Logbook - For a more detailed log without dates, ask to see my project construction document.

Name: Sara Waqas
Grade: 9

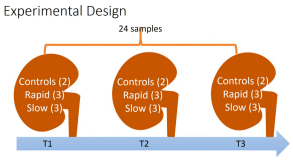
Project Type: Study
Project Title: Unraveling the Success of ADA and PDK1 Overexpressed CAR T cells in Clearing Colorectal Adenocarcinoma Through RNA Sequencing Analysis

Date:	Notes:
Oct. 22nd, 2023	Brainstormed ideas for research. Want to be based on bioinformatics, but not very clear what. Want to research colorectal cancer as the tissue is very interesting. It is heterogenous. It is also on the rise so treatment and biomarkers for it are very important.
Oct. 24th, 2023	Worked on trying to define a research question. Maybe looking at very specific biomarkers or pathways. Issue is: DATA AND WHAT SPECIFICALLY. Thinking of confirming a study, but conducting my own research would be so much more rewarding. Prevalence of Young Onset Colorectal Cancer, and identifying genetic biomarkers for its surge. First one: https://www.sciencedirect.com/science/article/abs/pii/S001650851941937 9 analyzes based on polygenic risk score. Use variables that are single nucleotide polymorph because it limits the variables. Okay so

	https://bmccancer.biomedcentral.com/articles/10.1186/s12885-021-07871-z talks about the Cadherin 1(CDH1) rs9929218 may act by increasing the risk of colorectal cancer, colorectal adenoma, or both. These studies, however, reported inconsistent associations. THIS IS PERFECT, I CAN DOUBLE CHECK THIS. Meta-analysis implied considerable association between CRC and rs9929218 (OR = 1.21, 95%CI 1.04–1.42 for GG versus AA; OR = 1.22, 95%CI 1.05–1.42 for GG/AG versus AA). In the subgroup analyses, significantly increased risks were found among Europeans n 8q23.3, 8q24.21, 9p24, 10p14, 11q23.1, 14q22.2, 15q13.3, 16q22.1, 18q21.1, 19q13.1, and 20p12.3 have been identified by GWAS, illustrating, the CRC as a complex genetic disease [1, 6, 8,9,10,11,12]. Among these SNPs, rs9929218 (16p22.1), located in the intron region of the gene cadherin 1 (CDH1), was identified to be associated with CRC risk [13]. In 201 which emphasizes a significant association between rs9929218 polymorphism and CRC susceptibility [13]. Nonetheless, its limitation is the absence of raw genotype data that reference dominant and recessive models. The rs9929218 has been identified as an aroused general interest for CRC susceptibility by recent genome-wide association studies and this polymorphism has shown that the G allele is associated with an increased risk of colorectal cancer. However, some of the literature has produced contrary results [14, 15]. So the issue is conflicting results. They have found a correlation between rs9929218 and CRC but there are some studies that disagree with the relationship. So the linked study is doing a meta analysis of all these studies and seeing if it is true. I need to decide what I will control and change. I guess I will control the environment, and race/age. But I will be changing the genotype. I also want to alter environment, and keep the rest as similar as possible of course that will be very diffcult tho. It is more prevelent in white people, so it may be environmental, but then again they are all from the US so its hard to say.

	Cohort effects are variations resulting from the unique experience/exposure of a group of subjects (cohort) as they move across time. The most commonly defined group in epidemiology is the birth cohort based on year of birth and it is described as difference in the risk of a health outcome based on birth year. So this means lifestyle changes (implicating generational lifestyle changes in the development of EOCRC Marx O, Mankarious M, Yochum G. Molecular genetics of early-onset colorectal cancer. World J Biol Chem 2023; 14(2): 13-27 [PMID: 37034132 DOI: 10.4331/wjbc.v14.i2.13]) is probably the most plausible. So the promotion of intestinal stem cell populations, insulin resistance, adipocye levels (Molecular links between obesity, metabolic disorders, and CRC have been suggested, including the promotion of intestinal stem cell populations[15,16], increased insulin resistance, adipocyte levels, and inflammation[17]. How EOCRC risk factors affect clinical presentation is still under investigation. One aspect of EOCRC clinical presentation of particular interest is tumor location[18].
Nov. 5th, 2023	Today I looked into molecular characteristics and how if we identify actual pathways and go to the molecular level we can identify what exactly, to the genome level, influences CRC and why it is worse on the left side. Research parts of colon later While left-sided colon cancer is more predominant in EOCRC, right-sided EOCRC is associated with lower overall survival compared to left-sided EOCRC (44% vs 61%)[20]. Several factors have been implicated in the difference in survival between right-sided and left-sided CRC. Marx O, Mankarious M, Yochum G. Molecular genetics of early-onset colorectal cancer. World J Biol Chem 2023; 14(2): 13-27 [PMID: 37034132 DOI: 10.4331/wjbc.v14.i2.13] Why does left side colorectal cancer have a lower survival rate? (44 percent to 61) ● During embryonic development, the proximal colon originates

	from the midgut while the distal colon originates from the hindgut. This developmental difference may impact cancer cell origins as well as the metastatic potential of tumors due to differences in vascularization. ● several microbiota changes have been characterized between the proximal and distal colon which may play a role in oncogenesis ● Proximal colonic tumors also have distinct histopathological features as they tend to be more mucinous with microsatellite instability and mismatch repair (MMR) deficiency compared to distal tumors In my own words: ● There are many steps of embryonic development: the germinal stage which is from fertilization to implantation (I actually think this may be wrong so we will cme back to that but during that stage the) proximal ● This means there may be molecular drivers of distal and proximal EOCRC tumors!! We need to investigate these. In genetics and bioinformatics, a single-nucleotide polymorphism is a germline substitution of a single nucleotide at a specific position in the genome that is present in a sufficiently large fraction of considered population molecular mechanisms involved in colorectal cancer (CRC) supports three main molecular pathways. The almost classical chromosomal instability (CIN) pathway is based on the seminal publication of Vogelstein and contains most of the kirsten rat sarcoma viral oncogene homolog (KRAS) mutated CRCs. The mismatch repair deficient or microsatellite instable (MSI) pathway was discovered through elucidation of the gene mutations responsible for Lynch syndrome and is characterized by a hypermutating state and frequent B-Raf proto-oncogene, serine/threonine kinase (BRAF) V600E mutation. The CpG island methylator phenotype (CIMP) pathway goes along with the occurrence of serrated precursor lesions and is also strongly related to the MSI pathway, notably through frequent methylation of the mutL homolog 1 promoter, which confers MSI-high status. I also researched more about DNA and RNA itself, so I could understand the CMS (consensus model). I researched epithelial cells too and how they may drive colorectal cancer progression. I have gained a grasp on basic

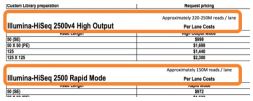
	nucleic acid function.
Nov. 10th, 2023	Today I researched about how I could research epithelial cells function and the genes they express that cause them to allow colorectal cancer to progress by looking into DNA sequencing. DNA IS TRANSCRIBED INTO MRNA, RNA, TRNA. Using rna seq u isolate rna esp mrna in tissues n take a snapshot of tissue. Every cell in body have same dna but become different cells because of the expresion. So we wanna know what genes are being expressed at this point. We cannot do protein profiling because we do not have the technology. So RNA sequencing is the closest we can get to seeing what genes are being expressed currently. Why not micro array? ● Have to preselect probe u wanna use ● Transcripts u can detect, u have a target ● W rna seq no preselect, just openly look at the genes in the cells and u can discover novel transcrips. ● Broader range of dynamic detection ● SOme genes have no expression – ● Higher specificity. Question will be specific so data should be too. What are differentially expressed genes between control n variable And what r the pathways involved? Can explain differences in disease progression 

Compare different groups at different times

SEQUENCING DEPTH:

High output or rapid mode

High output

220 million 

U don't need much depth if ur just finding differentially expressed genes

Look at literature and see how much reads they got.

SINGLE END:

PAIR END:

SMORNA – ISN GLE IS ENOUGH

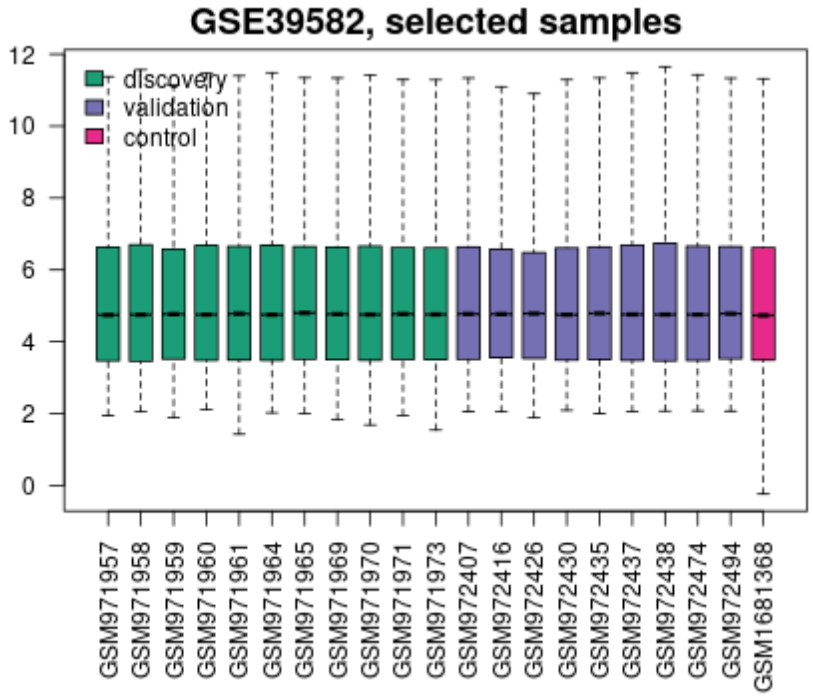
MRNA U NEED PAIR END

Double end reduces false positives because there is a chance it only mathces one end of the read

1. Which genes do these reads belong to?
- How many reads align to a specific gene.

Not that we know that, we want to know if the different sample groups express genes differentially.

Not much flexibility

	FASTQ – header- then sequence Base qualities: ● Computer how confidence it is to call it an A. ● Higher confidence/lower error score the higher quality score: Downlaod reference genome and map your read to that genome Ensembl https://useast.ensembl.org/info/data/ftp/index.html Fasta and GTF – annotation file- chromosone x to position __ __ wjether it is x on or protein seq Chromosome number, then position, plus string or minor string and then gene id Created a specific research question: "How does the Consensus Molecular Subtype (CMS) classification in colorectal cancer, primarily derived from bulk transcriptomics, correlate with the single-cell transcriptomic profiles of epithelial cell diversity within tumors?"
Nov. 12th, 2023	Attempted to utilize the GEO ANALYZR tool. Did not work. 

	Decided to research alternative ways to find DEGs. Learned that a gene is declared differentially expressed if an observed difference or change in read counts or expression levels between two experimental conditions is statistically significant.
Nov. 13th, 2023	I downloaded the SRA toolkit, a way to find DEGs and a way to analyze RNA sequencing data.
Nov. 14th, 2023	Learned about the NCBI bioproject database through endlessly searching about epithelial cells. Very, very helpful as it has possible articles and just very recent projects with raw data. Here were my top picks: https://www.ncbi.nlm.nih.gov/bioproject/PRJEB55156 https://www.ncbi.nlm.nih.gov/Traces/study/?uids=29535734%2C29535735%2C29535736%2C29535745%2C29535744%2C29535743%2C29535742%2C29535741%2C29535740%2C29535739%2C29535738%2C29535737&o=acc_s%3Aa https://trace.ncbi.nlm.nih.gov/Traces/?view=run_browser&acc=ERR10026303&display=metadata https://trace.ncbi.nlm.nih.gov/Traces/?view=study&acc=ERP140037 Metastatis https://www.ncbi.nlm.nih.gov/bioproject/PRJEB53814 https://www.ncbi.nlm.nih.gov/bioproject/PRJNA759644 Circulating tumor cells uscs Settled on this one: https://www.ncbi.nlm.nih.gov/bioproject/PRJEB64127 Single cell sequencing of colorectal tumors and adjacent non-malignant colon tissue Single cell RNA-sequencing has been applied to core and border regions of 9 colorectal tumors as well as to matched adjacent non-malignant colon tissue for the purpose of generating a cellular map of colorectal tumors and their tumor microenvironment. Actually it was single not paired ugly rat liers so im changing it

- Objective of the Study: The study aims to validate the genomic-guided predictive efficacy of immunotherapy in colorectal cancer and understand the interactions between tumor cells and immune-system cells.
- Patient Information: Two treatment-naive (not yet received treatment colorectal cancer patients were recruited. One patient had MSI-high/TIB-high, and the other had POLE-mutant/TMB-UH. These patients received a single dose of pembrolizumab.
- Sampling: Paired endoscopic biopsies of the tumor and adjacent normal tissue were collected. This sampling was done at baseline and after two courses of neoadjuvant therapy.
- Sequencing Method: Single cell sequencing was performed to analyze the genomic information at the single-cell level.
- Treatment: Patients received a single dose of pembrolizumab, which is an immunotherapy drug.
- Monitoring: The study involves monitoring the patients' response to the treatment by analyzing changes in the genomic profiles of tumor and normal tissues.

This type of study can provide valuable insights into the effectiveness of i

Identifying Molecular and genetic factors influencing response and spread of colorectal cancer patients with single cell rna sequencing analysis.
https://www.ncbi.nlm.nih.gov/Traces/study/?query_key=44&WebEnv=MCID_65539021ecadc040f54b08dc&o=acc_s%3Aa&s=SRR22998014,SRR22998015,SRR22998016,SRR22998017,SRR22998018,SRR22998019,SRR22998020,SRR22998021,SRR22998022,SRR22998023,SRR22998024,SRR22998025

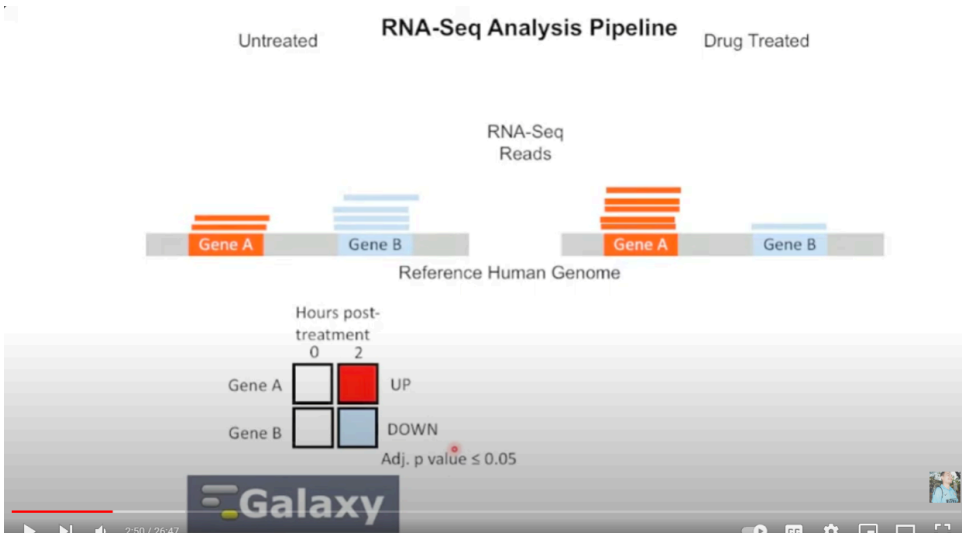
https://www.ncbi.nlm.nih.gov/bioproject/PRJNA919183

So I wanna still do something on the spread. I can do that later but yeah this can be very helpful.

https://www.ncbi.nlm.nih.gov/books/NBK546616/#:~:text=Pembrolizumab%20is%20an%20FDA%2Dapproved,advanced%20melanoma%20in%20September%202014.

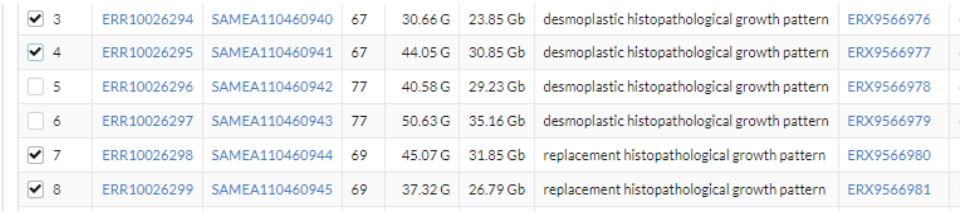
So pembrolizuma is what was induced.

https://pubmed.ncbi.nlm.nih.gov/37146911/

	Pembrolizumab is an immunotherapy that basically supresses the PD-1 expression so that the cancer cells cannot hide from the T cells.  So we see if it is upregulated or downregulated. If there is adiff the p value will increase. Gene differentiatiopn will be apparent and dependent on upregulation of genome or mutation. By using rna sequencing at the single cell we can identify specific mutations that are specific to the tissue. We have sigmoid cancer and the rectal one so there may be a difference in expression based on response to KEYDANA. Found a very helpful tutorial on using a website called Galaxy for RNA sequencing analysis: https://www.youtube.com/watch?v=KVh98S89yUU&ab_channel=Arman Ghodsinia Uploaded data from https://www.ncbi.nlm.nih.gov/bioproject/PRJEB64127
Nov. 18th, 2023	Data finished uploaded today and I preformed FASTQC quality control. Data was alright quality, but some were low so I preformed trimmomatic to remove any reads under 20 PHRED score.
Nov. 19th, 2023	Finished trimmomatic. Had to rerun a few times due to errors, but now onto HISAT2. Again had to rerun for SRAS ending with 036+, but got it working eventually.

Nov. 20th, 2023	Downloaded the gtff3 reference annotation and preformed stringtie, then stringtie merge Now another round of stringtie to actually align the reads to new reference file created by my data.
Nov. 26th, 2023	Renaming files to prepare them for DESEQ2 (actually shows me the log2fc and the DEGs. However, I made a fatal mistake. I did not separate the sigmoid progressive and the rectal total response. Will now be separating them I have actually two experiments so im gonna repeat this process for each of my experiments control will be compared to the sigmoid progressive and the rectal response First one is sigmoid progressive compared to control I created a new history to separate sigmoid progressive so I must delete everything from stringtie merge step and up luckily it didn't take anymore space which is miracalous idk how to spell leabe me alone ok im so happy rn I couild cry Okay im doing stringtie merge for sigmoid progressive Now im switching to rectal complete response Okay ive done both. Next ill do stringtie for both using my BAM files. Now second stringtie for sigmoid progressive Done sigmoid progressive
Nov. 30th, 2023	Renamed gene counts files.
Dec. 4th, 2023	So my pc1 was like 94 I'm rerunning just in case, but honestly it may be good its saying its all due to one factor, Ok tomorrow doing annotate deseq2 but im honestly feeling unmoored sad because I'm unsure if I messed up. Might be avoiding it because I don't wanna reveal how absolutely terribly I messed up. Ok lets just need to research a lot more like what everything would mean and how to interpret.

	• Comparison 1: Sigmoid Cancer (PD) vs. Control (Rectal Adjacent Normal):
	  ○ GSM6919590 (Sigmoid Cancer, PD)
	  ○ GSM6919589 (Rectal Adjacent Normal)
	• Comparison 2: Rectal Cancer (CR) vs. Control (Rectal Adjacent Normal):
	  ○ GSM6919588 (Rectal Cancer, CR)
	  ○ GSM6919589 (Rectal Adjacent Normal)
	That was my experiment

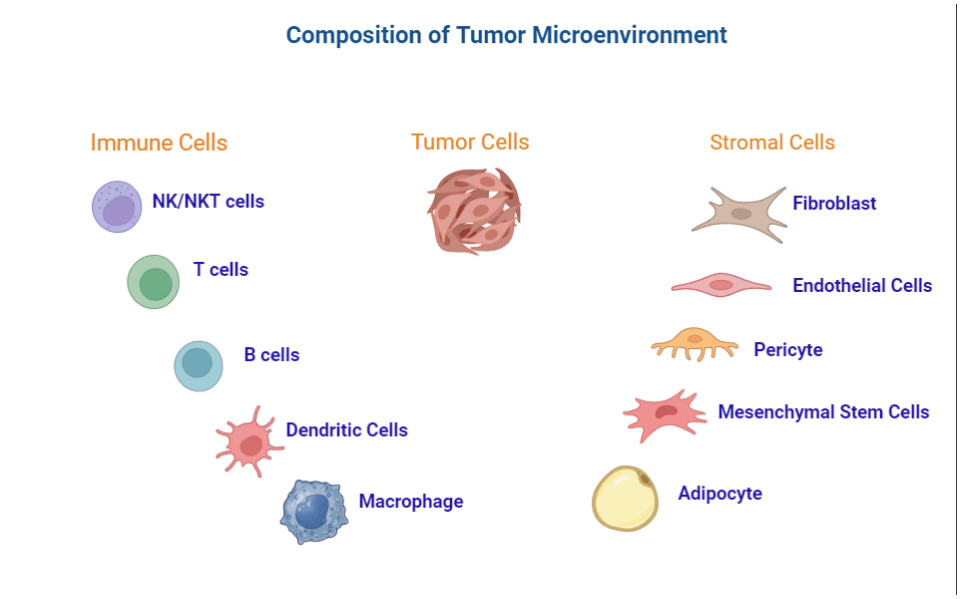
Dec. 8th, 2023	My suspicions were correct and my issue was that the rectal adjacent could not be a control. As these are all separate patients, it would be illogical to try to compare to a separate control. The data was pretty low quality too. The data and results are unusable. I must find another one. So SRA accession 12/8/2023 from https://www.ncbi.nlm.nih.gov/Traces/study/?query_key=12&WebEnv=MCID_657397bf32988251df3498ec&o=acc_s%3Aa&s=ERR10026298,ERR10026299 Bioproject https://www.ncbi.nlm.nih.gov/bioproject/PRJEB55156 Im doing  For now but I may redo w the rest later
Dec. 9th, 2023	Decided on another data set that investigates the overexpression of ADA and PDK1 on CAR T cells https://www.ncbi.nlm.nih.gov/Traces/study/?query_key=47&WebEnv=MCID_65748ba6b7719f7e04fd290c&o=acc_s%3Aa

☑ 3	ERR10026294	SAMEA110460940	67	30.66 G	23.85 Gb	desmoplastic histopathological growth pattern	ERX9566976
☑ 4	ERR10026295	SAMEA110460941	67	44.05 G	30.85 Gb	desmoplastic histopathological growth pattern	ERX9566977
☐ 5	ERR10026296	SAMEA110460942	77	40.58 G	29.23 Gb	desmoplastic histopathological growth pattern	ERX9566978
☐ 6	ERR10026297	SAMEA110460943	77	50.63 G	35.16 Gb	desmoplastic histopathological growth pattern	ERX9566979
☑ 7	ERR10026298	SAMEA110460944	69	45.07 G	31.85 Gb	replacement histopathological growth pattern	ERX9566980
☑ 8	ERR10026299	SAMEA110460945	69	37.32 G	26.79 Gb	replacement histopathological growth pattern	ERX9566981

	So basically they genetically altered t cells and They want to explore the possibility of using genetic reprogramming to modify T cells. Specifically, they aim to take advantage of a common mechanism that cancer cells use to suppress the function of T cells. This suppression occurs by creating an unfavorable metabolic environment in the tumor, known as the tumor microenvironment (TME). This environment hinders the ability of t cells to attack (TME CONSISTS OF RAPIDLY PROLIFERATING CANCER CELLS, it is heterogenous so contains immune, stomal, and tumor cells) always involving. (healthy cells are infiltrating the tumor mass) Researchers mention that they conducted an "in silico screen." "In silico" refers to computer-based simulations or experiments. So, they used computer-based methods to screen and identify two specific genes, ADA and PDK1, as metabolic regulators. These genes are believed to play a role in the metabolic processes that influence the tumor microenvironment. Mutations occuring have a direct impact on nature and function of immune cells (may express detectable antigens and the immune cells will take note and be able to locate and destroy or can create an immunosuppressive microenvirnment Wnt singling /β-catenin pathway which limit infiltration of cytotoxic t cells, or once intered tumor a mutation can supress immune activation. Car t cell limits: Cytokin release syndrome https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6003181/ Immune system floods the bloodstream with cytokines (they control cell growth and neurotoxicity. Cytokin release syndrom has been helped with IL-6 receptor blockade but neurotoxicity has not been combatted both still can get out of hand Steroids are not targetted Resistance to car t cell therapy: solid tumors – rarely see objective and meaningful responses and complete remission for solid tumors due to

tumor microenvironment being so heterogenous

https://molecular-cancer.biomedcentral.com/articles/10.1186/



What is car t cell therapy?

Immune system is split into specific and nonspecific

Innate (non specific) is the quickest response by immune system not specific to any type of infection

Adaptive: b and t cells are fundamental to adaptive.

B cells: humoral mediated immunity (fluids of body) (b cells secrete antibodies to circulate in blood and other parts. If it reaches cell it is ineffective

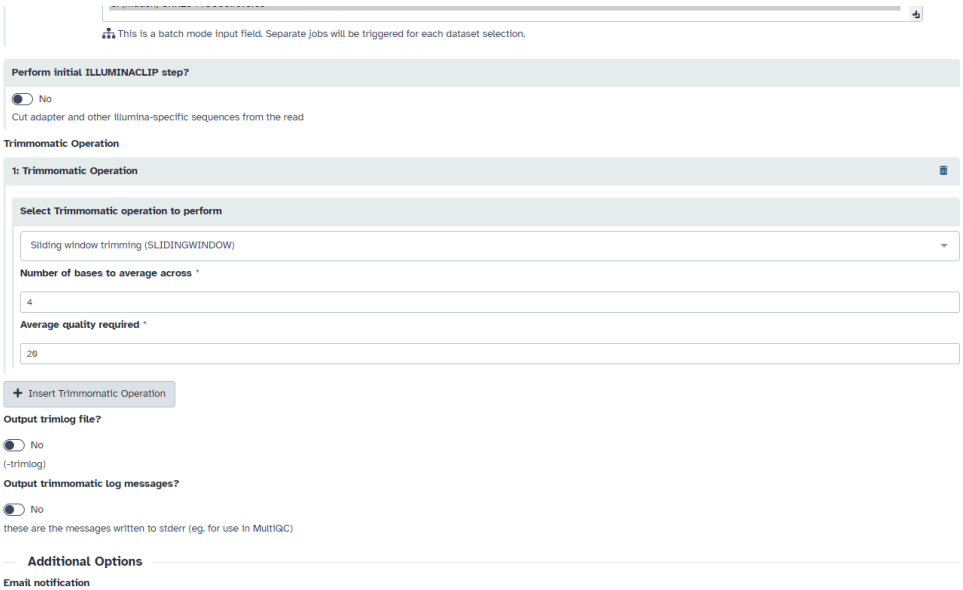
- Secrete antibodies (proteins that bnind to bacteria and viruses prevents infections from spreading. Blocks bacteria from growing colonies. In viruses, bind to the viruses and prevent it from inflintrating cells (OPSINOZATION) marks bacteria or viruses for destruction (innate)

T cells: cell mediated immunity – protect body from infections that reach the inside cell (kill affected cell)

If a t cell finds and T cellr ecepter (TCR on surface) recepts it it can release cytotoxic molecules killing the cell

Same t cell may be infecctive and unable to bind and release cytotoxic molecules. Can onyl respond to one type of bivurs.

	T cells signal other immune cells B cells and NK to assist in immune response Cytotoxic T cell: CD8+ CD8 glycoprotein Recognize antigens attached to MHC class 1 molecule. Most infected patogens express MHC class 1 so the t cell recognizes it. On a hunt for cancer cells Find antige CD8 cytotoxic T cells can kill other cells CD8 can kill cancer cells Adaptive provides through body. T cells: white blood cells that Disease causing agents are pathogens Adaptive is focused on one specific pathogen Antigen binding site: binding site is where the cell can bind to on antibody different each antibodyt ype Cancer cells have antigens that our T Cells receptors are unable to recognize as abnormal and thus destroy, so they are unable to fight against cancer cells. Chimeric antigen receptor T cells are genetically engineered to contain a receptor shaped to bind to cancer kills and kill them.
Dec. 10th, 2023	Performed FASTERQ dump, now completed, did FASTQC for quality control and read up on what makes good data so it look all good. As for ADA same thing. Very high quality – both usually have phred scores of 36/40 which is insanely good Doing trimmomatic now

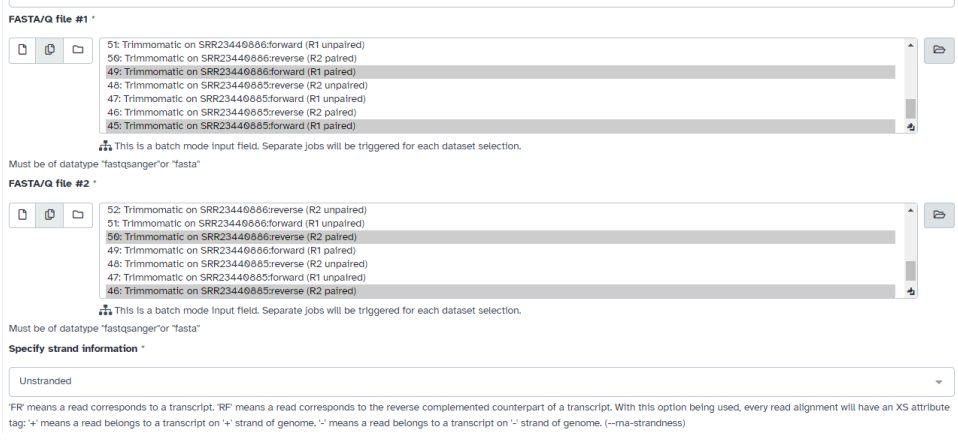


Put in my FORWARD in R1 (odd) and REVERSE in R2 (even) default parameters. (PDK1)

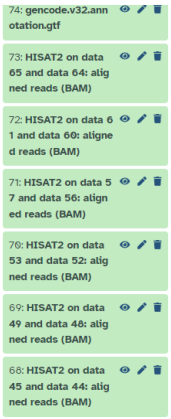
ADA IS ODD IS REVERSE AND EVEN IS FORWARD

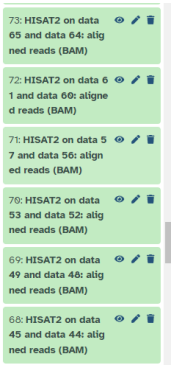
Sequence alignment – HISAT ON PDK1 OE first

Reference genome – hg38 is being aligned to 😊



HISAT2 guide – SHOULD BE FORWARD REVERSE

	Ran both for PDK1 and ADA Done HISAT2 now doing stringtie Stringtie complete now stringtie merge Having issues with my second stringtie
Dec. 16th, 2023	So SRA start at 118 and goes 73-68: 118 – data 73 SRR23440893 ADA 3 121 – data 72 SRR23440892 ADA 2 124 – data 71 SRR23440891 ADA 1 127 – data 70 SRR23440890 CONTROL 3 130 – data 69 SRR23440889 CONTROL 2 133 – data 68 – 45 and 44 SRR23440888 CONTROL 1  PDK1 OE NAMING 112 – data 72 SRR23440889 CONTROL 2 115 – data 71 SRR23440888 CONTROL 1 118 – data 70 SRR23440887 PDK1 3 121 – data 69 SRR23440886 PDK1 2 124 – data 68 SRR23440885 PDK1 1 127 – data 73 SRR23440890 CONTROL 3

	 DESEQ 2 FOR PDK1 AND ADA OE
Dec. 17th, 2023	Put data in excel. Since I did not utilize stringtie to avoid alignment errors and over annotation, I must convert ENSEMBL IDs to official gene symbols. Using https://www.biotools.fr/human/ensembl_symbol_converter to achieve this. On ADA OE data: A B C D E F G gene id base mea l2fc stderror waldstat pval adpval I am filtering ADPVAL (Benjimini method) to only show me statistically significant values (0.05). Deselecting NA A B C D 1 gene id l2fc adpval 2 ENSG0000 9.558072 2.22E-64 3 ENSG0000 3.330522 2.22E-64 4 ENSG0000 3.078075 8.05E-53 5 ENSG0000 3.785398 2.75E-46 6 ENSG0000 2.135811 3.70E-35 7 ENSG0000 3.035397 7.43E-33

	Made a new table to make life easier only contains gene ID, l2fc, and adpval Filtering the l2fc that are now only greater than 0 to find upregulation Gonna use GPROFILER First though I must copy my codes and define the actual gene. Maybe novel isoforms. 575 DEGS in total to research.
Dec. 18th, 2023	I was unable to identify 75 upregulated ADA OE genes. Possibly novel isoforms as human genome is very well annotated.

Dec. 26th, 2023	SO COULD FIND 319 UPREG! DOWNREG –759 originally – 448 after Lets study pathways now FOR PDK1 Example from PDK1 upregulation – the conversion. So lost some bcs they have been unannotated For ADA Upreg - 155 Downreg – 204 Made the cut Originally – upreg – 241 Downreg – 322 https://www.genome.jp/kegg/pathway.html what was used to understand the pathways Ok now im gonna research more about ADA and PDK1 and how it helps for now then pathways all of them we will analyze create a table w most

	uprefgulated and functions and genespart of an upregulated pathway and their functions Ok nvm lets see the Top 10 Most Upregulated Genes: https://rgd.mcw.edu/rgdweb/report/gene/main.html?id=16570585 Used this to learn about genes and novel isoforms.
Dec. 28th, 2023	Created tables with top 10 most upregulated and down regulated genes for both ADA and PDK1 and began researching their functions.
Dec. 29th, 2023	Decided to uninclude novel isoforms as they were irrelevant to study as I was unable to research them. They were marked as N/A. Made tables with p values.
Dec. 30th, 2023	Continued to research functions of genes and added a section of research onto the function of the gene in the context of colorectal cancer and cognate leukemia (the PDK1 scope).
Jan. 1st, 2024	A bit more of the article from the bioproject in which the data I accessed was released, so I decided to read up on it and understand fully how they preformed their experiment, their results, and their interpretations. It is a preprint, so I had to be wary.
Jan. 2nd, 2024	Began pathway enrichment analysis using gProfiler. PATHWAY ANALYSIS Enriched Pathways (ADA) Up reg Cell Cycle: P val 0.036 Down reg Metabolic Pathways: p val 0.042 Pathways Enriched in Biological Processes of Upregulated Genes From ADA Overexpressed Car-T Cells Pathways Enriched of Upregulated Genes From ADA Overexpressed Car-T Cells Using KEGG Analysis

Jan. 4th, 2024	Continued pathway analysis using gProfiler and tried to interpret my results better. Additionally, I created an abstract: Chimeric antigen receptor (CAR) T cell therapy has been widely successful in treating patients with hematological malignancies, due to their homogenous tumor nature allowing a single antigen to be targeted by CAR-T cells. Conversely, solid tumors, like those formed with colorectal cancer, have heterogenous phenotypes, genetic material profiles, and proteins among the affected area and patients themselves, making it difficult to identify a single target antigen, and prevent immunosuppression within the metabolically unfavorable tumor microenvironment. Colorectal cancer is the third most common cancer globally, predominantly affecting individuals 50 and older due to the extensive growth time of colorectal polyps. However, colorectal cancer incidence has significantly increased for Canadians under 50, underscoring the importance of discovering effective treatment. The data obtained for this project is derived from Renauer et al's study which aimed to genetically modify T cells to combat a tumor-intrinsic evasion mechanism in which cancer cells utilize a metabolically unfavorable tumor microenvironment (TME) to suppress T cell function. Using an in silico screen, they identified Pyruvate Dehydrogenase Kinase 1 (PDK1) and Adenosine deaminase (ADA) as metabolic regulators, as gene overexpression (OE) strengthened the cytolysis of CD19-specific CD8 CAR-T cells against leukemia cells, with PDK1 or ADA deficiency decreasing the cytolysis. Moreover, ADA-OE in α-HER2 CAR-t cells was found to increase proliferation, memory, improve cancer cytolysis, and decrease exhaustion when tested on in vivo human colorectal cancer tumors. In turn, ADA-OE improved tumor infiltration and clearance by α-HER2 CAR-t cells. By identifying the pathways and genes associated with the success of ADA-OE α-HER2 CAR-T cells in eliminating colorectal adenocarcinomas, and other solid tumors, identifying potential therapeutic targets for the treatment of colorectal adenocarcinomas and other solid tumors is possible. This project identifies differentially expressed genes and enriched pathways associated with ADA-OE α-HER2 CAR-T cells in comparison to CD19-speicifc CD8 CAR-T cells, through RNA sequencing analysis, and determines their role in the enhanced anti-colorectal cancer tumor response observed. RNA sequencing analysis was also performed on PDK1-engineered CAR-T cells, and instead the differentially expressed genes and enriched pathway's role in enhanced antitumor behavior were studied in the context of colorectal cancer and cognate leukemia. I also outlined my steps for the rest of the write up: Introduction:

	● Car t cell therapy and how it is like created the cells ● Issues with solid tumor ● Ada and pdk1 and why they were overexpressed (like what specifically, more than metabolic regulation) ● HER2 and its effectivity and why ada was expressed with it ● PDK1 and cognate leukemia ● A bit on RNA sequencing analysis Methodology ● Workflow ● WHy I made certain decisions ● Everything Quality Assurance/Control ● Not using the stringtie b cs human genome is already well annotated, so i can avoid misalignment ● Utilized Trimmomatic tool to ensure reads were high quality ● FASTQC quality control results (most 36/40 on PHRED score) Results ● Discussion ● Implications ● Basically what's next
Jan. 5th, 2024	Finished pathway enrichment analysis and researched in context of cancer and anti-tumor behavior as well as cell proliferation and cell cycle maintenance. PDK1 Pathways Enriched in Molecular Functions of Downregulated Genes From PDK1 Overexpressed Car-T Cells Steroid Binding 0.003129 Protein Binding 0.00387 Modification-Dependent Protein Binding 0.02305 K63-Linked Polyubiquitin Modification-Dependent Protein Binding 0.02325

	Pathways Enriched in Biological Processes of Downregulated Genes From PDK1 Overexpressed Car-T Cells Organelle Organization 0.006436 Organonitrogen Compound Metabolic Process 0.01646 Regulation of Primary Metabolic Process 0.01678 Regulation of Nitrogen Compound Metabolic Process 0.04759 Cellular Components With Enriched Pathways of Downregulated Genes From PDK1 Overexpressed Car-T Cells Cytoplasm 0.00000000000000000000000000004072 Nucleocytoplasm 0.0000002064 Pathways Enriched in Molecular Functions of Upregulated Genes From PDK1 Overexpressed Car-T Cells Protein Binding 0.00006696 Oxidoreduction-Driven Active Transmembrane Transporter Activity 0.01289 Pathways Enriched in Biological Processes of Upregulated Genes From PDK1 Overexpressed Car-T Cells Mitotic Cell Cycle Process 0.007895 Response to Stress 0.001204 Organonitrogen Compound Metabolic Process 0.01284 Phosphorylation 0.01293 Phosphate-Containing Compound Metabolic Process 0.01418 Localization 0.01508 Vesicle-Mediated Transport 0.01519 Phosphorus Metabolic Process 0.01875 Intracellular Signal Transduction 0.02401 Positive Regulation of Cellular Process 0.03159 Cellular Components With Enriched Pathways of Upregulated Genes From PDK1 Overexpressed Car-T Cells Cytoplasm 0.000000000000954 Cytosol 0.000004751

	Organelle Membrane 0.003366 Respiratory Chain Complex 0.00474 Intracellular Anatomical Structure 0.005268 Endomembrane System 0.005785 Inner Mitochondrial Membrane Protein Complex 0.007565 Respirasome 0.01075 Envelope 0.01607 Organelle Envelope 0.01607 Sarcoplasm 0.03412 Cytochrome Complex 0.03543 Mitochondrion 0.04103 Membrane 0.04116 Cell Projection 0.04139 Mitochondrial Respirasome 0.04784 Pathways Enriched of Upregulated Genes From PDK1 Overexpressed Car-T Cells Using KEGG Analysis Cardiac muscle contraction 0.001035 Chemical carcinogenesis - reactive oxygen species 0.02459 Oxidative phosphorylation 0.0309 Diabetic cardiomyopathy 0.0438 Outlined my methodology: Cardiac muscle contraction 0.026 Method – Found data on NCBI gene database ----> Sent to GALAXY ----> FASTQC dump -----> FASQC Quality Control ----> Trimmomatic ----> HISAT ----> featureCounts ----> deseq2 ----> g:Profiler and DAVID to convert Ensembl IDs to gene names (did not quantify reads using stringtie or utilize DESEQ2 annotation to avoid faulty alignment, quantification, and annotation. Additionally, featureCounts is more accurate than StringTie for pair-ended reads, but many choose to utilize stringTie as DESEQ2 annotation is compatible and they do not require an external source to convert their Ensembl IDs to gene symbols). ---> g:Profiler for pathway enrichment analysis. —> NCBI gene database to analyze individual genes —> KEGG pathway database
Jan. 6th, 2024	Realized some of my assertions and conclusions about pathways and genes were not the best - it is okay if overexpression is occurring of pathways and genes that usually help tumor growth, as this is a localized cell and the cell's expression. It is not on the tumor's effect on the cell, so I need to ensure I understand something I labeled as not very effective or less

	effective/promoting tumor growth may be quite contrary as it may assist in the actual proliferation of the cell. Began working on further researching the 40 top DEGs and interpreting them.
Jan. 7th, 2024	Took a break from researching the DEGs and decided to focus on them while completing my write up. I edited the abstract to include citations and began on introduction. Aiming to finish the introduction and methodology today. Possibly quality assurance.
Jan. 11th, 2024	Left write up because I realized it is less important and easier to synthesize. Finding other ways to interpret and represent my data as its the main component of my work.
Jan. 21st, 2024	Consulted my friend's dad, a bioinformatic, regarding how I should interpret my data. He said if I have already done visualization (already done by GALAXY) I should focus on interpreting my results.
Jan. 26th, 2024	Again interpreting my data and realized that it does have a slight correlation to colorectal cancer spread if it downregulates genes that relate to its progression, as local cells affect the global gene landscape. Created lists of functions and implications of my top 10's, and will in-detail interpret. I also created a game plan for what I have to get done now to complete this project: - Refine list with implications to ensure it is comprehensive - Confirm that surrounding cells affect global gene expression and write a small blurb about it and find a credible source to confirm - Finish write up - Understand how the data is visualized and be able to explain it well - Start working on poster - Print out posters - Practice presentations
Jan. 27th, 2024	Finally finished all the functions and implications, and will compile later today and research a bit more to see if I'm missing anything. Then, I will continue write up.
January 30th - February 4th, 2024	Decided I was not detailed enough and wanted to really understand my results by juicing out as much information as possible. Continued interpreting my 40 genes, and looking in the context of CRC, the tumor microenvironment, T cell efficiency, and global gene landscape. Example

	of one: HECW2, an integral component of the E3 ubiquitin ligases, intricately regulates neural crest cell function as a regulator of glial cell line-derived neurotrophic factor. E3 ubiquitin ligases, including HECW2, are recognized for their multifaceted roles in modulating protein stability, degradation, and cellular responses. HECW2 has been found to be prognostic in colorectal cancer progression, because of its ability to mediate the ubiquitin-proteasome degradation of lamin B1, which activates AKT/mTOR signalinghttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC10539569/ . The AKT/mTOR signaling pathway has been an indicator of colorectal cancer because of its role in regulating proliferation, invasion and the metabolism of cells in the tumor microenvironmenthttps://pubmed.ncbi.nlm.nih.gov/31215384/ . On the other hand, lamin B1 is crucial in inhibiting colorectal cancer progression through senescence, and increasing their sensitivity to immune responseshttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC9321645/ . This means the downregulation of HECW2 may be linked to the effectiveness of ADA overexpressed CAR T cells. Moreover, HECW2 has been linked to promoting resistance to drug treatments like chemotherapy, which may be involved in similar mechanisms used by CAR T cellshttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC10539569/ .
February 6th - 7th, 2024	Finished pathway analysis and determined novel methods for T cell activation, and continued proliferation and thriving through novel metabolism methods aimed to conserve energy. My school fair is the 23rd, so I am cutting it pretty close. I must finish my trifold design first, but I am making posters so I just need to print them all out.
February 11th, 2024	Created the title for my project: Unraveling the Success of ADA and PDK1 Overexpressed CAR T cells in Clearing Colorectal Adenocarcinoma Through RNA Sequencing Analysis. I also began looking for how I will create my trifold. I will do a flow chart methodology, and this is the layout: First Section: Abstract Background Information Objectives Second Section Guiding Question Methodology ● Data, pipeline, etc

	Results Last: Analysis Conclusion Sources of error What's Next?
February 14th, 2024	Measured trifold to create posters (31x60) made my posters 30x60. Made them half (15x30) and two for each
February 14th-19th, 2024:	Designing tri fold posters. Aim to print them out by the 20th so I can begin sticking them on. Need to print out log book, as well.
February 21st, 2024	Sticking posters on trifold, and practicing pitch for school fair. Finished compiling citations.

February 23rd, 2024	School fair! Chosen to attend CYSF, and given valuable advice on presentation. Will work to refine my trifold before the fair. The days preceding the fair will just be updating my portal and my trifold.

Research on Top 10 Most Upregulated DEGs:

PDK1 upreg:

PRAL (Non-Annotated Long Intergenic Non-Protein Coding RNA):
PRAL, a non-annotated long intergenic non-protein coding RNA, participates in the modulation of chromatin structure, RNA stabilization, and transcriptional regulation. Upregulation of PRAL within PDK1-overexpressed CAR T cells implies an enhanced orchestration of these molecular processes. This heightened expression could lead to a more refined chromatin structure, stabilized RNA molecules, and precise transcriptional regulation. Downregulation of PRAL has been observed as a potential biomarker for oral and lung cancer (both solid tumors), which may be indicative of PDK1-overexpressed CAR T cells success in clearance. The optimized molecular environment created by PRAL upregulation potentially translates to improved gene expression control. Additionally, PRAL inhibited hepatocellular carcinoma growth, translating to success across solid tumors. Huang, Z., Zhou, J. K., Peng, Y., He, W., & Huang, C. (2020). The role of long noncoding RNAs in hepatocellular carcinoma. Molecular cancer, 19(1), 77 . https://doi.org/10.1186/s12943-020-01188-4

PARPBP (Predicted to Enable DNA Binding):
PARPBP, predicted to enable DNA binding, assists in negatively regulating double-strand break repair through homologous recombination. The upregulation of PARPBP in PDK1-overexpressed CAR T cells signifies an orchestrated enhancement in DNA binding processes, contributing to a more controlled genomic maintenance mechanism. This heightened expression potentially optimizes the negative regulation of double-strand break repair, promoting enhanced efficiency in maintaining genomic stability. The upregulation of PARPBP may foster a cellular environment with reduced error rates in DNA repair. However, its hypomethylation was found to cause resistance towards the oxaliplatin drug in colorectal cancer patients https://pubmed.ncbi.nlm.nih.gov/34508743/. Further investigation is required to determine if PARPBP is involved in unfavorable prognosis of leukemia and colorectal cancer, but currently, it is not. By exerting a negative regulatory influence on repair processes, PARPBP could play a crucial role in minimizing errors and aberrations, bolstering survival gene expression and cytolytic functions in the context of colorectal cancer treatment.

EEF1A1 (Eukaryotic Elongation Factor 1 Complex Isoform A1):

EEF1A1, an isoform of the Eukaryotic Elongation Factor 1 Complex, is intricately involved in the enzymatic delivery of aminoacyl tRNAs to the ribosome. The upregulation of EEF1A1 in PDK1-overexpressed CAR T cells denotes a strategic elevation in the cellular machinery responsible for protein synthesis. This orchestrated increase in EEF1A1 expression suggests an optimization of translation processes, potentially enhancing the overall efficiency of protein synthesis within the CAR T cells. The heightened levels of EEF1A1 may contribute to an increased rate of aminoacyl tRNA delivery, supporting robust and sustained protein production essential for the cytolytic functions and survival gene expression.Although EEF1A1 is observed to prevent the inhibition of proliferation and cell cycle block in many cancers including colorectal cancer, its overexpression on the cellular level is unlikely to promote cancer growth, and the PDK1 overexpressed CAR T cells may be utilizing a successful tumor growth mechanism to promote their own proliferation. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9844609/ . Additionally, EEF1A1 is essential in successful T cell activity, and its downregulation contributes to weakened functionhttps://journals.aai.org/jimmunol/article/209/6/1189/234140/A-Rapid-Translational-Immune-Response-Program-in .

MT-RNR1 (Mitochondrially Encoded 12S RNA):

MT-RNR1, responsible for encoding mitochondrial 12S ribosomal RNA, plays a pivotal role in various cellular processes, including DNA binding and transcription factor binding activity. In the context of PDK1-overexpressed CAR T cells, the upregulation of MT-RNR1 indicates an enhancement in cellular processes associated with mitochondrial activity. The increased expression of MT-RNR1 potentially optimizes mitochondrial functions , leading to improved cellular energetics and phosphate metabolic processes. This optimization in mitochondrial activity aligns with the broader goal of PDK1-overexpressed CAR T cells, where enhanced energy production is critical for sustaining the metabolic demands associated with cytolytic functions and prolonged T cell survival. Furthermore, the upregulation of MT-RNR1 may contribute to a more controlled regulation of cellular processes. The mitochondria plays roles in lipid synthesis, calcium regulation, signaling, and cell cycle progression. In the context of T cells, they contribute in an anabolic way to provide materials for activation, clonal expansion, and differentiation of T cells https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8002030/.

MAPKAPK3 (MAP Kinase-Activated Protein Kinase 3):
MAPKAPK3, encoding a protein within the Ser/Thr kinase family, operates as a mitogen-activated protein kinase (MAP kinase)-activated protein kinase. In the context of PDK1-overexpressed CAR T cells, the upregulation of MAPKAPK3 suggests an orchestrated enhancement in kinase activity, contributing to effective T cell functionhttps://www.ncbi.nlm.nih.gov/gene/7867 . The heightened kinase activity may lead to optimized signal transduction cascades, influencing and promoting cellular processes associated with proliferation, determination, and differentiationhttps://www.ncbi.nlm.nih.gov/gene/7867 .This optimized signaling aligns with the therapeutic goals of PDK1-overexpressed CAR T cells, as effective signal transduction is vital for robust T cell responses against colorectal cancer cells. Additionally, MAPKAPK3 prevents CAR T cell exhaustion and death as it, along with MAPKAPK2, positively regulates

starvation-induced macroautophagy by adding a phosphate group to the crucial ATG protein, Beclin 1https://elifesciences.org/articles/05289 . With MAPKAPK3 upregulated in both ADA and PDK1 overexpressed CAR T cells, it is likely a leading reason for the increased efficiency of their function and should be investigated in its potential for CAR T cell genetic design.

SECTM1 (Transmembrane Protein in Hematopoietic and Immune System Processes):
SECTM1, encoding a transmembrane protein with predicted involvement in hematopoietic and immune system processes, demonstrates upregulation in PDK1-overexpressed CAR T cells. This upregulation suggests an orchestrated enhancement in SECTM1 expression, contributing to the modulation of immune responses. The transmembrane nature of SECTM1 implies its role in cellular communication and signal transduction. The elevated expression in PDK1-overexpressed CAR T cells indicates an optimized immune response regulation. SECTM1's broad expression in specific tissues signifies a targeted impact, potentially influencing the cellular microenvironment during the combat against colorectal cancer. Moreover, SECTM1 is associated with positive immunotherapy response, implying it has largely positive effects on global gene expressionhttps://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=DetailsSearch&Term=6398. This is due to its strong correlation in the costimulation of CD8 T cell proliferation and induction of IFN-γ productionhttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC32893999/ . IFN-γ primarily activates macrophages to enhance their phagocytic activity, tumoricidal capabilities, and ability to internally eliminate pathogens is vital for promoting anti-tumor behavior https://www.sciencedirect.com/topics/neuroscience/interferon-gamma#:~:text=A%20primary%20role%20for%20IFN,reactive%20oxygen%20and%20nitrogen%20intermediates. .

MLX (Regulator of Proliferation, Determination, and Differentiation):
MLX, a gene involved in lipid and glucose metabolism, contributing to proliferation, determination, and differentiationhttps://www.alliancegenome.org/gene/HGNC:11645 , exhibits upregulation in PDK1-overexpressed CAR T cells. The upregulation of MLX implies an orchestrated enhancement in its regulatory role, influencing cellular processes crucial for anti-cancer activity. MLX forms heterodimers with Mad proteins, and its upregulation may contribute to a more controlled and targeted regulation of proliferation and differentiation pathways by effectively processing energy and lipids, which contribute to T cell proliferation https://www.alliancegenome.org/gene/HGNC:11645. This heightened regulatory activity aligns with the desired therapeutic goals of PDK1-overexpressed CAR T cells. The upregulation of MLX signifies an optimized cellular response to stimuli, contributing to enhanced functionality and anti-cancer effects in PDK1-overexpressed CAR T cells.

TBC1D17 (Regulator of GTPase Activity and Retrograde Transport):
TBC1D17, a gene predicted to enable GTPase activator activity and involved in retrograde transport within cells, demonstrates upregulation in PDK1-overexpressed CAR T cells https://www.ncbi.nlm.nih.gov/gene/79735. This upregulation suggests an orchestrated

enhancement in the regulation of cellular processes associated with intracellular transport and GTPase activity. The heightened GTPase activator activity may contribute to a more controlled modulation of retrograde transport, ensuring efficient movement of cellular components within PDK1-overexpressed CAR T cells. Additionally, the involvement of TBC1D17 in retrograde transport signifies a potential impact on intracellular dynamics of PDK1-overexpressed CAR T cells, enhancing their adaptability and responsiveness.

PDK1 (3-Phosphoinositide-Dependent Protein Kinase 1):
PDK1, a pivotal kinase in cellular signaling pathways, undergoes upregulation in PDK1-overexpressed CAR T cells. This upregulation plays a crucial role in enhancing T cell metabolism, cytolysis of cancer cells, and the expression of survival genes. This is showcased in the context of CD28, a corereceptor on T cells, and T cell antigen receptors require PDK1 to integrate their signaling in order to activate the T cells. When PDK1 was removed, TCR-CD28 signals could not induce NF-κB activation or protein kinase C θ phosphorylation, which are essential for T cell activation. Upregulation of PDK1 leads to an optimized metabolic state within CAR T cells and positive global gene expression as a metabolic regulator, ensuring a robust and sustained energy supply for their anti-cancer activities. The increased expression of survival genes further supports the longevity and efficacy of these CAR T cells in the challenging tumor microenvironment. Consequently, the upregulation of PDK1 in CAR T cells not only enhances their immediate cytolytic capabilities but also contributes to sustained functionality, fostering a more effective and durable response against colorectal cancer.

HELLPAR (Long Intergenic Non-Protein Coding RNA):
HELLPAR, a long intergenic non-protein coding RNA, experiences upregulation in PDK1-overexpressed CAR T cells. This upregulation assumes a pivotal role in chromatin remodeling, RNA stabilization, and transcription regulation https://www.ncbi.nlm.nih.gov/gene/101101692. The heightened expression of HELLPAR signifies an orchestrated enhancement in these molecular processes, fostering a more controlled gene expression profilehttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC1820769/ . This optimized chromatin structure ensures precise transcriptional regulation, contributing to a finely-tuned and efficient cellular environment within PDK1-overexpressed CAR T cells. The upregulation of HELLPAR, with its involvement in RNA stabilization and transcriptional control, potentially amplifies the anti-cancer efficacy of these CAR T cells https://www.nature.com/articles/nrm.2017.104. Moreover, HELLPAR was overexpressed in ADA overexpressed CAR T cells as well, serving as a potential biomarker for successful CAR T cell activity for leukemia and colorectal cancer.

Downreg PDK1

MTERF4:
Function and Implications:
MTERF4 is involved in RNA binding and plays a predicted role in rRNA transcriptional regulation and processing. Downregulation of MTERF4 in ADA-overexpressed CAR T cells suggests an

optimized environment for transcriptional activity. Dysregulation in RNA binding and transcriptional control can lead to cellular stress and dysfunction. By downregulating MTERF4, ADA-overexpressed CAR T cells aim to maintain a more controlled gene expression profile, reducing the potential harmful effects associated with uncontrolled transcriptional activity https://pubmed.ncbi.nlm.nih.gov/21531335/ . This refined transcriptional regulation could contribute to improved cellular function. However, loss of MTERF4 also leads to a decrease in mitochondrial translation, which is essential to increased mitochondrial mass and function of CD8* T cells during a time when endogenous or exogenous pyrogens elevate the body's thermoregulatory set-point https://www.pnas.org/doi/10.1073/pnas.2023752118#:~:text=Through%20genetic%20and%20pharmacological%20approaches,cells%20exposed%20to%20febrile%20temperature . Downregulation may be indicative of damage being caused by the PDK1 overexpression, and explains why it was ineffective against colorectal cancer, as blood cancers are easier to treat.

PTGER2:
Function and Implications:
PTGER2 encodes a receptor for prostaglandin E2, and PTGER2 is associated with microsatellite instability (MSI)-high in colorectal cancerhttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC2837535/ . In the context of ADA-overexpressed CAR T cells treating colorectal cancer, downregulation of PTGER2 signifies a strategic modulation of prostaglandin E2 signaling https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2837535/ . MSI-high is linked to increased tumor cell proliferation, angiogenesis, and evasion of immune surveillance in colorectal cancer. Moreover, PTGER2 was found to suppress cytotoxic T lymphocyte survival and functionalityhttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC4505619/ . This is heightened when cells are affected by viruses, as it is upregulated during viral infiltration, causing the cytotoxic T cells to deteriorate https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4505619/ . By downregulating PTGER2, ADA-overexpressed CAR T cells aim to avoid activating these pathways, enhancing their anti-cancer efficacy. The intentional disruption of prostaglandin signaling contributes to a targeted and controlled cellular response, potentially minimizing harmful effects associated with unregulated signaling in the tumor microenvironment.

SMG6:
Function and Implications:
SMG6 encodes a component of the telomerase ribonucleoprotein complex, playing a vital role in replicating and maintaining chromosome ends. In ADA-overexpressed CAR T cells designed to combat colorectal cancer, downregulation of SMG6 offers a deliberate adjustment in telomerase and chromosome maintenancehttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC2440797/ . This fine-tuning aims to optimize the balance between cell proliferation and survival, contributing to an environment where ADA-overexpressed CAR T cells can efficiently target cancer cells. SMG6 has a secondary role in the nonsense-mediated mRNA decay (NMD) pathway, which provides endonuclease activity near premature translation termination codon required for initiating NMD, which prevents the translation of mutant mRNA's with these premature termination codonshttps://pubmed.ncbi.nlm.nih.gov/19060897/ . NMD has been observed to

suppress immune response, which suggests its downregulation can lead to an optimized and broader mRNA landscapehttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC2440797/ . This landscape could contribute to several positive outcomes, as it allows the preferential translation of mRNAs encoding proteins that are crucial for effective anti-cancer responses. Additionally, the modulation of NMD through SMG6 downregulation might lead to increased translation of specific immune-related genes, potentially enhancing the overall immune response.

TSC22D1-AS1:
Function and Implications:
TSC22D1-AS1 is a non-coding gene intricately involved in gene expression regulation, chromatin remodeling, and modulation of protein function. In the context of ADA-overexpressed CAR T cells for colorectal cancer treatment, the downregulation of TSC22D1-AS1 is harmful and promotes colorectal tumor progression when globally downregulated by inducing cell proliferationhttps://www.sciencedirect.com/science/article/abs/pii/S0006291X18304170 . This is consistent in other forms of cancer as the TSC22 domain family has been a cancer suppressor gene. https://www.sciencedirect.com/science/article/abs/pii/S0006291X18304170 , and is likely a side effect of PDK1 overexpression in CAR T cells.

LINGO3:
Function and Implications:
LINGO3, predicted to be active in the extracellular matrix and space, plays a crucial role in cell membrane compositionhttps://www.proteinatlas.org/ENSG00000220008-LINGO3 . Its down regulation in PDK1 overexpressed CAR T cells is negative, as LINGO3 functions in the intestine to regulate mucosal tissue regeneration and the normal intestinal structure https://pubmed.ncbi.nlm.nih.gov/33941035/. This is important to
maintain proper barrier function and absorption of nutrients in the gastrointestinal tract.

ACAD8:
Function and Implications:
ACAD8, encodes genetic information for a member of the acyl-CoA dehydrogenase group that catalyzes the dehydrogenation of acyl-CoA derivatives during fatty acid metabolism for cellular energy. In the context of ADA-overexpressed CAR T cells combating colorectal cancer, the downregulation of ACAD8 is negative, as ACADS are downregulated in colorectal cancer tissues, and ACADS expression is positively associated with B cells, CD4+ T cells, CD8+ T cells, **M1 macrophages, neutrophil**s, and
Tregshttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC8579304/. ACAD8, and ACAD groups in general can become a therapeutic target for CAR T cells, and are prognostic for tumor progressionhttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC8579304/.

NUDCD2:
Function and Implications:
NUDCD2's main function is encoding a receptor for prostaglandin E2, which serves as a metabolite of arachidonic acid. It is also predicted to be capable of binding to unfolded proteins,

and protein folding in the cytosol, intercellular bridge, and mitotic spindle. While this downregulation might suggest a modulation of protein folding processes. Although there is little information regarding NUDCD2 and prognosis in the context of colorectal cancer, it is overexpressed in Head and Neck squamous cell carcinoma, a similar solid cancer, implying it may have detrimental effects to colorectal cancer progression and thus was silenced for more effective immune response NUDCD2 is overexpressed in head and neck squamous cell carcinoma and is involved in nuclear division. Additionally, unfolded protein response, activated when excess unfolded proteins accumulate in the endoplasmic reticulum, increases cancer cell viability and survival during critical moments**https://rdcu.be/dxNma**. As NUDCD2 is downregulated, it will bind less to unfolded proteins, and as the unfolded protein response is critical in the endoplasmic reticulum, its potential benefits are nullified **https://rdcu.be/dxNma**. NUDCD2 is downregulated in both ADA overexpressed CAR T cells and PDK1 overexpressed CAR T cells, implying its suppression is crucial for effective CAR T cell activity.

PIGF:
Function and Implications:
PIGF provides the genetic information for 2 proteins involved in **GPI-anchor biosynthesis**, is a gene with potential implications for ADA-overexpressed CAR T cells in the context of treating colorectal cancer. PIGF was found to allow the development of resistance to antiangiogenic treatment of colorectal tumors, and is being looked at as a possible therapeutic target to enhance treatmenthttps://pubmed.ncbi.nlm.nih.gov/31608707/. Moreover, PIGF has been involved in many hematological malignancies, including the pathogenesis of leukemia and tumor cell proliferationhttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC5916812/. Downregulation of PIGF suggests a modulation in GPI-anchor biosynthesis processes. This alteration may influence the composition of cellular membranes and their interactions. In the specific environment of ADA-overexpressed CAR T cells, this could contribute to a cellular milieu less conducive to colorectal cancer progression.

TENM1:
Function and Implications:
TENM1, a gene expressed in neurons and possibly acting as a cellular signal transducer, holds significance in the landscape of ADA-overexpressed CAR T cells targeting colorectal cancer. The downregulation of TENM1 could impact cellular signaling, particularly in the context of immune response and anti-cancer activity. TENM1 was recurrently mutated in colorectal cancer tumor tissue, and it's family, the teneurins, are being investigated as potential treatment targetshttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC7383341/. Furthermore, TENM1 and its teneurin family were found to be involved in drug resistance, and tumor initiation and progression in many cancers, including leukemia https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7956758/.

DDIT4-AS1:
Function and Implications:

DDIT4-AS1 is a non-coding gene involved in gene expression regulation, chromatin remodeling, and modulation of protein function, and plays a crucial role in regulating gene expression within ADA-overexpressed CAR T cells. However, its overexpression has been associated with colorectal cancer metastasis, and more aggressive tumorshttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC8191213/. Additionally, it is generally associated with cancer stem cells by interacting with other RNA molecules and influencing their global gene expressionhttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC9915130/.

ADA UPREG

HEMK1:
Function and Implications:
HEMK1 works in the methyl repair system and mismatch repair system of DNA by catalyzing the transfer of methyl groups to DNA and plays a crucial role in maintaining genome stability. Tumors displaying MSI high instability had a deficiency in the mismatch repair system, creating an elevation in mutation frequency, as the mismatch repair system corrects replication errors in synthesized DNA, as well as prevents the combination of homeologous DNA sequencinghttps://www.sciencedirect.com/science/article/abs/pii/S1568786418303094. The mismatch repair system also directs cell cycle checkpoint and apoptosis activation to different types of DNA damage https://www.sciencedirect.com/science/article/abs/pii/S1568786418303094 .This may mean that ADA overexpressed CAR T cells require this increased ability to silence and repair newly synthesized DNA, as without it, the CAR T cell is prone to avoidable exhaustion and harmful mutations. Moreover, many cancer types utilize the mechanisms HEMK1 is capable of to allow for effective metabolism, including cellular detoxification, cell and tissue growth, neurotransmitter synthesis, and gene expressionhttps://pubmed.ncbi.nlm.nih.gov/20920744/ . ADA overexpressed CAR T-cells potentially benefit from this methylation as the TMI's dynamic environment can be combatted through the silencing of tumor progression genes https://www.nature.com/articles/nrd2974 . This may imply that ADA overexpressed CAR T cells owe their efficiency to methylation allowing them to recognize harmful sequences and thereby silence them without affecting surrounding, healthy sequences. Methylation capabilities are also important in sustaining memory in CD4 T and CD8 T cells, as well as human lymphocytes in general, allowing them to recognize and therefore destroy previously encountered pathogens, improving immune response https://clinicalepigeneticsjournal.biomedcentral.com/articles/10.1186/s13148-022-01399-0. Unique DNA methylation patterns are also observed in terminally differentiated effector memory CD8 T cells (TEMRA) compared to other CD8 T memory cell subtypes, emphasizing the role of DNA methylation in defining distinct memory cell populationshttps://clinicalepigeneticsjournal.biomedcentral.com/articles/10.1186/s13148-022-01399-0 . As methylation is an epigenetic mechanism utilized by CAR T cells post operation and integration into the patient's body, to prevent exhaustion, its upregulation allows the cells to employ this mechanism readily and avoid exhaustion https://www.nature.com/articles/s41375-023-01966-1.

ADA:
Function and Implications:
ADA (Adenosine Deaminase), encoding an enzyme catalyzing the hydrolysis of adenosine to inosine, plays a pivotal role in the purine catabolic pathway. In ADA-overexpressed CAR T cells, the increased activity of ADA leads to a higher rate of adenosine breakdown. This surge in ADA activity contributes to the regulation of purine levels, potentially creating an unfavorable environment for cancer cells. Additionally, ADA is known to promote the proliferation of T, B, and NK lymphocytes. The upregulation of ADA in CAR T cells reinforces the immune response, facilitating robust anti-cancer activity. The concerted action of ADA, by both regulating purine metabolism and enhancing immune cell proliferation, synergistically supports the effectiveness of CAR T cells in combating colorectal cancer.

MAPKAPK3:
Function and Implications:
MAPKAPK3, encoding a protein within the Ser/Thr kinase family, operates as a mitogen-activated protein kinase (MAP kinase)-activated protein kinase. In the context of PDK1-overexpressed CAR T cells, the upregulation of MAPKAPK3 suggests an orchestrated enhancement in kinase activity, contributing to effective T cell functionhttps://www.ncbi.nlm.nih.gov/gene/7867 . The heightened kinase activity may lead to optimized signal transduction cascades, influencing and promoting cellular processes associated with proliferation, determination, and differentiationhttps://www.ncbi.nlm.nih.gov/gene/7867 .This optimized signaling aligns with the therapeutic goals of PDK1-overexpressed CAR T cells, as effective signal transduction is vital for robust T cell responses against colorectal cancer cells. Additionally, MAPKAPK3 prevents CAR T cell exhaustion and death as it, along with MAPKAPK2, positively regulates starvation-induced macroautophagy by adding a phosphate group to the crucial ATG protein, Beclin 1https://elifesciences.org/articles/05289 . With MAPKAPK3 upregulated in both ADA and PDK1 overexpressed CAR T cells, it is likely a leading reason for the increased efficiency of their function and should be investigated in its potential for CAR T cell genetic design.

EEF1A1:
Function and Implications:
EEF1A1, an isoform of the Eukaryotic Elongation Factor 1 Complex, is intricately involved in the enzymatic delivery of aminoacyl tRNAs to the ribosome. The upregulation of EEF1A1 in PDK1-overexpressed CAR T cells denotes a strategic elevation in the cellular machinery responsible for protein synthesis. This orchestrated increase in EEF1A1 expression suggests an optimization of translation processes, potentially enhancing the overall efficiency of protein synthesis within the CAR T cells. Moreover, heightened levels of EEF1A1 may contribute to an increased rate of aminoacyl tRNA delivery, supporting robust and sustained protein production essential for the cytolytic functions and survival gene expression. Although EEF1A1 is observed to prevent the inhibition of proliferation and cell cycle block in many cancers including colorectal cancer, its overexpression on the cellular level is unlikely to promote cancer growth, and the PDK1 overexpressed CAR T cells may be utilizing a successful tumor growth mechanism to promote their own proliferation. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9844609/ .

Additionally, EEF1A1 is essential in successful T cell activity, and its downregulation contributes to weakened function https://journals.aai.org/jimmunol/article/209/6/1189/234140/A-Rapid-Translational-Immune-Response-Program-in . As EEF1A1 was upregulated in both PDK1 overexpressed CAR T cells and ADA overexpressed CAR T cells, it likely is a result of the distinct overexpression, and can be attributed to their success in clearing tumors. Understanding EEF1A1's function in the context of exhaustion, and more in depth regarding development is crucial when determining its potential for CAR T cell genetic design.

HBB:
Function and Implications:
HBB, which encodes the beta chains in hemoglobin, is a critical component in determining the structure of polypeptide chains in hemoglobin. Upregulation of HBB is often associated with promoting cell survival. In this scenario, ADA overexpression potentially assists in the increased production of HBB, leading to improved cell viability. HBB, the gene encoding beta chains in hemoglobin, is typically downregulated in cancer patients. However, its upregulation, as observed in ADA-overexpressed CAR T cells, has been linked to promoting cell survival. The increased expression of HBB may contribute to enhanced cellular resilience and viability. This could be attributed to HBB's involvement in maintaining proper oxygen transport, crucial for sustaining cellular processes. Moreover, HBB and HBA1, both involved in hemoglobin synthesis have been observed as tumor suppressor genes, as they have been observed downregulated in acute myeloid leukemia, as they inhibited proliferation, induced apoptosis, and silenced cell cycle processes at the G2/M phase, in tumor cells https://www.tandfonline.com/doi/full/10.1080/16078454.2022.2117186 . Tumor cells are heavily reliant on the G2/M phase to pause the cell cycle process in order to repair DNA damagehttps://www.tandfonline.com/doi/full/10.1080/15384101.2021.1922806. As for colorectal cancer, upregulation of the cell division associated 5 process in general, and specifically the G2/M phase has been associated with poor prognosis and is highly expressed, meaning HBB upregulation can leave tumor cells vulnerable, and unable to perform cell integrity processes, allowing the ADA overexpressed CAR T cells to clear them efficiently.

HELLPAR:
HELLPAR, a long intergenic non-protein coding RNA, experiences upregulation in PDK1-overexpressed CAR T cells. This upregulation assumes a pivotal role in chromatin remodeling, RNA stabilization, and transcription regulation https://www.ncbi.nlm.nih.gov/gene/101101692. The heightened expression of HELLPAR signifies an orchestrated enhancement in these molecular processes, fostering a more controlled gene expression profilehttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC1820769/ . This optimized chromatin structure ensures precise transcriptional regulation, contributing to a finely-tuned and efficient cellular environment within PDK1-overexpressed CAR T cells. The upregulation of HELLPAR, with its involvement in RNA stabilization and transcriptional control, potentially amplifies the anti-cancer efficacy of these CAR T cells

https://www.nature.com/articles/nrm.2017.104. Moreover, HELLPAR was overexpressed in ADA overexpressed CAR T cells as well, serving as a potential biomarker for successful CAR T cell activity for leukemia and colorectal cancer.

MTREX:
MTREX, enables ATP binding and acts as an RNA helicase, participating in RNA catabolic processes by responding to cellular DNA damage, and is localized in the nucleoplasm. In ADA-overexpressed CAR T cells designed for combatting colorectal cancer, the upregulation of MTREX suggests a regulation of RNA turnover. The enzymatic activity of MTREX, as an RNA helicase, contributes to the unwinding of RNA structures, facilitating their degradation. This controlled RNA degradation process is beneficial as it allows the cell to promptly eliminate unwanted or damaged RNA molecules. Additionally, disrupting nuclear RNA catabolism leads to defects in RNAPII elongation, decreased expression of long genes, and a dedifferentiation state characterized by defects in cell identity and developmental potency https://www.cell.com/molecular-cell/pdf/S1097-2765(23)00903-6.pdf . RNAPII elongation is important for mRNA synthesis, and the regulation of gene expression https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/rna-polymerase-ii . This means MTREX's role in RNA catabolism appears to be a core regulatory module that safeguards important cellular processes, including RNAPII activity, expression of endogenous retroviruses (ERVs), and maintenance of cell identity during embryonic development https://www.cell.com/molecular-cell/pdf/S1097-2765(23)00903-6.pdf .

MLX: (Regulator of Proliferation, Determination, and Differentiation):
MLX, a gene involved in coding a member of the helix-loop-helix leucine zipper (bHLH-Zip) transcription factor family that is involved in lipid and glucose metabolism. These proteins also assist in proliferation, determination, and differentiationhttps://www.alliancegenome.org/gene/HGNC:11645 , exhibits upregulation in PDK1-overexpressed CAR T cells. The upregulation of MLX implies an orchestrated enhancement in its regulatory role, influencing cellular processes crucial for anti-cancer activity. MLX forms heterodimers with Mad proteins, and its upregulation may contribute to a more controlled and targeted regulation of proliferation and differentiation pathways by effectively processing energy and lipids, which contribute to T cell proliferation https://www.alliancegenome.org/gene/HGNC:11645. This heightened regulatory activity aligns with the desired therapeutic goals of PDK1-overexpressed CAR T cells. The upregulation of MLX signifies an optimized cellular response to stimuli, contributing to enhanced functionality and anti-cancer effects in PDK1-overexpressed CAR T cells.

CYP1B1-AS1, an enzyme within the cytochrome family, plays a crucial role in the breakdown of drugs and the synthesis of specific lipids. Upregulation of CYP1B1-AS1 in ADA-overexpressed CAR T cells may influence drug metabolism and lipid production. This could potentially contribute to the modulation of the cellular microenvironment, creating conditions that favor the anti-cancer activity of ADA-overexpressed CAR T

cellshttps://www.researchgate.net/figure/Upregulation-of-CYP1B1-AS1-inhibits-cell-proliferation-and-induces-apoptosis-a_fig3_373480558. It was observed that CYP1B1-AS1 was significantly downregulated in breast cancer, and inhibited cancer cell proliferation and induced apoptosis, partly by inhibiting neddylation https://www.researchgate.net/figure/Upregulation-of-CYP1B1-AS1-inhibits-cell-proliferation-and-induces-apoptosis-a_fig3_373480558. Neddylation modulates many essential biological processes, allowing for tumorigenesis, and is globally overexpressed in the tumor microenvironment https://molecular-cancer.biomedcentral.com/articles/10.1186/s12943-019-0979-1 .

MPPE1, predicted to enable GPI anchor binding and involved in the GPI anchor biosynthetic process, plays a critical role in attaching specific proteins to the cell membrane. Upregulation of MPPE1 in ADA-overexpressed CAR T cells suggests an increased capacity for anchoring proteins to the cell membrane. This heightened activity may influence the overall composition of the cell membrane and contribute to alterations in cellular signaling and interactions. Although MPPE1 has been found prognostic for melanoma tumor progression, it is unrelated to colorectal cancer progression, and therefore analysis into its cellular role becomes pivotal https://www.proteinatlas.org/ENSG00000154889-MPPE1/pathology . Proteins involved in GPI anchor binding were found to allow the phosphorylation and palmitoylation of Linker for Activation (LAT) of T cells, in T cells https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5835848/ . This protein is crucial in cell signaling, and it requires **phosphorylation** and **palmitoylation** to localize and function https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5835848/ . Moreover, LAT activity and GPI anchor binding proteins were found to synergize the proliferation of Jurkat cells, an immortalized line of T cell lymphocytes https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5835848/ .

ADA DOWNREG

GIGYF2:
GIGYF2, encoding a protein with multiple polyglutamine (a chain of amino acids where the amino acid glutamine is repeated multiple times in a row) stretches, plays a role in cellular processes influenced by polyglutamine-containing proteins. Polyglutamine expansions are linked to alterations in the cellular environment, impacting cellular processes such as signal transduction, protein folding, and GIGYF2 is involved in regulating these processes.
This modulation may lead to nuanced changes in the cellular landscape, potentially affecting the dynamics of signaling cascades or protein-protein interactionshttps://pubmed.ncbi.nlm.nih.gov/20670374/ . Polyglutamine expansions are considered detrimental for disease progression and tumor progression, and decreased CRE-mediated transcription, which is vital for gene expressionhttps://academic.oup.com/hmg/article/10/17/1829/642975 . By downregulating GIGYF2, a protein involved in these processes, negative effects such as aggregate formation, caspase-dependent cell death, and decreased neurite outgrowth can be mitigatedhttps://academic.oup.com/hmg/article/10/17/1829/642975 . By strategically influencing

polyglutamine-associated cellular processes, GIGYF2 may assist in creating an environment that hinders colorectal cancer progression, but it remains non prognostic in most cancers, and little research is related to its role it T cells and the tumor microenvironmenthttps://www.proteinatlas.org/ENSG00000154889-GIGYF2/cell+line .

MTERF4:
MTERF4, a gene allowing RNA binding, is predicted to have roles in rRNA transcriptional regulation and processing, as well as mitochondrial transcription and ribosome assembly. Dysregulation in RNA binding and transcriptional control can lead to cellular stress and dysfunction. By downregulating MTERF4, ADA-overexpressed CAR T cells aim to maintain a more controlled gene expression profile, reducing the potential harmful effects associated with uncontrolled transcriptional activityhttps://pubmed.ncbi.nlm.nih.gov/21531335/. The downregulation of MTERF4 in ADA-overexpressed CAR T cells indicates a targeted impact on RNA-related processes, potentially altering cellular bioenergetics and protein synthesishttps://www.sciencedirect.com/science/article/abs/pii/S0006291X1530173X . As MTERF4 is involved in the assembly of mitochondrial ribosomes, its downregulation is contextually beneficial, as it can prevent proliferation in cancer cells by limiting energy productionhttps://www.sciencedirect.com/science/article/abs/pii/S1044579X17300962?via%3Dihub.

PTGER2:
PTGER2 encodes a receptor for prostaglandin E2, and PTGER2 is associated with microsatellite instability (MSI)-high in colorectal cancer https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2837535/ . In the context of ADA-overexpressed CAR T cells treating colorectal cancer, downregulation of PTGER2 signifies a strategic modulation of prostaglandin E2 signalinghttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC2837535/. MSI-high is linked to increased tumor cell proliferation, angiogenesis, and evasion of immune surveillance in colorectal cancer. Moreover, PTGER2 was found to suppress cytotoxic T lymphocyte survival and functionalityhttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC4505619/. This is heightened when cells are affected by viruses, as it is upregulated during viral infiltration, causing the cytotoxic T cells to deterioratehttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC4505619/. By downregulating PTGER2, ADA-overexpressed CAR T cells aim to avoid activating these pathways, enhancing their anti-cancer efficacy. The intentional disruption of prostaglandin signaling contributes to a targeted and controlled cellular response, potentially minimizing harmful effects associated with unregulated signaling in the tumor microenvironment.

NUDCD2:
NUDCD2's main function is encoding a receptor for prostaglandin E2, which serves as a metabolite of arachidonic acid. It is also predicted to be capable of binding to unfolded proteins, and protein folding in the cytosol, intercellular bridge, and mitotic spindle. While this downregulation might suggest a modulation of protein folding processes. Although there is little information regarding NUDCD2 and prognosis in the context of colorectal cancer, it is overexpressed in Head and Neck squamous cell carcinoma, a similar solid cancer, implying it

may have detrimental effects to colorectal cancer progression and thus was silenced for more effective immune response NUDCD2 is overexpressed in head and neck squamous cell carcinoma and is involved in nuclear division. Additionally, unfolded protein response, activated when excess unfolded proteins accumulate in the endoplasmic reticulum, increases cancer cell viability and survival during critical moments**https://rdcu.be/dxNma**. As NUDCD2 is downregulated, it will bind less to unfolded proteins, and as the unfolded protein response is critical in the endoplasmic reticulum, its potential benefits are nullified **https://rdcu.be/dxNma**.

HECW2:
HECW2, an integral component of the E3 ubiquitin ligases, intricately regulates neural crest cell function as a regulator of glial cell line-derived neurotrophic factor. E3 ubiquitin ligases, including HECW2, are recognized for their multifaceted roles in modulating protein stability, degradation, and cellular responses, but overexpression has been found to increase genomic instability, which is negative https://www.sciencedirect.com/science/article/pii/S0167488918300922 . HECW2 has been found to be prognostic in colorectal cancer progression, because of its ability to mediate the ubiquitin-proteasome degradation of lamin B1, which activates AKT/mTOR signalinghttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC10539569/ . The AKT/mTOR signaling pathway has been an indicator of colorectal cancer because of its role in regulating proliferation, invasion and the metabolism of cells in the tumor microenvironmenthttps://pubmed.ncbi.nlm.nih.gov/31215384/ . On the other hand, lamin B1 is crucial in inhibiting colorectal cancer progression through senescence, and increasing their sensitivity to immune responseshttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC9321645/ . This means the downregulation of HECW2 may be linked to the effectiveness of ADA overexpressed CAR T cells. Moreover, HECW2 has been linked to promoting resistance to drug treatments like chemotherapy, which may be involved in similar mechanisms used by CAR T cellshttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC10539569/ .

KCTD20:
KCTD20 is involved in exhibiting the ability for identical protein binding and the positive regulation of phosphorylation processes. Phosphorylation is the addition of a phosphoryl group to an ion, and it is used in cellular storage and the transfer of available energy. KCTD20 is also involved in promoting the Akt/mTOR signaling pathway by binding to all of its isoforms, promoting its phosphorylation and therefore functionhttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC3827329/ . This implies that KCTD20 may be involved in regulating death and growth, and its downregulation is to prevent the CAR T cells from entering an exhausted state or activation-induced cell deathhttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC3827329/, https://link.springer.com/article/10.1007/s13577-022-00670-z . Additionally, KCTD20 is being investigated as an unfavorable prognostic factor for colorectal cancer, and assisting in its progressionhttps://www.proteinatlas.org/ENSG00000112078-KCTD20/pathology/colorectal+cancer . BTBD10, a gene with functions extremely similar to KCTD20 that has been determined as an unfavorable biomarker for most solid cancers, increases proliferation of cancer cells and invasion, which may be similar to how KCTD20 increases colorectal cancer progressionhttps://www.frontiersin.org/articles/10.3389/fmolb.2021.762541/full .

LINGO3:
LINGO3 is predicted to be active in the extracellular matrix and space, and plays a crucial role in cell membrane composition https://www.protein atlas.org/ENSG00000220008-LINGO 3. ADA overexpression influencing the downregulation of LINGO3 might impact the structural integrity of the extracellular matrix and cellular membranes. This modulation can potentially alter cellular interactions, affecting processes like adhesion and communication. Considering LINGO3's involvement in the cell membrane, its downregulation may influence cell surface interactions and signaling. Although there is limited information regarding LINGO3's involvement in colorectal cancer, its function in composing cell membrane may be the reason for its suppression, as "membrane therapy" has emerged to suppress cancer growth factors by regulating signaling and regulate transport of materials in and out of the cellshttps://www.jlr.org/article/S0022-2275(21)00006-7/fulltext . Constant composition may disrupt this process, and suppression may regulate aberrant cell construction found in colorectal cancer https://www.jlr.org/article/S0022-2275(21)00006-7/fulltext.

ACAD8:
ACAD8, encodes genetic information for a member of the acyl-CoA dehydrogenase group that catalyzes the dehydrogenation of acyl-CoA derivatives during fatty acid metabolism for cellular energy. In the context of ADA-overexpressed CAR T cells combating colorectal cancer, the downregulation of ACAD8 is negative, as ACADS are downregulated in colorectal cancer tissues, and ACADS expression is positively associated with B cells, CD4+ T cells, CD8+ T cells, **M1 macrophages, neutrophil**s, and Tregshttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC8579304/. ACAD8 is also favorably prognostic in many solid tumors, including colorectal tumors. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8579304/.

MXRA8:
MXRA8 is predicted to contribute to establishing the neuroglial blood-brain barrier, located in the extracellular exosome, enabling beta-galactosidase function and participating in carbohydrate metabolism. The downregulation of MXRA8 suggests a potential modulation of immune-related processes and interactions with the tumor microenvironment. The expression of MXRA8 is associated with CD8+ T cell infiltration, specifically in colorectal cancer, as it is involving in cancer-related signaling processes https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9871988/. Furthermore, it led to metastasis through enabling cell migration and maintaining tumor purityhttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC9871988/ . The downregulation implies that ADA overexpressed CAR T cells successfully suppressed MXRA8's function, and may be partly how they were able to clear colorectal tumors and limit progression (cite original study).

GLB1L3 is predicted to involve beta-galactosidase activity by catalyzing the hydrolysis of lactose into glucose and galactose, and is also predicted to be involved in carbohydrate metabolism. This involvement in carbohydrate metabolism suggests that GLB1L3 downregulation may limit the availability of essential nutrients for tumor growth, potentially hindering the energy production processes within cancer cells

https://www.mdpi.com/2075-4418/12/10/2309. Moreover, beta-galactosidase activity is known as a tumor biomarker, as its increased activity correlates with the activity of malignant cells, and is two times more overexpressed in colorectal cancerhttps://www.mdpi.com/2075-4418/12/10/2309.

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