

tRNA dosage regulates lineage dependency and resistance in prostate cancer

Corresponding Author: Dr Andrew Hsieh

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)

Kim et al discover that a specific tRNA isodecoder tRNA Arg UCU-1 is a key regulator of lineage plasticity and therapy resistance in prostate cancer. The authors show that this isodecoder is both necessary and sufficient for lineage plasticity and its overexpression sensitizes cells to AR pathway-targeted therapies. Several transcription factors, especially TARDBP and ZSCAN29 directly regulate expression of the isodecoder. tRNA-Arg-UCU-1 enhances the translational efficiency of AGA codon-enriched mRNA, which include SWI/SNF chromatin remodeling machinery. Clinical studies validate that tRNA-Arg-UCU-1 expression correlates with significant reduction in NE prostate cancer and shorter time to bone metastases as well as reduced overall survival. Overall, this is an impressive study, that very clearly validates the function of a single tRNA isodecoder in prostate cancer and therapy resistance in a variety of complementary models. The clinical data is also a very strong part of the study.

There are some points of clarification below.

Major points

1. Since tRNA-Arg-UCU-4 isodecoder does not change in the transition from LP to AD, it would strengthen the conclusion if the authors used it as a negative control, and demonstrated that the observed phenotypes are specific to tRNA-Arg-UCU-1.
2. Please show the phenotypic characteristics (e.g., AR activity, NE features, sensitivity to AR inhibitors) of the cell line models used for MSR-Seq. Currently, the cited reference is a bioRxiv preprint, which has not undergone peer review.
3. Is it possible to treat the Hi-Myc;nTr22 +/- mice with AR-targeted therapies? (This is not a required experiments as the aging of the mice takes 9 months and might be too long for a review process of a reasonable length)
4. The authors identified TAPDBP and ZSCAN29 as transcription factors regulating tRNA-Arg-UCU-1 transcription. According to the ChIP-seq results, they appear to share binding sites. Do these factors act cooperatively or competitively at the locus?
5. What is the mechanism underlying the decrease of these transcription factors in NE states? Further clarification would help to link TF regulation with lineage plasticity.
6. The SWI/SNF components that are biased for AGA codons should be validated further, with a codon-switching reporter. At the moment the evidence is based on correlation and on qPCR and western blotting and does not directly validate that translational changes are directly driven by codon-bias.

Minor points

1. In the Introduction, the authors refer to "post-transcriptional gene regulation" in lineage plasticity. While translation falls under this category, the phrase is often interpreted as regulation at the RNA levels including alternative splicing, RNA modification, and miRNA. The authors should clarify and specify the scope of the term based on their focus.
2. In Figure 1, please clarify the difference between ARG.6 and ARG.10?
3. Does lineage conversion via decreased tRNA-Arg-UCU-1 levels actually promote prostate cancer metastasis in vivo? This could strengthen the clinical relevance of the findings (Figure 6).

4. Upon knockdown of tRNA-Arg-UCU-1, do protein levels of NE pathway components increase?
5. How do cells respond to NE-targeted therapies upon depletion or restoration of tRNA-Arg-UCU-1?
6. In Figure 5e, are the enriched genes overall biased toward AGA codons?
7. Biological replicates should be included for Figure 4e and 4h.
8. Please show the correlation between tRNA-Arg-UCU-1 levels and ZSCAN29 levels in patient specimens similar to Supplementary Figure 5e.
9. Please clarify the specificity of the shRNA, qPCR primers and Northern blot probe for tRNA-Arg-UCU-1.

Referee #2

(Remarks to the Author)

A correlation to the physiological needs in protein production, e.g. by changes in the expression of tRNA isoacceptor sets (tRNAs charged with the same amino acid but bearing different anticodons) is well understood. However, changes in the expression levels of individual isodecoders (tRNAs with the same anticodon) are less understood. In this work, Kim et al. have identified via unbiased screening and then determined the role of particular tRNAArg(UCU)-1 isodecoder in cell physiology and pathology.

I found this work very balanced, intriguing and timely. Starting from basic biology it spreads into pathophysiology. I only have few issues to be fixed.

- 1) Controls: although the data clearly points on tRNAArg(UCU)-1, I would like to see other, closely related isodecoders, to be tested in overexpression studies. What is the unique difference of isodecoder 1 to others? According to Supp. Info, most features are shared. I think it is appropriate to test others since they could be more expressed in other cell types to higher levels.
- 2) The results of tRNAArg(UCU)-1 depletion are encouraging yet needs revising. Does this depletion also affects other tRNA ARG isodecoders or even other genes?
- 3) The reporter contains many ARG codons in the row, what would happen if they are separated? I do not think that such high cluster of ARG codons exists in endogenous mRNAs
- 4) Enzalutamide is used. However, I believe that it has some pleiotropic effects, please use yet another chemotherapy drug to complement the findings
- 5) Please use other ARG isoacceptor gene as control for GOF and LOS studies
- 6) TARDBP and ZSCAN29 regulate tRNAArg(UCU)-1 dosage: actual ChIP assay is needed to complement depletion studies. Also, do both proteins are need or their function is redundant in this context?
- 7) tRNA cleavage was only tested earlier in the workflow. I would like to see actual NB pictures including species below mature tRNAs. Please provide them as part of Supp. Info

Referee #3

(Remarks to the Author)

A. This well written manuscript describes a highly novel area of prostate cancer biology focused on a tRNA, tRNAArg(UCU)-1, that the authors claim is central to the lineage plasticity reported in advanced prostate cancer that associates with treatment resistance, visceral metastatic disease and poor prognosis. The authors claim that decreased expression of tRNAArg(UCU)-1 results in a change in SWI/SNF complex function associated with altered SMARCC2 protein expression with this regulation occurring at a protein translation level due to altered tRNAArg(UCU)-1 expression.

B. This is a highly novel piece of work. I am not aware of any other work reporting similar findings. Indeed this area of biology is very novel to the (prostate) cancer research field.

C. The approaches taken are valid and the presentation excellent; while I am not deeply familiar with tRNA biology this work is well described and the claims made appear bolstered by sound data although I believe this manuscript can be strengthened by replies to my comments. The data quality appears high and validated by the use of multiple different models (a major challenge in prostate cancer research) and some limited clinical data.

D. Stats use appears appropriate.

E. Conclusions: The authors appear to make reasonable conclusions

F. Suggested improvements:

- 1) The gene for tRNAArg(UCU)-1 appears to lie on chromosome 17p, a locus that is commonly altered in prostate cancer

and includes multiple key genes; can the authors confirm this? Can they also report on the frequency of neuroendocrine prostate cancer or histopathological evidence of lineage plasticity eg AR low in these tumors.

2) The authors claim that TARDBP and ZSCAN29 are transcription factors that are key regulators of tRNAArg(UCU)-1 which they now report as a major controller of lineage plasticity in prostate cancer. Can the authors present data from prostate cancer biopsy RNAseq data validating associations between the expression of these genes and key lineage plasticity factors including ASCL1, SOX2 and/or SNAI1/SNAI2/ZEB1. Are the RNAseq pathways associated with these TFs validating their claim?

3. The authors should make clear that the two cell lines they initially focus on are essentially derived from each other, limiting their ability to generalize their cell line findings (ie C42B is derived from LnCaP). This reviewer would also like the authors to better justify their use of the genomic alterations induced in these models which are multiple and include TP53 R175H, MYCN, ASCL1, as well as SRRM4, NR0B2, BCL2, and KRAS G12V. Were all these genomic changes really necessary to induce lineage plasticity? Are these changes truly representative of clinical lineage plasticity in PCa?

4. Can the authors in their conclusions comment on how to clinically translate their findings? How can this biology be drugged to impart clinical benefit?

5. The impacts of decreased tRNAArg(UCU)-1 expression may be broad. Have the authors excluded molecular changes beyond impact on the SWI/SNF complex? Also could the impact on PCa cell biology be broader than an impact on lineage plasticity? What do RNAseq data after knock in and knockout (and knock back in) studies indicate?

6. Do the authors have any evidence that changes in tRNAArg(UCU)-1 expression have homeostatic roles? In embryogenesis?

7. Why did tRNATyr GUA drop out in C4-2B, when this cell line is a derivative of LnCaP?

8. Can the authors make more clear whether they found, in clinical samples, any other tRNAs whose expression were differentially expressed between adenocarcinoma of the prostate and NEPC?

Referee #4

(Remarks to the Author)

The manuscript "tRNA dosage regulates lineage dependency and therapy resistance in prostate cancer" by Kim et al. proposes that a single tRNA isodecoder, tRNAArg(UCU)-1, is a dosage-sensitive regulator of lineage dependence and ARPI drug response in prostate cancer. The application of several targeted approaches, including MSR-seq, Basescope, polysome RNA-seq and the use of engineered cell and mouse models are major strengths of the present study. Utilizing these methods, the authors argue that (1) tRNAArg(UCU)-1 decreases during tumor cell lineage progression (AD -> LP/NE transitions), (2) that decreases in tRNAArg(UCU)-1 abundance promotes AR-independence and ARPI resistance, (3) that restoring tRNAArg(UCU)-1 reverses lineage state and drug response, (4) that TDP43 and Zscan29 control the tRNA-Arg-TCT-1-1 gene locus, and (5) that, mechanistically, tRNAArg(UCU)-1 levels have downstream consequences on AGA-codon-biased translation, particularly for SMARCC2 and potentially other SWI/SNF components.

While specific concerns related to experimental design choices and analyses are shared below, a general concern relates to conclusions on the specificity of tRNAArg(UCU)-1 as a modulator of lineage dependency and therapy resistance. To truly separate tRNAArg(UCU)-1 as a unique player in this respect, one would need to examine the individual effects of all tRNA isodecoders (e.g. using a CRISPR screen) and show that this particular isodecoder stands out in a significant way. In the absence of this, a simpler alternative model is that the observed lineage effects may reflect broader perturbations in tRNA pools, where changes in tRNAArg(UCU)-1 abundance are representative, but not necessarily unique. In other words, it remains possible that other UCU isodecoders and other tRNAs could contribute similarly if perturbed. The present data indicates tRNAArg(UCU)-1 as a potential marker and functional participant in lineage plasticity, but whether it is unique in this role requires additional comparative evidence. If tRNAArg(UCU)-1 is not unique, it raises many questions about whether the consequences are related to general perturbations of translation, rather than the TDP43/Zscan29 -> tRNAArg(UCU)-1 -> SMARCC2 model proposed by the authors. On a related note, the evidence supporting TDP43 and Zscan29 as regulators of tRNAArg(UCU)-1 are similarly missing a comparative framework – are these factors enriched at tRNAArg(UCU)-1 compared to other tRNA genes? Experiments on TDP43 are also confounded by the established roles of TDP43 in RNA-binding, which may conceivably bind to a multitude of tRNAs and affect their steady-state abundance.

Major points:

1. A fundamental concern relates to the analysis and interpretation of tRNA^{Arg}(UCU)-1 changes identified by MSR-seq. It appears that the method used does not include the use of unique molecular identifiers (UMIs), and that PCR amplification is used in the generation of sequencing libraries. Given that the authors then appear to use a restricted sequence space for alignment and thereafter use counts per million (CPM) as their quantification metric, PCR bias is highly likely to shape the CPM estimates in panels 1b, 1d, 1h, etc, and any downstream metric (e.g. % tRNA UCU-1 in tRNA UCU group, etc.). This becomes a serious concern when all downstream experiments are limited to a single tRNA isodecoder with this underlying premise.
2. It is not clear from the methods descriptions what constitutes a “high scoring” tRNA. The authors should specify these criteria in the manuscript in more detail, as well as a complete list of tRNA sequences used for alignment (Line 650–651).
3. In all differential analyses (e.g. y-axis label in Figure 1a, 1c) it appears that the authors have not accounted for multiple hypothesis testing, the authors need to adjust p-values (e.g. using FDR) and re-assess accordingly.
4. Figure 1h is impossible to interpret for reasons described above (it is very rare for authors to make interpretations on CPM values for good reason), in addition to the lack of replicates and estimates of variance.
5. Figure 1j – my understanding is that Basescope is not quantitative to the level of “copies per cell”, particularly for high abundance RNAs, can the authors clarify? There is also the possibility that highly structured and modified tRNAs may affect probe binding efficiency – can the authors rule out that differences in tRNA modifications underlying the changes in tRNA^{Arg}(UCU)-1 estimates?
6. Figure 1l – it is not clear what ARG.10 represents or how it was calculated. Regardless, this panel applies a Pearson’s correlation even though this method is sensitive to outliers and that the assumption/test for linearity is unlikely. The authors should really apply Spearman’s rank correlation, as they did in later figures.
7. Figure 1j-l is restricted to analysis of tRNA^{Arg}(UCU)-1, yet comparisons to other tRNA^{Arg}(UCU) isodecoders (and other isoacceptors) would be informative here in order to begin establishing the tRNA^{Arg}(UCU)-1 centered model proposed by the authors.
8. Figure 2 (tRNA^{Arg}(UCU)-1 depletion drives lineage independence and therapeutic resistance in prostate cancer) – the authors establish shRNA-seq approaches to deplete tRNA^{Arg}(UCU)-1, however looking at the supplemental figure, this isodecoder appears to have only 2 tRNA-specific variants that are separated towards the 5’ and 3’ ends. How are the authors controlling for off-target depletion of the other isodecoders? There is no validation here that the intended depletion of tRNA^{Arg}(UCU)-1 is specific. A northern blot of the other tRNA^{Arg}(UCU) tRNAs is necessary.
9. Figure 2d – it is unclear why a paired t-test was chosen for this comparison, which would imply that individual samples were treated first with scramble and then with UCU depletion. The authors should instead perform a two-way ANOVA.
10. In Figure 2e, a northern blot comparing total tRNA-UCU-1 between the two mouse strains is needed to confirm that single-copy animals truly have lower tRNA-UCU-1 abundance.
11. In Figure 2g, the numbers of hi-myc/nTr22^{+/+} versus single-copy mice are unbalanced. More importantly, Welch’s t-test assumes normality, yet the distribution of hi-myc/nTr22^{+/-} is skewed (the mean is sensitive to the outliers).
12. In Figure 2h, control H&E images for the hi-myc/nTr22^{+/+} group should be shown. The Methods should also specify how high-grade PIN was quantified.
13. In Figure 3a, as in Figure 2a, a blot for other UCU isodecoders is needed to demonstrate specificity.
14. In Figure 3b, the color bar should be defined explicitly (z-score, log2 fold-change, etc.).
15. Figure 4 – the authors are drawing on eye-test analysis of available ChIP-seq data and comparative analysis of CPM values (again flawed) to establish a small predictive set of tRNA-UCU-1 regulators. First, differential mRNA expression analyses require a differential framework, not unpaired Welch’s t-test on CPM values. Even so, the tenuous link between observational ChIP signal at one gene, a CPM change in AD vs. LP, and ensuing changes in tRNA-UCU-1 abundance in TDP43 and Zscan9 depletion experiments is far from sufficient to establish that “TARDBP and ZSCAN29 regulate tRNA^{Arg}(UCU)-1 expression in prostate cancer”. Instead, the authors would need to (a) Perform a broad regulatory analysis of individual factors at all tRNA genes (is TDP43 at all tRNA genes? Many tRNA genes? Which factors are statistically enriched at tRNA^{Arg}(UCU)-1 compared to an empirical null distribution?) and (b) Establish that such factors localize to the tRNA^{Arg}(UCU)-1 gene locus and are differential in AD and LP conditions (e.g., differential CUT&RUN), and that transcription-centered approaches (e.g. nascent-seq) capture changes in RNA polymerase III transcription. These analyses would need to be repeated following functional depletion of such factors.
16. For polysome RNA-seq, it is easy to imagine that RNA populations in the monosome and polysome fractions differ significantly, more so than the assumptions that edgeR and DE-seq2 allow for. Nevertheless, this is how the authors define high and low translation efficiency mRNAs. Then, we are given that Arg-AGA codons are slightly different between AD, LP, and UCU conditions (Figure 5b-d). Surprisingly, other codons appear to diverge more meaningfully, though no statistics are

provided for AGA or others. I recommend that the authors apply a permutation framework to empirically determine the significance of AGA representation in high vs. low TE mRNAs.

17. Figure 5g-h – it is unclear why SMARCC2 levels were not shown for AD. Nevertheless, there are several leaps here that are tenuous at best, but the basis for significant conclusions of the study.

Referee #5

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #6

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)

I thank the authors for their careful and complete response. All of my concerns have been addressed, and I have no remaining comments.

Referee #2

(Remarks to the Author)

All my critiques has been addressed and I believe that this interesting work should be accepted for publication

Referee #3

(Remarks to the Author)

I have no further comments for the authors.

Referee #4

(Remarks to the Author)

The authors have made significant efforts in their revision that address our previous concerns and strengthen the claims of the manuscript. In addition to other new experiments and analyses, the permutation framework helps confirm that Arg-AGA codon frequency is significantly enriched among high-translational-efficiency transcripts in AD and UCU cells but not in LP cells (Fig. 5b-d). The authors might consider commenting on AAA (Lys) and GAA (Glu), which show numerically larger differences in codon frequency between high- and low-TE gene sets in both AD and UCU cells (and perhaps include the FDR for all codons, including these) – however this is a relatively minor point.

Referee #6

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

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Summary of Key Revisions: We would like to thank the reviewers for their detailed and thoughtful comments on our manuscript. We appreciate the time and effort invested in evaluating our work. We have carefully considered all the feedback provided and have made the necessary revisions to improve the clarity, accuracy, and overall quality of the manuscript. Below, we provide a summary and a detailed point-by-point response to each of the reviewer's comments and outline the corresponding revisions made in the manuscript.

1) *tRNA^{Arg}(UCU)-1-specific impacts on prostate cancer lineage transitions*. To address reviewers' comments on the specificity of tRNA^{Arg}(UCU)-1 in regulating lineage plasticity in prostate cancer, we have generated the following additional models:

- Four new stable overexpression cell lines expressing all other tRNA^{Arg}(UCU) isodecoders, as well as tRNA^{Arg} isodecoders from three additional isoacceptor families (tRNA^{Arg}(ACG)-1, tRNA^{Arg}(CCG)-2, tRNA^{Arg}(CCU)-4).
- Two additional tRNA^{Arg} isodecoder knockdown cell lines targeting tRNA^{Arg}(CCG)-2 and tRNA^{Arg}(UCG)-3.
- Three CRISPR-engineered cell lines: control (sgAAVS1), *tRNA-Arg-TCT-1-1* knockout, *tRNA-Arg-TCT-1-1* knockout with tRNA^{Arg}(UCU)-1 re-expression.

We found that neither overexpression nor knockdown of any other tRNA isodecoder recapitulated the molecular phenotypes induced by tRNA^{Arg}(UCU)-1, including changes in AR and neuroendocrine gene expression. We also demonstrated that tRNA^{Arg}(UCU)-1 knockdown does not produce off-target effects on the remaining tRNA^{Arg}(UCU) isodecoders by using isodecoder-specific detection probes. Lastly, using *tRNA-Arg-TCT-1-1* knockout cell lines, we validated the specificity of both our northern blot probe and qPCR primers for tRNA^{Arg}(UCU)-1.

2) *UMI-based MSR-seq analysis with multiple-comparisons testing*. We have compared UMI-based counts with PCR amplified read counts and observed a strong concordance between the two quantification methods. In addition, we performed appropriate multiple-comparison testing across all MSR-seq datasets, and the revised statistical analyses support the conclusions presented in the manuscript.

3) *TARDBP/ZSCAN29-dependent tRNA-Arg-TCT-1-1 transcription by RNA polymerase III*. To address reviewers' comments regarding TARDBP- and ZSCAN29-dependent regulation of *tRNA-Arg-TCT-1-1* transcription, we performed the following experiments:

- CUT&RUN profiling of TARDBP, ZSCAN29, H3K4me3, and RNA polymerase III (POLR3A) in LNCaP AD and LP cell lines
- ChIP-qPCR analysis after *TARDBP* or *ZSCAN29* depletion in LNCaP and C4-2B lines
- Northern blot analysis of all tRNA^{Arg}(UCU) isodecoders upon KD of *TARDBP* or *ZSCAN29*
- Combined knockdown of *TARDBP* and *ZSCAN29*

We discovered that the *tRNA-Arg-TCT-1-1* gene locus exists in a H3K4me3-enriched chromatin state that preferentially recruits TARDBP, ZSCAN29, and RNA polymerase III. Loss of *TARDBP* or *ZSCAN29* selectively reduced expression of tRNA^{Arg}(UCU)-1 without affecting the remaining tRNA^{Arg}(UCU) isodecoders, establishing a locus-specific mechanism of Pol III transcription.

4) *Arg-AGA-codon-dependent translation of SMARCC2 mRNA*. We re-analyzed the relative codon frequency in AD, LP, and UCU add-back cells using a permutation framework with multiple-comparison correction and found that Arg-AGA codons are significantly preferred in AD and UCU, but not in LP cells. We also conducted a codon-switch reporter assay by replacing Arg-AGA codons in SMARCC2 with Arg-CGC and found that tRNA^{Arg}(UCU)-1 addback selectively enhances the decoding of Arg-AGA codons within SMARCC2 mRNA. Finally, we tested the enzalutamide sensitivity in LNCaP UCU shScramble and shSMARCC2 cells.

Referees' comments:

Referee #1 (Remarks to the Author):

Kim et al discover that a specific tRNA isodecoder tRNA Arg UCU-1 is a key regulator of lineage plasticity and therapy resistance in prostate cancer. The authors show that this isodecoder is both necessary and sufficient for lineage plasticity and its overexpression sensitizes cells to AR pathway-targeted therapies. Several transcription factors, especially TARDBP and ZSCAN29 directly regulate expression of the isodecoder. tRNA-Arg-UCU-1 enhances the translational efficiency of AGA codon-enriched mRNA, which include SWI/SNF chromatin remodeling machinery. Clinical studies validate that tRNA-Arg-UCU-1 expression correlates with significant reduction in NE prostate cancer and shorter time to bone metastases as well as reduced overall survival. Overall, this is an impressive study, that very clearly validates the function of a single tRNA isodecoder in prostate cancer and therapy resistance in a variety of complementary models. The clinical data is also a very strong part of the study.

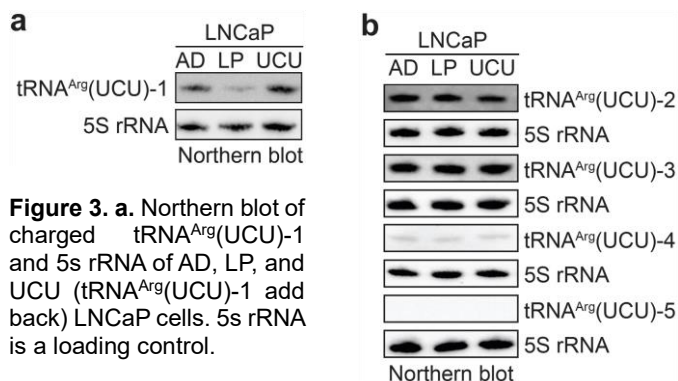
There are some points of clarification below.

We are thankful that the reviewer found our study “impressive” and “very clearly validates the function of a single tRNA isodecoder in prostate cancer and therapy resistance in a variety of complementary models.” The comments provided were very helpful and we have experimentally addressed them below.

Major points

1. Since tRNA-Arg-UCU-4 isodecoder does not change in the transition from LP to AD, it would strengthen the conclusion if the authors used it as a negative control, and demonstrated that the observed phenotypes are specific to tRNA-Arg-UCU-1.

We thank the reviewer for this suggestion and have included tRNA^{Arg}(UCU)-4 as well as the other UCU isodecoders as negative controls in the revised manuscript. We first conducted isodecoder specific northern blot analyses and confirmed that tRNA^{Arg}(UCU)-2, tRNA^{Arg}(UCU)-3, tRNA^{Arg}(UCU)-4, and tRNA^{Arg}(UCU)-5 levels do not change between AD and LP cells or following re-expression of tRNA^{Arg}(UCU)-1 (Fig. 3a & Extended Data Fig. 6b). These findings demonstrate that the changes we see in tRNA^{Arg}(UCU)-1 expression in AD, LP, and tRNA^{Arg}(UCU)-1 addback cells are specific to tRNA^{Arg}(UCU)-1 and not the other isodecoders which confirm our MSR-seq data (Fig. 1b).



Extended Data Figure 6. b. Northern blot of charged tRNA^{Arg}(UCU)-2, -3, -4, -5 of LNCaP AD, LP, and UCU cells. 5S rRNA is a loading control.

Next, we generated tRNA^{Arg}(UCU)-2, tRNA^{Arg}(UCU)-3, tRNA^{Arg}(UCU)-4, and tRNA^{Arg}(UCU)-5 overexpression plasmids by inserting three tandem repeats of each tRNA sequence plus 200 base-pairs of its flanking regulatory regions from the endogenous gene locus, into a promoterless lentiviral vector and made a series of stable overexpressing LP (lineage plastic) cell lines (**Extended Data Fig. 6e**). Importantly, overexpression of tRNA^{Arg}(UCU)-1 was sufficient to increase androgen receptor-driven genes expression (**Fig. 3b & Extended Data Fig. 6d**). However, this was not observed with overexpression of tRNA^{Arg}(UCU)-2, tRNA^{Arg}(UCU)-3, tRNA^{Arg}(UCU)-4, or tRNA^{Arg}(UCU)-5 (**Extended Data Fig. 6f**). These new results demonstrate that restoration of AR activity in LP cells is specific to tRNA^{Arg}(UCU)-1.

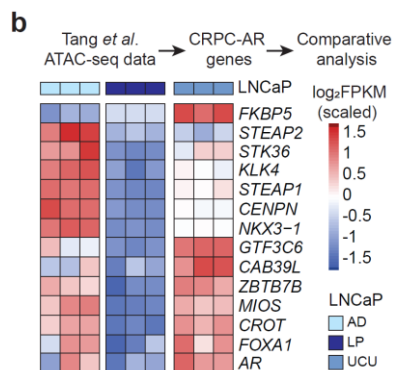
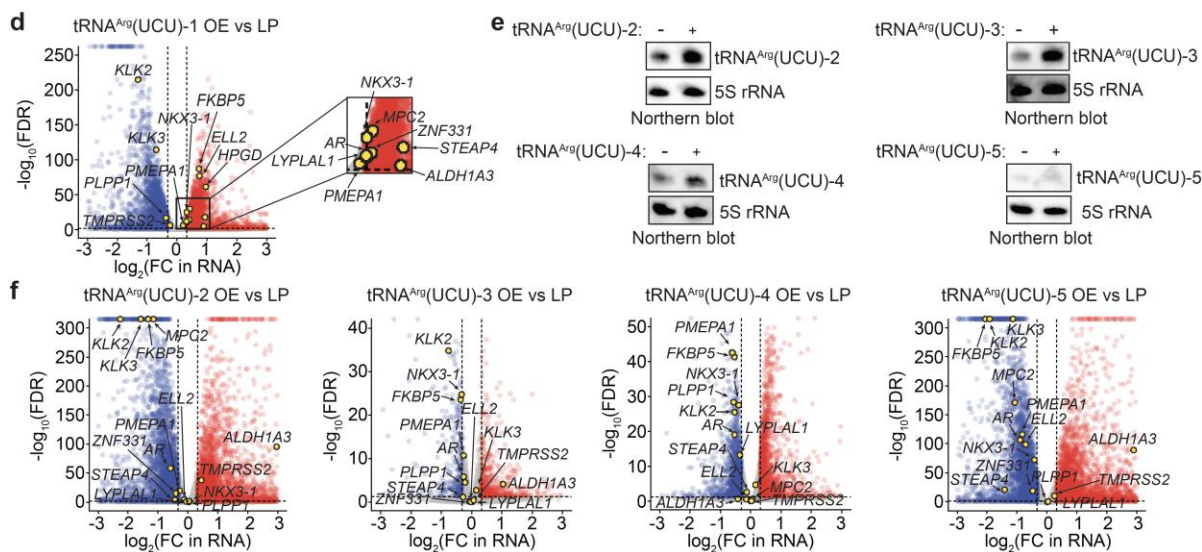


Figure 3. b. Heatmap of RNA-seq showing expression of a subset of CRPC-AR signature genes (*FKBP5*, *STEAP2*, *STK36*, *KLK4*, *STEAP1*, *CENPN*, *NKX3-1*, *GTF3C6*, *CAB39L*, *ZBTB7B*, *MIOS*, *CROT*, *FOXA1*, *AR*) across LNCaP AD, LP, and UCU cells. Scale bar represents z-score normalized log₂FPKM gene expression.



Extended Data Figure 6. d. Volcano plot of RNA-seq from LNCaP LP and UCU cells. Select AR pathway genes from ARG.10 and Tang *et al.* ATAC-seq data (*ELL2*, *HPGD*, *LYPLAL1*, *MPC2*, *ZNF331*)¹ are highlighted in yellow. **e.** Northern blot analysis of tRNA^{Arg}(UCU)-2, -3, -4, -5 and 5S rRNA in LNCaP LP and LP with tRNA^{Arg}(UCU)-2, -3, -4, -5 overexpression cells, respectively. 5S rRNA is a loading control. **f.** Volcano plot of RNA-seq from LNCaP LP and LP cells with tRNA^{Arg}(UCU)-2, -3, -4, -5 overexpression. The same AR pathway genes from **Extended Data Fig. 6d** are labeled in yellow.

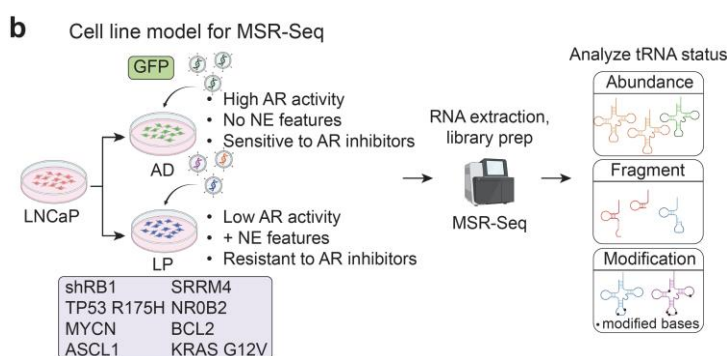
In addition to the new figures, we added new text in lines 212-215:

“Lastly, we overexpressed tRNA^{Arg}(UCU) isodecoders 2-5, tRNA^{Arg}(ACG)-1, tRNA^{Arg}(CCG)-2, or tRNA^{Arg}(CCU)-4 in LNCaP LP cells and observed no increase in AR pathway genes as we

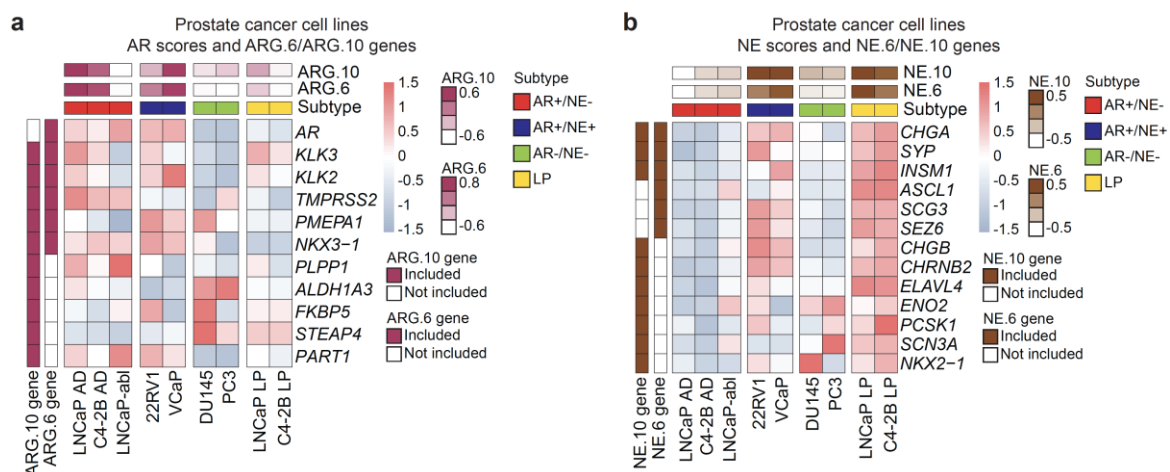
observed with $tRNA^{Arg}(UCU)-1$ re-expression (Fig. 3b & Extended Data Fig. 6d-h & Supplementary Table 4).”

2. Please show the phenotypic characteristics (e.g., AR activity, NE features, sensitivity to AR inhibitors) of the cell line models used for MSR-Seq. Currently, the cited reference is a bioRxiv preprint, which has not undergone peer review.

Thank you for pointing this out. To describe the characteristics of our cell line models more clearly, we have provided a schematic illustrating the factors used to drive AD to LP transdifferentiation (Extended Data Fig. 1b), and we have added heatmaps of androgen receptor (AR) and neuroendocrine (NE) gene expression signatures from AD and LP cells compared to commonly used prostate cancer cell lines (Extended Data Fig. 2a-b). Furthermore, we have validated these RNA-seq findings via qPCR (Fig. 3c and Extended Data Fig. 6c) and demonstrated sensitivity and resistance to the AR inhibitor enzalutamide (Fig. 3e). Lastly, our bioRxiv paper has undergone peer review and is now published in eLife (DOI:10.7554/eLife.106511.1).



Extended Data Figure 1. b. Global mapping of all tRNA species during lineage plasticity. LNCaP cells transduced with GFP lentivirus (green box) are referred to as adenocarcinoma (AD), and those transduced with a pool of factors for neuroendocrine transdifferentiation (purple box) as lineage plastic (LP). MSR-seq using RNA samples from the model captures three aspects of tRNA biology including tRNA abundance, fragmentation, select modifications.



Extended Data Figure 2. a. Heatmap illustrating AR scores (ARG.6 and ARG.10) and normalized AR pathway gene expression of *AR*, *KLK3*, *KLK2*, *TMPRSS2*, *PMEPA1*, *NKX3-1*, *PLPP1*, *ALDH1A3*, *FKBP5*, *STEAP4*, and *PART1* across different molecular subtypes of prostate cancer cell lines. Four prostate cancer subtypes (AR+/NE- = ARPC, AR+/NE+ = amphicrine, AR-/NE- = double negative, LP = lineage plastic) are shown. **b.** Heatmap showing NE scores (NE.6 and NE.10) and normalized NE pathway gene expression of *CHGA*, *SYP*, *INSM1*, *ASCL1*, *SCG3*, *SEZ6*, *CHGB*, *CHRNB2*, *ELAVL4*, *ENO2*, *PCSK1*, *SCN3A*, and *NKX2-1* across different molecular subtypes of prostate cancer cell lines. Four prostate cancer subtypes (AR+/NE- = ARPC, AR+/NE+ = amphicrine, AR-/NE- = double negative, LP = lineage plastic) are shown.

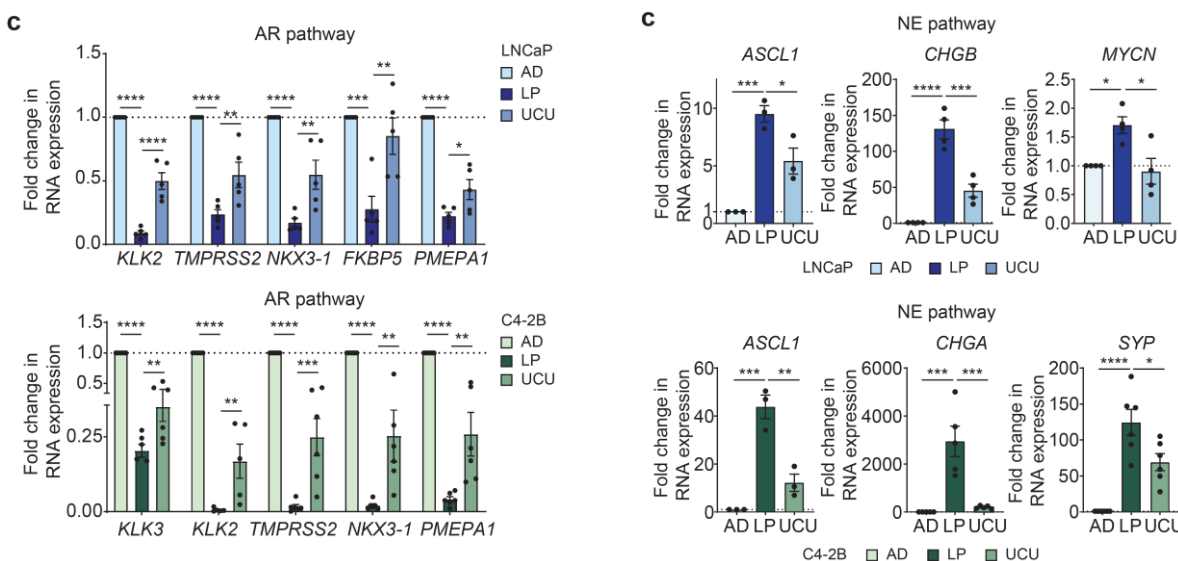


Figure 3. c. AR pathway gene expression analysis by qPCR in AD, LP and UCU LNCaP (n = 5 replicates, above) and C4-2B (n = 6 replicates, below) models; mean $\pm$ SEM. p-values, one-way ANOVA with Sidak's multiple comparisons test (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$).

Extended Data Figure 6. c. NE pathway gene expression analysis by qPCR in LNCaP AD, LP, and UCU cells (n = 5 replicates, above) and C4-2B AD, LP, and UCU cells (n = 6, below); mean $\pm$ SEM. P-values, one-way ANOVA with Sidak's multiple comparisons test (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$).

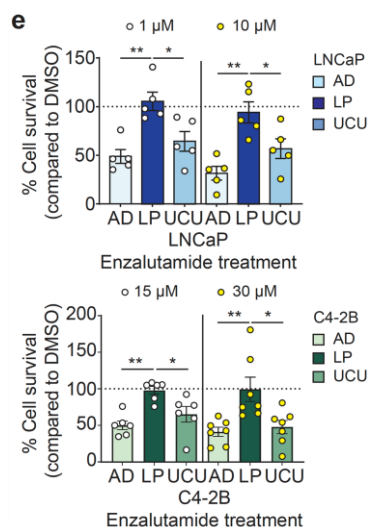
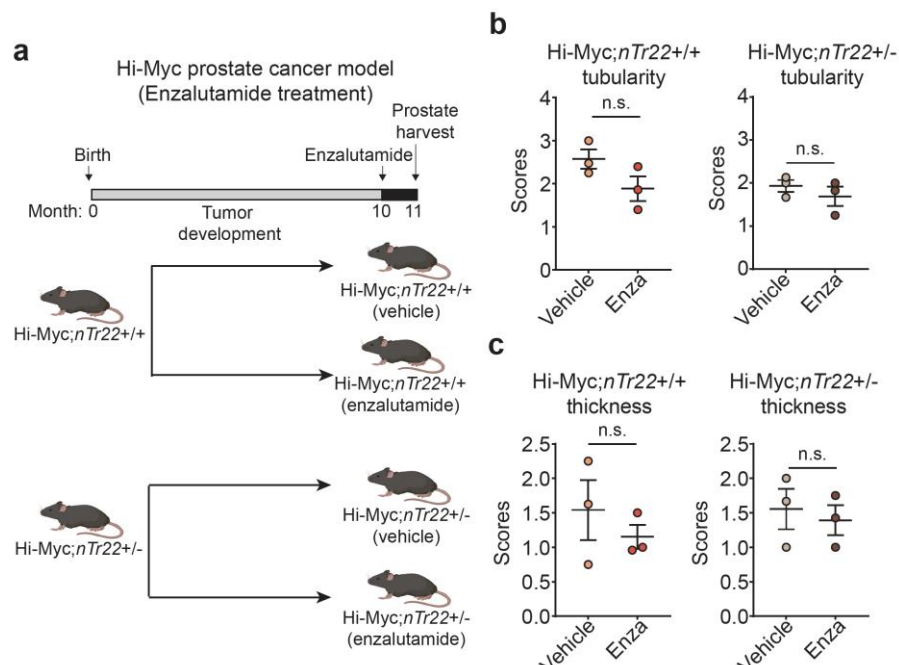


Fig 3. e. Cell survival calculated by dividing baseline-normalized luminescence readings in enzalutamide-treated conditions by baseline-normalized luminescence readings in DMSO-treated conditions for each biological replicate. 1 or 10 μM enzalutamide treatment in LNCaP AD, LP, and UCU cells (n = 5-6 replicates, above) and 15 or 30 μM enzalutamide treatment in C4-2B AD, LP, and UCU cells (n = 6-7 replicates, below); mean ± SEM. p-values, one-way ANOVA with Tukey's multiple comparisons test (* = p < 0.05; ** = p < 0.01; *** = p < 0.001).

3. Is it possible to treat the *Hi-Myc;n-Tr22 +/-* mice with AR-targeted therapies? (This is not a required experiments as the aging of the mice takes 9 months and might be too long for a review process of a reasonable length)

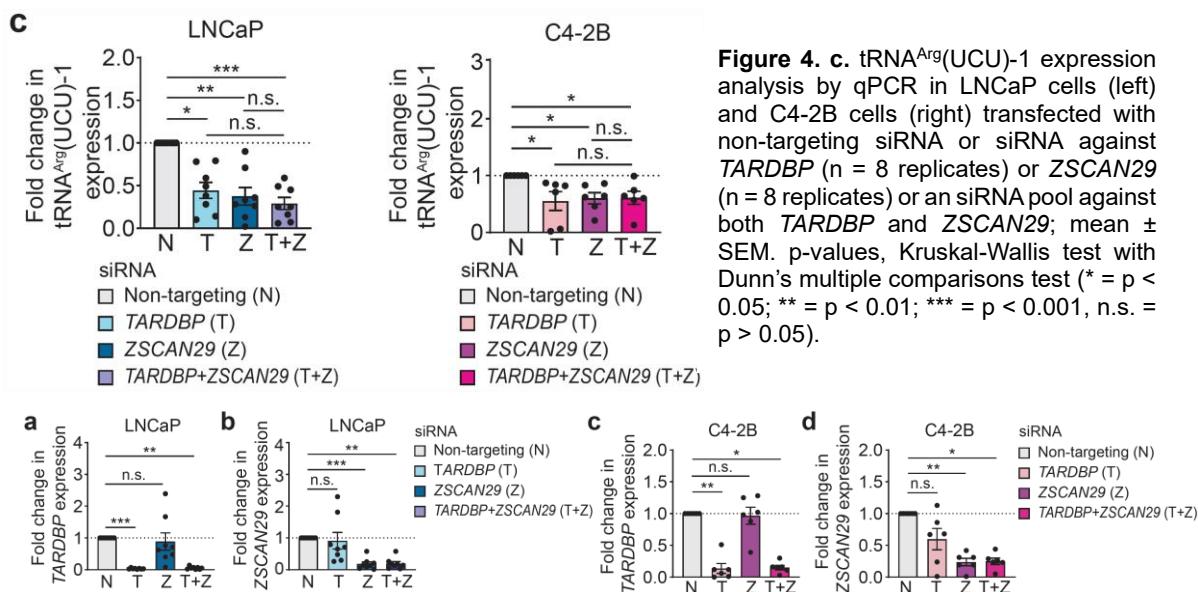
This is an important clinical question. We treated 10-month-old *Hi-Myc;n-Tr22+/+* and *Hi-Myc;n-Tr22+/-* mice with enzalutamide at 50 mg/kg by daily oral gavage (**Reviewer 1. Fig. 1a**). After one month of treatment, we harvested the mouse prostates and carefully examined H&E images for signs of tumor aggressiveness. We scored tumor regions based on the thickness and tubularity (degree of protrusion into the lumen) of the luminal cell layer. We found a decrease in thickness and tubularity following enzalutamide treatment that was more prominent in *Hi-Myc;n-Tr22+/+* prostates compared to *Hi-Myc;n-Tr22+/-* prostates. However, this trend was not statistically significant (**Reviewer 1. Fig. 1b-c**). Based on this small sample size, we conclude that enzalutamide decreases prostate cancer progression more so in *Hi-Myc;n-Tr22+/+* mice compared to *Hi-Myc;n-Tr22+/-* mice, but the effect was not as significant as castration (**Fig. 2h-i**). We postulate that these varying outcomes are related to the difference between castration and androgen receptor inhibition. Castration significantly abrogates androgen production whereas enzalutamide only blocks AR activity and does not eliminate circulating androgens. Therefore, the effects on tumor growth are more pronounced in the context of castration versus chemical inhibition.



Reviewer 1. Figure 1. a. Timeline of enzalutamide treatment and prostate harvest. Hi-Myc; nTr22+/+ and Hi-Myc; nTr22+/- mice were aged for 10 months for prostate tumor development. For each genotype, mice were randomized to receive either vehicle control or enzalutamide (50 mg/kg) daily for 1 month. **b-c.** Semi-quantitative histopathological scoring of tumor aggressiveness based on tubularity (**b**) and thickness (**c**) in the anterior prostate between vehicle and enzalutamide treated groups in Hi-Myc; nTr22+/+ and Hi-Myc; nTr22+/-; mean $\pm$ SEM. p-values, Welch's t-test (n.s. = $p > 0.05$).

4. The authors identified TAPDBP and ZSCAN29 as transcription factors regulating tRNA-Arg-UCU-1 transcription. According to the ChIP-seq results, they appear to share binding sites. Do these factors act cooperatively or competitively at the locus?

Thank you for raising this question. To address this, we conducted two complementary studies. First, we performed siRNA-mediated knockdown of TAPDBP, ZSCAN29, and TAPDBP+ZSCAN29 in both LNCaP and C4-2B cells and observed that knockdown of both transcription factors did not further decrease tRNA^{Arg}(UCU)-1 expression more than knockdown of either individual transcription factor alone (**Fig. 4c**). Thus, TAPDBP and ZSCAN29 do not act cooperatively at this gene locus. Next, we conducted TAPDBP and ZSCAN29 qPCR and observed that neither TAPDBP nor ZSCAN29 knockdown affects the expression of the other transcription factor (**Extended Data Fig. 8a-d**). Thus, TAPDBP and ZSCAN29 do not appear to act competitively at the tRNA-Arg-TCT-1-1 locus.



Extended Data Figure 8. a. *TARDBP* expression analysis by qPCR in LNCaP cells transfected with non-targeting siRNA or siRNA against *TARDBP* (n = 8 replicates) or *ZSCAN29* (n = 8 replicates) or an siRNA pool against both *TARDBP* and *ZSCAN29* (n = 8 replicates); mean $\pm$ SEM. p-values. **b.** *ZSCAN29* expression analysis by qPCR in LNCaP cells transfected with non-targeting siRNA or siRNA against *TARDBP* (n = 8 replicates) or *ZSCAN29* (n = 8 replicates) or an siRNA pool against both *TARDBP* and *ZSCAN29* (n = 8 replicates); mean $\pm$ SEM. **c.** *TARDBP* expression analysis by qPCR in C4-2B cells transfected with non-targeting siRNA or siRNA against *TARDBP* (n = 6 replicates) or *ZSCAN29* (n = 6 replicates) or an siRNA pool against both *TARDBP* and *ZSCAN29* (n = 6 replicates); mean $\pm$ SEM. **d.** *ZSCAN29* expression analysis by qPCR in C4-2B cells transfected with non-targeting siRNA or siRNA against *TARDBP* (n = 6 replicates) or *ZSCAN29* (n = 6 replicates) or an siRNA pool against both *TARDBP* and *ZSCAN29* (n = 6 replicates); mean $\pm$ SEM. p-values, Kruskal-Wallis test with Dunn's multiple comparisons test (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; n.s. = $p > 0.05$).

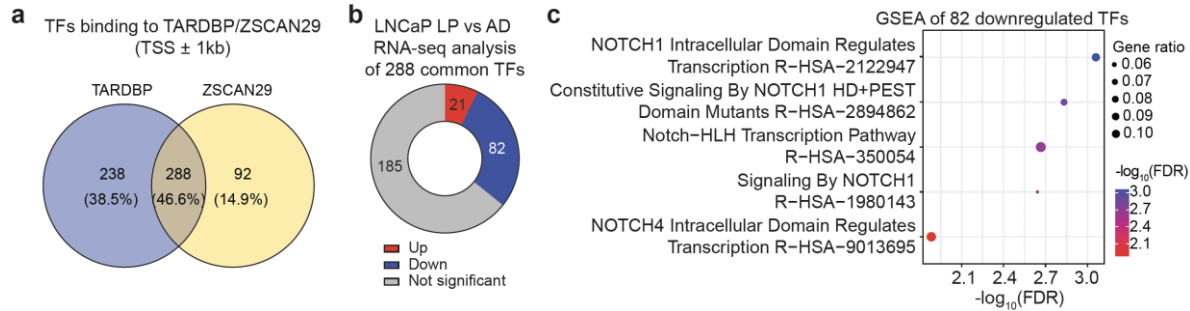
In addition to the new figures, we added new text in lines 265-269:

*“To determine whether these factors regulate $tRNA^{Arg}(UCU)-1$ expression, we individually silenced *ASH2L*, *TARDBP*, *ZSCAN29*, or *ZBTB40* and quantified $tRNA^{Arg}(UCU)-1$ abundance. *TARDBP* and *ZSCAN29* silencing led to a significant decrease in both LNCaP and C4-2B cells, but their combined knockdown did not further reduce $tRNA^{Arg}(UCU)-1$ levels (Fig. 4c-d & Extended Data Fig. 8a-d).”*

5. What is the mechanism underlying the decrease of these transcription factors in NE states? Further clarification would help to link TF regulation with lineage plasticity.

We thank the reviewer for asking this question as *TARDBP* and *ZSCAN29* have not been described as lineage plasticity-associated factors in prostate cancer. Of note, we demonstrate that *TARDBP* and *ZSCAN29* decrease at the transcript and protein levels in the context of lineage plasticity (Fig. 4b & Extended Data Fig. 8i). Therefore, to address this question, we need to understand what regulates *TARDBP* and *ZSCAN29* expression. To this end, we first investigated the ENCODE transcription factor dataset and curated a list of 288 transcription factors that bind to the genomic loci of *TARDBP* and *ZSCAN29*. (Extended Data Fig. 14a). We reasoned that decreased expression of these common transcription factors could reduce *TARDBP* and *ZSCAN29* expression in the context of lineage plasticity. Next, using RNA-seq data from LNCaP AD and LP cells, we discovered that 82 of 288 transcription factors were significantly downregulated in LP cells (Extended Data Fig. 14b). GSEA of these transcription factors

revealed that they converge on the Notch signaling pathway (**Extended Data Fig. 14c**). Importantly, the Notch signaling pathway has been shown to suppress neuroendocrine transdifferentiation in prostate cancer^{2,3}. In conclusion, our analysis demonstrates that decreased Notch signaling during lineage transitions may lead to transcriptional downregulation of *TARDBP* and *ZSCAN29*.



Extended Data Figure 14. **a.** Venn diagram of transcription factors that binding to the *TARDBP* or *ZSCAN29* gene loci. **b.** Pie chart describing the expression patterns of the 288 transcription factors that bind to the *TARDBP* and *ZSCAN29* loci derived from LNCaP AD and LP cells. **c.** GSEA of 82 downregulated transcription factors found in **Extended Data Fig. 14b**. Five categories in Reactome involve Notch signaling pathways (NOTCH1 Intracellular Domain Regulates Transcription R-HSA-2122947, Constitutive Signaling By NOTCH1 HD+PEST Domain Mutants R-HSA-2894862, Notch-HLH Transcription Pathway R-HSA-350054, Signaling By NOTCH1 R-HSA-1980143, NOTCH4 Intracellular Domain Regulates Transcription R-HSA-9013695) with FDRs of (0.000871727, 0.001474023, 0.002158648, 0.002282211, 0.013087535) were identified.

In addition to the new figures, we added new text in lines 449-452 in the discussion:

*“Although the upstream signals governing *TARDBP* and *ZSCAN29* expression remain to be defined, integrative analysis identified Notch signaling as a candidate regulatory pathway, consistent with the established loss of Notch activity during neuroendocrine prostate cancer progression (**Extended Data Fig. 14a-c**)^{2,3}.”*

6. The SWI/SNF components that are biased for AGA codons should be validated further, with a codon-switching reporter. At the moment the evidence is based on correlation and on qPCR and western blotting and does not directly validate that translational changes are directly driven by codon-bias.

We thank the reviewer for this comment. We designed a codon-switching reporter in which Arg-AGA codons in the SMARCC2 cDNA sequence were either wild-type or switched to Arg-CGC. The FLAG-tagged SMARCC2 cDNA plasmids were stably expressed in AD, LP, and UCU cells and protein expression of each reporter line was measured by western blot. We found that wild-type FLAG-tagged SMARCC2 with Arg-AGA codons is tRNA^{Arg}(UCU)-1-dependent, but SMARCC2 with Arg-AGA codons switched to Arg-CGC did not show changes at the protein level (**Fig. 5h**). This new data confirms that translation of AGA-containing SMARCC2 mRNA is uniquely sensitive to tRNA^{Arg}(UCU)-1 levels.

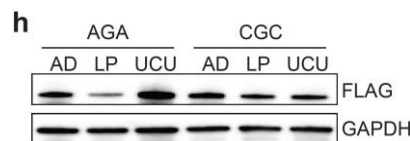


Figure 5. h. Immunoblot of flag-tagged wild-type (AGA) and AGA-codon switched (CGC) SMARCC2 in LNCaP AD, LP, and UCU cells. GAPDH is a loading control.

In addition to the new figures, we added new text in lines 369-377:

*“To determine if SMARCC2 sensitivity to $tRNA^{Arg}(UCU)$ -1 abundance was mediated by encoded AGA codons, we generated a codon switching reporter in which Arg-AGA codons in SMARCC2 were replaced with Arg-CGC codons. Wild-type and codon-switched reporters were introduced into AD, LP, and $tRNA^{Arg}(UCU)$ -1 add back cells. LP cells transduced with the Arg-AGA reporter showed reduced SMARCC2 protein abundance compared to AD cells, whereas $tRNA^{Arg}(UCU)$ -1 re-expression restored SMARCC2 mRNA translation (**Fig. 5h**). In contrast, the Arg-CGC reporter was insensitive to lineage state, and $tRNA^{Arg}(UCU)$ -1 overexpression did not enhance SMARCC2 protein synthesis (**Fig. 5h**). These findings indicate that efficient SMARCC2 translation depends on $tRNA^{Arg}(UCU)$ -1 availability through AGA codon usage.”*

Minor points

1. In the Introduction, the authors refer to “post-transcriptional gene regulation” in lineage plasticity. While translation falls under this category, the phrase is often interpreted as regulation at the RNA levels including alternative splicing, RNA modification, and miRNA. The authors should clarify and specify the scope of the term based on their focus.

We thank the reviewer for this comment and apologize for the lack of clarity. We have edited this statement for clarity and focus.

Introduction, lines 32-35 & 54-55:

“Despite significant progress in defining the epigenetic and transcriptional mechanisms underlying lineage dependencies and transitions in cancer, the role of gene regulation at the level of mRNA translation in controlling cell fate remains largely unexplored—despite its profound implications for therapy resistance and patient outcomes^{4,5}.”

“Addressing these questions will provide a unified framework in our fundamental understanding of translation dynamics and its role in shaping the phenotypic diversity of cancer.”

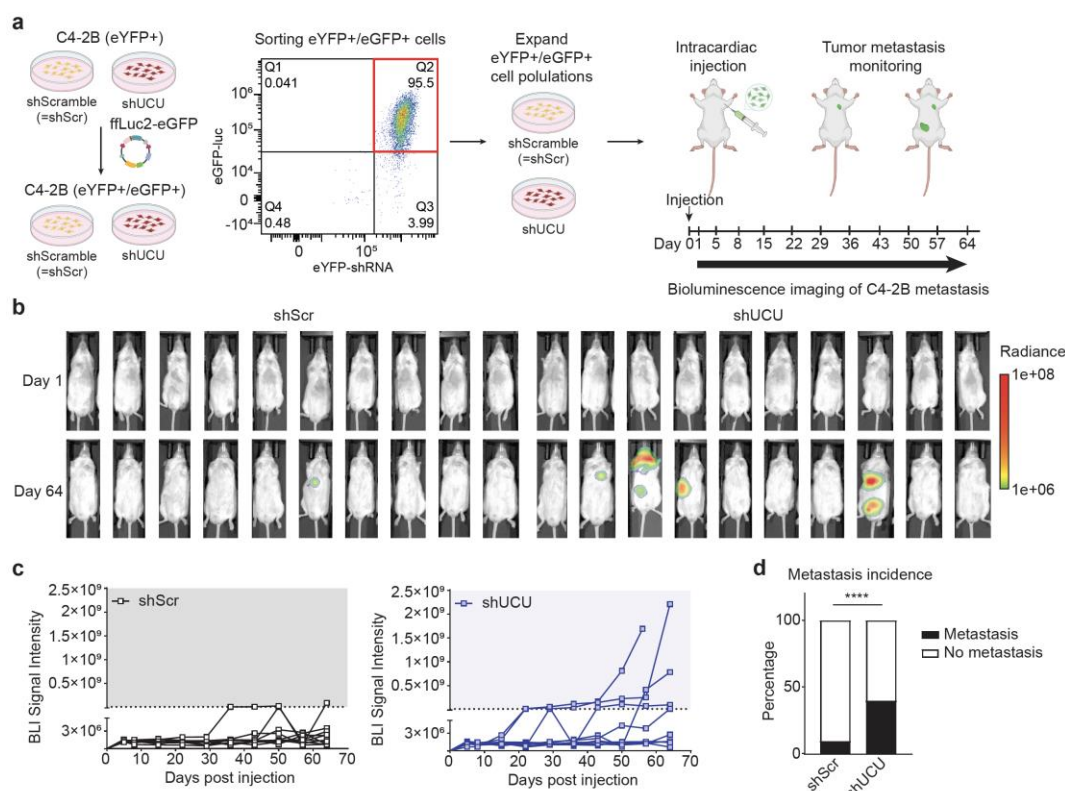
2. In Figure 1, please clarify the difference between ARG.6 and ARG.10?

We realize that ARG.6 and ARG.10 were not clearly explained in the original manuscript. We have added a description of ARG.6 and ARG.10 in the methods section. ARG.10 is a group of ten genes (*KLK3*, *KLK2*, *TMPRSS2*, *PMEPA1*, *NKX3-1*, *PLPP1*, *ALDH1A3*, *FKBP5*, *STEAP4*, and *PART1*) associated with AR-pathway activation⁶. ARG.6 is a subset derived from ARG.10 and includes *AR*, *KLK3*, *KLK2*, *NKX3-1*, *TMPRSS2*, *PMEPA1*¹⁷. These signatures were originally derived from prostate cancer patient tissue and has been historically used by the prostate cancer field to

quantify AR activity^{6,8} To quantify ARG.6 and ARG.10 we used log₂FPKM values from our RNA-seq dataset as an input for GSVA to calculate the AR score⁶.

3. Does lineage conversion via decreased tRNA-Arg-UCU-1 levels actually promote prostate cancer metastasis *in vivo*? This could strengthen the clinical relevance of the findings (Figure 6).

This is an interesting question and highly relevant to disease progression in patients with prostate cancer. To address it, we generated luciferase-expressing C4-2B shScramble (shScr) and shUCU cell lines and introduced them into NSG mice (n=10 per group) via intracardiac injection (**Extended Data Fig. 12a**). We monitored metastasis *in vivo* using the LagoX bioluminescence imaging system once a week for 10 weeks. We found that C4-2B shUCU xenografts exhibited accelerated metastasis compared to the shScramble group. In particular, tRNA^{Arg}(UCU)-1 knockdown significantly increased the incidence of metastasis (4/10 in the shUCU group vs. 1/10 in the shScramble group) and the size of metastasis as measured by luminescence signal intensity (**Extended Data Fig. 12b-d**). These findings demonstrate that tRNA^{Arg}(UCU)-1 not only influences lineage state but also impacts the metastatic capacity of prostate cancer. This is consistent with our clinical finding in **Fig. 6a** demonstrating that patients with lower levels of



Extended Data Figure 12. **a.** Overview of *in vivo* metastasis assay. C4-2B shScr and shUCU cells were transduced with a fLuc2-eGFP construct containing lentivirus. Following recovery, transduced cell populations with both eYFP-shRNA positive (shScr or shUCU expressing) and eGFP-positive (fLuc2-eGFP) were sorted and expanded. Cells were collected, resuspended in PBS and injected into the hearts of 8–12-week-old NSG male mice. Tumor metastasis was monitored by bioluminescence imaging weekly for 10 weeks. **b.** Representative images of bioluminescence signal from individual mice in the shScr and shUCU groups. **c.** Time-course measurement of bioluminescence signal from the shScr (left) and shUCU (right) groups. Individual lines indicate each mouse. **d.** Stacked bar graph showing the percentage of mice in shScr (n = 10) and shUCU (n = 10) groups that developed metastasis; two-sided Fisher's exact test (**** = p < 0.0001).

tRNA^{Arg}(UCU)-1 have more rapid onset of metastases. The new data has been included in the results section.

In addition to the new figures, we added new text in lines 402-409:

“Based on these clinical correlations and the previous observations that tRNA^{Arg}(UCU)-1 depletion in AR+ prostate cancer increase metastasis-associated genes (Extended Data Fig. 4e-f), we performed an in vivo metastasis assay using luciferase-expressing C4-2B shScr and shUCU cells (Extended Data Fig. 12a). Following intracardiac injection, we monitored bioluminescence signals for 10 weeks (Extended Data Fig. 12b) and found that tRNA^{Arg}(UCU)-1 depletion led to a 4-fold increase in metastasis compared to the shScr group (Extended Data Fig. 12c-d). These findings demonstrate tRNA^{Arg}(UCU)-1 levels not only impact AR dependence and therapy response in prostate cancer but also metastatic progression of the disease in vivo.”

4. Upon knockdown of tRNA-Arg-UCU-1, do protein levels of NE pathway components increase?

We have shown that NE pathway gene expression increases upon knockdown of tRNA^{Arg}(UCU)-1 (Fig. 2d-e). To determine if this leads to an increase in protein levels of NE pathway components, we conducted a western blot for ASCL1, a transcription factor and driver of neuroendocrine differentiation^{9,10}. We observed that knockdown of tRNA^{Arg}(UCU)-1 increased ASCL1 protein levels (Extended Data Fig. 4g). To determine if this was functional, we analyzed RNA-seq data from the same cell lines which revealed enrichment of ASCL1 target gene expression (NOURUZI_NEPC_ASCL1_TARGETS) (Extended Data Fig. 4f). Together, these findings demonstrate that reduced tRNA^{Arg}(UCU)-1 expression activates the neuroendocrine-associated ASCL1 pathway in prostate cancer.

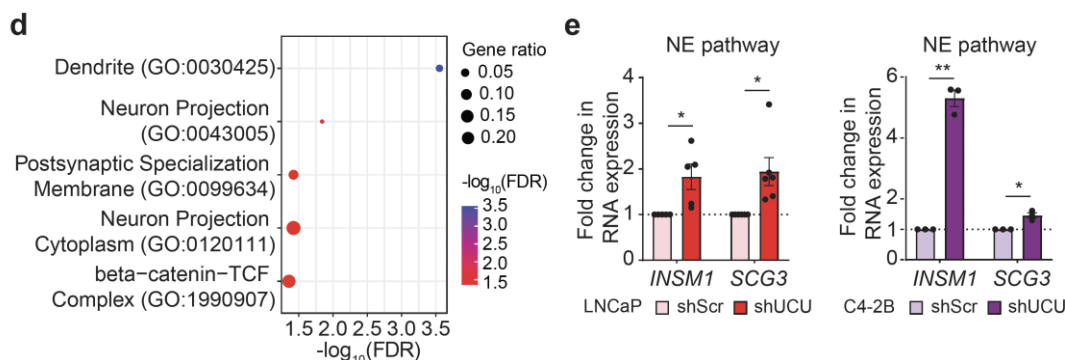
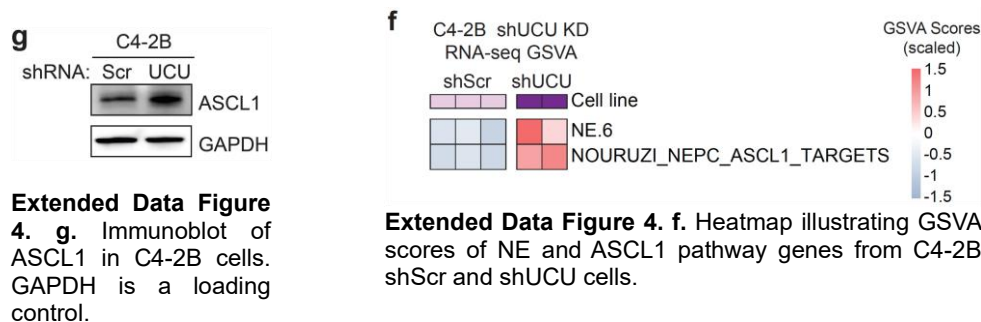


Figure 2. d. GSEA of genes commonly upregulated in LNCaP shUCU and C4-2B shUCU. e. NE pathway gene expression analysis by qPCR of shScr and shUCU LNCaP (n = 5-6 replicates, left) and C4-2B (n = 3, right) cells; mean $\pm$ SEM. p-values, unpaired Welch's t-test (* = p < 0.05; ** = p < 0.01).



Extended Data Figure 4. g. Immunoblot of ASCL1 in C4-2B cells. GAPDH is a loading control.

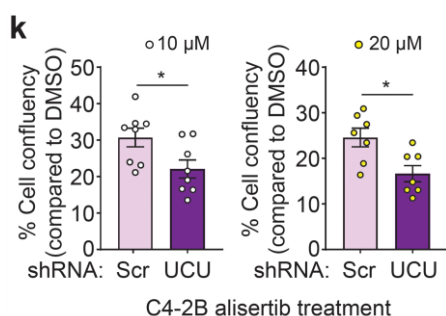
Extended Data Figure 4. f. Heatmap illustrating GSEA scores of NE and ASCL1 pathway genes from C4-2B shScr and shUCU cells.

In addition to the new figures, we added new text in lines 152-156:

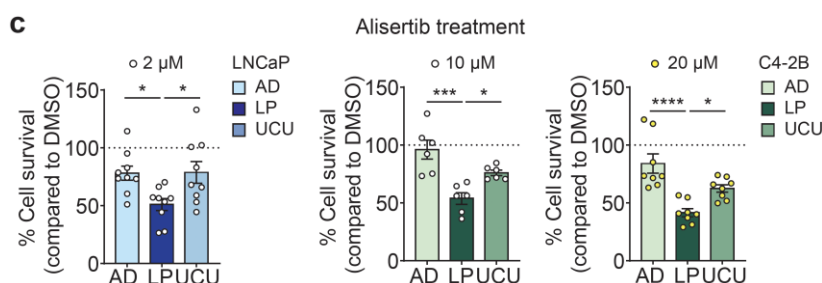
“Knockdown of tRNA^{Arg}(UCU)-1 led to a decrease in AR pathway genes expression at both the transcript (Fig. 2b & Extended Data Fig. 4d-f & Supplementary Table 3) and protein levels (Fig. 2c), alongside increased expression of genes in neuron-related and neuroendocrine pathways (Fig. 2d-e & Extended Data Fig. 4e-g).”

5. How do cells respond to NE-targeted therapies upon depletion or restoration of tRNA-Arg-UCU-1?

We appreciate this comment especially because neuroendocrine prostate cancer (NEPC) is a particularly aggressive disease and does not respond to standard androgen deprivation therapy. Currently, there are no FDA approved NEPC-specific targeted therapies. However, the kinase AURKA is overexpressed in a subset of NEPC and associated with disease aggressiveness¹¹. Moreover, some NEPC patients have demonstrated significant therapeutic responses to pharmacologic inhibition of AURKA¹¹. Thus, we tested the AURKA inhibitor, alisertib, in our cell line models to determine the impact of tRNA^{Arg}(UCU)-1 levels on sensitivity to the inhibitor. tRNA^{Arg}(UCU)-1 knockdown cells were more sensitive to alisertib compared to shScr cells (Extended Data Fig. 4k). In addition, tRNA^{Arg}(UCU)-1 addback in LP cells increased resistance to alisertib (Extended Data Fig. 7c). Thus, tRNA^{Arg}(UCU)-1 levels were negatively associated with sensitivity to AURKA inhibition. We have added the new data to the results section.



Extended Data Figure 4. k. Percent cell confluency calculated by dividing baseline-normalized confluence in alisertib-treated wells by baseline-normalized confluence in DMSO-treated wells at day 7 for each biological replicate. shScr and shUCU C4-2B cells were treated with 10 μM (n = 8 replicates) or 20 μM (n = 7 replicates) of alisertib; mean ± SEM. p-values, unpaired Welch's t-test (* = p < 0.05).



Extended Data Figure 7. c. Cell survival was calculated by dividing baseline-normalized luminescence readings in alisertib-treated conditions by baseline-normalized luminescence readings in DMSO-treated conditions for each biological replicate. 2 μM alisertib treatment in LNCaP AD, LP, and UCU cells (n = 9 replicates, left) and 10 or 20 μM alisertib treatment in C4-2B AD, LP, and UCU cells (n = 6-8 replicates, right); mean ± SEM. p-values, one-way ANOVA with Tukey's multiple comparisons test (* = p < 0.05; *** = p < 0.001; **** = p < 0.0001).

In addition to the new figures, we added new text in lines 167-168 & 222-223:

“Conversely, $tRNA^{Arg}(UCU)$ -1 knockdown increased sensitivity to the AURKA inhibitor, alisertib, which has been shown to have activity in NEPC (**Extended Data Fig. 4k**)¹¹.”

“In contrast, AD and UCU cells were less sensitive to alisertib treatment than LP cells (**Extended Data Fig. 7c**).”

6. In Figure 5e, are the enriched genes overall biased toward AGA codons?

We agree with the need for clarification. In **Fig. 5b-d**, we have now used a permutation framework to study AGA-usage frequency in high TE transcript. We demonstrate that the high TE genes are enriched for AGA codons in AD and LP cells with $tRNA^{Arg}(UCU)$ -1 addback (**Fig. 5b,d**). However, we do not observe this enrichment in LP cells (**Fig. 5c**). Thus, enriched genes in the adenocarcinoma and $tRNA^{Arg}(UCU)$ -1 re-expression setting are biased toward AGA codons.

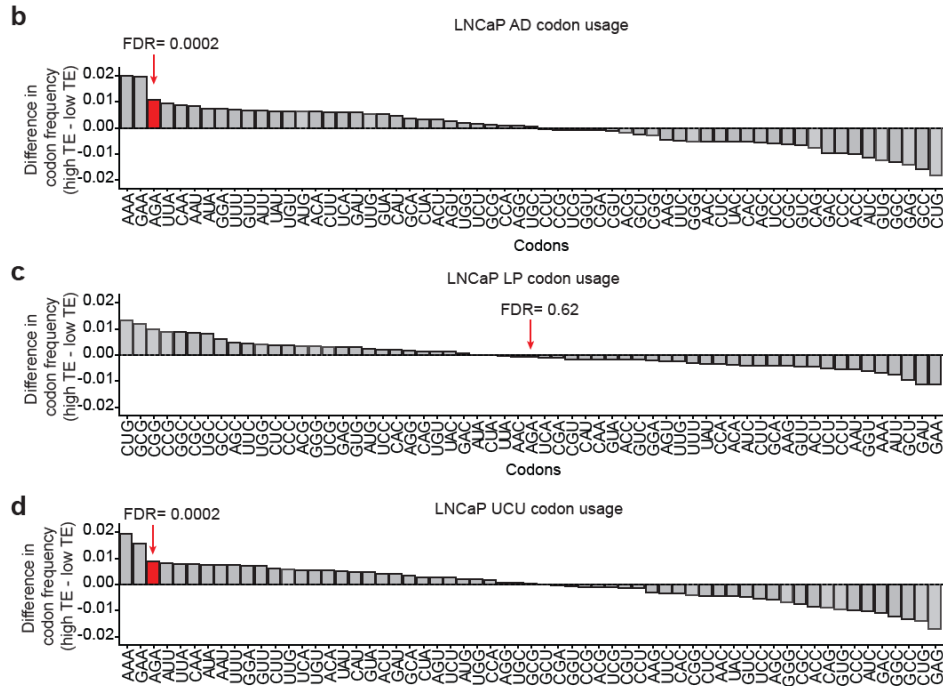
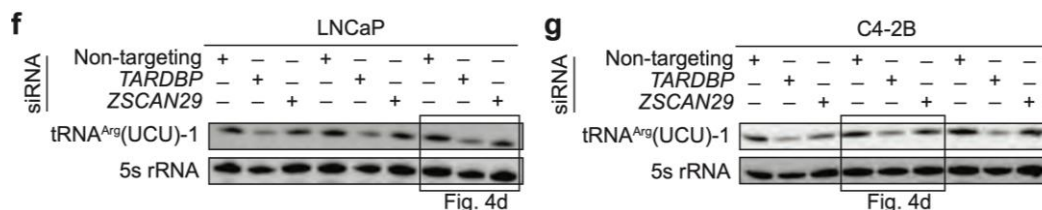


Figure 5. **b.** Bar plot showing codon usage in LNCaP AD cells. Codon frequency was calculated by analyzing codon compositions in genes with high vs. low translational efficiency in AD cells. **c.** Bar plot showing codon usage in LNCaP LP cells. **d.** Bar plot showing codon usage in LNCaP UCU cells. (**b-d**)The Arg-AGA codon is highlighted in red with FDR shown above the red arrow.

7. Biological replicates should be included for Figure 4e and 4h.

We have now included the entire northern blot with 3 biological replicates in **Extended Data Fig. 9f-g**. The previous Fig. 4e and 4h have now been combined into **Fig. 4d**.



Extended Data Figure 9. f. Northern blot of tRNA^{Arg}(UCU)-1 and 5s rRNA in LNCaP cells transfected with non-targeting siRNA or siRNA against *TARDBP* or *ZSCAN29* (n = 3 replicates). 5s rRNA is a loading control. The representative northern blot image used in **Fig. 4d** is highlighted in the black box. **g.** Northern blot of tRNA^{Arg}(UCU)-1 and 5s rRNA in C4-2B cells transfected with non-targeting siRNA or siRNA against *TARDBP* or *ZSCAN29* (n = 3 replicates). 5s rRNA is a loading control. The representative northern blot image used in **Fig. 4d** is highlighted in the black box.

8. Please show the correlation between tRNA-Arg-UCU-1 levels and ZSCAN29 levels in patient specimens similar to Supplementary Figure 5e.

Using the total area of tRNA^{Arg}(UCU)-1 puncta in patient tissues, we found a strong positive correlation between puncta size and ZSCAN29 levels similar to what we observed with *TARDBP*. We have added the correlation plot to **Fig. 4e**.

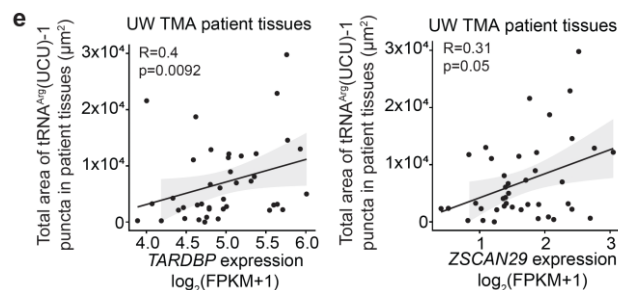


Figure 4. e. Correlation plot between normalized *TARDBP* (left) and *ZSCAN29* (right) expression and total area of tRNA puncta from the BaseScope assay with Spearman's correlation statistics shown.

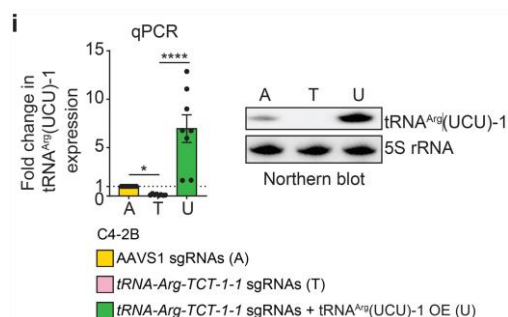
In addition to the new figures, we added new text in lines 271-274:

“Moreover, TARDBP and ZSCAN29 expression correlated with tRNA^{Arg}(UCU)-1 abundance in prostate cancer patient specimens (Spearman's correlation coefficient R = 0.4, p = 0.0092 for TARDBP; Spearman's correlation coefficient R = 0.31, p = 0.05 for ZSCAN29) (Fig. 4e).”

9. Please clarify the specificity of the shRNA, qPCR primers and Northern blot probe for tRNA-Arg-UCU-1.

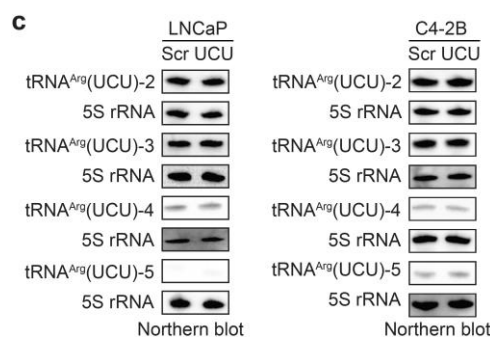
We thank the reviewer for this valuable suggestion as the tRNA^{Arg}(UCU) isodecoders share highly similar sequences and the specificity of our probes and primers is key to demonstrating tRNA^{Arg}(UCU)-1-specific impact on lineage conversion in prostate cancer.

To determine the specificity of our tRNA^{Arg}(UCU)-1 qPCR primers and northern blot probe, we generated a CRISPR KO cell line using 2 sgRNAs for selective deletion of the *tRNA-Arg-TCT-1-1* gene in C4-2B cells. As a control, we generated a KO cell line with AAVS1 sgRNAs. Following complete knockout, we re-introduced tRNA^{Arg}(UCU)-1 using the addback plasmid. Using C4-2B sgAAVS1 control KO (A), *tRNA-Arg-TCT-1-1* KO (T), and *tRNA-Arg-TCT-1-1* KO with tRNA^{Arg}(UCU)-1 addback (U) cell lines, we performed qPCR and northern blot analysis. We found that *tRNA-Arg-TCT-1-1* KO cells did not express tRNA^{Arg}(UCU)-1 by qPCR and northern blot analysis. This could be rescued by re-expression of tRNA^{Arg}(UCU)-1 (**Extended Data Fig. 1i**). These findings demonstrate the specificity of our qPCR primers and north blot oligonucleotide for tRNA^{Arg}(UCU)-1.



Extended Data Figure 1. i. Validation of specificity of qPCR primers (left) and northern blot probe (right) for tRNA^{Arg}(UCU)-1 using C4-2B CRISPR KO and CRISPR KO with tRNA^{Arg}(UCU)-1 overexpression cell lines. Compared to the control (A), cells nucleotransfected with tRNA-Arg-TCT-1-1 sgRNAs (T) showed complete knockout of tRNA^{Arg}(UCU)-1 expression. tRNA^{Arg}(UCU)-1 overexpression (U) restored the tRNA levels as measured by qPCR (n = 8 replicates); mean ± SEM. P-values, Kruskal-Wallis test with Dunn's multiple comparisons test (* = p < 0.05; **** = p < 0.0001). and northern blot.

To determine the specificity of our shRNA against tRNA^{Arg}(UCU)-1, we conducted northern blot analysis of knockdown cells using probes that target the other tRNA^{Arg}(UCU) isodecoders. None of the other tRNA^{Arg}(UCU) isodecoders showed expression changes at the tRNA level (**Extended Data Fig. 4c**). Thus, our shRNA that targets tRNA^{Arg}(UCU)-1 does not impact the expression levels of all other tRNA^{Arg}(UCU) isodecoders.



Extended Data Figure 4. c. Northern blot of charged tRNA^{Arg}(UCU)-2, -3, -4, -5 of shScr and shUCU LNCaP (left panel) and C4-2B (right panel) cell lines. 5S rRNA is a loading control.

Referee #2 (Remarks to the Author):

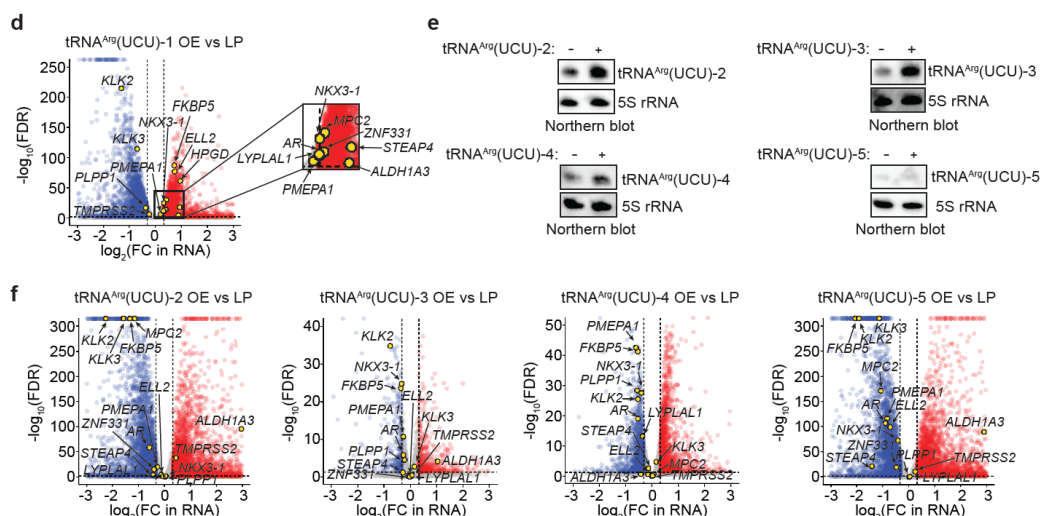
A correlation to the physiological needs in protein production, e.g. by changes in the expression of tRNA isoacceptor sets (tRNAs charged with the same amino acid but bearing different anticodons) is well understood. However, changes in the expression levels of individual isodecoders (tRNAs with the same anticodon) are less understood. In this work, Kim *et al.* have identified via unbiased screening and then determined the role of particular tRNA^{Arg}(UCU)-1 isodecoder in cell physiology and pathology.

I found this work very balanced, intriguing and timely. Starting from basic biology it spreads into pathophysiology. I only have few issues to be fixed.

We are grateful that the reviewer found our work “very balanced, intriguing and timely.” We have carefully considered all comments and have experimentally addressed them below.

1a) Controls: although the data clearly points on tRNA^{Arg}(UCU)-1, I would like to see other, closely related isodecoders, to be tested in overexpression studies.

We thank the reviewer for highlighting the controls in the manuscript given that our study demonstrates tRNA^{Arg}(UCU)-1-dependent lineage plasticity in prostate cancer. To address this comment, we designed overexpression plasmids using the other 4 tRNA^{Arg}(UCU) isodecoder and generated stable cell lines by lentivirally transducing LP cells. We confirmed that overexpression of tRNA^{Arg}(UCU)-2, -3, -4, and -5 by northern blot (**Extended Data Fig. 6e**). Using these overexpression cell lines, we conducted RNA-seq to probe for changes in the AR pathway gene expression. The results showed that only tRNA^{Arg}(UCU)-1, but not tRNA^{Arg}(UCU)-2, -3, -4, -5 consistently restored AR activity (**Extended Data Fig. 6d&f**). This confirms that the lineage conversion we observed in prostate cancer is specific to tRNA^{Arg}(UCU)-1 and overexpression of other tRNA^{Arg}(UCU) isodecoders is insufficient to recapitulate this phenotype. We have included the new data in the results section.



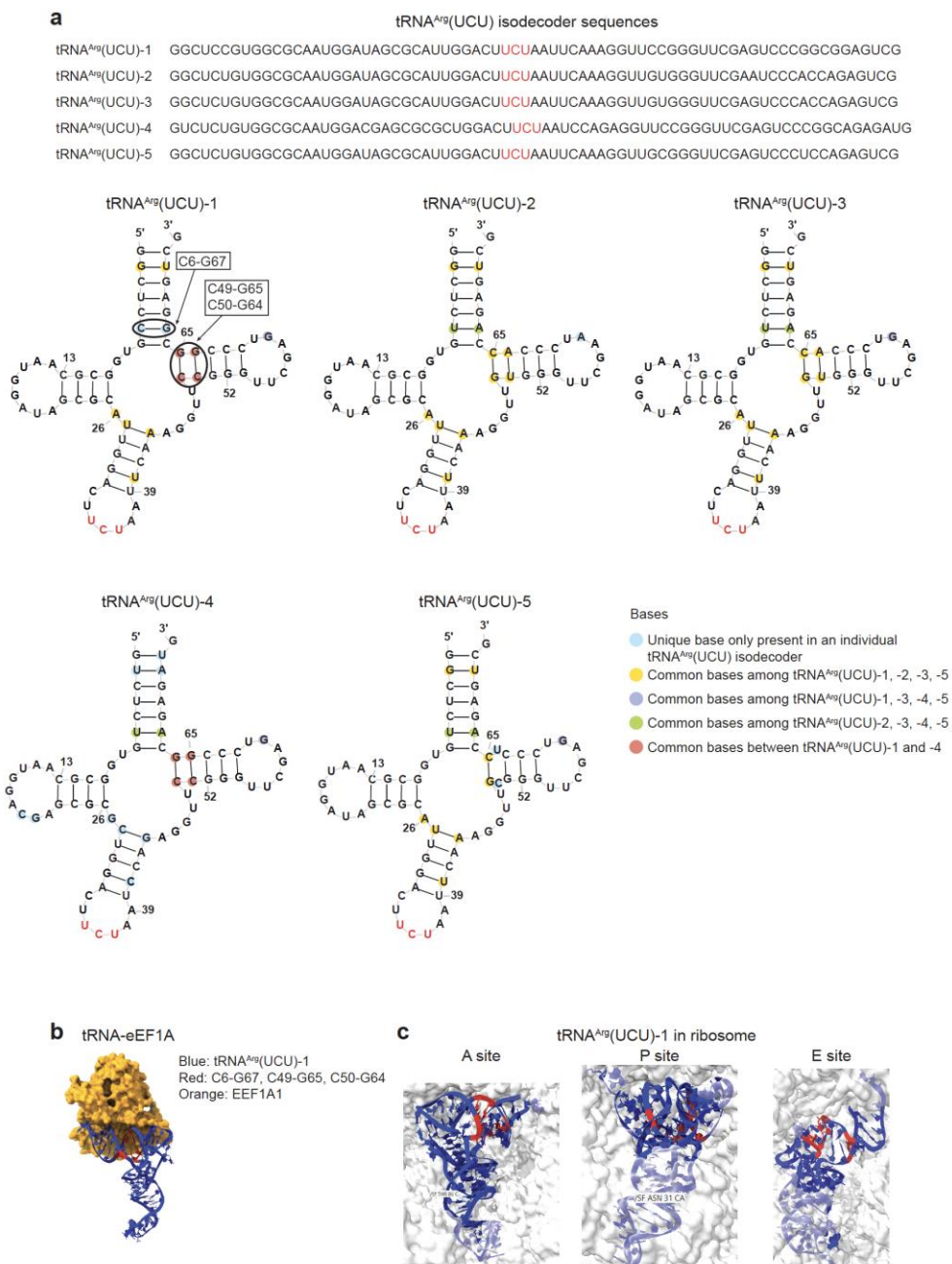
Extended Data Figure 6. d. Volcano plot of RNA-seq from LNCaP LP and UCU cells. AR pathway genes from ARG.10 and Tang *et al.* ATAC-seq data (*ELL2*, *HPGD*, *LYPLAL1*, *MPC2*, *ZNF331*)¹ are highlighted in yellow. e. Northern blot analysis of tRNA^{Arg}(UCU)-2, -3, -4, -5 and 5s rRNA in LNCaP LP and LP with tRNA^{Arg}(UCU)-2, -3, -4, -5 overexpression cells, respectively. 5s rRNA is a loading control. f. Volcano plot of RNA-seq from LNCaP LP and LP cells with tRNA^{Arg}(UCU)-2, -3, -4, -5 overexpression. The same AR pathway genes from **Extended Data Fig. 6d** are labeled in yellow.

In addition to the new figures, we added new text in lines 212-215:

“Lastly, we overexpressed tRNA^{Arg}(UCU) isodecoders 2-5, tRNA^{Arg}(ACG)-1, tRNA^{Arg}(CCG)-2, or tRNA^{Arg}(CCU)-4 in LNCaP LP cells and observed nearly no increase in AR pathway genes as we observed with tRNA^{Arg}(UCU)-1 re-expression (Fig. 3b & Extended Data Fig. 6d-h & Supplementary Table 4).”

1b) What is the unique difference of isodecoder 1 to others? According to Supp. Info, most features are shared. I think it is appropriate to test others since they could be more expressed in other cell types to higher levels.

tRNA^{Arg}(UCU)-1 differs from tRNA^{Arg}(UCU)-2, -3, and -5 at three base pair sequences (**Extended Data Fig. 13a**), one in the acceptor stem (C6-G67 versus U6-A67) and two in the T stem (C49-G65 versus G49-C65, C50-G64 versus U50-A64/C50-U64). tRNA^{Arg}(UCU)-4 differs from tRNA^{Arg}(UCU)-1 throughout the tRNA body sequence, much more extensively than tRNA^{Arg}(UCU)-2, -3, and -5, we therefore focused on comparing tRNA^{Arg}(UCU)-1 to tRNA^{Arg}(UCU)-2, -3, and -5 on potential reasons why tRNA^{Arg}(UCU)-1 may be a more efficient decoder in translation. Based on the cryo-EM structures, the 3 base pairs that differ between tRNA^{Arg}(UCU)-1 and tRNA^{Arg}(UCU)-2, -3, and -5 interact with eEF1A (**Extended Data Fig. 13b**) that delivers tRNA to the ribosome, and directly interacts with the ribosome in the A, P, and E sites (**Extended Data Fig. 13c**). These features suggest that both eEF1A and ribosome interactions may make a difference in translation among these tRNA^{Arg}(UCU) isodecoders.



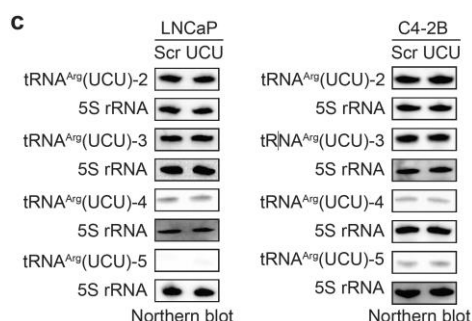
Extended Figure 13. a. Sequences of tRNA^{Arg}(UCU) isodecoder members. The anticodon UCU is highlighted in red (above). Structures of tRNA^{Arg}(UCU) isodecoders with unique and common bases labeled (below). Unique base pairs in tRNA^{Arg}(UCU)-1 but not in tRNA^{Arg}(UCU)-2, -3, or -5 are circled. **b.** Cryo-EM image of direct interactions of the 3 base pairs that differ among tRNA^{Arg}(UCU)-1 and tRNA^{Arg}(UCU)-2, -3, and -5 in the eEF1A-tRNA complex. A-site tRNA interaction with eEF1A1 (pdb 8G60), highlighting C6-G67, C49-G65, and C50-G64 pairs in red. **c.** Cryo-EM images showing direct interactions of the 3 base pairs in ribosome A (left), P (middle), E (right) sites. Left, A-site tRNA interaction with ribosome (6Y0G), highlighting C6-G67, C49-G65, and C50-G64 pairs in red. Middle, P-site tRNA interaction with ribosome (8G60), highlighting C6-G67, C49-G65, and C50-G64 pairs in red. Right, E-site tRNA interaction with ribosome (6QZP), highlighting C6-G67, C49-G65, and C50-G64 pairs in red.

In addition to the new figures, we added new text in lines 433-442:

“Another important question raised by our findings is what distinguishes $tRNA^{Arg}(UCU)$ -1 from the other $tRNA^{Arg}(UCU)$ isodecoders. Although $tRNA^{Arg}(UCU)$ -4 is highly divergent from $tRNA^{Arg}(UCU)$ -1, $tRNA^{Arg}(UCU)$ -1 differs from $tRNA^{Arg}(UCU)$ -2, -3, and -5 by three base pairs (**Extended Data Fig. 13a**): one in the acceptor stem (C6-G67 versus U6-A67) and two in the T stem (C49-G65 versus G49-C65, C50-G64 versus U50-A64/C50-U64). These positions contact eukaryotic translation elongation factor 1A (eEF1A), which mediates aminoacyl-tRNA delivery into the ribosome, as well as the ribosome itself in the A, P, and E sites (**Extended Data Fig. 13b,c**). We therefore speculate that these sequence differences influence interactions with the translation machinery, providing a potential structural basis for the isodecoder-specific translational activity of $tRNA^{Arg}(UCU)$ -1.”

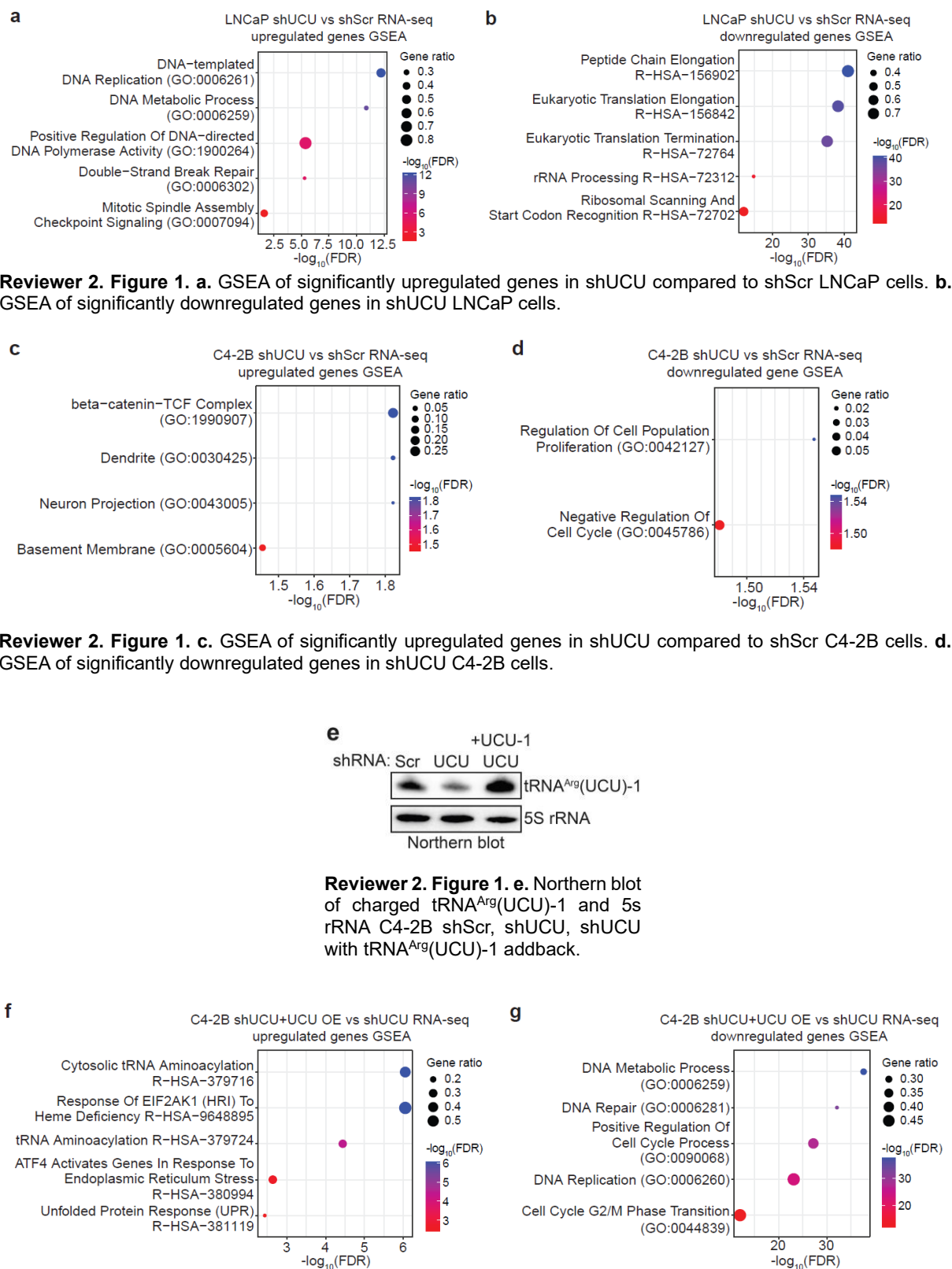
2) The results of $tRNA^{Arg}(UCU)$ -1 depletion are encouraging yet needs revising. Does this depletion also affect other $tRNA^{Arg}$ isodecoders or even other genes?

We thank the reviewer for this question. To determine if shRNA depletion of $tRNA^{Arg}(UCU)$ -1 also affects other $tRNA^{Arg}(UCU)$ isodecoders, we conducted northern blot analysis. We did not observe any changes in $tRNA^{Arg}(UCU)$ -2, -3, -4, and -5 levels upon $tRNA^{Arg}(UCU)$ -1 knockdown in LNCaP and C4-2B cells (**Extended Data Fig. 4c**). These findings demonstrate the specificity of our knockdown method.



Extended Data Figure 4. c. Northern blot of charged $tRNA^{Arg}(UCU)$ -2, -3, -4, -5 of shScr and shUCU LNCaP (left panel) and C4-2B (right panel) cell lines. 5s rRNA is a loading control.

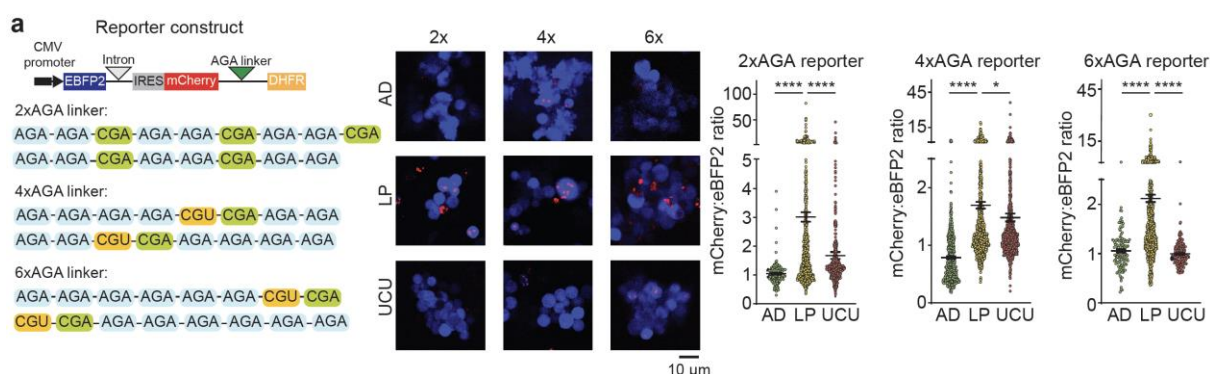
Next the reviewer asked if $tRNA^{Arg}(UCU)$ -1 knockdown affects other genes. To probe for transcriptome-wide changes on gene expression, we conducted RNA-seq on LNCaP and C4-2B cells with and without $tRNA^{Arg}(UCU)$ -1 knockdown. In LNCaP cells, $tRNA^{Arg}(UCU)$ -1 knockdown decreased the expression of genes involved in mRNA translation and increased those that regulate DNA replication and the cell cycle (**Reviewer 2. Fig. 1a-b**). In C4-2B cells, $tRNA^{Arg}(UCU)$ -1 depletion increased the abundance of neuronal program genes and decreased negative regulators of the cell cycle (**Reviewer 2. Fig. 1c-d**). We also generated a C4-2B rescue cell line by lentiviral transduction of a $tRNA^{Arg}(UCU)$ -1 construct into shUCU cells (**Reviewer 2. Fig. 1e**). Following $tRNA^{Arg}(UCU)$ -1 addback (C4-2B shUCU+UCU), genes involved in tRNA metabolism and the integrated stress response pathways were induced (**Reviewer 2. Fig. 1f**). Conversely, cell cycle and DNA replication-related genes showed a marked reduction in expression (**Reviewer 2. Fig. 1g**). Together, these transcriptome-wide studies reveal that $tRNA^{Arg}(UCU)$ -1 dosage can influence the expression of proliferation regulators, translation factors, and other lineage markers.



Reviewer 2. Figure 1. f. GSEA of significantly upregulated genes from C4-2B shUCU+UCU OE versus shUCU comparison. **g.** GSEA of significantly downregulated genes from C4-2B shUCU+UCU OE versus shUCU comparison.

3) *The reporter contains many ARG codons in the row, what would happen if they are separated? I do not think that such high cluster of ARG codons exists in endogenous mRNAs*

This is an excellent suggestion. We have now made different versions of the reporter construct with 2x, 4x, 6x AGA repeats separated by different Arg codons – CGA and CGU (**Extended Data Fig. 11a, left**). Following stable cell line generation using these new reporters, we took fluorescence images of cells using confocal microscopy and quantified fluorescence intensity using the Imaris 10.2 imaging analysis software. We have included a detailed description in the Methods section. The new results confirmed that LP cells with 2x, 4x, 6x AGA codons had higher mCherry signals (**Extended Data Fig. 11a, middle and right**) compared to AD or UCU cells demonstrating impaired translation of AGA codons in the context of low tRNA^{Arg}(UCU)-1 levels. We have added these findings to the results section.



Extended Data Figure 11. a. Schematic of the AGA reporter constructs with 2x, 4x, 6x AGA linkers (left). Representative images of LNCaP AD, LP, UCU cells expressing the 2x, 4x, 6x AGA reporter constructs (middle). Quantification of mCherry:EBFP2 signal ratio using Imaris image analysis software (right); mean ± SEM. p-values, Kruskal-Wallis test with Dunn's multiple comparisons test (* = p < 0.05; **** = p < 0.0001).

In addition to the new figures, we added text in lines 338-340:

"We repeated the assay with different combinations of AGA codon repeats (2x, 4x, 6x) and observed the same trends (Extended Data Fig. 11a)."

4) *Enzalutamide is used. However, I believe that it has some pleiotropic effects, please use yet another chemotherapy drug to complement the findings.*

We thank the reviewer for this comment and have extended our study to include two other clinically used androgen receptor inhibitors, apalutamide and darolutamide. Consistent with our findings with enzalutamide (**Fig. 3e**), LNCaP and C4-2B LP cells were more resistant to apalutamide and darolutamide than AD cells. In addition, tRNA^{Arg}(UCU)-1 addback reduced cell viability compared to LNCaP and C4-2B LP cells treated with these inhibitors (**Extended Data Fig. 7a-b**). We also conducted similar experiments in the LNCaP and C4-2B tRNA^{Arg}(UCU)-1 knockdown. Similar to our enzalutamide study, depletion of tRNA^{Arg}(UCU)-1 increased resistance to apalutamide and darolutamide (**Extended Data Fig. 4j**).

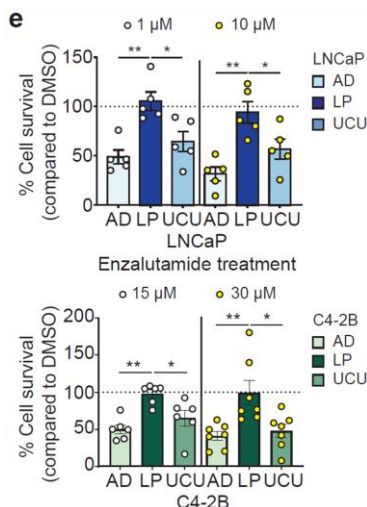
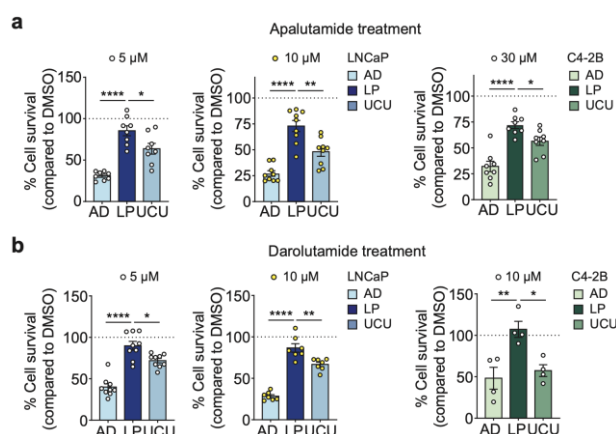


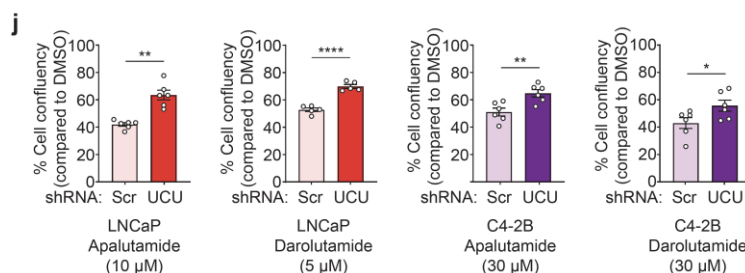
Fig 3. e. Cell survival calculated by dividing baseline-normalized luminescence readings in enzalutamide-treated conditions by baseline-normalized luminescence readings in DMSO-treated conditions for each biological replicate. 1 or 10 μM enzalutamide treatment in LNCaP AD, LP, and UCU cells (n = 5-6 replicates, above) and 15 or 30 μM enzalutamide treatment in C4-2B AD, LP, and UCU cells (n = 6-7 replicates, below); mean ± SEM. p-values, one-way ANOVA with Tukey's multiple comparisons test (* = p < 0.05; ** = p < 0.01).



Extended Data Figure 7. a. Cell survival was calculated by dividing baseline-normalized luminescence readings in apalutamide-treated conditions by baseline-normalized luminescence readings in DMSO-treated conditions for each biological replicate. 5 or 10 μM apalutamide treatment in LNCaP AD, LP, and UCU cells (n = 8-9 replicates, left) and 30 μM apalutamide treatment in C4-2B AD, LP, and UCU cells (n = 8 replicates, right); mean ± SEM. p-values, one-way ANOVA with Tukey's multiple comparisons test (* = p < 0.05; ** = p < 0.01; **** = p < 0.0001). **b.** 5 or 10 μM darolutamide treatment in LNCaP AD, LP, and UCU cells (n = 7-9 replicates, left) and 10 μM darolutamide treatment in C4-2B AD, LP, and UCU cells (n = 4 replicates, right); mean ± SEM. p-values, one-way ANOVA with Tukey's multiple comparisons test (* = p < 0.05; ** = p < 0.01; **** = p < 0.0001).

In addition to the new figures, we included new text in lines 218-222:

“To this end, we treated LNCaP and C4-2B AD, LP, and UCU cells with ARPIs, enzalutamide, apalutamide, or darolutamide. Relative to AD cells, LP cells were resistant to AR inhibition. However, addback of tRNA^{Arg}(UCU)-1 was sufficient to restore sensitivity to ARPIs (Fig. 3e & Extended Data Fig. 7a-b).”



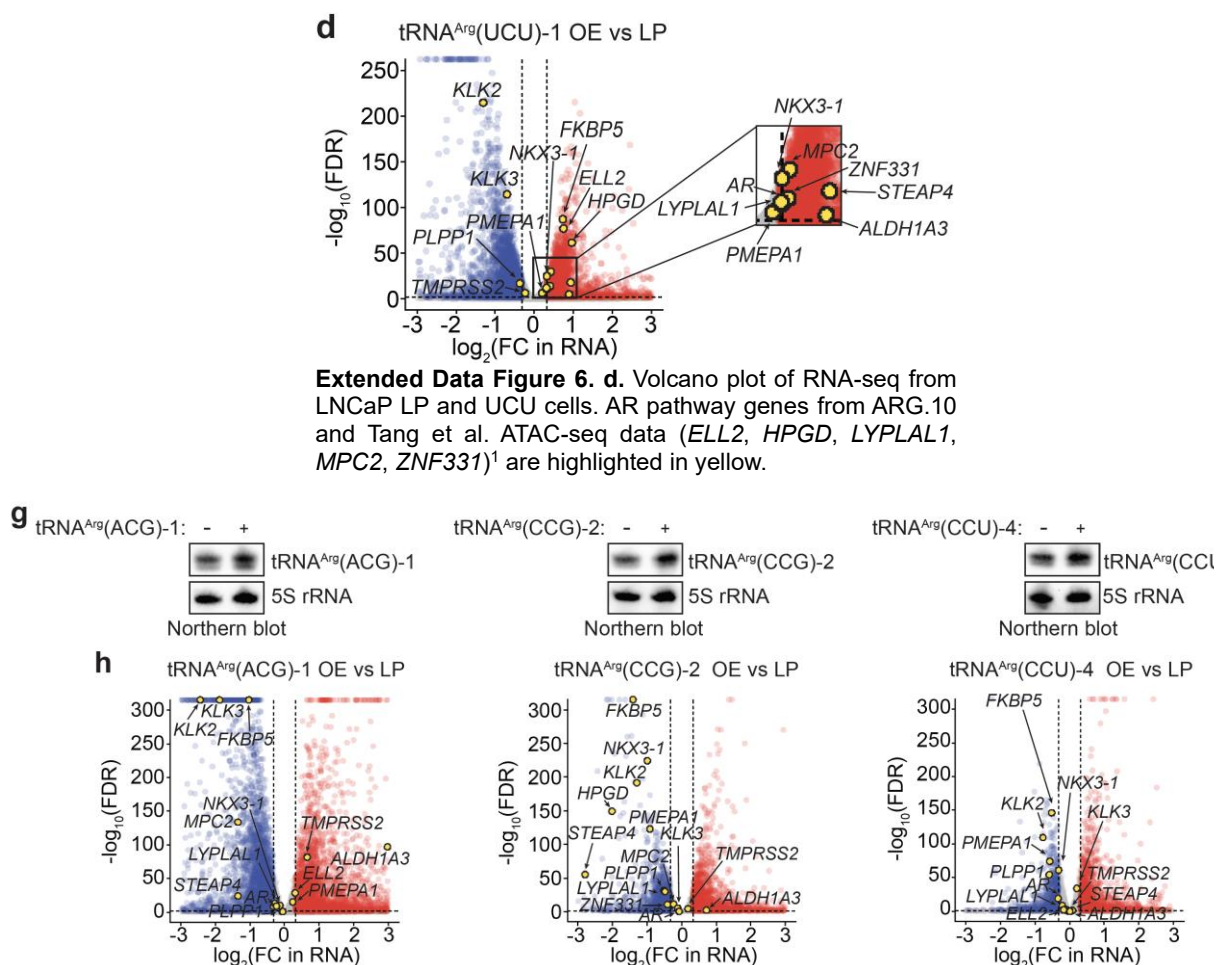
Extended Data Figure 4. j. Percent cell confluency calculated by dividing baseline-normalized confluence in apalutamide or darolutamide-treated wells by baseline-normalized confluence in DMSO-treated wells at day 7 for each biological replicate. shScr and shUCU LNCaP cells (left) were treated with 10 μ M apalutamide (n = 6 replicates) or 5 μ M darolutamide (n = 5 replicates), and shScr and shUCU C4-2B cells (right) were treated with 30 μ M apalutamide (n = 6 replicates) or 30 μ M darolutamide (n = 6 replicates); mean $\pm$ SEM. p-values, unpaired Welch's t-test (* = p < 0.05; ** = p < 0.01; **** = p < 0.0001).

In addition to the new figures, we added new text in lines 161-167:

“Our findings raise the important question of whether this phenotypic switch driven by a decrease in tRNA^{Arg}(UCU)-1 can impact the cellular response to therapeutics commonly used in prostate cancer patients to target the AR. To address this question, shScr and shUCU cells were treated with the FDA-approved AR pathway inhibitors (ARPI) enzalutamide, apalutamide, or darolutamide^{12,13,14}. Notably, shUCU cells were significantly more resistant to all these ARPIs across multiple concentrations in both LNCaP and C4-2B models (Fig. 2f & Extended Data Fig. 4j).”

5) Please use other ARG isoacceptor gene as control for GOF and LOS studies

These are important controls. We have previously shown that overexpression of tRNA^{Arg}(UCU)-1 in LP cells leads to an upregulation of androgen receptor pathway genes (Extended Data Fig. 6d). To address this comment, we generated tRNA^{Arg}(ACG)-1, tRNA^{Arg}(CCG)-2, and tRNA^{Arg}(CCU)-4 overexpression plasmids by inserting three tandem repeats of each genomic tRNA sequence flanked by 200 upstream and 200 downstream bases into a promoterless lentiviral vector and made a series of stable overexpressing LP (lineage plastic) cell lines which we confirmed by northern blot analysis (Extended Data Fig. 6g). We subsequently conducted RNA-seq on the new tRNA^{Arg} isoacceptor overexpression cell lines. Remarkably, overexpression of other tRNA^{Arg} isoacceptor did not induce androgen receptor pathway gene expression (Extended Data Fig. 6h).



Extended Data Figure 6. g. Northern blots of $tRNA^{Arg}(ACG)$ -1, $tRNA^{Arg}(CCG)$ -2, $tRNA^{Arg}(CCU)$ -4 and 5s rRNA in LNCaP LP cells and LP cells with $tRNA^{Arg}(ACG)$ -1, $tRNA^{Arg}(CCG)$ -2, and $tRNA^{Arg}(CCU)$ -4 overexpression, respectively. 5s rRNA is a loading control. **h.** Volcano plot of RNA-seq from LNCaP LP and LP cells with $tRNA^{Arg}(ACG)$ -1, $tRNA^{Arg}(CCG)$ -2, or $tRNA^{Arg}(CCU)$ -4 overexpression. The same AR pathway genes from **Extended Data Fig. 6d** are highlighted in yellow.

In addition to the new figures, we added new text in lines 212-215:

“Lastly, we overexpressed $tRNA^{Arg}(UCU)$ isodecoders 2-5, $tRNA^{Arg}(ACG)$ -1, $tRNA^{Arg}(CCG)$ -2, or $tRNA^{Arg}(CCU)$ -4 in LNCaP LP cells and observed nearly no increase in AR pathway genes as we observed with $tRNA^{Arg}(UCU)$ -1 re-expression (Fig. 3b & Extended Data Fig. 6d-h & Supplementary Table 4).”

We also performed a reciprocal loss of function study by shRNA-mediated knockdown of $tRNA^{Arg}(CCG)$ -2 and $tRNA^{Arg}(UCG)$ -3 in C4-2B cells. While knockdown of $tRNA^{Arg}(UCU)$ -1 decreased androgen receptor pathway gene expression (Fig. 2b), $tRNA^{Arg}(CCG)$ -2 or $tRNA^{Arg}(UCG)$ -3 depletion did not (Extended Data Fig. 4h-i). This new data confirms that androgen receptor pathway dependency in prostate cancer is $tRNA^{Arg}(UCU)$ -1-specific and other Arg isoacceptors cannot replicate this function.

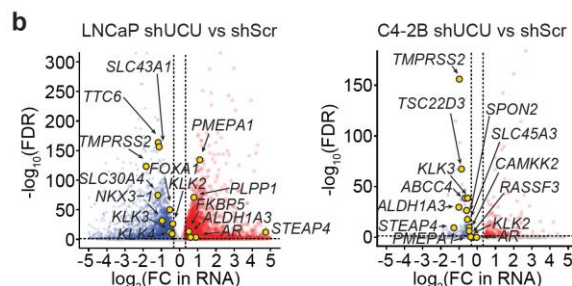
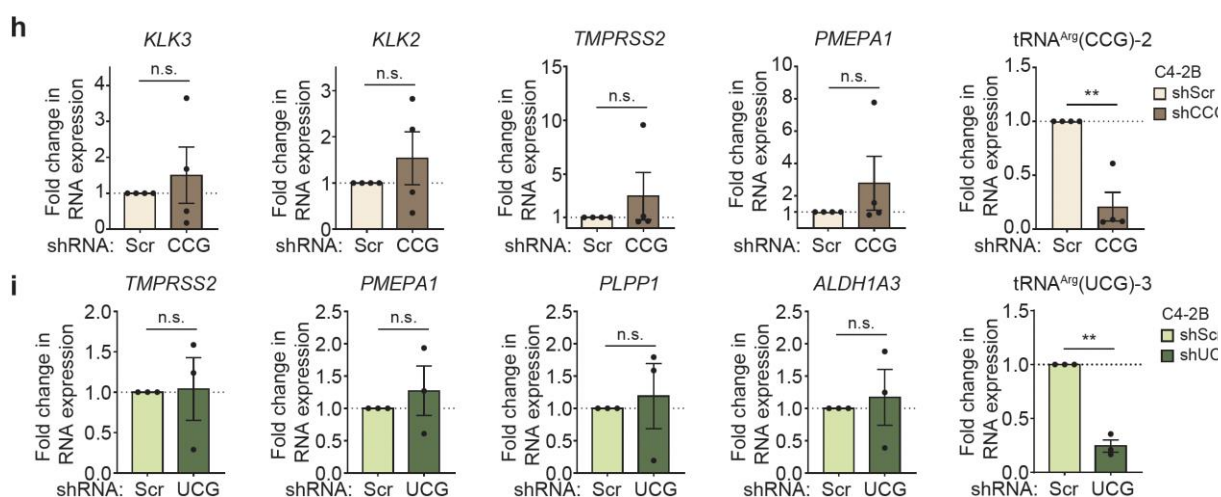


Figure 2. b. Volcano plot of RNA-seq from LNCaP shScr and shUCU (left) and C4-2B shScr and shUCU cells (right). AR pathway genes from ARG.10 and Tang et al. ATAC-seq data (SLC43A1, TTC6, SLC30A4, KLK4, TSC22D3, SPON2, SLC45A3, CAMKK2, RASSF3, ABCC4)¹ are highlighted in yellow.



Extended Data Figure 4. h. AR pathway and $tRNA^{Arg}(CCG)-2$ expression analysis by qPCR of shScr and shCCG C4-2B ($n = 4$ replicate) cells; mean $\pm$ SEM. p-values, unpaired Welch's t-test (** = $p < 0.01$; n.s. = $p > 0.05$). **i.** AR pathway and $tRNA^{Arg}(UCG)-3$ expression analysis by qPCR of shScr and shUCG C4-2B ($n = 3$ replicate) cells; mean $\pm$ SEM. p-values, unpaired Welch's t-test (** = $p < 0.01$; n.s. = $p > 0.05$).

In addition to the new figures, we added new text in lines 156-159:

“Furthermore, these changes were specific to $tRNA^{Arg}(UCU)-1$ because knockdown of $tRNA^{Arg}(CCG)-2$ and $tRNA^{Arg}(UCG)-3$ did not alter AR pathway gene expression (**Extended Data Fig. 4h-i**). Together, these findings indicate that reducing $tRNA^{Arg}(UCU)-1$ dosage promotes phenotypic plasticity in androgen-responsive cells.”

6a) TARDBP and ZSCAN29 regulate $tRNA^{Arg}(UCU)-1$ dosage: actual ChIP assay is needed to complement depletion studies.

We thank the review for this comment. We agree that we need to validate TARDBP and ZSCAN29 occupancy at the $tRNA-Arg-TCT-1-1$ locus. To this end, we performed CUT&RUN profiling of TARDBP and ZSCAN29 across all tRNA genomic loci in LNCaP AD and LP cells. Among all tRNA genes, $tRNA-Arg-TCT-1-1$ was the only locus that exhibited coordinated reductions in TARDBP and ZSCAN29 occupancy together with decreased mature tRNA abundance during lineage plasticity (**Extended Data Fig. 9a-c**). Within the six $tRNA-Arg-TCT$ isodecoder loci, only $tRNA-$

Arg-TCT-1-1 showed significantly reduced occupancy of both TARDBP ($\log_2FC = -4.811$, FDR = 0.006) and ZSCAN29 ($\log_2FC = -3.858$, FDR = 0.015). Although *tRNA-Arg-TCT-2-1* also exhibited reduced TARDBP occupancy ($\log_2FC = -3.573$, FDR = 0.038), ZSCAN29 occupancy was increased in LP cells ($\log_2FC = 0.532$, FDR = 0.015). No significant differences in TARDBP or ZSCAN29 occupancy were detected at the remaining *tRNA-Arg-TCT* loci (*tRNA-Arg-TCT-3-1*, -3-2, -4-1, and -5-1) (Fig. 4f & Extended Data Fig. 9a & Supplementary Table 6).

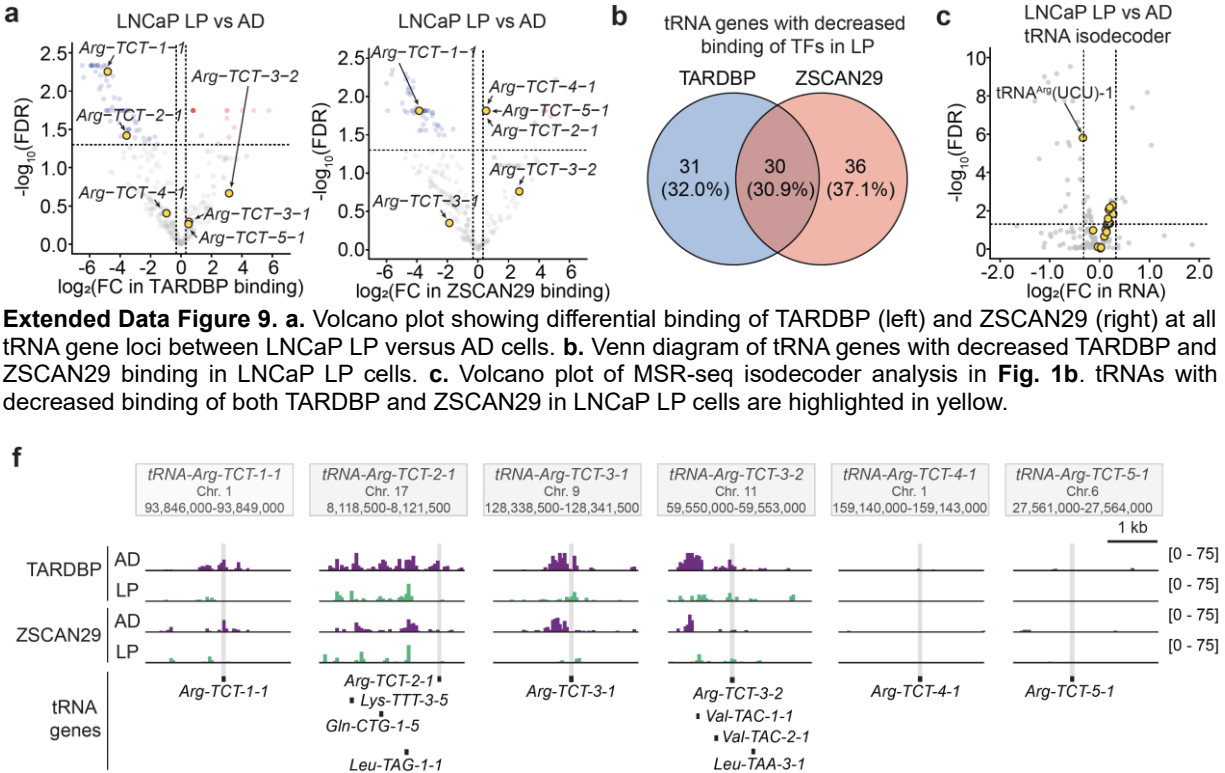


Figure 4. f. CUT&RUN IgG normalized tracks of TARDBP and ZSCAN29 across all *tRNA-Arg-TCT* gene loci in LNCaP AD and LP cells. Grey boxes denote the location of *tRNA-Arg-TCT* genes.

Given that RNA polymerase III is necessary for the transcription of tRNA, we performed POLR3A ChIP-qPCR using LNCaP and C4-2B cells with and without knockdown of *TARDBP* or *ZSCAN29* (Fig. 4j). We observed that:

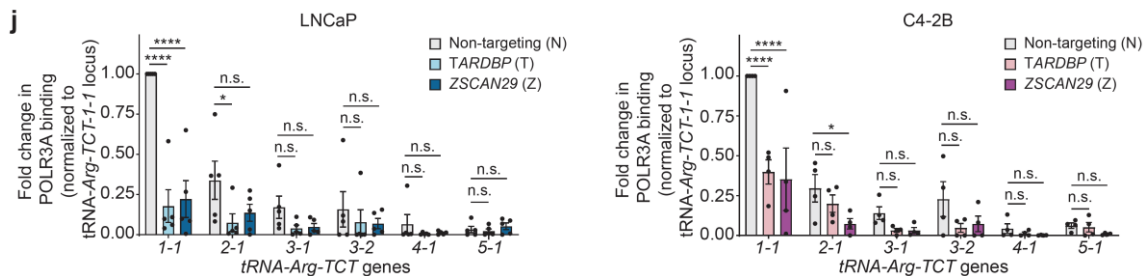
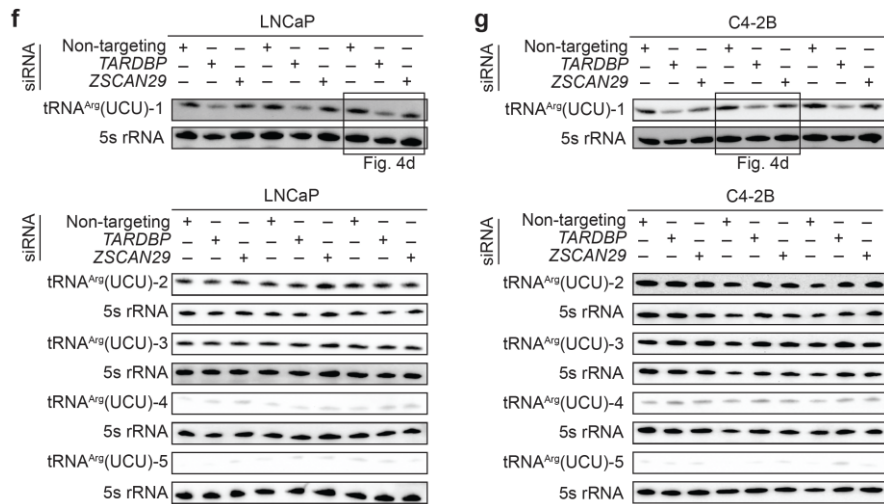


Figure 4. j. RNA polymerase III binding analysis at *tRNA-Arg-TCT* gene loci by ChIP-qPCR in LNCaP cells (left; n = 5 replicates) and C4-2B cells (right; n = 4 replicates) transfected with non-targeting siRNA or siRNA against *TARDBP* or *ZSCAN29*; mean $\pm$ SEM. p-values, two-way ANOVA with Tukey's multiple comparisons test (* = $p < 0.05$; **** = $p < 0.0001$).

- POLR3A binding is most prevalent at the *tRNA-Arg-TCT-1-1* locus compared to 2-1, 3-1, 3-2, 4-1, and 5-1.
- Knockdown of *TARDBP* or *ZSCAN29* led to the largest decrease in POLR3A binding at the *tRNA-Arg-TCT-1-1* locus compared to 2-1, 3-1, 3-2, 4-1, and 5-1.

Lastly, to further demonstrate specificity for *tRNA^{Arg}(UCU)-1*, we probed for *tRNA^{Arg}(UCU)-2*, -3, -4, and -5 after *TARDBP* or *ZSCAN29* knockdown. Unlike *tRNA^{Arg}(UCU)-1*, we observed no changes in tRNA expression levels amongst the other isodecoders (**Extended Data Fig. 9f-g**). These findings highlight the specificity by which *TARDBP* and *ZSCAN29* regulate *tRNA^{Arg}(UCU)-1* expression.



Extended Data Figure 9. f. Northern blot of *tRNA^{Arg}(UCU)-1* (above), -2, -3, -4, -5, and 5s rRNA (below) in LNCaP cells transfected with non-targeting siRNA or siRNA against *TARDBP* or *ZSCAN29* ($n = 3$ replicates). 5s rRNA is a loading control. The representative northern blot image used in **Fig. 4d** is highlighted in the black box. **g.** Northern blot of *tRNA^{Arg}(UCU)-1* (above), -2, -3, -4, -5, and 5s rRNA (below) in C4-2B cells transfected with non-targeting siRNA or siRNA against *TARDBP* or *ZSCAN29* ($n = 3$ replicates). 5s rRNA is a loading control. The representative northern blot image used in **Fig. 4d** is highlighted in the black box.

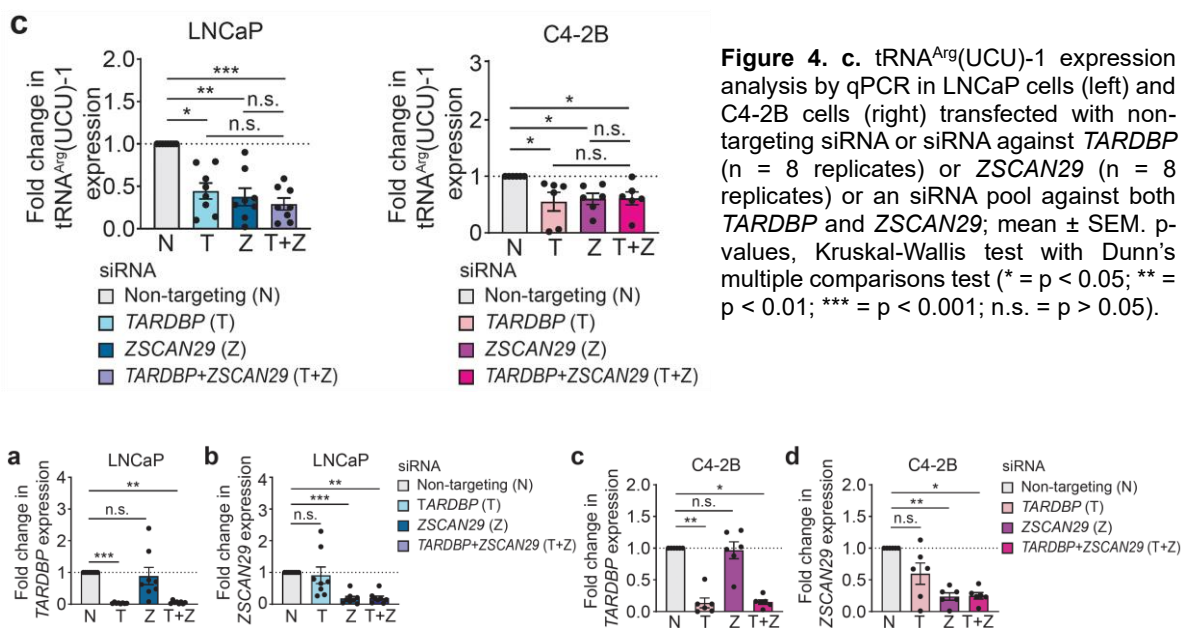
In addition to the new figures, we have written new text in lines 278-288 & 303-312:

“Next, we performed CUT&RUN profiling of *TARDBP* and *ZSCAN29* across all tRNA genomic loci in LNCaP AD and LP cells. Among all tRNA genes, *tRNA-Arg-TCT-1-1* was the only locus that exhibited coordinated reductions in *TARDBP* and *ZSCAN29* occupancy together with decreased mature tRNA abundance during lineage plasticity (**Extended Data Fig. 9a-c**). Within the six *tRNA-Arg-TCT* isodecoder loci, only *tRNA-Arg-TCT-1-1* showed significantly reduced occupancy of both *TARDBP* ($\log_2FC = -4.811$, $FDR = 0.006$) and *ZSCAN29* ($\log_2FC = -3.858$, $FDR = 0.015$). Although *tRNA-Arg-TCT-2-1* also exhibited reduced *TARDBP* occupancy ($\log_2FC = -3.573$, $FDR = 0.038$), *ZSCAN29* occupancy was increased in LP ($\log_2FC = 0.532$, $FDR = 0.015$). No significant differences in *TARDBP* or *ZSCAN29* occupancy were detected at the remaining *tRNA-Arg-TCT* loci (*tRNA-Arg-TCT-3-1*, -3-2, -4-1, and -5-1) (**Fig. 4f & Extended Data Fig. 9a & Supplementary Table 6**).”

“Based on these finding, we tested whether TARDBP and ZSCAN29 preferentially regulate expression of the H3K4me3-enriched *tRNA*-Arg-TCT-1-1 locus compared to all other *tRNA*-Arg-TCT loci. Knockdown of TARDBP or ZSCAN29 in LNCaP and C4-2B cells selectively reduced expression of *tRNA*^{Arg}(UCU)-1, whereas the remaining *tRNA*^{Arg}(UCU) isodecoders were unaffected (**Extended Data Fig. 9f-g**). Consistent with these findings, depletion of TARDBP or ZSCAN29 significantly reduced RNA polymerase III occupancy only at the *tRNA*-Arg-TCT-1-1 locus in both cell lines (**Fig. 4j & Extended Data Fig. 9h-i**). Together, these findings demonstrate that TARDBP and ZSCAN29 preferentially maintain RNA polymerase III occupancy at the H3K4me3-enriched *tRNA*-Arg-TCT-1-1 locus, thereby selectively promoting *tRNA*^{Arg}(UCU)-1 transcription (**Extended Data Fig. 9j**).”

6b) Also, do both proteins are needed or their function is redundant in this context?

To determine whether both proteins are needed for transcriptional regulation of *tRNA*-Arg-TCT-1-1, we conducted siRNA knockdown of both TARDBP and ZSCAN29 and measured *tRNA*^{Arg}(UCU)-1 levels. The combined knockdown did not further decrease *tRNA*^{Arg}(UCU)-1 levels compared to depletion of either TARDBP or ZSCAN29 alone (**Fig. 4c & Extended Data Fig. 8a-d**). These data suggest that TARDBP and ZSCAN29 do not act cooperatively and function in a redundant manner.

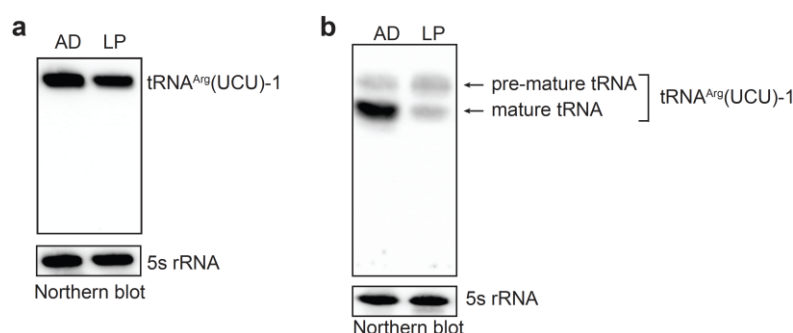


Extended Data Figure 8. a. TARDBP expression analysis by qPCR in LNCaP cells transfected with non-targeting siRNA or siRNA against TARDBP (n = 8 replicates) or ZSCAN29 (n = 8 replicates) or an siRNA pool against both TARDBP and ZSCAN29 (n = 8 replicates); mean ± SEM. p-values. **b.** ZSCAN29 expression analysis by qPCR in LNCaP cells transfected with non-targeting siRNA or siRNA against TARDBP (n = 8 replicates) or ZSCAN29 (n = 8 replicates) or an siRNA pool against both TARDBP and ZSCAN29 (n = 8 replicates); mean ± SEM. **c.** TARDBP expression analysis by qPCR in C4-2B cells transfected with non-targeting siRNA or siRNA against TARDBP (n = 6 replicates) or ZSCAN29 (n = 6 replicates) or an siRNA pool against both TARDBP and ZSCAN29 (n = 6 replicates); mean ± SEM. **d.** ZSCAN29 expression analysis by qPCR in C4-2B cells transfected with non-targeting siRNA or siRNA against TARDBP (n = 6 replicates) or ZSCAN29 (n = 6 replicates) or an siRNA pool against both TARDBP and ZSCAN29 (n = 6 replicates); mean ± SEM. p-values, Kruskal-Wallis test with Dunn's multiple comparisons test (* = p < 0.05; ** = p < 0.01; *** = p < 0.001; n.s. = p > 0.05).

7) *tRNA cleavage was only tested earlier in the workflow. I would like to see actual NB pictures including species below mature tRNAs. Please provide them as part of Supp.Imfo*

We thank the reviewer for this comment. We conducted northern blots with 10 µg of total RNA from LNCaP AD and LP cells and were able to detect only full-length tRNA^{Arg}(UCU)-1 (**Reviewer 2. Fig. 2a-b**). The original probe we used for northern blotting targets the region between bases 48 to 73 of tRNA^{Arg}(UCU)-1 (**Reviewer 2. Fig. 2a**), which has the greatest number of unique bases among tRNA^{Arg}(UCU) isodecoders.

To determine if the lack of fragments was a result of our probe, we also designed and tested a probe that targets the entire body sequence of tRNA^{Arg}(UCU)-1 to capture any fragments from all tRNA^{Arg}(UCU) isodecoders within the lysates. However, we observed pre-mature and mature tRNA^{Arg}(UCU)-1 with no fragment bands of smaller size below them (**Reviewer 2. Fig. 2b**). We conclude from these studies that LNCaP AD and LP cells have a very low level of tRNA^{Arg}(UCU)-1-derived fragments.



Reviewer 2. Figure. 2. **a.** Northern blot of tRNA^{Arg}(UCU)-1 using a probe that targets the 3' end of the tRNA. 5s rRNA is a loading control. **b.** Northern blot of tRNA^{Arg}(UCU)-1 using a probe that targets the entire tRNA body. 5s rRNA is a loading control.

Referee #3 (Remarks to the Author):

A. This well written manuscript describes a highly novel area of prostate cancer biology focused on a tRNA, tRNAArg(UCU)-1, that the authors claim is central to the lineage plasticity reported in advanced prostate cancer that associates with treatment resistance, visceral metastatic disease and poor prognosis. The authors claim that decreased expression of tRNAArg(UCU)-1 results in a change in SWI/SNF complex function associated with altered SMARCC2 protein expression with this regulation occurring at a protein translation level due to altered tRNAArg(UCU)-1 expression.

B. This is a highly novel piece of work. I am not aware of any other work reporting similar findings. Indeed this area of biology is very novel to the (prostate) cancer research field.

C. The approaches taken are valid and the presentation excellent; while I am not deeply familiar with tRNA biology this work is well described and the claims made appear bolstered by sound data although I believe this manuscript can be strengthened by replies to my comments. The data quality appears high and validated by the use of multiple different models (a major challenge in prostate cancer research) and some limited clinical data.

D. Stats use appears appropriate.

E. Conclusions: The authors appear to make reasonable conclusions

We are thrilled that the reviewer found that our manuscript “describes a highly novel area of prostate cancer biology.” We appreciate the comments provided by the reviewer and have addressed them experimentally point-by-point below.

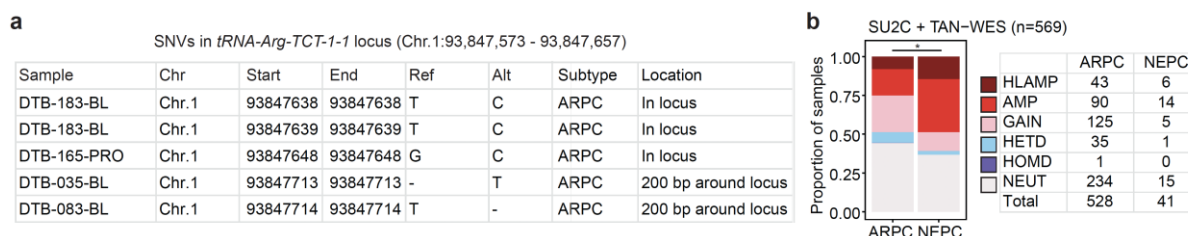
F. Suggested improvements:

1)The gene for tRNAArg(UCU)-1 appears to lie on chromosome 17p, a locus that is commonly altered in prostate cancer and includes multiple key genes; can the authors confirm this? Can they also report on the frequency of neuroendocrine prostate cancer or histopathological evidence of lineage plasticity eg AR low in these tumors.

We appreciate the opportunity to clarify this point. The *tRNA-Arg-TCT-1-1* gene resides in Chr.1: 93,847,573-93,847,657. Within a 20 kilobase window there is only one neighboring gene called BCAR3 which has not been implicated in prostate cancer. However, it is completely unknown if this gene locus is prone to mutations. Thus, to determine if the *tRNA-Arg-TCT-1-1* gene locus possesses mutations or copy number alterations in prostate cancer, we analyzed whole genome sequencing (WGS) and whole exome sequencing (WES) data from the University of Washington Prostate Cancer Rapid Autopsy cohort and the West Coast SU2C cohort. Importantly, these tissues come from advanced stage prostate cancer metastases and metastatic biopsies, respectively¹⁵⁻¹⁸. Studying the WGS data (n = 197), we found that three patients with androgen receptor positive prostate cancer had single nucleotide alterations within the gene body and two had mutations within 200 bases of the gene body (**Reviewer 3. Fig. 1a**). We did not observe any mutations of this gene in patients with neuroendocrine prostate cancer (NEPC).

We next performed copy number variation (CNV) analysis focusing on the *tRNA-Arg-TCT-1-1* gene locus using WES data and enumerated the types of CNVs in both prostate adenocarcinoma (n = 528) and NEPC (n = 41) tumors. NEPC tumors exhibited a statistically significant increase in amplifications in the *tRNA-Arg-TCT-1-1* locus compared to adenocarcinomas (**Reviewer 3. Fig.**

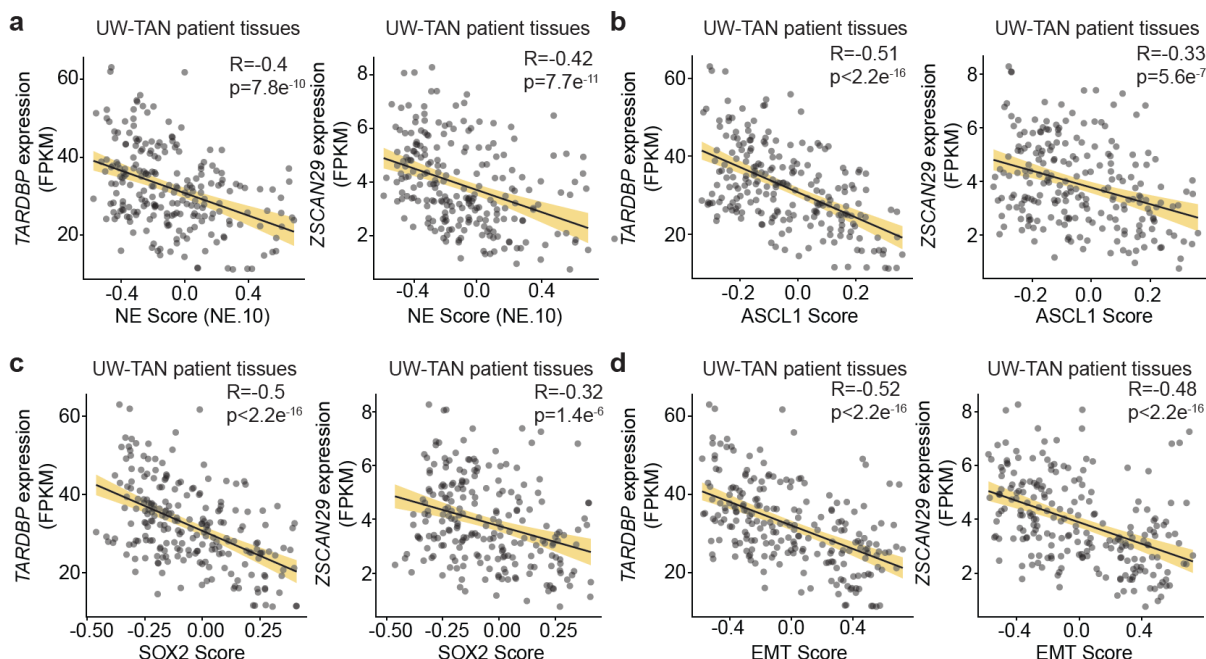
1b). In contrast, deletion events (heterozygous deletion, homozygous deletion) were not common in this region. Together, our data suggests that the decrease in $tRNA^{Arg}(UCU)$ -1 levels we observe in NEPC patient specimens were not due to genomic mutations or a loss of the *tRNA-Arg-TCT-1-1* gene but rather a transcriptional mechanism which we elucidated in **Fig. 4**.



Reviewer 3. Figure 1. a. Table of SNVs found in the *tRNA-Arg-TCT-1-1* gene locus. **b.** Bar graph of CNV categories in ARPC (AR+/NE-, AR+/NE+, ARlow/NE-) and NEPC (AR-/NE+); Chi-square test (* = $p < 0.05$).

2) The authors claim that *TARDBP* and *ZSCAN29* are transcription factors that are key regulators of *tRNA^{Arg}(UCU)*-1 which they now report as a major controller of lineage plasticity in prostate cancer. Can the authors present data from prostate cancer biopsy RNAseq data validating associations between the expression of these genes and key lineage plasticity factors including *ASCL1*, *SOX2* and/or *SNAI1/SNAI2/ZEB1*. Are the RNAseq pathways associated with these TFs validating their claim?

We thank the reviewer for this valuable comment as *TARDBP* and *ZSCAN29* have not been previously described as lineage plasticity regulators in prostate cancer. Using the University of Washington Prostate Cancer Rapid Autopsy RNA-seq dataset composed of end stage patient tumors^{19,20}, we first compared the transcript levels of *TARDBP* and *ZSCAN29* with a published neuroendocrine (NE) score⁶ and observed a significant negative correlation (**Extended Data Fig. 10a**). Next, we derived *ASCL1* and *SOX2* pathway scores based on publicly available gene sets and correlated the score with *TARDBP* and *ZSCAN29* levels. Like the NE score, we observe that lower *TARDBP* and *ZSCAN29* expression were significantly associated with higher *ASCL1* and *SOX2* pathway scores (**Extended Data Fig. 10b-c**). Lastly, to determine how *TARDBP* and *ZSCAN29* correlate with epithelial-to-mesenchymal transition (EMT), we utilized an EMT gene set to create an EMT score and observed a significant negative correlation (**Extended Data Fig. 10d**). As such, *TARDBP* and *ZSCAN29* transcript levels strongly associate with lineage plasticity programs in advanced prostate cancer patients.



Extended Data Figure 10. **a.** Correlation plot between the NE score (NE.10) and TARDBP (left) or ZSCAN29 (right) expression with Spearman's correlation statistics shown. **b.** Correlation plot between the ASCL1 score and TARDBP (left) or ZSCAN29 (right) expression with Spearman's correlation statistics shown. **c.** Correlation plot between the SOX2 pathway score and TARDBP (left) or ZSCAN29 (right) expression with Spearman's correlation statistics shown ($n = 222$ patient tissues). **d.** Correlation plot between the EMT pathway score and TARDBP (left) or ZSCAN29 (right) expression with Spearman's correlation statistics shown ($n = 222$ patient tissues).

In addition to the new figures, we added new text in lines 314-321:

Given the role of *tRNA^{Arg}(UCU)-1* in regulating prostate cancer lineage plasticity, we next investigated whether TARDBP and ZSCAN29 are associated with lineage states in patient tumors. Using RNA-seq data from the University of Washington metastatic castration-resistant prostate cancer patient cohort¹⁹⁻²¹, we found that TARDBP and ZSCAN29 expression was strongly inversely correlated with neuroendocrine and epithelial-to-mesenchymal transition (EMT) gene signatures (**Extended Data Fig. 10a-d**). These findings extend our experimental observation to human disease and establish that TARDBP and ZSCAN29 are associated with lineage plasticity in patients.

3a. The authors should make clear that the two cell lines they initially focus on are essentially derived from each other, limiting their ability to generalize their cell line findings (ie C4-2B is derived from LNCaP).

We thank the reviewer for pointing this out. Indeed, C4-2B cells were derived from a metastatic xenograft of LNCaP parental cells^{22,23}. To clarify this in the manuscript, we now state that, C4-2B cells are a metastatic derivative of LNCaP parental cells (**line 103**). We would like to emphasize that while the LNCaP and C4-2B models were used as our primary *in vitro* system, we further corroborated these findings across multiple model systems and tissues including:

- Other prostate cancer cell lines: VCaP, 22RV1, DU145, PC3, and LNCaP-abl (**Fig. 1e, 3f-h**).
- The LTL331 PDX model developed by Dr. YZ Wang which transdifferentiates after castration(**Fig. 1f**)²⁴.

- Patient specimens from the University of Washington Prostate Cancer Rapid Autopsy Program¹⁹ (**Fig. 1g-k**).
- An autochthonous prostate-specific MYC over-expressing mouse model haploinsufficient for *tRNA-Arg-TCT-1-1* (**Fig. 2h-i**).
- The TRAMP prostate cancer mouse model with overexpression of *tRNA^{Arg}(UCU)-1* (**Fig. 3i**).

Thus, we sought to generalize our findings through multiple orthogonal systems.

In addition to these figures, we added following text in lines 136-139:

*“Together, these observations across multiple model systems and patient tissues collectively establish *tRNA^{Arg}(UCU)-1* as a lineage-associated isodecoder whose abundance decreases with the transition from AR-driven adenocarcinoma to a lineage plastic, therapy-resistant state in prostate cancer.”*

3b) This reviewer would also like the authors to better justify their use of the genomic alterations induced in these models which are multiple and include TP53 R175H, MYCN, ASCL1, as well as SRRM4, NR0B2, BCL2, and KRAS G12V. Were all these genomic changes really necessary to induce lineage plasticity? Are these changes truly representative of clinical lineage plasticity in PCa?

This cell-based system of transdifferentiation was developed by Dr. John Lee (a collaborator on this project and co-author) and recently published in eLIFE (**DOI: 10.7554/eLife.106511.1**). The candidate factors that were chosen to enforce lineage plasticity in LNCaP and C4-2B cells were based off of their extensive experience driving lineage changes in the prostate epithelium²⁵, the published literature, as well as the cellular requirements to overcome the stress of differentiation.

Here is the stated rationale for the design of this system as published in the eLIFE paper:

“Loss of TP53 and RB1 as well as amplification of MYCN have been identified as common genetic features associated with NEPC²⁵⁻²⁸. SRRM4 is a splicing regulator that has been shown to inactivate the RE-1 silencing transcription factor (REST) which functions to suppress a major neural gene program. Loss of REST has been implicated as a common feature in NEPC that has been reported to functionally drive [neuroendocrine transdifferentiation] of [prostate cancer]^{29,30}. In addition, the pioneer neural transcription factor ASCL1 is expressed in most NEPC and appears to define a transcriptional and epigenetic subtype of the disease³¹. NR0B2, otherwise known as short heterodimer partner (SHP), is an orphan nuclear receptor that is highly expressed in NEPC that interacts with AR and inhibits AR activity³². Lastly, BCL2 and KRAS G12V were included to potentially reduce apoptosis and enhance reprogramming efficiencies^{33,34}”

Thus, with the exception of BCL2 and KRAS G12V which are necessary for efficient pre-programming, all of the other genes have a firm clinical rationale for their inclusion in promoting transdifferentiation. However, we acknowledge that there is no such thing as a perfect model of human prostate cancer which is why we extensively validated our findings using orthogonal systems including patient derive xenografts (**Fig. 1f**), autochthonous mouse models (**Fig. 2h-i & Fig. 3i**), other prostate cancer cell lines (**Fig. 1e & 3h**), and patient tissue specimens (**Fig. 1g-k**).

4. Can the authors in their conclusions comment on how to clinically translate their findings? How can this biology be drugged to impart clinical benefit?

These are important questions. We believe there are two ways in which our findings can be clinically translated.

1) We have developed an RNA *in situ*-based method to accurately measure tRNA^{Arg}(UCU)-1 levels in archival patient specimens (**Fig. 1g-k**). We observed that patients with lower intensity of tRNA^{Arg}(UCU)-1 puncta more rapidly developed androgen independence and metastasis (**Fig. 6a-d**). This assay could be used as a biomarker for disease aggressiveness and a need for treatment escalation. However, this would need to be tested in large patient cohorts retrospectively and prospectively.

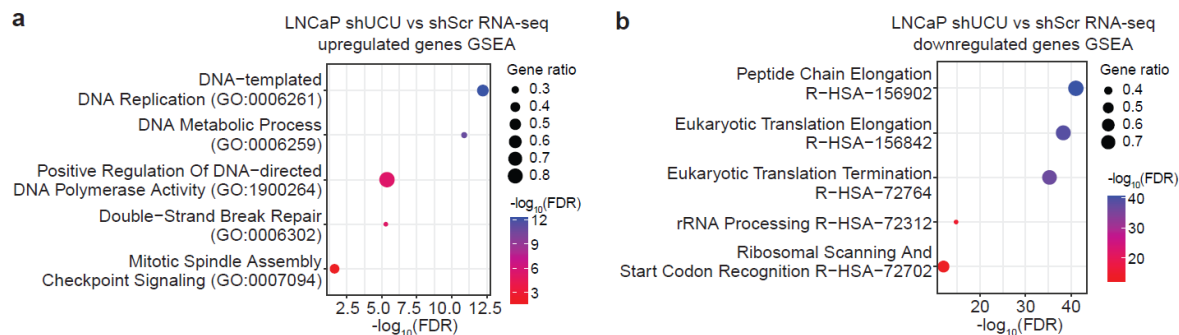
2) Two reports have demonstrated the feasibility of tRNA overexpression *in vivo* using adenoviral systems^{35,36}. If these methods could be harnessed for safe delivery into patients, it may be possible to engineer methods to restore tRNA^{Arg}(UCU)-1 levels in men with advanced prostate cancer to reverse lineage plasticity and regain sensitivity to androgen receptor pathway inhibitors.

Both concepts have been included in the discussion in lines 475-484:

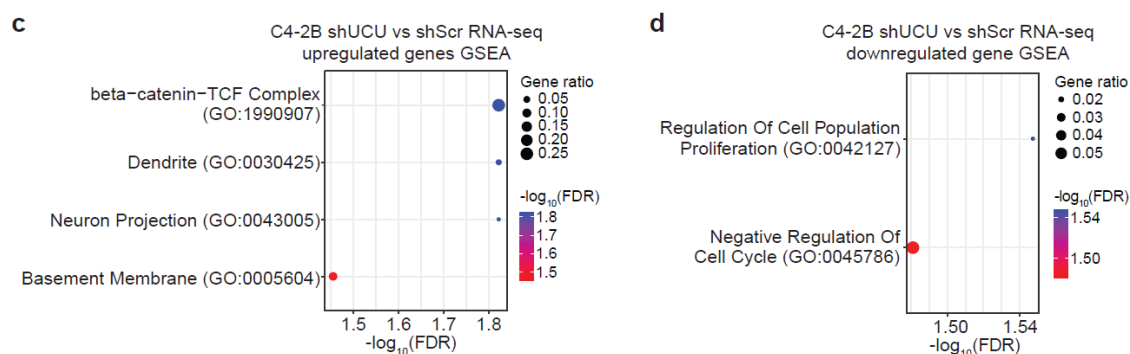
“While these clinical associations are derived from retrospective datasets, prospective studies will be required to assess the predictive utility of tRNA isodecoders as biomarkers of AR-targeted therapy resistance. Beyond its potential as a biomarker, there is growing interest in the therapeutic application of tRNAs. Notably, adeno-associated virus (AAV)-mediated delivery of suppressor tRNAs has been used to restore protein function in models of mucopolysaccharidosis type I³⁶, and lipid nanoparticle-based tRNA delivery has enabled re-expression of CFTR in patient-derived epithelia from individuals with cystic fibrosis³⁵. These emerging technologies offer a potential framework for restoring tumor-suppressive tRNA isodecoders such as tRNA^{Arg}(UCU)-1 to reprogram lineage dependency and re-sensitize tumors to existing therapies in advanced prostate cancer patients.”

5. The impacts of decreased tRNA^{Arg}(UCU)-1 expression may be broad. Have the authors excluded molecular changes beyond impact on the SWI/SNF complex? Also could the impact on PCa cell biology be broader than an impact on lineage plasticity? What do RNAseq data after knock in and knockout (and knock back in) studies indicate?

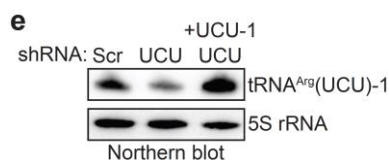
We thank the reviewer for this insightful comment. Decreased expression of tRNA^{Arg}(UCU)-1 likely has a broader impact on prostate cancer than only lineage plasticity. To address this question, we conducted RNA-seq on tRNA^{Arg}(UCU)-1 knockdown cells and knock back in cell lines (overexpression of tRNA^{Arg}(UCU)-1 following knockdown). In LNCaP cells, tRNA^{Arg}(UCU)-1 knockdown decreased the expression of genes involved in mRNA translation and increased those that regulate DNA replication and the cell cycle (**Reviewer 3. Fig. 2a-b**). In C4-2B cells, tRNA^{Arg}(UCU)-1 depletion increased the abundance of neuronal program genes and decreased negative regulators of the cell cycle (**Reviewer 3. Fig. 2c-d**). We also generated a C4-2B rescue cell line by lentiviral transduction of a tRNA^{Arg}(UCU)-1 construct into shUCU cells. Following tRNA^{Arg}(UCU)-1 addback (C4-2B shUCU+UCU OE), genes involved in tRNA metabolism and the integrated stress response pathways were induced. Conversely, cell cycle and DNA replication-related genes showed a marked reduction in expression. (**Reviewer 3. Fig. 2e-g**). Together, these transcriptome-wide studies reveal that tRNA^{Arg}(UCU)-1 dosage can influence of the expression of proliferation regulators, translation factors, and other lineage markers.



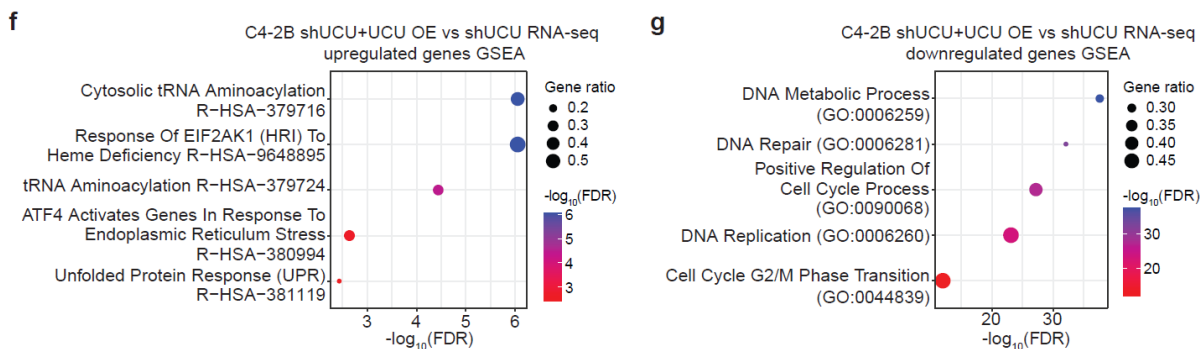
Reviewer 3. Figure 2. a. GSEA of significantly upregulated genes in shUCU compared to shScr LNCaP cells. **b.** GSEA of significantly downregulated genes in shUCU LNCaP cells.



Reviewer 3. Figure 2. c. GSEA of significantly upregulated genes in shUCU compared to shScr C4-2B cells. **d.** GSEA of significantly downregulated genes in shUCU C4-2B cells.



Reviewer 3. Figure 2. e. Northern blot of charged tRNA^{Arg}(UCU)-1 and 5S rRNA C4-2B shScr, shUCU, shUCU with tRNA^{Arg}(UCU)-1 addback.



Reviewer 3. Figure 2. f. GSEA of significantly upregulated genes from C4-2B shUCU+UCU OE cells versus shUCU cells. **g.** GSEA of significantly downregulated genes from C4-2B shUCU+UCU OE cells versus shUCU cells.

6. Do the authors have any evidence that changes in *tRNA^{Arg}(UCU)-1* expression have homeostatic roles? In embryogenesis?

We appreciate the reviewer's comment. Much of what we know about the physiologic role of *tRNA^{Arg}(UCU)-1* has been derived from mice. Remarkably, mice with whole body deletion of *tRNA-Arg-TCT-1-1* are viable and are born at Mendelian ratios (data from our collaborator and co-author Dr. Susan Ackerman)³⁷. *tRNA^{Arg}(UCU)-1* has been extensively profiled in the murine brain and its expression varies widely depending on the cell type. For example, astrocytes, oligodendrocytes, microglia, and pericytes express high levels of *tRNA^{Arg}(UCU)-1*, whereas neurons have comparatively low levels³⁷. Instead, neurons abundantly expression *tRNA^{Arg}(UCU)-4* whose body sequence differs extensively from *tRNA^{Arg}(UCU)-1*. Thus, in the context of normal physiology, *tRNA^{Arg}(UCU)-1* levels vary in a cell-type and tissue-specific manner, and isodecoders may "compensate" for each other to maintain consistent levels of each isoacceptor family.

To highlight the tissue-specificity of *tRNA^{Arg}(UCU)-1* we added the following text in lines 425-431:

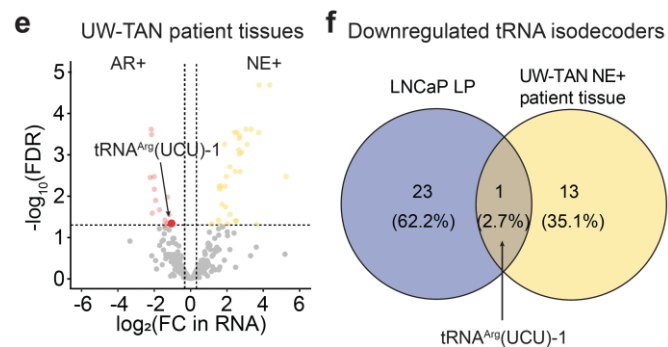
"While expression of certain tRNA isodecoders, such as tRNA^{Arg}(UCU)-4³⁷, are functionally associated with neurons, this tRNA was not altered during lineage plasticity. Notably, a comprehensive analysis comparing tRNAs in neurons and non-neuronal cells revealed that tRNA^{Arg}(UCU)-1 exhibited the most significant decrease among all isodecoders^{37,38}. This finding suggests that low tRNA^{Arg}(UCU)-1 expression could be a common feature of neuronal gene expression programs. It remains to be determined how tRNA^{Arg}(UCU)-1 controls neuronal expression networks."

7. Why did *tRNA^{Tyr} GUA* drop out in C4-2B, when this cell line is a derivative of LnCaP?

Thank you for highlighting the other tRNA isoacceptor found in the initial screen. We have re-analyzed our MSR-seq data using UMI-based counts to avoid PCR biases during library preparation in response to Reviewer 4. In addition, we performed multiple-comparison analyses of the isoacceptor dataset using the DESeq2 and edgeR packages to rigorously assess the statistical significance of differential isoacceptor expression between AD and LP cells (**Fig. 1a & Extended Data Fig. 1c**). In these new analyses *tRNA^{Tyr}(GUA)* is no longer significantly decreased.

8. Can the authors make more clear whether they found, in clinical samples, any other tRNAs whose expression were differentially expressed between adenocarcinoma of the prostate and NEPC?

We appreciate the reviewer's comment regarding differential tRNA expression in patient tissues. To address this point and extend our analysis to clinical specimens, we performed MSR-seq on a subset of AR+ (n = 3) and NE+ (n = 4) prostate cancer samples obtained from the University of Washington Rapid Autopsy Program. We identified 14 isodecoders that were downregulated in NEPC, including: *tRNA^{Arg}(UCU)-1*, *tRNA^{Ala}(AGC)-4*, *tRNA^{Arg}(ACG)-2*, *tRNA^{Arg}(UCG)-3*, *tRNA^{Gly}(UCC)-2*, *tRNA^{Ile}(AAU)-4*, *tRNA^{Ile}(AAU)-5*, *tRNA^{Ile}(AAU)-7*, *tRNA^{Ile}(AAU)-8*, *tRNA^{Ile}(UAU)-2*, *tRNA^{Lys}(CUU)-3*, *tRNA^{Lys}(CCU)-4*, *tRNA^{Tyr}(GUA)-2*, and *tRNA^{Tyr}(GUA)-5* (**Extended Data Fig. 3e**). Importantly, comparison of the LNCaP and patient MSR-seq datasets revealed that *tRNA^{Arg}(UCU)-1* was the only isodecoder significantly downregulated in both datasets (**Extended Data Fig. 3f**). These findings further support investigation of the molecular and cellular functions of *tRNA^{Arg}(UCU)-1*. However, whether additional downregulated tRNAs contribute functionally to prostate cancer progression remains unclear and warrants future investigation.



Extended Data Figure 3. e. Volcano plot showing differential tRNA isodecoder expression between AR+ and NE+ UW rapid autopsy patient tissues. **f.** Venn diagram of downregulated tRNA isodecoders between LNCaP LP cells and UW rapid autopsy NE+ patient tissues.

In addition to the new figures, we added the following text in lines 130-132:

“Furthermore, the only tRNA isodecoder downregulated in both the lineage plastic LNCaP cell line model and patient tissue samples was $tRNA^{\text{Arg}}(\text{UCU})-1$ (Extended Data Fig. 3e-f & Supplementary Table 2).”

Referee #4 (Remarks to the Author):

The manuscript “tRNA dosage regulates lineage dependency and therapy resistance in prostate cancer” by Kim et al. proposes that a single tRNA isodecoder, tRNA^{Arg}(UCU)-1, is a dosage-sensitive regulator of lineage dependence and ARPI drug response in prostate cancer. The application of several targeted approaches, including MSR-seq, Basescope, polysome RNA-seq and the use of engineered cell and mouse models are major strengths of the present study. Utilizing these methods, the authors argue that (1) tRNA^{Arg}(UCU)-1 decreases during tumor cell lineage progression (AD -> LP/NE transitions), (2) that decreases in tRNA^{Arg}(UCU)-1 abundance promotes AR-independence and ARPI resistance, (3) that restoring tRNA^{Arg}(UCU)-1 reverses lineage state and drug response, (4) that TDP43 and Zscan29 control the tRNA-Arg-TCT-1-1 gene locus, and (5) that, mechanistically, tRNA^{Arg}(UCU)-1 levels have downstream consequences on AGA-codon-biased translation, particularly for SMARCC2 and potentially other SWI/SNF components.

0.1. While specific concerns related to experimental design choices and analyses are shared below, a general concern relates to conclusions on the specificity of tRNA^{Arg}(UCU)-1 as a modulator of lineage dependency and therapy resistance. To truly separate tRNA^{Arg}(UCU)-1 as a unique player in this respect, one would need to examine the individual effects of all tRNA isodecoders (e.g. using a CRISPR screen) and show that this particular isodecoder stands out in a significant way. In the absence of this, a simpler alternative model is that the observed lineage effects may reflect broader perturbations in tRNA pools, where changes in tRNA^{Arg}(UCU)-1 abundance are representative, but not necessarily unique. In other words, it remains possible that other UCU isodecoders and other tRNAs could contribute similarly if perturbed. The present data indicates tRNA^{Arg}(UCU)-1 as a potential marker and functional participant in lineage plasticity, but whether it is unique in this role requires additional comparative evidence. If tRNA^{Arg}(UCU)-1 is not unique, it raises many questions about whether the consequences are related to general perturbations of translation, rather than the TDP43/Zscan29 -> tRNA^{Arg}(UCU)-1 -> SMARCC2 model proposed by the authors.

We thank the reviewer for providing us with thoughtful and constructive comments on our manuscript. The reviewer's feedback has helped us improve the manuscript, and we have made the necessary revisions to address each point raised. Below we provide detailed responses to each of the comments.

The reviewer raises the important point of the specificity of tRNA^{Arg}(UCU)-1 in our study. To address this, we performed isodecoder specific northern blots and confirmed that tRNA^{Arg}(UCU)-2, tRNA^{Arg}(UCU)-3, tRNA^{Arg}(UCU)-4, and tRNA^{Arg}(UCU)-5 levels do not change between adenocarcinoma (AD) and lineage plastic (LP) cells or following re-expression of tRNA^{Arg}(UCU)-1 (**Fig. 3a & Extended Data Fig. 6b**). These findings demonstrate that the changes we see in tRNA^{Arg}(UCU)-1 expression in AD, LP, and tRNA^{Arg}(UCU)-1 addback cells are specific to tRNA^{Arg}(UCU)-1 and not the other isodecoders which confirm our MSR-seq data (**Fig. 1b**).

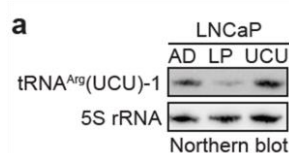
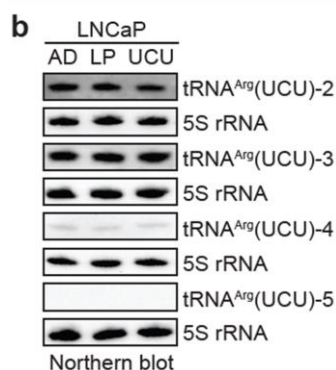


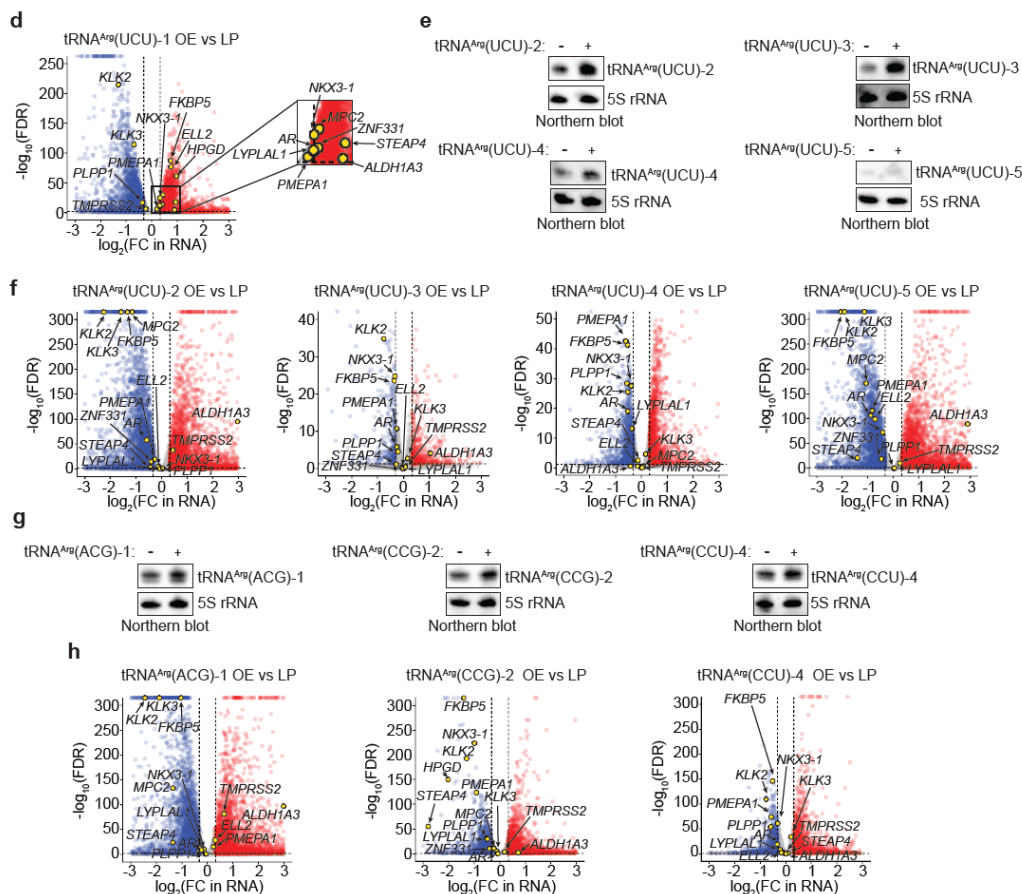
Figure 3. a. Northern blot of charged tRNA^{Arg}(UCU)-1 and 5S rRNA of AD, LP, and UCU (tRNA^{Arg}(UCU)-1 add back) LNCaP cells. 5S rRNA is a loading control.



Extended Data Figure 6. b.

Northern blot of charged tRNA^{Arg}(UCU)-2, -3, -4, and -5 of LNCaP AD, LP, and UCU cells. 5S rRNA is a loading control.

Next, we generated tRNA^{Arg}(UCU)-2, tRNA^{Arg}(UCU)-3, tRNA^{Arg}(UCU)-4, and tRNA^{Arg}(UCU)-5 constructs and established a series of stable overexpression LP (lineage plastic) cell lines (**Extended Data Fig. 6e**). We previously demonstrated that overexpression of tRNA^{Arg}(UCU)-1 in LP cells was sufficient to increase androgen receptor (AR)-driven gene expression (**Extended Data Fig. 6d**). In contrast, overexpression of tRNA^{Arg}(UCU)-2, -3, -4, or -5 failed to restore AR pathway activity (**Extended Data Fig. 6f**). As additional controls, we generated LP cells overexpressing tRNA^{Arg}(ACG)-1, tRNA^{Arg}(CCG)-2, and tRNA^{Arg}(CCU)-4, none of which increased AR pathway gene expression (**Extended Data Fig. 6g-h**). Together, these new experiments further support that tRNA^{Arg}(UCU)-1 uniquely influences cell state in prostate cancer.



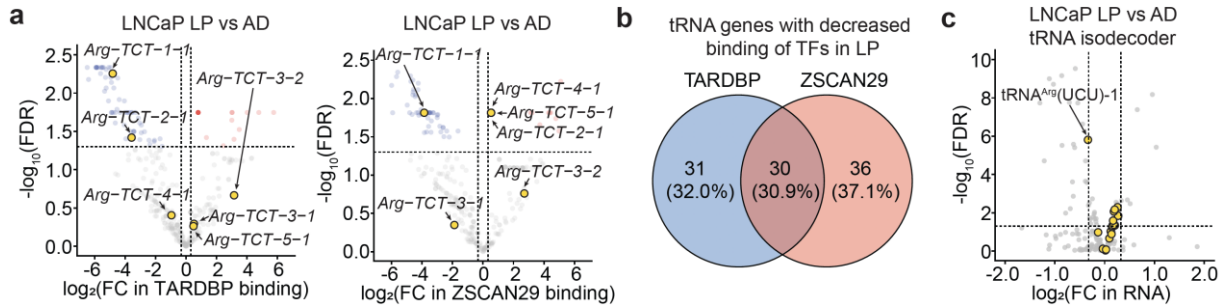
Extended Data Figure 6. d. Volcano plot of RNA-seq from LNCaP LP and UCU cells. AR pathway genes from ARG.10 and Tang et al. ATAC-seq data (*ELL2*, *HPGD*, *LYPLAL1*, *MPC2*, *ZNF331*)¹ are highlighted in yellow. **e.** Northern blot of *tRNA^{Arg}(UCU)*-2, -3, -4, -5 and 5s rRNA in LNCaP LP and LP with *tRNA^{Arg}(UCU)*-2, -3, -4, -5 overexpression cells, respectively. 5s rRNA is a loading control. **f.** Volcano plot of RNA-seq from LNCaP LP and LP cells with *tRNA^{Arg}(UCU)*-2, -3, -4, -5 overexpression. The same AR pathway genes from Extended Data Fig. 6d are highlighted in yellow. **g.** Northern blot of *tRNA^{Arg}(ACG)*-1, *tRNA^{Arg}(CCG)*-2, *tRNA^{Arg}(CCU)*-4 and 5s rRNA in LNCaP LP and LP with *tRNA^{Arg}(ACG)*-1, *tRNA^{Arg}(CCG)*-2, *tRNA^{Arg}(CCU)*-4 overexpression cells, respectively. 5s rRNA is a loading control. **h.** Volcano plot of RNA-seq from LNCaP LP and LP cells with *tRNA^{Arg}(ACG)*-1, *tRNA^{Arg}(CCG)*-2, or *tRNA^{Arg}(CCU)*-4 overexpression. The same AR pathway genes from Extended Data Fig. 6d are highlighted in yellow.

In addition to these new figures, we have included the following text in lines 212-215:

“Lastly, we overexpressed *tRNA^{Arg}(UCU)* isodecoders 2-5, *tRNA^{Arg}(ACG)*-1, *tRNA^{Arg}(CCG)*-2, or *tRNA^{Arg}(CCU)*-4 in LNCaP LP cells and observed nearly no increase in AR pathway genes as we observed with *tRNA^{Arg}(UCU)*-1 re-expression (Fig. 3b & Extended Data Fig. 6d-h & Supplementary Table 4).”

0.2. On a related note, the evidence supporting *TDP43* and *Zscan29* as regulators of *tRNA^{Arg}(UCU)*-1 are similarly missing a comparative framework – are these factors enriched at *tRNA^{Arg}(UCU)*-1 compared to other *tRNA* genes?

We thank the review for this comment. To study TARDBP and ZSCAN29 in a comparative framework, we conducted a series of new CUT&RUN, ChIP, and molecular studies. First, we performed CUT&RUN profiling of TARDBP and ZSCAN29 across all tRNA genomic loci in LNCaP AD and LP cells. Among all tRNA genes, *tRNA-Arg-TCT-1-1* was the only locus that exhibited coordinated reductions in TARDBP and ZSCAN29 occupancy together with decreased mature tRNA abundance during lineage plasticity (**Extended Data Fig. 9a-c**). Within the six *tRNA-Arg-TCT* isodecoder loci, only *tRNA-Arg-TCT-1-1* showed significantly reduced occupancy of both TARDBP ($\log_2FC = -4.811$, FDR = 0.006) and ZSCAN29 ($\log_2FC = -3.858$, FDR = 0.015). Although *tRNA-Arg-TCT-2-1* also exhibited reduced TARDBP occupancy ($\log_2FC = -3.573$, FDR = 0.038), ZSCAN29 occupancy was increased in LP cells ($\log_2FC = 0.532$, FDR = 0.015). No significant differences in TARDBP or ZSCAN29 occupancy were detected at the remaining *tRNA-Arg-TCT* loci (*tRNA-Arg-TCT-3-1*, *-3-2*, *-4-1*, and *-5-1*) (**Fig. 4f & Extended Data Fig. 9a & Supplementary Table 6**).



Extended Data Figure 9. a. Volcano plot showing differential binding of TARDBP (left) and ZSCAN29 (right) at all tRNA gene loci between LNCaP LP versus AD cells. **b.** Venn diagram of tRNA genes with decreased TARDBP and ZSCAN29 binding in LNCaP LP cells. **c.** Volcano plot of MSR-seq isodecoder analysis in **Fig. 1b**. tRNAs with decreased binding of both TARDBP and ZSCAN29 in LNCaP LP cells are highlighted in yellow.

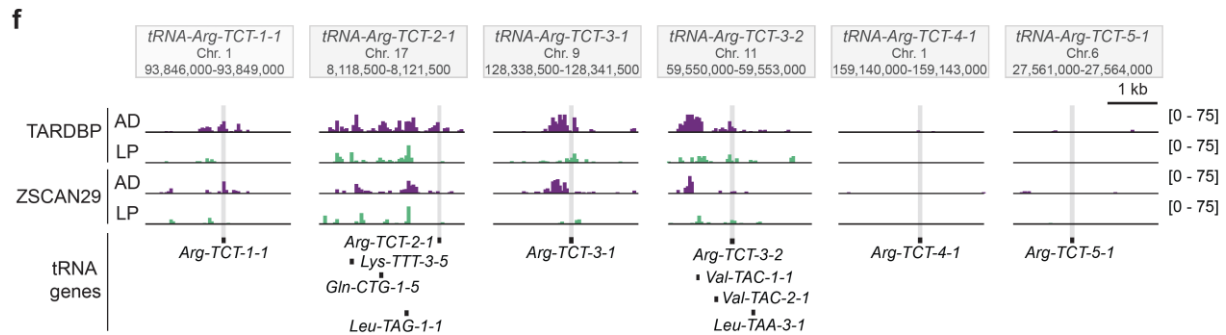


Figure 4. f. CUT&RUN IgG normalized tracks of TARDBP and ZSCAN29 across all *tRNA-Arg-TCT* gene loci in LNCaP AD and LP cells. Grey boxes denote the location of *tRNA-Arg-TCT* genes.

Given that TARDBP and ZSCAN29 occupancy was reduced at the *tRNA-Arg-TCT-1-1* and *tRNA-Arg-TCT-2-1* loci, we sought to determine why only *tRNA^{Arg}(UCU)-1* levels decreased during lineage transition (**Fig. 1b**). Because H3K4me3 is associated with active RNA polymerase II and III transcriptional activity³⁹⁻⁴², we examined H3K4me3 abundance across the *tRNA-Arg-TCT* loci. Notably, the *tRNA-Arg-TCT-1-1* locus exhibited the highest level of H3K4me3 compared to all the other loci combined (**Fig. 4g-h**). Moreover, we observed a decrease in *tRNA-Arg-TCT-1-1* H3K4me3, but not in *tRNA-Arg-TCT-2-1* H3K4me3 following lineage conversion (**Fig. 4i & Extended Data Fig. 9d**). Lastly, we measured RNA polymerase III activity by POLR3A CUT&RUN and found that transcriptional activity specifically decreased at the *tRNA-Arg-TCT-1-1* locus in the context of lineage plasticity, but not at the other *tRNA-Arg-TCT* loci (**Extended Data Fig. 9e**).

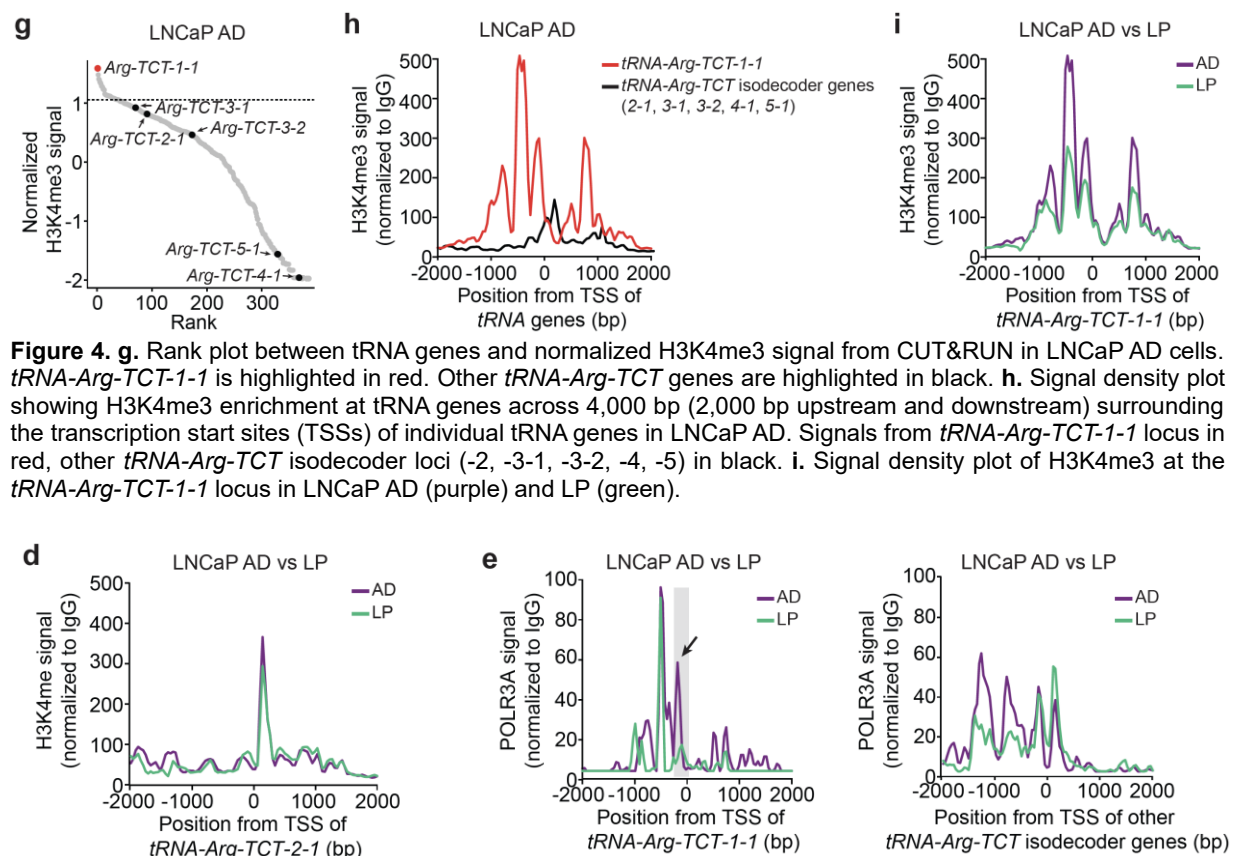
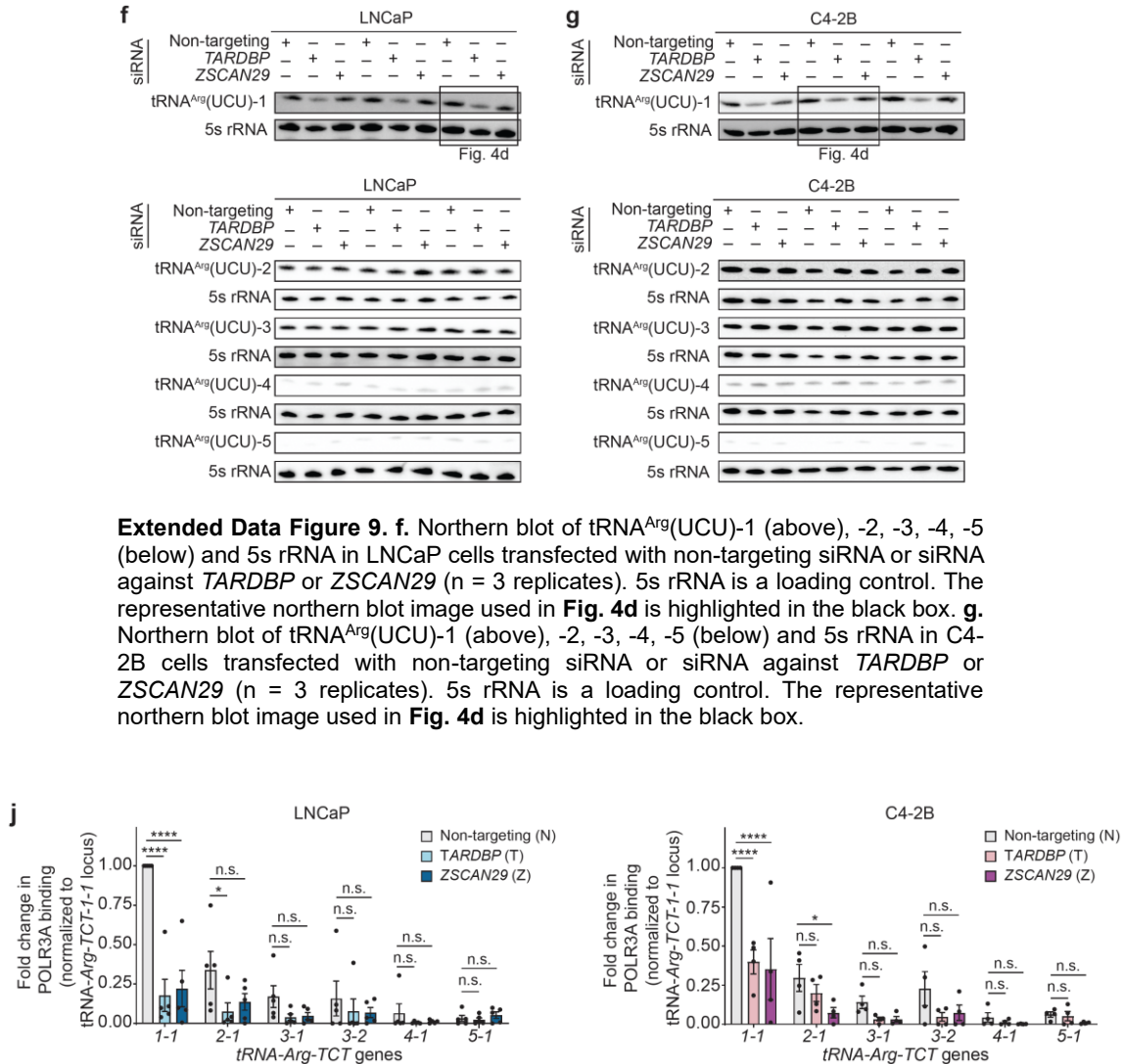


Figure 4. g. Rank plot between tRNA genes and normalized H3K4me3 signal from CUT&RUN in LNCaP AD cells. *tRNA-Arg-TCT-1-1* is highlighted in red. Other *tRNA-Arg-TCT* genes are highlighted in black. h. Signal density plot showing H3K4me3 enrichment at tRNA genes across 4,000 bp (2,000 bp upstream and downstream) surrounding the transcription start sites (TSSs) of individual tRNA genes in LNCaP AD. Signals from *tRNA-Arg-TCT-1-1* locus in red, other *tRNA-Arg-TCT* isodecoder loci (-2, -3-1, -3-2, -4, -5) in black. i. Signal density plot of H3K4me3 at the *tRNA-Arg-TCT-1-1* locus in LNCaP AD (purple) and LP (green).

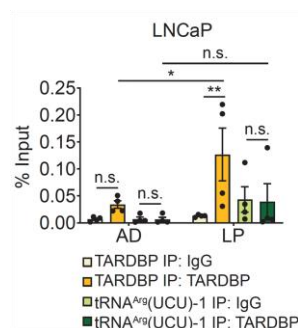
Extended Data Figure 9. d. H3K4me3 signal density plot at the *tRNA-Arg-TCT-2-1* locus. LNCaP AD is purple, and LP is green. e. POLR3A signal density plot at the *tRNA-Arg-TCT-1-1* locus (left) and at other *tRNA-Arg-TCT* isodecoder loci (-2, -3, -3-2, -4, -5) (right). LNCaP AD is purple, and LP is green.

Based on these findings, we tested whether TARDBP and ZSCAN29 preferentially regulate expression of the H3K4me3-enriched *tRNA-Arg-TCT-1-1* locus compared to all other *tRNA-Arg-TCT* loci. Knockdown of TARDBP or ZSCAN29 in LNCaP and C4-2B cells selectively reduced expression of tRNA^{Arg}(UCU)-1, whereas the remaining tRNA^{Arg}(UCU) isodecoders were unaffected (Extended Data Fig. 9f-g). Consistent with these findings, depletion of TARDBP or ZSCAN29 significantly reduced RNA polymerase III occupancy only at the *tRNA-Arg-TCT-1-1* locus in both cell lines (Fig. 4j). Together, these findings demonstrate that TARDBP and ZSCAN29 preferentially maintain RNA polymerase III occupancy at the H3K4me3-enriched *tRNA-Arg-TCT-1-1* locus, thereby selectively promoting tRNA^{Arg}(UCU)-1 transcription.



0.3. Experiments on *TDP43* are also confounded by the established roles of *TDP43* in RNA-binding, which may conceivably bind to a multitude of tRNAs and affect their steady-state abundance.

We agree with the reviewer that *TARDBP* could potentially bind to tRNA and affect steady state abundance. This possibility is particularly relevant for tRNA^{Arg}(UCU)-1, which is decreased during lineage plasticity. To address this concern, we conducted RNA immunoprecipitation followed by qPCR (RIP-qPCR) to determine whether *TARDBP* directly binds tRNA^{Arg}(UCU)-1 in our LNCaP cell line models. *TARDBP* immunoprecipitation from both LNCaP AD and LP cell lysates did not enrich tRNA^{Arg}(UCU)-1 relative to the IgG control (**Reviewer 4. Fig. 1**). In contrast, *TARDBP* mRNA, a well-established *TARDBP* target⁴³⁻⁴⁵, was robustly enriched and served as a positive control (**Reviewer 4. Fig. 1**). These findings indicate that the reduction in tRNA^{Arg}(UCU)-1 abundance is unlikely to result from direct *TARDBP* binding.

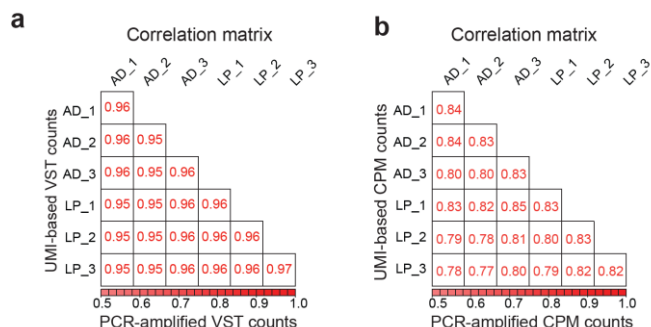


Reviewer 4. Figure 1. Analysis of TARDBP-tRNA^{Arg}(UCU)-1 interactions by RIP-qPCR in LNCaP AD and LP cells (n = 4 replicates); mean ± SEM. p-values, two-way ANOVA with Sidak's multiple comparisons test (* = p < 0.05; ** = p < 0.01; n.s. = p > 0.05).

Major points:

1. A fundamental concern relates to the analysis and interpretation of tRNA^{Arg}(UCU)-1 changes identified by MSR-seq. It appears that the method used does not include the use of unique molecular identifiers (UMIs), and that PCR amplification is used in the generation of sequencing libraries. Given that the authors then appear to use a restricted sequence space for alignment and thereafter use counts per million (CPM) as their quantification metric, PCR bias is highly likely to shape the CPM estimates in panels 1b, 1d, 1h, etc, and any downstream metric (e.g. % tRNA UCU-1 in tRNA UCU group, etc.). This becomes a serious concern when all downstream experiments are limited to a single tRNA isodecoder with this underlying premise.

We thank the reviewer for raising this concern. MSR-seq incorporates UMIs during library construction, enabling removal of PCR duplicates prior to quantification. To directly address the possibility that PCR amplification bias influenced our results, we reanalyzed our MSR-seq datasets using UMI-collapsed counts compared to PCR-amplified counts following either CPM or VST normalization. UMI-derived and PCR-amplified counts showed a strong positive correlation (**Reviewer 4. Fig 2a-b**), indicating that PCR amplification did not substantially affect quantification. Importantly, analysis using UMI-collapsed counts confirmed that the tRNA^{Arg}(UCU) family was the only isoacceptor significantly downregulated during lineage plasticity. Furthermore, among the five tRNA^{Arg}(UCU) isodecoders, only tRNA^{Arg}(UCU)-1 remained significantly decreased following multiple-comparison correction using two independent analytical methods (**Fig. 1a-b, Extended Data Fig. 1c,e**). These findings demonstrate that our conclusions are robust and are not driven by PCR amplification bias. We have updated the manuscript to incorporate the UMI-based analyses.



Reviewer 4. Figure 2. a. Pearson correlation matrix comparing VST-normalized counts generated using UMI-collapsed and PCR-amplified MSR-seq reads. **b.** Pearson correlation matrix comparing CPM-normalized counts generated using UMI-collapsed and PCR-amplified MSR-seq reads.

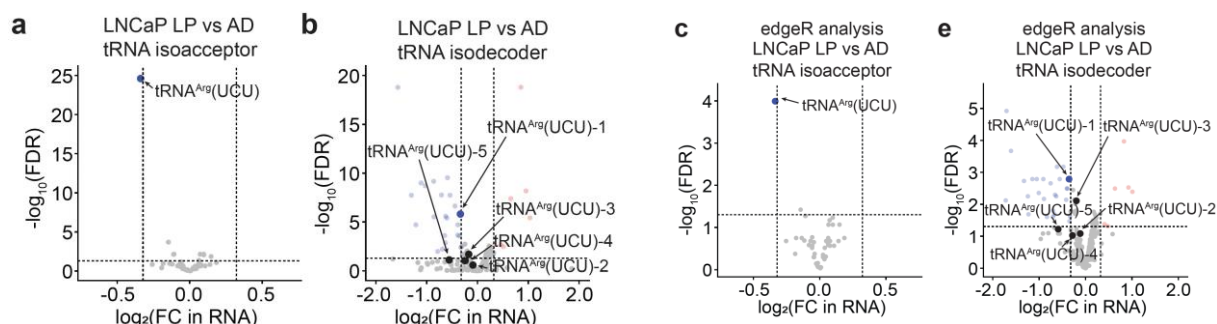


Figure 1. a. Volcano plot showing reduced expression of tRNA^{Arg}(UCU) isoacceptor in lineage plastic (LP) versus adenocarcinoma (AD) LNCaP cells. **b.** Volcano plot of isodecoder abundance changes between AD and LP cells. These DESeq2 analyses were based off of UMI-collapsed counts.

Extended Data Figure 1. c. Volcano plot showing tRNA isoacceptor edgeR analysis of MSR-seq data from LNCaP AD and LP cells. **e.** Volcano plot showing tRNA isodecoder edgeR analysis of MSR-seq data from LNCaP AD and LP cells. These analyses were based off of UMI-collapsed counts.

2. It is not clear from the methods descriptions what constitutes a “high scoring” tRNA. The authors should specify these criteria in the manuscript in more detail, as well as a complete list of tRNA sequences used for alignment (Line 650–651).

We appreciate the reviewer’s comment. We used the Genomic tRNA Database (GtRNAdb) for alignment in our manuscript, which catalogs predicted tRNAs across multiple species with primary sequences, multi-gene alignments, and predicted secondary structures and modifications of tRNAs^{46,47}. This is the most commonly used tRNA sequence database in the field. High-scoring tRNAs in the database are high-confidence, functional genes with a score of 50 or higher in the tRNAscan-SE analysis for human tRNAs (<https://gttrnadb.ucsc.edu/docs/tRNAgene/#trna-alignments>). Out of more than 600 predicted tRNA genes in the human genome, we curated a custom tRNA list that contains nonredundant, high confidence tRNAs (261 nuclear tRNAs and 22 mitochondrial tRNAs). The hg38 tRNA reference file we used is now included as **Supplementary Table 10**.

We updated the Methods to make this clear in lines 809-813:

“Barcode demultiplexing and sequence alignment were performed as previously described⁴⁸. Briefly, the libraries were aligned to a custom curated GRCh38/hg38 human transcriptome of

nonredundant, high confidence mature tRNA sequences from the Genomic tRNA Database (GtRNAdb)^{46,47} plus the 22 mitochondrially encoded tRNA sequences (**Supplementary Table 10**) using bowtie2 (v.2.5.4) and converted to .bam files by SAMtools.”

3. In all differential analyses (e.g. y-axis label in Figure 1a, 1c) it appears that the authors have not accounted for multiple hypothesis testing, the authors need to adjust p-values (e.g. using FDR) and re-assess accordingly.

We thank the reviewer for this comment. As described above, we reanalyzed the MSR-seq datasets used UMI-collapsed counts and applied FDR correction for multiple hypothesis testing using both DESeq2 and edgeR packages. Importantly, both analytical approaches yielded consistent results. The tRNA^{Arg}(UCU) family remained the only tRNA isoacceptor significantly altered during lineage plasticity (**Fig. 1a and Extended Data Fig. 1c**). Furthermore, of all the tRNA^{Arg}(UCU) isodecoders, only tRNA^{Arg}(UCU)-1 remained significantly downregulated following multiple-comparison correction ($\log_2FC = -0.336$, FDR = 1.55E-06 for DESeq2, $\log_2FC = -0.357$, FDR = 1.63E-03 for edgeR) (**Fig 1b and Extended Data Fig 1e**). Thus, correction for multiple hypothesis testing does not alter the conclusion of our study.

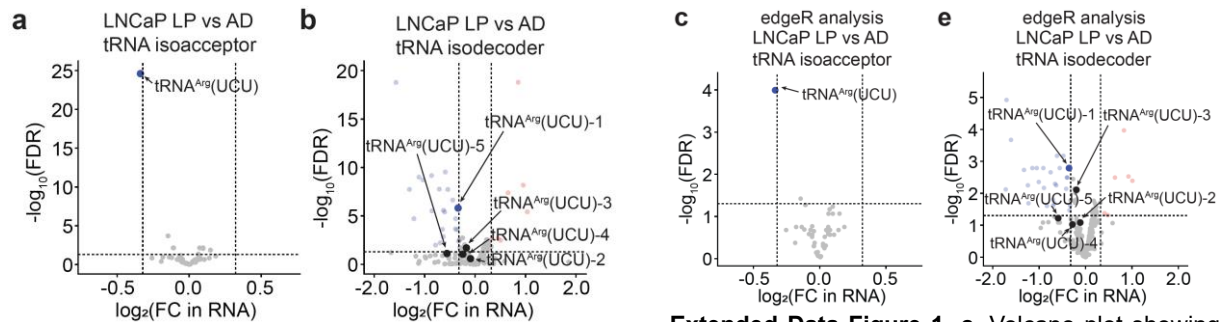


Figure 1. a. Volcano plot showing reduced expression of tRNA^{Arg}(UCU) isoacceptor in lineage plastic (LP) versus adenocarcinoma (AD) LNCaP cells. **b.** Volcano plot of isodecoder abundance changes between AD and LP cells. These DESeq2 analyses were based off of UMI-collapsed counts.

Extended Data Figure 1. c. Volcano plot showing tRNA isoacceptor edgeR analysis of MSR-seq data from LNCaP AD and LP cells. **e.** Volcano plot showing tRNA isodecoder edgeR analysis of MSR-seq data from LNCaP AD and LP cells. These analyses were based off of UMI-collapsed counts.

In addition to the new figures, we added new text in lines 91-94 & 97-100:

“Among 49 isoacceptors, only tRNA^{Arg}(UCU) was significantly downregulated during the lineage transition in LNCaP cells ($\log_2FC -0.338$, FDR = 2.42E-25 in **Fig. 1a** and $\log_2FC -0.336$, FDR = 1.02E-4 in **Extended Data Fig. 1c & Supplementary Table 1**).”

“Moreover, tRNA^{Arg}(UCU)-1 was the sole tRNA^{Arg}(UCU) isodecoder that substantially decreased in the context of lineage plasticity ($\log_2FC -0.336$, FDR = 1.55E-06 in **Fig. 1b** and $\log_2FC -0.357$, FDR = 1.626E-03 in **Extended Data Fig. 1e**).”

4. Figure 1h is impossible to interpret for reasons described above (it is very rare for authors to make interpretations on CPM values for good reason), in addition to the lack of replicates and estimates of variance.

We thank the reviewer for this comment. We have acquired additional PDX tumor samples and conducted northern blot analysis to determine if tRNA^{Arg}(UCU)-1 expression progressively

decreases during neuroendocrine transdifferentiation. Indeed, using three biological replicates, we found that tRNA^{Arg}(UCU)-1 consistently decreased as prostate adenocarcinoma PDX tumors differentiated into neuroendocrine tumors after castration (**Fig. 1f & Extended Data Fig. 2c**).

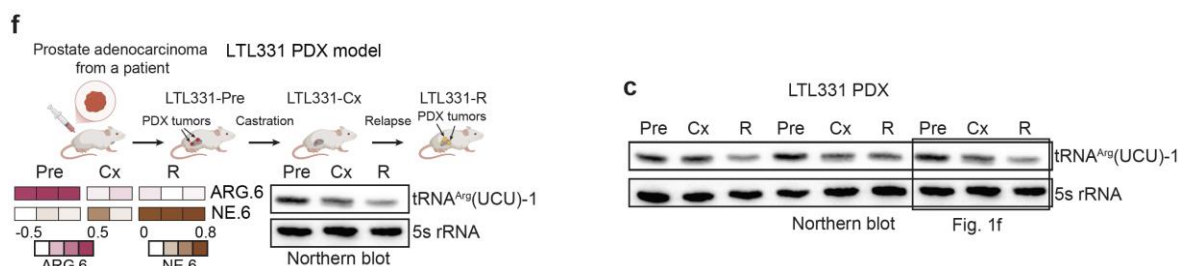


Figure 1. f. Schematic of LTL331 PDX model. (Above) LTL331 PDX derived from a patient with prostate adenocarcinoma (= Pre) becomes castration resistant following androgen deprivation through castration (= Cx). (Lower left) AR scores (ARG.6) and NE scores (NE.6) among Pre, Cx, R samples. (Lower right) Representative northern blot of charged tRNA^{Arg}(UCU)-1 and 5s rRNA from LTL331 tumors.

Extended Data Figure 2. c. Northern blot of tRNA^{Arg}(UCU)-1 and 5s rRNA from LTL331 tumor tissues. Three biological replicates are shown. Box demarcates northern blot shown in **Fig. 1f**.

In addition to the new figures, we added new text in lines 114-120:

*“We next examined tRNA^{Arg}(UCU)-1 expression in the LTL331 patient derived xenograft (PDX) model, a well-established PDX derived from prostate adenocarcinoma tissue that transdifferentiates to neuroendocrine prostate cancer (NEPC) upon castration (**Fig. 1f**)²⁴. We found a stepwise reduction in tRNA^{Arg}(UCU)-1 abundance from pre-castration (Pre) to post-castration (Cx) to relapse (R) samples (**Fig. 1f & Extended Data Fig. 2c**), coinciding with the loss of AR signaling and acquisition of neuroendocrine features (**Fig. 1f & Extended Data Fig. 2d-e**).”*

5a. Figure 1j – my understanding is that Basescope is not quantitative to the level of “copies per cell”, particularly for high abundance RNAs, can the authors clarify?

We thank the reviewer for this comment. RNA *in situ* hybridization slides were analyzed using HALO quantitative image analysis software, which quantified hybridization signals using a per-cell and per-area spot-counting approach. In HALO, “copies per cell” is not measured by directly counting RNA molecules. It refers to the image-derived proxy count of RNA puncta/dots per segmented cell. Therefore, the “copies per cell” metric is not an absolute number of RNA molecules within each cell, but a semi-quantitative score based on the intensity and size of individual puncta detected on the slides. To make this clear to readers, we have revised the y-axes in **Fig. 1h & j-k** to “Avg. number of tRNA^{Arg}(UCU)-1 puncta in TMA tissues.”

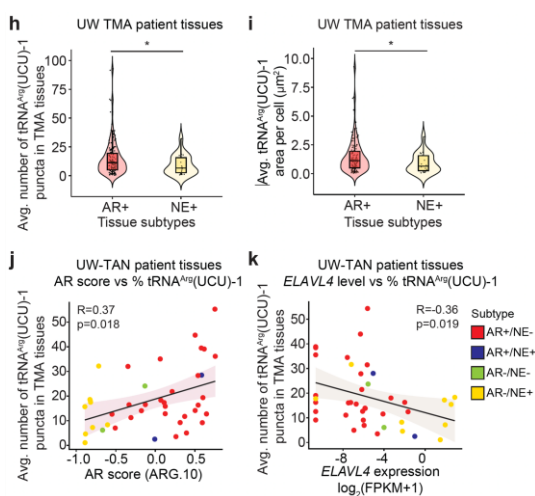


Figure 1. h. Violin plots showing distribution of average tRNA puncta per cell in AR+ (n = 137 tumors) and NE+ (n=18) tumors; mean $\pm$ quartile. p-values, unpaired Welch's t-test (* = $p < 0.05$). i. Violin plots showing distribution of average area of tRNA per cell in AR+ (n = 137 tumors) and NE+ (n=18) tumors; mean $\pm$ quartile. P-values, unpaired Welch's t-test (* = $p < 0.05$). j. Correlation plot between AR score (ARG.10) and average number of tRNA^{Arg}(UCU)-1 puncta per cell in patient tissues with Spearman's correlation statistics shown (n = 41 patient tissues had RNA-seq data available). k. Correlation plot between ELAVL4 gene expression and average number of tRNA^{Arg}(UCU)-1 puncta per cell in patient tissues with Spearman's correlation statistics shown (n = 41 patient tissues had RNA-seq data available).

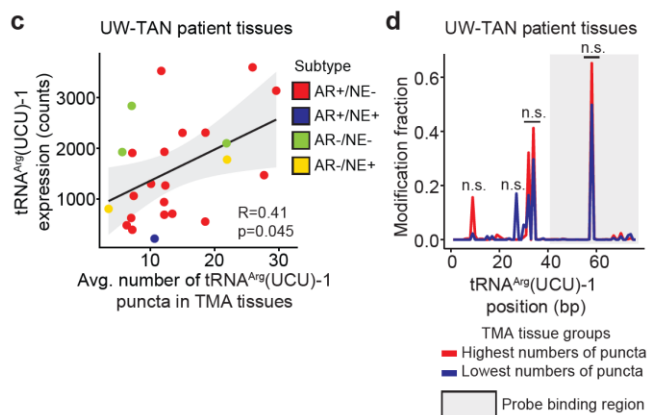
In addition to the new figures, we added new text in lines 124-127 & 132-136:

“Analyzing 56 patients from the University of Washington prostate cancer rapid autopsy cohort⁶, we observed that AR+ tumors have a significantly higher number and cellular area of tRNA^{Arg}(UCU)-1 puncta per cell compared to NE+ tumors (Fig. 1h-i).

“Moreover, tRNA^{Arg}(UCU)-1 abundance positively correlated with AR activity indicating that tRNA^{Arg}(UCU)-1 expression reflects tumor lineage state (Spearman's correlation coefficient $R = 0.37$, $p = 0.018$) (Fig. 1j). Conversely, we also found a negative correlation between the NE marker gene, ELAVL4⁶, and tRNA^{Arg}(UCU)-1 levels (Spearman's correlation coefficient $R = -0.36$, $p = 0.019$) (Fig. 1k).”

5b. There is also the possibility that highly structured and modified tRNAs may affect probe binding efficiency – can the authors rule out that differences in tRNA modifications underlying the changes in tRNA^{Arg}(UCU)-1 estimates?

We agree with the reviewer that structure and tRNA modifications may affect probe binding efficiency. To address this comment, we have conducted MSR-seq on a subset of the same patient tissue samples we used for RNA *in situ* hybridization (RISH). In comparing these orthogonal methods, we found a strong positive correlation between tRNA^{Arg}(UCU)-1 abundance as measured by RISH and MSR-seq (Extended Data Fig. 3c). Furthermore, we quantified tRNA modifications at the probe-binding region of tRNA^{Arg}(UCU)-1 and we found no statistically significant difference between high puncta and low puncta prostate cancer tissues (Extended Data Fig. 3d). Together, these new MSR-seq data suggest that the differential expression of tRNA^{Arg}(UCU)-1 in patient tissues of two different histological subtypes (AD and NE) reflects a downregulation of tRNA^{Arg}(UCU)-1 in lineage plasticity and not differential RISH probe binding secondary to structure or tRNA modifications.



Extended Data Figure 3. c. Correlation plot between average $tRNA^{Arg}(UCU)-1$ tRNA puncta from the BaseScope assay and $tRNA^{Arg}(UCU)-1$ abundance from MSR-seq ($n = 24$) with Spearman's correlation statistics shown. **d.** Modification frequency derived from MSR-seq of individual bases in $tRNA^{Arg}(UCU)-1$ between samples with the highest and lowest number of $tRNA^{Arg}(UCU)-1$ puncta from the UW rapid autopsy cohort ($n = 3$ for each group). P-values, Wilcoxon rank-sum test (n.s. (not significant) = $p > 0.05$).

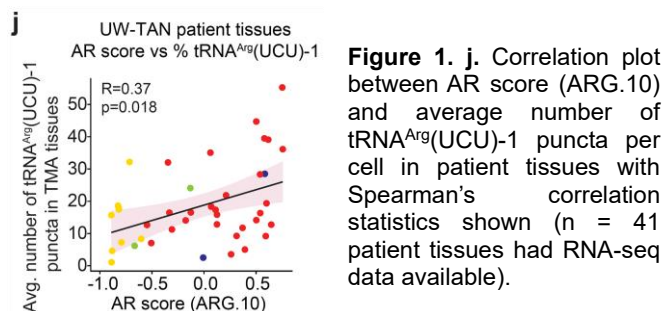
In addition to the new figures, we added the following text in lines 127-130:

"This correlated with MSR-seq quantification of a subset of tumors (Spearman's correlation coefficient $R = 0.41$, $p = 0.045$) (Extended Data Fig. 3c) and not with tRNA chemical modifications at the probe binding site (Extended Data Fig. 3d)."

6. Figure 1l – it is not clear what ARG.10 represents or how it was calculated. Regardless, this panel applies a Pearson's correlation even though this method is sensitive to outliers and that the assumption/test for linearity is unlikely. The authors should really apply Spearman's rank correlation, as they did in later figures.

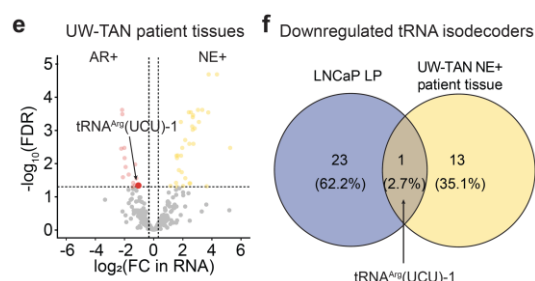
We appreciate the opportunity to clarify the androgen receptor (AR) score (ARG.10) used in the manuscript. ARG.10 is a group of 10 genes (*KLK3*, *KLK2*, *TMPRSS2*, *PMEPA1*, *NKX3-1*, *PLPP1*, *ALDH1A3*, *FKBP5*, *STEAP4*, and *PART1*) associated with AR-pathway activation⁶. It was originally derived from prostate cancer patient tissue and has been historically used by the prostate cancer field to quantify AR activity. To quantify ARG.10 we used $\log_2$ FPKM values from our RNA-seq dataset as an input for Gene Set Variation Analysis (GSVA)⁶ to develop the AR score.

We have re-analyzed the previous Fig. 1l (now Fig. 1j) using a Spearman's rank correlation and found a statistically significant positive correlation between the AR score (ARG.10) and $tRNA^{Arg}(UCU)-1$ expression in patient tissues. This new finding supports our original claim that $tRNA^{Arg}(UCU)-1$ positively correlates with AR activity. We have updated this in the manuscript.



7. Figure 1j-l is restricted to analysis of tRNA^{Arg}(UCU)-1, yet comparisons to other tRNA^{Arg}(UCU) isodecoders (and other isoacceptors) would be informative here in order to begin establishing the tRNA^{Arg}(UCU)-1 centered model proposed by the authors.

We thank the reviewer for pointing this out. We have conducted additional MSR-seq on a subset of these patient specimens to address the reviewer's comment. MSR-seq analysis of prostatic adenocarcinoma (AD) and neuroendocrine prostate cancer (NEPC) patient tissue specimens showed that tRNA^{Arg}(UCU)-1 expression is significantly lower in NEPC compared to AD (**Extended Data Fig. 3e**). The other four tRNA^{Arg}(UCU) isodecoders were not differentially expressed in AD and NEPC tumors. These new results confirm our previous observations from prostate cancer cell line models, the LTL331 PDX model, and patient tissues that tRNA^{Arg}(UCU)-1 is downregulated in the context of lineage plasticity (**Fig. 1a-c, f, h-i**). We observed that 13 additional isodecoders were downregulated in NEPC samples including: tRNA^{Ala}(AGC)-4, tRNA^{Arg}(ACG)-2, tRNA^{Arg}(UCG)-3, tRNA^{Gly}(UCC)-2, tRNA^{Ile}(AAU)-4, tRNA^{Ile}(AAU)-5, tRNA^{Ile}(AAU)-7, tRNA^{Ile}(AAU)-8, tRNA^{Ile}(UAU)-2, tRNA^{Lys}(CUU)-3, tRNA^{Lys}(CCU)-4, tRNA^{Tyr}(GUA)-2, and tRNA^{Tyr}(GUA)-5. This is not surprising because in the LNCaP LP cell line, we found 24 downregulated isodecoders (**Fig. 1b**). However, the only tRNA that was consistently downregulated in both datasets was tRNA^{Arg}(UCU)-1 (**Extended Data Fig. 3f**). These data strengthen our claim that tRNA^{Arg}(UCU)-1 abundance correlates with lineage plasticity in prostate cancer and have been included in the manuscript.



Extended Data Figure 3. e. Volcano plot showing differential tRNA isodecoder expression between AR+ and NE+ UW rapid autopsy patient tissues. **f.** Venn diagram of downregulated tRNA isodecoders between LNCaP LP cells and UW rapid autopsy NE+ patient tissues.

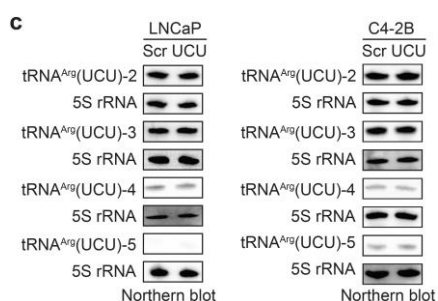
In addition to the new figures, we added new text in lines 130-132 & 136-139:

“Furthermore, the only tRNA isodecoder downregulated in both the lineage plastic LNCaP cell line model and patient tissue samples was tRNA^{Arg}(UCU)-1 (Extended Data Fig. 3e-f & Supplementary Table 2).”

“Together, these observations across multiple model systems and patient tissues collectively establish tRNA^{Arg}(UCU)-1 as a lineage-associated isodecoder whose abundance decreases with the transition from AR-driven adenocarcinoma to a lineage plastic, therapy-resistant state in prostate cancer.”

8. Figure 2 (tRNA^{Arg}(UCU)-1 depletion drives lineage independence and therapeutic resistance in prostate cancer) – the authors establish shRNA-seq approaches to deplete tRNA^{Arg}(UCU)-1, however looking at the supplemental figure, this isodecoder appears to have only 2 tRNA-specific variants that are separated towards the 5' and 3' ends. How are the authors controlling for off-target depletion of the other isodecoders? There is no validation here that the intended depletion of tRNA^{Arg}(UCU)-1 is specific. A northern blot of the other tRNA^{Arg}(UCU) tRNAs is necessary.

We appreciate the reviewer's suggestion to demonstrate the specificity of tRNA^{Arg}(UCU)-1 depletion in our cell line models. To address this, we conducted northern blot analysis of the other tRNA^{Arg}(UCU) isodecoders (-2, -3, -4, and -5) in LNCaP and C4-2B cells with and without tRNA^{Arg}(UCU)-1 knockdown. We observed no change in expression of the other isodecoders, confirming the highly selective nature of our knockdown strategy (Extended Data Fig. 4c).



Extended Data Fig. 4. c. Northern blot of charged tRNA^{Arg}(UCU)-2, -3, -4, -5 of shScr and shUCU LNCaP (left panel) and C4-2B (right panel) cell lines. 5s rRNA is a loading control.

In addition to the new figures, we added new text in lines 150-151:

“Knockdown was specific for tRNA^{Arg}(UCU)-1 and did not affect any other tRNA^{Arg}(UCU) isodecoder (Extended Data Fig. 4c).”

9. Figure 2d – it is unclear why a paired t-test was chosen for this comparison, which would imply that individual samples were treated first with scramble and then with UCU depletion. The authors should instead perform a two-way ANOVA.

We appreciate the reviewer's feedback and apologize for any confusion. In this experiment, scramble (Scr) and UCU-1 knockdown cells were treated with enzalutamide at the same concentration. This allowed us to calculate the percent confluence of cells upon drug treatment in a pairwise fashion. For rigor we tested enzalutamide at multiple concentrations. To clarify this, we have now separated the graphs into four distinct observations and performed a Welch's t-test to

determine statistical significance between shScr and shUCU-1 groups at each enzalutamide concentration (**Fig. 2f**). This has been updated in the manuscript.

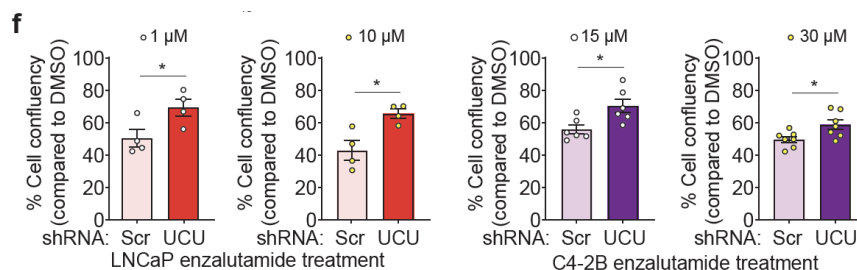
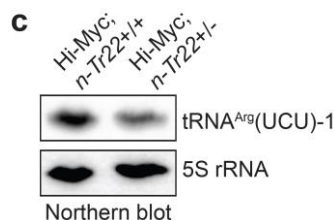


Figure 2. f. Percent cell confluency calculated by dividing baseline-normalized confluence in enzalutamide-treated wells by baseline-normalized confluence in DMSO-treated wells at day 7 for each biological replicate. shScr and shUCU LNCaP cells (left) were treated with enzalutamide 1 μM (n = 4 replicates) or 10 μM (n = 4 replicates), and shScr and shUCU C4-2B cells (right) were treated with enzalutamide 15 μM (n = 5 replicates) or 30 μM (n = 5 replicates); mean ± SEM. p-values, unpaired Welch's t-test (* = p < 0.05).

10. In Figure 2e, a northern blot comparing total *tRNA-UCU-1* between the two mouse strains is needed to confirm that single-copy animals truly have lower *tRNA-UCU-1* abundance.

We thank the reviewer for this comment. To confirm that the mouse with one copy of the *tRNA-Arg-TCT-1-1* gene (*Hi-Myc;n-Tr22+/-*) has lower expression of *tRNA^{Arg}(UCU)-1* compared to the mouse with two copies of the *tRNA-Arg-TCT-1-1* gene (*Hi-Myc;n-Tr22+/+*), we performed northern blot analysis and found a reduction in *tRNA^{Arg}(UCU)-1* levels in *Hi-myc;n-Tr22+/-* derived organoids compared to *Hi-Myc;n-Tr22+/+* organoids (**Extended Data Fig. 5c**).



Extended Data Figure 5. c. Representative northern blot of charged *tRNA^{Arg}(UCU)-1* of prostate organoids from *Hi-Myc;n-Tr22+/+* and *Hi-Myc;n-Tr22+/-* mice. 5s rRNA is a loading control.

In addition to the new figures, we have included new text in lines 178-180:

*“Myc protein levels were equivalent between the two genotypes and *Hi-Myc;n-Tr22+/-* organoids had reduced *tRNA^{Arg}(UCU)-1* expression (**Extended Data Fig. 5b-c**).”*

11. In Figure 2g, the numbers of *hi-myc/n-Tr22+/+* versus single-copy mice are unbalanced. More importantly, Welch's *t*-test assumes normality, yet the distribution of *hi-myc/n-Tr22+/-* is skewed (the mean is sensitive to the outliers).

We thank the reviewer for the careful evaluation of our statistical analysis of **Fig. 2g (new Fig. 2h)**. We acknowledge that the sample sizes between the two groups are unequal and that the distribution of the Hi-Myc;*n-Tr22*^{+/-} cohort may not fully satisfy the assumptions underlying Welch's t-test. To address these concerns, we re-analyzed the data using the non-parametric Mann-Whitney U test, which does not assume normality and is less sensitive to outliers. Consistent with our original findings, ventral prostate lobe weight remained significantly different between the two groups in the castrated setting (**new Fig. 2h**). We have revised the figure legend accordingly.

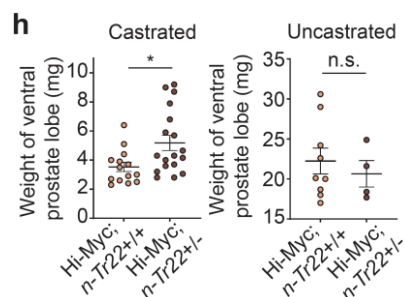
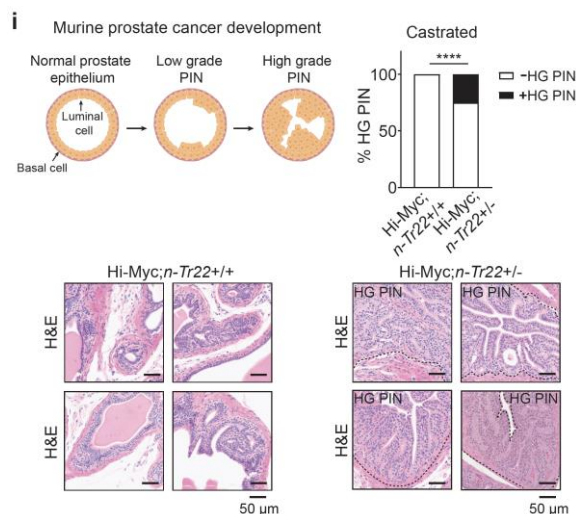


Figure 2. h. (Left) Distribution of weights for ventral prostate lobes from castrated mice (Hi-Myc;*n-Tr22*^{+/+} (n = 14), Hi-Myc;*n-Tr22*^{+/-} (n = 18)); mean ± SEM. P-values, unpaired Mann-Whitney U test (* = p < 0.05). (Right) Distribution of weights for ventral prostate lobes from uncastrated mice (Hi-Myc;*n-Tr22*^{+/+} (n = 9), Hi-Myc;*n-Tr22*^{+/-} (n = 4)); mean ± SEM. P-values, unpaired Welch's t-test (n.s. = p > 0.05).

12. In Figure 2h, control H&E images for the hi-myc/*n-Tr22*^{+/+} group should be shown. The Methods should also specify how high-grade PIN was quantified.

Thank you for your comment. We have now included Hi-Myc;*n-Tr22*^{+/+} H&E images in **Fig. 2i** (previously **Fig. 2h**) in the revised manuscript. We have also included a detailed explanation of how high-grade PIN was quantified in the Methods section.



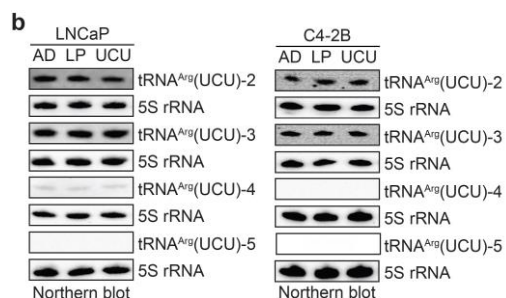
Revised description in the Methods, lines 957-965:

Mouse prostate PIN analysis

PIN regions in mouse prostate were histologically examined based on key features including epithelial stratification and crowding, cribriform architecture, and protrusion of luminal layers to the lumen. Compared to normal mouse prostate, low grade PIN is characterized by epithelial tufting and partial lumen crowding^{49,50}. High-grade PIN is a strongly premalignant lesion described by dense epithelial piling, nearly filled lumens, complex papillary growth, and the presence of cribriform histology. Histopathological evaluation was performed in a blind manner by three independent reviewers for the detection of high-grade PIN in mouse anterior prostates.

13. In Figure 3a, as in Figure 2a, a blot for other UCU isodecoders is needed to demonstrate specificity.

We appreciate the reviewer's suggestion to demonstrate the specificity of tRNA^{Arg}(UCU)-1 add back in our cell line models. We conducted northern blot analysis using oligonucleotide probes specific to the other tRNA^{Arg}(UCU) isodecoders (-2, -3, -4, and -5) and found no difference in their expression between AD, LP, and LP with tRNA^{Arg}(UCU)-1 add back cells (**Extended Data Fig. 6b**). These findings demonstrate the specificity of the re-expression of tRNA^{Arg}(UCU)-1.



Extended Data Figure 6. b. Northern blot of charged tRNA^{Arg}(UCU)-2, -3, -4, and -5 of LNCaP AD, LP, and UCU cells (left) and C4-2B AD, LP, and UCU cells (right). 5s rRNA is a loading control.

In addition to the new figures, we added new text in lines 203-205:

“Moreover, increasing tRNA^{Arg}(UCU)-1 abundance did not affect expression levels of the other tRNA^{Arg}(UCU) isodecoders (Extended Data Fig. 6b).”

14. In Figure 3b, the color bar should be defined explicitly (z-score, log2 fold-change, etc.).

Thank you for pointing this out. The color bar represents z-score normalized FPKM gene expression. We have now included this description in the Figure legend (Fig. 3b).

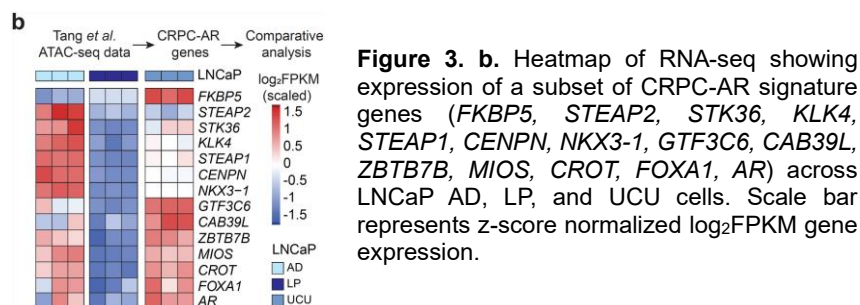


Figure 3. b. Heatmap of RNA-seq showing expression of a subset of CRPC-AR signature genes (*FKBP5*, *STEAP2*, *STK36*, *KLK4*, *STEAP1*, *CENPN*, *NKX3-1*, *GTF3C6*, *CAB39L*, *ZBTB7B*, *MIOS*, *CROT*, *FOXA1*, *AR*) across LNCaP AD, LP, and UCU cells. Scale bar represents z-score normalized log₂FPKM gene expression.

15. Figure 4 – the authors are drawing on eye-test analysis of available ChIP-seq data and comparative analysis of CPM values (again flawed) to establish a small predictive set of tRNA-UCU-1 regulators. First, differential mRNA expression analyses require a differential framework, not unpaired Welch’s t-test on CPM values. Even so, the tenuous link between observational ChIP signal at one gene, a CPM change in AD vs. LP, and ensuing changes in tRNA-UCU-1 abundance in TDP43 and Zscan9 depletion experiments is far from sufficient to establish that “TARDBP and ZSCAN29 regulate tRNA^{Arg}(UCU)-1 expression in prostate cancer”. Instead, the authors would need to (a) Perform a broad regulatory analysis of individual factors at all tRNA genes (is TDP43 at all tRNA genes? Many tRNA genes? Which factors are statistically enriched at tRNA^{Arg}(UCU)-1 compared to an empirical null distribution?) and (b) Establish that such factors localize to the tRNA^{Arg}(UCU)-1 gene locus and are differential in AD and LP conditions (e.g., differential CUT&RUN), and that transcription-centered approaches (e.g. nascent-seq) capture changes in RNA polymerase III transcription. These analyses would need to be repeated following functional depletion of such factors.

We thank the reviewer for raising these important points, which prompted us to conduct new experiments and substantially improve our understanding of how TARDBP and ZSCAN29 regulate tRNA^{Arg}(UCU)-1 expression. First, we have conducted differential analysis of RNA-seq from LNCaP AD and LP cells as suggested by the reviewer and found that four transcription factors (*TARDBP* ($\log_2FC = -0.418$, FDR = 0.0136) *ZSCAN29* ($\log_2FC = -0.79$, FDR = 0.0212), *ASH2L* ($\log_2FC = -0.399$, FDR = 0.0407), *ZBTB40* ($\log_2FC = -0.637$, FDR = 0.009) were significantly decreased in the context of lineage plasticity compared to all the other transcription factors that bind to the *tRNA-Arg-TCT-1-1* locus (**Fig. 4b**). We previously showed through knockdown experiments that TARDBP and ZSCAN29, but not ASH2L and ZBTB40 regulate tRNA^{Arg}(UCU)-1 abundance (**Fig. 4c,d & Extended Data Fig. 8e-h**).

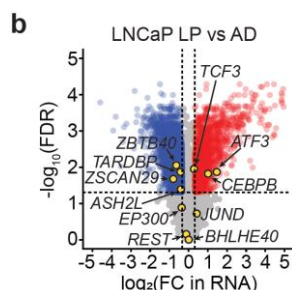
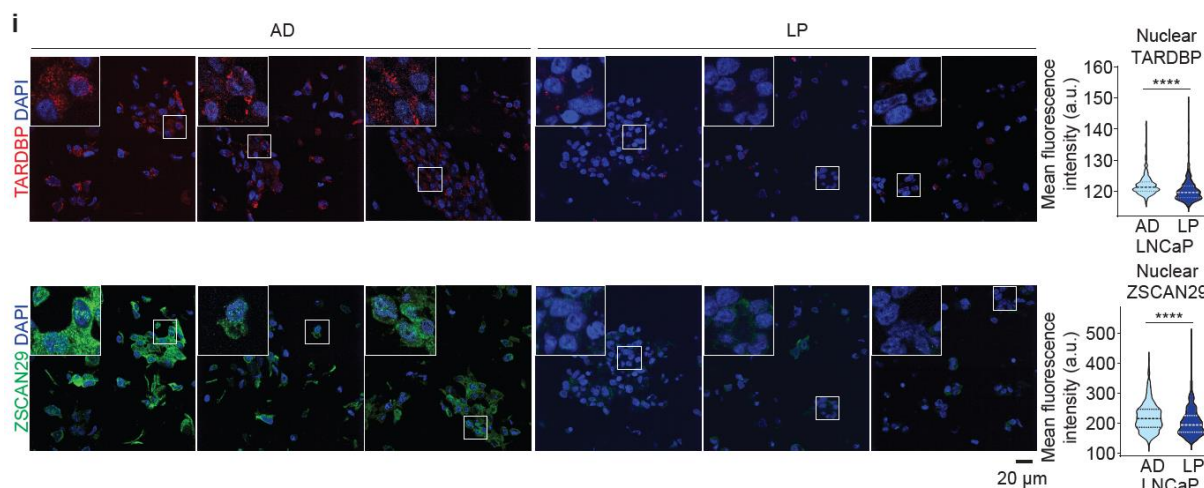


Figure 4. b. RNA-seq of LNCaP AD and LP showing expression changes of transcription factors (highlighted in yellow) in **Fig. 4a**.

In addition to the RNA-seq data, we have now confirmed that nuclear TARDBP and ZSCAN29 expression significantly decreases in the context of lineage plasticity (**Extended Data Fig. 8i**). Together, these observations suggest that LNCaP AD cells are enriched for these two transcription factors, but lineage transition leads to a reduction in TARDBP and ZSCAN29 in LP cells.



Extended Data Figure 8. i. Representative immunofluorescence images of TARDBP (red; above) and ZSCAN29 (green; below) with nuclear staining (DAPI) from LNCaP AD and LP cells. Quantification of nuclear mean fluorescence intensity (right); mean $\pm$ SEM. P-values, unpaired Welch's t-test (**** = $p < 0.0001$). Scale bar 20 μm .

Second, we performed CUT&RUN profiling of TARDBP and ZSCAN29 across all tRNA genomic loci in LNCaP AD and LP cells. Among all tRNA genes, *tRNA-Arg-TCT-1-1* was the only locus that exhibited coordinated reductions in TARDBP and ZSCAN29 occupancy together with decreased mature tRNA abundance during lineage plasticity (**Extended Data Fig. 9a-c**). Within the six *tRNA-Arg-TCT* isodecoder loci, only *tRNA-Arg-TCT-1-1* showed significantly reduced occupancy of both TARDBP ($\log_2\text{FC} = -4.811$, $\text{FDR} = 0.006$) and ZSCAN29 ($\log_2\text{FC} = -3.858$, $\text{FDR} = 0.015$). Although *tRNA-Arg-TCT-2-1* also exhibited reduced TARDBP occupancy ($\log_2\text{FC} = -3.573$, $\text{FDR} = 0.038$), ZSCAN29 occupancy was increased in LP cells ($\log_2\text{FC} = 0.532$, $\text{FDR} = 0.015$). No significant differences in TARDBP or ZSCAN29 occupancy were detected at the remaining *tRNA-Arg-TCT* loci (*tRNA-Arg-TCT-3-1*, *-3-2*, *-4-1*, and *-5-1*) (**Fig. 4f & Extended Data Fig. 9a & Supplementary Table 6**).

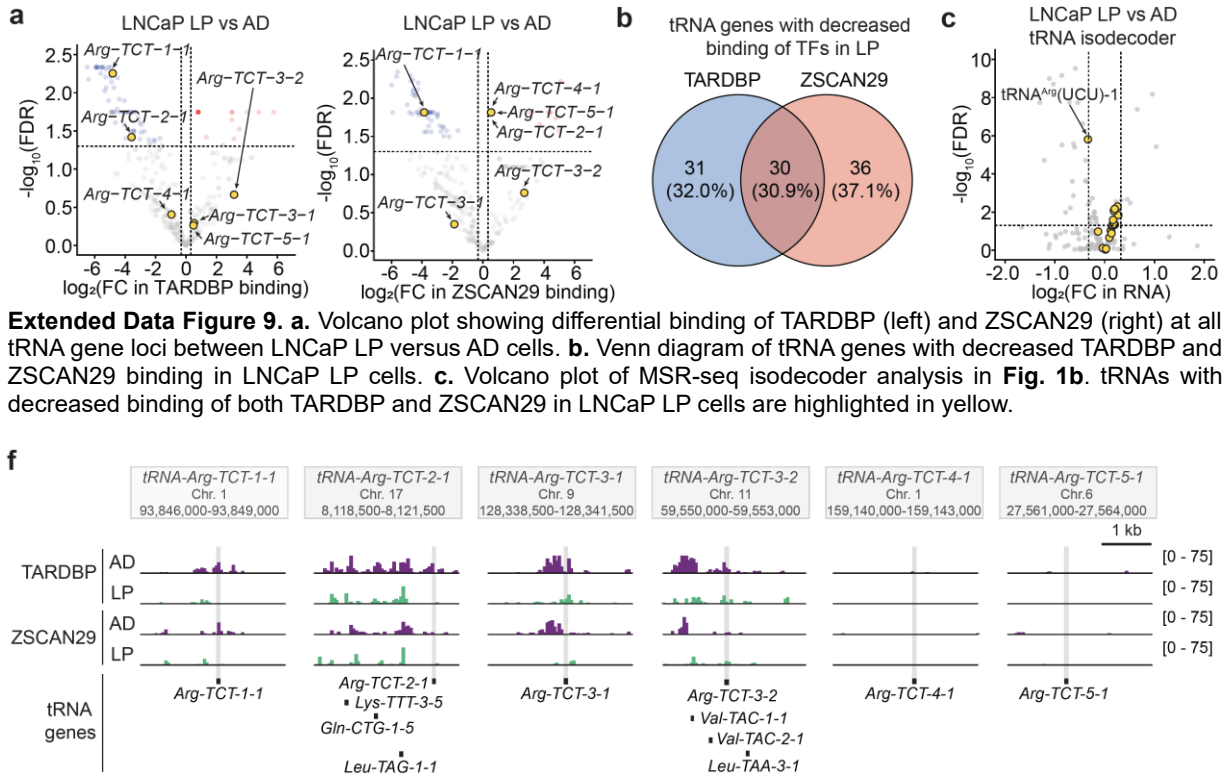


Figure 4. f. CUT&RUN IgG normalized tracks of TARDBP and ZSCAN29 across all *tRNA-Arg-TCT* gene loci in LNCaP AD and LP cells. Grey boxes denote the location of *tRNA-Arg-TCT* genes.

Given that TARDBP occupancy was reduced at both the *tRNA-Arg-TCT-1-1* and *tRNA-Arg-TCT-2-1* loci, whereas ZSCAN29 occupancy was reduced only at *tRNA-Arg-TCT-1-1*, we sought to determine why only *tRNA^{Arg}(UCU)-1* levels decreased during lineage plasticity (**Fig. 1b**). Because H3K4me3 is associated with active RNA polymerase II and III transcriptional activity³⁹⁻⁴², we examined H3K4me3 enrichment across the *tRNA-Arg-TCT* loci. Notably, the *tRNA-Arg-TCT-1-1* locus exhibited substantially higher H3K4me3 enrichment than any of the other *tRNA-Arg-TCT* loci (**Fig. 4g-h**). Moreover, H3K4me3 abundance decreased selectively at the *tRNA-Arg-TCT-1-1* locus following lineage conversion, whereas no significant changes were observed at the *tRNA-Arg-TCT-2-1* locus (**Fig. 4i & Extended Data Fig. 9d**). Consistent with these chromatin changes, POLR3A CUT&RUN demonstrated a selective reduction in RNA polymerase III occupancy at the

tRNA-Arg-TCT-1-1 locus during lineage plasticity, with no corresponding changes at the remaining *tRNA-Arg-TCT* loci (**Extended Data Fig. 9e**).

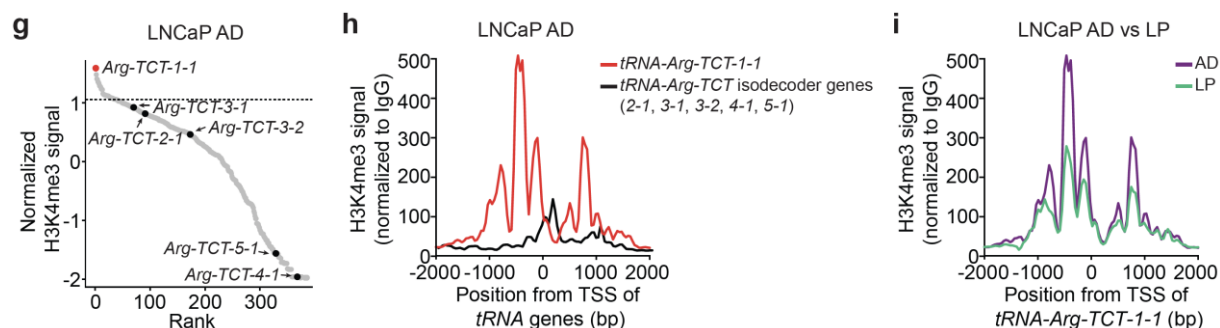
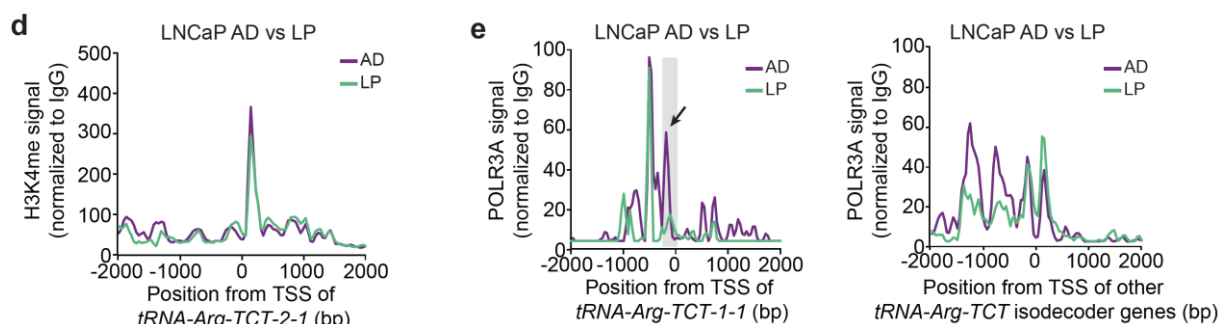
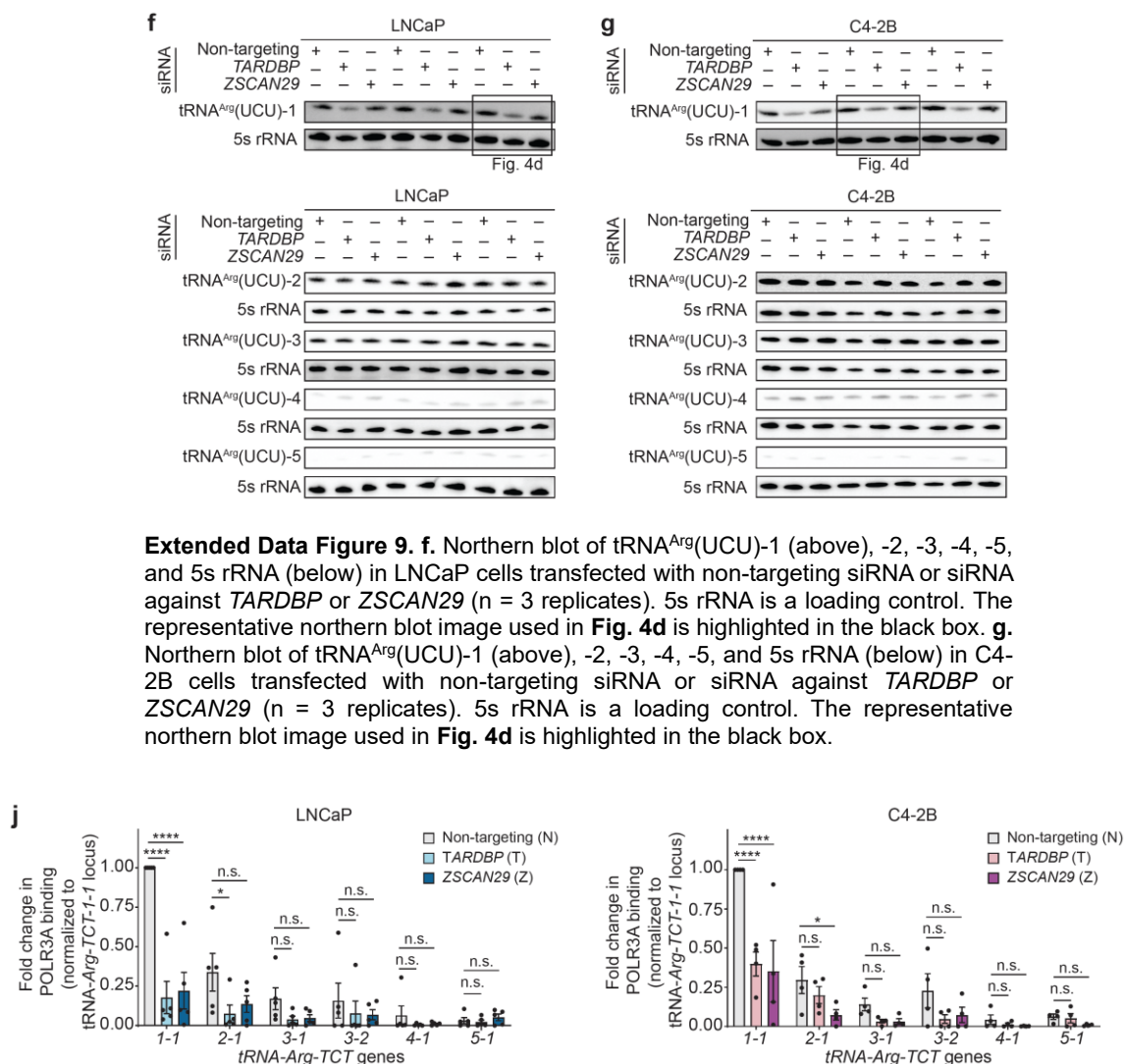


Figure 4. g. Rank plot between tRNA genes and normalized H3K4me3 signal from CUT&RUN in LNCaP AD cells. *tRNA-Arg-TCT-1-1* is highlighted in red. Other *tRNA-Arg-TCT* genes are highlighted in black. h. Signal density plot showing H3K4me3 enrichment at tRNA genes across 4,000 bp (2,000 bp upstream and downstream) surrounding the transcription start sites (TSSs) of individual tRNA genes in LNCaP AD. Signals from *tRNA-Arg-TCT-1-1* locus in red, other *tRNA-Arg-TCT* isodecoder loci (-2, -3-1, -3-2, -4, -5) in black. i. Signal density plot of H3K4me3 at the *tRNA-Arg-TCT-1-1* locus in LNCaP AD (purple) and LP (green).



Extended Data Figure 9. d. H3K4me3 signal density plot at the *tRNA-Arg-TCT-2-1* locus. LNCaP AD is purple, and LP is green. e. POLR3A signal density plot at the *tRNA-Arg-TCT-1-1* locus (left) and at other *tRNA-Arg-TCT* isodecoder loci (-2, -3, -3-2, -4, -5) (right). LNCaP AD is purple, and LP is green.

Third, based on these findings, we tested whether TARDBP and ZSCAN29 preferentially regulate expression of the H3K4me3-enriched *tRNA-Arg-TCT-1-1* locus compared to all other *tRNA-Arg-TCT* loci. Knockdown of TARDBP or ZSCAN29 in LNCaP and C4-2B cells selectively reduced expression of tRNA^{Arg}(UCU)-1, whereas the remaining tRNA^{Arg}(UCU) isodecoders were unaffected (**Extended Data Fig. 9f-g**). Consistent with these findings, depletion of TARDBP or ZSCAN29 significantly reduced RNA polymerase III occupancy only at the *tRNA-Arg-TCT-1-1* locus in both cell lines (**Fig. 4j**). Together, these findings demonstrate that TARDBP and ZSCAN29 preferentially support RNA polymerase III activity at the H3K4me3-enriched *tRNA-Arg-TCT-1-1* locus.



In addition to the new figures, new text has been added in lines 265-312.

16. For polysome RNA-seq, it is easy to imagine that RNA populations in the monosome and polysome fractions differ significantly, more so than the assumptions that edgeR and DE-seq2 allow for. Nevertheless, this is how the authors define high and low translation efficiency mRNAs. Then, we are given that Arg-AGA codons are slightly different between AD, LP, and UCU conditions (Figure 5b-d). Surprisingly, other codons appear to diverge more meaningfully, though no statistics are provided for AGA or others. I recommend that the authors apply a permutation framework to empirically determine the significance of AGA representation in high vs. low TE mRNAs.

We appreciate the reviewer for the valuable suggestion. To address this question, we have used the permutation framework described in the Methods section and similarly observed that Arg-AGA

codon frequency is significantly increased in the high translation efficiency genes in LNCaP AD and UCU cells but not in LP cells (Fig. 5b-d).

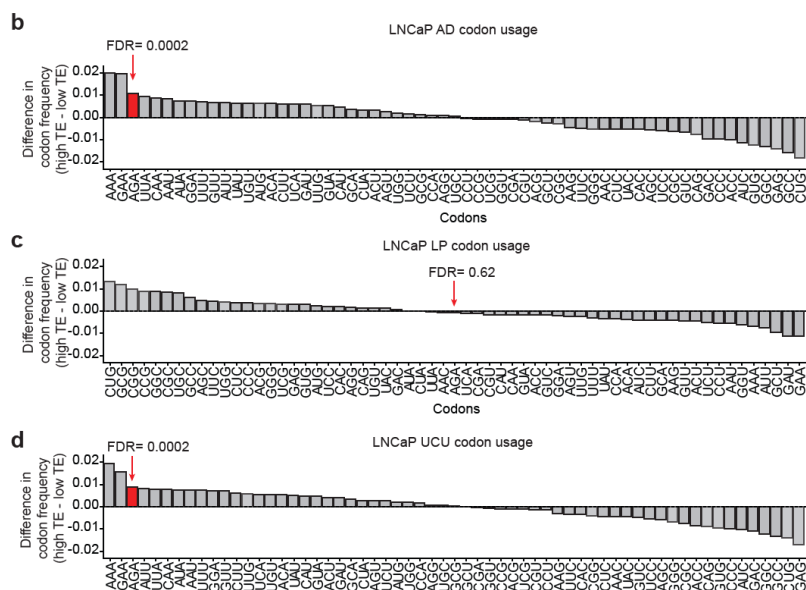


Figure 5. **b.** Bar plot showing codon usage in LNCaP AD cells. Codon frequency was calculated by analyzing codon compositions in genes with high vs. low translational efficiency in AD cells. **c.** Bar plot showing codon usage in LNCaP LP cells. **d.** Bar plot showing codon usage in LNCaP UCU cells. **(b-d)** The Arg-AGA codon is highlighted in red with FDR shown above the red arrow.

Revised description in the Methods, lines 1166-1183:

Codon usage analysis

For each cell type (AD, LP, UCU), genes with the highest and genes with the lowest translation efficiency were selected from Xtail analysis (v.1.2.0). Codon frequencies for each gene were calculated from MANE Select transcripts (v1.4) using GENCODE v46 protein-coding transcript sequences, as the fraction of each sense codon relative to total sense codons in the coding sequence. To control for amino acid composition differences between gene sets, codon frequencies were normalized within each amino acid family by dividing each codon's frequency by the sum of frequencies of all synonymous codons for the same amino acid, yielding relative codon usage frequencies. Methionine (ATG) and tryptophan (TGG), each encoded by a single codon, were excluded as uninformative. For each of the 59 remaining sense codons in each cell type, we computed the observed difference in mean relative codon frequency between the high-TE and low-TE gene sets. To generate a null distribution, we pooled the genes and randomly permuted the high/low TE labels 10,000 times, recomputing the mean relative frequency difference for each permutation. Two-sided empirical *P* values were calculated as $(m + 1)/(N + 1)$, where *m* is the number of permutations with an absolute difference greater than or equal to the observed absolute difference and *N* is the total number of permutations. *P* values were corrected for multiple testing using the Benjamini-Hochberg method across 59 codons within each cell type.

In addition to the new figures and updated methods, we added new text in the results section in lines 343-354:

*“We next performed polysome RNA-seq to see if we could observe a similar effect on endogenous mRNAs. Polysome RNA-seq is a technique in which ribosome-bound mRNAs are fractionated by density and sequenced to determine the translation efficiency (TE) of each mRNA transcriptome-wide (**Extended Data Fig. 11b & Supplementary Table7**). We compared codon usage among AD, LP, and UCU cells by assessing the codon composition of mRNA with the highest and lowest statistically significant TEs (adjusted p-value < 0.05). In AD cells, the Arg-AGA codon was one of the most significantly abundant codons in high TE mRNAs suggesting efficient translation within the adenocarcinoma model (FDR = 0.0002). However, in LP cells Arg-AGA codons were no longer enriched (FDR = 0.62) (**Fig. 5c**). Importantly, addback of tRNA^{Arg}(UCU)-1 restored AGA codon translation (FDR = 0.0002) (**Fig. 5d**). These findings reveal that tRNA^{Arg}(UCU)-1 dosage influences the translation of endogenous Arg-AGA-enriched mRNAs which provides a potential mechanism for gene-specific translation and regulation of lineage dependence.”*

17. Figure 5g-h – it is unclear why SMARCC2 levels were not shown for AD. Nevertheless, there are several leaps here that are tenuous at best, but the basis for significant conclusions of the study.

We appreciate the reviewer’s feedback. We have updated old **Fig. 5g-h** and have now included the AD cell line to show that SMARCC2 protein levels are lowest in the lineage plastic LP cell line with no significant changes at the mRNA level (new figures **Fig. 5f-g**). We agree that more mechanistic studies are necessary to determine if SMARCC2 is translationally regulated by tRNA^{Arg}(UCU)-1. We hypothesize that SMARCC2 protein levels are sensitive to tRNA^{Arg}(UCU)-1 levels because of the AGA codon content encoded in its coding sequence. To test this hypothesis, we designed a codon switching reporter in which Arg-AGA codons in the SMARCC2 cDNA sequence were switched to Arg-CGC. The wild-type and mutant reporters were then transduced into AD, LP, and LP tRNA^{Arg}(UCU)-1 add back cell lines. LP cells transduced with the Arg-AGA reporter showed low levels of SMARCC2 protein compared to AD cells. Also, re-expression of tRNA^{Arg}(UCU)-1 in LP cells rescued SMARCC2 mRNA translation (**Fig. 5h**). However, cells transduced with the mutant Arg-CGC SMARCC2 reporter did not show this trend and tRNA^{Arg}(UCU)-1 overexpression failed to enhance protein synthesis of mutant SMARCC2 (**Fig.**

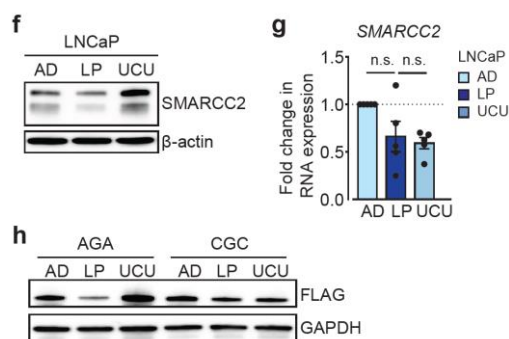


Figure 5. f. Immunoblot of SMARCC2 in LNCaP AD, LP, and UCU cells. β -actin is a loading control. **g.** SMARCC2 expression analysis by qPCR in LNCaP AD, LP, and UCU cells ($n = 5$ replicates); mean $\pm$ SEM. p-values, one-way ANOVA with Sidak’s multiple comparisons test (n.s. = $p > 0.05$). **h.** Immunoblot of wild-type (AGA) or codon-switched (CGC) flag-tagged SMARCC2 in LNCaP AD, LP, and UCU cells. GAPDH is a loading control.

5h). Together, these data support a codon-dependent mechanism whereby tRNA^{Arg}(UCU)-1 promotes efficient translation of SMARCC2 through AGA codons in its coding sequence.

SMARCC2 is a core component of SWI/SNF chromatin-remodeling complex, which has been shown to facilitate AR-dependent transcription in prostate cancer^{51,52}. Thus, we next silenced SMARCC2 in LP tRNA^{Arg}(UCU)-1 addback cells which led to a decrease in AR pathway activity at the mRNA and protein levels (**Fig. 5i-j**). Lastly, we asked if this down regulation of AR activity decreases the sensitivity of these cells to pharmacologic inhibition of AR. Remarkably, LNCaP LP cells with restored expression of tRNA^{Arg}(UCU)-1 and sensitivity to enzalutamide were rendered resistant upon SMARCC2 knockdown (**Fig. 5k**). Together, these findings demonstrate that tRNA^{Arg}(UCU)-1 regulates the translation of SMARCC2 which can impact lineage fate and sensitive to AR pathway inhibitors.

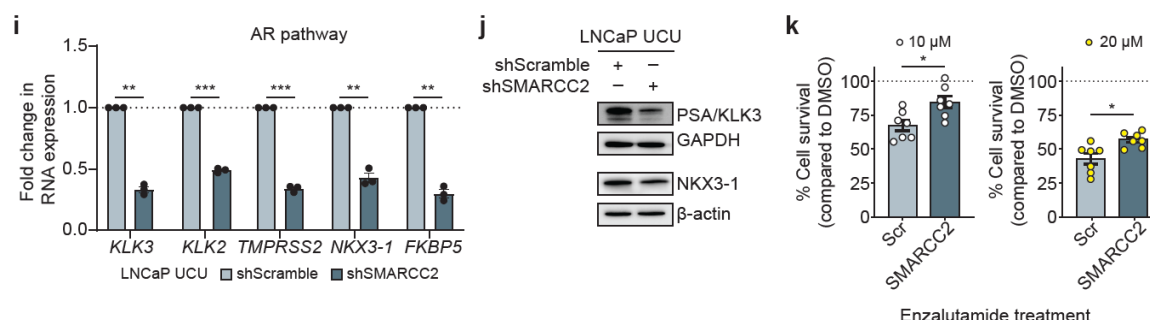


Figure 5. i. AR pathway expression analysis by qPCR in LNCaP LP UCU cells after shScramble or shSMARCC2 transduction (n = 3 replicates); mean ± SEM. p-values, unpaired Welch's t-test (** = p < 0.01; *** = p < 0.001). **j.** Immunoblot of PSA/KLK3 (above) and NKX3-1 (below) in LNCaP LP UCU cells after shScramble or shSMARCC2 transduction. GAPDH and β-actin are loading controls, respectively. **k.** Cell survival was calculated by dividing baseline-normalized luminescence readings in enzalutamide-treated conditions by baseline-normalized luminescence readings in DMSO-treated conditions for each biological replicate. LNCaP LP UCU shScramble and shSMARCC2 cells (n = 7 replicates) were treated with 10 or 20 μM enzalutamide; mean ± SEM. p-values, unpaired Welch's t-test (* = p < 0.05).

In addition to the new figures, we added new text in lines 369-377 & 379-386:

*"To determine if SMARCC2 sensitivity to tRNA^{Arg}(UCU)-1 abundance was mediated by encoded AGA codons, we generated a codon switching reporter in which Arg-AGA codons in SMARCC2 were replaced with Arg-CGC codons. Wild-type and codon-switched reporters were introduced into AD, LP, and tRNA^{Arg}(UCU)-1 add back cells. LP cells transduced with the Arg-AGA reporter showed reduced SMARCC2 protein abundance compared to AD cells, whereas tRNA^{Arg}(UCU)-1 re-expression restored SMARCC2 mRNA translation (**Fig. 5h**). In contrast, the Arg-CGC reporter was insensitive to lineage state, and tRNA^{Arg}(UCU)-1 overexpression did not enhance SMARCC2 protein synthesis (**Fig. 5h**). These findings indicate that efficient SMARCC2 translation depends on tRNA^{Arg}(UCU)-1 availability through AGA codon usage."*

*"We next examined whether SMARCC2 mediates the lineage effects of tRNA^{Arg}(UCU)-1. Silencing SMARCC2 in LNCaP LP tRNA^{Arg}(UCU)-1 addback cells reduced AR pathway activity at both the mRNA and protein levels, thereby reversing lineage features induced by tRNA^{Arg}(UCU)-1 re-expression (**Fig. 5i-j & Extended Data Fig. 11e-f**). Next, we asked whether this reduction in AR activity altered sensitivity to pharmacologic AR inhibition. Notably, LNCaP LP cells with tRNA^{Arg}(UCU)-1 restoration became resistant to enzalutamide treatment following SMARCC2 knockdown (**Fig. 5k**). Together, these data reveal that tRNA^{Arg}(UCU)-1 regulates the translation of SMARCC2 to impact lineage state, AR signaling, and sensitivity to AR pathway inhibition."*

Response to Reviewer Comments for Kim *et al.*

Referee #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

We appreciate the time and effort the reviewer has taken to read our manuscript and provide valuable suggestions.

Referee #6 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

We would like to thank the reviewer for their thoughtful and constructive comments.

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- 1 Tang, F. *et al.* Chromatin profiles classify castration-resistant prostate cancers suggesting therapeutic targets. *Science* **376**, eabe1505 (2022).
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- 2 Ku, S. Y. *et al.* Notch signaling suppresses neuroendocrine differentiation and alters the immune microenvironment in advanced prostate cancer. *J Clin Invest* **134** (2024).
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We thank the reviewers for their comments and suggestions.

Referees' comments:

Referee #1 (Remarks to the Author):

I thank the authors for their careful and complete response. All of my concerns have been addressed, and I have no remaining comments.

We are thankful for the referee's comments which strengthened our manuscript.

Referee #2 (Remarks to the Author):

All my critiques has been addressed and I believe that this interesting work should be accepted for publication

We are grateful that the reviewer "believes that this interesting work should be accepted for publication." The comments provided enhanced our story.

Referee #3 (Remarks to the Author):

I have no further comments for the authors.

We appreciate the reviewer's constructive comments.

Referee #4 (Remarks to the Author):

The authors have made significant efforts in their revision that address our previous concerns and strengthen the claims of the manuscript. In addition to other new experiments and analyses, the permutation framework helps confirm that Arg-AGA codon frequency is significantly enriched among high-translational-efficiency transcripts in AD and UCU cells but not in LP cells (Fig. 5b-d). The authors might consider commenting on AAA (Lys) and GAA (Glu), which show numerically larger differences in codon frequency between high- and low-TE gene sets in both AD and UCU cells (and perhaps include the FDR for all codons, including these) – however this is a relatively minor point.

We thank the reviewer for providing important feedback throughout the review process. Their comments were very helpful. As suggested, we have now commented on AAA (Lys) and GAA (Glu) in Lines 301-303 and have included the FDRs for all the codons in **Fig. 5b-d** in **Supplementary Table 7**.

Referee #6 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

We thank referee 6 for co-reviewing our manuscript.