Polycystins regulate ezrin function and cleavage to control renal cell and tubular morphology

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Abstract

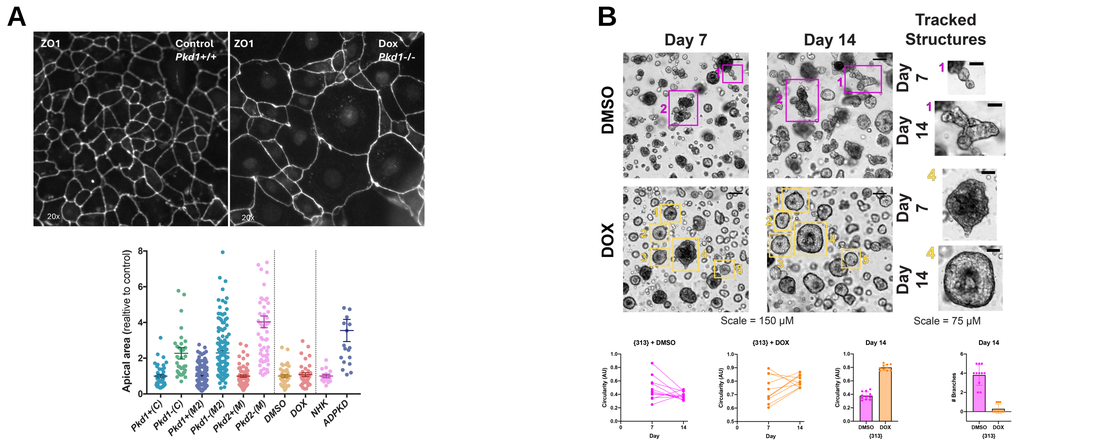
Background: Renal cyst formation in ADPKD requires altered cell and tubular morphology. Previously, we used inducible inactivation of Pkd2 in a 3D-tubuloid model to measure acute changes in tubuloid shape after Pkd2 loss and observed alterations in tubule morphology before changes in overall volume, arguing that morphological changes are among the first consequences of polycystin loss. We hypothesized a direct role for the polycystin complex in regulating the apical cytoskeleton protein, ezrin (EZR), to control cell and tubular morphology.

Methods: We used clonal cell lines derived from the kidneys of Pkd1 or Pkd2 Pax8rtTA TetOCre mice crossed with the SV40 immorto-mouse: Pkd1-iKO (#312) and Pkd2-iKO (#125). Pkd1/2 can be efficiently deleted with addition of Doxycyline (DOX) to the cell media.

Results: We observed a strong correlation between the loss of Pkd1/2 and increased apical cell area, as defined by ZO1, in 2D Pkd1/2-iKO cells. The acute loss of Pkd1/2 also resulted in decreased ezrin abundance and altered localization. Although Pkd1/2 deletion resulted in decreased ezrin we found upregulation of Ezr mRNA in both the DOX treated Pkd2-iKO cells and in human ADPKD cystic tissue, leading us to hypothesize that PC1/2 regulates ezrin protein more directly. Immunoprecipitation experiments of HEK293 cells transfected with ezrin, Myc-PC2 or Flag-PC1 showed both PC1 and PC2 successfully pulled down ezrin suggesting a protein interaction. Interestingly, the pull down efficiency was greater with the full length PC1 than the CTF- PC1 fragment. To confirm an endogenous interaction between ezrin and PC1 we isolated primary renal epithelial cells from a transgenic Pkd1-HA mouse and found again HA-PC1 successfully pulled down ezrin. Mechanistically, ezrin activity is regulated by cystine proteases calpain 1 and 2. We observed a substantial increase in the N-terminal 55KDa ezrin cleavage product after acute Pkd2 loss in the DOX treated Pkd2-iKO cells as well in human APDKD cystic tissue. Chemical inhibition of either calpain 1 or calpain 2 led to increased ezrin cleavage in control cells mimicking Pkd2 loss. However, inhibition of both calpain 1 and 2 reduced ezrin cleavage in DOX treated Pkd2-iKO cells to levels comparable to control cells.

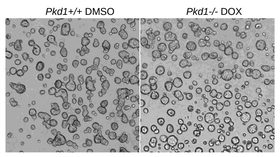
Conclusion: The polycystin proteins regulate ezrin function and cleavage to control renal cell and tubular morphology.

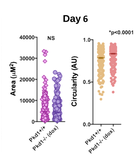
Background: Loss of Polycystin Changes Cell and Tubule Morphology



(A): A similar observation occurs when Pkd2 or Pkd1 is knocked out with DOX treatment in 2D cell culture where Pkd2 or Pkd1 loss results in a significant increase in cell apical area. Increased apical area is also observed in primary ADPKD cells compared to control. **(B)** Kidney tubuloid 3D structures tracked over 14 days treated with DMSO or DOX to knockout Pkd1. Complex structures occur in normal kidney epithelial cells and is lost following Pkd1 loss. DOX treatment leads to a significant increase in structure circularity and a significant decrease in tubuloid branching.

Background: Disruption of the Apical Compartment Occurs Early in Cytogenesis

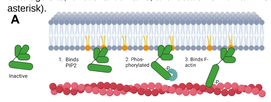


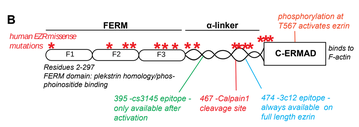


Day six in 3D culture, Pkd1 clone 313 demonstrates loss of Pkd1 does not alter overall size of tubuloid structures; but the circularity is significantly increased in the Pkd1-/- tubuloids suggesting morphology change may precede significant changes in proliferation or fluid secretion.

Ezrin (EZR) activation and topography

(A) Scheme for Ezrin activation. **(B)** Ezrin topography including antibody epitopes, cleavage site, functional domains, and location of human mis-sense variants (red asterisk).





Ezrin is a major regulator of the apical compartment

Hypothesis: Polycystins influence cell and tubular morphology through regulation of ezrin and the apical actin cytoskeleton

1) 3D Tubuloids Lacking Pkd1 or 2 Exhibit Ezrin Mislocalization and Significant Loss of Protein Expression

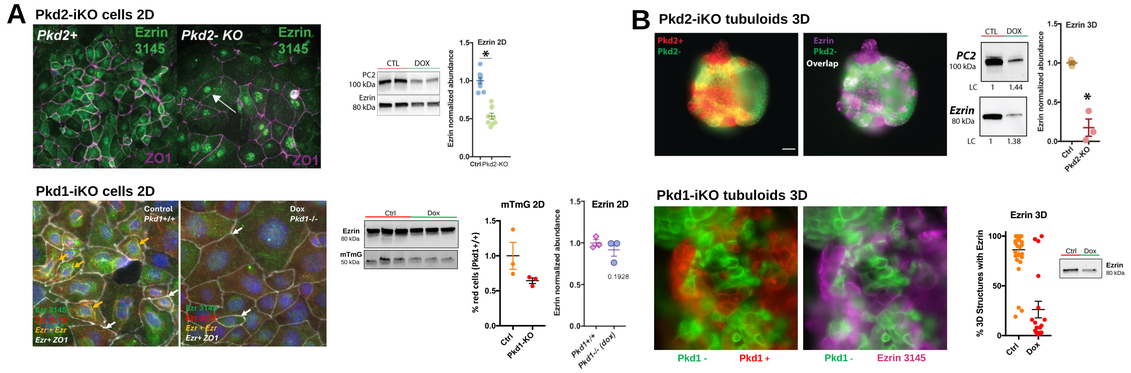
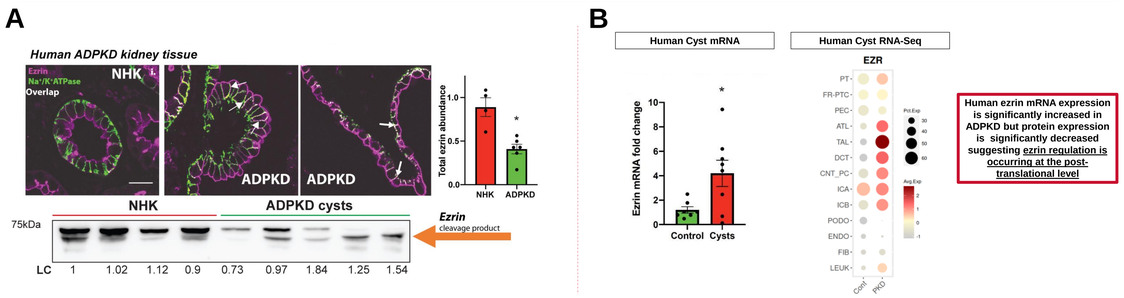


Figure 1: Pkd2-iKO cells treated with/without DOX (10ug/ml) to induce Pkd2 or Pkd1 loss in 2D and 3D. **(A)** Loss of Pkd2 or Pkd1 in 2D causes decreased protein abundance and nuclear translocation of ezrin. **(B)** Tubule fragments from Pkd2 fl/fl;Pax8rtTA;TetOCre;mTmG mice were used to generate 3D tubuloids. Immunofluorescent images of the mTmG reporter (Pkd2 + red; Pkd2 - green) following DOX treatment and stained with ezrin 3145 (purple). Ezrin is completely absent in cells lacking Pkd2 but present in Pkd2+ cells. Western blot analysis of 3D tubuloids reveal a significant decrease in Pkd2 and ezrin protein abundance in Pkd2 -/- cells. **(C)** Tubule fragments from Pkd1 fl/fl;Pax8rtTA;TetOCre;mTmG mice were used to generate 3D tubuloids. Immunofluorescent images of the mTmG reporter (Pkd1 + red; Pkd1 - green) following treatment of DOX and stained with ezrin 3145 (purple). Ezrin is completely absent in cells lacking Pkd1 but present in Pkd1+ cells.

2) Ezrin is Regulated at the Protein Level



Human ezrin mRNA expression is significantly increased in ADPKD but protein expression is significantly decreased suggesting ezrin regulation is occurring at the post-translational level

Figure 2: (A) Human ADPKD tissue displays altered ezrin localization. Ezrin is localized to the apical membrane in normal human kidney (NHK). In cystic tissue ezrin localization extended to the lateral membrane (arrows) and co-localized with the basolateral membrane marker Na+/K+ ATPase indicated in white. Ezrin protein abundance is significantly decrease in ADPKD cysts compared to NHK. ADPKD cysts also have increased ezrin cleavage product (orange arrow) indicative of ezrin inactivation. (NHK; male N=4/ ADPKD; male N=5). **(B)** Ezrin (Ezr) gene expression is significantly increased in ADPKD cystic tissue in the proximal tubule, ascending thin limb and distal tubule segments of the kidney in (Muto et al. 2022).

3) Ezrin Interacts with Full-length Polycystin 1 and Polycystin 2 in Overexpressed HEK and Primary Kidney Cells

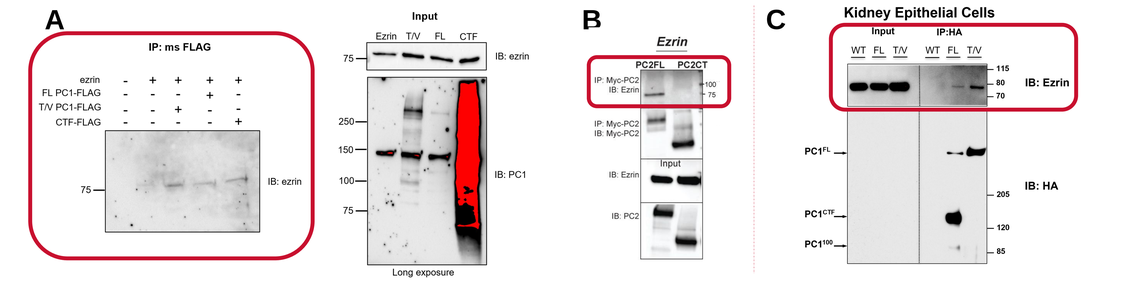


Figure 3: (A) HEK cells were transfected with C-terminal Flag tagged full length PC1, uncleavable GPS domain T/V mutant, or CTF. Cell lysates were immunoprecipitated using a monoclonal Flag antibody and probed with ezrin. Ezrin interacts with both full length PC1 and the CTF. **(B)** HEK cells were transfected with N-terminal Myc tagged full length PC2 or the CTF (final TM domain and C-terminus). Cell lysates were immunoprecipitated using a Myc antibody and probed with ezrin. Ezrin interacts only with the full length version of PC2 and not the CTF region. **(C)** Primary kidney cell lysates were used to immunoprecipitate ezrin with full-length PC1 or the uncleavable GPS domain T/V mutant. Ezrin interacts with full-length PC1 and T/V mutant.

4) Calpain 1 and 2 Mediate Ezrin Cleavage in ADPKD

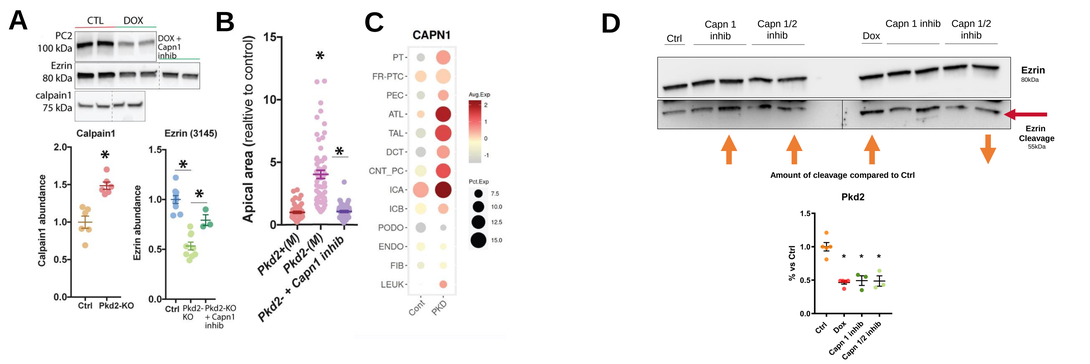


Figure 4: (A) Representative WB from Pkd2-iKO cells treated with/without DOX (10ug/ml) to induce knocked and probed with PC2, ezrin and calpain 1 antibodies. DOX treatment significantly decreases both PC2 and ezrin protein abundance but increases calpain 1. PC2-iKO cells treated with a calpain 1 specific inhibitor (Calpain IV 1.0uM) significantly increases ezrin protein abundance. Ezrin abundance is rescued after calpain IV (calpain 1 inhibitor) treatment. (B) Comparison of apical area delineated by ZO-1 in the same Pkd2-iKO cells treated with calpain inhibitor IV. Inhibition of calpain 1 rescues the increased apical area in Pkd2-iKO cells. (C) CAPN1 gene expression is increased primarily throughout the entire distal nephron in ADPKD kidneys (Muto et al 2022). **(D)** Representative western blot from Pkd2-iKO cells with/without DOX and treated either with calpain inhibitor IV (calpain 1 inhibitor) or with calpain 1 and 2 inhibitor. DOX treatment increases ezrin cleavage indicative of inactivation. Calpain 1 inhibitor has no effect on ezrin cleavage compared to DOX only treatment. Inhibition of both calpain 1 and 2 results in decreased ezrin cleavage and is comparable to control cells. Pkd2 protein abundance is significantly decreased in DOX treated cells compared to control.

Conclusions

- Altered cell and tubule morphology due to polycystin loss precedes proliferative expansion
- Ezrin protein expression and localization are significantly decreased and altered only where polycystin expression is lost
- Immunoprecipitation experiments reveal PC1 and PC2 both interact with ezrin
- Increased ezrin cleavage and decreased protein expression suggest ezrin is regulated at the post-translational level
- Calpains 1 and 2 likely mediate ezrin cleavage and inactivation

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