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14. ABSTRACT

The mitochondrial deoxynucleotide triphosphate (dNTP) is maintained by the mitochondrial deoxynucleoside salvage pathway and dedicated for the mitochondrial DNA (mtDNA) homeostasis, and the mitochondrial deoxyguanosine kinase (DGUOK) is a rate-limiting enzyme in this pathway. Here, we investigated the role of the DGUOK in the self-renewal of lung cancer stem-like cells (CSC). Our data support that DGUOK overexpression strongly correlates with cancer progression and patient survival. The depletion of DGUOK robustly inhibited lung adenocarcinoma tumor growth, metastasis, and CSC self-renewal. Mechanistically, DGUOK is required for the biogenesis of respiratory complex I and mitochondrial OXPHOS, which in turn regulates CSC self-renewal through AMPK- YAP1 signaling. The restoration of mitochondrial OXPHOS in DGUOK KO lung cancer cells using NDI1 was able to prevent AMPK-mediated phosphorylation of YAP and to rescue CSC stemness. Genetic targeting of DGUOK using doxycycline-inducible CRISPR/Cas9 was able to markedly induce tumor regression. Our findings reveal a novel role for mitochondrial dNTP metabolism in lung cancer tumor growth and progression, and implicate that the mitochondrial deoxynucleotide salvage pathway could be potentially targeted to prevent CSC-mediated therapy resistance and metastatic recurrence.

15. SUBJECT TERMS
DGUOK regulates lung adenocarcinoma stemness; DGUOK regulates mitochondrial deoxynucleoside salvage pathway; Inhibit DGUOK may prevent metastatic recurrence.

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

We propose that the dormant metastatic non-small cell lung cancer (NSCLC) cells rely on the mitochondrial deoxyguanosine kinase (DGUOK) to maintain mitochondrial homeostasis, which could be targeted to prevent metastatic recurrence. We reason that the non-oncogene addiction of NSCLC on DGUOK is a therapeutic vulnerability that could be exploited to prevent lung cancer metastasis.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

DGUOK, NSCLC, cancer stem cell, metastasis, mitochondria, didanosine

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Our major goals are:

1. To define the role of DGUOK in the self-renewal of NSCLC stem-like cell.

2. To define DGUOK as a target to inhibit NSCLC tumor growth and prevent metastasis.

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

MAJOR ACTIVITIES

Major task 1:

1. We have used limiting dilution tumor initiation assay and tumor sphere formation assay to determine the role of DGUOK in the self-renewal abilities of NSCLC stem-like cells.
2. We have used FACS and flow cytometry to determine the CSC population in control and DGUOK depleted NSCLC cells. Several cancer stem cell marker assays, including ALDEFLOR (for ALDH+ population), side population assay and CD166, CD49 were used.
3. We have determined the effects of DGUOK depletion on mitochondrial oxidative phosphorylation using mitostress assay. We have also determined the effects of DGUOK depletion on mtDNA levels, complex I biogenesis. Yeast NDI1 was used to restore mitochondrial oxphos in DGUOK KO lung cancer cells and the effect of NDI1 rescue on CSC self renewal were determined in cell culture and mouse models.

Major task 2**:**

1. We have examined the effects of pharmacological inhibition of DGUOK with DDI on CSC self-renewal and mtDNA levels. We have also determined the potential synergistic effects of DGUOK inhibition (with KO or DDI) and phenformin treatment. We have determined the effects of DGUOK KO on lung cancer metastasis in animal.
2. We have determined the effects of DGUOK KO and DDI treatment on tumor growth using lung cancer cell lines and patient-derived lung cancer cells.

SPECIFIC OBJECTIVES

1. To define the role of dGK in the self-renewal of NSCLC stem-like cell.
2. To define dGK as a target to inhibit NSCLC tumor growth and prevent metastasis.

SIGNIFICANT RESULTS AND KEY OUTCOMES

Major task 1: To define the role of dGK in the self-renewal of NSCLC stem-like cell.

1. DGUOK depletion significantly reduced CSC population in NSCLC. ALDH activity positive ($ALDH^+$) is lung cancer CSC markers. H1650 and LLC cells were loaded with ALDEFLUOR, a fluorescent substrate for aldehyde dehydrogenase, and effects of DGUOK depletion on the proportions of CSC with high ALDH activities were determined by flow cytometry. As shown in Fig 1A, DGUOK depletion in H1650 and LLC cells decreased the $ALDH^+$ population by twofold or five-fold, respectively. CD166, CD49 and side populations are also lung cancer CSC markers. We performed the flow cytometry with these standard lung cancer stem-like cell markers and the results of these markers were consist with ALDH activity result when we depleted DGUOK in the cells (data not shown). Thus, our data showed that DGUOK knock out robustly decrease the proportion of CSC in lung cancer. Therefore, we choose to knock out DGUOK instead of sorting out $ALDH^+$ population. The sorted cells lose ALDH+ phenotype after adherent culture for passages.

2. DGUOK depletion significantly inhibited CSC self-renewal in cell culture and animal models. The ability of individual lung cancer cells to form tumor sphere under non-adherent condition in serum-free medium is considered a surrogate assay for the self-renewal abilities of cancer cell stemness. To further investigate the role of DGUOK in CSC self-renewal, we determine the effects of DGUOK depletion on lung sphere formation in lung cancer cell lines and. The expression levels of DGUOK were comparable between established lung cancer cell lines. As shown in Figs 2B and C, the depletion of DGUOK dramatically inhibited tumor sphere formation in LLC, H1650, A549. Limiting dilution tumor initiation assay is the gold standard assay to determine cancer cell stemness. Serially diluted LLC cells were injected subcutaneously into syngeneic Albino BL6 mice, and the formation of

palpable tumor was determined 26 days after injection. Tumor-negative mice were further tracked for more than 4 months to confirm the inability of these mice to form tumor at the injection sites. As shown in Fig 1D and 1E, the tumor initiation probabilities for control LLC cells were 100% (8 out of 8), 25% (2 out of 8), and 0% (0 out of 8) at 1×10^5, 1×10^4, and 1×10^3 cells per injection, respectively. No tumor formation was detected in the DGUOK KO group at any dilution (Figs 1D and 1E), supporting an essential role for DGUOK in the self-renewal of lung cancer CSC. Ectopic expression of DGUOK in DGUOK KO LLC cells was able to partially restore tumor initiation at 1×10^5 dilution (7 out of 8) (Fig 1D and 1E).

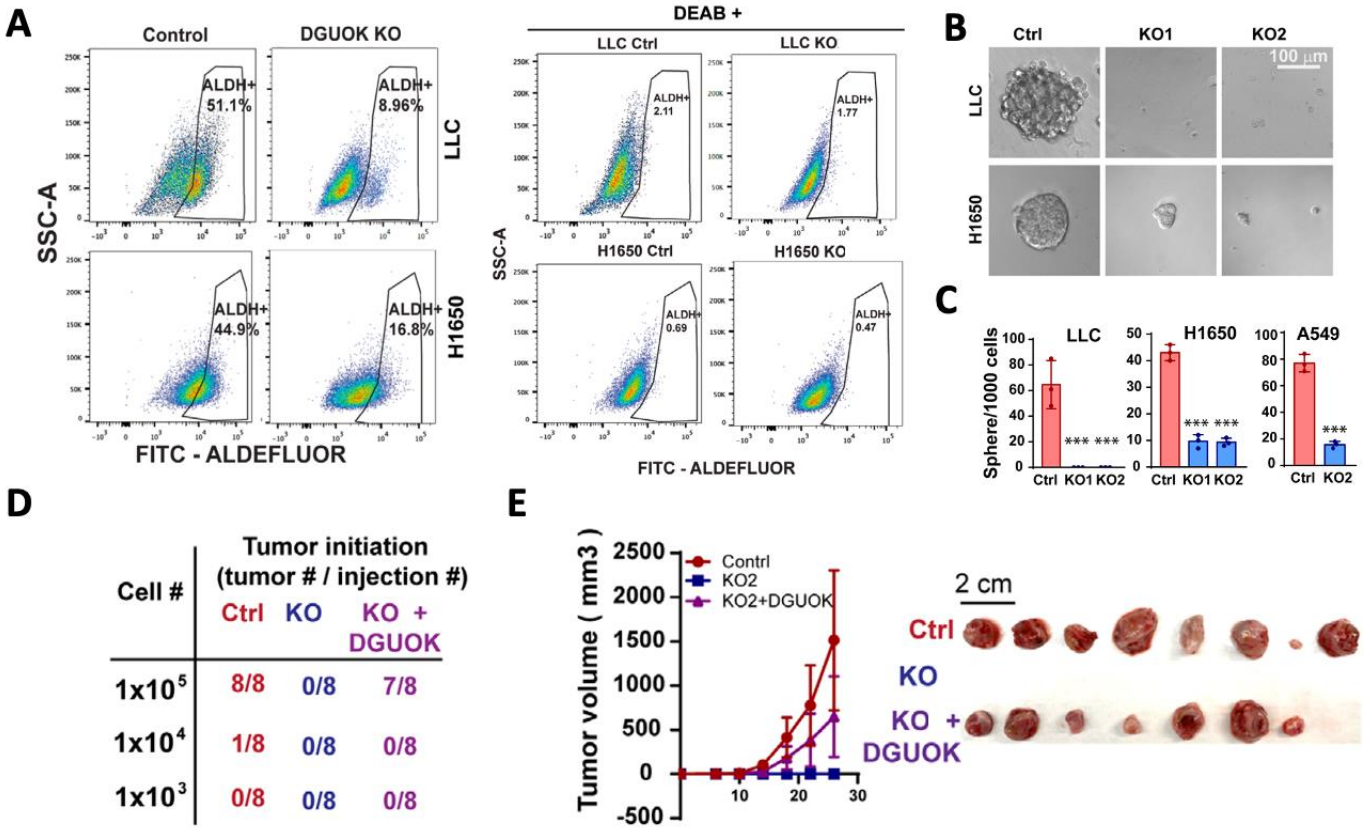

Figure 1. DGUOK regulates the self-renewal ability of NSCLC stem-like cells.

A. Flow cytometric assay using ALDEFLUOR showing the effects of DGUOK depletion on the proportion of ALDH+ cells in lung adenocarcinoma cell lines. Negative control for the ALDEFLUOR assay data. DEAB (diethylaminobenzaldehyde), an aldehyde dehydrogenase inhibitor, was used to inhibit ALDH in lung adenocarcinoma cells.

B,C. Representative images and quantitation of the effects of DGUOK depletion on lung tumor sphere formation by lung adenocarcinoma cells.

D. Limiting dilution tumor initiation assay to determine the effects of DGUOK depletion and DGUOK rescue on CSC self-renewal in LLC cells.

E. the effects of ectopic DGUOK in DGUOK KO LLC cells on tumor growth (1×10^5 cells). Data are shown as mean ± SD (n = 8). Mice were ended on day 26. Left panel, tumor growth curves from each group of mice. Right panel, the image of harvested LLC allografts tumors after euthanasia of mice on day 26 (1×10^5 cell groups).

3. <u>Our data indicated that DGUOK regulates CSC self-renewal in NSCLC through mitochondrial OXPHOS.</u>

First, DGUOK knockout reduced the mtDNA levels by 50% (Figs 2A), suggesting DGUOK is critical for mtDNA homeostasis in NSCLC.There is evidence that disruption of mtDNA homeostasis might either increase or decrease the pool of cytosolic dNTP. Since DGUOK KO decreased the mtDNA levels in lung adenocarcinoma cells, we examined the effects of DGUOK KO on the levels of cellular dNTP pools. As shown in Fig 2B, the dNTP levels all decreased by 50–70%, suggesting a role for DGUOK in the regulation of cytosolic dNTP metabolism, presumably through mtDNA homeostasis and mitochondrial metabolism.

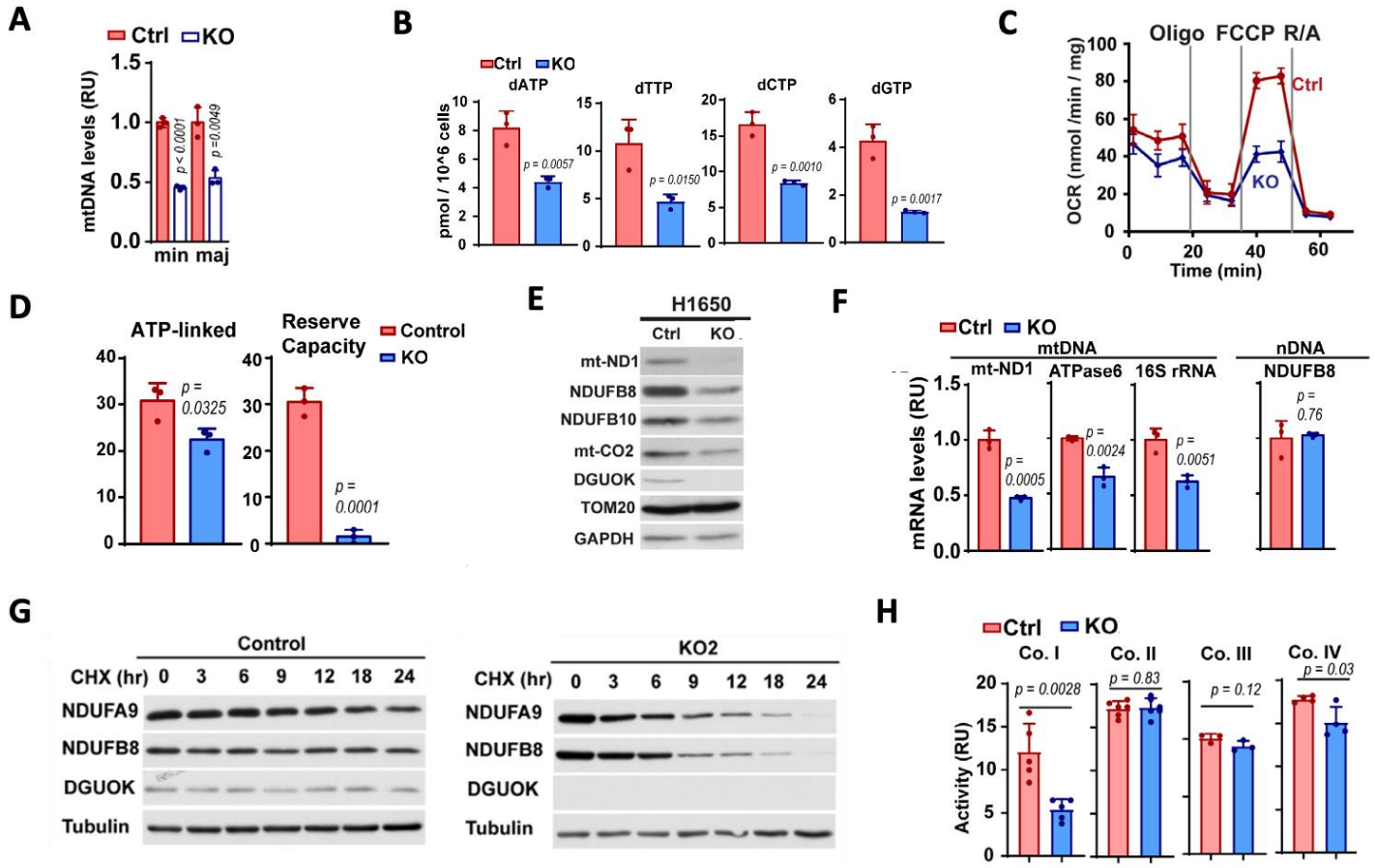

Figure 2. DGUOK is critical to the mitochondrial functionality in lung cancer.

A. The effects of DGUOK KO on mtDNA copies in H1650 cells. Two pairs of primers recognizing minor Arc (minArc) and major Arc (majArc) region of mtDNA were used for the qPCR quantitation.

B. The effects of DGUOK KO on cellular dNTP levels in H1650 cells.

C. The effects of DGUOK depletion on mitochondrial oxygen consumption rate in H1650 cells. n = 3 independent replicates per group.

D. Quantification of the effects of DGUOK depletion on ATP-linked OCR and reserve capacity OCR in H1650 cells.

E. Western blotting showing the decrease in respiration complex I subunits in DGUOK KO H1650 cells.

F. The effects of DGUOK depletion on the levels of mtDNA mRNA and nuclear DNA-encoded complex I subunits.

G. The protein stabilities of two nuclear DNA-encoded complex I subunits (NDUFA9 and NDUFB8) in controls or DGUOK KO H1650 cells. Cells were treated with 50 μg/ml cycloheximide (CHX) for indicated time to block protein translation, and the levels of remaining proteins were detected by Western blotting.

H. The effect of DGUOK depletion on the activities of respiratory complexes I, II, III, and IV.

To determine the effect of DGUOK depletion on mitochondrial OXPHOS, we used Mito Stress tests to evaluate the oxygen consumption rate (OCR) of H1650 cells in the presence of DGUOK knockout reduced the mtDNA levels and levels of mtDNA-encoded RNA by 50–70% respiratory complex inhibitors. DGUOK depletion inhibited ATP- linked OCR and reserve capacity OCR, suggesting a

crucial role for DGUOK in the mitochondrial metabolism in lung cancer cells (Figs 2C and 2D).

Since the OCR level was inhibited by DGUOK depletion, the respiration chain might be affected. To investigate the effects of DGUOK depletion on mitochondrial respiratory complexes, we used western blotting to determine the levels of complex I and complex IV subunits in control and DGUOK KO H1650 cells. As shown in Fig 2E, the expression levels of several complex I and complex IV proteins (mt-ND1, NDUFB8, NDUFB10, mt-CO2) are remarkably decreased in DGUOK KO cells. The reduced expression of complex I proteins was not due to global inhibition of mitochondrial biogenesis, since the protein levels of TOM20 (a mitochondrial outer membrane protein) were not affected (Fig 2E). The decrease in respiratory complex proteins was further confirmed when a different sgRNA was used to knockout DGUOK in lung cancer cells (Fig 2F). Although DGUOK depletion had no effects on the mRNA levels of nuclear- encoded complex I subunits (Fig 2F), the protein stability of nuclear-encoded NDUFA9 and NDUFB8 was reduced in DGUOK KO cells (Fig 2G). It is possible that the decrease in mtDNA encoded proteins inhibits the assembly of respiratory complex I, which in turn reduces the stability of nuclear-encoded subunits.

By using four different mitochondrial complexes activity kits, we also observed 56% reduction in complex I activity in DGUOK KO cells, which was consistent with the marked reduction in complex I protein levels (Fig 2H). Complex IV activities were decreased by 15%, while the activities of complexes II and III were not affected in DGUOK KO cells. It is interesting to note that 7 of the 13 mtDNA-encoded proteins are complex I subunits, which could explain the more severe inhibition of complex I activities in DGUOK KO cells.

Taken together, our data support that DGUOK is crucial for mitochondrial OXPHOS in lung adenocarcinoma.

To determine whether DGUOK regulates CSC self-renewal through mitochondrial OXPHOS, we took two independent approaches. In the first approach, we ectopically express human DGUOK in DGUOK knockout LLC (a murine lung adenocarcinoma line) cells. In the second approach, we used NDI1, a single subunit NADH: ubiquinone oxidoreductase from yeast. When expressed in DGUOK KO cells, ectopic DGUOK or NDI1 was able to restore basal, ATP-linked, and maximum capacity OCR (Figs 3A and 3B). The ectopic expression NDI1 in DGUOK KO lung cancer cells was able to at least partially restore the proportion of $ALDH^+$ population (Fig 3C), $CD166^+$ population, and "side population" (data not shown) when compared to control cells.

The ability of individual lung cancer cells to form tumor sphere under non-adherent condition in serum-free medium is considered a surrogate assay for the self-renewal abilities of cancer cell stemness. To further investigate the role of DGUOK in CSC self-renewal, we determined the effects of DGUOK depletion on lung sphere formation in lung cancer cell lines. Ectopic DGUOK and NDI1 also restore the lung sphere formation in DGUOK KO cells (Fig 3D), indicating the restoration of mitochondrial OXOPHOS was able to rescue CSC self-renewal.

To further critically evaluate the role of DGUOK and mitochondrial OXPHOS in CSC self- renewal, we used a limiting dilution tumor initiation assay to determine the effects of DGUOK depletion, DGUOK rescue, and NDI1 rescue on cancer cell stemness. Serially diluted LLC cells were injected subcutaneously into syngeneic Albino BL6 mice, and the formation of palpable tumor was determined 26 days after injection. Tumor-negative mice were further tracked for more than 4 months to confirm the inability of these mice to form tumor at the injection sites. As shown in Fig 3E, the tumor initiation probabilities for control LLC cells were 100% (8 out of 8), 25% (2 out of 8), and 0% (0 out of 8) at 1×10^5, 1×10^4, and 1×10^3 cells per injection, respectively. No tumor formation was detected in the DGUOK KO group at any dilution (Figs 3E and Fig1D), supporting an essential role for DGUOK in the self-renewal of lung cancer CSC. The ectopic expression of NDI1 in DGUOK KO LLC cells was

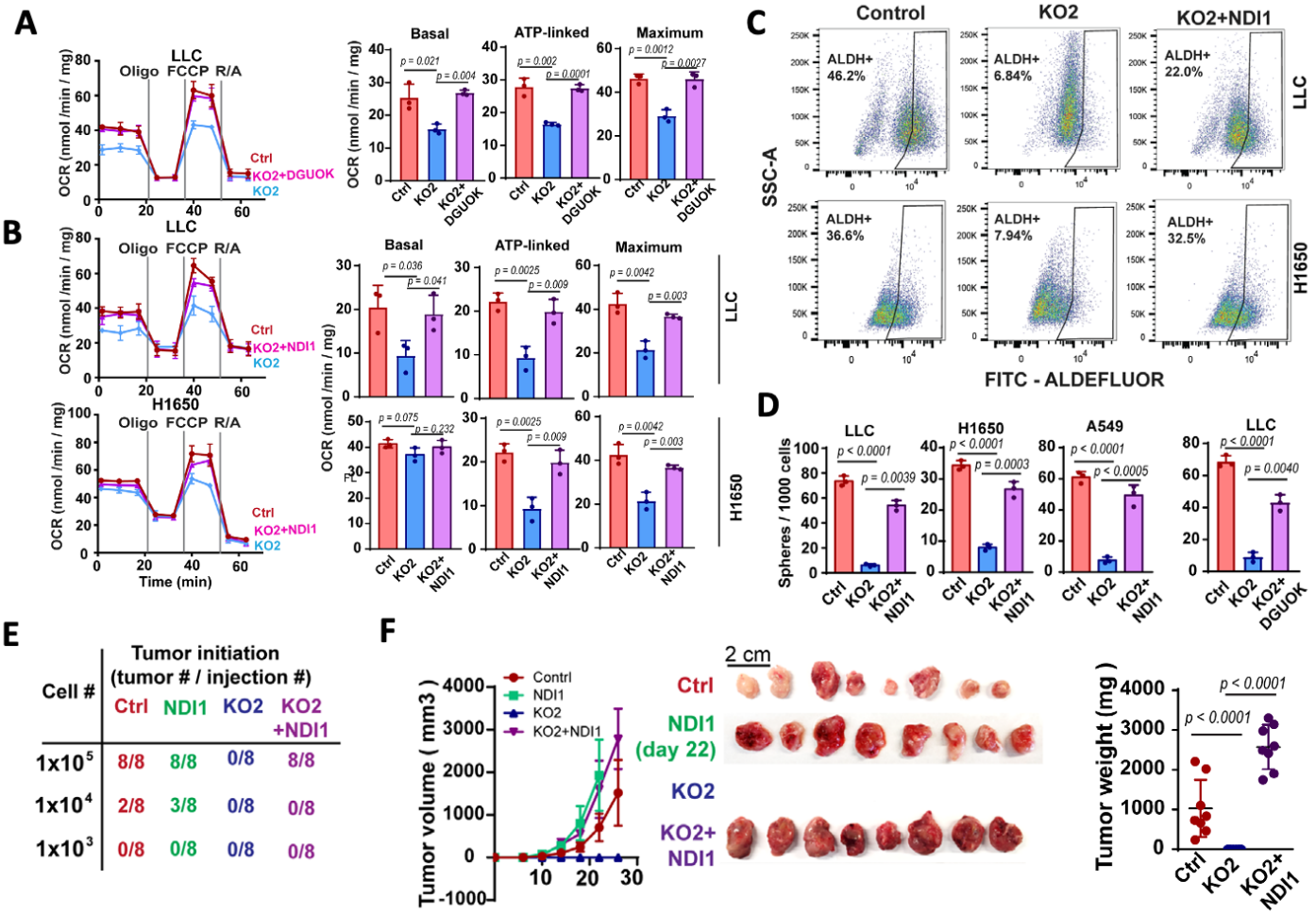

Figure 3. DGUOK regulates cancer stem cell self-renewal through mitochondrial Oxidative Phosphorylation.

A. The effects of ectopic DGUOK on mitochondrial OCR in DGUOK KO LLC, as determined by Mito Stress assay.

B. The effects of ectopic yeast NDI1 on mitochondrial OCR in DGUOK KO LLC and H1650 cells, as determined by Mito Stress assay.

C. The ectopic expression of yeast NDI1 (A) and DGUOK (B) in DGUOK KO H1650 and LLC cells was able to partially restore the levels of $ALDH^+$ CSC population, as determined by flow cytometric and ALDEFLUOR assay.

D. The ectopic expression NDI1 (D) and DGUOK (E) was able to restore lung tumor sphere formation in DGUOK KO lung adenocarcinoma cells. Data are shown as mean ± SD (n = 3 independent replicates per group).

E. Limiting dilution tumor initiation assay to determine the effects of DGUOK depletion and NDI1 rescue on CSC self-renewal in LLC cells.

F. The effects of NDI1 in DGUOK KO LLC cells on tumor growth (1×10^5 cells). Data are shown as mean ± SD (n = 8). Experiment in NDI alone group was ended on day 22 due to tumor ulceration. Other groups were ended on day 26. Left panel, tumor growth curves from each group of mice. Middle panel, the image of harvested LLC allografts tumors after euthanasia of mice. Right panel, the quantitation of tumor weight.

able to rescue tumor formation at the 1×10^5 cell dilution (8 out of 8), but not at higher dilutions (1×10^4 or 1×10^3) suggesting at least partial rescue of cancer cell stemness in DGUOK KO cells by restoration of mitochondrial OXPHOS (Fig 3E). NDI1 overexpression in control cells modestly increased tumor initiation in 1×10^4 group (3 out of 8) (Fig 3E). Similarly, ectopic expression of DGUOK in DGUOK KO LLC cells was able to partially restore tumor initiation at 1×10^5 dilution (7 out of 8) (Fig 1B). Consistent with the tumor initiation experiment, ectopic NDI1 and DGUOK were able to restore tumor growth in DGUOK KO LLC cells (Figs 3E and 3F). Intriguingly, the tumors from the NDI1 OE groups

(1×10^5 dilution, in either control or DGUOK KO LLC cells) were moderately larger than the control group (Fig 3F), indicating a role of mitochondrial OXPHOS in tumor growth.

Major task 2: To define dGK as a target to inhibit NSCLC tumor growth and prevent metastasis.

1. <u>Pharmacological inhibition of DGUOK with DDI decreased mtDNA copies and inhibited tumor sphere formation in cell culture.</u> DDI treatment partially inhibited tumor growth in xenograft mouse models.

   Next, we sought to determine whether the over-reliance of lung CSC on DGUOK could be exploited to inhibit tumor growth and to prevent tumor initiation. There is currently no specific DGUOK inhibitor available; however, several FDA-approved anti-HIV reverse transcriptase inhibitors such as didanosine (DDI) and Zidovudine have been shown to inhibit DGUOK and mtDNA homeostasis in cell culture and mouse models (Sun, Eriksson et al., 2014b, Sun, Eriksson et al., 2014c). Interestingly, there is anecdotal evidence that DDI treatment might be responsible for the long term survival in a HIV positive small cell lung cancer patient (Kato, Ieki et al., 2005). We investigated whether DDI could be repurposed as a DGUOK inhibitor to suppress CSC self-renewal in lung adenocarcinoma. As shown in Fig. 4A and 4B, DDI treatment in H1650 and three patient-derived lung adenocarcinoma lines (PDC027, PDC236 and PDC251) resulted in remarkable reduction in mtDNA copies and the protein levels of DGUOK, confirming earlier observations (Sun et al., 2014b). DDI treatment also robustly decreased the ability of PDAC cells to form tumor sphere (Fig. 4C and 4D). The administration DDI to Albino BL6 mice two days after the subcutaneous implantation of LLC cells was able to modestly inhibited tumor growth by approximately 50% (Figs 4E-4G). However, DDI

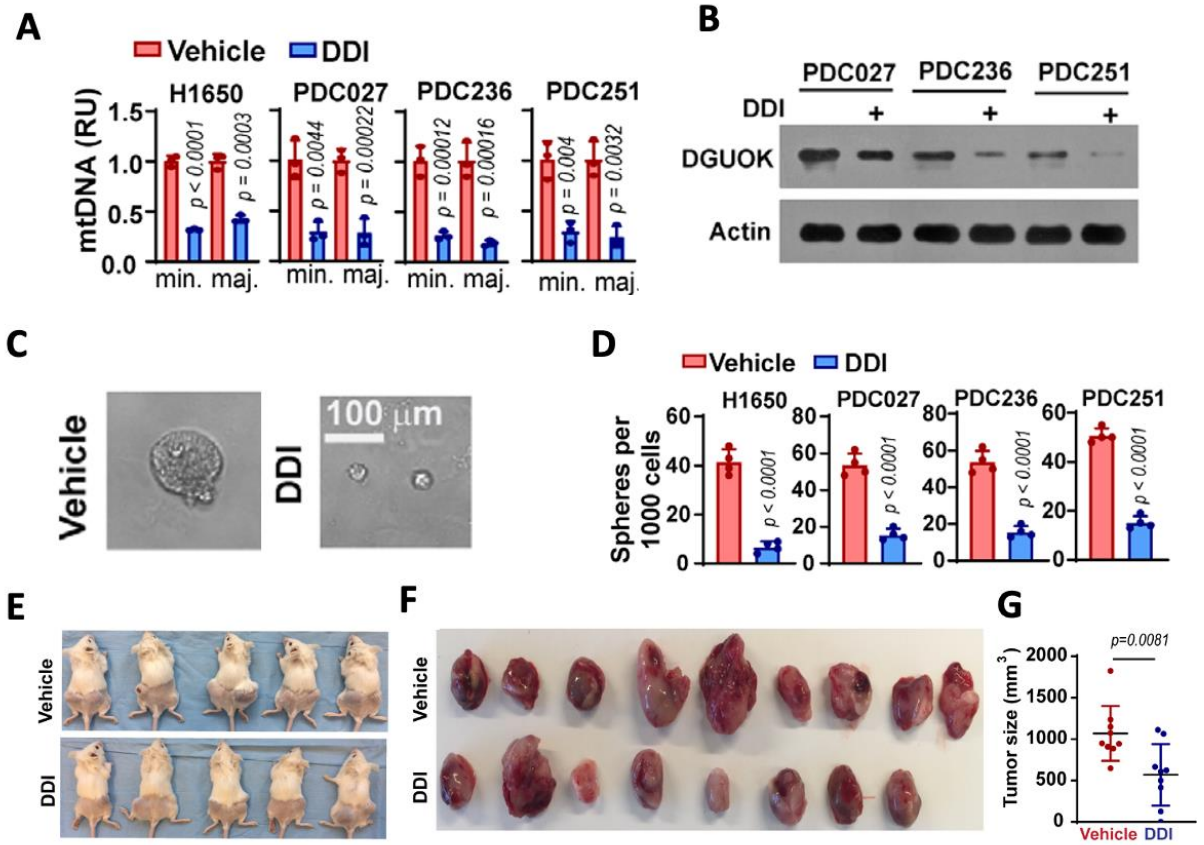

Figure 4. DDI inhibit tumor growth by targeting DGUOK.

A. The effect of DDI treatment on mtDNA copies in H1650 cells and three patient-derived cancer cells from lung adenocarcinoma (PDC027, PDC236, and PDC251). Two pairs of primers recognizing minor Arc (minArc) and major Arc (majArc) region of mtDNA were used for the qPCR quantitation. Data are shown as mean ± SD (n = 3 per group).

B. The effect of DDI treatment on DGUOK protein levels in the three patient-derived cancer cell lines.

C,D. Representative images (C) and quantitation data (D) showing the effects of DDI treatment on lung sphere formation in H1650 and patient-derived lung cancer cells. Data are shown as mean ± SD (n = 4 independent samples per group).

E-G. Images of control or DDI-treated tumor bearing mice (D), resected tumor (E), and measurement of tumor size (F) at the time of euthanasia, (n=5 inoculations per group into both flank of female Albino BL6 mice).

treatment didn't significantly affect mtDNA copies in harvested LLC allograft tumor, suggesting incomplete inhibition of DGUOK in the xenograft animal model (data not shown).

2. Genetic targeting of DGUOK using CRISPR/Cas9 was sufficient to inhibit NSCLC tumor growth and lung metastasis.

To determine whether DGUOK targeting could prevent tumor initiation, we used a Tet-On CRISPR/Cas9 system, where doxycycline treatment could efficiently induce the expression of Cas9 and the depletion of DGUOK protein levels in approximately one week (Fig. 5A). Luciferase-labeled LLC cells expressing Tet-On Cas9 and DGUOK sgRNA were injected into Albino BL6 mice. Two days after injection, mice were randomized into two groups, with one group provided with doxycycline chow to induce the depletion of DGUOK in cancer cells. As shown in Fig. 5B, doxycycline-induced DGUOK depletion inhibited 90% of tumor initiation. To evaluate the efficacies of DGUOK targeting in established tumors, LLC allograft expressing Tet-On Cas9 and DGUOK sgRNA were allowed to grow for 12 days until palpable tumors were developed. As control LLC allografts without DGUOK sgRNA were used. The mice were then randomized into two groups and provided with regular chow or doxycycline chow 12 days post implantation. As shown in Fig. 5C, 7 of the 10 LLC tumor allografts in the DGUOK KO2 group start to regress about one week after switching to doxycycline chow (when most tumor reach 50-100 mm^3). In contrast doxycycline chow had no effect on tumor growth in control LLC control group (Fig. 5C), suggesting that the tumor regression in the KO group was due to doxycycline-induced depletion of DGUOK. When the experiment ends two weeks after the doxycycline chow treatment, most of the regressing tumors have reduced to a size too small for tissue harvesting. The three tumors that didn't regress also grew slower than average tumors in the control group (Fig. 5C-5E). The western blotting analysis of homogenized tumors demonstrated about 50% decreases in DGUOK protein levels in the three remaining tumors in the doxycycline group, suggesting partial DGUOK depletion in these tumors (Fig. 5F). Doxy-induced depletion of DGUOK also reduced the levels of NDUFB8 and YAP1, as determined by Western blotting (Fig. 5F). IHC staining of harvested tumor tissues from the LLC model further confirmed the reduction of YAP1 levels from DGUOK depletion (Fig. 5G). Our data suggest that genetic targeting of DGUOK induced tumor regression through inhibition of mitochondrial OXPHOS and YAP1 signaling. Therefore, the dysregulated mitochondrial dNTP metabolism in lung adenocarcinoma CSC could be potentially targeted in lung cancer.

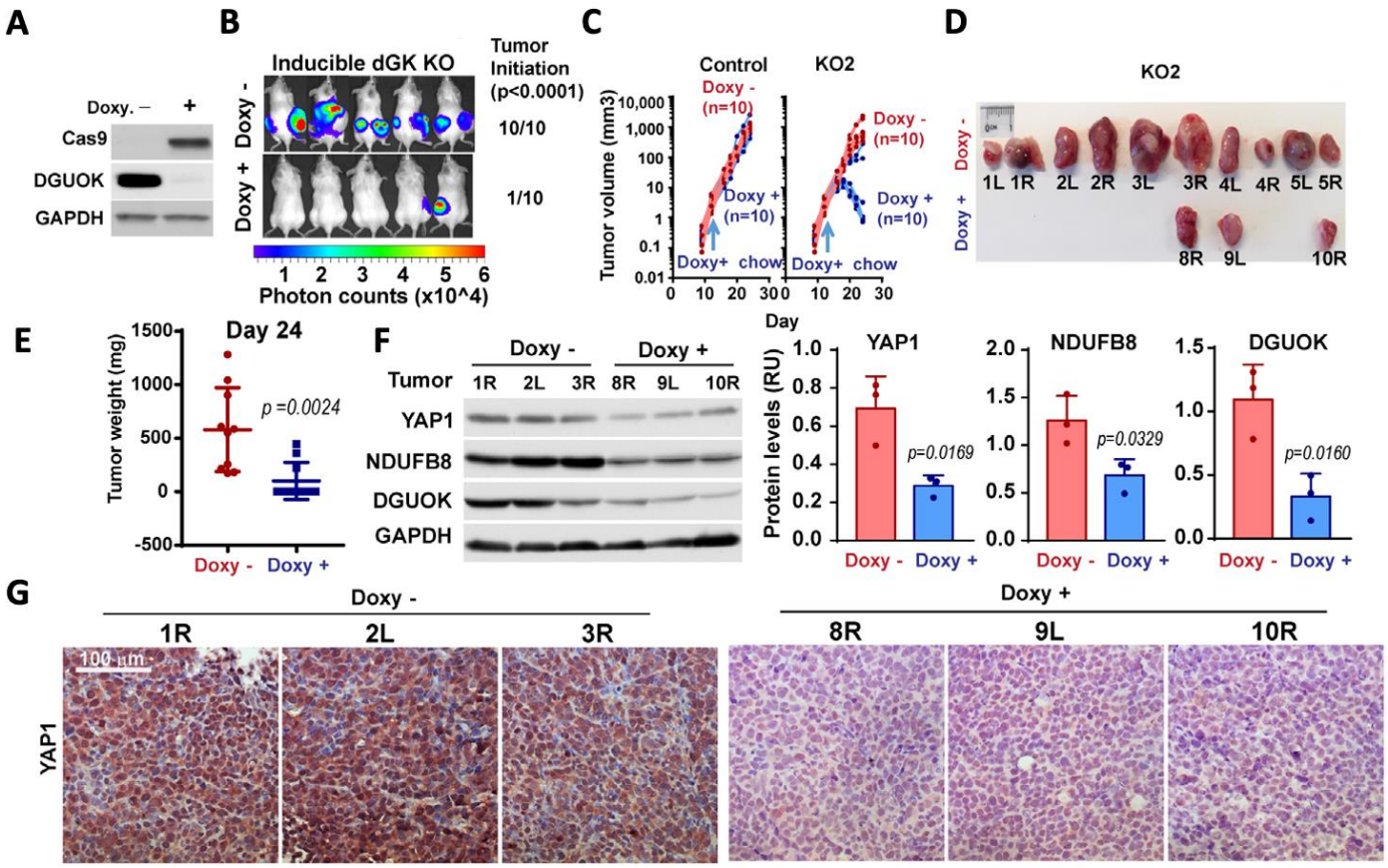

Figure 5. Genetic targeting of DGUOK inhibits tumor initiation and induces tumor regression..

A. Western blotting showing the doxycycline (doxy)-induced expression of Cas9 and the deletion of DGUOK protein expression after 7 days of treatment.

B. 1 × 10^5 LLC cells expressing Tet-On Cas9 and DGUOK sgRNA5 (KO2) were inoculated via s.c. into both flanks of Albino BL6 mice. One group of mice (n = 5 mice, 10 inoculations per group) were provided with doxycycline chow 2 days after inoculation. The formation of tumor was monitored by bioluminescence imaging28 days postinoculation. P < 0.0001, n = 10 mice per group, Fisher's exact test.

C. 1 × 10^5 LLC cells stably expressing Tet-On Cas9 and with DGUOK sgRNA (KO2 group) or without sgRNA (control group) were inoculated via s.c. into Albino BL6 mice. Mice were provided with doxycycline chow (doxy+ group), with regular chow as control (doxy+ group), 12 days (blue arrow) after inoculation (n = 5 mice, 10 inoculations per group). Tumor growth was measured using a caliper starting from day 9.

D,E. Harvested tumors (H) and quantitation of tumor weight (I) from euthanized KO2 mice from experiment in (G). Data are shown as mean ± SD (n = 5 mice).

F. Western blotting to determine the expression levels of YAP1, NDUFB8, and DGUOK in three tumors each from the doxy- and doxy+ group in (D). Densitometry quantitation of relative protein expression levels (normalized against GAPDH) is shown as bar graphs on the right. Data are shown as mean ± SD (n = 3).

G. Immunohistochemistry staining to detect the expression levels of YAP1 in tumors harvested from doxy- and doxy+ experimental groups.

3. <u>Genetic KO of DGUOK didn't have synergistic effects with phenformin treatment in NSCLC cells.</u>

It has been previously reported that defective mitochondrial OXPHOS sensitize cancer cells to metabolic stress such as phenformin and metformin. Since DGUOK ablation significantly inhibited mitochondrial OXPHOS, we examine the effect of DGUOK KO on biguanide sensitivities. As shown

in Fig. 6A, DGUOK KO had no effect on sensitivities to phenformin-induced cell death, suggesting that there was not synergistic effect between DGUOK inhibition and phenformin treatment.

Figure 6. DGUOK has no synergistic effects with phenformin treatment.

A,B. Dead cell stained with PI. Merged bright field and red fluorescence images show that DGUOK KO did not have synergistic effects with phenformin treatment (40 μM) in H1650 cell. Data are shown as mean ± SD (n = 3).

Goals not met:

We didn't determine the potential synergistic effects between DGUOK inhibition/ ablation in mouse models because our experiments in cell culture revealed that, surprisingly, DGUOK KO had no effect on the sensitivities of NSCLC cells to phenformin treatment, although DGUOK KO did inhibited mitochondrial OXPHOS.

Our data indicate that DDI treatment was able to decrease mtDNA copies and inhibit lung sphere formation in lung adenocarcinoma cell lines and patient-derived cancer cells. Although DDI as an anti-cancer agent in Lewi's lung cancer allograft model was only modestly effective, it is possible that more potent and specific DGUOK inhibitors based on these anti- HIV nucleotide analogs could be developed as therapeutics for lung adenocarcinoma patients.

In summary, our study has reached these significant accomplishments:

1. Our results indicate that depletion of DGUOK inhibits mitochondrial OXPHOS, decreased mtDNA copies and inhibited the expression and activities of the mitochondrial respiratory Complex I.

2. Multiple CSC marker was used to determine the effects of DGUOK depletion on CSC population and the self-renewal abilities of CSC. Our results indicated that DGUOK depletion remarkably decreased CSC population and inhibited CSC self-renewal in cell culture.

3. Limiting dilution tumor initiation approach was used to evaluate the role of DGUOK in CSC self-renewal. Our results indicated that DGUOK depletion abrogate the ability of lung cancer CSC to initiate tumor formation.

4. We further determined the role of mitochondrial OXPHOS in DGUOK-mediated control of CSC self-renewal. Our results indicate that restoration of mitochondrial OXPHOS in DGUOK was able to restore CSC self-renewal.

5. We used an inducible CRISPR/Cas9 system and doxycycline chow to mimic targeting of DGUOK in lung

cancer xenograft models. Our results indicate that inducible inhibition of DGUOK was able to induce tumor regression in established lung cancer xenograft.

6. We also examined the DDI inhibition in lung cancer growth. Our results indicated that DGUOK could be a pharmacological target to inhibit tumor growth. More selective and potent DGUOK ihibitors could be useful in NSCLC treatment.

Taken all together, our data support that DGUOK is required for CSC self-renewal in lung adenocarcinoma. Genetic or pharmacological targeting of DGUOK in lung adenocarcinoma cells robustly reduced the CSC population and inhibited CSC self-renewal.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

I have been invited for an oral presentation in AACR 2019 meeting. This is my first time to present my study in the biggest international meeting. I have significantly improved my oral English and the skill for presentation.

I got a scholar-in-training award in AACR 2019 meeting.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

The findings from this project has been presented at the 2019 AACR Annual Conference as an oral presentation. A manuscript summarizing the results from this project is been published.
Lin S, Huang C, Sun J, Bollt O, Wang X, Martine E, Kang J, Taylor M, Fang B, Singh PK, Koomen J, Hao J[#], **Yang S[#]**. The mitochondrial deoxyguanosine kinase is required for cancer cell stemness in lung adenocarcinoma. ***EMBO Mol. Med***. 2019 Oct 21:e10849. doi: 10.15252/emmm.201910849. [Epub ahead of print] PMID: 31633874; PMCID: PMC6895611

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to Report

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The success of proposed study is paradigm shifting in two important aspects: (1) how metastatic lung cancer cells survive dormancy and establish metastatic colonization is poorly understood and there is currently no effective approach to eliminate dormant metastatic cancer; (2) the role of deoxynucleoside salvage pathway in lung cancer metastasis is an uncharted territory.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to Report

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Authors: Shengchen Lin, Chongbiao Huang, Oana Bollt, Xiuchao Wang, Jiaxin Kang, Matthew D Taylor, Bin Fang, Pankaj K Singh, John Koomen, Jihui Hao, Shengyu Yang. The mitochondrial deoxyguanosine kinase is required for cancer cell stemness in lung adenocarcinoma. EMBO Molecular Medicine. 2019 Dec; volume 1; (12):e10849. doi: 10.15252/emmm.201910849. Epub 2019 Oct 21.
  Status of publication: Published.
  Acknowledgement of federal support: Yes.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Conference publication and oral presentation in 2019 AACR international annual meeting Abstract #973 The mitochondrial deoxyguanosine kinase regulates lung adenocarcinoma cancer stem like cells through AMPK/YAP1 signaling. Shengchen Lin etc. al.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

<u>Example:</u>

Name: *Mary Smith*

Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): 1234567
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: *Shengchen Lin*
Project Role: *PI*
Researcher Identifier (e.g. ORCID ID): 0000-0003-1488-8680
Nearest person month worked: *15*
Contribution to Project: *I have performed in experimental design and all the experiments of this project.*
Funding Support:

Name: *Shengyu Yang*
Project Role: *co-I*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *0*
Contribution to Project: *Dr. Yang participate in experimental design, data interpretation and the preparation of this report*
Funding Support: *NIH/NCI, the Pardee Foundation.*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

1R01 CA233844 (Yang) 12/1/2018-11/30/2023 3.0 cm
National Institutes of Health
A novel role of fascin in cancer metastasis
The goal of this grant is to determine how fascin controls metastatic colonization through remodeling of the mitochondrial actin filaments and augmenting mitochondrial OXPHOS.
Role: PI
Overlap: none

201303 (Yang) 10/1/2018-9/30/2019 1.8 cm
Elsa U. Pardee Foundation
Targeting a novel fascin-Nrf2 signaling circuit in metastatic lung cancer
The major goal of this grant is to investigate a novel fascin-Nrf2 signaling circuit in lung cancer metastasis.
Role: PI
Overlap: none

Penn State Cancer Institute Bridge funding 08/01/2018-07/31/2019
The Metabolic Role of Fascin in Cancer Metastasis
The major goal of this grant is to provide bridge funding to facilitate the resubmission of an R01 proposal for the PI to study the metabolic role of fascin in cancer metastasis.
Role: PI
Overlap: none

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*

- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*