Supporting Information

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SI Materials and Methods

Gnotobiotic Mice. Germ-free C57BL/6 mice were maintained in plastic gnotobiotic isolators as previously described (1). For monocolonization experiments, age-matched germ-free C57BL/6 mice were orally gavaged with 10^8 CFU of stationary-phase bacterial culture. The following bacterial strains were used to colonize germ-free mice: *Bacteroides fragilis* strain 9343 and its isogenic mutant Δ*tsr15*M8 (2), *Bacteroides thetaiotaomicron* strain VPI-5482, and *Salmonella typhimurium* strain SL1433 and its isogenic mutant ΔSPI-1 (3). Mice were killed after 48 h and intestinal tissues were snap-frozen for RNA extraction. Small-intestinal colonization levels were measured by dilution plating of luminal contents. Bacterial levels in spleen and mesenteric lymph nodes were determined by dilution plating of homogenized tissue.

Bone Marrow Reconstitution. Ly5.2 recipient mice (wt or *MyD88*$^{-/-}$) were γ-irradiated twice with 5 Gy and reconstituted with 5×10^6 bone marrow cells from Ly5.1 donor mice (6–8 weeks of age). At 8 weeks after reconstitution, mice were tested for chimerism and analyzed for expression of antimicrobial factors and bacterial translocation to mesenteric lymph nodes. Reconstitution efficiencies were >90% as determined by FACS staining of blood leukocytes with antibodies against Ly5.1 and Ly5.2.

LPS Administration Studies. For LPS administration experiments, age-matched germ-free C57BL/6 mice were orally gavaged with 1 mg or 10 mg of LPS from *E. coli* serotype 026:B6 (Sigma). After 48 h, mice were killed and intestinal tissues were snap frozen for RNA extraction.

Supporting Description Accompanying Fig. S5. Paneth cells detect bacteria that closely associate with the mucosal surface. Although the vast majority of bacteria are sequestered in the gut lumen, a subpopulation of commensals localizes close to the mucosal surface, in or beneath the mucus layer that overlies the entire epithelium (4). Surface-associated bacteria are those most likely to opportunistically invade across the epithelial barrier and threaten host health, and indeed, increases in mucosa-associated bacterial densities are linked to pathologies such as inflammatory bowel disease (4). Our data in Figs. 3 and 4 show that Paneth cells control bacterial penetration across the mucosal barrier rather than regulating luminal bacterial numbers. A plausible model to explain these observations is that Paneth cells regulate surface bacterial numbers through production of antimicrobial factors (Fig. 5). This model raises the question of whether Paneth cells also specifically detect bacteria that are closely associated with the mucosal surface rather than luminal bacterial populations. To assess whether Paneth cells selectively detect surface mucosa-associated bacteria, we analyzed three gnotobiotic models in which bacterial-mucosal interactions are promoted through distinct mechanisms.

Commensals can become closely associated with mucosal surfaces through mechanisms that include aggregation of bacteria at the epithelial surface (5) and mucus binding (6). In the first model we used *Bacteroides fragilis* and its isogenic mutant (Δ*tsr15 M8*), which autoaggregates on surfaces *in vitro* (2) and *in vivo* (L.E. Comstock, personal communication) as a result of mutation of a key regulator of surface protein expression, tsr15. Oral inoculation of the wild-type and Δ*tsr15* mutant strains into germ-free mice resulted in similar luminal bacterial loads (Fig. S5*A*). However, there was a ≈10-fold increase in the numbers of mutant bacteria recovered from MLN (Fig. S5*A*), consistent with enhanced mucosal association. Concurrently we found that expression of RegIIIγ, RegIIIβ, CRP-ductin, and RELMβ were all significantly higher in Paneth cells from mice colonized with the Δ*tsr15* mutant (Fig. S5*B*).

Host mucosal defenses such as secretory immunoglobulin A inhibit close association between bacteria and the mucosal surface epithelium (7), and thus increased numbers of mucosa-associated bacteria are observed in immunodeficient mice (8–10). We found that the related commensal *Bacteroides thetaiotaomicron* triggered significantly enhanced antimicrobial factor expression in Paneth cells from *RAG1*$^{-/-}$ mice as compared with wild-type mice (Fig. S5*D*). Although *B. thetaiotaomicron* colonization of wild-type mice elicited RegIIIγ, RegIIIβ, and RELMβ mRNA levels similar to those observed in conventional mice, these expression levels were enhanced in *RAG1*$^{-/-}$ mice (Fig. S5*D*), which lack a functional adaptive immune system (11). These differences were not due to disparities in luminal bacterial loads, which were similar between the two hosts (Fig. S5*C*). Rather, enhanced expression correlated with increased translocation to MLNs (Fig. S5*C*), consistent with the increased surface bacterial loads characteristic of immunodeficient mice (10).

In contrast to commensals, enteric pathogens frequently harbor mechanisms for specific epithelial attachment, giving them a foothold in the densely colonized intestinal ecosystem and facilitating invasion of host tissues (12). *Salmonella typhimurium* interacts directly with gut epithelia as a first step in barrier translocation and systemic dissemination (12). We compared Paneth cell responses to wild-type *S. typhimurium* and ΔSPI-1, an isogenic mutant of *Salmonella* that lacks genes necessary for direct interaction with epithelial cells (3). Following oral inoculation into germ-free wild-type mice, ΔSPI-1 elicited antimicrobial factor expression similar in magnitude to that observed in conventional mice (Fig. S5*F*). However, wild-type *Salmonella* triggered markedly higher expression of these factors (Fig. S5 *F* and *G*). Luminal colonization levels did not account for these differences (Fig. S5*E*). However, 30-fold fewer mutant than wild-type *Salmonella* were recovered from MLN (Fig. S5*E*), consistent with the epithelial interaction defect of ΔSPI-1 (13).

Collectively, these findings suggest that Paneth cells are selectively sensitive to bacteria that closely associate with the mucosal surface, regardless of whether these bacteria are commensals or pathogens. Enhanced activation of MyD88-dependent responses occurs irrespective of whether association is through surface aggregation, host immunodeficiency, or direct epithelial attachment, suggesting that activation does not depend on a species-specific bacterial factor. It is unlikely that overt entry of bacteria into epithelial cells is required to activate the MyD88-dependent antimicrobial program of Paneth cells, as these responses are triggered by commensals, such as *B. thetaiotaomicron*, that lack mechanisms for epithelial entry (14). It is furthermore unlikely that there is a requirement for direct bacterial binding to Paneth cells, as *Bacteroides* species also lack mechanisms for direct epithelial attachment (14). Rather, we propose that Paneth cells sense mucosa-associated bacterial density as a function of microbe-associated molecular patterns (MAMP) concentration at the mucosal surface, resulting in concentration-dependent activation of TLRs (Fig. 5). These data further show that challenge with a single Gram-negative species is sufficient to activate a complex antimicrobial response that includes antimicrobial factors directed against both Gram-

positive and Gram-negative bacteria. This likely reflects the fact that the intestinal epithelium is normally confronted with a complex microbial challenge that requires a complex antimicrobial response.

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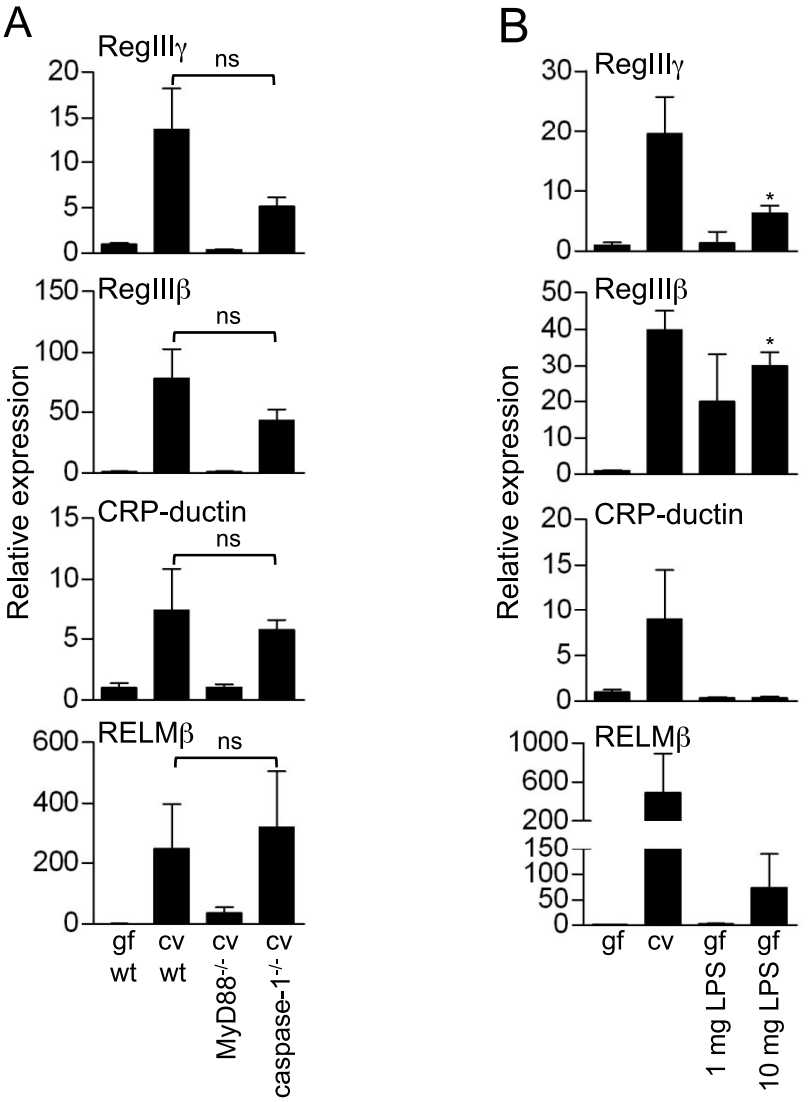

Fig. S1. MyD88-dependent antimicrobial factor expression is caspase-1 independent and inducible by LPS. (*A*) Expression of antimicrobial factors was compared between germ-free (gf) wild-type (wt), conventional (cv) wild-type, cv *MyD88*$^{-/-}$, and cv *caspase-1*$^{-/-}$ mice. Transcript levels were assayed by Q-PCR of small intestine ($n = 3$ mice per group). Results were normalized to 18S rRNA levels and expressed relative to germ-free (gf) controls. Error bars indicate ± SEM. ns, not significant ($P > 0.05$). (*B*) Expression of *RegIIIγ*, *RegIIIβ*, and *RELMβ* are induced by lipopolysaccharide (LPS). LPS was administered orally to germ-free mice, and transcript levels were assayed 48 h later by Q-PCR of small intestine ($n = 3$ mice per group; $P < 0.05$).

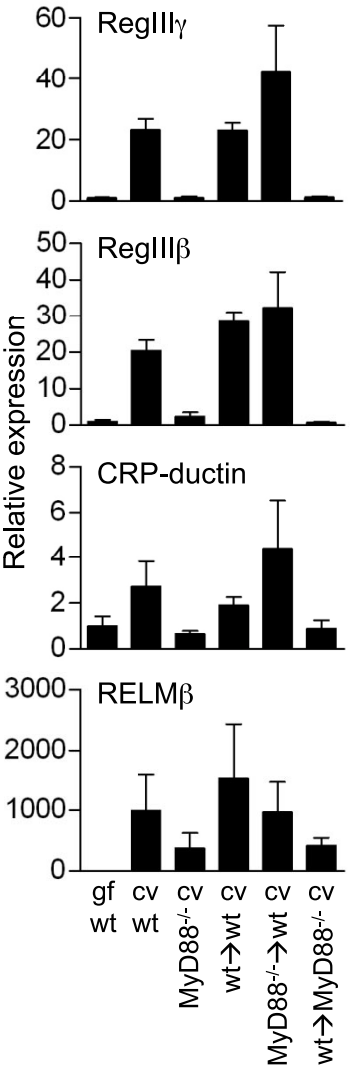

Fig. S2. Activation of the MyD88-dependent antimicrobial program does not require MyD88 expression in hematopoietic lineages. mRNA expression levels were quantitated in small intestines of wild-type (wt) or *MyD88*$^{-/-}$ mice transplanted with bone marrow from wild-type or *MyD88*$^{-/-}$ donors (3–4 mice per group). Q-PCR assays were run in triplicate and are shown as mean values normalized to 18S ribosomal RNA. gf, germ free; cv, conventional. Relative expression levels were calculated relative to gf controls. Error bars indicate ± SEM. wt → wt, wild-type donor to wild-type recipient; *MyD88*$^{-/-}$ → wt, *MyD88*$^{-/-}$ donor to wt recipient; wt → *MyD88*$^{-/-}$, wild-type donor to *MyD88*$^{-/-}$ recipient.

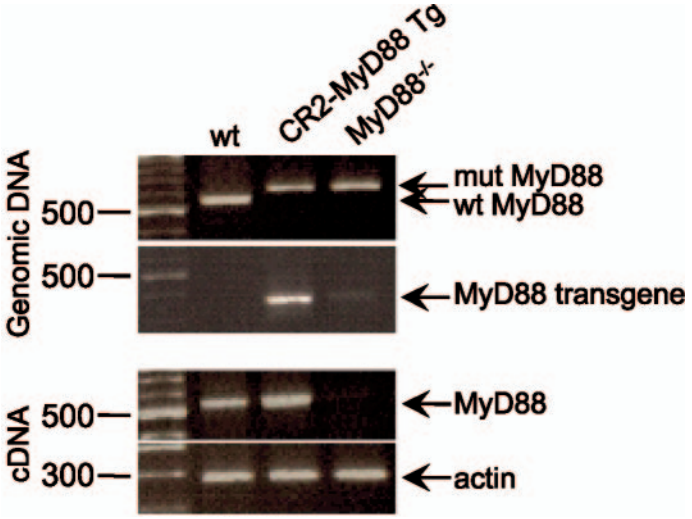

Fig. S3. Analysis of CR2-MyD88 transgenic mice. MyD88 wild-type, knockout, or transgenic alleles were identified by PCR of genomic DNA. Expression of the MyD88 transgene was assessed by RT-PCR of small intestinal RNA. Primer sequences are given in Table S1. CR2-MyD88 Tg denotes mice harboring the CR2-FLAG-MyD88 transgene on a *MyD88*$^{-/-}$ background.

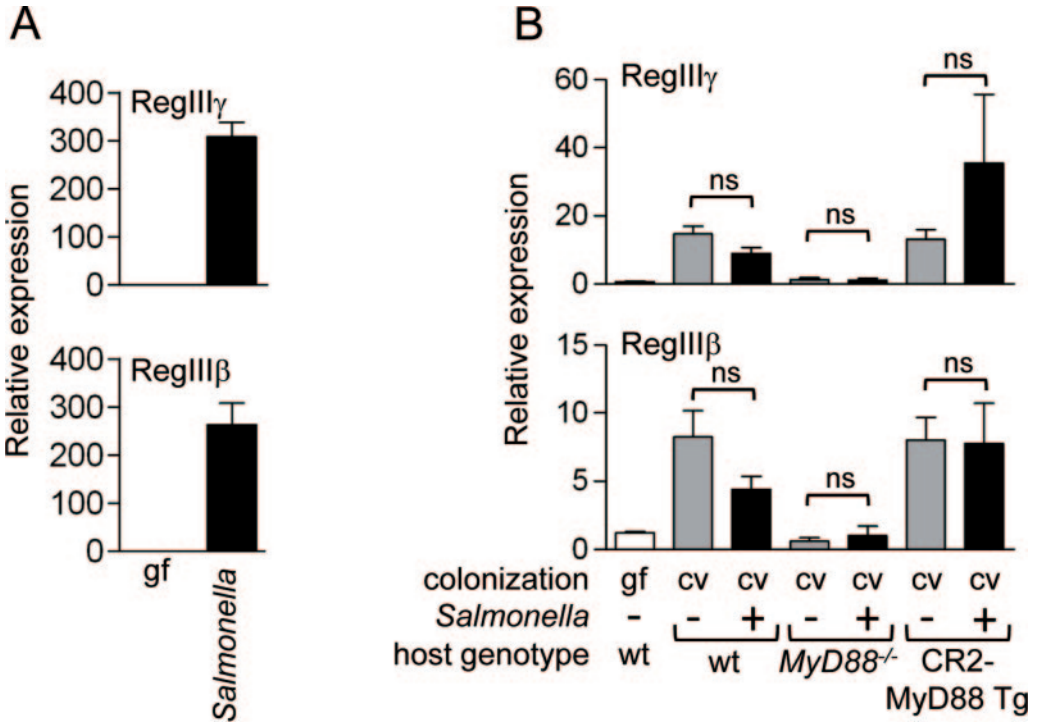

Fig. S4. MyD88-dependent antimicrobial responses following *Salmonella* challenge. (*A*) 10^8 cfu of wild-type *Salmonella typhimurium* were introduced orally into germ-free wild-type C57BL/6 mice. Mice were killed 48 h later. Q-PCR assays were performed as described in Fig. 1. $n = 3$–4 mice per group; error bars indicate ± SEM. This shows that *Salmonella* can elicit expression of RegIIIγ and RegIIIβ. (*B*) Conventionally raised wt, *MyD88*$^{-/-}$, or CR2-MyD88 Tg mice were orally inoculated with 10^8 cfu of *S. typhimurium*. Numbers of *S. typhimurium* in small intestine, MLN, and spleen were quantitated after 48 h and are shown in Fig. 3. *Salmonella* infected mice revealed no statistically significant increases ($P > 0.05$) in RegIIIβ and RegIIIγ expression over the levels observed in conventional uninfected mice. This is consistent with the low numbers of colonizing *Salmonella* relative to commensal bacteria shown in Fig. 3*B*. However, as shown in Fig. 3*B*, Paneth cell-intrinsic MyD88 protects against *Salmonella* penetration. Thus, the protection afforded against *Salmonella* penetration and dissemination is likely the result of commensal stimulation of MyD88-dependent responses. This suggests that commensals protect their host against pathogen invasion not only through colonization resistance but also by activating MyD88-dependent signaling pathways in the host epithelium. Q-PCR assays were performed as described in Fig. 1. Error bars indicate ± SEM. ns, not significant.

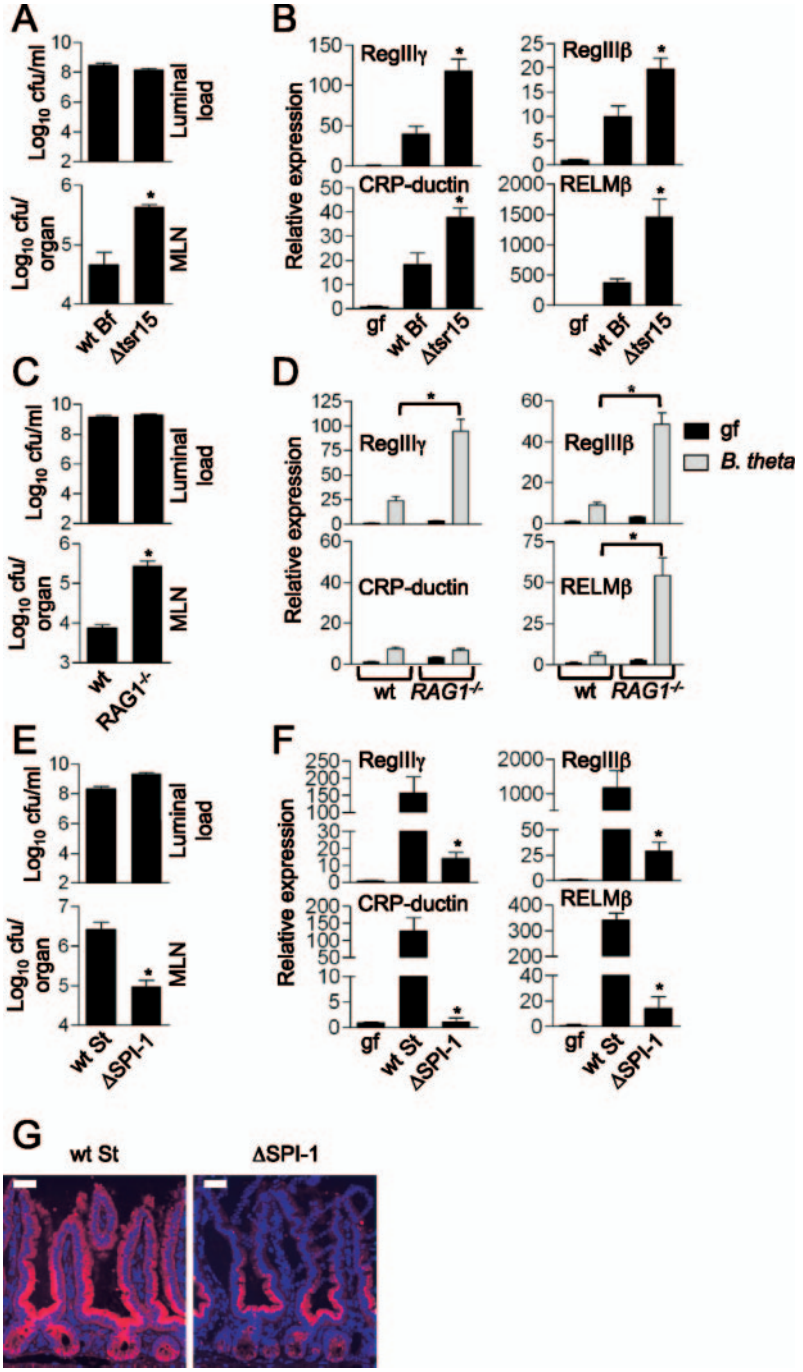

Fig. S5. Paneth cells detect bacteria that closely associate with the mucosal surface. (*A* and *B*) 10^8 cfu of wild-type *Bacteroides fragilis* (Bf) or the isogenic mutant Δ*tsr15 M8* were introduced orally into germ-free wild-type C57BL/6 mice and killed after 48 h. (*A*) Bacterial numbers were quantitated in small intestinal lumen and mesenteric lymph nodes (MLN). (*B*) Q-PCR assays on Paneth cells recovered by laser capture microdissection from small intestinal tissues. (*C* and *D*) 10^8 cfu of *Bacteroides thetaiotaomicron* were orally inoculated into germ-free wild-type (wt) or *RAG1*$^{-/-}$ C57BL/6 mice and killed after 48 h. (*C*) Quantitation of bacterial numbers and (*D*) Q-PCR analysis was performed as in (*A*) and (*B*), respectively. Note that although CRP-ductin expression is elicited following *B. thetaiotaomicron* colonization of wild-type mice, we do not observe enhanced expression following colonization of *RAG1*$^{-/-}$ mice. It is not clear why CRP-ductin differs in this way from the other three transcripts, although we note that CRP-ductin is unique in that it is unresponsive to oral administration of LPS (Fig. S1*B*). (*E* and *F*) 10^8 cfu of wild-type *S. typhimurium* (wt St) or the isogenic mutant ΔSPI-1 were introduced orally into germ-free wild-type C57BL/6 mice. (*E*) Quantitation of bacterial numbers and (*F*) Q-PCR analysis was performed as in (*A*) and (*B*), respectively. $n = 3$–4 mice per group; error bars indicate ± SEM. *$P < 0.05$. (*G*) Visualization of RegIIIγ expression in small intestinal epithelium from mice orally challenged with wt St or the isogenic mutant ΔSPI-1. Sections were counterstained with Hoechst dye (blue). (Scale bar: 50 μm.)

Table S1. Primers used in this study

Gene	Forward primer	Reverse primer
18S rDNA	CATTCGAACGTCTGCCCTATC	CCTGCTGCCTTCCTTGGA
RegIIIγ	TTCCTGTCCTCCATGATCAAAA	CATCCACCTCTGTTGGGTTCA
RegIIIβ	TACTGCCTTAGACCGTGCTTTCTG	GACATAGGGCAACTTCACCTCACA
CRP-ductin	TGAACCGTGTGACAGTGGTCTTCA	TCTCCTTGTCACACTGCCATCTGT
RELMβ	AGCTCTCAGTCGTCAAGAGCCTAA	CACAAGCACATCCAGTGACAACCA
Defcr-rs-10	ATCATCCAGGTGATTCCCAGCCAT	TTCCGGGTCTCCAAAGGAAACAGA
FLAG-MyD88	CAAGATCTATGGACTACAAAGACGATGACGACAAG	CAAGATCTAGGGCAGGGACAAAGCCTTGGCAAGGC
MyD88 transgene	CCTTGCTGGCGGAGGAGATGGGC	GTCAGAAACAACCACCACCATGC
MyD88 gene	TGGCATGCCTCCATCATAGTTAACC	GTCAGAAACAACCACCACCATGC
neoR		ATCGCCTTCTATCGCCTTCTTGACG
actin	GAAGTACCCCATTGAACATGGC	GACACCGTCCCCAGAATCC