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Abstract approved:

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Andrey Morgun

Cervical cancer is the fourth most common cancer in women worldwide with human papillomavirus (HPV) being the main cause of the disease. Currently available treatment methods are limited and emphasize the need for discovery of new therapies that improve patient outcome. Chromosomal amplifications have been identified as a source of upregulation for cervical cancer driver genes but cannot fully explain the increased expression of immune genes in invasive carcinoma. Insight into additional factors that cause a shift from immune tolerance of HPV to the elimination of the virus, such as local microbiota, may improve diagnostic markers. Furthermore, identification of strategies for selection in combinatorial targeted therapies will allow development of efficient methods to combat the disease. In this work we shed the light on both issues.

In our first chapter we examined the currently known roles of microbiota in cancers triggered by viral infections. We provide a model of microbiome contribution to the development of oncogenic viral infections and virus associated cancers, give examples of this process in human tumors, and describe the challenges that prevent progress in the field as well as their potential solutions.

In our second chapter we investigated whether microbiota affect molecular pathways in cervical carcinogenesis by performing microbiome analysis, via sequencing 16S rRNA in tumor biopsies from 121 patients. While we detected many intra-tumor taxa (289 operational taxonomic units (OTUs)), we focused on the 38 most abundantly represented microbes. To search for microbes and host genes potentially involved in the interaction, we reconstructed a transkingdom network by integrating a previously discovered cervical cancer gene expression network with our bacterial co-abundance network and employed the bipartite betweenness centrality metric. The top ranked microbes were represented by the families Bacillaceae, Halobacteriaceae, and *Prevotellaceae*. While we could not define the first two families to a species level, *Prevotellaceae* was assigned to *Prevotella bivia*. By coculturing a cervical cancer cell line with *P. bivia*, we confirmed that three out of the ten top predicted genes in the transkingdom network (lysosomal associated membrane protein 3 (LAMP3), STAT1, TAP1) - all regulators of immunological pathways- were upregulated by this microorganism. Therefore, we propose that intra-tumor microbiota may contribute to cervical carcinogenesis through the induction of immune response drivers, including the well-known cancer gene LAMP3.

In our third chapter we identified the strategies for combining two cancer drivers as targets for gene therapies to inhibit cell growth. We use a gene co-expression network to model the disease state. Two major theories exist on how to combine regulating genes to gain control over biological network: the first strategy is to take control over as many genes in the network as possible (distantly located regulator nodes that control different parts of the network); in contrast, the second strategy is to manipulate a localized but critical portion of the network (closely located regulator nodes that control same parts of the network). To test which of the two strategies is superior, we first screened 34 potential proliferation drivers using a cervical cancer cell line to identify true regulators of cell growth. In the second step, we reconstructed a gene co-expression network from the union of targets from eight confirmed proliferation regulators (DTL, S100PBP, TPX2,

EXO1, CDCA8, NEK2, ITGB3BP, ANP32E); in addition, we identified that 5-38% of driver targeted genes were correlated with cell proliferation. Average shortest path values between each pair of proliferation associated drivers' targets were chosen as a measure of distance between two drivers in the network. In the next phase of our research, we performed double knock downs for each possible pair of drivers (total – 28 pairs) to see the inhibition effect on cell growth. We found that the average shortest path between drivers and number of unique proliferation associated targets both predict the degree of proliferation inhibition. The best performing driver pairs inhibited cell growth below 50% of the control; these were DTL+S100PBP, DTL+TPX2, and S100PBP+CDCA8. Based on our results, we suggest that gaining control over a large but localized portion of the network responsible for the phenotype of interest provides more control biological processes compared to methods controlling as many nodes in the whole network as possible.

Identification of Cervical Cancer Key Regulators using Network Biology Approach

by
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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Dariia Vyshenska, Author

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DEDICATION

TO MY DAD.
For teaching me independent learning and helping me to open the world.

CHAPTER 1: INTRODUCTION

According to the latest WHO report, cervical cancer ranks the fourth most common cancer among women worldwide for both incidence (6.6%) and mortality (7.5%) [1]. Approximately 311,000 deaths [2] and 570,000 new cases were estimated for 2018 [3], making cervical cancer a gruesome burden for the human population.

Human papillomavirus (HPV) infection is necessary, although not sufficient alone, to trigger the disease. HPV remains the most common viral infection of the reproductive tract [4] and it is estimated that 80% of people will be infected with HPV during their lifetime [5]. While anti-HPV vaccinations have been effective in decreasing the number of new infections, it is not readily available to vulnerable populations that lack access to health care in the US and throughout the world [6, 7].

Even though HPV is established as a causal agent for cervical cancer, other players such as vaginal bacteria, may contribute to the disease progression. For example, recent work implicated microbiota in the development of gastrointestinal and some internal organ tumors [8]. Moreover, a crosstalk between bacterial microbiota and viruses has emerged as a possible player in virus-associated cancers such as in liver and head and neck cancer [9]. Studies of vaginal microbiome and HPV infection confirm changes in overall diversity and some taxa associated with the disease [10] suggesting that bacteria can impact HPV elimination/integration affecting cancer progression. Understanding the role and mechanism of the microbiome in development or progression of cervical cancer can provide crucial insights on preventative strategies and personalized therapeutic techniques for cancer treatment.

Despite the grave statistics, therapies for this cancer have not evolved much over the last decade and usually employs the general anti-cancer toolkit (surgery, chemotherapy, and radiation therapy). Unfortunately, available therapy options not only show low efficacy, but they also cause tremendous side effects from the accumulation of toxic agents in healthy organs. Furthermore, the significant heterogeneity [11] of this cancer makes the success of standard treatment challenging and calls for application of new therapeutic strategies[12].

Because cancer pathways are frequently redundant, it is widely accepted that targeting more than one master regulator simultaneously may be required to achieve efficient therapeutic response [13]. A systems approach provides a unique framework for finding master regulators that can serve as therapeutic targets [14] as well as for identifying tumor-specific targets [15] that would enable combinatorial treatment.

To understand the disease and to pave the path for development of new therapies to treat cervical cancer, in this work we investigate the disease from two prospective methods: how interaction with local microbiome contribute to disease progression, and testing strategies of combining pairs of cancer drivers as potential drug targets in order to inhibit cervical cancer proliferation. In the first chapter, we provide information of the current knowledge on how local microbiome and pathogenic bacteria can contribute to the development and progression of viral-induced cancers, including cervical cancer. In the second chapter, we identify the bacteria *Prevotella bivia* as a possible player in progression of cervical cancer through upregulation of host genes, further identified as regulators of immunological pathways (LAMP3, STAT1, TAP1). In chapter three, we tested a hypothesis whether in gene co-expression network, disease model of cervical cancer, the distance between cancer drivers can be used to pair these drivers to achieve better control over the phenotype of interest. For

this purpose, we count the total number of phenotype-correlated targets and measure distances between proliferation-associated targets of paired cancer drivers in the network. We identify both, the number of targets and the average distance between them, as predictors of cervical cancer cell growth inhibition effect for the knock down of driver pair gene expression.

CHAPTER 2: INTERPLAY BETWEEN VIRUSES AND BACTERIAL MICROBIOTA IN CANCER DEVELOPMENT

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Introduction

As the human lifespan lengthens, the incidence of cancer worldwide is also increasing. The World Health Organization predicts the frequency of cancer occurrence to increase by 70% over the next two decades [16, 17], indicating a rise in the global cancer epidemic. One of the established causes of cancer is viral infection, which is responsible for 20% of the global cancer burden [18]. Among these infections, the most common are Human Papilloma Virus (HPV) and Hepatitis C/B viruses with other less frequent contributors being Epstein - Barr virus (EBV), Human Immunodeficiency Virus (HIV), and Kaposi Sarcoma Herpesviruses (KSHV) [19]. These viruses use two different strategies to cause cancer: first, by directly affecting host cell machinery (e.g. HPV); and second, indirectly, by inhibiting the human immune system (e.g. HIV) [20, 21]. It is common knowledge that the development of some cancers require viral infection, such as HPV for cervical cancer. It is less known, however, why most people infected with oncogenic viruses will never develop cancer.

A hint in solving this puzzle may come from studies demonstrating the crucial role of microbiota (collection of microorganisms living with the host) in the course of viral infections [22-25]. Moreover, microbiota have been recently implicated in different diseases associated with aberrant immune responses ranging from diabetes and autoimmunity to obesity and cancer [26, 27]. For example, a recent epidemiologic study reported an association between antibiotic exposure and the development of several malignancies such as esophageal, gastric, pancreatic, lung, prostate, and breast cancers [28].

Studies thus far have placed emphasis on gastrointestinal microbiota and its role in the development and progression of gut-associated malignancies [29]. For example, *Helicobacter pylori* causes gastric adenocarcinoma and is a classic

case of oncogenic bacteria [30]. Intestinal infections with other bacteria such as *Salmonella typhi* [31] and *Streptococcus gallolyticus* (*bovis*) [32] were also linked to the development of hepatobiliary and colorectal cancers, respectively. These studies represent additional support for the hypothesis that some microbiota members as an understudied environmental factor contributing to protection from or the development of virus-associated cancers. Even though the gut microbiome represents the majority of bacteria in the human microbiome [33], other body sites such as the vagina and oral cavity have been explored for their participation in cancer development and progression.

Although oncogenic properties of virus and bacteria, individually, are popular topics, the interaction between these in the context of cancer has not been well investigated. In recent years, we have witnessed an increasing number of attempts to interrogate this three-way interaction, particularly the influence of microbiota on the progression and acquisition of oncogenic viral infections. However, the question whether bacteria are beneficial or harmful in this context remains unanswered for many cancers. In this review, we provide a model of microbiome contribution to the development of oncogenic viral infections, discuss examples of this process in human tumors, and describe obstacles in the field and their potential solutions.

Model Description

The role of bacteria (and bacterial microbiota) in viral infections leading to cancer can be assigned to two broad categories: bacteria that influence viral particles, and bacteria that affect host interaction with viral infection.

On the one hand, healthy microbiota was shown to contribute to infections by interacting with viruses directly and through bacterial by-products [22-25]. It was reported that commensal microbiota augment the transmission of mouse mammary tumor virus [34, 35], bacterial lipopolysaccharides enhance virion stability of poliovirus [36], and enteric bacteria promote norovirus infection through histo-blood group antigen-like substances [24, 37]. On the other hand, healthy microbiota are critical for immune system development, especially on mucosal surfaces [38]. Antibiotic-treated mice exposed to mucosal influenza virus were observed to have impaired innate and adaptive antiviral immune responses and delayed clearance of the virus [39]. These and other studies define microbiota as a putatively important factor for the development of virus-associated cancers.

Herein we propose a model for the three-way interaction between bacteria, virus, and mammalian host, highlighting two distinct mechanisms for the contribution of microbiota to virus-associated cancers. The first involves the direct effect of interaction of bacteria and bacterial products on viruses, primarily affecting their infectivity (Fig. 1A). The second involves bacteria-host interactions leading to changes in host gene expression and subsequent activation/repression of viral expression or direct promotion of inflammation synergizing with the tumorigenic effects of a virus (Fig. 1B). There is evidence to suggest that the role of bacteria can be positive or negative in terms of disease progression with each of these cases. In this review, we discuss conventional tumor viruses and explore the role of gut, vaginal, and oral microbiota components in both of these mechanisms.

Gastrointestinal Microbiome

The gut microbiome is the largest microbial community in the human body. Recent discoveries show its involvement in a variety of functions, including immune system training and metabolism regulation [40-42]. Separate members of gut microbiota as well as dysbiosis (i.e. non-specific alterations of mammalian microbial communities) have been implicated in disease development and progression. Among most prominent examples are diabetes [43], irritable bowel disease [40], and cancer [44, 45]. Additionally, intestinal microbiota has been implicated in modulating the effect of different anti-cancer treatments [46-48]. Helicobacter species, in particular *Helicobacter pylori*, is the most well studied bacterial member of the gut community that causes cancer [30].

Hepatitis Viruses

The second leading cause of cancer mortality is liver cancer [49]. The most prevalent histologic type of primary liver cancer is hepatocellular carcinoma (HCC) [50] primarily caused by chronic infection with hepatitis B (HBV) or hepatitis C (HCV) virus [51]. Although both viruses can cause cancer, HCV currently attracts stronger interest from the scientific community possibly due to the absence of a vaccine against HCV.

The pathogenicity of both HBV and HCV involves a combination of direct and indirect mechanisms. The HCV encoded core, nonstructural protein 5A (NS5A) and NS3, and HBV encoded X antigen (HBx) are able to promote host cell proliferation. Both viruses are similarly capable of blocking cell immune response, inhibiting apoptosis while promoting angiogenesis and metastasis. By contrast, chronic inflammation caused by oxidative stress also contributes to the process of carcinogenesis [21].

While HCV is oncogenic, not all patients suffering from chronic hepatitis C will develop cancer. One of the first indications that bacteria may be a critical parameter in liver cancer came with the observation that mice infected with Helicobacter were developing strong inflammatory responses leading to hepatocellular carcinoma [52]. Another group later found an association between *H. pylori* specific antibody levels and HCV associated hepatocellular carcinoma [53]. Helicobacter DNA was also found in liver and was associated with hepatitis C induced cirrhosis [54], which indicates the ability of *H. pylori* to invade the liver and putatively contribute to disease development (Fig. 2B). However, a more recent study conducted on HCV transgenic mice colonized with *H. pylori* found no indication of bacteria translocation into the liver and no promotion of tumorigenesis [55]. Whether this experimental system failed to promote carcinogenesis, or *H. pylori* does not contribute to HCV-associated liver cancer, remains unknown.

Another *Helicobacter* species, *Helicobacter hepaticus,* has been shown to cause chronic hepatitis and liver cancer in rodents [52]. In the following study, Fox *et al.* have shown that presence of *H. hepaticus* in the gut lumen promotes development of hepatocellular carcinoma in HCV infected mice, acting synergistically with viral infection [56]. This process did not require bacterial invasion. Fox *et al.* also showed that in an aflatoxin B1 HCC mouse model, *H. hepaticus* induced the nuclear factor-κB (NF-κB) along with downstream innate and Th1-type adaptive immunity. In a HCV transgenic mouse model, they also observed increased gene expression of an NF-kB-dependent inflammatory chemokine (CXCL9) in mice colonized with *H. hepaticus* [21, 56, 57] (Fig. 2C, 2D). Concordantly in both models, tumor growth was accelerated in the presence of *H. hepaticus*. These bacteria, detected in human intestine [58] and liver [59-61], is suspected to contribute not only to cirrhosis [62] and HCC [59], but also to

a set of other conditions such as inflammatory bowel disease [58] and prostate cancer [63]. Consequently, this data suggests a synergistic relationship between *H. hepaticus* and HCV in human cancers.

The link between HBV related oncogenesis and gut microbiota has also been reported. In 2011, Chen *et al.* found that enteric fungi diversity was positively correlated with a worsened disease state in chronic HBV infection [64]. More recently, Chou *et al.* showed that in adult C3H/HeN mice transfected with HBV, elimination of gut microbiota with antibiotics resulted in viral persistence phenotypes, including prolonged HBV surface antigens (HBsAg) presence, impaired anti-HBs production, and limited HBV core antigen (HBcAg)-specific IFN-γ–secreting splenocytes [65]. Although these results suggest that the persistence of HBV infection in antibiotic-treated mice is attributed to an ineffective adaptive humoral and cellular immune response, specific bacteria were not identified in this study. Other studies observed that a decrease in fecal *Bifidobacterium* populations was associated with liver disease progression of HBV infection in humans [66, 67]. In addition, *Bifidobacterium* species have been shown to decrease the protein and transcript levels of HBsAg in a HBV-transfected human hepatoma cell line [68]. Interestingly, Bifidobacterium can also increase host gene expression of IFN-signaling components such as STAT1 [68] and lower serum cholesterol levels [69-71]. Furthermore, IFN stimulation have been shown to downregulate lipid metabolic pathways [42], known in host cells to be necessary for HBV production [72, 73]. Therefore, we propose that *Bifidobacterium* species are able to stimulate IFN-dependent pathways which results in a downregulation of lipid metabolism and a reduction in HBV infection (Fig. 2A). Further experimentation is warranted to establish how the antiviral and antitumor effects of these bacteria contribute to overall protection against malignancy.

Cervicovaginal Microbiome

Despite the fact that microbiota in the vagina is less diverse than in the gut it can also contribute to protection against or susceptibility to some illnesses, especially sexually transmitted diseases. Healthy stable vaginal microbiome is thought to be a first line of defense [74, 75] against diseases caused by opportunistic and true pathogens: outcompeting dangerous bacteria or protecting the host with bacterial by-products, in particular lactic acid [76-79] and hydrogen peroxide [75, 80, 81].

Lactobacilli, commonly considered as such beneficial microbes, represent the dominant genus [82, 83] in healthy vaginal microbiota. However, this does not seem to be universally applicable as Gardnerella-dominated microbiomes were observed as another frequent type in some populations [84]. Disruption of healthy vaginal microbiota (bacterial vaginosis - BV) [82] may increase risks of sexually transmitted infections (STI) [85], and even contribute to the disease progression, putatively, including cancer development.

Human Papillomavirus

Human papillomavirus (HPV) is the most common STI in the United States and although the majority of HPV types are non-carcinogenic, there are at least 13 high-risk oncogenic types (hrHPV), with HPV16 and HPV18 leading the list [86, 87]. One of the most common cancers caused by HPV infection remains cervical squamous cell carcinoma. However, HPV is also linked to anal, vulvar, vaginal, penile and head-and-neck carcinomas [88].

hrHPV infections remain the main predictors for cervical intraepithelial neoplasia (CIN), precursor to tumor, and cervical cancer development itself [89-91]. HPV infects basal epithelial cells. After infection HPV either exist in episomal form or can integrate into the cell genome causing genomic instability. Expression of HPV oncogenes E6 and E7 is also dependent on integration: in episomal form expression of E2 protein keeps expression levels of E6/7 low, but during integration open reading frame of E2 gene is disrupted and E2 no longer is able to control HPV oncogenes. E6, E7 and E5 HPV oncoproteins retain keratinocytes in proliferative state, avoiding apoptosis and clearance by the immune system. It was also reported that HPV is able to promote angiogenesis and deregulate cellular energetics [92].

Although hrHPV type infections are necessary for development of cervical cancer, in most cases the virus is cleared by the host. Only 0.3-1.2% of women eventually develop cervical cancer [93] which means that additional risk factors contribute to the disease progression and vaginal microbiota is possibly one of them. It appears that both, the protective role of normal vaginal microbiome and the contribution of certain pathogens, may play a role in development of cervical intraepithelial lesions and cancer.

While *Lactobacillus gasseri*-dominated vaginal bacterial communities are associated with faster clearance of HPV infection [94], dysbiosis, and bacterial vaginosis are associated with CIN development and progression [95, 96] (Fig. 3D). Furthermore, it is unclear whether bacteria in the disrupted vaginal microbiome affect host susceptibility, virus survival, or infectivity. Some evidence (discussed below), however, points to the possible involvement of *Prevotella* genus that contains *Prevotella bivia,* a microbe known to be associated with bacterial vaginosis.

Normally inhibited by Lactobacillus [80], *Prevotella* species may become abundant when the homeostasis of the vaginal microbial community is disrupted by such factors as diet or hormone status [97] (Fig. 3E). Recent findings also suggest host genetics as an important factor in *Prevotella* outgrowth [97]. Increasing in abundance, *Prevotella* species may provide nutrients (e.g. ammonia and amino acids), to other members of microbial community such as *Gardnerella vaginalis* and *Peptostreptococcus anaerobius* [98, 99], and thus diversify the vaginal microbial landscape [84] (Fig. 3F). Furthermore, multiple studies of *Prevotella* associations with bacterial vaginosis and cervicitis [83, 97, 100] point to *Prevotella* as a conductor orchestrating the state of vaginal microbiomes. Additionally, a clear link between *Prevotella* genus and HPV infection [101], in particular with high risk HPV types [102], has been established. Adding to the potential importance of these microbes, *Prevotella* was cultured from cervical cancer samples in 1993 [103], and more recently we detected it as the most abundant genus in cervical cancer biopsies (unpublished). Finally, increased expression of NF-κB, Toll-like receptor (TLR), NOD-like receptor, and TNF-α signaling pathways in antigen presenting cells from blood, and increased levels of pro-inflammatory cytokines from vaginal lavage have been associated with microbial communities that include *Prevotella* [84]. Thus, it is likely that *Prevotella* or *Prevotella*-driven vaginal microbiome may act in favor of persistent HPV infection promoting cervical cancer development through upregulation of cell proliferation and chronic inflammation.

Aside from the common members of vaginal microbiota, pathogens have also been suspected in the promotion of HPV infection. For example, *Chlamydia trachomatis*, has been studied for some time as a potential co-factor of HPV in the process of tumorigenesis [104, 105]. Investigation of the potential mechanism of how *C. trachomatis* infection may influence HPV infection and cancer

development are underway. For example, it was demonstrated that *C. trachomatis* can decrease the expression of caveolin-1 (tumor suppressor) and increase C-myc mRNA levels (oncogene) [106]. A study conducted by Paba *et al.* found a correlation between *C. trichomonas* infection and upregulation of cytoplasmic and nuclear NF-kB, VEGF-c and survivin in HPV-positive CINs and cervical cancer [107], which points out to the possibility that *C. trachomatis* can also act through the NF-kB pathway, promoting local inflammation, cell survival and proliferation (Fig. 3G). Despite many studies and even mechanistic research, the scientific community has not yet reached a consensus about whether *C. trachomatis* plays a causal role in aiding HPV in carcinogenesis. Thus epidemiological studies that would investigate the temporal relationship between *C. trachomatis* and HPV infections are required to settle this long standing debate.

Human Immunodeficiency Virus

Human Immunodeficiency Virus (HIV) is a carcinogenic virus in its ability to act as a co-factor for EBV and KSHV in carcinogenesis [108]. Immunosuppression, chronic antigenic stimulation, and cytokine dysregulation are reported to contribute to HIV-associated cancer development. However, the main cause of HIV-related cancer development remains immunosuppression: the inability of the immune system to recognize and clear host cells infected with potentially oncogenic viruses enables those viruses to express oncogenic proteins, which in its turn leads to cancer development [21]. Due to the absence of vaccines and ways to eliminate HIV, understanding risk factors contributing to the pathogenesis of HIV infections becomes a priority.

Sexual transmission is one of the most common ways to acquire HIV, suggesting that vaginal microbiota may influence this process. Indeed, several studies found an association between the risk of HIV infection and bacterial vaginosis [109-111]. However, it is still unclear whether the absence or overgrowth of some bacteria in the vaginal microbiota would make women more susceptible to or protected from HIV infection.

In 2013, Aldunate *et al.* showed that physiological concentrations of lactic acid can inactivate different HIV subtypes [112]. Thus, *Lactobacillus* spp. which is known to produce lactic acid, may play an important role in protection against sexually transmitted diseases, including HIV [113] (Fig. 3A). In contrast, bacterial vaginosis predisposes females to sexually transmitted diseases [114], and can reactivate latent HIV infection [115, 116]. Evidence suggests that bacteria are not acting directly on HIV, but rather via bacterial by-products (e.g. butyric acid) [117, 118]. Specifically, immunosuppression caused by HIV is an important step in the establishment of lifelong latent infection and avoidance of host immune response. Latently infected cells harbor the HIV proviral DNA genome integrated into heterochromatin, allowing the persistence of transcriptionally silent proviruses [119]. Hypoacetylation of histone proteins by histone deacetylases (HDAC) is involved in the maintenance of HIV latency by repressing viral transcription [120, 121]. Interestingly, butyric acid-producing bacteria (*Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Clostridium cochlearium*, *Eubacterium multiforme*, and *Anaerococcus tetradius*), residing in different mucosal environments (gut, vaginal, and oral cavities), are capable of reactivating latent HIV infection through host HDAC pathways [117, 118] (Figs. 3B,4B). Butyric acid is known to inhibit the enzymatic activity of HDAC by directly competing with HDAC substrate at the catalytic center of the enzyme [122] (Fig. 4A). Butyrate originating from microbiota has been shown to promote

immunosuppressive/immunoregulatory effects [123]. Considering this, it is plausible that in addition to reactivating viruses, butyric acid can inhibit antiviral immunity in the host directly.

Additionally, the epidemiological synergy of sexually transmitted infections (STI) with HIV transmission was reported in multiple studies. Ferreira et al. found that stimulation of HIV infected T-cells with TLR3, 4, or 5 ligands leads to enhanced HIV-1 replication via direct activation of HIV long terminal repeats, or by inducing secreted factors that promote HIV replication [124]. Among the pathogens suspected to contribute to HIV infection and reactivation, one player stands out - *Neisseria gonorrhoeae*: it was found to be highly associated with HIV infection [125] and activation of HIV expression [124, 126, 127]. Not only does *N. gonorrhoeae* drives an increase of activated CD4+ T-cells [126] and pro-inflammatory cytokines in genital epithelial cells such as IL-6, IL-8 and TNFα [124], but exposure to the pathogen directly drives HIV expression in T cells in NF-kB and AP-1 dependent manner [124, 126]. Malott *et al.* showed that *N. gonorrhoeae* by-product heptose-monophosphate is necessary for invoking these host responses [126] (Fig. 3C). Thus, the course of HIV infection can be altered negatively (by lactate producers) or positively (by butyrate producers or *N. gonorrhoeae*), preventing or favoring, respectively, development of immunodeficiency, which may increase susceptibility to EBV and KSHV infection, which are responsible for a substantial proportion of HIV-related cancers.

Oral Microbiome

Although many studies have been devoted to HIV in the genital tract, the connection between HIV, oral microbiome, and periodontal disease has also drawn the attention of the scientific community [128-131] [117, 118]. Recent works have found associations between periodontitis, a chronic inflammatory disease of the periodontium occurring in response to bacterial infection, and several types of oral cancers [132-135]. Periodontal disease is marked by a disruption in the oral microbiome and is often associated with a shift to anaerobic bacteria such as *Porphyromonas gingivalis*, that has also been directly implicated in cancers [136, 137]. Although common risk factors for these cancers include smoking and alcohol abuse, there are an increasing number of cases where no significant smoking or drinking history has been reported. Among other risk factors are viral infections HIV, HPV, herpesviruses (EBV, HCMV, KSHV) and oral hygiene [138]. Herpesviruses, particularly EBV and KSHV, are known to cause cancer in the context of immunodeficiency caused by HIV/AIDS [139].

Herpesviruses

Herpesviruses have recently been implicated in the progression of periodontitis in the oral cavity [140-144]. Among these viruses, KSHV, EBV, and HCMV have also been associated with head and neck cancers, primarily those found in the mouth. A hallmark of herpesviruses is their ability to establish life-long latent infection in host cells. During latent infection, certain viral genes are repressed and viral progenies are not produced. The reactivation of lytic cycle genes in virus-infected cells marks the production of viral progeny which ultimately leads to host cell lysis. Both latent and lytic cycle genes are critical for tumorigenesis and the evasion of host immune response. In addition, studies

exploring co-infection and crosstalk between these viruses as well as with HIV has shown direct (virus-virus) and indirect (through host immunity) interactions, most notably affecting the transition between latent and lytic viral cycles [139, 145].

Human cytomegalovirus (HCMV) is not usually regarded as an oncogenic virus. However, HCMV infections have been implicated in malignant diseases from different cancer entities and can cause fatal diseases in immunocompromised patients [146]. Some evidence also suggests its role in sustaining chronic inflammation in the progression of cancer [147]. However, in the case of oral squamous cell carcinomas, Saravani *et al.* reported that only 6.3% of 48 patient samples were found to have detectable HCMV [148]. Although this patient population had low incidence of HCMV detection, it does not discount the possibility of synergy between HCMV and periodontal disease (not explored) in the development of oral cancer subtypes. In fact, HCMV has been associated with active periodontal disease and *P. gingivalis* [141-144]. Thus, strengthening the argument to keep HCMV among potential co-factors contributing to chronic inflammation that leads to oral tumorigenesis.

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is a herpesvirus that has become a well-known oncovirus in immunocompromised individuals infected with HIV [149]. This virus is consistently associated with Kaposi’s sarcoma, a cancer developed from cells that line the lymph or blood vessels and usually appears as a tumor on the skin or mucosal [150]. Latent transcripts in KSHV include genes and miRNAs that favor viral persistence and replication while promoting host-cell proliferation and survival. Furthermore, lytic cycle genes favor viral replication by affecting the DNA damage response, reprogramming metabolism, promoting survival, and mediating immune evasion, all of which have a role in promoting cancer

development [21]. In 2007, Morris *et al.* explored the effects of supernatants from cultures of different bacteria on KSHV infected BCLBL-1 cells [151]. They found that metabolic end products from pathogens (gram-negative anaerobic periodontopathogens *F. nucleatum* and *P. gingivalis*) induced lytic replication of KSHV through the activation of a stress-activated MAPK pathway in host cells. More specifically, butyric acid from bacteria supernatants were found to inhibit cellular HDACs and activate the p38 kinases pathway, resulting in hyperacetylation of histones on immediate early viral promoters (Fig. 4C). The targeting of HDACs by *P. gingivalis* is also seen in the reactivation of HIV as we discussed previously.

Epstein–Barr virus (EBV) was the first virus shown to cause cancer in humans and is associated with a wide range of human cancers originating from epithelial cells, lymphocytes and mesenchymal cells [152]. Infection is transmitted from host to host via salivary contact, and the virus passes through the oropharyngeal epithelium to B lymphocytes, where it establishes a lifelong latent infection. EBV has three main latency patterns, each of which have a role in avoiding host response while promoting B-cell survival/proliferation [21]. Although not deeply studied, there is increasing evidence that EBV early lytic genes, particularly those encoding homologues of Bcl-2 (BALF1), IL-10 (BCRF1) and c-fms receptor (BARF1), may be involved in oncogenesis as well as in promoting viral infection. Reactivation of the virus and production of progeny contributes to several human diseases including nasopharyngeal carcinomas and lymphomas [153]. Similar to KSHV and HIV, the latent virus was found to be reactivated upon stimulation with supernatant from *P. gingivalis* (containing high concentrations of butyric acid) through the inhibition of HDACs [153]. This inhibition resulted in increased acetylation of adjacent histone and transcriptional activation of the BZLF1 promoter, whose gene product ZEBRA is known to be

the master regulator in EBV transition from latency to lytic state [153] (Fig. 4D). Taken together with the case of KSHV, there is compelling evidence for the ability of butyric acid producing bacteria such as *P. gingivalis* (main player in periodontitis) to reactivate latent herpesvirus infection and contribute to the development of cancer in the oral cavity.

As described above, butyric-acid producing bacteria (especially the periodontopathogens *P. gingivalis*) can regulate the viral life cycle in host cells. Periodontopathogens can induce a reactivation of HIV which in turn may lead to the opportunistic infection of herpesviruses due to immunodeficiency. Latent infection of EBV and KSHV can be reactivated by the same bacteria leading to the induction of oncogenes and transformation to malignancy. Independent of bacteria, a model of KSHV and EBV co-infection *in vitro* has shown complementing lytic activation [154], suggesting a more complex model of reactivation in which periodontopathogens may act in tandem with direct inter-viral interaction to regulate infection and the development of cancers. Although HCMV has not been studied extensively in the same context, its association with *P. gingivalis* and periodontal disease offers hints of viral-bacterial interaction potentially involving mechanisms used by other herpesviruses.

Human Endogenous Retroviruses

Endogenous retroviruses (ERVs) are endogenous viral elements located in the genome of jawed vertebrates, including humans, and are thought to be relics of ancestral infectious retroviruses [155]. Human endogenous retroviruses (HERVs) compose about 4-8% of human genome [156]. While there is not enough evidence to solidify the role of HERVs in causing human cancers, the

connection between the two is constantly been researched [155]. Reactivation of HERV expression in cancers [155, 157] can contribute to genome instability and is suspected to be a prominent player in disease development. Furthermore, resurrection of murine leukemia virus (MLV) in immunocompromised mice was found to be dependent on intestinal microbiota [158]. In humans, environmental and intestinal microbes were able to modulate the transcriptional activity of endogenous retroviruses [159]. It is not clear whether this process contributes to cancer development. Nonetheless, multiple studies show that upregulated HERV expression in cancerous cells can drive inflammatory responses by upregulating type I interferon pathways [160-162] and eliciting T-cell specific antitumor immunity [163]. These findings may indicate the role of the immune system in recognizing the reactivation of "sleeping" HERVs as a sign of cell transformation and clearing the potential threat before it develops into malignancies.

Risk Factors of Virus-associated Cancers and Microbiome

Risk factors for cancer, such as age, lifestyle, diet and genetics, have been identified but the mechanisms underlying their contribution to disease are not always well understood [164, 165]. The discovery of the role of commensal microbiota and pathobionts in cancer development and progression suggests that at least some risk factors may be linked to the disease through microbiota. For example, while smoking is a risk factor for nasopharyngeal carcinoma [166], it is also associated with dysbiosis in oral microbiota [167], which in its turn can contribute to periodontal disease leading to cancer development [168]. In cervical cancer, a high number of sexual partners is a well-known risk factor. This association has been commonly attributed to increased chances to be exposed to

high risk HPV [169]. However, women with multiple sexual partners also present disruption of vaginal microbiota (bacterial vaginosis) [170, 171] that may facilitate chronic HPV infection and cervical cancer as discussed earlier (section 4.1). Another example is liver cancer, for which diabetes and obesity have been identified as risk factors [164, 172]. Interestingly, in obese people among many changes in the gut microbiome, a decrease in *Bifidobacterium* was reported [173]. Because this bacterium has been shown to play a positive role in elimination of HBV (as described in section 3.1), this may partially explain the association between diet-induced obesity and liver cancer. Although environmental and hereditary factors make virus-bacteria-host interactions even more complex to investigate, it is crucial to remember that understanding the role of these external factors may become a powerful tool for cancer prevention and treatment.

Summary

It has recently become evident that progress in the field of virus-associated cancers can be enhanced by elucidation of how bacterial microbiota contributes to virus-host interaction. Although the dissection of these transkingdom interactions is evolving [174], we are still in the early stages of this journey. Indeed, epidemiological and even some mechanistic studies are accumulating. However, the scientific community has not yet reached a unanimous agreement on whether any specific cancer requires bacteria-virus interaction for carcinogenesis.

Herein, we have described a model of bacteria-virus interactions in the development of cancer. We identify two main mechanisms; the first is accomplished by the interaction between bacteria and virus resulting in changes in the course of viral infection. The most notable example of such mechanism is the

inactivation of HIV via bacteria-derived lactic acid [112]. The second mechanism is an effect of the bacteria on the host resulting in alterations of host susceptibility to viral infection. This can happen in several ways: a) bacteria inducing pro-tumor chronic inflammation (e.g. activation of NF-kB pathway [21, 56, 84, 107, 124, 126]), b) commensal bacteria promoting antiviral and antitumor immunity (e.g. Bifidobacterium in in HBV [68, 175]), and c) bacterial metabolite reactivating oncoviruses (butyric acid inhibition of host HDAC pathways reactivating HIV [117, 118], EBV [153], KSHV [151]) (Table 1).

In addition to proposing the model, we identified key questions that have to be answered in order to move the field forward (Fig. 5) and major technological/logistical solutions that are required to answer these questions (Fig. 6).

Finally, treatment of cancer with chemicals and radiation are currently the most popular and efficient strategies [176, 177]. More recently, our armamentarium was advanced with vaccines against oncoviruses [178] and immunotherapy [179], both using the immune system to prevent or kill cancer. Unfortunately, neither of these strategies allowed us to eliminate completely any oncovirus, nor to cure some cancers.

Therefore, novel approaches are required to significantly change the status quo of this field. We believe that this change should come with methods promoting a healthy microbiome, development of next generation antibiotics targeting individual bacteria, new probiotics, and companion diagnostics that will define a course of personalized/precision medicine for each individual patient based on their respective transcriptome and microbiome.

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CHAPTER 3: TRANSKINGDOM NETWORK REVEALS BACTERIAL PLAYERS ASSOCIATED WITH CERVICAL CANCER GENE EXPRESSION PROGRAM

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Introduction

Cervical cancer remains the fourth most common cancer in women worldwide, and the world's second highest cause of female cancer mortality [180]. Persistent infection with high-risk human papillomavirus (hrHPV) is a causative factor in cervical carcinogenesis [86]. The HPV vaccination is expected to decrease the cancer incidence, however, in Central and Eastern Europe and developing countries where there is a lack of systematic screening and vaccination programs, morbidity and mortality are anticipated to remain high. Thus, cervical carcinogenesis will remain a major threat to women's health. Most women who are infected with HPV never develop cancer. Hence, persistent HPV infection is necessary but may not be sufficient to trigger cancer development. A better understanding of the additional factors required for carcinogenesis would improve identification of the high-risk cases and would represent a major step towards personalized medicine in cervical cancer [93].

According to the current model of cervical carcinogenesis, hrHPV exists in the episomal state within basal cells in the epithelium during early disease onset [181]. The viral gene E2, while expressed, suppresses expression of E6 and E7 viral oncogenes. The longer the infection persists, more HPV integrates into the host genome. Upon integration, the open reading frame of E2 is disrupted which reduces the control of E6 and E7 and promotes cell proliferation [93]. Therefore, the key event that turns chronic HPV infection into cancer is elimination of the episomal form of virus as it is the only source of the oncosuppressor E2. The host immune system may eliminate episomal HPV, giving the cells with an integrated form of virus a growth advantage [182]. Hence, although insufficient antiviral immunity enables persistent HPV infection at an early state, proceeding to the integrated state seems to require activation of the

woman's immune system. Chromosomal amplifications in the infected cells have recently been identified as one of the key sources of upregulation of driver genes in this disease [183]. However, these genomic alterations cannot fully explain increased expression of immune genes in invasive carcinoma. Additional factors that may tip the balance from making the immune system tolerate HPV to eliminate virus are so far unknown.

Microbiota can have a significant role in disease, contributing to development of metabolic (diabetes, enteropathy associated with common variable immunodeficiency, etc.) and immune (IBD, asthma, allergy) disorders as well as cancer (colorectal, gastric, lung, pancreatic etc.) [26, 184-193]. Microbial communities have also been found to specifically contribute to virus induced carcinogenesis [9]. In particular, multiple studies confirm vaginal dysbiosis (bacterial vaginosis) to be a risk factor for HPV infection [194] and its progression [112, 195]. Studies of vaginal [10] and cervical [9] microbiota have found associations between changes in microbial community (e.g. overall diversity, abundances of particular taxa) and cancer development. Furthermore, the location of this cancer suggests a possible role of common members of cervico-vaginal microbiota in cervical carcinogenesis. While almost 25 years ago, bacteria cultured from cervical cancer biopsies were proposed to contribute to cancer progression, we are still far from understanding the role of non-viral microbes in this disease [103].

In this study, we aimed to investigate whether microbiota may affect molecular pathways in cervical carcinogenesis. We reconstructed a transkingdom network that integrates microbiome and host transcriptome data in tumor samples from patients to infer key bacterial players. We demonstrate the significance of this approach by identifying several bacterial candidates and show that one of

them (*Prevotella bivia)* upregulates a well-known human cancer driver, lysosomal associated membrane protein 3 (LAMP3).

Materials & Methods

Patients

Tumor specimens were retrieved from 123 patients with locally advanced squamous cell carcinoma of the uterine cervix. One to 4 biopsies were taken at different locations of the tumor at the time of diagnosis, immediately frozen in liquid nitrogen, and stored at −80°C. DNA and RNA from different biopsies of the same tumor were pooled. The clinical protocol was approved by the Regional Committee for Medical Research Ethics in southern Norway (REC no. S-01129). Written informed consent was obtained from all patients (Supplemental Form S1). DNA was isolated according to a standard protocol with proteinase K, phenol, chloroform, and isoamylalcohol [196] . Purified DNA quality and concentration were assessed using the Quant-iT™PicoGreen® dsDNA Assay Kit (Life Technologies, Carlsbad, CA, USA).

Gene expression

Gene expression profiling of 123 tumors used in this study was performed using the Illumina HumanWG-6 v3 Expression BeadArrays with approximately 48,000 transcripts (Illumina Inc., San Diego, CA). All samples had more than 50% tumor cells in hematoxylin and eosin stained sections. This selection may have led to some bias in the results, but was chosen to reduce the influence of

different normal cell proportion across the samples. Total RNA was isolated by the use of Trizol reagent (Life Technologies) followed by LiCl precipitation. Hybridization, scanning, signal extraction and normalization were performed as described [197].

Bacterial DNA quantification

Bacterial content was quantified using QuantiFast SYBR Green mix (Qiagen, Germantown, MD) and universal bacterial primers, UniF340 (5'-ACTCCTACGGGAGGCAGCAGT) and UniR514 (5'-ATTACCGCGGCTGCTGGC). Standards were created from serial dilutions of extracted DNA from bacteria grown from mouse cecum contents.

16S rRNA library preparation and sequencing

For MiSeq Illumina sequencing, total genomic DNA was subjected to PCR amplification targeting the 16S rRNA variable region 4 (V4) using the bacterial primers 515F/806R. Test reactions were performed with samples with varying amounts of bacterial DNA to determine the threshold for successful library preparation, defined as a positive band on an agarose gel. 40-250 ng of total template DNA were used for each PCR reaction, performed in triplicates per sample. Each set of triplicate PCR reactions was pooled and purified using the MinElute PCR Purification Kit (Qiagen). The pools were checked for proper band size of 382 bp by gel electrophoresis using 2% agarose pre-cast E-gels (Life Technologies). Negative controls consisted of samples without template for DNA extraction and PCR amplification. The pools were then quantified using the Qubit

dsDNA BR Assay Kit (Life Technologies). Barcoded amplicons were pooled at equal volumes and concentrations (2 uL of 5 ng/uL DNA). The total pool was sequenced using the Illumina MiSeq 2000 sequencing platform at the Center for Genome Research and Biocomputing at Oregon State University (OSU) to generate pair-ended 250 nt reads. The dataset generated and analyzed during the current study is available in Sequence Read Archive under accession no. SRP131188.

Processing of raw 16S rRNA reads

Raw forward-end fastq reads from the Illumina sequencing output were quality-filtered, demultiplexed, and analyzed using quantitative insights into microbial ecology (QIIME) [198] . Reads were quality filtered using default QIIME parameters; reads with a Phred quality score of <20, ambiguous base calls, and fewer than 187 nt (75% of 250 nt) of consecutive high-quality base calls were discarded. Additionally, truncation occurred on reads with three consecutive low-quality bases. The samples were demultiplexed using 12 bp barcodes, allowing for a maximum of 1.5 errors in the barcode.

Reads were clustered using UCLUST [199] at 97% similarity into operational taxonomic units (OTUs) at QIIME default parameters. A representative set of sequences from each OTU were selected for taxonomic identification by selecting the cluster seeds (first read assigned to that OTU). Representative sequences for each OTU were aligned using BLAST (e-value < 0.001) to Greengenes (version 13.8) OTU reference sequences (97% similarity) to obtain taxonomy assignments. OTUs were filtered for singletons (only found in one sample) and relative abundance was quantified by dividing raw read counts

by total number of reads for each sample. Alpha diversity rarefaction curves using the Shannon index and a heatmap of OTU frequencies were obtained from QIIME scripts using default parameters.

Comparing bacterial communities from different body sites

HMP data. High-quality fastq reads for region v3 and v5 were obtained from (ftp://public-ftp.ihmpdcc.org/HMQCP/seqs_v35.fna.gz). Female patients from the first visit were retained if they had samples from vagina (Posterior_fornix) and at least stool or skin (Left and Right of Antecubital fossa and Retroauricular crease) to allow multi-site comparisons for the same patient. In case there were multiple samples from the same site of a patient, we retained the sample with highest number of sequences assigned to OTUs. This resulted a total of 380 samples from 77 females.

Healthy cervix. Raw fastq data for 17 HPV neg samples was obtained from European Nucleotide Archive, Study PRJEB1872, and used as healthy cervix samples. High-quality reads (Phred score > 19) were retained using QIIME's split_libraries_fastq.py command and a Phred offset of 33.

To allow comparisons between studies, reads for the 455 samples (Table S2) were assigned to OTUs at 97% sequence similarity using UCLUST and closed reference OTU picking. The reverse strand match was enabled for the HMP data during OTU picking. The studies were merged using common OTU IDs. Singleton OTUs were removed. The combined OTU table had 455 samples and 2,516,046 sequences assigned to 444 OTUs (Table S2). The table was rarefied with a sequence threshold of 1000 sequences (Table S2) and used for diversity analysis. Beta diversity was calculated using weighted and unweighted

unifrac and used for PCoA analysis. Alpha diversities were compared using Shannon diversity index.

Cervical cancer samples with greater than 1000 sequences (based on the above threshold used for rarefaction) assigned to OTUs were retained and singleton OTUs were removed. The resulting OTU table had 432 OTUs for 52 cervical cancer samples (Table S2), was relativised and used to create a heat tree at the genus level using Metacoder [200].

OTU collapsing

An initial quality check of *de novo* cervical cancer OTUs were made against healthy vaginal samples from the Human Microbiome Project (as described below). OTU representative sequences, OTU table, and mapping file for 16S rRNA V3-V5 sequencing were downloaded from the Human Microbiome Project (HMP) QIIME Community Profiling datasets publicly available [201][29][29](2012b). Representative sequences from cervical cancer *de novo* picked OTUs were aligned to HMP representative sequences using USEARCH. Sequences with identity matches greater than 0.97 were collapsed together, keeping HMP OTU identification, taxonomy, and representative sequences as appropriate; if multiple cervical cancer OTUs matched, their read counts were summed for each sample. These new “collapsed” OTUs were used for subsequent analyses.

Transkingdom network reconstruction

Pairwise Spearman correlations were calculated for each OTU with mean relative abundance >0.5%. Spearman correlations were additionally calculated between top OTUs and 738 differentially expressed genes (quantile-normalized gene expression microarray, raw data available in Gene Expression Omnibus under accession no. GSE68339) found in previous cancer gene regulatory network [183]. Correlations with $P < 0.001$ and FDR < 0.1 were imported into igraph R package for network reconstruction. Gene-OTU correlations were integrated with OTU-OTU correlations along with the previously published gene-gene network. Multiple edges and self-loops were removed. Visualization was performed in Cytoscape.

Bipartite betweenness centrality (biBC) is the measure of probability for nodes belonging to one subnetwork to be bottlenecks in the transfer of signal to the nodes in another subnetwork, and vice versa. It was calculated as previously described [202] between the microbial subnetwork and each differentially expressed gene (DEG) subnetwork as well as all DEGs. Relativized biBC values were calculated by dividing the value of a particular node to the sum of all nodes in that metric. Relativized biBC values were then log transformed as followed:

log2((biBC*10^6)+1)

Prevotella OTU alignment

Representative sequences were extracted for all original cancer *de novo* OTUs that were collapsed into OTU_97.1949. These sequences were aligned to SILVA 16S rRNA sequences for *Prevotella* using USEARCH. Matches with length >200 bp and mismatch ≤40 bp were kept for quantifications. For each species, the numbers of hits for each representative sequence were summed after

normalization to the total number of hits for that representative sequence. This method was used due to multiple database matches for each given representative sequence due to high similarity and low specificity of the query sequence.

Human cell culture

HeLa cervical cancer cell line was acquired from ATCC and maintained in Modifed Eagle's Medium (EMEM) with Earle's Balanced Salt Solution, L-Glutamine, and Non-Essential Amino Acids, without Calcium. EMEM was supplemented with 5% Heat-Inactivated Fetal Bovine Serum (FBS) and 1% Penicillin Streptomycin solution (PEST). Frozen cells stored in 10% DMSO in liquid nitrogen were thawed in a 37°C water bath until thawed (approx. 3 min), added to 4 mL of fresh media, and centrifuged for 3 minutes at 2500 rpm to pellet cells. The media solution was aspirated, and cells were resuspended in 15 mL of fresh media. Cells were grown in 5% CO_2 at 37°C in 75 cm^2 treated, vented cap flat bottom culture flasks. Cells were passaged at approximately 80% confluency using 3 mL of 0.25% Trypsin supplemented with 2.21 mM EDTA without sodium bicarbonate to detach adherent cells.

Bacteria cell culture

The following bacteria were used in this study: *Prevotella bivia* (ATCC 29303) and *Lactobacillus crispatus* (ATCC 33197). All bacteria were grown on Brucella blood agar plates supplemented with hemin and vitamin K (Hardy Diagnostics) at 37°C in anaerobic conditions using the GasPak™ EZ Container

System (BD Diagnostics). Cultures were stored in 25% glycerol at -80°C. Bacterial plates were restreaked once every 2-3 days as needed for experiments.

Human-bacteria co-culture

Bacterial cultures were taken from frozen stocks, plated on Brucella blood agar plates, and restreaked after 3 days. After incubation for 2 days, bacteria were suspended in 3 mL of PBS and quantified by optical density at OD600 nm; correlations between OD600 and Colony Forming Units (CFU) were made prior to the experiments. HeLa cells were resuspended in EMEM media without PEST, counted using a hemocytometer, seeded at 75,000 cells/well in 24-well flat bottom culture plates, and incubated at 37°C in 5% CO_2 24 hours prior to bacterial treatment. Bacteria were adjusted to appropriate concentration for 40 uL treatments at a multiplicity of infection (MOI) of 10 in replicates of 3 or more, using sterile PBS as the negative control. Bacteria-treated HeLa cells were incubated at 37°C in anaerobic conditions using GasPak™ EZ Anaerobe Container System Sachets for either 24 hours, followed by on-plate lysis using RLT Lysis Buffer (Qiagen) and lysate storage at -80°C.

RT-qPCR for co-culture experiment

RNA was extracted from cell lysate using the RNeasy Mini Kit (Qiagen) with on-column DNase digestion. RNA was quantified using Qubit RNA BR Assay Kit (Life Technologies) and reverse transcribed using qScript cDNA Synthesis Kit (Quantabio, Beverly, MA). RT-qPCR was performed using PerfeCTA SYBR Green FastMix (Quantabio) and human gene primers (Table

S5). RT-qPCR set up was as follows: sample was heated to 95°C, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. Fold change was calculated by normalizing to 18S rRNA housekeeping gene.

Results

Varying amounts of bacterial DNA were detected by quantitative PCR (qPCR) in 121 out of 123 tumor samples from cervical cancer patients (Table S1). Sufficient amounts for 16S rRNA library preparation and sequencing was found in 58 samples. After 16S rRNA sequencing and quality filtering, we obtained 3,975,755 high-quality reads with a median of 87,320 reads/sample.

As a first step of analysis, we evaluated our cancer microbiome data in relation to publicly available datasets for adjacent healthy body sites [15, 83, 97, 101, 203-207] and sites sampled by the Human Microbiome Project [201, 208][29, 40][29, 40](2012a; 2012b). While such analysis is limited since the samples were not matched (i.e. not the same individuals), it provides general information about the microbiota in cervical cancer compared to other tissues. Unsupervised principal coordinate analysis using common OTUs showed that the cervical cancer samples had a different microbiome composition than samples from healthy cervix, vagina, stool, or skin samples (Fig. 7A). Compared to healthy uterine cervix and vagina, the cancer microbiome showed increased alpha diversity (Fig. S1) and high abundance of genera such as *Prevotella* (27.3%), *Fusobacterium* (17%), and *Peptoniphilus* (10.8%) as well as a low abundance of *Lactobacillus* (0.5%) (Fig. 7B, 7C; Table S2).

To investigate whether the bacteria influence molecular pathways in the tumors, we built a transkingdom network that integrated microbial abundance and

cancer gene expression networks. First, OTUs with an abundance of at least 0.5% were selected, and a bacterial co-abundance network that contained 38 nodes and 54 edges was reconstructed. For the human portion of the transkingdom network, we selected the network based on meta-analysis of transcriptomes from five cohorts of cervical cancer patients [93]. In that study, we reconstructed a gene expression meta-network, identifying three pathways with up-regulated cell cycle and proinflammatory/antiviral genes, and down-regulated epithelial cell differentiation genes as key features of cervical carcinogenesis. To integrate the microbial and human subnetworks we calculated correlations between microbial abundances and gene expression from the cancer network measured in the same tumor samples as bacteria. The network contained 21 taxa and 698 human genes connected by 19 transkingdom edges (Fig. 8A).

The inflammatory/antiviral genes are the most plausible targets of regulation by bacteria. In addition, antiviral gene expression is of special interest in cervical cancer due to its involvement in elimination of episomal HPV that leads to tumor growth [9, 209]. Therefore, to infer bacteria that may drive changes in cancer gene expression we searched for 'bottleneck' bacterial nodes that link microbial and inflammatory/antiviral subnetworks using the bipartite betweenness centrality (BiBC) metric [202, 210, 211]. The top three microbes with highest BiBC represented OTUs from families *Bacillaceae* and *Halobacteriaceae* and the genus *Prevotella* (Fig. 8B).

To confirm that our approach indeed allowed us to identify bacterial regulators of cervical cancer we decided to test *in vitro* if bacteria with the highest BiBC score can influence cervical cancer gene expression. For this purpose, we needed to identify one of the top bacteria candidates to the species level. Unfortunately, due to limitations of 16S rRNA sequencing (limited resolution and sensitivity if compared to metagenomic data analysis [212]), QIIME was unable

to assign two of the top three BiBC ranked OTUs to specific species. However, the *Prevotella* OTU had more advanced assignment, which were assigned to family level. An alternative bioinformatics approach was therefore used to identify bacterial species that were represented by the *Prevotella*-related OTU. We aligned representative sequences from all OTUs that were collapsed into the *Prevotella* OTU (OTU_97.1949) to the SILVA 16S rRNA database containing sequences of *Prevotella* genus (Fig. 8C). While matches for seven different species from this genus were found, *P. bivia* had the most hits, indicating that this species was the most probable bacterium connected to cancer gene expression. Review of recent literature with publicly available 16S rRNA data of healthy cervix and vagina showed that *Prevotella* had much higher abundance in our cervical cancer cohort than in five studies of healthy vagina [83, 204-207] and four studies of healthy cervix [15, 97, 101, 203] (Fig. 8D, Table S4). Altogether these results provided *P. bivia* as a candidate bacterium that may contribute to upregulation of proinflammatory/antiviral genes in cervical cancer.

Cervical carcinomas consist of a mixture of stromal, immune and cancer cells [213, 214]. Therefore, the bacteria harbored by tumor might have direct and indirect effects on cancer cells. To test if bacteria predicted by our analysis can drive cervical cancer gene expression program directly, we performed *in vitro* co-incubation of *P. bivia* with a cervical cancer cell line (HeLa) and measured expression of selected genes. We used *Lactobacillus crispatus* as a control because it is a common standard in co-culture studies for bacterial induction of innate immunity [215, 216]. While *L. crispatus* was among top most abundant bacteria detected in cancer samples, it showed decreased abundance in cervical cancer compared to other body sights (Fig. S2, Table S4) and it was not connected to any cancer genes in the transkingdom network (BiBC=0) indicating no influence on cancer gene expression program. To evaluate which genes might be

the most affected by bacteria we calculated BiBC between the microbial portion of the transkingdom network and inflammatory/antiviral subnetwork and selected top ten genes for further analysis (Fig. 9A). Our gene expression data was generated from whole tissue samples; therefore, it was not surprising that two (GZMB, CXCL10) out of ten selected genes were not detected in the co-incubation experiments with the cancer cell line. Indeed, GZMB is mostly expressed by T lymphocytes and NK cells [217, 218] and probable sources of CXCL10 expression are endothelial and stromal cells [219, 220]. Among the eight remaining genes we found that three (LAMP3, STAT1, and TAP1) were upregulated by *P. bivia* (Fig. 9B, 9C, 9D) whereas no genes were downregulated by this bacterium (Fig 9B, 9C, 9D, Fig. S3). These results further support that intra-tumor bacteria like *Prevotella* may be involved in control of the gene expression program of cervical cancer.

Discussion

The use of transkingdom network analysis in our study provided novel insight into the host-microbiome relationship in cervical cancer. The method has previously proved to be successful for identification of members of the microbial community responsible for a variety of pathological situations, such as enteropathy associated with common variable immunodeficiency [184], diabetes [26], as well as microbes and microbial genes that regulate the effect of antibiotics on the intestine [210]. Our work demonstrates a new approach where transkingdom network is used to elucidate the pathogenic role of microbiota in cancer. We combined a bacterial co-abundance network with gene expression of key pathways in cervical carcinogenesis [183], increasing the probability of

detecting host-microbiome relationships of relevance for cancer development. The results strongly support a role of microbes from the *Bacillaceae* and *Halobacteriaceae* families and the genus *Prevotella* in regulation of a pro-inflammatory pathway that is activated in cervical cancer [183]. This opens the possibility that bacteria are involved in the elimination of episomal HPV in cervical cancer and thereby play a role in carcinogenesis.

Results from the transkingdom network analysis were further tested and showed that the most promising candidate, *P. bivia,* up-regulated three genes (LAMP3, STAT1, and TAP1) in the pro-inflammatory pathway [183]. Noteworthy, LAMP3 is one of the key drivers of this pathway that controls expression of STAT1 and several other antiviral genes [183]. Overexpression of LAMP3 has been shown to promote metastasis in cervical cancer xenografts and to associate with poor treatment outcome in clinical studies [221]. Moreover, LAMP3 is induced by hypoxic conditions [222, 223] and potentially stimulates hypoxia dependent cancer metastasis [223, 224]. Co-culture of a cervical cancer cell line with *P. bivia* in our work showed that this bacterium indeed upregulates LAMP3 under anaerobic conditions. It is therefore reasonable to hypothesize that *P. bivia* (and possibly anaerobes) attracted by hypoxic environment, infiltrates the tumor and induces a pro-inflammatory gene expression program via up-regulation of LAMP3. LAMP3 itself seems to play a crucial role in cervical cancer. Similarly to its role in other cancer types [225, 226], it promotes metastases and, by driving expression of multiple antiviral genes, it is potentially involved in elimination of episomal HPV which leads to overexpression of the E6 and E7 HPV oncogenes and disease progression [227]. To better understand the mechanism of LAMP3 mediated effects in cervical cancer further studies are needed that would use techniques of knockdown/knockout and overexpression of LAMP3 in combination with *P. bivia* co-culture.

Our study is a first step towards connecting intra-tumor microbiome to molecular pathways operating in cervical cancer. We analyzed bulk gene expression of the tumor containing a mixture of transcriptomes of different cell types. Therefore, it might not be surprising that genes such as GZMB and CXCL10, which are expressed by non-cancerous cells, were among top ones predicted to be induced by intra-tumor microbiota. Therefore, future studies may take advantage of single cell sequencing allowing for identification of indirect communication between cancer cells and bacteria, for example via infiltrating immune cells. In addition, while our results pointed to several bacteria that might be important, we were able to focus only on one of them, *P. bivia*. It is commonly believed that microbiota may affect its host through combined effect of several taxa rather than as single bacterium [228, 229]. Better species identification can be achieved by employing shotgun together with 16S rRNA sequencing since this approach was shown to improve detection and overall diversity of bacterial species [184, 230, 231]. Therefore, whole genome shotgun sequencing should be considered in future studies.

Conclusions

A possible involvement of bacteria in the carcinogenesis of cervical cancer could have implications for women presenting with persistent HPV infection. Unraveling the crosstalk between cervico-vaginal microbiota and the woman body may allow development of personalized preventative measures against the infection through shaping the microbiome to favor fast virus elimination [232]. It could also help to identify single bacterial or community markers of disease progression for better diagnostics. When used together with HPV testing, such

markers may lead to more precise evaluation of a woman's risk of developing cancer that would be especially useful in undeveloped countries where access to the healthcare system is limited [233]. Additionally, new evidence points out to cervical microbiome as an important player in development [234] and treatment of cervical intraepithelial neoplasia [235] that needs to be investigated and considered. Pinpointing the role of bacteria in treatment outcomes at later stages of the disease [46, 236, 237] could also empower identification of bacterial and host gene targets for new anticancer therapies. Our results open a new direction for the development of companion diagnostics that would evaluate intra-tumor microbiome and guide the administration of antibiotics as an adjuvant to standard therapy.

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CHAPTER 4: INFERRING A COMBINATION OF MASTER REGULATORS TO CONTROL CERVICAL CANCER NETWORK

Dariia Vyshenska, Kaito Hioki, Kimberly White, Richard Rodrigues, Oleh Taratula, Natalia Shulzhenko, Andrey Morgun

Introduction

Cervical cancer remains the fourth most frequent cancer among women worldwide [1]. In the United States alone, about 13,170 new invasive cases and 4,250 deaths are estimated to arise from cervical cancer in 2019 [238]. Patients diagnosed with this disease face a five-year survival rate of 66% [239] and must endure severe side effects from conventional anti-cancer therapies [239]. New treatment methods are in urgent need to ease the reactions from available therapies and improve patient outcomes.

With all the challenges in treating cancer, a combinatorial approach is one of the most promising new strategies that may help to combat drug resistance, reduce tumor growth, and reduce metastatic potential [240]. Because cancer pathways are frequently redundant, it is widely accepted that simultaneous administration of more than one drug may be required to achieve efficient therapeutic response [13]. Indeed, Sahin et al. achieved inhibition of invasiveness for a breast cancer cell line by simultaneous inhibition of Erb2 and MEK1 [241]; another group succeeded to suppress cell growth of ovarian cancer *in vitro* by concomitant knock down of two genes of the same family (DNMT3b and DNMT1) that control DNA methylation [242]. However, the absence of established strategies for pinpointing successful drug target combinations is the major obstacle to the introduction of combinatorial therapies into the clinical setting.

In recent years, gene co-expression networks have proved to be an efficient tool for the dissection of biological systems on different levels – such as species, cellular, protein, or mRNA. [243-247]. As a successful tool for finding key regulators in these systems [248, 249], network biology already aids in identification of cancer biomarkers [250, 251] and key regulators [252-254].

Therefore, it may be possible to apply the network approach to discover new combinatorial treatments.

From the network biology perspective this goal can be reformulated as the pinpointing of regulator node combinations, in such a way that targeting these nodes would allow the shutdown of the whole disease network. No consensus exists on the strategies of how this can be achieved, however there are two potential methods. One strategy is to identify and take control over a small but critical area of the network [255], and the second strategy is to control as many nodes of the network as possible [256]. Both approaches are equally appealing but lack experimental evidence to claim which one is superior.

Therefore, in this research we have tested both approaches for the selection of proliferation-regulating driver pairs identified in cervical cancer. For this purpose, we reconstructed a gene co-expression network as a model of cancer proliferation by these drivers. Drivers that control a similar portion of the network have their targets more closely located compared to drivers that control different portions of the network. To assess the distance between two portions of the network controlled by driver pairs we used the average shortest path metric (Fig. 10). By measuring the effect of knock down for driver pairs, we show that drivers that control more proliferation associated targets and have these targets located closer to each other perform better at inhibiting cell growth. Thus, our results demonstrate that taking control of a large but localized portion of the network is the superior approach between the two existing strategies.

Materials & Methods

Cell culture

An ME180 cell line, kindly provided by Dr. Pulivarthi H. Rao, was maintained in RPMI 1640 (THermoFisher) supplemented with 10% heat-inactivated fetal bovine serum (VWR) and 1% penicillin streptomycin solution (Corning). Cells were grown at 37°C and 5% CO2 conditions; subculturing and cryopreservation procedures were performed according to ATCC protocol. Cells were used at passages 7-9 for all following experiments.

siRNA knock down experiments

Evaluation of siRNA efficacy. ME180 cells were seeded 4000 cells per well in 96 well flat bottom plates according to ATCC protocol in 200 uL of cell culture media. 24 hours after seeding, each well was transfected with either one of 34 targeting siRNA or a scramble siRNA control (Table S6). Before transfection, 100 uL of media was removed from each well. Transfection procedures were done according to the Lipofectamine RNAiMAX Reagent protocol (Protocol Pub. No. MAN0007825 Rev. 1.0). 3 pM of siRNA and 0.6 uL of Lipofectamine were delivered to each well in 20 uL solution. 80 uL of fresh cell culture media was added to each well. Cells were collected 72 hours after transfection using the RLT Cell Lysis Buffer from the RNeasy Mini Kit (QIAGEN). RNA extraction was done using the RNeasy Mini Kit (QIAGEN) following the manufacturer's protocol; DNase treatment was not applied. Concentrations of RNA yield were measured with Qubit RNA BR Assay Kit (Life Technologies). cDNA was prepared with qScript cDNA Synthesis Kit (Quanta Biosciences) following the manufacturer's protocol.

Primers and reporter dye solutions were prepared for Quantitative Real-Time PCR using the PerfeCTa SYBR Green PCR Kit (QuantaBio) and GAPDH

as a housekeeping control gene (Table S7). qRT-PCR was performed using the StepOnePlus Real-Time PCR system and software (Applied Biosystems) with the following protocol: samples were heated to 95°C, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec.

Single driver proliferation assay screening. Cell growth was evaluated using the xCELLigence RTCA DP system (ACEA Biosciences Inc.) according to the manufacturer's specifications. ME180 cells were seeded at 4000 cells per well (E-Plate 16) in 200 uL of cell culture media. 24 hours after seeding, the experiment was paused for transfection procedures. Before preparation of transfection solutions, 100 uL of media was removed from each well. Transfection solutions were prepared according to the Lipofectamine RNAiMAX Reagent protocol (Protocol Pub. No. MAN0007825 Rev. 1.0). 3 pM of siRNA with 0.6 uL of Lipofectamine were delivered in 20uL solution; 80 uL of fresh cell culture media was added to each well. Plates were placed back into the slots and cell growth was evaluated for another 72 hours. For each driver knock down condition, relative growth of the cells were evaluated as a percentage of the scrambled control condition:

$$\%\ \text{of control} = 100 * \frac{\text{siRNA knock down Cell Index}}{\text{scrambled control Cell Index}}$$

Double siRNA Knockdown experiments. ME180 cells were seeded in 96-well clear, flat bottom plates at 4000 cells per well in 200uL of cell culture media. 24 hours after seeding, 100uL of media was removed from each well. siRNA and transfection reagents were prepared according to the Lipofectamine RNAiMAX Reagent protocol (Protocol Pub. No. MAN0007825 Rev. 1.0). Reagent volumes were reduced to avoid toxicity: for single knockdown conditions, 1.5uM of siRNA and 0.3uL of Lipofectamine were delivered to each well in 10uL solution; 90uL of fresh cell culture media was added to each well. For double knockdown conditions, 10uL of each siRNA solution of the driver pair was added to the well;

80uL of fresh cell culture media was added to each well. Plates were returned to the incubator for 72 hours. Cell viability was assessed using the AlamarBlue Cell Viability Assay (G-BioSciences), following the protocol provided by the supplier. In brief, 100uL of media was removed from each well followed by the addition of 10uL of AlamarBlue. Plates were returned to the incubator for 2 hours. Absorbance was read at 570nm and 600nm using the Synergy HT Multi-Mode Microplate Reader (BioTek), and percentage of AlamarBlue reduction was calculated as described in the protocol. For each driver knockdown condition, cell viability was evaluated as a percentage of the proper volume scrambled control (see above).

RNA sequencing

RNA samples from single knock down experiments, transfected with either scramble control RNA (n=12) or siRNA targeting one of eight (DTL, S100PBP, TPX2, EXO1, CDCA8, NEK2, ITGB3BP, ANP32E) confirmed drivers (n=3 for each) were used for transcriptome sequencing. Libraries were prepared with QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen) using the standard protocol and 30 ng of total RNA input. Library concentrations were measured using the Qubit dsDNA HS Assay Kit (Life Technologies), and RNA quality was assessed using the High Sensitivity D5000 ScreenTape and TapeStation Analysis Software A.02.02 (Agilent Technologies, Inc.). Barcoded amplicons were pooled at equal volumes and 4 nM concentrations. The total pool was sequenced using the Illumina HiSeq 2000 platform to generate single-ended 250 nt reads.

Processing raw RNASeq reads.

Raw forward-end FASTQ reads from the Illumina sequencing output were demultiplexed using generic Illumina software. Quality filtering and adapter trimming were performed using the BBDuk tool from the BBTools suite (parameters: k=13 ktrim=r forcetrimleft=12 useshortkmers=t mink=5 qtrim=r trimq=15 minlength=20). Alignment was performed using TopHat (v2.1.1) with disabled coverage-based search for junctions and the UCSC provided human genome (GRCh37/hg19). Conversion of .bam files into .sam files was performed using Samtools (v1.9) and count tables were acquired with htseq-count software (v0.9.1) (parameters: -s no -a 10 -f sam --stranded=no -o).

Network reconstruction and interrogation.

Identification of DEGs. Read counts for each gene in each sample was normalized as:

$$log_2\left(\left(\frac{\text{gene reads per sample}}{\text{total reads per sample}}\right) * 10^6 + 1\right)$$

Next, the gene count tables were quantile normalized. Differential gene expression was found for each knock down driver using BRB-ArrayTools (v4.6.0). DEGs were identified as those having differential expression with a p-value less or equal to 0.01. The union of DEGs (3126 unique Ensembl IDs) for each driver was used in gene co-expression network reconstruction.

Network reconstruction. Network reconstruction was performed using workflow as described previously [202]. Transcriptome sequencing samples from

single knock down experiments were pooled the following way: group 1 with 12 scramble control samples, group 2 with 24 samples of targeted knock down and 3 samples where the KPNA2 driver was knocked down but did not show inhibition of growth. The Spearman rank-order correlation test was performed separately for group 1 and group 2 on all union DEGs (3126 Ensemble IDs). The following filtering criteria were applied: directionality filtering of correlation coefficient for both groups (if the correlation coefficient signs were equal between group 1 and group 2 – the Ensemble ID would pass, otherwise – not pass); the p-value threshold for correlation coefficients were less or equal to 0.005 for both groups 1 and 2. Combined p-values for group 1 and group 2 were calculated using the Fisher method, with FDR calculated for Fisher combined p-values. Gene IDs with Fisher combined p-values less or equal to 0.005 and FDR less or equal to 0.05 were selected. Next, for the genes that were consistently regulated across different knock down conditions (same direction of fold change across all drivers regulating this gene), we calculated the Proportion of Unexpected Correlations (PUC) [257] and those that did not pass PUC criteria were filtered out. The network was visualized using Cytoscape (v3.5.1).

Correlation of gene expression with proliferation. Spearman rank-order correlation was calculated between all Ensembl IDs and cell index (units of cell growth from xCELLigence; averages of technical replicates) for group 1, group 2, and a pooled group (pooled group 1 and 2). Only gene IDs that showed the same direction of correlation across three groups were used in the next steps. Fisher combined p-values were calculated using Spearman correlation p-values for group 1 and group 2, as well as FDR for fisher combined p-values. Gene IDs that had fisher combined p-value less or equal to 0.05 and FDR values less or equal to 0.1 were considered to be correlated, and details about their correlation with

proliferation (correlation coefficient median, Fisher combined p-value, and FDR) were added as attribute values to the reconstructed co-expression network.

Identification of subnetwork clusters. Three subnetwork clusters were identified using the Molecular Complex Detection Cytoscape app (MCODE v1.5.1) with default parameters (no Haircut, with Fluff). Subnetwork 1 was selected as the first default cluster with maximum density (score 14.774), Subnetwork 2 – as the third default cluster (score 10.771), and Subnetwork 3 – as the fifth default cluster (score 7.825). Enrichment analysis for each subnetwork was performed using InnateDB resources (www.innatedb.com). We used the gene list of each subnetwork and performed "Pathway analysis" with pre-set settings.

Average shortest paths length calculations. The function "shortest_paths" from the igraph python package was used to calculate all possible shortest paths between targets of two drivers. Averages of all shortest path lengths were then calculated.

Results

Cervical cancer drivers' testing. Evolutionary forces within cancer lead to the rewiring of multiple vital processes such as angiogenesis, immunosuppression, and metabolism [258]. Although desirable, targeting all processes at the same time may not be feasible; therefore, we are focusing our research on one cancer feature that: a) is central in enabling the disease to adapt and survive; b) can be reliably modeled and measured in laboratory settings. Of the cancer hallmarks, cell proliferation fits our criteria the best – it is essential for cancer adaptation and can be easily measured *in vitro*. Therefore, in the first step of our study we asked which driver genes directly affect cell proliferation. From

our previous studies we pinpointed 49 genes that may play critical roles in the disease progression using two different complementary approaches [183, 211]. The first set of 35 drivers was found analyzing frequency of chromosomal aberrations with nodal degree in a gene co-expression network [183]. These genes were categorized as “true” regulators of cancer. The second set consisting of 14 genes was pinpointed using differentially correlated gene analysis, and was considered to be the key bottleneck genes between “true” regulators and the rest of the disease network [211].

Out of these 49 potential cervical cancer drivers, 43 genes were upregulated in cancer and were identified as potential tumor promoters. As our interest is in oncogenes, we excluded the six downregulated candidates identified as potential tumor suppressors. Since we were performing our following screen using an *in vitro* model where immune processes cannot be simulated, we also removed 9 genes identified as drivers of the immune system for the next steps of the research.

To determine which genes out of 34 candidate drivers influence cell proliferation, we used the ME180 cervical cancer cell line and knocked down each candidate gene individually using small interfering RNAs (siRNA’s) and evaluated the effect on proliferation 72 hours later. Each siRNA had at least 50% knock down efficacy (Fig. S5). Knock down of ten tested drivers (CEP70, CKS1B, CDC20, RFC4, DEPDC1, RPA2, BIRC5, MCM2, MTFR1, RAD54B) resulted in an increase of cell proliferation during the first screen. Nine genes showed similar results during the second screen (PSMB4, RAD21, TOPBP1, CACYBP, HNRNPR, CENPN, NAT13(NAA50), GMPS, NUP155) (Fig. 11, Table S8). The genes which knock down resulted in increased proliferation (potential tumor suppressors) were not fulfilling our criteria and were excluded

from next screening experiments, due to our primary focus on drivers that may in the future be targeted as part of anti-cancer knockdown therapies [211].

After this first experimental screen, we found nine drivers (DTL, S100PBP, TPX2, EXO1, CDCA8, NEK2, ITGB3BP, ANP32E, SEPHS1) that showed consistent inhibition of cell proliferation when downregulated (Fig. 11, Table S8). Out of them, 8 genes were confirmed for the next steps (SEPHS1 was excluded due to technical reasons) as proliferation-regulating cervical cancer drivers.

Driver network reconstruction and interrogation. Next, in order to identify the direct and indirect targets of confirmed proliferation drivers, we performed transcriptome sequencing of the knock down samples. For each driver, the number of target genes ranged from 2775 to 2902 (p-value threshold 0.01); in total 3126 unique differentially expressed genes (DEGs) were identified as potential driver targets (Fig. 12A).

Since our primary interest centered at regulation of cell proliferation, we reconstructed a gene co-expression network aimed to represent the phenotype of interest at a gene expression level. For this purpose, we used the total union of driver potential targets. As all 8 drivers control proliferation to some extent, we expected these pathways and nodes to be interconnected and core for proliferation. Thus, genes/nodes that were filtered out of the network were considered as peripheral to our main goal and the remaining network was used for further investigation.

After application of filtering criteria (FDR 0.05, other details can be in see M&M section) the number of nodes in the network was narrowed down to 1379 with 4572 edges linking them (Fig 12B, Table S9, S10, File S1). The network had the following properties: density 0.005, heterogeneity 1.305, centralization 0.038, and average number of neighbors 6.631. Each driver controlled a range between

9-35% of the full network. On average, 49% of the targets of each driver were regulated by one driver and the other 51% were regulated by multiple drivers (number ranged 41-56%) (Fig. 13A).

Noteworthily, except for a few, the nodes in the network were either consistently regulated by multiple drivers or had only one regulating driver (Fig. 13B, 13C). This finding suggests that the same biological processes are regulated by different drivers in the similar manner. Next, we verified if the best performing drivers in our first screen also regulated more targets and found no correlation between the degree of proliferation inhibition and total number of genes controlled by an individual driver (Fig. 14A). This finding suggests that each identified driver has multiple functions, where control of cell proliferation is just one of them and not necessary the primary.

Because the focus of our study was to identify drivers of cancer proliferation, we then decided to investigate if gene expression of driver targets were correlated with cell growth independently from knockdown (see details in M&M). We found a union of 211 genes to be positively associated and 75 genes to be negatively associated with proliferation (total of 286 genes, FDR 0.1). A range between 13 and 108 genes for individual drivers were found associated with proliferation (positively and negatively regulated gene ranged between 10-85 and 3-23, respectfully) (Fig. 14B, 14C). Accordingly, in contrast to all driver-regulated genes, the percentage of targeted genes correlated with proliferation (for each driver, a range between 5-38%) were associated with cell growth inhibition by the driver's knock down, indicating that the driver with the larger portions of its targets associated with proliferation is more specialized to maintain this particular phenotypic feature compared to other drivers (Fig. 14D).

We then examined the network for the presence of subnetworks and identified three clusters of nodes, which we will refer to as: sNW1, sNW2 and

sNW3. sNW1 consisted of 244 nodes and 1795 edges, sNW2 – 71 nodes and 377 edges, sNW3 – 58 nodes and 223 edges. Gene enrichment analysis showed that sNW1 mostly contained genes involved in metabolic pathways, and sNW2 for DNA replication pathways (Table S11). It was also surprising to find enrichment in proliferation associated genes in sNW1 and sNW2 (59% and 32% respectfully), and only 3% in sNW3 (chi-square p-value<0.05) (Fig. 15).

Next, we moved to the main question of our study investigating how to control a regulatory network. To test whether one of the two strategies (covering many nodes in the network or controlling a focused portion of the network) works better we used a distance metric to categorize each drivers' pair based on the distance between their targets. For this purpose, we used the average shortest path between targets of two drivers in the pair and calculated it three-ways, between: a) paired drivers' targets associated with proliferation (Fig. 16), b) paired drivers' targets not associated with proliferation, c) all paired drivers' targets (Table S12).

Driver pair knock downs. To see whether average shortest path is predictable of driver pair performance, we carried out double knock down experiments for each possible pair of drivers (total 28). Due to a significant cell growth inhibition effect by knock down of several drivers, we decreased the amount of each targeting siRNA for paired knock downs by half (Fig. S6) to obtain a wider window of detection for the effect.

The effect of double knock down on growth inhibition ranged from 49% to 0% with three pairs having no statistically significant difference compared to control samples (Fig. 17, Table S13). The best performing driver pairs were identified as: S100PBP+CDCA8, DTL+S100PBP, and DTL+TPX2; the worst performing pairs were: ITGB3BP+ANP32E , EXO1+ANP32E, and NEK2+ANP32E.

Distance and number of unique proliferation associated targets as predictors of driver pair knock down performance. Next, we verified the relationship between the average shortest path calculated for all targets and effect of double knock down on cell growth. We observed a weak trend of positive correlation in this analysis (Fig. 18A). Encouraged by this result and accounting to our previous results in single knockdowns, we verified whether distances between proliferation-related target genes will be associated with inhibition efficiency.

Indeed, average shortest path lengths between proliferation associated targets strongly correlated with cell growth inhibition (the shorter the path between targets of the pair, the more inhibition we observe during knock down of the two drivers (Fig. 18B)). For example, one of the best performing pairs, S100PBP+DTL, has more co-localized targets (Fig. 19A) than ITGB3BP+ANP32E (Fig. 19B), one of the worst performing driver pairs. This finding suggests that accounting for all targets dilutes the signal with noise and is not representative of the performance of the pair at controlling the phenotypic feature of interest. More importantly, our result strongly supports the idea that control over a narrow area of the regulatory network has a higher chance of desirable effects on specific cellular phenotypes.

In addition, we found that the performance of driver pair knocks down also depend on the number of unique targets associated with proliferation for each pair. We then performed model fitting using ANOVA for the full model (2 variables with interaction: shortest path and number of targets) and reduced model (2 variables without interaction: shortest path and number of targets). We found reduced model to be preferable (ANOVA p-value 0.8426). None of the two variables could be excluded from the model (ANOVA p-value < 0.05). After performing multiple linear regression analysis, we found both shortest path and

the number of proliferation correlated targets for the pair to be statistically significant predictors of growth inhibition for the driver pair knock down (p-value < 0.05, Fig. 20):

$$y = 0.05 * x_1 + 5.35 * x_2$$

where y – average growth percent from control for driver pair knock down, x_1 – number of unique proliferation correlated targets for the driver pair, and x_2 – average shortest path between proliferation associated targets of paired drivers.

Discussion

Reversion of disease phenotypes is one of the central topics in Medical Biology. From the perspective of a network approach this goal can be rephrased as taking the biological network of the disease under control [259]. Little research exists that would investigate the best approaches of regulating biological networks [255, 256, 260, 261]. However, the pressing need for combinatorial therapies to treat diseases such as antibiotic resistant infections [262, 263] or drug-resistant cancers [264] requires unraveling the best strategies of controlling highly redundant biological networks.

While addressing the issue of controlling a disease network in this research we discovered several findings. First, the disease drivers identified for cervical cancer control multiple cancer features, but each phenotypic feature may be controlled by a limited number of cancer drivers. Therefore, if all drivers are tested for control of only one cancer feature the proportion of drivers that pass validation is low. Second, if the main goal is to gain control over the specific phenotype, it is critical to identify the portion of the network that regulates this phenotype (in our case – proliferation). Although the network itself is a fair model

of the disease state, other critical targets for the disease features may add unnecessary noise. Accordingly, targeting separate cancer hallmarks at a time may be the preferable strategy over attempts to control all features at once to treat cancer. Third, we show that combinations of drivers that control a closely located portion of the network and also control more targets associated with the phenotype of interest perform better at controlling the phenotypic feature, compared to drivers regulating distant portions of the network. Thus, gaining firm control over a large and focused portion of the network associated with the phenotype is superior than attempts to control as many nodes in the whole network as possible.

Our findings may have broad implications for the Network Biology field and for cancer research, however, additional research is needed to understand if the same strategies would apply to other cancer features or other cancer models, and if our findings may be useful for other disease types. Nonetheless, our data may be instrumental in developing new combinatorial treatment options for cervical cancer patients that would use siRNAs and their combinations. Further, understanding how heterogeneity of cervical cancer may influence performance of driver combinations is critical. Identification of patient groups that could benefit from such therapies, and/or efficient methodology of determining the best gene combinations for each patient would be one of the next steps before bringing this approach into the clinical setting.

Conclusions

Gene co-expression networks can be instrumental in controlling the phenotype of interest. Driver genes of the disease regulate multiple disease

features, although each feature is controlled by a small fraction of these drivers. Identification of the network portion directly associated with the targeted phenotypic feature is critical for predicting the best performing driver combinations. Gaining stronger control over large but focused portions of the network, through controlling drivers which targets are closely located gives superior results at controlling the phenotype of interest.

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CHAPTER 5: CONCLUSIONS

Absence of efficient treatment options for cervical cancer patients may partially be attributed to lack of clear understanding of all players involved in the cancer progression. To investigate whether microbiome can be one of the factors for the disease advancement we first performed a thorough literature review in Chapter 2 and identified two major strategies of bacteria influencing development of virus-induced cancers: through direct interaction with the virus (by facilitating infectivity or by destroying viral particles); and through interaction with the host (upregulating or downregulating certain molecular pathways involved in the disease progression). In Chapter 3 we then used a transkingdom network as a model of microbiota-host interaction in cervical cancer. We found key bacteria and host genes that serve as bottlenecks for bacteria-cancer communication. We identified *Bacillaceae*, *Halobacteriaceae*, and *Prevotella* to be the top three microbial bottleneck candidates. For the next steps of our research we needed to classify at least one of these top bacteria to the species level. An alternative analysis resulted in pinpointing *Prevotella* as *Prevotella bivia*. To confirm if the top bacteria can influence cervical cancer gene expression, we performed *in vitro* experiments by co-culturing *P. bivia* with cervical cancer cell line and measuring gene expression of top 10 ranked bottleneck host immune genes. Out of the 8 expressed in the cell line genes, 3 were upregulated by *P. bivia* (LAMP3, STAT1, TAP1) and 5 were unchanged, confirming that *P. bivia* is one but not the only bacteria influencing cervical cancer gene expression. Interestingly, LAMP3, upregulated by *P. bivia* gene, is a well-known oncogene that is able to promote metastasis in cervical cancer [221]. Therefore, our findings support the prediction that local microbes can be involved in the development and progression of cervical cancer.

Considering this information more research is needed to confirm other microbial players as possible regulators of the cervical cancer gene expression program. Metagenomics sequencing instead of 16S rRNA could improve the species level resolution for bacterial community analysis and aid in identification of bacterial pathways involved in cancer-bacteria interaction. Moreover, bacterial combinations rather than single bacteria should also be considered and tested as cancer regulators to identify if there is any synergistic effect present between cancer driving bacteria.

To move personalized medicine for cervical cancer patients forward it may be necessary to also establish microenvironmental conditions under which host-microbiome interactions manifest and can be manipulated. For example, special attention should be paid to hypoxia, one of the main predictors of cancer aggressiveness [224, 265, 266], and its cause-effect relationships with cancer-bacteria interactions. Unraveling the mechanism of microbiome-host interaction can aid in development of better biomarkers and possibly introduce antibiotics as adjuvant therapies for patients, broadening the toolkit of anti-cancer therapy.

Lack of combinatorial therapies is another area of improvement that can be tackled by computational biology. Targeting duplicates or even triplicates of disease drivers may be a solution that allows treatments to bypass redundancy and heterogeneity of cervical cancer. To find a strategy of combining cancer regulators, in Chapter 4 we used previously identified cervical cancer drivers and pinpointed nine drivers as cell growth regulators. We reconstructed a gene co-expression network out of eight drivers' targets. The network was used to test the hypothesis that the distance between the drivers in the network is associated with the effect of driver pair knock down on the cell proliferation. Our research showed that in order to predict to what extent a driver or a driver pair influences the cell growth we must first identify the portion of the network directly

associated with the targeted phenotype. We confirmed that the smaller the average shortest paths between proliferation associated drivers' targets (metric that represents the distance between drivers in the network) the more growth inhibition we observe during the knock down of this driver pair. However, we also established that the more phenotype associated targets are controlled by a drivers' pair the more proliferation inhibition we observe too. This finding indicates that both predictors, average shortest path and number of targets associated with phenotype, are important in predicting to what extent the drivers' pair will influence cell growth in knock down experiments.

Due to our research being done on one cervical cancer cell line (ME180), a similar approach should be applied to investigate if our findings can be generalized to other cervical cancer cell lines and, possibly, primary tumors. To test the best performing drivers' pairs for cytotoxicity additional experiments must be done using primary cell lines and mouse xenograft models. Nanocarrier-based delivery systems should be considered as delivery agents for siRNAs targeting driver pairs due to their ability to improve drug release and limit side effects of the delivered drug [267]. In addition, the developed strategy for pinpointing disease-driving gene combinations can also be instrumental in developing combinatorial therapies for other cancers, that may lack diversity in anti-cancer approaches or are characterized by frequently occurring drug resistance.

FIGURES

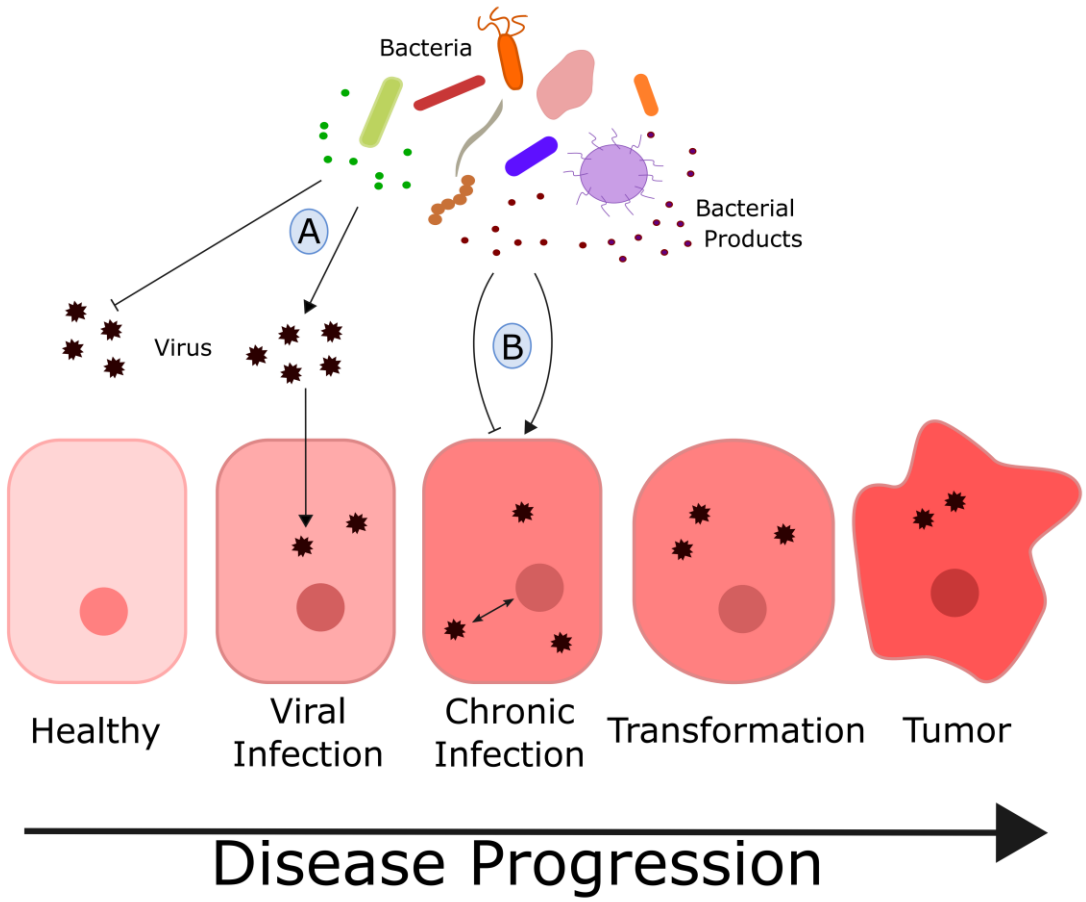


Fig. 1. Model of bacteria-virus interactions in cancer development and progression: A) direct interaction between bacteria or bacterial by-products and virus resulting in inhibition or promotion of viral infection into host cell; B) indirect interaction between bacteria and virus mediated by host response to bacterial stimuli through activation of various pattern recognition receptors.

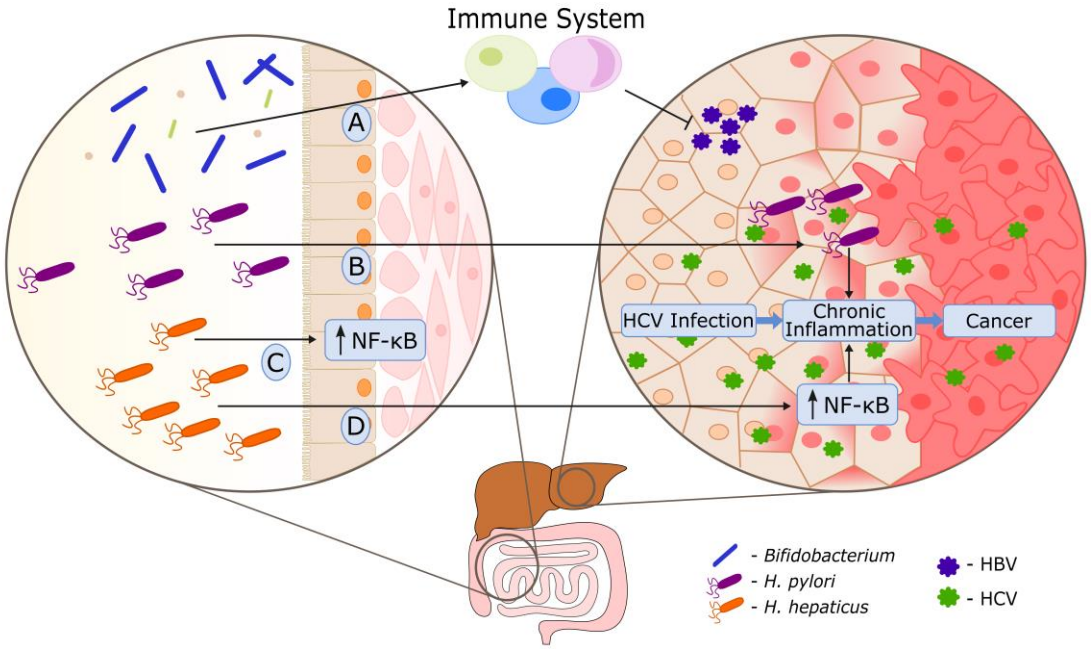


Fig. 2. The interplay between gut microbiome, host and human hepatitis viruses (HCV and HBV): A) healthy gut microbiota stimulates host immune system resulting in HBV infection clearance; B) Helicobacter pylori invades liver and contributes to chronic inflammation induced by HCV; C, D) Helicobacter hepaticus upregulates NF-κB dependent pathways in mouse gut (C) and liver (D), synergizing with HCV-related inflammation in the development of hepatocellular carcinoma.

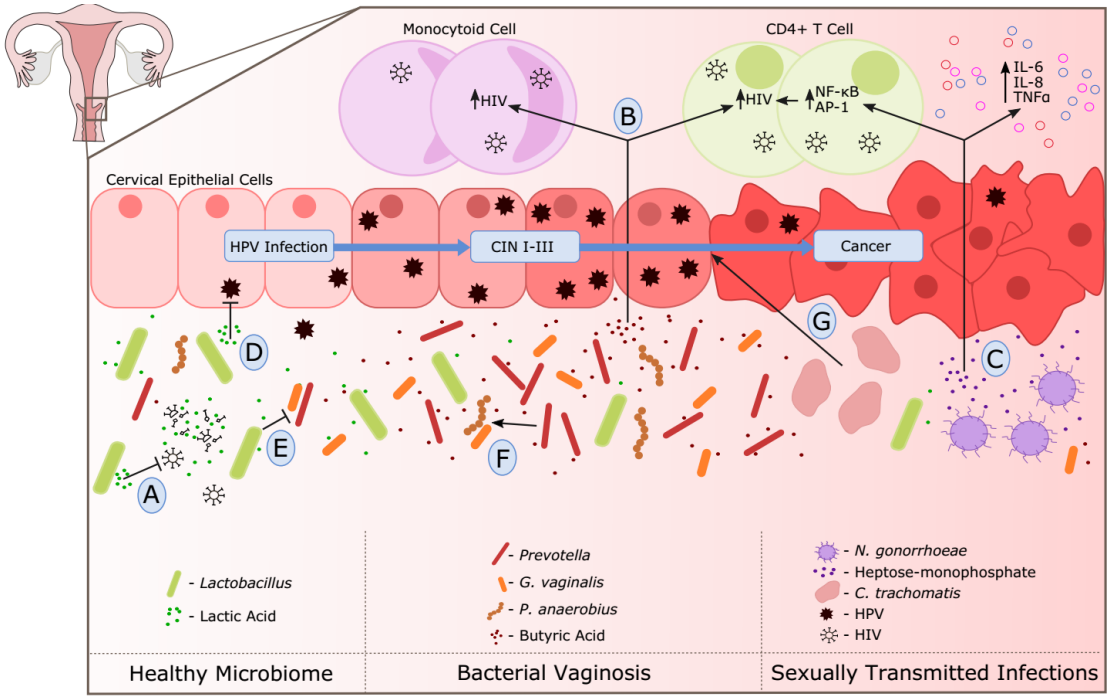


Fig. 3. Cervicovaginal microbiota and pathogens can influence the progression of viral infection associated with cancer development: A) Lactobacillus spp., predominant bacteria in the vaginal microbiome, secretes lactic acid capable of inactivating HIV; B) butyric acid secreted by vaginal microbiota induces HIV replication in Human CD4+ T lymphocyte and macrophage/monocyte cell lines harboring latent HIV; C) Neisseria gonorrhoeae induces IL-6, IL-8 and TNFα production in genital epithelial cells and upregulates HIV gene expression in T-cells via heptose-monophosphate induced NF-kB and AP-1 pathways; D) domination of vaginal microbiota by Lactobacillus gasseri is associated with faster HPV clearance; E) Lactobacillus spp. is able to inhibit the viability of Gardnerella vaginalis and *Prevotella bivia*; F) *Prevotella spp.* produces ammonia and different amino acids that benefit G. vaginalis and Peptostreptococcus anaerobius, contributing to bacterial vaginosis; G) Chlamydia trichomatis may influence cervical cancer development, either through synergy with HPV or by contributing to local chronic inflammation.

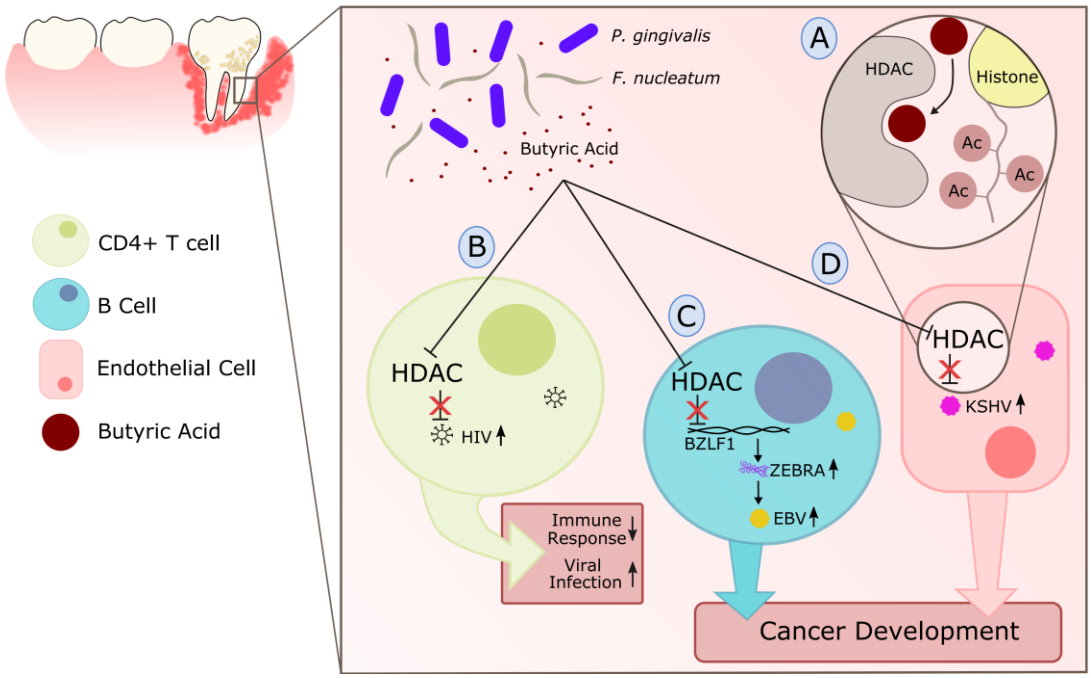


Fig. 4. The role of periodontal disease in head and neck cancers can be mediated by the effects of HIV and herpesviruses (EBV and KSHV): A) butyric acid produced by periodontal pathogens such as Fusobacterium nucleatum and Porphyromonas gingivalis competitively inhibit Histone Deacetylases (HDACs), resulting in the reduced deacetylation of histone proteins and sustaining viral gene expression B) activation of HIV proviral gene expression through HDAC inhibition increases viral progeny production; HIV infection suppresses systemic immune responses and aides in the acquisition of other viral infections; C) inhibition of HDAC causes transcriptional activation of the BZLF1 promoter in EBV infected cells and results in production of ZEBRA, known switch for lytic cycle activation; D) inhibition of HDACs in KSHV-associated cells result in the induction of MAPK and expression of lytic genes. Both EBV (C) and KSHV (D) mechanisms promote the spread of viral infection and contributes to tumor development.

Box 1

- Which shifts in microbial communities (presence/absence of particular bacterial species or alterations in microbial community structures) can enhance oncogenic virus infection progression or, on the contrary, help to eliminate it?

- Which host molecular pathways involved in viral infections and tumorigenesis are altered by microbiota?

- Can host genetics shape microbiota toward being anti- or pro-tumorigenic?

- How do microbiota affect the success of anticancer therapies?

- How do antitumor treatments affect microbiota? Are these effects relevant for efficient treatments and treatment-related co-morbidities?

Fig. 5. Current trending questions.

Box 2

Emerging technologies and other solutions:

- Experimental approaches for the generation of different types of omics data that would allow simultaneous assessment of functional states of each of viruses, host and bacteria (e.g. new and improved single cell technologies for both eukaryotic and prokaryotic cells).

- Computational tools for the analysis of multi-omics datasets that would provide robust predictions of regulatory relationships between all three kingdoms (e.g. transkingdom networks [202, 210], LEfSe [268]).

- Generation of new experimental models: a) humanized animal models (e.g. gnotobiotic animal models for cancer caused by oncogenic viruses harboring human microbiota and immune system); b) cell lines and tissues with virus and bacteria present (e.g. differentiated 3D cell aggregates colonized with specific bacteria [269-274]); c) next generation *in vitro* models such as organ-on-chip and human-on-chip microfluidic devices that allow application of robotic systems.

- Creation of multidisciplinary teams (involving oncologists with expertise in basic and clinical science, systems biologist, virologist, microbiologists, and engineers) through special funding mechanisms or even the creation of new institutions.

Fig. 6. Emerging technologies and other solutions.

Fig. 7. Community composition in cervical cancer and healthy adjacent sites: A) PCoA of unweighted unifrac comparing microbiota of samples from cervical cancer (n = 52), healthy cervix (n = 17), vagina (n = 76), stool (n = 28), and skin (n = 55). B) Bar chart of mean relative abundance of genera in cervical cancer biopsies, cytobrush from healthy ectocervical mucosa, and swab from healthy posterior vaginal fornix. Genera are arranged in descending order of mean relative abundance in cervical cancer samples from bottom to top of the bar chart. Genera with mean relative abundance <0.5% across the three sites are grouped into "Other" found at bottom of bar chart. C) Phylogenetic tree indicating the relationship and mean relative abundance (blue color intensity) of various genera in cervical cancer samples. The size of node and its label indicate the number of OTUs belonging to that taxonomy.

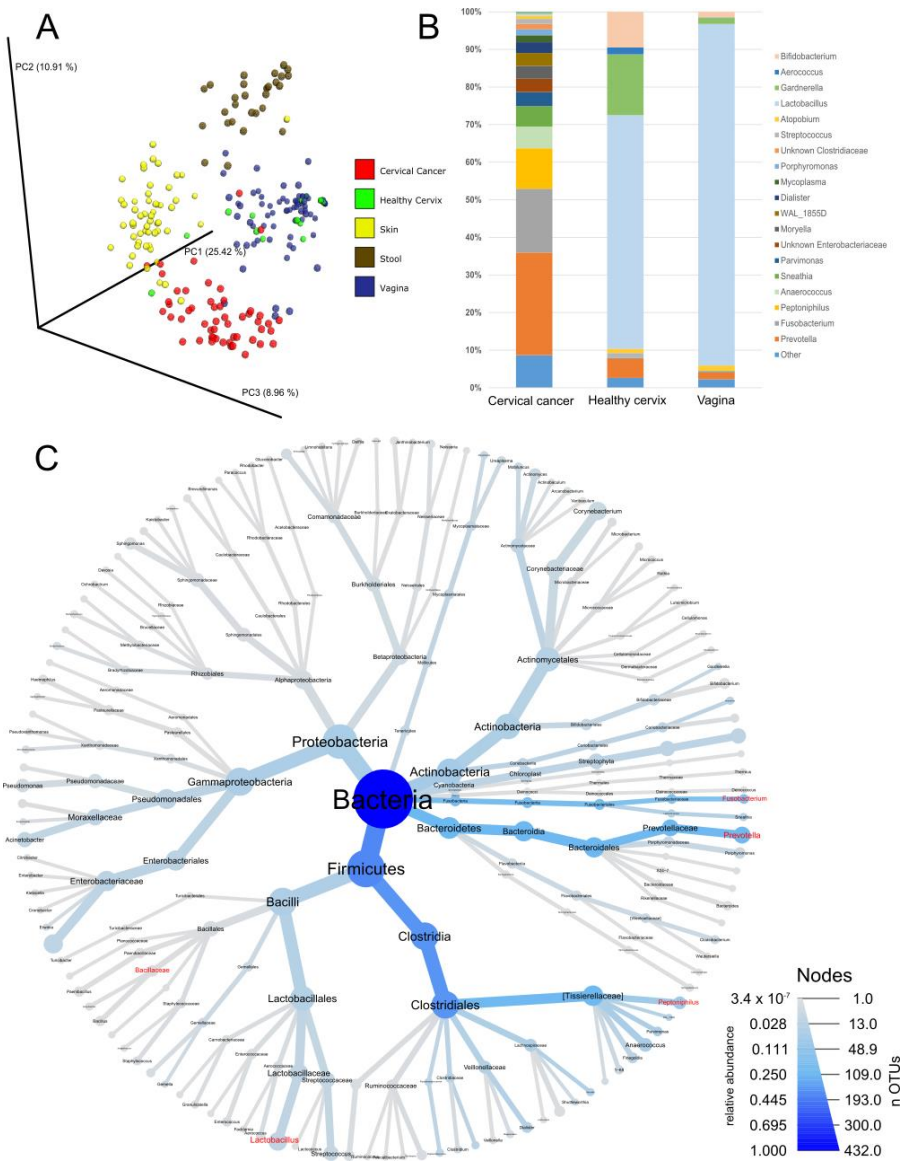


Fig. 7. Community composition in cervical cancer and healthy adjacent sites

Fig. 8. Transkingdom microbe-gene regulatory network: A) Transkingdom correlation network ($p < 0.001$; FDR < 0.1) between microbial network (21 OTUs, 50 edges) and previously described (Mine et al., 2013) tumor differentially expressed genes (698 DEGs, 3,066 edges) connected by 19 edges. Edge-weighted spring layout was performed in Cytoscape. Nodes represent: orange—bacteria; green—antiviral genes; purple—epithelial cell differentiation genes; blue—cell cycle genes; and gray—genes not assigned to specific subnetwork or function. Lines indicate: blue—positive of correlation between nodes; red—negative of correlation between nodes. Orange star indicates *Prevotella* OTU with high BiBC whereas dashed lines connecting the node and its name designate the top five BiBC scored bacteria OTU. B) Top BiBC OTUs (15/38) calculated between microbial subnetwork and antiviral subnetwork. C) Top *Prevotella* species in SILVA 16S rRNA database matched to representative sequences assigned to OTU_97.1949 (match length >200 bp, mismatch = 40 bp). D) *Prevotella* mean abundance in cervical cancer (CC) compared with previous 16S studies for healthy adjacent sites: HPV negative cervix (HPV-cervix) and healthy vaginal microbiome (healthy vagina). (PMID numbers of the source article specified for each column).

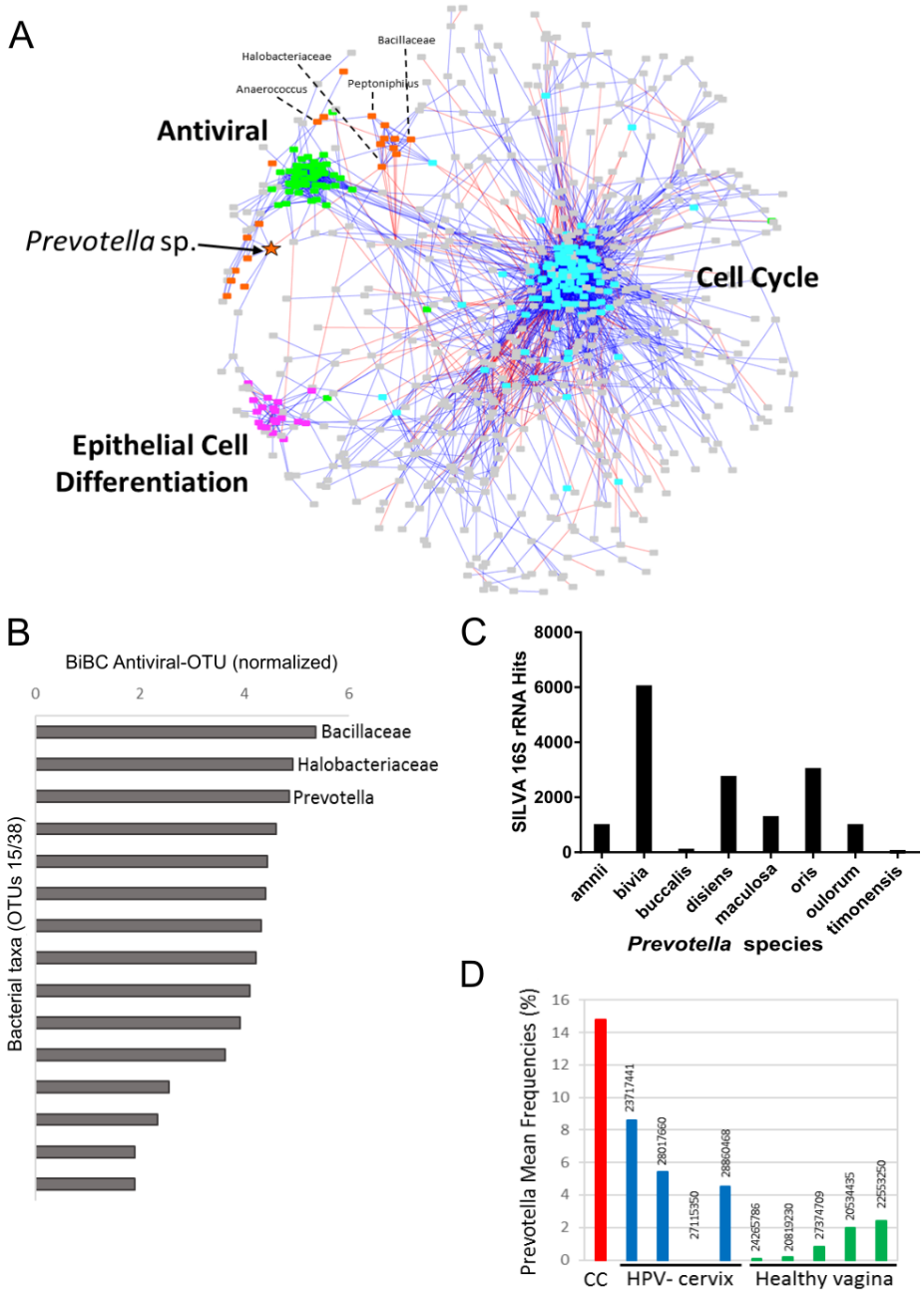


Fig. 8. Transkingdom microbe-gene-regulatory network.

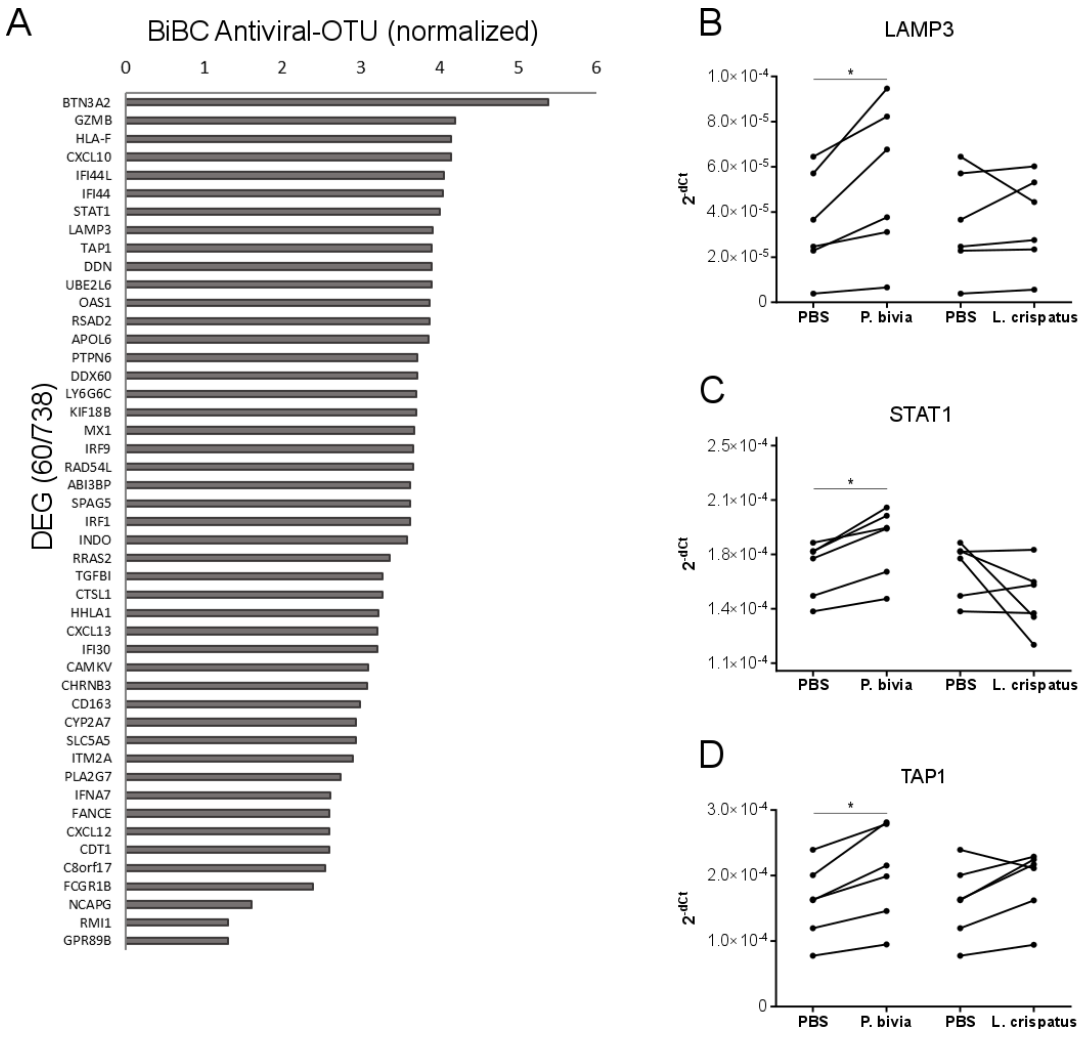


Fig. 9. Host gene expression regulated by *P. bivia*: A) Top DEGs (60/738) ranked by BiBC centrality calculated between bacteria and antiviral genes in transkingdom network (normalized BiBC = log2((BiBC*106) + 1)). B–D) RT-qPCR for cervical cancer top BiBC genes (LAMP3, STAT1, and TAP1), for which gene expression was upregulated in HeLa cells by *P. bivia* but not *L. crispatus* co-culture compared to negative treatment (PBS). mRNA levels were normalized to 18S rRNA gene expression. (*p-value < 0.05, one-tailed Wilcoxon matched-pairs signed rank test).

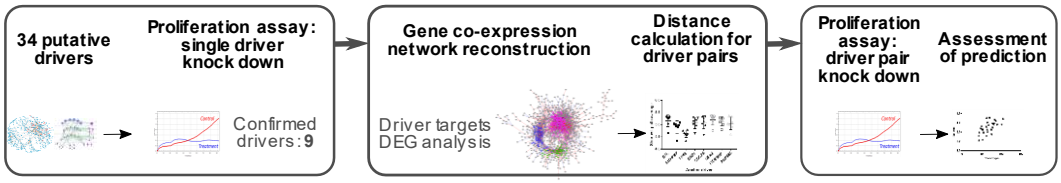


Fig. 10. Research workflow.

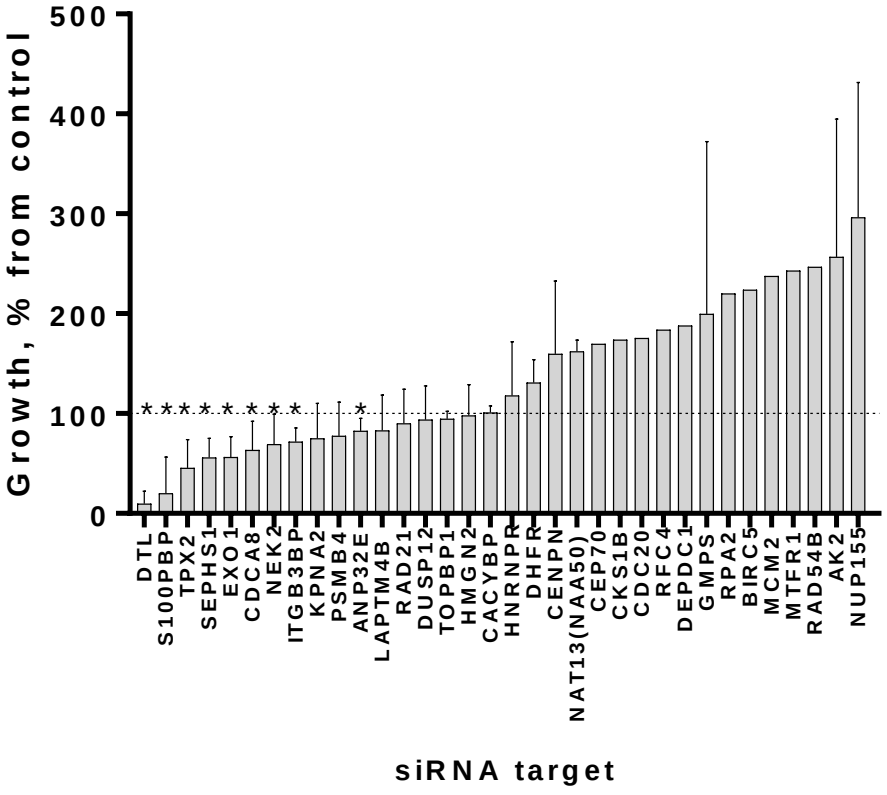


Fig. 11. Single driver proliferation screening. Cell growth (percent of control cells, treated with scrambled control siRNA) for 34 single drivers knocked down using siRNA (n =1-8). (*p-value < 0.05, one-tailed Wilcoxon matched-pairs signed rank paired test)

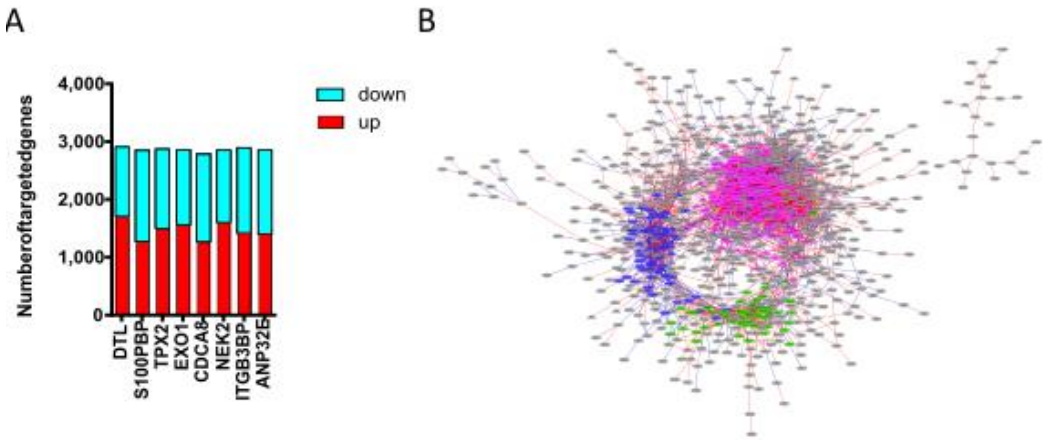


Fig. 12. Gene co-expression network reconstruction: A) Number of putative driver's targets upregulated and downregulated when the respective driver is knocked down with siRNA. B) Gene co-expression network reconstructed from the union of genes targeted by eight proliferation regulating gene drivers.

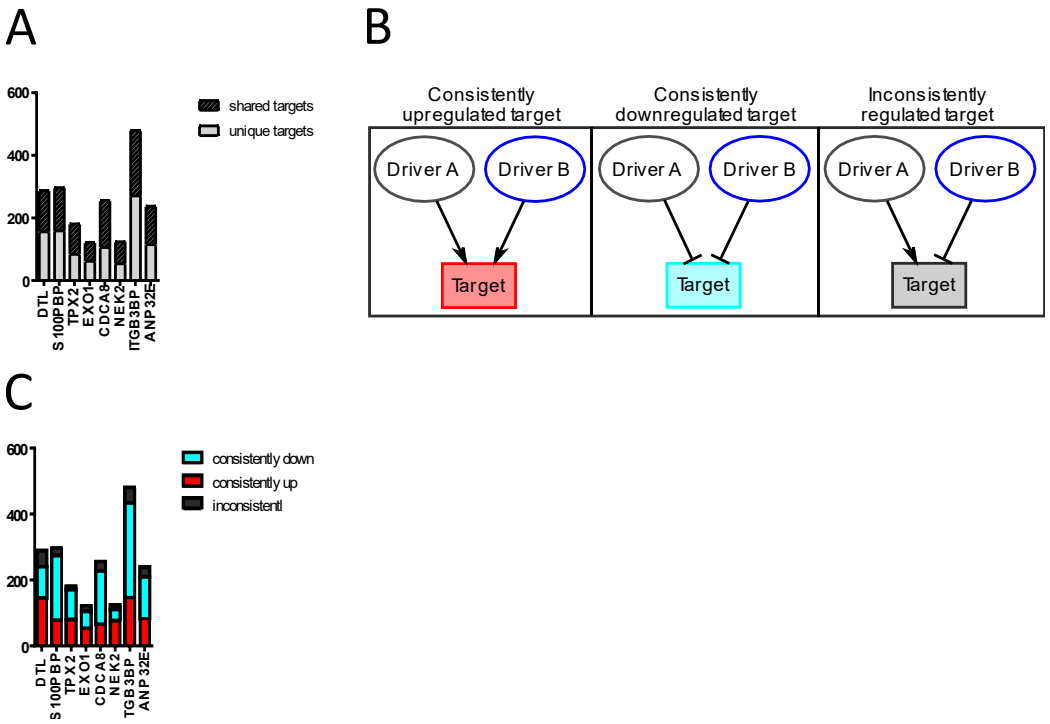


Fig. 13. Characterization of driver targets: A) Number of targets either regulated uniquely by respective driver (unique targets) or regulated by multiple drivers (shared targets). B) Visual representation of consistently and inconsistently regulated targets by multiple drivers. C) Number of targets either consistently upregulated, downregulated or inconsistently regulated for each driver.

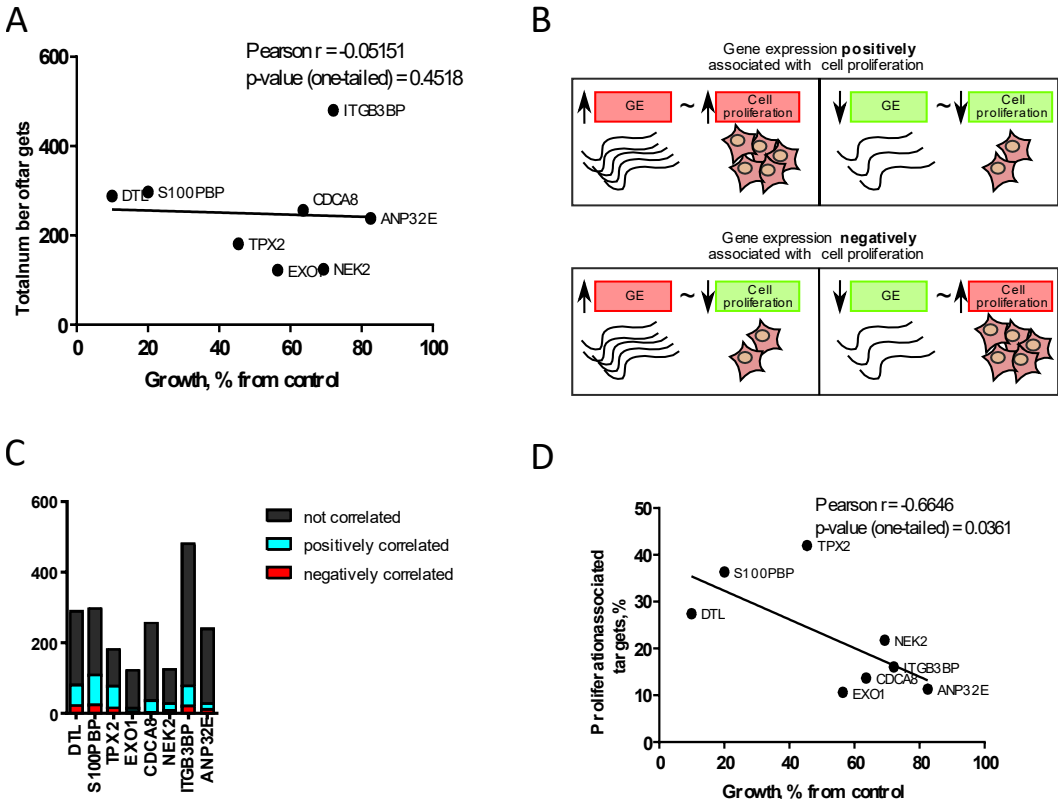


Fig. 14. Degree of cell growth inhibition for a single driver is associated with the percentage of targets correlated with proliferation but not the total number of regulated genes: A) The total number of genes a single driver regulates does not correlate with cell growth inhibition effect of respective driver knock down. B) Visual representation of target gene expression positively and negatively associated with cell proliferation. C) Number of targets whose gene expression is either positively associated, negatively associated, or not associated with cell proliferation. D) The percent of a driver's targets that correlate with proliferation is associated with cell growth inhibition effect by knock down of its respective driver.

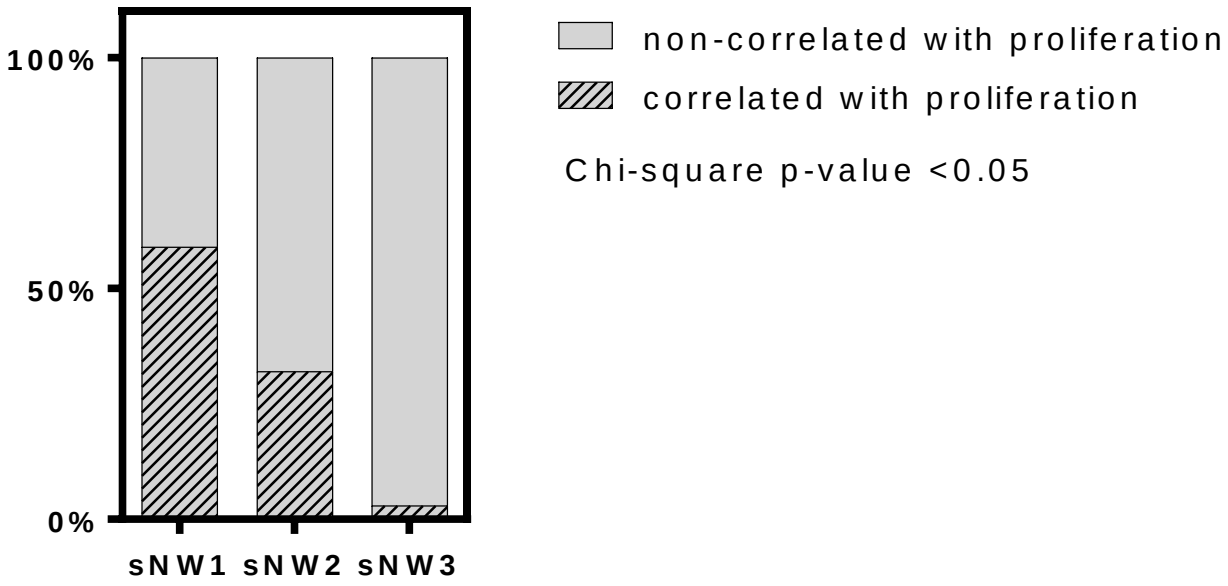


Fig. 15. Proportion of nodes associated and not associated with proliferation in three subnetworks.

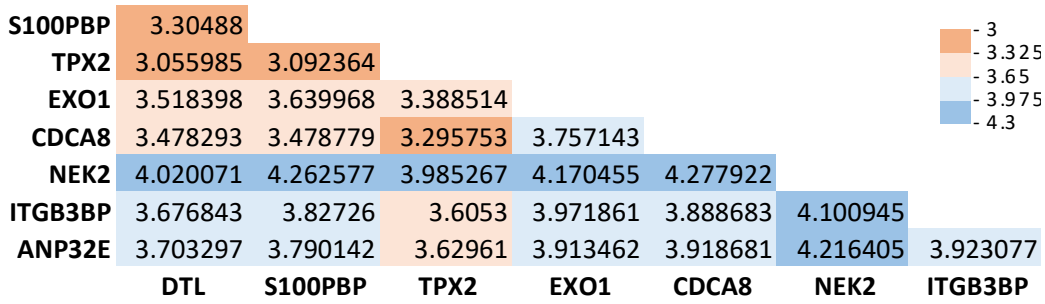


Fig. 16. Average shortest path between proliferation associated targets of each possible driver pair.

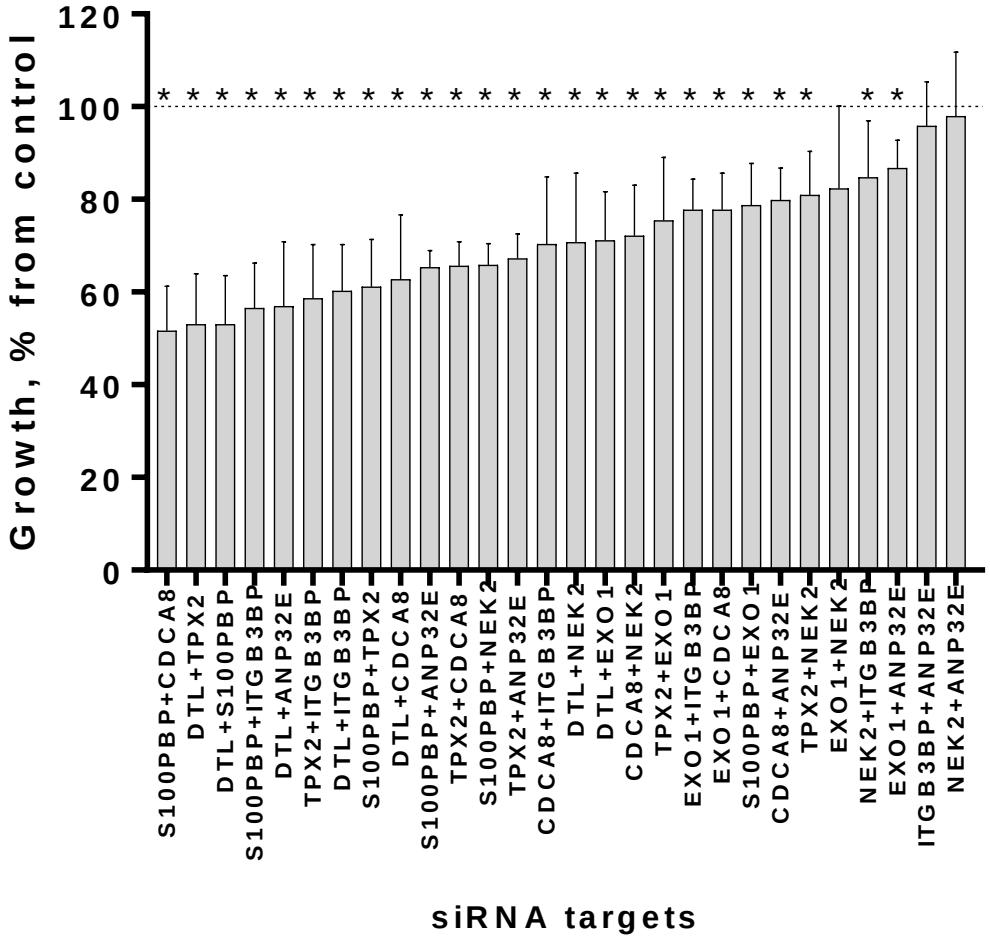


Fig. 17. Driver pair proliferation screening. Cell growth (percent of control cells, treated with scrambled control siRNA) for 28 driver pairs where two drivers were knocked down simultaneously using siRNA (n = 5). (*p-value < 0.05, two-tailed Welch t-test)

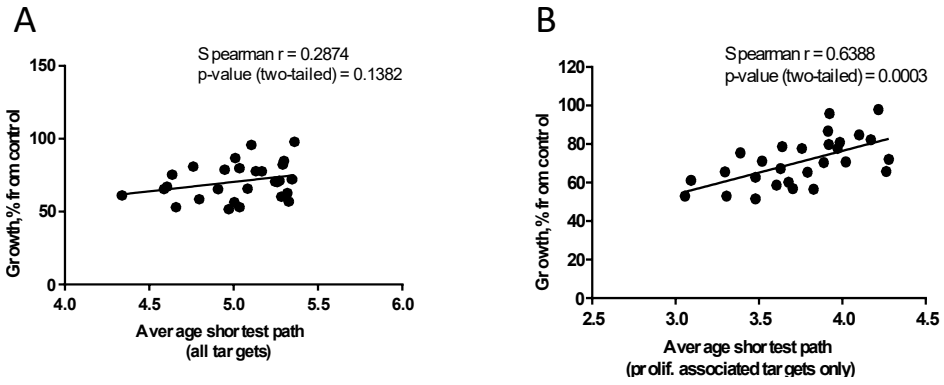
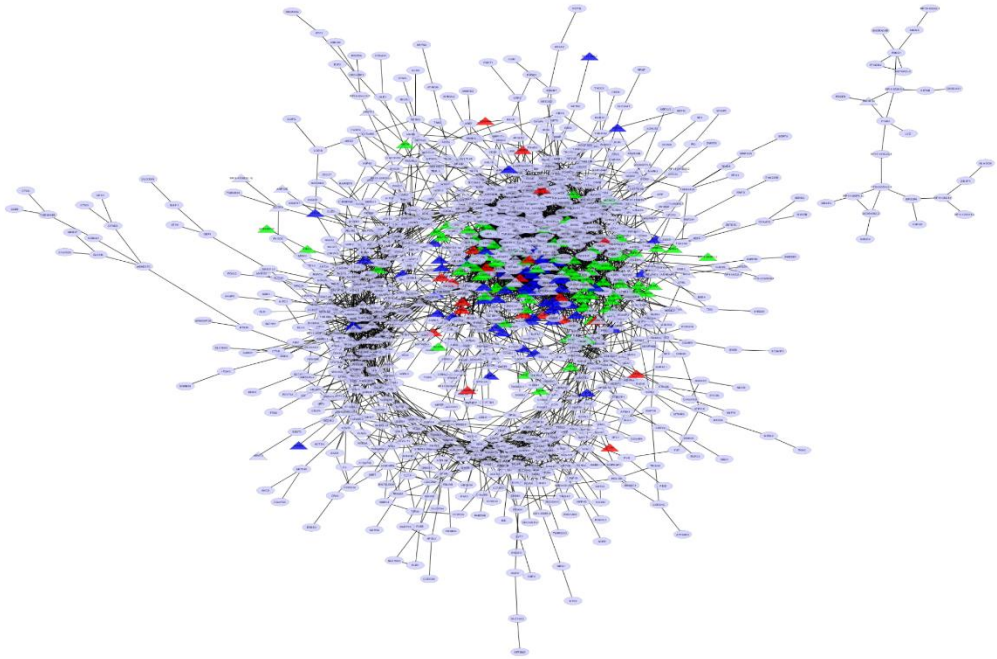


Fig. 18. Correlation analysis between average shortest path metrics for each driver pair and the degree of cell growth inhibition in the respective driver pair knock down: A) Average shortest path calculated between all targets of two drivers or B) Average shortest path calculated between proliferation associated targets only does correlate with performance of the driver pair in double knock down experiments.

Fig. 19. Localization of proliferation associated targets in the network for the best and worst performing driver pairs at inhibiting cell growth: A) best performing pair - DTL and S100PBP worst performing pair - ITGB3BP and ANP32E. (color code: blue nodes are uniquely regulated by either DTL or ITGB3BP, and green – S100PBP or ANP32E; red nodes are regulated by both drivers in the pair).

A



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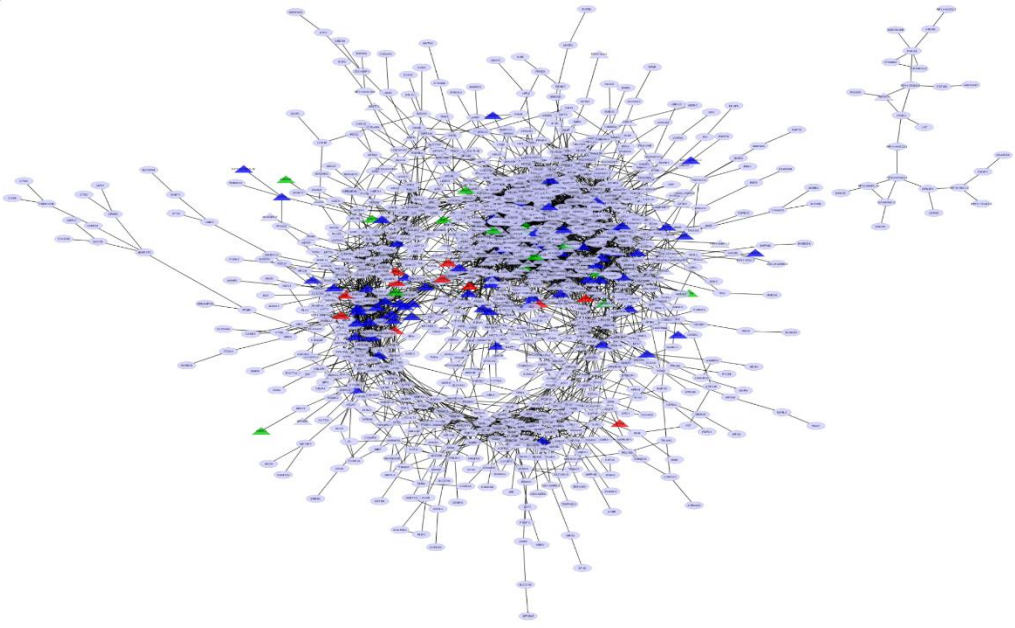

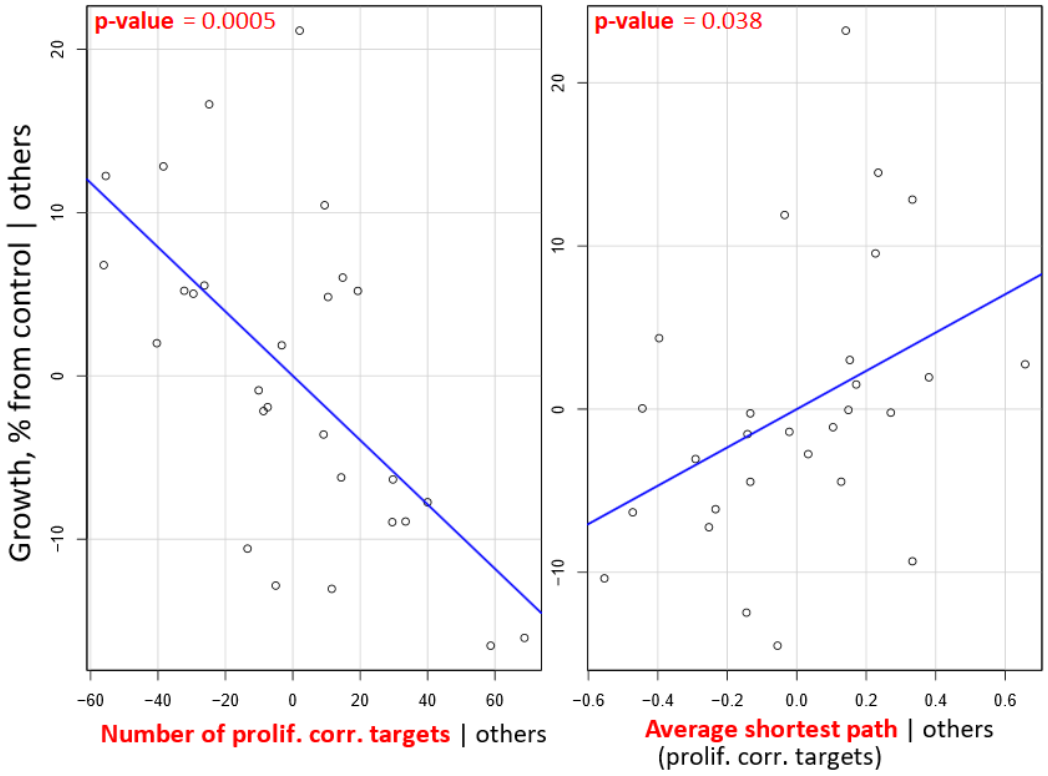

Fig. 20. Added-variable plots of multiple linear regression model for driver pair knock down growth inhibition prediction.

TABLES

Table 1. Summary of findings for each virus discussed in this review. Information presented is limited to the scope of the article.

Virus	Viral Location	Cancer Type	Bacteria-Virus Interaction	Microbiome Location	Bacteria	Bacterial By-products	Bacteria Impact on Host
HCV	liver	hepatocellular carcinoma	indirect	gut, liver	*Helicobacter pylori* [52-54], *Helicobacter hepaticus* [52, 56]		activation of NF-κB [21, 56] cirrhosis [62] HCC [59]
HBV	liver	hepatocellular carcinoma	indirect	gut	Bifidobacterium [66, 67]		HBV clearance [65, 68] anti-tumor immunity [175] up-regulation of IFN-dependent pathways [68] lower serum cholesterol [69-71]
HPV	vaginal	cervical	indirect	vaginal	*Lactobacillus gasseri* [94] BV-associated [95, 96] *Chlamydia trachomatis* [89-91, 104, 275, 276] *Prevotella* [101, 102]		decrease caveolin-1 and increase C-myc [106] upregulation of NF-kB, VEGF-c and survivin [107] upregulation of NF-κB, Toll-like receptor (TLR), NOD-like receptor, and TNF-α signaling pathways [84]
HIV	lymphoid cells	head & neck, cervical, Kaposi's sarcoma	direct, indirect	gut, oral, vaginal	BV-associated [115, 116] *Neisseria gonorrhoeae* [124-127] butyric acid producing bacteria [117, 118]	lactic acid [112] heptose-monophosphate [126] butyric acid [117, 118]	TLR3,4,5 [124] activated CD4+ T-cells [126] pro-inflammatory cytokines [124] NF-kB and AP-1 [124, 126] inhibit HDAC [117, 118]
HCMV	oral	head & neck		oral	*Porphyromonas gingivalis* [141-144]		
KSHV	oral	Kaposi's sarcoma	indirect	oral	*Porphyromonas gingivalis* [140-143, 151] *Fusobacterium nucleatum* [151]	butyric acid [151]	inhibit HDAC and activate p38 [151]

Table 1 (Continued)

EBV	B lymphocytes	nasopharyngeal carcinoma	indirect (host)	oral	*Porphyromonas gingivalis* [153]	butyric acid [153]	inhibit HDAC and activate BZLF1 promoter [153]
HERVs			indirect	gut			upregulation of type I interferon pathways [160-162] T-cells specific antitumor immunity [163]

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Consortium Leidos Biomedical Massachusetts General Hospital McDonnell Genome Institute at Washington University Medical College of Wisconsin Medical University of South Carolina Memorial Sloan Kettering Cancer Center Montefiore Medical Center NantOmics National Cancer Institute National Hospital, Abuja, Nigeria National Human Genome Research Institute National Institute of Environmental Health Sciences National Institute on Deafness &Other Communication Disorders Ontario Tumour Bank, London Health Sciences Centre Ontario Tumour Bank, Ontario Institute for Cancer Research Ontario Tumour Bank, The Ottawa Hospital Oregon Health &Science University Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center SRA International St Joseph's Candler Health System Eli &Edythe L. Broad Institute of Massachusetts Institute of Technology &Harvard University Research Institute at Nationwide Children's Hospital Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University University of Bergen University of Texas MD Anderson Cancer Center University of Abuja Teaching Hospital University of Alabama at Birmingham University of California, Irvine University of California Santa Cruz University of Kansas Medical Center University of Lausanne University of New Mexico Health Sciences Center University of North Carolina at Chapel Hill University of Oklahoma Health Sciences Center University of Pittsburgh University of São Paulo, Ribeir ão Preto Medical School University of Southern California University of Washington University of Wisconsin School of Medicine &Public Health Van Andel Research Institute Washington University in St Louis CGARNAECoMABSBCHBCoMBRIoCoHBIfR: **Integrated genomic and molecular characterization of cervical cancer**. *Nature* 2017, **543**(7645):378-384.

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APPENDIX

APPENDIX FIGURES

Vedlegg 2

Pasient ID: **J 5**

PASIENTINFORMASJON

Blod og vevsprøver ved livmorhalskreft

Vi vil spørre om du kan akseptere at det blir tatt ekstra blods- og vevsprøver til forskning i forbindelse med utredning og behandling av livmorhalskreft.
I tilfelle du skal ha strålebehandling vil dette medføre en ekstra gynekologisk undersøkelse i narkose etter fem strålebehandlinger. Du vil også bli spurt om å fylle ut et selvregistreringsskjema, hvor du selv vurderer hvilke plager du har. Dette gjøres før du starter strålebehandlingen samt en gang hvert halve år i 2 år, og deretter årlig inntil 5 år etter strålebehandlingen.

HENSIKTEN MED STUDIEN

Leger og forskere ved Radiumhospitalet arbeider hele tiden for å forbedre behandlingen av livmorhalskreft. For å kunne gjøre dette trenger vi tilgang til vevsprøver fra svulstvevet samt blodprøver. Disse prøvene vil bli brukt til dette formål.

PERSONVERN OG TILGANG TIL JOURNALOPPLYSNINGER

For å vurdere resultatene fra studiene vil laboratoriedata bli sammenholdt med data vedrørende behandling og oppfølgning. Alle opplysninger vil bli behandlet i anonym form og konfidensielt.

Dersom det senere skulle bli nødvendig å innhente ytterligere opplysninger om din sykdom, vil dette skje via din lege ved DNR, og alle opplysninger blir behandlet konfidensielt.

Resultatene fra studiene vil bli publisert i anonym form i internasjonale medisinske tidsskrifter.

DELTAGELSE I STUDIEN

Din deltagelse i disse undersøkelsene vil ikke få betydning for din behandling eller sykdom, men kan gi oss verdifull kunnskap til bruk i fremtiden. Din deltagelse er selvfølgelig helt frivillig. Du kan når som helst trekke deg uten at det får konsekvenser for den videre behandlingen.

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Pasient ID:

SAMTYKKEERKLÆRING **Blod og vevsprøver ved livmorhalskreft**

Jeg er innforstått med at blodprøver/vevsprøver kan bli anvendt til forskningsformål.
Jeg er informert om at deltakelsen er frivillig, og at jeg når som helst kan trekke min samtykke uten å oppgi grunn og uten at dette påvirker den videre behandlingen.
Jeg er innforstått med at mine personlige/kliniske data blir behandlet strengt konfidensielt.

Pasientens navn: _____

Pasientens underskrift: _____ Dato: _____

Underskrift ansvarlig lege: _____ Dato: _____

Kopi av denne pasientinformasjonen er utlevert pasienten.

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Fig. S1. Consent form signed by patients.

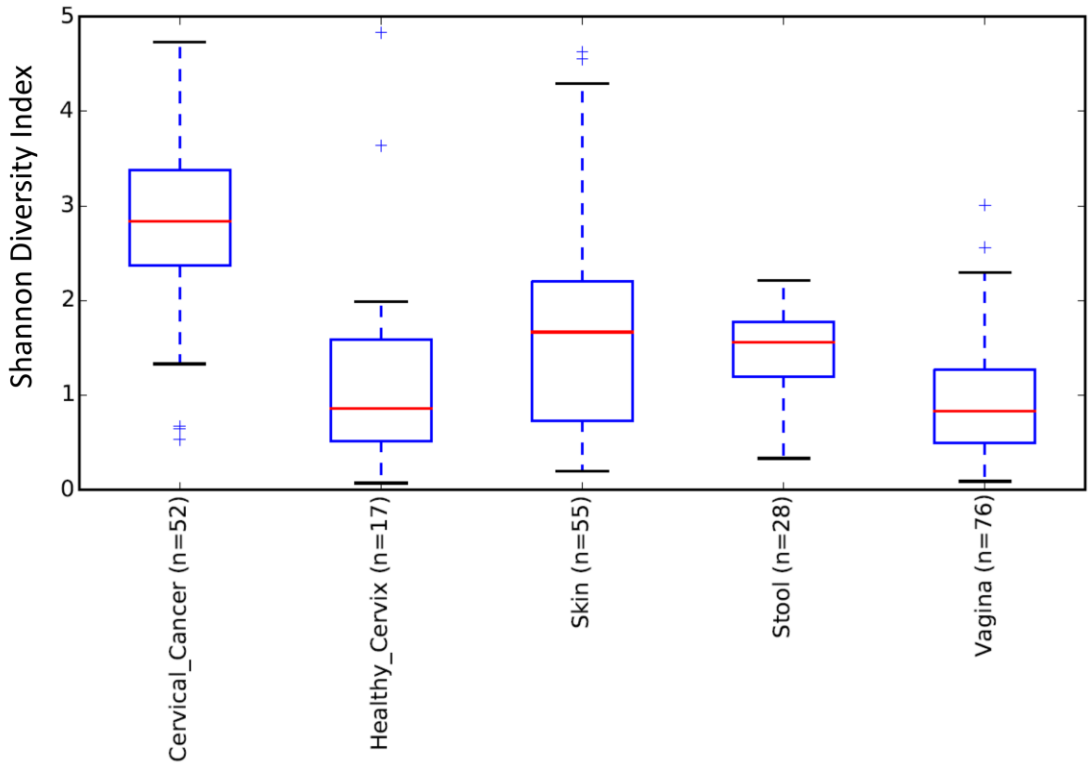

Fig. S2. Shannon Alpha Diversity Index for different tissues.

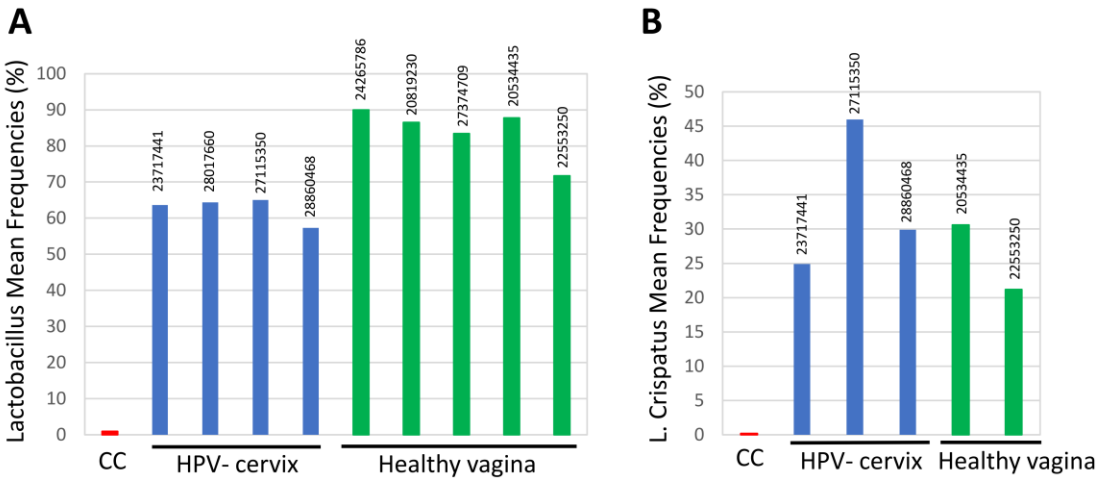

Fig. S3. Abundances of Lactobacillus and L. crispatus in cervical cancer, HPV negative cervix and healthy vaginal microbiome. Abundances of Lactobacillus (A) and *L. crispatus* (B) in cervical cancer (CC) (our data), HPV negative cervix (HPV-cervix) and healthy vaginal microbiome (Healthy vagina). Abundances are represented by mean frequencies (%) data reported by published articles (PMID numbers of the source article specified for each column).

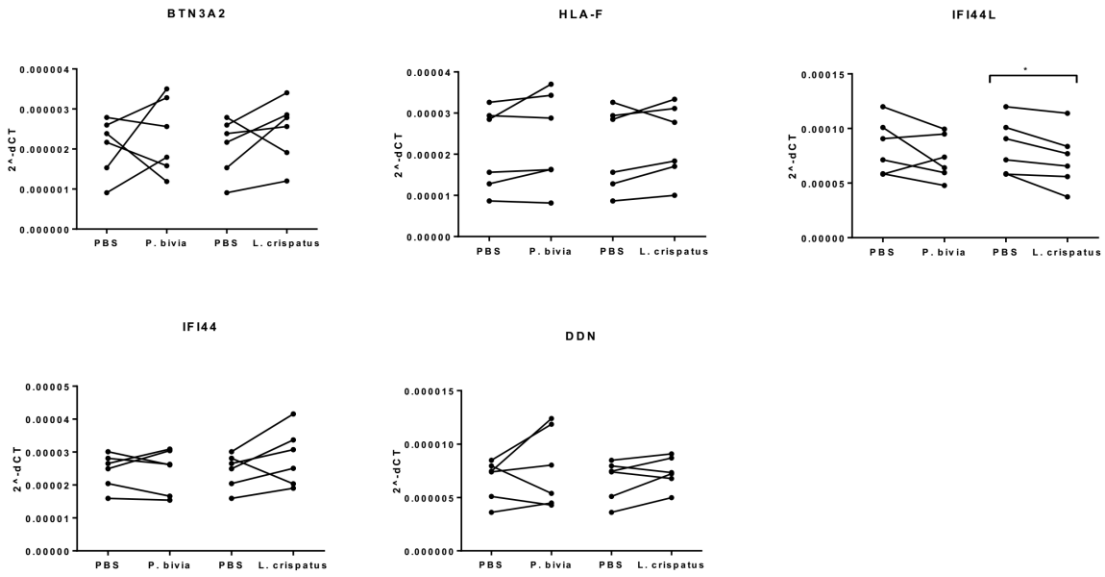

Fig. S4. RT-qPCR for cervical cancer top BiBC genes. HeLa cells were co-cultured with either *P. bivia* or *L. crispatus* and gene expression was compared to negative treatment (PBS) of Hela cells. mRNA levels were normalized to 18S rRNA gene expression. (*p-value < 0.05, one-tailed Wilcoxon matched-pairs signed rank test).

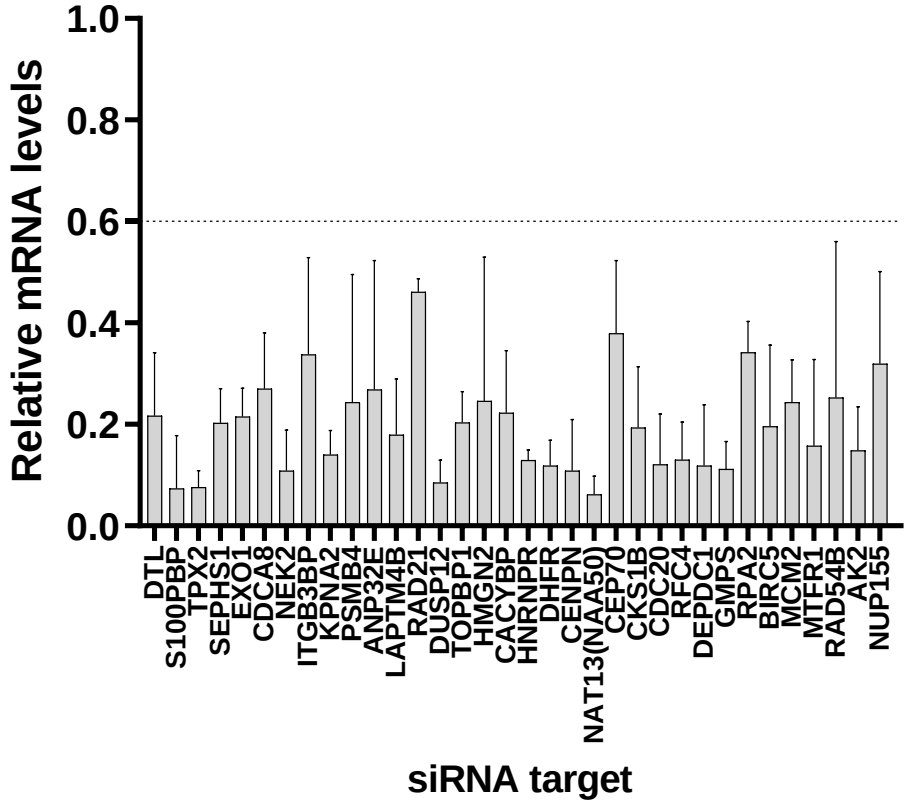

Fig. S5. qRT-PCR gene expression results for single driver knock down (n ≥2).

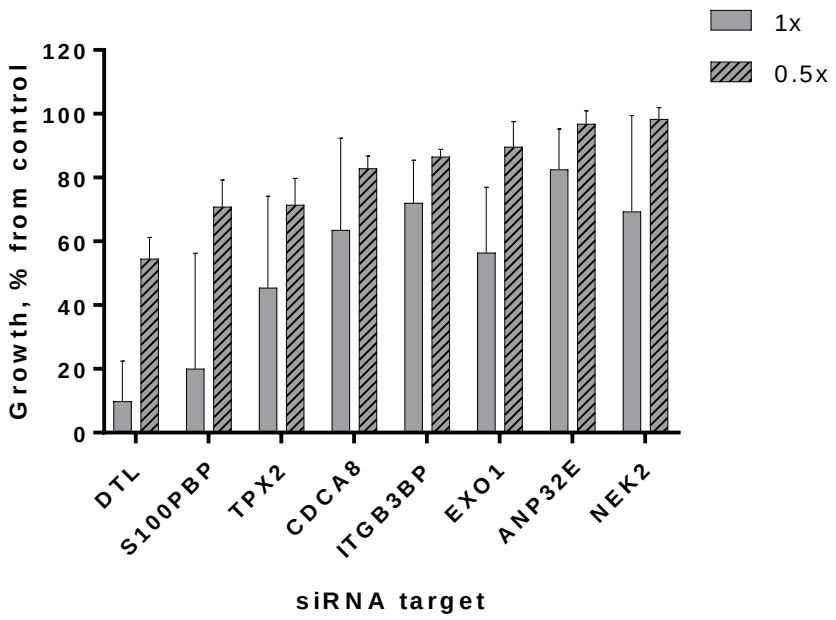

Fig. S6. Decrease in siRNA amount results in smaller inhibition effect on proliferation in single driver knock down conditions ($n \geq 5$).