

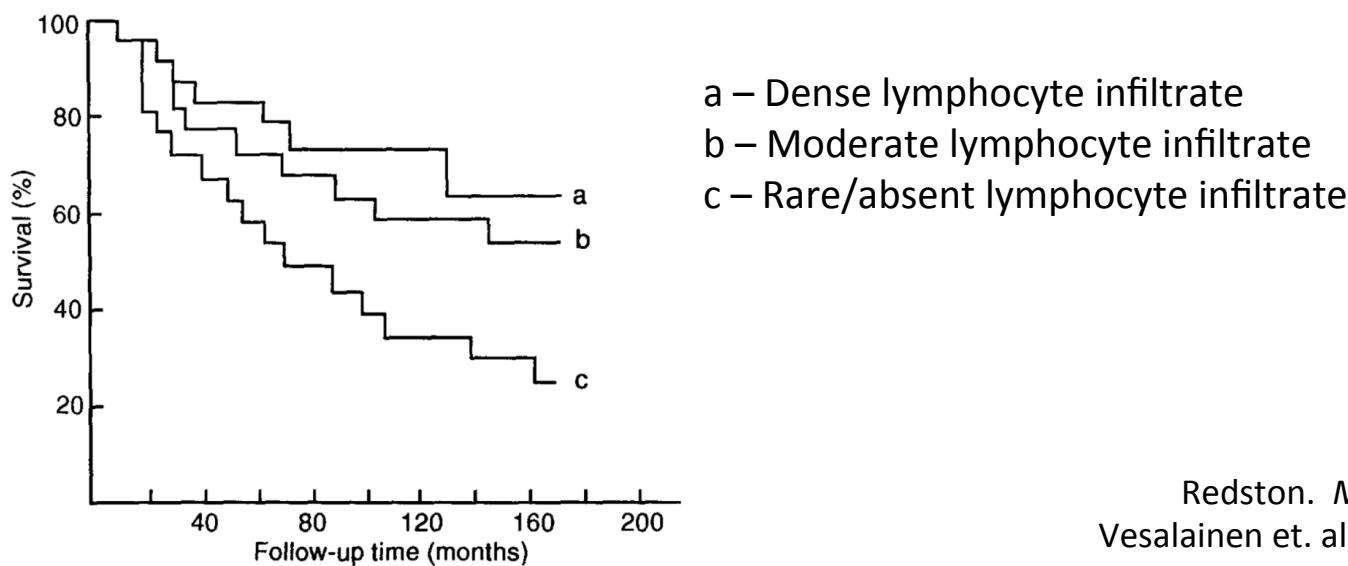
Influence of PI3K pathway activation on T cell-inflamed signature in metastatic prostate cancer

Jess Fessler

Patnaik Lab Meeting, August 23rd 2016

Prostate Cancer Immunology

- PCa: bad deal
- New therapies for mCRPC provide minimal survival benefit
- Potential responsiveness to immunotherapy
 - Corresponds to increased infiltration



Redston. *Mod Pathol.* (2001)
Vesalainen et. al. *Eur J Canc* (1994)

PTEN Loss in PCa

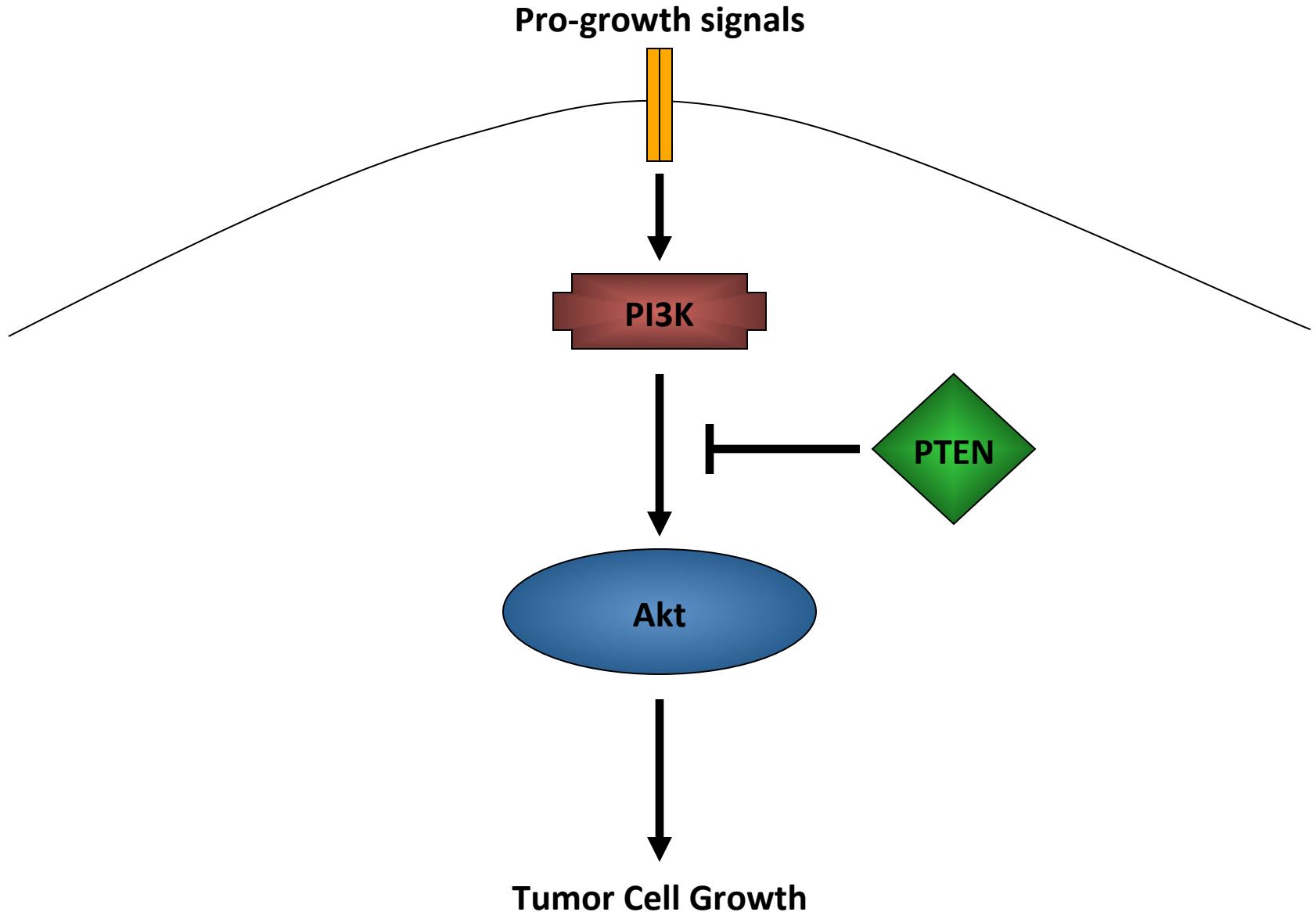
- Lost in up to 50% of primary, 75% of mets
 - Sufficient for development of disease
- PTEN loss associated with:
 - Increased immunosuppressive cytokine expression
 - Decreased T cell infiltration/function
 - Enhanced Treg function
 - Non T-cell inflammed phenotype (in melanoma...preliminary data in PCa)

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Can we target consequences of PTEN loss to reverse this immunosuppression, leading to a pro-inflammatory tumor microenvironment and enhanced responsiveness of immunotherapy?

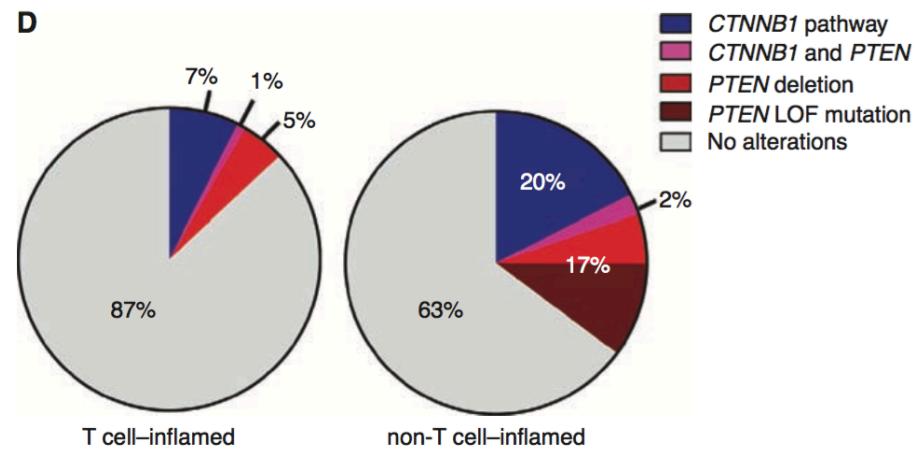
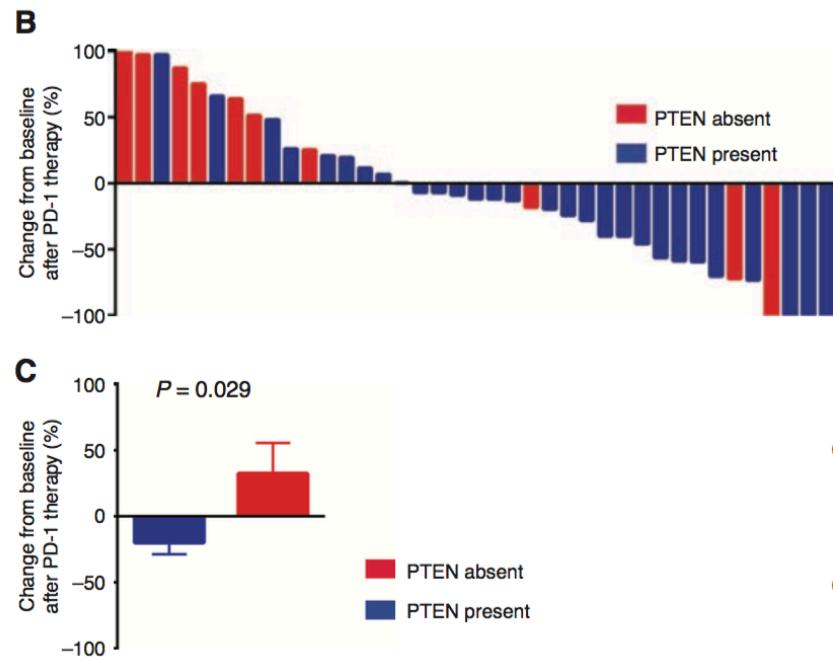
PI3K-PTEN-AKT Signaling Pathway



PI3K Inhibition

- Several class I PI3K inhibitors have entered clinical testing
- Pan-PI3K inhibitors (i.e. GSK'458, BKM120), PI3K α inhibitors have efficacy, but also on-target, off-tumor effects
 - Decreased T cell function
- PI3K β inhibitors
 - Central to PCa tumorigenesis, critical for PTEN-deficient tumor growth
 - Does not inhibit T cell function; synergizes with checkpoint blockade in PTEN-deficient melanoma

Loss of PTEN Promotes Resistance to T Cell-Mediated Immunotherapy



PCF Challenge Award

1. To determine if PTEN loss in mCRPC correlates with a lack of a T cell-inflamed tumor microenvironment, relative to PTEN-proficient mCRPC.
2. To determine whether PI3K β inhibition promotes an inflammatory tumor microenvironment and enhances the efficacy of PD-1 blockade in PTEN/p53 and PTEN-deficient genetically engineered mouse models of CRPC.
3. To evaluate the safety and maximum tolerated dose of GSK2636771 in combination with nivolumab, and whether this strategy promotes a T-cell inflamed tumor microenvironment and enhanced immunotherapy efficacy in PTEN-deficient mCRPC patients.

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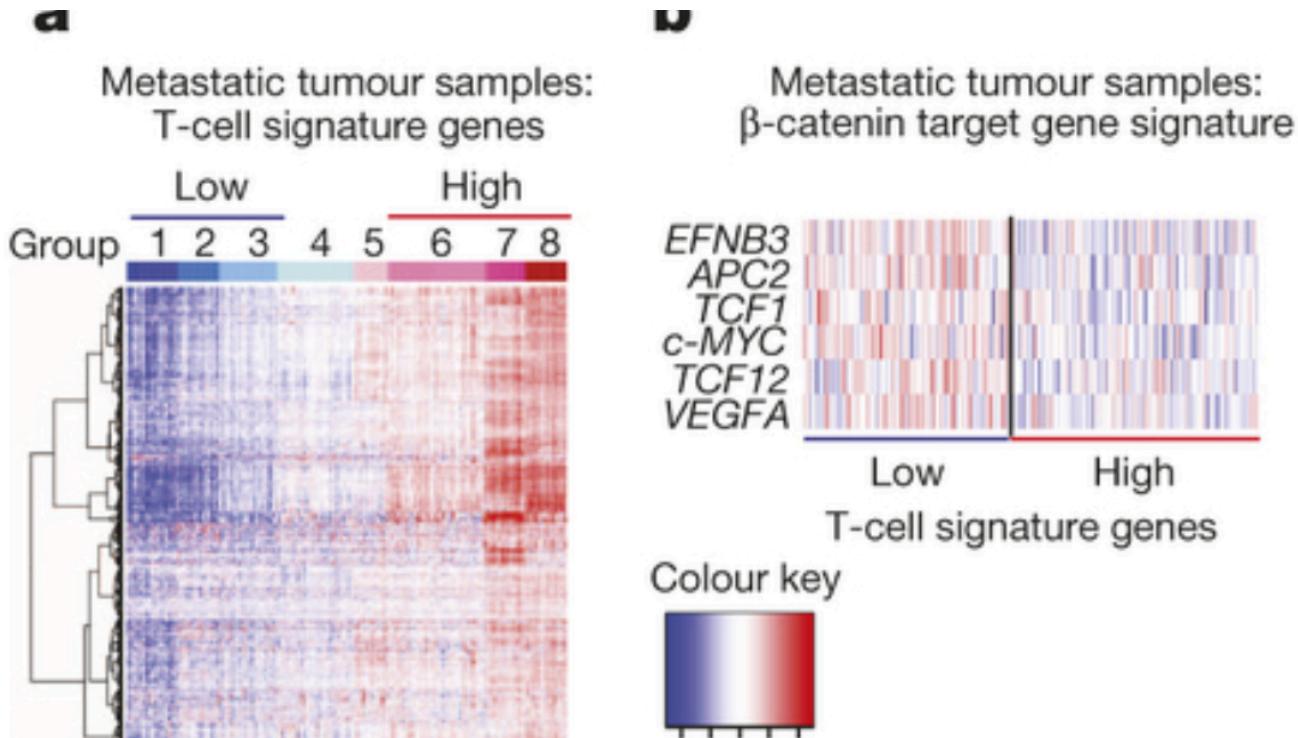
Melanoma-intrinsic β -catenin signalling prevents anti-tumour immunity

Stefani Spranger, Riyue Bao & Thomas F. Gajewski

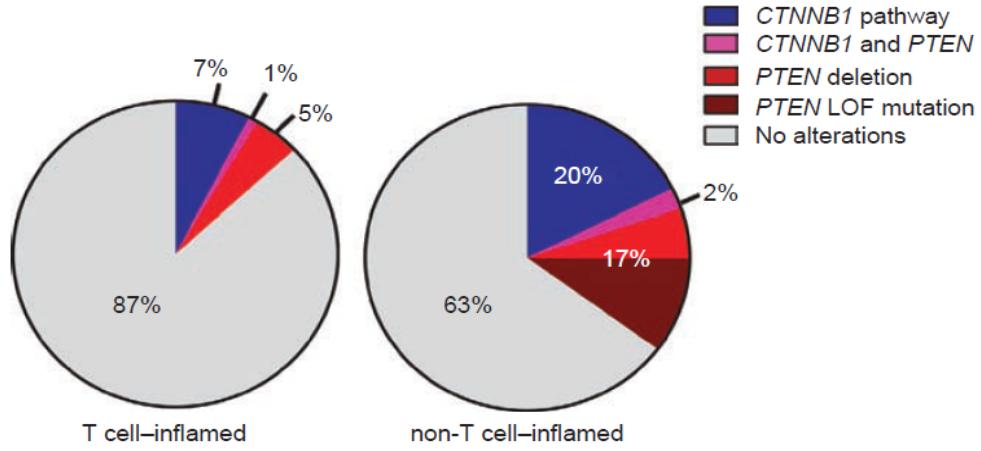
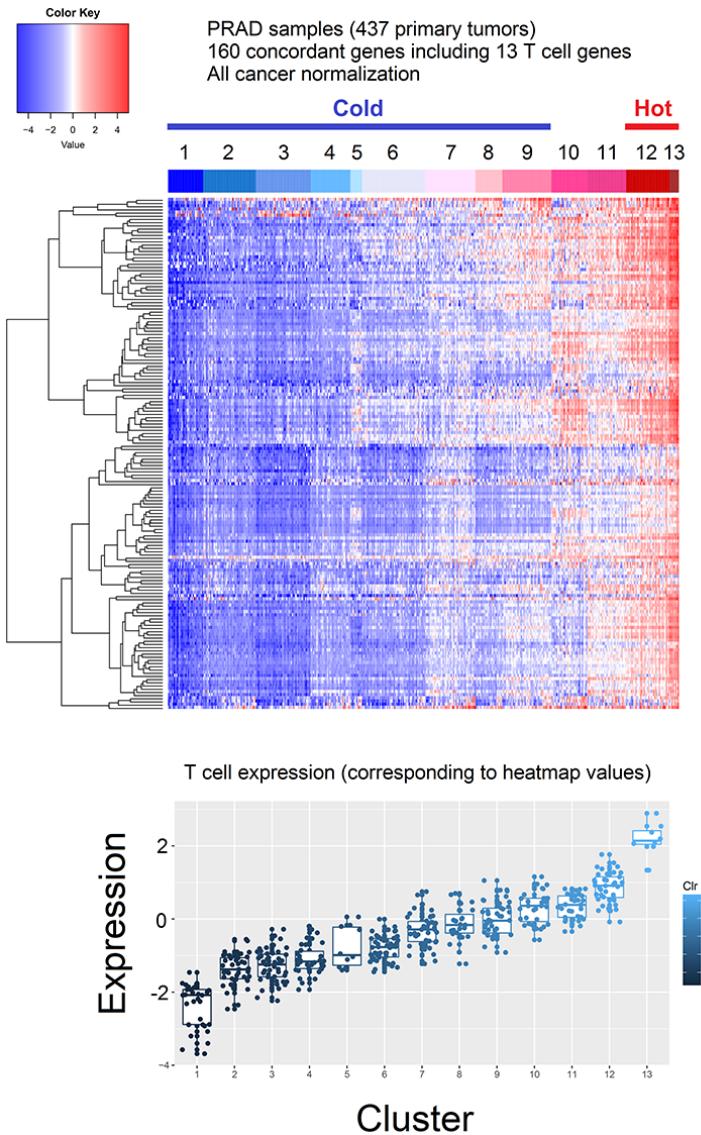
[Affiliations](#) | [Contributions](#) | [Corresponding author](#)

Nature 523, 231–235 (09 July 2015) | doi:10.1038/nature14404

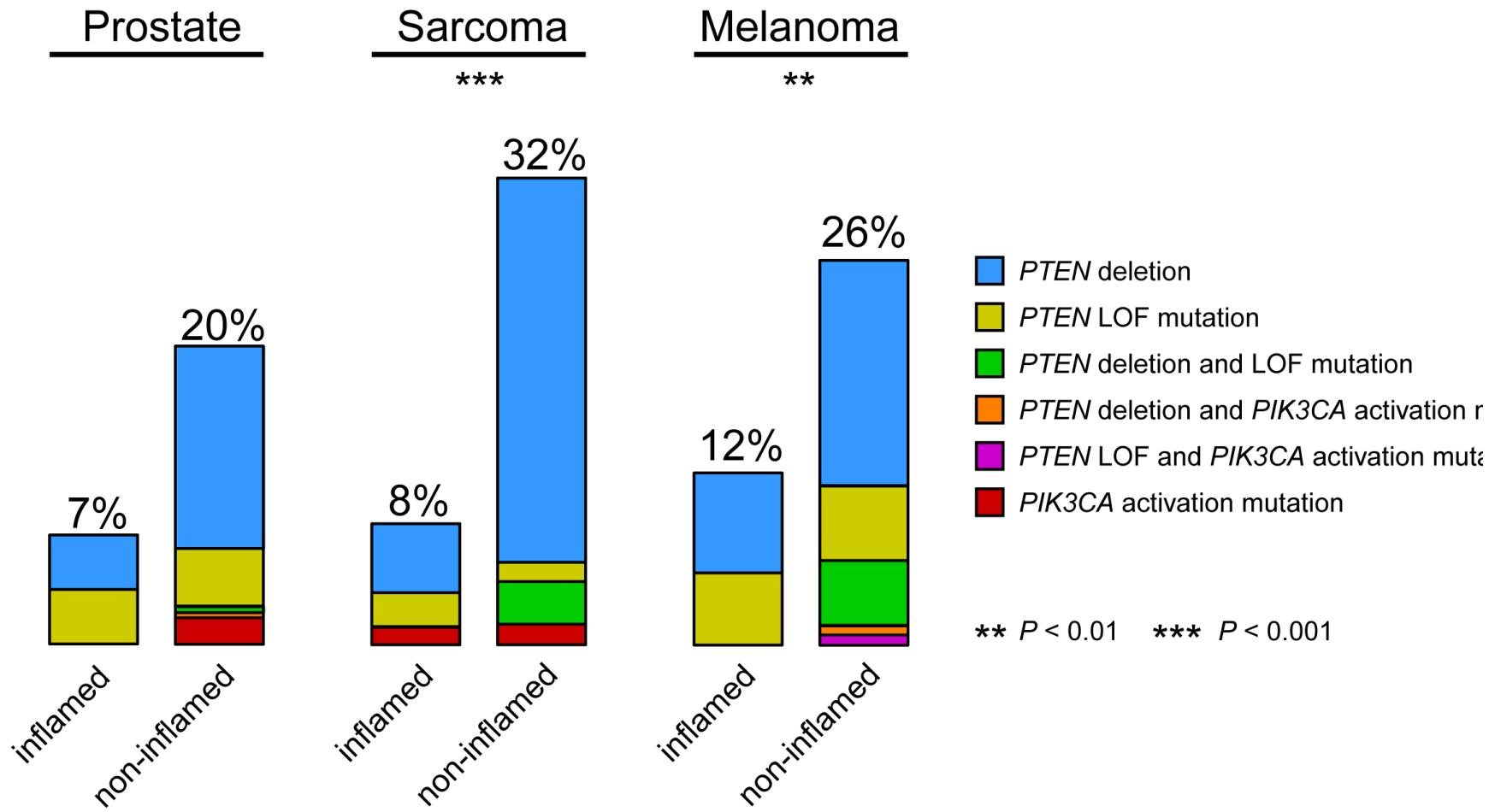
Received 15 August 2014 | Accepted 05 March 2015 | Published online 11 May 2015



PTEN-Loss Associated with Non T Cell-Inflamed Gene Signature in primary prostate samples



Frequencies of genomic alterations in PTEN and its target gene PIK3CA between T cell-inflamed and non-inflamed tumors





THIS IS WHERE **THE END OF CANCER BEGINS**

→ SU2C SCIENTIFIC RESEARCH TEAMS

→ SU2C-PCF Dream Team: Precision Therapy for Advanced Prostate Cancer

- 150 metastatic prostate cancer samples
- RNAseq, CNV, and mutation information
- Analyzed 43 from U Michigan

Bioinformatics pipeline for analyzing T-cell inflamed signature

- Remove genes that have signal in less than 50% of samples
- Log2 transform data, upper quartile normalize, and scale data
- Generate heatmap using entire gene set, clustering both samples and genes
- Cut cluster dendrogram at predetermined K
 - ($K = \text{number of clusters}$)

Bioinformatics pipeline for analyzing T-cell inflamed signature (cont.)

- Select clusters that contain the 13 T cell signature genes
- Re-cluster **samples** using Consensus Clustering
- Based on consensus clusters, classify sample as either hot or cold
- Compare PTEN/PIK3CA status in hot vs. cold groups

Complete heat map – gene expression for ALL genes

13 T cell signature

genes:

CCL2

CCL3

CCL4

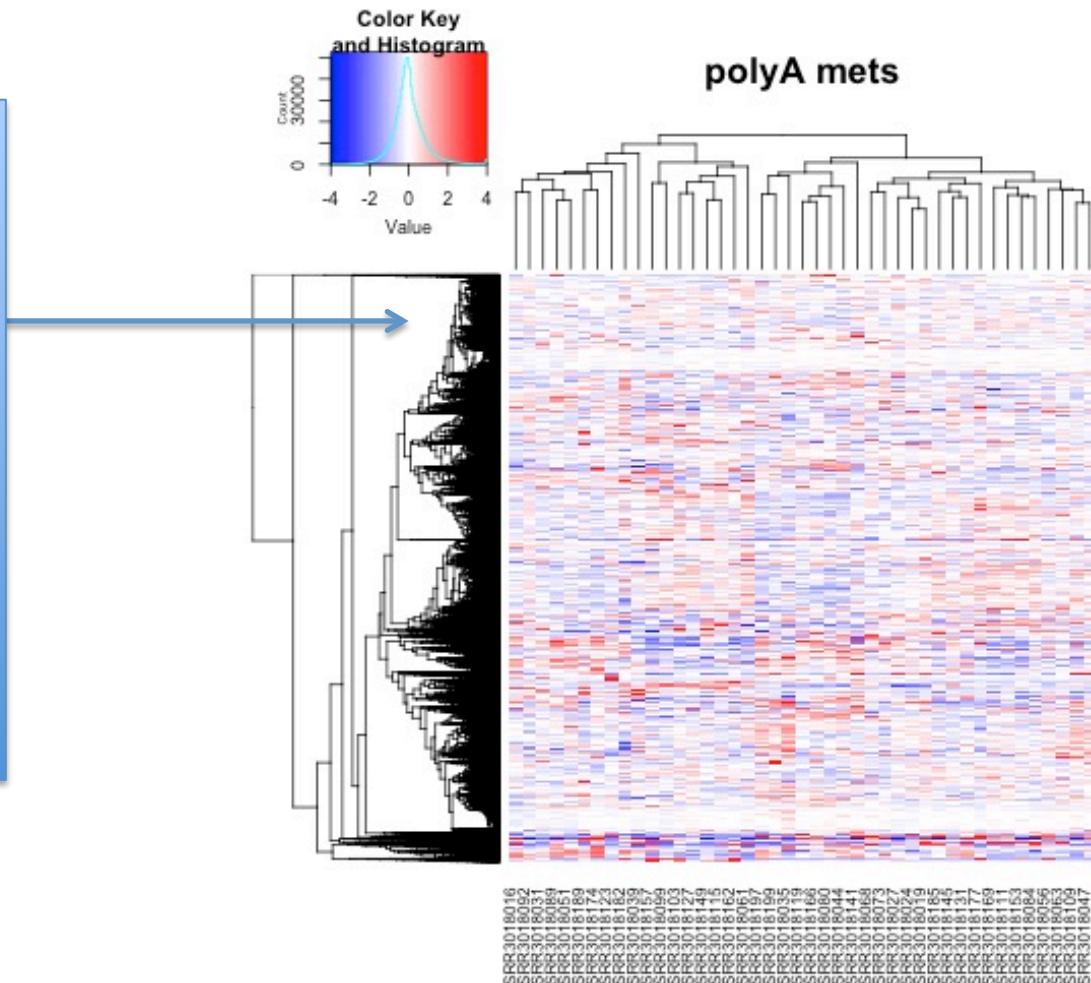
CXCL9

CXCL1

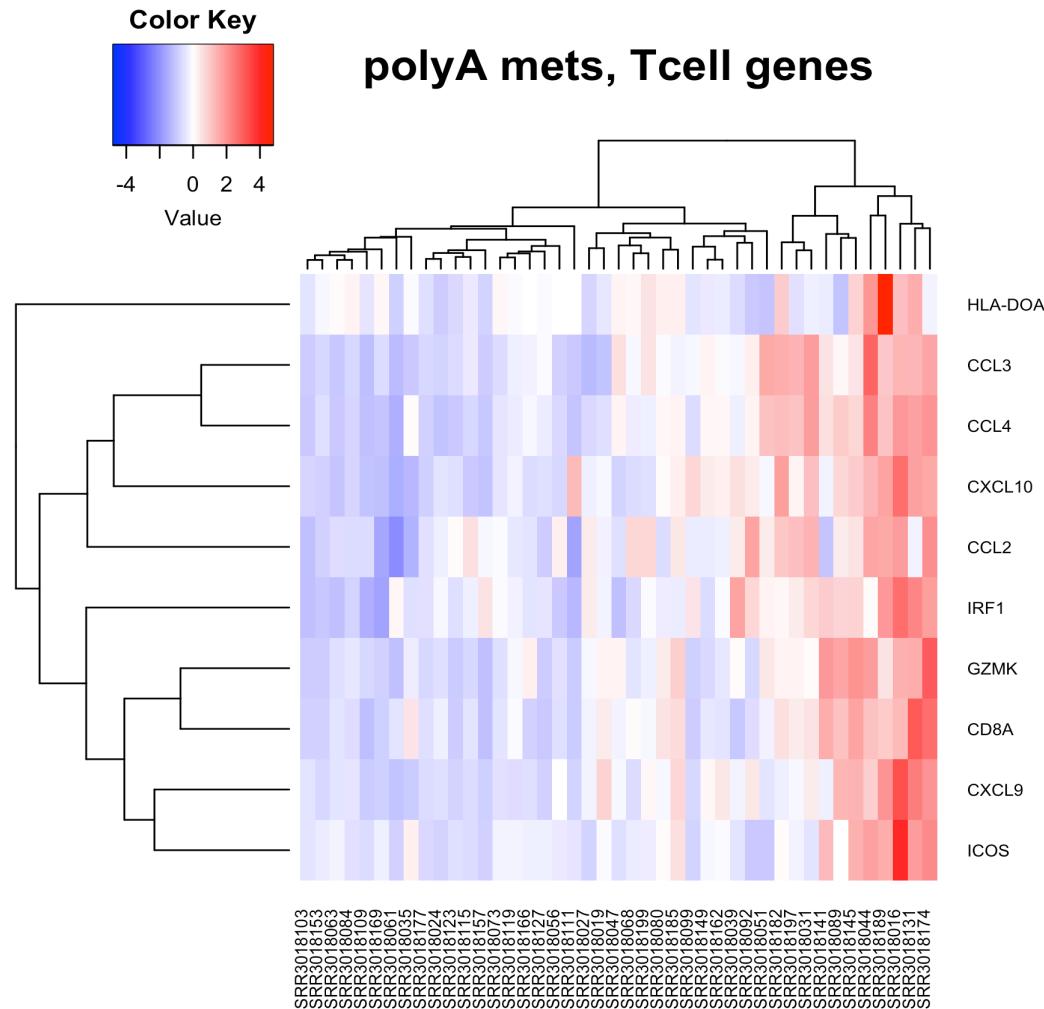
ICOS

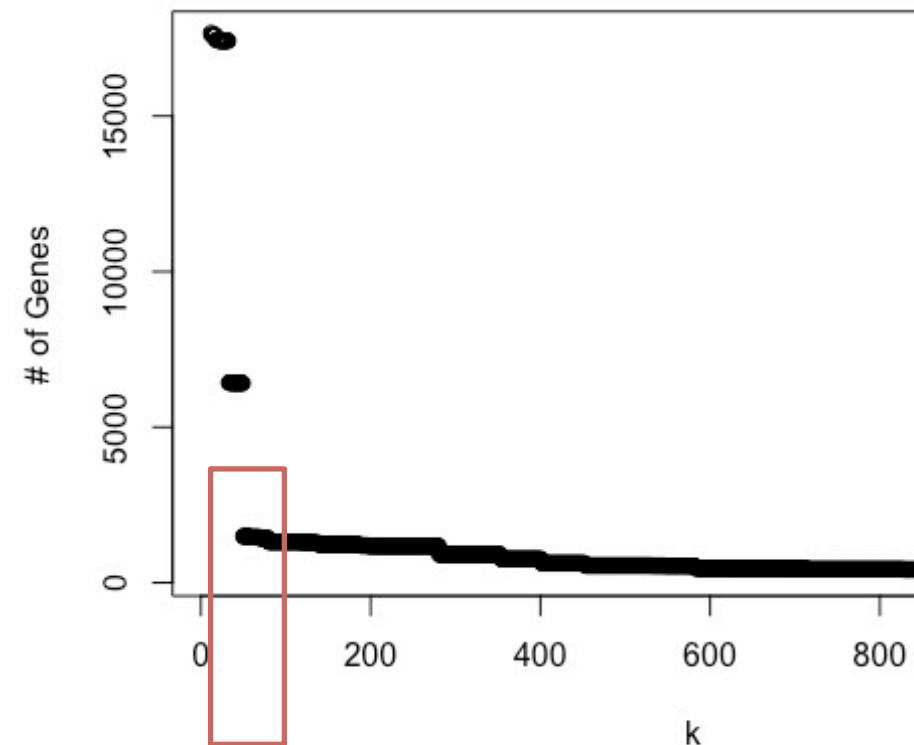
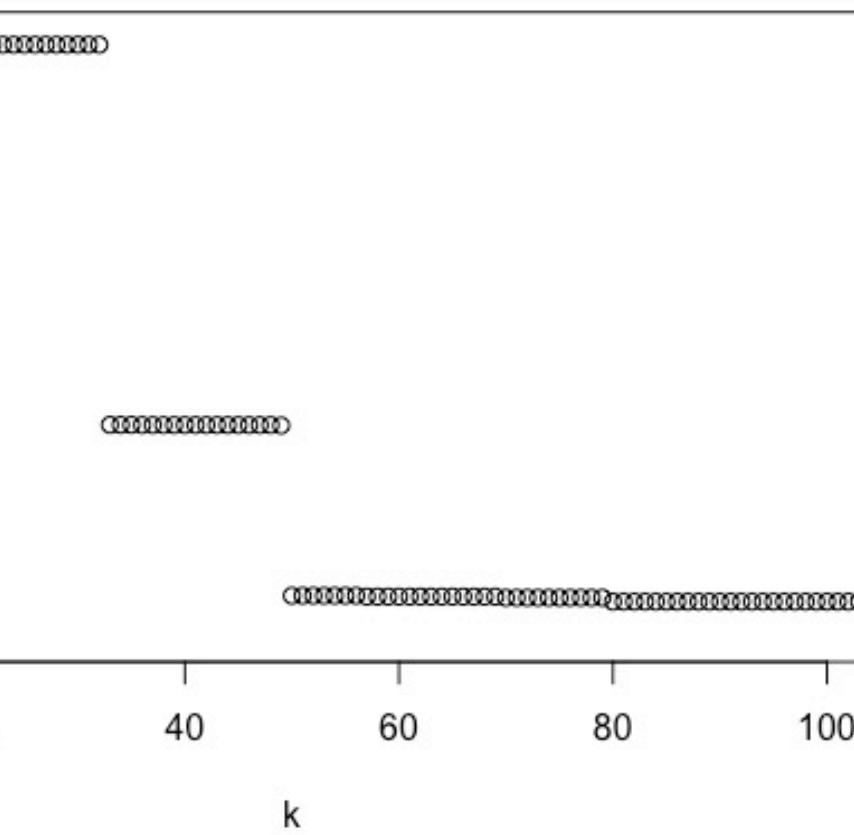
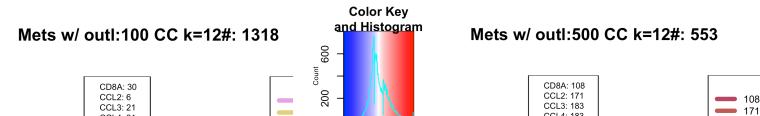
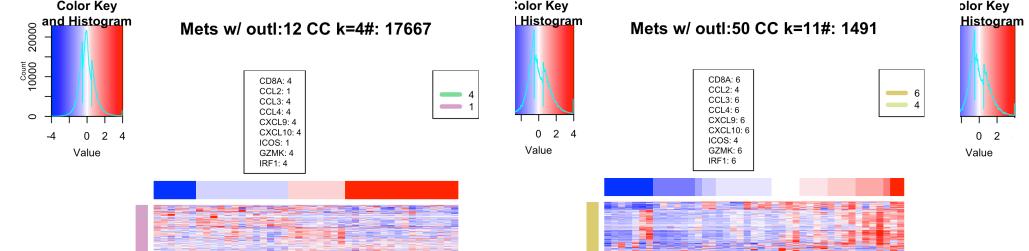
GZMK

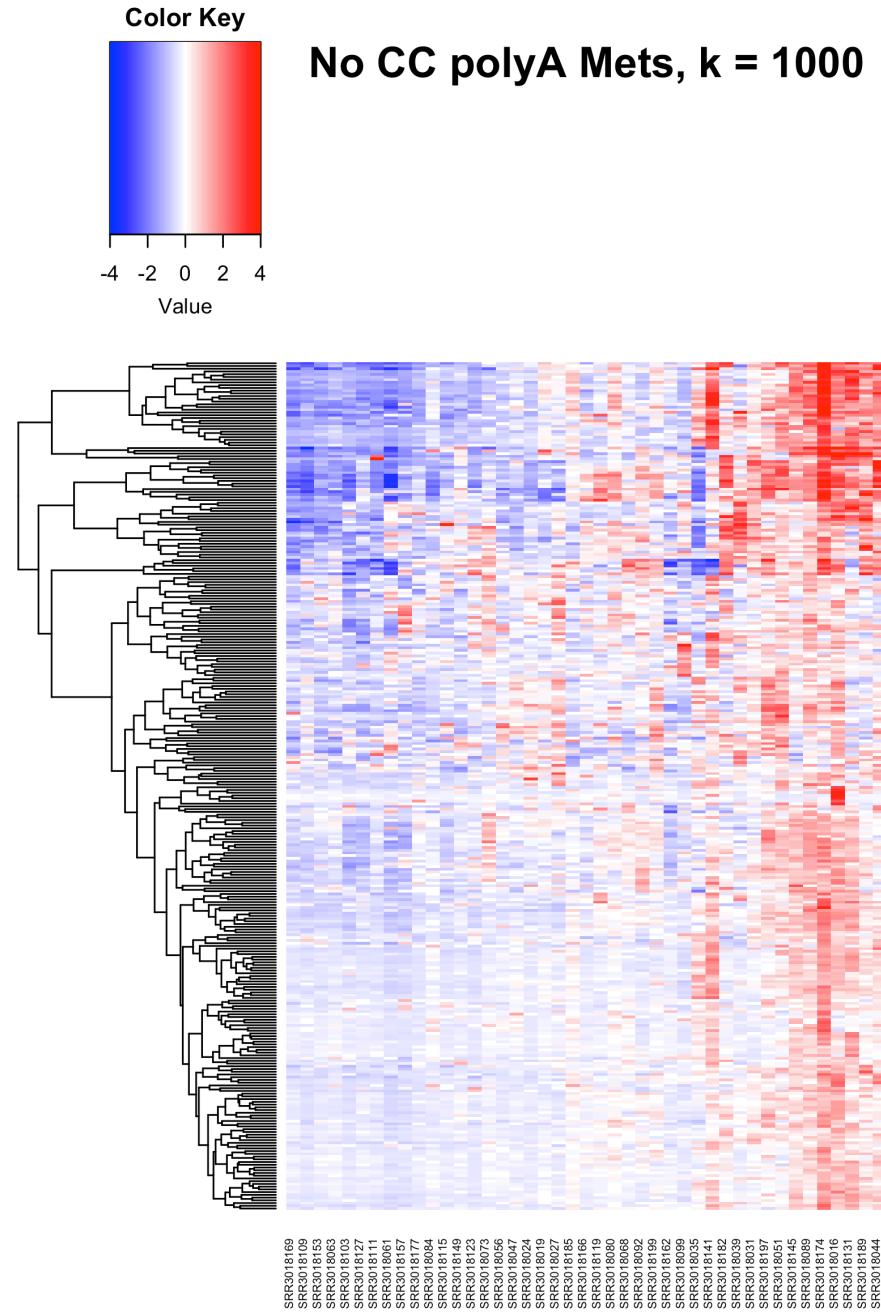
*HLA-DOA, HLA-DOB,
HLA-DMA, HLA-DMB*



T-cell signature genes show small percentage of metastatic samples are hot

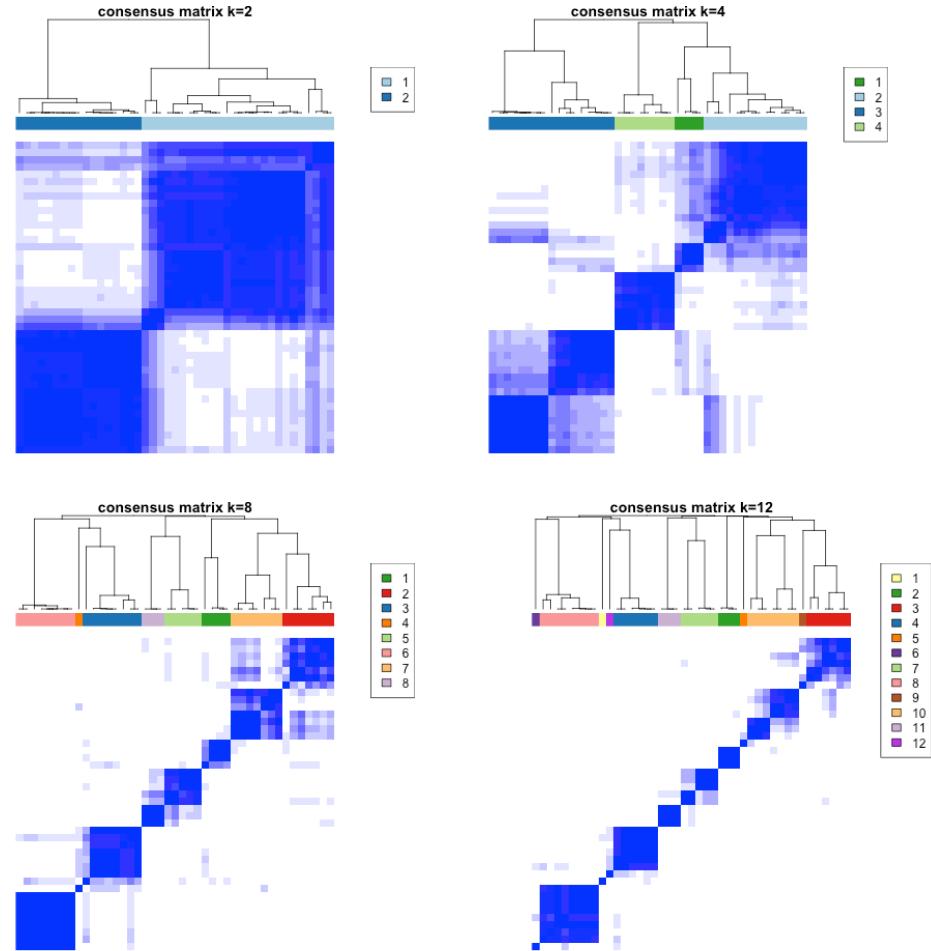


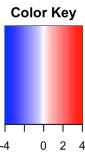




Consensus Clustering

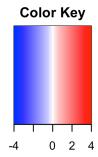
- Tool used for unsupervised class discovery





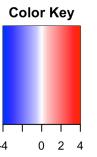
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CD8A: 376
CCL2: 283
CCL3: 310
CCL4: 310
CXCL9: 400
CXCL10: 488
ICOS: 38
GZMK: 376
IRF1: 180



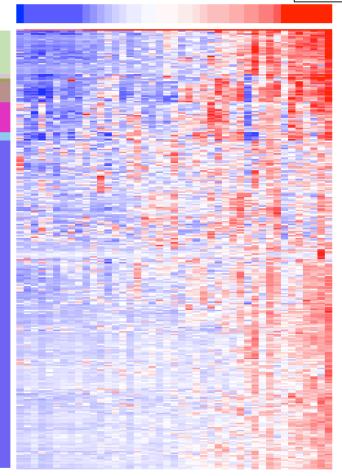
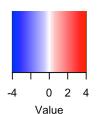
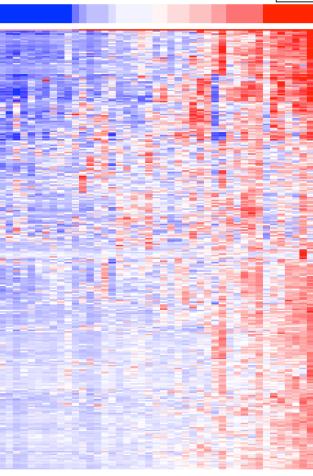
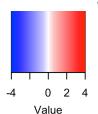
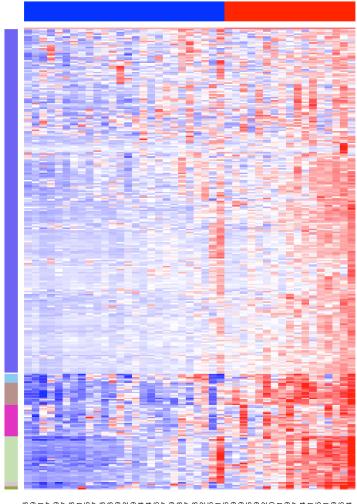
Mets w/ outl:1000 CC k=4#: 310

CD8A: 376
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CCL3: 310
CCL4: 310
CXCL9: 400
CXCL10: 488
ICOS: 38
GZMK: 376
IRF1: 180



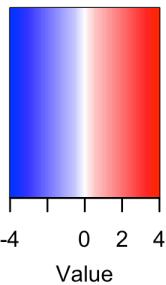
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GZMK: 376
IRF1: 180

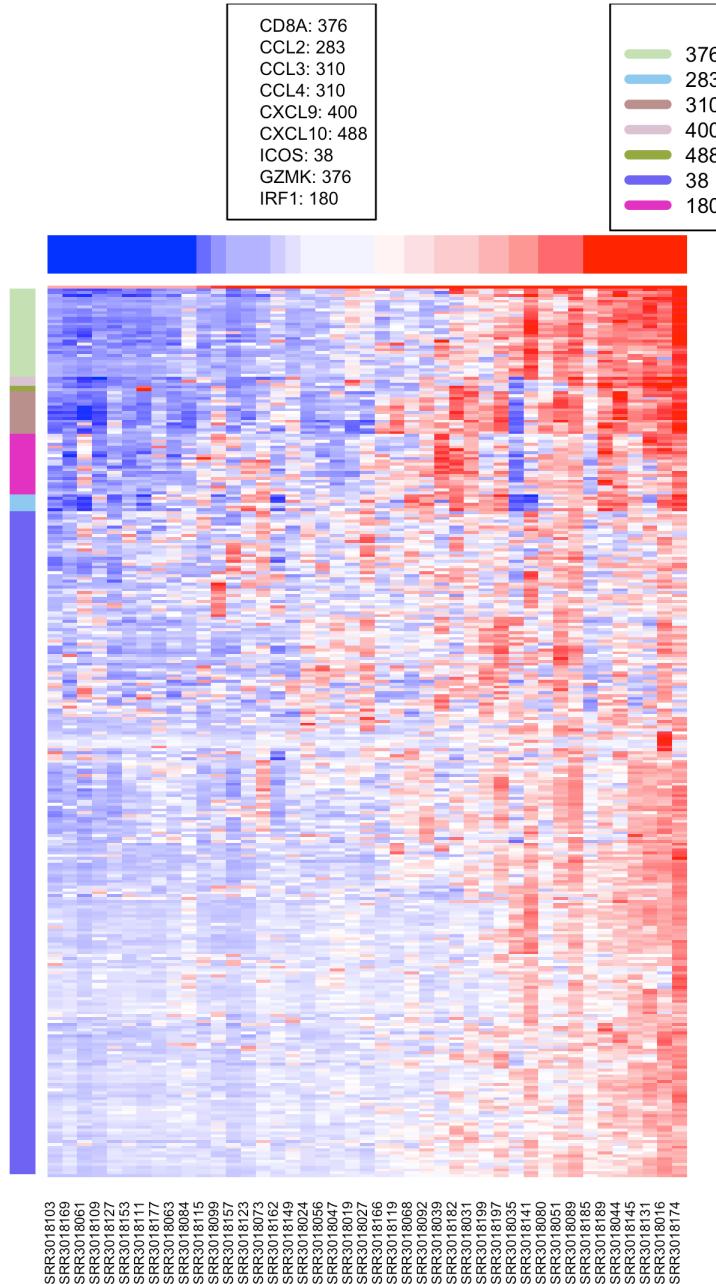


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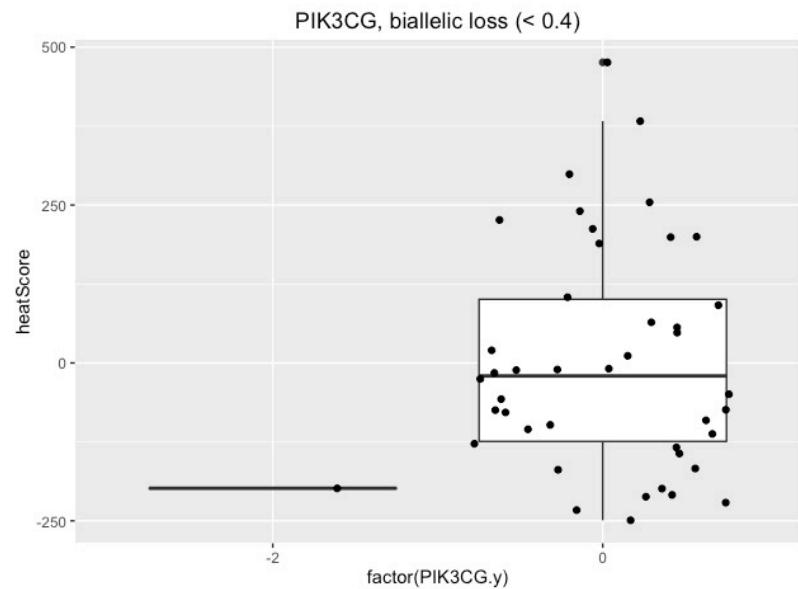
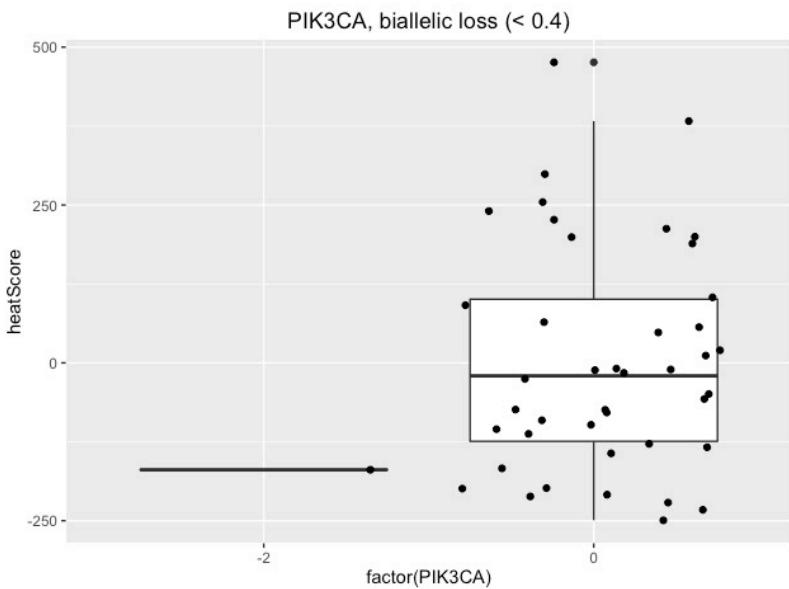
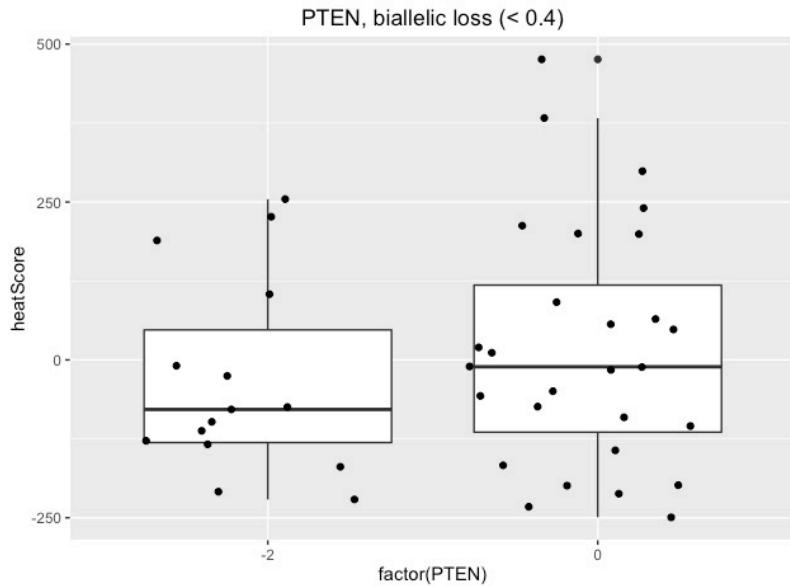
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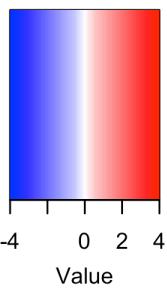
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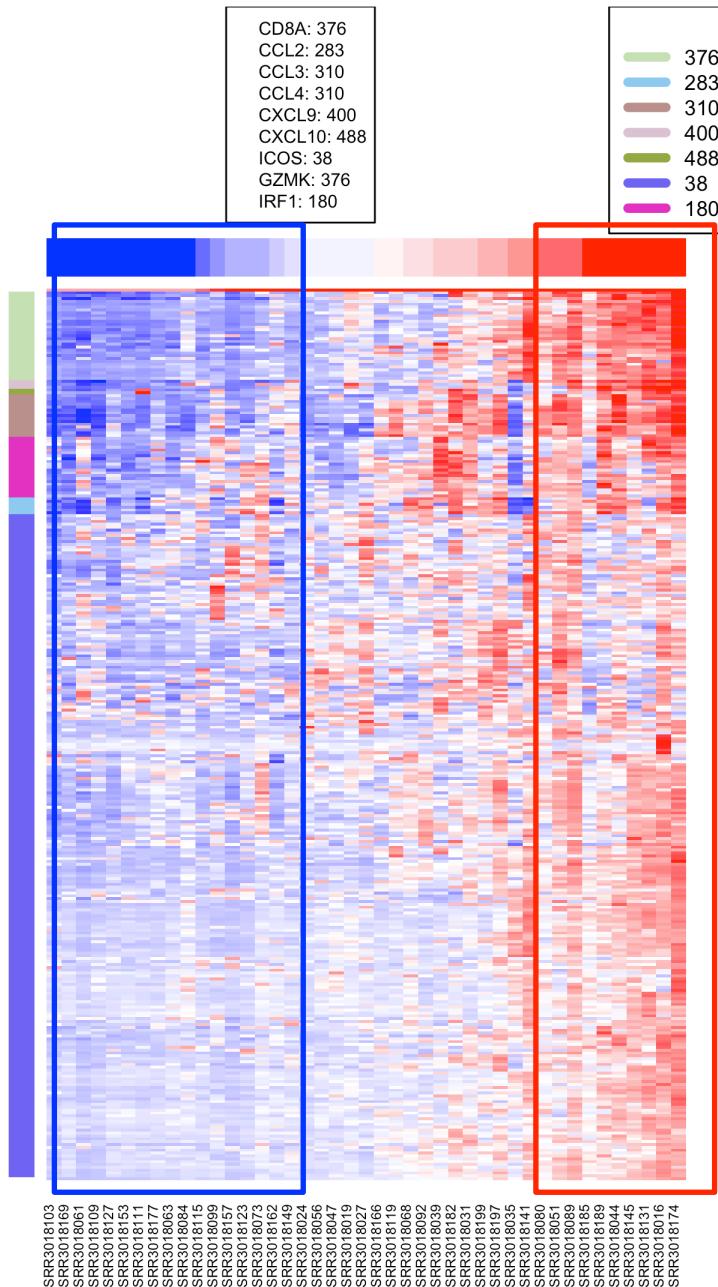
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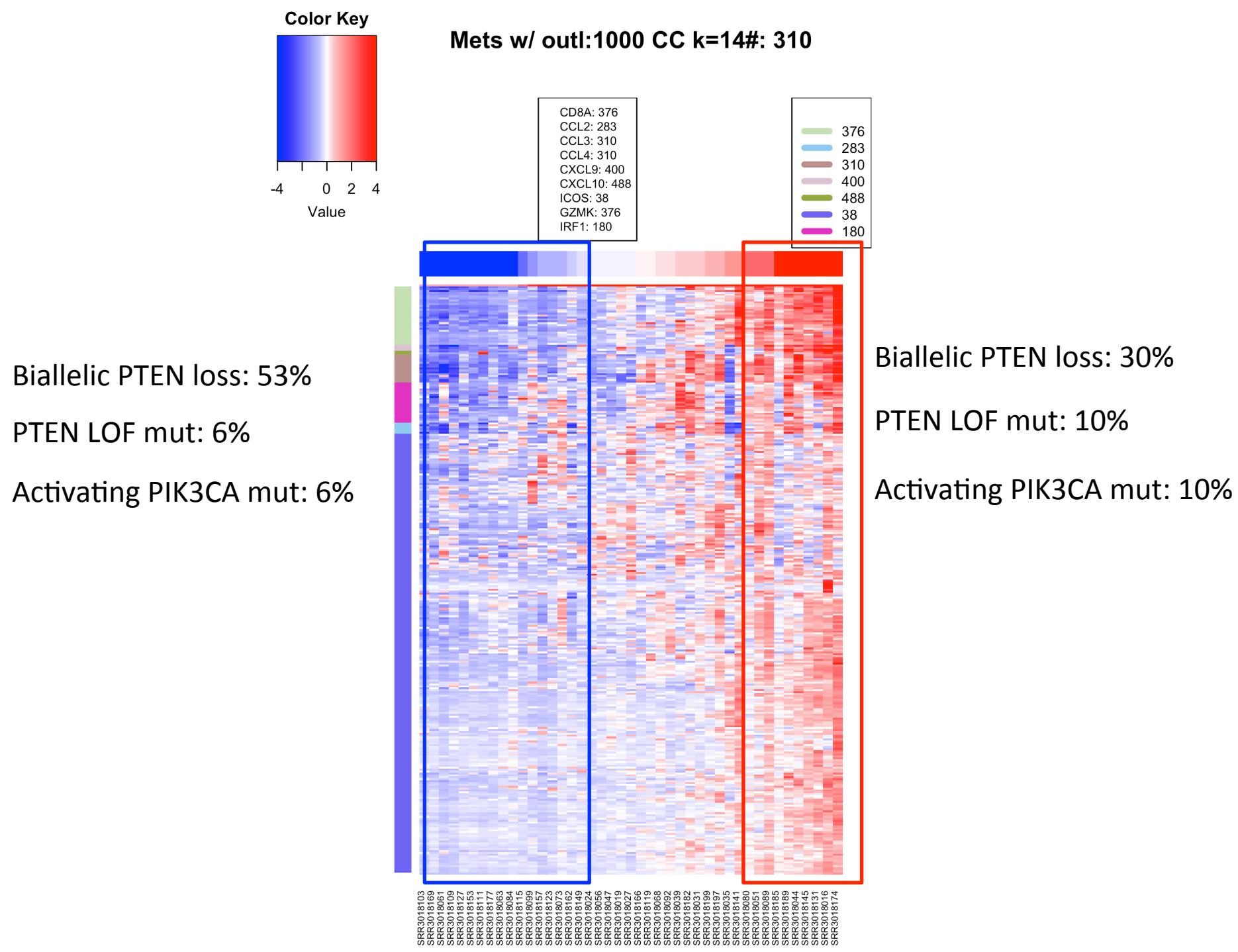


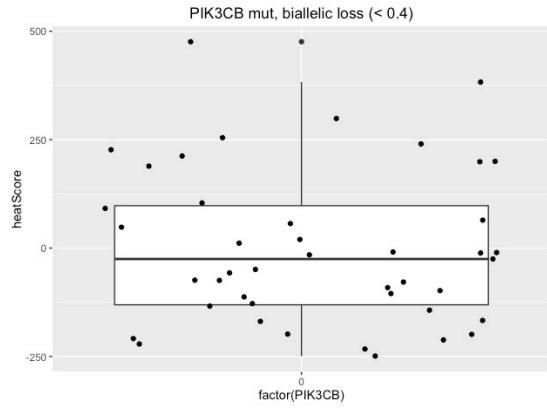
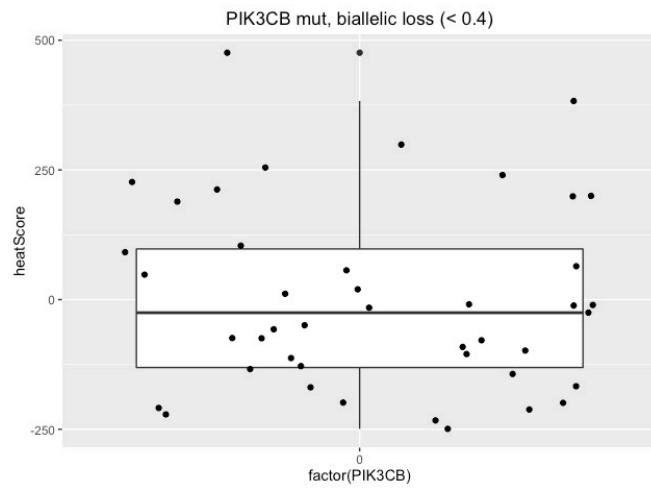
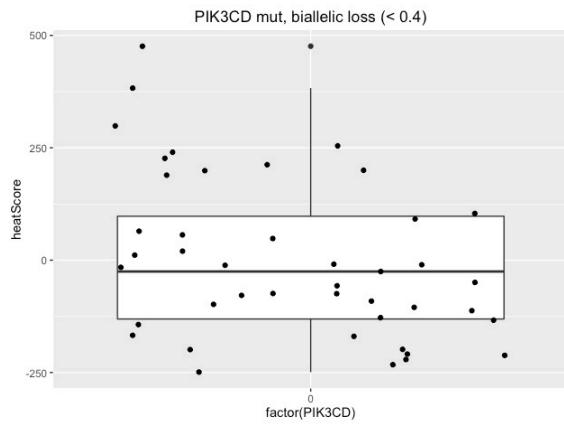
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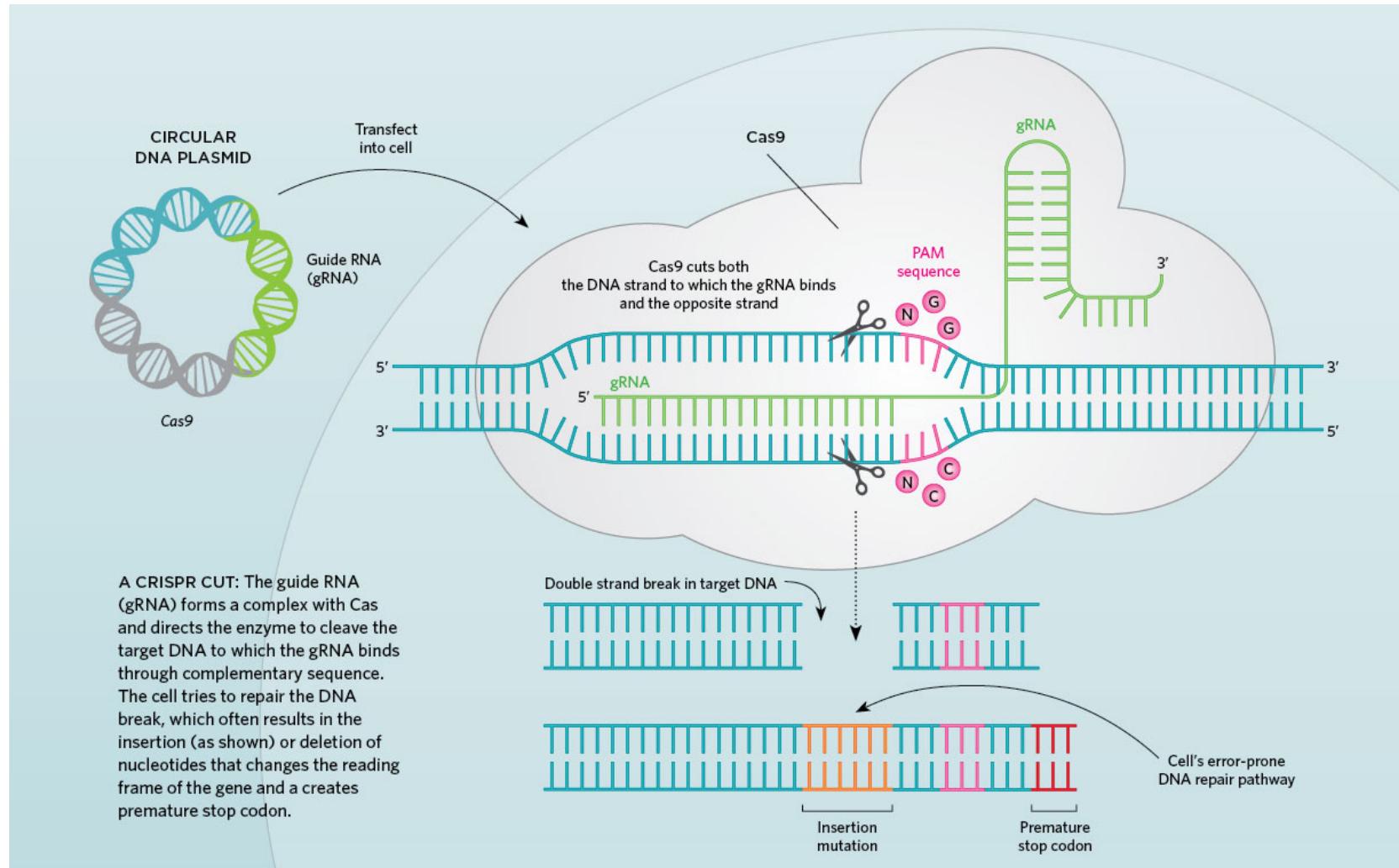
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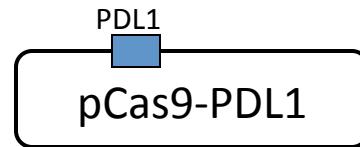
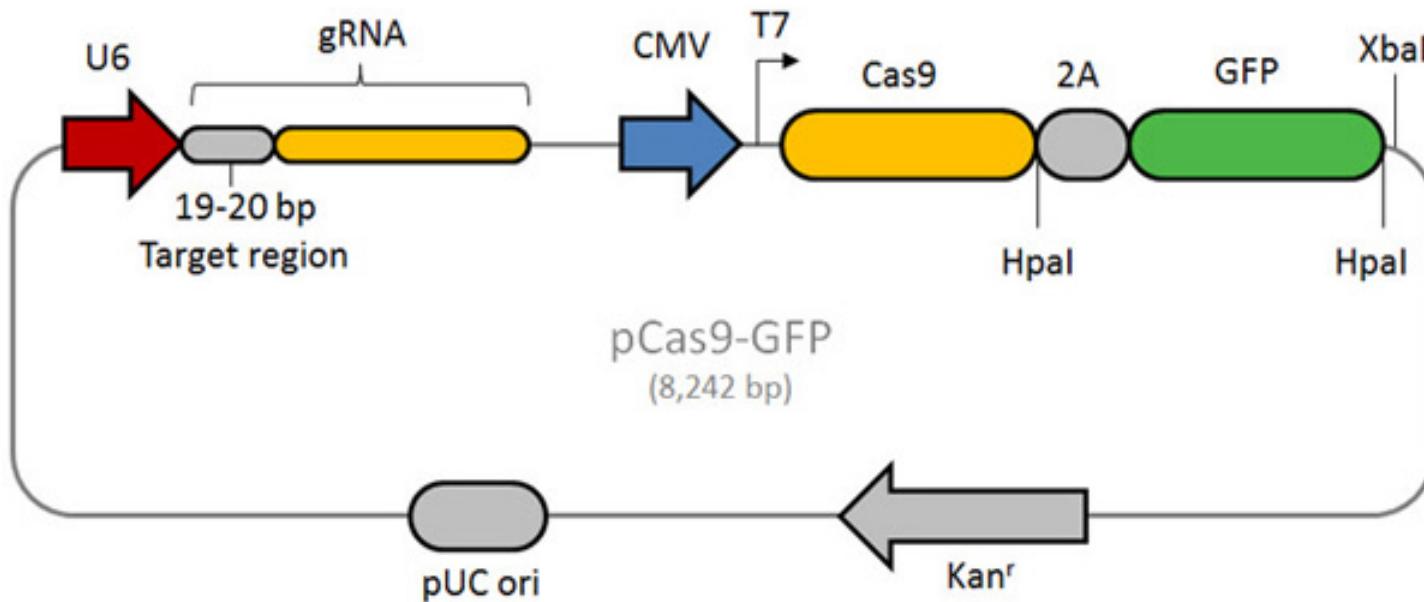




Generating TRAMP-C1^{ΔPTEN} CRISPR-Cas Gene Editing (Clustered Regularly Interspaced Short Palindromic Repeats)

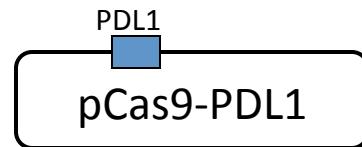


Sigma pCas9 CRISPR Plasmid



CRISPR-PD-L1 → CRISPR-PTEN

Site Directed Mutagenesis

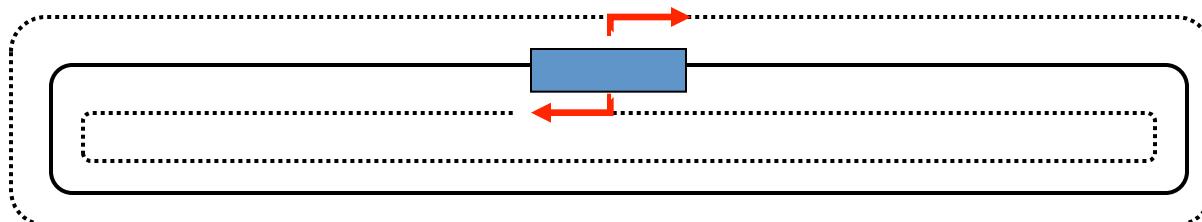


PDL1: TTTACTATCACGGCTCCAA

PTEN: AGATCGTTAGCAGAAACAAA

A schematic diagram of the PTEN gene. It shows two pink circles, each containing a capital letter 'P', with arrows pointing away from the gene sequence. The gene sequence is shown in red: AGATCGTTAGCAGAAACAAA.

Xue et. al, *Nature* (2014)

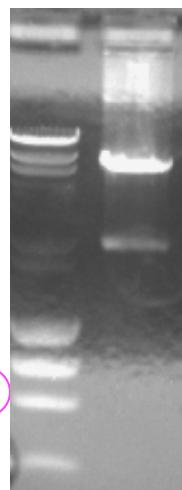


↓
Site-directed mutagenesis PCR

P
CAGAAACAAA
GTCTT GTTT

AGATCGTTAG
TCTAGCAATC

P

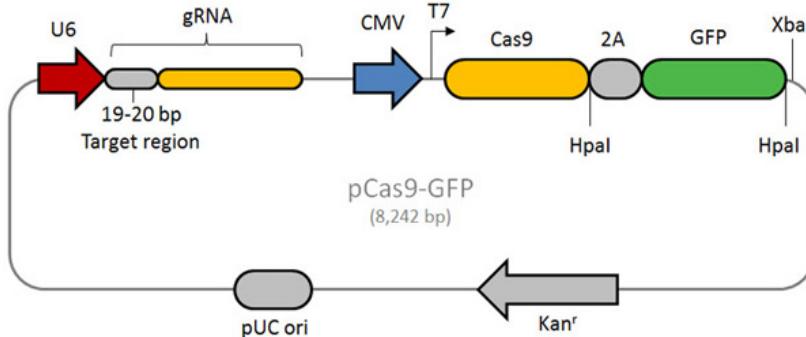


U6 Promoter

Guide RNA Target

CMV Promoter

ACTATCATATGCTTACCGTAAC TGAAAGTATT CGATTCTGGCTTATATATCTTGTGGAAA
GGACGAAACACCGGTTTACTATCACGGCTCCAA GTTTTAGAGCTAGAAATAGCAAGTTAAAATAA
GGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTCTAGGCCAGCTT
CTTGTACAAAGTTGGCATTAACCGTGTGACATTGATTATTGACTAGTTATTAA**TAGTAATCAATT**
ACGGGGTCATTAGTTCATAGCCC ATATATGGAGTTCCCGCGTTACATAACTACGGTAAATGGCCC
GCCTGGCTGACCGCCAACGACCCCCGCCATTGACGTCAATAATGACGTATGTTCCCAGTAA
CGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTACGGTAAACTGCCACTTGGCA
GTACATCAAGTGTATCATATGCCAAGTACGCCCTATTGACGTCAATGACGGTAAATGGCCCGC
CTGGCATTATGCCAGTACATGACCTTATGGGACTTCCATTGGCAGTACATCAATGGCGTGGATAGCGGTTGAC
TCATCGCTATTACCATGGTGATGCGGTTTGGCAGTACATCAATGGCGTGGATAGCGGTTGAC
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PTEN_CRISPR_MUT_FWD:

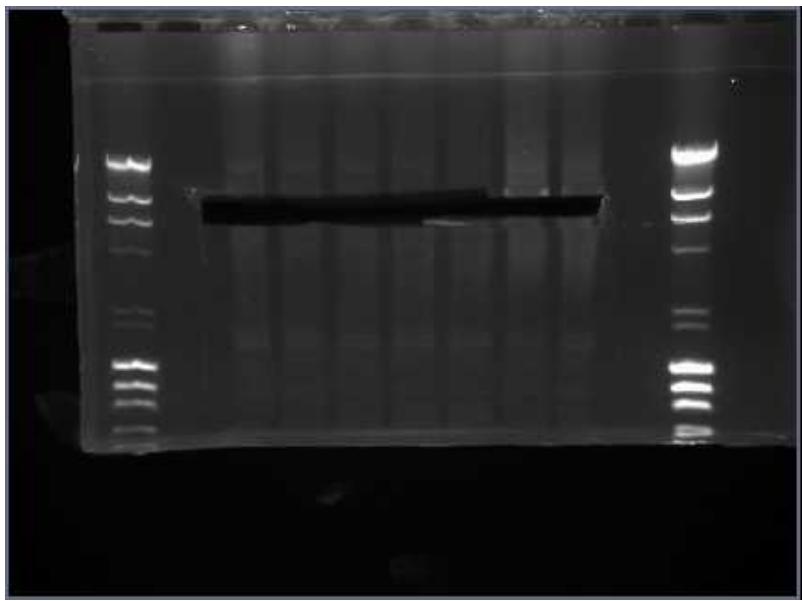
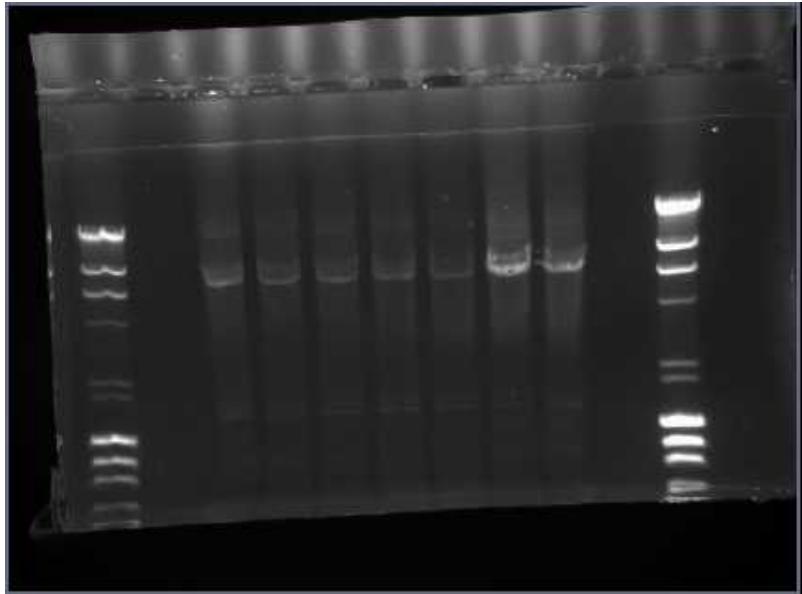
5PHOS-CA GAA ACA AAG TTT TAG AGC
TAG AAA TAG CAA GTT AAA ATA AG

PTEN_CRISPR_MUT_REV:

5PHOS-CT AAC GAT CTC CGG TGT
TTC GTC CTT TCC

PTEN_CRISPR_PCR_FWD:

GGA GAT CGT TAG CAG AAA CAA AG



Gel purification

PCR
mix



agarose
gel

+ buffer NT



binding



washing



elution



Sequence

pCas9-PTEN and CMV_Rev alignment

DNAMAN File: Multiple Alignment			
pCas9-pten_part1	ACTATCATATGCTTACCGTAACITGAAAGTATTTCGATT	40	
cmv_clean		0	
Consensus			
pCas9-pten_part1	CTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGGA	80	
cmv_clean		0	
Consensus			
pCas9-pten_part1	GATCGTTACAGAAAACAAGCTTTAGAGCTAGAAATAGC	120	
cmv_cleanCAGAAAACAAGTTTAGAGCTAGAAATAGC	30	
Consensus	cagaacaaagtttagagctagaaatagc		
pCas9-pten_part1	AAGTTAAAATAAGGCCTACTCCGTATCAACTTGAAAAAGT	160	
cmv_clean	AAGTTAAAATAAGGTACTCCGTATCAACTTGAAAAAGT	70	
Consensus	aagttaaaataaggtagtccgttatcaacttgaaaaagt		
pCas9-pten_part1	GGCACCGAGTCGGTCTTTTCTAGGCCAGCTTCTT	200	
cmv_clean	GGCACCGAGTCGGTCTTTTCTAGGCCAGCTTCTT	110	
Consensus	ggcacccgactcggtgtcttttctagggccagcttctt		
pCas9-pten_part1	GTACAAAGTTGGCATTAACGCGITGACATTGATTATTGAC	240	
cmv_clean	GTACAAAGTTGGCATTAACGCGITGACATTGATTATTGAC	190	
Consensus	gtacaaagttggcattaacggttgacattgttattgac		
pCas9-pten_part1	TAGTTATTAATAGTAATCAATTACGGGGTCATTACTTCAT	280	
cmv_clean	TAGTTATTAATAGTAATCAATTACGGGGTCATTACTTCAT	190	
Consensus	tagttataatagtaatcaattacggggtcattagtcat		
pCas9-pten_part1	AGCCCATATATGGAGTTCCCGCGTACATAACTTACGGTAA	320	
cmv_clean	AGCCCATATATGGAGTTCCCGCGTACATAACTTACGGTAA	230	
Consensus	agcccatatatggattcccggttacataacttacggtaa		
pCas9-pten_part1	ATGGCCCGCCTGGCTGACCGGCCAACGACCCCCGCCATT	360	
cmv_clean	ATGGCCCGCCTGGCTGACCGGCCAACGACCCCCGCCATT	270	
Consensus	atggcccgccctggctgaccgcoccaacgacccccggccatt		
pCas9-pten_part1	GACGTCAATAATGACGTATGTCCTCATAGTAACGCCAATA	400	
cmv_clean	GACGTCAATAATGACGTATGTCCTCATAGTAACGCCAATA	310	
Consensus	gacgtcaataatgacgtatgtcccatagtaacgc当地		
pCas9-pten_part1	GGGACTTTCCAATGACGICAATGGGTGGAGTATTIACGGT	440	
cmv_clean	GGGACTTTCCAATGACGICAATGGGTGGAGTATTIACGGT	350	
Consensus	gggactttccattgacgtcaatgggtggagttacgggt		
pCas9-pten_part1	AAACTGCCACTTGGCACTACATCAAGTGTATCATATGCC	480	
cmv_clean	AAACTGCCACTTGGCACTACATCAAGTGTATCATATGCC	390	
Consensus	aaactgccacttggcgtatcatcaagtgtatcatatgcc		
pCas9-pten_part1	AAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCC	520	
cmv_clean	AAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCC	430	
Consensus	aagtacgccccctatgacgtcaatgacggtaaatggccc		
pCas9-pten_part1	GCCTGGCATTATGCCCACTACATGACCTTATGGGACTTTC	560	
cmv_clean	GCCTGGCATTATGCCCACTACATGACCTTATGGGACTTTC	470	
Consensus	gcctggcattatgcccaactacatgacccattatgggacttcc		
pCas9-pten_part1	CTACTTGGCAGTACATCTACGTATTAGTCATCGCTTAC	600	
cmv_clean	CTACTTGGCAGTACATCTACGTATTAGTCATCGCTTAC	485	
Consensus	ctacttggcagatcacatctacgtattagtcatcgcttac		

pCas9-PTEN and pCas9-PDL1 alignment

DNAMAN File: Multiple Alignment			
pCas9-pdl1_part1	ACTATCATATGCTTACCGTAACITGAAAGTATTTCGATT	40	
pCas9-pten_part1	ACTATCATATGCTTACCGTAACITGAAAGTATTTCGATT	40	
Consensus	actatcatatgttacccgtaaacttggaaagtatttcgattt		
pCas9-pdl1_part1	CTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGGT	80	
pCas9-pten_part1	CTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGGT	80	
Consensus	cttggctttatatatcttggaaaggacgaaacacccgg		
pCas9-pdl1_part1	.TACTATICAAGGCCTAAAGTTAGCTAGAAATAGC	118	
pCas9-pten_part1	GATCGTAGCACAAACAAGGTTTAGCTAGAAATAGC	120	
Consensus	t t ca a g tttagactgactaaatagc		
pCas9-pdl1_part1	AAGTTAAAATAAGGCCTACTCCGTATCAACTTGAAAAAGT	158	
pCas9-pten_part1	AAGTTAAAATAAGGCCTACTCCGTATCAACTTGAAAAAGT	160	
Consensus	aagttaaaataaggctagtccgttatcaacttgaaaaagt		
pCas9-pdl1_part1	GGCACCGAGTCGGTCTTTTCTAGGCCAGCTTCTT	198	
pCas9-pten_part1	GGCACCGAGTCGGTCTTTTCTAGGCCAGCTTCTT	200	
Consensus	ggcacccgactcggtgtcttttctagggccagcttctt		
pCas9-pdl1_part1	GTACAAAGTTGGCATTAACGCGITGACATTGATTATTGAC	238	
pCas9-pten_part1	GTACAAAGTTGGCATTAACGCGITGACATTGATTATTGAC	240	
Consensus	gtacaaagttggcattaacggttgacatttgatttattgac		
pCas9-pdl1_part1	TAGTTATTAATAGTAATCAATTACGGGGTCATTACTTCAT	278	
pCas9-pten_part1	TAGTTATTAATAGTAATCAATTACGGGGTCATTACTTCAT	280	
Consensus	tagttataatagtaatcaattacggggtcattagtcat		
pCas9-pdl1_part1	AGCCCATATATGGAGTTCCCGCGTACATAACTTACGGTAA	318	
pCas9-pten_part1	AGCCCATATATGGAGTTCCCGCGTACATAACTTACGGTAA	320	
Consensus	agcccatatatggattcccggttacataacttacggtaa		
pCas9-pdl1_part1	ATGGCCCGCCTGGCTGACCGGCCAACGACCCCCGCCATT	358	
pCas9-pten_part1	ATGGCCCGCCTGGCTGACCGGCCAACGACCCCCGCCATT	360	
Consensus	atggcccgccctggctgaccgcoccaacgacccccggccatt		
pCas9-pdl1_part1	GACGTCAATAATGACGTATGTCCTCATAGTAACGCCAATA	398	
pCas9-pten_part1	GACGTCAATAATGACGTATGTCCTCATAGTAACGCCAATA	400	
Consensus	gacgtcaataatgacgtatgtcccatagtaacgc当地		
pCas9-pdl1_part1	GGGACTTTCCAATGACGICAATGGGTGGAGTATTIACGGT	438	
pCas9-pten_part1	GGGACTTTCCAATGACGICAATGGGTGGAGTATTIACGGT	440	
Consensus	gggactttccattgacgtcaatgggtggagttacgggt		
pCas9-pdl1_part1	AAACTGCCACTTGGCACTACATCAAGTGTATCATATGCC	478	
pCas9-pten_part1	AAACTGCCACTTGGCACTACATCAAGTGTATCATATGCC	480	
Consensus	aaactgccacttggcgtatcatcaagtgtatcatatgcc		
pCas9-pdl1_part1	AAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCC	518	
pCas9-pten_part1	AAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCC	520	
Consensus	aagtacgccccctatgacgtcaatgacggtaaatggccc		
pCas9-pdl1_part1	GCCTGGCATTATGCCCACTACATGACCTTATGGGACTTTC	558	
pCas9-pten_part1	GCCTGGCATTATGCCCACTACATGACCTTATGGGACTTTC	560	
Consensus	gcctggcattatgcccaactacatgacccattatgggacttcc		
pCas9-pdl1_part1	CTACTTGGCAGTACATCTACGTATTAGTCATCGCTTAC	598	
pCas9-pten_part1	CTACTTGGCAGTACATCTACGTATTAGTCATCGCTTAC	600	
Consensus	ctacttggcagatcacatctacgtattagtcatcgcttac		

pCas9-PTEN and PTEN primer alignment

DNAMAN File: Multiple Alignment			
pcas9-pten_parti	ACTATCATATGCTTACCGTAACITGAAGATTTGATT	40	
pten	0	
Consensus		
pcas9-pten_parti	CTTGGCTTTATATATCTIGGAAAGGACGAAACACCGGA	80	
pten	0	
Consensus		
pcas9-pten_parti	CATOGTTAGCAGAAAAGGTITTAGAGCTAGAAAATAGC	120	
pten	0	
Consensus		
pcas9-pten_parti	AAGTAAAATAAGGCTACTCCGTTATCAACTTGAAAAAGT	160	
pten	.. AGTAAAATAAGGCTACTCCGTTATCAACTTGAAAAAGT	38	
Consensus	t aaataaggctagtccggttatcaacttgaaaaagt		
pcas9-pten_parti	GGCACCGAGTCGGTGCTTTCTAGGCCAGCITTCTT	200	
pten	GGCACCGAGTCGGTGCTTTCTAGGCCAGCITTCTT	78	
Consensus	ggcacccgagtcggtgctttctaggcccagcittctt		
pcas9-pten_parti	CTACAAAGTTGCCATTAACCGGITGACATGATTATTGAC	240	
pten	CTACAAAGTTGCCATTAACCGGITGACATGATTATTGAC	118	
Consensus	gtacaaaagtggatttaacgcgttgacattgattttagtac		
pcas9-pten_parti	TAGTTATTAATAGTAATCAATTACGGGGICATTACTTCAT	280	
pten	TAGTTATTAATAGTAATCAATTACGGGGICATTACTTCAT	158	
Consensus	tagttatataatgtatcaattacggggtcatttagttcat		
pcas9-pten_parti	AGCCCATAATCGAGTTCCCGGITACATAACTTACGGTAA	320	
pten	AGCCCATAATCGAGTTCCCGGITACATAACTTACGGTAA	198	
Consensus	agcccaataatatggatcccggttacataacttacggtaa		
pcas9-pten_parti	ATGGCCCGCCTGGTGACCGGCCAACGACCCCCGCCATT	360	
pten	ATGGCCCGCCTGGNTGACCGGCCAACGACCCCCGCCATT	238	
Consensus	atggcccgccctgg tgaccggccaaacgacccccggccatt		
pcas9-pten_parti	GACGTCAATAATGACCTATGTTCCCATACTAACGCCAATA	400	
pten	GACGTCAATAATGAONATGTTCCCATACTAACGCCAATA	278	
Consensus	gacgtcaataatgac tatgttcccatacgtaaacgccaaata		
pcas9-pten_parti	GGGACTTTCCAATGACCTGCAATGGTGGACTATTIACGGT	440	
pten	GGGACTTTCCAATGACCTGCAATGGTGGACATTIACNGT	318	
Consensus	gggactttccattgac caatgggtggag attac gt		
pcas9-pten_parti	AAACTGCCACTTGGCACTACATCAACTTATCATATGCC	480	
pten	AAACTGCCACTTGGCACTACATCAAGNNNATNATATGCC	358	
Consensus	aaactgcccacttggca tacatcaag g at atatgcc		
pcas9-pten_parti	AAAGTACCCCCCTATTGACGTCAATGACCGTAAATGGCC	520	
pten	NN .TACCNCCCCCTANTGACNNCAATGANNNNNAAANGCCC	397	
Consensus	tagc cccta tgac caatga aa ggccc		
pcas9-pten_parti	CCCTGCTTAAATGCCACTACATACGCTTATGGCACTTTC	560	
pten	NNCTGCTTAAATGCCACTACATGNNNNNNNGNAC....	433	
Consensus	ctgg attatgcccagatacatg c t g ac		
pcas9-pten_parti	CTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTAC	600	
pten	433	
Consensus		

pCas9-PTEN and PTEN_mut fwd alignment

DNAMAN File: Multiple Alignment					
pcas9-pten_parti	ACTATCATATGCTTACCGTAACITGAAAGTATTTCGATT	40	pcas9-pten_parti	CATGGTATGCCGTTTTCGCAGTACATCAATGGCGTGGGA	640
pten_mut_fwd	0	pten_mut_fwd	CATGGTATGCCGTTTTCGCAGTACATCAATGGCGTGGGA	508
Consensus		Consensus	catggtatgcgttttggcagtcataatcaatggcgctgga	
pcas9-pten_parti	CTTGGCTTATATATCTIGTGGAAAGGACGAAACACCGGA	80	pcas9-pten_parti	TAGCGGTTTGCACGGGATITCCAACGCTCCACCCCCA	680
pten_mut_fwd	0	pten_mut_fwd	TAGCGGTTTGCACGGGATITCCAACGCTCCACCCCCA	548
Consensus		Consensus	tagcggtttactacacgggatttccaagtctccacccca	
pcas9-pten_parti	GATCGTTAGCAGAAAACAAGGTTTAGACCTAGAAATAGC	120	pcas9-pten_parti	TTGACGTCAATGGAGTTGTTTGGACCAAAATICA	717
pten_mut_fwd	0	pten_mut_fwd	TTGACGTCAATGGAGTTGTTTGGACCAAAATICAACG	588
Consensus		Consensus	ttgacgtcaatggagtttgttgg accaaaatca	
pcas9-pten_parti	AAGTTAAAATAACGCTAGTCGGTTATCAACTTGAAAAAGT	160	pcas9-pten_parti	717
pten_mut_fwd	28	pten_mut_fwd	GGACTTTCCAAAATGTCNNAAACAACCCGCCCCATTGACN	628
Consensus	g agtcgttatcaacttgaaaaagt		Consensus		
pcas9-pten_parti	GGCACCGAGTCGGTGCCTTTCTAGGCCAGCTTCTT	200	pcas9-pten_parti	717
pten_mut_fwd	GGCACCGAGTCGGTGCCTTTCTAGGCCAGCTTCTT	68	pten_mut_fwd	CANATNGGCGAGGCTGNACNGTNGAGGTCTNATAAGC	668
Consensus	ggcacccgaggcgggtttttctaggcccagcttctt		Consensus		
pcas9-pten_parti	GTACAAAGTTGCCATTAACCGGTTGACATTGATTATTGAC	240	pcas9-pten_parti	717
pten_mut_fwd	GTACAAAGTTGCCATTAACCGGTTGACATTGATTATTGAC	108	pten_mut_fwd	AAANNTCTCTGGCTNANTAGAGAANNCTGCTTACTGGCT	708
Consensus	gtacaaagggttgcattacgcgttgacattgttgcac		Consensus		
pcas9-pten_parti	TAGTTATTAAATAGTAATCAATTACGGGGTCATTACTTCAT	280	pcas9-pten_parti	...	717
pten_mut_fwd	TAGTTATTAAATAGTAATCAATTACGGGGTCATTACTTCAT	148	pten_mut_fwd	CTA	711
Consensus	tagtttaatagaatcaattacgggttcattatgttcat		Consensus		
pcas9-pten_parti	AGCCCATAATGGAGTTCGGTACATAACTTACGGTAA	320			
pten_mut_fwd	AGCCCATAATGGAGTTCGGTACATAACTTACGGTAA	188			
Consensus	agcccatatatggagttcccggttacataacttacggtaa				
pcas9-pten_parti	ATGGCCCGOCTGGCTGACCGCCCAACGACCCCCGCCATT	360			
pten_mut_fwd	ATGGCCCGOCTGGCTGACCGCCCAACGACCCCCGCCATT	228			
Consensus	atggcccgccctggctgaccggcccaacgacccccggccatt				
pcas9-pten_parti	GACGTCAATAATGACGTATGTCGGCATAGTAACGCCATA	400			
pten_mut_fwd	GACGTCAATAATGACGTATGTCGGCATAGTAACGCCATA	268			
Consensus	gacgtcaataatgacgtatgttcccatagtaacgcctaata				
pcas9-pten_parti	GGGACTTTCCAATGACGTCAATGGGGAGTATTACGGT	440			
pten_mut_fwd	GGGACTTTCCAATGACGTCAATGGGGAGTATTACGGT	308			
Consensus	gggactttccattgacgtcaatgggtggagtttacggt				
pcas9-pten_parti	AAACTGCCACTTGGCACTACATCAAGTCTATCATATGCC	480			
pten_mut_fwd	AAACTGCCACTTGGCACTACATCAAGTCTATCATATGCC	348			
Consensus	aaactggccacttggcagtcataatcaagtgtatcatatgcc				
pcas9-pten_parti	AAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCC	520			
pten_mut_fwd	AAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCC	388			
Consensus	aaatgacgtccctatttgacgtcaatgacggtaatggccc				
pcas9-pten_parti	GCCTGGCATTATGCCCACTACATGACCTTATGGGACTTTC	560			
pten_mut_fwd	GCCTGGCATTATGCCCACTACATGACCTTATGGGACTTTC	428			
Consensus	gcctggcattatggccagtcataatgacgttgcatttttc				
pcas9-pten_parti	CTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTAC	600			
pten_mut_fwd	CTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTAC	468			
Consensus	ctacttggcagtcataatctacgtatgttgcattttac				

pCas9-PTEN and PTEN_mut rev alignment

DNAMAN File: Multiple Alignment

```

pcas9-pten parti ..... CCTATGGAAAAANNCAGCAACGGGCCTTTACGGTTC
pten mut rev_rc
Consensus

pcas9-pten parti ..... CTGGCCITTCGICGGCCITTCGCACAGTTCTIGCTGC
pten mut rev_rc
Consensus

pcas9-pten parti ..... ITCGCGATGTACGGGCCAGATAACGCGIAAGGICGGGCA
pten mut rev_rc
Consensus

pcas9-pten parti ..... GGAAGACGGCCIATTCCCATGAITCCTICATAIITGCAT
pten mut rev_rc
Consensus

pcas9-pten parti ..... ATACGAIACAAGGCTGIIAGAGAGATAATAGAATAATT
pten mut rev_rc
Consensus

pcas9-pten parti ..... TIGACTGIAAACACAAAGATATTAGTACATAATAACGTGACG
pten mut rev_rc
Consensus

pcas9-pten parti ..... IAGAAAAGTAATAATTCTGGGIAAGTTCCAGTTAAAAA
pten mut rev_rc
Consensus

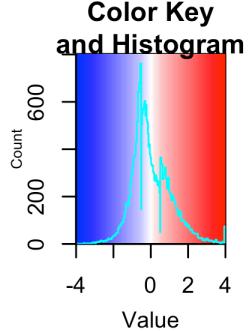
pcas9-pten parti ..... ACTATCAIATGCITACCGTAACTT
pten mut rev_rc
Consensus
ACTATCAIATGCITACCGTAACTT
TATGTTAAATGGACTATCAIATGCITACCGTAACTT
actatcatatgcttaccgttaactt

pcas9-pten parti ..... GAAAGIATTTCGATTTCITGGCTTATATATCTTGGAAT
pten mut rev_rc
Consensus
GAAAGIATTTCGATTTCITGGCTTATATATCTTGGAAT
caaagtatttcgattttctggcttatatat

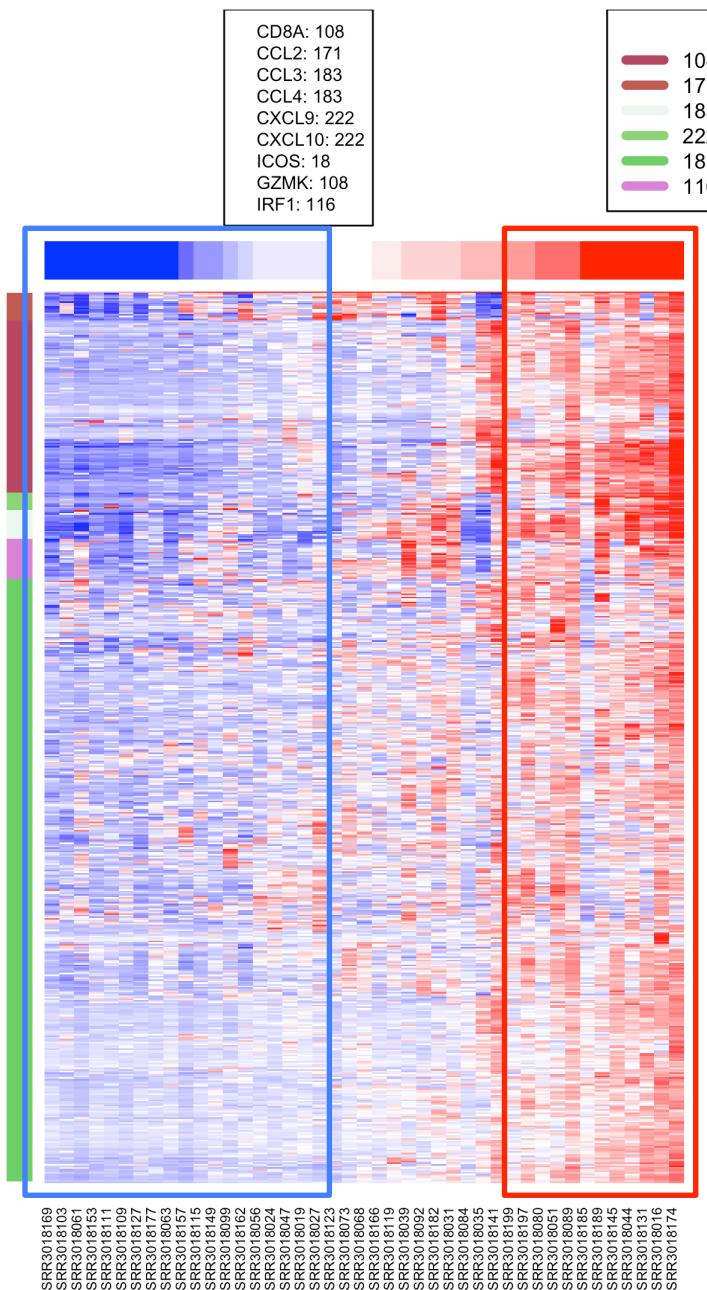
pcas9-pten parti ..... AGGACGAAACACCGGAGATCGTIAGCAGAACAAAG
pten mut rev_rc
Consensus
.....
```

pCas9-PTEN and U6_Fwd alignment

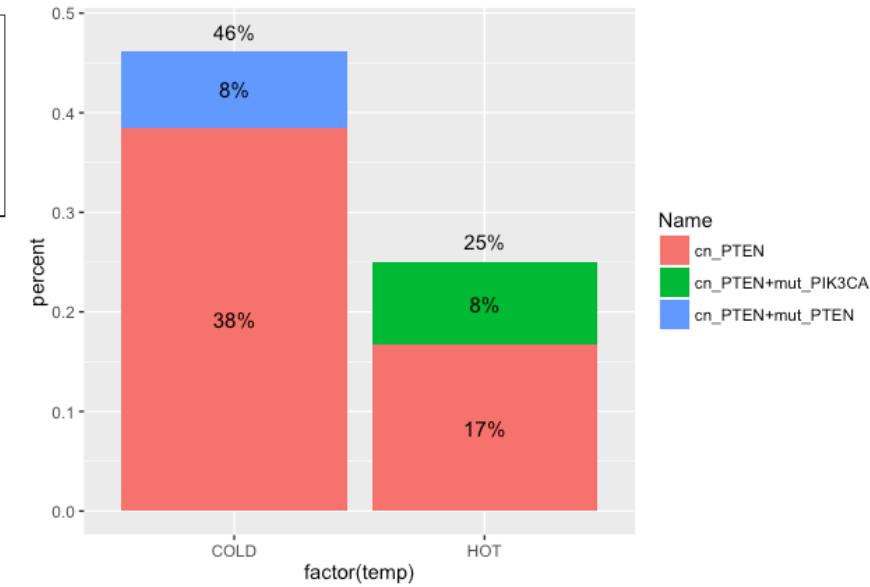
DNAMAN File: Multiple Alignment			
pcas9-pten_parti	ACTATCATATGCTTACCGTAACITGAAAGTATTTCGATTT	40	
u6_clean	CATTT	5
Consensus	attt	
pcas9-pten_parti	CTTGGCTTATATATCTTGAAAGGACGAAAACACCGGA	80	
u6_clean	CTTGGCTTATATATCTTGAAAGGACGAAAACACCGGA	45	
Consensus	cttggcttataatatcttgaaaggacgaaaacacccgga		
pcas9-pten_parti	CATCGTTAGCAAAACAAAGCTTTAGAGCTAGAAATAGC	120	
u6_clean	NATCGTTAGAGCTCCCAA.....GTTTAGAGCTAGAAATAGC	83	
Consensus	atcgttag g caa gtttagagctagaataatgc		
pcas9-pten_parti	AAGTTAAAAATAAGGCTACTCGTTATCAACTTGAAGAAGT	160	
u6_clean	AAGTTAAAAATAAGGCTACTCGTTATCAACTTGAAGAAGT	123	
Consensus	aagttaaaaaaaaggctagtcgttatcaacttgaaaaagt		
1 pcas9-pten_parti	GGCACCGAGTCCGTGCTTTTCTAGGCCAGCTTCTT	200	
u6_clean	GGCACCGAGTCCGTGCTTTTCTAGGCCAGCTTCTT	163	
Consensus	ggcacccaggatcggtgtttttctaggcccaagtttctt		
1 pcas9-pten_parti	GTACAAAGTGGCATTACCGCGTTGACATTGATTATTGAC	240	
u6_clean	GTACAAAGTGGCATTACCGCGTTGACATTGATTATTGAC	203	
Consensus	gtacaaaatggcattacccgttgcacattgttttgcac		
2 pcas9-pten_parti	TAGTTATTAATGTAATCAATTACCGGTCATTAGTCAT	280	
u6_clean	TATTTTACNT2NNAAANCAATTACNGNGTNTTNTTCAT	243	
Consensus	ta tta t ta aa caattac g gtc tt ttcat		
pcas9-pten_parti	AGCCCCATATATCAGATTCCCGTTAGATAACCTTACGGTAA	320	
u6_clean	ANCCCCACANANINGAGTCNNCGINACATAACCTTACGNTAA	283	
Consensus	acccca a a gagttc cgt ac taacttag taa		
2 pcas9-pten_parti	ATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCATT	360	
u6_clean	A.....	284	
Consensus	a		
2 pcas9-pten_parti	GACGTCATAATGACGTATGTTCCCATAAGTAACGCCAATA	400	
u6_clean	284	
Consensus			
3 pcas9-pten_parti	GGGACTTCCATTGACGTCAATGGTGGAGTTAACGGT	440	
u6_clean	284	
Consensus			
pcas9-pten_parti	AAACTGCCACTTGGCACTACATCAAGTGTATCATATGCC	480	
u6_clean	284	
Consensus			
pcas9-pten_parti	AAAGTACGCCCTATTGACGTCAATGACGGTAAATGGCCC	520	
u6_clean	284	
Consensus			
3 pcas9-pten_parti	GCCTGGCATTATGCCACTACATGACCTTATGGGACTTTC	560	
u6_clean	284	
Consensus			
pcas9-pten_parti	CTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTAC	600	
u6_clean	284	
Consensus			



Mets w/ outl:500 CC k=13#: 553



K=500, cc=13, cold:5 hot:3
Cold = 13 # Hot = 12



cn_PTEN: 0.248
changed unchanged

COLD 7 7

HOT 3 9

mut_PIK3CA: 0.462
changed unchanged

HOT 1 11

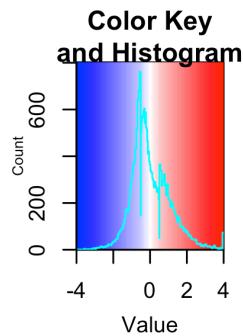
COLD 0 14

mut_PTEN: 1
changed unchanged

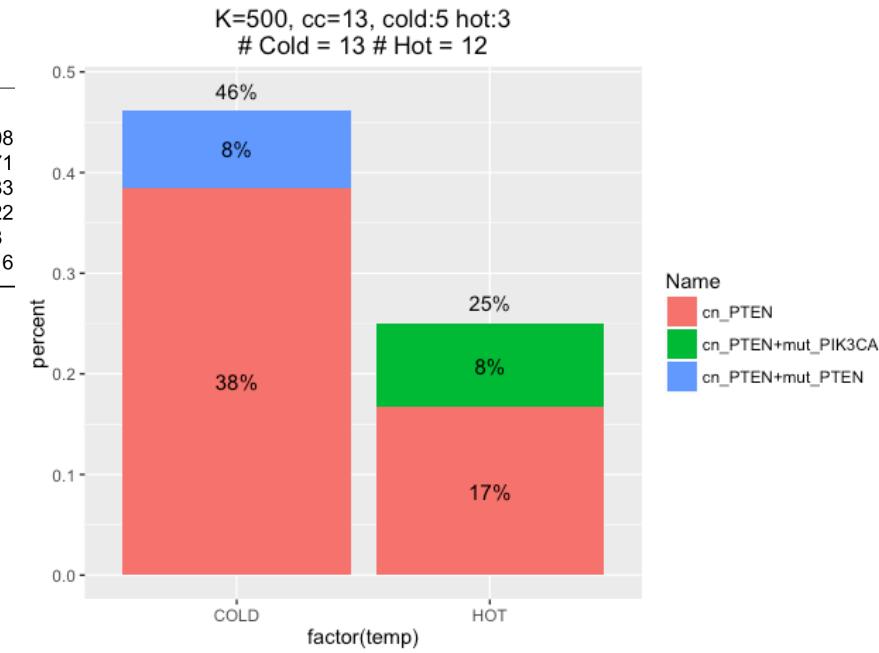
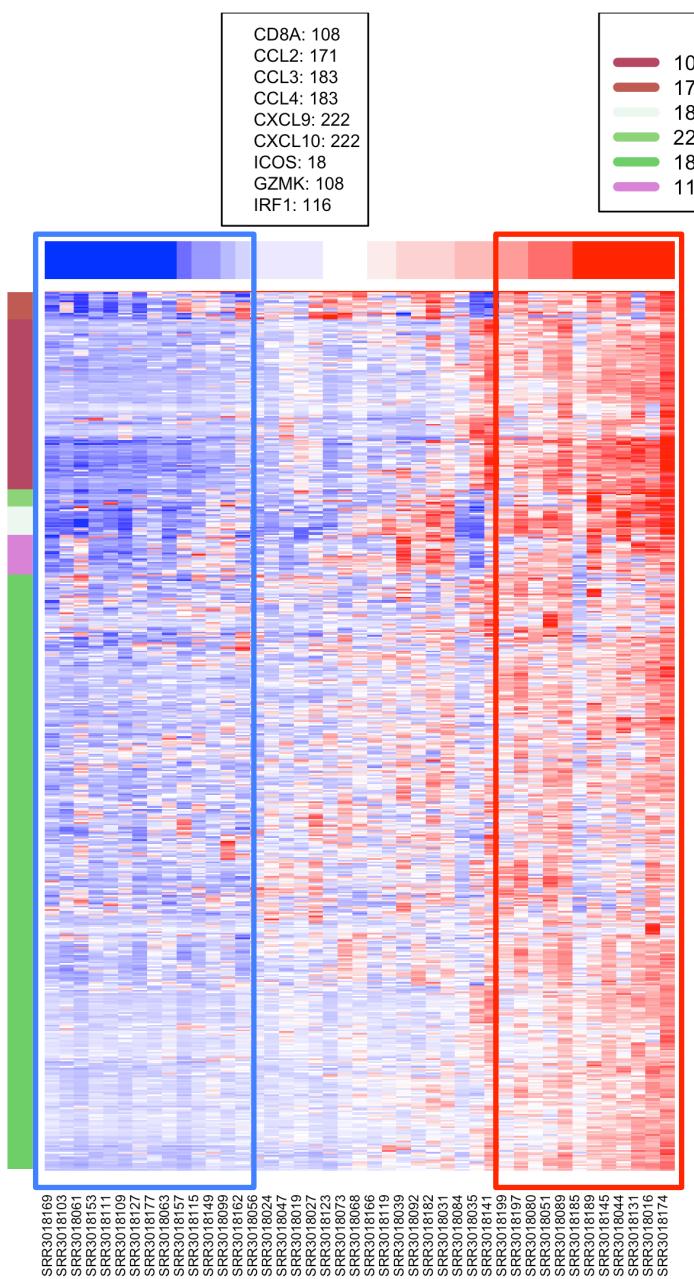
COLD 1 13

HOT 0 12

for all activating mutations, p-value=0.248



Mets w/ outl:500 CC k=13#: 553



cn_PTEN: 0.411
changed unchanged

COLD 6 7
HOT 3 9

mut_PIK3CA: 0.48
changed unchanged

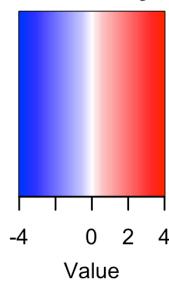
HOT 1 11
COLD 0 13

mut_PTEN: 1
changed unchanged

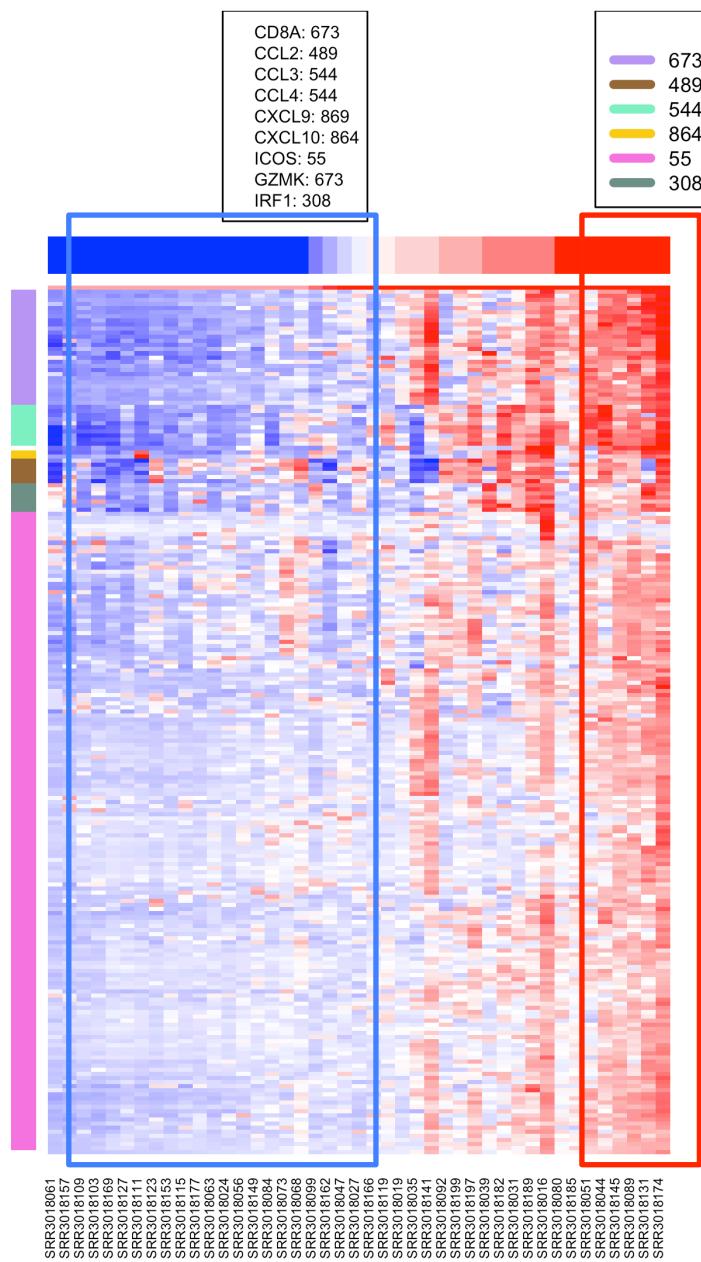
COLD 1 12
HOT 0 12

for all activating mutations, p-value=0.411

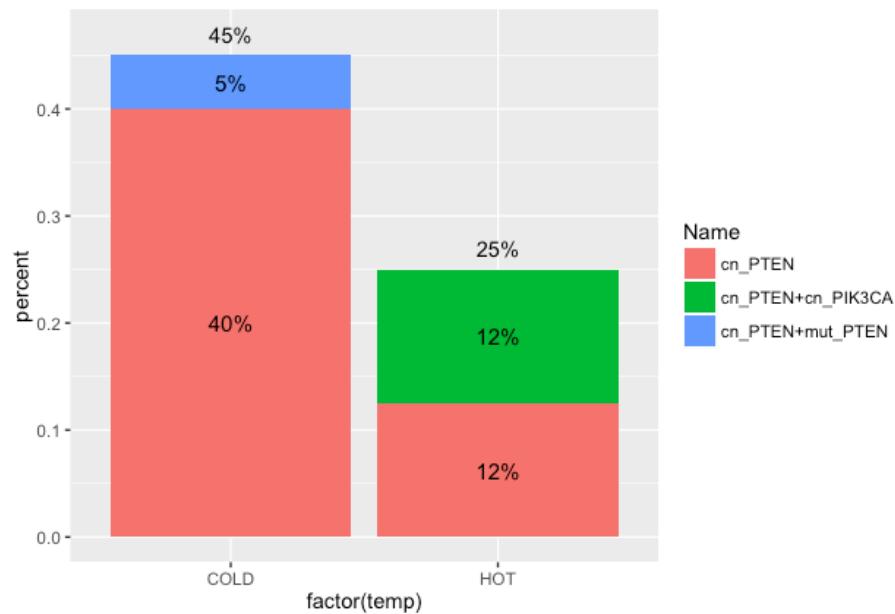
Color Key



Mets w/ outl:2000 CC k=10#: 209



K=2000, cc=10, cold:4 hot:1
Cold = 20 # Hot = 8



cn_PTEN: 0.419
changed unchanged

COLD 9 11

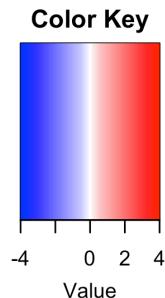
HOT 2 6

mut_PTEN: 1
changed unchanged

COLD 1 19

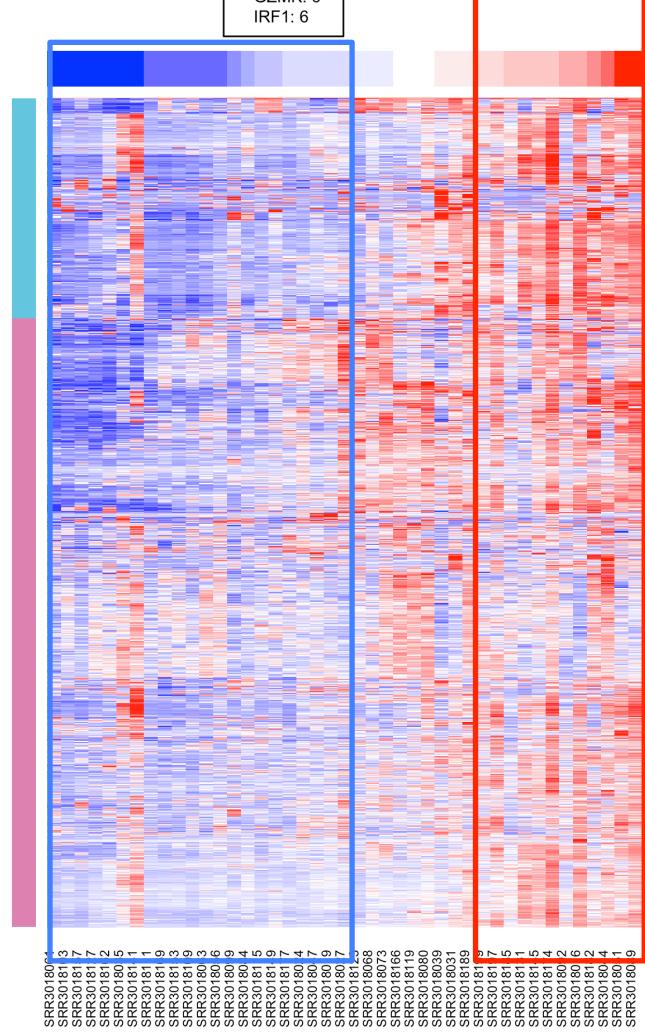
HOT 0 8

for all activating mutations, pvalue=0.419

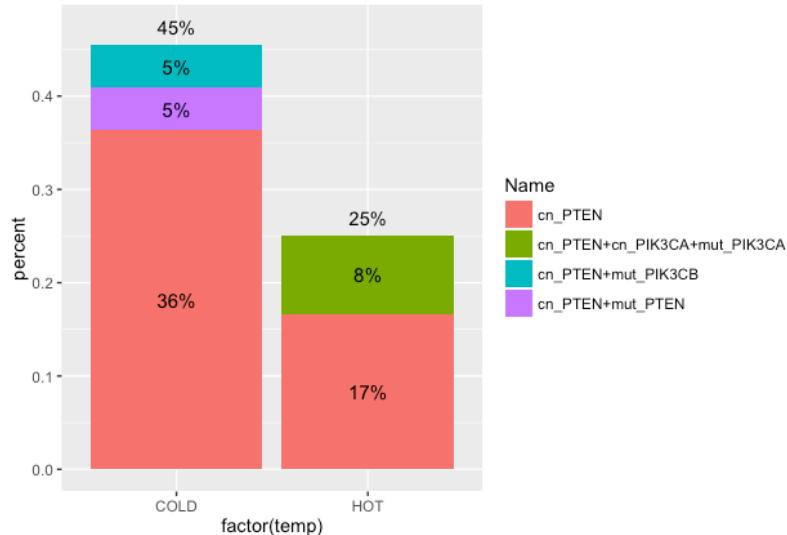


Mets w/ outl:50 CC k=15#: 1491

CD8A: 6
CCL2: 4
CCL3: 6
CCL4: 6
CXCL9: 6
CXCL10: 6
ICOS: 4
GZMK: 6
IRF1: 6



K=50, cc=15, cold:6 hot:6
Cold = 22 # Hot = 12



```
[1] "cn_PTEN: 0.292"  
     changed unchanged
```

COLD 10 12

HOT

[1] "cn PIK3CA: 0.

changed unchanged

HOT 1 11

COLD 0 22

```
[1] "mut_PIK3CA: 0.353"  
     changed unchanged
```

changed uncha

HOT 1 11

COLD 0 22
[1] "mut_PIK3CB: 1"

[±] mat_FIRSEB:
changed uncha

changed uncl

COLD 1 21
HOT 0 12

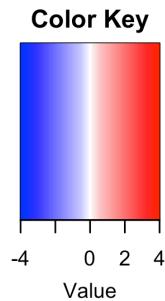
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[1] "mut PTEN: 1"
```

[–] was uncha

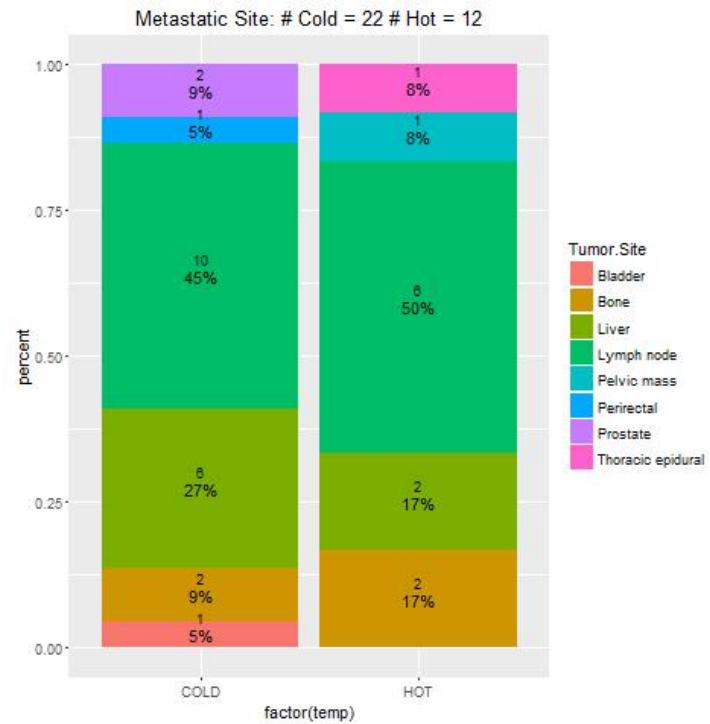
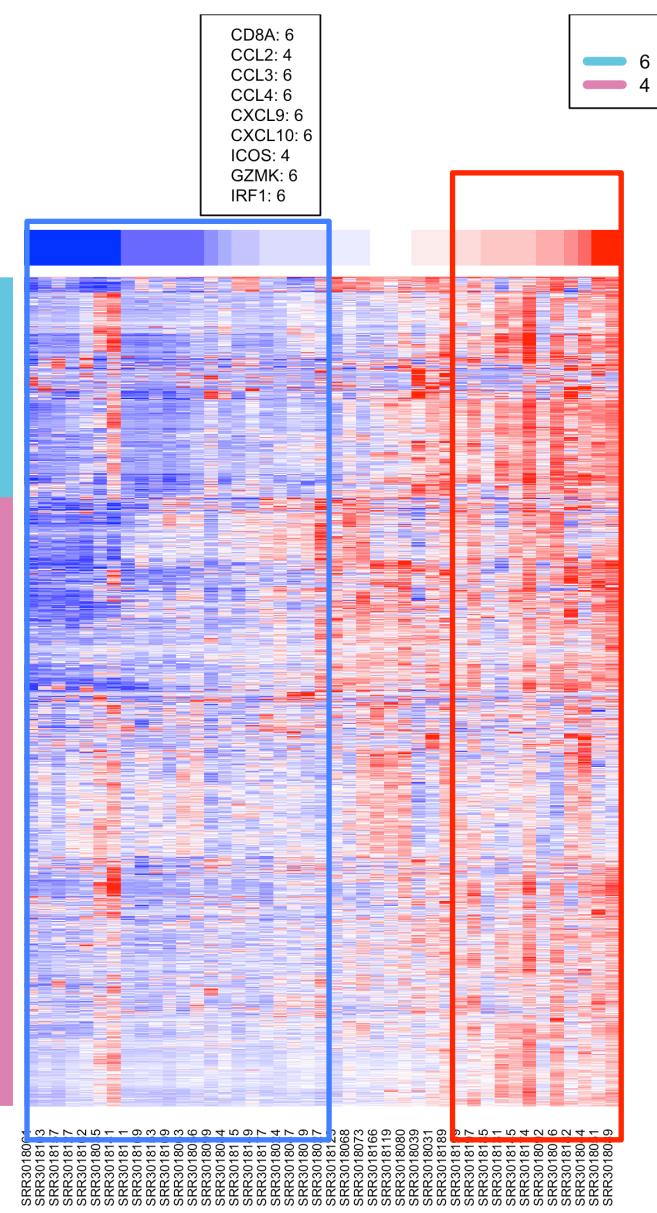
changed uncharged
COLD 1 21

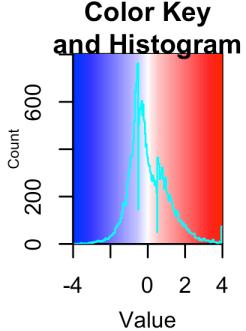
HOT 0 12

```
[1] "for all activation functions
```

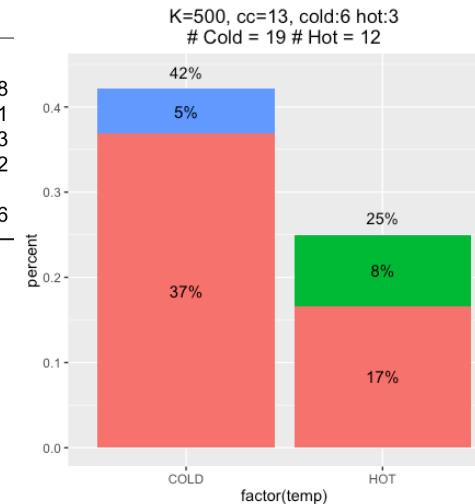
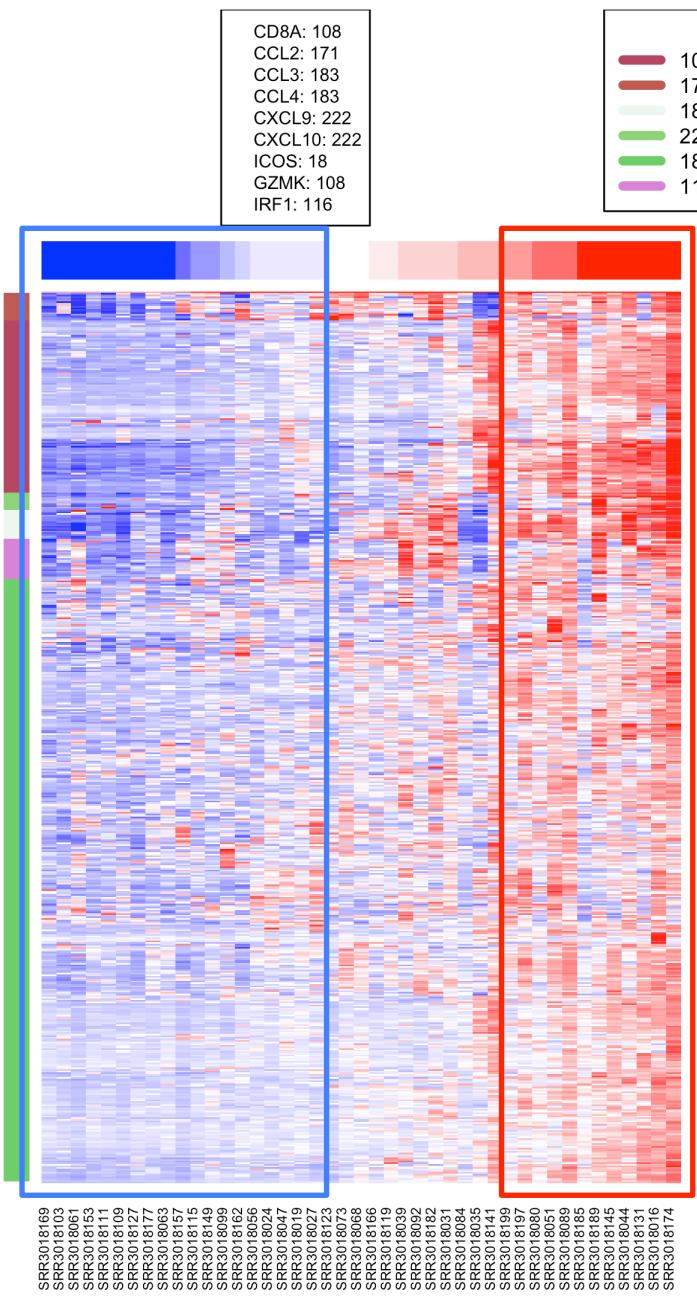


Mets w/ outl:50 CC k=15#: 1491





Mets w/ outl:500 CC k=13#: 553



[1] "cn_PTEN: 0.452"
changed unchanged

COLD 8 11

HOT 3 9

[1] "mut_PIK3CA: 0.387"
changed unchanged

HOT 1 11

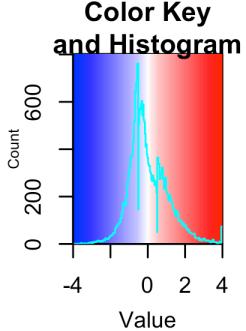
COLD 0 19

[1] "mut_PTEN: 1"
changed unchanged

COLD 1 18

HOT 0 12

[1] "for all activating mutations, p-value=0.452"



Mets w/ outl:500 CC k=13#: 553

