Setd2 loss results in retained introns that may be translated

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Abstract

SETD2 is often mutated in a subset of therapeutically intractable cancers, including 10% of primary clear cell Renal Cell Carcinoma (ccRCC), papillary RCC (pRCC), and also 30% of metastatic ccRCC. We have shown that Setd2 mutations cause intron retention and may result in protein misfolding due to aberrant translation of introns.

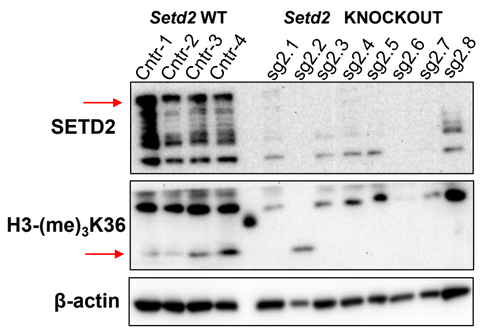
Using CRISPR we created Setd2 knock-out (KO) murine Renal Cell Carcinoma cells (RENCA) and performed RNA-sequencing. We compared prevalence of transcripts with retained introns between *Setd2*-KO vs control clones. *Setd2*-KO cells demonstrated increased number of retained introns as well as significant increase of intron retention level over control cells ($p<1x10-15$).

We hypothesized that if the retained introns translate to peptides, these peptides would improperly fold and activate the Unfolded Protein Response (UPR) via accumulation of unfolded proteins in the endoplasmic reticulum. Hallmark Gene Set Enrichment Analysis (GSEA) of transcriptomes revealed significant enrichment of UPR transcriptional signature in *Setd2*-KO cells compared to negative control (NES ≥ 1.46, q<0.1). Similarly, UPR pathway was strongly enriched in 51 SETD2-mutant ccRCC samples vs 196 *SETD2*-WT tumor cases contained in TCGA (NES 1.57, q=0.012). GSEA also revealed strong enrichment of many immune inflammatory pathways. This suggests that SETD2-mutant tumors are inflamed, possibly related to excessive presence of misfolded proteins.

We confirmed activation of the UPR in vitro by detecting such indicators of UPR activation as ATF6 nuclear translocation and increase of cleaved ATF6, ATF4 and Xbp1 levels in Setd2-deficient vs. unedited RENCA cells. *SETD2*-mutant human tumors also demonstrated nuclearized ATF6, whereas *SETD2*-WT tumors and stroma from both genotypes had no ATF6 nuclear localization.

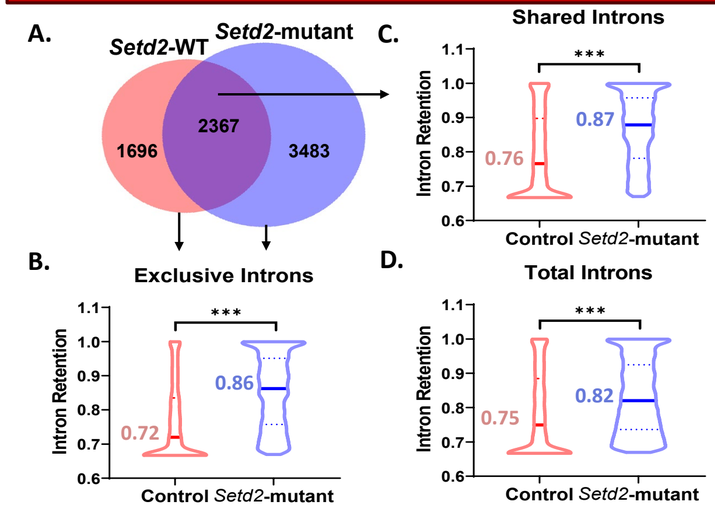
Taken together our findings support the hypothesis that SETD2 loss results in retained introns, which may be translated. We are now exploring this possibility using proteomics approaches.

CRISPR-mediated Setd2 knock out in RENCA



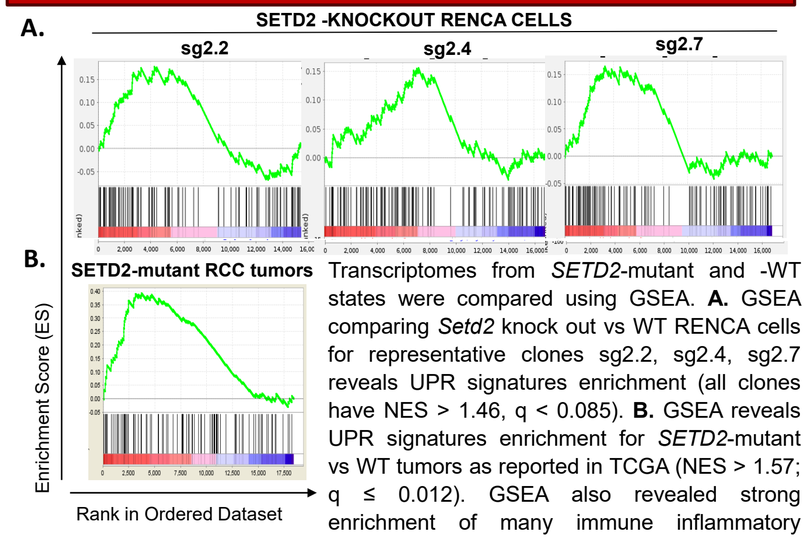
Using several single guide RNAs (sgRNA) and RENCA cells, we created RCC cells harboring *Setd2* knock out (KO) cells. Upon immunoblot, the 250 kDa Setd2 protein and trimethyl-H3K36 are absent in KO clones. Representative data from clones targeted by sg2 are presented; sg3 clones harbored similar phenotype.

Setd2-deficient cells retain introns



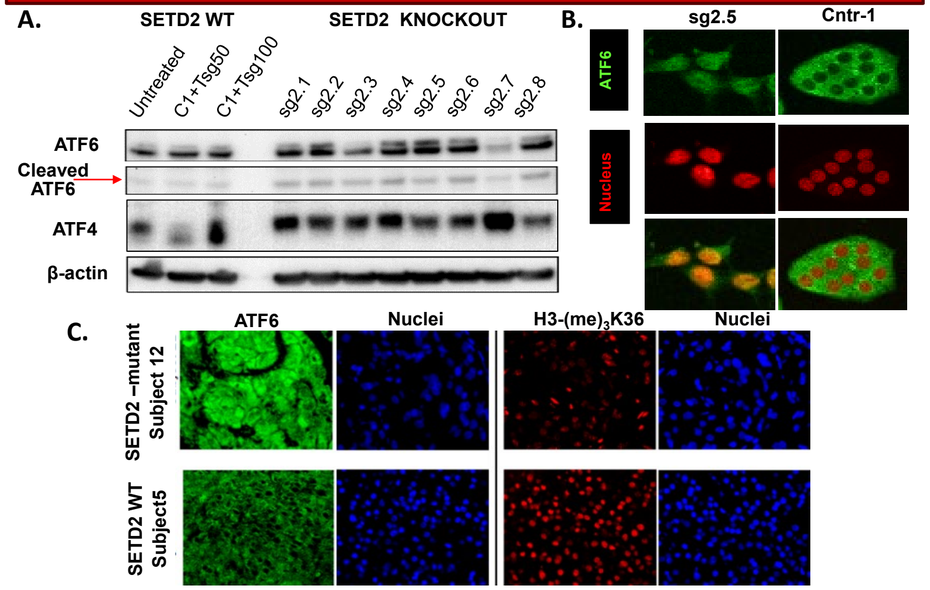
RNAseq data were analyzed using eXpress and KMA algorithm to estimate level of retained introns. Each retained intron was given a score from 0-1 which is representative of the degree of retention. *Setd2-KO* cells demonstrate have higher number of introns overall (**A**), higher intron retention score for uniquely retained introns (**B**) and even for introns that are shared in both control and knockout cells (**C**), as well as across all retained introns (**D;** ***, Student's two-sided t test, $p < 1x10^{-15}$; median is shown).

Setd2 loss strongly associates with activated Unfolded Protein Response (UPR) transcriptional signature



Transcriptomes from *SETD2*-mutant and -WT states were compared using GSEA. **A.** GSEA comparing *Setd2* knock out vs WT RENCA cells for representative clones sg2.2, sg2.4, sg2.7 reveals UPR signatures enrichment (all clones have NES > 1.46, q < 0.085). **B.** GSEA reveals UPR signatures enrichment for *SETD2*-mutant vs WT tumors as reported in TCGA (NES > 1.57; q ≤ 0.012). GSEA also revealed strong enrichment of many immune inflammatory pathways (not shown), suggesting that *SETD2*-mutant tumors are inflamed, possibly related to excessive presence of misfolded proteins.

Setd2 loss activates UPR in cells and RCC tumors



Setd2-WT cells were treated with 0, 50 and 100 μM thapsigargin (Tsg), which induces the UPR as a positive control; *Setd2 KO* causes ATF6 cleavage and induces ATF4 (**A**) and ATF6 nuclear translocation (**B**), which are markers of UPR pathway activation. Representative data for sg2 clones are shown, results for sg3 are similar.

Additionally, *SETD2*-mutant tumors (from patients receiving treatment at FCCC) display cytoplasmic and nuclear localization of ATF6 and lack of H3-K36 trimethylation (**C,** top). Note that H3-K36-trimethylation-positive nuclei in this sample are mostly elongated and therefore likely stromal. In contrast, a *SETD2* WT tumor (**C,** bottom) lacks ATF6 nuclear localization but has strong H3-(me)3-K36 signal. ATF6 and H3-K36 trimethylation were assessed on consecutive sections.

Conclusion and Future Direction

- *Setd2* mutation causes decrease in H3K36 trimethylation and as result aberrant intron retention in RENCA cells and likely in human tumors
- We confirmed increase in both intron retention level and number of retained introns in *Setd*-KO cells compared vs. *Setd*-WT RCC cells
- We have shown, that *Setd2* (and *SETD2*) loss enriches the transcriptional signature of UPR in vitro and in human tumor samples
- Activation of UPR supports the hypothesis, that introns retained due to SETD2 loss might be translated and result an abundance of misfolded proteins in RCC cells
- We are currently identifying translated introns using proteomics approaches and we hypothesize that *SETD2*-mutant tumors might be inflamed due to presence of these misfolded proteins

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