Neuronal Wiskott Aldrich Syndrome Protein (N-WASP) is required for podocyte foot process stabilization

Supplemental Materials & Methods

Histology and scanning- / transmission electron microscopy and immuno-gold labelling

Sclerosis score was adapted from El Nahas (el Nahas, Bassett et al. 1991): grade I describing normal appearing glomeruli, grade II with mild to moderate alterations such as mesangial hypercellularity and grade III defined by sclerosed glomeruli. Up to 60 glomeruli per individual animal were assessed and counted (total number of animals analyzed 2-4 per timepoint). For scanning electron microscopy samples were fixated with 4% glutaraldehyde for 4 days and were then subsequently dehydrated (EtOH 50, 70, 80, 90 and 100%; 1:1 EtOH and HMDS for 1 hour and 30 minutes 100% HMDS, afterwards solvent was allowed to evaporate) and coated with Gold (Zeiss Semco Nanolab7, Polaron Cool Sputter Coater E 5100, Balzer Cpd 020). Image acquisition was performed using a Leo 1450 VP scanning electron microscope.

High Pressure Freeze Elelctron Microscopy

Freshly prepared tissue samples of 1 week and 2 week old wild type and mutant mice were frozen in 200 µm deep aluminium platelets (Microscopy Services, Flintbek) using 20 % BSA in PBS as a freezing medium. The samples were frozen with a BalTec HPM 10. Freeze substitution was carried out in a Leica EM AFS at -90 °C for 24 h in 0.1 % tannic acid, followed by 2 % OsO_4 for 22 h (each w/v in dry acetone) with slowly increasing temperature, according to Rostaing (Rostaing, Real et al. 2006). 50 nm sections were cut using a Leica UC6 ultra microtome. Sections were transferred on Formvar-coated copper single slot-grids and stained for 45 min on drops of 4 % (w/v) uranyl acetate (diluted in distilled water) and then washed in distilled water. After air drying the grids were incubated on drops of lead citrate (Reynolds, 1963) for 2 min in a CO_2-free atmosphere, and rinsed in distilled water. Specimens were examined in a Zeiss EM 902A, equipped with a 1024 × 1024 CCD detector (Proscan CCD HSS 512/1024; Proscan Electronic Systems, Scheuring, Germany).

Immunofluorescence and confocal microscopy

Nuclei were detected by using Hoechst 33342. Slides were mounted with ProLong Gold Antifade (Invitrogen). Image acquisition was done either by using a Zeis Axioscope 40FL microscope, equipped with AxioCam MRc5 digital video camera and immunofluorescence apparatus (Carl Zeis SpA) or a confocal imaging set up using a Zeiss LSM 510 upright

microscope (Zeiss, Germany), equipped with a Plan-Apochromat 63x/1.4 Oil M27 objective. Image recording was performed via Axiovision 4.3 or Zen black Software (Zeiss).

Primary cell isolation and cultivation

In brief glomeruli were isolated from 10 day old pups and cultivated in standard podocyte culture medium (RPMI, 10% FCS, Penicillin/Streptomycin and Insulin/Transferrin/Selenite all from Roche). After an expansion periode of 4-5 days cells were trypsinized and transferred to HBSS + 0,1 % BSA (Hanks Buffered Salt Solution). Cell solution was then FACS sorted and only EGFP positive cells were used for further experiments (MoFlo cell sorter, Beckmann Coulter, Germany). Further cultivation was performed using standard podocyte culture medium and cells were plated on collagen IV (Sigma Aldrich) coated flasks or dishs (all Corning). Doxycycline in a final concentration of 2µg/ml were added to culture medium to inducible cell lines and was replaced every 48 h.

Preparation of glomerular RNA and RT-PCR

Glomeruli were isolated as described under Western Blot experiments from 3 week old mice. Glomeruli were lysed using GTC buffer and RNA was extracted using the phenol-chloroform method. Equal amounts of RNA (100ng) were applied to RT-PCR using random nucleotide primers. Spleen from adult animals was processed in a similar manner. Specific primers for WASP (forward: 5´-gcctctgagacctcctgttg-3´, reverse: 5´-ccccagacactggagtagga-3´), N-WASP (forward: 5´-cagaagtgacgaccctggac-3´, reverse: 5´-tgcacttctttgcccacata-3´) and GAPDH (forward: 5´-acccagaagactgtggatgg-3´, reverse: 5´-aggtggaagagtgggagttg-3´) were used in PCR experiments (annealing temperature 60°, 40 cycles). Representative images were recorded using a digital gel documentation system.

Migration assays and live cell imaging

To evaluate single cell migration cells were seeded on ibidi µ-treat (Ibidi) dishes and cultivated in standard medium. Phase contrast observation of cells was performed using a Nikon Biosstation device, 20x objective (37° Celsius, 5% CO_2) for 6 – 10 hours. Further analysis and evaluation was done by using the chemotaxis and cell migration plugin for NIH Image J. Velocity for all cells is expressed as µm/hour. Experiments were performed in quadruplicate with 15-25 cells per experiment (n = 3). Lamellipodia formation upon growth factor stimulation was performed using a live cell imaging set up: Zeiss Cell Observer equipped with table heating, CO_2 controlled atmosphere, definite focus device and 2 cooled CCD cameras (Axiocam Rev.3, 1300x1000). Image acquisition was performed using a 100x/1.45 oil Alpha-Plan-Fluar objective with phase contrast, every 2 seconds for a total

observational time o 15 minutes. Cells were serum starved for 4 hours and EGF 20ng/ml was applied at defined timepoints to the medium.

Dorsal ruffle assays

For dorsal ruffle assays cells were seeded on collagen IV coated coverslips and were cultivated for 24h in standard culture medium. After starvation in medium without growth factor supplements for 12-16h hours cells were chased with EGF (20ng/ml – Invitrogen) for indicated time periods. Cells were subsequently fixated with PFA 4% for 2 min, permeabilized with Triton-X-100 0,1% and stained with phalloidin-Alexa-548 (Invitrogen) for 25 minutes. After mounting cells were examined using a Zeiss epifluorescence microscope, 20x objective. Between 150-200 cells per condition were analyzed and cells with definite circular dorsal ruffles (CDRs) were counted as positive. Experiments were repeated between 3-5 times with different individual cell lines. For Wiskostatin (Sigma) inhibitor experiments cells were pre-treated with indicated concentrations of Wiskostatin and subsequent stimulation with EGF (20 ng/ml) was then performed. DMSO was used as a control in these experiments and image analysis was equal to the described protocol above. To measure the diameter of respective CDRs experiments were performed as described above and individual cells (10-20) per experiment (repeated at least 3 times for each genotype) were recorded using a Zeiss Axioscope 40FL microscope, equipped with AxioCam MRc5 digital video camera and 63x objective. Diameters were measured using the Axiovision 4.3 software.

Dextran uptake

To detect dextran uptake in primary podocytes full culture medium was removed and replaced with serum-free RPMI for 12-16 hours. EGF (final concentration of 20ng/ml) and fluorescently labelled Dextran (final concentration 25ng/ml – Dextran-Rhodamine, Invitrogen) was added for 5 minutes. Cells were then subsequently washed with ice-cold PBS two times to remove residual dextran and fixed with 4% paraformaldehyde and embedded in ProLong® Gold Antifade Reagent (Invitrogen). Further analysis was performed using a confocal microscopy set up Zeiss LSM 510 upright microscope (Zeiss, Germany), equipped with a Plan-Apochromat 63x/1.4 Oil M27 objective. Measurement of individual vesicles and size distribution was performed by using analyze particle plug-in of Image J NIH software Version 1.44. Each experiment was repeated 3 times with individual cell lines and 10-15 cells per condition were assessed.

Supplemental Figure Legends:

S-Figure 1: ***Localization of actin nucleation machinery in podocytes***

(a-f) Confocal microscopy with respective specific antibodies for N-WASP, the ARP2 subunit and CORTACTIN display colocalization with the foot process marker protein NEPHRIN. (a-b) Comparison between 3 week old wildtype and respective N-WASP KO mice exhibits a loss of N-WASP protein, while the localization pattern of NEPHRIN appears unaffected. (c-f) No differences between wildtype and knockout animals were detected with regards to the localization of ARP2 and Cortactin. (g) As in murine kidneys also in human glomeruli a colocalization of CORTACTIN and NEPRHIN was observed by confocal microscopy. (h-i) Cortactin immunogold labeling reveals an intense decoration of immunogold positive particles in foot processes of human podocytes (FP – foot process, GBM – glomerular basement membrane).

S-Figure 2: ***Deletion of N-WASP at early postnatal developmental time points***

(a-b) Confocal immunofluorescence staining of P2 old glomeruli from respective wildtype and knockout animals. Nephrin as a specific podocyte marker was used to visualize podocytes (indicated by P). Comparison of N-WASP fluorescence intensity and localization indicates a clear reduction of N-WASP protein, although some residual positivity is detectable at this age (white arrows). (c-d) With one week N-WASP is not detectable in podocytes in respective knockout glomeruli (white arrows), whereas a clear signal is present in the mesangium (M). The podocyte marker Nephrin appears not to be affected at both postnatal developmental time points.

S-Figure 3: ***N-Wasp deletion leads to glomerular sclerosis and renal failure***

(a-b) At 3 weeks of age first signs of proteinuria are detected in N-WASP knockout kidneys (arrows indicate proteinaceous casts in dilated tubules). While glomerular morphology displays no major abnormalities. (c-d) At 9 weeks of age many glomeruli exhibit total sclerosis in *N-WASPfl/fl*Nphs2Cre* animals (indicated by black arrows, white asterisks marks proteinaceous casts – d). Higher magnification reveals matrix expansion (white asterisks) and intraglomerular cystic degeneration (black arrows). (e-f) Sclerosis index indicates significant sclerosis in 9 week old KO animals, whereas at 3 weeks the majority of glomeruli appeared normal (no difference between wildtype and KO animals). (g-h) Desmin as a marker of mesangial matrix expansion shows accumulation in NWASP KO mice.

S-Figure 4: ***Localization of classical podocyte markers in N-WASP KO mice***

Confocal images of classical podocyte markers NEPHRIN, PODOCIN and SYNAPTOPODIN exhibit no obvious differences between wildtype and respective knockout animals (a-d).

S-Figure 5: ***Generation of a primary podocyte culture system***

(a) As podocytes are embedded in the multicellular complex of glomeruli (including parietal epithelial, mesangial and endothelial cells) an efficient method for specific cell enrichment is required. Intercrossing of the *Tomato* reporter allele allows selection for only *Nphs2-Cre* expressing cells (genetically defined as podocytes). After isolation of glomeruli, cultivation phase is performed and FACS sort results in a highly purified population of EGFP positive cells. (b) Doxycycline application results in activation of Cre-recombinase *in vitro* and results in loss of N-WASP protein as shown by confocal microscopy (c-f). (g-h) Focal adhesions appear not to be altered in NWASP KO podocytes as assessed by staining for VINCULIN. (j) Single cell tracking revealed slightly increased cell migration velocity in NWASP KO induced podocytes (* $p<0.05$).

S-Figure 6: ***Involvement of NWASP in dorsal ruffle size of primary podocytes***

(a) Long and short axis diameters were assessed in individual cells of wildtype and respective mutant podocytes after application with EGF. In those mutant podocytes, which showed intact CDRs, the majority of cells exhibited smaller sized structures. (b) Quantification of mutant and wildtype primary podocytes for long and short axis diameters of individual CDRs (* $p<0.05$; ** $p<0.001$). The distribution analysis shows a shift to smaller sized CDRs in mutant podocytes. (c) After application of doxycycline in *NWASPfl/fl*Nphs2rtTA*TetOCre*Tomato* mice activity of Cre-recombinase results in recombinatorial events in up to 60-70% in all glomeruli.

S-Figure 7: ***WASP does not compensate for loss of N-WASP in podocytes***

(a) RT-PCR experiments on spleen tissue, total kidney and isolated podocytes as well as non-podocytes. N-WASP is abundantly present in all tested samples, whereas WASP is only specifically expressed in spleen tissue. (b) WASP is not detectable in either cDNA from wildtype or knockout glomeruli; GAPDH was used as a loading control. (c) Also in western blot experiments using a specific antibody directed against WASP, no signals were present in glomerular lysates from wildtype and knockout animals.

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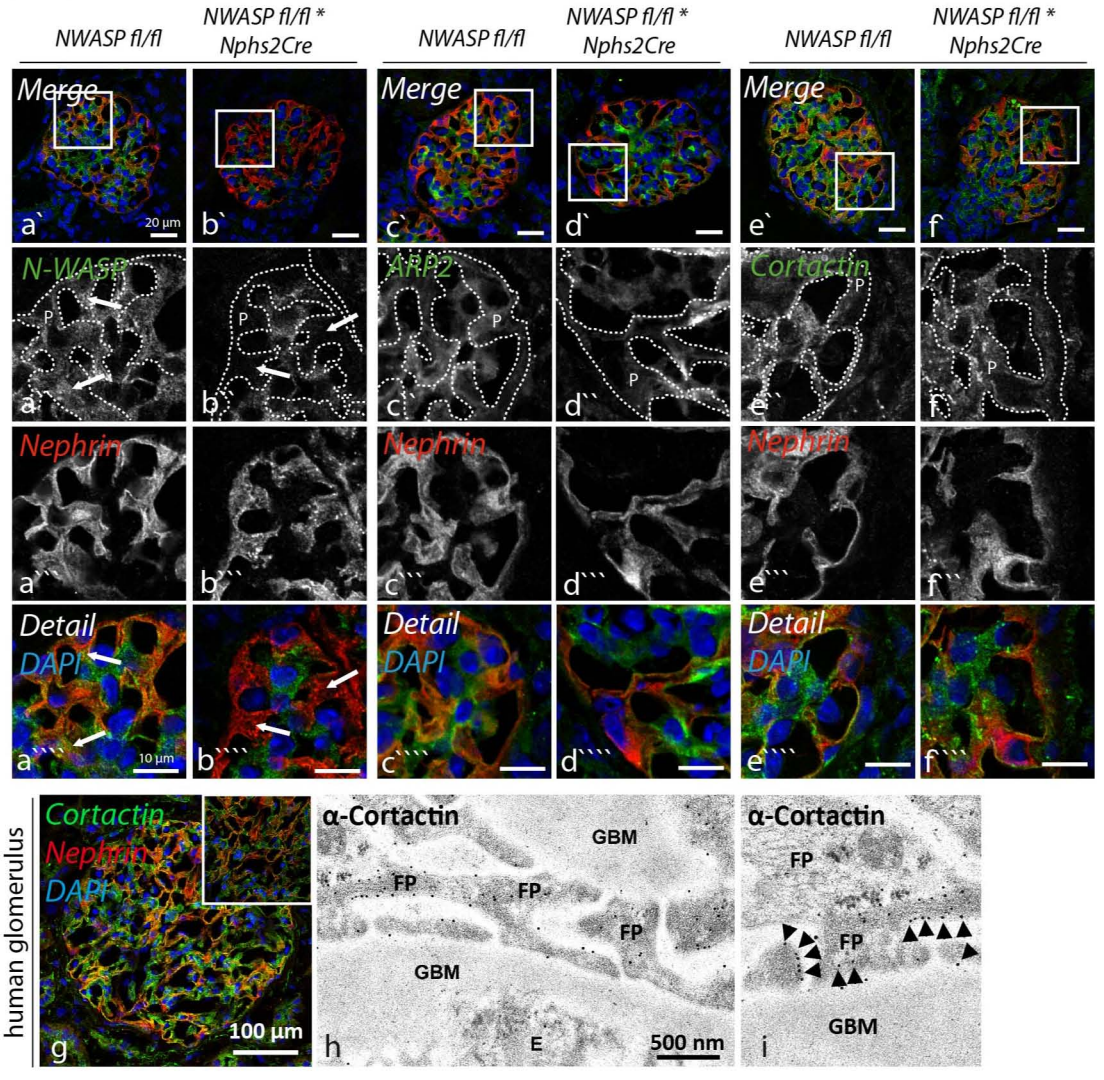

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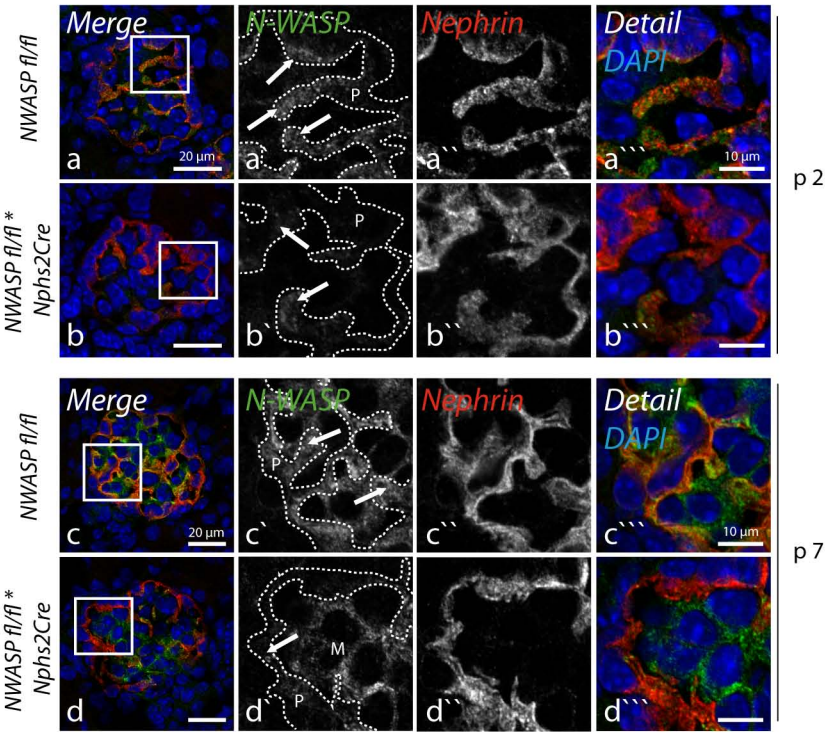
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NWASP fl/fl * Nphs2Cre
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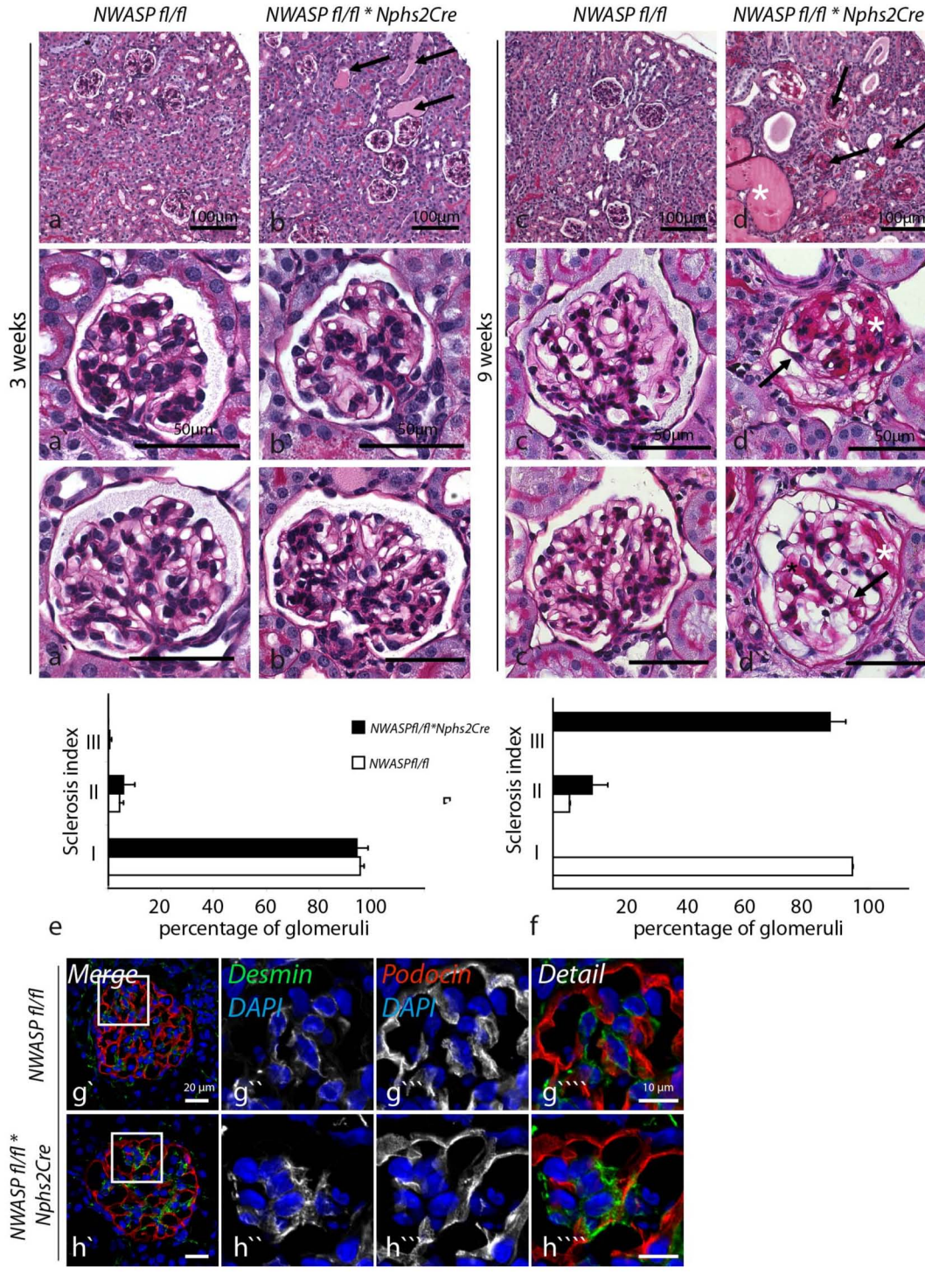

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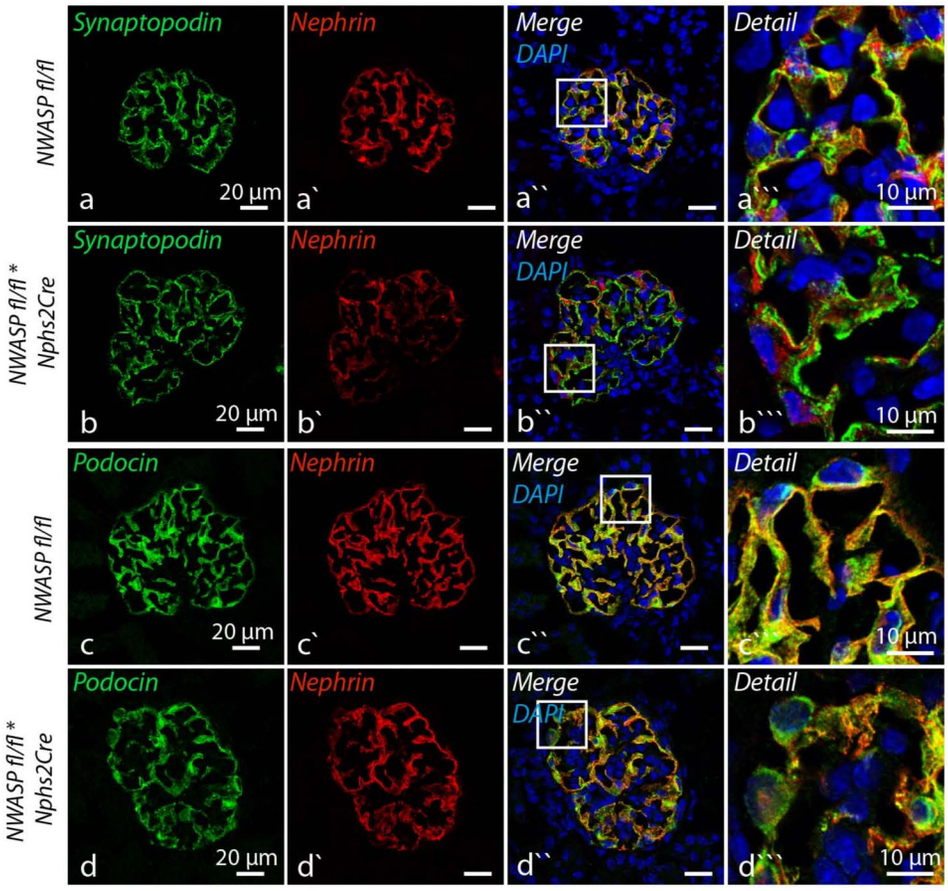

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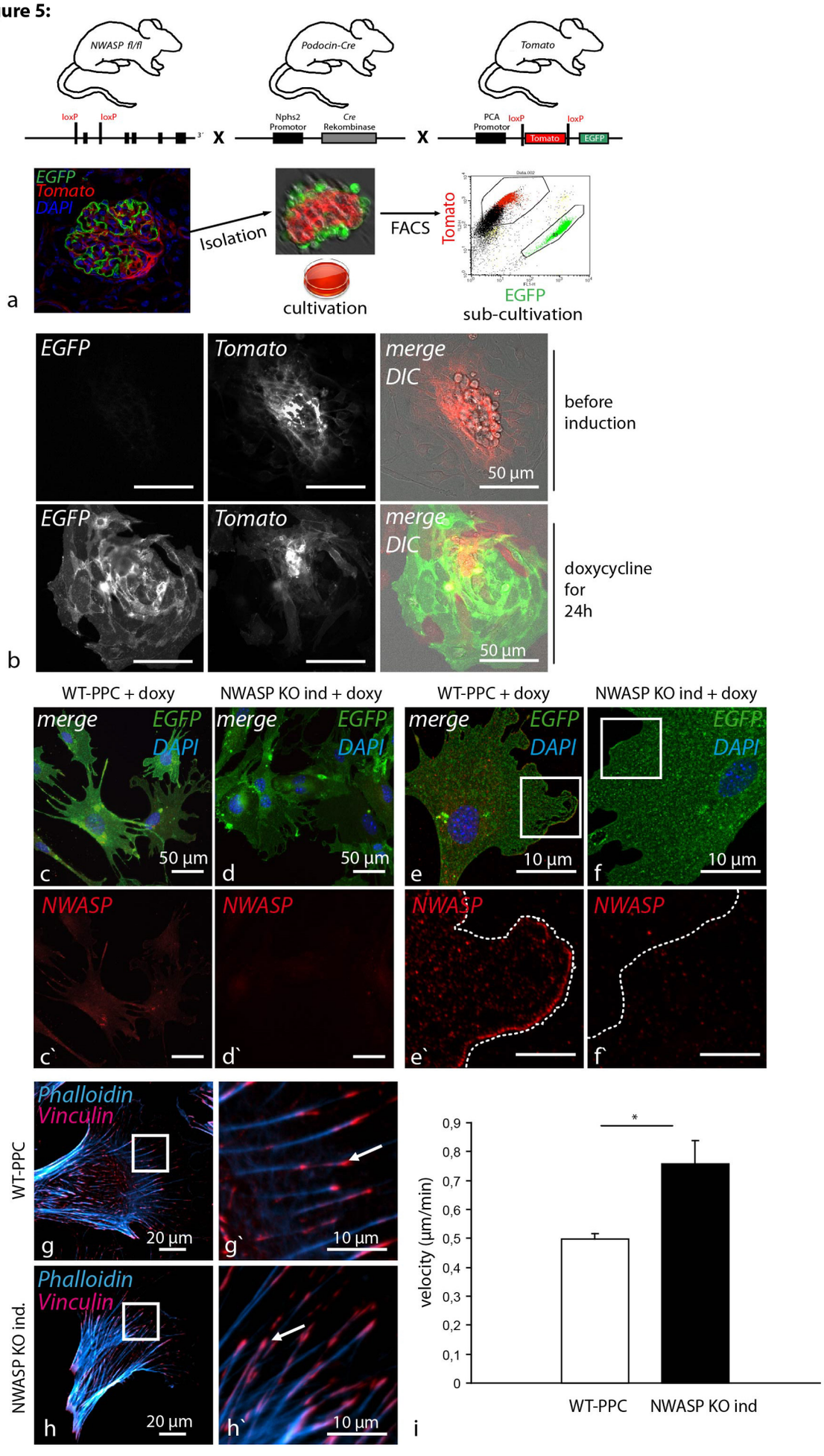

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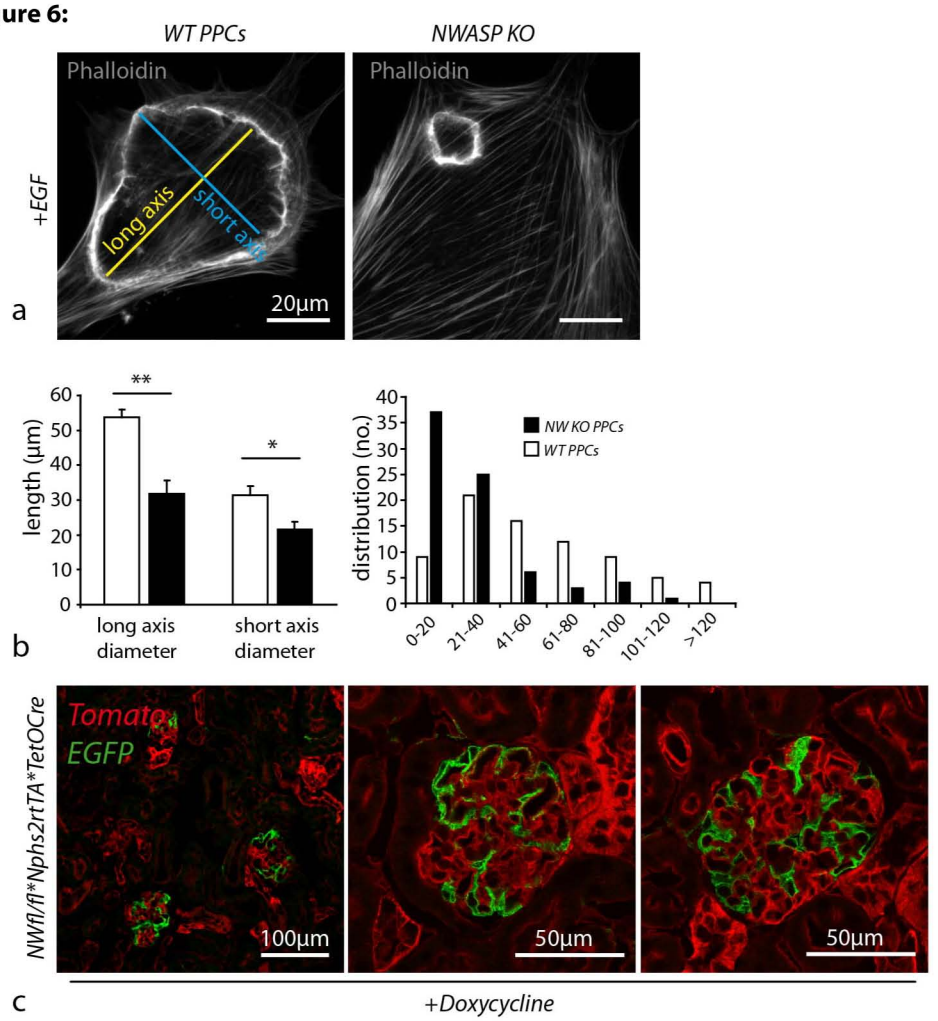

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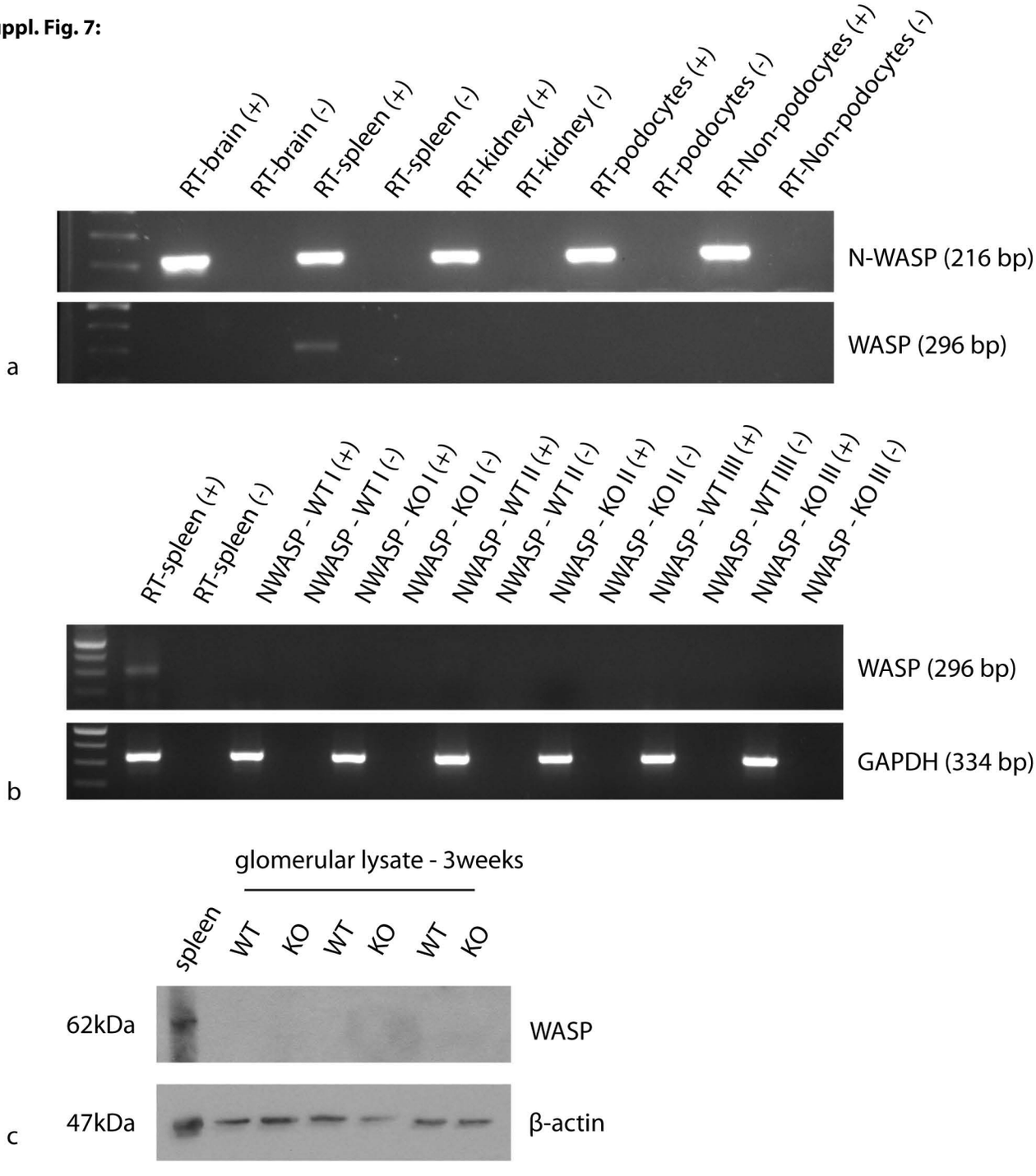
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RT-podocytes (+)
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RT-Non-podocytes (+)
RT-Non-podocytes (-)
N-WASP (216 bp)
WASP (296 bp)
a
RT-spleen (+)
RT-spleen (-)
NWASP - WT I (+)
NWASP - WT I (-)
NWASP - KO I (+)
NWASP - KO I (-)
NWASP - WT II (+)
NWASP - WT II (-)
NWASP - KO II (+)
NWASP - KO II (-)
NWASP - WT IIII (+)
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NWASP - KO III (+)
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GAPDH (334 bp)
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glomerular lysate - 3weeks
spleen
WT
KO
WT
KO
WT
KO
62kDa
WASP
47kDa
β-actin
c