RESEARCH ARTICLE

Talin regulates integrin β1-dependent and -independent cell functions in ureteric bud development

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ABSTRACT

Kidney collecting system development requires integrin-dependent cell-extracellular matrix interactions. Integrins are heterodimeric transmembrane receptors consisting of α and β subunits; crucial integrins in the kidney collecting system express the β1 subunit. The β1 cytoplasmic tail has two NPxY motifs that mediate functions by binding to cytoplasmic signaling and scaffolding molecules. Talins, scaffolding proteins that bind to the membrane proximal NPxY motif, are proposed to activate integrins and to link them to the actin cytoskeleton. We have defined the role of talin binding to the β1 proximal NPxY motif in the developing kidney collecting system in mice that selectively express a Y-to-A mutation in this motif. The mice developed a hypoplastic dysplastic collecting system. Collecting duct cells expressing this Y-to-A mutation had severe abnormalities in cell adhesion, migration, proliferation and growth factor-dependent signaling. In contrast, mice carrying the YY/AA mutation in this motif. The integrin β1 cytoplasmic tail contains two well-defined NPxY motifs (Y783 at the membrane proximal and Y795 at the membrane distal sites) (Czuchra et al., 2006). The functional significance of these domains was addressed by mutagenesis studies where both Y783 and Y795 were mutated to alanine (YY/AA). YY/AA mutations selectively induced in the skin epidermis showed the same abnormalities as mice lacking the β1 subunit; however, mice carrying the single β1 Y783A mutation exhibited only minor defects (Meves et al., 2011). In contrast, mice carrying the YY/AA mutations in the developing UB displayed a significantly less severe phenotype than mice lacking the β1 subunit (Mathew et al., 2012b). The effect of the β1 Y783A mutation in the UB is unknown.

The β1 tail NPxY motifs bind crucial cytosolic proteins, with talins representing the most studied (Ye et al., 2014). There are two talin isoforms in mammals, talin1 and talin2, which have the same domain structure and 76% amino acid identity. In some tissues, these isoforms are differentially expressed, whereas in others, such as the kidney collecting system, both are expressed (Prackel et al., 2012). Talins are required for integrin activation and they provide a direct link between integrins and the actin cytoskeleton by acting as a scaffold for the recruitment of other proteins, such as vinculin and actin (Albige-Rizo et al., 2009; Calderwood et al., 2013). Talins are thought to induce integrin activation by first binding to the membrane-proximal NPxY of the β1 integrin tail after which they interact with a more membrane proximal binding site to destabilize the putative integrin salt bridge and then stabilize the helical structure of the membrane proximal region of the β1 integrin tail (Wegener et al., 2007). Talins are required for normal development and constitutive deletion of talin1 causes E8.5 lethality due to gastrulation defects (Monkley et al., 2000). Talin1 was also shown to be required for integrin αIIbβ3 activation (Calderwood et al., 1999), and platelet aggregation and clotting (Nieswandt et al., 2007; Petrich et al., 2007). Talin1 is important for the normal function of podocytes in the glomeruli of the kidneys, where it regulates the actin cytoskeleton rather than integrin activation (Tian et al., 2014). Talin2 is more dispensable than talin1. Global talin2-null mice are viable with only a mild muscle phenotype (Debrandt et al., 2012). Both talins are required for normal muscle development. Mice lacking both isoforms in muscle have a fatal perinatal phenotype.

KEY WORDS: Kidney, Signaling, Nuclear magnetic resonance, Tubules

INTRODUCTION

The kidney consists of numerous nephrons that drain into the multibranched collecting system. Embryologically, the nephrons are derived from the metanephric mesenchyme, while the collecting system is formed by iterative branching of the ureteric bud (UB), a process regulated by multiple factors, including integrin-dependent cell-extracellular matrix (ECM) interactions (Mathew et al., 2012a).

Integrins, transmembrane receptors that mediate ECM interactions, are essential for multiple cell functions. There are 24 integrins composed of αβ heterodimers (Pozzi and Zent, 2013). Among the eight β subunits, integrin β1 is most abundantly expressed and pairs with 12 α subunits, including laminin- (α3, α6) and collagen- (α1, α2) binding subunits, thereby forming the principal integrins expressed by the kidney (Mathew et al., 2012a). Integrin β1 is required for normal kidney collecting system development and its selective deletion in the UB (E10.5) causes a severe branching morphogenesis defect (Zhang et al., 2009).

The integrin β1 cytoplasmic tail contains two well-defined NPxY motifs (Y783 at the membrane proximal and Y795 at the membrane distal sites) (Czuchra et al., 2006). The functional significance of these domains was addressed by mutagenesis studies where both Y783 and Y795 were mutated to alanine (YY/AA). YY/AA mutations selectively induced in the skin epidermis showed the same abnormalities as mice lacking the β1 subunit; however, mice carrying the single β1 Y783A mutation exhibited only minor defects (Meves et al., 2011). In contrast, mice carrying the YY/AA mutations in the developing UB displayed a significantly less severe phenotype than mice lacking the β1 subunit (Mathew et al., 2012b). The effect of the β1 Y783A mutation in the UB is unknown.

The β1 tail NPxY motifs bind crucial cytosolic proteins, with talins representing the most studied (Ye et al., 2014). There are two talin isoforms in mammals, talin1 and talin2, which have the same domain structure and 76% amino acid identity. In some tissues, these isoforms are differentially expressed, whereas in others, such as the kidney collecting system, both are expressed (Prackel et al., 2012). Talins are required for integrin activation and they provide a direct link between integrins and the actin cytoskeleton by acting as a scaffold for the recruitment of other proteins, such as vinculin and actin (Albige-Rizo et al., 2009; Calderwood et al., 2013). Talins are thought to induce integrin activation by first binding to the membrane-proximal NPxY of the β1 integrin tail after which they interact with a more membrane proximal binding site to destabilize the putative integrin salt bridge and then stabilize the helical structure of the membrane proximal region of the β1 integrin tail (Wegener et al., 2007). Talins are required for normal development and constitutive deletion of talin1 causes E8.5 lethality due to gastrulation defects (Monkley et al., 2000). Talin1 was also shown to be required for integrin αIIbβ3 activation (Calderwood et al., 1999), and platelet aggregation and clotting (Nieswandt et al., 2007; Petrich et al., 2007). Talin1 is important for the normal function of podocytes in the glomeruli of the kidneys, where it regulates the actin cytoskeleton rather than integrin activation (Tian et al., 2014). Talin2 is more dispensable than talin1. Global talin2-null mice are viable with only a mild muscle phenotype (Debrandt et al., 2012). Both talins are required for normal muscle development. Mice lacking both isoforms in muscle have a fatal perinatal phenotype,
which was not because muscle cells cannot activate their integrins but due to their inability to generate force across integrins (Conti et al., 2008, 2009; Manso et al., 2013).

The kidney tubules, including the collecting ducts (CDs), have very high levels of talin 1 and talin 2 (Praekelt et al., 2012); however, their importance in development is unknown. In this study, we have defined the function of talins and the role of talin binding to the β1 integrin subunit in UB development.

RESULTS
Disrupting the NPxY talin binding site on integrin β1 leads to defective UB development

To define the role of β1 integrin-talin interactions in UB development, we generated mice that selectively carry the Y783A substitution (Czuchra et al., 2006) in the developing UB. Mice expressing the Y/A mutation were bred by intercrossing heterozygous Y/A mutant mice with floxed β1 integrin mice and transgenic mice expressing Cre recombinase under the hoxB7 promoter (Yu et al., 2002). The offspring had deletion of the floxed β1 allele starting at E10.5 in the UB and expressed only the Y/A mutant allele. Y/A mutant mice were born in a normal Mendelian ratio, but at 3 weeks of age, they were significantly smaller than wild-type controls (4.9±0.8 versus 9.95±0.54 g, P<0.05), and at 6 months they were sacrificed due to failure to thrive. The phenotype was equally penetrant in both male and female mice. The kidneys of 4-week-old Y783A mice were significantly smaller than in wild-type mice (Fig. 1A-F) and ∼20% of the medullary and cortical collecting ducts were dilated. By 3 months there was increased cellularity and destruction of epithelia within the collecting ducts (Fig. 1G,J) and by 6 months the mice developed hypercellularity and fibrosis, resulting in an end-stage kidney (Fig. 1H,I). High-powered microscopy of the medulla of the kidneys after staining with Dolichos biflorus, which stains the CDs and antibodies directed against Tamm-Horsfall protein (which stains the thick ascending limb of the loop of Henle), revealed that the dilated tubules with increased cellularity were CDs (Fig. 1K,L).

The adult phenotype of the Y783A mice was consistent with defects in development. We therefore determined when this became identifiable by characterizing mutant kidneys at E12.5, E15.5 and E17.5 (Fig. 2A-F). At E12.5, the size of the wild-type and Y783A mouse embryos was similar (798±4 mm versus 796±8 mm) as were the kidneys. Hematoxylin and Eosin-stained E12.5 kidneys suggested the mutant UB had undergone less branching morphogenesis than the wild-type mice. This early branching defect was verified by performing Wnt11 in situ hybridization on whole-mount kidneys at E12.5. Whereas wild-type kidneys had undergone several rounds of branching, all Y783A kidneys analyzed (n=6) appeared to be arrested at the T-bud stage (Fig. 2C,D). At E15.5, branching morphogenesis had progressed, although the Y783A kidneys were still significantly smaller than wild type with fewer collecting ducts visible (Fig. 2E-F). At E17.5, the differences in kidney size between wild type and Y783A mutants were even more obvious (Fig. 2G-H) with fewer dilated collecting ducts present (Fig. 2I,J).

Owing to the branching morphogenesis phenotype in the Y783A kidney, we investigated the defects at E15.5 in more detail. Although the size of the Y783A and wild-type mice was not significantly different (990±17 mm versus 883±28 mm), the Y783A kidneys were significantly smaller than wild type with fewer collecting ducts visible (Fig. 2E-F). At E15.5, although the Y783A kidneys were still significantly smaller than wild type with fewer collecting ducts visible (Fig. 2E-F). At E17.5, the differences in kidney size between wild type and Y783A mutants were even more obvious (Fig. 2G-H) with fewer dilated collecting ducts present (Fig. 2I,J).
and wild-type kidneys of mice from the same litters, they do not assess absolute branch number within the developing UB. When we examined cell proliferation and apoptosis, no difference in apoptosis (as assessed by TUNEL staining) was present (data not shown). There was, however, a significant decrease in the overall number of Ki67-positive UB cells in the Y783A mutants at E15.5 (15% versus 34%, \( P < 0.05 \), Fig. 3I-K). As most proliferation occurs in the UB tips, these data suggest that decreased cell proliferation is at least in part responsible for the branching phenotype in the Y783A mutants. Thus, we conclude that Y783 of the membrane-proximal NPxY motif of the \( \beta_1 \) integrin subunit regulates UB branching morphogenesis.

The membrane-proximal NPxY of integrin \( \beta_1 \) regulates CD cell function

We generated CD cells expressing the Y783A mutant to identify the cellular mechanisms underlying the in vivo development phenotypes. \( \beta_1 \)-null CD cells were transfected with either \( \beta_1 \)-WT or \( \beta_1 \)-Y783A mutant human cDNA and flow sorted for equal levels of expression (data not shown) (Mathew et al., 2012b). Y783A and wild-type CD cells spread equally and looked identical when grown on glass coverslips with 10% serum (Fig. 4A). When the Y783A CD cells were placed in 3D collagen/matriact gel substrates, they did not form tubules and grew as cysts (Fig. 4C,D). We next defined which crucial cell functions required for tubulogenesis were affected by the Y783A mutation by performing cell-adhesion, migration and proliferation assays on collagen I and laminin-511 (a major component of the kidney tubular basement membranes) (Fig. 4E-G). Y783A mutant cell adhesion (Fig. 4E) and migration (Fig. 4F) were decreased by about 75% and 50% on collagen I and Ln-511, respectively. By contrast, Y783A cell proliferation was reduced by only \( \sim 25\% \) on each of these substrates (Fig. 4G). Thus, the Y783A mutation did not alter CD cell morphology under normal culture conditions but CD cells are unable to undergo 3-D tubulogenesis. This was primarily caused by decreased cell adhesion and migration, and to a lesser extent by reduced proliferation on \( \beta_1 \) integrin-dependent substrates.

The membrane-proximal NPxY of integrin \( \beta_1 \) regulates CD cell signaling

A key requirement for cell adhesion is the ability of activated integrins to bind their ligands. We assessed this by determining the binding of antibody 12G10 (which binds to active integrins) relative to an antibody that binds integrins irrespective of their conformation (Mould et al., 1995). The integrin activation index of Y783A CD cells was decreased by about 75% and 50% on collagen I and Ln-511, respectively. By contrast, Y783A cell proliferation was reduced by only \( \sim 25\% \) on each of these substrates (Fig. 4G). Thus, the Y783A mutation did not alter CD cell morphology under normal culture conditions but CD cells are unable to undergo 3-D tubulogenesis. This was primarily caused by decreased cell adhesion and migration, and to a lesser extent by reduced proliferation on \( \beta_1 \) integrin-dependent substrates.
adhesion on Ln-511 were assessed, there was no difference in FAK (Fig. 5B,C) or Erk (Fig. 5D,E) activation between the cells, although there was a significant decrease in Akt (Fig. 5F,G) activation in the Y783A mutants. Thus, the Y783A mutation decreased β1 integrin binding to the affinity-dependent 12G10 antibody and adhesion-dependent Akt activation, but did not alter FAK and Erk signaling.

Gial cell line-derived neurotrophic factor (GDNF) is crucial for the initiation of UB development and fibroblast growth factors (FGFs) play a major role in later UB branching morphogenesis (Dressler, 2006). As downstream signaling of both these factors in CD cells is dependent on integrin β1 (Zhang et al., 2009), we investigated whether this is reliant on the membrane proximal NPxY motif. We treated wild-type or Y783A CD cells adherent on laminin-511 with either GDNF (Fig. 5H-M) or FGF10 (Fig. 5N-S) and determined their signaling responses over time. In wild-type cells, FAK (Fig. 5H,I,N,O), Akt (Fig. 5J,K,P,Q) and Erk (Fig. 5L,M and Fig. 4R,S) phosphorylation was strongly induced by GDNF and FGF-10. Peak activation was at 15 min and was sustained to 45 min for FAK, but diminished over time for Akt and Erk. When the Y783A CD cells were stimulated with GDNF or FGF10, FAK was stimulated and sustained to the same level as in the wild-type CD cells (Fig. 5H,I,N,O). By contrast, the level and duration of Akt activation was significantly decreased in the Y783A CD cells after either GDNF or FGF10 stimulation (Fig. 5J,K,P,Q). Following GDNF stimulation, Erk activation was significantly less in the Y783A CD cells than in wild-type cells at all time points measured (Fig. 5L,M,R,S). Erk activation was similar in both Y783A and wild-type CD cells at 15 min following FGF10 stimulation; however, it was not sustained in the Y783A CD cells (Fig. 5L,M,R,S). These data indicate that the membrane proximal NPxY motif of the β1 integrin modulates the response of Akt and Erk, but not FAK signaling, in CD cells in response to GDNF and FGF10.

The membrane proximal NPxY motif is the principal talin-binding site of the integrin β1 cytoplasmic tail

The relatively mild phenotype resulting from the Y783A mutation was somewhat unexpected as this mutation is predicted to prevent interactions between integrin β1 and talins, which are crucial for integrin function (Calderwood et al., 1999; Monkley et al., 2000; Nieswandt et al., 2007; Petrich et al., 2007a,b). An explanation for this result is that sufficient interactions between talins and integrins occur for function. We therefore used nuclear magnetic resonance (NMR), a highly sensitive method that can define the affinities of protein binding in a phospholipid bilayer, to test how efficiently this result is that sufficient interactions between talins and integrins occur for function. We therefore used nuclear magnetic resonance (NMR), a highly sensitive method that can define the affinities of protein binding in a phospholipid bilayer, to test how efficiently

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**Fig. 4. Y783A mutations in the β1-integrin tail alter integrin-dependent cell functions.** (A,B) Wild-type and Y783A mutant CD cells grown on plastic in the presence of 10% FBS, stained with rhodamine-phalloidin and visualized by immunofluorescent microscopy. Scale bars: 50 μm. (C,D) Wild-type and Y783A mutant CD cells placed in 3D collagen I/Matrigel gels for 7 days in the presence of 5% FBS, stained with rhodamine-phalloidin and visualized by confocal microscopy. Scale bars: 50 μm. (E) CD cell populations were allowed to adhere to collagen I (Col-1) or laminin 511 (Ln-511) for 1 h. Data are mean±s.d. of three experiments in triplicate. *P<0.05 (between wild type and mutant). (F) CD cells were plated on transwells coated with Col-1 or Ln-511, and migration, measured as cells per high powered field (hpf), was evaluated after 4 h. Data are mean±s.d. of three experiments in triplicate. *P<0.05 (between wild type and mutant). (G) CD cell populations were plated on collagen I (Col-1) or laminin 511 (Ln-511). After 24 h, the cells were treated with [3H] thymidine and incubated for a further 24 h, [3H] thymidine incorporation (in cpm) was then determined. Data are mean±s.d. of three experiments in triplicate. *P<0.05 (between wild type and Y783A integrins).
Based on studies showing that the full talin 1 head domain (F0-F3) is needed for integrin β1 activation (Bouaouina et al., 2008), we examined binding of this domain (1-433 amino acids, 55 kDa) to the combined transmembrane/cytoplasmic domain (TM/CTD) of integrin β1 subunit in phospholipid bicelles (Fig. 6). Phospholipid bicelles provide an ideal model membrane that maintains the integrin TM domain in solution and ensures its native-like orientation relative to the attached cytosolic domain. Binding of the talin F0-F3 domain to the β1TM/CTD results in gradual peak disappearance, characteristic of binding kinetics that are in the slow exchange NMR time scale regime (Fig. 6). The fact that binding appears to saturate when the F0-F3 domain:β1TM/CTD ratio exceeds 1:3 suggests that β1 in bicelles is homotrimeric (Li et al., 2001) and it is only the first binding event in the process of 3:3 complex formation that is spectroscopically detectable. The fact that a new set of peaks does not appear upon complex formation suggests that the complex formed between the homotrimeric integrin β1 in bicelles and a single subunit of the F0-F3 domain is so large that its NMR resonances are undetectably broad. Plots of reductions in peak intensities from β1 integrin Y783, V790 and V791 as a function of talin concentration were therefore fitted by a model for single-site binding (based on the assumption that one F0-F3 domain binds one integrin homotrimer) (Fig. 6B). The results indicate that K_d for the binding of F0-F3 to β1 TM/CTD is 1.9±1.2 μM (Fig. 6B). The binding affinity of the talin F0-F3 domain to the β1 Y783A mutant was greatly reduced. K_d could not be determined because binding was not saturated at the highest level of F0-F3 tested (Fig. 6C and Fig. S1), indicating that K_d>100 μM. This indicates that Y783 in the proximal NPxY motif of integrin β1 is critically involved in the association between these proteins in membrane-mimicking phospholipid bicelles, and there is virtually no interaction between talin and the Y783A mutant β1 cytoplasmic tail.
Talin is crucial for kidney development

Our in vivo and in vitro data indicate that disrupting β1 binding to talins causes a moderate renal collecting system developmental phenotype. We therefore tested the hypothesis that ablating talins would phenocopy the Y783A mutation (Morse et al., 2014). Talins 1 and 2 are closely related, compensate for each other (Calderwood et al., 2013) and are highly expressed in the mouse collecting system (Praekelt et al., 2012). We deleted both isoforms in the UB by crossing the talin 1flox/flox mouse (Conti et al., 2008) with the constitutive talin 2-null mouse (Debrand et al., 2012). These double homozygote mice were crossed with the hoxB7-cre mouse to delete talin 1 specifically in the UB. In contrast to the wild-type mice (data not shown), the talin 1/2 KO cells were round, never adhered to plastic and did not develop stress fibers, even when grown in the presence of 10% serum (Fig. 8A,B). Talin 1flox/flox/talin 2-null mouse (Debrand et al., 2012) was characterized and shown to have consistent cell behavior.

To determine the cause of the agenesis in the talin mutants, we performed in situ hybridization on isolated urogenital systems at E11 and 12.5 with a probe for Ret. Although a T-shaped UB structure was visible in the E11 wild-type urogenital system, the Talin mutants contained an unbranched UB (Fig. 7B-C). At E12.5, although the UB had branched several times in the wild-type mice, the mutant UB was halted at a T-shaped stage in all mutants assessed (Fig. 7D,E, n=4). The halted development was confirmed when the UB of E12.5 kidneys was stained with E-cadherin and confocal microscopy on whole mounts was performed (Fig. 7F-G) (Movies 3 and 4). This result suggests that talins are required for early UB branching that occurs soon after induction from the Wolffian duct.

Double talin-null CD cells have a major adhesion defect

To define the mechanisms underlying this unexpected phenotype, we derived CD cells from the talin 1flox/flox mouse and deleted the talin 1 gene in vitro using adeno-Cre virus to produce talin 1/2 knockout (KO) CD cells. At least three clones were characterized and shown to have consistent cell behavior. Talin 1flox/flox/talin 2 null CD (wild-type) cells were used as controls as they behaved phenotypically like CD cells isolated from wild-type mice (data not shown). The talin 1/2 KO cells were round, never adhered to plastic and did not develop stress fibers, even when grown in the presence of 10% serum (Fig. 8A,B). Talin 1/2 knockout CD cells placed in 3D collagen-I/MG gels were unable to develop tubules and formed clumps of rounded cells that proliferated slowly (Fig. 8C,D). Expression of β1, α1, α2, α3 and α6 integrin subunits in talin 1/2 KO and wild-type CD cells were similar (data not shown). The severe adhesion defect precluded adhesion and migration assays on 2D substrates, but we could measure the β1 integrin activation index, which was about 25% of the control cells (Fig. 8E). We confirmed a severe proliferation defect in the talin 1/2 KO CD cells grown on LM-511 in the presence of 1% serum (Fig. 8F). Thus, deleting talin 1 and 2 in CD cells causes a severe defect in the ability of β1 integrins to bind ligand, as well as adhesion, spreading and proliferation abnormalities resulting in their inability to undergo tubulogenesis.

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Talin-null CD cells have a major polarity defect

The phenotypical abnormalities of the talin1/2 KO CD cells suggested they have polarity abnormalities. When we grew wild-type and talin1/2 KO cells on transwells, the wild-type CD cells develop a polarized monolayer, whereas talin1/2 KO CD cells grew as non-confluent multilayered cell populations as seen in z-stacks and cross-sections of CD cells stained with rhodamine phalloidin (Fig. 9A-D). The amount of the tight junction protein ZO-1 was significantly reduced in the talin1/2 KO CD cells (Fig. 9M) and it was found in punctate regions rather than the expected cell membrane staining (Fig. 9E-F). Similarly, there was much less E-cadherin, which was also not localized to the cell membrane (Fig. 9G-H). The talin 1/2 null CD cells were stained with E-cadherin and visualized by confocal microscopy. Scale bars: 100 μm. At least three embryos of each phenotype were investigated with similar results.

DISCUSSION

 Binding of the talin head to the membrane proximal NPxY motif of the β1 integrin tail regulates integrin activation and function (Calderwood et al., 1999; Ginsberg, 2014; Moser et al., 2009; Nieswandt et al., 2007; Petrich et al., 2007a; Ye et al., 2014). In this study, we directly investigated the importance of the talin-β1 integrin NPxY motif interaction in UB development and CD duct cell tubulogenesis. We showed that introducing a β1 integrin Y783A mutation into the UB in mice and CD cells resulted in moderate UB branching morphogenesis and tubulogenesis defects, which were much less severe than when β1 integrin was deleted (Wu et al., 2009; Zhang et al., 2009). The Y783A mutation was shown to reduce the affinity for the talin 1 head by a factor of at least 50. However, co-deletion of the talins from the developing UB resulted in total absence of kidneys due to a major branching morphogenesis defect apparent at E12.5. In addition to severe abnormalities of integrin-dependent functions, talin null CD cells were unable to polarize normally and the actin cytoskeleton was malformed. Thus, we conclude that talins are essential for kidney CD development via mechanisms that are both dependent and independent of their ability to bind to the β1 integrin subunit NPxY.

We have previously shown that deleting β1 integrin in the developing UB caused fatal hypoplastic dysplastic kidneys due to decreased UB branching morphogenesis and nephron formation.
Defects in the Y783A CD cells were also less severe than in epithelium (Marose et al., 2008; Yu et al., 2002; Zhao et al., 2002). Our results are also consistent with those of Mathew et al. (2012b), who showed that YY/AA CD cells have a milder phenotype than their YY/AA counterparts, and our two previous studies. This strategy helps justify the validity of our conclusion that the difference in the phenotypes is due to distinct functions of the β1 integrin in the YY/AA CD cells, in every aspect examined (Mathew et al., 2012b). Thus, the membrane-proximal and -distal NPxY motifs differentially regulate UB development and CD cell function and signaling.

The progressively less severe effects of β1 integrin deletion, the YY/AA and the Y783A mutations in UB development, and CD cell tubulogenesis contrasts with other cell types. Integrin β1-null mice, as well as mice with β1 Y783A and YY/AA mutations constitutively knocked in, die at the pre-implantation stage (Meves et al., 2013), and their embryonic stem cells and fibroblasts have similar integrin activation and adhesion defects. By contrast, β1 Y783A expression in keratinocytes causes a milder phenotype than that observed in mice with either the β1 integrin gene deletion or the YY/AA substitutions (Meves et al., 2013). A Y/A mutation in the integrin β3 membrane proximal NPxY motif caused significantly less bleeding than that detected in integrin β3-null animals (Petrich et al., 2007b), although this mutation results in the absolute inability of integrin αIIbβ3 to bind fibrinogen (Petrich et al., 2007b).

The phenotype of the talin 1/2-null mice and CD cells was more severe than those detected in both the YY/AA (Mathew et al., 2012b) and β1 null (Zhang et al., 2009) mice and CD cells, suggesting that the defects caused by talin deletion are integrin β1 dependent and independent. Our data differ from mice where platelet talin 1 (the only talin expressed in platelets) was deleted (Nieswandt et al., 2007; Petrich et al., 2007b), or where mutations abrogating talin-β3 binding (Y747A and L746A corresponding to Y783A in the β1 subunit) were introduced (Petrich et al., 2007b). Fibrinogen-binding ability was affected equally in platelets from both sets of mice, although the bleeding phenotype was worse in the talin 1-null mice (Petrich et al., 2007a,b). A moderate adhesion and spreading defect was present in talin 1-null platelets on collagen 1, although platelet morphology was normal (Nieswandt et al., 2007; Petrich et al., 2007b). While the equivalent fibrinogen-binding defects in platelets from both the talin 1-null and Y747A and L746A mice were ascribed to abnormal talin 1-mediated β3 integrin function, the worse bleeding in the talin 1-null mice was proposed to be from altered activation/function of both β1 and β3 integrins. Another explanation proposed was that residual talin binding to integrin was still present despite the presence of disrupting mutations in the membrane proximal NPxY mutation (Nieswandt et al., 2007). It is possible the UB defect in the Y783A mice is less severe than the talin 1/2-null phenotype because it expresses other non-β1 integrins. However, no UB developmental abnormalities are described in integrin αV-null mice and the β1-null UB and CD phenotype (Zhang et al., 2009) was significantly less severe than in the talin 1/2 null mice and CD cells. Furthermore, our NMR data indicates an at least 50-fold reduction of affinity in the Y783A integrin versus the wildtype.

We later showed that YY/AA mutations in the membrane-proximal and -distal NPxY motifs caused both a moderately severe UB branching defect, intratubular cell proliferation and end-stage tubulointerstitial fibrosis of the kidney (Mathew et al., 2012b). The Y783A mutation described in this study results in a less serious phenotype than the YY/AA mutant with severe UB branching defect, intratubular cell proliferation and end-stage tubulointerstitial fibrosis of the kidney (Zhang et al., 2009). We later showed that YY/AA mutations in UB development, and CD cell function and signaling.

The progressively less severe effects of β1 integrin deletion, the YY/AA and the Y783A mutations in UB development, and CD cell tubulogenesis contrasts with other cell types. Integrin β1-null mice, as well as mice with β1 Y783A and YY/AA mutations constitutively knocked in, die at the pre-implantation stage (Meves et al., 2013), and their embryonic stem cells and fibroblasts have similar integrin activation and adhesion defects. By contrast, β1 Y783A expression in keratinocytes causes a milder phenotype than that observed in mice with either the β1 integrin gene deletion or the YY/AA substitutions (Meves et al., 2013). A Y/A mutation in the integrin β3 membrane proximal NPxY motif caused significantly less bleeding than that detected in integrin β3-null animals (Petrich et al., 2007b), although this mutation results in the absolute inability of integrin αIIbβ3 to bind fibrinogen (Petrich et al., 2007b).

The phenotype of the talin 1/2-null mice and CD cells was more severe than those detected in both the YY/AA (Mathew et al., 2012b) and β1 null (Zhang et al., 2009) mice and CD cells, suggesting that the defects caused by talin deletion are integrin β1 dependent and independent. Our data differ from mice where platelet talin 1 (the only talin expressed in platelets) was deleted (Nieswandt et al., 2007; Petrich et al., 2007b), or where mutations abrogating talin-β3 binding (Y747A and L746A corresponding to Y783A in the β1 subunit) were introduced (Petrich et al., 2007b). Fibrinogen-binding ability was affected equally in platelets from both sets of mice, although the bleeding phenotype was worse in the talin 1-null mice (Petrich et al., 2007a,b). A moderate adhesion and spreading defect was present in talin 1-null platelets on collagen 1, although platelet morphology was normal (Nieswandt et al., 2007; Petrich et al., 2007b). While the equivalent fibrinogen-binding defects in platelets from both the talin 1-null and Y747A and L746A mice were ascribed to abnormal talin 1-mediated β3 integrin function, the worse bleeding in the talin 1-null mice was proposed to be from altered activation/function of both β1 and β3 integrins. Another explanation proposed was that residual talin binding to integrin was still present despite the presence of disrupting mutations in the membrane proximal NPxY mutation (Nieswandt et al., 2007). It is possible the UB defect in the Y783A mice is less severe than the talin 1/2-null phenotype because it expresses other non-β1 integrins. However, no UB developmental abnormalities are described in integrin αV-null mice and the β1-null UB and CD phenotype (Zhang et al., 2009) was significantly less severe than in the talin 1/2 null mice and CD cells. Furthermore, our NMR data indicates an at least 50-fold reduction of affinity in the Y783A integrin versus the wildtype.

Our data suggest that the severe defect in UB development of the talin 1/2 null mouse is not due solely to its inability to activate integrins. This is consistent with studies where mice lacking talin 1 in podocytes died of kidney failure, and talin 1 depletion in podocytes caused a modest reduction in β1 integrin activation, podocyte adhesion and spreading that were accompanied by abnormalities in the actin cytoskeleton (Tian et al., 2014). Similarly, mice lacking both talin isoforms in muscle have a severe cardiomyopathy and die soon after birth, a defect that was not due to the inability of the muscle cells to activate integrins (Conti et al., 2008, 2009; Manso et al., 2013). Thus, unlike β3 integrins, which require talin for their activation and function, it appears that talin exerts many of its functions by mechanisms that are...
downregulation of talin 1 with siRNAs did not compromise
by siRNA (Tian et al., 2014), it contrasted with the persistent ability
were similar to that of podocytes in which talin 1 was downregulated
cytoskeleton alterations observed in the talin 1/2 null CD cells
and ZO-1 helps with the recruitment of actomyosin regulators, such
required to control PTEN protein expression (Subauste et al., 2005);
talin 1-talin head interactions required for integrins to bind their
β
1 integrins.

Generation of integrin β1 cell lines
β1-null collecting-duct (CD) cells described previously (Zhang et al., 2009)
were transfected with either full-length human integrin β1 (WT) or β1 integrins carrying the Y783A mutation. Equal surface expression of the β1 integrin subunits was obtained by flow cytometry sorting using the antibody ALIB2. The talin 1- and talin 2-null cells were isolated from the CD5- to 6-week-old talin 1β (β TS mouse/talin 2-null mice and immortalized with

Cell adhesion, migration, proliferation and tubule formation
Laminin-511 was made as previously described (Yazlovitskaya et al., 2015)
rat tail collagen I was purchased from Sigma-Aldrich. Cell adhesion,
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and harvested 0, 30, 45 and CD cells were seeded onto laminin 511-coated

MATERIALS AND METHODS

Generation of mice
Experiments were approved by the Vanderbilt University Institutional
in the actin cytoskeleton, cell polarity and expression of
and tight junction proteins. These results suggest that
talin 1 antibody (BD Pharmingen, 55531), pAKT (Cell Signaling 9271), AKT (Cell Signaling 9272), pErk (Cell Signaling 9101), ERK (Cell Signaling 9102).

Protein production
Protein purification of the integrin β1 TM/CTD domain and talin head (F0-
were composed of q=0.3 D7PC/POPC/POPS, where q is the lipid (POPC
and rat tail collagen I was purchased from Sigma-Aldrich. Cell adhesion,
Surface expression of the F0-F3 domain was determined using

Nuclear magnetic resonance spectroscopy
Peak assignments in the NMR spectrum of integrin
β
1 subunit. The activation

Cell signaling
CD cells were serum starved for 12 h and plated on LM-511 (0.25 μg/ml),
and harvested 0, 30, 45 and CD cells were seeded onto laminin 511-coated

Morphological and immunohistochemical analysis
Whole mouse embryos or kidneys were evaluated by light microscopy. Kidney
staining and scoring was performed as previously described (Mathew et al.,
were fixed in 4% PFA overnight and subjected to whole-mount immuno-fluorescent staining with primary antibody E-cadherin (Life

4156

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controls talin engagement with the actomyosin machinery. Nat. Commun. 6, 10038.


