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Award Number: W81XWH-16-1-0568

TITLE: RUNX1T1 Amplification Induces "Small Cell" Cancer

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REPORT DATE: December 2018

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

X Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE:	**2. REPORT TYPE:**	**3. DATES COVERED** *(From - To)*
December 2018	FINAL	1 Sep 2016 - 31 Aug 2018

4. TITLE AND SUBTITLE:

RUNX1T1 amplification induces 'small cell' cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-1-0568

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S):

Afshin Dowlati, M.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Case Western Reserve University
10900 Euclid Ave
Cleveland, OH 44106

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel
Command Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Small cell lung cancer (SCLC) is one of the deadliest cancers encountered by oncologists, with 5-year survival rates of less than 2% for patients with metastatic disease. Current thinking is that small cell lung cancer (SCLC) arises from a small population of specific neuroendocrine-like cells in the lung and is driven principally by concurrent mutation of two genes, TP53 and RB1. While this may be true for the majority of 'every-day' SCLC patients, there are two other clinically-important subgroups of cancer patients with 'small cell' disease; so-called combined small cell lung cancer and extra-pulmonary small cell cancer. In combined SCLC the tumors consist of both a SCLC component and a second subtype of lung cancer, such as adenocarcinoma, and it is believed that the second, more differentiated component has transformed into a small cell cancer. Similarly, extra-pulmonary small cell tumors have primary tumors that arise outside the lung, such as in the prostate or GI tract, and transform into a small cell cancer. So in reality the term 'small cell' simply describes a microscopic appearance, or phenotype. Clinically, however, this 'small cell' phenotype is of great importance because it is treated the same, regardless of whether it is pulmonary, combined or extra-pulmonary and predicts the same aggressive disease course with high mortality.

Here we seek to validate one potential pathway leading to the formation of a 'small cell' phenotype: through amplification of a gene called *RUNX1T1*, which we observed only in the 'small cell' component of two combined SCLC tumors. We will do this by investigating more combined SCLC tumors for *RUNX1T1* amplification, as well as by over-expressing *RUNX1T1* in various cancer cell lines to see if it transforms them into a 'small cell' phenotype. These studies may provide new ideas for treating SCLC itself, as well as other 'small cell' cancer subtypes outside the lung, such as some prostate cancers, which have broad military relevance beyond smoking-related diseases.

15. SUBJECT TERMS
Small cell, lung cancer, RUNX1T1, non-small cell, amplification

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
a. REPORT Not classified	**b. ABSTRACT** Not classified	**c. THIS PAGE** Not classified		2	**19b. TELEPHONE NUMBER** *(include area code)*

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1. Introduction:

The goal of this study was to explore a potential role for *RUNX1T1* amplification as a mechanism for transforming non-small cell lung cancer (NSCLC) into small cell lung cancer (SCLC). This idea was based on our finding of *RUNX1T1* amplification in the SCLC component of two 'combined' SCLC tumors that was not present in the matching NSCLC component. This was of interest because *RUNX1T1* plays an oncogenic role in some forms of leukemia as the C-terminal partner of the fusion protein ETO and because *RUNX1T1* demonstrates much higher mRNA expression levels in SCLC compared to NSCLC cells in the Cancer Cell Line Encyclopedia (CCLE) database (https://portals.broadinstitute.org/ccle). Therefore, the aims of this study were to: 1) determine the effect of RUNX1T1 overexpression in NSCLC cell lines, and 2) investigate *RUNX1T1* amplification in a larger cohort of >22 archived 'combined' SCLC tumors by CNV analyses. This grant has received a no-cost extension for a year to allow us to continue pursuing these aims. This represents a final report and incorporates our progress for Years 1 and 2 as it relates to our revised SOW dated 02/12/18 (see Appendix).

2. Keywords:

Small cell lung cancer, SCLC, combined small cell lung cancer, combined sclc, RUNX1T1, ETO, gene amplification, phenotype, non-small cell lung cancer, NSCLC, copy number variation, CNV, transformation

3. Accomplishments:

- **What were the major goals of the project? What was accomplished under these goals?** The goals are listed as stated in the revised SOW and are followed by relevant accomplishments.

<u>Specific Aim 1: To overexpress *RUNX1T1* in NSCLC cells as a surrogate mechanism for gene amplification and look for phenotypic changes consistent with a SCLC phenotype</u>:

Major Task 1: To obtain and prepare RUNX1T1 lentiviral constructs and make stable clones of six NSCLC cell lines:

Subtask 1: Obtain and prepare RUNX1T1 lentivirus. We purchased pre-made lentivirus particles from GeneCopoeia that carried a lentiviral vector encoding variant 3 of *RUNX1T1* with an N-terminal FLAG tag and the *neo* antibiotic selection marker. A complicating factor was that *RUNX1T1* has at least 15 mRNA transcripts encoding 6 different protein isoforms, all differing in the N-terminus. We chose to over-express variant 3 because it represented the shortest protein isoform while still retaining all of the annotated protein functional domains of RUNX1T1.

Subtask 2: Isolate stable clones expressing RUNX1T1. Initially we infected four NSCLC cell lines: PC-9, H1650, H1869 and A549. We isolated stable pools of cells by antibiotic selection. We did not observe any obvious change in cell phenotype by eye in these pools of selected NSCLC cells. We then used qPCR to validate over-expression in these stable pools and found that while endogenous *RUNX1T1* mRNA levels were nearly undetectable in parental cells (C_T ~ cycle 35-36), the pools of selected NSCLC cells demonstrated a minimal ~256-fold over-expression of *RUNX1T1* mRNA relative to parental cells (C_T ~ cycle 26-27) (Figure 1). For comparison, the C_T for β-actin was about 25 for all NSCLC cells tested, both parental and stable pools.

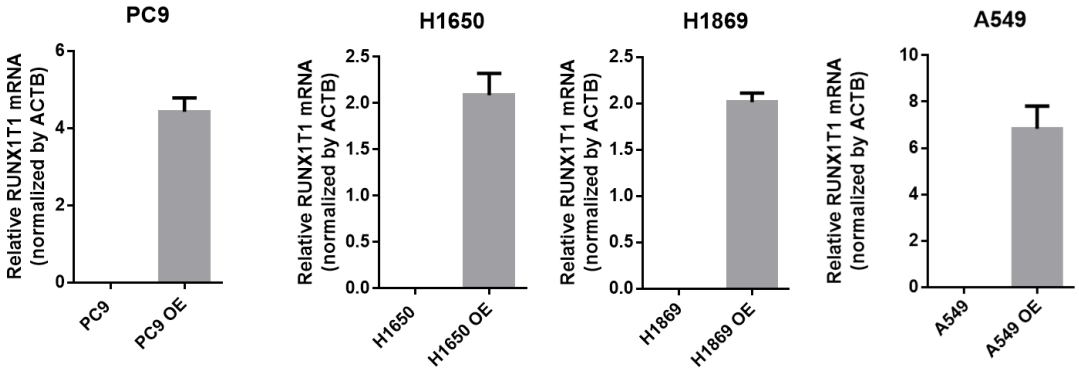

Figure 1: RT-qPCR of RUNX1T1 over-expression in NSCLC. Levels of endogenous mRNA for *RUNX1T1* were undetectable in all four cell lines. β-actin (ACTB) was a positive control. The average of triplicate determinations is shown.

Major Task 2: To analyze NSCLC cells stably over-expressing RUNX1T1:

Subtask 1: Make protein lysates for western blot analyses. We examined whole cell protein lysates from parental and *RUNX1T1* over-expressing NSCLC cells and could not demonstrate any RUNX1T1 expression in either the parental or *RUNX1T1* over-expressing NSCLC cell lines using either a Cell Signaling (CST) anti-RUNX1T1 antibody or an anti-FLAG antibody. Subsequently, as a control, we infected HEK293T cells with *RUNX1T1* lentivirus and obtained cells with robust *RUNX1T1* protein expression, validating our protein detection methods (data not shown). We also infected a SCLC cell line with *RUNX1T1* lentivirus, SW1271, which in our hands is easily transfected, and after G418 selection obtained pools of cells with increased *RUNX1T1* mRNA (C_T ~ cycle 22-24) and protein expression (see Figure 2).

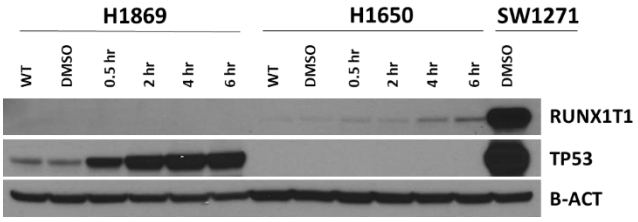

Figure 2: Effect MG132 on RUNX1T1 over-expression. Western blot results of protein lysates using antibodies indicated to right. WT: protein lysate from parental cell line. DMSO: vehicle for MG132, incubated for 6 hr. Protein lysate for SW1271 over-expressing RUNX1T1 served as a positive control in this blot. H1650 does not express TP53. β-actin (B-ACT) was a loading control. PC-9 cells gave results similar to H1869.

Taken together these results suggested that RUNX1T1 protein expression was under post-translational repression in NSCLC cells. To explore this idea further, we incubated three NSCLC cell lines over-expressing *RUNX1T1* mRNA with the proteasome inhibitor MG132 (10 μM) for increasing amounts of time. We found that while RUNX1T1 expression was increased to detectable levels in H1650 cells, this was not true for H1869 or PC-9 cells (see Figure 2). The increase in RUNX1T1 protein expression in H1650 cells, however, was small compared to the baseline over-expression of RUNX1T1 in SW1271 cells. We used expression of TP53 as a control to show the efficacy of proteasome inhibition. We concluded that although RUNX1T1 protein may undergo proteasomal degradation, this is not the main reason for its lack of over-expression in NSCLC cells.

Based on these unexpected results we infected additional lung cancer cell lines with *RUNX1T1* lentivirus and made stable pools of both NSCLC (H1299) and SCLC (H841, H82, H526, H446) cells to determine if RUNX1T1 protein could only be over-expressed in SCLC, but not NSCLC cells. Once again we found that all new stable pools of cells, both NSCLC (not shown) and SCLC (see Figure 3), demonstrated robust increases in *RUNX1T1* mRNA compared to parental controls by qPCR. One major difference, however, was that SCLC cell lines demonstrated measurable endogenous *RUNX1T1* mRNA expression (C_T ~ cycle 26-27) whereas NSCLC cells

did not (C_T ~ cycle 35), validating the results of the CCLE database reporting high expression of endogenous *RUNX1T1* only in SCLC, but not NSCLC.

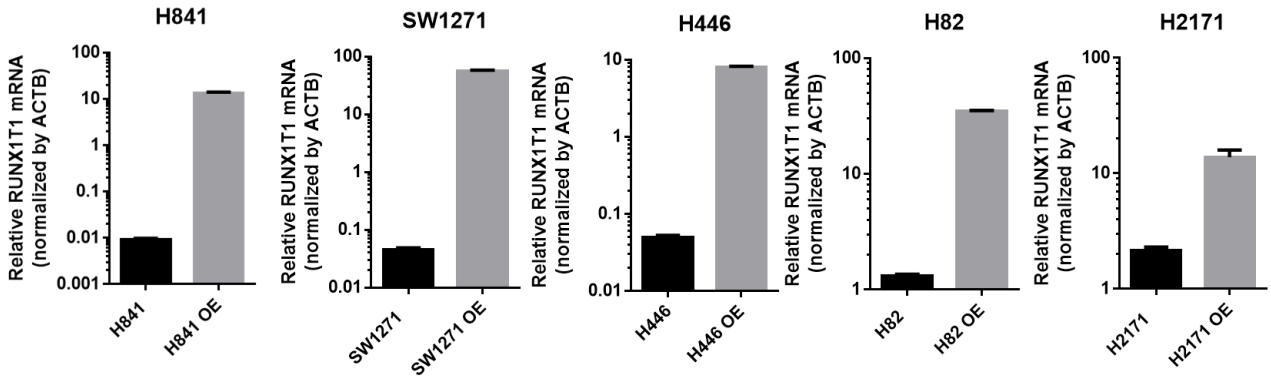

Figure 3: RT-qPCR of RUNX1T1 over-expression in SCLC. Levels of endogenous mRNA for *RUNX1T1* were undetectable in all five cell lines. β-actin (ACTB) was a positive control. The average of triplicate determinations is shown.

By the time of these SCLC RT-qPCR experiments, we had also improved the sensitivity of our RUNX1T1 protein detection in western blots by switching to an anti-RUNX1T1 antibody from Novus. Using the Novus antibody we could now show endogenous expression of RUNX1T1 in SCLC cell lines, but not in NSCLC cells (Figure 4).

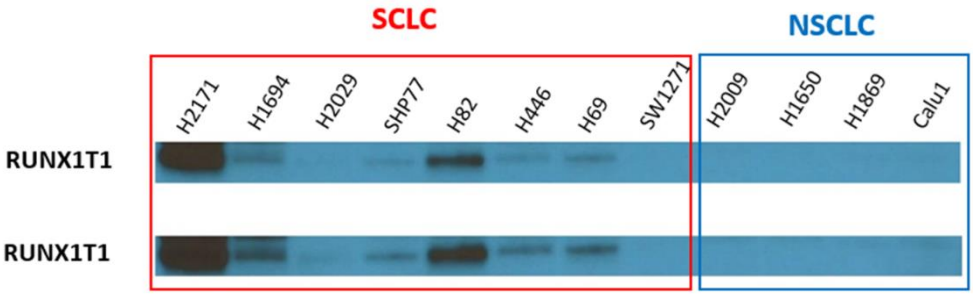

Figure 4: Endogenous expression RUNX1T1 in lung cancer cell lines. Western blots of protein lysates using new Novus antibody. NSCLC cells under blue text, SCLC cells under red text. Two different exposures are shown. SCLC cells are arranged, left to right, from high to low *RUNX1T1* mRNA levels as reported in CCLE.

When we re-examined RUNX1T1 over-expression among *all* the cells using the sensitive Novus antibody. we found that RUNX1T1 protein over-expression could be detected in most cells, although with great variability (see Figure 5 - endogenous expression of RUNX1T1 protein in SCLC cells could not be detected at this exposure)

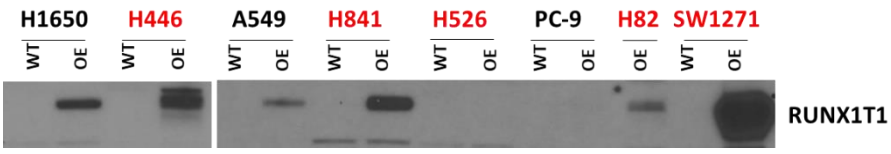

Figure 5: Stable RUNX1T1 over-expression in lung cancer cell lines. Western blots of protein lysates using new Novus anti-RUNX1T1 antibody. WT: parental cells. OE: RUNX1T1 over-expressing stable cell pools. NSCLC names in black text. SCLC names in red text.

To date, we have not observed any obvious phenotypic changes in NSCLC or SCLC cells infected with *RUNX1T1* lentivirus. Never-the-less we did attempt to detect newly emergent neuroendocrine (NE) gene expression typical of SCLC (ASCL1, NEUROD1, synaptophysin, INSM1 and TTF1) using several NSCLC cell lines with detectable RUNX1T1 protein over-expression, but could detect none by western blotting, even in H1299 cells which over-express very high levels of RUNX1T1 (see Figure 6). Two SCLC cell lines, DMS79 and H446, with endogenous NE expression were used as positive controls in these experiments.

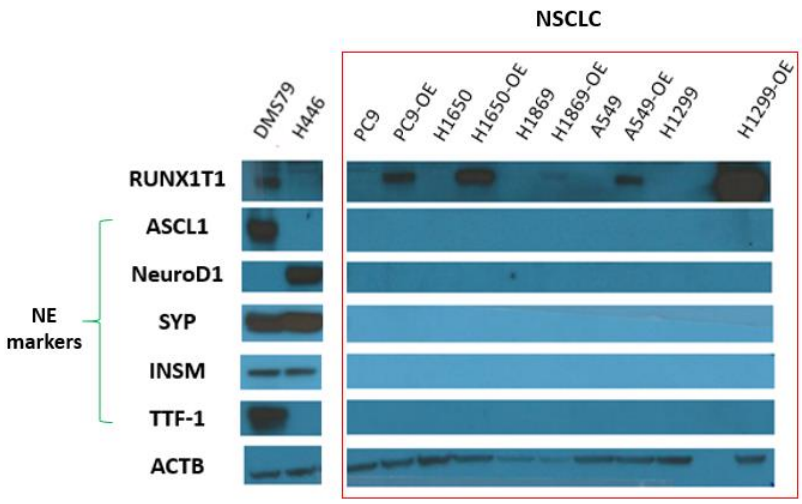

Figure 6: NE expression in lung cancer cell lines with stable RUNX1T1 over-expression. Western blots of protein lysates using antibodies indicated to left. Results shown for parental cells and RUNX1T1 over-expressing (OE) stable cell pools. NSCLC cells in red box. Results for endogenous NE expression in SCLC DMS79 and H446 cells shown to left as positive controls.

We reasoned that NE expression may be suppressed in RUNX1T1 OE NSCLC cells by NOTCH pathway signaling that is uniquely present in NSCLC, but not in SCLC. Thus, we incubated PC9 NSCLC cells (WT and RUNX1T1 OE) with the NOTCH signaling inhibitor DAPT. While DAPT effectively inhibited NOTCH1 receptor cleavage and generation of its downstream signaling mediator HES1, it caused an unexpected decrease in RUNX1T1 over-expression (Figure 7). This was also observed to a lesser extent in H1650 cells (data not shown). Because RUNX1T1 over-expression decreased with DAPT, we did not look to see if NE expression increased. We also proposed in our grant that RUNX1T1 over-expression might decrease NOTCH signaling to promote a SCLC phenotype, however, there did not seem to be any difference in basal HES1 levels between WT and RUNX1T1 OE PC9 cells (Figure 7). Thus, although reduced NOTCH signaling may not be behind our proposed *RUNX1T1*-induced NSCLC to SCLC transformation, if indeed it happens at all, the activity of this pathway clearly regulates RUNX1T1 expression, similar to the proteasome pathway (Figure 2). These results clearly point to our limited knowledge on the biology of RUNX1T1 and the need to further study it.

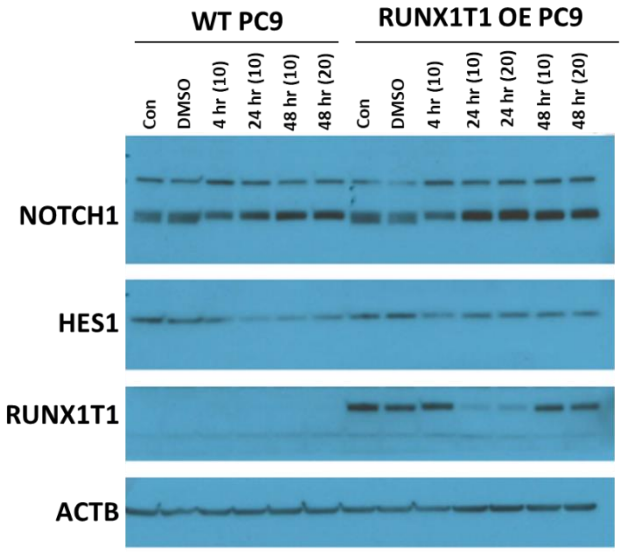

Figure 7: Effect of NOTCH inhibitor DAPT on PC9 cells with/without stable RUNX1T1 over-expression. Western blots of protein lysates using antibodies indicated to left. Results shown for WT cells and RUNX1T1 over-expressing (OE) stable cell pools. The concentration of drug, in µM, is shown in parentheses after duration of drug treatment, in hr. DMSO was vehicle control for DAPT.

Subtask 2: Isolate RNA and perform gene expression profiling. We isolated RNA from 5 pairs (lentiviral control vs RUNX1T1 OE) of cell lines for microarray analysis: two NSCLC (H1650, H1299) and three SCLC (SW1271, H82, H2171). The samples were profiled using the Clariom S Human microarray by Affymatrix.

Subtask 3: Bioinformatic analysis of gene expression profiling. The samples were normalized using the Robust Multiarray Average (RMA) method and collapsed to gene-level using the GSEA Collapse Dataset function. The final output was 19402 genes available for analysis. A quick look at the results validated *RUNX1T1* OE in all cell lines profiled: H1650 (49.8-fold increase), H1299 (215.4-fold), SW1271 (119.0-fold), H82 (4.2-fold) and H2171 (1.7-fold). The greater fold differences in NSCLC vs SCLC reflect the lack of basal expression in NSCLC cells. These results also match what was observed by western blotting (Figures 5 and 6). Gene set enrichment profiles were compared between control vs *RUNX1T1* OE in the cell lines using GSEA Hallmark and KEGG gene sets. The most intriguing and useful results were the

identification of Hallmark gene sets that were depleted in all *RUNX1T1* OE cells, shown in Table 1, as well as gene sets specifically depleted in NSCLC, shown in Table 2 (those highlighted in yellow are highly significant in both Tables). These analyses revealed that one of the most significantly perturbed pathways was E2F, which is uniquely disrupted in most SCLC tumors by either *RB1* chromosomal loss or gene mutation. This finding supported our hypothesis that *RUNX1T1* amplification may play a role in NSCLC to SCLC transformation in combined SCLC tumors.

Table 1: All RUNX1T1 OE cells.

NAME	SIZE	NES	NOM p-val
HALLMARK_E2F_TARGETS	185	-2.3909	0.0000
HALLMARK_INTERFERON_ALPHA_RESPONSE	91	-2.3311	0.0000
HALLMARK_G2M_CHECKPOINT	188	-2.0883	0.0000
HALLMARK_MYC_TARGETS_V2	51	-2.0649	0.0000
HALLMARK_INTERFERON_GAMMA_RESPONSE	192	-2.0530	0.0000
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	191	-1.9321	0.0000
HALLMARK_MYC_TARGETS_V1	178	-1.8589	0.0000
HALLMARK_UV_RESPONSE_UP	154	-1.7959	0.0000
HALLMARK_COAGULATION	132	-1.7601	0.0000
HALLMARK_INFLAMMATORY_RESPONSE	193	-1.7519	0.0000
HALLMARK_KRAS_SIGNALING_UP	190	-1.7283	0.0000
HALLMARK_TNFA_SIGNALING_VIA_NFKB	193	-1.7143	0.0000
HALLMARK_TGF_BETA_SIGNALING	53	-1.5609	0.0101
HALLMARK_DNA_REPAIR	135	-1.5505	0.0020
HALLMARK_P53_PATHWAY	190	-1.5305	0.0039
HALLMARK_ESTROGEN_RESPONSE_LATE	193	-1.4659	0.0020
HALLMARK_XENOBIOTIC_METABOLISM	192	-1.3595	0.0192
HALLMARK_APOPTOSIS	154	-1.3487	0.0100
HALLMARK_ESTROGEN_RESPONSE_EARLY	191	-1.3050	0.0277
HALLMARK_UV_RESPONSE_DN	136	-1.2986	0.0467
HALLMARK_COMPLEMENT	187	-1.2691	0.0444
HALLMARK_IL2_STAT5_SIGNALING	192	-1.1512	0.1429
HALLMARK_APICAL_JUNCTION	192	-1.1345	0.1759
HALLMARK_IL6_JAK_STAT3_SIGNALING	85	-1.1108	0.2570
HALLMARK_SPERMATOGENESIS	130	-1.0494	0.3360
HALLMARK_ANGIOGENESIS	34	-1.0071	0.4380
HALLMARK_MITOTIC_SPINDLE	194	-0.9190	0.7082
HALLMARK_ANDROGEN_RESPONSE	95	-0.8958	0.6546
HALLMARK_NOTCH_SIGNALING	31	-0.7899	0.7896
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	100	-0.7687	0.9259
HALLMARK_WNT_BETA_CATENIN_SIGNALING	41	-0.7585	0.8565
HALLMARK_APICAL_SURFACE	43	-0.7250	0.8922

Table 2: NSCLC RUNX1T1 OE cells only.

NAME	SIZE	NES	NOM p-val
HALLMARK_TGF_BETA_SIGNALING	53	-2.1849	0.0000
HALLMARK_E2F_TARGETS	185	-2.1334	0.0000
HALLMARK_INTERFERON_ALPHA_RESPONSE	91	-2.0112	0.0000
HALLMARK_MYC_TARGETS_V2	51	-1.9478	0.0000
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	191	-1.9200	0.0000
HALLMARK_G2M_CHECKPOINT	188	-1.7047	0.0000
HALLMARK_COAGULATION	132	-1.6676	0.0000
HALLMARK_MYC_TARGETS_V1	178	-1.6617	0.0000
HALLMARK_UV_RESPONSE_UP	154	-1.6474	0.0000
HALLMARK_ESTROGEN_RESPONSE_LATE	193	-1.6428	0.0000
HALLMARK_APOPTOSIS	154	-1.5884	0.0000
HALLMARK_ESTROGEN_RESPONSE_EARLY	191	-1.5671	0.0000
HALLMARK_P53_PATHWAY	190	-1.5262	0.0000
HALLMARK_INTERFERON_GAMMA_RESPONSE	192	-1.4981	0.0044
HALLMARK_INFLAMMATORY_RESPONSE	193	-1.4068	0.0065
HALLMARK_DNA_REPAIR	135	-1.3981	0.0149
HALLMARK_KRAS_SIGNALING_UP	190	-1.3876	0.0067
HALLMARK_ANGIOGENESIS	34	-1.3492	0.1102
HALLMARK_IL2_STAT5_SIGNALING	192	-1.2806	0.0373
HALLMARK_TNFA_SIGNALING_VIA_NFKB	193	-1.2462	0.0549
HALLMARK_XENOBIOTIC_METABOLISM	192	-1.2215	0.0875
HALLMARK_APICAL_JUNCTION	192	-1.1468	0.1557
HALLMARK_COMPLEMENT	187	-1.1427	0.1579
HALLMARK_UV_RESPONSE_DN	136	-1.1127	0.2348
HALLMARK_MITOTIC_SPINDLE	194	-1.1076	0.2255
HALLMARK_GLYCOLYSIS	192	-1.0478	0.3320
HALLMARK_PANCREAS_BETA_CELLS	39	-1.0367	0.3931
HALLMARK_HEDGEHOG_SIGNALING	33	-1.0082	0.4534
HALLMARK_BILE_ACID_METABOLISM	110	-0.9961	0.4421
HALLMARK_NOTCH_SIGNALING	31	-0.9784	0.4778
HALLMARK_HEME_METABOLISM	188	-0.9506	0.5787
HALLMARK_PEROXISOME	98	-0.9413	0.6022
HALLMARK_PI3K_AKT_MTOR_SIGNALING	101	-0.9048	0.6858

We sought to validate a change in E2F pathway activity associated with RUNX1T1 OE by two methods: 1) using E2F-luciferase assays, and 2) by western blotting for changes in RB1 and phospho-RB1. An E2F-luciferase reporter kit was bought from Qiagen (Cignal Reporter Assay) and transiently transfected into control and RUNX1T1 OE cells. The majority of RUNX1T1 OE cells showed significantly increased E2F-reporter activity (A549, H1299, H841, SW1271) (Figure 8). These experiments included cell lines which were not analyzed by microarray to obtain a broader view of the effect of RUNX1T1 OE on E2F activity in cells (A549, H841, PC9).

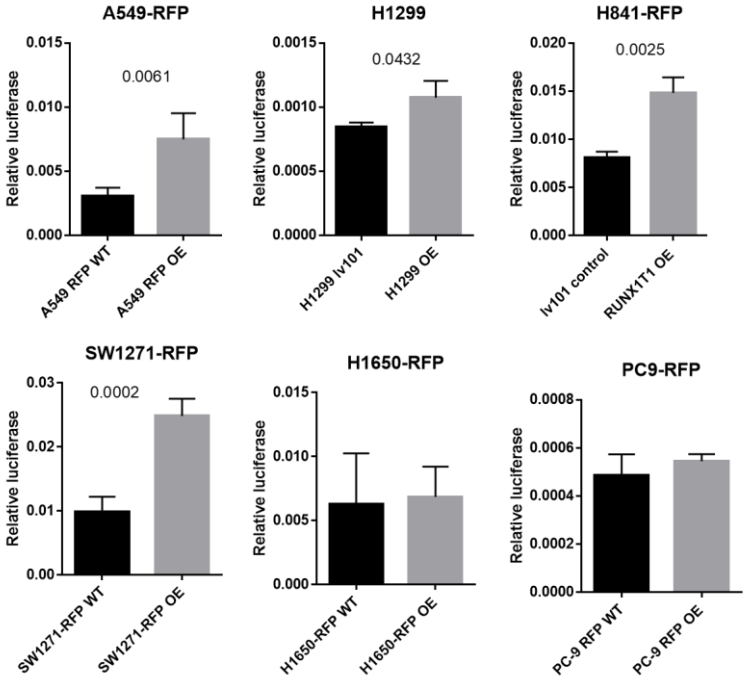

Figure 8: E2F-luciferase activity in cell lines with/without stable RUNX1T1 over-expression. E2F-luciferase activity is shown under basal conditions 24 hr after transfection. Firefly luciferase activity is normalized to co-transfected Rinella luciferase activity. The average of quadruplicate determinations are shown, along with *p* values comparing controls to RUNX1T1 OE cells (no value = not significant). SW1271 and H841 are SCLC cell lines while all the others are NSCLC cell lines.

In parallel, we prepared protein lysates from the same six paired cell lines and analyzed them by western blotting for total RB1 and phospho-RB1 (Ser^{780}) expression. Total RB1 expression was

decreased in A549, H841 and H1650 cell lines by RUNX1T1 OE, and increased in H1299 and SW1271 cells (Figure 9). Parallel blots showed that phospho-RB1 results generally paralleled those of total RB1 (data not shown). Taken together, bioinformatic analyses, luciferase assays and western blotting results all support a role for RUNX1T1 in regulating E2F pathway activity. Our preliminary results show this is not due to a change in expression of upstream pathway regulators such as cyclins and CDKs. We have also found that RUNX1T1 OE does not produce any large change in cell proliferation rates using Cell TiterGlo and FACS assays (data not shown). We are now trying to find the mechanism(s) behind these RUNX1T1-induced changes in RB1/E2F activity and are searching for the emergence of other new phenotypes suggestive of a SCLC transformation, such as chemo-sensitivity.

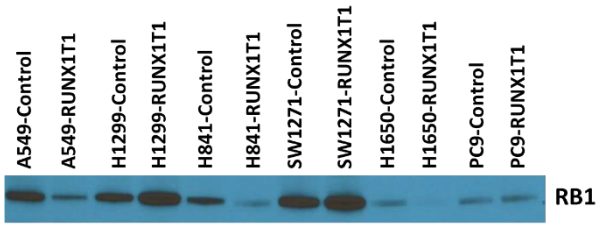

Figure 9: Effect RUNX1T1 OE on RB1 expression in lung cancer cells. Western blots of protein lysates using antibody against total RB1. Results shown for matched control cells and RUNX1T1 over-expressing stable cell pools.

Major Task 3: To analyze SCLC cells stably over-expressing RUNX1T1: Because of our initial difficulty in detecting RUNX1T1 over-expression in NSCLC cells, we began exploring what this protein does in SCLC- does it play any role in maintaining the neuroendocrine phenotype? This question was of interest because so little is known about the functional role of RUNX1T1 as a *non-fusion* protein, that is, separate from ETO.

Subtask 1: Make protein lysates for western blot analyses. We explored this question initially in the stable RUNX1T1 OE cells we described above (Figures 3 and 5). Western blots of protein lysates showed that RUNX1T1 OE does elicit changes in the level of expression of NE proteins, however these changes seemed to be cell-type specific (see Figure 10). For example, NEUROD1 expression increases in H82 and H2171 cells but decreases in H446 cells after RUNX1T1 OE. Synaptophysin (SYP) shows a completely different pattern of change. These divergent changes in NE expression made it hard to follow-up these results so we sought to use microarray analyses to identify more consistent, global changes in gene expression pathways.

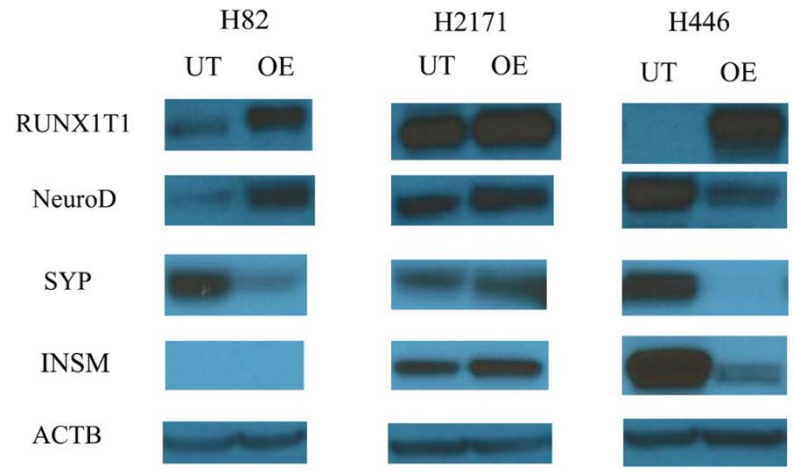

Figure 10: Effect RUNX1T1 OE on NE expression in SCLC cells. Western blots of protein lysates using antibodies shown at left. Results shown for matched control cells (UT) and RUNX1T1 over-expressing (OE) stable cell pools.

Subtask 2: Isolate RNA and perform gene expression profiling. As mentioned above, H82 and H2171 cells with RUNX1T1 OE were analyzed by microarray along with NSCLC RUNX1T1 OE cells.

Subtask 3: Bioinformatic analysis of gene expression profiling. When analyzed by themselves, RUNX1T1 OE in H82 and H2171 produced many changes in gene set expression similar to those shown Tables 1 (all cells) and 2 (NSCLC only), even though the fold increase in RUNX1T1 expression was much lower. One notable exception was that no change in E2F targets was observed, likely because neither of these two SCLC cell lines has RB1 protein expression. Some of these results are shown in Table 3. There were no significant increases in Hallmark gene set expression in SCLC cells (data not shown). We have not performed any follow-up experiments to these GSEA pathway results because we are focused on exploring the effects of RUNX1T1 OE in NSCLC cells, which is the central question posed in our grant.

Table 3: SCLC RUNX1T1 OE cells only.

NAME	SIZE	NES	NOM p-val
HALLMARK_INTERFERON_ALPHA_RESPONSE	91	-1.9790298	0
HALLMARK_TGF_BETA_SIGNALING	53	-1.8792305	0
HALLMARK_HEDGEHOG_SIGNALING	33	-1.6346115	0.00759013
HALLMARK_APOPTOSIS	154	-1.5881705	0.00172117
HALLMARK_TNFA_SIGNALING_VIA_NFKB	193	-1.5490748	0
HALLMARK_IL2_STAT5_SIGNALING	192	-1.5261596	0
HALLMARK_INFLAMMATORY_RESPONSE	193	-1.4503263	0.01025641
HALLMARK_ESTROGEN_RESPONSE_LATE	193	-1.4467113	0.00720721
HALLMARK_ESTROGEN_RESPONSE_EARLY	191	-1.3594646	0.01386482
HALLMARK_COAGULATION	132	-1.3041772	0.05347594
HALLMARK_BILE_ACID_METABOLISM	110	-1.2970712	0.06151142
HALLMARK_PANCREAS_BETA_CELLS	39	-1.2854459	0.11870503
HALLMARK_INTERFERON_GAMMA_RESPONSE	192	-1.2562695	0.05583756
HALLMARK_MYOGENESIS	192	-1.2521503	0.06420546
HALLMARK_IL6_JAK_STAT3_SIGNALING	85	-1.241492	0.11636364

Major Task 4: To analyze SCLC cells with stable knockdown of RUNX1T1: As part of our attempt to determine the role of endogenous RUNX1T1 in SCLC cells, we produced stable knockouts (KO) of this gene in H1694, H2171 and H82 cells using CRISPR/Cas9 lentivirus we purchased from GenScript.

Subtask 1: Make protein lysates for western blot analyses. Western blots of protein lysates showed that *RUNX1T1* KO does elicit changes in the level of expression of NE proteins, however once again these changes seemed to be cell-type specific (see Figure 11). In addition, we looked at changes in other proteins (cMYC and phospho-ERK) and again observed divergent results among different cells. Again, we sought to use microarray analyses to identify more consistent, global changes in gene expression pathways.

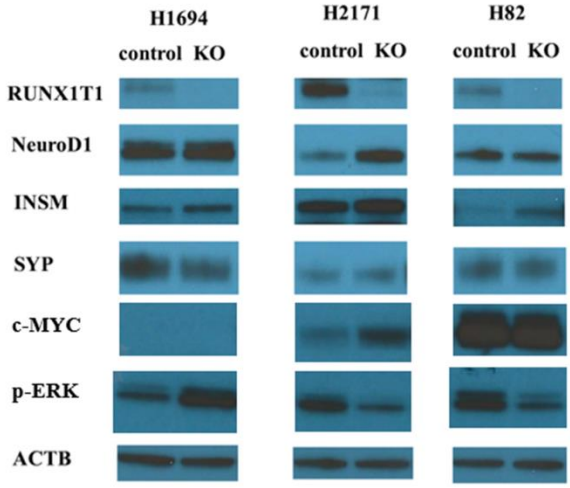

Figure 11: Effect *RUNX1T1* KO on gene expression in SCLC cells. Western blots of protein lysates using antibodies shown at left. Results shown for matched control cells (con) and *RUNX1T1* knockout (KO) stable cell pools.

Subtask 2: Isolate RNA and perform gene expression profiling. H82 and H2171 cells with *RUNX1T1* KO were analyzed by microarray along with SCLC and NSCLC RUNX1T1 OE cells.

Subtask 3: Bioinformatic analysis of gene expression profiling. Although microarray analysis did not demonstrate significant changes in *RUNX1T1* KO in H82 (1.2-fold increase) and H2171 (0.8-fold decrease) cells, this is not an uncommon result with CRISPR/Cas9 due to technical reasons and our western blot results confirmed that RUNX1T1 protein expression was indeed decreased in these cells (Figure 11). Interestingly, when analyzed by themselves, RUNX1T1 KO in H82 and H2171 produced almost identical changes in gene set expression similar to those shown Tables 1 (all cells) and 2 (NSCLC only), but in an opposite direction, which might be expected. Most important, E2F targets were increased. The results are shown in Table 4. We intend to perform follow-up experiments to these GSEA pathway results to complement our results on the effects of RUNX1T1 OE in NSCLC cells.

Table 4: SCLC *RUNX1T1* KO cells only.

NAME	SIZE	NES	NOM p-val
HALLMARK_E2F_TARGETS	185	1.5276	0.0000
HALLMARK_G2M_CHECKPOINT	188	1.5059	0.0000
HALLMARK_MYC_TARGETS_V1	178	1.4599	0.0000
HALLMARK_COMPLEMENT	187	1.0959	0.2271
HALLMARK_MTORC1_SIGNALING	194	0.9868	0.4829
HALLMARK_PANCREAS_BETA_CELLS	39	0.9416	0.5585
HALLMARK_MYC_TARGETS_V2	51	0.9305	0.5845
HALLMARK_APICAL_JUNCTION	192	0.9140	0.7036
HALLMARK_UV_RESPONSE_UP	154	0.8710	0.8333
HALLMARK_ANGIOGENESIS	34	0.8394	0.7256
HALLMARK_HEDGEHOG_SIGNALING	33	0.6579	0.9777
HALLMARK_OXIDATIVE_PHOSPHORYLATIO	191	0.6260	1.0000
HALLMARK_UNFOLDED_PROTEIN_RESPON	100	0.4133	1.0000

<u>Specific Aim 2: To interrogate our SCLC cohort of combined SCLC samples for evidence of RUNX1T1 amplification at the DNA and protein levels</u>:

Major Task 1: Obtain tissues blocks of tumor specimens and quality control using IHC markers. With the help of our collaborating thoracic pathologist, Dr. Michael Yang, we have searched for tumor specimens from patients annotated in our SCLC database as having 'combined' SCLC. Starting from a list of 19 initial patients, we now have in our possession 7 archived tumor specimens available for analysis. On a practical level this low number of specimens meant that it was not worthwhile making tissue microarrays (TMAs) from the

'combined' tumor components, as we had originally proposed in the grant, and we also did also not have enough tissue to extract RNA/DNA for mRNA/CNV analysis of *RUNX1T1* expression. Instead, we began to develop in-situ hybridization (ISH) techniques for mRNA expression analyses.

Major Task 2: Prepare TMAs of SCLC and NSCLC cell lines for methods development. See response to Major Task 1. However, we did construct a new TMA consisting of only lung cancer cell lines (29 SCLC, 15 NSCLC, 6 mesothelioma, 1 normal), to help us trouble-shoot future methods to detect *RUNX1T1* expression.

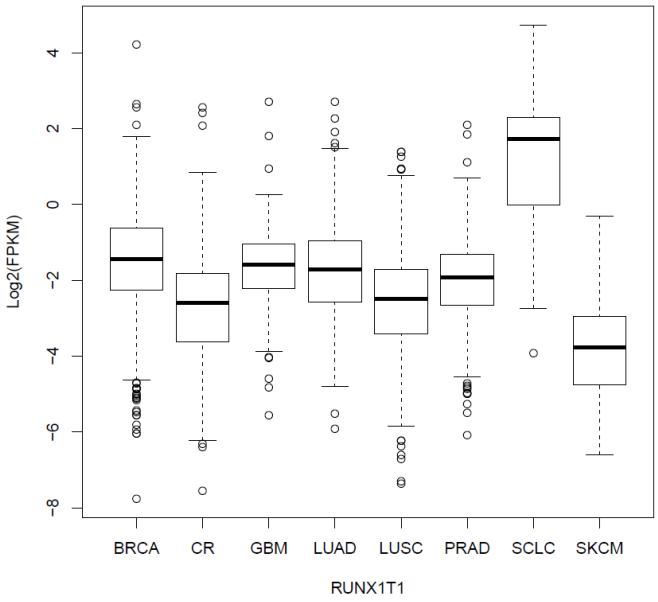

Figure 12: *RUNX1T1* mRNA expression in tumors. Determined from RNAseq data. BRCA: breast cancer, CR: colorectal cancer, GBM: glioblastoma, LUAD: lung adenoma NSCLC, LUSC: lung squamous NSCLC, PRAD: prostate adenoma cancer, SKCM: skin melanoma cancer.

Major Task 3: Perform IHC, RNAscope and ISH analyses of tumor specimens. See response to Major Task 1. We initially attempted to determine RUNX1T1 protein expression using an IHC-only anti-RUNX1T1 antibody (LS BIO) using a lung cancer TMA we had previously constructed (22 SCLC, 12 NSCLC, 3 normal lung specimens, with 2-4 cores from each specimen). Unfortunately, Dr. Michael Yang did not find the staining to be specific for SCLC compared with NSCLC and it was also not nuclear, as we expected since RUNX1T1 is a transcriptional co-repressor. Thus, we sought to confirm the differential expression of *RUNX1T1* in SCLC compared to NSCLC tumors, as well as to other cancers. First, we used RNAseq data from the TCGA as well as a SCLC genomics study (Rudin et al, Nat Genetics 44:1111-1116, 2012) and found that SCLC easily expressed the highest levels of *RUNX1T1* mRNA among all tumors examined (Figure 12).

We then sought to confirm the specific expression of endogenous RUNX1T1 protein in SCLC vs NSCLC. We used western blotting with the sensitive Novus antibody on whole cell protein lysates, as well as nuclear fractions, prepared from 14 SCLC and 4 NSCLC cell lines. The results, some of which are shown in Figure 13, demonstrated that the majority of SCLC cell lines express endogenous RUNX1T1 protein, although to variable levels, whereas no endogenous RUNX1T1 protein was ever detected in any NSCLC cell line. The level of endogenous RUNX1T1 protein in SCLC, however, was much less than that observed for over-expressed RUNX1T1 in SW1271 cells. These results were consistent with the mRNA expression data in the CCLE and our previous qPCR (Figures 1 and 3) and western blotting results (Figure 4). Taken together, these results in lung cancer cell lines validated the use of our lung cancer cell line TMA as a good tool to trouble-shoot future detection methods for *RUNX1T1* expression.

Figure 13: Endogenous RUNX1T1 expression in lung cancer cell lines. Western blot results of protein lysates using new Novus anti-RUNX1T1 antibody. Wcl: whole cell lysate. Nuc: nuclear fraction. Space: empty lane. OE SW1271: stable pool of RUNX1T1 over-expressing cells used as positive control. NSCLC names in black text. SCLC names in red text.

We now believe that the best way to detect *RUNX1T1* expression is at the mRNA level by RNAscope. This is due to the limited amount of tumor tissue available, the lack of specificity of anti-RUNX1T1 antibodies in IHC, the greater specificity of nucleic acid over protein detection in general, and the reality that *RUNX1T1* amplification in combined SCLC should manifest itself as increased *RUNX1T1* mRNA levels to be functionally important. We purchased a kit to perform RNAscope detection of *RUNX1T1* mRNA and *MYC* mRNA (to be used as a positive control since 30% SCLC cells show *MYC* amplification and expression). Initially we optimized our methods using H1299 NSCLC with dramatic RUNX1T1 OE (see Figure 6) but no MYC expression and H2171 SCLC cells exhibiting high endogenous RUNX1T1 (see Figures 4 and 13) as well as harboring *MYC* amplification. The results we obtained by RNAscope reflected the expression levels expected for these two genes (Figure 14). We are now using these optimized conditions to probe the cell line TMA and our SCLC/NSCLC tumor TMA to hopefully demonstrate specific expression of both *RUNX1T1* and *MYC* only in SCLC. If successful, we will then probe our more precious combined SCLC tumor specimens.

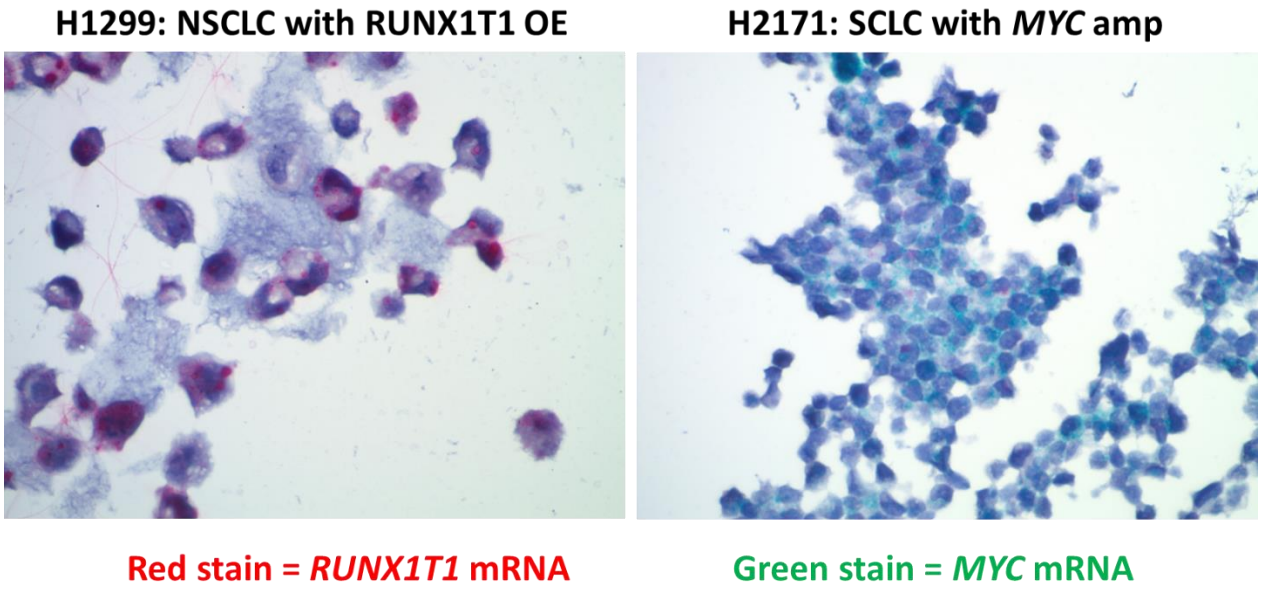

Figure 14: RNAscope detection of *RUNX1T1* and *MYC* expression in lung cancer cell lines. Cytoplasmic detection of *RUNX1T1* mRNA is visualized as red staining while *MYC* mRNA is visualized as green staining. The NSCLC H1299 cell with RUNX1T1 OE show intense red but no green staining. The SCLC H2171 cells show scattered red but universal green staining. Cell pellets were formaldehyde-fixed and paraffin-embedded (FFPE) prior to RNAscope analysis. The magnification is 600x.

- **What opportunities for training and professional development has the project provided?**

Although this project was not intended to provide training opportunities, the majority of research results have been produced by Tian He, a graduate student in the Department of Biochemistry at Case Western Reserve University as part of her PhD thesis.

- **How were the results disseminated to communities of interest?**

Although none of this work has been published yet, we anticipate submitting a manuscript in the first half of 2019 after we answer questions on the underlying mechanism of RUNX1T1 action in RUNX1T1 OE NSCLC cells and *RUNX1T1* KO SCLC cells, and obtaining final results for *RUNX1T1* mRNA in-situ hybridization (ISH) in human tumor specimens of pure and combined SCLC.

- **What do you plan to do during the next reporting period to accomplish the goals?**

N/A

4. Impact:

- **What was the impact on the development of the principal discipline(s) of the project?**

To date, we have:

- confirmed the specific expression of *RUNX1T1* in SCLC, but not in NSCLC or other solid tumors at an mRNA and protein level
- shown that RUNX1T1 expression is likely subject to post-translational regulation
- demonstrated that *RUNX1T1* regulates NE gene expression in SCLC cell lines
- shown that RUNXT1 OE in NSCLC elicits changes in E2F target gene expression opposite that of *RUNX1T1* KO in SCLC
- Taken together, these results support our hypothesis that *RUNX1T1* may play a role in the phenotypic transformation of NSCLC to SCLC in combined SCLC

- **What was the impact on other disciplines?**

Our results may suggest a role for *RUNX1T1* in the transformation of other extra-pulmonary cancers into a SCLC phenotype, such as prostate cancer. This may lead to the development of improved therapies against SCLC of any origin.

- **What was the impact on technology transfer?**

Nothing to report.

- **What was the impact on society beyond science and technology?**

Nothing to report.

5. Changes/problems:

- **Changes in approach and reasons for change.**

We only had to change our approach to analyzing *RUNX1T1* expression in tumor samples to RNAscope, as stated in Specific Aim 2, major task 3.

- **Actual or anticipated problems or delays and actions or plans to resolve them.**

The only delay has been in our ability to obtain and analyze tumor specimens of combined SCLC from the Pathology archives. We now have the specimens in hand and an optimized RNAscope method to proceed with the analysis of *RUNX1T1* expression. Although we may only have 7 tumor specimens, this should be more than enough to draw conclusions about *RUNX1T1* expression in this subtype of SCLC.

- **Changes that had a significant impact on expenditures.**

Nothing to report.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.**

Nothing to report.

6. Products:

Nothing to report.

7. Participation & other collaborating organizations:

- **What individuals have worked on the project?**

Name:	Afshin Dowlati, MD
Project role:	PI
Researcher identifier:	Orchid # 0000-0003-4535-6839
Nearest person months worked:	24
Contribution to project:	PI- oversight and direction.
Funding support:	This and grants listed below and University Hospitals of Cleveland research support.

Name:	Karen McColl, BS
Project role:	Lab manager
Researcher identifier:	N/A
Nearest person months worked:	12
Contribution to project:	Ms. McColl has worked on all aspects of this project.
Funding support:	This and University Hospitals of Cleveland research support.

Name:	Tian He, BS
Project role:	Graduate student.
Researcher identifier:	N/A
Nearest person months worked:	21
Contribution to project:	Ms. He has worked on all aspects of this project.
Funding support:	University Hospitals of Cleveland research support.

Name:	Gary Wildey, PhD
Project role:	Program manager
Researcher identifier:	Orchid # 0000-0001-7105-1313
Nearest person months worked:	24
Contribution to project:	Research advise and report writing.
Funding support:	University Hospitals of Cleveland research support.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Yes, one new grant was funded (R21), one was non-competitively renewed (U24), one received no-cost extension (DoD Concept):

Active Grants:

R21 (Dowlati, PI) 07/01/18-06/30/20 1.2 calendar
NIH/NCI $275,000
Identification and targeting of chemotherapy refractory small cell lung cancer

Here we propose to determine whether *RB1* mutation status and/or protein expression identifies chemo-refractory patients in small-cell lung cancer (SCLC). A secondary aim is to determine whether *RB1* mutation status and/or protein expression predicts sensitivity to CDK4/6 inhibitors in SCLC models.
Overlap: None.

U24 (Rudin, PI) 02/20/17-01/31/22 0.6 calendar
NIH/NCI $75,000 (sub only)
Small-cell Lung Cancer (SCLC) Consortium: Coordinating Center
Subproject: *Construction of SCLC tissue micro-arrays*

Here we propose to construct multiple tissue micro-arrays from de-identified small-cell lung cancer tumor specimens and provide matching genomic profiling data on tumor specimens with sufficient tissue. Additional data on patient and tumor features will be provided for each tumor specimen placed in the TMA.
Role: Leader, tissue micro-array subproject.
Overlap: None- no funds are requested to make the TMAs used in the current application, as stated in the Budget Justification.

Concept Award (Dowlati, PI) 09/01/17-08/31/18 1.2 calendar
DOD $100,000
Targeting Chemo-resistance in Small Cell Lung Cancer

This proposal is focused on a gene, *HEPACAM2*, which appears to be uniquely and highly expressed only in SCLC. Because HEPACAM2 likely is required for cell division and is poly(ADP-ribosylated) by the enzyme tankyrase, we propose that disruption of its function by tankyrase inhibitors may provide a unique strategy to selectively inhibit SCLC growth and proliferation.
Overlap: None.

- **What other organizations were involved as partners?**

Nothing to report.

8. Special reporting requirements:

Nothing to report.

9. Appendix:

STATEMENT OF WORK – 02/12/2018
PROPOSED START DATE Sept 01, 2016

Site 1: Case Western Reserve University
11100 Euclid Ave
Cleveland, OH 44106
PI: Afshin Dowlati

Specific Aim 1: **To overexpress RUNX1T1 in NSCLC cells as a surrogate mechanism for gene amplification and look for phenotypic changes consistent with a SCLC phenotype:**	**Timeline** **(Months)**	
Major Task 1: To obtain and prepare RUNX1T1 lentiviral constructs and make stable clones of six NSCLC cell lines**:**		
Subtask 1: Obtain and prepare RUNX1T1 lentivirus.	1-2	Karen McColl
Subtask 2: Isolate stable clones expressing RUNX1T1.	3-18	Karen McColl
Major Task 2: To analyze NSCLC cells stably over-expressing RUNX1T1:		
Subtask 1: Make protein lysates for western blot analyses.	5-18	Karen McColl
Subtask 2: Isolate RNA and perform gene expression profiling.	18-20	Karen McColl
Subtask 3: Bioinformatic analysis of gene expression profiling.	20-24	Gene Expression core
Major Task 3: To analyze SCLC cells stably over-expressing RUNX1T1:		
Subtask 1: Make protein lysates for western blot analyses.	6-18	Tian He
Subtask 2: Isolate RNA and perform gene expression profiling.	18-20	Tian He
Subtask 3: Bioinformatic analysis of gene expression profiling.	20-24	Gene Expression core
Major Task 4: To analyze SCLC cells with stable knockdown of RUNX1T1:		

Subtask 1: Make protein lysates for western blot analyses.	13-18	Tian He
Subtask 2: Isolate RNA and perform gene expression profiling.	18-20	Tian He
Subtask 3: Bioinformatic analysis of gene expression profiling.	20-24	Gene Expression core
Milestone(s) Achieved:		
Specific Aim 2: **To interrogate our SCLC cohort of combined SCLC samples for evidence of RUNX1T1 amplification at the DNA and protein levels:**		
Major Task 1: Obtain tissues blocks of tumor specimens and quality control using IHC markers.	1-20	Dr. Dowlati, Pathology core
Major Task 2: Prepare TMAs of SCLC and NSCLC cell lines for methods development.	12-20	Dr. Dowlati, Pathology core
Major Task 3: Perform IHC, RNAscope and ISH analyses of tumor specimens.	20-24	Dr. Dowlati, Pathology core
Milestone(s) Achieved:		
Milestone Achieved: HRPO/ACURO Approval:	05/05/16	