Objectives: Dr. Babaian has discovered that colorectal cancers (CRC) possess an '*onco-ribosome'* – a population of ribosomes lacking an ultra-conserved 18S rRNA nucleobase modification situated at the decoding center of the ribosome [1]. Partial loss of this RNA modification is widespread in CRC and present in 22+ other cancer types. Decreased rRNA modification is associated with a CRC proliferation and translational dysregulation, yet the mechanism underlying this aberration is unknown and is the primary objective of this research proposal. The secondary objective is to explore if this molecularity distinct and cancer-specific onco-ribosome can be exploited as a chemotherapeutic target.

Significance: Colorectal carcinoma will be newly diagnosed in 26,900 Canadians and kill 9,700 in 2020, making it the 2nd and 3rd leading cause of cancer deaths in men and women, respectively [2]. While CRC mainly affects older patients, there has been a sharp 2-4 fold increased incidence in Millennials [3]. As these young adults age, CRC is projected to increase as a major health burden. Thus, there is an urgent need to advance our knowledge of CRC biology and develop safer therapies which mitigate the long-term cytotoxic damage of standard treatments, especially in treating the young.

Context: The ribosome is an RNA-protein complex essential for protein synthesis. The human 80S ribosome contains 4 structural and catalytic ribosomal RNAs (rRNAs): 5S, 5.8S, 18S and 28S; and ~80 ribosomal proteins (RPs). The genes encoding rRNAs, *RNA5S* and *RNA45S* are found in repetitive arrays (80-800 copies/cell) which due to technical limitations have been excluded from the reference human genomes [4] and consortia sequencing projects (i.e. 1000 Genomes Project, The Cancer Genome Atlas (TCGA)). However, the concept that ribosomes are a homogenous set of ribonucleoprotein complexes has recently become fundamentally challenged [5,6], and research into the molecular underpinnings which distinguishes heterogenous classes of ribosomes has flourished.

Ribosomal heterogeneity occurs at multiple levels. Ribosomes from separate cellular compartments associate with differing sets of mRNA [7], the stoichiometry of the RP sub-units can vary across tissues and cancer [8,9], and the constellation of >100 rRNA modifications can vary substantially [10–12]. RP genes are also mutational drivers of several cancers: 8-10% of T-cell Acute Lymphocytic Leukemias possess *RPL5* or *RPL10* mutations, and 20% of Chronic Lymphocytic Leukemias have *RPS15* mutations [13,14]. At the rDNA sequence level, Dr. Babaian and others have expanded the known reservoir of rDNA genetic diversity in human populations [15,16]. Altogether this constructs a model of ribosomes as heterogeneous units with functional specializations [6,8,17].

In an exploratory analysis of rRNA sequence variation in CRC using RNA-seq, Dr. Babaian identified a single, cancer-specific point variant within 18S rRNA *in 45% of patients* (N = 66 pairs, p_{adj} = 3.8e-8, Fig 1a) and reproducible across 5 independent CRC cohorts [1], one *not* reflected in the rDNA sequence (Fig 1b). For context, the *KRAS* proto-oncogene is mutated in only ~35% of CRC patients [18]. Specifically, the rRNA variant occurs at the uridine at 18S.1248, a nucleobase that is normally hyper-modified to macpΨ (1-methyl-3-(α-amino-α-carboxyl-propyl) pseudouridine) [19]. This modification hinders Watson-Crick base pairing and introduces a reverse transcriptase (RT) read "error signature" (a U to C signature present in all >12,000 RNA-seq libraries tested to date) which quantifies the extent to which 18S.1248.U is modified to unmodified (Fig 1c; reviewed in [20]). Thus these tumors contain an overall decrease in macpΨ.1248 modification rRNA, termed "hypo-macpΨ".

Dr. Warren is a leading expert on the structure-function of the ribosome in the context of human disease, including cancer. Together we will bridge the molecular (epi-)genetics of rRNA variation and ribosome structure-function analyses in the proposed research to directly expand the understanding of ribosomal heterogeneity in the domain of cancer biology. One fruition of this research is to explore these cancer-specific onco-ribosomes as a drug target and ultimately develop therapies to improve the treatment of Canadian cancer patients.

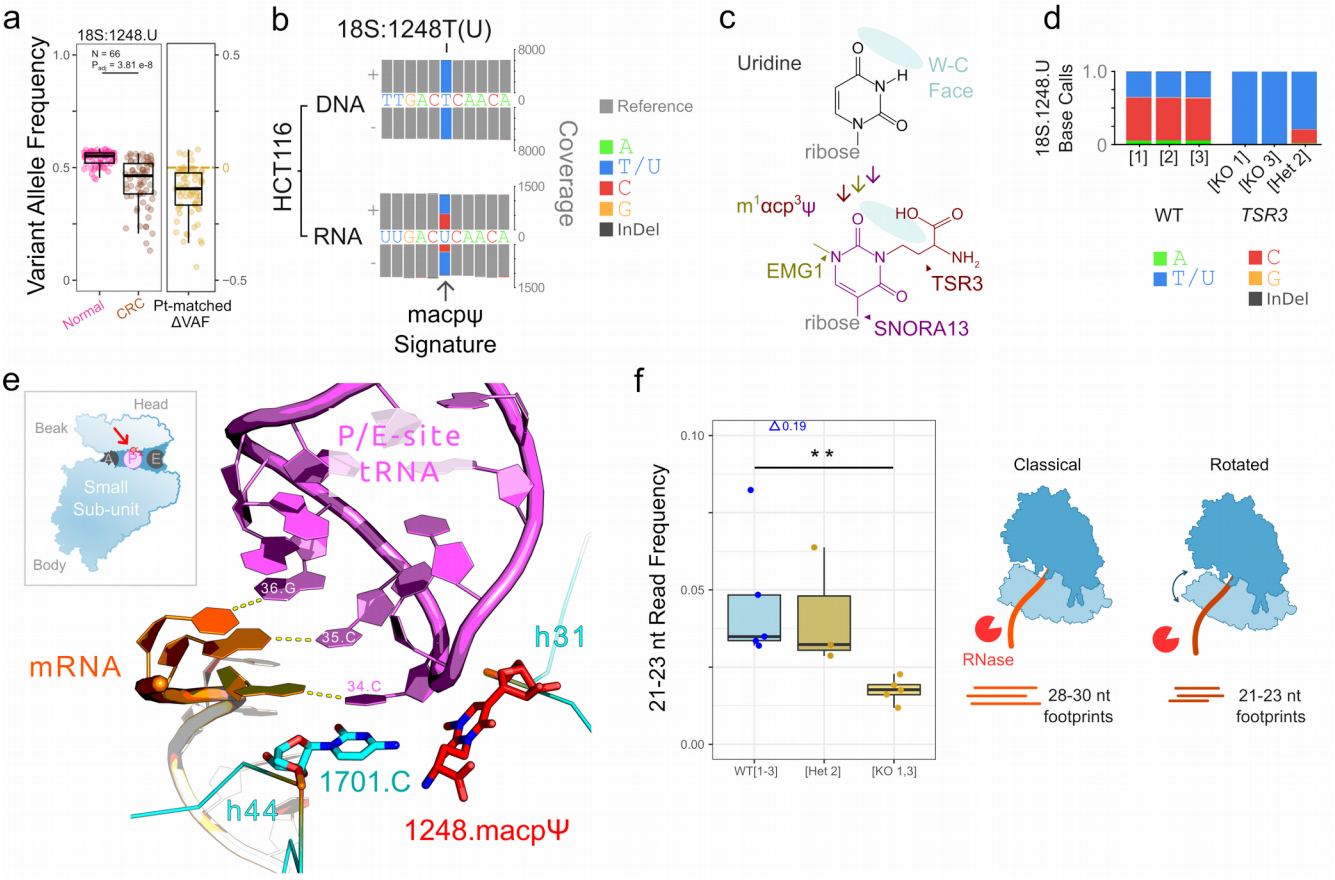

Figure 1 **a)** RNA-seq read variants at 18S.1248.U from CRC tumors and matched-normal adjacent tissue. **b)** The variant-signature is absent from all DNA-seq (representative of N ~1,500) and present in all RNA-seq (representative of N ~12,000). **c)** The uridine at this position is hyper-modified to macpΨ in three enzymatic steps. **d)** *TSR3* Knockout (KO) ameliorates the acp-modification, shown by RNA-seq read variant analysis. **e)** Cryo-EM structure (6EKO) with P/E-site tRNA showing contact point with 18S.1248.macpΨ. **f)** Relative frequency of short (21-23 nt) ribosomal footprinting fragments corresponding to rotated ribosome state.

Rationale: The ribosome is a validated drug target. Many common anti-bacterial, -fungal and -parasitic drugs inhibit contagion ribosomes relative to host mammalian ribosomes [21]. Since macpΨ-deficient onco-ribosomes possess a structural difference potentially amenable to small molecule inhibition, we will investigate the biological consequences of this hypo-macpΨ onco-ribosome as a first step to validating it as therapeutic target in cancer.

Aim 1: Delineate the translational impact of 18S.1248.macpΨ in a CRC cell line model

Hypothesis: Hypo-macpΨ CRC cells contain an onco-ribosome which imparts a tumourigenic translational property relative to physio-normal macpΨ CRC cells

To define the physio-normal function of macpΨ.1248 and distinguish how macpΨ-loss impacts the ribosomes translational capacity, I will compare macpΨ-null CRC cells, to isogenic rescues and normo-macpΨ CRC cells via molecular reporter assays and multi-omic translational analyses. Molecular insights will be validated by state of the art whole-ribosome cryogenic electron microscopy (cryo-EM). This integrative approach will identify the molecular significance of the hypo-macpΨ phenotype observed in patients as a first step in validating the onco-ribosome as a therapeutic target.

Little is known about the function of 18S.1248.macpΨ. This modification only occurs at 18S.1248.U, and both the base and modification are perfectly conserved in all eukaryotes (~1.5 *billion* years), implying an ancient, conserved function [22]. The macpΨ is created in three enzymatic steps: SNORA13 guided pseudouridylation, EMG1-mediated methylation, and TSR3-mediated amino-carboxyl-propylation (acp) (Fig 1c, [23]).

Starting from parental HCT116 CRC cells, I applied CRISPR-Cas9 and created two *TSR3* knockout [KO] clones and a *TSR3* heterozygous knockout [Het], with total and partial (hypo) loss of macpΨ modification, respectively. Surprisingly, combined RNA-seq, Ribo-Seq (also called ribosome footprinting) and global proteomics of *TSR3*[KO/Het] relative to three wildtype control clones showed a marked increase in the translational efficiency of a sub-set of genes, specifically enrichment for mRNA encoding ribosomal proteins. While *TSR3*[KO/Het] cells showed no difference in proliferation or translational output as measured by global puromycin incorporation assay [1], this comparison was made at steady-state and thus confounded by clonal genetic variation.

To measure the effect of 18S.1248.macpΨ in a well controlled system, *TSR3*[KO] clones will be transfected with a an empty pcDNA3.1 (control) or pcDNA3.1-*TSR3* (pTSR3) plasmid to rescue TSR3 and macpΨ-modification within an isogenic background. Modification levels between pTSR3 and control cells will be validated by the acp-RT-PCR assay (invented for this purpose by Dr. Babaian, [1]) over a 96 hour time course. Proliferation and global translation rates of pTSR3 or control treated cells will be compared, with pTSR3 cells expected to show a decrease in translation and proliferation rates associated with an inhibition of RP translation. Upon establishing this system, matched RNA-seq and tandem mass tag (TMT) labeling and mass spectrometry will be performed in biological triplicate, with an expectant decrease in RP translation efficiency, the inverse of the knockout phenotypes. This system is less suseceptible to biological variation relative to long-term clonal expansions separating the genetic knock-outs, and thus more power can be achieved to determine differential translation in the presence of macpΨ.1248. A narrower set of transcripts is critical in determining the underlying mRNA sequence motifs, like programmed ribosomal slippage sites, are associated with macpΨ.1248 functions.

Our molecular dynamic simulations implicate the macpΨ.1248 base in direct interaction with the P-site tRNA, adjacent to the codon:anti-codon interface (Fig 1d, [1]). Further, *TSR3*[KO] clones contained a significant and reproducible decrease in ribosome-protected RNA fragments between 21-23 nt long (Fig 1e), consistent with a decrease in ribosome stability in the post-peptide bond rotated state [24]. A role for the macpΨ.1248 modification in the elongation phase of protein synthesis when this rotation state occurs would reconcile these findings. Functionally this would implicate the maintenance of the codon reading-frame during elongation which can be tested with reporter assays. Cloning a set of ribosomal frameshift signals such as those in HIV, Murine Leukemia Virus, coronaviruses, or from endogenous genes such as *ROBO1* into a dual-luciferase reporter plasmid backbone is a rapid way to measure frameshifting [25]. A panel of these constructs will be made and measured in both WT versus *TSR3*[KO] cells and *TSR3*[KO] + pcDNA3.1 versus *TSR3*[KO] + p*TSR3* cells.

Whole ribosome cryo-EM reaches 2Å in the ribosome core. The aforementioned molecular reporters narrow down the potential states in which 18S.1248.macpΨ exerts function. Contrasting cryo-EM structures of ribosomes from WT, TSR3[KO], and pTSR3 rescue cells will directly validate proposed mechanisms and lays the foundation for the design of rational drug inhibition of the onco-ribosome.

Evolutionary, structure-function, and cancer-recurrence data all strongly support a biologically significant role for hypo-macpΨ in cancer biology, either as a neomorphic oncogenic function or loss of tumour suppressor activity. Determining the molecular mechanism of this modification in translational biology will inform us how this aberration in cancer patients impacts tumor biology.

Aim 2: Explore the hypo-macpΨ phenotype in cancers can be exploited as a target for small molecule inhibition and/or biomarker

Hypothesis: Loss of macpΨ.1248 confers drug hypersensitivity to CRC cells relative to controls

The amino-carboxyl-propyl modification on 18S.1248.macpΨ is physically accessible to drugs in the mRNA channel of the ribosomal small sub-unit (Fig 1e). Loss of this modification in a major fraction of ribosomes within cancer cells, and it's ubiquitous presence in normal tissues raises the possibility that this facet of the ribosome may be a target or biomarker for small molecule inhibition.

Previously it has been shown that yeast *tsr3*[KO] cells were specifically hypersensitive to the FDA-approved aminoglycosides Paromomycin and Hygromycin B [23], although this was not recapitulated in our human *TSR3*[KO] cells, relative to wildtype controls [1]. While aminoglycosides or other ribosome-inhibiting compounds are not known to bind the P-site adjacent to macpΨ.1248, we postulate that these cancer ribosomes may be less resilient to pharmacological perturbation (thus serve as a biomarker). This is supported by multiple studies reporting paradoxical hypersensitivity of cancerous cells to common ribosome targeting antibiotics such as cycloheximide, doxycycline, puromycin and tetracycline [26–29], although the macpΨ.1248 modification status in those studies is unknown.

We have identified 14 FDA-approved ribosome inhibiting compounds, and at least >50 unapproved but commercially available compounds. As research our understanding the mechanism of action of 18S.1248.macpΨ unfolds and the knockout and rescue lines are established, these model systems will be screened in parallel for hypo-macpΨ associated drug hypersensitivity. Initially we will determine the 50% inhibitory concentration (IC_{50}) for each compound in wildtype and compare it to *TSR3*[KO] and *TSR3*-rescue as measured by standard MTT colomertic assay.

These straight forward experiments directly test our hypothesis, providing the data to catalyze the acquisition of a project- or even program-grants for the expansion of this research into a multi-disciplinary drug development collaboration. Tumors from at least 22 distinct cancer types show some extent of hypo-macpΨ, including 45% of CRC and 25.5% of Diffuse Large B-cell Lymphoma patients [1]. Identifying a compound with a pharmacological window, even if initially small, can have profound consequences and lead to the development of a new class of chemotherapies.

The ribosome has been a drug target since the discovery of streptomycin in 1944. Understanding the hypo-macpΨ cancerous variant of the human ribosome has paradigm-shifting potential for both aberrant protein synthesis in cancer, and for exploring the potential of the onco-ribosome as a novel chemotherapeutic target. Thus, this innovative research stream has true translational potential.

Environment: Dr. Alan Warren's laboratory at the University of Cambridge is the ideal environment to execute this research proposal. Dr. Babaian has demonstrably been a driving force in research on the onco-ribosome as the lead/corresponding author on the hypo-macpΨ discovery paper recently published in *Cell Reports*. By joining the Warren lab, we will combine orthogonal approaches stemming from biochemical/structure-function analysis to compliment the genetics/computational background of Dr. Babaian. This inter-disciplinary approach reflects the underlying philosophy of the Cambridge Institute for Medical Research (CIMR) which offers world-class research facilities, including a cryo-EM facility, and a community fostering collaborations. In parallel to this wet-work, an ongoing collaboration with Dr. Dylan Girodat at the University of Arizona will provide whole ribosome molecular dynamic simulations to probe how macpΨ modifications alter interaction energies of the elongating ribosome.

It is through collaborations and complimenting expertise that the full impact of this research can be achieved, both from a scientific and societal perspective.