Role of FGFR2 in bladder urothelial injury

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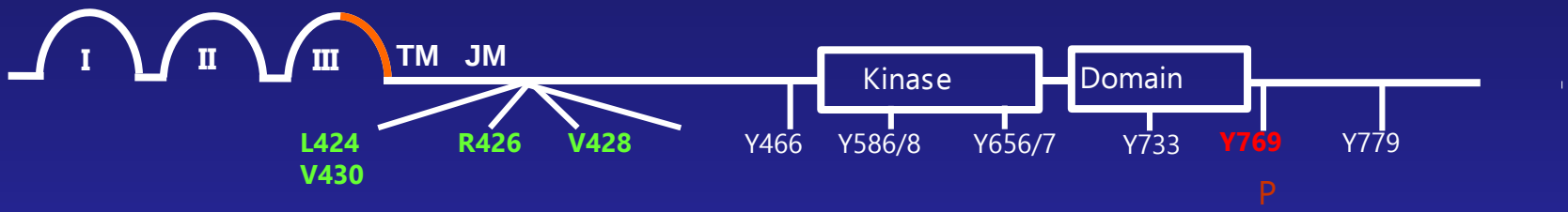
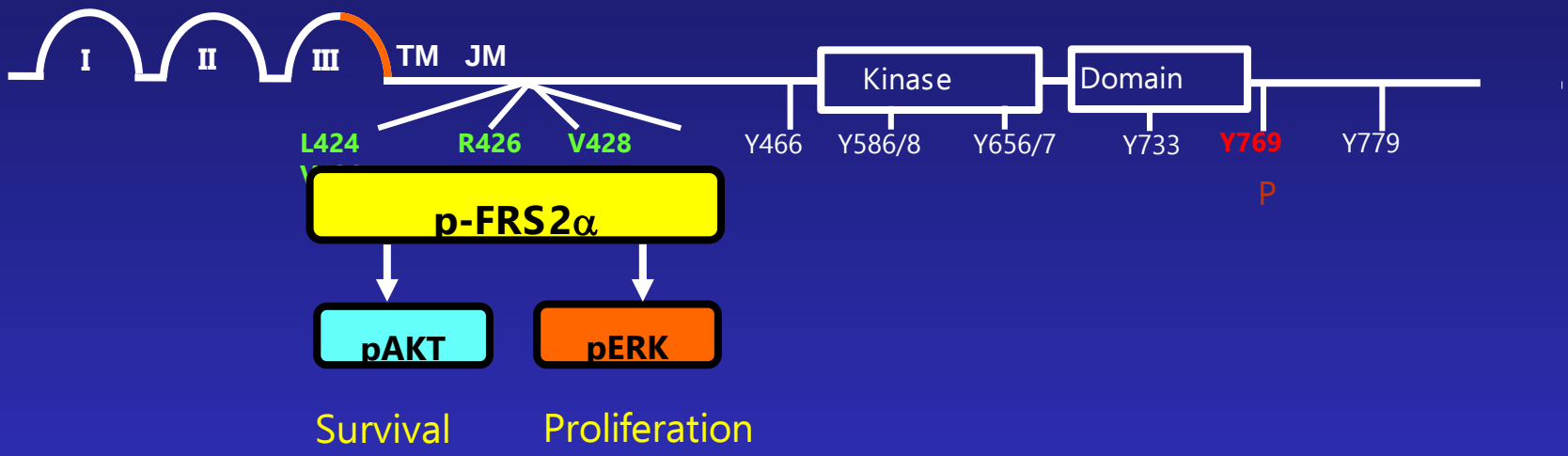
Our major focus:

- Role of fibroblast growth factor receptors (FGFRs) (receptor tyrosine kinases) in the:
  - developing and postnatal kidney
  - more recently in postnatal bladder urothelial injury

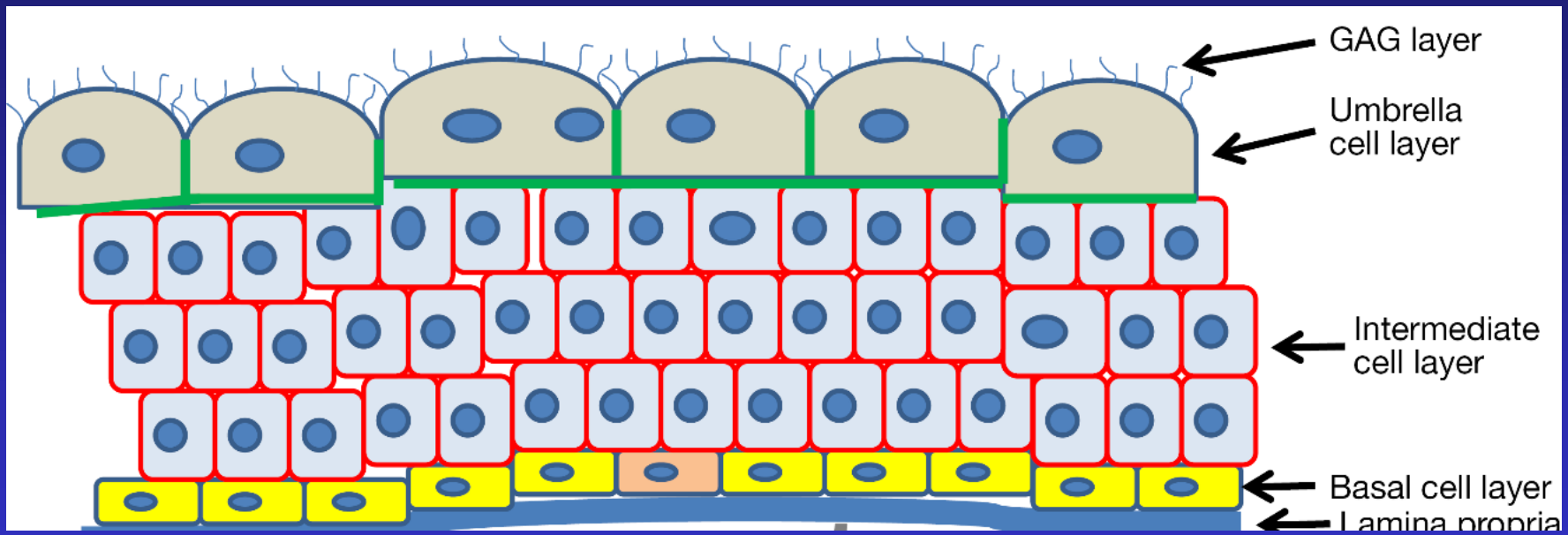
FGFRs signal through binding proteins/adapters



FGFRs signal through docking proteins/adapters



Urothelium



Hurst et al, 2015 Translational Andrology and Urology

Are there roles for FGFR2 in urothelial injury/regeneration?

- We examined Cyclophosphamide (CPP) and radiation - induced injury
- CPP causes necrosis of Superficial cells (2-6 hours) and apoptosis of deeper urothelium (4-24 hours)
  - While much of the repair is done by 28 days, still see regen 6 m
- Radiation induces DNA damage and full thickness urothelial apoptosis
  - Limited data on regen, but known in literature to last months

Clinical problems in the bladder from CPP/radiation

- Hemorrhagic cystitis:
  - Potentially life threatening hemorrhage
  - May lead to chronic fibrosis, contractures, vesicoureteral reflux

- Bladder cancer:
  - Patients given CPP for lymphoma had 4.5 fold increased risk of bladder cancer J Natl Cancer Inst 1995; 87(7): 524-30

Rationale for examining FGFR2 signaling in bladder urothelial injury

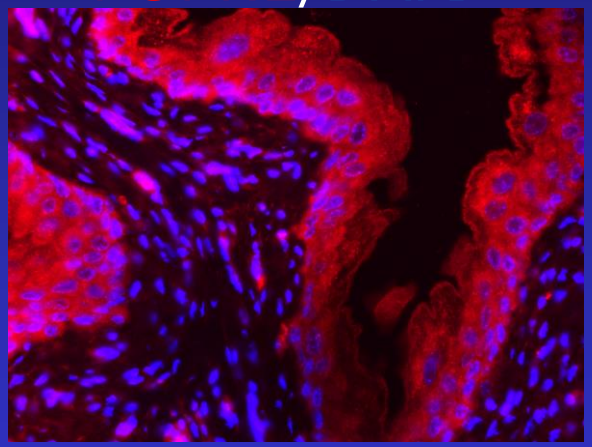
- FGFR2 is highly expressed in bladder urothelium
- Systemic human KGF (FGF7- ligand for FGFR2)
  - Led to a better urothelial structure when given before CPP (unclear if from proliferation/regeneration or reduced injury).

    Cancer research, 1997. 57(3): p. 472-5
- Published abstract reported that inducible loss of *Fgfr2IIIb* isoform led to prolonged injury after CPP

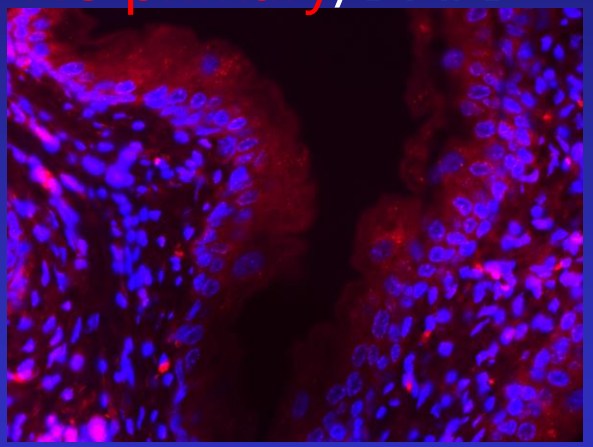
  The Journal of Urology, 2011. 165(4S): p. e547-8

We confirmed that FGFR2 appears to be expressed in all adult bladder urothelial layers

FGFR2/DAPI



No primary/DAPI



Two questions we had about FGFR2 in bladder injury

- What is the role of KGF in ameliorating CPP or radiation- induced bladder injury/driving regeneration?

- What is the role of endogenous FGFR2 signaling in regeneration of bladder urothelium after CPP-injury?

Two questions we had about FGFR2 in bladder injury

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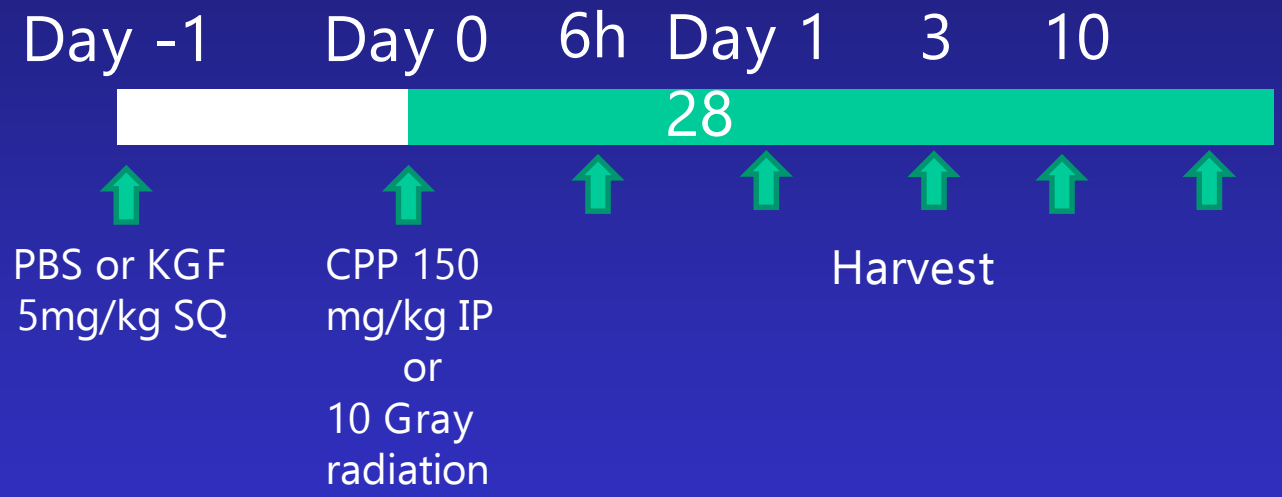
How does KGF administration affect injury?

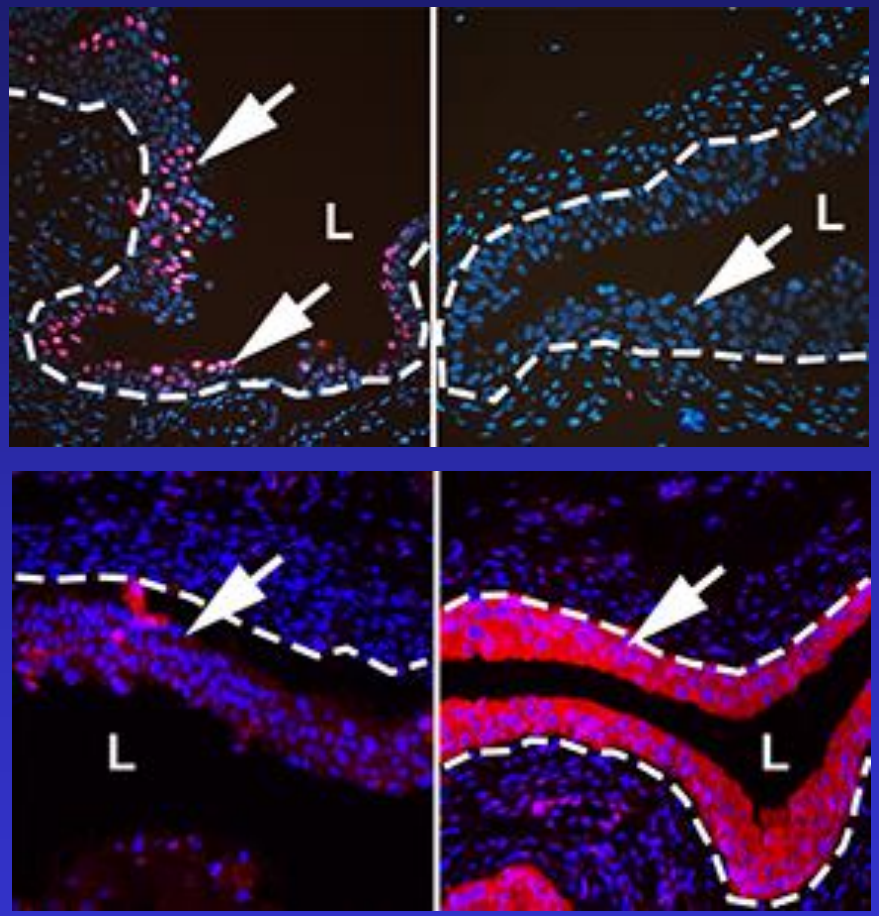


KGF-pretreatment protects deeper cell apoptosis likely via activation of AKT – 24 hours post CPP

PBS KGF

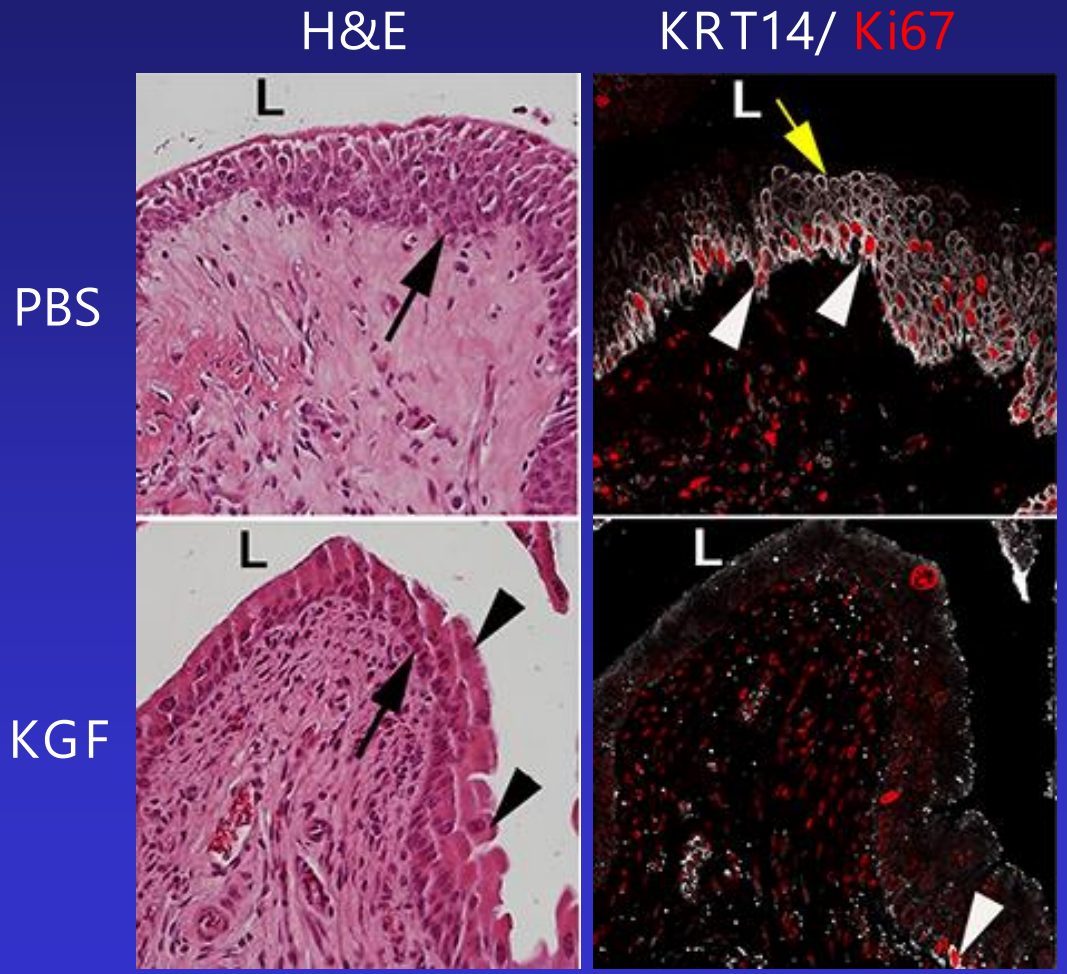
TUNEL/DAPI

pAKT/DAPI

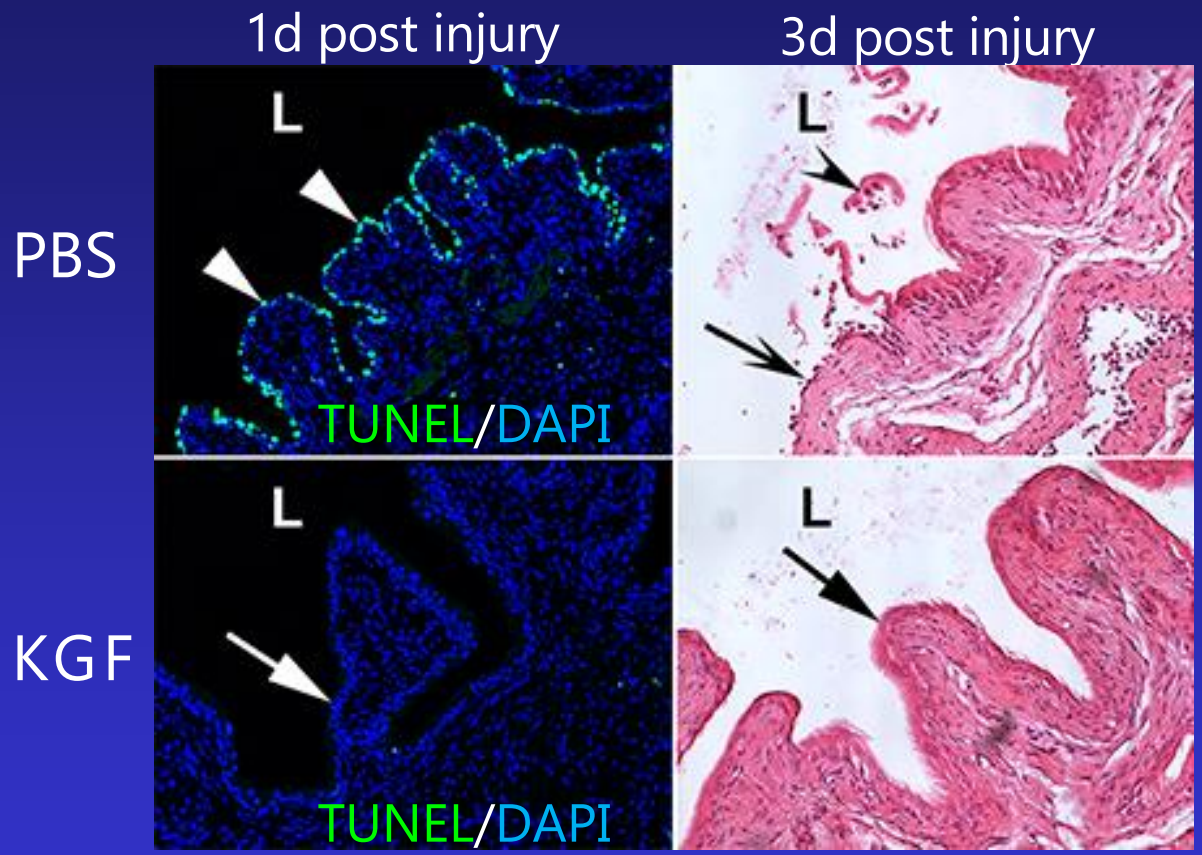


pERK levels were low in both PBS/KGF groups 24 hours after CPP

KGF-pretreatment leads to faster and higher fidelity repair- 10 days post CPP

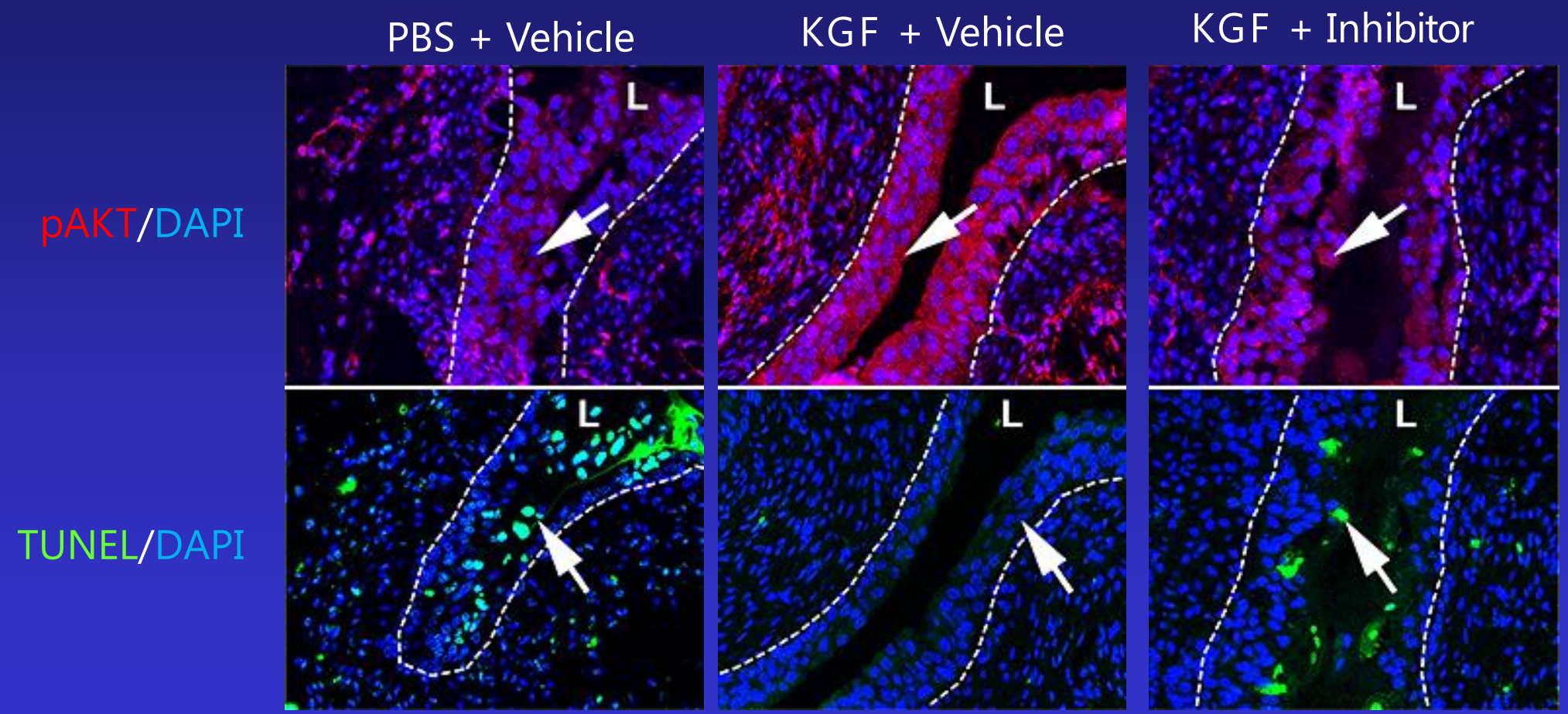


KGF blocks urothelial apoptosis after radiation :

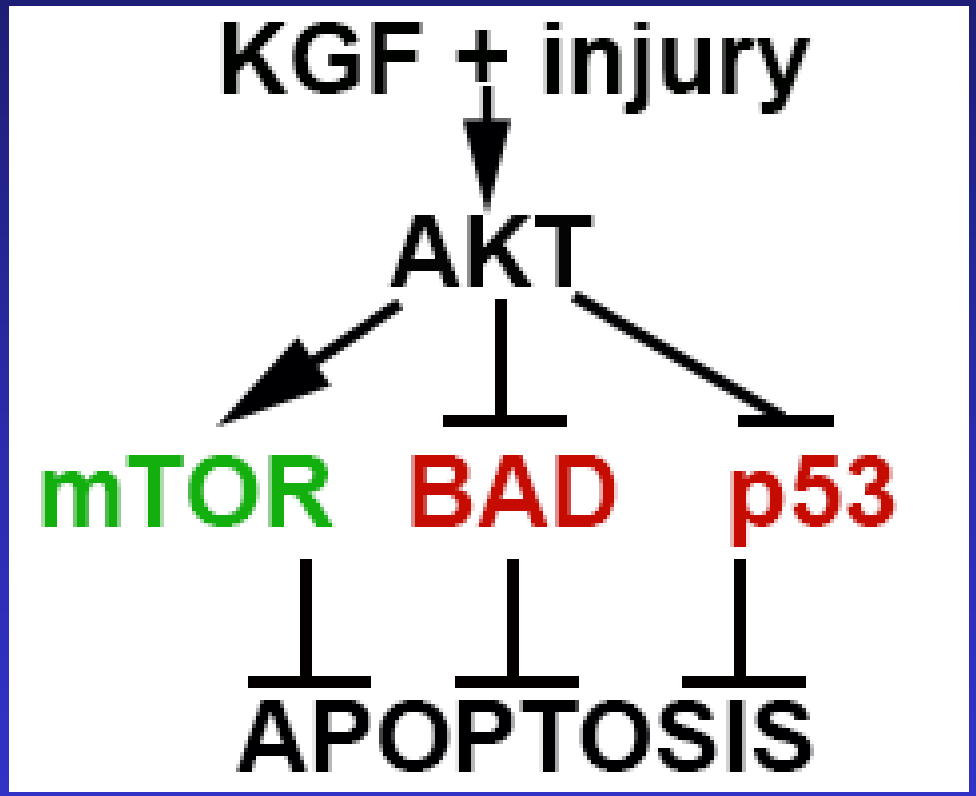


- Appears to be AKT-mediated as with CPP

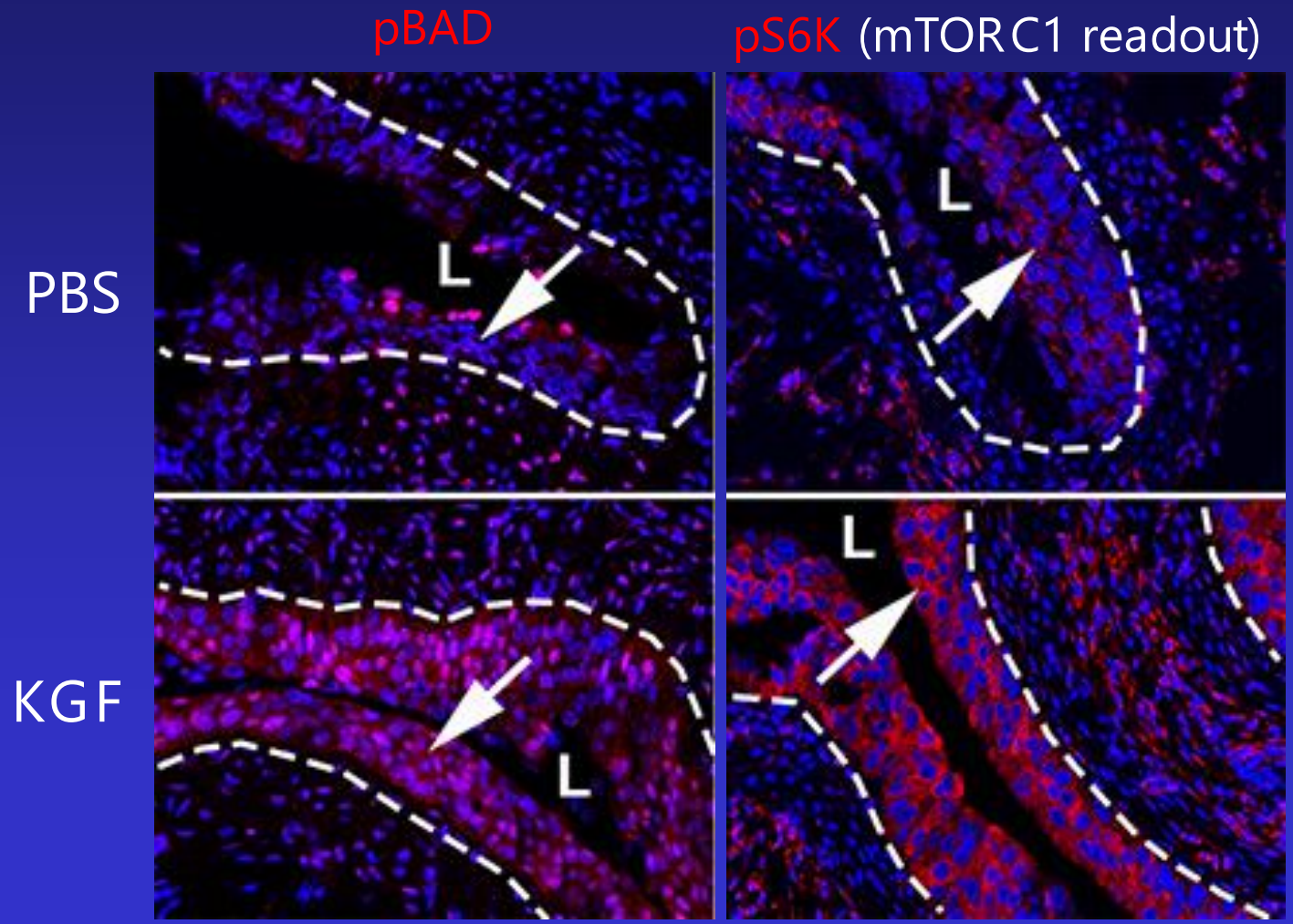
AKT inhibitor partially blocks pAKT and KGF-driven cytoprotection 12 hours post-CPP



How does KGF-AKT block apoptosis?



KGF-AKT drives pBAD staining and mTORC1 activity in CPP injured urothelium.



No changes with p53

KGF-AKT drives pBAD staining and inhibits p53 activity in radiation injured urothelium.

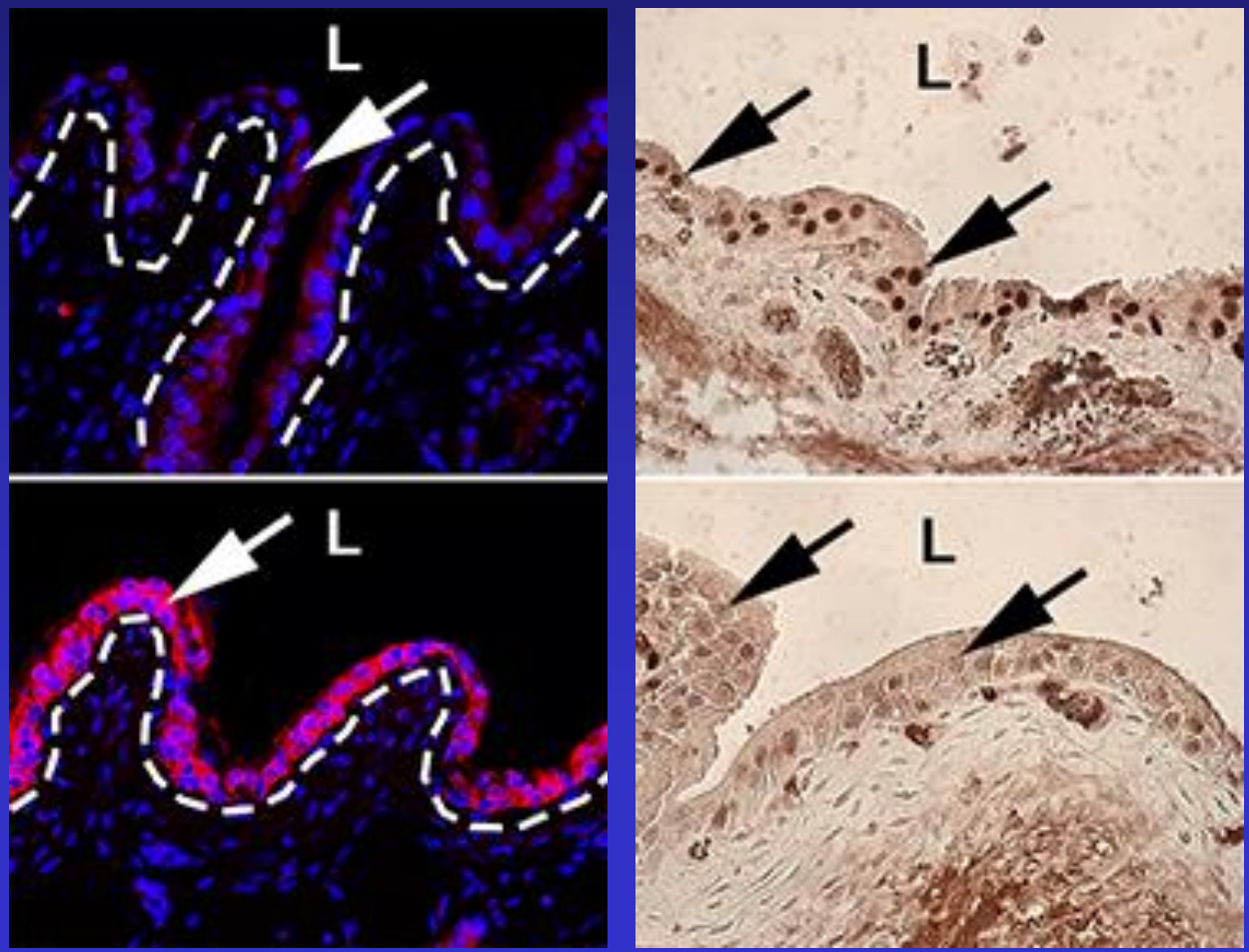
pBAD

p21 (p53 readout)

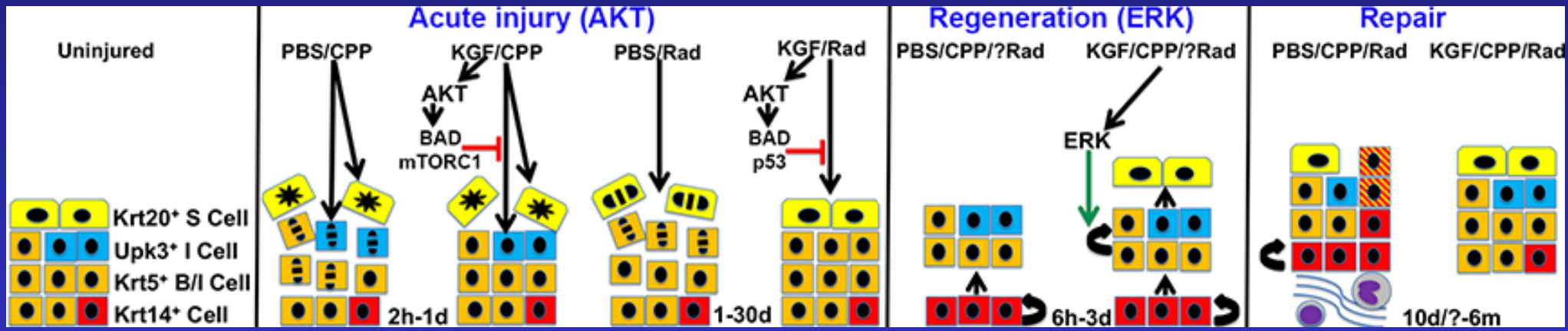
No changes with mTORC1

PBS

KGF



Working model-KGF and bladder injury



KGF also appears to block urothelial apoptosis in a spinal cord injury/neurogenic bladder model

Two questions we had about FGFR2 in bladder injury

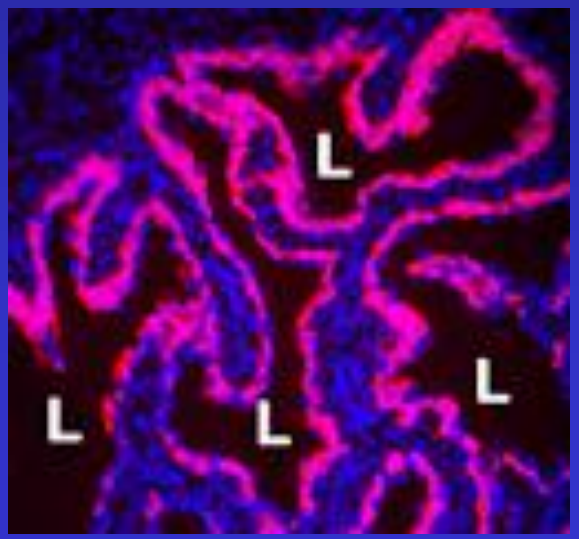
- What is the role of KGF in ameliorating CPP-induced bladder injury/driving regeneration?

- What is the role of endogenous FGFR2 signaling in regeneration of bladder urothelium after CPP-injury?

Role of *Fgfr2* in CPP-injured urothelium?

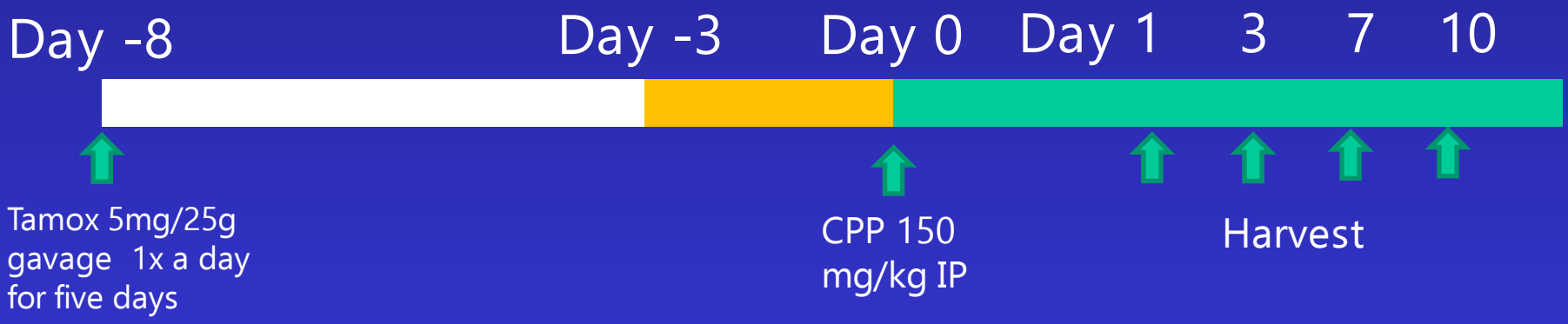
Using a tamoxifen-inducible ShhCre line-bred to a CAG reporter to visualize tdTomato **RFP** expression/**DAPI**

ShhCre *CAG$^{fl/fl}$* *ShhCreCAG$^{fl/+}$*

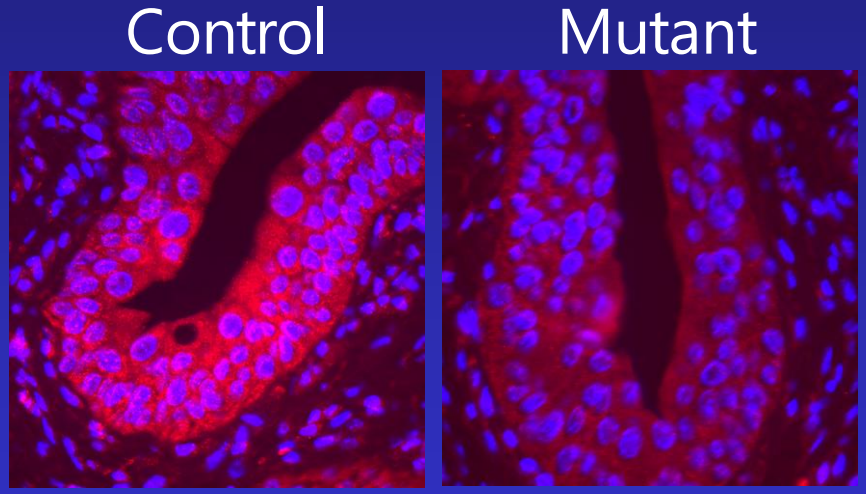


Role of FGFR2 in postnatal Cytoxan-induced urothelial injury?
Breeding Scheme/Experimental Design

ShhCreFgfr2$^{fl/+}$ X *Fgfr2*$^{fl/fl}$ → *ShhCreFgfr2*$^{fl/fl}$



ShhCreFgfr2$^{fl/fl}$ (mutant) mice have significant knockdown of FGFR2 expression in urothelium vs. controls

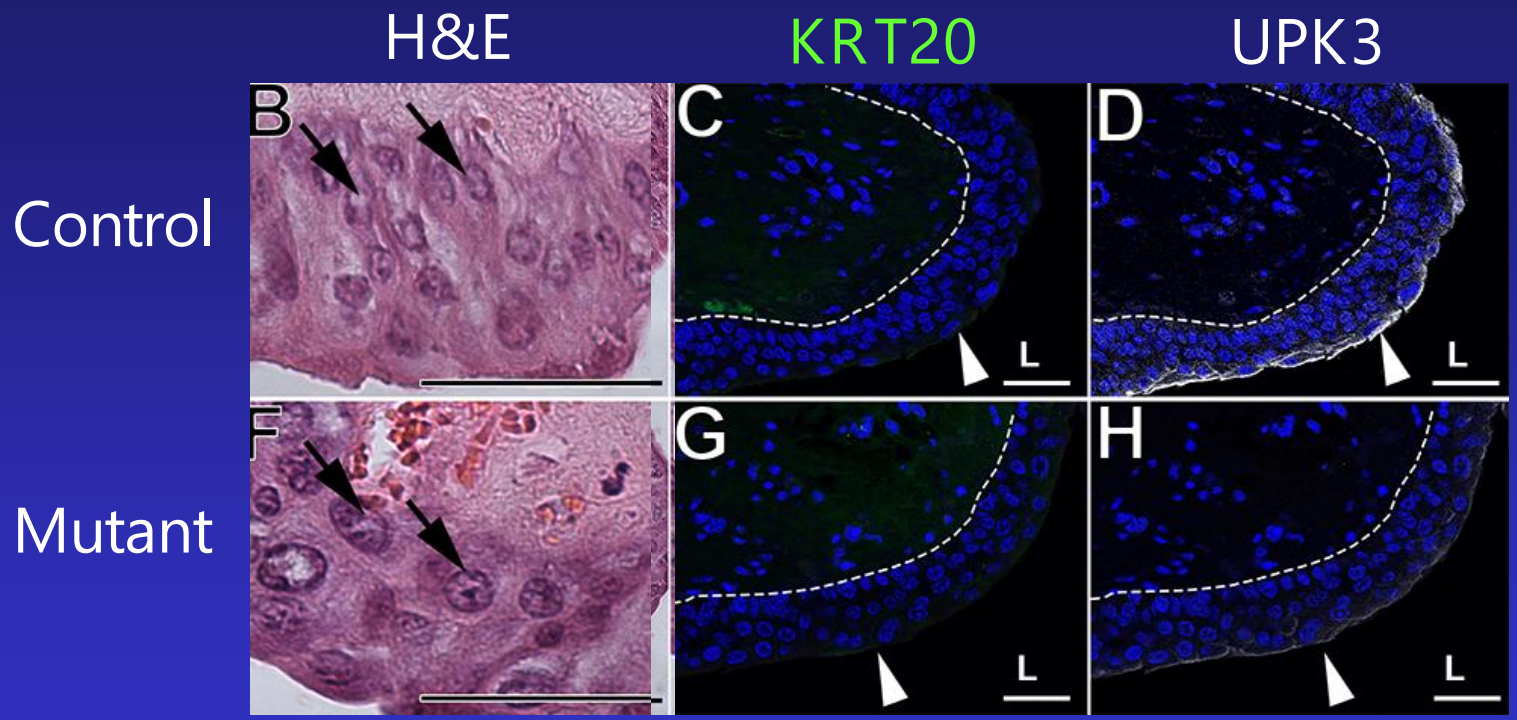


Mutant urothelium appears normal in the absence of CPP

24 hours after CPP:

- Mutants did not appear to be more injured than controls
  - Similar levels of apoptosis, loss of urothelial markers
- Mutants had a similar level of a regenerative response
  - Similar rates of $Ki67^+$ (proliferating) cells, including $KRT14^+$ cells (to our surprise)
  - No excesses in numbers of $\gamma H2AX^+$ cells (marker of DNA damage/replication stress)

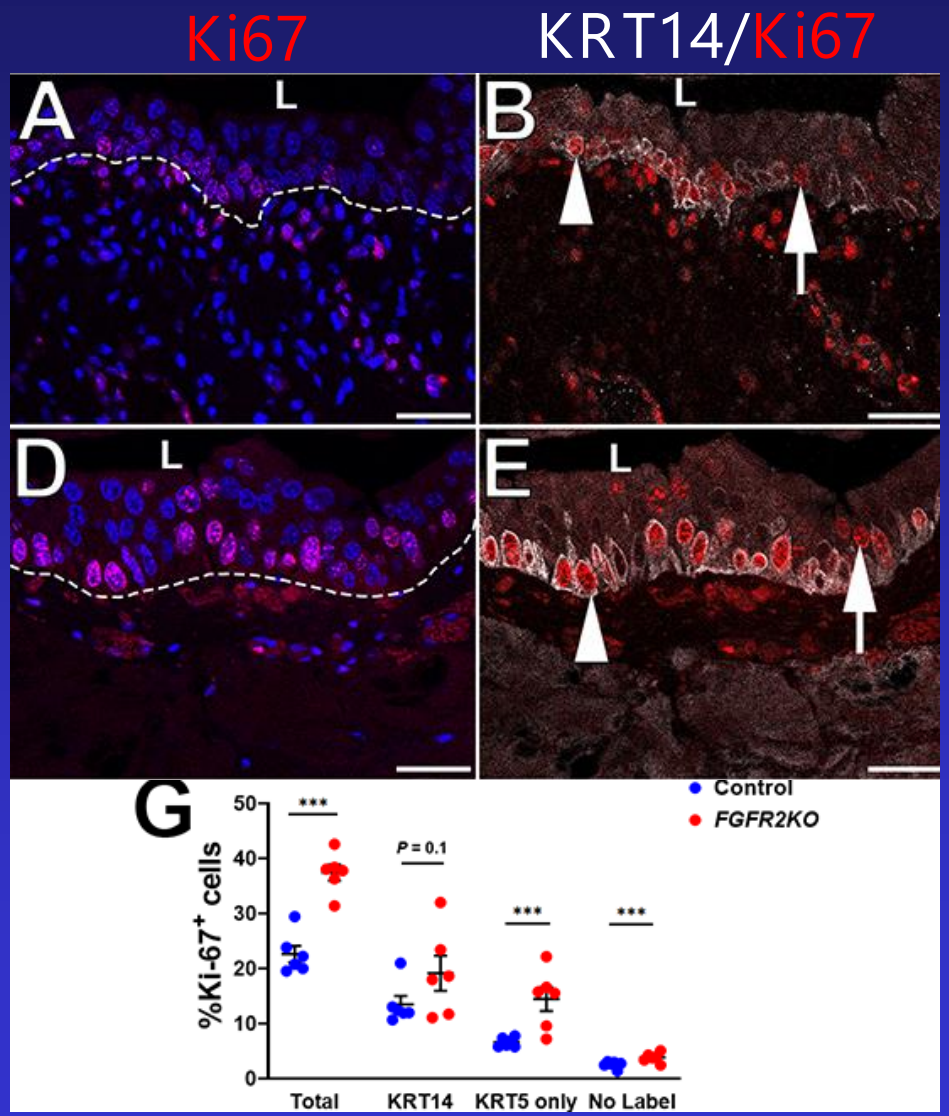
Three days post CPP, mutants have more hemorrhage and impaired UPK3^{+} cell regeneration



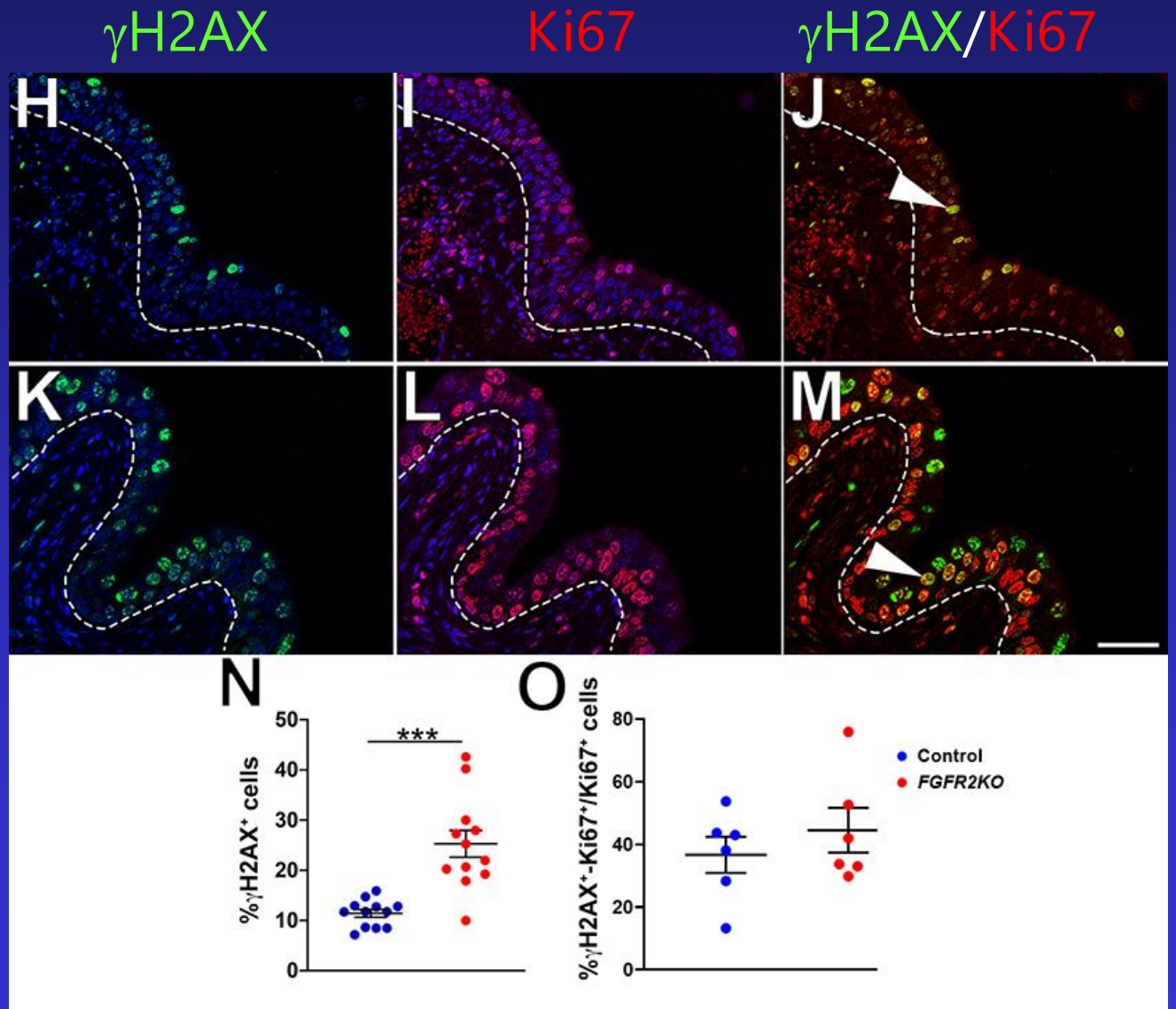
High power H&E images show that mutants have enlarged Basal cells/nuclei and fewer urothelial cell layers

Do the 3-day injured mutants have impaired KRT14^{+} or other urothelial cell proliferation causing the reduction in urothelial cell layer number vs. controls?

Three days post-CPP, mutants actually have ***increased*** numbers of "proliferating" urothelial cells (most of which are larger KRT14$^+$ cells)



Three days post-CPP, mutants have more replication stress/DNA damage than mutants, much of which is in the proliferating cells

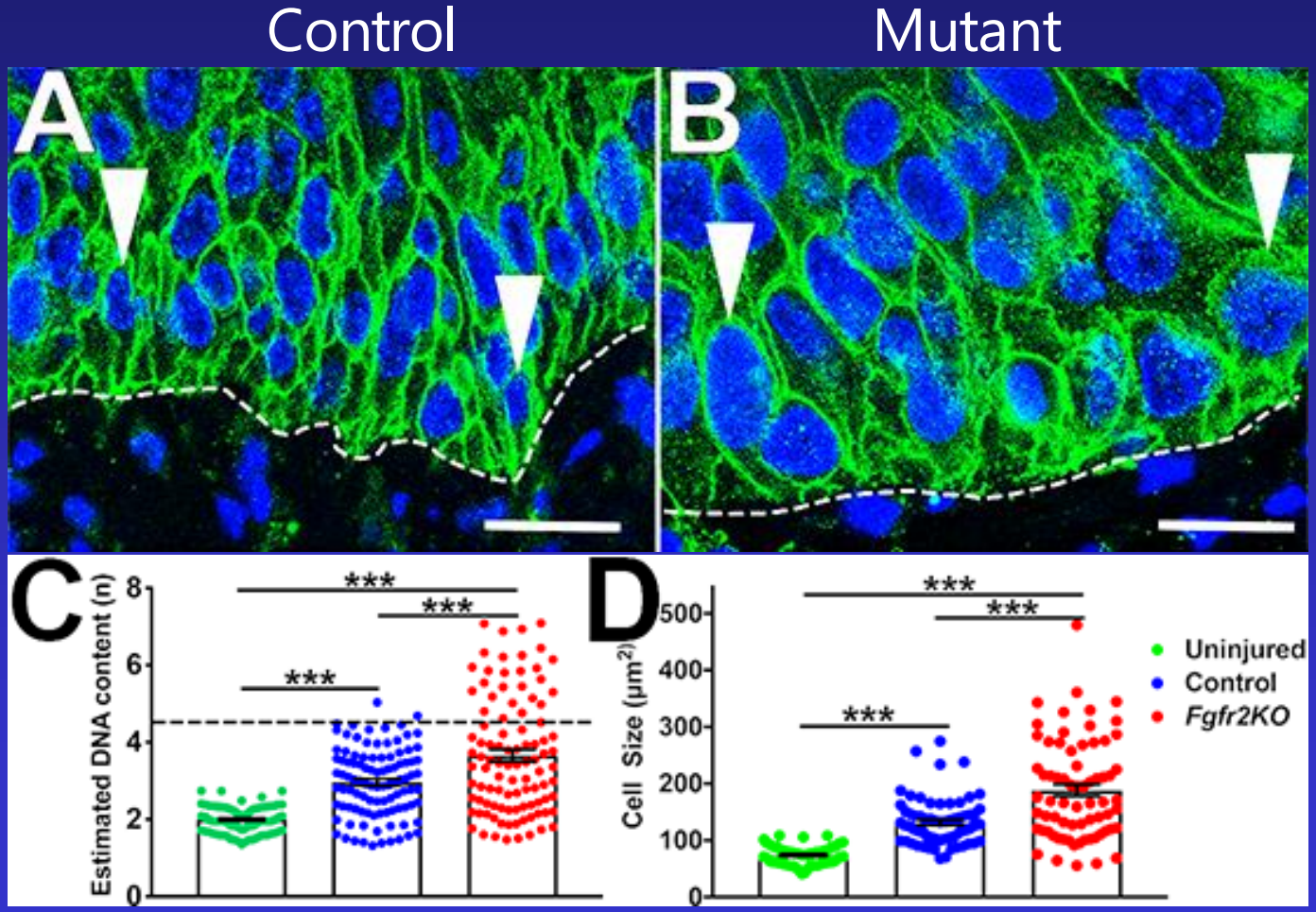


Summary-Endogenous FGFR2-1

- *Fgfr2* mutants have no increases in acute injury CPP from 4 to 24 hours after CPP
- Three days after CPP, mutants do have impaired regeneration
  - Impaired UPK3^{+} cell regeneration
  - More submucosal hemorrhage and inflammation (neutrophil infiltration) c/w more barrier defects and urinary backleak
  - Fewer urothelial cell layers with larger Basal cells/nuclei
- 3-day injured mutants have apparent ***increases*** in (including possibly KRT14^{+} cell) proliferation and more DNA damage/replication stress
- The increase in mutant urothelial "proliferation" is incongruent with fewer urothelial cells and larger KRT14^{+} Basal cells 3 days post-CPP
- We wondered if mutants had pathological endoreplication (cell cycle activity bypassing mitosis) leading to aberrant regeneration

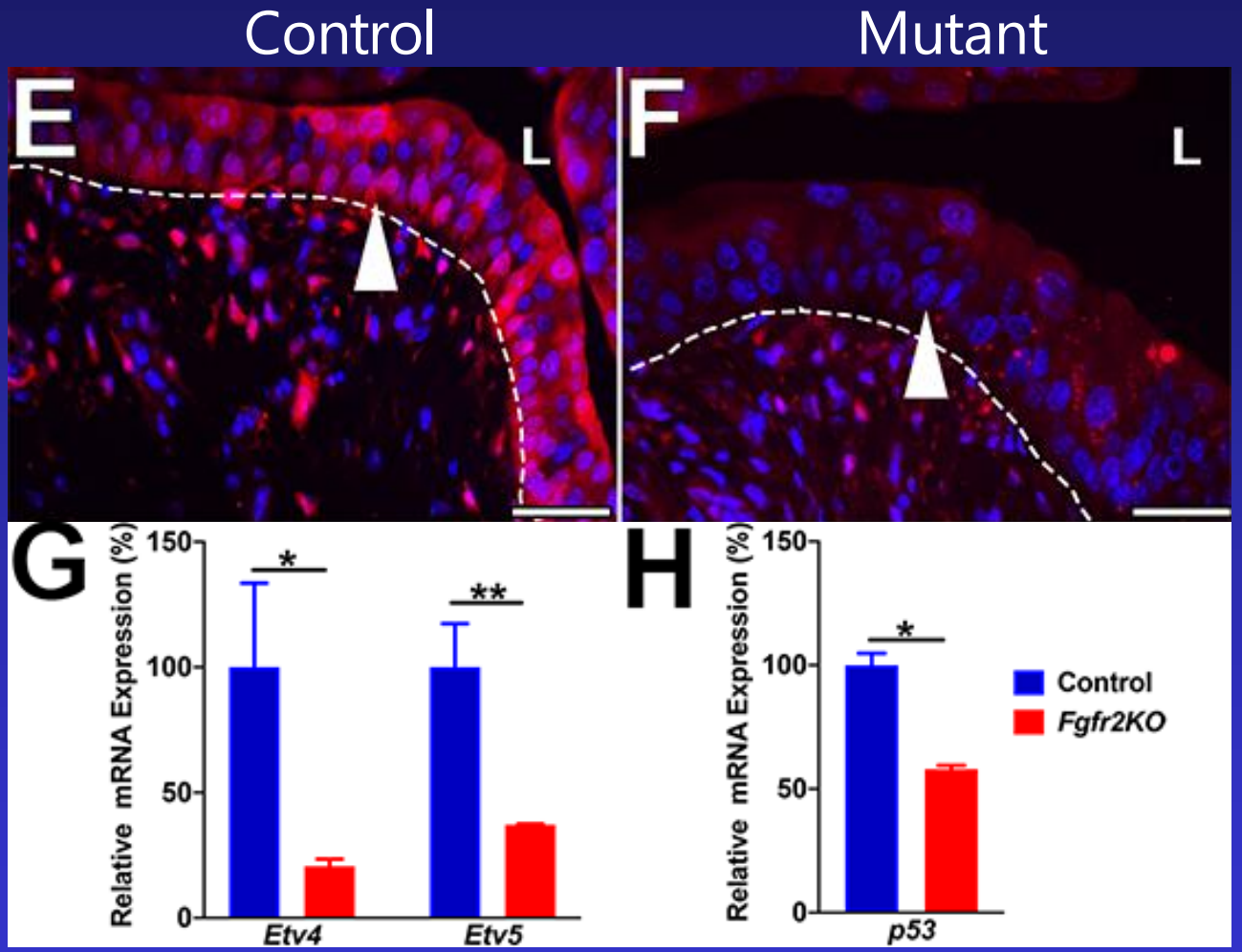
Confocal analysis of DAPI intensity reveals increased DNA content in mutants 3 days post CPP, consistent with endoreplication:

DAPI/E-cadherin



Mutant endoreplication appears to be from loss of ERK activity: pERK/DAPI staining and qPCR readouts of ERK activity

pERK/DAPI

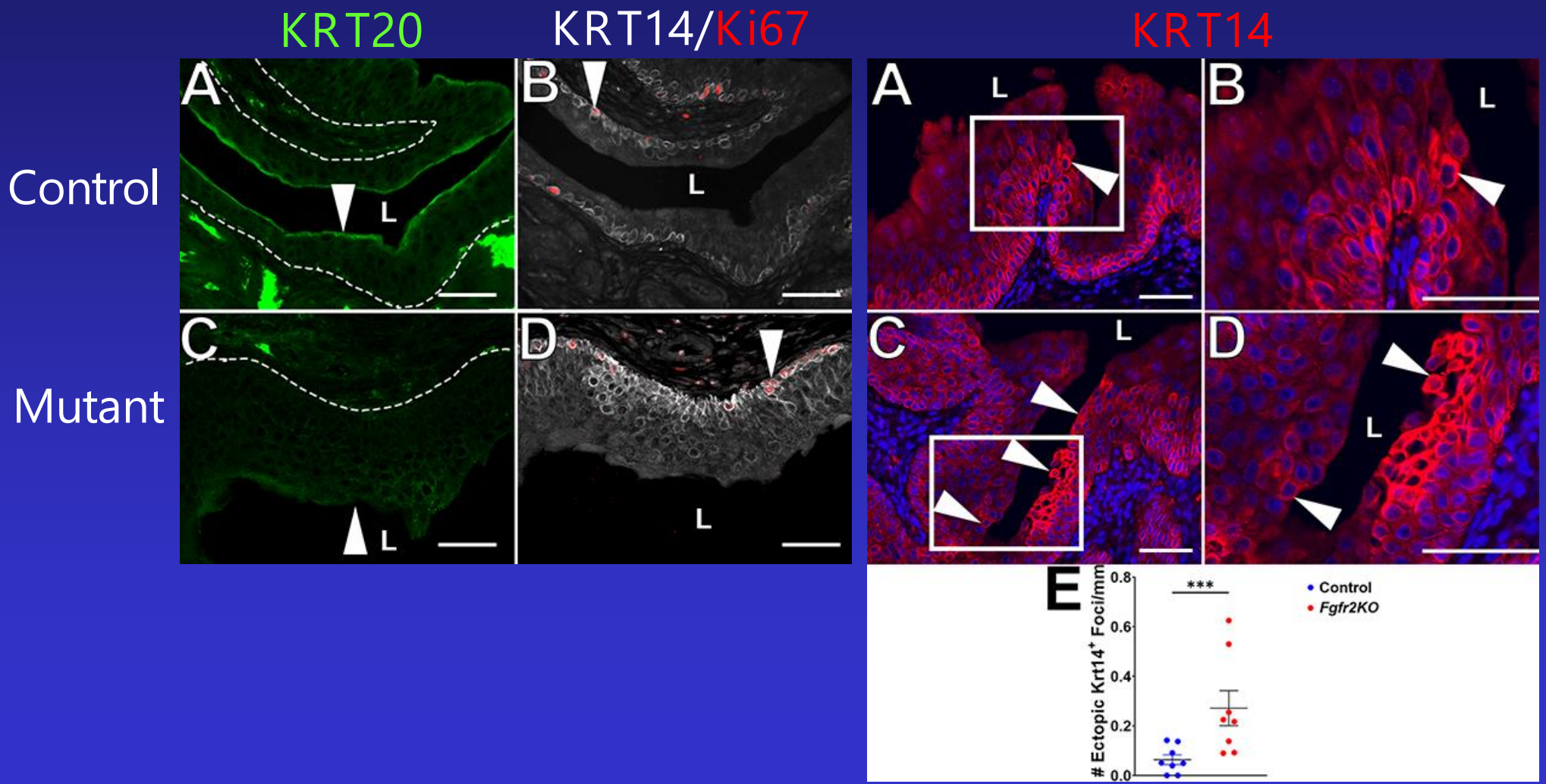


pAKT levels are low in both mutants and controls 3 days after CPP

Mutant regeneration defects persisted at 10 and 28 days after injury:

- Poor regeneration of outer Intermediate and Superficial cells (UPK3$^+$ and/or KRT20$^+$ cells)
- Stromal hemorrhage and inflammation (likely in part from poor barrier function and backleak of urine)
- Persistence of large KRT14+ cells, many marked by Ki67 (proliferation….likely ongoing pathological endoreplication)
- More ectopic lumenal KRT14$^+$ cells than controls

6 months post CPP, mutants continue to have regeneration defects, KRT14$^+$ cell proliferation and more ectopic lumenal KRT14$^+$ cells vs controls

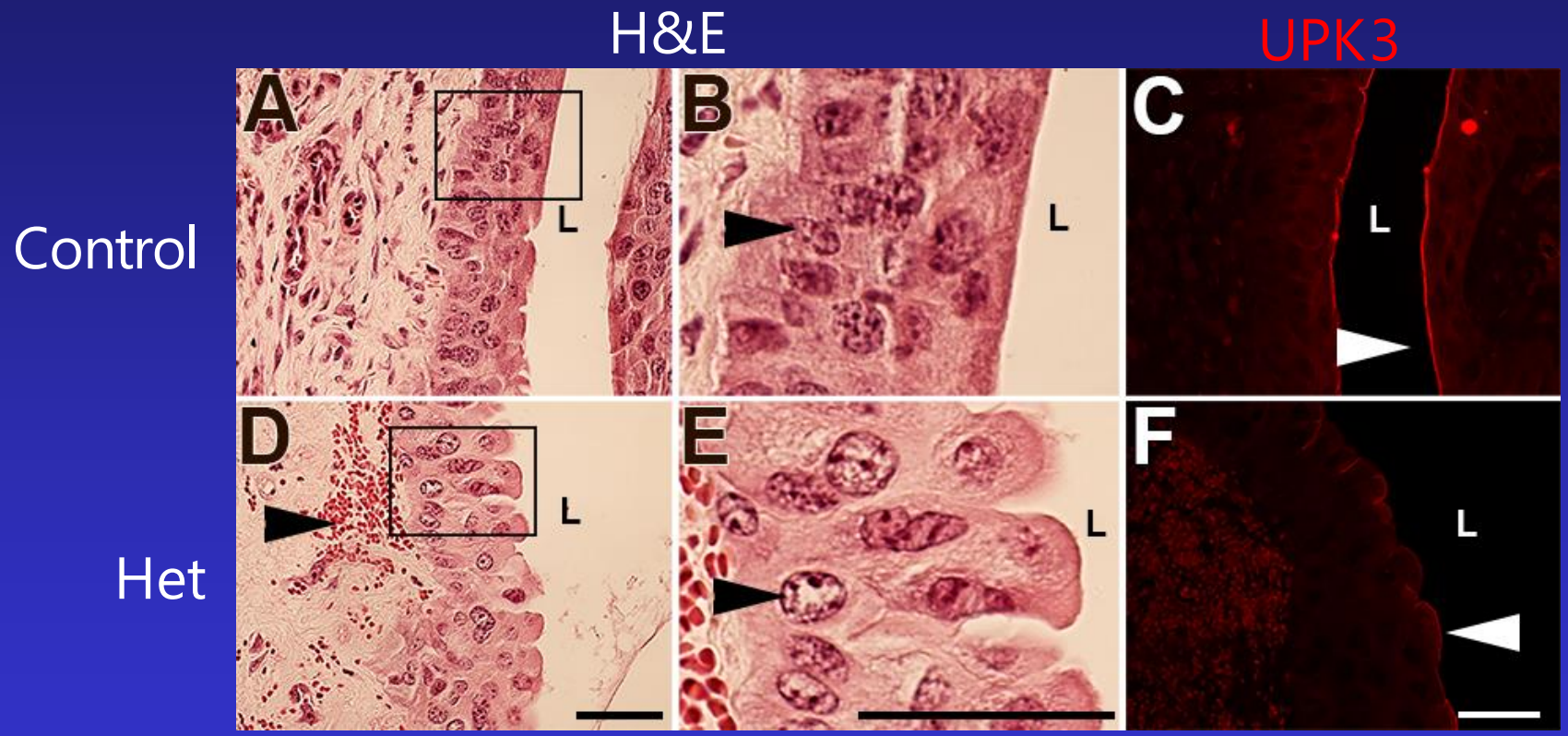


We wondered if the heterozygous mice (*ShhcreFgfr2*$^{fl/+}$) had an aberrant response to CPP

- Thus, we challenged the heterozygous mice to 150 mg/kg of IP Cyclophosphamide

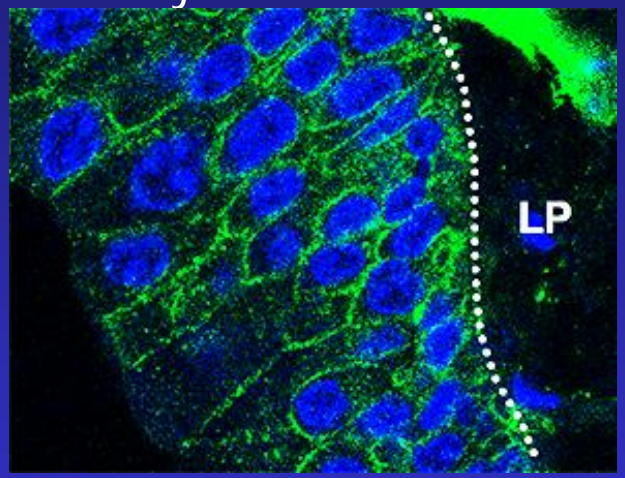
- We assessed mice at 3 days post injury:

Fgfr2 heterozygotes more hemorrhage, larger Basal cells/nuclei and UPK3^{+} cell regeneration defects vs. controls 3 days post CPP



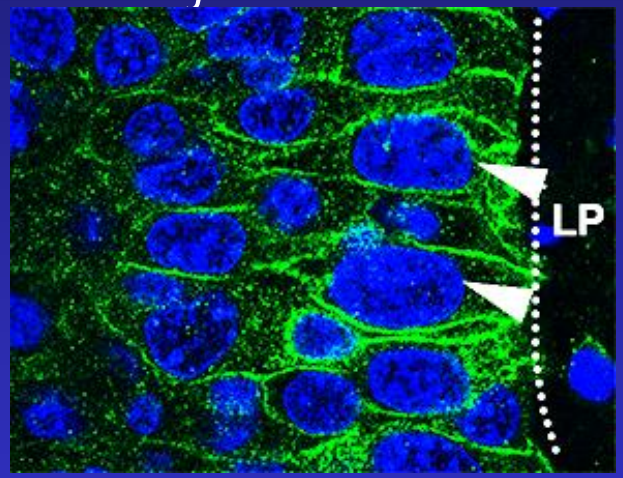
Confocal analysis of DAPI intensity reveals increased DNA content in heterozygotes 3 days post CPP, consistent with endoreplication (as in the mutants):

Injured Control



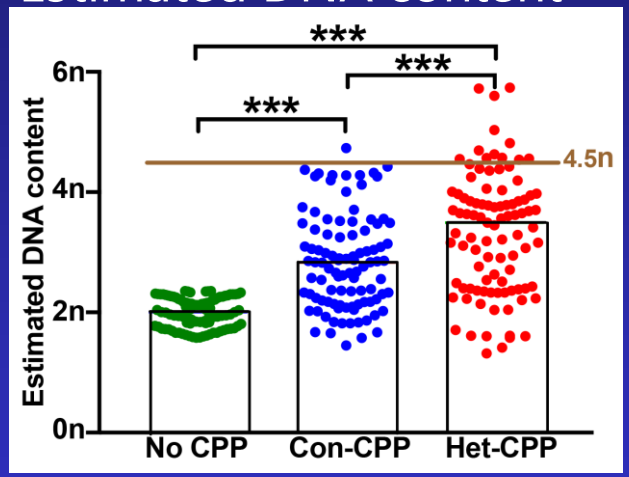
DAPI/E-cadherin

Injured Het



DAPI/E-cadherin

Estimated DNA content



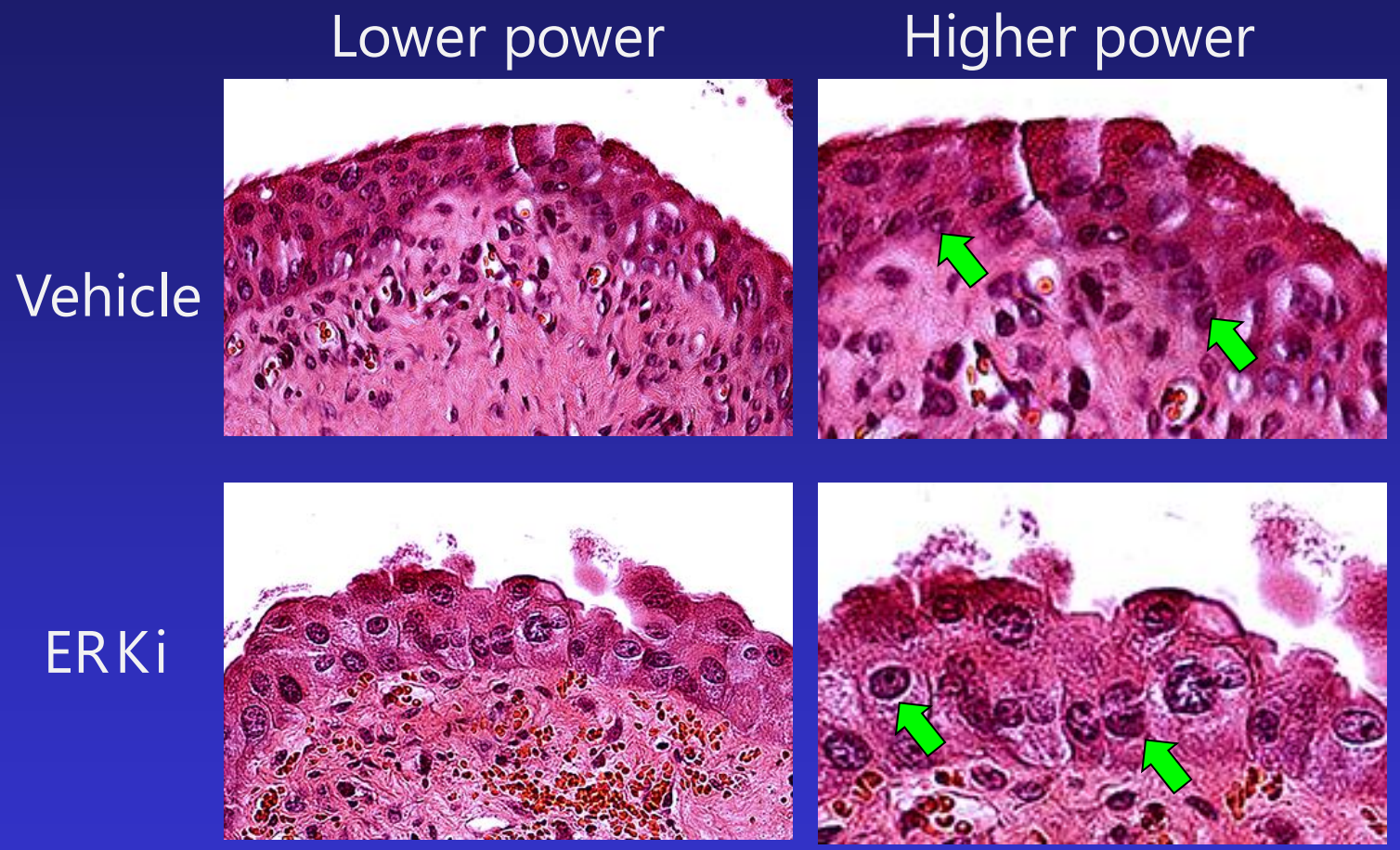
The injured heterozygotes have ~13% of nuclei that are > 4.5n compared to injured homozygous mutants that have ~28% of nuclei that are >4.5n

This is clinically relevant as a mouse (or person) can live and appear normal with a one allele loss of FGFR2 (or knockdown of that pathway)

We next wanted to establish causal links between loss of ERK activity with aberrant urothelial endoreplication after CPP

- We have done preliminary work using a systemic ERK inhibitor (ERKi) (SCH772984) in wildtype mice given CPP
  - Administers 12.5mg/kg of ERKi (or vehicle) IP concurrently with CPP and then every 12 hours up to 72 hours

ERKi-treated mice have larger Basal cell nuclei 3 days after CPP: H&E staining



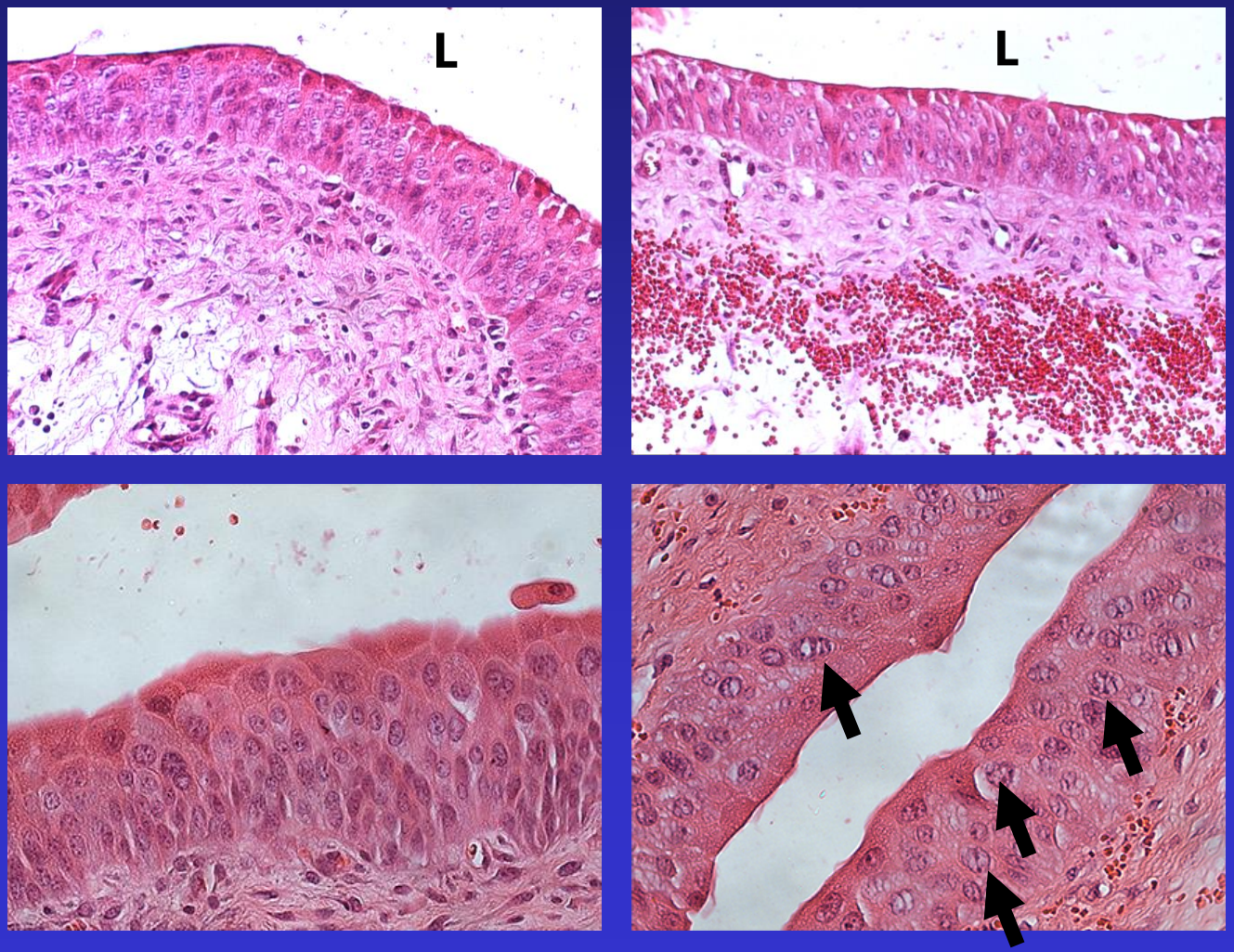
There appear to be more Ki67^{+} cells in the ERKi-treated mice too

Given the possible links between maladaptive DNA repair in the *Fgfr2* mutants, we next challenged *Fan1-/-* (Fan1KO) mutants with CPP

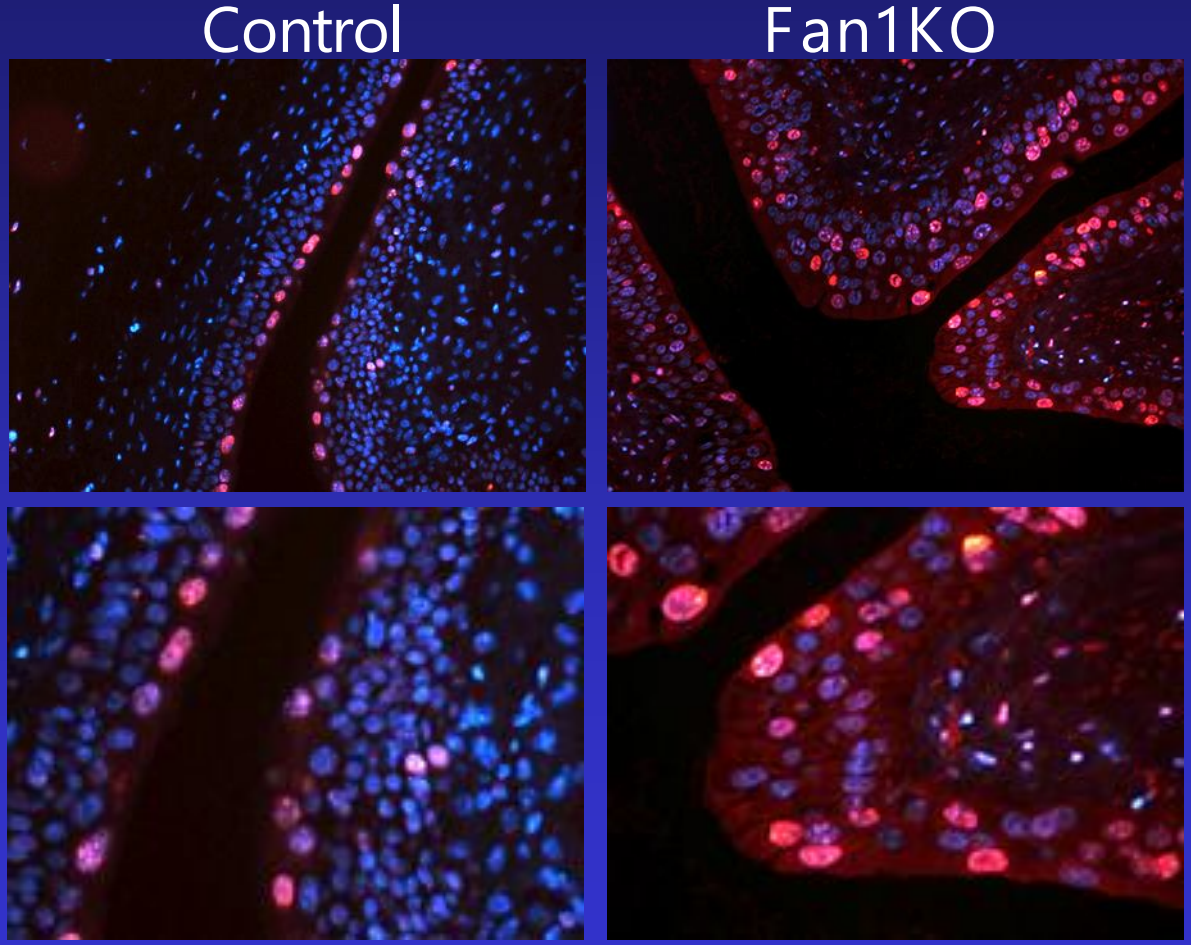
3 days post CPP, *Fan1KO* have increased inflammation/ hemorrhage and larger Basal cell nuclei vs. controls (similar to *Fgfr2* mutants): H&E staining

Control

Fan1KO



Fan1KO mutants appear to have increased DNA damage/stress in multiple layers and larger nuclei 3 days post CPP vs controls (like FGFR2 mutants): **γH2AX/DAPI** staining



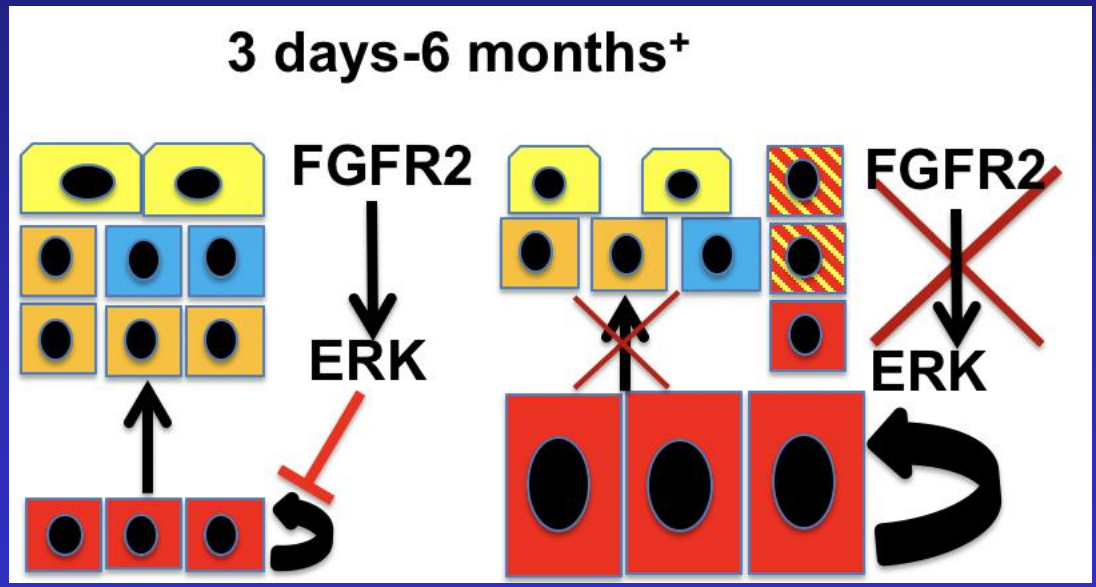
Summary-Endogenous FGFR2 (FAN1)-2

- A one allele loss of *Fgfr2* leads to endoreplication and replication stress
  - This adds clinical relevance in that a one allele loss of FGFR2 is compatible with life
  - People with polymorphisms leading to lower FGF7-FGFR2 signaling may be at risk for significant regeneration defects (even cancer) after CPP

- *Fgfr2*$^{LR/LR}$ and ERKi studies supports a causal link between FGFR2-driven ERK activation that suppresses endoreplication

- *Fan1KO* mice have a similar response to CPP as *Fgfr2* mutants
  - This may mean that other mutations in DNA damage/cell cycle regulating genes would lead to abnormal endoreplication after CPP
  - This is relevant to patients with mutations that would make them likely to develop lymphomas that could mean exposure to CPP (e.g. ATM)

Proposed actions of endogenous FGFR2 signaling in bladder urothelium after Cyclophosphamide



Acknowledgements:

Current lab members:

- **Sridhar Narla**
- **Dan Bushnell**
- **Joanne Duara**

Past lab members:

- Caitlin Schaefer
- Katie Schwabenbauer
- Pawan Puri
- Sunder Sims-Lucas
- Valeria Di Giovanni
- Surbhi Chouhan
- Whitney Sunseri
- David Hains
- Andreas Correa
- Janelle Fox
- Dan Casella
- Christina Nguyen

University of Pittsburgh

- Rannar Airik
- Gerry Apodaca
- Tony Kanai
- Irina Zabbarova
- Youko Ikeda
- Glen Cannon
- Steve Docimo
- Chet DeGroat
- George Gittes

Other Universities:

- David Ornitz
- Zubaida Saifudeen
- Jordan Kreidberg
- Brad Yoder
- Fen Wang
- Janet Rossant
- Jon Epstein
- Mark de Caestecker
- Kirk McHugh
- Joseph Schlessinger
- V.P.Eswarakumar
- Indra Gupta

Funding sources:

- NIDDK: R01 DK095748 and R01 DK121493-01A1
- NIDDK: P30 DK079307
- Univ of Pittsburgh Vascular Medicine Institute/CTSI

Thank You!