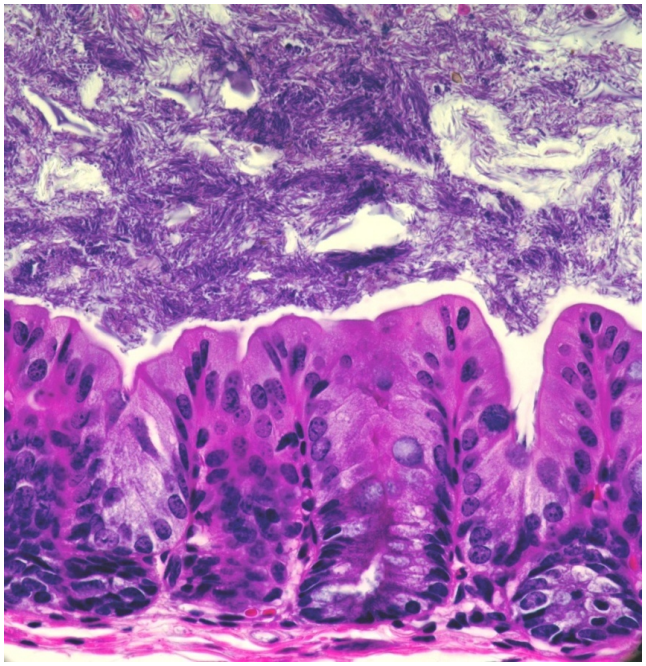
Control of Epithelial Homeostasis by the Microbiota



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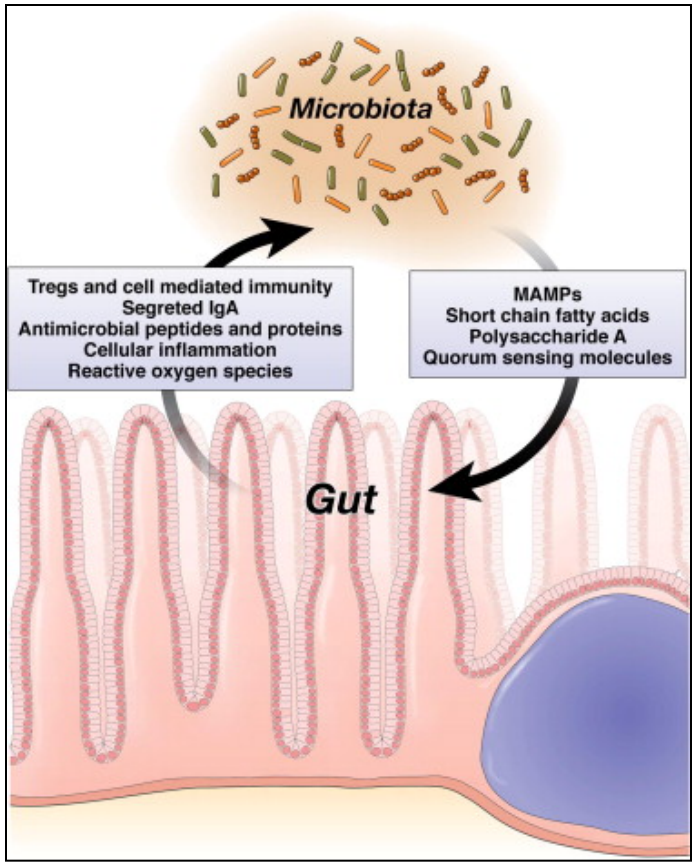
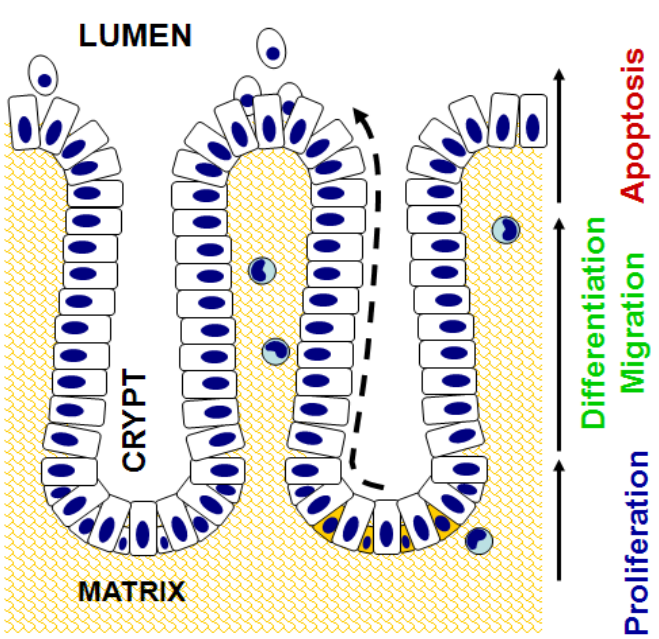
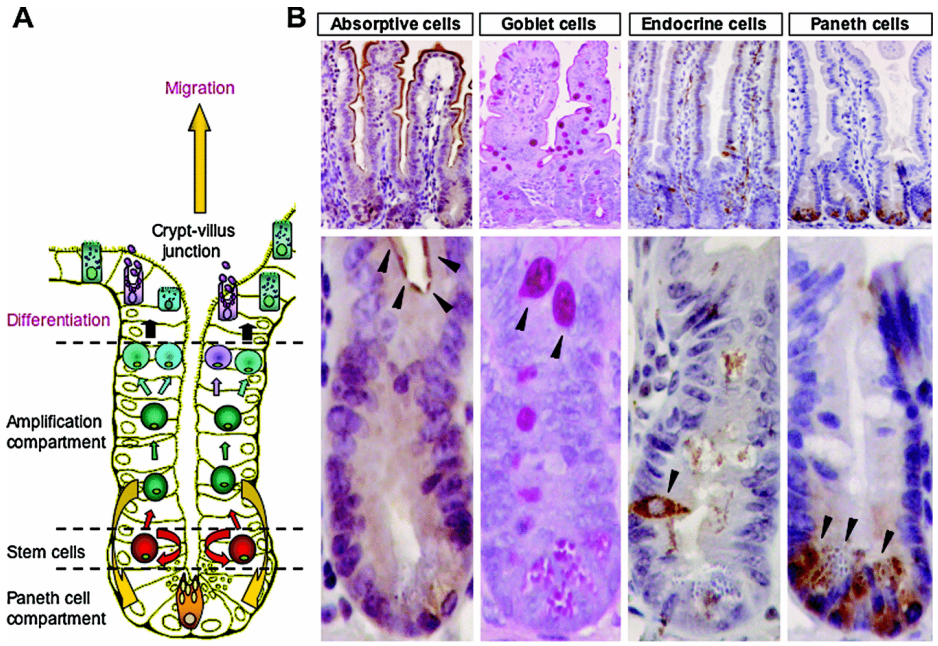
Effects of the microbiota on the gut

- Competitive exclusion of pathogens
- Metabolic/nutritional/energy utilization
  - Vitamin synthesis
  - SCFA as energy source- role in obesity
- Adaptive Immune Regulation,
  - Induction of Immunosuppressive T cells (Tregs)
- Innate Immune Regulation
  - Dampening of inflammatory responses
- Epithelial development and survival
  - Cytoprotective effects of PRR signaling
  - Stimulation of barrier function, IEC restitution, proliferation



The Intestinal Epithelia (motility, proliferation, differentiation)





Germ-free studies

- Small intestinal crypts exhibit a slower turnover of the epithelial cells, with crypt to villus transit time doubling
- Markedly attenuated regenerative responses to colonic injury

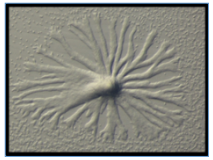
The microbiota can influence normal homeostasis of the gut aside from traditional innate immune responses

Question:

How the normal microbiota mechanistically interacts with the epithelia is not well understood

How the microbiota can influence epithelial growth and proliferation is not well understood

Influence of Reactive Oxygen Species (ROS) on cell proliferation/differentiation



Bloomfield G, et al. **Superoxide signaling required for multicellular development of *Dictyostelium*.** 2003, *J. Cell Sci.* 116:3387

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Owusu-Ansah E, Banerjee U. **ROS prime Drosophila haematopoietic progenitors for differentiation.** 2009, *Nature.* 461:537

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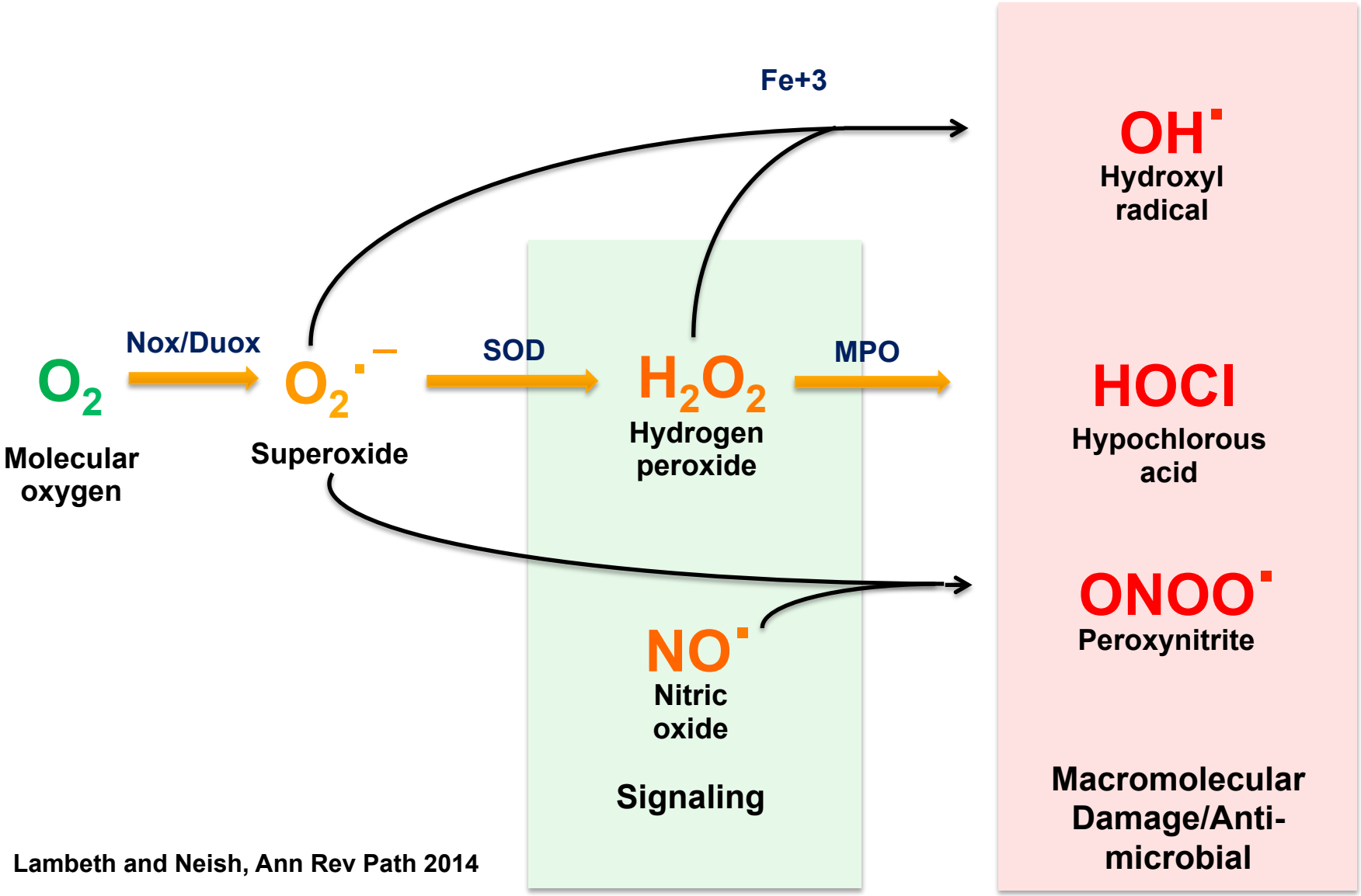
Morimoto H, et al. **ROS Are Required for Mouse Spermatogonial Stem Cell Self-Renewal.** 2013, Cell Stem Cell. 12:774

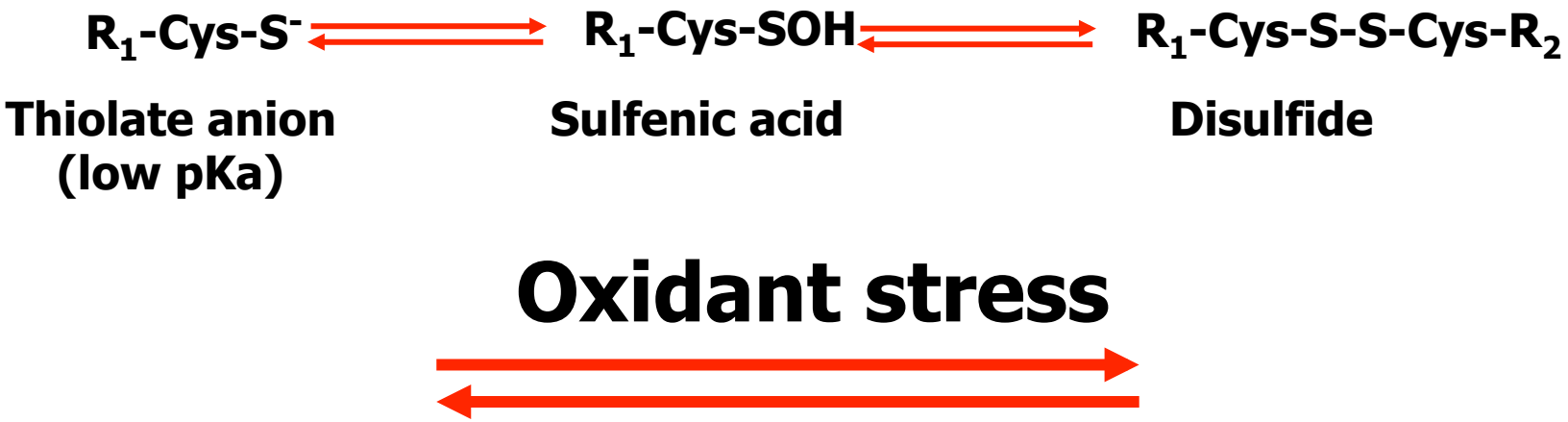
Reactive Oxygen Species



Lambeth and Neish, Ann Rev Path 2014

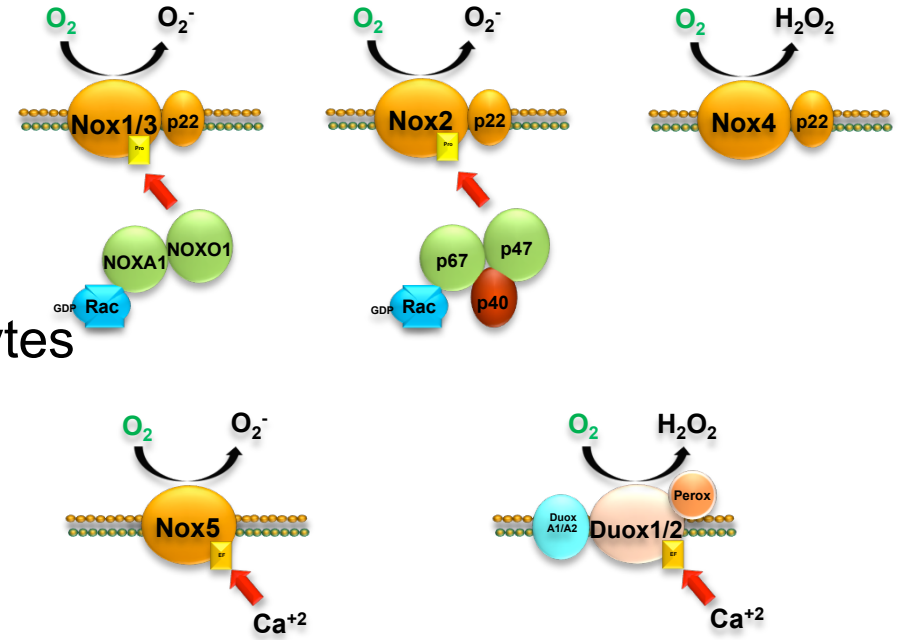
Redox regulation of enzymes

- Mediated by transient oxidation of low pKa catalytic cysteines
- Rapid, reversible and highly localized
- Known target enzymes:
  - Ub like protein ligases; SUMO, Ubc12
  - Dual specificity protein kinases
  - LMW-PTPases
  - Keap1



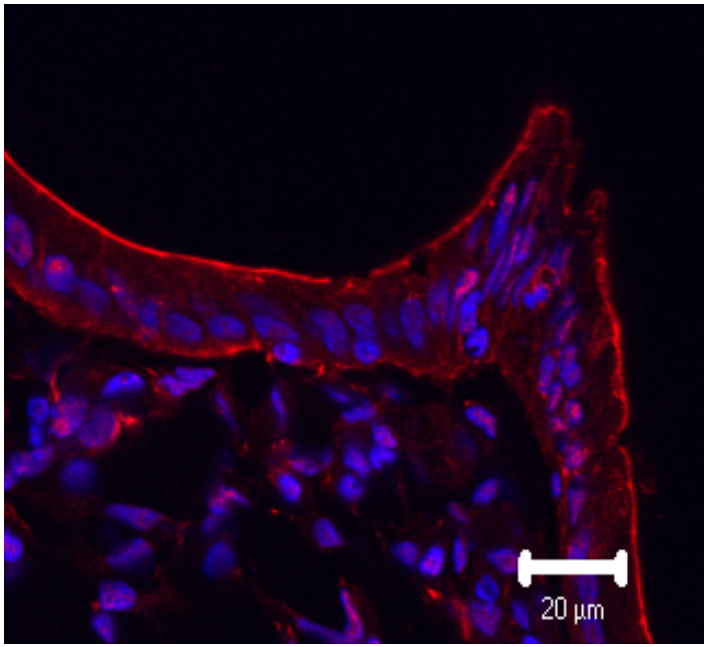
Induced ROS as a response to microbes

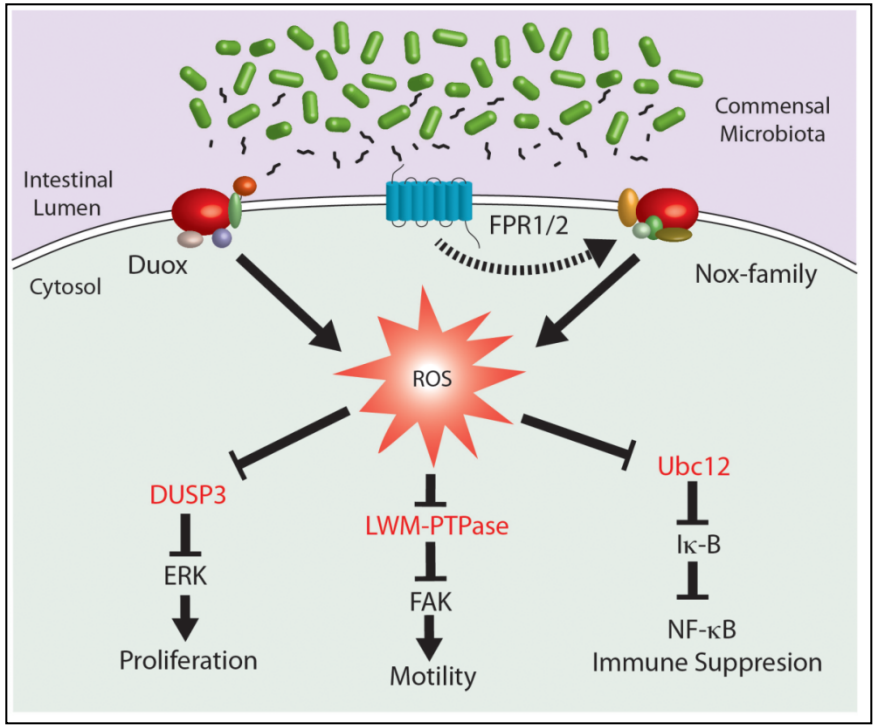
- Plants
  - Defensive: Immediate response, lignification response
  - Root development
- C. elegans
  - Defensive: anti-microbial, ceDuox/BLI-3
  - Cuticle development
- Drosophila
  - Anti-microbial (dDuox)
  - Gut epithelial homeostasis (Nox)
- Mammalian “professional” phagocytes
  - Microbiocidal oxidant burst (Nox2)
- Mammalian barrier epithelia
  - Signaling and homeostatic function (Nox1)



- **Strong conservation of NADPH generating enzymes (Nox’s)**

The Formyl Peptide Receptor pathway





Prokaryotic regulation of epithelial responses by inhibition of IkB-a ubiquitination, Science. 2000; 289, 1560-1563.
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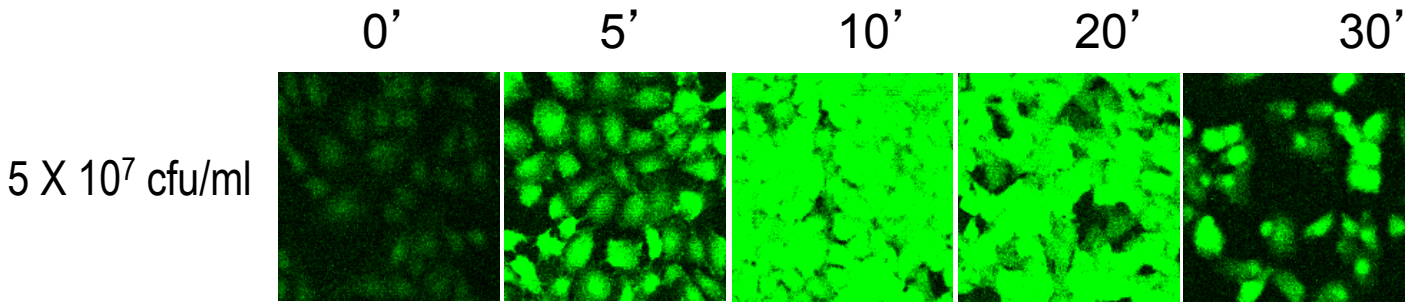
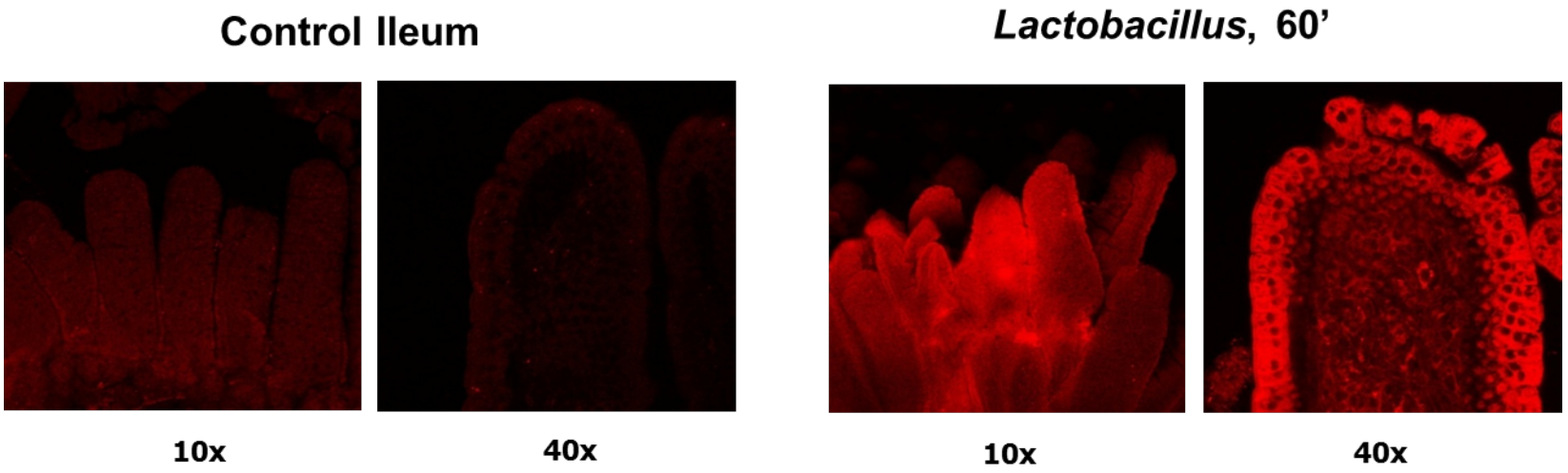
Enteric commensal bacteria potentiates epithelial restitution via ROS-mediated inactivation of FAK phosphatases. Proc. Natl. Acad. Sci. 2011;108:8803-8
Enteric commensal bacteria induced ERK via FPR dependent redox modulation of DUSP3. J. Biol. Chem, 2011; 286:38448-38455.
Annexin A1- FPR1-Nox1 dependent redox signaling promotes epithelial wound repair. J Clin Invest, 2013; 123(1):443-54

Commensal bacteria induce rapid epithelial ROS generation *in vitro* and *in vivo*

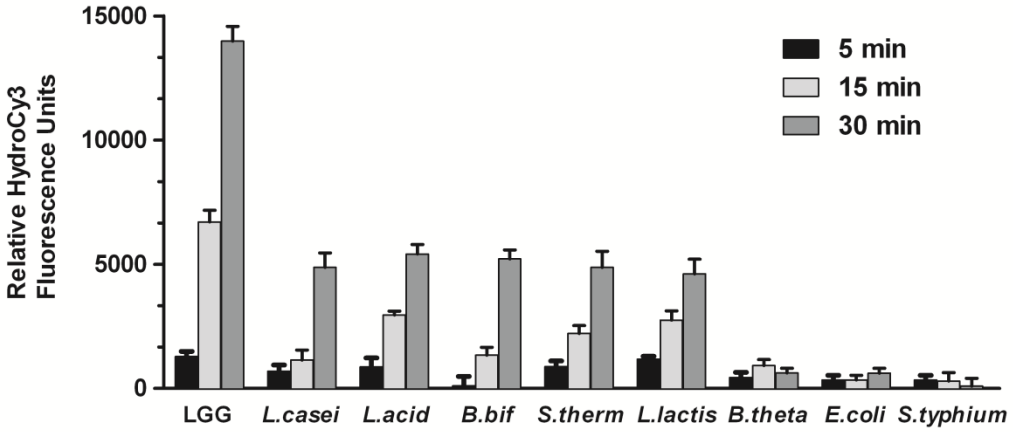


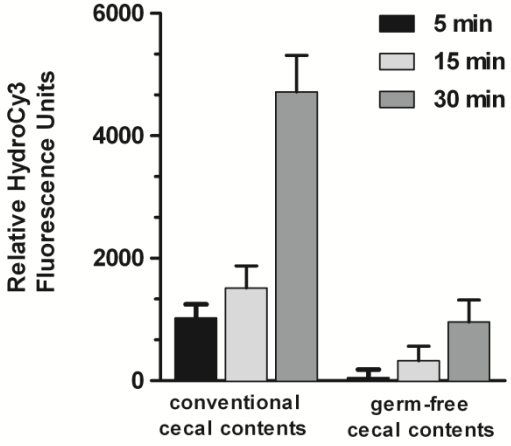
Lactobacillus **colonized onto IEC-6 monolayers prior to DCF detection of ROS**

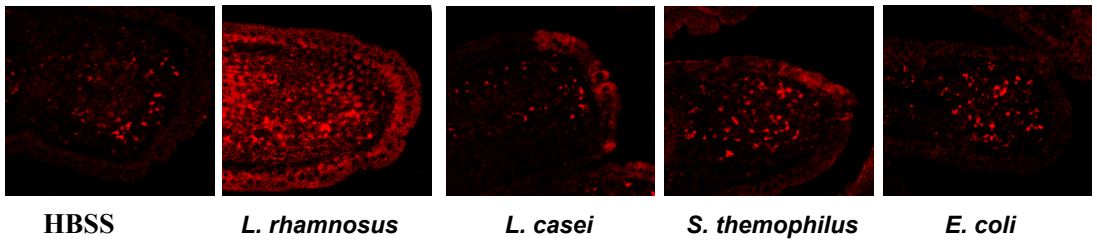


Conventional 10 week female mice were fed cultures of *Lactobacillus* or sterile PBS control for 60 minutes. Hydrocyanine-Cy3 reagent dosed i.p. 20 min prior.

Differential ROS induction by distinct taxa of commensal bacteria *in vitro* and *in vivo*



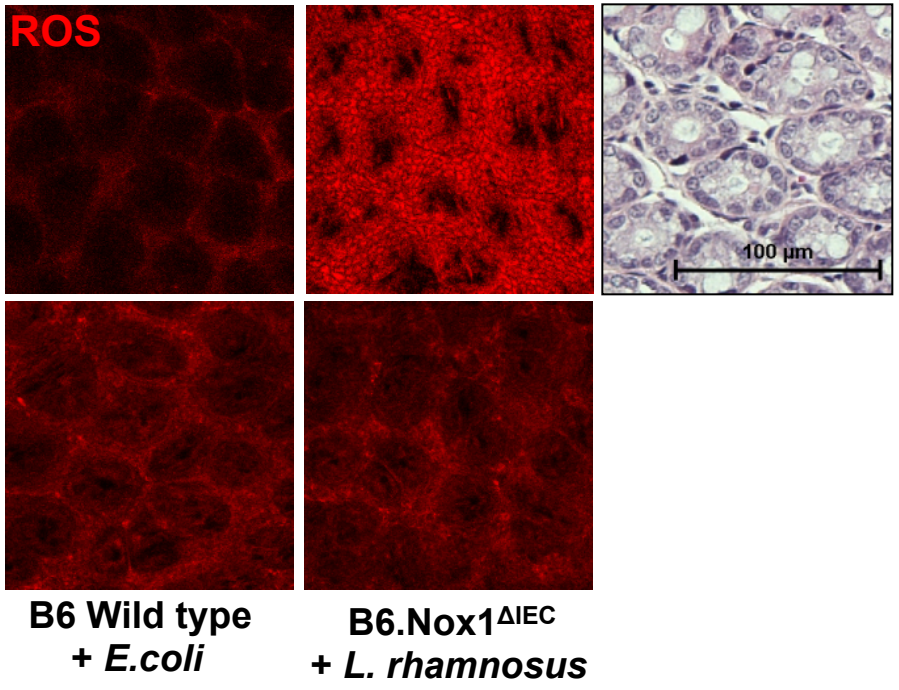




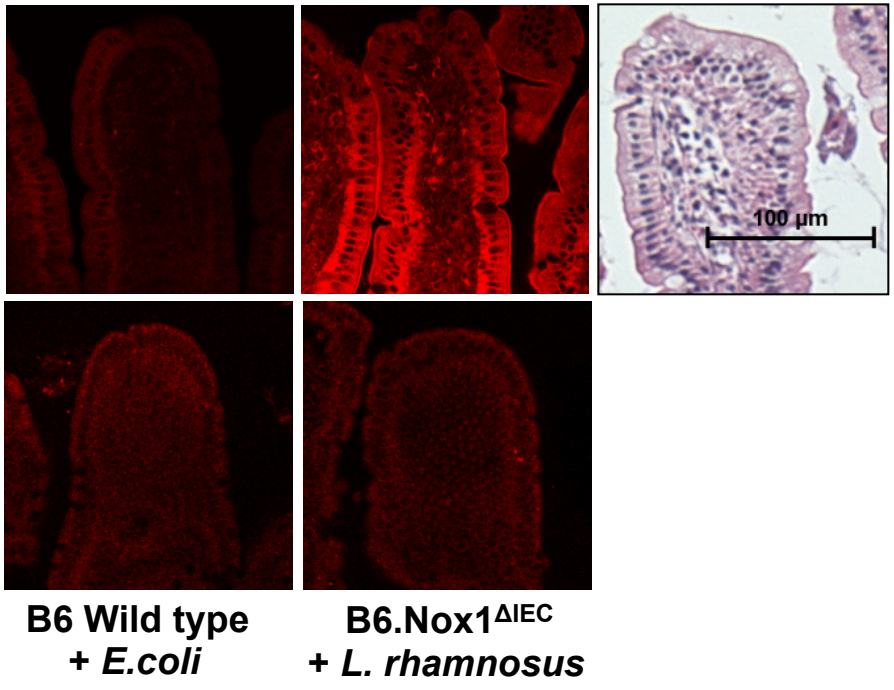
Mouse jejunum

Colonization of intestine with *Lactobacillus* induces Nox1-dependent generation of cellular ROS

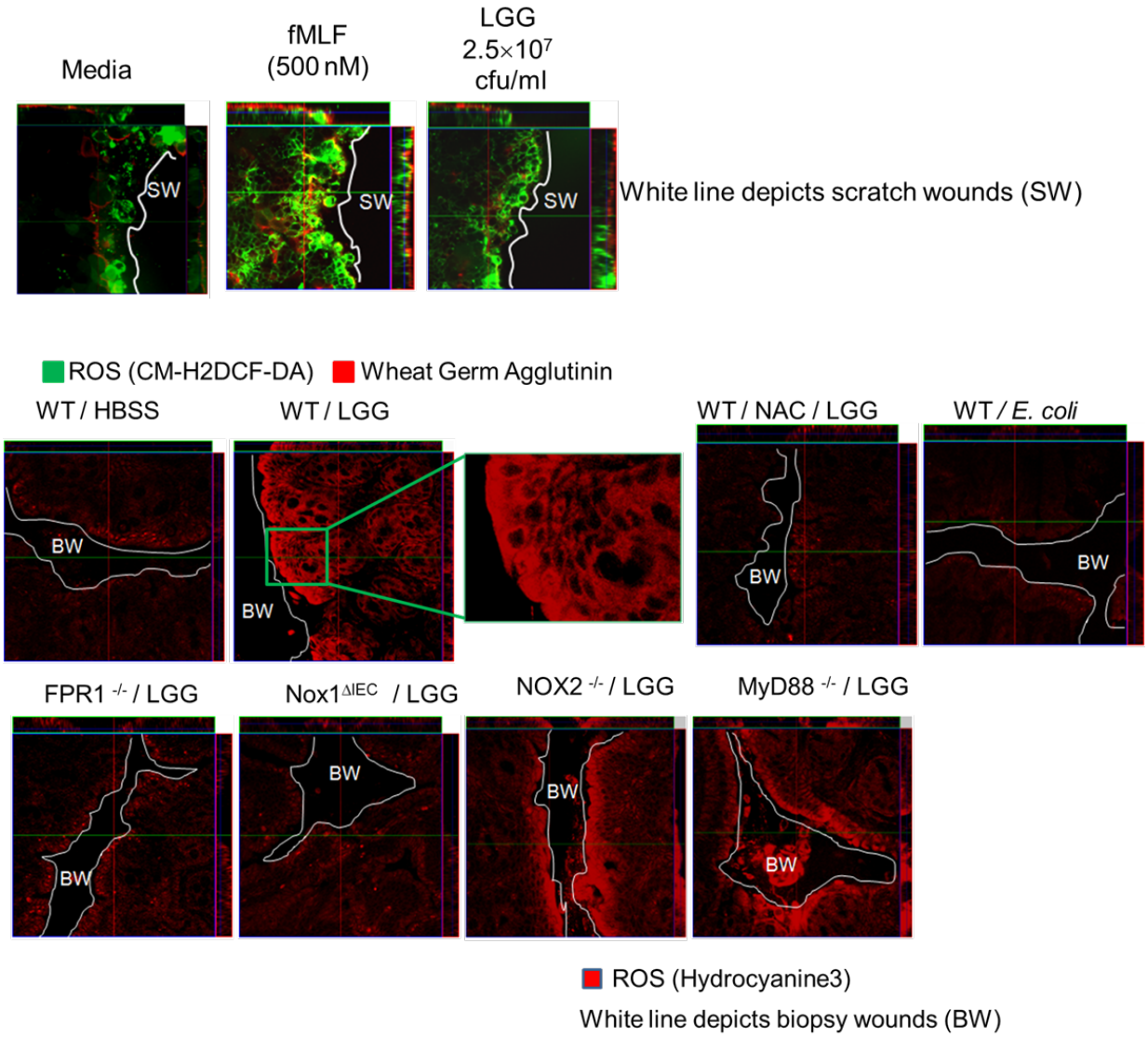
6-week-old colonic tissue



6-week-old distal small intestine tissue

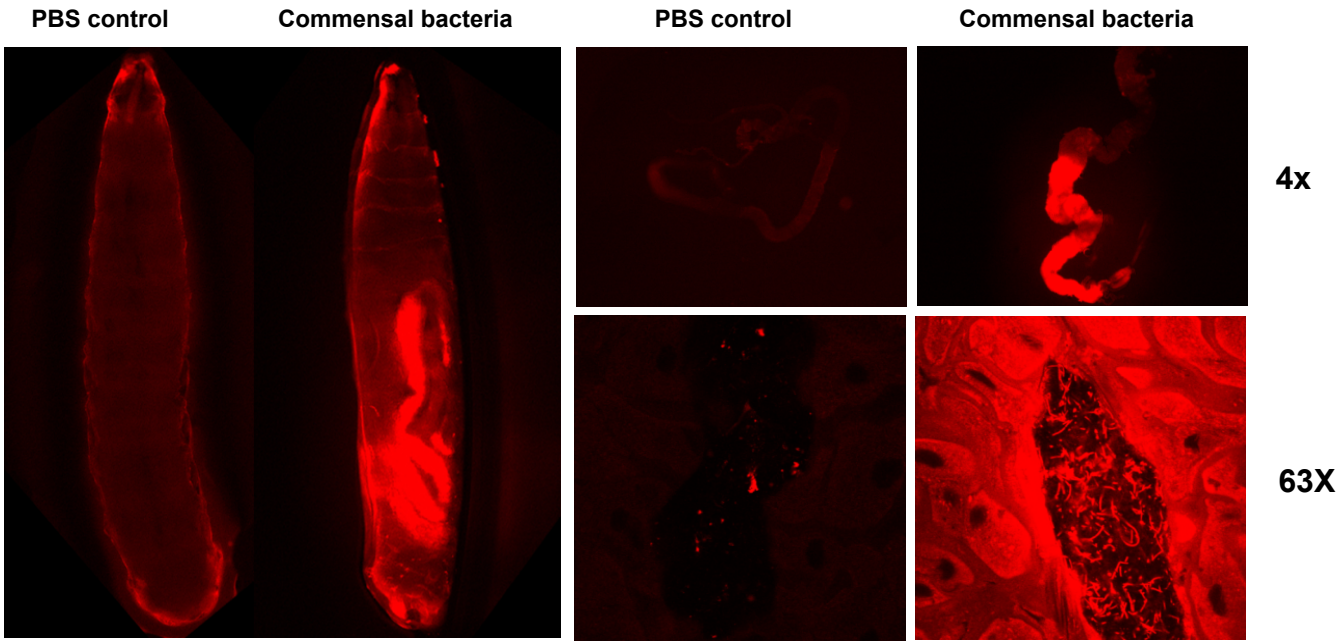


Lactobacilli induces rapid epithelial ROS generation *in vitro and in vivo* at wound edges



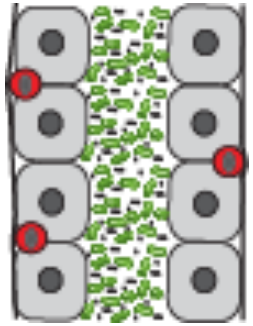
Commensal bacteria induce rapid epithelial ROS generation *in vivo* in the Drosophila gut

Third instar Drosophila larvae

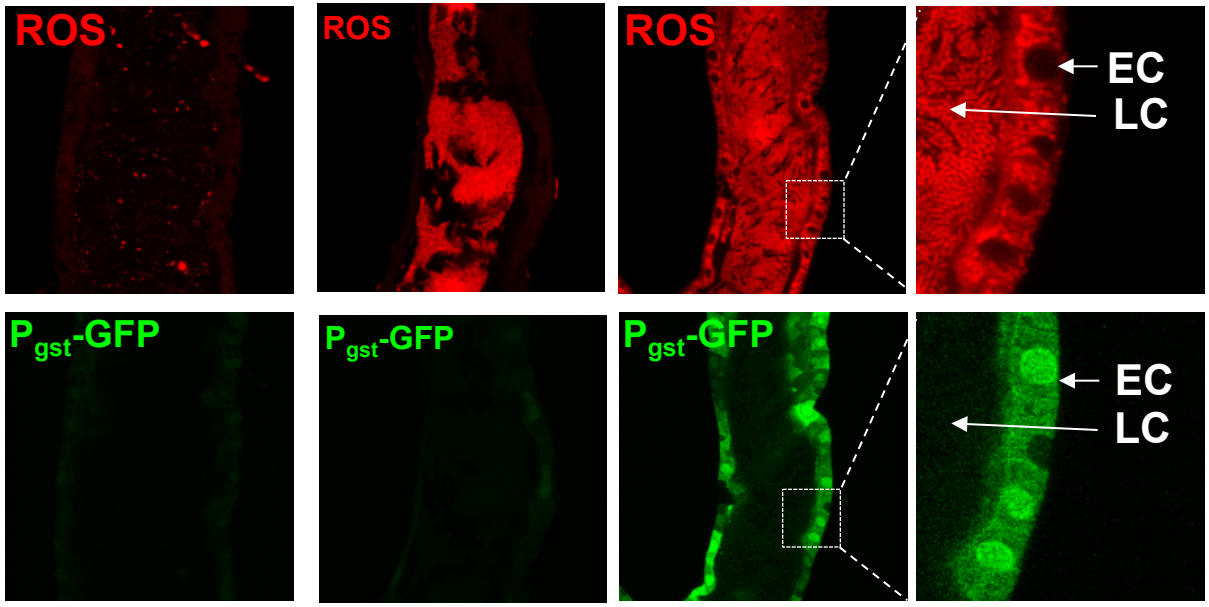


Axenic third instar larvae were fed cultures of *Lactobacillus plantarum* or sterile PBS control for 30 minutes Both *L. plantarum* and PBS cultures included hydrocyanine-Cy3 reagent.

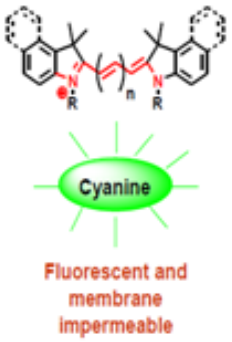
Colonization of the first instar *Drosophila* midgut by *Lactobacillus plantarum* induces cellular ROS generation

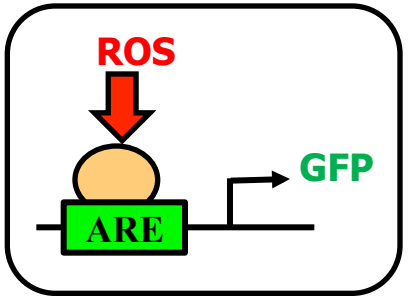


Germ-free | *E. coli* | *L. plantarum*

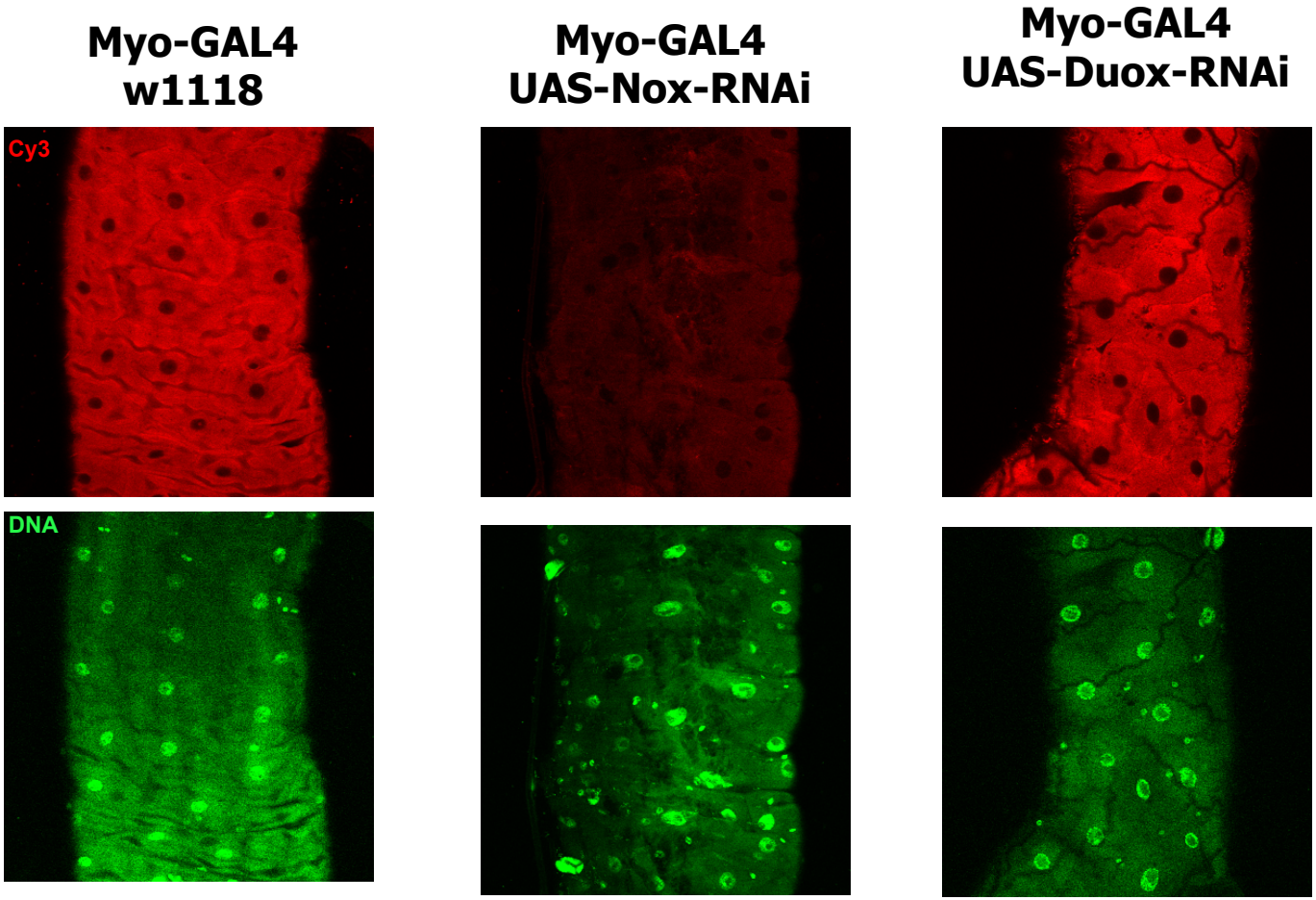


First instar Drosophila larvae



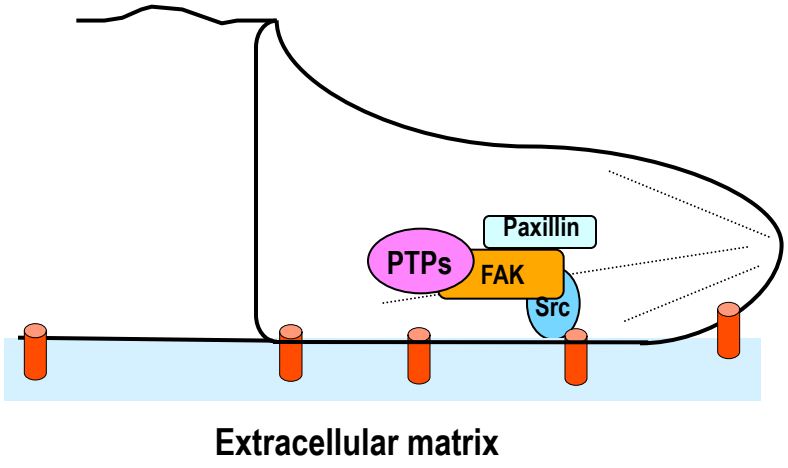


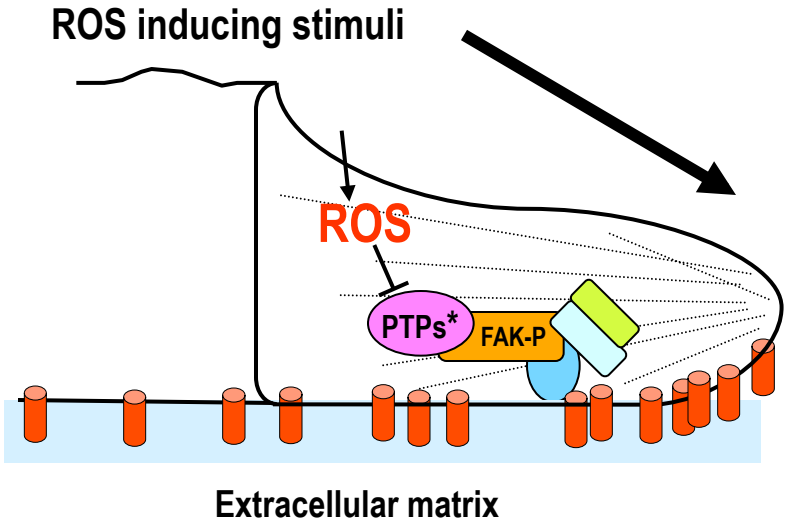
Fly dNox is required for ROS generation



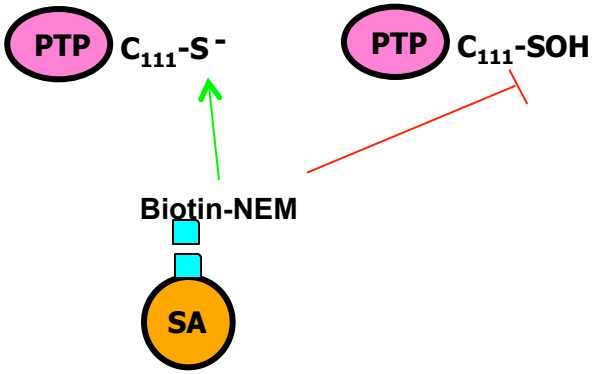
Larval guts fed LGG for 30min with hydro cy3

ROS signaling stimulates epithelial movements

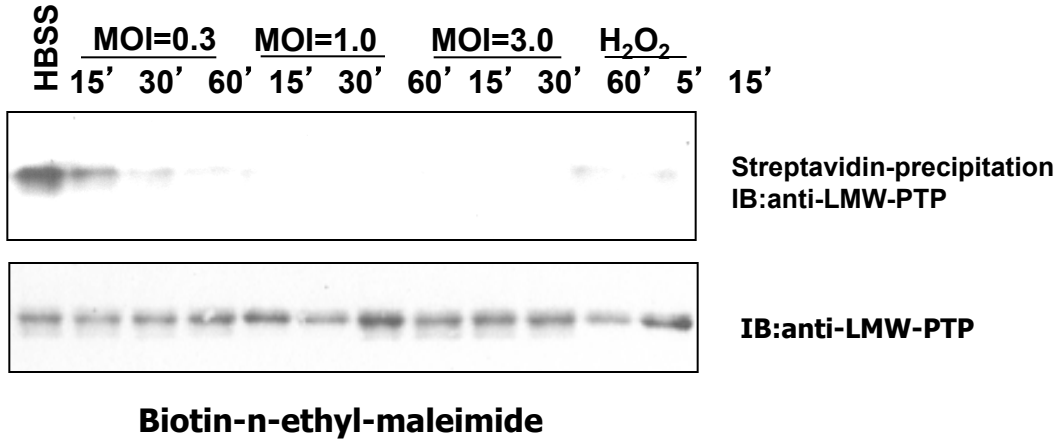


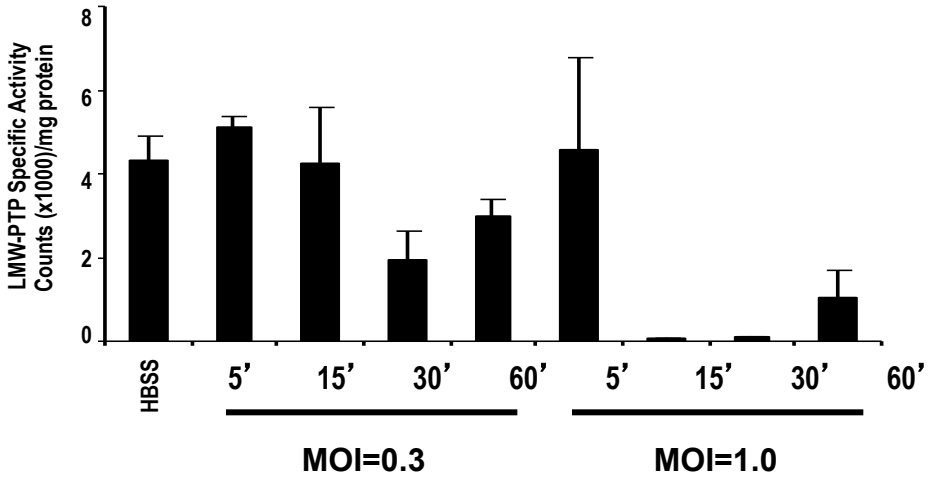


Commensal colonization elicits oxidation and inactivation of FAK phosphatase, LMW-PTPase

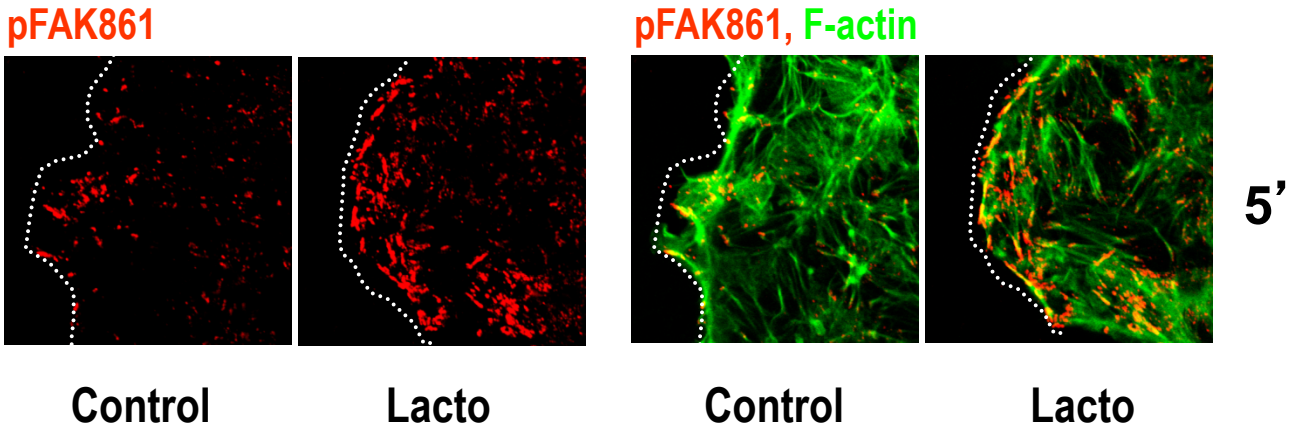


Precipitate, immunoblot/assay for PTPase

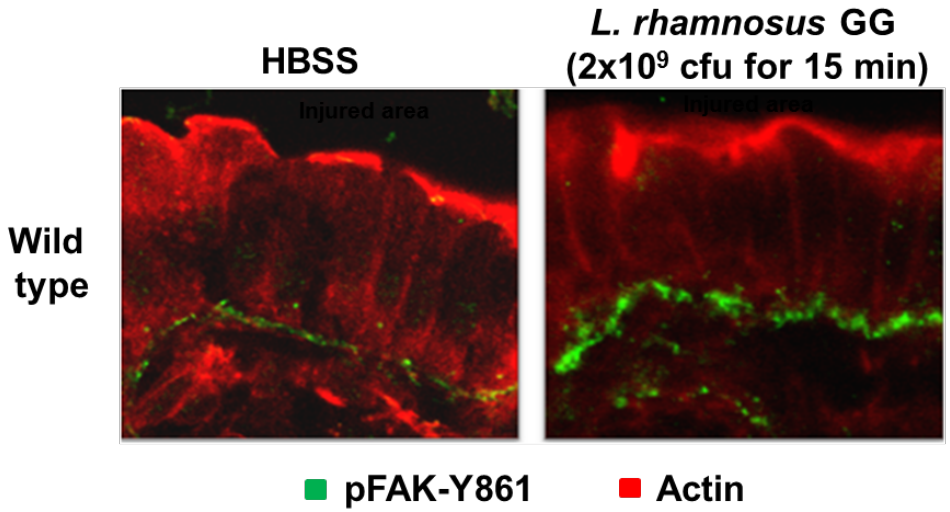




Commensal colonization elicits rapid phosphorylation of FAK

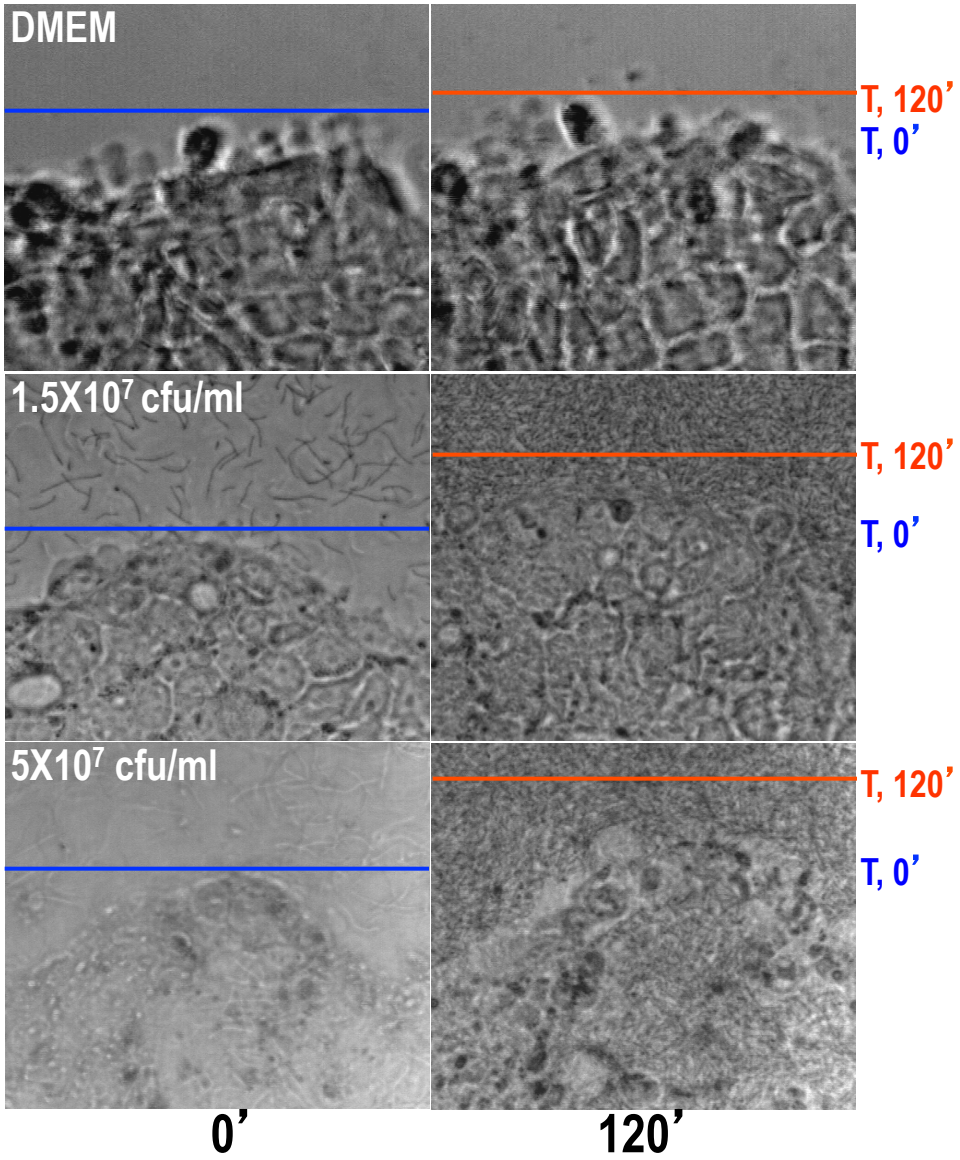


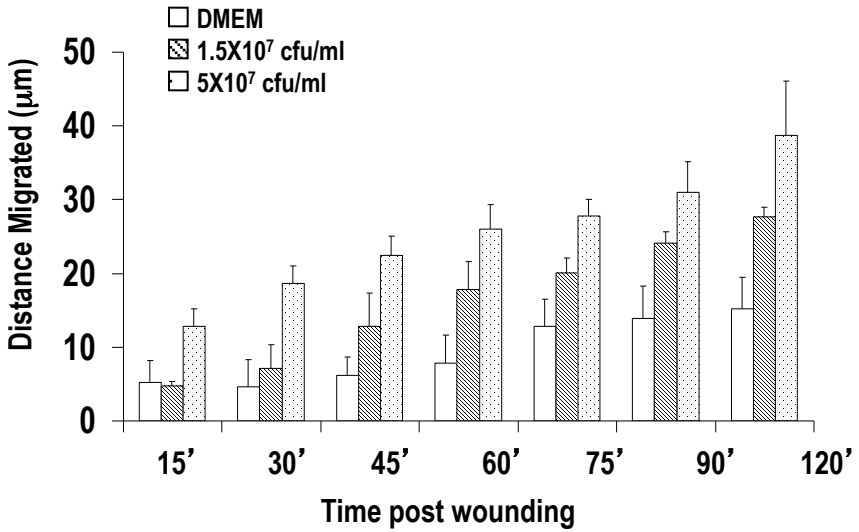
Immunofluorescence of F-actin and phosphorylated FAK-Tyr-861reorganizatio induced by LGG.

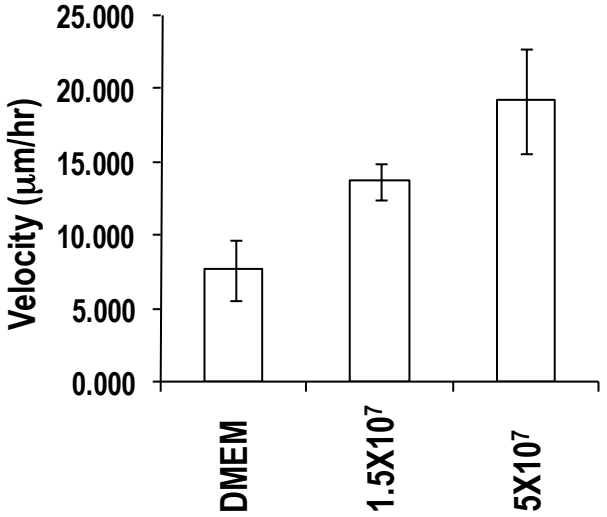


En face image of the wound bed

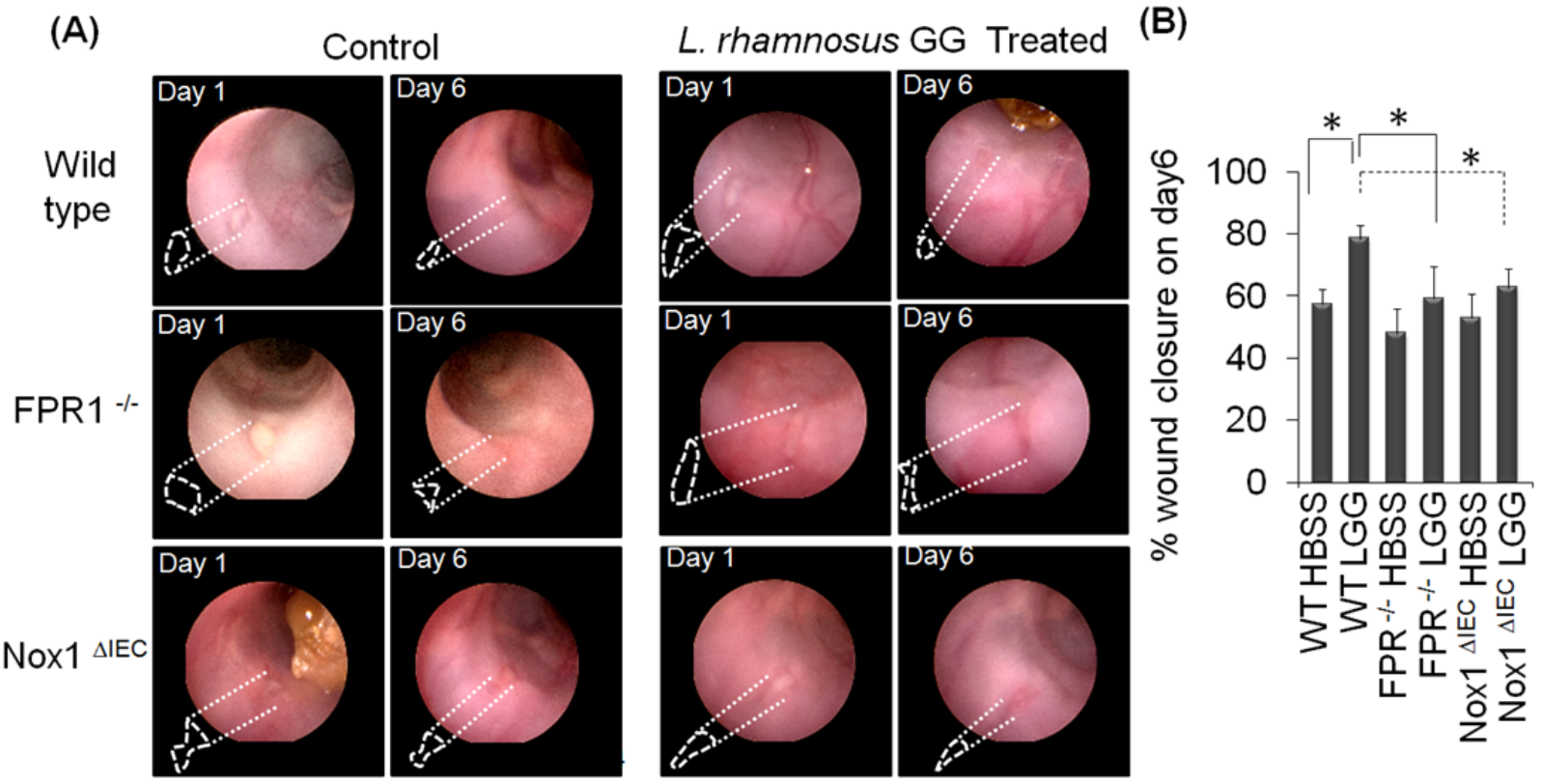
Commensal colonization elicits increased migration of model epithelia





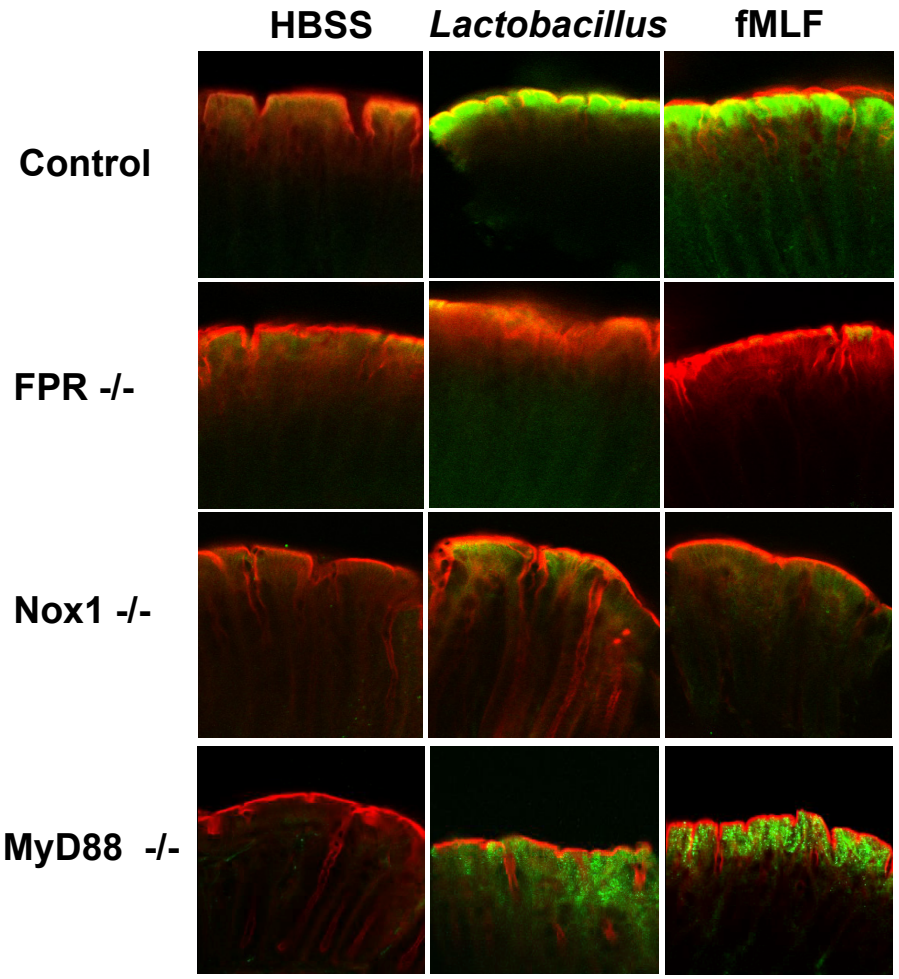


Lactobacilli enhances epithelial wound healing *in vivo*



Swanson et al. **Enteric commensal bacteria potentiate epithelial restitution via ROS-mediated inactivation of focal adhesion kinase phosphatases.** PNAS. 2011 24;108(21):8803-8.

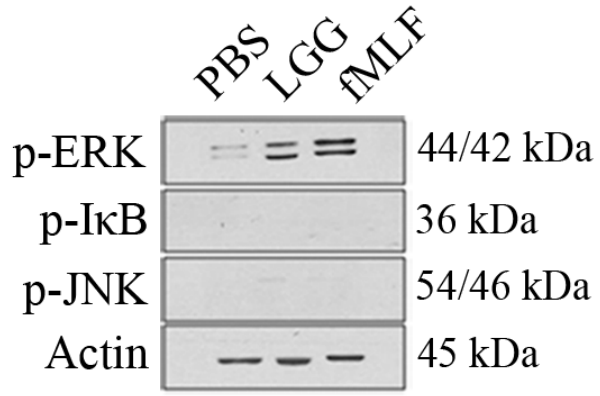
Microbiota stimulates phosphorylation of ERK in vivo in a FPR and Nox1 dependent manner



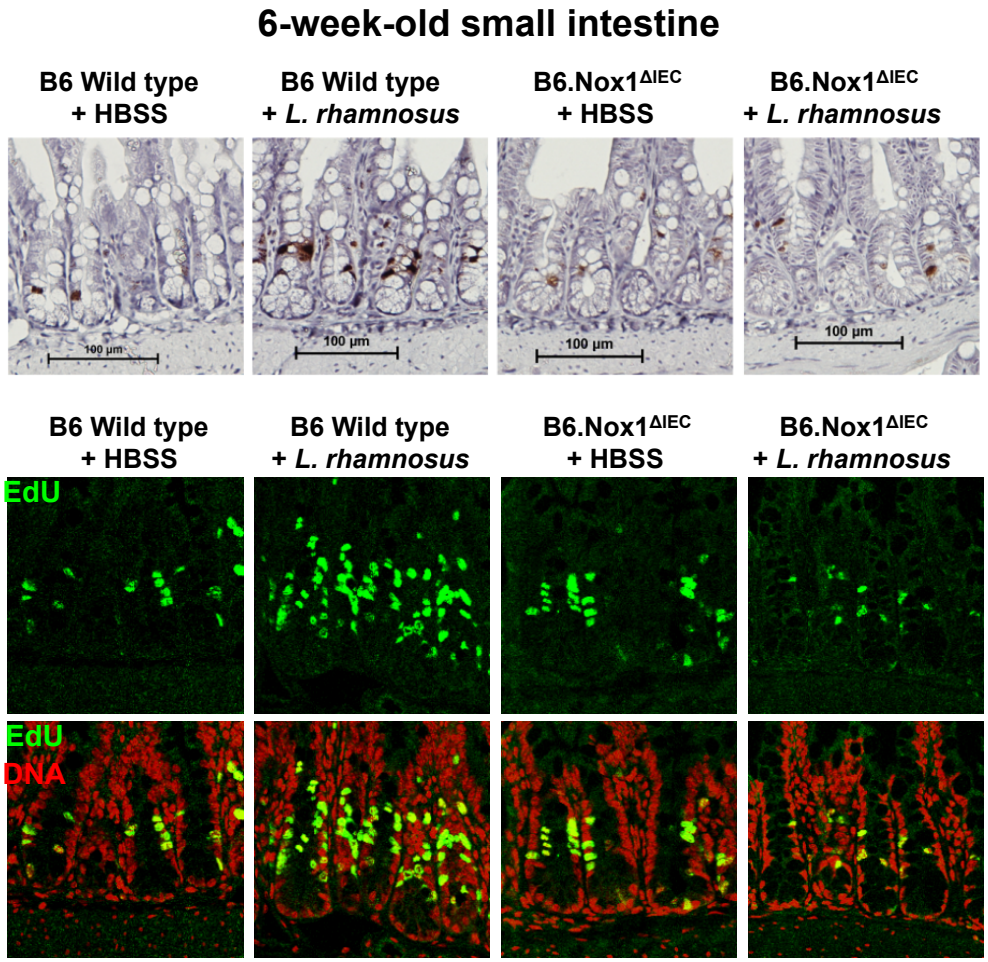
Actin

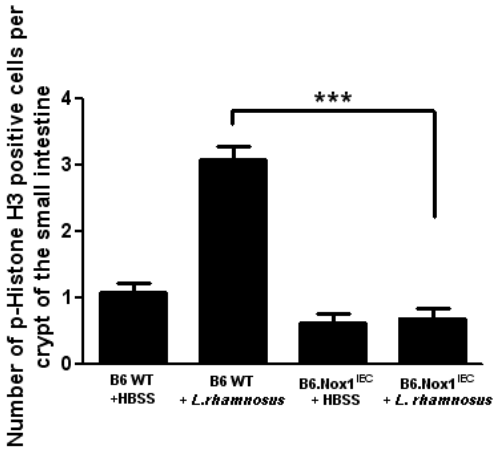
Phospho-ERK

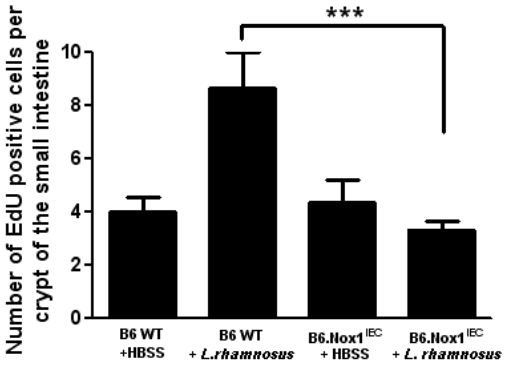
Intraluminal *Lactobacillus* activates ERK in murine colon in vivo within 15 min



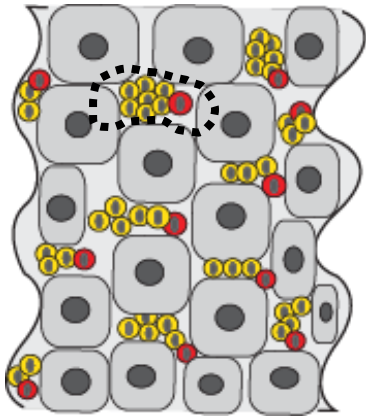
Lactobacilli stimulates crypt proliferation in mice in a Nox1 dependent manner



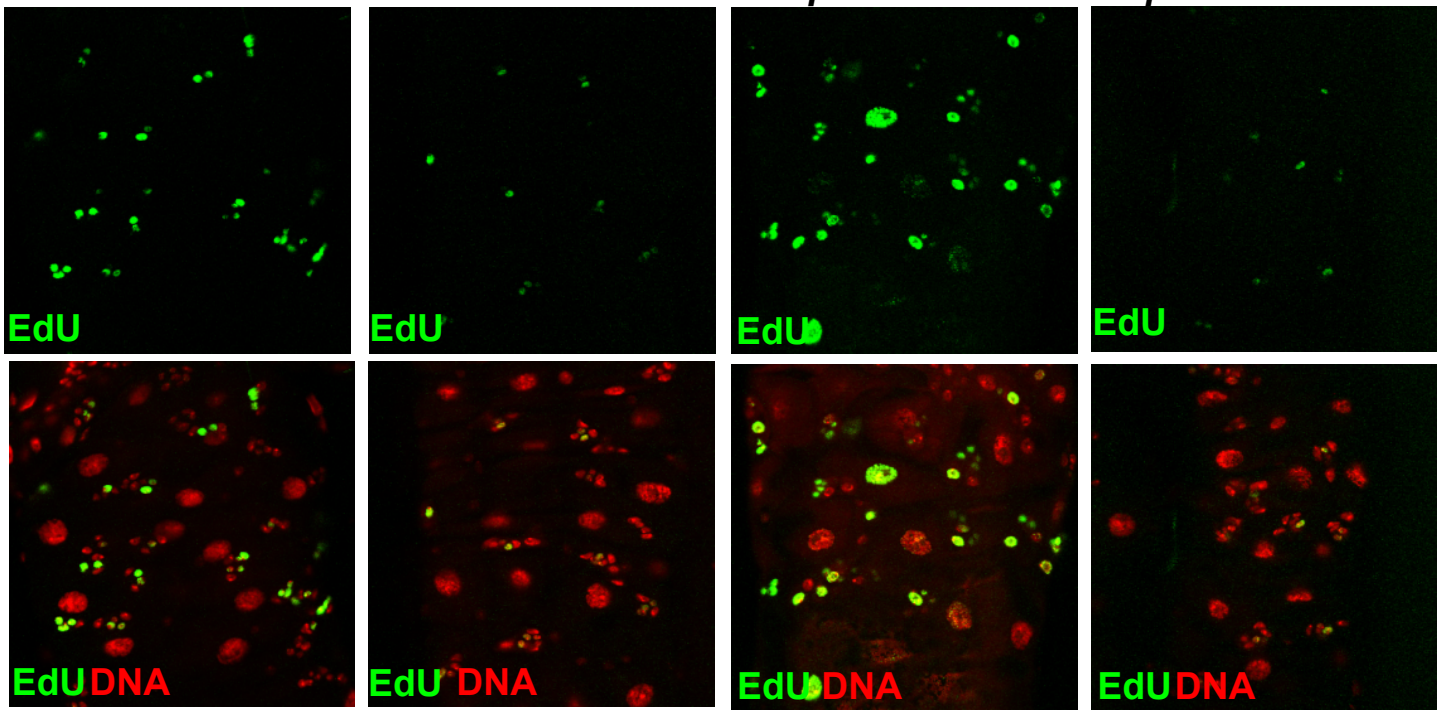


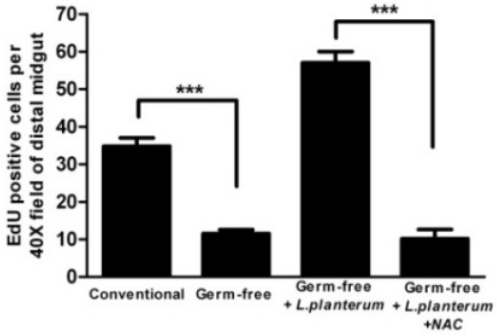


Lactobacilli induces ROS-dependent cellular proliferation in the *Drosophila* intestine

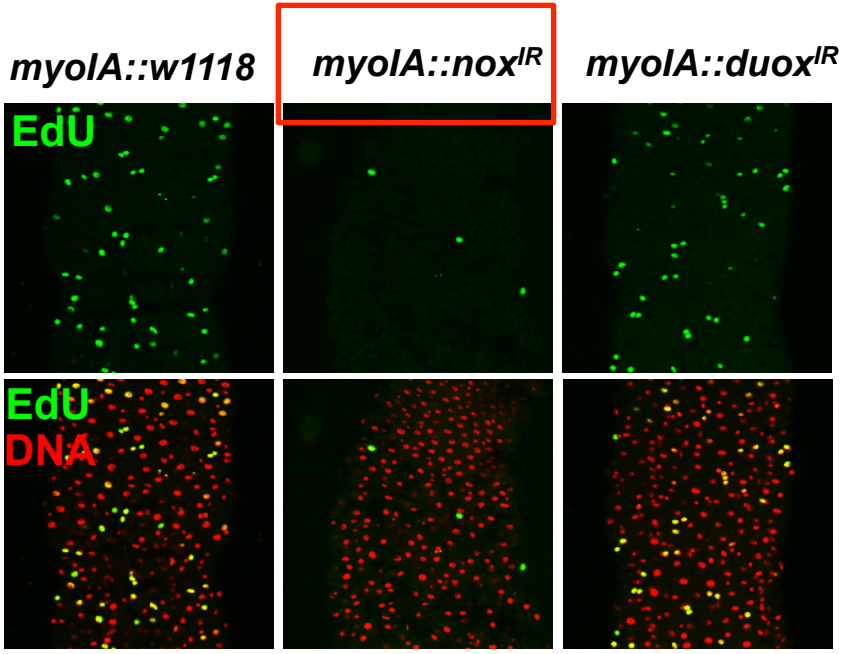


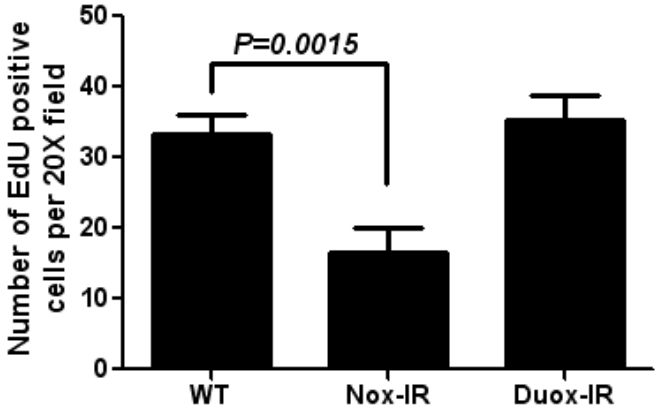
Conventional | Germ-free | Germ-free + *L. plantarum 4h* | Germ-free + *L. plantarum* + NAC





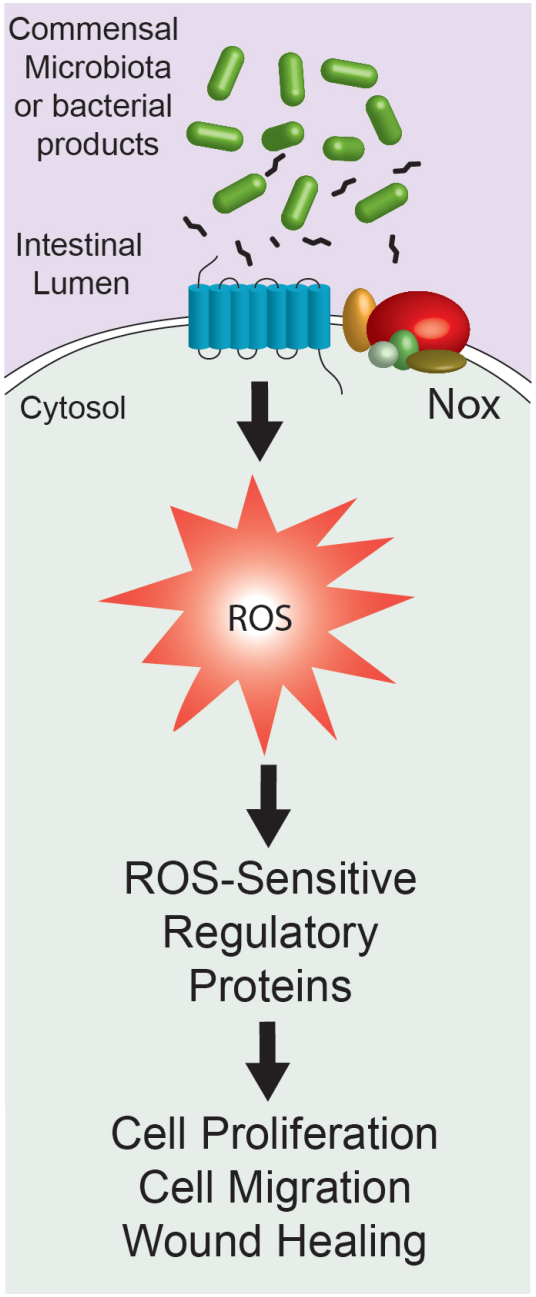
Proliferation in the *Drosophila* epithelia is Nox dependent





Conclusion :

Nox dependent generation of physiological levels of ROS by *Lactobacillus* (and likely other bacteria) is a novel signaling mechanism for transducing bacterial signals into host regulatory events that mediate intestinal homeostasis, proliferation and restitution



Future Challenges

- Novel ROS sensitive proteins/pathways
  - ROS dependent signaling eg Nrf2/ARE pathway
- Correlation with innate immunity and PRR signaling
- Correlation with microbial determinants and bacteria taxonomy
- Role in normal gut development
- Role in wound healing
- Role in oncogenesis

Gaps

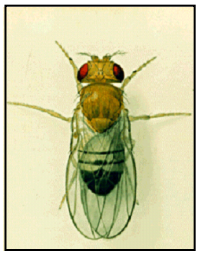
- Epithelial cell biology as a host-microbial system
- Cooperation between model systems workers
- “Comparative metagenomics”

Acknowledgments

- Neish Lab
  - Ashfaq Alam
  - Huixia Wu
  - Chirayu Desai
  - Jeff Mercante
  - Phil Swanson
- RheinaIlt Jones
  - Liping Luo
- Asma Nusrat
  - Giovanna Leoni

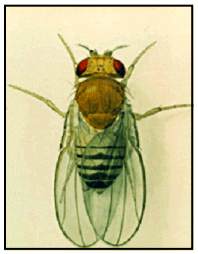
- Supported by NIAID RO1 AI 64462 and NIDDK R01 DK 89763

Expression of RNAi against Nox and Duox in fly enterocytes



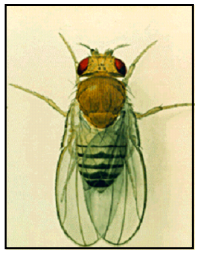
enhancer-Gal4

X

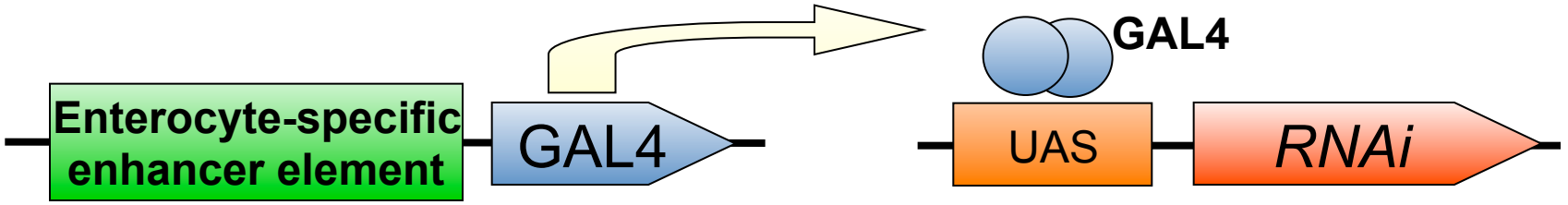


UAS-Nox^{IR}

OR

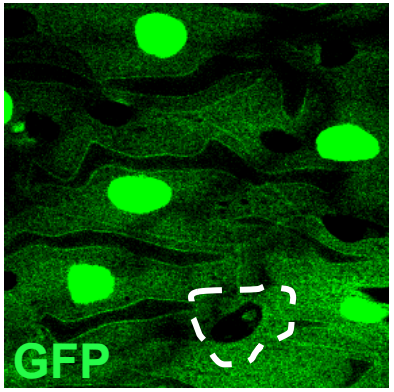


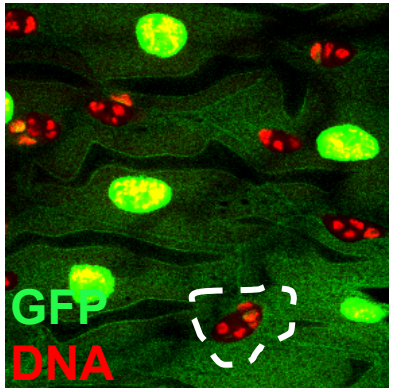
UAS-Duox^{IR}

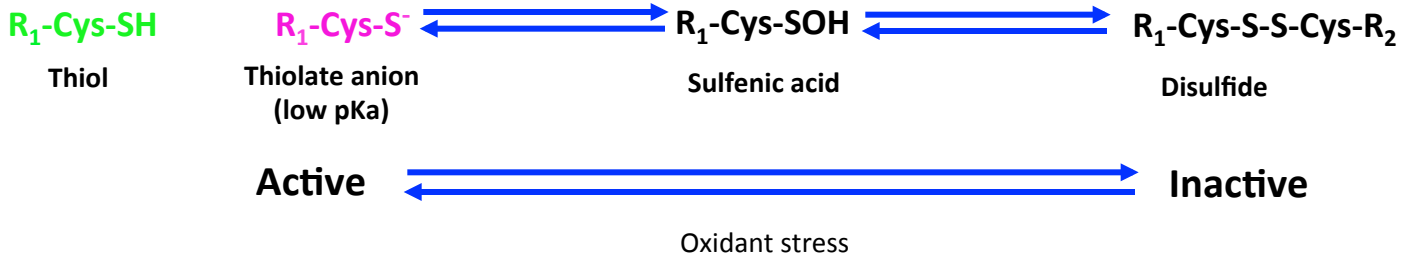


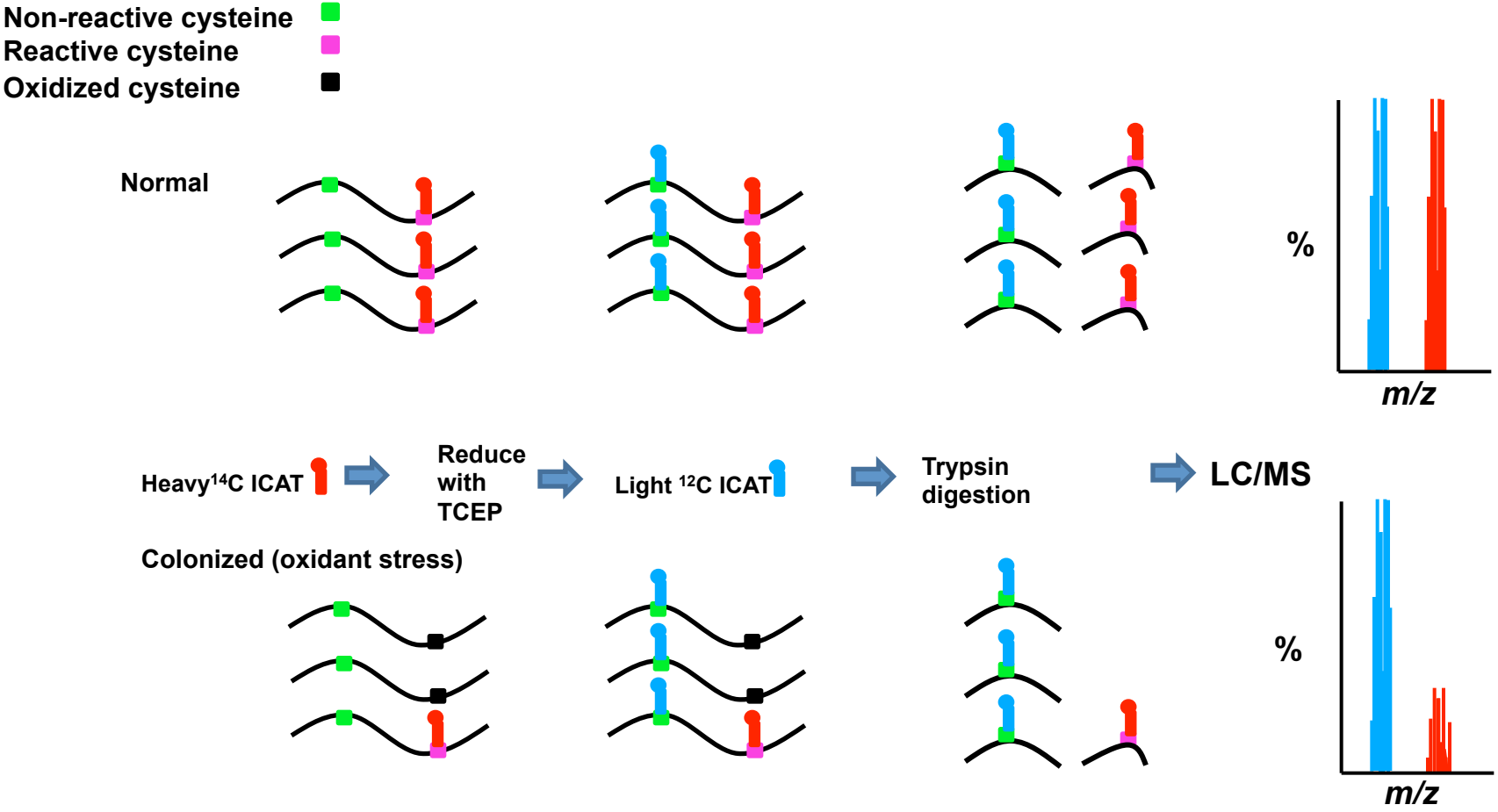
myo-GAL4
EC
specific driver

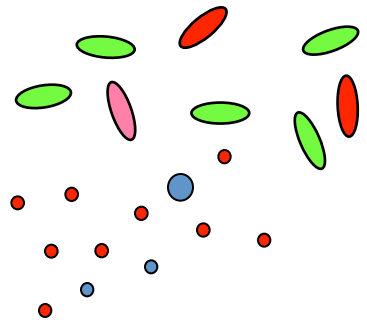
Myo-GAL4, UAS-nlsGFP






R1-Cys-SH
Thiol
R1-Cys-S-
Thiolate anion
(low pKa)
R1-Cys-SOH
Sulfenic acid
R1-Cys-S-S-Cys-R2
Disulfide
Active
Inactive
Oxidant stress


Non-reactive cysteine
Reactive cysteine
Oxidized cysteine
Normal
%
m/z
Heavy14C ICAT
Reduce with TCEP
Light 12C ICAT
Trypsin digestion
LC/MS
Colonized (oxidant stress)
%
m/z



Identify, characterize redox sensitive regulatory targets

Characterize and validate redox sensitive physiologic pathways and processes

1. Epithelial cell culture
2. Drosophila
3. Mice

Identify, characterize ROS inducing commensals, bacterial products, small molecules

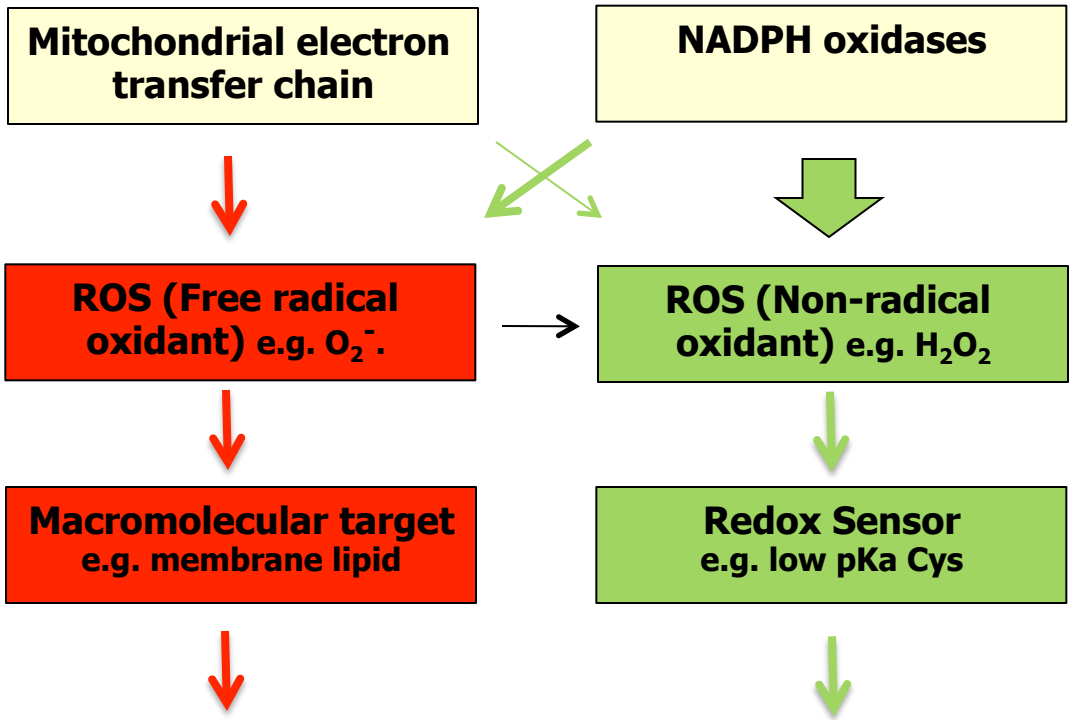
Extracellular

Cytoplasm

ROS production	(Hydro Cy3, etc)
Oxidation of regulatory proteins	BIAM/IAA (candidate) ICAT (high throughput)
Experimental validation	Enzymatic, signaling assays
Homeostatic and biochemical alterations	Metabolic and phenotypic analyses
Systems analysis	Redox proteomic database, redox pathway maps

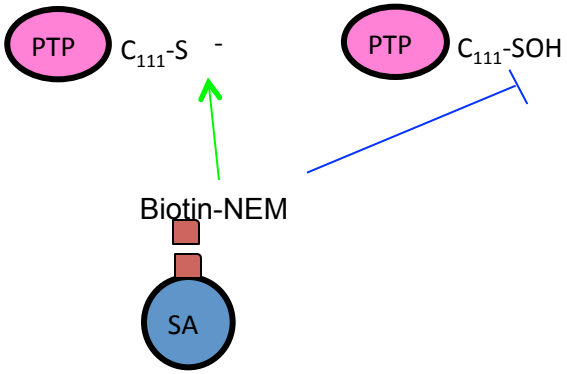
ROS effects on the cell

Upstream signals/stress

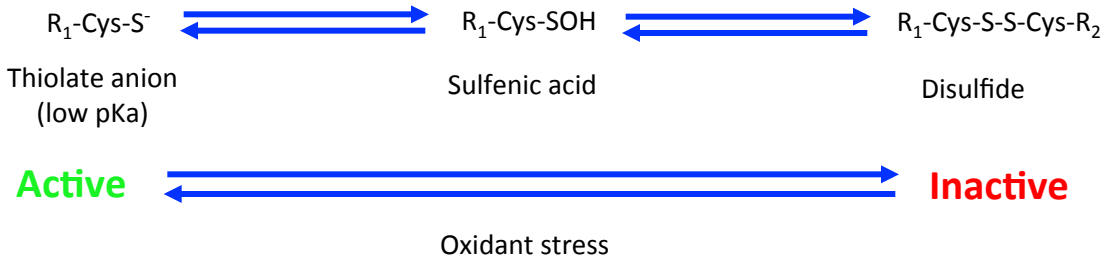


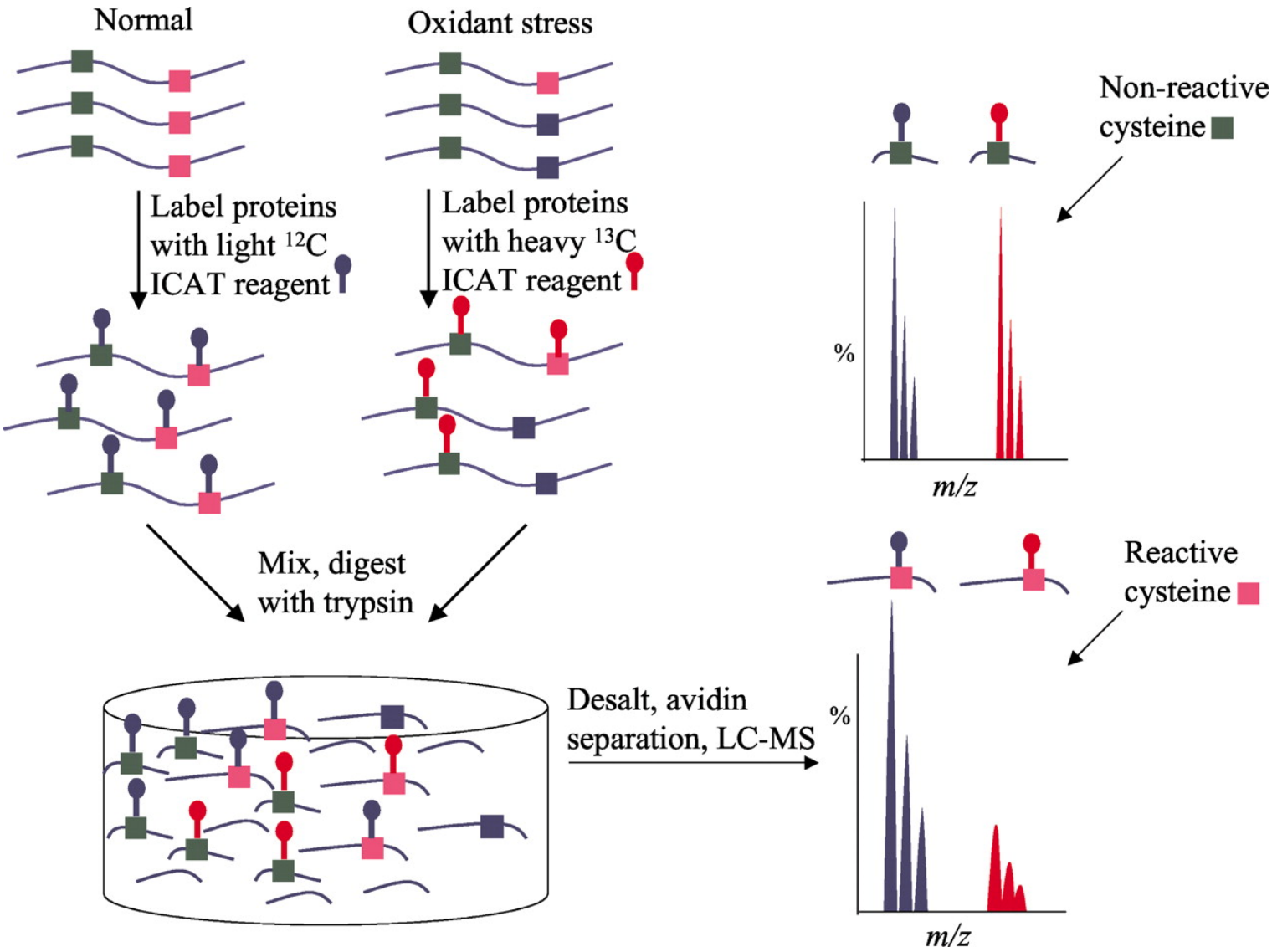
Oxidative damage
(Pathologic** or **microbiocidal)

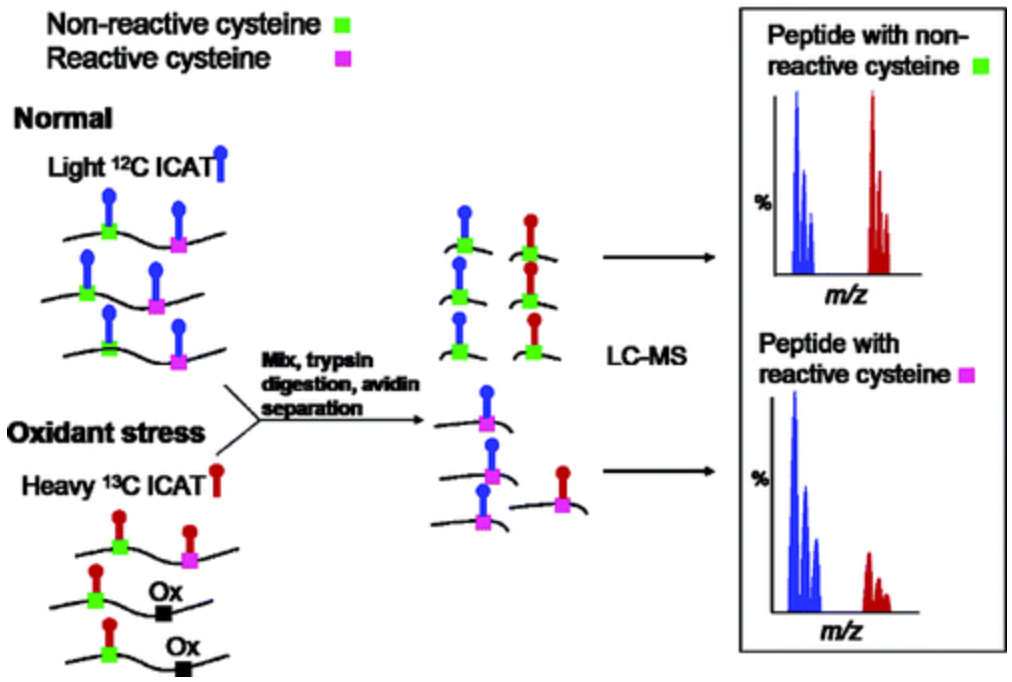
Redox signaling ***(Physiologic signaling)***



Precipitate, immunoblot/assay for PTPase




Normal
Oxidant stress
Label proteins with light ^{12}C ICAT reagent
Label proteins with heavy ^{13}C ICAT reagent
Mix, digest with trypsin
Desalt, avidin separation, LC-MS
Non-reactive cysteine
Reactive cysteine
%
m/z
%
m/z



(a)

Cell extract

↓

Modify thiols with H-ICAT

↓

Reduce with TCEP

↓

Modify thiols with L-ICAT

↓

Treat with trypsin

↓

LC²-MS/MS to determine % reduced for Cys peptides

(b)

Protein Name

Protein metabolism
Elongation factor1-α

Elongation factor-2

Glu-tRNA synthetase
Ubiquitin-activating E
Calreticulin

Signal transduction
Protein phosphatase
G-protein,β subunit

Detoxification
Peroxiredoxin 6
GSH-S-transferase

Stress Response
Hsp 70

Hsp 90

Cell Structure/Motility
Cofilin
β-Tubulin

α-tubulin

(c)

Database chemic

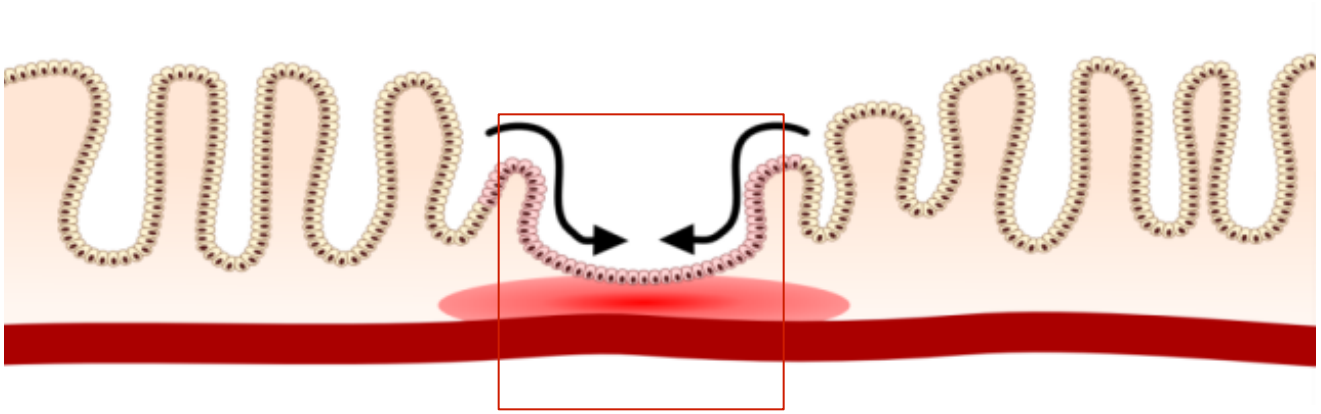
Redox prote

Thiol redox circuitry maps

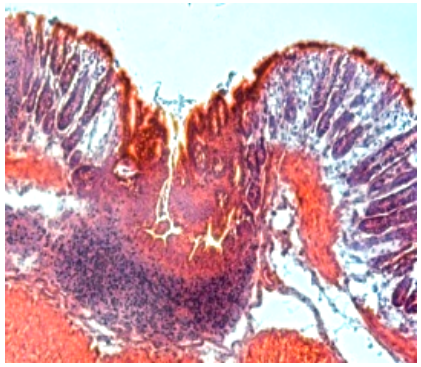
Conclusions

- These data suggest an intriguing role for intestinal bacteria in the modulation of the cellular homeostatic pathways via ROS generation
- Possible mechanism for commensal “crosstalk” with the host
- Possible mechanism for probiotic effects

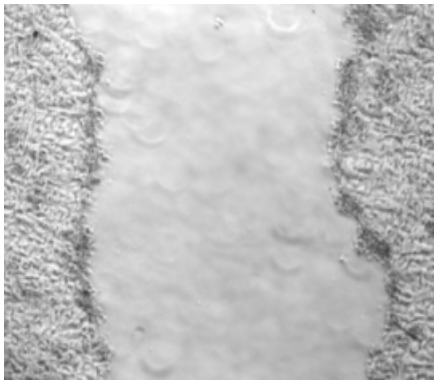
Restitution: Involves coordinated epithelial proliferation and migration



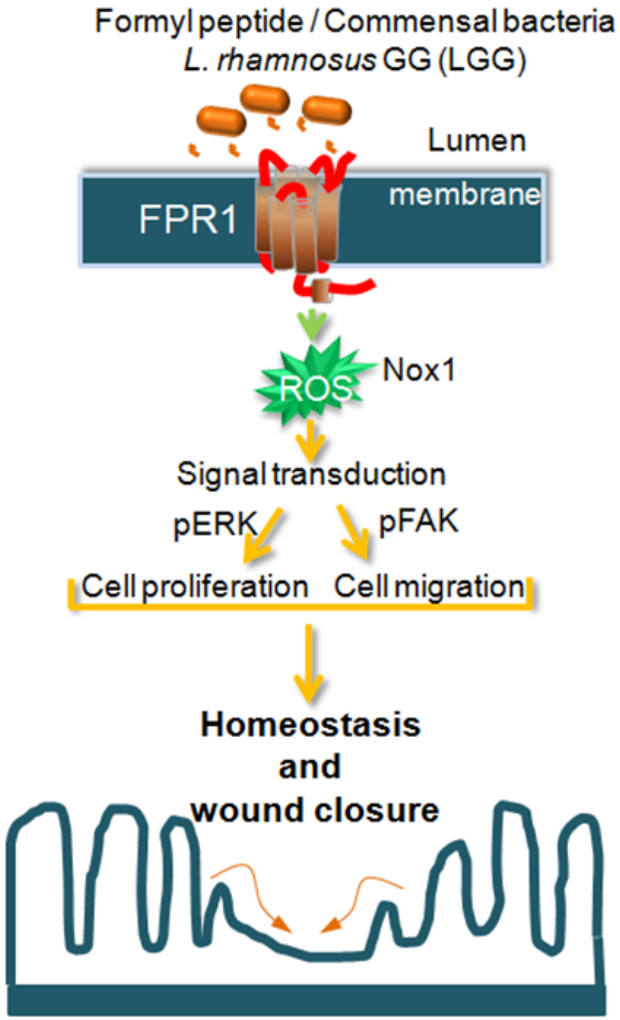
In vivo



In vitro



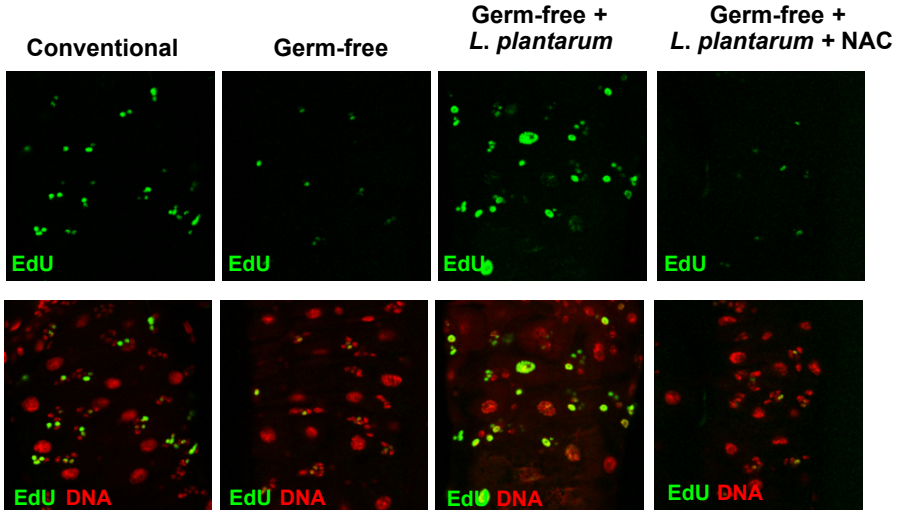
ROS mediated signaling regulates multiple homeostatic pathways

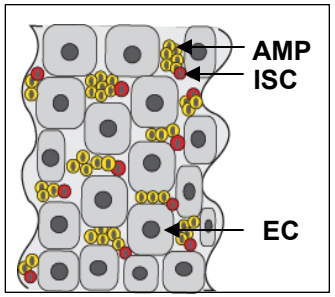


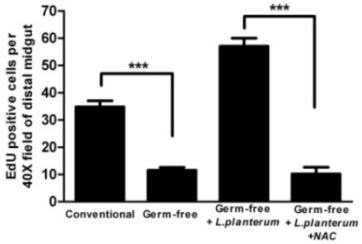
Summary

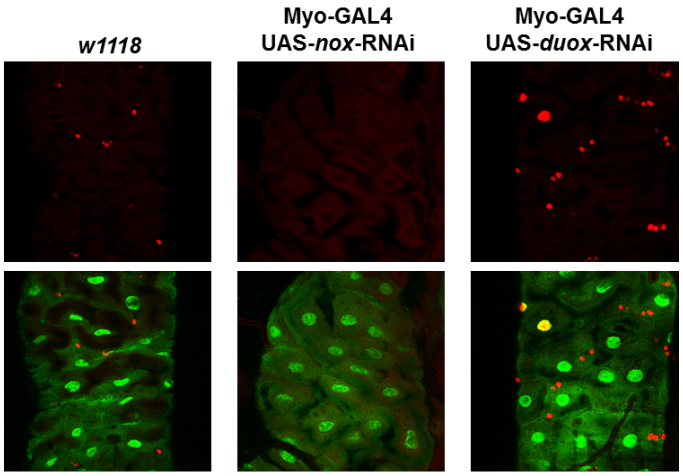
- Specific commensals (and commonly used probiotics) can stimulate non proinflammatory signaling in the gut
- Signaling results in increased epithelial proliferation and migration
- Physiological “deliberate” generation of ROS is involved in these processes
- Possible mechanism for beneficial homeostatic effects of probiotics

Microbiota stimulates crypt proliferation in flies in a Nox1 dependent manner







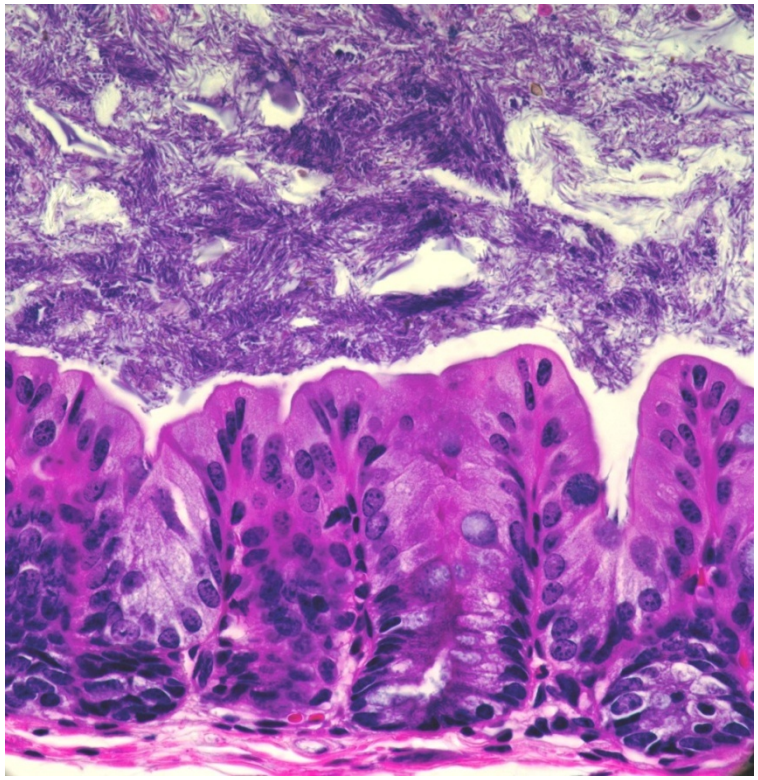


Myo-GAL4 in combination with GAL80ts at 29C for 60hours

The Microbiota

- 10^{14} organisms, mostly in colon
  - 10x more than humans cells!
- Acquired at birth
- Approximately 500 genera represented
  - Most in two Divisions, *Bacteroidetes* and *Firmicutes*
- Diverse metabolic abilities
  - Alternate energy sources and fermentative products

- **Symbiotic with host**



ROS generation as a response to bacteria

- Oxidant or “respiratory burst” in phagocytes in response to FPR signaling
- High degree of evolutionary conservation in wide variety of animals and plants
- Strong conservation of NADPH generating enzymes (Nox’s)