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Epithelial responses to Porphyromonas gingivalis in the community context.

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EPITHELIAL RESPONSES TO *PORPHYROMONAS GINGIVALIS* IN THE COMMUNITY CONTEXT

By

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ABSTRACT

EPITHELIAL RESPONSES TO *PORPHYROMONAS GINGIVALIS* IN THE COMMUNITY CONTEXT

Zackary Ray Fitzsimonds

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Porphyromonas gingivalis, a keystone pathogen for periodontal disease, initiates a complex molecular dialogue with gingival epithelial cells, leading ultimately to disruption of host signaling pathways. Dysbiotic host responses are also thought to contribute to the initiation and progression of oral squamous cell carcinomas. Recently, the homeostatic commensal *Streptococcus gordonii* has been shown to antagonize *P. gingivalis*-induced epithelial cell signaling events in host cells, such as proliferation and migration. The aim of this study was to characterize pathways that *P. gingivalis* targets to disturb host signaling, with a specific focus on pathways that *S. gordonii* can restore to homeostatic levels in the presence of *P. gingivalis*. RNAseq analysis of gingival epithelial cells challenged with *P. gingivalis* revealed a transcriptional pattern reflecting activation of Notch signaling. Signaling is activated by proteolytic cleavage of Notch and Jag1 by gingipain proteases and leads to upregulation of Olfactomedin 4 (OLFM4). This activation is antagonized by *S. gordonii* through the secretion of hydrogen peroxide, which inactivates the gingipains, preventing proteolytic cleavage of Notch1 and Jag1, ultimately blocking OLFM4 upregulation. OLFM4 is required for *P. gingivalis*-induced epithelial cell

migration. RNAseq analysis identified an OLFM4-independent immune signature that included increased expression of CXCL8, CXCL10, and CXCL11 in epithelial cells transfected with siRNA and then challenged with *P. gingivalis*. In addition, a tissue culture model revealed that OLFM4 was required for *P. gingivalis*-induced epithelial cell migration. OLFM4 therefore represents a new axis by which *P. gingivalis* disturbs homeostasis at the mucosal barrier, as well as an additional mechanism for ameliorating this disruption by *S. gordonii*. This study provides insight into the complex molecular dialogue at the mucosal-microbe interface, and as a result expands on the need for new therapeutic approaches to prevent and identify those at high-risk for oral squamous cell carcinoma.

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CHAPTER 1 INTRODUCTION

Worldwide, approximately 405,000 patients are diagnosed with oral cancer each year with a the 5-year survival rate of just 50% [1]. This poor prognosis can be partially explained by the characteristic asymptomatic presentation in the early stages; by the time the patient has developed painful symptoms the carcinoma is in its late stages [2]. Oral cancers can originate as lymphomas in the lymphatic tissue of the tonsils and base of tongue; as carcinomas within salivary gland tissue; but most commonly as squamous cell carcinomas in areas of the mouth containing stratified squamous epithelium. Carcinomas of the oropharynx (including the base of the tongue) are generally referred to as oropharyngeal squamous cell carcinomas (OPSCC), and in OPSCC human papillomavirus (HPV) infection is a major risk factor [3]. Cancers of the oral squamous cells (OSCC), which account for about 90% of oral carcinomas, present most frequently on the tongue, lips, floor of the mouth, and gingiva [4]. Around 75% of OSCC can be attributed to tobacco smoking, which increases the risk for developing oral cancer by 6-fold [2]. Smoking also provides an encouraging environment for periodontal pathogens, and is an independent risk factor for periodontal disease [3]. Alcohol is another significant risk factor for oral cancer, and the combined risk for those who smoke and drink is increased fifteen-fold [2]. Gingival squamous cell carcinoma (GSCC) is particularly interesting because the traditional risk factors of smoking and alcohol consumption are not associated with this malignancy, and as the lesions mimic the appearance of periodontal disease they tend to

go untreated [5]. Tooth loss as a result of bone loss in periodontal disease is an independent risk factor for head and neck, gastric, and colorectal cancer [6, 7]. Additionally, colonization by periodontal pathogens has recently been identified as a risk factor for OSCC independent of alcohol, smoking, and HPV [3], and increased colonization by the periodontal pathogen *Porphyromonas gingivalis* has been correlated with GSCC [8]. The association between the oral microbiota (the collection of microorganisms found in the oral cavity) and cancers of the head and neck region has been extensively studied in recent years, using both culture dependent and independent methodology [9,10]. What has emerged is a picture of enrichment of particular organisms such as *Fusobacterium nucleatum*, *Treponema denticola*, and *P. gingivalis,* along with a decrease in the oral streptococci (with the exception of *S. anginosus*) [9, 10].

The notion that bacteria may be involved in the development of oral tumors is not new. For example *Treponema pallidum* was considered an etiological agent of OSCC back in the early part of the 20th century. However, with the subsequent recognition of the importance of viruses in carcinogenesis the idea fell in to abeyance, and it was not until *Helicobacter pylori* was established as a cause of gastric cancer in the 1990s that the potential for a carcinogenic role of bacteria became rehabilitated [11, 12]. Mechanistically, there are a number of broadly defined categories by which bacteria could contribute to tumor growth and development. These include modulation of the balance of host cell proliferation and death; disruption of immune surveillance; and alteration of the metabolism of host produced compounds, nutritional substrates or pharmaceuticals [13]. Oral bacteria such as *P. gingivalis, F. nucleatum* and *T. denticola* exhibit properties

consistent with these criteria, and can increase epithelial cell proliferation while inhibiting apoptosis, alter the inflammatory microenvironment and produce carcinogenic metabolites. These organisms will be discussed and referenced below due to their positive correlation with OSCC, as well the availability of mechanistic studies that have shown cancer-associated phenotypes.

Porphyromonas gingivalis

P. gingivalis is a keystone pathogen in periodontitis [14, 15] and many of the pathogenic mechanisms that impinge upon tissue integrity and disrupt protective immune responses are potentially relevant to tumorigenesis. These pathogenic mechanisms are summarized in Fig. 1.

Moreover, immunohistochemistry and other detection methods have identified increased colonization of *P. gingivalis* in OSCC, esophageal squamous cell carcinoma (ESCC), and GSCC [8, 16, 17]. In vitro, *P. gingivalis* engages gingival epithelial cells (GECs) in a complex molecular dialogue, a major thread of which involves subversion of host signaling pathways by bacterial effectors, such as the FimA-component fimbriae and the SerB serine phosphatase, to promote bacterial entry, intracellular trafficking and survival [18-20]. Comprehensive analyses of host transcriptional response to *P. gingivalis* invoke a pattern of enhanced cell survival and proliferation [21-23] phenotypes that have been verified by a number of laboratories.

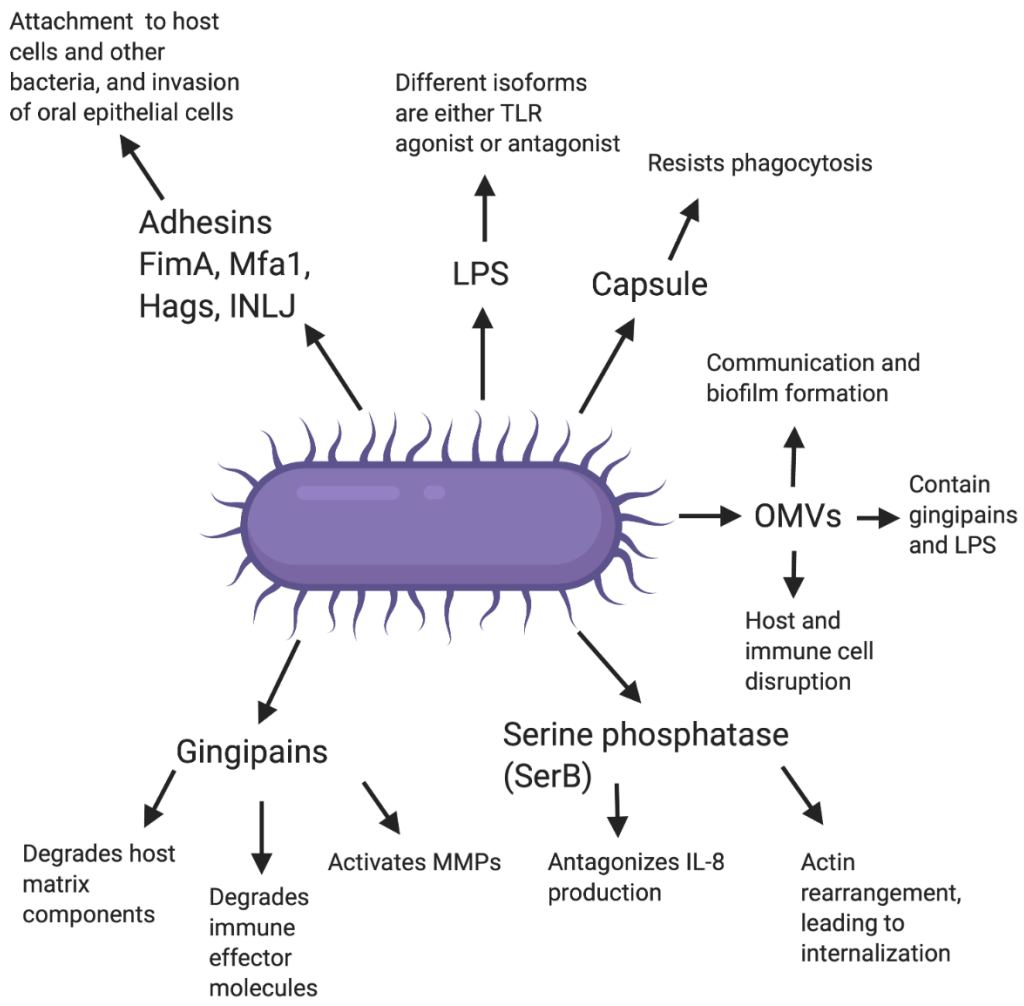


Figure 1: Summary of *P. gingivalis* virulence factors involved in host signaling manipulation and their targets.

Indeed, *P. gingivalis* utilizes multiple mechanisms to suppress host cell death and stimulate proliferation. In primary cultures of GECs, *P. gingivalis* activates the Jak1/Akt/Stat3 signaling hub that controls intrinsic mitochondrial apoptosis pathways [24, 25], and at the mitochondrial membrane the activity of proapoptotic effectors such as Bad is reduced, while the ratio of antiapoptotic factors, such as Bcl2, to proapoptotic

factors is increased [26]. Downstream caspases including caspase-9 and the executioner caspase-3 are consequently suppressed. Additionally, *P. gingivalis* can modulate expression of microRNAs (miRs) in epithelial cells, and up-regulation of miR-203 leads to reduction of the proapoptotic signaling molecule SOCS3 [27]. A major antiapoptotic effector molecule of *P. gingivalis* is the secreted enzyme nucleoside diphosphate kinase (NDK), which can function as an ATPase and prevent ATP-dependent apoptosis mediated through the purinergic receptor P2X7 [28]. Another antiapoptotic function of NDK involves phosphorylation of Heat-shock-protein-27 (HSP27) which curtails cytochrome C release and caspase-9 activation [29]. Recently it has become apparent that *P. gingivalis* possesses a variety of kinase and phosphatase enzymes, some of which can function within host cells. Of particular relevance to cell survival, activation of the multi-purpose transcriptional regulator FOXO1 by dephosphorylation of serine residues induces antiapoptotic programs in epithelial cells, and knockdown of FOXO1 abrogates *P. gingivalis*-induced resistance to cell death [30].

Along with prolonged cell survival, increased proliferation is a feature of *P. gingivalis* infected epithelial cells. Signaling induced by the FimA fimbrial protein accelerates progression of primary GECs through the S-phase of the cell cycle by manipulation of cyclin/CDK (cyclin-dependent kinase) activity and by reducing the level of the p53 tumor suppressor [31]. The gingipains of *P. gingivalis* may also contribute to cell proliferation through proteolytic activation of β-catenin and disassociation of the β-catenin destruction complex. The accumulation of active β-catenin fragments in the nucleus drives the activity of the β-catenin-dependent, pro-proliferative TCF/LEF

promoter [32]. In oral squamous carcinoma cells, *P. gingivalis* can increase cell proliferation by regulating cyclin D1 expression through the miR-21/PDCD4/AP-1 negative feedback signaling pathway [33]. Additionally, in oral tumor cells *P. gingivalis* can increase expression of α-defensins which have been found to elevate proliferation through effects on epidermal growth factor receptor (EGFR) signaling [34].

Another role for *P. gingivalis* in carcinogenesis relates to its ability to influence the epithelial to mesenchymal transition (EMT) in GECs, as shown in Fig. 2. EMT is a cellular program through which epithelial cells shed their tight junctions in favor of an individual, mesenchymal phenotype. EMT is important for embryogenesis, and wound healing, but, if uncontrolled, ultimately leads to increased migration/invasion and cancer cell stemness. As befits its importance to the cell, EMT is controlled by a complex regulatory network involving a series of transcription factors such as Zinc Finger E-Box Binding Homeobox 1 (ZEB1), Zinc Finger E-Box Binding Homeobox 2 (ZEB2), Snail Family Transcriptional Repressor 1 (SNAI1), and Twist Family BHLH Transcription Factor 1 (TWIST1). These factors induce EMT by downregulating epithelial cell tight junction proteins (e.g. E- cadherin (CDH1), Zona Occludens Protein 1 (ZO-1)) while upregulating mesenchymal characteristics (e.g. N-cadherin (CDH2*)*, matrix metallopeptidase (MMP) 9 Vimentin (VIM)) [17, 35-38].

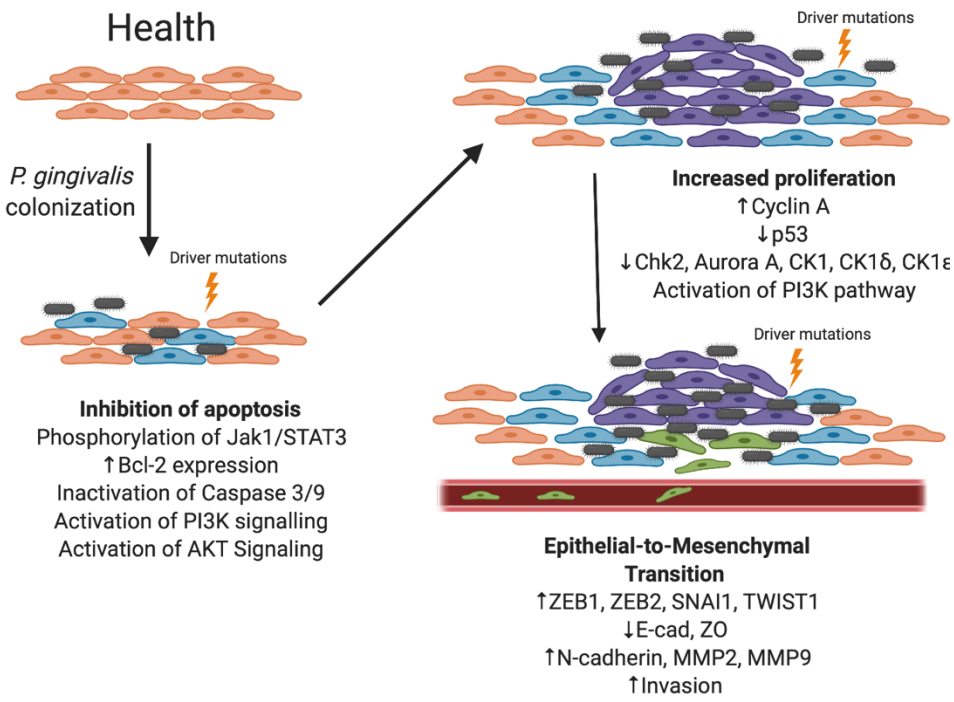


Figure 2: Potential mechanisms by which *P. gingivalis* (dark green rods) could impact cancer associated processes in gingival epithelial cells. Peach healthy epithelial cells, Blue epithelial cells that have acquired an anti-apoptotic phenotype, Purple epithelial cells that have acquired an accelerated proliferation phenotype, and Green epithelial cells that have undergone EMT, and have acquired an invasive phenotype. For simplicity other contributing host and environmental factors are not depicted.

ZEB1 is upregulated in a FimA-dependent manner through a pathway that involves GSK-3β in primary GECS [17, 37]. ZEB2, on the other hand, is regulated in a FimA-independent manner involving gingipain processing and activation of β-catenin along with dephosphorylation and activation of FOXO1 [38]. Although the extent and duration of EMT induced by *P. gingivalis* remains to be determined, epithelial cell infection leads to an increase in stemness, as evidenced by upregulation of the stem cell markers CD44 and CD133 and enhanced migration [17, 35-38]. Invasion, and potentially metastasis of

epithelial cells, can be facilitated by host MMP enzymes which degrade extracellular matrix and basement components. *P. gingivalis* has been shown to upregulate production of several MMPs including MMP-1, MMP-2, MMP-7, MMP-9 and MMP-10 from primary and transformed oral epithelial cells [17, 36, 37, 39, 40]. In invasive OSCC lines, *P. gingivalis* gingipains can stimulate proteinase-activated receptor 2 (PAR2) and PAR4 to increase MMP-9 proenzyme expression through ERK1/2-Ets1, p38/HSP27, and NF-kB pathways [40]. Subsequently, in a two-hit mechanism, gingipains process the proenzyme to active MMP9 ensuring an increase in cellular invasion [40, 41]

P. gingivalis has also been shown to activate Notch signaling through its secreted gingipains. Notch signaling is another pathway that can be activated to inhibit apoptosis, accelerate cell cycle progression, and induce EMT. Al-Attar et al. have shown that *P. gingivalis* activates Notch signaling specifically through Notch1, and the triple gingipain knockout mutant was unable to stimulate the pathway [42]. These investigators primarily focused on Notch activation leading to secretion of PLA_2-IIA, which is an anti-microbial peptide utilized by *P. gingivalis* to shift to a more pathogenic microbial community [42].

While a role for *P. gingivalis* induced Notch signaling has been defined in terms of anti-microbial peptide regulation, traditional Notch regulated phenotypes associated with transformation of epithelial cells have not been characterized. Notch signaling has also been shown to regulate Olfactomedin 4 (OLFM4), which is a secreted glycoprotein that was first characterized by its role in inhibition of apoptosis through binding of the mitochondrial protein GRIM19 [43]. Since that initial study, OLFM4 has been shown to

additionally accelerate cell-cycle progression, activate EMT, positively regulate MMPs, and dampen the innate immune response [44-47].

Liu et. al. showed that *H. pylori* upregulates OLFM4 in the stomach, and this regulation allows for efficient colonization [47]. Moreover, upon deletion of OLFM4, *H. pylori* was efficiently cleared by an unimpaired innate immune response. Another study sought to characterize the secretome of non-neoplastic, immortalized epithelial cells and neoplastic oral derived cell lines [48]. OLFM4 was the most secreted protein (12-fold increase) in tested supernatants of neoplasm-derived cell lines compared to the supernatant of non-neoplasm, immortalized cells, although its extracellular function has not been characterized [48] .

Chronic inflammation has emerged as a major contributor to tumor growth and metastasis, mainly through modulation of the tumor microenvironment by cytokines and chemokines, and through differential receptor expression [49]. The ability of *P. gingivalis* to incite prolonged, dysregulated inflammation could also contribute to the epidemiological associations between periodontitis and OSCC [49, 50]. In both OSCC cell lines and primary GECS *P. gingivalis* can upregulate programmed death-ligand 1 (PD-L1, B7-H1) and B7-DC, receptors that lead to anergy and apoptosis of activated T cells, and contribute to tumor cells' resistance to host immune responses [51]. In OSCC cells *P. gingivalis* stimulates the release of a variety of chemokines/cytokines including IL-8, IL-6, TGF-β1, and TNF-α [35, 39, 52]. IL-8 can increase MMP production and cell invasiveness, as well as stimulate proliferation through transactivation of the Epidermal growth factor (EFG)receptor [53]. In addition, the IL-23/IL-17 axis, which is strongly pro-tumorigenic in

colorectal cancer [54], can be induced by *P. gingivalis* [55]. Interestingly, in primary GECs *P. gingivalis* adopts a more stealth-like behavior through a process known as localized immune paralysis [56]. Release of a serine phosphatase (SerB) intracellularly results in antagonism of IL-8 production through dephosphorylation of the serine 538 residue of the p65 subunit of NF-κB [57]. Dephosphorylation of the p65 subunit prevents dimerization of the subunits, thus inactivating NF-κB. While this may restrain tumor progression, the effect may be offset by inhibition of the angiostatic chemokines CXCL9, CXCL10 and CXCL11 that would otherwise promote neovascularization of tumors and increased tumor growth or metastasis [49, 58].

In vivo evidence also supports a role for *P. gingivalis* in the development of oral carcinomas. In the 4-nitroquinoline-1-oxide (4NQO) tongue squamous cell carcinoma model, *P. gingivalis*-treated mice developed more and larger tumors on the tongue compared to the carcinogen alone group [59-62]. The development of squamous cell carcinoma was associated with enhanced free fatty acid production both in the tongue and in the serum of 4NQO treated mice, which is a shift also seen in oral cancer.

Fusobacterium nucleatum

While *F. nucleatum* is prevalent in a healthy microbiota, several studies have found that *F. nucleatum* is significantly enriched in patients with disease, whether that be periodontal disease, preterm delivery of low birth weight infants, head and neck cancer, or colorectal cancer (CRC) [63]. The potential role of *F. nucleatum* in cancers has been investigated in both *in vitro* and *in vivo* studies. *F. nucleatum* produces an adhesin, FadA, which is crucial for attachment and subsequent invasion of epithelial cells [64]. FadA is

thought to play a major role in CRC by binding to E–cadherin on CRC cells, thus activating β-catenin signaling and differentially regulating inflammatory and oncogenic responses [65]. The FadA-E-cadherin axis also upregulates Annexin A1, a modulator of Wnt/β-catenin-based proliferative signaling in CRC cells [66]. Localization of what is primarily an oral organism with developing tumors in the GI tract may be accomplished by another fusobacterial adhesin, Fap2, which binds to Gal-GalNac, abundant on CRC cell surfaces [67]. Fap2 can also immunosuppress tumor infiltrating lymphocytes, which are essential for immune responses to tumors. Specifically, Fap2 binds and activates the inhibitory immunoreceptor TIGIT which is expressed by T and Natural Killer (NK) cells [68]. Further compromising anti-tumor immunity, *F. nucleatum* activates the human inhibitory receptor CEACAM1 which also suppresses T and NK cells activities [69]. Clearly then, *F. nucleatum* can significantly impact cell signaling and tumor immunity with relevance to CRC. The extent to which these properties may pertain to OSCC is a vein of information ready to be mined. Studies that have been performed establish the ability of *F. nucleatum* to induce nuclear localization of NF-κB in GECs, while also increasing secretion of IL-1β via activation of the NLRP3 inflammasome and caspase-1 [70]. Release of endogenous danger-associated molecular patterns (DAMPs) such as apoptosis-associated speck-like protein (ASC) and high-mobility group box 1 protein (HMGB1) further amplifies inflammation. P38 is also activated by *F. nucleatum*, which leads to increased secretion of MMP9 and MMP13 [71]. Moreover, *F. nucleatum* can induce an EMT program in OSCC cells through upregulation of TGF-β, TNFα, and EGF signaling [35]. In conclusion, *F.*

nucleatum induces proliferation of CRC cells, suppresses T and NK cell activity, and increases secretion of MMP9 and MMP13, all of which contribute to carcinogenesis.

Coinfection with *F. nucleatum* and *P. gingivalis* exacerbated *in vivo* tumor development in the murine 4NQO tongue squamous cell carcinoma model [60]. The infected group had larger, more invasive tumors, with increased expression of cell cycle progression marker Cyclin D1 [60]. There was also an increase in phosphorylation of STAT3 in the infected group, which led to increased expression of IL-6 [60]. Further, in a colorectal cancer model *F. nucleatum* increases the size and number of tumors which develop in C57Bl/6 *Apc*$^{min/+}$ mice [72]. In summary, *F. nucleatum* enhances tumorigenesis in OSCC and CRC murine models, and with *P. gingivalis* is of critical importance in understanding microbial contributions to oral carcinogenesis.

Treponema denticola

In a healthy person's oral cavity, *T. denticola* is found in low abundance; however, in periodontal disease, *T. denticola* is one of the most abundant organisms [73]. Similarly, an increased abundance of *T. denticola* has been associated with ESCC and OSCC, and similarly correlated with an increased risk of CRC [74]. *T. denticola* is highly proteolytic, with dentilisin (chymotrypsin-like proteinase) being the primary secreted protease [75]. The presence of dentilisin is strongly correlated with early-stage mobile tongue squamous cell carcinoma, and high expression of dentilisin is associated with increased tumor invasion, tumor size, and recurrence in patients less than 60 years old [76]. Dentilisin can degrade IL-8 and TNFα [77, 78], and cleaves pro-MMP8 and pro-MMP9 to their active forms [10]. In a two-hit mechanism, dentilisin also degrades tissue inhibitors of MMPs,

TIMP1 and TIMP2, contributing to overall more proteolytic environment favoring invasion of epithelial cells. To summarize the above, *T. denticola* is associated with ESCC, OSCC, and CRC, and its secreted protease dentilisin is responsible for degradation of IL-8, TNFα, TIMP1, and TIMP2, all of which could contribute to an environment capable of inducing transformation of epithelial cells.

Oral Microbial Communities

The preceding text describes how individual species impact carcinogenesis; however, in the oral cavity, bacteria assemble into multi-species, spatially constrained communities known as biofilms. Within these communities, functional specialization of bacterial species emerges, and in periodontitis it is pathogenicity at the community level, or nososymbiocity, which is thought to determine the potential for disease [15]. Metabolic interactions can drive the spatial and temporal arrangement of organisms in the oral polymicrobial community, and individual species tend to associate with partners that are physiologically compatible [79]. Interestingly, a bioinformatics study of 11 oral bacteria identified a large redundancy in the metabolic potential of the community, and metabolic capabilities varied among early and late colonizers [80]. Furthermore, a recent meta- transcriptomic analysis of diseased periodontal sites as compared with patient matched healthy sites determined the metabolic profile of the community was highly conserved, whereas individual species associated with this profile were interchangeable. In other words, the overall metabolic profile is more consistent than the presence or absence of individual species. These studies suggest that the overall metabolic potential of the community may correlate better with disease than the presence of individual

species [81]. Overall metabolic stability is likely driven by shared ecology and stresses, and community homeostasis is a balance between metabolic redundancy and metabolic cross-feeding. If the metabolic capabilities and community metabolic necessities overlapped to a large extent, this would result in constant competition among individual species for nutrients. Instead, the conservation of core metabolic functions and mutualistic metabolism reduces antagonistic interactions between integral community participants and promotes community homeostasis.

Spatial Determinants Between Oral Microbes

Within a subgingival biofilm, the stable environment and intimate proximity facilitate nutrient transfer mechanisms among microbial constituents. For example, spatial imaging of plaque biofilms shows that *Porphyromonas gingivalis* and *Fusobacterium nucleatum* are often in close association [82]. In addition, *F. nucleatum* enhances the growth of *P. gingivalis* in the presence of oxygen, which suggests that the more aerotolerant *F. nucleatum* consumes oxygen, thereby providing a microenvironment with lower redox potential to benefit the growth of more oxygen-sensitive organisms [83].

In a study by Palmer et al. [84], *Actinomyces naeslundii* was incubated in minimal medium that did not support its growth; however, it displayed robust growth and coaggregation when cocultured with *Streptococcus oralis. Streptococcus gordonii* and related mitis group streptococci participate in several instances of cooperative metabolism. *S. gordonii* catabolizes carbohydrates and secretes H_2O_2 and L-lactate, a major carbon source for lactate-utilizing bacteria such as *Veillonella atypica* and

Aggregatibacter actinomycetemcomitans [85, 86]. Consequently, *S. gordonii* and related organisms can promote *A. actinomycetemcomitans* growth, biofilm formation, and in vivo pathogenicity [87, 88]. *V. atypica* induces expression of the *amyB* gene, encoding an α-amylase, of *S. gordonii* [89]; and thus *S. gordonii* grows better in association with *V. atypica* and secretes more lactate due to carbohydrate utilization, to the benefit of both organisms. Moreover, cooperative metabolism may have global benefits to other community members, as the lactate-utilizing *V. atypica* and *A. actinomycetemcomitans* neutralize acidification of the oral environment, thus protecting more acid-sensitive species, such as *P. gingivalis*.

Metabolic Cooperation between *A. actinomycetemcomitans* and *S. gordonii*

The relationship between *A. actinomycetemcomitans* and *S. gordonii* is more complex and involves a balance between synergistic cross-feeding and antagonistic interactions. Stacy et al. [90] demonstrated that *A. actinomycetemcomitans* spatially positions itself far enough away to allow detoxification of H_2O_2 through the production of catalase, but close enough to *S. gordonii* to benefit from the secretion of L-lactate. Optimal positioning of *A. actinomycetemcomitans* within the biofilm matrix is achieved through the production of dispersin B, an enzyme that hydrolyzes polysaccharides and is upregulated in the presence of *S. gordonii* [91]. Additionally, *S. gordonii* increases the availability of oxygen for *A. actinomycetemcomitans* to use as a terminal electron acceptor. In this way, *S. gordonii* shifts the metabolism of *A. actinomycetemcomitans* from fermentative to oxidative respiration [92]. This process, termed “cross-respiration,” also enhances the growth and fitness of *A. actinomycetemcomitans*. Similar homeostatic

mechanisms to maximize energy gains from metabolic cross-feeding, while reducing antagonistic interactions, may represent a fundamental force in shaping the spatial organization of dental plaque. *A. actinomycetemcomitans* possesses the capability to influence global regulation of community development, and its presence in an in vitro multispecies biofilm was shown to modulate the proteomic profile of the entire complex community [95]. Further investigation identified the community regulator as the histone-like family of nucleoid-structuring (H-NS) proteins of *A. actinomycetemcomitans.* H-NS proteins act as translational silencers with global regulatory potential in many Gram-negative bacteria. In *Escherichia coli*, deletion of the *hns* gene attenuates biofilm development [93], similar to its role in *A. actinomycetemcomitans*, where it promotes biofilm development as well as adhesin production [94]. Proteomic analysis of an *in vitro* polymicrobial community with the *hns* deletion strain of *A. actinomycetemcomitans* suggested that H-NS globally regulates community metabolic pathways involved in peptide, carbohydrate, and malate metabolism [95].

Metabolic Cooperation Between Oral Streptococci and *P. gingivalis*

Oral streptococci also have a significant impact on the properties and pathogenic potential of *P. gingivalis*, and these organisms are located in close proximity in vivo [82]. Interactions occur on multiple levels and many involve metabolic communication. Oral streptococci efficiently metabolize sugars, and a reduction in galactose in the biofilm milieu could alter physiology, cell shape, and intracellular granulation of *P. gingivalis* [96]. *S. gordonii* promotes increased biofilm development and synergistic pathogenicity in rodent models of periodontitis with *P. gingivalis* [97-99]. The *S. gordonii* genes *spxB* and

cbe are among those instrumental in promoting biofilm development with *P. gingivalis* [100]. SpxB (pyruvate oxidase) produces acetyl phosphate and H_2O_2 from pyruvate under aerobic conditions, but its role in promoting biofilm development with *P. gingivalis* remains unclear. Cbe (chorismate- binding enzyme) synthesizes para-aminobenzoic acid (pABA) for secretion and folate biosynthesis. Recently, pABA was determined to promote fitness and colonization of *P. gingivalis in vivo* with a mouse oral model [101]. Moreover, pABA reduced the overall stress of *P. gingivalis* while promoting fimbrial production. Metabolic and proteomic analyses of *P. gingivalis* responses to pABA showed that *P. gingivalis* can utilize pABA for folate biosynthesis. Surprisingly, pABA reduced the production of exopolysaccharide and attenuated the virulence of *P. gingivalis*. These metabolic interactions may regulate community density and spatial configuration, again supporting the developing narrative that metabolic communication promotes structure and polymicrobial synergy within the oral biofilm [102]. It is important to note that individual species of oral streptococci can affect *P. gingivalis* synergistically or antagonistically through distinct process. For example, *Streptococcus cristatus* and *Staphylococcus intermedius* are antagonistic to *P. gingivalis* [103, 104], in part through removal of the biofilm-promoting amino acid arginine by streptococcal arginine deiminase enzymes [103].

T. denticola and *P. gingivalis* Metabolic Interactions

Most of the examples discussed so far involve early colonizing commensal bacteria and more pathogenic later colonizers; however, interactions among these secondary colonizers are also manifold [105]. One example of cooperative metabolism between

periodontal pathogens is the metabolic cross-talk between *Treponema denticola* and *P. gingivalis*. When cultured together, *P. gingivalis* and *T. denticola* coaggregate and display enhanced growth [106-108]. Gas-liquid chromatography analysis of culture supernatants demonstrated that *P. gingivalis* produces isobutyric acid, which stimulates the growth of *T. denticola*, while *T. denticola* secretes succinate, which is utilized by *P. gingivalis* [106]. Additional studies demonstrated that *T. denticola* induces the production and secretion of glycine by *P. gingivalis* to promote its own growth in mixed culture [107]. Transcriptome analysis also suggests that *P. gingivalis* produces thiamine pyrophosphate, an essential nutrient for *T. denticola*; however, direct cross-feeding of this metabolite has yet to be established. Recently, *in vivo* metatranscriptomic studies showed that these cooperative metabolic interactions occur during periodontitis and may be a significant contributor to synergistic pathogenicity [109, 110].

Polymicrobial Synergy and Dysbiosis in Cancer

One current theory of periodontal disease etiology, the polymicrobial synergy and dysbiosis (PSD) model, holds that synergistic interactions within the polymicrobial community shape and stabilize a dysbiotic microbiota which perturbs host homeostasis. Disease is caused by reciprocally reinforced interactions between such physically and metabolically integrated polymicrobial communities and a dysregulated host inflammatory response [102]. While periodontitis and cancer are clearly distinct diseases, they share an underlying similarity in that they are, in essence, wounds that fail to heal [111]. Community perturbations consistent with a PSD model have been proposed for tumor development [112, 113]. Combinations of oral bacterial species are consistently

identified in OSCC lesions, resonating with the idea that community-wide properties may promote tumorigenesis.

In the 4NQO model, *F. nucleatum* and *P. gingivalis* synergistically promote cancer progression [60]. Another feature of the PSD model is that the microbial roster is of less relevance to nososymbiocity than the presence of combinations of functional genes, as communities of different compositions can exhibit similar functions [102]. Support for this concept in OSCC comes from a study of the microbiota and transcriptome in the 4NQO mouse model. Whereas variability in community dynamics was observed, the metatranscriptome revealed patterns of metabolic signatures consistently present in OSCC. These include nitrogen transport, response to stress, interspecies interactions, Wnt pathway modulation, and amino acid and lipid biosynthesis [62]. Similarly, a pilot study of human OSCC tumors found metabolic activities better correlated with disease than did community microbial composition [114], and a comparison of microbiotas associated with OSCC in different countries reveals functional rather than compositional similarities [115]. Fig. 3 summarizes a proposed role for PSD in OSCC.

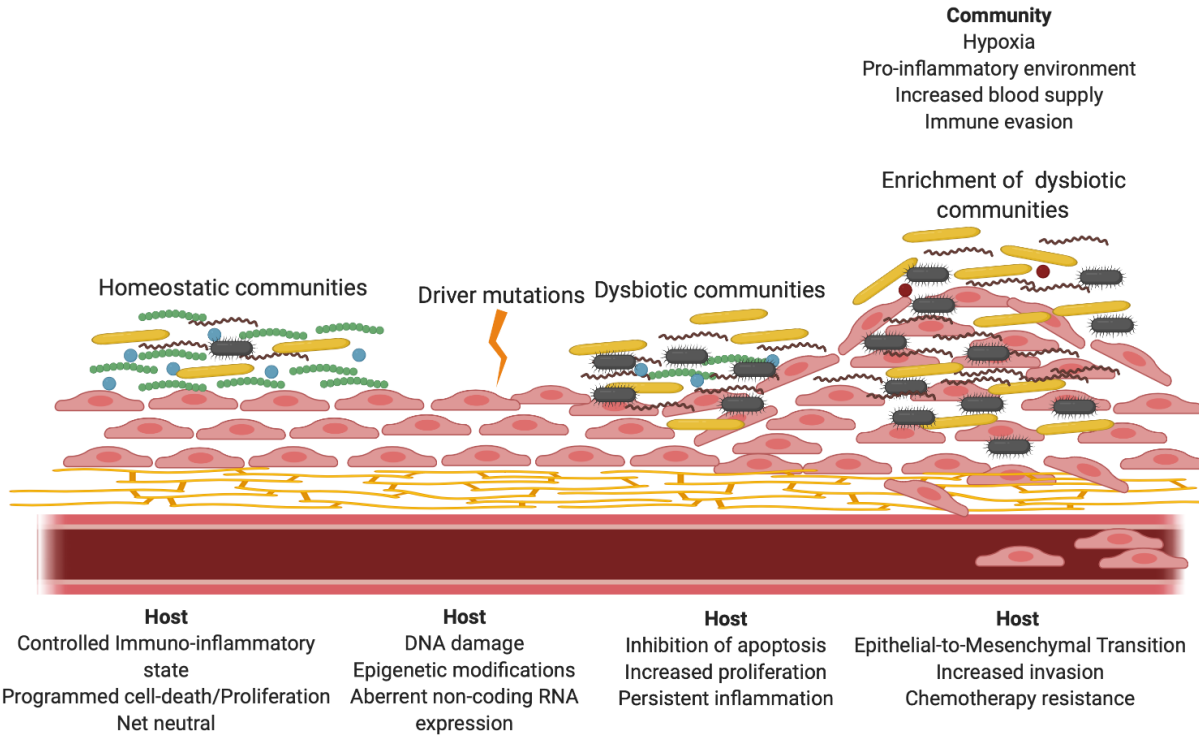


Figure 3: Schematic representation of polymicrobial synergy and dysbiosis model for oral squamous cell carcinoma. In health, host cell responses to homeostatic communities are a eubiotic balance of proliferation with programmed cell death. Driver mutations lead to dysregulation of host processes, which can also be manipulated by organisms associated with a dysbiotic community. As the tumor microenvironment is established anaerobic, gram-negative organisms are enriched as a result of hypoxic, pro-inflammatory conditions. As organisms such as *P. gingivalis, F. nucleatum* and *T. denticola* accumulate, the tumor cells can acquire an invasive phenotype through EMT, as well as increased resistance to chemotherapeutic drugs.

Interactions among bacterial constituents of communities can be antagonistic as well as synergistic, and numerous cases of antagonisms have been documented among oral bacteria [38, 102, 116] Although there are conflicting reports in the literature [117], in general, most oral streptococcal species tend to be underrepresented in the microbiotas associated with OSCC [62, 118]. This can be interpreted to indicate reduced fitness of these organisms in a tumorigenic environment. However, a further

interpretation is that these organisms are eubiotic and help maintain homeostasis at mucosal membranes; in their absence the microbiota becomes increasingly tumorigenic. In that regard, *S. gordonii* can reprogram epithelial cell global transcriptional patterns such that the subsequent response to *P. gingivalis* is diminished, and, further, *S. gordonii* can prevent *P. gingivalis*-induced gingival epithelial cell proliferation [102]. Moreover, *S. gordonii* can antagonize *P. gingivalis*-induced ZEB2 production and associated cell migration by inhibiting the activation of the FOXO1 transcription factor through the TAK1-NLK negative regulatory pathway, as shown in Fig. 4 [38]. Hence, while *S. gordonii* is an accessory pathogen in periodontal disease [102], this species may be a homeostatic commensal in oral cancer, an illustration of the importance of environmental context for bacterial functionality.

The goal of this study was to identify signaling events manipulated by periodontal pathogen *P. gingivalis* that contribute to homeostatic disruption, specifically focusing on pathways overridden by homeostatic commensal *S. gordonii* in gingival epithelial cells. We hypothesized that *P. gingivalis* would activate genes involved in tissue disruption and dampen innate immune signaling, whereas *S. gordonii* would either program the cells to resist this activation or interact with other inhibitory pathways. We characterized the differences in transcriptomic responses of epithelial cells challenged with *P. gingivalis* and/or *S. gordonii* using RNAseq. The transcriptome data revealed OLFM4, a gene involved in apoptosis, proliferation, migration, and dysregulation of innate immunity, all of which are differentially influenced by the microbes, as a potential axis that is disturbed by *P. gingivalis* and restored by *S. gordonii*. The initial identification of

OLFM4 was followed by characterization of the mechanism for upregulation by *P. gingivalis*, inhibition by *S. gordonii*, and ultimately the phenotypic outcome of this differential regulation.

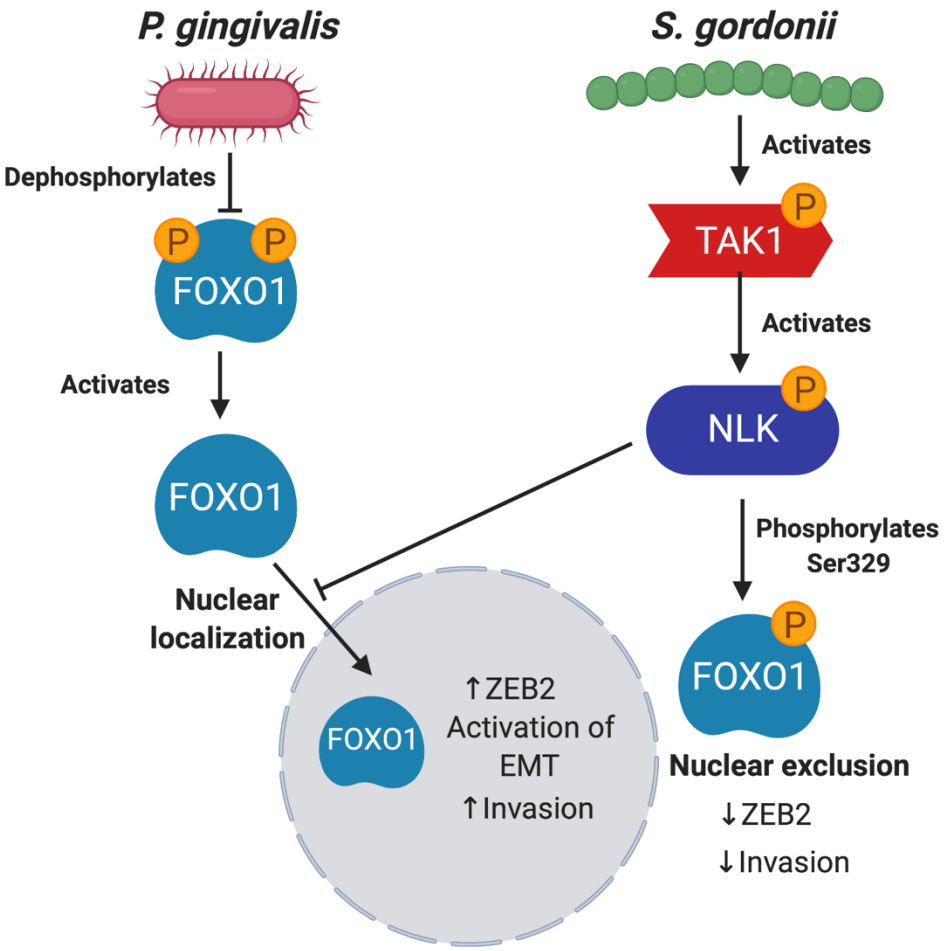


Figure 4: Antagonistic interactions of *S. gordonii* on *P. gingivalis* through activation of the TAK1-NLK host kinase cascade. *P. gingivalis* can dephosphorylate FOXO1 on serine residues which prevents translocation form the nucleus to the cytoplasm thus enhancing activity. When *S. gordonii* is present the TAK1-NLK1 pathway is activated which

supersedes the effect of *P. gingivalis* and increases phosphorylation of FOXO1 on Ser329 thus allowing translocation of FOXO1 to the cytoplasm where it is inactive.

CHAPTER 2 MATERIALS AND METHODS

Eukaryotic Cells, Bacterial Strains, Growth and Infection Conditions. Telomerase immortalized gingival keratinocytes (TIGKs) [119] and OKF6/TERT2 cells [120] were maintained with Dermalife-K Serum free culture medium supplemented with DermaLife K LifeFactors Kit. SCC9 cells from ATCC were grown in 1:1 DMEM: Ham's F12 medium supplemented with 400 ng/ml hydrocortisone and 10% fetal bovine serum. ESCC9706 cells [121] were grown using DMEM supplemented with 10% fetal bovine serum. Cells were cultured at 37°C with 5% CO_2. *Porphyromonas gingivalis* ATCC 33277, HG66, W83, 381, A1A7-28, and low passage clinical isolate MP4-504 were grown in trypticase soy broth (TSB) supplemented with 1 mg/mL yeast extract, 5 μg/mL hemin and 1 ug/mL menadione. Deletional mutants Δ*fimA*, Δ*mfa1*, Δ*serB*, and Δ*porK* [57, 122-124] were grown in the presence of 10 μg/mL erythromycin, Δ*kgp* was grown in the presence of 20 ug/mL chloramphenicol, Δ*ltp1*/Δ*php1* and Δ*rgpA/B* were grown in the presence of erythromycin and 1ug/mL tetracycline, and Δ*kgp/rgpA/B* were grown in the presence of erythromycin, tetracycline, and chloramphenicol [125]. *Streptococcus gordonii* DL1, *S. mutans* KSPK2, *S. oralis* 10557, *S. constellatus* ATCC 27823, and *S. sanguinis* 10556 were grown in brain heart infusion broth (BHI) supplemented with 5 mg/ml yeast extract, *S. gordonii* Δ*spxB* CΔ*spxB*, Δ*sspA/B*, Δ*cbe*, [100] were grown in the presence of 5 μg/mL erythromycin, and CΔ*spxB* was grown with 10 μg/mL tetracycline. *Fusobacterium nucleatum* ATCC 25586 was grown in BHI supplemented with 1 mg/mL yeast extract, 5

μg/mL hemin, and 1 μg/mL menadione. *Treponema denticola* ATCC 35405 was grown in new oral spirochete broth (NOS), and *Filifactor alocis* ATCC 35896 was grown in BHI supplemented with 5 mg/mL yeast extract, 5 μg/mL hemin, 1 μg/mL menadione, and 20% L- arginine. All bacteria were grown to mid-log phase at 37°C under standard anaerobic conditions of 85% N_2, 10% H_2, 5% CO_2.

Cells were grown to ~80% confluence and challenged with bacteria at an MOI of 100 for 1 h, followed by 23 h culture in fresh medium, unless otherwise noted.

Antibodies and Reagents

OLFM4, Activated Notch1, and GAPDH antibodies were from Abcam. Phalloidin- Texas Red, Alexa Fluor 488, and DAPI were from Thermofisher. OLFM4 and Notch1 ELISAs were from Abcam, and Jagged1 ELISA was from Thermofisher. OLFM4, Notch1, Notch2, Notch3, Notch4, Jagged1, Jagged2, DLL1, DLL3, DLL4, SMAD3, SMAD7, ZEB2, FOXO1, β-catenin, GSK3β, ADAM10, ADAM17, and NLK siRNA were from Origene. TLCK, FH535, and chromogenic substrate L-BAPNA were from Sigma Aldrich. Recombinant chimera Jagged1-FC was from RND systems. Gamma secretase inhibitor LY-374973 (DAPT) was from Sigma Aldrich, and ADAM/TACE inhibitor TAPI-2 was from Tocris.

Quantitative Reverse Transcription-PCR

Cells were harvested, and RNA was purified using RNAeasy plus kit from Qiagen. 2ug RNA was reverse transcribed using high capacity reverse transcription kit (Thermofisher).

Applied Biosystems Taqman fast universal master mix and Taqman gene expression assays for OLFM4, GAPDH, HES5, Notch1, Notch2, Notch3, Notch4, Jagged1, Jagged2, DLL1, DLL3, DLL4, SMAD3, SMAD7, ZEB2, FOXO1, β-catenin, GSK3β, ADAM10, ADAM17, and NLK were from Thermofisher. qRT-PCR was performed using an Applied Biosystems QuantStudio 3. Thresholds were automatically calculated using Applied Biosystems software, and Cycle threshold (Ct) values were normalized to GAPDH. Fold changes were calculated using 2-(delta delta)Ct.

Immunoblots and ELISAs

Cells were lysed using RIPA buffer containing Protease and PhosSTOP phosphatase inhibitor (Roche). Protein concentration was estimated by a bicinchoninic acid (BCA) assay. Proteins (20 µg) were separated on a 10% SDS-PAGE gel, and transferred to a nitrocellulose membrane (0.2 µm) by electroblotting for 1h at 100V. Nitrocellulose membranes were blocked with 5% bovine serum albumin (BSA) in Tris buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature. The membrane was probed with primary antibodies in TBST overnight at 4^0C. Membranes were washed 3 times with TBST, and probed with HRP-conjugated secondary antibody at room temperature for 1 h. ECL substrate (ThermoFisher) was used to visualize the proteins, and images were generated using a ChemiDoc XRS Plus (BioRad).

Cell culture supernatant was centrifuged for 20 min at 20,000xg at 4^0C, filtered (0.4 µm), and concentrated using Amicon Ultra concentrators. For ELISA, 25 µl of supernatant per well was used per manufacturer's guidelines.

Plasmid preparation, RNA interference, Transfections, and Luciferase assay

OLFM4 promoter-luciferase reporter construct was made by amplification of a 500 bp fragment upstream of the OLFM4 coding sequence, which was cloned into pGL3-basic plasmid (Promega). All constructs were confirmed by sequencing at the University of Louisville Sequencing Core. The internal control reporter was pRL vector that provides constitutive expression of Renilla luciferase (Promega). For siRNA cells were grown to 50-60% confluence, and cells for plasmid transfection were grown to 60-70% confluence. Transfection was for 48 h using Lipojet transfection agent (SignaGen), and after 24h medium was changed to allow cells to recover before bacterial challenge. Confirmations of knockdowns can be found in Fig. S1. Luciferase reporter assays were performed using a Stop & Glo Dual luciferase reporter kit from Promega. Luciferase activity was measured using a 10s integration time in a Luminometer (Molecular Devices). Firefly luciferase activity was normalized to Renilla luciferase activity from the same lysates.

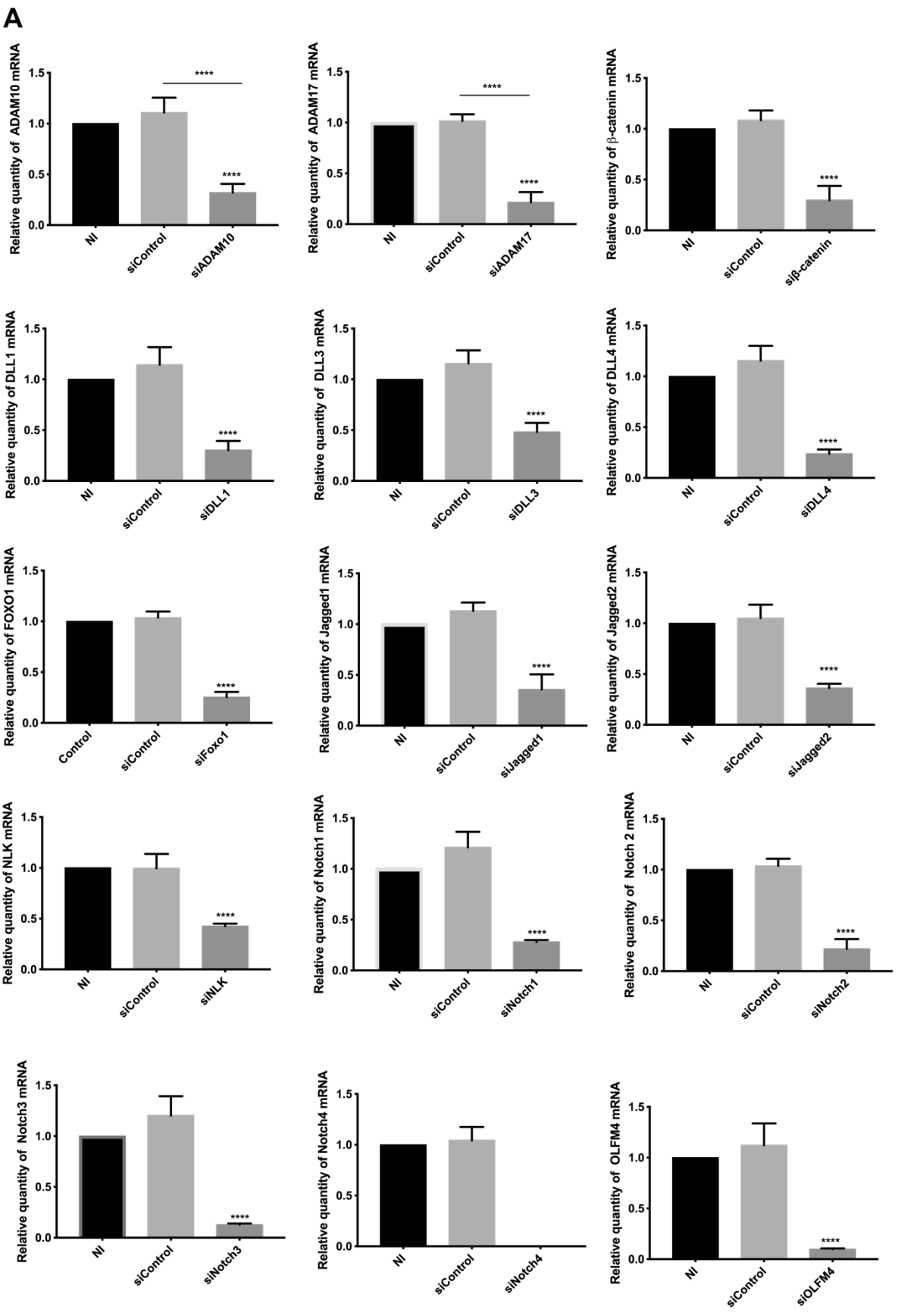

A
Relative quantity of ADAM10 mRNA
NI
siControl
siADAM10
Relative quantity of ADAM17 mRNA
siADAM17
Relative quantity of β-catenin mRNA
siβ-catenin
Relative quantity of DLL1 mRNA
siDLL1
Relative quantity of DLL3 mRNA
siDLL3
Relative quantity of DLL4 mRNA
siDLL4
Relative quantity of FOXO1 mRNA
Control
siFoxo1
Relative quantity of Jagged1 mRNA
siJagged1
Relative quantity of Jagged2 mRNA
siJagged2
Relative quantity of NLK mRNA
siNLK
Relative quantity of Notch1 mRNA
siNotch1
Relative quantity of Notch 2 mRNA
siNotch2
Relative quantity of Notch3 mRNA
siNotch3
Relative quantity of Notch4 mRNA
siNotch4
Relative quantity of OLFM4 mRNA
siOLFM4
1.5
1.0
0.5
0.0
****

Figure S1: Confirmation of siRNA transfections. Target genes for qRT-PCR were normalized to GAPDH, and fold changes were calculated using the ΔΔCT method. Significance was calculated using a One-way ANOVA, and *** P<.005 and **** P<.001.

Immunofluorescence and Confocal Laser Scanning Microscopy

TIGK cells were grown on Lab-Tek II chamber slides, and after bacterial challenge cells were washed twice with phosphate buffered saline (PBS), fixed and permeabilized with 4% paraformaldehyde and 0.1% Triton x-100 for 10 min at room temperature. Goat serum (10%) in TBST was used to block the cells for 20 min at room temperature. Cells were incubated with primary antibody in TBST with 10% goat serum overnight at 4^{O}C. Cells were washed 3 times with TBST, then incubated with Alexa Fluor 488 secondary antibody (1:200) for 2 h at room temperature in the dark. After washing as before and blocking with 10% goat serum, cells were incubated with Texas Red-Phalloidin (1:100) for 2 h at room temperature in the dark. Cells were washed 3 times, and coverslips were mounted using ProLong Gold (Invitrogen) with 4'6-diamidino-2-phenylindole (DAPI) overnight. Slides were scanned with a Leica SP8 confocal microscope, and images were analyzed with Volocity 6.3 Software (PerkinElmer).

Nuclear localization was quantified using the 3D image processing software IMARIS (Bitplane AG). The Surfaces function in IMARIS was used to create a 3D model of the nuclei from the DAPI channel. IMARIS surfaces then calculated Alexa Fluor 488 voxels within each nucleus, which was normalized to the volume of the nucleus. Total normalized Alexa Fluor 488 was then divided by the number of cells analyzed.

Transwell Assays

For studying bacterial interactions with the epithelial cells, $1x10^5$ cells were grown only in the lower chamber of a 0.4 µm transwell filter plate. Cells were challenged with *P. gingivalis*, and the upper chamber contained cell culture media and either *S. gordonii*, *S. mutans*, or *S. oralis*.

For studying epithelial cell to epithelial cell interactions, TIGKs were cultured in both the lower chamber and on the transwell insert in the upper chamber. *P. gingivalis* was added to the upper chamber only.

To measure the motility of TIGK cells we used a matrigel migration assay (BD Biosciences). Cells ($2x10^5$) cells were seeded onto the matrigel insert in the upper chamber, with cell culture medium in the lower chamber, and cultured for 18 h. The filter was removed, and the surface was scraped with a cotton swab to remove any cells that did not migrate. Cells that migrated through the filter were fixed with 1% methanol, and stained with toluidine blue. Cells were counted from 3 random fields at 20x using a Nikon Eclipse T100 microscope.

RNAseq

RNA was extracted using the RNAqueous-Micro Total RNA Isolation kit (ThermoFisher). The TruSeq Stranded Total RNA with RiboZero Plus kit (Illumina) was used to generate a sequencing library from 1 µg of total RNA. Paired-end sequencing was performed on an

Illumina Nextseq 500 at the University of Louisville Core using the Nextseq 500 High-Output Kit (150 cycles) (Illumina). Base calls were made using the BaseSpace FastQ Version 1.0.0 application (Illumina, Inc.). Raw gene counts with a minimum of two counts per million in at least one sample were used as the initial dataset for downstream RNASeq analyses. These raw counts were used for the determination of differentially expressed genes via the DESeq2 Bioconductor/R package (DOI: 10.18129/B9.bioc.DESeq2) using the recommended guidelines. Output was in the form of log2 fold change expression values and p-values adjusted for multiple comparisons using the Benjamini-Hochberg procedure. Differentially expressed genes were defined using cutoffs of 1 for the log2 fold change and an adjusted p-value of 0.05. This output was used as input for the generation of volcano plots using the EnhancedVolcano Bioconductor/R package (DOI: 10.18129/B9.bioc.EnhancedVolcano). This output was also used as input for functional enrichment analysis through the String Database version 11 using an FDR stringency of 1 percent and a minimum interaction confidence score of 0.400 for network generation. For principal component analysis (PCA) and heatmap generation, the raw count data were made homoscedastic using a regularized logarithm transformation. PCA was conducted using base R and PCA plots generated using the ggfortify R package. Gene count data for heatmap generation was further converted into z-scores and used as input into the ComplexHeatmap Bioconductor/R package.

Gingipain Activity Assay

Culture supernatant from *P. gingivalis* was filtered (0.4 μm) and combined with filtered supernatant from with either aerobic or anaerobic cultures of *S. gordonii* or *S. mutans*. For experiments using H_2O_2, culture supernatant from *P. gingivalis* was incubated with H_2O_2 for 30 min. Gingipain activity was visualized using the chromogenic substrate L-BAPNA. The rate of substrate hydrolysis and the accumulation of *p*-nitroanilide were monitored spectrophotometrically at 405 nm over time in a Spectramax M5 reader (Molecular Devices).

Statistical Analysis

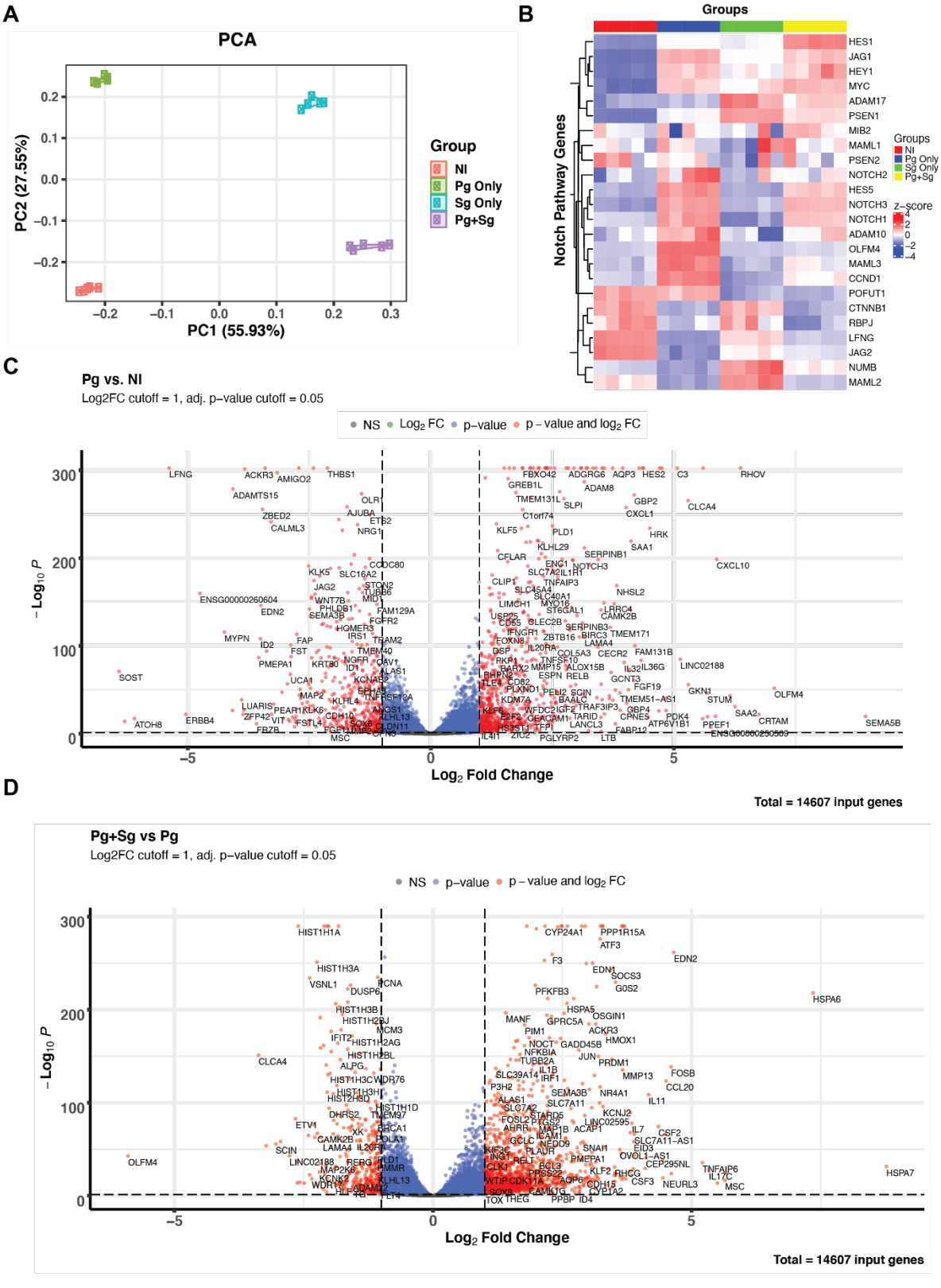
Each analysis is representative of at least 3 biological replicates with technical duplicates, and confocal images are representative of 3 biological replicates with at least 3 randomly scanned areas of the chamberslide. ANOVA with Tukey's multiple comparison test were conducted using GraphPad Prism V8. Statistical analyses of RNA-Seq data are described above.

CHAPTER 3 RESULTS

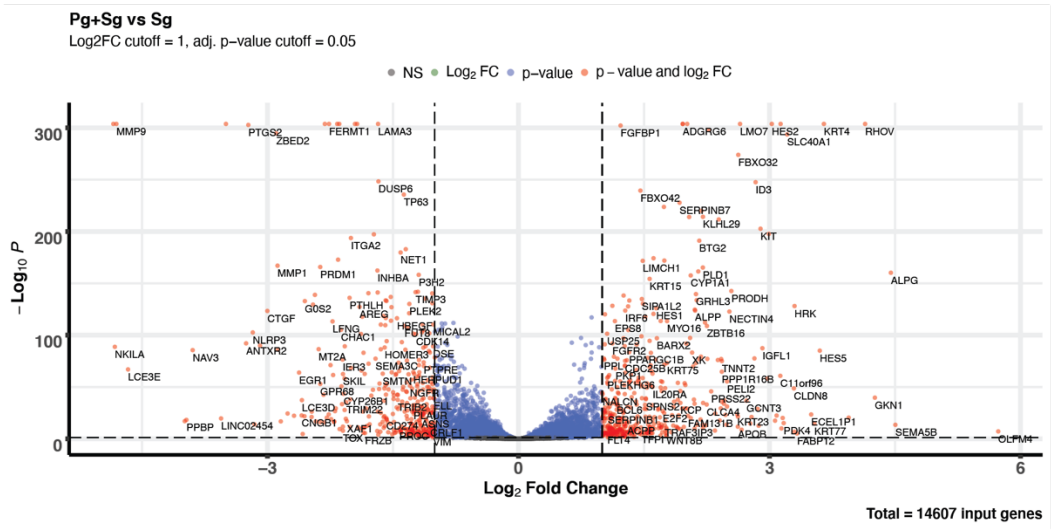
To characterize the interplay between *P. gingivalis* and *S. gordonii* at the epithelial barrier we first employed RNAseq to identify host genes that are differentially regulated in the presence of each organism alone versus the two species present together. Telomerase immortalized gingival keratinocytes (TIGKs) were challenged with either *P. gingivalis*, *S. gordonii*, or a dual infection with both organisms. Fig. 5A shows the PCA plot for the RNAseq, where we observed tight clustering of samples within the same group and separation between each of the groups, indicating distinct transcriptional responses. We enriched for genes involved in Notch signaling in Fig. 5B, which was recently shown to be activated by *P. gingivalis* [42]. We found in Fig. 5B that the Notch1 and Notch3 receptors, Jagged1 ligand (Jag1), and downstream targets OLFM4 and Hes5 were all upregulated by *P. gingivalis*.

OLFM4 is an antiapoptotic glycoprotein which promotes tumor growth [43], and is selectively expressed in inflamed epithelium [47]. In the dual infection group, expression of all of these genes was significantly reduced compared *P. gingivalis* alone, and differential expression of the Notch pathway in response to *P. gingivalis* (Pg) did not occur in the presence of *S. gordonii* (Sg). The results for the Pg vs. NI, Pg+Sg vs. Pg, Pg+Sg vs. Sg, and Pg vs. Sg comparisons from the RNAseq were also summarized as a volcano plot in Fig. 5C-F, and shared differentially expressed genes summarized as upset charts in Fig. 5G-H. The transcriptome data on *P. gingivalis* induction of OLFM4 was corroborated

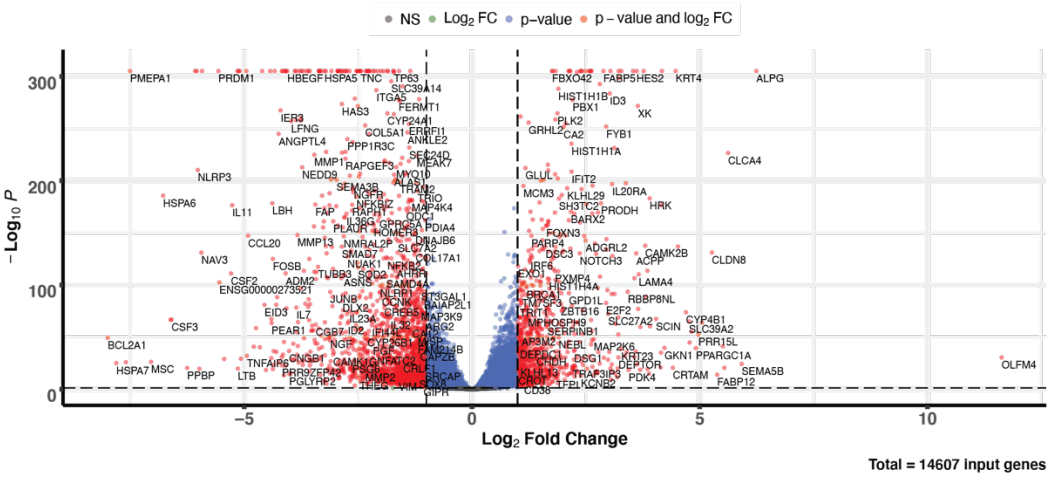
in a dose and time dependent manner at the transcriptional (Fig. 6A) level, and at 24 h the protein level by Western blotting (Fig. 6B), ELISA (Fig. 6C) and confocal microscopy (Fig. 6D), all showed an increase in OLFM4 protein in response to *P. gingivalis.* These mRNA and protein analyses identify that *P. gingivalis*-induced upregulation of OLFM4 through Notch signaling is reduced when *S. gordonii* is present.


A
PCA
PC2 (27.55%)
PC1 (55.93%)
Group
NI
Pg Only
Sg Only
Pg+Sg
B
Groups
Notch Pathway Genes
HES1
JAG1
HEY1
MYC
ADAM17
PSEN1
MIB2
MAML1
PSEN2
NOTCH2
HES5
NOTCH3
NOTCH1
ADAM10
OLFM4
MAML3
CCND1
POFUT1
CTNNB1
RBPJ
LFNG
JAG2
NUMB
MAML2
Groups
NI
Pg Only
Sg Only
Pg+Sg
Z-score
C
Pg vs. NI
Log2FC cutoff = 1, adj. p-value cutoff = 0.05
NS
Log2 FC
p-value
p - value and log2 FC
−Log10 P
Log2 Fold Change
Total = 14607 input genes
D
Pg+Sg vs Pg
Log2FC cutoff = 1, adj. p-value cutoff = 0.05
NS
p-value
p - value and log2 FC
−Log10 P
Log2 Fold Change
Total = 14607 input genes

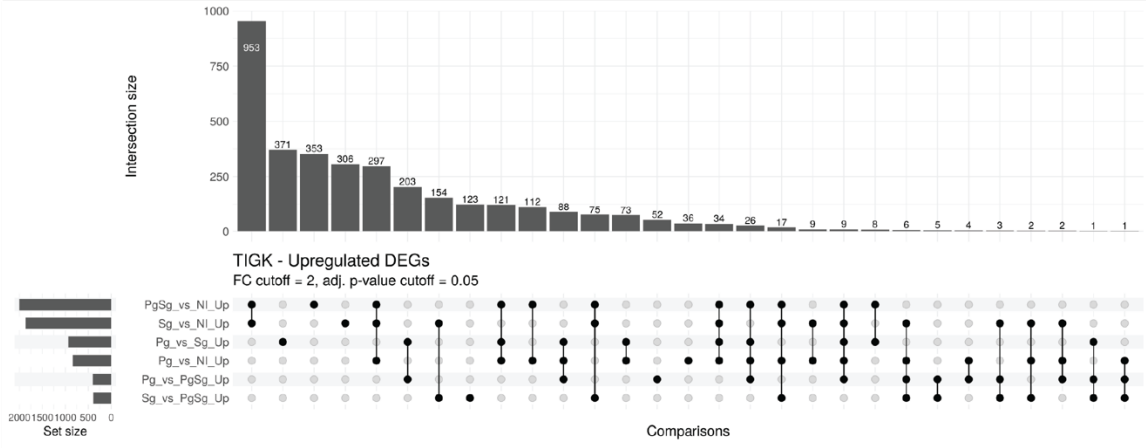
E


Pg+Sg vs Sg
Log2FC cutoff = 1, adj. p–value cutoff = 0.05
NS
Log2 FC
p–value
p – value and log2 FC
– Log10 P
Log2 Fold Change
Total = 14607 input genes

F


Pg vs Sg
Log2FC cutoff = 1, adj. p–value cutoff = 0.05
NS
Log2 FC
p–value
p – value and log2 FC
– Log10 P
Log2 Fold Change
Total = 14607 input genes

G


Intersection size
TIGK - Upregulated DEGs
FC cutoff = 2, adj. p-value cutoff = 0.05
PgSg_vs_NI_Up
Sg_vs_NI_Up
Pg_vs_Sg_Up
Pg_vs_NI_Up
Pg_vs_PgSg_Up
Sg_vs_PgSg_Up
Set size
Comparisons

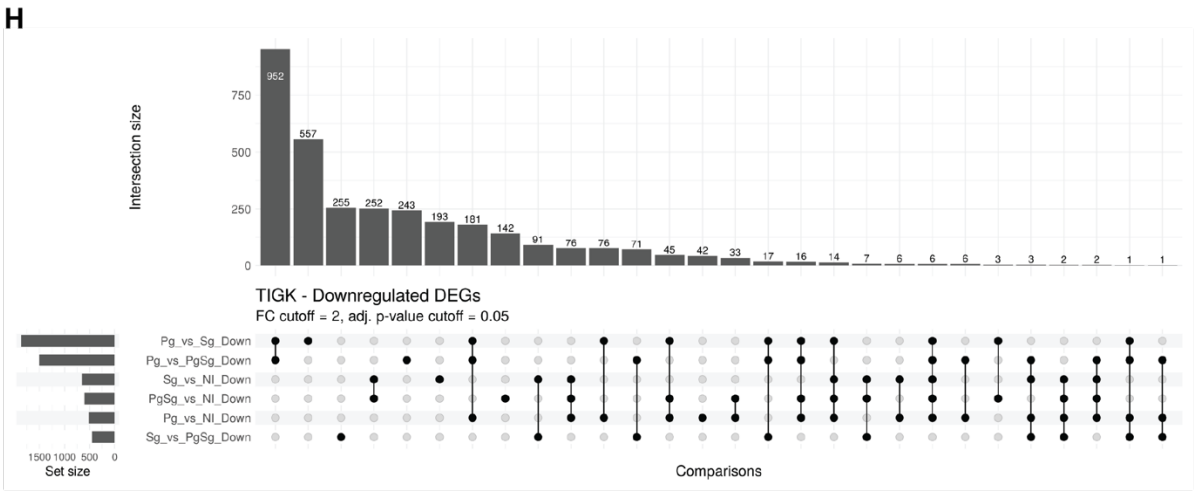


Figure 5: RNAseq of TIGK cells challenged with *P. gingivalis* and/or *S. gordonii*
TIGK cells were challenged with *P. gingivalis* (MOI 100) and/or *S. gordonii* (MOI 50) for 1h, then the media was changed to remove any non-adherent bacteria. Cells were harvested at 24h, at which point RNA was harvested, and libraries were prepared for RNAseq. The PCA plot (A) shows distinct separation of non-infected (NI), *P. gingivalis* (Pg), *S. gordonii* (Sg), and *P. gingivalis* co-challenged with *S. gordonii* (Pg+Sg) groups, as well as a tight clustering of individual samples within each group. Notch signaling is transcriptionally regulated by *P. gingivalis*, and *S. gordonii* interrupts activation by *P. gingivalis*, specifically reducing Notch1, Notch3, Jagged1, Hes5, and OLFM4 (B). Graphical comparisons of gene expression are represented by volcano plots for *P. gingivalis* vs. NI (C), *P. gingivalis* + *S. gordonii* vs. *P. gingivalis* alone (D), *P. gingivalis* + *S. gordonii* vs. *S. gordonii* alone (E), and *P. gingivalis* alone vs. *S. gordonii* alone (F). A summary of the shared upregulated (G) and downregulated (H) genes for each comparison have been presented as upset charts.

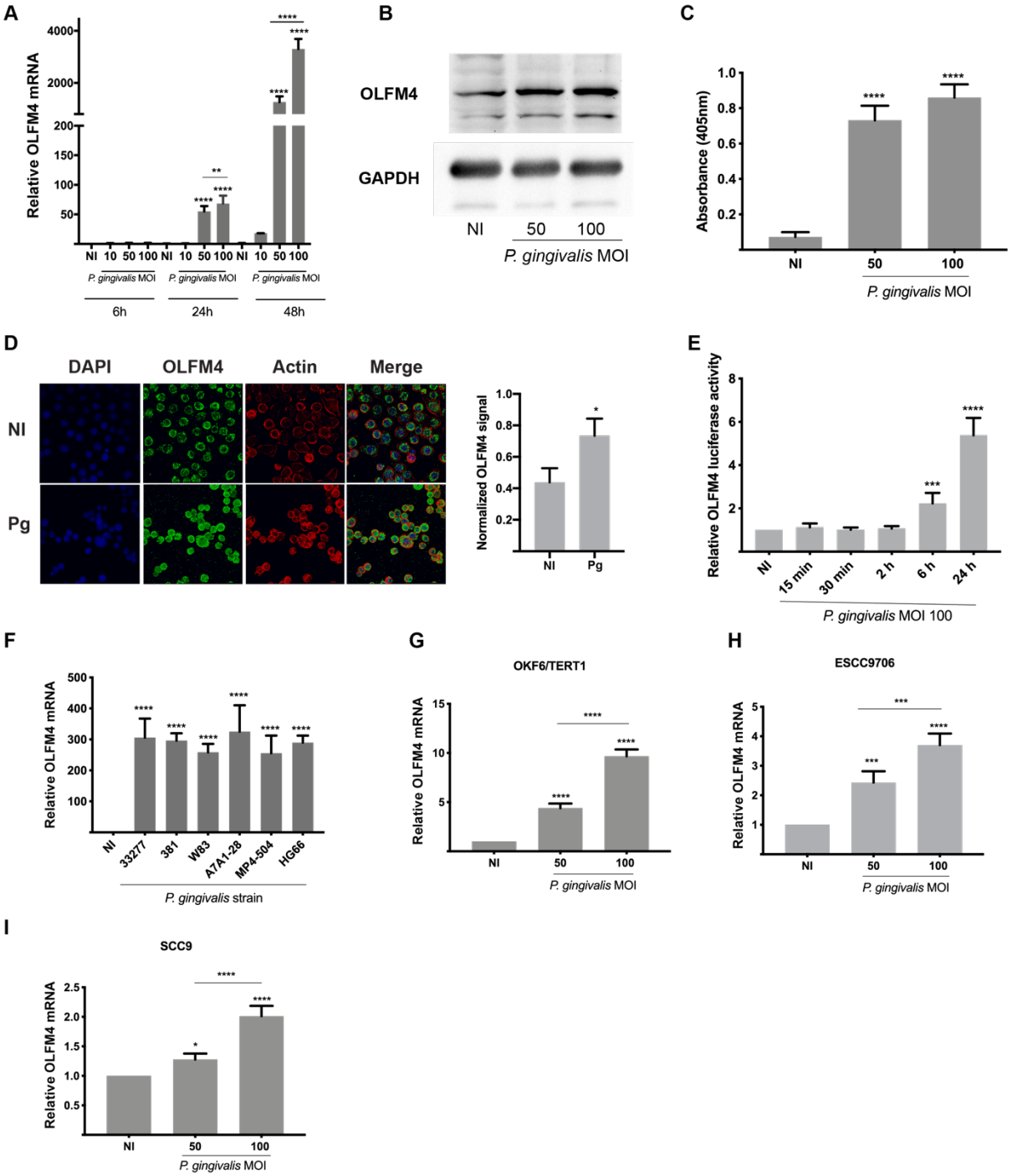

A
Relative OLFM4 mRNA
4000
2000
200
150
100
50
NI 10 50 100 NI 10 50 100 NI 10 50 100
P. gingivalis MOI
6h
24h
48h
B
OLFM4
GAPDH
NI 50 100
P. gingivalis MOI
C
Absorbance (405nm)
1.0
0.8
0.6
0.4
0.2
NI 50 100
P. gingivalis MOI
D
DAPI
OLFM4
Actin
Merge
NI
Pg
Normalized OLFM4 signal
NI Pg
E
Relative OLFM4 luciferase activity
NI 15 min 30 min 2 h 6 h 24 h
P. gingivalis MOI 100
F
Relative OLFM4 mRNA
NI 33277 381 W83 A7A1-28 MP4-504 HG66
P. gingivalis strain
G
OKF6/TERT1
Relative OLFM4 mRNA
NI 50 100
P. gingivalis MOI
H
ESCC9706
Relative OLFM4 mRNA
NI 50 100
P. gingivalis MOI
I
SCC9
Relative OLFM4 mRNA
NI 50 100
P. gingivalis MOI

Figure 6: Regulation of OLFM4 by *P. gingivalis*
TIGK cells were challenged with *P. gingivalis* ATCC 33277 at an MOI of either 10, 50, or 100 for 6 h, 24 h, or 48 h, then RNA was harvested and reverse transcribed into cDNA for qRT-PCR (A). OLFM4 upregulation was first observed at 24 h, and significantly increases in a dose and time dependent manner. Cells were harvested as previously described at 24 h, and western blot confirmed increases OLFM4 protein expression (B). As OLFM4 is a secreted protein, verification of increased secretion was investigated by ELISA of cell culture supernatants. Cells were challenged with *P. gingivalis* at indicated MOIs, then supernatants were collected, filtered, and concentrated (C). Cells were grown on chamber slides and challenged for 24h at an MOI 100, then probed with OLFM4 antibodies (green), phalloidin labeling actin (red), and lastly the nucleus was stained with DAPI (blue). *P. gingivalis* significantly increased OLFM4 intensity, which was normalized to the size of each nucleus (D). 500 bp upstream of OLFM4 was cloned into the promoterless PGL3-Firefly luciferase construct, and was used to measure promoter activity of TIGK cells challenged with *P. gingivalis* at an MOI of 100 for the indicated times. *P. gingivalis* increases promoter activity at 6 h, with the greatest increase at 24 h (E). Multiple strains of *P. gingivalis* were tested at an MOI of 100 for 24 h, including the encapsulated W83 and low passage clinical isolate MP4-504 (F). Each strain was equally able to stimulate OLFM4 expression. *P. gingivalis* upregulated OLFM4 in OKF6/TERT1 cells, which are immortalized non-cancerous mucosal cells (G), as well as the esophageal cancer derived cell line ESCC9706 (H) and tongue squamous cell carcinoma derived SCC9 cell line (I). Target genes for qRT-PCR were normalized to GAPDH, and fold changes were calculated using the ΔΔCT method. Significance was calculated using a One-way ANOVA, and *P<.05, ** P< .01, *** P<.005, and **** P<.001.

To confirm regulation of OLFM4 at the transcriptional level, TIGKs were transfected with a luciferase-reporter construct containing the regulatory fragment spanning 500bp upstream of the coding region [126]. In a luciferase assay challenge with *P. gingivalis* increased OLFM4 promoter activity beginning at 6 h (Fig. 6E). We explored multiple *P. gingivalis* strains to verify upregulation of OLFM4 is a property conserved across the species. In Fig. 6F we show all tested strains of *P. gingivalis* induced elevated OLFM4 expression, including the fimbriated/non-encapsulated lineage (33277, 381, and the gingipain hypersecreting HG66), the encapsulated/afimbrial lineage (W83), the fimbriated/encapsulated lineage (A7A128) and the low-passage clinical isolate MP4-504

[127-130]. Next, we determined whether OLFM4 regulation by *P. gingivalis* is specific to gingival epithelial cells. The OKF6 line are telomerase immortalized buccal mucosa cells, and we found that *P. gingivalis* upregulates OLFM4 in these cells, albeit to lower levels than occur in TIGKs in Fig. 6G. Tongue (SCC9) and esophageal (ESCC9706) squamous cell carcinoma cells also displayed an increase in OLFM4 expression in response to *P. gingivalis* challenge, again at a substantially lesser magnitude compared to TIGK cells in Fig. 6H-J. These results indicate that epithelial cells of the gingiva, which is the primary in vivo habitat of *P. gingivalis,* are more responsive to challenge with the organism, at least in terms of OLFM4 regulation, as compared to cells derived from other sites in the oral and esophageal regions.

Because genes involved in Notch signaling and OLMF4 were coordinately upregulated by *P. gingivalis* exposure, and normalized when *S. gordonii* was present, we further explored the role of Notch signaling in the regulation of OLFM4 in TIGK cells. There are four Notch receptors which initiate signaling through the pathway, and quantitative (q) RT-PCR showed that expression of Notch1 and Notch3 is enhanced by infection with *P. gingivalis* in Fig. 7A. To elucidate the role of these Notch receptors in OLFM4 upregulation, expression of each receptor was suppressed using siRNA. Knock down of Notch1 led to significantly decreased OLFM4 mRNA levels in the *P. gingivalis* challenged cells in Fig. 7B, indicative of a role for Notch1 in the signaling pathway exploited by *P. gingivalis* to regulate OLFM4. Knock down of Notch 2 and 4 had no effect on OLFM4 expression, whereas knock down Notch3 significantly increased the level of OLFM4, possibly due to increased availability or expression of Notch1. Notch signaling is activated

by the ligands Jag1, Jag2, DLL1, DLL3, and DLL4, and by qRT-PCR *P. gingivalis* upregulates expression of Jag1 and downregulates Jag2 in Fig. 7C. As shown in Fig. 7D knock down of Jag1 led to decreased induction of OLFM4 by *P. gingivalis*, whereas knocking down Jag2 increased OLFM4, indicating that Jag2 expression may interfere with signaling through Notch1. Suppression of DLL 1, 3, or 4 did not negatively influence OLFM4 mRNA levels in response to *P. gingivalis*. Knock down of Notch1 and Jag1 also diminished *P. gingivalis*-dependent regulation of an additional target of the Notch signaling pathways, Hes5 (Fig. 7E),. Collectively these data corroborate the importance of the Jag1-Notch1 signaling pathway in TIGK responses to *P. gingivalis*.

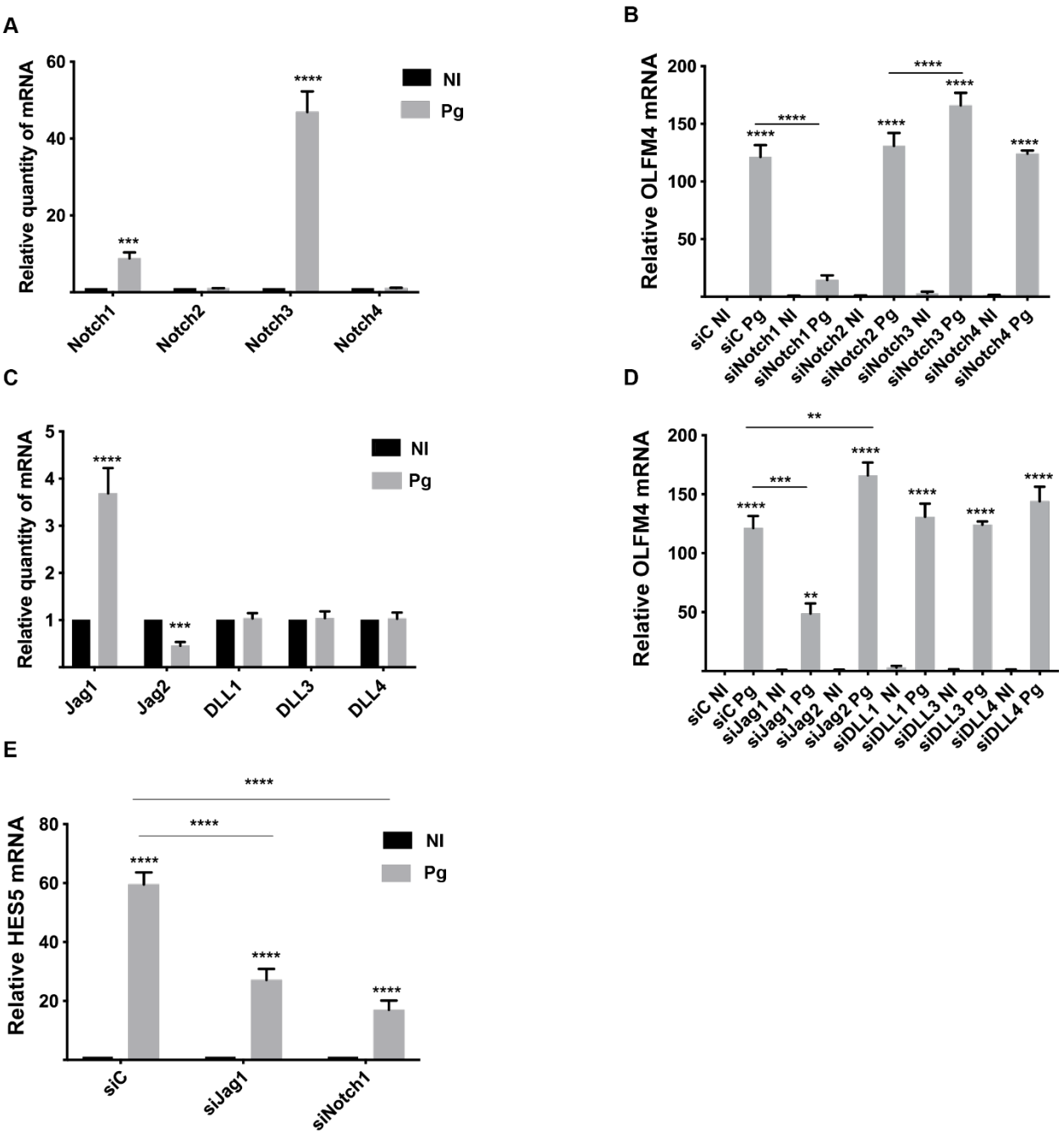


Figure 7: Regulation of Notch signaling by *P. gingivalis*

P. gingivalis upregulates Notch1 and Notch3 receptors, but not Notch2 and Notch4 at MOI 100 for 24 h in TIGK cells by qRT-PCR (A). Notch1-4 receptors were individually knocked down through siRNA transfection, and scrambled siRNA was used as a control. *P. gingivalis* requires Notch1, but not Notch2-4 for OLFM4 upregulation (B). Similarly, we investigated regulation of Notch ligands Jag1, Jag2, DLL1, DLL3, and DLL4 by *P. gingivalis* as in (A) and found *P. gingivalis* significantly upregulates Jag1, downregulates Jag2, and does not regulate DLL1, DLL3, and DLL4 (C). Employing siRNA transfections as in (B) for Notch ligands we found that Jagged1 was required for OLFM4 upregulation by *P.*

gingivalis, but not Jag2, DLL1, DLL3, or DLL4 (D). HES5 is also a downstream target of Notch signaling, and in a similar fashion to OLFM4 requires Notch1 and Jag1 for optimal regulation of HES5 mRNA (E). Target genes for qRT-PCR were normalized to GAPDH, and fold changes were calculated using the ΔΔCT method. Significance was calculated using a One-way ANOVA, and ** P< .01, *** P<.005, and **** P<.001.

It has been shown that endothelial cells are able to cleave Jag1 and release a soluble form of Jag1 that can then bind to Notch receptors on colorectal cancer cells and activate signaling [131]. To explore whether a similar mechanism of activation occurs in our model, we utilized a transwell system to determine whether challenge with *P. gingivalis* causes release of soluble signaling factors. TIGKs were cultured both on the transwell filter in the upper chamber, as well as in the lower chamber. When cells in the upper chamber were challenged with *P. gingivalis*, OLFM4 transcription was upregulated in the lower chamber cells in Figure 8.

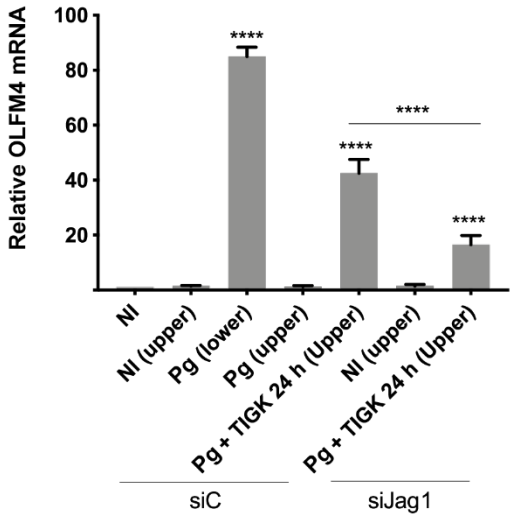


Figure 8: *P. gingivalis* induces paracrine activation of Notch signaling through soluble Jagged1
TIGK cells were grown in the lower chamber of a 0.4um transwell plate, as well as on top of the transwell filter. Only cells above the transwell filter were transfected with either

control siRNA or siJag1. Cells were then challenged with *P. gingivalis* either above or below the transwell filter as indicated, and only cells below the transwell filter were harvested for RNA and reverse transcribed for qRT-PCR. *P. gingivalis* alone was not enough to stimulate the lower TIGK cells in the upper chamber of the transwell filter, but when TIGK cells were seeded onto the upper chamber activation of OLFM4 in the lower chamber was maintained. Additionally, specifically knocking down Jag1 reduced OLFM4 activation in the lower chamber cells, which implicates a soluble form of Jag1 being involved in this paracrine activation mechanism. Target genes for qRT-PCR were normalized to GAPDH, and fold changes were calculated using the ΔΔCT method. Significance was calculated using a One-way ANOVA, and **** P<.001.

To evaluate the role of Jag1 in this regulation Jag1 was knocked down by siRNA in cells in the upper chamber. After challenge of upper chamber cells with *P. gingivalis* there was significantly less OLFM4 expression in the lower chamber cells compared to the siControl condition as shown in Figure 8. These results indicate that *P. gingivalis* can activate the Notch pathway in a paracrine manner by increasing production of Jag1.

OLFM4 is also a target gene of the Wnt/β-catenin pathway and of the NF-κB transcription factor [126]. As *P. gingivalis* can activate Wnt/β-catenin signaling, but not NF-κB [32, 57], we thus questioned whether the Wnt/β-catenin pathway is also involved in *P. gingivalis*-induced expression of OLFM4. However, neither pharmacological inhibition of Wnt nor siRNA knockdown of β-catenin had an affect on OLFM4 induction in response to *P. gingivalis* as shown in Fig. 9 A-B.

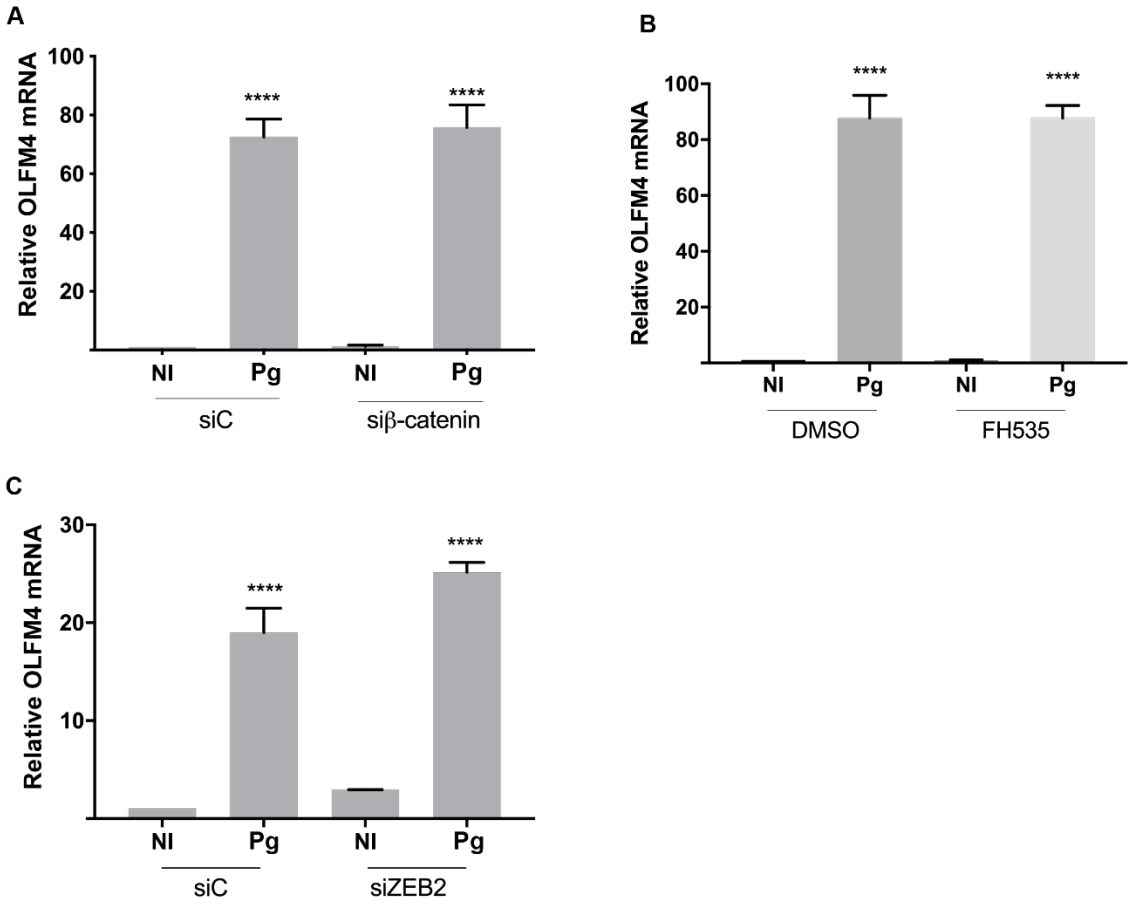


Figure 9: Wnt/β-catenin and ZEB2 are not required for activation of OLFM4 by *P. gingivalis* *P. gingivalis* activates Wnt/B-catenin signaling, and this pathway has been implicated in OLFM4 regulation. We utilized siRNA targeting β-catenin (A) and a pharmacological inhibitor of Wnt signaling, FH535 (B) in TIGK cells. Cells were then challenged with *P. gingivalis* at an MOI of 100 for 24 h, and RNA was harvested for QRT-PCR. β-catenin knock and pharmacological inhibition of Wnt signaling did not impede the regulation of OLFM4. *P. gingivalis* also activates the epithelial to mesenchymal transition transcription factor ZEB2, which could potentially bind the promoter region based on a consensus sequence found upstream of the coding region of OLFM4. Knocking down ZEB2 did not prevent regulation of OLFM4 by *P. gingivalis* (C). Target genes for qRT-PCR were normalized to GAPDH, and fold changes were calculated using the ΔΔCT method. Significance was calculated using a One-way ANOVA, **** P<.001.

In silico interrogation of the promoter region of OLFM4 using the eukaryotic promoter database, revealed the presence of a Zeb2 consensus binding element. Zeb2 activity is enhanced by *P. gingivalis*, hence we examined its potential involvement in OLFM4 regulation. As shown in Fig. 9C, siRNA knock down of Zeb2 did not prevent increased OLFM4 mRNA production in response to *P. gingivalis*. This finding further supports the importance of Notch signaling in the regulation of OLFM4 by *P. gingivalis*.

One mechanism for transcriptional regulation of Jag1 is through the integration of Notch and TGF-β signaling. SMAD3, a component of the TGF-β transcriptional activation complex, has been shown to activate Notch signaling through upregulation of Jag1 and Hey1 [132]. We show in Fig. 10 that SMAD3 is involved in *P. gingivalis*-induced Jag1 transcriptional regulation.

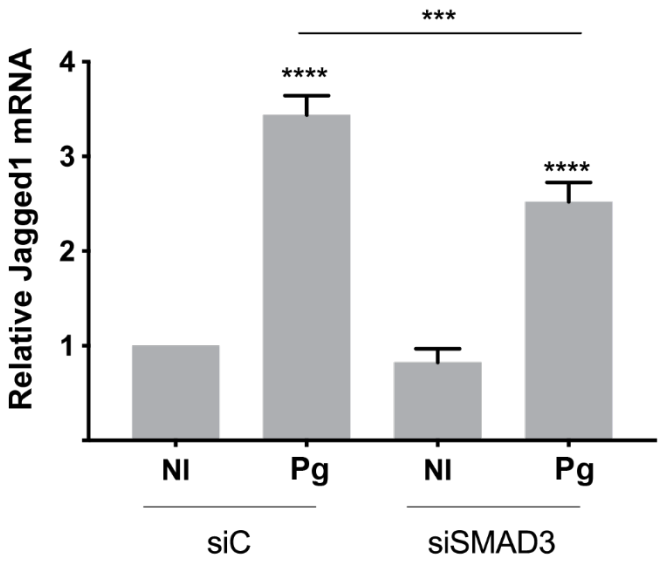


Figure 10: SMAD3 enhances upregulation of Jagged1 by *P. gingivalis*

TGF-β signaling intersect at the SMAD3-Jag1 signaling axis. SMAD3 has been shown to transcriptionally regulate Jag1, leading to activation of Notch signaling. Here we knocked down SMAD3 with siRNA in TIGK cells, and used scrambled siRNA as a control. TIGK cells were then challenged with *P. gingivalis* for 24 h, and RNA was harvested for qRT-PCR. SMAD3 knockdown significantly reduced Jag1 transcript, which implicates TGF-β signaling as a potential activator of Notch signaling. SMAD3 transcript was normalized to GAPDH, and fold changes were calculated using the ΔΔCT method. Significance was calculated using a One-way ANOVA, *** P<.005, and **** P<.001.

To identify the effector molecules of *P. gingivalis* responsible for activating Notch signaling, we examined a panel of strains with mutations in established virulence or colonization factors. As shown in Fig. 11A, loss of fimbrial adhesins or of serine and tyrosine phosphatases did not impact the ability of *P. gingivalis* to regulate OLFM4. In contrast, the Δ*rgpAB*Δ*kgp* mutant, which is deficient in the production of the arginine specific (RgpA and RgpB) and lysine specific (Kgp) gingipain proteases, was unable to stimulate OFLM4 production.

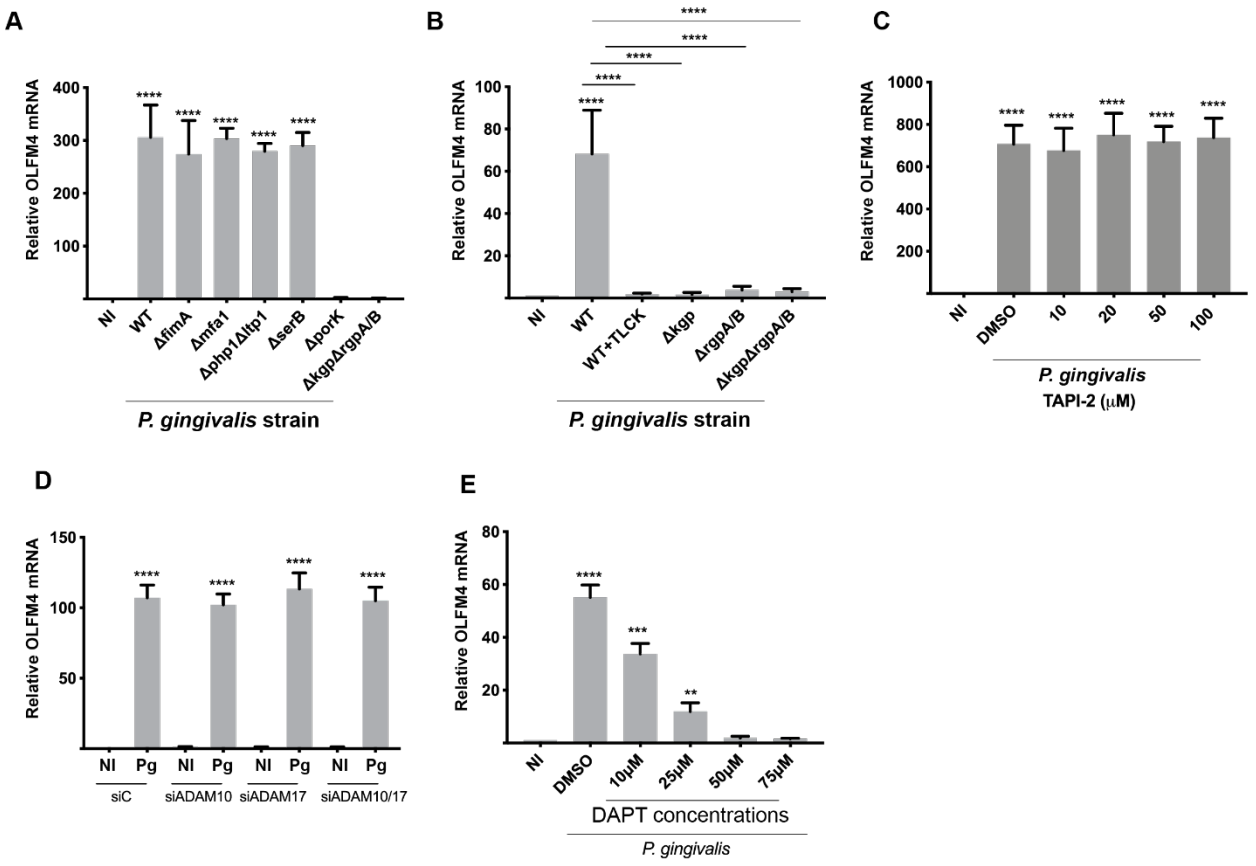


Figure 11: Activation of Notch signaling by *P. gingivalis* gingipains
TIGK cells were challenged for 24 h at an MOI of 100 with ATCC 33277 wildtype (WT), as well as deletional mutants of adhesins (*mfa1, fimA*), tyrosine and serine phosphatase network (*ltp1/php1, serB*), and type 9 secretion system (T9SS) machinery and effectors (*porK, kgp/rgpA/rgpB*). Loss of *P. gingivalis* adhesins or phosphatases did not interfere with regulation of *OLFM4*, but loss of T9SS regulatory protein PorK and T9SS cargo *kgp/rgpA/rgpB* significantly reduced levels of OLFM4 (A). We then challenged TIGK cells with *P. gingivalis* wildtype treated with the gingipain inhibitor TLCK, as *kgp, rgpA/B*, and *kgp/rgpA/rgpB* deletional mutants (B). RNA was harvested, and *OLFM4* transcript measured by qRT-PCR. TLCK treated *P. gingivalis* was unable to stimulate *OLFM4* transcription, and an unimpaired proteolytic load was required for *OLFM4* regulation. The Notch receptors are sequentially cleaved after binding Notch ligands first by extracellular ADAM10/17 proteases, then by intracellular gamma-secretase. TIGK cells were treated with a pharmacological inhibitor of the extracellular ADAM10/17 proteases TAPI-2 at indicated concentrations, then challenged with *P. gingivalis* for 24 h (C). Pharmacological inhibition of ADAM10/17 (C) nor siRNA knockdown of *ADAM10* and/or *ADAM17* (D) impeded *P. gingivalis* induced *OLFM4* by qRT-PCR, implicating *P. gingivalis* produced extracellular gingipain proteases in activation of Notch signaling. *P. gingivalis* can also invade epithelial cells, so we also investigated the potential role of intracellular gingipains in cleavage of intracellular Notch. Gamma-secretase inhibitor DAPT was used at indicated concentrations, and in a dose dependent manner reduced *P. gingivalis* induction of

OLFM4 in TIGK cells (E). This excludes the role of gingipains involvement of Notch activation in an intracellular capacity. Target genes for qRT-PCR were normalized to GAPDH, and fold changes were calculated using the ΔΔCT method. Significance was calculated using a One-way ANOVA, ** P< .01, *** P<.005, and **** P<.001.

Gingipains are secreted through the type IX secretion system, and loss of PorK, which is required for formation of a functional secretion pore [133] also abrogated the ability of *P. gingivalis* to enhance OLFM4 transcription. We further investigated the role of gingipains by challenging cells with individual gingipain mutants, which revealed that loss of either Kgp or RgpA/B was sufficient to prevent upregulation of OLFM4 (Fig. 11B). Additionally, pretreatment of parental *P. gingivalis* with the gingipain inhibitor TLCK prevented OLFM4 synthesis. Engagement of the Notch receptor by Jag1 induces a conformational change in the Notch which exposes an extracellular region of that can be cleaved by the extracellular proteases ADAM10 and ADAM17 [134]. On the basis of results with gingipains, we hypothesized that these proteolytic enzymes of *P. gingivalis* activate signaling through cleavage of the extracellular domain of Notch. To test this notion, we used TAPI-2, a pharmacological inhibitor of the ADAM10 and ADAM17 proteases [135]. We found that *P. gingivalis* remained capable of activating Notch in the presence of TAPI-2 as shown in Fig. 11C, suggesting that the gingipains can functionally compensate for the loss of the ADAM10 and ADAM17 proteases. This was corroborated by siRNA targeting ADAM10 and ADAM17, which also failed to prevent activation of Notch and upregulation of OLFM4 (Fig. 11D). In contrast, DAPT, which is an inhibitor of gamma secretase, the enzyme that cleaves the intracellular domain of the Notch receptors, completely inhibited stimulation of OLFM4 and HES5 transcription by *P. gingivalis* (Fig.

11E). Thus, the gingipains are unable to complement cleavage of the intracellular domain of Notch, and function extracellularly to activate Notch. Although *P. gingivalis* can internalize and survive inside gingival epithelial cells [136], production of the gingipains is downregulated intracellularly [137] and may be insufficient function on a membrane protein. Collectively, the results show that *P. gingivalis* can activate Notch1 signaling in a two-hit mechanism, by proteolytic cleavage of the extracellular domain and by increasing production of the Jag1 agonist.

The ecosystem of the oral cavity in which *P. gingivalis* resides is a complex because multispecies community partners engage *P. gingivalis* in multilevel interspecies interactions, which can be either synergistic or antagonistic [102]. Hence, we next investigated the impact of several community partners of *P. gingivalis* on OLFM4 regulation. Co-infection of *P. gingivalis* with either *Filifactor alocis*, *Fusobacterium nucleatum*, or *Treponema denticola*, had no influence on regulation of OLFM4 by *P. gingivalis* (Fig. 12A).

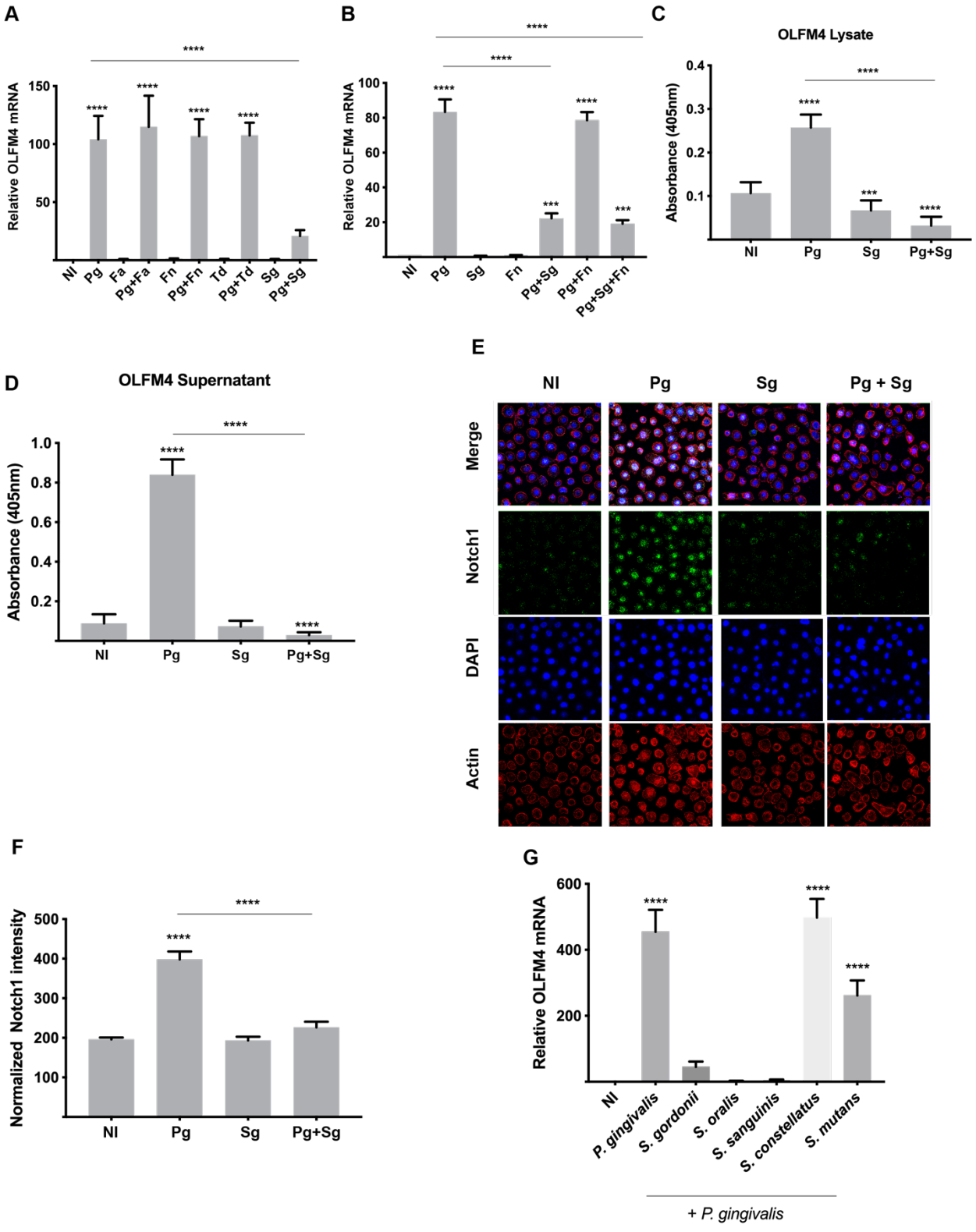


Figure 12: Antagonism of *P. gingivalis* induced OLFM4 by *S. gordonii*

P. gingivalis is generally found in communities, so we investigated the role these interactions in regulation of OLFM4 in TIGK cells. Cells were challenged with an MOI of 100 of either *P. gingivalis* and/or *Filifactor alocis* (Fa), *Fusobacterium nucleatum* (Fn), *Treponema denticola* (Td), and *Streptococcus gordonii* (Sg). Regulation of OLFM4 was unaffected by Fa, Fn, or Td, but *S. gordonii* significantly reduced OLFM4 regulation by *P. gingivalis* (A). We also investigated the role of multispecies interactions by challenging TIGK cells with Pg (MOI 50), Fn (MOI 50), and Sg (MOI 10), and found that Sg maintained the ability to inhibit OLFM4 (B). Confirmation of protein expression was verified by using either cell lysates (C) or supernatants (D) with an OLFM4 ELISA. *P. gingivalis* significantly increased protein expression in both cell lysates and supernatants, and *S. gordonii* significantly reduced OLFM4 protein in both cell lysates and supernatants in combination with *P. gingivalis*. Activated Notch localizes to the nucleus, so we then investigated localization of Notch1 with *P. gingivalis* and/or *S. gordonii* (E). *P. gingivalis* significantly increased localization of Notch1 to the nucleus, but *S. gordonii* prevented activation and localization of Notch (F). We then investigated the role of other potential oral streptococci in manipulation of *P. gingivalis* signaling. We found that organisms that produce H_2O_2 i.e. *S. gordonii*, *S. oralis*, and *S. sanguinis* significantly reduced *P. gingivalis* mediated OLFM4 upregulation, whereas *Streptococci* that are unable to produce H_2O_2 showed a reduced ability to inhibit OLFM4 (G). Target genes for qRT-PCR were normalized to GAPDH, and fold changes were calculated using the ΔΔCT method. Significance was calculated using a One-way ANOVA, *** P<.005, and **** P<.001.

However, co-infection with *S. gordonii* impeded OLFM4 induction by *P. gingivalis*, and in a three species consortium of *P. gingivalis*, *F. nucleatum* and *S. gordonii*, the antagonistic effect of *S. gordonii* was dominant (Fig. 12B). We verified OLFM4 expression changes at the protein level using an ELISA, which showed that *S. gordonii* can antagonize production of OLFM4 as reflected both the intracellular and extracellular amounts of the protein (Fig. 12C-D).

Following cleavage by (gamma)-secretase the intracellular domain of the Notch receptor localizes to the nucleus, where it binds and activates the transcription factor RBP-JK, leading to upregulation of Notch target genes. We utilized confocal microscopy to determine the localization pattern of cleaved Notch in TIGK cells challenged with *P. gingivalis* and/or *S. gordonii*. Fig. 12E-F shows that *P. gingivalis* induces nuclear

localization of Notch, which is antagonized in the presence of *S. gordonii*. These results suggest that *S. gordonii* prevents *P. gingivalis*-induced upregulation of OLFM4 by blocking activation of Notch signaling. *S. gordonii* is one of several species of oral streptococci with the potential to coaggregate with *P. gingivalis in vivo*. A number of additional oral streptococcal species were thus tested for antagonistic properties, and we found that *S. oralis* and *S. sanguinis* were also capable of inhibiting OLFM4 expression (Fig. 12G). In contrast, *S. constellatus* and *S. mutans* did not suppress *P. gingivalis*-induction OLFM4 upregulation. One phenotypic property of these streptococcal species which tracks with antagonism of *P. gingivalis*, is production of hydrogen peroxide (H_2O_2) as a metabolic by product [138, 139].

We have previously shown that *S. gordonii* can antagonize the action of *P. gingivalis* by activating Nemo-like Kinase (NLK) which in turn suppresses the FOXO1 pathway. We confirmed that the antagonistic action of *S. gordonii* is independent of NLK by siRNA knockdown. As shown in Fig. 13, a reduction in NLK levels did not impact the ability of *S. gordonii* to prevent *P. gingivalis* induction of OLFM4. Additionally, we investigated the role of exogenous Jag1 in the role of OLFM4 transcriptionally activation, and determined whether *S. gordonii* could prevent activation through host cell manipulation. Exogenous treatment with recombinant Jag1 was enough to stimulate OLFM4 transcription (Fig. 14), however *S. gordonii* was unable to impede this activation, which indicates this inhibition could be driven through *P. gingivalis*-*S.gordonii* signaling, rather than *S. gordonii*-host signaling.

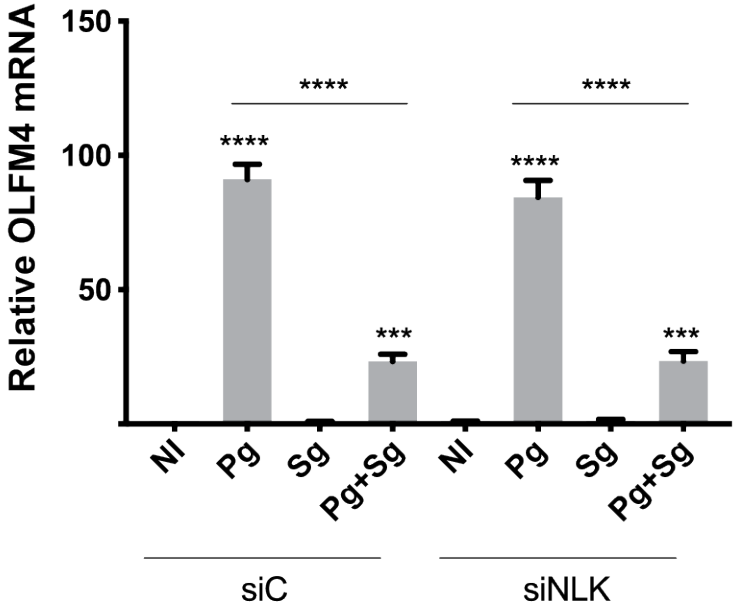


Figure 13: NLK-FOXO1 signaling is not required for inhibition of OLFM4
S. gordonii inhibits manipulation of host signaling by *P. gingivalis* through activation of host kinase NLK, which then phosphorylates and inactivates transcription factor Foxo1 (activated by *P. gingivalis*). TIGK cells were transfected with siRNA targeting NLK, then challenged with *P. gingivalis* (MOI 100) and/or *S. gordonii* (MOI 50) for 24h. RNA was then harvested, and expression of OLFM4 measured by qRT-PCR. Knocking down NLK had no effect on OLFM4 inhibition by *S. gordonii*. OLFM4 was normalized to GAPDH, and fold changes were calculated using the ΔΔCT method. Significance was calculated using a One-way ANOVA, *** P<.005, and **** P<.001.

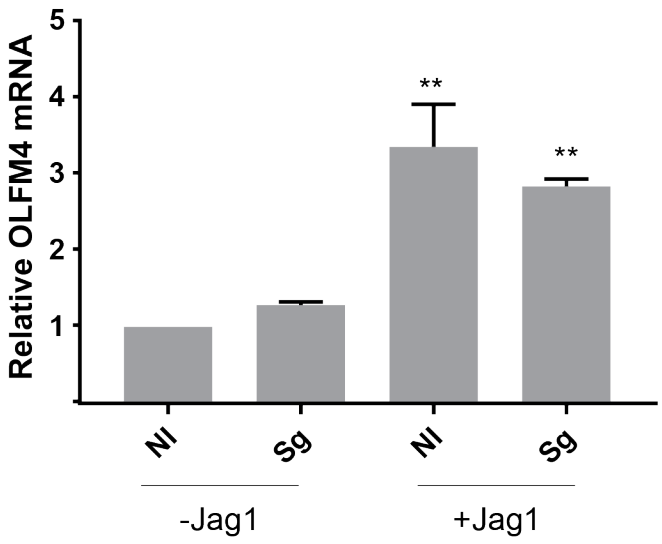


Figure 14: *S. gordonii* cannot inhibit Notch signaling activated with exogenous Jagged1 TIGK cells were seeded on wells either coated with protein G or Jag1-FC, then at 24h challenged with *S. gordonii*. Exogenous Jag1 was enough to stimulate OLFM4 transcription, however *S. gordonii* was unable to impede this activation. OLFM4 CT values were normalized to GAPDH, and fold changes were calculated using the ΔΔCT method. Significance was calculated using a One-way ANOVA, ** P< .01

To test whether secreted metabolites were sufficient to inhibit OLFM4 expression, we utilized a transwell assay. TIGK cells in the lower chamber were challenged with *P. gingivalis* in the lower chamber along with either *S. gordonii*, S. *oralis*, or S. *mutans* in the upper chamber. Both *S. gordonii* and S. *oralis* were able to inhibit *P. gingivalis*-induced OLFM4 expression whereas *S. mutans* had no effect (Fig. 15A). These results show that functional activity resides in a product secreted from *S. gordonii* and *S. oralis.*

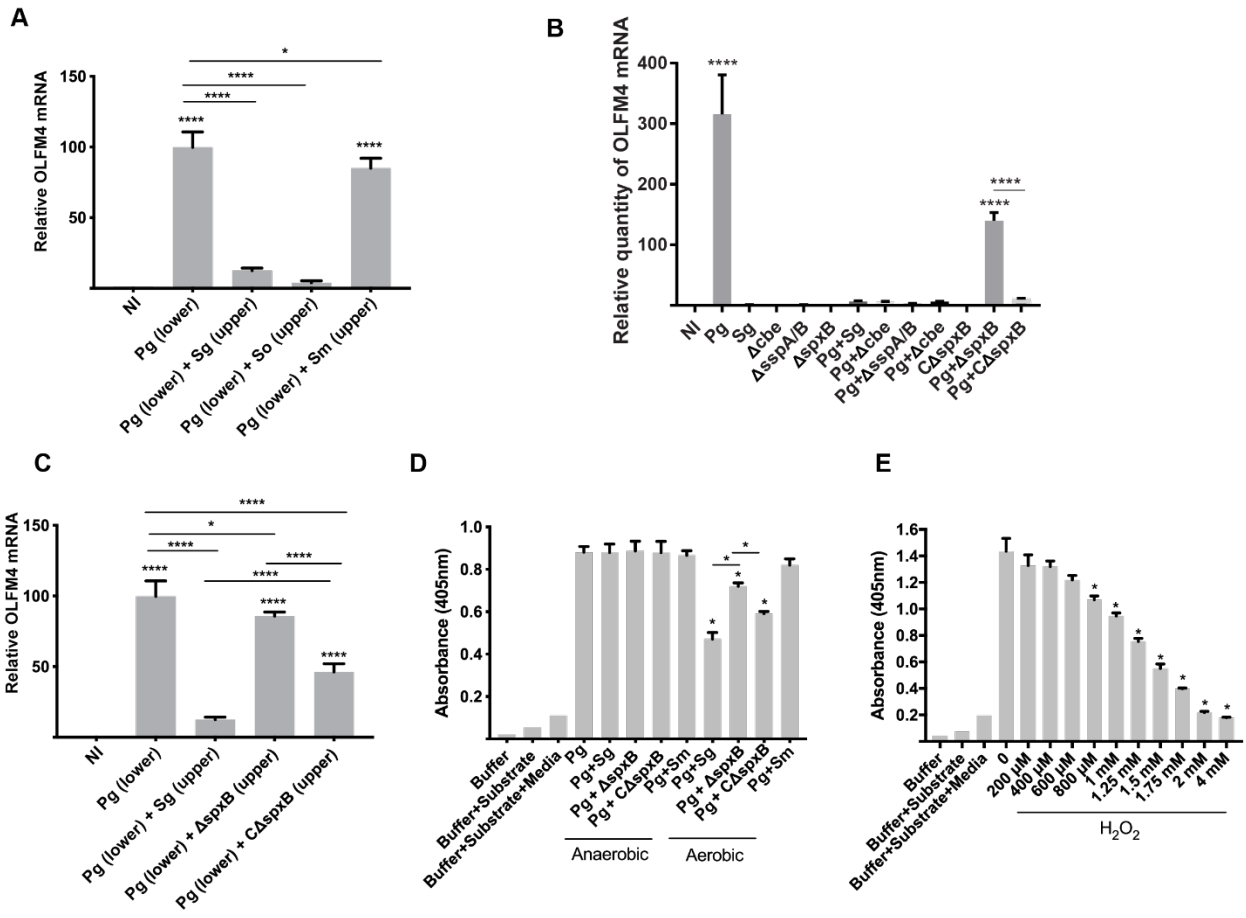


Figure 15: Inhibition of gingipains by *Streptococci* produced H_2O_2

To follow up the role of H_2O_2 in the inhibition of OLFM4 by *P. gingivalis* we utilized the 0.4 um transwell system. TIGK cells were grown in the lower chamber, then challenged with *P. gingivalis* (MOI 100) in the lower chamber where indicated and *S. gordonii* (Sg), *S. oralis* (So), or *S. mutans* (Sm) in the upper chamber (A). *S. gordonii* and *S. oralis* were able to inhibit OLFM4 independent of contact with either *P. gingivalis* or TIGK cells, and *S. mutans* did not affect *P. gingivalis* induced OLFM4. *S. gordonii* interacts with *P. gingivalis* through direct binding with adhesins (SspA/B), as well as through secreted metabolites pABA and H_2O_2. Inhibition of OLFM4 expression by *P. gingivalis* was not ameliorated with adhesin or pABA secretion deficient mutants (*sspA/B, cbe*), however the H_2O_2 deficient mutant (*spxB*) showed significantly less inhibition. This inhibition was restored by complementing *spxB* (B). The role of secreted H_2O_2 was confirmed by utilizing 0.4um transwell filters. TIGK cells were grown in the lower chamber, then challenged with *P. gingivalis* in the lower chamber or the indicated strains of *S. gordonii* in the upper chamber. The deletional *spxB* mutant was unable to inhibit OLFM4, whereas the complemented strain was partially restored to wildtype level inhibition (C). Gingipains require a reduced environment for optimal protease activity, so we investigated the role of H_2O_2 in oxidizing and therefore inhibiting activity of RgpA/B using a chromogenic substrate assay. Supernatant was taken from *P. gingivalis* grown to mid-log phase by spinning the cells down and filtering the

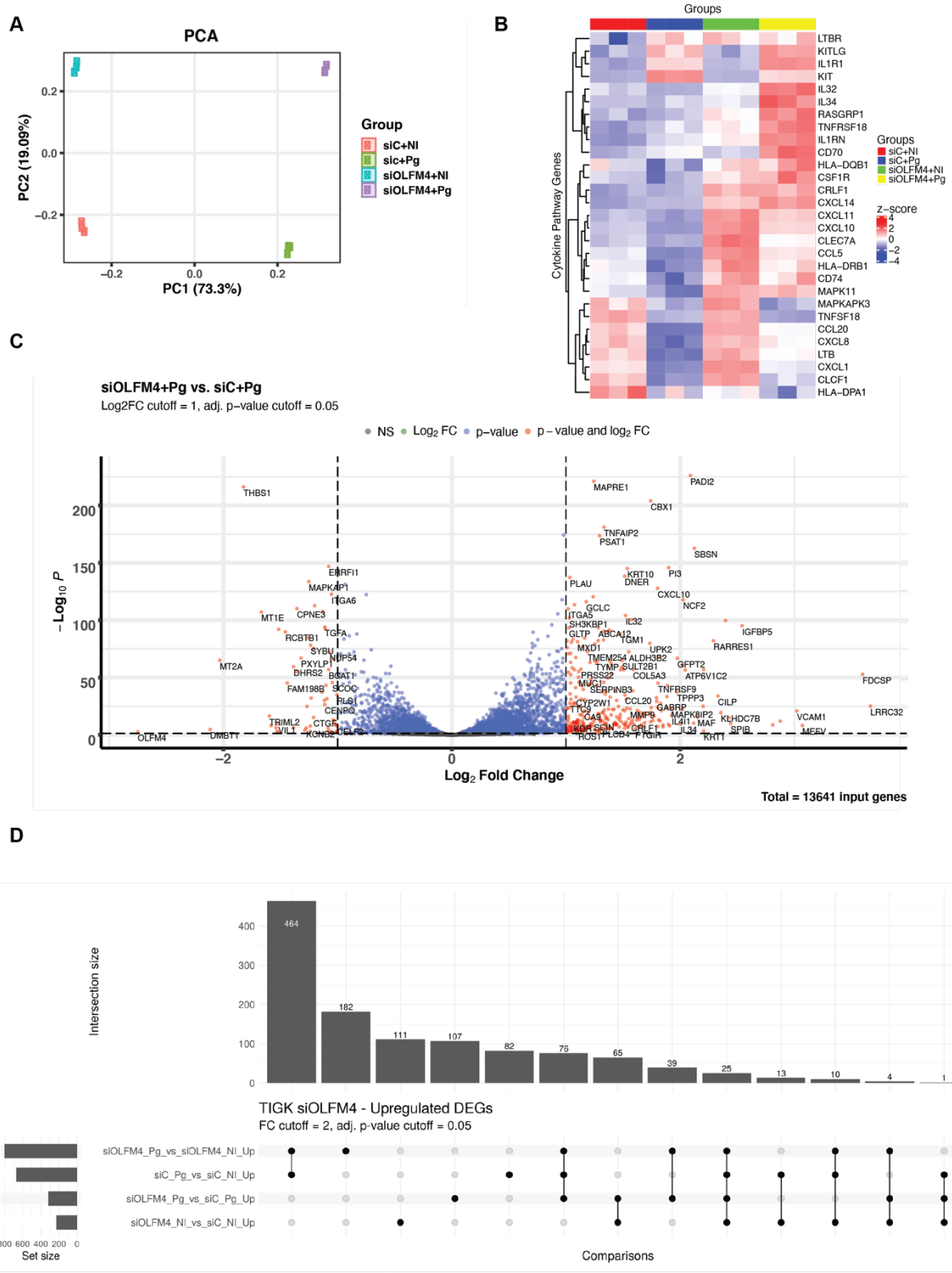
supernatant. *P. gingivalis* supernatant was then incubated with metabolically active anaerobic or aerobic *S. gordonii* or *S. mutans*. Supernatant was then collected and filtered, then activity of RgpA/B was measured through cleavage of a chromogenic substrate (D). Anaerobic cultures did not impact activity of RgpA/B, but aerobically grown *S. gordonii* did reduce activity of RgpA/B by about 50%. This inhibition was ameliorated in the *spxB* deletional mutant, and partially restored by the complemented spxB strain. *S. mutans* did not inhibit RgpA/B activity in either anaerobic or aerobic cultures. We then confirmed inhibition of RgpA/B activity by H_2O_2 by incubating *P. gingivalis* supernatant with indicated concentrations, and measuring activity by cleavage of a chromogenic substrate. Significant inhibition is observed at 800 µm, and activity is reduced in a dose dependent manner until 4 mM (E). Target genes for qRT-PCR were normalized to GAPDH, and fold changes were calculated using the ΔΔCT method. Significance was calculated using a One-way ANOVA, and *P<.05, *** P<.005, and **** P<.001.

To provide additional evidence of a role for H_2O_2 we utilized a pyruvate oxidase mutant (Δ*spxB*), in which H_2O_2 production is reduced by approximately 90% through loss of conversion of pyruvate to acetate with H_2O_2 as a byproduct. As shown in Fig. 15B, the Δ*spxB* mutant was unable to suppress OLFM4 transcription in a co-infection assay. In contrast, mutants of *S. gordonii* unable to bind (Δ*sspA/B*) or accumulate (Δ*cbe*) with *P. gingivalis* retained the ability to impede the increase in OLFM4. Similarly, in a transwell assay with *P. gingivalis* in the lower chamber and *S. gordonii* in the upper chamber, the Δ*spxB* mutant had no impact on induction of OLFM4 mRNA by *P. gingivalis*, whereas parental and complemented Δ*spxB* mutant were antagonistic (Fig. 15C). Complementation of the mutation with the *spxB* gene in trans (CΔ*spxB*) restored the antagonistic phenotype. Collectively, these results strongly implicate secreted H_2O_2 as the effector of streptococcal antagonism of *P. gingivalis*-induced OLFM4 regulation.

Interestingly, gingipains require a reduced environment for activity to maintain the cysteine catalytic domain [140]. Thus, we hypothesized that oxidation by H_2O_2 was impairing the activity of the gingipains. To test this, we used a chromogenic assay to

measure gingipain activity in the supernatant of *P. gingivalis* cultures incubated with streptococci either capable or unable to produce H_2O_2 (Fig. 15D). Aerobically cultured *S. gordonii* WT and the complemented mutant CΔ*spxB* significantly reduced gingipain activity. Activity was partially reduced by the Δ*spxB* mutant, possibly due to residual H_2O_2 production, and was unaffected by *S. mutans*. In contrast, following anaerobic culture of the streptococcal strains, in which H_2O_2 is not produced, there was no inhibition of gingipain activity in any condition. We then sought to test concentrations of H_2O_2 that could inhibit gingipain activity. As shown in Fig. 15E, 800 μM was the lowest dose at which significant inhibition of gingipain activity was observed, with the greater reduction occurring between 1-2 mM, which is the range of H_2O_2 amounts that can be produced extracellularly by streptococci [141]. Taken together, our data support the concept that H_2O_2 produced by certain species of oral streptococci such as *S. gordonii* inhibit the activity of gingipains and consequently impede activation of the Notch pathway by *P. gingivalis*.

Many of the epithelial cell responses to *P. gingivalis* revolve around the axis of survival/proliferation/migration [12]. OLFM4 is an antiapoptotic factor that promotes tumor growth [43]. Thus, we next investigated the extent to which OLFM4 participates in epithelial cell responses to *P. gingivalis* by knocking down OLFM4 and performing RNA Seq. The PCA plot for this RNA seq showed clustering of individual biological replicates in each group, as well as distinct separation between conditions (Fig. 16A).


A
PCA
PC2 (19.09%)
PC1 (73.3%)
Group
siC+NI
sic+Pg
siOLFM4+NI
siOLFM4+Pg
B
Groups
Cytokine Pathway Genes
z−score
C
siOLFM4+Pg vs. siC+Pg
Log2FC cutoff = 1, adj. p−value cutoff = 0.05
NS Log2 FC p−value p − value and log2 FC
−Log10 P
Log2 Fold Change
Total = 13641 input genes
D
Intersection size
TIGK siOLFM4 - Upregulated DEGs
FC cutoff = 2, adj. p-value cutoff = 0.05
siOLFM4_Pg_vs_siOLFM4_NI_Up
siC_Pg_vs_siC_NI_Up
siOLFM4_Pg_vs_siC_Pg_Up
siOLFM4_NI_vs_siC_NI_Up
Set size
Comparisons

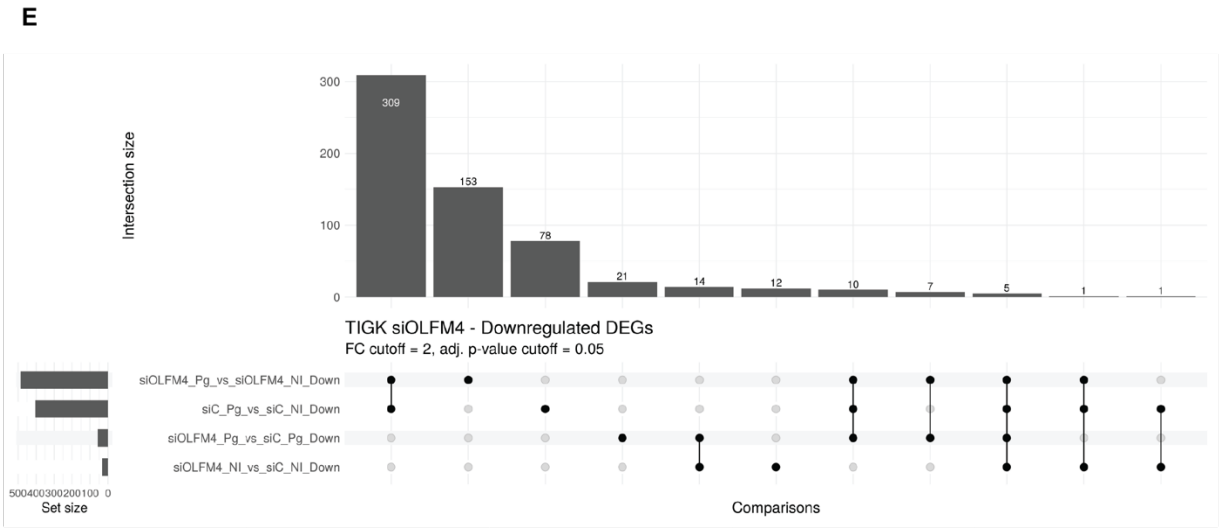


Figure 16: Knocking down OLFM4 reveals altered inflammatory transcriptomic profile
TIGK cells were transfected with either scrambled siRNA or siOLFM4, then challenged with *P. gingivalis* for 24h. Libraries were prepped as described in Figure 5. The PCA plot (A) shows tight clustering of individual samples within groups, and distinct clustering of each group. A heatmap of pro-inflammatory cytokines/chemokines reveals significantly upregulated pro-inflammatory markers in the siOLFM4 Pg group compared to the siControl Pg group (B). (C) shows a volcano plot that graphically depicts differential gene expression in siOLFM4 Pg and siControl Pg cells, and (D) summarizes the comparison of each individual group for upregulated and down regulated genes.

Enrichment for pro-inflammatory gene within each group in Fig. 16B revealed a distinct signature in the siOLFM4 conditions, specifically genes that were are known to be downregulated by *P. gingivalis* were upregulated, including CXCL8, CXCL10, and CXCL11. We have summarized the transcriptional changes in the siOLFM4 *P. gingivalis* treated cells and siControl *P. gingivalis* treated cells as a volcano plot in Fig. 16C and shared differentially expressed genes are summarized as upset charts in Fig. 16D.

OLFM4 manipulates multiple host processes through protein-protein interactions that *P. gingivalis* is also known to impact, such as migration. We investigated the role for OLFM4 in migration of the epithelial cells. In Fig. 17, we knocked down OLFM4 in epithelial

cells, then challenged with *P. gingivalis*. We found that reduction of OLFM4 significantly diminished migration of TIGKs in response to *P. gingivalis*. .

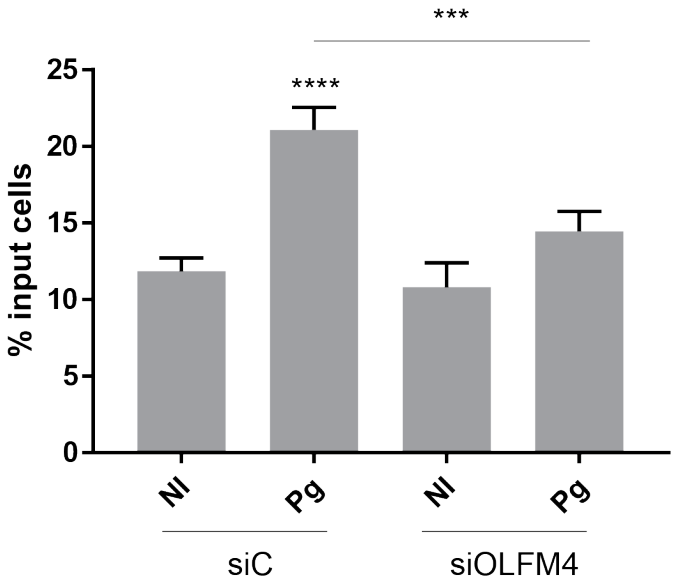


Figure 17: *P. gingivalis* promotes migration of epithelial cells through OLFM4
TIGK cells were transfected with either scrambled siRNA or siRNA targeting OLFM4. 48 h after transfection cells were challenged with an MOI of 100 *P. gingivalis*, then seeded over a Matrigel transwell insert for 18h. After incubation cells were scraped off the surface of the membrane, and the cells that had invaded were fixed with methanol and stained with toluidine blue. Cells were enumerated in at least 3 fields, and percentages calculated in comparison to an insert without Matrigel coating. Significance was calculated using a One-way ANOVA, *** P<.005, and **** P<.001.

The regulatory mechanism for OLFM4 by *P. gingivalis*, as well as the method for antagonism by *S. gordonii* is summarized in Figure 18.

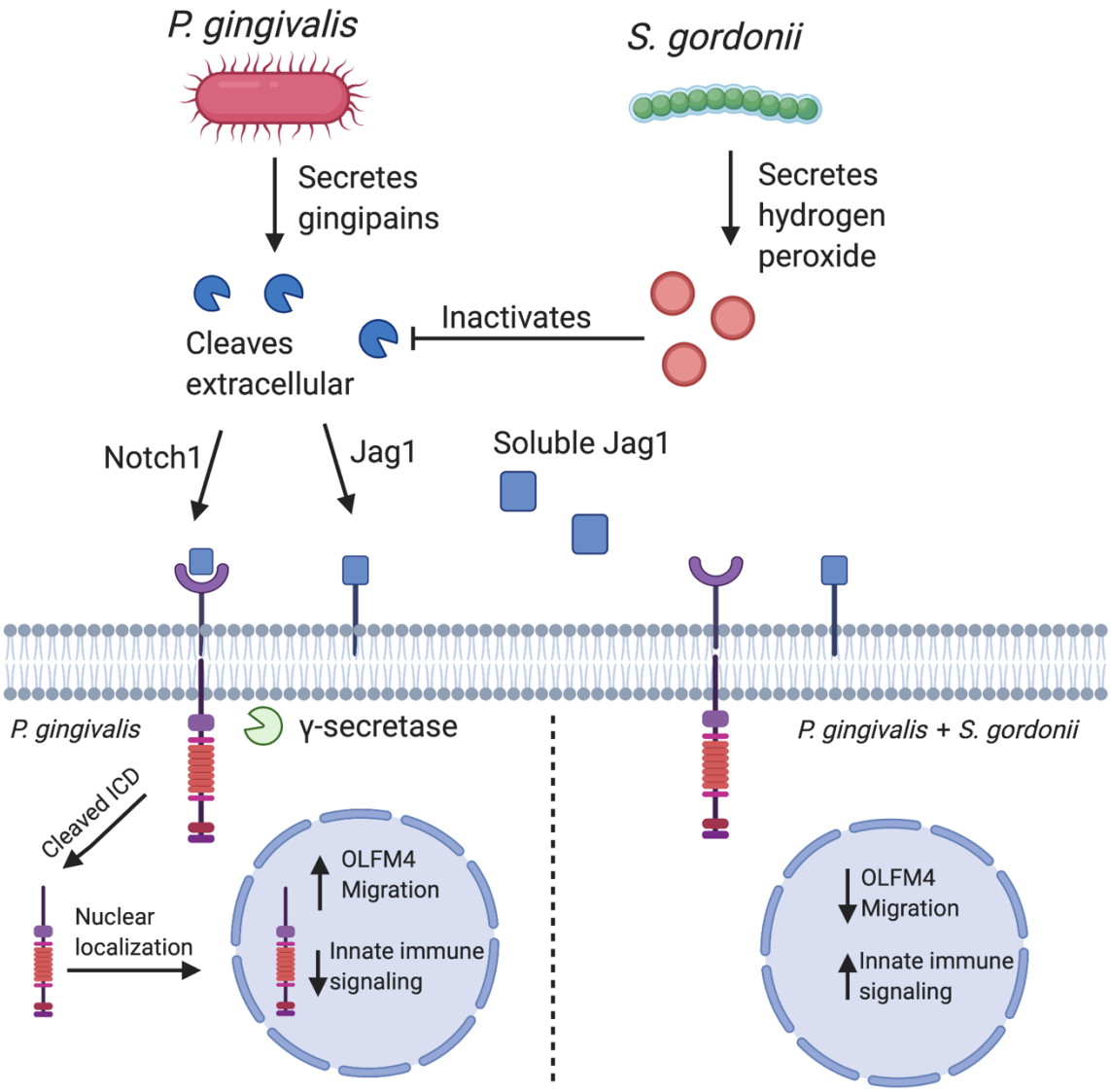


Figure 18: Graphic summarizing the pathway by which *P. gingivalis* activates Notch signaling, and OLFM4 as a downstream target of activation. *S. gordonii* inhibits this activation through secretion of H_2O_2, which inhibits RgpA/B activity, preventing activation and downstream activation of OLFM4.

CHAPTER 4 DISCUSSION

The idea of bacteria causing cancer has been fervently studied since the classification of *Helicobacter pylori* as a causative agent for stomach cancer in 1984. Oral squamous cell carcinoma has a 5-year mortality rate of approximately 50%, in part because diagnosis tends to be elusive due to its asymptomatic progression. Identification of microorganisms, or a microbial profile, that increases the probability of OSCC would help in identifying those who are high risk, and would represent a unique preventative treatment modality. One candidate bacterium that has been associated with OSCC for decades is *P. gingivalis*, which has the capacity to inhibit apoptosis, enhance proliferation, and induce an epithelial-to-mesenchymal like invasive phenotype in epithelial cells. *P. gingivalis* has been characterized as a keystone pathogen for periodontal disease by its ability to directly manipulate the immune response through its own virulence factors, as well as by driving changes in the microbial community that leads to a dysbiotic relationship with the host immune system, leading to persistent inflammation. This changing microenvironment can be a driving force in the transformation of epithelial cells, as byproducts of inflammation produces a nutrient rich environment and dysregulates the tumor immune microenvironment, which favors persistent colonization by anaerobes. Recent studies have focused on relative abundance of bacteria on sites of OSCC compared to contralateral healthy controls and have reported that numbers of periodontal pathogens like *P. gingivalis* tend to be higher, whereas commensals such as

some streptococci tend to be decreased [142] . While this likely reflects fitness within the tumor microenvironment, in the absence of homeostatic commensals such as *S. gordonii,* the tumorigenic potential of *P. gingivalis* will be unconstrained. For example, *P. gingivalis* regulates EMT factor ZEB2 through dephosphorylation of host transcription factor FoxO1. This interaction is antagonized by *S. gordonii* which induces phosphorylation of FOXO1 through activation of host TAK/NLK kinase cascade. These properties are made more interesting because these organisms interact directly to enhance each other's virulence through attachment and metabolic synergy. However, in the host epithelial environment, these organisms antagonize one another through dysregulation of host signaling cascades.

The goal of this study was to identify signaling events impacted by periodontal pathogen *P. gingivalis* that contribute to homeostatic disruption, specifically focusing on pathways overridden by homeostatic commensal *S. gordonii* in gingival epithelial cells. We hypothesized that *P. gingivalis* would activate genes involved in tissue disruption and dampening of innate immune signaling, whereas *S. gordonii* would either program the cells to resist this activation or interact with other inhibitory pathways. We approached this by characterizing differences in transcriptomic responses of epithelial cells challenged with *P. gingivalis* and/or *S. gordonii* using RNAseq, revealing OLFM4, a gene involved in apoptosis, proliferation, migration, and dysregulation of innate immunity, all of which are differentially influenced by the microbes, as a potential axis that is disturbed by *P. gingivalis* and restored by *S. gordonii*. The initial identification of OLFM4 was followed by

characterization of the mechanism for upregulation by *P. gingivalis*, inhibition by *S. gordonii*, and ultimately the phenotypic outcome of this differential regulation.

Currently, there are few studies that characterize the complete transcriptional fingerprint left by oral microbes on gingival epithelial cells. Hasegawa et. al. focused on transcriptional changes using a microarray of epithelial cells challenged with either *F. nucleatum* or *S. gordonii,* which identified activation of MAPK signaling by both organisms [143]. However, they reported that *F. nucleatum* increased IL-6 and IL-8 production, whereas *S. gordonii* inhibited production of both. Handfield et. al. focused on the individual challenge of epithelial cells with either *P. gingivalis* or *A. actinomycetemcomitans* [23]. This report showed that *P. gingivalis* activated p53 signaling, leading to inhibition of apoptosis, whereas *A. actinomycetemcomitans* inactivated p53 signaling, thereby inducing apoptosis [23]. Using gene array analysis in human immortalized gingival keratinocytes (HIGKs), Mans et. al. [144] reported that *S. gordonii* overrides transcriptional changes in cell cycle regulatory genes, opposing *P. gingivalis* mediated cell cycle progression in favor of cell cycle arrest. While this study provided significant insight into the regulatory mechanisms for cell cycle activity, microarray technology has since been superseded by deep sequencing approaches, and thus new, previously unknown targets can be identified. Additionally, as *S. gordonii* can antagonize activation of transcription factor FOXO1 by *P. gingivalis* in gingival epithelial cells (discussed above), there are likely to be comprehensive transcriptional changes when *P. gingivalis* and *S. gordonii* are co-infected.

In this study, we identified unique complete transcriptomic profiles between gingival epithelial cells challenged with *P. gingivalis* and/or *S. gordonii* through RNAseq, which characterized the interaction between the co-infected group to be more similar to the individual *S. gordonii* group than the *P. gingivalis* alone group. We started by investigating genes that were differentially regulated by *P. gingivalis*, but the opposite trend was observed in the *P. gingivalis* + *S. gordonii* group. This led us to OLFM4, which was upregulated by *P. gingivalis*, downregulated by *S. gordonii*, and was significantly reduced compared to the *P. gingivalis* alone group.

OLFM4, also known as GW112, was originally characterized by its function in binding the mitochondrial protein GRIM19 [43], and attenuating interferon-β mediated apoptosis in prostate cancer cells [43]. OLFM4 has since been implicated in EMT, cell cycle activity, and modulation of innate immune signaling [44, 45, 47, 126]. In non-small cell lung cancer, under hypoxic conditions, OLFM4 is significantly upregulated, and can then positively regulate HIF-1α to promote EMT [45]. In hepatocellular carcinoma, OLFM4 enhances activation of STAT3, which then increases proliferation and induces EMT in HepG2 cells [145]. *H. pylori* induces OLFM4 regulation in gastric epithelial cells, which upon stimulation binds NOD1/NOD2, inhibiting activation of NF-κB. Knockout OLFM4 mice could effectively eliminate *H. pylori* from the stomach, whereas wildtype mice experienced persistent colonization [47]. Further investigation revealed a unique cytokine profile in OLFM4 knockout mouse stomach, specifically increased transcriptional and protein levels of IL-1β, IL-5, IL-12, and MIP-1α [47]. In summary, OLFM4 inhibits apoptosis, increases proliferation and invasion, and manipulates host immune signaling, which are

all characteristic phenotypes of epithelial cells challenged with *P. gingivalis.* Additionally, *S. gordonii* inhibits *P. gingivalis* induced proliferation and invasion in epithelial cells, which points the potential role for an OLFM4 regulatory axis.

OLFM4 is primarily regulated through the Notch1-Jagged1 signaling cascade. Notch signaling was significantly enriched in the *P. gingivalis* alone RNAseq, with, for example, Notch1, Jagged1, and the Notch target gene Hes5 were upregulated. Interestingly, Notch1, Jagged1, and Hes5 were all significantly lower in the *P. gingivalis* + *S. gordonii* compared to the *P. gingivalis* alone group. Recently, Al-Attar et. al. found that *P. gingivalis* activates Notch signaling in oral mucosal and gingival epithelial cells, leading to increased transcription, translation, and secretion of the anti-microbial protein PLA_2-IIA, which is one factor responsible for the dysbiotic shift observed after colonization by *P. gingivalis*. We similarly observed that *P. gingivalis* induces nuclear localization of Notch1 in epithelial cells. In contrast, not only does *S. gordonii* not induce nuclear localization, but it inhibits Notch localization by *P. gingivalis*. Al-Attar et. al. found that *P. gingivalis* induced nuclear localization of Notch1, whereas *S. gordonii* and *F. nucleatum* did not. This study also revealed upregulation of HES1 and HEY1 by *P. gingivalis* in mucosal cells, which is recapitulated in our epithelial cell RNAseq. Additionally, the gingipain deficient *P. gingivalis* could not stimulate Notch1 in OKF6 cells. Previously, OLFM4 had been shown to require Notch1, and stimulation with exogenous Jagged1 in order to increase OLFM4 protein. We corroborated this finding, and additionally we excluded Notch ligands Jagged2 and DLL1-4, as well as Notch2-4 from a role in regulating OLFM4.

P. gingivalis produced proteolytic enzymes, the Kgp, RgpA, and RgpB gingipains, are key virulence factors in the manipulation of host immunity and tissue homeostasis. Each of the gingipains is capable of inhibiting classical, lectin, and alternative pathways of the complement cascade through the proteolytic degradation of C3. The gingipains also target C5b, which attenuates the formation of the membrane attack complex. *P. gingivalis* induces non-canonical activation of β-catenin through cleavage by the gingipains into functional fragments, which are able to translocate to the nucleus. Functional β-catenin and gingipains are required for upregulation of ZEB2, and β-catenin is also likely involved in the regulation of proliferation and production of MMP7. Additionally, *P. gingivalis* cleaves MMP-1, -3, and -9 into their active forms with the gingipains, which leads to increased destruction of the host periodontium and potentially increased motility of epithelial cells. *P. gingivalis* induces a local chemokine paralysis through degradation of CXCL8, CXCL9, and CXCL10, which is mediated through a secreted proteolytic enzyme (likely the gingipains.) Additionally, the gingipains have been shown to proteolytically degrade β-catenin into its functional form, which is capable of nuclear localization. This proteolytic activation was capable of being induced with purified gingipains, in the absence of any whole cell *P. gingivalis* whole cells. β-catenin regulates cell proliferation, as well as MMPs, which implicates an additional role for the gingipains in these phenotypes. *P. gingivalis* gingipains activate PAR2 and PAR4, leading to the phosphorylation of IκB, the nuclear translocation of NF-κB, and increased transcription of proMMP9 (50, 51). In addition, gingipains can cleave proMMP9, generating the mature active enzyme (52), which is important for cancer cell invasion and metastasis. Gingipains

can also proteolytically process proteins on the epithelial cell surface, causing release and redistribution, with consequent effects on signal transduction and inflammatory responses (53, 54). Intracellularly, *P. gingivalis* gingipains can degrade mammalian target of rapamycin (mTOR), thus disrupting the mTOR pathway which regulates the cytoskeleton, as well as cleave β-actin directly (55, 56). In trophoblasts, gingipains can degrade p53 and the E3 ubiquitin protein ligase homolog protein (MDM2) and modulate the activity of multiple signaling pathways, resulting in both cell cycle arrest and cell death (57). The capacity of gingipains to regulate the expression of inflammatory mediators at the mRNA level has also been demonstrated. In gingival fibroblasts, gingipains increase TGFβ gene expression, while suppressing the expression of CXCL8 (58). Elevated signaling through TGFβ and STAT3 could also contribute to tumorigenesis.

We found here that the gingipains are required for the activation of Notch signaling through a previously unknown mechanism. Our data support a role for each of the gingipains in cleaving the transmembrane protein Jagged1 into a soluble form, allowing for paracrine activation of Notch signaling, as well as acting in place of the extracellular ADAM10/17 proteases in cleaving Notch1 into its active form. We propose a role for OLFM4 in the regulation of invasion in gingival epithelial cells, as well as dampens epithelial pro-inflammatory genes, which are both functions that require gingipains. Fig. 19 integrates OLFM4 signaling into the context of other epithelial cell signaling pathways dysregulated by *P. gingivalis*.

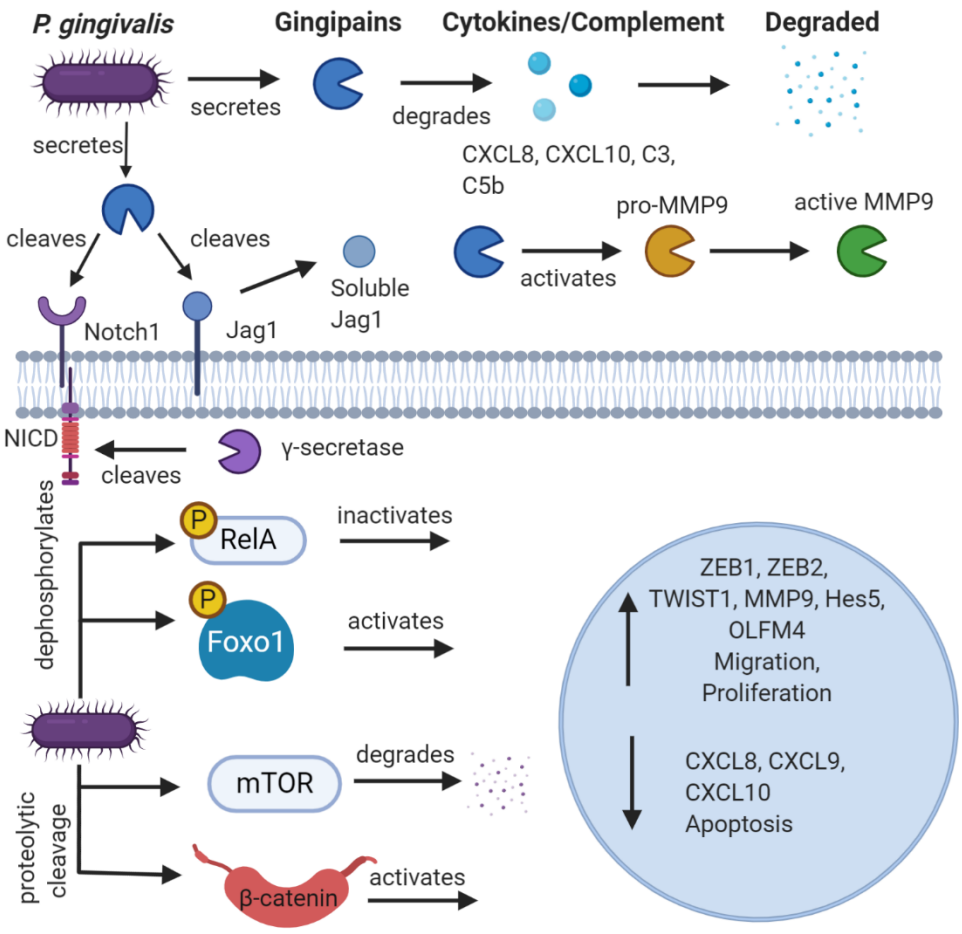


Figure 19: Graphic summarizing signaling pathways manipulated by *P. gingivalis*

While studying organisms individually reveals phenotypes specific to the organisms, studying interactions in the context of communities provides greater biological relevance, as it is more similar to the organization of microbes in the oral cavity. Communities in the oral cavity tend to be complex, reducing the possibility for rogue, individual species to interact with host cells. There are many advantages to this lifestyle, such as protection from environmental stresses, metabolic cross-feeding, antibiotic resistance, increased protection from immune surveillance, and an increased ability to colonize the host through binding the biofilm substratum and community members

through adhesins. *P. gingivalis* interacts with multiple bacteria in the oral cavity, specifically *F. alocis*, *S. gordonii*, and *T. denticola*. A key feature for colonization is the ability to attach to members of the community, and benefit each other through metabolic cross-feeding. *F. alocis* is an emerging pathogen that is capable of localizing to gingival epithelial cells, as well as attaching to and developing a heterotypic community with *P. gingivalis*. These organisms have opposing interactions with epithelial cells, specifically in regards to apoptosis and inflammation. Similar to *F. alocis*, *T. denticola* also interacts with epithelial cells and *P. gingivalis*. *T. denticola* has been shown to interact with epithelial cells through the secretion of a chymotrypsin like protease capable of degrading host cytokines and activating MMPs. Interestingly, this protease was not sufficient to activate Notch signaling in gingival epithelial cells, alluding to a more specific interactions being required for Notch1 cleavage by *P. gingivalis*.

As a primary colonizer, *S. gordonii* interacts with a multitude of organisms. *S. gordonii* plays a key role in the structure and regulation of pathogenicity in oral biofilms through its secretion of H_2O_2. While this would normally be toxic to the anaerobic organisms that are prevalent in periodontal diseases, there are organisms within the biofilm that are able to detoxify H_2O_2, such as *A. actinomycetemcomitans*. *A. actinomycetemcomitans* senses H_2O_2 through the oxygen sensitive OxyR transcriptional regulator, which then activates catalase (KatA) and Dispersin B. Catalase detoxifies H_2O_2, and Dispersin B is used to optimally distance itself from *S. gordonii.* Oral streptococci can be stratified into health-associated species that produce H_2O_2, such as *S. gordonii* and *S. oralis*, and cariogenic species that do not produce H_2O_2, such as *S. mutans*. *S. gordonii* and

S. oralis contribute to a healthy community through H_2O_2 induced cell death of *S. mutans* [146]. Liu et. al. characterized the production of H_2O_2 in a biofilm by *S. gordonii*, specifically finding that H_2O_2 is produced at 0.7 mM-1.6 mM over a period of 2-8 h which is sufficient to significantly impair RgpA/B activity, with inhibition starting at 0.8 mM and maximal inhibition occurring at 2 mM. Not only does H_2O_2 modulate the composition of biofilms, but it is also impacts survival and pathogenicity of other organisms. In defined media, *P. gingivalis* survives in up to 3 mM of H_2O_2, with *S. gordonii* and *S. oralis* producing H_2O_2 at about 1-2 mM. *P. gingivalis* has two characterized tyrosine phosphatases, which respond differently to H_2O_2. Ltp1 activates the transcriptional regulator CdhR, which inhibits *mfa1* and *luxS*, having an overall net reduction in community development. H_2O_2 inhibits Ltp1 activity, inactivating CdhR, promoting *mfa1* and *luxS*, leading to an overall net gain in community development. Additionally, *P. gingivalis* controls community development with *S. gordonii* through another tyrosine phosphatase Php1. Php1 positively regulates exopolysaccharide (EPS) production, which is an important feature of biofilm substrata, acting a glue for monospecies and multispecies interactions. This enzyme dephosphorylates Ptk1, a *P. gingivalis* tyrosine kinase, which is another key regulator of EPS production and community development.

Gingipains are cysteine proteases that require a reducing environment for optimal activity. Because H_2O_2 would oxidize the disulfide bonds, leading to inactivation of the gingipains, we characterized and titrated this inhibition. Consistent with our hypothesis, the gingipains were inactivated by the H2O2-producing *S. gordonii*, but not *S. mutans*. This inhibition of gingipain activity is pivotal for the antagonism of Notch activation by *S.*

gordonii. The communication via secreted metabolites is interesting in that there needn't be direct contact with the microbes to each other, or to the epithelial cells.

With an increased interest in pathogenic microorganisms, or microbial profiles, that can act as carcinogenic agents, investigators up until now have generally overlooked the role of commensal organisms maintaining homeostatic interactions in the host. Here we provide additional evidence for the role of *S. gordonii* as a homeostatic commensal in the context of epithelial cell transformation, whereas in periodontal disease it is considered an accessory pathogen. This system is unique in that these organisms do not necessarily need to be directly associated with the epithelial cells, but instead can mediate host signaling through secreted proteases/metabolites. It's particularly interesting that *P. gingivalis* can perpetuate Notch signaling through cleavage of Jagged1, so cells that had never interacted with *P. gingivalis* are able to induce Notch signaling and OLFM4 expression. Interestingly, recent studies have found that not only is *P. gingivalis* significantly enriched in OSCC, but *Streptococci* are significantly underrepresented under the same conditions. This study provides insight on the complex molecular dialogue between the commensals and pathogens of the microbiome, and particularly expands on the role of *S. gordonii* through direct inactivation of *P. gingivalis* secreted proteases, in addition to the previously studied subversion of host pathways to impede *P. gingivalis* induced signaling.

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CURRICULUM VITA

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2. Education

2016 – 2023	D.M.D. / Ph.D. Department of Oral Immunology and Infectious Diseases, University of Louisville School of Dentistry, University of Louisville, Louisville, KY Mentor: Dr. Richard J. Lamont.
2012 - 2015	B.S. in Biology (concentration in Physiology), Chemistry minor, College of Basic and Applied Science, Middle Tennessee State University, Murfreesboro, TN

3. Training and Experience

During my PhD training I have gained experience in: Cell culturing, isolating and growing primary gingival epithelial cells from whole human tissue, siRNA transfection, plasmid construction, overexpression in epithelial cells, PCR, qRT-PCR, SDS-PAGE gels, Western Blots, confocal microscopy, luciferase promoter assays, growth of anaerobic bacteria, genetic manipulation of bacteria, Matrigel invasion assays, protein co-immunoprecipitation, protein-DNA chromatin immunoprecipitation (ChIP), RNAseq, RNAseq pathway analysis, mouse models including 4NQO induced OSCC and Baker's model for periodontitis, classifying mouse tongue biopsies histologically, and mouse euthanasia.

4. Funding, Awards, and Other Honors

4.1. Funding

2018-2022 Ruth L. Kirschstein Nation Research Service Awards for Individual Predoctoral Fellows, NIDCR, NIH, "Regulation of ZEB1 by *Porphyromonas gingivalis* in gingival epithelial cells", F30DE028166

4.2 Awards

2019 Arnold Bleiweis Travel Award, IADR
2019 AADR Bloc Travel Grant
2018 2nd Place, Research! Louisville
2018 University of Louisville School of Dentistry Summer Research Program
2017 Delegate, ADA Colgate Dental Students' Conference on Research
2016 NCI R25 Cancer Education Program Travel Grant
2016 NCI R25 Cancer Education Program

4.3 Memberships

2017-Current IADR

5. Bibliography

5.1 Manuscripts

1. Ohshima J*, Wang Q*, **Fitzsimonds ZR**, Miller DP, Sztukowska MN, Jung YJ, Hayashi M, Whiteley M, Lamont RJ. *Streptococcus gordonii* programs epithelial cells to resist ZEB2 induction by *Porphyromonas gingivalis*. Proc Natl Acad Sci U S A. 2019 Apr 23;116(17):8544-8553.
2. Miller DP, **Fitzsimonds ZR**, Lamont RJ. Metabolic Signaling and Spatial Interactions in the Oral Polymicrobial Community. J Dent Res. 2019 Nov;98(12):1308-1314.
3. Jung YJ, Miller DP, Perpich JD, **Fitzsimonds ZR**, Shen D, Ohshima J, Lamont RJ. Porphyromonas gingivalis Tyrosine Phosphatase Php1 Promotes Community Development and Pathogenicity. mBio. 2019 Sep 24;10(5).
4. **Fitzsimonds ZR**, Rodriguez-Hernandez CJ, Bagaitkar J, Lamont RJ. From Beyond the Pale to the Pale Riders: The Emerging Association of Bacteria with Oral Cancer. J Dent Res. 2020 Jun;99(6):604-612.

5.2 Poster presentations

1. **Fitzsimonds ZR**, Oshima J, Wang Q, Miller DP, Sztukowska M, Jung YJ, Hayashi M, Whiteley M, Lamont RJ. *Streptococcus gordonii* antagonizes *Porphyromonas gingivalis*-mediated

signaling in epithelial cells 2019. Penn Periodontal Conference 2019. Philadelphia, PA. 2019.

2. **Fitzsimonds ZR**, Oshima J, Lamont RJ. *Streptococcus gordonii* inhibits *Porphyromonas gingivalis* induced ZEB2 upregulation in gingival epithelial cells through long non-coding RNA ESRG. Research! Louisville. 2018.
3. **Fitzsimonds ZR**, Sztukowska M, Metzler M, Darling D, Rouchka E, Kalbfleisch T, Lamont RJ. Differential regulation of long non-coding RNAs in gingival epithelial cells by *Porphyromonas gingivalis*. IADR. San Francisco, CA. 2017.

5.3 Oral Presentations

"Streptococcus gordonii antagonizes Porphyromonas gingivalis-mediated signaling in epithelial cells." IADR. Vancouver, Canada. 2019.

"Streptococcus gordonii antagonizes Porphyromonas gingivalis-mediated signaling in epithelial cells." Penn Periodontology 2019. Philadelphia, PA. 2019.

6. Mentorship

Ruth MacIntyre (MS)- University of Louisville School of Dentistry
Steven Chapman (MD)- University of Louisville School of Medicine
Ankur Patel (BS)- University of Louisville
Daonan Shen (MD-PhD)- University of Louisville