

# Preventing Engrailed-1 activation in fibroblasts yields wound regeneration without scarring

Shamik Mascharak, Heather E. desJardins-Park, Michael F. Davitt, Michelle Griffin, Mimi R. Borrelli, Alessandra L. Moore, Kellen Chen, Bryan Duoto, Malini Chinta, Deshka S. Foster, Abra H. Shen, Michael Januszyk, Sun Hyung Kwon, Gerlinde Wernig, Derrick C. Wan, H. Peter Lorenz, Geoffrey C. Gurtner, Michael T. Longaker

Paper Presentation // Marie Messeritsch

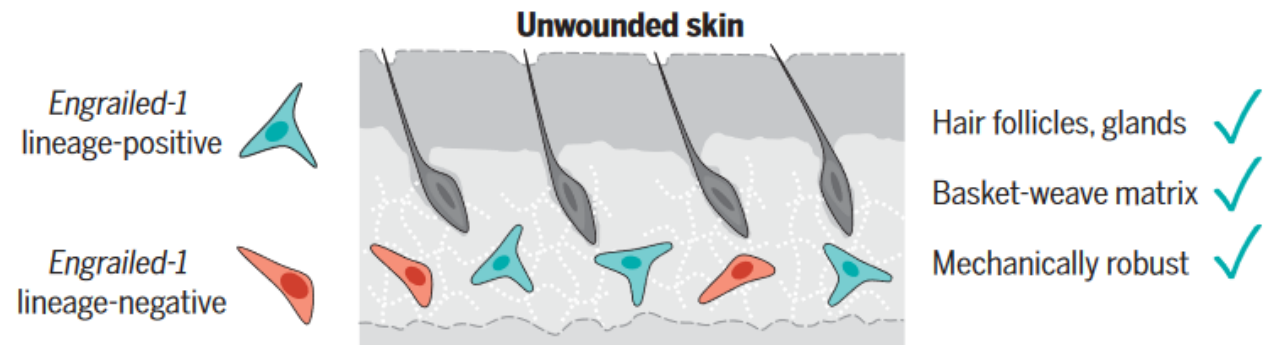
# Scarring

Fibrotic scar tissue:

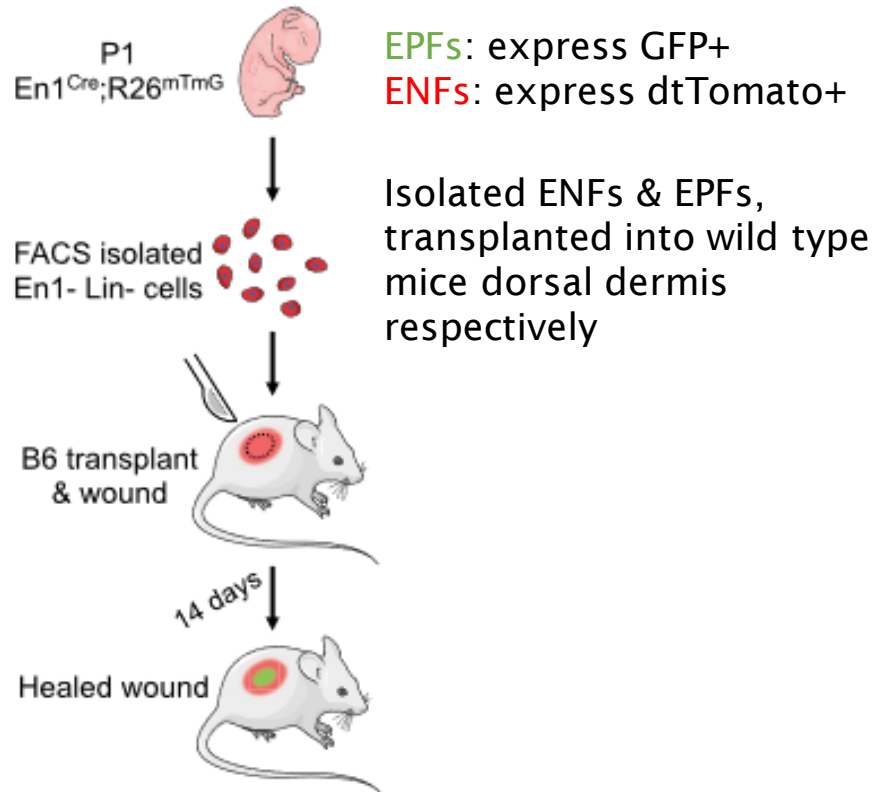
- Lack of dermal appendages (hair, glands, etc.)
- ECM with dense, parallel fibers
- Altered fiber structure → weaker

Key mediators of scarring: **Fibroblasts (FBs)**

- EPFs: Engrailed-1 lineage positive
- ENFs: Engrailed-1 lineage negative
- eEPFs: embryonic EPFs (emerge during normal development in utero)
- pEPFs: postnatally derived EPFs



# Transplanted ENFs express En1 to become pEPFs within wound environment

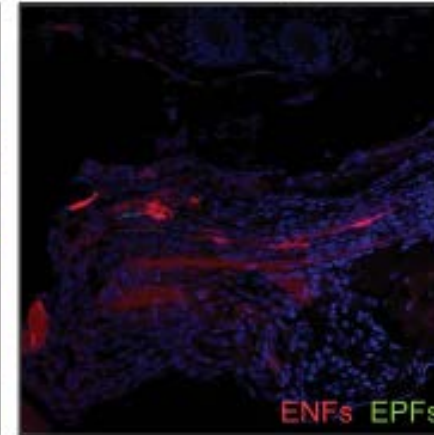
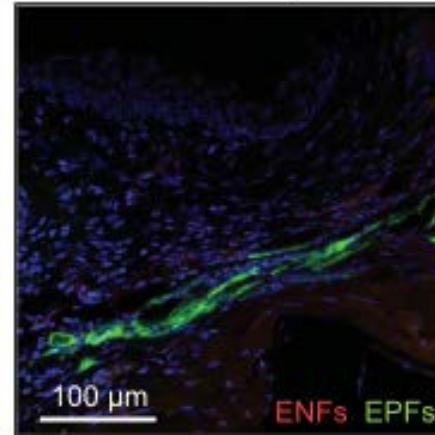


Confocal  
imaging

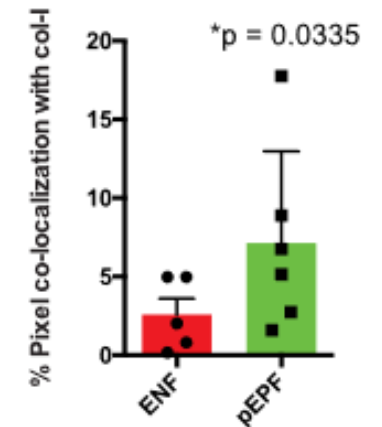
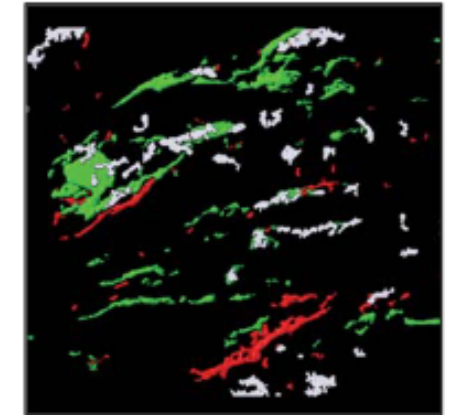
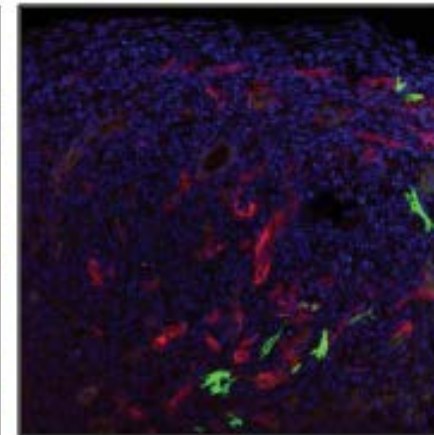
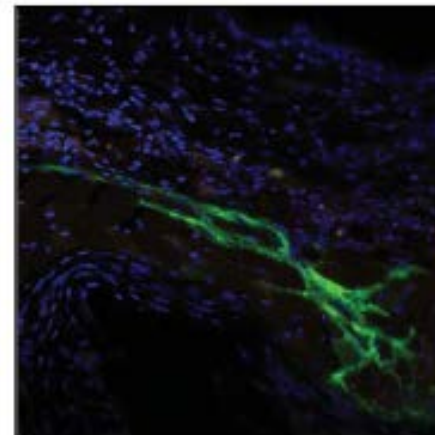
EPF Transplant

ENF Transplant

Unwounded



Wounded (14 days)

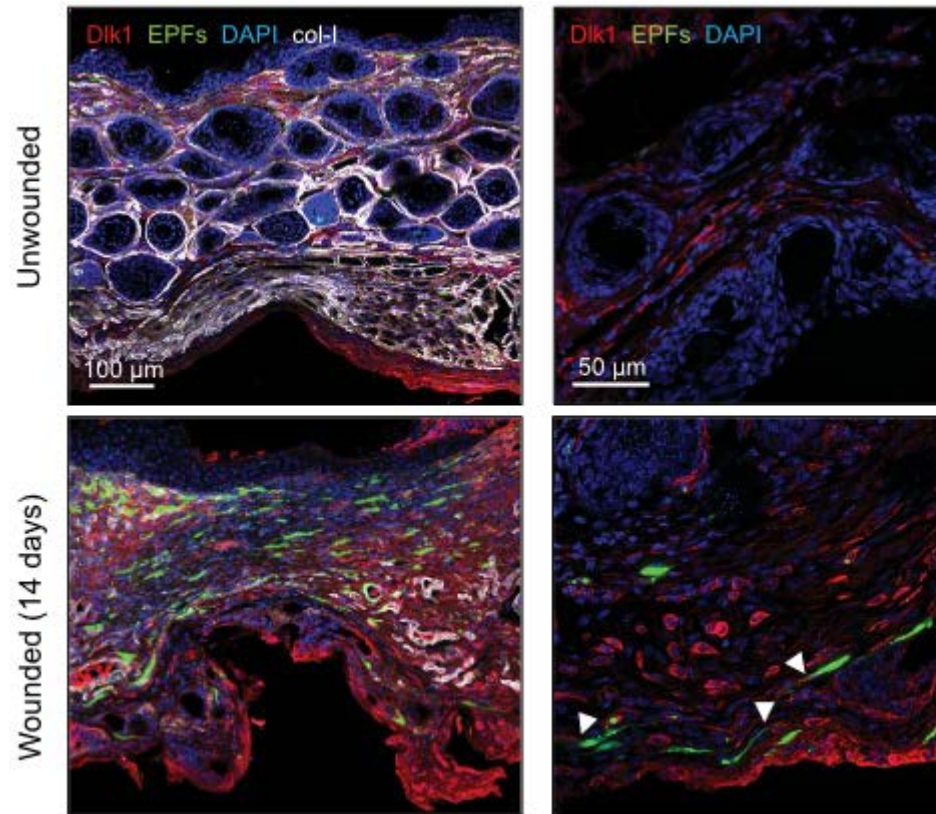
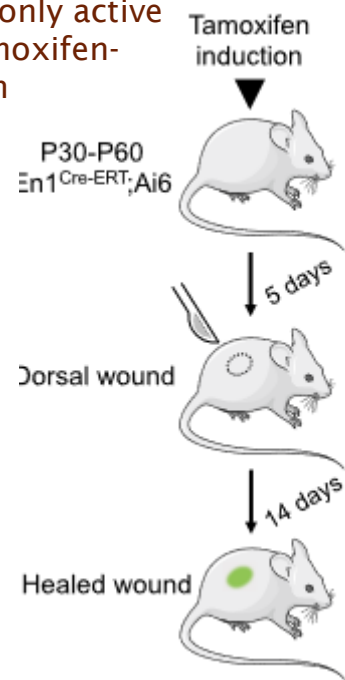


Transplanted ENFs express En1 to become pEPFs within wound environment

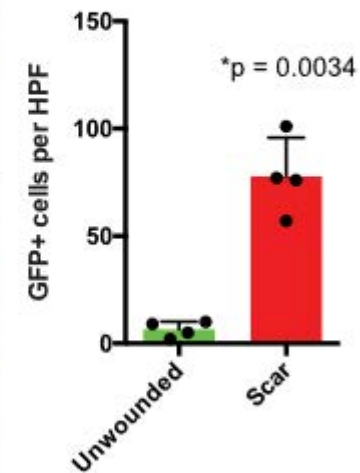
pEPF overlap with col-1 > than overlap of col-1 with ENFs that had not activated En1 → increased collagen-production from En1 expressing cells

# Postnatal En1-Activation is specific to wound setting

En1 (cre) only active after Tamoxifen-induction



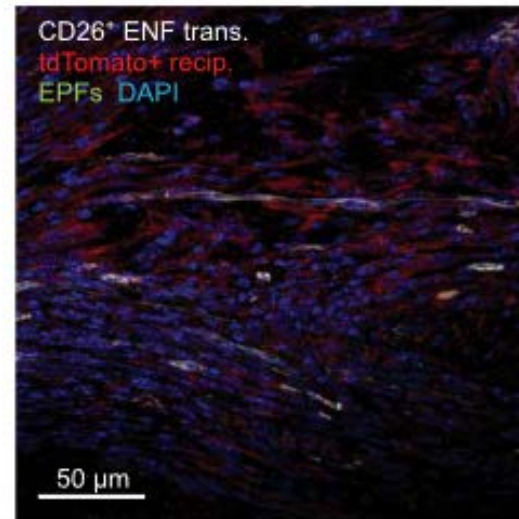
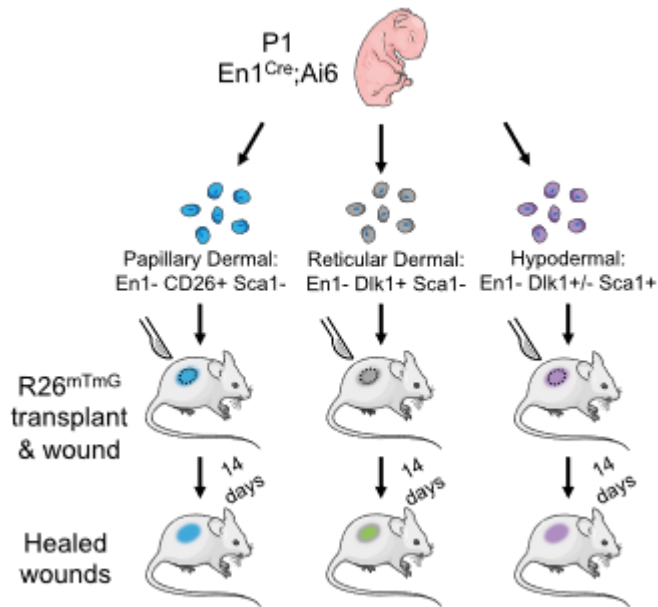
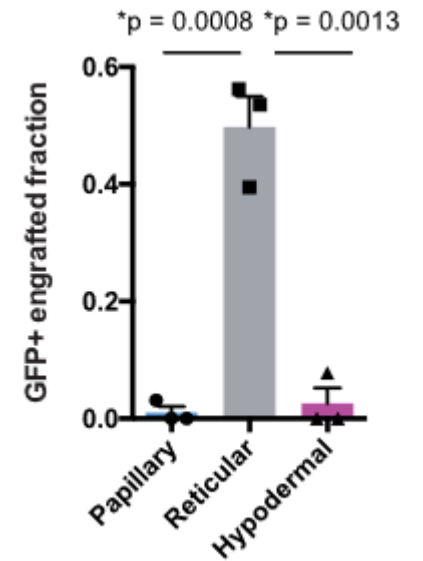
Unwounded: only few GFP+ cells  
Wounded: ~40% of FBs were GFP+



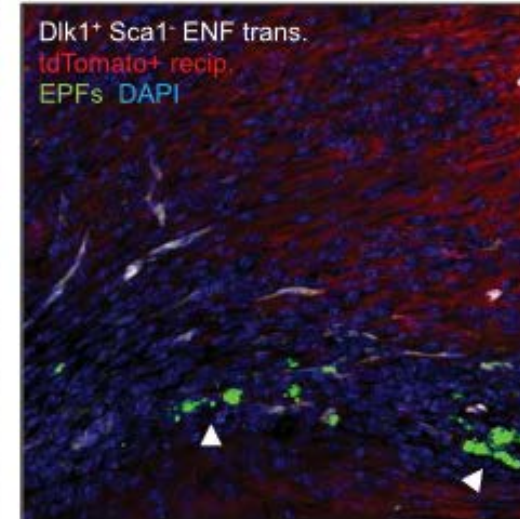
Postnatal ENF-to-EPF transition generates substantial fraction of scar producing EPFs

# Reticular ENFs expand & activate En1 after injury

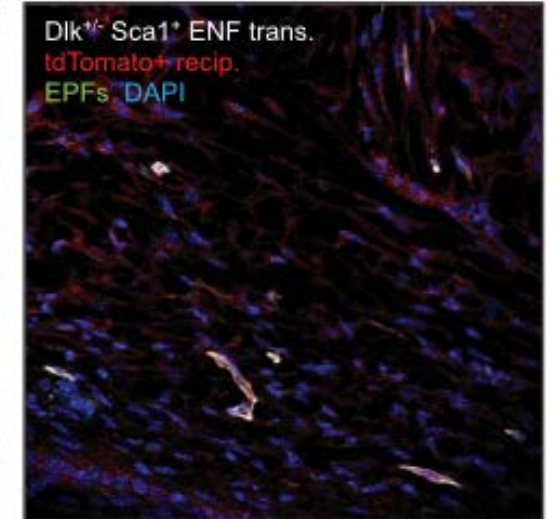
ENFs: multiple subpopulations;  
**different wound phenotypes**  
 corresponding to anatomical  
 subpopulations?



papillary



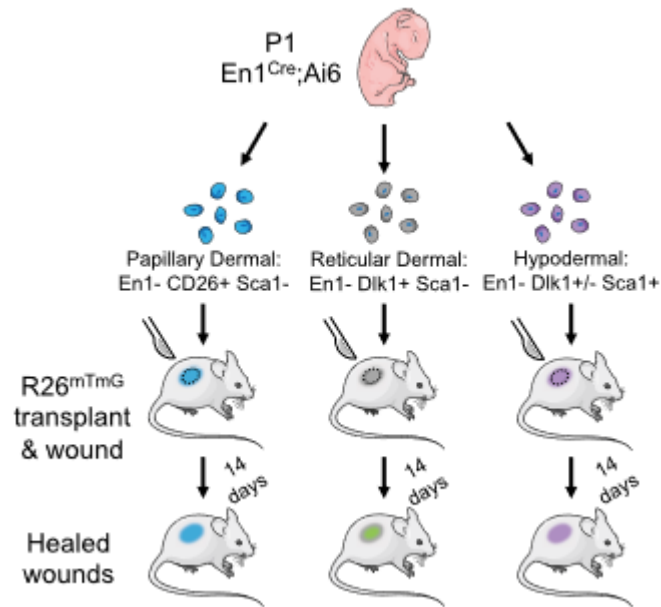
reticular



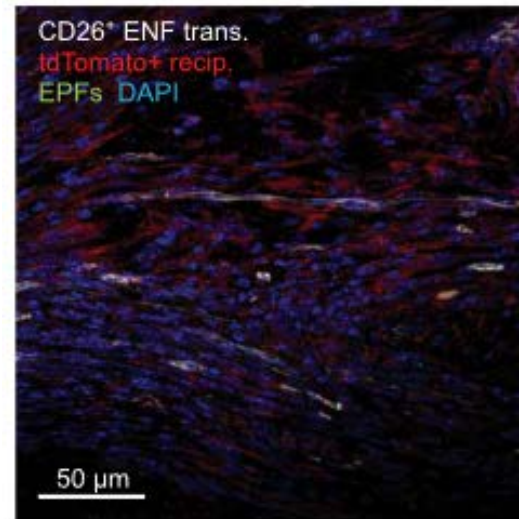
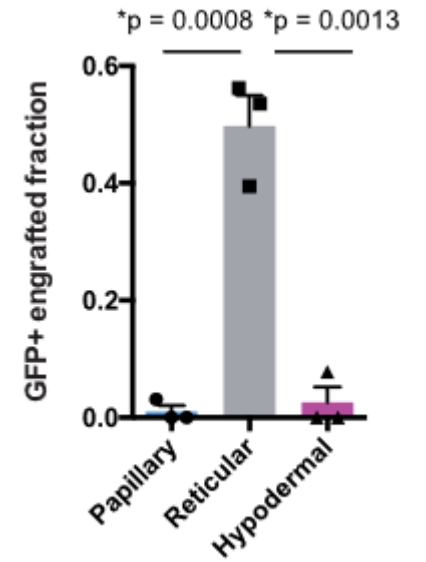
hypodermal

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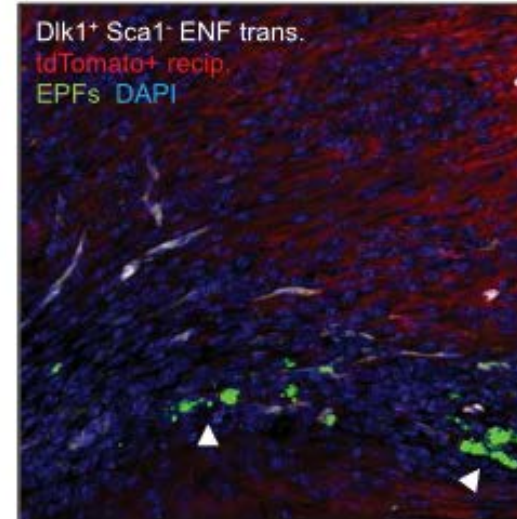
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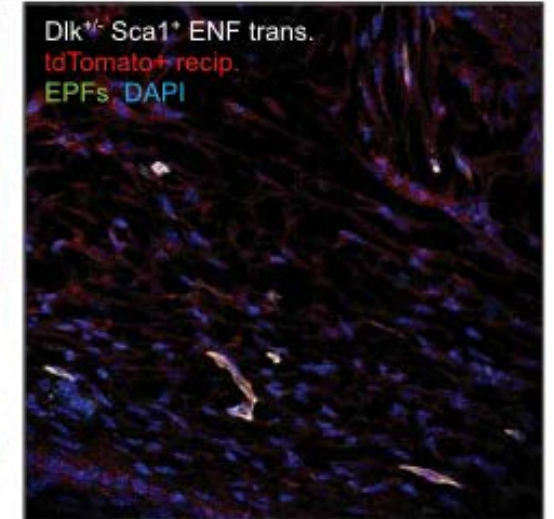
Primary subpopulation capable of En1 activation



papillary



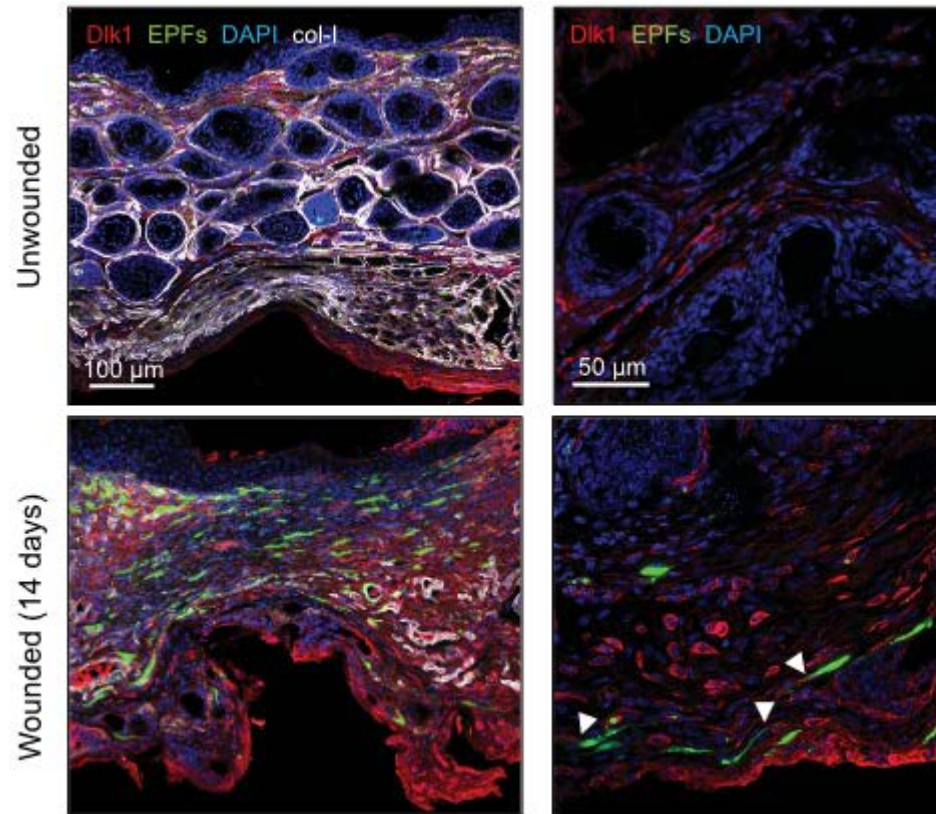
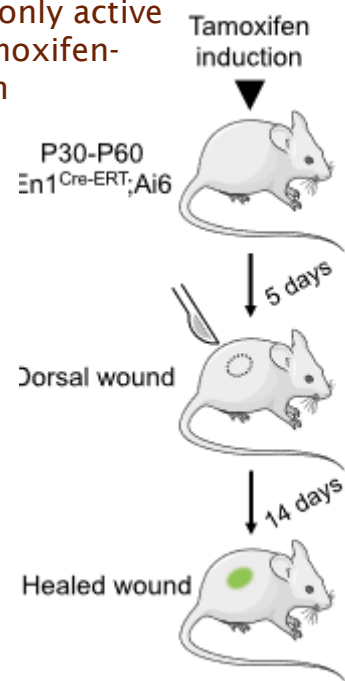
reticular



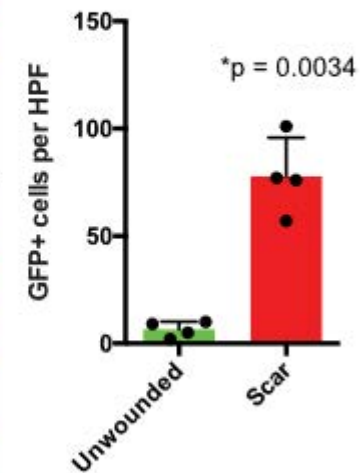
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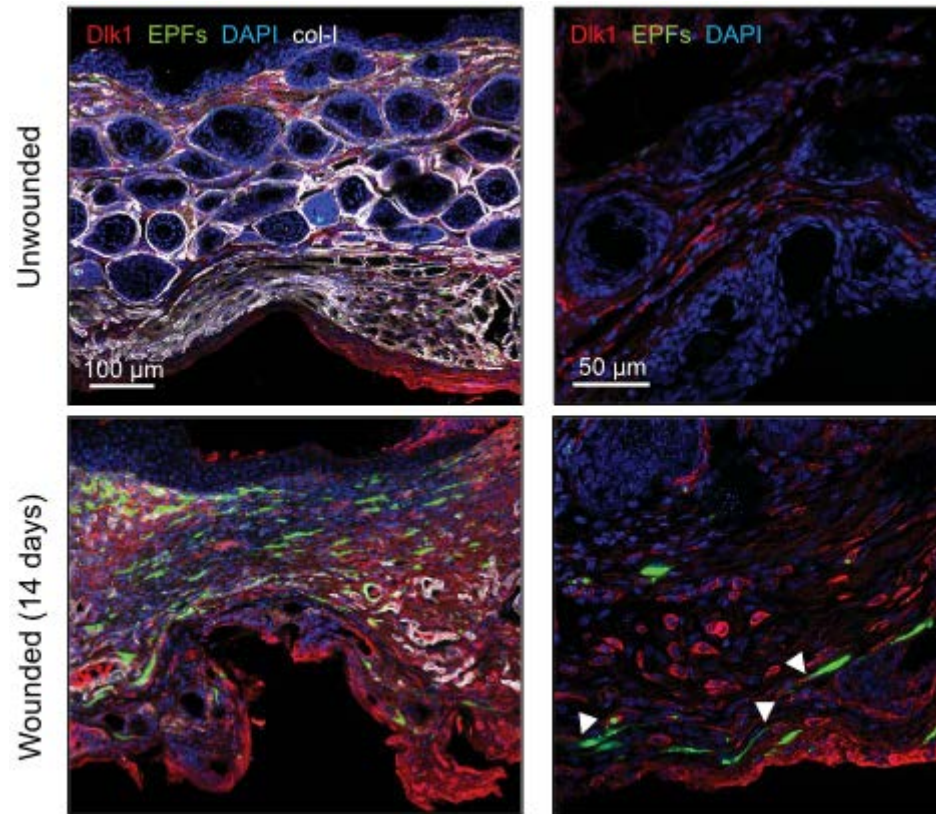
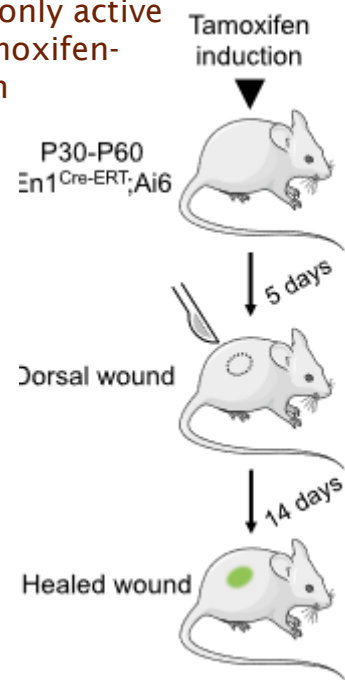
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Wounded: ~40% of FBs were GFP+



Postnatal ENF-to-EPF transition generates substantial fraction of scar producing EPFs

# Postnatal En1-Activation is specific to wound setting

En1 (cre) only active after Tamoxifen-induction



**Dik1:** reticular deep dermal FB marker

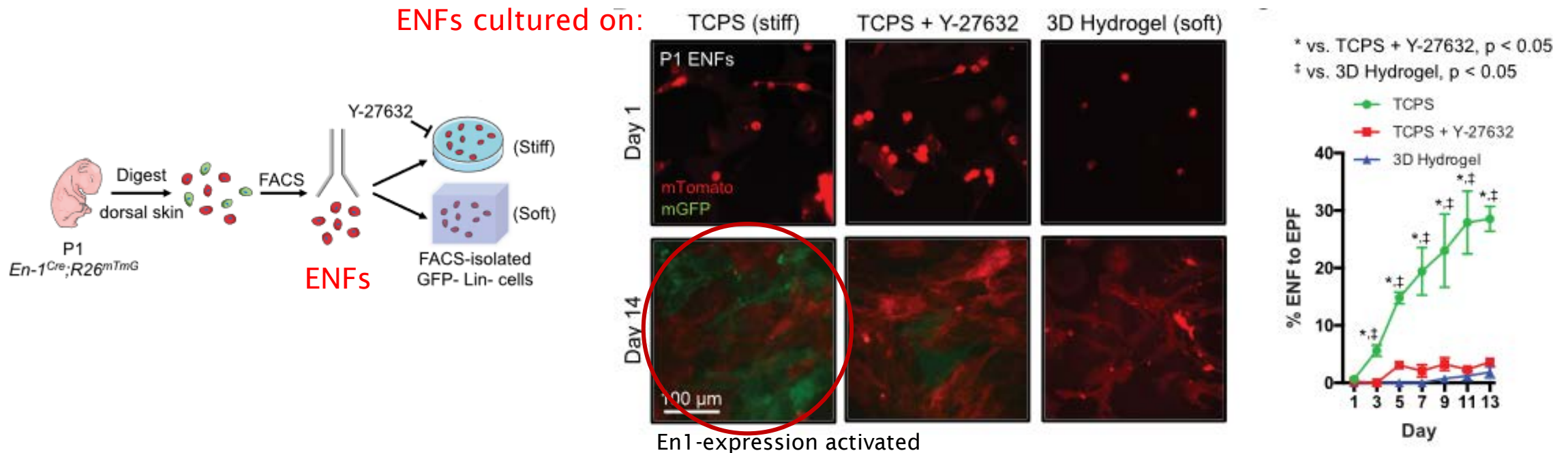
- **Unwounded skin:** expression in deep dermis
- **Scars:** expressed throughout dermis

reticular ENFs expand & activate En1 after injury to contribute to scarring



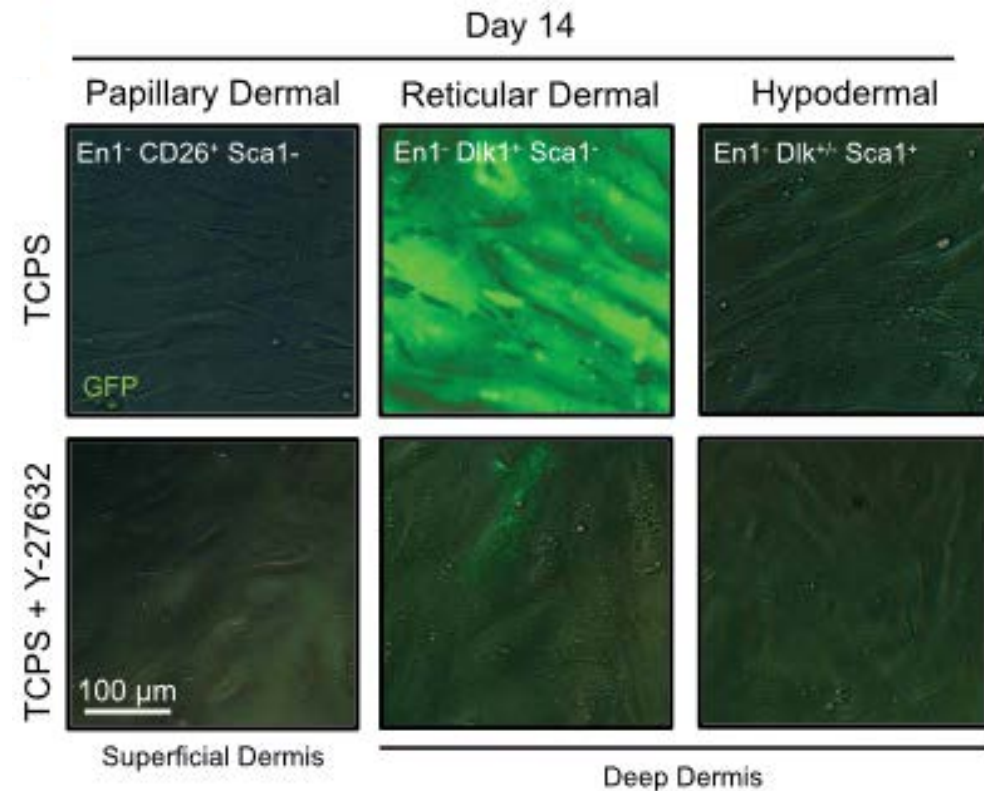
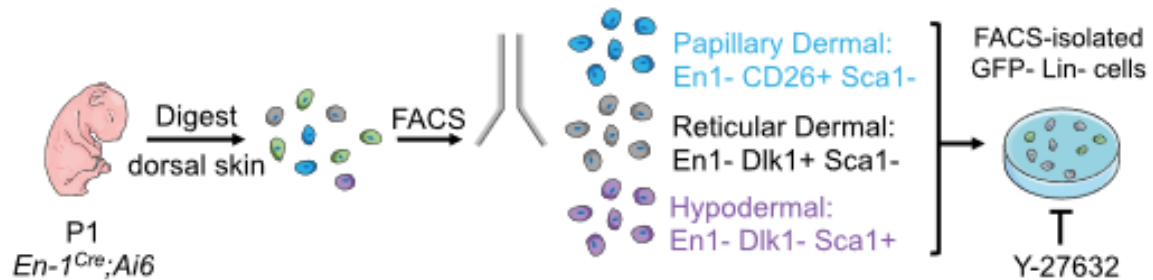
# Postnatal Engrailed-1 activation is mechanoresponsive

- ROCK: *Rho/Rho associated protein kinase signalling*  
(ROCK: key mediator of actin organization → regulator of cell migration)
- Mechanotransduction modulates wound-resident cells
- **Hypothesis:** mechanical cues activate ENFs to express En1 → pEPFs



# Reticular ENFs: near complete conversion to pEPFs after 14 days

- ENF subpopulations on TCPS with or without block
- Consistent with in vivo findings:



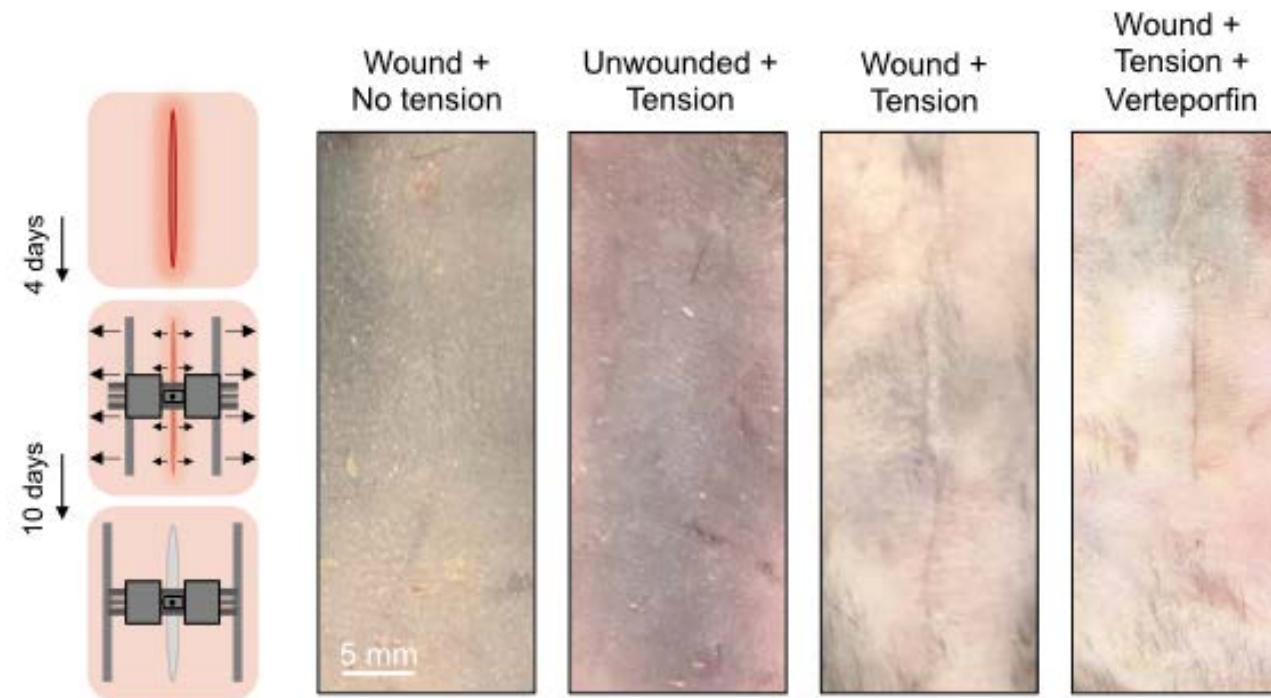
Conversion blocked  
by ROCK-inhibitor

# FBs within all subpopulations are capable of En1-expression

- RNA-seq of papillary, reticular & hypodermal ENFs after 2, 7, and 14 days on TCPS
- All expressed low-level En1 at 2 days
- FBs within all subpopulations can express En1
- Day 14:
  - Papillary ENFs: no evidence of mechanical activation
  - Reticular & hypodermal ENFs: up-regulated integrin-related terms (→ active mechanotransduction)
    - Reticular ENFs: activated En1 + collagen GSEA (Gene set enrichment analysis) terms
    - Hypodermal ENFs: activation of Wnt/TGF- $\beta$  pathway, lipid- & collagen related terms
- only reticular ENFs expressed fibrogenic transcriptional program (as in in vivo)

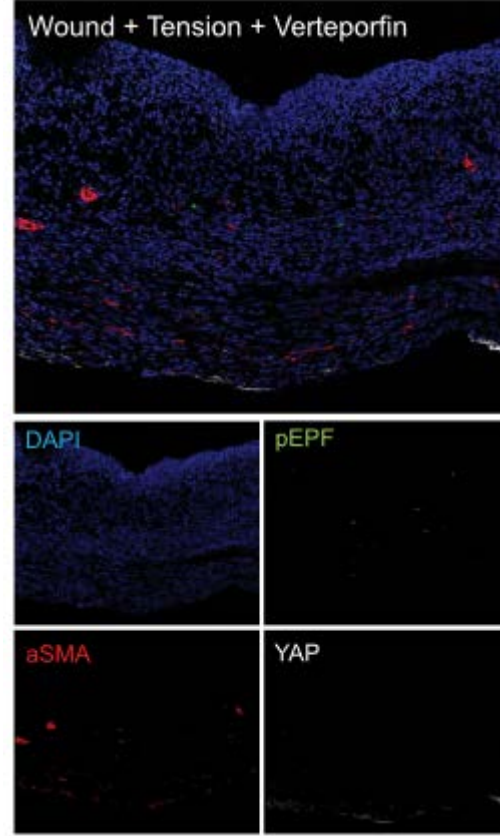
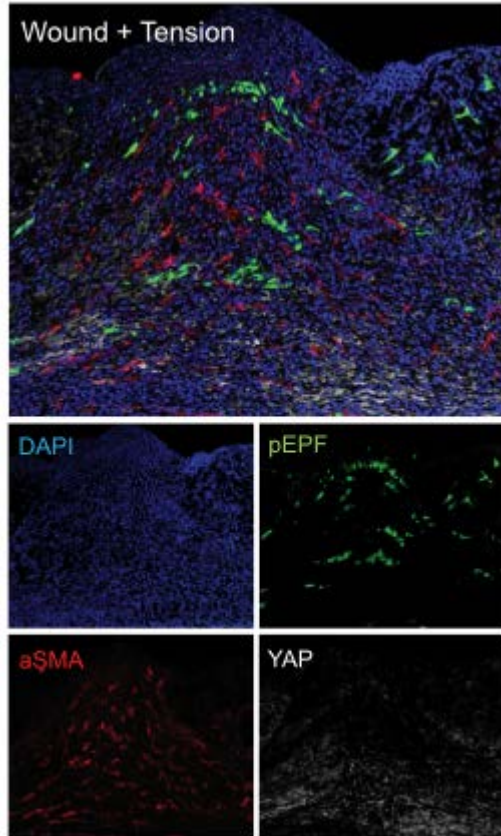
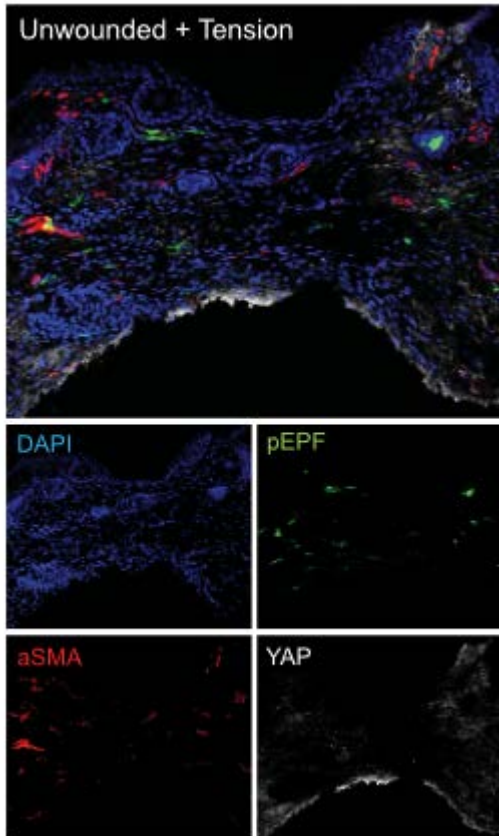
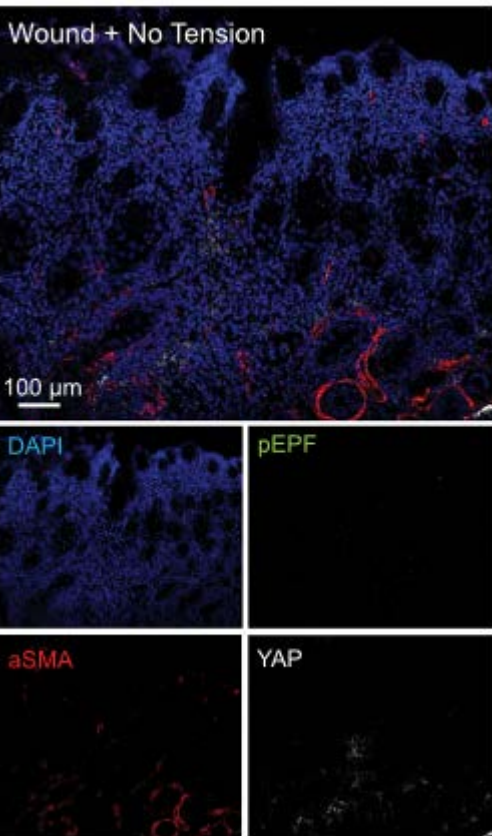
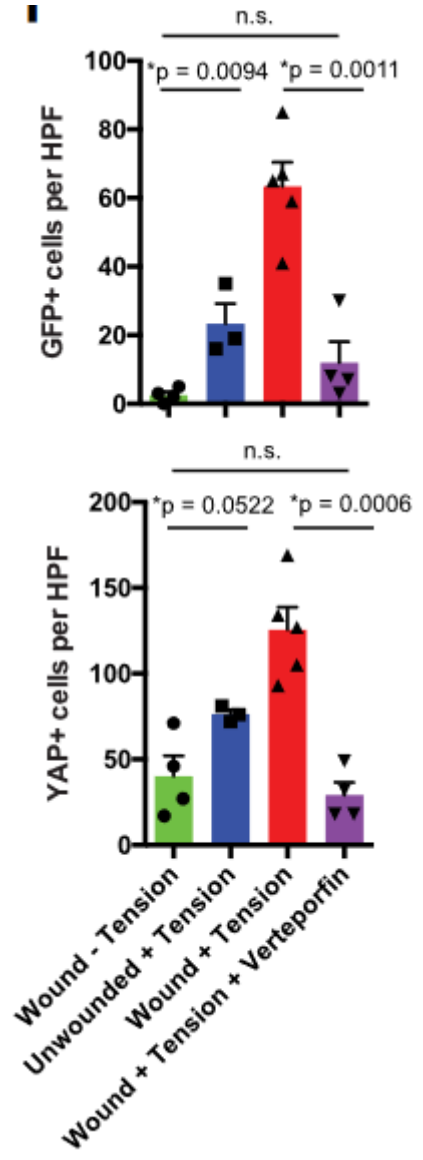
# Role of mechanical cues in vivo

- Tamoxifen-induced En1 (Cre-ERT);Ai6 mice
- Affixed distraction devices: expanded over 10 days





**Mechanically loaded scars:**  
grossly thickened,  
raised, greatest YAP  
& αSMA expression





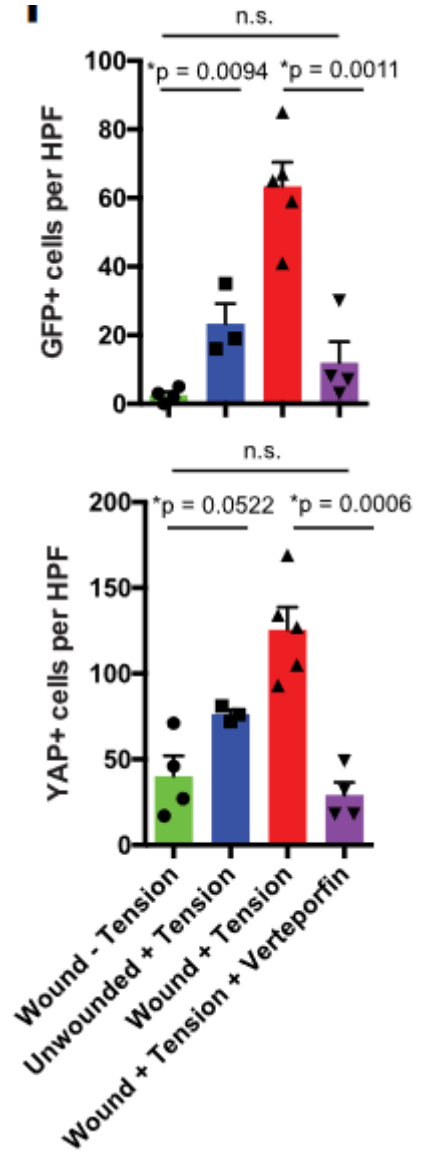
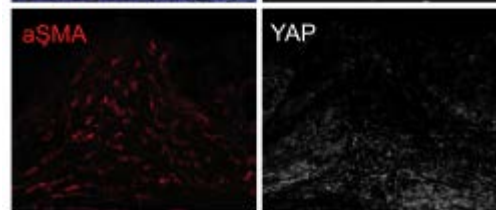
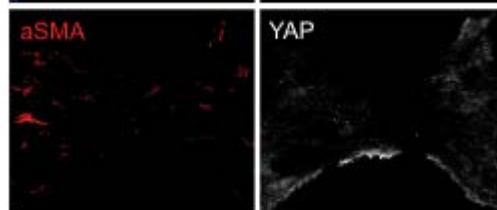
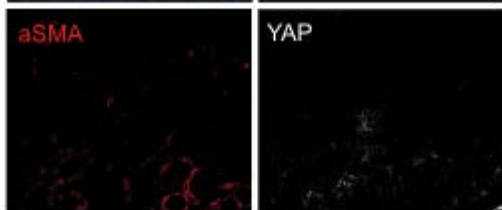
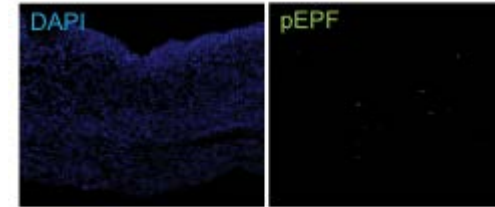
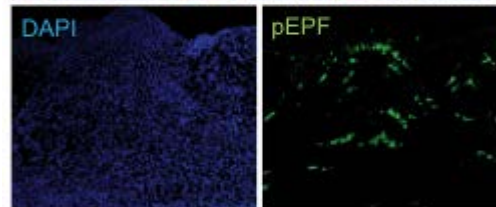
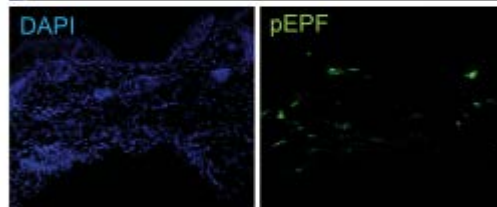
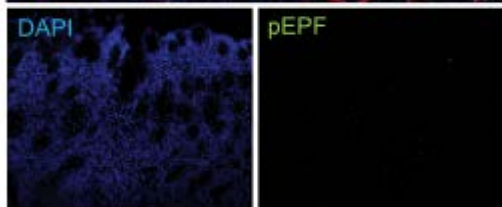
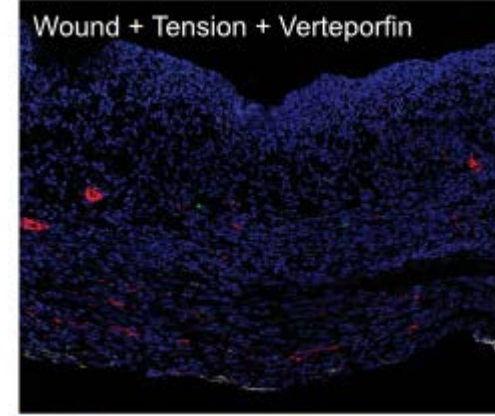
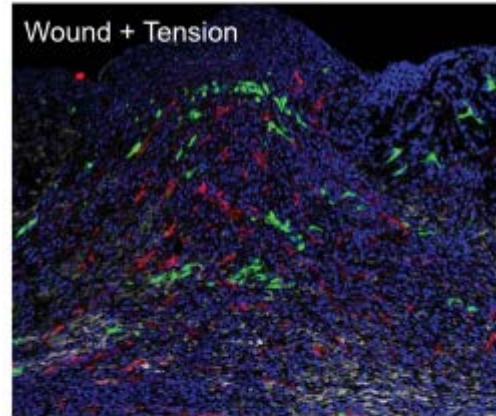
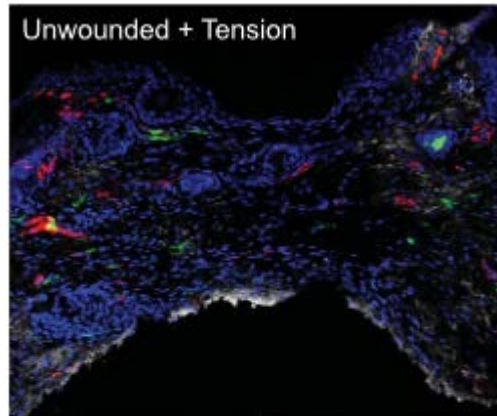
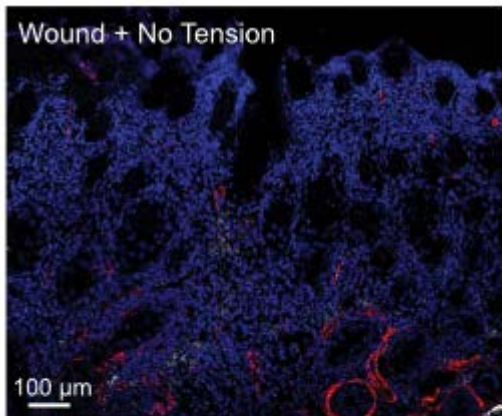
**$\alpha$ -SMA:** smooth muscle protein, for cellstructure, -motility, -contractility



**YAP:** transcriptional regulator (activates transcription of genes involved in cell proliferation & suppressing apoptotic genes)



**Mechanically loaded scars:** grossly thickened, raised, greatest YAP &  $\alpha$ SMA expression



# YAP mechanotransductional signalling plays a dominant role in ENFs

Mechanical stimulation → YAP ad nucleus → activation of proliferation- & migration-related genes

En1Cre-ERT;Ai6 YAP immunostaining through colocalization analysis pipeline:

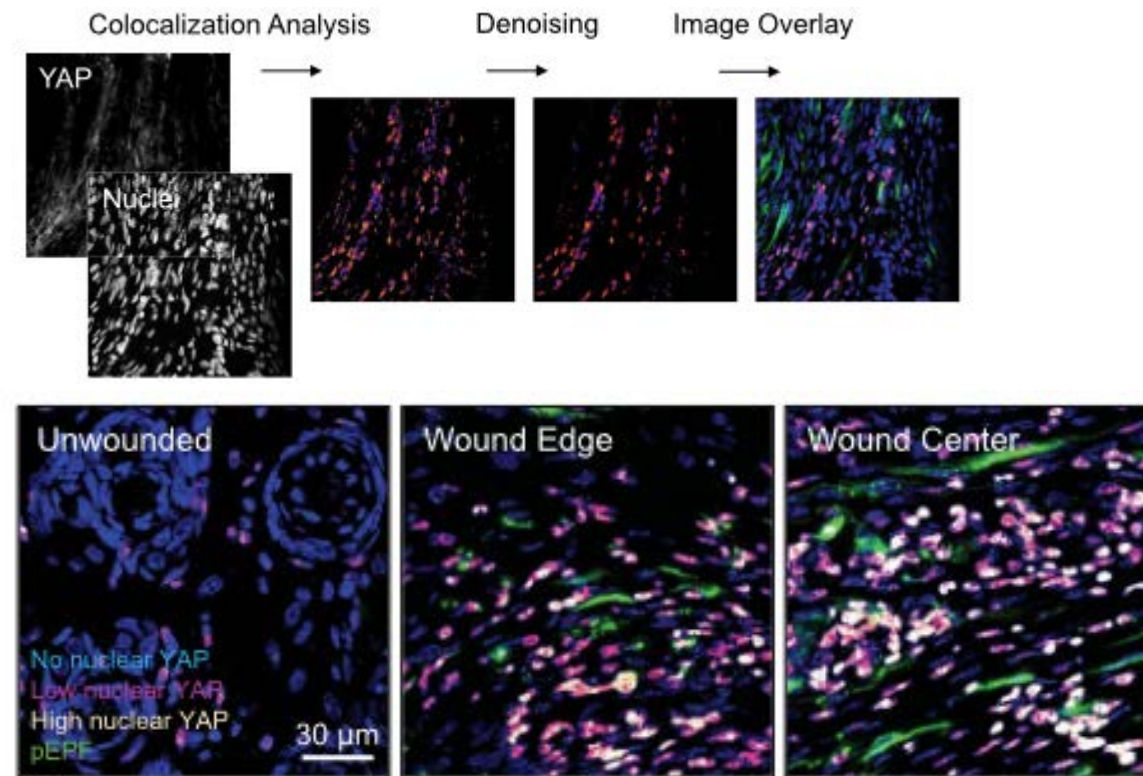
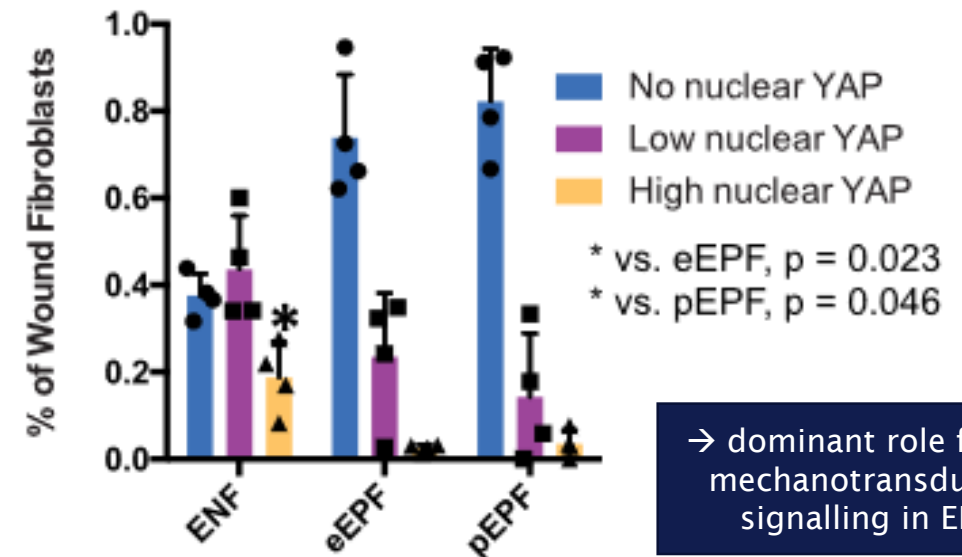


Image-analysis-pipeline to compare YAP-signaling in ENFs & EPFs across time-course of healing:

- EPFs: most no YAP at all time
- ENFs: rapidly activated YAP-signalling, retained high nuclear YAP

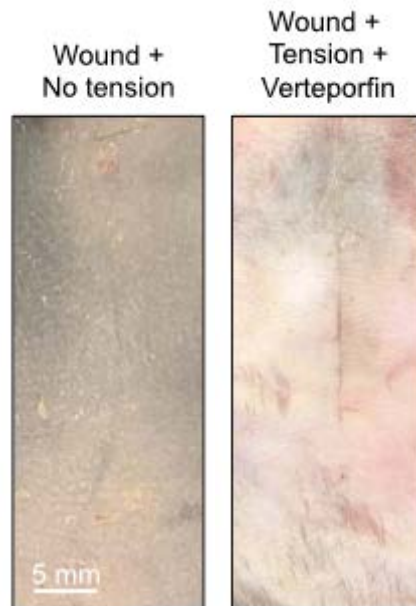


→ dominant role for YAP mechanotransduction signalling in ENFs

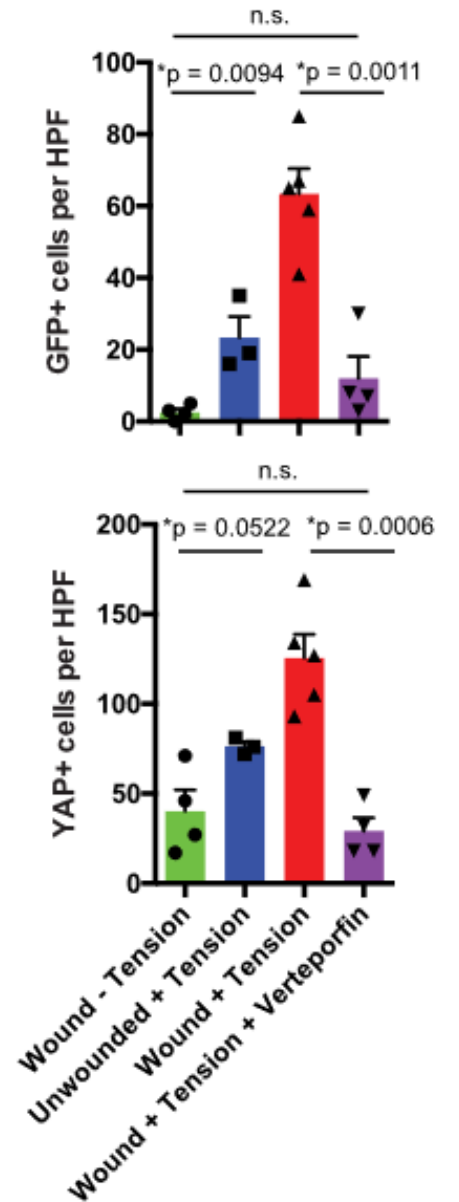
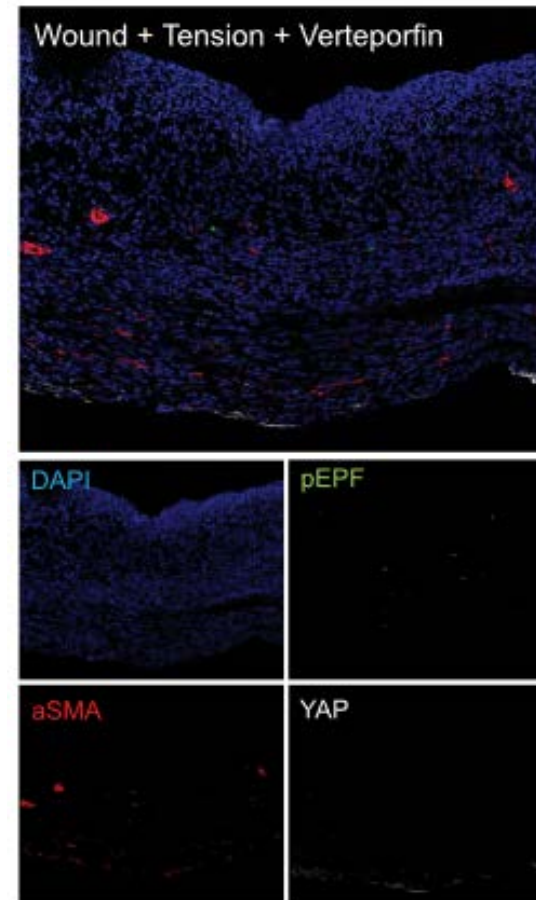
# Blocking the mechanotransductional pathway reduces ENF-to-EPF transition

**Hypothesis:** YAP promotes scarring by driving mechanoresponsive ENF-to-pEPF transition

→ Treated wounds with **verteporfin** (= YAP-inhibitor):  
*mitigated effects of tension*



Wounds resembled non-mechanically loaded wounds

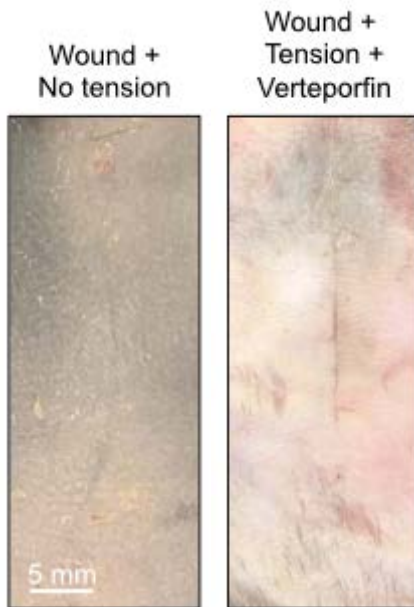




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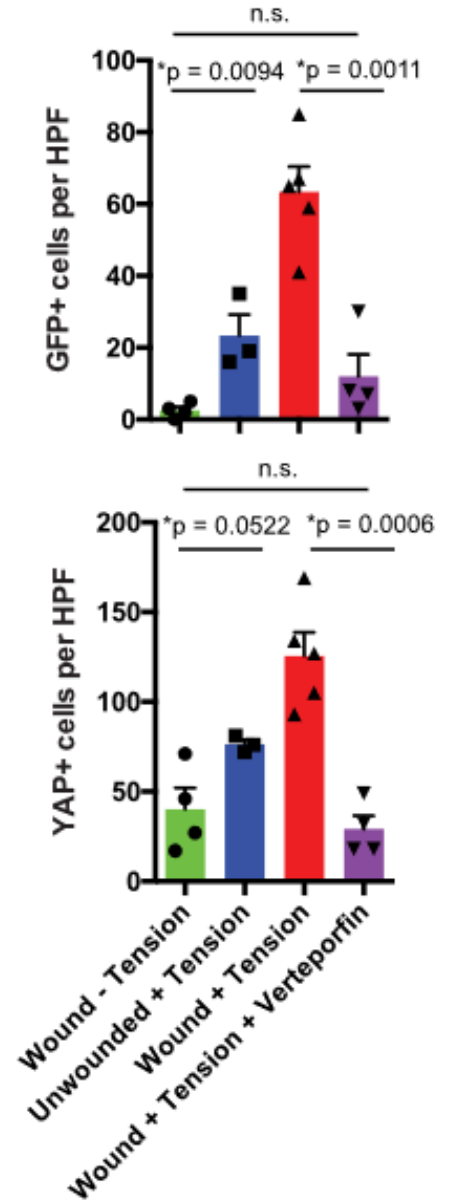
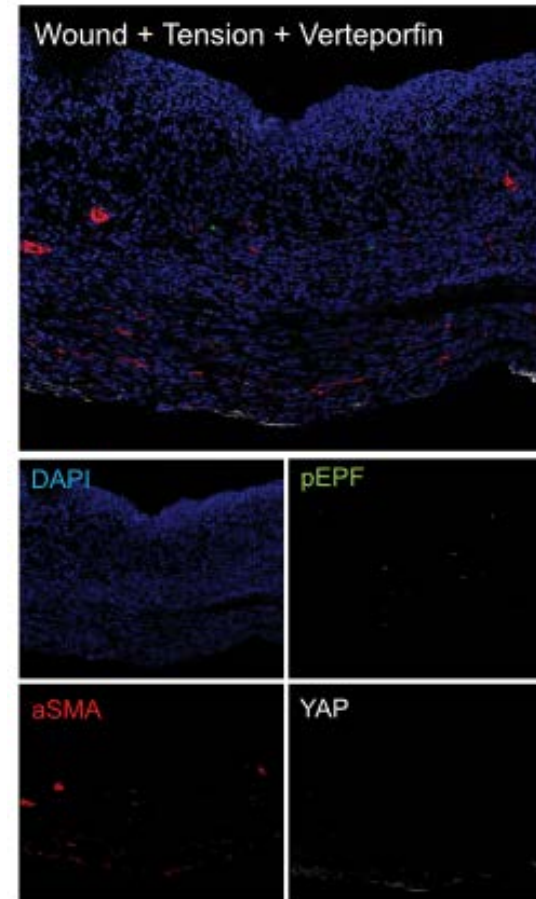
→ Treated wounds with **verteporfin** (= YAP-inhibitor):  
*mitigated effects of tension*



Blocked mechanotransductional pathways → fewer pEPFs

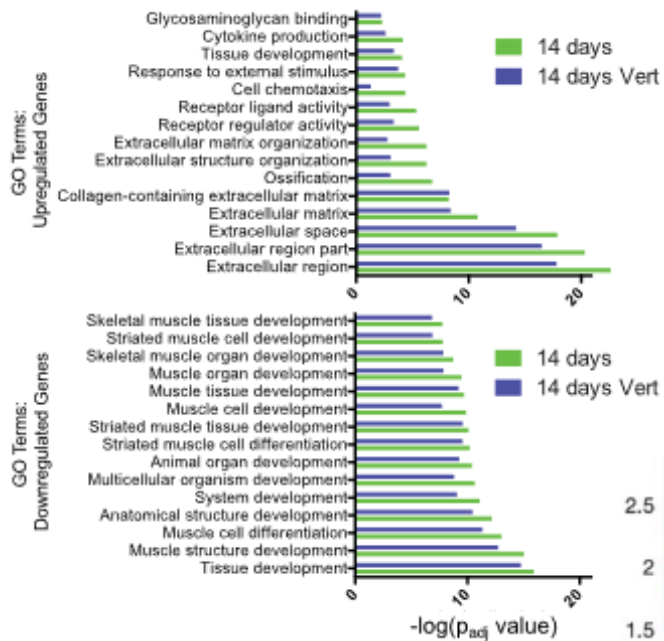
Mechanical tension drives ENF-to-EPF transition

Wounds resembled non-mechanically loaded wounds



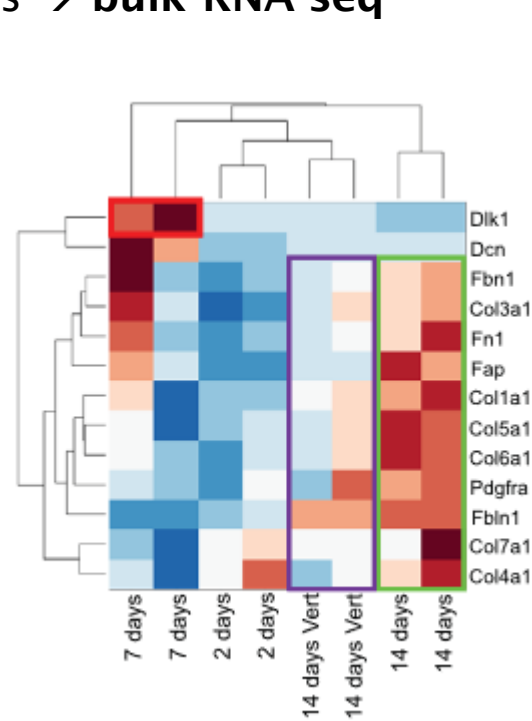
# In vitro investigation of transcriptomic changes reveal gene shift after 14 days, mitigated by Verteporfin

Investigate transcriptomic changes during postnatal En1 activation:  
ENFs grown on TCPS for 2, 7 or 14 days → **bulk RNA-seq**

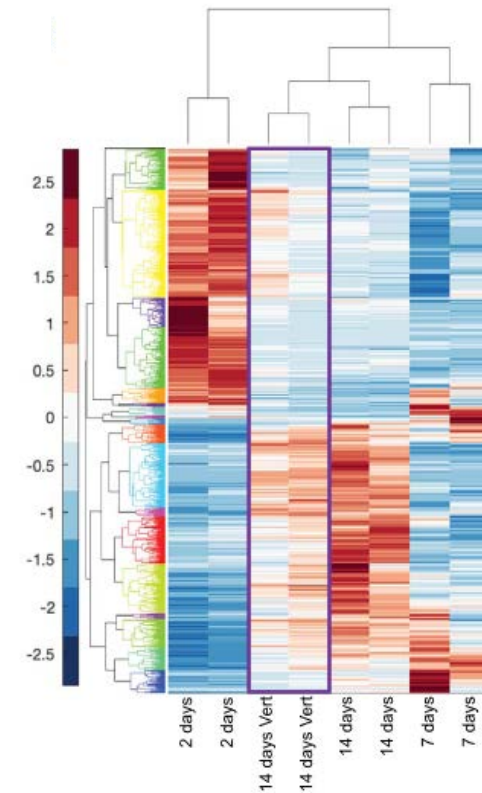


**Upregulation:** ECM deposition related terms (→ profibrotic changes)

**Downregulation:** muscle development related terms (*native ENFs express muscle related genes – might be lost upon mechanical activation*)



**Dlk1** expression up-regulated in ENFs at 7 d  
Profibrotic/matrix genes upregulated at 14d  
Mitigated by verteporfin



Verteporfin-treatment: mitigated transcriptomic shift after 14d

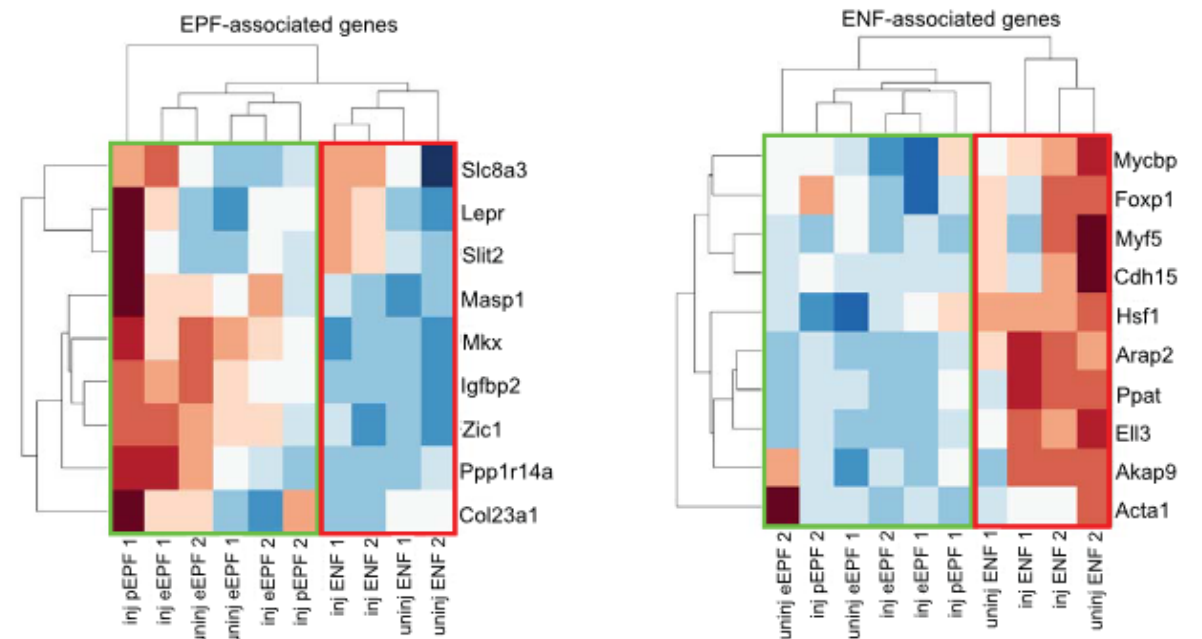
PCA: Verteporfin-treated ENFs resembled untreated cells that had been in culture for 2 days → **closer retention of native ENF identity**

# In vivo transcriptional changes confirm that pEPFs recapitulate eEPF signatures

- Hierarchical clustering of DEGs & PCA
- pEPFs clustered more closely with eEPFs than with ENFs
- Compared transcriptional activity of genes previously reported to differentiate ENFs & pEPFs → pEPFs gene profile resembled that of eEPFs

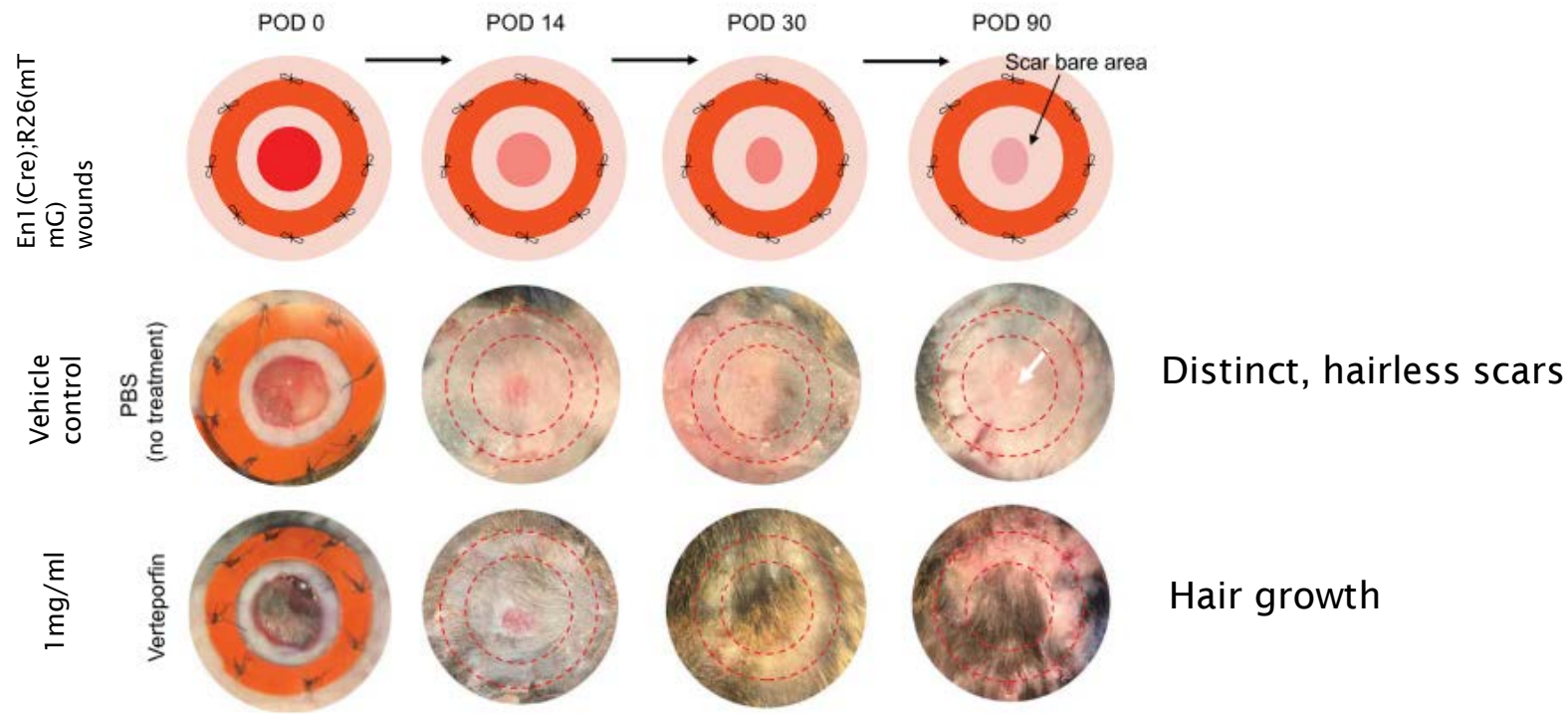


pENF *En1* activation requires a profibrotic, eEPF-like transcriptome (in vitro and in vivo)

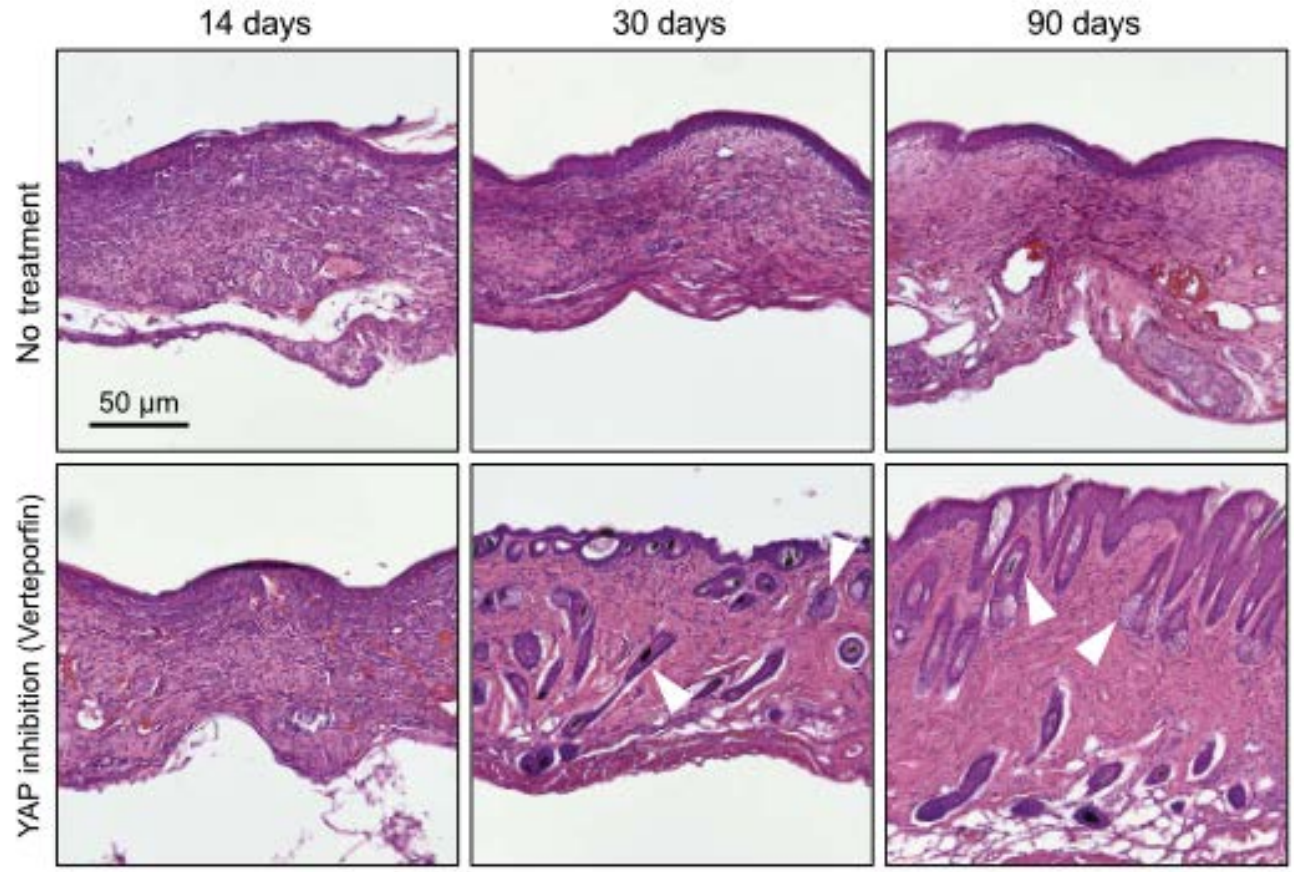


# Modulating YAP signaling promotes regenerative ENF-mediated wound healing

- En1-activation: profibrotic phenotype, blocked by YAP inhibition in vitro
- Can YAP inhibition block En1 activation in vitro → reduced scarring?



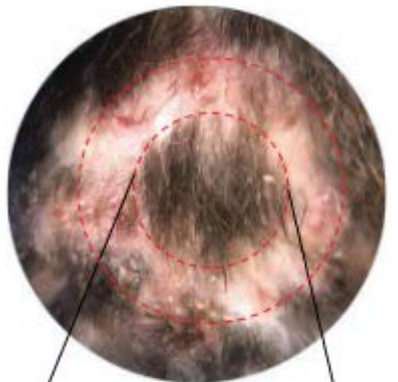
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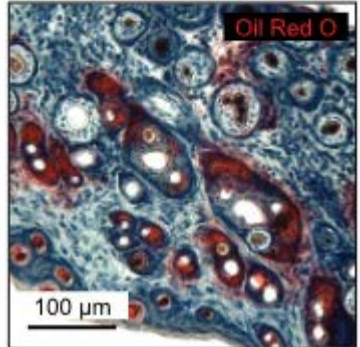
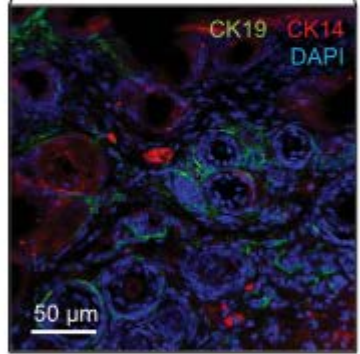
Dense, parallel collagen  
No HFs/SGs

Reduced fibrosis  
Numerous HF/SG-like structures

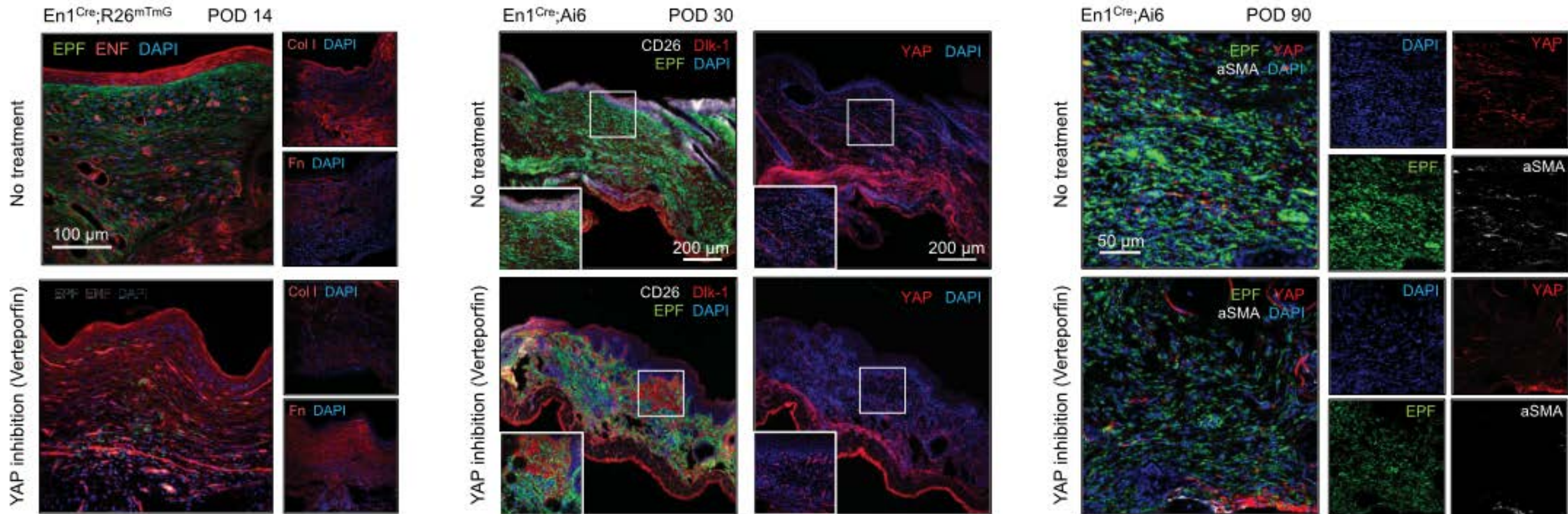
CK14/19-expression + pos. Oil Red O lipid staining  
→ **functional regenerated HF & SG**



YAP inhibition (Verteporfin)



# Fluorescent histology of control- or verteporfin treated wounds



Blocked transition of ENFs into profibrotic pEPFs

Mechanoresponsive Dlk1+/Ska- ENFs proliferate & migrate after wounding → activate En1 to become fibrogenic pEPFs

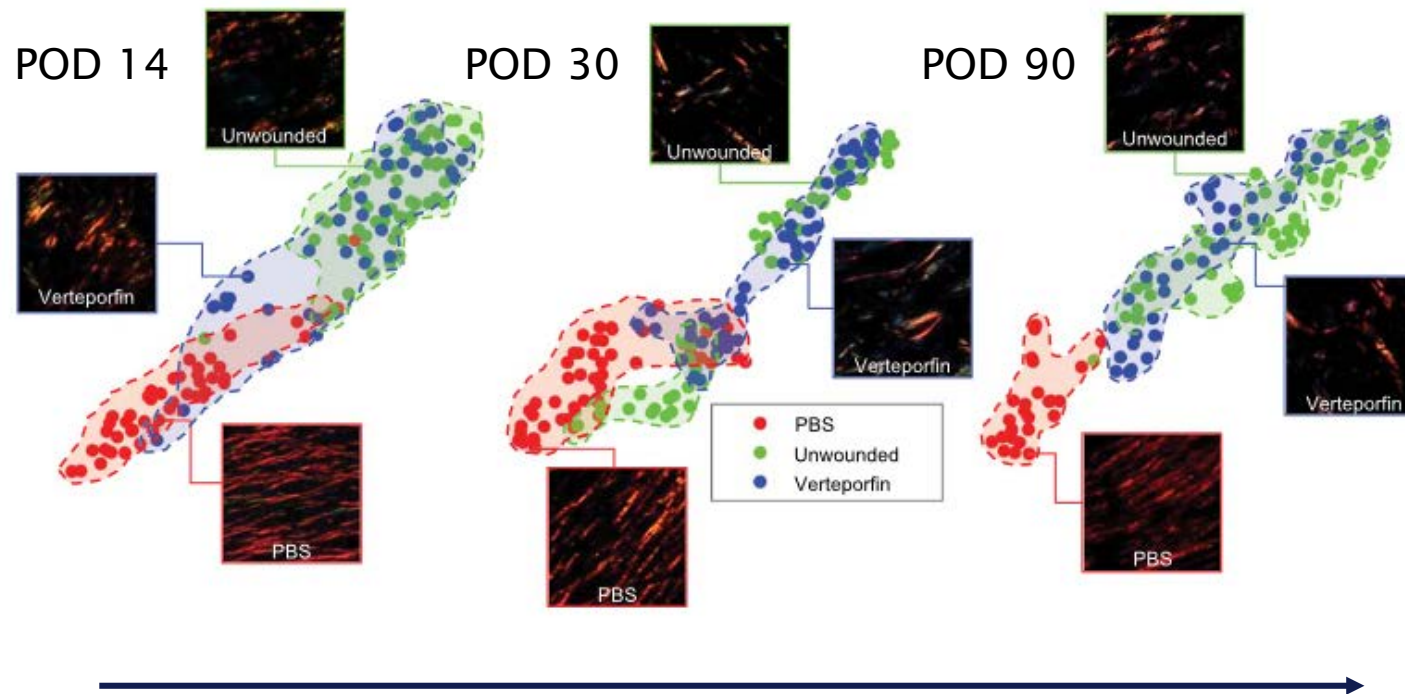
Blocking ENF mechanical activation yields ENF-driven wound regeneration

# YAP inhibition promotes wound regeneration

- **Machine-learning algorithm** to quantify tissue ultrastructure: POD 14 verteporfin-treated wounds distinct from control wounds, **comparable to unwounded skin**
  - Verteporfin treatment at POD 0 yields **long term regenerative remodeling**
- Quantitative analysis confirmed that **YAP inhibition promotes wound regeneration**

## PCA of ECM-parameters:

strong, increasing **overlap** between verteporfin and unwounded skin clusters



# Verteporfin-effect on wounds depends on dosing

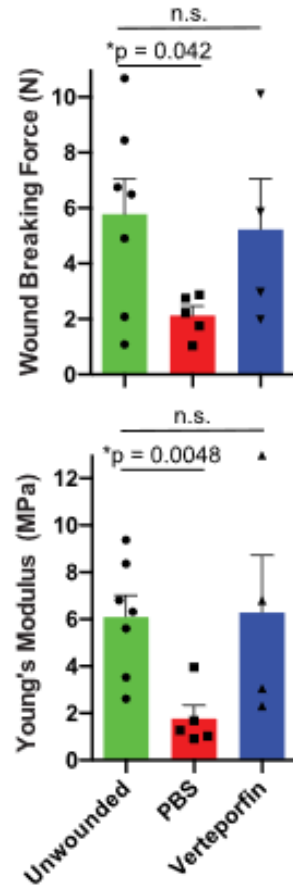
- **2 Doses** (POD 0, 4): healing rates comparable to single-dose effects
- **4 Doses** (POD 0, 4, 8, 12): EPFs almost fully depleted, but:
  - Wound closure delayed
  - Hair growth reduced
  - ECM features not as in unwounded skin

→ **Harmful effects upon excessive dosing**



# Verteporin allows restoration of unwound-like skin strength

- Scars: <80% of skins' strength
- POD 30:



**PBS:** reduced tensile strength

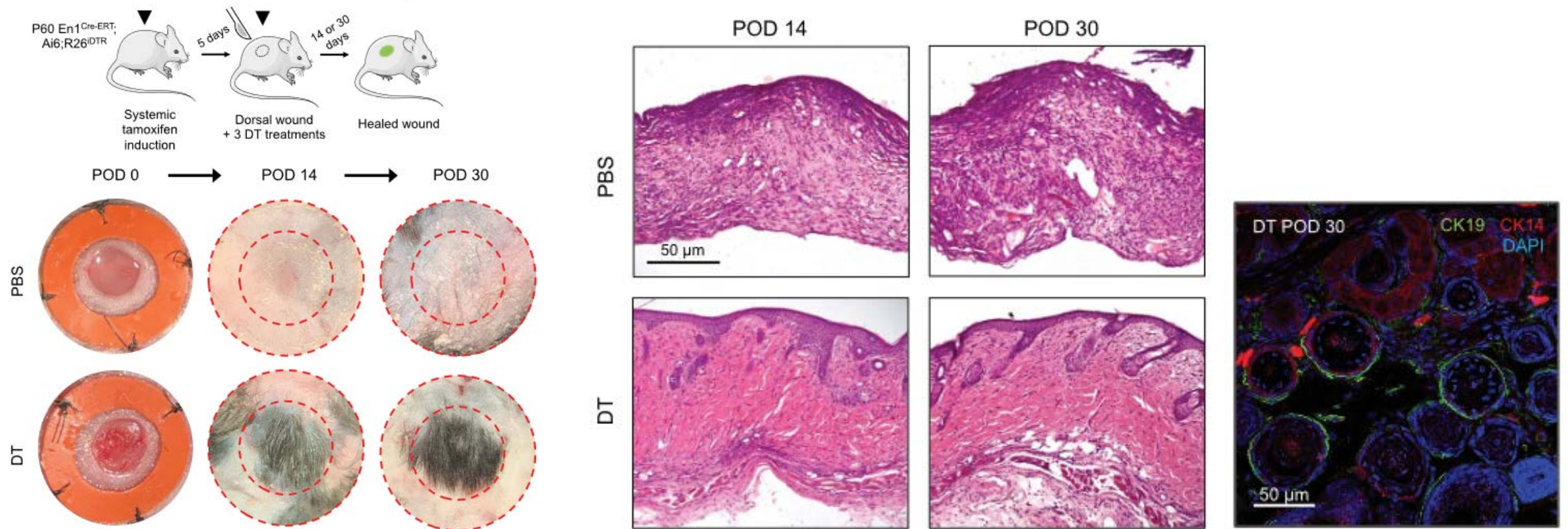
**Verteporfin-group:** restoration of unwound-like strength

# Investigation of En1 in vitro using En1 knockdown in ENFs

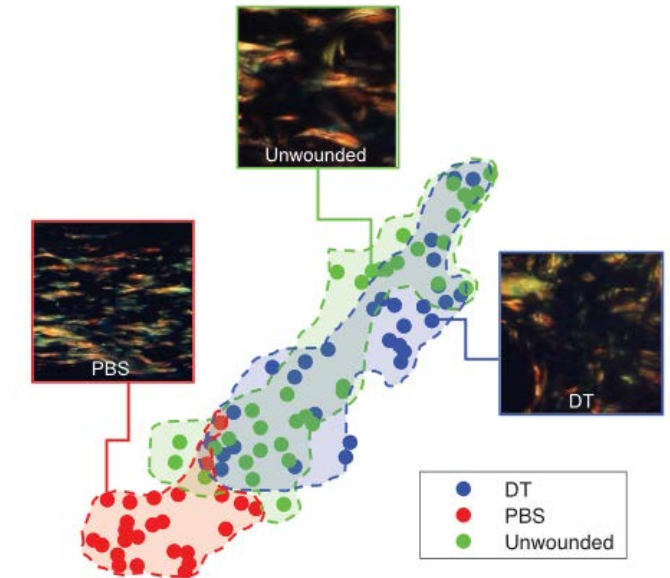
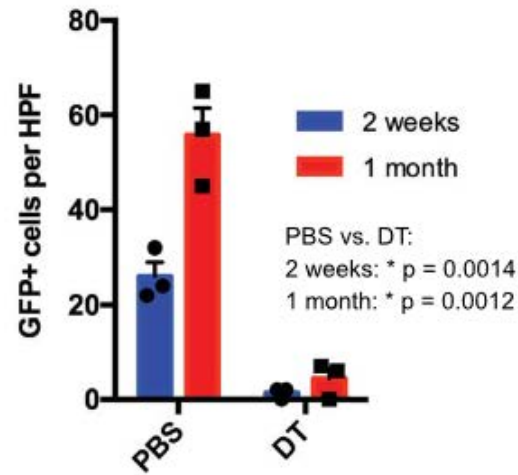
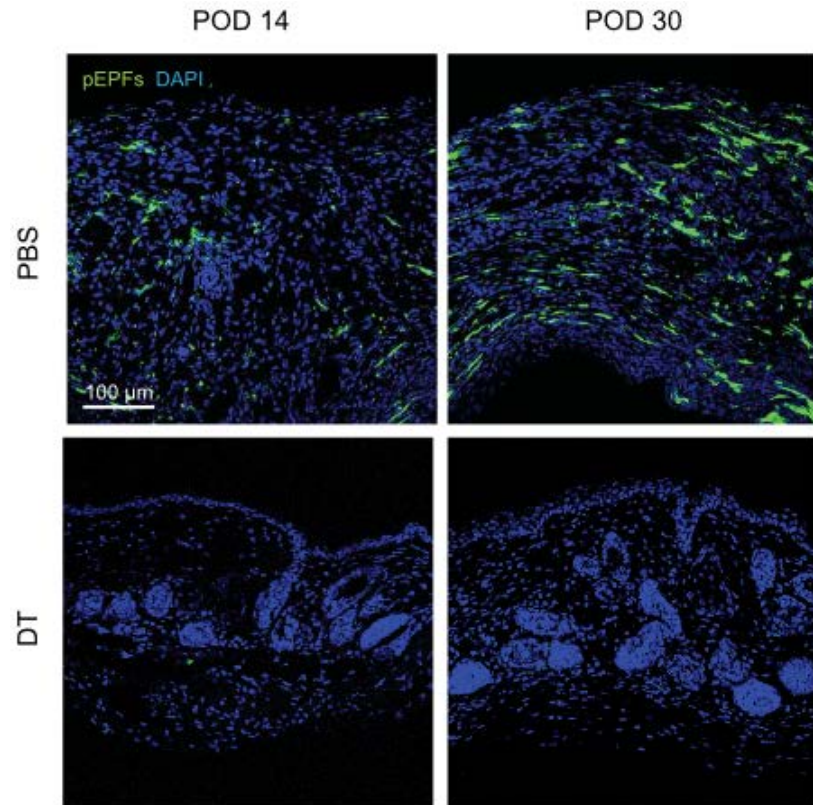
- shRNA to achieve long-term En1 knockdown in ENFs over 14 days on TCPS
  - RNA-seq: comparison to ENFs treated with nontargeting control shRNA
    - Decreased ECM production & deposition
    - GSEA: Downregulation across mechanotransduction (Rho/Notch/Hippo) + fibrosis (Jun/TGF $\beta$ ) pathways
- En1 knockdown decreases mechanically induced fibrogenic changes

# Investigation of En1 in vivo: diphtheria toxin ablation of En1-expressing FBs

Diphtheria toxin (DT) → Ablation of pEPFs in En1(Cre-ERT);Ai6;R26 iDTR mice



# Investigation of En1 in vivo: diphtheria toxin ablation of En1-expressing FBs



**ECM architecture:**

**PBS:** minimal to no overlap with unwounded skin  
**DT-treated wounds** virtually complete overlap with unwounded skin

Blocking En1-activating FBs is sufficient for wound regeneration  
 En1 is itself a mechanoresponsive master regulator of FB activation

# Confirmation of previous results with YAP knockout-mice

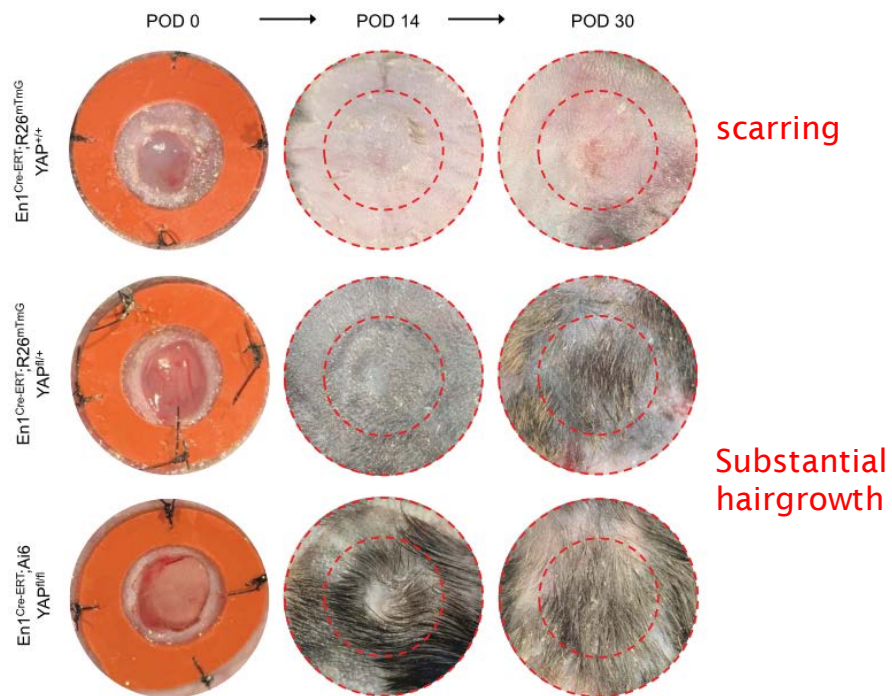
Confirm that regeneration under verteporfin actually resulted from modulated mechanotransduction & outrule off-target drug effects →  
**knockout mice**

- En1Cre-ERT;R26 mTmG;YAP fl/+ (**YAP fl/+** )
- En1 Cre-ERT;Ai6;YAP fl/fl (**YAP fl/fl**)
- En1Cre-ERT;R26 mTmG;YAP+/+ (**YAP+/+**; *control*)
- Tamoxifen induced, harvested wounds POD 14 & 30

# Confirmation of previous results with YAP knockout-mice

Confirm that regeneration under verteporfin actually resulted from modulated mechanotransduction & outrule off-target drug effects → **knockout mice**

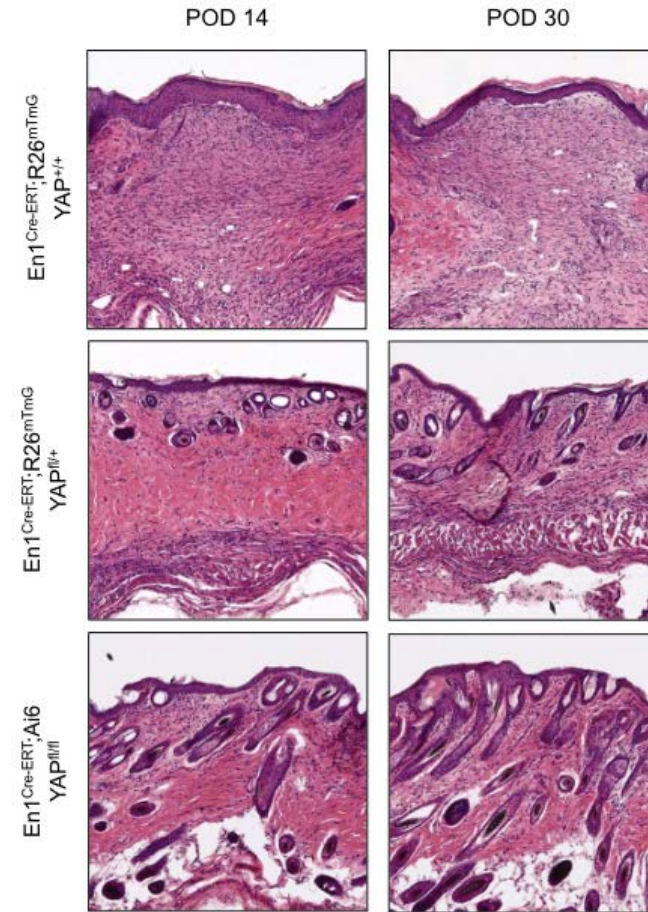
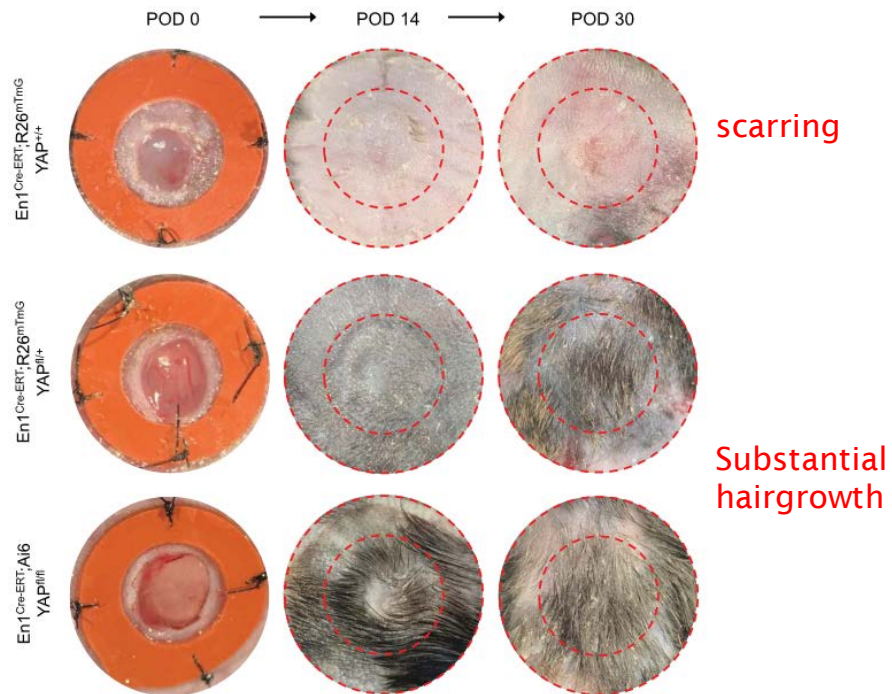
- En1<sup>Cre-ERT</sup>;R26<sup>mTmG</sup>;YAP<sup>fl/+</sup> (**YAP fl/+**)
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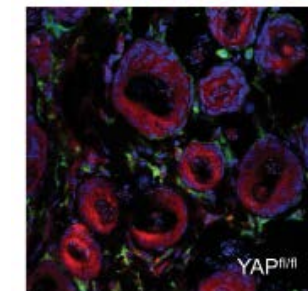
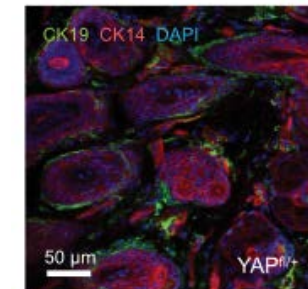
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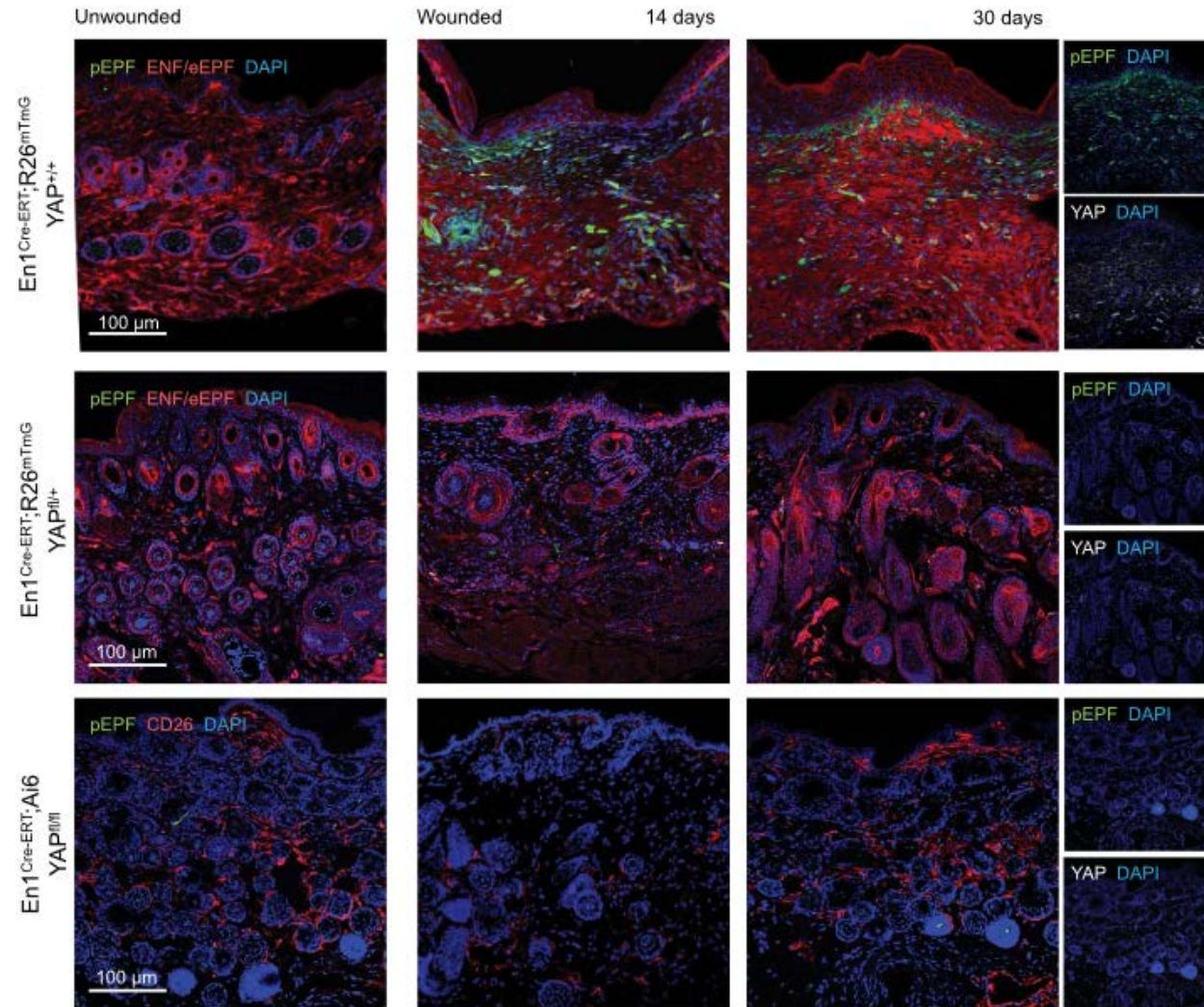


## Knockout mice:

- **POD 14:** regenerated HF/SG-like structures
- **POD 30:** fully developed CK14/19+ appendages



# YAP is required for postnatal En1-activation



*Unwounded skin:* no pEPFs

*Wounded:*

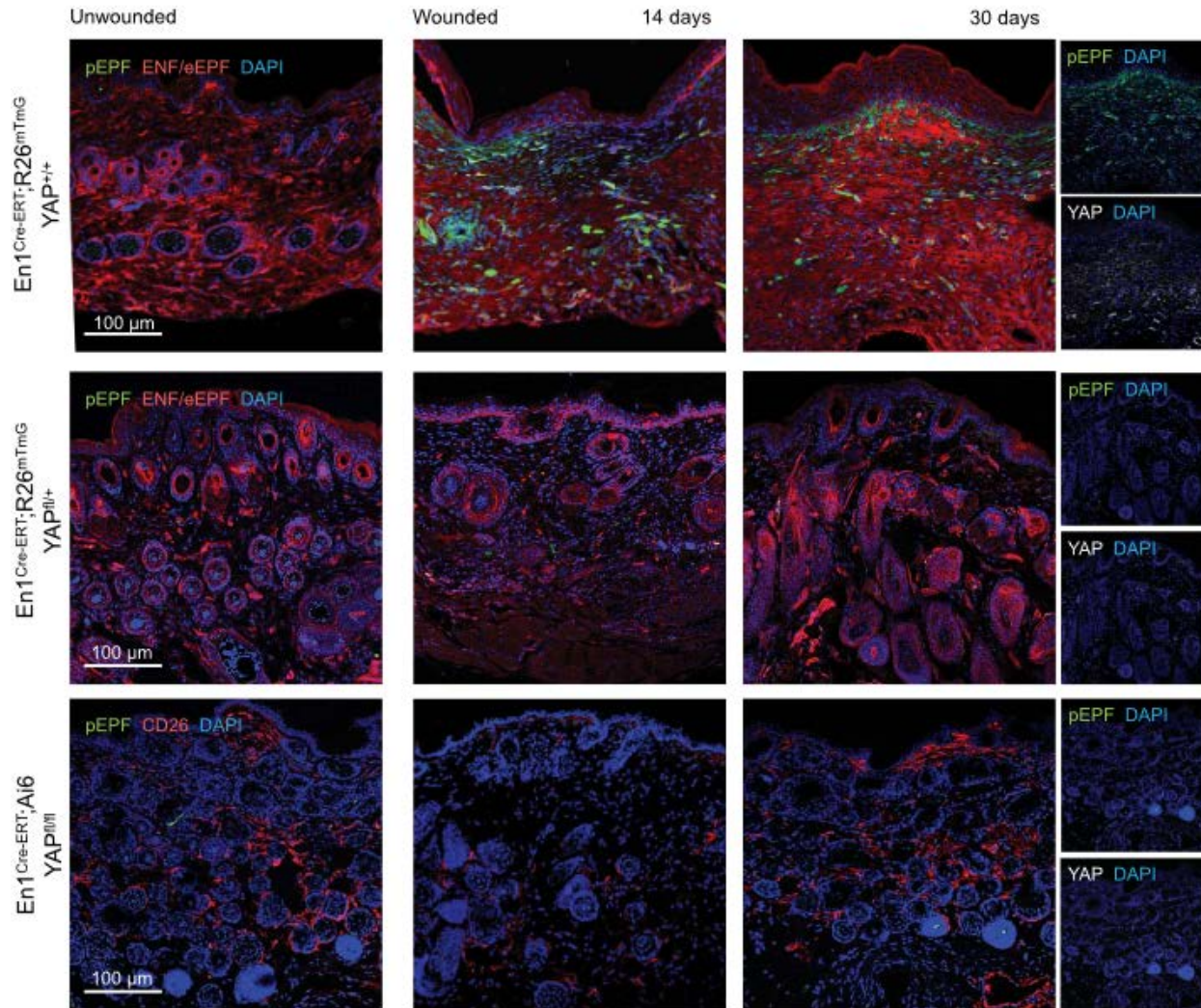
- YAP<sup>+/+</sup>: numerous pEPFs
- YAP<sup>fl/+</sup> & YAP<sup>fl/fl</sup>: rare pEPFs

→ YAP required for postnatal En1 activation

Confirmed by immunofluorescent histology



# YAP is required for postnatal En1-activation

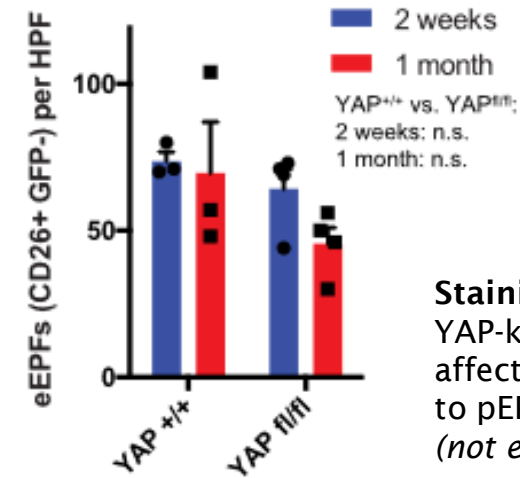


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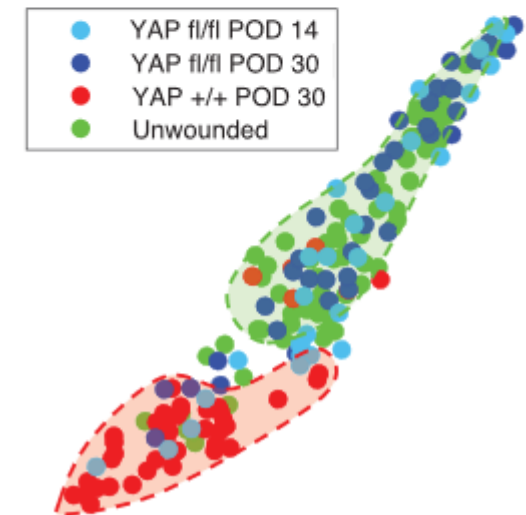
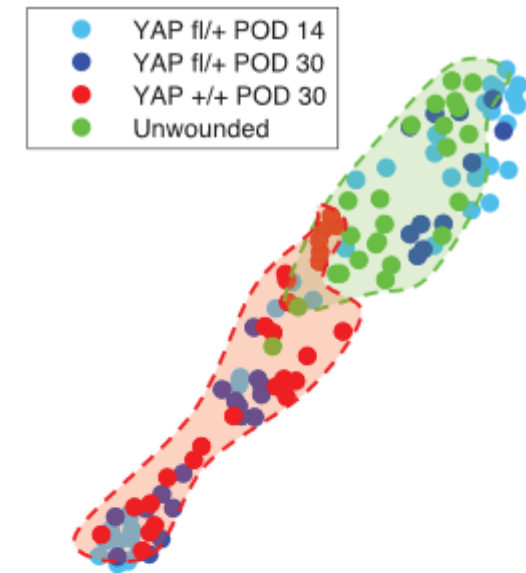


Staining for CD26 (Dpp4):  
YAP-knockout primarily affects ENFs & conversion to pEPFs  
(not existing eEPFs)

Confirmed by immunofluorescent histology

# ECM ultrastructure quantitation

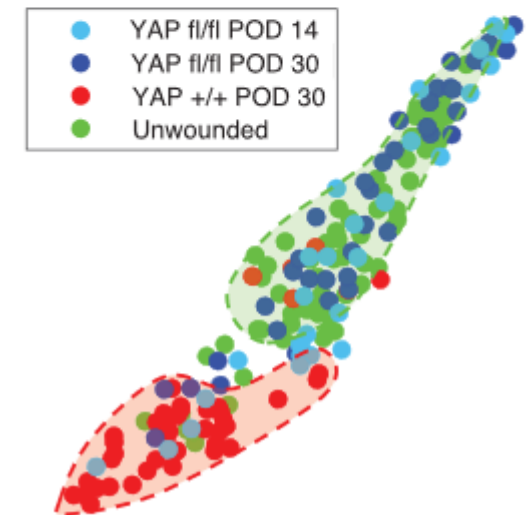
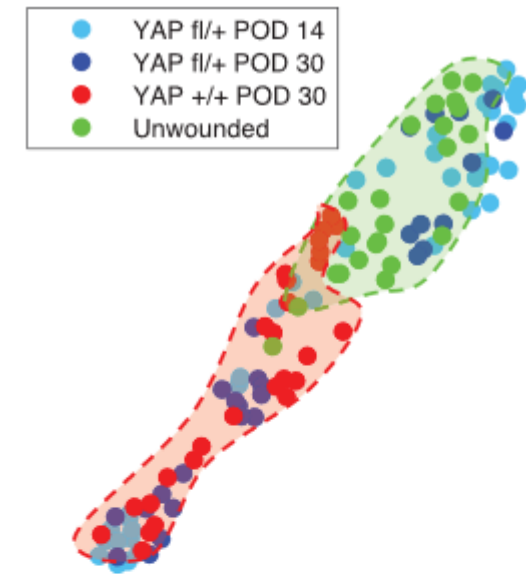
- YAP fl/+ wounds more similar to unwounded skin than YAP +/- wounds
- **Heterozygous** YAP-deletion insufficient for complete ultrastructural regeneration
- **Homozygous** wounds indistinguishable from unwounded skin
- May reflect YAP signaling „*dose-responsiveness*“



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“These findings support a functional role for YAP signaling in postnatal En1 activation and scarring, because genetic YAP blockade in mechanoresponsive fibroblasts resulted in fewer pEPFs and regeneration.”



# What they did

- FB-transplantation & transgenic mouse models to trace En1 expression
- Studies FBs responses to mechanical forces in vivo & in vitro
- Chemical (verteporfin) & transgenic inhibition of mechanotransduction signaling (diphtheria toxin ablation of En1-expressing FBs, floxed YAP knockout) to modulate En1 expression during wound healing
- Compared wounds to unwounded skin & scars by RNA-seq, quantitative histopathological comparison, mechanical strength testing

# What they found out

- ENFs in wound environment generate **~40-50% of scar FBs**
- ENF-to-pEPF transition depends on **mechanical cues**
- En1 regulates a **wide array of genes** related to skin fibrosis (comparison of ENFs with En1 expressing and En1 knockdown FBs by RNA-seq)
- Healing wounds: YAP inhibition by verteporfin blocks En1 activation, promotes ENF mediated repair → **skin regeneration in 30days with recovery of HF + SG**
- YAP inhibition induces recovery of **normal mechanical breaking strength**
- DT mediated ablation of pEPFs & FB targeted transgenic YAP knockout promoted recovery of normal skin structures → **modulation of En1 activation (direct & indirect) yields wound regeneration**

# Discussion

Thank you!