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This work was supported in part by an Avenir Program from INSERM, a grant from the French Kidney Foundation, Association pour l'Utilisation du Rein Artificiel (AURA), Assistance Publique des Hôpitaux de Paris (AP-HP, Programme hospitalier de recherche clinique) and a grant from the Fondation pour la Recherche Médicale (FRM).

The authors have declared that no conflict of interest exists

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Abstract

The mechanisms of podocyte disorders in idiopathic nephrotic syndrome (INS) are complex and remain incompletely elucidated. Abnormal regulation of NF-κB might play a key role in the pathophysiology of these podocyte diseases but it has little been investigated so far. We report here that induction of c-mip in podocytes of patients with INS is associated with a downregulation of RelA, a potent anti-apoptotic factor belonging to the NF-κB family. Overexpression of c-mip in differentiated podocytes promotes apoptosis by inducing caspaces-3 activity, an upregulation of the pro-apoptotic protein Bax, while the abundance of the anti-apoptotic protein Bcl-2 was decreased. Concomitant overexpression of RelA prevented the pro-apoptotic effects of c-mip. We show that targeted induction of c-mip in podocytes *in vivo* inhibits the expression of the RelA protein, and increases the Bax/Bcl2 ratio. The expression of c-mip and active caspase 3 is increased in FSGS biopsies and both proteins display a close spatial relationships. These results suggest that alterations of NF-κB activity might result from upregulation of c-mip and likely contribute to podocyte disorders in INS.

Introduction

Idiopathic nephrotic syndrome (INS) defines several entities including minimal change nephrotic syndrome (MCNS), and focal and segmental glomerulosclerosis (FSGS), which are considered as prototypic podocyte diseases. [1] Podocytes are terminally differentiated cells that line the outer aspect of the glomerular basement membrane (GBM) and constitute the ultimate barrier to urinary protein loss by the formation and maintenance of the podocyte foot processes (FP) and the interposed slit diaphragm. [2] Podocyte diseases might result from genetic defects of proteins playing a key structural and/or regulatory role in the integrity of the glomerular filtration barrier. [3] Regardless of the underlying cause, the early podocyte damages are characterized by ultrastructural alterations of the slit diaphragm. Cellular injuries may progress through a reversilble stage to podocyte depletion and glomerulosclerosis, which commonly complicate the outcome of chronic glomerular diseases in INS. In acquired INS, the mechanisms preceding these changes remain to be elucidated.

The NF-κB family of transcription factors plays a central role in many cellular processes through the regulation of genes involved in immunity, inflammation, cell proliferation, differentiation and apoptosis. In mammalian cells, the NF-κB family consists of five members, NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB and cRel. [4] The hallmark of NF-κB family is the presence in all members of a Rel-homology domain (RHD) at the NH2-teminus, responsible for DNA binding, dimerization and association with the I-κB inhibitory proteins. [5] Among the NF-κB proteins, only RelA, RelB and c-Rel have a COOH-terminus transactivation domain, which is lacking in p50 and p52, so that RelA, RelB and c-Rel homo- or heterodimers, function as transcriptional activators, whereas p50 and p52 homodimers function as repressors. [4] Although in most cases, Rel proteins act as transcriptional activators, they may repress some target genes. [6,7] Because of its implication in

many biological processes and the adverse consequences of its dysregulation, the activity of NF-κB is tightly regulated at multiple, translational and post-translational levels. The most common regulatory mechanism relies on the actions of the inhibitory I-κB proteins. [8] In resting cells, I-κB protein binds to nuclear localization site of the RHD, preventing the nuclear translocation of NF-κB protein and thereby its transcriptional activity. Upon activation, I-κB protein is phosphorylated, which allows its ubiquitination and subsequent degradation by proteasome. We recently shown that c-mip interacts with RelA and prevents its dissociation from the NFκB/IκBα complexes, resulting in the inhibition of nuclear translocation of NFκB and stabilization of IκBα. [9]

In human and experimental glomerular diseases, several mediators regulated by NF-κB such as cytokines and adhesion molecules have been reported to play pathogenic roles in inflammatory and proliferative glomerular diseases. [10] In non-proliferative glomerular diseases, such as minimal change disease and membranous nephropathy, characterized by heavy proteinuria, it has been suggested that the excess of proteins in the tubules and their subsequent degradation induce tubular expression of many proinflammatory genes through NF-κB activation. [11]

To our knowledge, the mechanisms of NF-┤ B regulation in the podocytes are little known. We recently reported that c-mip is overproduced in the podocytes during glomerular damages and interferes with signaling pathways playing a key role in podocyte function. Whether c-mip influences NF-┤ B activity has not been investigated.

We report here that c-mip represses *in vitro and in vivo* NF-κB activity by downregulating the expression of RelA. This effect was only observed in podocytes, while tubular cells displayed a contrasting increase of NF-kB activity. Moreover, c-mip increases the levels of Bax and enhances caspase 3 activity, while it reduces the Bcl-2 level, suggesting that c-mip exerts a proapoptotic function.

Materials and Methods

Patients

The diagnosis of kidney disease was carried out by renal biopsy, which was performed before the start of treatment. All patients had proteinuria above 3 g/24 h and severe hypo-albuminemia at the time of blood sampling,. MCNS and FSGS were clinically classified as idiopathic in all cases.

Control for c-mip screening includes adult patients with glomerular diseases (IgA, lupus nephritis) who exhibited a nephrotic syndrome. Normal renal samples were supplied by the hospital tissue bank (platform of biological resources, Henri Mondor hospital) from patients undergoing nephrectomy for polar kidney tumor.

Reverse transcription and real time quantitative PCR (RT-QPCR), and plasmid constructs

The expression plasmids for c-mip and RelA have been previously described. [12,13] Mouse total RNA was prepared from glomerular fractions isolated by graded sieving, using RNeasy kit (Qiagen, France). RT-QPCR for RelA was performed using the oligonucleotides sense (5'-TGT TAC CAT CAG GGC AGA TC–3') and anti-sense (5'-CAG GGT ACT CCA TCA GCA TG-3'). The samples (2 µl of the RT reaction mixture, corresponding to 20 ng of total RNA) were amplified in a 20-µl reaction mixture containing 0.5 mM of each primer and 1X Light Cycler DNA master SYBR green buffer (Roche Molecular Biochemical, Mannheim, Germany). RT-QPCR conditions include an initial denaturing step at 95°C for 15 min, followed by 40 cycles (denaturing: 95°C, 15 sec; annealing: 59°C, 30 sec; extension, 72°C, 35 sec).

Generation of c-mip stable podocyte cell lines

Conditionally immortalized mouse podocytes have been described elsewhere. [14] Before transfection, podocytes were maintained at 60% confluence, under permissive conditions (cells were cultured in RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 U/ml γ-INF, at 33°C). The full-length human c-mip was inserted into pDEST40 plasmid (Invitrogen, Inc, CA). Proliferating podocytes were transfected with c-mip expression plasmid (1 μg/10^6 cells), using the Amaxa system. Following, transfection, cells were maintained in complete medium for 24 hours, then geneticin antibiotic was added at 400 μg/ml (100% lethal to untransfected cells). Stably transfected cells were selected in serial passages using geneticin, then stored in liquid nitrogen or expanded under permissive conditions in the presence of geneticin until they were in sufficient number. Podocytes clones were isolated by limiting dilution and stored in liquid nitrogen. For transient transfection, podocytes were transfected with c-mip expression plasmid (pDEST40) alone or cotransfected with empty vector or RelA, using the nanofectine method according the instructions provided by the manufacturer (PAA, Austria). In some experiments, podocytes were incubated with 2 μM lactacystin (Sigma-Aldrich; St Louis, MO) for 20 hours, then protein lysates were prepared as previously described [13].

Caspase-3 activity assays.

Caspase 3 activity was monitored in a quantitative assay using a fluorogenic substrate. Podocytes stably tranfected with c-mip or expressing empty vector (~200,000 cells) were grown on 60-mm dishes at 37°C for 96 hours. Floating cells were collected and centrifuged, and the pellet was lysed in 50 μl of lysis buffer containing 50 mM HEPES, pH 7.4, 100 mM NaCl, 1% Nonidet P-40, 1 mM EDTA (pH 8.0), 1 mM dithiothreitol, 2 μg/ml leupeptin, 2

μg/ml aprotinin. Adherent cells were washed three times with cold PBS and lysed for 10 min on ice in 0.2 ml of lysis buffer. The lysates from adherent and floating cells were pooled and centrifuged, and the supernatants were collected. The activity of caspase recognizing the DEVD (Asp-Glu-Val-Asp) motif (DEVDase activity) was measured in 200 μl of assay buffer, containing 100 mM HEPES, pH 7.4, 10% sucrose, 10 mM dithiothreitol, 500 μM EDTA, 50 μg of protein, and 20 μM N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (AC-DEVD-AFC) as fluorogenic substrate (Biomol, Tebu, France). After 3 h at 37°C, the fluorescence of the reaction mixture was monitored every 30 min until seven hours, using a spectrofluorometer (FL600 microplate fluorescence reader, Biotek Instruments, Inc, Winooski, USA), with excitation and emission wavelengths of 400 and 530 nm, respectively.

TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) assays.

Paraffin-embedded kidney tissue sections were dewaxed, rehydrated, then treated with proteinase K solution (10 μg/ml in 10 mM Tris-HCl, ph7,4) for 30min at room temperature. The sections were rinsed twice with PBS and the In Situ Cell Death Detection Kit, POD (Roche Diagnostics GmbH, Germany).was used to label apoptotic cells. The sections were incubated with 50 μl TUNEL reaction mixture for 60 min at 37^{o}C in a humidified atmosphere in the dark. Terminal transferase was omitted for the negative control. As positive control, one section was incubated with DNase I (2000 U/ml in 50 mM Tris-HCl, ph 7,5, 10 mM MgCl2 and 1mg/ml BSA) for 10 min at RT to induce DNA strand breaks, prior the labeling procedures. The sections were rinsed 3 times with PBS, then incubated with 50 μl Converter-POD on sample, for 30min at 37^{o}C. The sections were rinsed 3 times with PBS, then 50 μl of DAB substrate and incubated for several minutes at room temperature. Finally, the sections were rinsed with PBS, mounted under glass coverslip and analyzed under light microscope.

Light microscopy and immunohistochemistry analysis

For light microscopy, the kidney sections from wild type and c-mip–transgenic mice were incubated for 16 hours in Dubosq Brazil, and subsequently dehydrated, paraffin-embedded and stained with periodic acid-Schiff reagent (PAS). For immunohistochemistry study, antigen retrieval was performed by immersing the slides in boiling 0.01 M citrate buffer in a 500 W microwave oven for 15 min. The endogenous peroxidase activity was blocked with 0,3% H2O2 in methanol for 30 min. Slides were incubated with the blocking reagents consisting of the Avidin–biotin solution for 30 min and the normal blocking serum for 20 min, then incubated overnight with specific polyclonal antibody. After washing with PBS, they were incubated with biotinylated goat anti-rabbit antibody. An avidin-biotinylated horseradish peroxidase complex (Vectastain ABC Reagent, Vector Laboratories; Burlingame, CA) and 3,3'-diaminobenzidine (Sigma Biochemical; St Louis, MO) as a chromogen were applied for visualization of the immunoreaction. The anti RelA, anti-cmip, [13] and anti-cleaved caspase 3 (Asp175) were used at a final concentration of 4 mg/ml, or at a 1/200 and 1/50 dilution, respectively. Omission of the primary antibody was considered as a negative control.

Immunofluorescence

Immunofluorescence studies on kidney tissues were performed with 4-μm thick cryostat sections fixed in acetone for 10 min, air-dried 30 min at room temperature, then kept in PBS for 3 min and blocked in 1% BSA-PBS. The sections were incubated with the indicated antibodies for one hour at room temperature, washed with PBS and incubated with FITC- or red fluorescent dye-conjugated secondary antibodies. For double fluorochrome labeling, the slides were simultaneously incubated with rabbit anti-c-mip antibody and mouse anti-nephrin antibody. After washing with PBS, the slides were simultaneously incubated with FITC-

conjugated goat anti-rabbit IgG and cyanine3-conjugated sheep anti-mouse IgG. Sections were examined by fluorescence microscopy (Zeiss, Germany) using red and green filters.

Antibodies and Western blotting

The primary antibodies used in this study included anti-RelA, anti-Tie2, anti-lamin B (Santa Cruz, Biotechnology, CA), anti-cleaved caspase 3 (Asp 75), anti-Bax, (cell signaling), anti-Bcl-2 (BD transduction Laboratories, USA), monoclonal anti-vimentin (Dako, Denmark), monoclonal anti-GAPDH (Abcam, Cambridge, UK), anti-actin (Sigma, Aldrich) and anti-nephrin (Progen, Heidelberg, Germany). The anti-c-mip polyclonal antibody has been previously described. [15] Western blotting was performed, using classical methods.

Generation of c-mip transgenic mice

The generation of c-mip transgenic mice has been previously described. [13] All experiments involving animals were conducted in accordance with French laws. . All the mice analyzed in this study were hemizygous males [Tg(+)] from the F8 to F12 generations.

Histological analysis

Morphological lesions were studied on 4 mm PAS-stained sections. Five kidney sections by mouse (n= 5 mice for each group) were analyzed. Between 20 and 30 glomeruli per kidney section were randomly selected.

Statistical Analysis

Statistical analysis of the data was performed using PRIZM 4 for Macintosh (Graphpad Software, Inc, USA). Unpaired or Paired Student t-tests were used. P value of less than 0,05 was considered significant.

Results

Expression of c-mip is associated with a downregulation of NF-κB in podocytes of patients with INS

Although the role of NF-κB in podocytes is poorly understood, it likely mediates important functions including regulation of signaling pathways and podocyte survival. [16] To investigate the possible influence of c-mip on NF-κB activity in podocytes of glomerular diseases, we studied the kidney biopsies of patients with INS. As previously reported, c-mip was clearly induced in kidney biopsies of patients with MCNS, while it was scarcely or not detected in normal human kidney. [13] The distribution pattern shows that c-mip was not confined to cytoplasmic compartment but it was also visualized in nuclei (Figure 1A). In contrast, we did not detect c-mip in glomeruli of IgA nephropathy or proliferative lupus nephritis. Immunohistochemistry analysis on the same kidney specimens (five normals and twelve MCNS) using an anti-RelA antibody showed that RelA was clearly detected in normal podocytes, but it was dramatically altered in the podocytes of MCNS (Figure 1A and 1B). Confocal microscopy analysis of normal glomeruli (Fig 2) shows that RelA: i) colocalized partially with nephrin but not with Tie2 suggesting that its expression is restricted to podocytes; ii) did not overlap with vimentin but mostly colocalized with lamin-B, a marker of nuclear membrane. In some podocytes, Rel A was also localized within the nucleus. By contrast, in glomeruli of patients with MCNS, the abundance of RelA was lower (Fig 2). These results suggest that induction of c-mip in podocytes is associated with a downregulation of RelA.

Expression of c-mip in vivo induces a downregulation of RelA at the post-transcriptional level

To understand the functional consequences of c-mip induction in podocytes, we generated transgenic mice expressing c-mip under nephrin promoter to restrict transgene expression to podocytes. C-mip-transgenic mice develop heavy proteinuria with foot processes effacement without inflammatory lesions or immune complex deposits. [13] The abundance of the RelA transcripts measured by quantitative RT-PCR did not significantly differ between wild type and transgenic mice (Figure 3A). In contrast, Western blotting of glomerular protein lysates showed that RelA abundance was significantly decreased in c-mip-transgenic mice, as compared with wild type mice (Figure 3B), which suggests that c-mip affects only the stability of the RelA protein. Indeed, coculture of c-mip-transfected podocytes in the presence of lactacystin, a potent proteasome inhibiteur, prevented the decrease of endogenous RelA (Figure 3C), suggesting that c-mip targets RelA through proteasome-mediated degradation.

C-mip displays proapoptotic properties

On the basis of the studies carried out in NF-κB-deficient mice, it is believed that NF-κB is a potent anti-apoptotic transcription factor. [17] Given our findings showing a downregulation of RelA, which is essential for NF-κB activity, we sought to determine whether c-mip influences the signaling pathways involved in apoptosis. Apoptosis is defined by morphological features including loss of adhesion, cell shrinkage and biochemical alterations such as upregulation of Bax and caspase-3 activity. Bax belongs to Bcl-2 family proteins, which include both anti-apoptotic (e.g. Bcl-2 and Bcl-xl) and pro-apoptotic (e.g. Bax, Bad) members. [18] The caspase-3 is one of the final downstream effectors of the apoptotic process and its activation is closely associated with mitochondrial dysfunction. [19] To assess whether c-mip is a proapoptotic molecule, we first transiently transfected c-mip alone in podocyte cell line and analyzed the expression level of Bax, Bcl2 and caspase 3. Overexpression of c-mip induced an upregulation of Bax and caspase 3, while Bcl2 abundance decreased when compared with

empty vector-transfected cells (Figure 4A). On the other hand, co-transfection of RelA antagonized the proapoptotic effects of c-mip. Then, we sought whether these changes are reproducible in differentiated podocytes stably transfected with c-mip or empty vector. We studied five independent c-mip stably transfected podocyte cell lines and two control clones stably transfected with empty vector. We performed quantitative Western-blot analysis to measure the abundance of the Bcl-2 and Bax proteins that were normalized to GAPDH loading. As shown in Figure 4B, Bax was overproduced in c-mip overexpressing podocytes, so that the Bax/Bcl-2 ratio was significantly increased (Figure 4B). Morphological analyses showed that empty vector-transfected cells displayed small morphological changes, while c-mip overexpressing podocytes exhibited severe cell shrinkage and most cells had detached. To assess whether these morphological changes involve caspase-3 activation, we performed a caspase-3 activity assay on stable transfectant cells. Podocytes overexpressing c-mip exhibited an activation of caspase-3 in a time-dependent manner, while no significant change was detected in the empty vector-transfected podocytes (Figure 4C).

The relevance of these findings was analyzed in c-mip transgenic mice. In this model, mice develop after 3 months of age progressive FSGS. [13] Histological analysis (Figure 5A) showed that the number of glomeruli displaying FSGS lesions was increased from three (4 ± 1,5%), six (7,5 ± 2,5%) to twelve months of age (23 ± 3,5%). Western-blot analysis from glomerular extracts revealed increased Bax in c-mip Tg mice, while Bcl-2 was reduced, with a Bax/Bcl-2 ratio that was increased by 3,1 fold, as compared with wild type mice (Figure 5B). We then used the TUNEL assay to detect DNA fragmentation in podocytes in vivo. In wild-type mice and in 3 month-old transgenic mice, TUNEL-positive nuclei were not detectable in glomeruli. However, in transgenic mice of 6 and 12 months of age, a number of podocytes became positive for TUNEL (Figure 6). Collectively, these results suggest that c-mip promotes apoptosis *in vitro* and *in vivo*.

FSGS lesions are characterized by dysregulation of podocyte function, leading to apoptosis. We have previously shown that c-mip abundance is increased in primary FSGS. Therefore, we investigated whether FSGS biopsy specimens exhibit active, cleaved caspase 3, a marker of cell apoptosis. Immunochemistry analysis was performed in seven FSGS biopsy specimens. Active caspase 3 was detected in all examined cases, at a variable degree, along the external side of some capillary loops, consistent with podocyte localization (Figure 7). The expression was decreased or disappeared in podocyte-depleted areas corresponding to severe FSGS lesions or glomerulosclerosis. (Figure 7). Interestingly, c-mip was increased in glomeruli in a pattern similar to that observed with active caspase 3.

Discussion

The role of NF-κB in glomerular biology and in pathophysiology of podocyte disease remains little explored. In the present work, we provide evidence that: i) increased expression of c-mip in podocytes of patients with INS is correlated with a downregulation of RelA; ii) c-mip destabilizes endogenous RelA protein *in vitro*, that is prevented by lactacystin, suggesting that c-mip targets RelA through proteasome-mediated degradation.; iii) Stable transfection of c-mip in the podocytes is associated with cell shrinkage and increased abundance of Bax and active caspase 3; iv) c-mip induces *in vivo* an upregulation of Bax and a decrease of Bcl-2, supporting the role of c-mip in apoptosis; v) c-mip transgenic mice develop progressive FSGS in the absence of overt tubular and interstitial lesions; vi) the abundance of c-mip and active caspase 3 was increased in human primary FSGS.

We have previously shown that c-mip interacts through its PH domain with Fyn and PI3 kinase regulatory subunit (p85), and exerts an inhibitory role in proximal signaling. [13,20] C-mip also binds to, and inhibits the activation of N-WASP, a major component of cytoskeleton that regulates the ARP2/3-mediated actin nucleation. In addition, we found that c-mip induces in vitro and in vivo cytoskeleton disorganization, [13] and increases the abundance of DAP kinase. [20] It has been shown that DAP kinase promotes apoptosis by disrupting integrin–mediated cell adhesion, alteration of cytoskeleton and loss of extracellular matrix-dependent survival signals. [21] Alteration of the actin network induces the production of reactive oxygen species *(*ROS*)* from mitochondria, leading to apoptosis. [22] We have previously found that c-mip binds RelA through its LRR-containing C-terminal domain. [9] We show here that c-mip may target RelA to proteasome-dependent degradation. Altogether, our results suggest that c-mip is a multifunctional protein and may promote apoptosis through different mechanisms, including cytoskeleton alterations, upregulation of DAP kinase and downregulation of RelA.

Several studies have shown that NF-κB hyperactivation is mainly correlated with the intensity of proteinuria and is restricted in the tubules of patients with progressive kidney diseases, including glomerular diseases. [23-25] Indeed, NF-κB activity is associated with increased tubular expression of the chemokines MCP-1, RANTES, osteopontin and the profibrogenic cytokines PDGF-BB and TGFβ, [23] leading to tubular apoptosis and fibrosis. In contrast, these studies fail to detect significant hyperactivation of NF-κB in podocyte in certain forms of glomerular diseases, suggesting that this mechanism does not prevail in these cells. It has been reported that NF-κB is expressed in a few mesangial cells and tubular epithelial cells in MCNS and MN, while it is detected in podocytes of IgA nephropathy and non-IgA mesangial proliferative glomerulonephritis. [24] These data are consistent with our results because the upregulation of c-mip might account for the non-detection of NF-κB in podocytes in INS. Conversely, in IgA nephropathy where the expression of NF-κB is found increased, c-mip was not detected in podocytes, according with previous data. [13] The downregulation of RelA in INS relapse may explain why these podocyte diseases are not associated with inflammatory processes in glomeruli. By contrast, activation of RelA in the podocytes of Par4-deficient mice is associated with severe glomerular and tubular inflammatory lesions. [26] Moreover, in pathological situations associated with sustained c-mip increase, the downregulation of NF-κB in glomeruli might represent a possible mechanism involved in podocyte apoptosis and may contribute to podocyte depletion in some podocyte diseases without early involvement of tubular and interstitial tissue injuries.

Two distinct signaling pathways are involved in the initiation of apoptosis: i) the extrinsic apoptotic pathway or receptor–linked pathway requires the binding of a ligand to a death receptor on the cell surface, such as TNF receptor; ii) the intrinsic apoptotic pathway that is mediated by mitochondrial damage and release of cytochrome C. [27] The development of the signaling cascade leading to apoptosis requires in most situations the upregulation of p53.

Despite several assays, we did not observe an upregulation of p53 in stable transfected podocytes overexpressing c-mip or in c-mip Tg mice (data not shown), which suggests that the proapoptotic function of c-mip does not require p53. Some studies have shown that the ability to trigger apoptotic pathways in the absence of p53 may result from the activation of Erk signaling. [28] However, the Erk pathway has multiple functions including pro and anti-apoptotic effects of which the mechanisms are still unclear. [29]

Induction of apoptosis following exposition to puromycin aminonucleoside (PAN), in cultured podocytes as well as in vivo, in a model of FSGS in rats, is associated with an increase of Bax and a reduction of Bcl-2. [19,30] The underlying mechanism involves an upregulation of p53 since, in this model, apoptosis is prevented by PFT-α and dexamethasone, which inhibit p53 activation. Indeed, two signaling pathways have been involved in podocyte apoptosis induced by PAN, depending of caspase-3 or apoptosis-inducing factor (AIF), respectively. Dexamethasone inactivates AIF by inhibiting its nuclear translocation. [19]

It has been shown that insulin growth factor-1 (IGF-1) protects from apoptosis induced by etoposide, an inhibitor of topoisomerase II, through a mechanism involving activation of PI3 kinase and Bad. [31] This conclusion is based on the fact that the inhibitors of PI3 kinase, Whormaninn and Ly294002, abrogate the anti-apoptotic effect of IGF1. Moreover, nephrin and VEGF inhibit apoptosis by recruiting the PI3 kinase-Akt signaling pathway. [32] Both VEGF and nephrin signaling pathways appear interconnected because mutation of nephrin, which disrupts the nephrin signaling, abrogates the anti-apoptotic effect of VEGF. [32]

The anti-apoptotic role of CD2AP, an adapter protein anchoring the nephrin-mediated proximal signals to actin cytoskeleton, has been evidenced by the phenotype of CD2AP-deficient mice, which develop nephrotic syndrome and renal failure caused by glomerulosclerosis. [33] In this model, proteinuria occurs in the context of accelerated apoptosis

resulting from hyperactivation of p38 mitogen activated protein kinase (p38 MAPK) by TGFβ1. [34,35] The mechanism by which CD2AP impedes the apoptotic process involves a downregulation of p38 MAPK via activation of PI3 kinase and Akt signaling. [35]

Direct evidence of the role of TGFβ1 in podocyte apoptosis comes from study of transgenic mice overexpressing TGFβ1 under control of the albumin promoter. [36] In this model, Tg mice display glomerulosclerosis and interstitial fibrosis with renal failure and death in 50% of offspring at 5-12 weeks of age. [34] Histological lesions observed in TGFβ1 and c-mip Tg models are different. Glomerulosclerosis and ECM expansion are present in the early stages (~ 2 weeks) in TGFβ1 Tg mice, while glomeruli are morphologically normal despite the presence of a nephrotic syndrome and foot process fusions in c-mip Tg mice, until 4-5 months of age. Renal failure and death are rare during the first year of live in c-mip Tg mice even though these complications are common in TGFβ1 Tg mice. However, like for c-mip Tg mice, podocyte apoptosis precedes mesangial expansion.

In conclusion, we reported here new functional aspects of c-mip in podocytes. We show that c-mip induces *in vitro* and *in vivo* the destabilization of the RelA protein in podocytes. The upregulation of c-mip and active caspase 3 in human FSGS suggest that sustained upregulationn of c-mip might result in podocyte depletion and progressive FSGS.

Acknowledgments

We are grateful to Dr Peter Mundel for providing us with the mouse podocyte cell line. This work was supported in part by an Avenir Program from INSERM, a grant from the French Kidney Foundation. V Ory was supported by grants of Ministère de la recherche and from the Société de Néphrologie-Laboratoire Fresenius. Q Fan was supported by grant from the Fondation pour la Recherche Médicale (FRM). N Hamdaoui was supported by joint grant from the Association Française contre les Myopathies (AFM) and the French Kidney Foundation.

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Legends

Figure 1. Induction of c-mip in MCNS relapse is associated with a downregulation of RelA. Representative immunohistochemical analysis of serial sections from normal human kidney (NHK) and kidney biopsy specimens. **A:** Expression of c-mip. Note that c-mip was below detection limits in normal glomeruli, while it was clearly visualized along the external side of the capillary loops of MCNS relapse. The expression of c-mip was not restricted to cytoplasm compartment but it is also distributed in the nuclei. Note that c-mip was not detected in IgA or in proliferative lupus nephritis. Scale bars, 20 mm. **B:** Expression of RelA. Note that RelA is highly abundant in normal podocytes, while it was reduced in MCNS relapse. Scale bars, 20 mm.

Figure 2. Confocal microscopy analysis. Detection of RelA (green) and nephrin, tie-2, vimentin, and lamin-B (red) in normal human kidney (NHK, left panel) and kidney biopsy specimens from MCNS relapse (right panel). Note that RelA partially colocalized with nephrin and lamin- but not with vimentin and tie-2 in normal glomeruli. The expression of RelA was hardly detected in MCNS relapse. Scale bars, 20 mm.

Figure 3. The RelA protein is destabilized in the presence of c-mip, in vivo and in vitro. Glomeruli from wild-type (WT, n=5) and 6 month-old c-mip transgenic mice [Tg(+), n=5] were isolated and the abundance of RelA was analyzed. **A:** Quantification of *RelA* transcripts by RT-QPCR. **B:** Quantitative Western blotting of RelA protein. Data are representative of three independent experiments. **C:** Podocytes were transiently transfected with c-mip or empty vector (Ev), cultured 20 hours with or without lactacystin (2 mM), then analyzed by

Western blotting. Note that lactacystin prevents the destabilization of endogenous RelA. Data are representative of two independent experiments.

Figure 4. Transient and stably overexpression of c-mip in podocytes promote apoptosis. **A,** Podocytes were singly transiently transfected with empty vector (Ev) and c-mip or cotransfected with Ev/c-mip or RelA/c-mip. The expression of Bax, Bcl2 and caspase 3 was analyzed by Western blotting on the protein lysates. Note that c-mip increases Bax and caspase 3 and downregulates Bcl2, these alterations being prevented by the coexpression of RelA. **B,** Podocytes were stably transfected with c-mip expression plasmid (pDest 40) or empty vector (Ev), then cloned by limiting dilution. Morphological and/or quantitative western blot data for six clones (five c-mip and one Ev clones) are shown. c-mip was revealed by anti-V5 tag monoclonal antibody. Quantification of c-mip was carried out by subtracting the background related to the empty vector. Note that c-mip abundance varies depending of the clone; higher c-mip abundance is correlated with severe cellular injury and an increase of the Bax/Bcl2 ratio. **C,** Differentiated podocytes stably transfected with c-mip or empty vector (Ev) were tested for caspase 3 activity assays. The caspase 3 activity was monitored every 30 min during seven hours.

Figure 5. C-mip promotes progressive FSGS in transgenic mice. A, left, Representative Periodic acid-Schiff (PAS) staining of kidney sections from 3 to 12 month-old wild type and proteinuric Tg mice. Scale bars: left: 50 mM, right, 20 mM. Glomerular alterations are apparent at 6 and 12 months characterized by FSGS lesions and mesangial expansion. The tubules appear normal and no inflammatory injuries can be seen. Right, percentage of glomeruli with FSGS. Five mice were analyzed by age range (≥ 300 glomeruli). Data represent the mean ± SEM. **B,** glomerular protein extracts from 12 month-old wild type and

proteinuric Tg mice were analyzed by Western blotting for the relative expression of Bax and Bcl-2;

Figure 6. c-mip promotes apoptosis in vivo. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (Tunel) assay on paraffin kidney sections of wild-type and c-mip transgenic mice. Note that tunnel positive cells were not detected in 3 month-old transgenic mice, whereas many podocytes became positive at 6 months of age

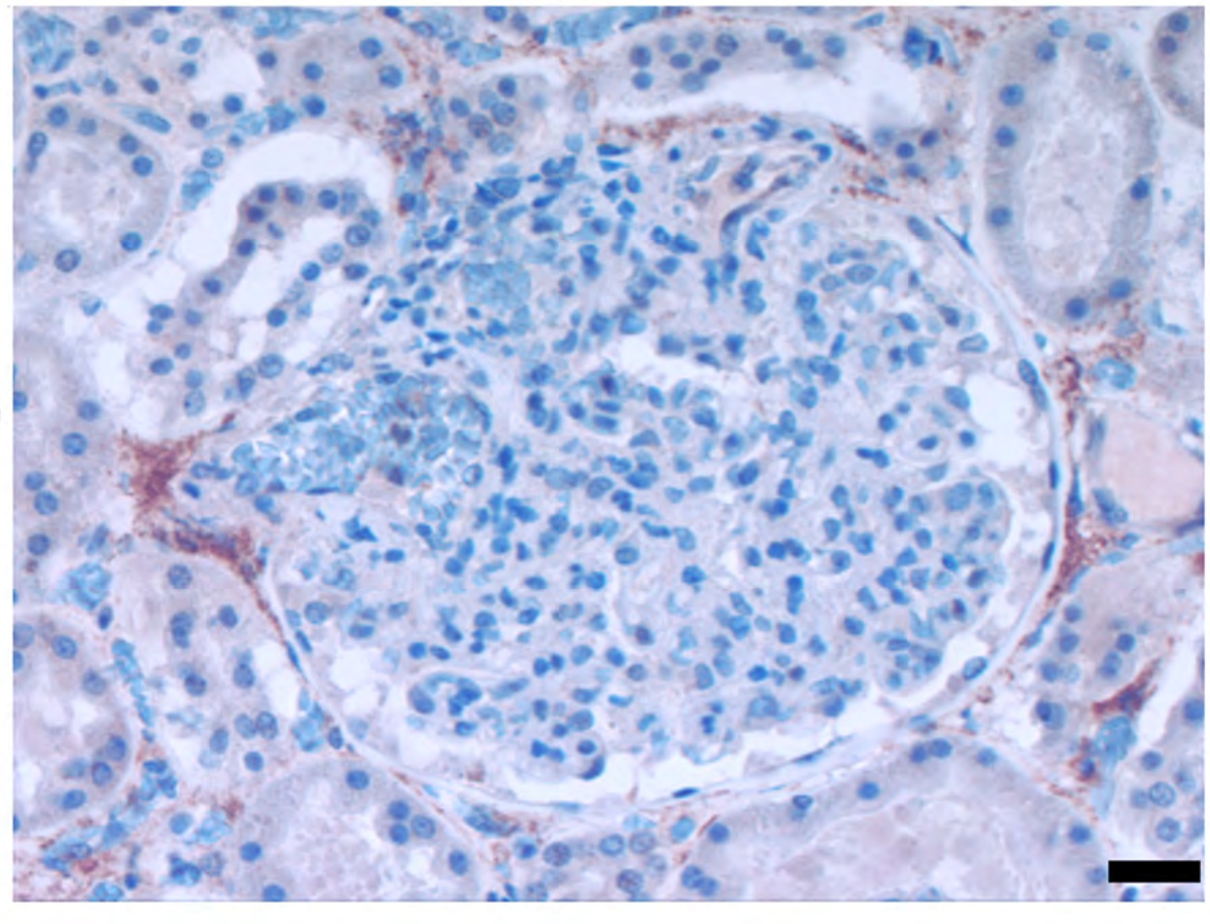
Figure 7. Expression of c-mip and caspase 3 in FSGS biopsies. Representative Immunostaining of c-mip and cleaved caspase 3 from FSGS biopsy specimens. In most cases, the localization of c-mip and active caspase 3 are observed in close proximity. The labels strongly decreases or disappears in podocyte-depleted areas corresponding to FSGS lesions or severe glomerulosclerosis. Scale bars, 20 mm.

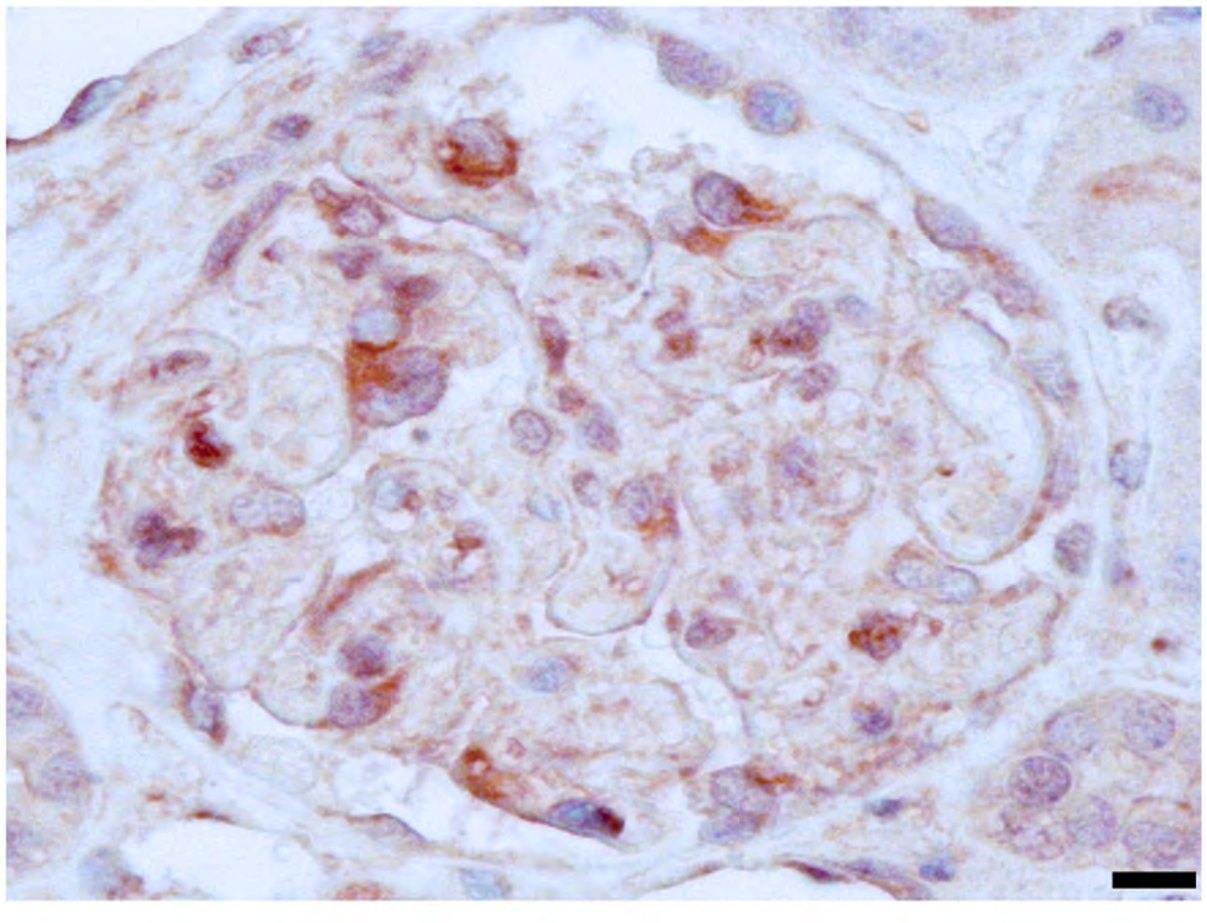
A

NHK

MCNS

c-mip

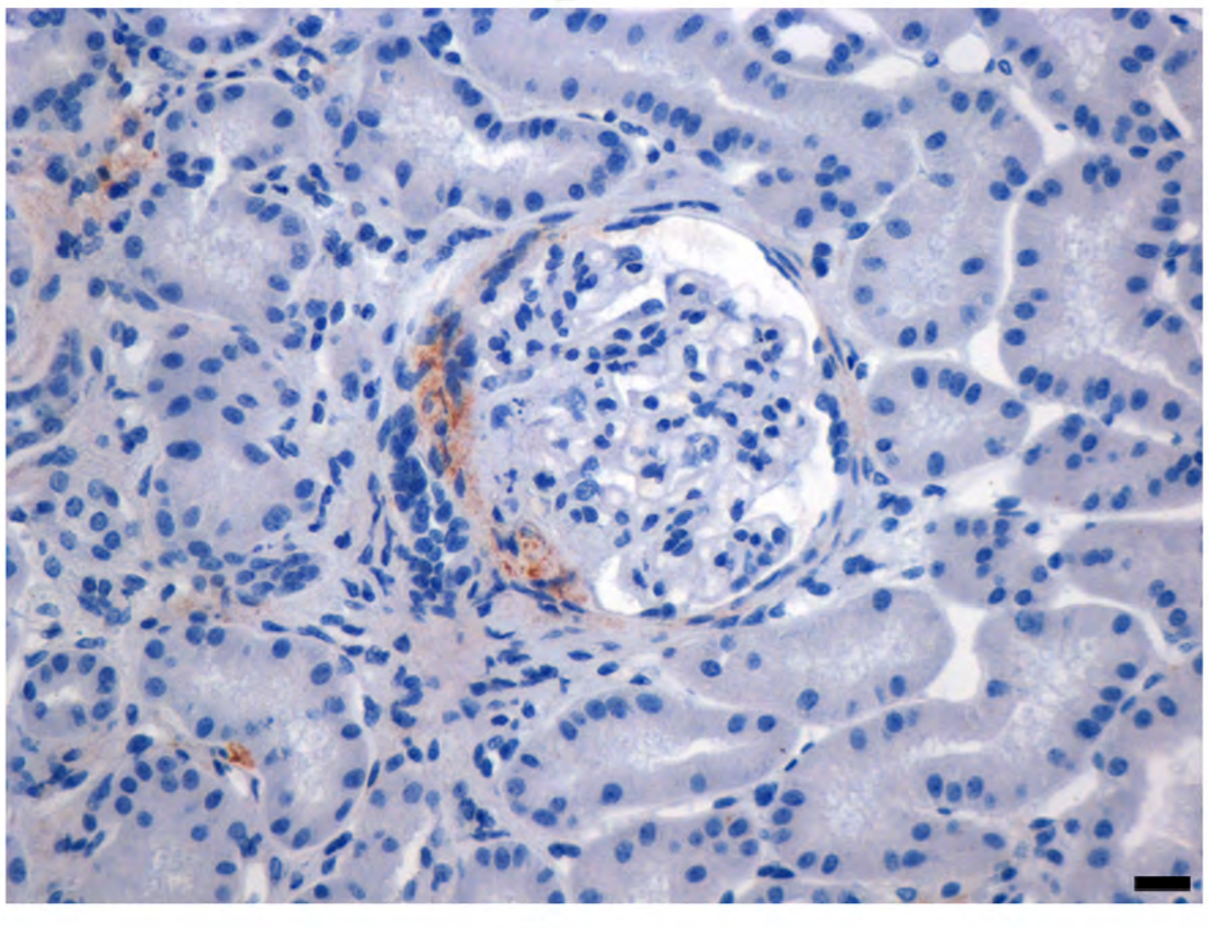


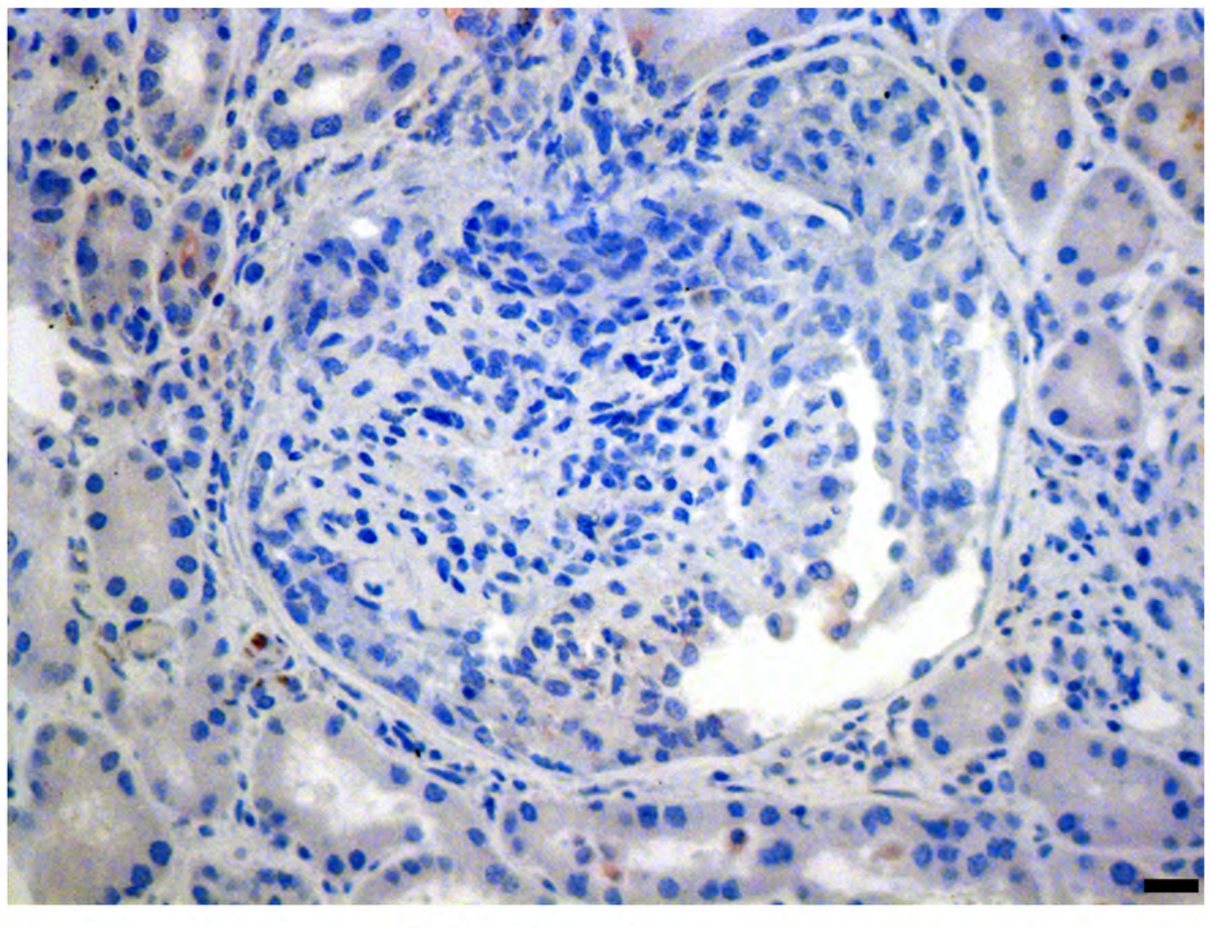


IgA

LN-IV

c-mip



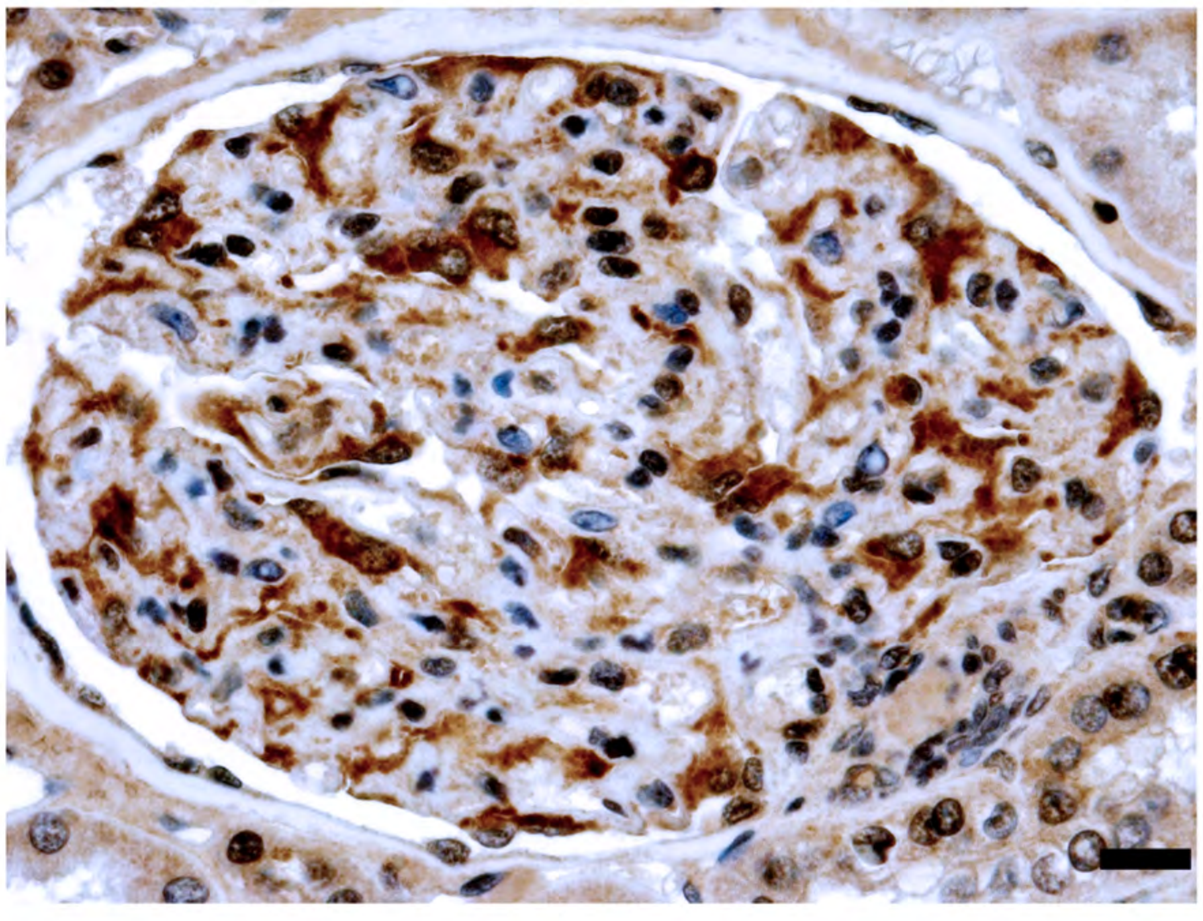


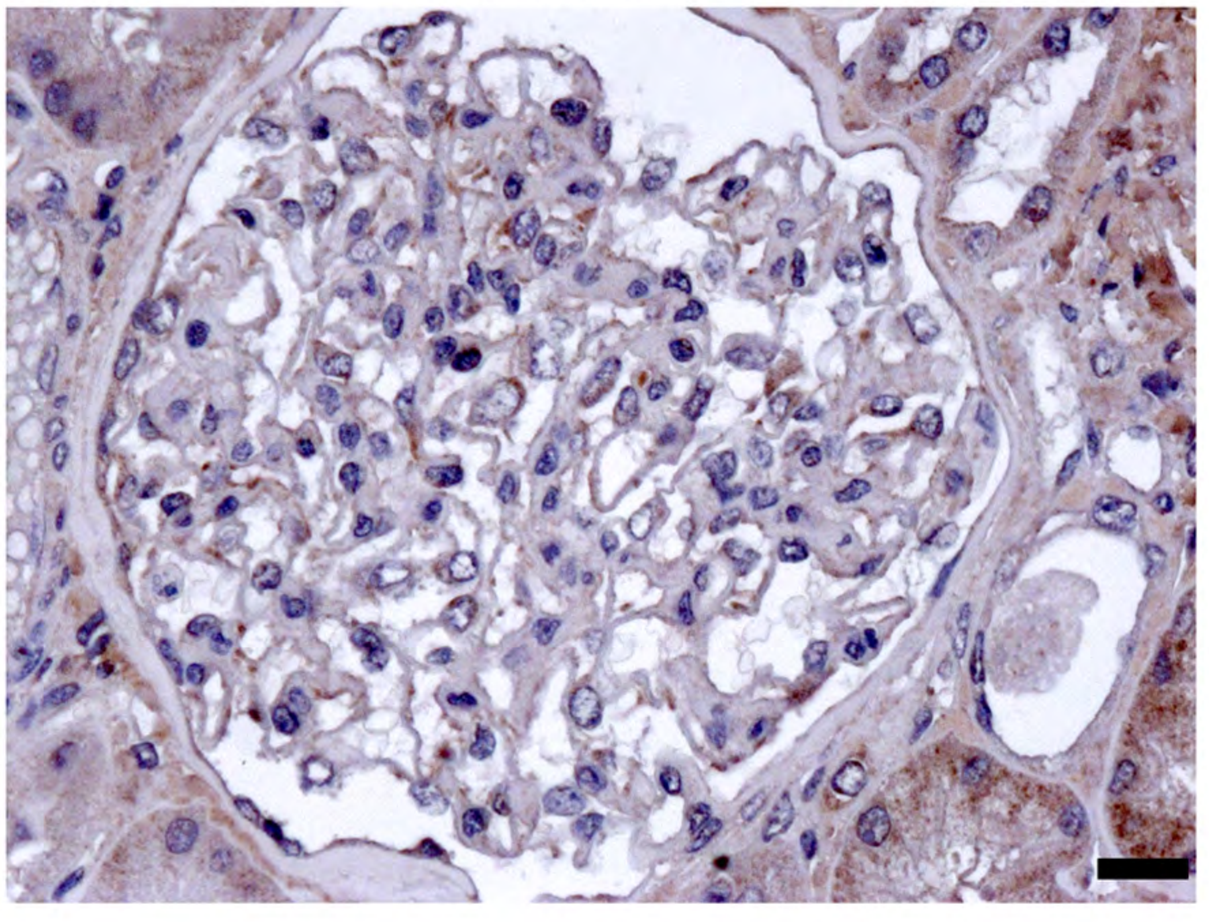
B

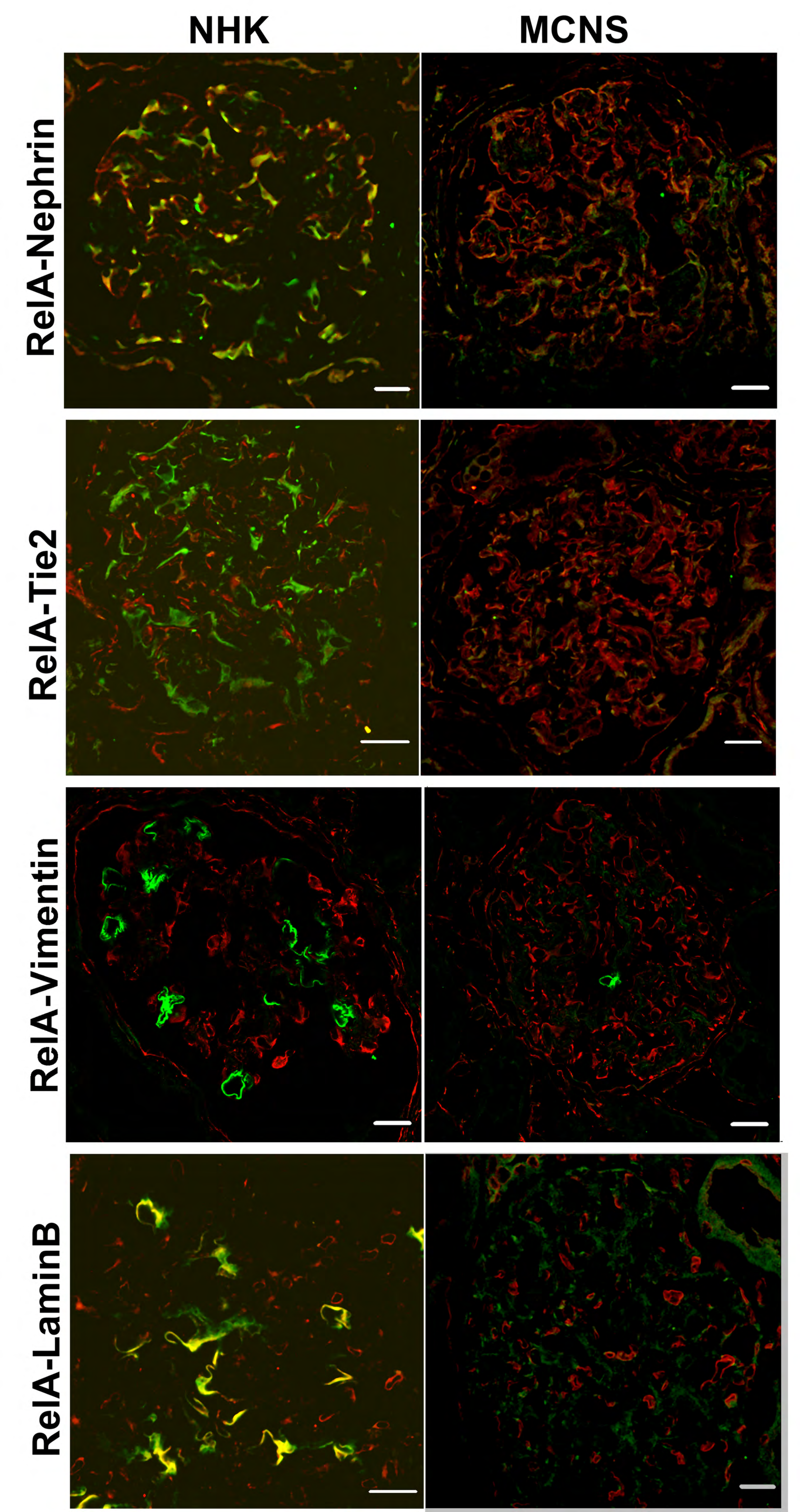
NHK

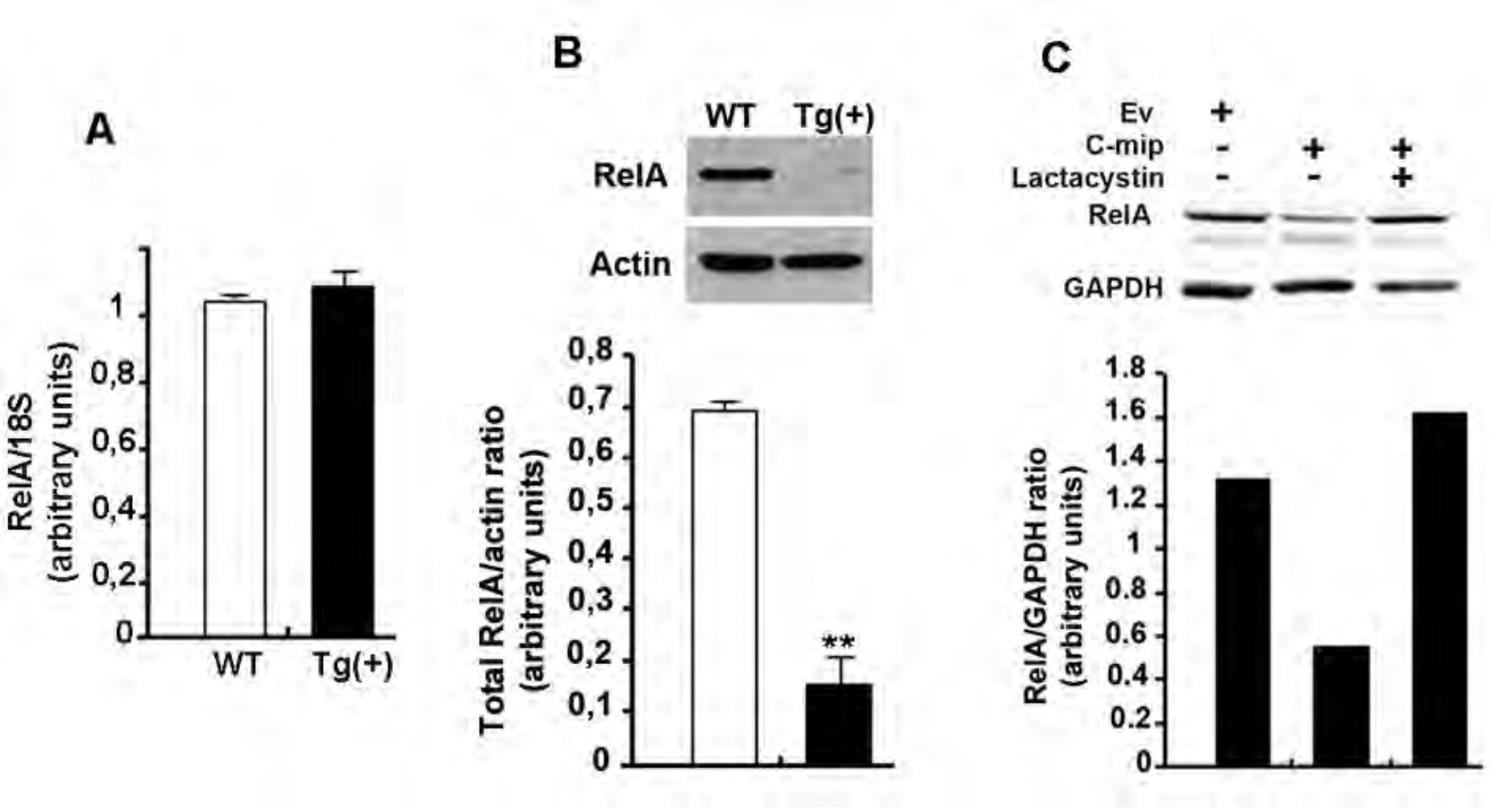
MCNS

Rel A

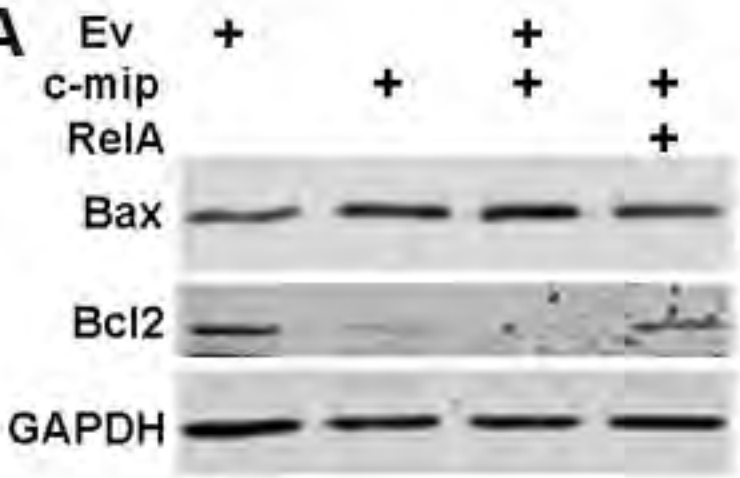


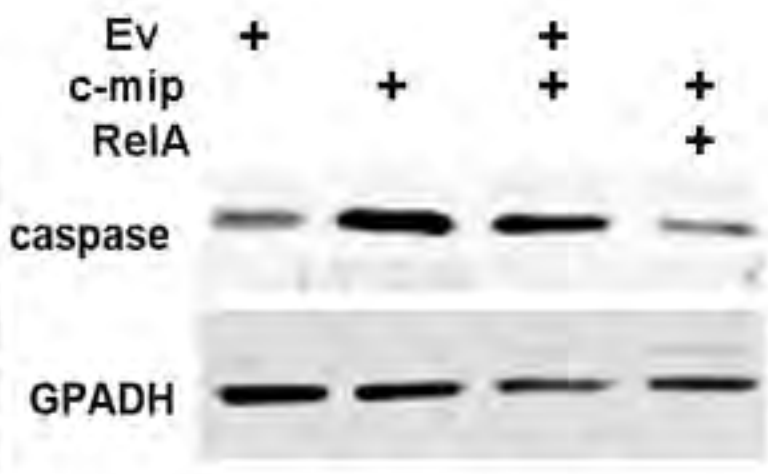


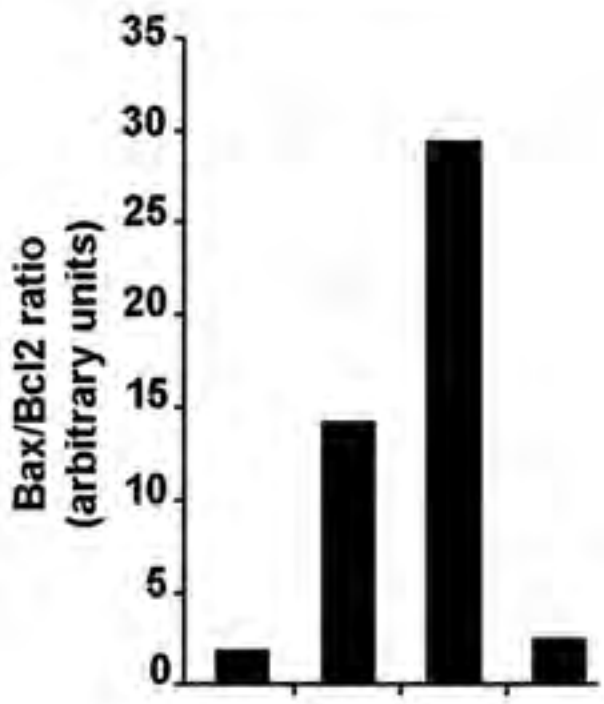

NHK
MCNS
RelA-Nephrin
RelA-Tie2
RelA-Vimentin
RelA-LaminB

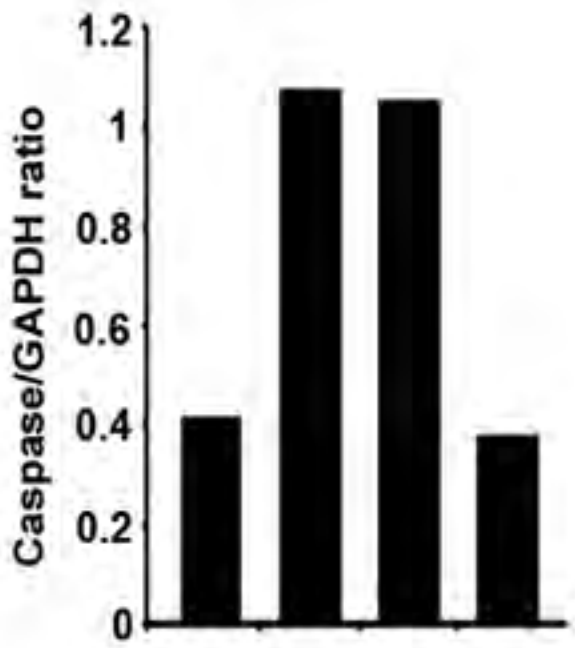

A
RelA/18S (arbitrary units)
1
0,8
0,6
0,4
0,2
0
WT
Tg(+)
B
WT
Tg(+)
RelA
Actin
Total RelA/actin ratio (arbitrary units)
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
**
C
Ev
C-mip
Lactacystin
RelA
GAPDH
RelA/GAPDH ratio (arbitrary units)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0

A

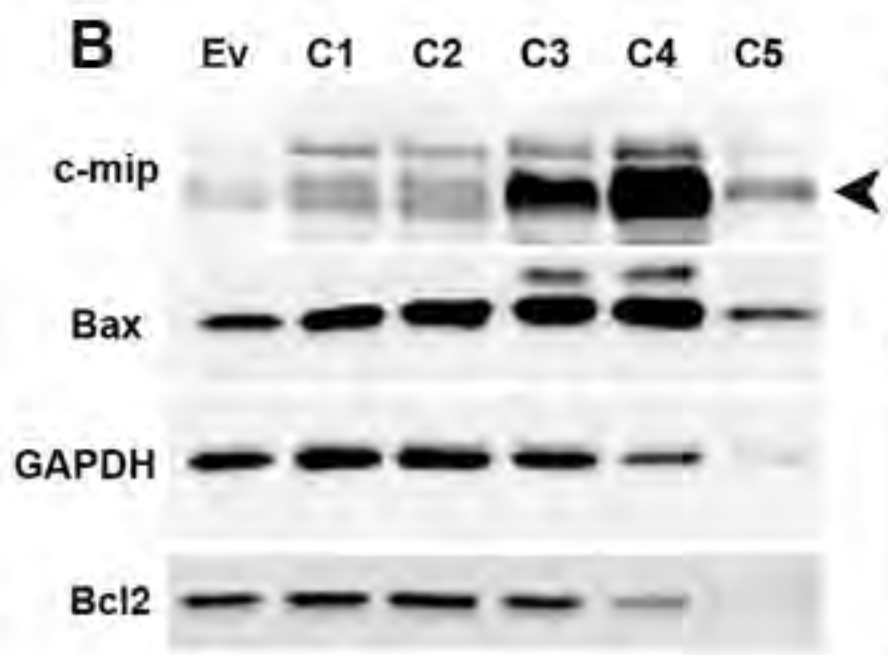

Ev
c-mip
RelA
Bax
Bcl2
GAPDH

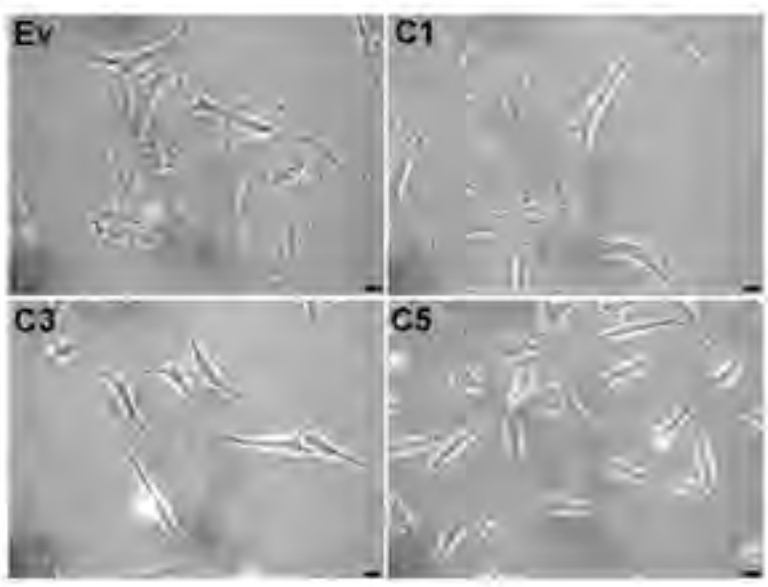

Ev
c-mip
RelA
caspase
GPADH

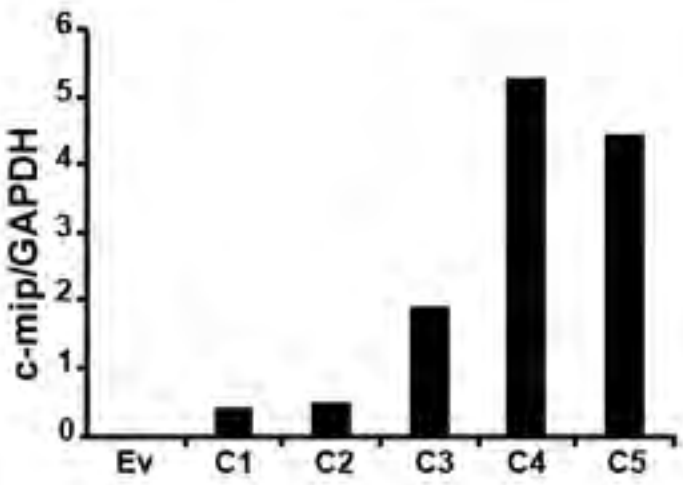

Bax/Bcl2 ratio
(arbitrary units)
35
30
25
20
15
10
5
0

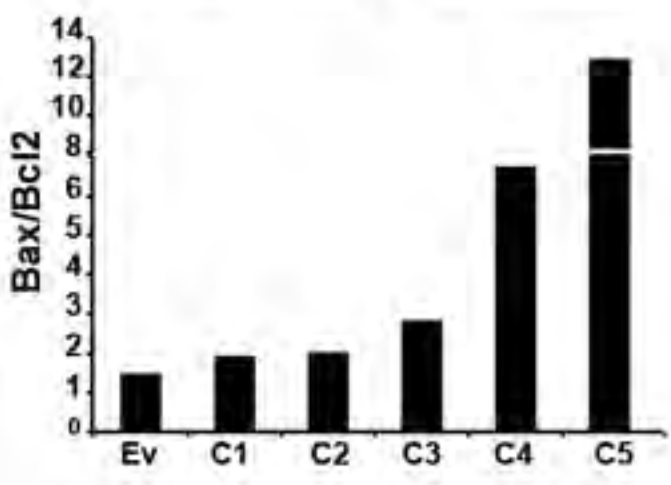

Caspase/GAPDH ratio
1.2
1
0.8
0.6
0.4
0.2
0

B

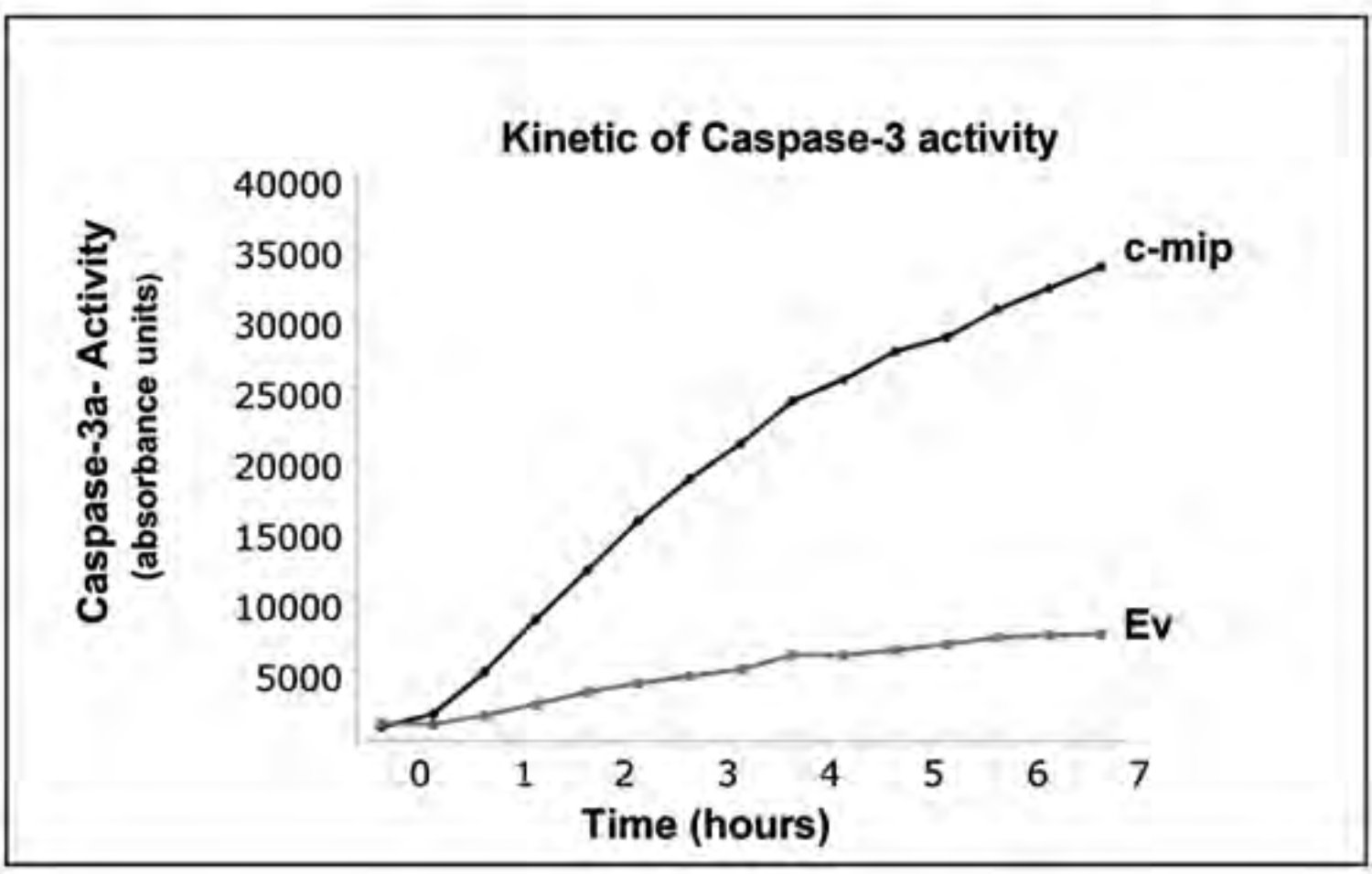

Ev
C1
C2
C3
C4
C5
c-mip
Bax
GAPDH
Bcl2


Ev
C1
C3
C5


c-mip/GAPDH
6
5
4
3
2
1
0
Ev
C1
C2
C3
C4
C5


Bax/Bcl2
14
12
10
8
6
5
4
3
2
1
0
Ev
C1
C2
C3
C4
C5

C


Kinetic of Caspase-3 activity
Caspase-3a- Activity
(absorbance units)
40000
35000
30000
25000
20000
15000
10000
5000
0
1
2
3
4
5
6
7
Time (hours)
c-mip
Ev

A

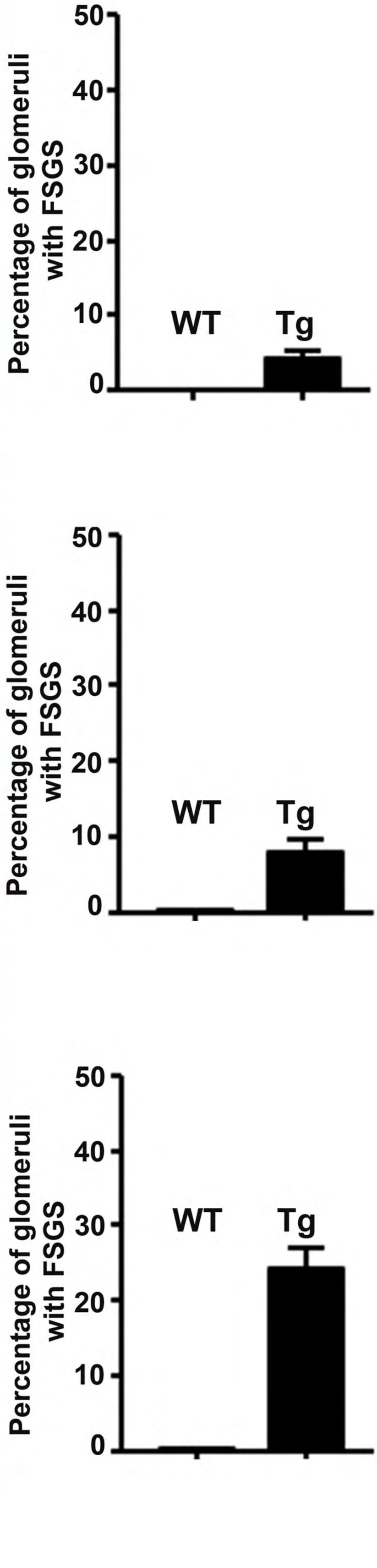
WT

Tg

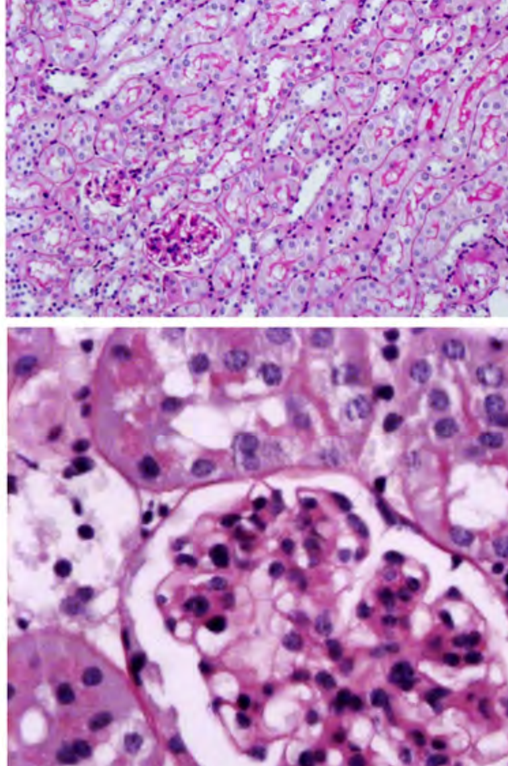
3m

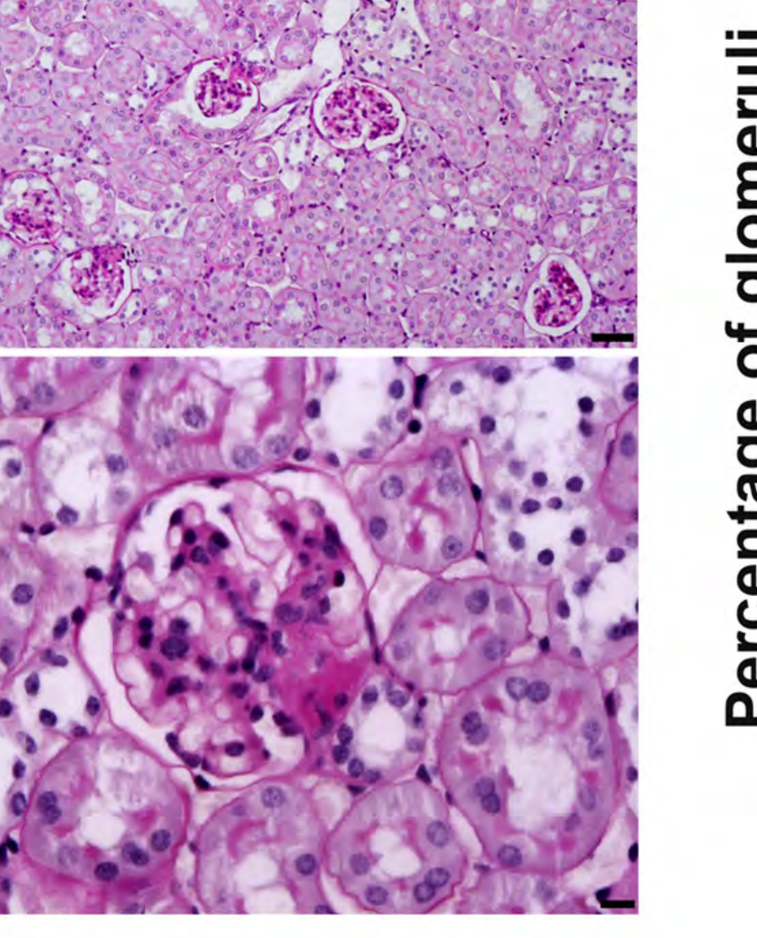
6m

12m



B





WT

Tg(+)

3 months

6 months

12 months

c-mip

Caspase3

FSGS1

FSGS2

FSGS3

FSGS4