels to become active in podocytes. Recall that active TRPC5 channels are not detected at any meaningful density following Ang II application except in cells or animals over-expressing AT1Rs [49,51,200].

Greka and coworkers originally proposed that TRPC6 and TRPC5 have opposing effects on cytoskeleton and cell motility in podocytes because they detected TRPC6 in a complex with the GTPase RhoA, and found TRPC5 in a complex with Rac1. Moreover, those GTPases have opposing effects on certain aspects of podocyte cytoskeleton and motile behavior [51]. In our view this model is simplistic insofar as it considers that the effects of Rac1 and RhoA on cytoskeleton supersede any other effects and because it assumes that both GTPases are exclusively downstream of the channels themselves. Those assumptions are not valid. Data already discussed has shown that TRPC6 activation in podocytes, certainly via the canonical lipid and GPCR pathways, occurs secondary to activation of Nox2 [44,48,52]. Nox2 activation requires formation of a complex with active Rac1 [215] and Nox2 itself is part of a larger complex with podocin and TRPC6 in podocytes [44]. Therefore, a model in which Rac1 is downstream of TRPC5 near the slit diaphragm [51] actually predicts that TRPC5 activation could promote mobilization of TRPC6 through generation of ROS through activation of Nox2 (as we will discuss further below; see also [207,208]). In addition, there is evidence that RhoA is required for activation of phospholipase Cε and can therefore play a role in TRPC6 activation via GPCR pathways, i.e. RhoA can also function upstream of TRPC6 [216]. It is possible that

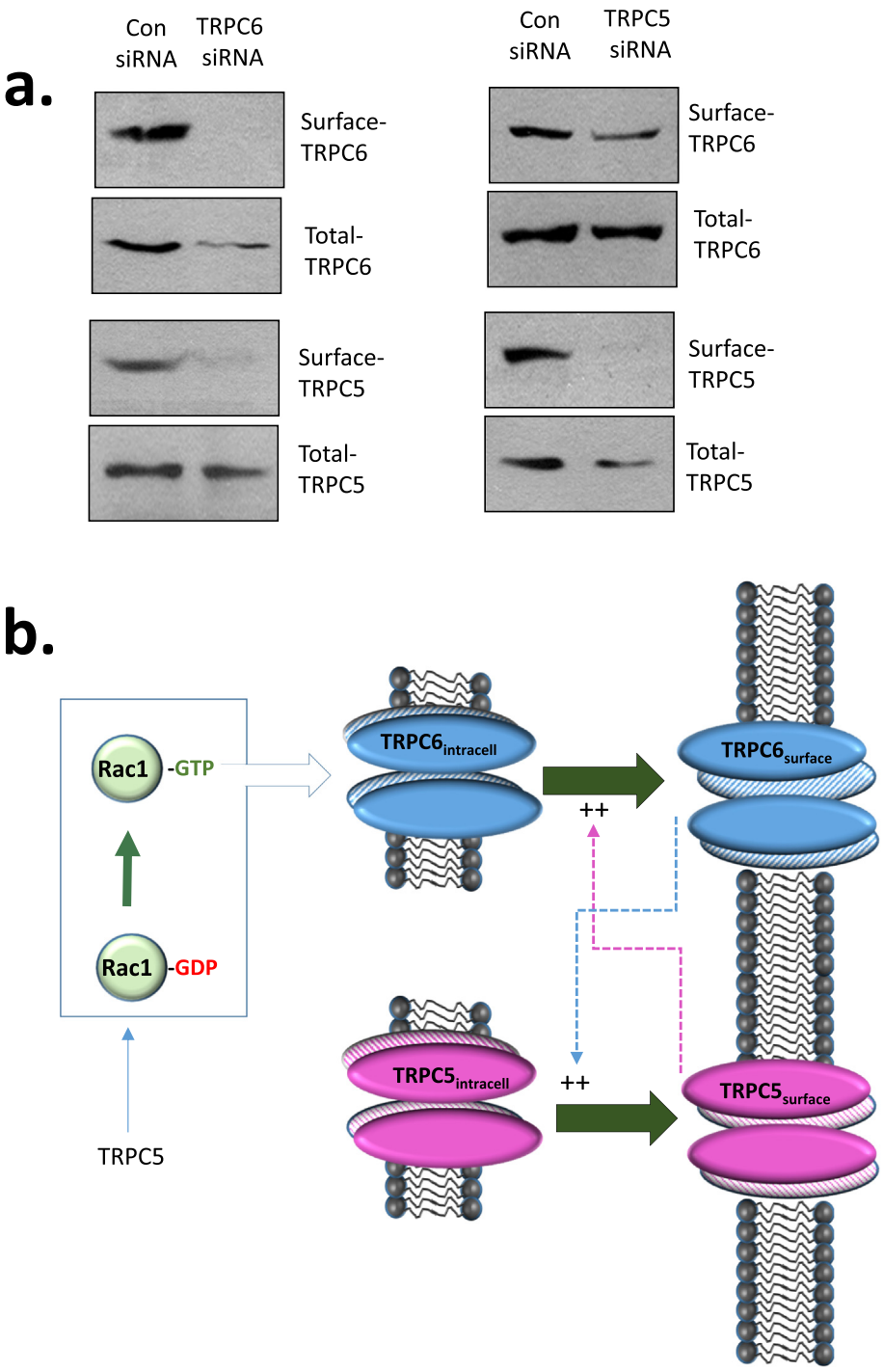

Fig. 10. Relationship between surface abundance of TRPC6 and TRPC5 in cultured podocytes. (a) Knockdown of TRPC6 using siRNA reduces steady-state abundance of both TRPC6 and TRPC5 (left), which is also seen following knockdown of TRPC5 (right). (b) Schematic diagram showing this relationship and a model for TRPC5 regulation of TRPC6. This is based on the observations that Rac1 is required for normal surface expression of TRPC6 [66] and TRPC5 is positively coupled to Rac1 [51].

RhoA could also contribute to TRPC5 activation by the same mechanism in the presence of proteins [217] known to be expressed in podocyte foot processes [218]. Consequently, experiments designed to dissect the role of small GTPases in pathways upstream and downstream of TRPC channels do not lend themselves to simple interpretation.

In summary, the role of TRPC5 in renal function and in kidney disease remains uncertain and requires additional study. It will be helpful for future investigations to examine the role of TRPC5 in additional kidney disease models, and especially in chronic disease models that more accurately reflect CKD in humans. Before intensive drug development efforts can proceed, it will be important to demonstrate that TRPC5 is in some way altered in human biopsy samples. It will also be useful to establish the cellular pathways that lead to TRPC5 activation in podocytes, and to determine if there are physiological connections between TRPC5 and TRPC6 other than the antagonistic relationship proposed originally based on behavior of an immortalized cell line in vitro [51]. Progress to date has been limited by the fact that

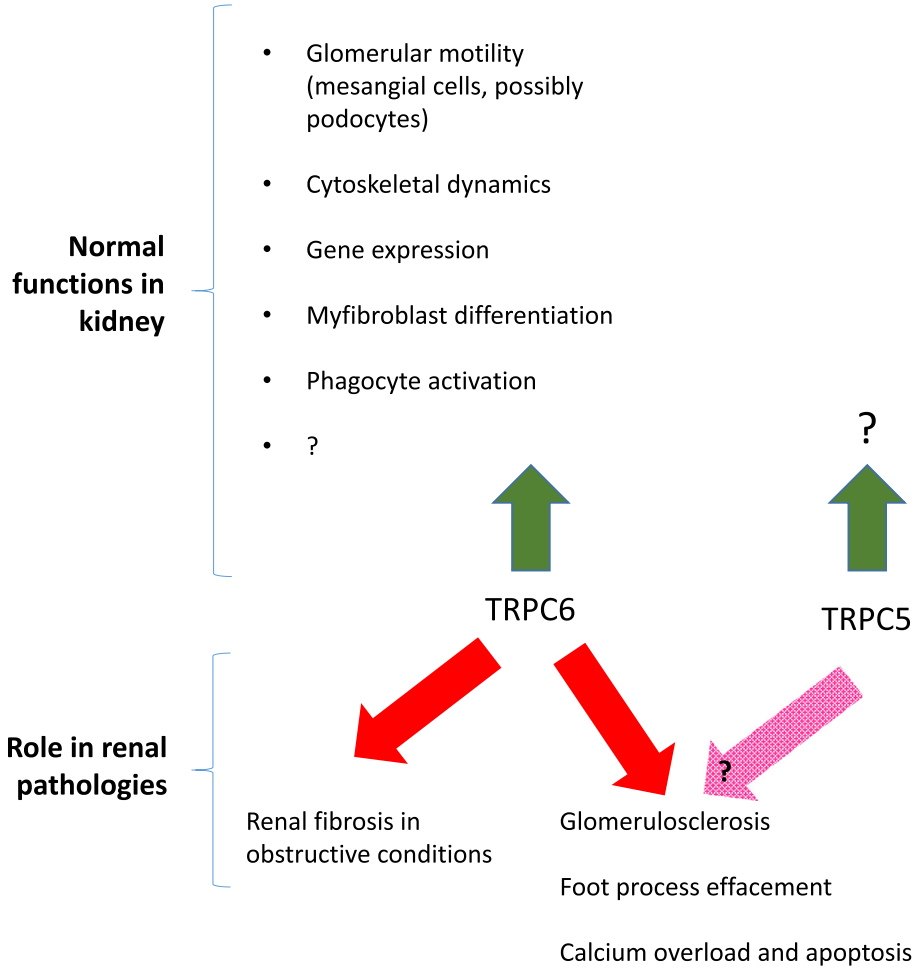

Fig. 11. Graphic summary of the current understanding of the role of TRPC6 and TRPC5 in renal pathology and their possible normal functions in the kidney.

TRPC5 knockout and transgenic animals currently exist only in mice on genetic backgrounds that markedly limit the CKD models that phenocopy human nephrotic syndromes.

13. Considerations for drug development and future studies

The preceding paragraphs have reviewed data supporting a role for TRPC6 and TRPC5 in the progression of kidney disease in animal models, and we have summarized several of the key points schematically in Fig. 11. The available data from humans support a role for TRPC6, without doubt in some familial forms of FSGS, and possibly in acquired forms of glomerular disease. This raises several questions as to the best approaches that might be followed to develop potential therapeutic agents. TRPC6 is closely related to TRPC3 (as well as TRPC7), and the majority of small molecule inhibitors that block TRPC6 also inhibit TRPC3 [219,220]. Most drug development efforts have proceeded under the assumption that more specific inhibitors would be better. However, there is evidence that TRPC3 can at least partially compensate for the loss of TRPC6, and in some tissues may actually over-compensate [144]. Therefore, while compounds such as various larixol derivatives [221–223] and SAR-7334 [224] exhibit marked selectivity for TRPC6 over TRPC3, it is certainly possible that better therapeutic responses would occur if both channels were blocked, which would occur with less specific compounds. In other words, it may not be advisable to exclude consideration of compounds with optimal pharmacokinetic properties just because they are able to block more than one type of TRPC channel.

TRPC6 and TRPC5 knockout animals seem relatively normal and breed well. However, it is possible that compensatory changes in related channels in TRPC knockout models mask complications that would ensue from acute pharmacological inhibition of any TRPC channels in vivo in humans. Bear in mind that small molecule channel blockade in vivo would occur too rapidly for any transcriptional compensation to take place. To date all available studies on TRPC6 and TRPC5 have been carried out in constitutive knockout or over-expression models. Inducible knockout models would avoid developmental issues, but even these transgenic systems require several days of induction with tamoxifen or tetracycline, and compensatory changes in the expression of other channels could easily occur over that time scale. Ultimately it will be necessary to identify TRPC6 inhibitors with well characterized pharmacokinetic properties and to test them in animal models that closely resemble human kidney diseases, ideally in more than one species. As already mentioned, this has been attempted with TRPC5, but to date a consensus has not been reached. Studies with small molecules should assess both albuminuria and preservation of renal structure and function.

All known TRPC channels have a fairly wide distribution in the body and it is certainly possible that acute pharmacological inhibition of either TRPC6 or TRPC5 could result in actions that immediately rule out their use in humans. For example, marked increases in bleeding time [225] or evidence that acute inhibition of these channels results in cognitive deficits [226–228] would certainly argue against further attempts to develop existing lead compounds into therapeutic agents. This can only established with certainty by subjecting existing lead compounds to additional testing in vivo.

Even if it turns out TRPC channels themselves cannot be targeted

directly for safety reasons, understanding pathways upstream and downstream of their activation in renal cells could lead to development of therapeutic agents. A particularly promising example of this is αvβ3-integrin [229] which is required for upregulation of TRPC6 in response to suPAR, TNF, and plasma from patients with recurrent FSGS [65,66]. Cilengitide is a well characterized inhibitor of αv-containing integrins [129]. It was originally developed as an anti-angiogenic compound, especially for treatment of aggressive glioblastomas. Cilengitide is well tolerated in humans [230] but unfortunately failed to produce therapeutic effects in newly diagnosed glioblastoma patients [231]. However it is possible that integrin inhibitors could be useful in primary FSGS or other nephrotic syndromes by preventing mobilization and upregulation of TRPC channels, and loss of podocin, especially if orally active forms could be developed. This idea is supported by recent studies in mice with deficiencies in β3-integrins [124] and mice over-expressing an endogenous integrin antagonist [130].

It also might be possible to target NADPH-oxidases that lie upstream of TRPC6 mobilization, and inhibitors of those enzymes have shown promise in animal models of diabetic nephropathy [232,233]. In fact, there may be many other ways to effectively target cell redox status and the thiol residues of TRPC channels, and these various approaches have been discussed in the context of neurodegenerative disorders [234]. Similarly, it is possible that agents such as calcineurin inhibitors are therapeutically effective in CKD part by blocking pathways downstream of podocyte TRPC channels [174]. This raises the possibility that other inhibitors of downstream pathways could be targeted. While cell-permeable NFAT inhibitors have been developed, and act by specifically preventing CN docking onto NFAT [235], these compounds are small peptides. Translating these into stable orally active therapeutic agents for CKD represents a substantial challenge, but it is possible that stable analogs could be developed.

A major unanswered question regarding TRPC channels, especially in glomeruli, pertains to their normal function. What is it that they actually do on a moment to moment basis in various glomerular cells? Do they induce some mechanical or motile response in podocytes or mesangial cells that alters filtration in some way, or which protects the system in the face of changes in transmural pressure? A major hurdle to understanding is the lack of inducible cell type-specific knockout models, which is especially problematic in studies of glomerular function. For example, TRPC6 channels are expressed in afferent arterioles, mesangial cells and podocytes. Changes in glomerular regulation that occur in constitutive global TRPC6 knockout animals could result from effects on any combination of these particular cells. This markedly limits what one might learn about, say, the function of podocyte TRPC6 channels, by any current experimental method. Similarly, TRPC5 is expressed in podocytes but is also present in vascular endothelial cells [208] and they have been suggested, among other things, to play a role in sensing pressures in vascular systems [236]. It is even possible that TRPC5 plays a role as a store-operated channel in certain contexts [237]. It will be important in the future to establish factors that cause TRPC5 channels to become active in glomerular cells.

Even if TRPC inhibitors are not eventually developed into therapeutic agents, their continued investigation will shed light on fundamental renal physiology and pathophysiology of large classes of diseases in which there is a large unmet need for new therapies.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgements

Work in our laboratory is supported by National Institutes of Health grant R01-DK104708. We have previously received support for this project from Pfizer Inc. and from a James Scherbensky award from the American Society of Nephrology.

Drs. Johannes Schlöndorff, Jochen Reiser, Moin Saleem, Peter Hoyer, Henning Hagmann, and Sanja Sever have provided materials and patient samples that have proved essential in forming ideas expressed in this review.

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