INDUCIBLE ACTIVATION OF sgRNA LIBRARIES IN TUMOR XENOGRAFTS EMPOWERS LARGE-SCALE IN VIVO TARGET DISCOVERY SCREENS

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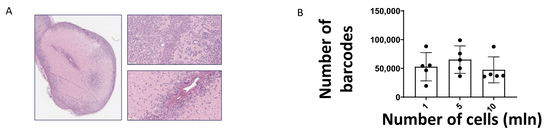
ABSTRACT

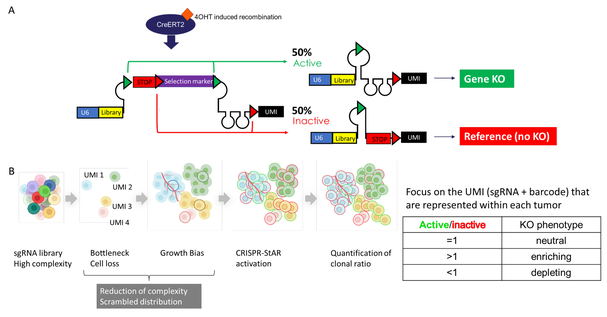
CRISPR-mediated genome editing is a powerful approach to understanding disease biology, including identifying genes essential for cancer cell proliferation, immune evasion and survival. We routinely use the technology to identify novel therapeutic targets for cancer using both *in vitro* and *in vivo* tumor models, focusing on targets that are synthetic lethal with tumor suppressor gene loss. Large-scale CRISPR screens are often conducted using *in vitro* cell culture systems as *in vivo* 'drop-out' screens face several bottlenecks resulting in poor sgRNA library representation and low signal to noise ratio. These inherent limitations of *in vivo* screens occur because only a small fraction of the injected cells contribute to xenograft tumor formation, and because of the uneven clonal expansion due to heterogenous growth conditions in the tumor microenvironment. Hence, many *in vivo* screens are underpowered for statistical analysis, resulting in a high rate of 'false negatives' or 'false positives' unless a large number of animals are used or the size of the sgRNA library is greatly reduced to overcome sampling noise. Here, we validate a novel *in vivo* screening technology, CRISPR-StAR (Stochastic Activation by Recombination), that overcomes these challenges by (i) activating the sgRNA library in established tumors and (ii) generating internally matched-pair controls for each sgRNA using molecular barcodes to capture the history of each clone within the tumor. Using this clonal information, we developed a robust computational pipeline that extracts meaningful target sgRNA-level data from individual tumors, despite the random under-representation of the larger library. Statistical (down-sampling) analysis revealed that CRISPR-StAR has a resolution of 1,000 sgRNAs per tumor which reduces the number of animals required by 7-fold to traditional approaches. Consequently, we have performed several druggable genome screens (~30,000 sgRNA) using just 30-40 individual tumors and identified a catalog of tumor suppressor genes that, when lost, strongly promote tumor growth *in vivo* without affecting cell proliferation *in vitro*. This group of genes is enriched with epigenetic modifiers, in particular multiple members of the COMPASS family and the SWI/SNF chromatin remodeling complexes. These results validate CRISPR-StAR as a powerful *in vivo* functional genomics platform for high throughput target discovery screens.

IN VIVO SCREENING IS LIMITED BY TUMOR HETEROGENEITY AND LOW ENGRAFTMENT



Heterogeneity of cancer cell growth and low engraftment rate in A549 xenograft limits in vivo dropout screening
A) Representative image of H&E staining of one tumor collected 10 days post injection. Areas of faster cell growth and areas of cell death are captured at higher magnification.
B) Independent of the number of cells injected, an average of about 50K cell clones were captured via semirandom barcodes. The NGS data also captured wide differences in clonal representation, reflecting the heterogenous cell proliferation within the tumor (not shown).

CRISPR-StAR SCREENING PLATFORM PROVIDES A SOLUTION

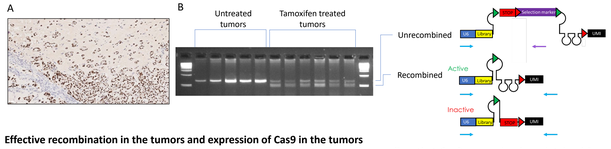


Focus on the UMI (sgRNA + barcode) that are represented within each tumor

Active/inactive	KO phenotype
=1	neutral
>1	enriching
<1	depleting

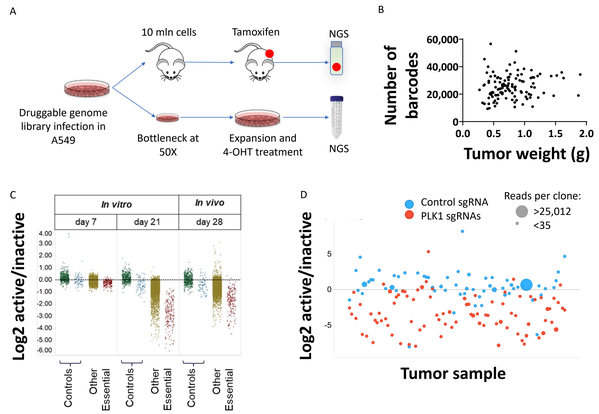
CRISPR-StAR (Stochastic Activation by Recombination) technology introduces internal controls for normalization
A) Induction of Cre recombinase activity by 4-OHT generates matched pair of gene KO and reference sample for each clone within the tumor. The inactive reference population captures the history of each single cancer cell clone.
B) CRISPR-StAR system is inducible. Recombination can be induced after the tumor is established. As a result, CRISPR-StAR screening allows faithful evaluation of gene requirement for tumor maintenance rather than combined effect that also includes in vitro phenotype and engraftment.

IN VIVO STOCHASTIC ACTIVATION OF sgRNAs EXCLUSIVELY OCCURS AFTER TAMOXIFEN ADMINISTRATION



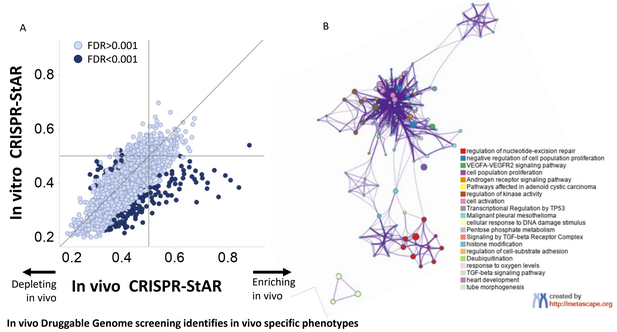
Effective recombination in the tumors and expression of Cas9 in the tumors
A) IHC staining of Cas9 in tumor section at time of induction with Tamoxifen confirm that Cas9 expression is maintained for gene editing after recombination.
B) PCR strategy to evaluate recombination with a three primers PCR. No recombination was detected at endpoint in tumors that were not treated with Tamoxifen. Recombination rate in tumors treated with Tamoxifen was 60%. The unrecombined construct is not amplified during library preparation for NGS.

CRISPR-StAR ENABLES LARGE SCALE IN VIVO SCREENS



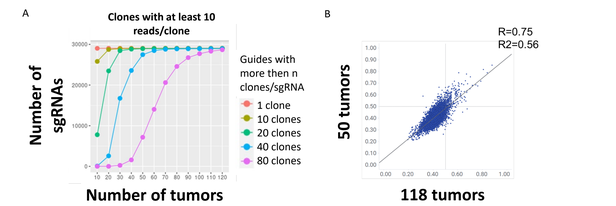
Scheme and QC in the Druggable Genome CRISPR-StAR screening
A) Druggable genome screening layout. Library size of 30,000 sgRNAs. Number of mice = 118.
B) 10% of the tumor mass was amplified for NGS library preparation. We infer our strategy did not introduce biases within the screening as we did not observe correlation between total tumor weight and number of barcodes identified.
C) Effective separation of essential genes and control sgRNAs both in vitro and in vivo at the end of the screening. The analysis includes all the tumor samples combined. The plotted active/inactive ratio is the average of the clonal ratios per gene.
D) Example of essential gene dropout in individual tumors compared to intron cutting control. The plotted active/inactive ratio is the average of the clonal ratios for all the sgRNAs per gene for each tumor.

NOVEL PHENOTYPES IDENTIFIED BY SCREENING IN VIVO VS IN VITRO



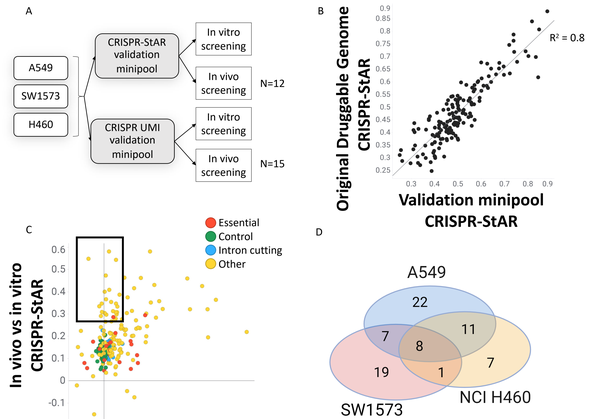
In vivo Druggable Genome screening identifies in vivo specific phenotypes
A) Bayesian statistical model comparison of the active vs inactive in vitro and in vivo (all samples combined). The correlation between the in vitro vs in vivo Bayesian model identifies a subset of gene KO that significantly (FDR<0.001) accelerate tumor growth or inhibit tumor growth in vivo.
B) Gene pathway analysis with Metascape of the significant top scoring hits in the in vivo screening vs in vitro screening. Gene pathways associated with epithelial to mesenchymal transition and epigenetic modifiers are predominant in the analysis.

CRISPR-StAR INCREASES IN VIVO SCREENING CAPACITY



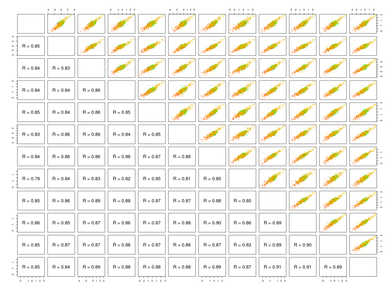
In vivo Druggable Genome screening at high resolution in A549 model using CRISPR-StAR
A) In silico downsampling analysis suggests 40 clones/sgRNA is sufficient to power Bayesian analysis. Therefore, for A549 model, only 40 tumors are needed for a 30,000 sgRNAs library.
B) Experimental validation with true biological replicate confirms downsampling analysis.

CRISPR-StAR IS INTERNALLY CONTROLLED AND HIGHLY REPRODUCIBLE



CRISPR-StAR is highly reproducible and probes new biology
A) A validation minipool library (1000 sgRNA) was designed to include the top enrichers and depleters in the previous druggable genome screening. The sgRNAs library was screened in the CRISPR-StAR system and conventional constitutive CRISPR-UMI system.
B) High reproducibility of the CRISPR-StAR platform, as shown by the correlation between the 2 screens in A549.
C) When comparing the in vivo vs in vitro scores for CRISPR-StAR screening and the CRISPR UMI screening in A549, a large portion of hits (box) are lost in the constitutive approach (CRISPR UMI), possibly relates to in vitro and engraftment effects.
D) Overlap of screening hits across cell lines.

ONE TUMOR IS SUFFICIENT FOR FUNCTIONALIZING 1000 sgRNAs



High correlation between tumors in the CRISPR-StAR screening
Strong correlation of the Log2 active/inactive ratio generated from each tumor.

SUMMARY

CRISPR-StAR enables highly efficient in vivo screen for novel target discovery

- **Druggable genome screening in 40 tumors from CDX models**
- **Inducible system in established tumor**
- **Clonal level resolution with internal sgRNA controls circumvent heterogeneity and engraftment bottlenecks**
- **Higher throughput and capabilities**
- **Batch effect correction allows reproducibility between experiments and flexibility to add on mice after preliminary analysis**

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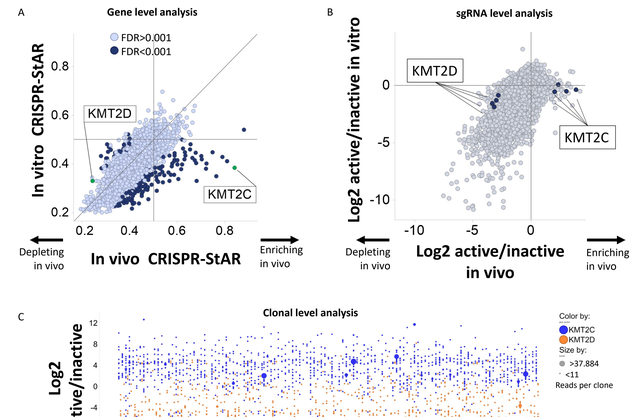
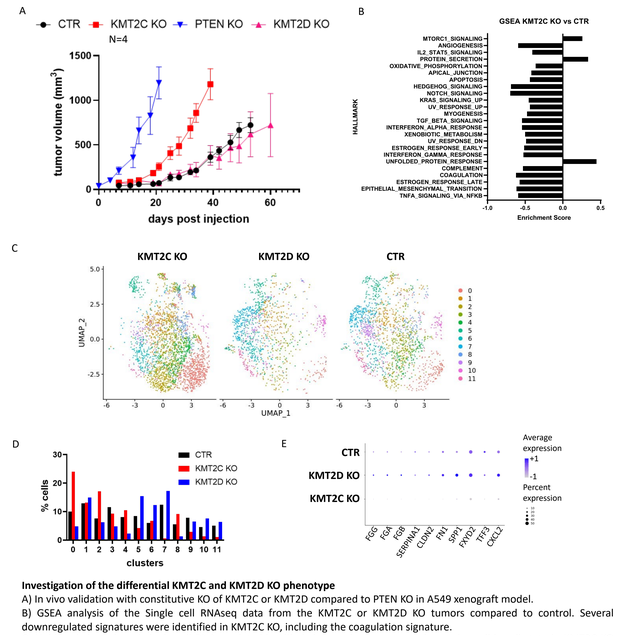
KMT2C AND KMT2D HAVE DIFFERENTIAL IN VIVO SPECIFIC PHENOTYPES IN A549



KMT2C and KMT2D in the Druggable Genome screening have opposite in vivo phenotypes
A) While KMT2C KO strongly promote tumor growth, KMT2D KO has a depletion phenotype both in vitro and in vivo.
B) Average of the clones' active/inactive ratios per sgRNAs in the in vitro and in vivo screening support the statistical conclusions in A, with all the sgRNAs performing similarly.
C) Active/inactive ratio for each clone per tumor sample pertaining KMT2C and KMT2D gene, all 4 sgRNAs are plotted. The in vivo effects of KMT2C and KMT2D KO are consistent regardless of individual reads number per clone.

INVESTIGATION OF THE DIFFERENTIAL KMT2C AND KMT2D KO PHENOTYPES



Investigation of the differential KMT2C and KMT2D KO phenotype
A) In vivo validation with constitutive KO of KMT2C or KMT2D compared to PTEN KO in A549 xenograft model.
B) GSEA analysis of the Single cell RNAseq data from the KMT2C or KMT2D KO tumors compared to control. Several downregulated signatures were identified in KMT2C KO, including the coagulation signature.
C) Unbiased clustering of single cell RNAseq data identified changes in gene expression and possibly cell state in KMT2C KO tumors, which were absent in control and KMT2D KO tumors. D) Quantification of clusters identified in C).
E) Differential genes that defines Cluster7, which is greatly under-represented in KMT2C KO and is consistent with the GSEA data which reflected the down regulation of coagulation pathway in B).