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Peptide YY: a Paneth cell antimicrobial peptide that maintains *Candida* gut commensalism

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Abstract

The mammalian gut secretes a family of multifunctional peptides that affect appetite, intestinal secretions, and motility, while some regulate the microbiota. We have found that peptide YY ($PYY_{1–36}$), but not endocrine $PYY_{3–36}$, acts as an antimicrobial peptide (AMP) expressed by gut epithelial Paneth Cells (PC). PC-PYY is packaged into secretory granules and is secreted into and retained by surface mucus, which optimizes PC-PYY activity. While PC-PYY shows some antibacterial activity, it displays selective antifungal activity against virulent *Candida albicans* hyphae, but not the yeast form. PC-PYY is a cationic molecule that interacts with the anionic surfaces of fungal hyphae to cause membrane disruption and transcriptional reprogramming that

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Author contributions: JFP and EBC were involved in every aspect of this study, including the discovery, conceptualization, experimentation, data analysis, and MS preparation; JW, DLT, LW, AMS, KGH, AK, XZ, YT, CMC and JFS performed experimentation and data analysis; BMP, OZ, HH, JCA, SMN, and VAL contributed to the conceptual, data analysis, and writing components of this study.

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selects for the yeast phenotype. Hence, PC-PYY is an antifungal AMP that contributes to the maintenance of gut fungal commensalism.

One Sentence Summary:

Paneth cell derived peptide YY, in its full-length form, checks the invasive potential of commensal fungi.

Keywords

Intestinal epithelial cells; antimicrobial peptide; antifungal; fungal virulence; gut mycobiome; mucus; mucosal barrier; commensalism; innate immunity

The coevolution of metazoans and microbes has been fundamental to the development of mutualistic beneficial relationships, not least in the digestive tract of animals. Gut microbes form region-specific stable and resilient communities essential for processes such as immune and metabolic development and overall intestinal homeostasis (1). Gut bacteria have been investigated in some detail, but less is known about gut fungi and archaea. The microbiota appear to be regulated in a healthy host, although, little is known about host surveillance and control of fungal populations in the gut.

Peptide YY (PYY) is a satiety hormone that is expressed and secreted by enteroendocrine cells (EECs) (2). In this work we found gut-specific Paneth cells (PCs) also express a form of PYY that functions as an antimicrobial peptide (AMP) with selective activity against *Candida albicans*. This fungus is found commonly as a commensal yeast, but becomes pathogenic upon transformation into its hyphal form (3). The antimicrobial function of PC-PYY plays a role in maintaining fungal commensalism by inhibiting the yeast-to-hypha transformation of opportunistic *Candida spp.*.

Results:

Presence of PYY observed in Paneth cells:

While visualizing mucosal enteroendocrine L-cells in murine distal ileum, we serendipitously observed the satiety regulating peptide PYY, immunostained with lysozyme (LYZ1) expressing PCs (Fig. 1A and 1B). This finding was surprising, as PCs are gut mucosal epithelial cells found in most mammals, which secrete AMPs against pathogens and regulate the local gut microbiota (4). We confirmed PYY immunolocalization in healthy human adult ileal PCs (Fig. S1A), observed antibody epitope specificity through recombinant peptide quenching (Fig. S1B,C), and identified PYY mRNA in PCs via fluorescent *in situ* hybridization (FISH; Fig. 1B). To achieve higher resolution, stimulated emission depletion (STED) and SP8 confocal microscopy were used to show that PYY and LYZ1 are packaged into discrete granules in the jejunum and ileum (Figs. 1C,D and S2A,B and Movie S1), raising the possibility that these peptides have different roles, sorting pathways, and regulation (5). Laser capture micro-dissection in the murine ileum followed by quantitative RT-PCR revealed robust PYY transcript levels in basal crypts in line with other PC-specific AMP transcripts (Figs. 1E and 1F). Reanalysis of a publicly deposited

murine small intestinal epithelial single-cell RNAseq dataset identified PYY expression in mature EEC populations that co-expressed with *Scg1*, *Cck*, and *Glp1*, but also in PCs, where it was co-expressed with *Dfa17*, *Atg16l1*, *Defa5*, and *Lyz1* (Figs. S3A,B) (6). The Human Protein Atlas single cell RNA database was searched for PYY, showing detection in PCs (Fig. S3C).

Anti-microbial functions of Paneth cell PYY:

PC expression of PYY indicated it might have an antimicrobial function. The predicted structure of PYY resembles the alpha-helical, amphipathic AMP, magainin-2 (Figs. 2A,B), produced in the skin of *Xenopus laevis*. PC-PYY is strictly the unmodified, full length $PYY_{1–36}$, whereas the circulating endocrine form, $PYY_{3–36}$, is formed by removal of two N-terminal amino acids by dipeptidyl peptidase IV (DPP-IV) (7). We initially assessed the antimicrobial activity of full length PYY peptide against representative Gram-positive and -negative bacteria, which showed less activity compared with more effective magainin-2 (Fig. 2L–N). We then tested full length PC-PYY on *C. albicans* growth and viability (8, 9). We induced transition of yeast into hyphal forms in the presence of 2.5% w/v porcine mucin. Propidium iodide (PI) was used to measure membrane permeabilization of $PYY_{1–36}$-treated *C. albicans* hyphae. Permeabilization of hyphae was observed at 30 μM $PYY_{1–36}$, but yeast were unaffected (Fig. 2C and Fig. S4A). $PYY_{1–36}$ - fluorescein isothiocyanate (FITC) tagged peptide co-localized with the PI signal within hyphae (Fig. 2C). We synthesized PYY using D-amino acids to generate a peptide stereoisomer, which disrupted hyphae in the same way as the native L-isoform (Fig. S4B). Less pronounced permeabilization was observed with the shorter endocrine $PYY_{3–36}$ (Fig. S4B,C). Similar staining with PI was found with hyphal forms of *C. tropicalis* and *C. dubliniensis* treated with $PYY_{1–36}$ (Fig. S5).

Given the observed hyphal specificity, we subjected *C. albicans* yeast and hyphae to increasing $PYY_{1–36}$ concentrations in media with or without 2.5% w/v mucin. $PYY_{1–36}$ did not affect yeast numbers or density (Fig. 2D and 2E); however, it elicited a decrease in hyphal respiration (Fig. 2F). Similar results were obtained for *C. tropicalis* (Fig. S6). The enteroendocrine form, $PYY_{3–36}$, showed less activity (Fig. S7A). Hyphal growth and survival were examined under increasing concentrations of $PYY_{1–36}$, where magainin-2 (positive) and scrambled (negative) peptides were used as controls. In aqueous conditions, hyphal growth and biofilm formation were significantly reduced at 20–50 μM $PYY_{1–36}$ (Figs. 2G and 2H). We observed a biphasic dose-response of $PYY_{1–36}$ action in aqueous (RPMI) buffer, similar to observations of other AMPs, with peak effect at ~30 μM and loss of activity by 100 μM (10, 11). We attribute this observation to concentration-dependent self-aggregation, or multimerization, of amphipathic alpha-helical AMP precipitates in aqueous medium, reducing bioactivity. Consistent with this hypothesis, a sigmoidal dose-response curve was observed in biofilm and colony forming units (CFUs) when 2.5% w/v mucin is present (Fig. 2H and 2I). No differences were observed for biofilm with $PYY_{3–36}$ (Fig. S7B). We surmise that mucin prevents self-aggregation of $PYY_{1–36}$, which allows its activity to be maintained.

To test whether the cationic properties of $PYY_{1–36}$ (as shown in Figs. 2A and 2B) allow binding to the anionic surface charge of fungi (5, 12) we used a cationic ferritin probe

and transmission electron microscopy to identify sites of anionic surface charge (Fig. 2J, white arrows). The cationic probe decorated the surface of hyphae, while the surfaces of yeast cells were not labelled. To further investigate charge interactions, we repeated PI membrane permeability assays with $PYY_{1–36}$ in the presence of sodium sulfate, to provide anionic quenching of cationic $PYY_{1–36}$ charge, which reduced $PYY_{1–36}$ induced permeability (Fig. S4D). Finally, scanning electron microscopy of yeast and hyphal forms of *C. albicans* showed that hyphal membranes exposed to $PYY_{1–36}$ develop surface blebbing and irregularities, while yeast membranes remain unaffected (Fig. 2K).

RNA-seq was used to measure the transcriptional response to $PYY_{1–36}$. The hyphal phenotype showed downregulation of cell wall synthesis, biofilm formation, and ribosome biogenesis pathways (Figs. S8A,B). By contrast, yeast cells showed fewer changes in gene expression, and those mostly affected were genes involved in yeast-to-hyphal transition and adhesion. In all, our evidence indicates $PYY_{1–36}$ has selective antimicrobial activity against the virulent, invasive hyphae of *C. albicans,* with limited effect on the blastoconidia.

Paneth cell PYY release and mucus localization:

PCs secrete AMPs into the gut lumen in response to the presence of microbial products (5). Although $PYY_{1–36}$ is produced by L-cells where it is released systemically, the ubiquitous serine protease DPP-IV rapidly cleaves it into endocrine $PYY_{3–36}$. We used *ex vivo* murine distal ileal loops to examine luminal PYY release (Fig. S9A–C). Following exposure to *C. albicans,* $PYY_{1–36}$ was measured by LC-MS (Fig. S10) in the lumen contents, the surface mucus, and mucosal tissue (Fig. 3A). At baseline, micromolar amounts of $PYY_{1–36}$ were detected in mucus following control incubations in this model (Fig. S11). However, introduction of *C. albicans* hyphae into ileal loops significantly increased $PYY_{1–36}$ levels. Less $PYY_{1–36}$ was observed when yeast were present or when cell free spent media were applied from cultures of either fungal morphology. $PYY_{3–36}$ was not detected in any of the lumen contents, mucus, or tissue compartments by MS, similar to previous findings (13, 14). These data indicate PC-PYY is released into overlying mucus where it is retained specifically in response to the presence of *C. albicans* hyphae. We confirmed this response using *in vivo* ileal ligated loops (Fig. 3B). Immunofluorescent studies confirmed *in vivo* depletion of PC PYY in the presence of hyphae (Fig. 3C; Fig. S12).

We used a 10 kDa MWCO filter to separate mucus and aqueous compartments to test whether the cationic amphipathic properties of $PYY_{1–36}$ has affinity to mucus (Fig. 3D). We found $PYY_{1–36}$ preferentially partitions into mucus, likely through charge interactions, hence explaining its retention on the mucosal surface. Since DPP-IV is present in the mucosal brush border (15) (Fig. 3E), we questioned why $PYY_{3–36}$ was not detected in overlying mucus. We incubated DPP-IV and $PYY_{1–36}$ with and without mucin, which showed $PYY_{3–36}$ generation in the absence of mucin, but none in the presence of mucin, suggesting proteolytic protection of $PYY_{1–36}$ within mucus layers. (Fig. 3F)

The effect of PYY on *Candida* at the intestinal mucosa:

Green fluorescent protein-tagged *C. albicans* hyphae (Fig. S13) were co-cultured with and without $PYY_{1–36}$ in human epithelial Caco-2 cells. In the presence of $PYY_{1–36}$, the numbers of hyphae attached to Caco-2 cells were significantly reduced (Figs. 4A, B).

Antibiotic induced chronic *C. albicans* intestinal colonization was established via oral gavage (4×10^6 CFU) in specific pathogen-free wild-type (WT) or PYY gene-deficient (PYY-KO) mice and used to investigate the effect of PYY on fungal populations *in vivo*. Oral gavage of colonized WT mice with $PYY_{1–36}$ decreased fungal titers in stool and intestinal contents as compared to mice given a scrambled peptide control (Fig. 4C and Fig. S14). Conversely, fungal colonization of PYY-KO mice was 2–3-fold higher than in WT counterparts (Fig. 4D). Total fecal output remained similar between groups, indicating comparable gut motility was maintained. A second intestinal colonization challenge experiment was performed in PYY-KO and WT animals with a higher *C. albicans* inoculation (2×10^7 CFU) under systemic and oral antibiotics to induce greater virulence. Here, PYY-KO animals exhibited elevated small intestinal *C. albicans* counts, more pronounced mucosal apoptosis, and significantly greater hyphal vs. yeast (36.3±12.1%) forms at the mucosal surface relative to WT counterparts (11.77±3.8%, $P<0.04$) (Fig 4E–I).

Given the current evidence that full-length PC-PYY localizes to the mucus layer, we isolated lumen- and mucus-associated samples from PYY-KO and WT littermates for mycobiota characterization under standard colony conditions. Young animals were used since PYY KO animals develop insulinemia and weight gain by 14 weeks of age, which could itself confound mycobiome results (2). Compared with the variable lumen fungal communities from either WT or PYY-KO, the mucus-associated layer harbored a distinct fungal population enriched for *Candida* (Figs. 4J and S15A–C). Within the mucus, PYY-KO animals, compared with WT counterparts, displayed elevated relative abundances of *Candida,* as well as of other members of *Saccharomycetaceae* (Fig. 4J, top panel and Fig. S15C). In contrast, bacteriome analysis showed minor relative abundance changes in *Ruminococcus, Coprococcus, Lachnospiraceae*, and *Prevotella*, as well as significantly altered beta diversity (Fig. 4, bottom panel and Fig. S15B). No differences were observed in EEC or PC density between genotypes (Fig. S16).

Discussion:

Fungi are normally present in the “healthy” gut microbiome. The yeast *Candida albicans* is found in 70% of humans (16), but it can transition into an opportunistic pathogen (17). PYY is highly conserved in vertebrate species (18) and its only known function to date is endocrine (19, 20). Here, we report that PC-PYY provides functionally-specific AMP activity against the invasive hyphal phenotype of *C. albicans* but not the yeast phenotype of this fungus, and limited antibacterial activity. Moreover, PC-PYY is packaged into secretory granules, distinct from those carrying Lyz1, and are released if mucosal surfaces are exposed to fungal hyphae, but not yeast. The specificity of cationic PC-PYY appears to lie in its electrostatic interaction with the anionic surface charge of *C. albicans* hyphae, determined by cationic probe imaging of the hyphal and yeast surface, and charge quenching experiments were performed with sulfate, which inhibited PC-PYY activity. Once

secreted, PC-PYY is retained in the overlying mucus of the gut lumen. Finally, we show PC-PYY drives transcriptional programming in *C. albicans* hyphae consistent with cell death and downregulation of virulence, whereas commensal yeast respond by downregulating pathways that promote the yeast-to-hypha transition. Thus, microbial selectivity, activation, and mucus compartmentalization of PC-PYY distinguishes it from other AMPs, which have broader ranges of activity against microorganisms (4, 21).

Our studies show that PYY is active against virulent forms of *C. albicans* and less so on other commensal microbes. We observed similar membrane disrupting effects of PC-PYY in other species of *Candida*, including *C. dubliniensis* and *C. tropicalis,* but not the respiratory tract pathogen, *Aspergillus fumigatus,* leading us to conclude these observations may be specific to intestinal fungal pathobionts. This property arguably plays a role in maintaining the gut mycobiome in a state of commensalism. This finding complements recent work showing anti-virulence properties of intestinal mucin glycans (22) and specific mucosal IgA responses to *C. albicans* hyphae, which similarly inhibit virulence and maintain commensalism (23).

This study has several important limitations. First, our *in vivo* model utilized a global PYY KO, which has disrupted endocrine PYY functions that could confound results compared with a PC specific PYY mutant. To limit this, young animals were used prior to metabolic disruption. Second, while PYY is found in several single cell RNAseq databases, not all report its presence, suggesting expression is at low abundance or regionally variable, consistent with our data. Finally, we employed *in vitro* PYY concentrations of 20–30 μM, whereas PYY was found to be 8–10 μM in intestinal mucus at baseline, but becomes elevated upon *C. albicans* challenge.

In summary, PC-PYY is a dual function intestinally secreted peptide, with an AMP structure that differs from its endocrine counterpart in biological action and function. The regulation and selectivity of PYY against gut invasive fungal phenotypes distinguishes it from other AMPs, implicating its function in gut mycobiome regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data and materials availability:

All data is available in the main text or the supplementary materials, including lead contact, materials, data, and code availability. Raw data for Candida RNAseq transcriptomics is available at NCBI GEO #GSE229566. Raw amplicon sequencing data for 16S and ITS is available at NCBI Short read achieve under BioProject IS: PRJNA631955.

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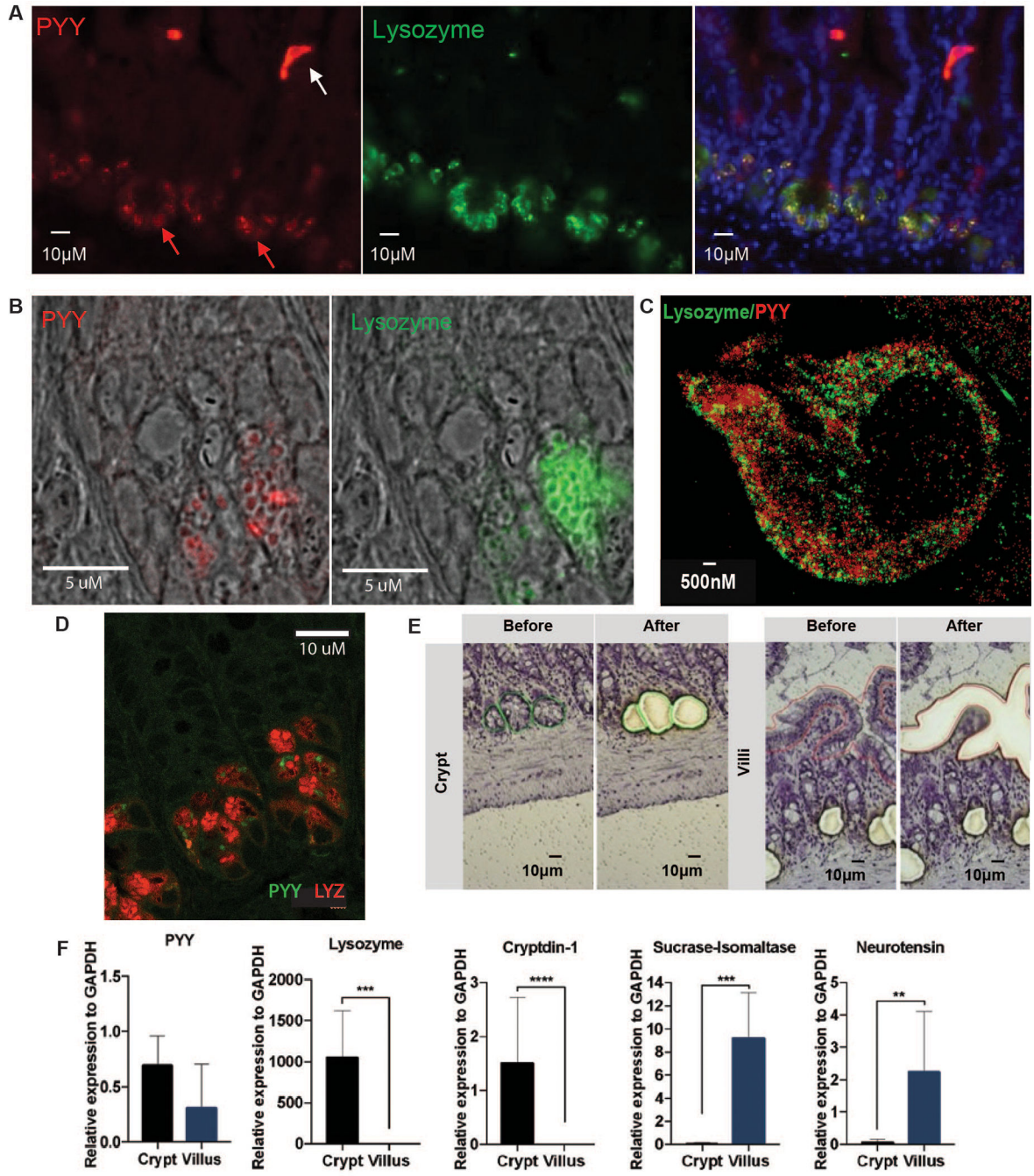

Fig. 1. Peptide YY (PYY) localizes to ileal Paneth cells.

(A) PYY (red) detection in Paneth cells (PC, red arrow) and L-cells (white arrow) via anti-PYY antibody immunofluorescence (IF). PC PYY staining co-localized (right panel) with PC Lysozyme (LYZ, green; middle panel). **(B)** Confirmation of PC PYY localization via mRNA Fluorescent *In Situ* Hybridization (red, left panel) and counterstaining with anti-LYZ antibody (green, right panel). **(C)** High-resolution stimulated emission depletion IF microscopy of PC PYY and LYZ packaging in discrete secretory granules within the cytosol. **(D)** SP8 confocal microscopy of PYY (green) and LYZ (red) in ileum PCs. **(E)**

Representative extractions from ileal crypts and villus epithelial cells via Laser-capture microdissection (LCM). **(F)** Relative expression of PYY and marker mRNA for crypt secretion compared with villus epithelium secretion estimated from LCM. Lysozyme and Cryptdin-1 are AMPs that are also secreted from PCs; Sucrase-isomaltase is released from the villus surface brush border; Neurotensin is a marker for enteroendocrine cells. Significance was determined using t-test (n=6, repeated twice, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

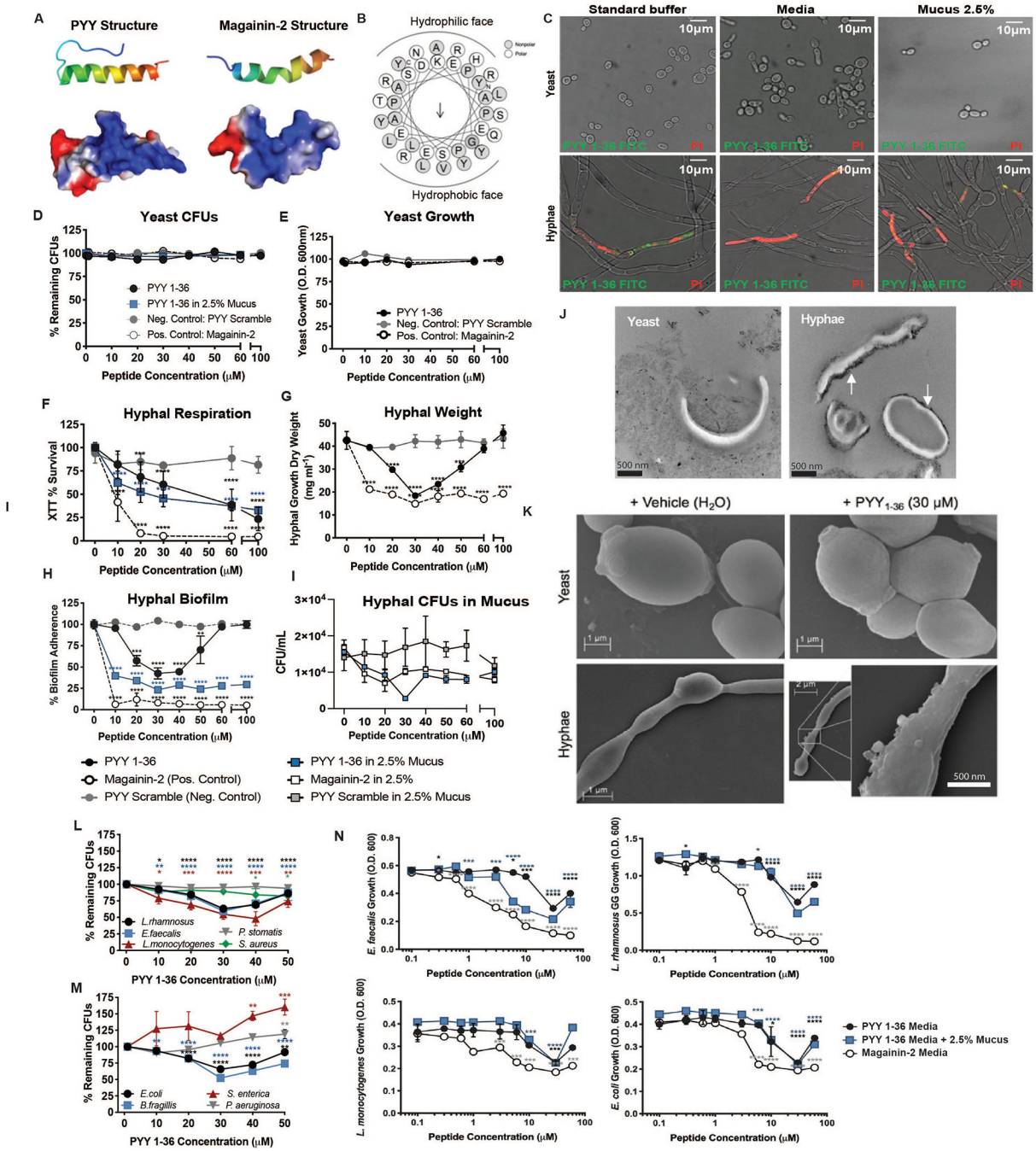

Fig. 2. PYY displays antimicrobial activity towards *Candida albicans* hyphae and some bacteria.

(**A**) Ribbon diagram (top) and space-filling model indicating electrostatic surface charge (bottom; red negative, blue positive) for amphipathic alpha-helix structures of $PYY_{1–36}$ and Magainin-2. **(B)** Amphipathic $PYY_{1–36}$ helical wheel projection displaying surface localization of residues and calculated hydrophobic dipole moment (μH 0.208, arrow). **(C)** Propidium iodide (PI) staining of *C. albicans* yeast and hyphae following exposure to $PYY_{1–36}$-FITC (green) in standard antimicrobial peptide assay buffer (AMP), preferred growth media for each form (YPD-yeast or RPMI-hyphae), or standard buffer + 2.5%

w/v porcine mucus. *C. albicans* yeast **(D) s**urvival (Colony Forming Units, CFUs) and **(E)** growth (Optical density, O.D.) following exposure to $PYY_{1–36}$ +/− mucus, PYY scramble, and Magainin-2 peptides. Pathogenic *C. albicans* hyphal **(F)** respiration by tetrazolium salt (XTT) assay **(G)** dry weight **(H)** biofilm adherence and (**I**) CFUs following exposure to $PYY_{1–36}$ +/− mucus, PYY scramble, and Magainin-2 peptides. **(J)** Transmission electron microscopy for visualization of cationic surfaces (black probe accumulation) in *C. albicans* yeast and hyphae membranes. **(K)** Scanning electron microscopy of *C. albicans* yeast and hyphae following 2-hour exposure to vehicle (H_2O) or $PYY_{1–36}$. Dose-dependent killing of Gram-positive (**L**) and Gram-negative (**M**) bacteria induced by $PYY_{1–36}$. CFUs remaining were normalized to 0 μM PYY. Significance was determined via ANOVA. (**N**) Impact of varying concentrations of $PYY_{1–36}$ +/− mucus versus Magainin-2 on bacteria growth. O.D. values were normalized to wells containing 0 μM PYY. All assays were performed in duplicate three times. Significance was determined via ANOVA (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

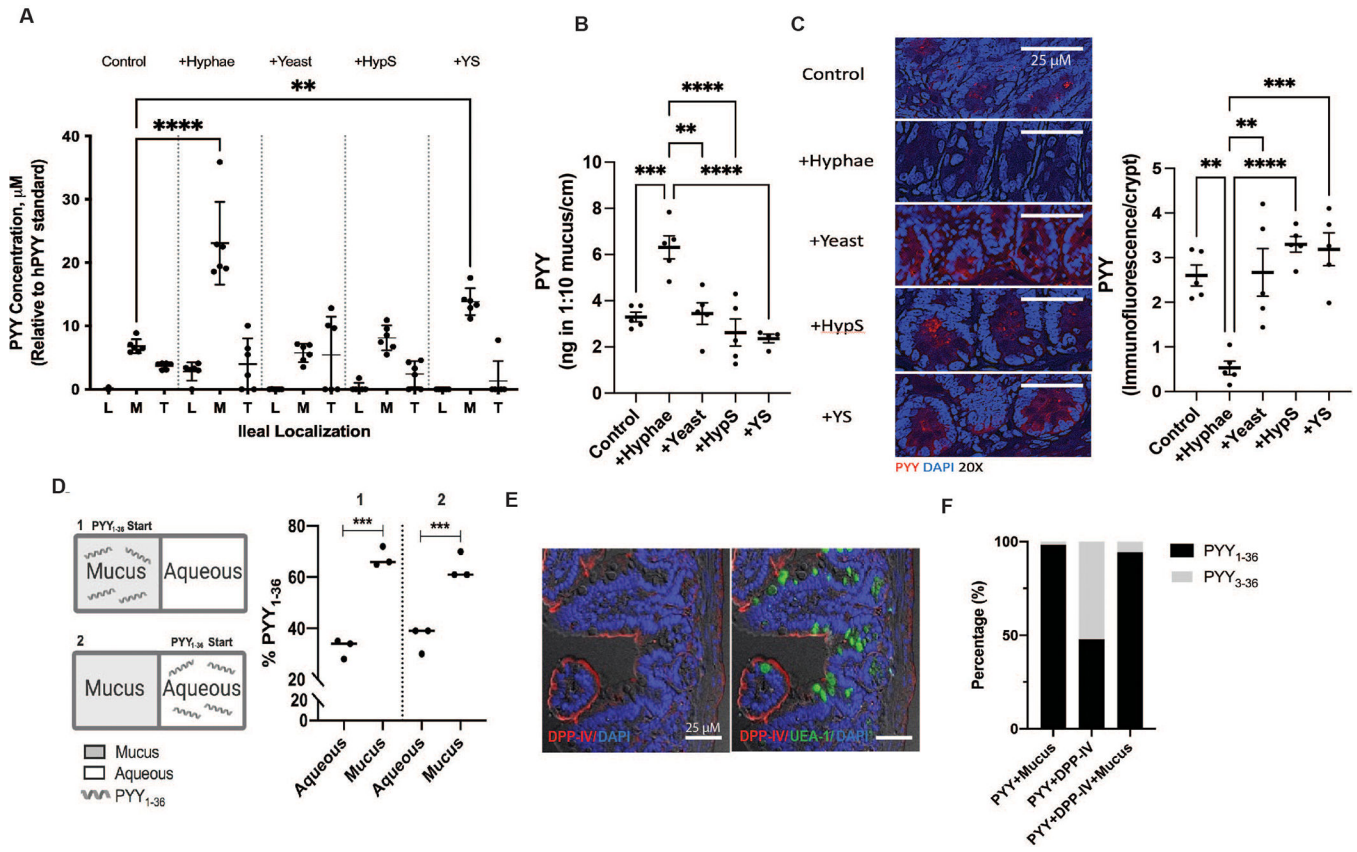

Fig 3. Exposure to *C. albicans* hyphae but not yeast enhances PYY localization into ileal mucus.

(**A**) $PYY_{1–36}$ quantification using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) in lumen (L), mucus (M), and tissue (T) of *ex vivo* ileal loops (n=6 /treatment) and in (**B**) mucus of *in vivo* ileal loops (n=5 /treatment) following stimulation with vehicle (Control), *C. albicans* hyphae (+Hyphae), *C. albicans* yeast (+Yeast), hyphae conditioned media (CM) supernatant (+HypS), or yeast CM supernatant (+YS). (**C**) Representative staining and quantification of PC PYY protein (red) following *in vivo* ileal loop stimulation with vehicle control, +Hyphae, +Yeast, +HypS, or +YS. Significance was measured using ANOVA and Dunnett's Multiple Comparisons Test; **p<0.01, *** p<0.005, **** p<0.001. (**D**) PYY mucus vs. aqueous partitioning *in vitro*. $PYY_{1–36}$ was added to either mucus (1) or aqueous (2) wells separated by a 10 kDa MWCO filter. Significance was measured using t-test; ***p<0.001. (**E**) Dipeptidyl peptidase IV (DDP-IV) immunofluorescence (IF) in murine ileum brush border. Lectin UEA-I-FITC (*Ulex europaeus)* counterstain = ileal mucus; DAPI = nuclei. (**F**) $PYY_{1–36}$ and $PYY_{3–36}$ exposure to DPP-IV +/− mucus to assess peptide degradation via LC-ESI-MS (n=3/treatment), expressed as a percent of total peptide. All experiments were done in triplicate with 3 replicate experiments.

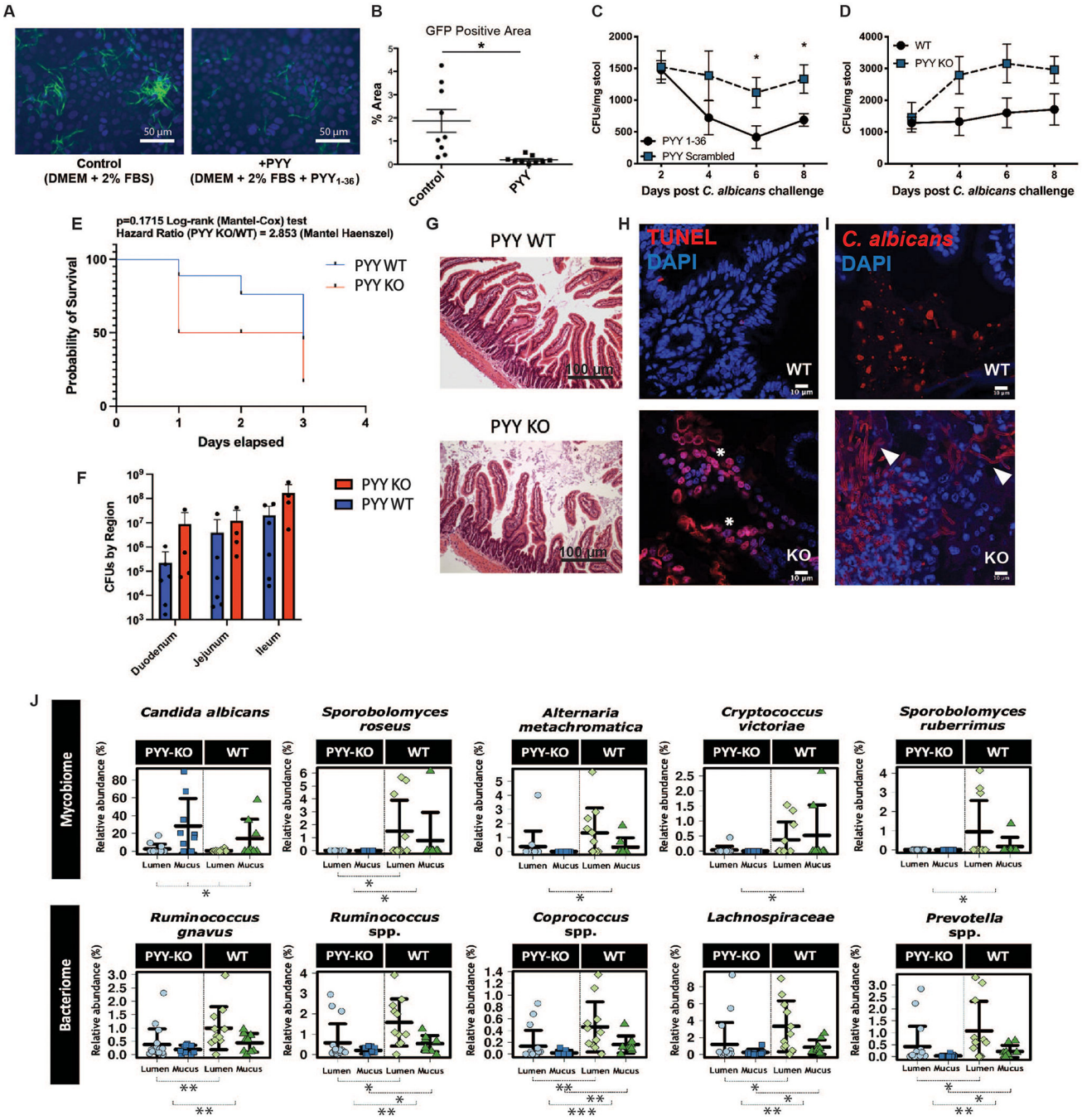

Fig 4. PYY reduces gastrointestinal colonization of *C. albicans in vitro* and *in vivo*.

(A, B) *C. albicans* hyphae abundance (Hyphae-GFP) on confluent Caco2 cells exposed to vehicle (Control) or $PYY_{1–36}$ (20µm) after 6 hours. (**C**) Fungal colony forming units (CFUs) in wild-type C57BL/6 mice (WT) +/− exogenously administered $PYY_{1–36}$ or scrambled peptide via oral gavage 8 days post *C. albicans* ($4×10^6$ CFU) challenge (n=8/group, *p<0.05 vs control). (**D**) *C. albicans* colonization ($4×10^6$ CFUs) in PYY-KO relative to WT mice (n=8–9/timepoint/group). (**E**) 72-hour PYY-KO and WT mouse survival after gavage with *C. albicans* ($2×10^7$ CFU; n=8/group, repeated twice with both males and females) under

cefoxitin and clindamycin (2 WT were humanely euthanized). (**F**) CFUs recovered from in small intestinal regions of PYY-KO and WT animals gavaged with *C. albicans* (2×10^7 CFU) (**G**) Intestinal mucosal gross morphology remained intact, while (**H**) epithelial cell apoptosis (red = TUNEL; white asterisk) and (**I**) virulent *Candida* morphology (hyphae; white arrows) was increased vs yeast in the mucosa of PYY-KO vs WT animals (36.3±12.1 vs. 11.77±3.8 %, $p<0.04$) following oral gavage challenge under clindamycin (2×10^7 CFU/mouse; $n=8$/group; repeated twice with both males and females, red = *Candida*). (**J**) Differentially altered fungal (top panel, $n=39$ samples) and bacterial (bottom panel, $n=46$ samples) populations from small intestinal mucus and lumen of PYY-KO vs. WT animals without Candida challenge as determined via Wilcoxon rank test (between groups) and Kruskal-Wallis test (across groups) *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. To account for uneven sequencing depth, data were transformed into relative abundances based on total sum scaling.