

ADPGK-AS1 long noncoding RNA switches macrophage metabolic and phenotypic state to promote lung cancer growth

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Abstract

Long noncoding RNAs (lncRNAs) influence the transcription of gene networks in many cell types, but their role in tumor-associated macrophages (TAMs) is still largely unknown. We found that the lncRNA ADPGK-AS1 was substantially upregulated in artificially induced M2-like human macrophages, macrophages exposed to lung cancer cells *in vitro*, and TAMs from human lung cancer tissue. ADPGK-AS1 is partly located within mitochondria and binds to the mitochondrial ribosomal protein MRPL35. Overexpression of ADPGK-AS1 in macrophages upregulates the tricarboxylic acid cycle and promotes mitochondrial fission, suggesting a phenotypic switch toward an M2-like, tumor-promoting cytokine release profile. Macrophage-specific knockdown of ADPGK-AS1 induces a metabolic and phenotypic switch (as judged by cytokine profile and production of reactive oxygen species) to a pro-inflammatory tumor-suppressive M1-like state, inhibiting lung tumor growth *in vitro* in tumor cell-macrophage cocultures, *ex vivo* in human tumor precision-cut lung slices, and *in vivo* in mice. Silencing ADPGK-AS1 in TAMs may thus offer a novel therapeutic strategy for lung cancer.

Keywords Long noncoding RNAs; Lung cancer; Metabolic remodeling; Tumor microenvironment; Tumor-associated macrophages

Subject Categories Cancer; Immunology; Respiratory System

DOI 10.15252/embj.2022111620 | Received 9 May 2022 | Revised 6 July 2023 |

Accepted 8 July 2023 | Published online 7 August 2023

The EMBO Journal (2023) 42: e111620

Introduction

Lung cancer remains the leading cause of cancer death worldwide for both men and women, with a 5-year survival rate of $\leq 15\%$ (Siegel *et al*, 2020). Histologically, lung cancer is categorized as either small-cell or nonsmall-cell lung carcinoma, accounting for 15 and 85% of cases, respectively (Travis *et al*, 2013, 2015). Several options are available for treatment, including chemotherapy, radiotherapy, and immunotherapy targeting particular immune-system components to activate the antitumor response (Seebacher *et al*, 2019). For example, immune checkpoint inhibitors, such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD1), have been incorporated into immune-based therapies for different cancer types (Topalian *et al*, 2012; Ock *et al*, 2017). However, lung cancer is usually diagnosed at late stages, and the current standard therapies rarely lead to a positive outcome (Brambilla & Gazdar, 2009) and indeed, the majority of patients remain unresponsive (Tan *et al*, 2020).

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The tumor microenvironment (TME), which consists of various immune cells together with the extracellular matrix, endothelial cells, and fibroblasts, influences cancer biology, including the response to immune checkpoint blockade, and is highly associated with patient survival (Fridman *et al*, 2012, 2017). TAMs are the most abundant cells in the TME and, based on their immune responsiveness and metabolic profile, can have two distinct activation states (Zheng *et al*, 2017). TAMs that inhibit tumor growth, migration, and angiogenesis are known as pro-inflammatory M1-like TAMs, whereas anti-inflammatory M2-like TAMs promote tumor growth, invasion, angiogenesis, and metastasis. These two phenotypes differ in their expression of marker genes and metabolic profiles (Galvan-Pena & O'Neill, 2014). The M1-like macrophages, which may be induced by lipopolysaccharide (LPS) and/or interferon- γ (IFN γ), are characterized by high expression of inflammatory markers, such as interleukin (IL-) 8, IL-12, or tumor necrosis factor- α (TNF α), and low expression of IL-10, macrophage mannose receptor (CD206), or colony-stimulating factor 1 receptor (CSF-1R). On the other hand, M2-like macrophages, such as those induced by IL-4 *in vitro*, have low expression of IL-8, IL-12, and TNF α , whereas the expression of IL-10, CD206, and CSF-1R is highly upregulated. Several metabolomics studies have shown that M1-like macrophages mainly employ aerobic glycolysis (Tannahill *et al*, 2013; Mills *et al*, 2016), whereas M2-like macrophages present with highly active mitochondria, enhanced fatty acid oxidation, and oxidative phosphorylation (OXPHOS) (Vats *et al*, 2006; Huang *et al*, 2014). Thus, understanding how TAMs are activated and regulated within the TME is extremely important for understanding cancer biology and harnessing TAMs for future anti-cancer strategies.

During recent decades, long noncoding RNAs (lncRNAs) have emerged from being considered “transcriptional noise” to being crucial regulators of various cellular processes. They represent a vast and diverse group of ncRNAs longer than 200 nucleotides and can interact with RNA, DNA, and proteins, which enables them to regulate the transcriptional and post-transcriptional processes through various mechanisms. lncRNAs were found to participate in diverse cellular processes ranging from normal development to disease contexts, such as cancer progression (Karger *et al*, 2021). RNA-based therapies targeting deregulated lncRNAs in diseases such as cancer may represent a promising approach, particularly for patients who are refractory to other treatment options. RNA therapeutics used *in vivo* include small interfering RNAs (siRNAs), antisense oligonucleotides (ASOs) such as a small hairpin RNA (shRNA), or the locked nucleic acid (LNA) GapmeR, which can trigger RNase H-mediated RNA degradation, or CRISPR/Cas9-mediated genome editing (Hu *et al*, 2020; Lee & Mendell, 2020; Martinez-Lage *et al*, 2020; Quemener *et al*, 2020).

Contrary to most other RNA transcripts, lncRNAs are expressed in a highly tissue-specific and cell type-specific manner (Statello *et al*, 2021). Few lncRNAs that act specifically in macrophages have hitherto been described, and those that are known to affect cell proliferation, differentiation, activation status, metabolic signaling, tissue infiltration, and cell–cell interactions with cancer cells. For example, the noncoding antisense RNA guanine nucleotide-binding protein, alpha stimulating (GNAS), also known as *GNAS-AS1*, is highly upregulated in tumor-promoting M2-like macrophages, resulting in enhanced expression of anti-inflammatory cytokines and tumor progression (Sun & Xu, 2019; Liu *et al*, 2020). In contrast,

growth arrest-specific 5 (*GAS5*) is expressed in M1-like macrophages, thereby promoting a pro-inflammatory phenotype (Wang *et al*, 2020).

The lncRNA *ADPGK-AS1* is a tumor-associated RNA in different cancer types, and it promotes tumor cell proliferation and migration (Song *et al*, 2018; Luo *et al*, 2019; Yang *et al*, 2019; Jiang & Wang, 2020). Additionally, the upregulation of *ADPGK-AS1* in gastric cancer seems to correlate negatively with patient survival (Huang & Yang, 2019). The role of *ADPGK-AS1* in macrophages of the TME, however, remains largely unknown. Here we report that *ADPGK-AS1* regulates mitochondrial metabolism and a phenotypic transition of TAMs in lung cancer, and experimental manipulation of its expression allows switching between tumor-promoting and tumor-suppressive TAMs. *ADPGK-AS1* was found to localize to mitochondria, where it could bind mitochondrial ribosomal proteins, ultimately resulting in the alteration of TAM metabolism.

Results

The lncRNA *ADPGK-AS1* is upregulated in TAMs in association with mitochondria

To explore *ADPGK-AS1*-mediated regulation of cellular processes during macrophage activation, human peripheral blood mononuclear cell (PBMC)-derived macrophages were first stimulated with LPS/IFN γ (to promote an M1-like state) or IL-4 (to promote an M2-like state). Then, whole-cell transcriptomics was used to analyze the M1 and M2 phenotypes. RNA sequencing results revealed 18,032 expressed genes, including 4,545 noncoding genes (Fig 1A). Noncoding genes were further subdivided into lncRNAs, of which 407 were differentially expressed genes, and other noncoding RNAs, such as microRNAs (miRNAs) or small nucleolar RNAs (snoRNAs) (Fig 1A). lncRNAs upregulated in M2-like macrophages (Fig 1B) were validated via quantitative PCR (qPCR) (Fig 1C). Candidates for further investigation were selected through various criteria, such as protein-coding probability, RNA length, histone marks at the promoter region, or available publications (Fig EV1A). Six candidates, including *ADPGK-AS1*, *RP4-644L1.2*, *RP11-184M15.1*, *LINC01800* (AC007880.1), *RP1-80N2.1*, and *KB-1991G5.1* were then further analyzed regarding their protein-coding ability and expression pattern in various cancer cell lines. *ADPGK antisense RNA 1* (*ADPGK-AS1*) was found to be predominantly expressed as transcript variant 1 (Fig EV1B and C) and showed nonprotein-coding ability in both *in vitro* transcription and translation (Fig EV1D and E). In addition, *ADPGK-AS1* was expressed at various levels among different cancer cells and tissues, being abundant in white blood cells and in particular in macrophages and their activated phenotypes (both M1 and M2; Fig EV1F and G). Additionally, *ADPGK-AS1* was more abundant in fluorescence-activated cell sorting (FACS) sorted macrophages (TAMs) from lung cancer patients compared to macrophages obtained from healthy lung tissue (NMs) (Fig 1D), as well as IL-4 stimulated PBMCs and THP1-derived M2-like macrophages (Fig EV1H and I). These findings suggest that *ADPGK-AS1* plays an important role in TAMs and possibly lung cancer progression. Therefore, we chose *ADPGK-AS1* for further characterization.

Fluorescence *in situ* hybridization (FISH) and subcellular fractionation revealed predominant cytoplasmic localization of

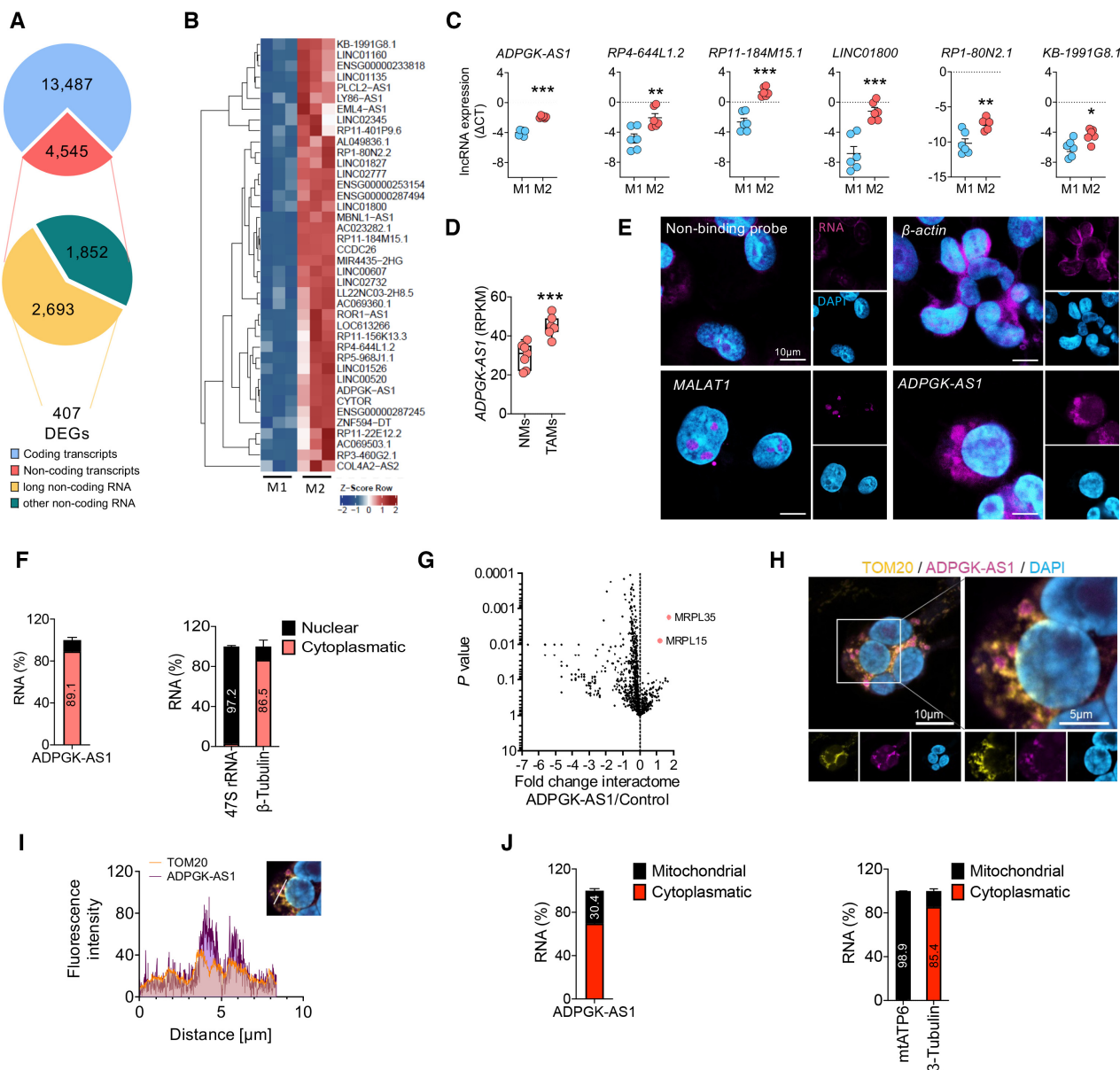


Figure 1. IncRNA *ADPGK-AS1* is upregulated in TAMs in association with an increase in mitochondrial abundance

A Pie charts of transcripts in M1-like (stimulated with LPS/IFN γ) and M2-like (stimulated with IL-4) macrophages categorized into coding and noncoding transcripts based on Ensembl gene types and the respective differentially expressed genes (DEGs) between M1 and M2 from RNA sequencing analysis.

B Heatmap of top 40 upregulated lncRNAs in M2-like macrophages (red) compared with M1-like macrophages (blue).

C Validation of upregulated lncRNAs (Δ CT) in PBMC-derived M2-like macrophages (stimulated with IL-4 for 24 h, M2). $n = 6$ biological replicates, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ compared with M1-like macrophages (LPS/IFN γ -stimulated for 24 h, M1).

D Expression analysis of *ADPGK-AS1* in TAMs sorted from healthy lung tissue (normal macrophages, NMs) or lung tumor tissue (TAMs). $n = 7$ biological replicates, $***P \leq 0.001$ compared with NMs.

E Representative images of fluorescence *in-situ* hybridization of *ADPGK-AS1* (magenta) and nuclei (blue) in IL-4 stimulated M2-like macrophages. β -actin served as the cytoplasmic control and *MALAT1* as the nuclear control. Scale bar = 10 μ m.

F RNA expression analysis of *ADPGK-AS1*, 47S ribosomal RNA (nuclear control), and β -Tubulin (cytoplasmic control) in IL-4-stimulated M2-like macrophages after subcellular fractionation. $n = 3$ independent experiments. The data represent the percentage of total detected RNA.

G Volcano plot showing the protein-interactome of *ADPGK-AS1* after RNA pulldown compared to a control RNA. $n = 5$ biological replicates. Red dots represent *ADPGK-AS1*-interacting MRPs (MRPL15, MRPL35).

H Costaining for *ADPGK-AS1* (magenta) and the mitochondrial marker TOM20 (yellow) in IL-4-stimulated M2-like macrophages. Scale bar = 10 μ m (left), 5 μ m (right).

I Fluorescence intensity profile across costained macrophages (see white line) for *ADPGK-AS1* (purple) and TOM20 (orange).

J Subcellular fractionation of IL-4-stimulated M2-like macrophages and isolation and analysis of *ADPGK-AS1* abundance in the cytoplasmic and mitochondrial fractions. The mitochondrial encoded RNA *mtATP6* served as the mitochondrial control and β -tubulin as the cytoplasmic control. $n = 3$ independent experiments, the data represent the percentage of total detected RNA.

ADPGK-AS1 in macrophages (Fig 1E and F). To identify proteins that interact with *ADPGK-AS1*, mass spectrometry analyses of proteins from *ADPGK-AS1* pulldown assays were performed (Fig EV1J). The results revealed that *ADPGK-AS1* could bind two mitochondrial ribosomal proteins (MRPs), namely MRPL35 and MRPL15 (Fig 1G; Appendix Table S1). Interestingly, costaining of macrophages for *ADPGK-AS1* and the mitochondrial marker TOM20 revealed overlapping staining, indicating that *ADPGK-AS1* can enter the mitochondria (Fig 1H and I). Subcellular fractionation of M2-like macrophages followed by RNA expression analysis revealed that ~30% of *ADPGK-AS1* resided in the mitochondrial fraction and ~70% in the cytoplasm (Figs 1J and EV1K). Taken together, these results suggest that *ADPGK-AS1* plays a role in the mitochondrial aspects of the M2-like macrophage phenotype.

MRPL35 is abundant in M2-like TAMs and is associated with poor survival in lung cancer patients

To determine whether MRPs interact with *ADPGK-AS1*, we performed RNA immunoprecipitation (RIP) with antibodies targeting MRPL15 and MRPL35, followed by RNA isolation and RT-qPCR for *ADPGK-AS1*. This analysis demonstrated that *ADPGK-AS1* was significantly enriched for MRPL35 (Fig 2A). Costaining of *ADPGK-AS1* and MRPs (MRPL35 and MRPL15; Fig 2B and C) also showed colocalization of *ADPGK-AS1* with MRPL35. We also analyzed MRPL15 and MRPL35 in tissue microarrays (TMAs) of lung cancer patients by multiplex immunofluorescence imaging (Fig 2D). Interestingly, MRPL35 was expressed mainly in M2-like TAMs ($CD68^+CD206^+TNF\alpha^-$) and not at all in M1-like TAMs ($CD68^+CD206^-TNF\alpha^+$) (Fig 2E). Besides macrophages, a few other cell types of the tumor microenvironment expressed a low level of MRPL35, and MRPL35 was not detected in the tumor-cell area (PanCK⁺). Additionally, we assessed the disease-free survival (DFS) and overall survival (OS) of patients with high or low expression of MRPL35 in M2 TAMs. Patients with high MRPL35 expression had significantly decreased DFS and OS (Fig 2F and G), suggesting that an abundance of MRPL35 in M2-like TAMs promotes lung cancer progression.

ADPGK-AS1 overexpression alters the regulation of mitochondrial metabolism

To further assess the function of *ADPGK-AS1* in cellular energy production and mitochondrial metabolism, we generated a stable *ADPGK-AS1* overexpression (OE) line in macrophages, and *ADPGK-AS1* OE was confirmed by qPCR and FISH (Fig 3A and B). Interestingly, *ADPGK-AS1* OE led to an increased number of mitochondria, as observed by staining the macrophages with MitoTracker and TOM20 (Fig 3C and D). Additionally, upregulated expression was observed for the three mitochondria-associated proteins TOM20, cytochrome C, and α b-crystalline, as well as for MRPL35 (Fig 3E). Mitochondrial DNA (mtDNA) was then isolated, and its ratio to genomic DNA, as well as a PCR analysis of the mitochondrial genes *NADH-ubiquinone oxidoreductase* (*ND1*, *ND2*, *ND4*), *ATP synthase membrane subunits* (*ATP6*, *ATP8*), *cytochrome C oxidase* (*CO1*, *CO2*), and *cytochrome B* (*CyB*) further confirmed the increased number of mitochondria in *ADPGK-AS1* OE macrophages (Fig 3F and G). In addition, the measurement of ATP production (Fig 3H) and oxygen consumption by the seahorse method revealed enhanced spare

capacity and oxygen consumption rate (OCR) in the seahorse assay (Fig 3I). Additionally, mitochondrial translational activity was seemingly elevated in *ADPGK-AS1* OE macrophages (Appendix Fig S1A–C). Furthermore, we observed downregulation of mitochondrial fusion markers (*Mfn1* and *Opa1*) and increased levels of mitochondrial fission marker via upregulation of phosphorylated dynamin-related protein-1 (pDrp1 at Ser616), and the phosphorylation of AMPK (pAMPK) was observed (Fig 3J). Notably, the upregulation of the mitochondrial fission markers upon *ADPGK-AS1* OE was reversed upon *ADPGK-AS1* knockdown (Fig EV2A). In addition, confocal microscopy images of TOM20-stained and transmission electron microscopy images of macrophages confirmed an increase in the numbers of smaller, fragmented mitochondria in *ADPGK-AS1* OE macrophages (Fig 3K and L). Overall, these findings suggest that *ADPGK-AS1* plays a fundamental role in mitochondrial dynamics in macrophages, including metabolic regulation and mitochondrial fragmentation.

ADPGK-AS1 impacts the macrophage metabolic state of macrophages

We also examined the role of *ADPGK-AS1* in metabolic signaling in macrophages. Metabolome analysis of *ADPGK-AS1* OE macrophages revealed increased levels of metabolites of the TCA cycle, such as malate, α -ketoglutarate (α KG), and 2-hydroxyglutarate (2-HG), in *ADPGK-AS1* OE macrophages, whereas lactate levels were decreased (Fig 4A). These results indicated an increase in the activity of mitochondrial metabolic pathways, which was confirmed by an increase in pyruvate dehydrogenase (PDH) protein levels (Fig EV2B), a decrease in lactate dehydrogenase (LDH) activity (Fig EV2C), and an increase in succinate dehydrogenase (SDH) and isocitrate dehydrogenase 1 (IDH1) activities (Fig EV2D–G). Analysis of the metabolome in *ADPGK-AS1*-deficient M2-like macrophages (Fig EV2H) revealed effects opposite to those of *ADPGK-AS1* OE (Fig EV2I). Furthermore, *ADPGK-AS1* OE increased mitochondrial membrane potential as determined by the accumulation of JC-1 aggregates (Fig 4B–D), which was rescued by *ADPGK-AS1* and MRPL35 knockdown. Furthermore, MitoSOX staining revealed decreased ROS levels in *ADPGK-AS1* OE macrophages, which increased again after the knockdown of *ADPGK-AS1* and MRPL35 (Fig 4E). Similarly, in the seahorse assay, the increased OCR in *ADPGK-AS1* OE macrophages was reduced after *ADPGK-AS1* knockdown as well as after MRPL35 knockdown (Fig 4F). These data suggested that *ADPGK-AS1* OE macrophages have highly active mitochondria, increased levels of TCA cycle metabolites, increased activities of TCA cycle enzymes, and reduced LDH activity. In summary, the results indicated that *ADPGK-AS1* functions through MRPL35 to determine the macrophage metabolic state.

Macrophages upregulate *ADPGK-AS1* and adapt their metabolic signaling pathways after cross talking with tumor cells

To further characterize the role of *ADPGK-AS1* in macrophages and the associated changes we identified in macrophage metabolism in the context of lung cancer, *ADPGK-AS1* expression in macrophages was examined after indirect coculture with the lung adenocarcinoma cell line A549 (Fig 5A). After coculture, the M1-like macrophage marker genes (*TNF α* and *IL-8*) were downregulated in the

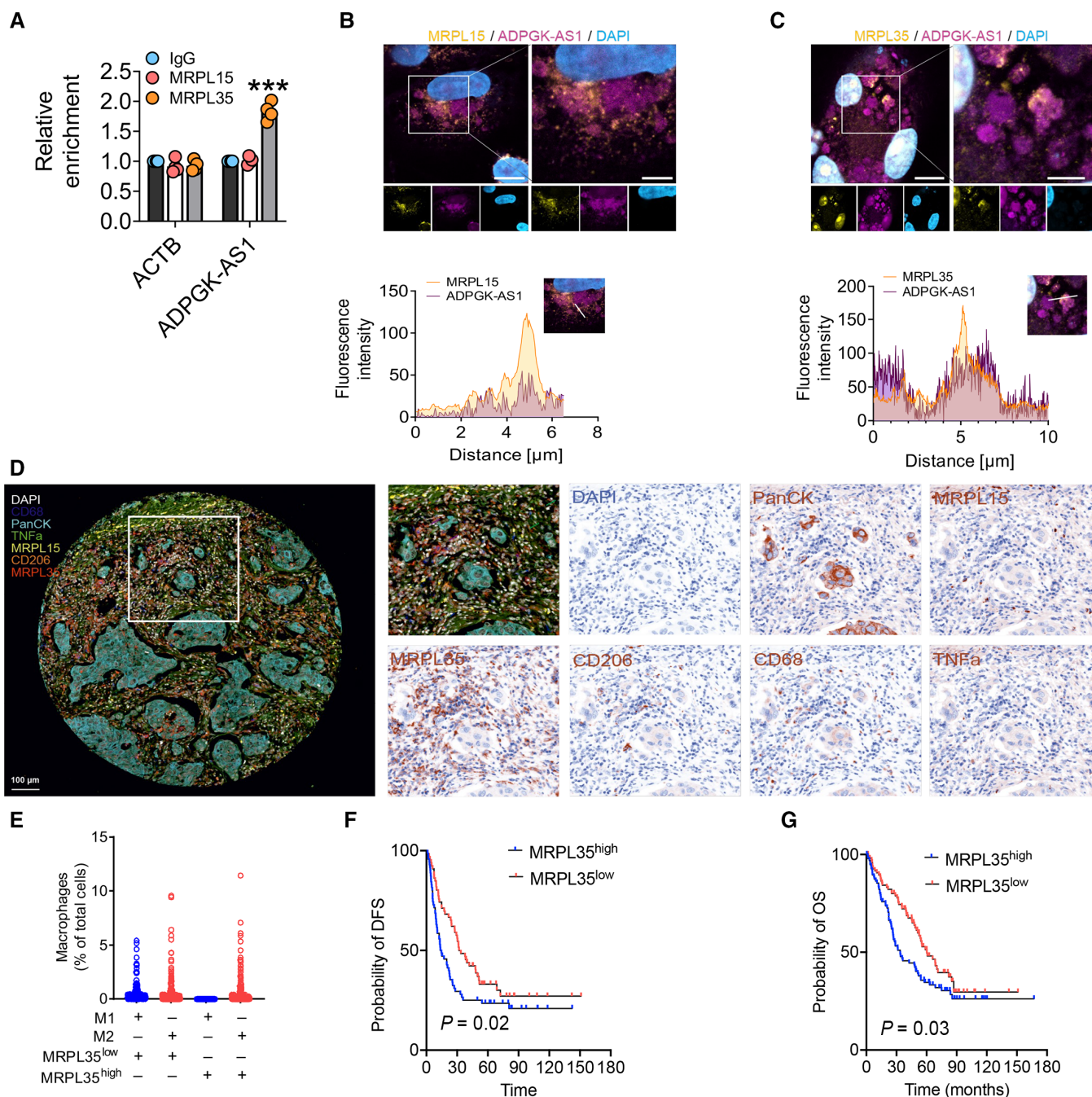


Figure 2. ADPGK-AS1 interacts with MRPL35, and increased expression of MRPL35 in M2-like TAMs is negatively associated with the survival of lung cancer patients.

- A** Validation of the ADPGK-AS1-interacting protein MRPL35 by RNA immunoprecipitation (RIP) followed by qPCR for ADPGK-AS1. $n = 4$ independent experiments; *** $P \leq 0.001$ compared with IgG control.
- B, C** Costaining for ADPGK-AS1 (magenta) and MRPL35 or MRPL15 (yellow) with respective fluorescence intensity across costained macrophages (see white line) for ADPGK-AS1 and MRPL35 or MRPL15. Scale bar = 10 μ m (left), 5 μ m (right).
- D** Multiplex immunofluorescence staining of TAMs of lung cancer tissues for CD68 (dark blue), PanCK (cyan), TNF α (green), MRPL15 (yellow), CD206 (orange), MRPL35 (red), and DAPI (white). $n = 200$ biological replicates.
- E** Number of M1-like or M2-like macrophages based on TAMs data for lung cancer patients with either high (above median) or low (below median) expression of MRPL35.
- F, G** Kaplan–Meier curve estimate of disease-free survival (DFS) or overall survival (OS) of patients with high (above median) or low (below median) expression of MRPL35 in M2-like macrophages. $n = 200$ biological replicates.

macrophages, whereas the M2-like macrophage marker genes (*CD206* and *CSF-1R*) were upregulated. This demonstrated that the cocultured macrophages inherited a tumor-promoting phenotype (Fig 5B). Cocultured macrophages with A549 cells also increased *ADPGK-AS1* expression in comparison with macrophage controls (Fig 5C), indicating that *ADPGK-AS1* is indeed associated with M2-like TAMs, with a potential impact on disease development. Metabolome analysis of lysates of cocultured macrophages revealed

upregulation of TCA cycle metabolites (Fig 5D), similar to that in macrophages overexpressing *ADPGK-AS1*.

Next, we analyzed the culture medium of the macrophage-A549 indirect coculture system to determine whether any secreted metabolites could be responsible for the induction of *ADPGK-AS1* expression in the macrophages. The results revealed significantly higher concentrations of α KG, gluconic acid, and prostaglandin E2 in the coculture medium, whereas lactate concentration was lower (Fig 5E).

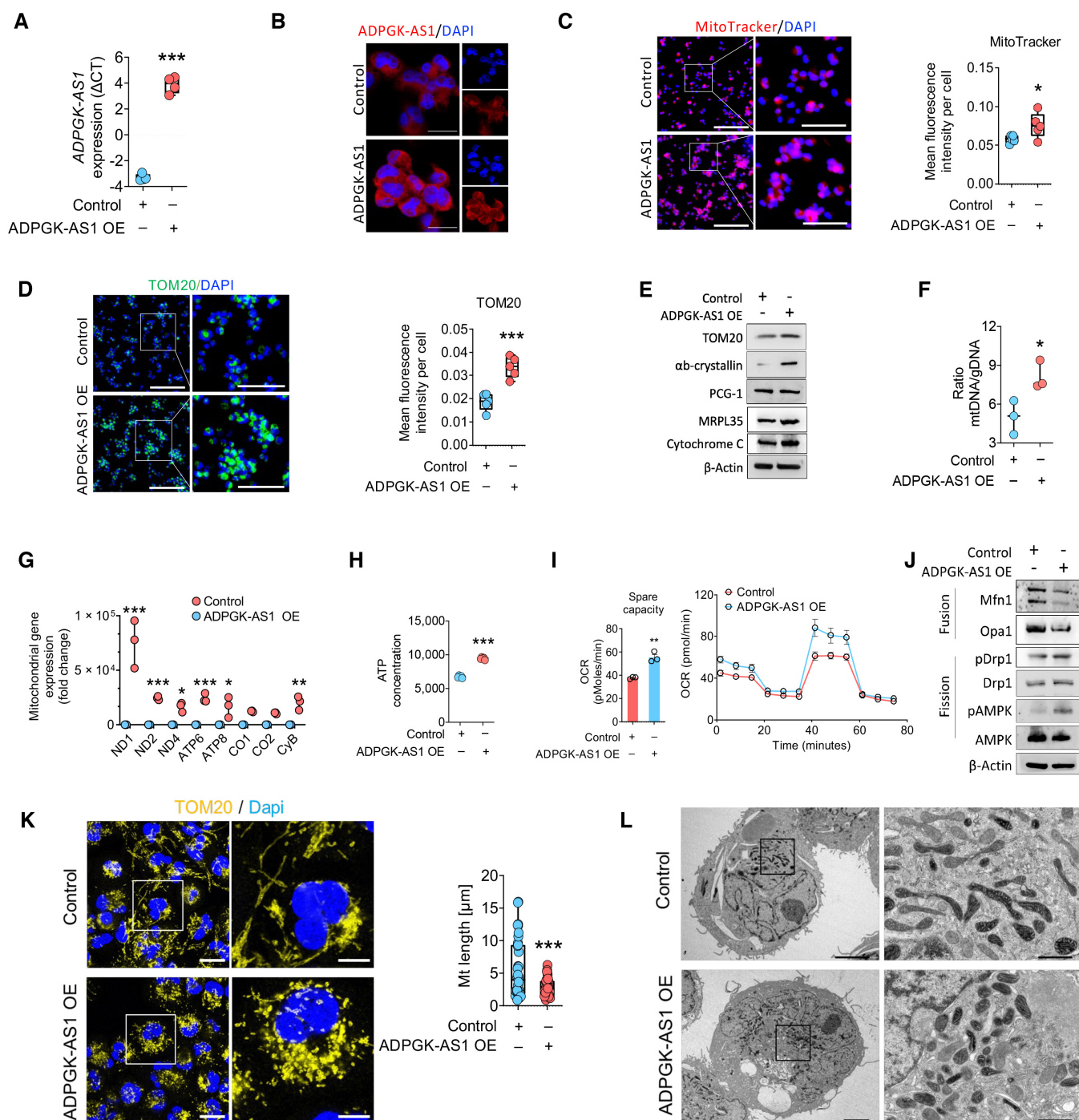


Figure 3.

Figure 3. ADPGK-AS1 overexpression alters the regulation of mitochondrial metabolism.

- A Expression of *ADPGK-AS1* lncRNA (Δ CT) in *ADPGK-AS1* overexpressing (OE) THP1 cells. $n = 4$ independent experiments. $***P \leq 0.001$ compared with control.
- B Representative FISH images of *ADPGK-AS1* (red) and nuclei (blue) in *ADPGK-AS1* OE THP1 cells. Scale bar = 25 μ m.
- C, D Representative images and quantification of mitochondria by MitoTracker (red) and TOM20 (green) in THP1 control and *ADPGK-AS1* OE cells. Nuclei were stained with DAPI (blue). Scale bar = 200 μ m (left), 100 μ m (right, magnification).
- E Representative immunoblot for mitochondrial protein expression (TOM20, α B-crystallin, PCG-1, MRPL15, MRPL35, cytochrome C) in THP1 control and *ADPGK-AS1* OE cells. $n = 4$ independent experiments.
- F Ratio of mitochondrial DNA (mtDNA) to genomic DNA (gDNA) content in THP1 control and *ADPGK-AS1* OE cells. $n = 3$ independent experiments. $*P \leq 0.05$ compared with control.
- G Gene expression analysis (fold change) of mitochondrially encoded genes (ND1, ND2, ND4, ATP6, ATP8, CO1, CO2, Cyb) after isolation of mtDNA from THP1 control and *ADPGK-AS1* OE cells. $n = 3$ independent experiments. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ compared with control.
- H, I ATP production ($n = 5$) and full oxygen consumption plot ($n = 8$) by the seahorse assay in THP1 control and *ADPGK-AS1* OE cells. $**P \leq 0.01$, $***P \leq 0.001$ compared with control.
- J Representative immunoblot of proteins involved in fission and fusion processes of mitochondria (Mfn1, Opa1, Drp1, pAMPK, AMPK). $n = 4$ independent experiments.
- K Representative confocal fluorescence images and quantification of mitochondrial shape with the mitochondrial marker TOM20 (yellow) in THP1 control and *ADPGK-AS1* OE cells. Nuclei were stained with DAPI (blue). $n = 35$ cells, Scale bar = 10 μ m (left), 5 μ m (magnification, right).
- L Transmission electron microscopy images of mitochondrial shapes in THP1 control and *ADPGK-AS1* OE cells. $n = 4$ cells, Scale bar = 5 μ m (left), 1 μ m (right, magnification).

Source data are available online for this figure.

Treatment of macrophages with the identified metabolites showed that only α KG significantly induced *ADPGK-AS1* expression (Fig 5F). To further analyze whether α KG could be involved in signaling upstream of macrophage *ADPGK-AS1* expression, we used A549 cells with a siRNA specific for IDH1, which is the enzyme mainly responsible for α KG production (Fig 5G). Macrophages that were treated with conditioned medium (CM) of IDH1 knockdown A549 cells had reduced induction of *ADPGK-AS1* expression compared with macrophages treated with A549 control-CM (Fig 5H), thereby supporting a potential role for α KG in *ADPGK-AS1* expression. Overall, these results suggest that *ADPGK-AS1* expression in macrophages is induced as a consequence of crosstalk between macrophages and tumor cells, with tumor-cell secreted α KG playing a major role. These results confirmed the induction of a tumor-promoting M2 macrophage phenotype and a related metabolic switch.

ADPGK-AS1 promotes a switch in macrophage phenotype from M1 like to M2-like, with an impact on cancer cell biology

Against the background of upregulated *ADPGK-AS1* in cancer cell cocultured macrophages and TAMs from patients with lung cancer, we hypothesized that *ADPGK-AS1*-induced changes in macrophages could impact the biology of adjacent cancer cells. We therefore examined macrophage activation marker genes in *ADPGK-AS1* OE macrophages (Fig 6A and B). M1-like marker genes, such as *TNF α* , *IL-8*, and *CXCL10* were downregulated, whereas M2-like marker genes, such as *CSF-1R*, *CD206*, *IL-10*, *ALOX15*, *TGF β* and *IL-1Ra*, were upregulated. CM from *ADPGK-AS1* OE macrophages did not affect tumor-cell proliferation (Figs 6C and EV3A), but it reduced the capacity of tumor cells to undergo apoptosis and increased tumor-cell migration compared with tumor cells incubated with macrophage control-CM (Figs 6D and E, and EV3B and C). These results demonstrated that *ADPGK-AS1* OE in macrophages is sufficient to induce a tumor-promoting M2-like phenotype. Interestingly, inhibition of translation in *ADPGK-AS1* OE macrophages led to rescue of the M2-like phenotype by upregulation of M1-like marker genes (*TNF α* and *IL-8*) and downregulation of M2-like marker genes (*CSF-1R* and *CD206*, Appendix Fig S1D), further indicating a

connection between *ADPGK-AS1*, mitochondrial dynamics, and macrophage polarization. Thus, the downregulation of *ADPGK-AS1* might serve as a novel approach to the development of lung cancer treatments. To examine this possibility, we isolated primary PBMC-derived macrophages, activated them to generate an M1-like or M2-like phenotype (Sarode *et al*, 2020b), and subsequently silenced *ADPGK-AS1* by LNA GapmeRs (Fig 6F). This approach resulted in the reversal of the effects of *ADPGK-AS1* OE. M1-like macrophage markers, such as *TNF α* , *IL-8*, *CD80*, and *CCR7*, were upregulated in M2-like *ADPGK-AS1* knockdown (KD) macrophages, whereas expression of *CSF-1R*, *CD206*, *IL-10*, and *TGF β* were downregulated (Fig 6G and H). CM from M2-like *ADPGK-AS1* GapmeR macrophages did not affect tumor cell proliferation, but increased tumor cell apoptosis and decreased migration capacity in comparison with tumor cells incubated with control CM (Figs 6I–K, and EV3D–F). To further demonstrate that *ADPGK-AS1* function is a consequence of its mitochondrial localization, we ruled out that it functions via ADP-dependent glucokinase (ADPGK), that is, its corresponding protein-coding gene (Fig EV3G). Indeed, the cellular abundance of ADPGK was elevated in macrophages overexpressing *ADPGK-AS1* or treated with GapmeR (Fig EV3H and I). However, a luciferase assay-based analysis did not reveal any direct regulation of the ADPGK promoter by *ADPGK-AS1* (Fig EV3J). Additionally, the treatment of macrophages with 8-bromo-AMP, an established inhibitor of ADPGK (Grudnik *et al*, 2018), did not have a similar effect on macrophage activation as *ADPGK-AS1* knockdown (Fig EV3K), including impact on tumor-cell functions (Fig EV3L). Finally, we investigated whether *ADPGK-AS1* OE or knockdown could also affect the M1-like macrophage phenotype. Although the expression of M1 marker genes increased after *ADPGK-AS1* knockdown in M1-like macrophages, the expression of M2 macrophage marker genes was not affected; moreover, there was no effect on tumor cell proliferation, apoptosis, or migration compared with the M1 control, suggesting that the observed effect on the modulation of *ADPGK-AS1* expression was specific to M2-like macrophages (Appendix Figs S2 and S3). These results demonstrated that *ADPGK-AS1* plays a crucial role in macrophage activation and phenotypic state, with a consequent impact on tumor cell apoptosis and migration.

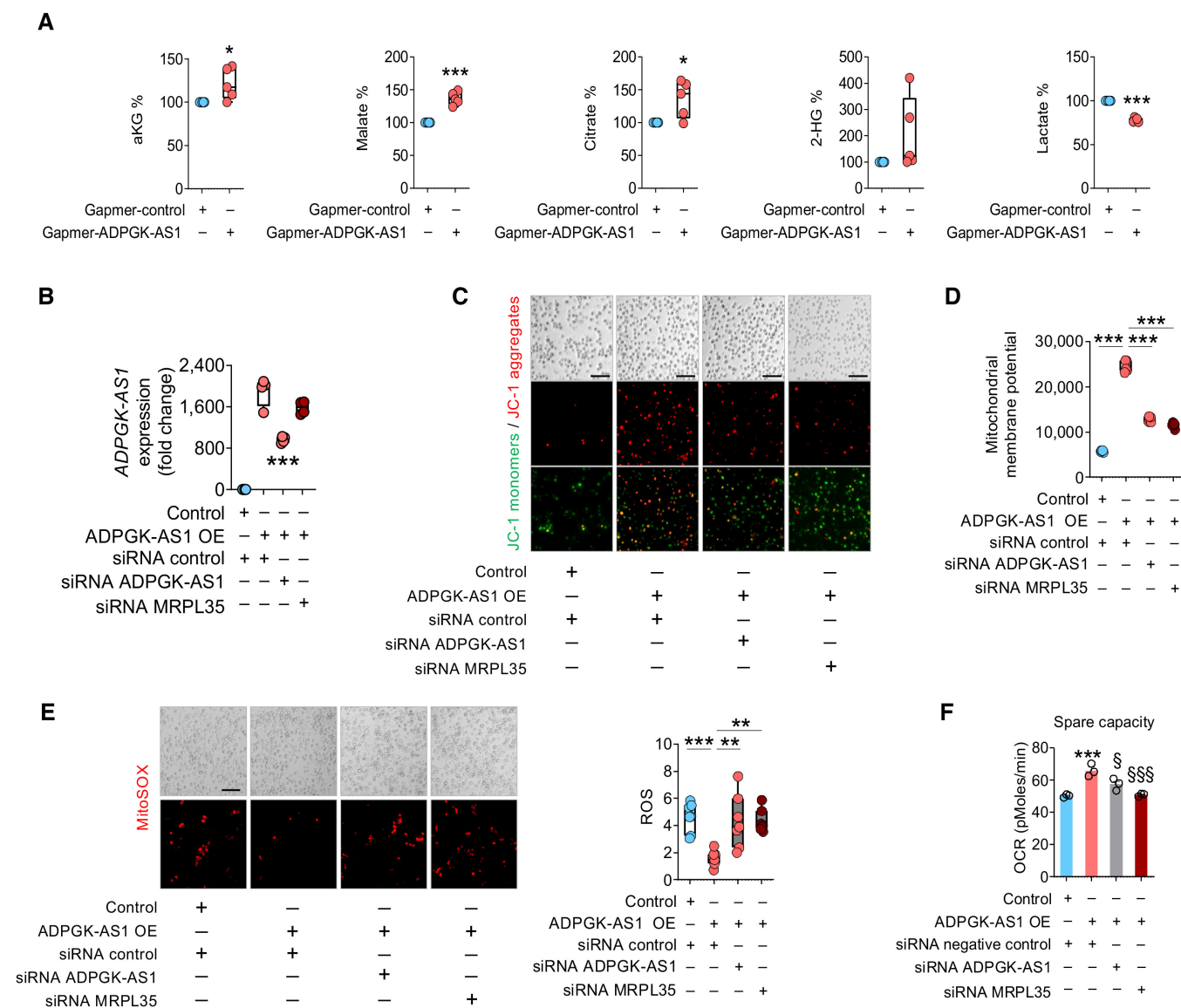


Figure 4. ADPGK-AS1 expression has an impact on macrophage metabolic state.

A LC-MS/MS analysis of deregulated metabolites in cell lysates of THP1 control and *ADPGK-AS1* OE macrophages. Results represent the percentage of the ratio of lysate to supernatant. $n = 5$ biological replicates. $^*P \leq 0.05$, $^{***}P \leq 0.001$ compared with control.

B Expression analysis of *ADPGK-AS1* and *MRPL35* (fold change) in THP1 control and *ADPGK-AS1* OE macrophages transfected with a siRNA against *ADPGK-AS1*, *MRPL35*, or a negative control. $n = 4$ independent experiments. $^{***}P \leq 0.001$ compared with the *ADPGK-AS1* OE siRNA control.

C Representative images of JC-1 monomers (green) and aggregates (red) in THP1 control and *ADPGK-AS1* overexpressing macrophages transfected with a siRNA against *ADPGK-AS1*, *MRPL35*, or a negative control. Scale bar = 100 μm .

D Mitochondrial membrane potential measured at 590 nm (JC-1 aggregates) in THP1 control and *ADPGK-AS1* OE macrophages transfected with a siRNA against *ADPGK-AS1*, *MRPL35*, or a negative control. $n = 5$ independent experiments. $^{***}P \leq 0.001$ compared with control.

E Representative images (left panel) and quantification (right panel) of ROS accumulation in THP1 control and *ADPGK-AS1* OE cells transfected with a siRNA against *ADPGK-AS1*, *MRPL35*, or a negative control. $n = 7$. $^{***}P \leq 0.001$ compared to control.

F Spare capacity (oxygen consumption rate) measured with the seahorse assay in THP1 control and *ADPGK-AS1* OE cells transfected with a siRNA against *ADPGK-AS1*, *MRPL35*, or a negative control. $n = 3$ independent experiments. $^{***}P \leq 0.001$ compared with control, $^sP \leq 0.05$, $^{sss}P \leq 0.001$ compared with *ADPGK-AS1* OE siRNA negative control.

ADPGK-AS1 knockdown inhibits tumor growth *in vivo*

To further understand the effect of *ADPGK-AS1* on macrophage biology and tumor growth and progression *in vivo*, we coinjected immunosuppressed mice (*NOD-scid IL2R γ manu* mice; NSG mice)

with A549 human lung cancer cells along with macrophages transfected with a vector for *ADPGK-AS1* knockdown (or negative control vector) and monitored tumor growth (note that *ADPGK-AS1* does not have an annotated ortholog in mice; Appendix Fig S4A and B). Tumor growth was enhanced in control mice as well as in mice

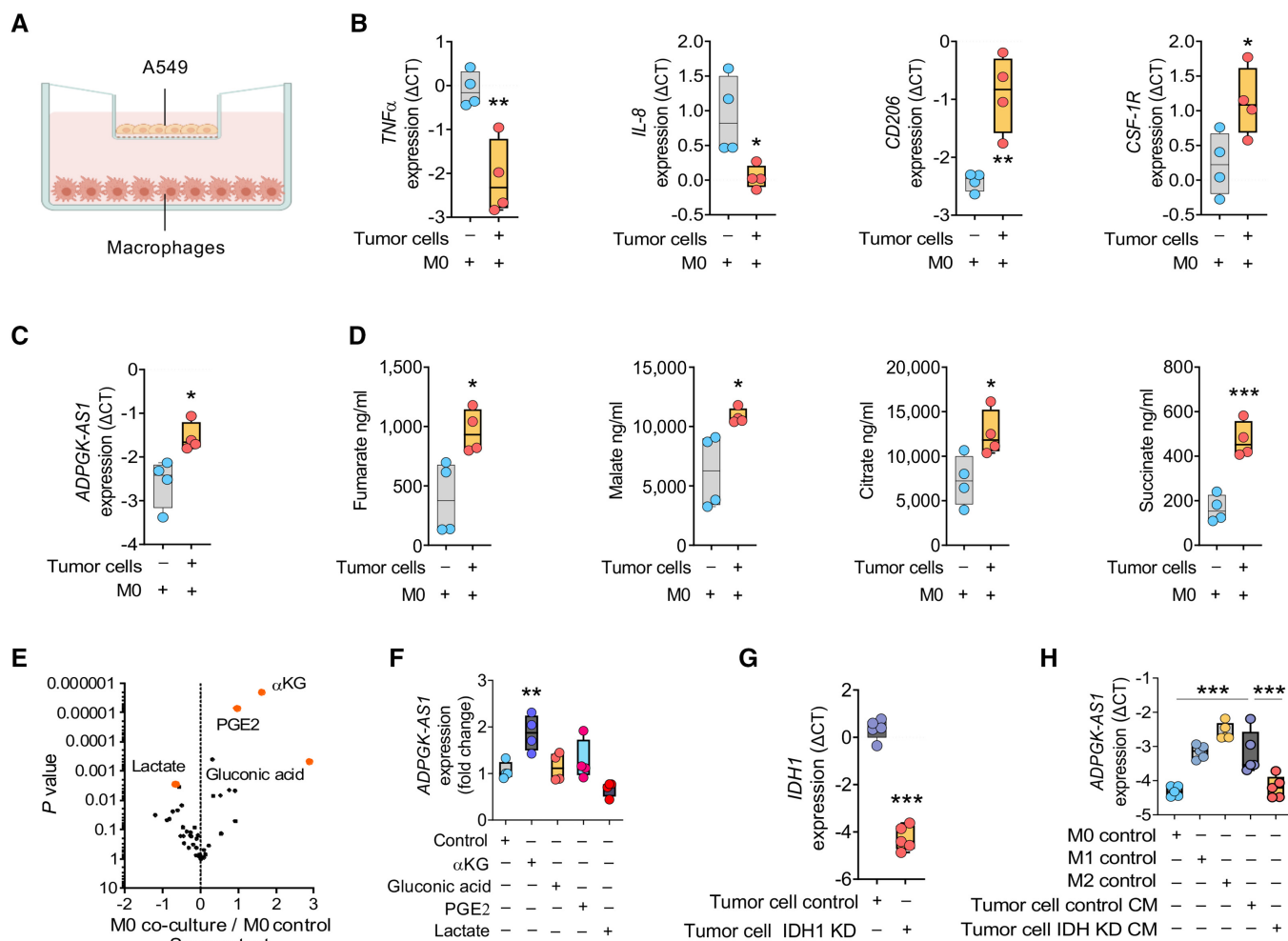


Figure 5. Macrophages upregulate *ADPGK-AS1* and adapt their metabolic signaling pathways after crosstalk with tumor cells.

- A Schematic of the experimental design of indirect co-culture of macrophages with A549 cells using a transwell system.
- B mRNA expression analysis (Δ CT) of macrophage markers (*TNF α* , *IL-8*, *CD206*, *CSF-1R*) in cocultured PBMC-derived macrophages with tumor cells or macrophage control (M0). $n = 4$ biological replicates. $*P \leq 0.05$, $**P \leq 0.01$ compared with M0.
- C Expression of *ADPGK-AS1* lncRNA in macrophages cocultured with tumor cells (A549) or macrophage controls (M0). $n = 4$ biological replicates. $*P \leq 0.05$, compared with M0.
- D Mass spectrometry analysis of TCA cycle metabolites (fumarate, malate, citrate, and succinate) in macrophages cocultured with tumor cells (A549) and macrophage control (M0). $n = 4$ biological replicates. $*P \leq 0.05$, $**P \leq 0.01$ compared with M0.
- E Volcano plot showing deregulated metabolites in the culture medium of a macrophage-tumor cell (A549)-cocultures compared with macrophage control medium (M0). $n = 6$ biological replicates. Red dots represent highly regulated metabolites (α KG, PGE2, gluconic acid, and lactate).
- F RNA expression analysis of *ADPGK-AS1* in macrophages treated with metabolites (α KG, gluconic acid, PGE2, lactate) for 24 h. $n = 4$ independent experiments. $**P \leq 0.01$ compared with M0 control.
- G Expression of *IDH1* mRNA in A549 cells transfected with siRNA specific for *IDH1* (IDH KD) or a negative control. $n = 5$ independent experiments. $***P \leq 0.001$ compared with the A549 control.
- H RNA expression analysis of *ADPGK-AS1* in THP1 macrophages stimulated to have an M1-like or M2-like phenotype, untreated (M0) or treated with conditioned medium (CM) of A549 cells transfected with a siRNA specific for *IDH* (IDH KD) or negative control. $n = 5$ biological replicates. $***P \leq 0.001$, compared with tumor cell control CM.

injected with M2-like control macrophages (Fig 7A–C). In contrast, mice coinjected with M1-like macrophages exhibited reduced tumor growth as well as reduced tumor weight. Co-injection of mice with M2-like macrophages and *ADPGK-AS1* knockdown macrophages resulted in reduced tumor growth in mice as well as low-weight tumors compared with mice in the M2-like control macrophage

group. Consistently, the analysis of the proliferation marker (Ki67) and apoptosis marker (cleaved caspase 3) using immunofluorescence staining confirmed these observations. The results revealed elevated levels of Ki67 in M2-like control coinjected mice but relatively low levels of Ki67 in M1-like control and M2-like *ADPGK-AS1* KD mice (Fig 7C and D). Also, M1-like control and M2-like

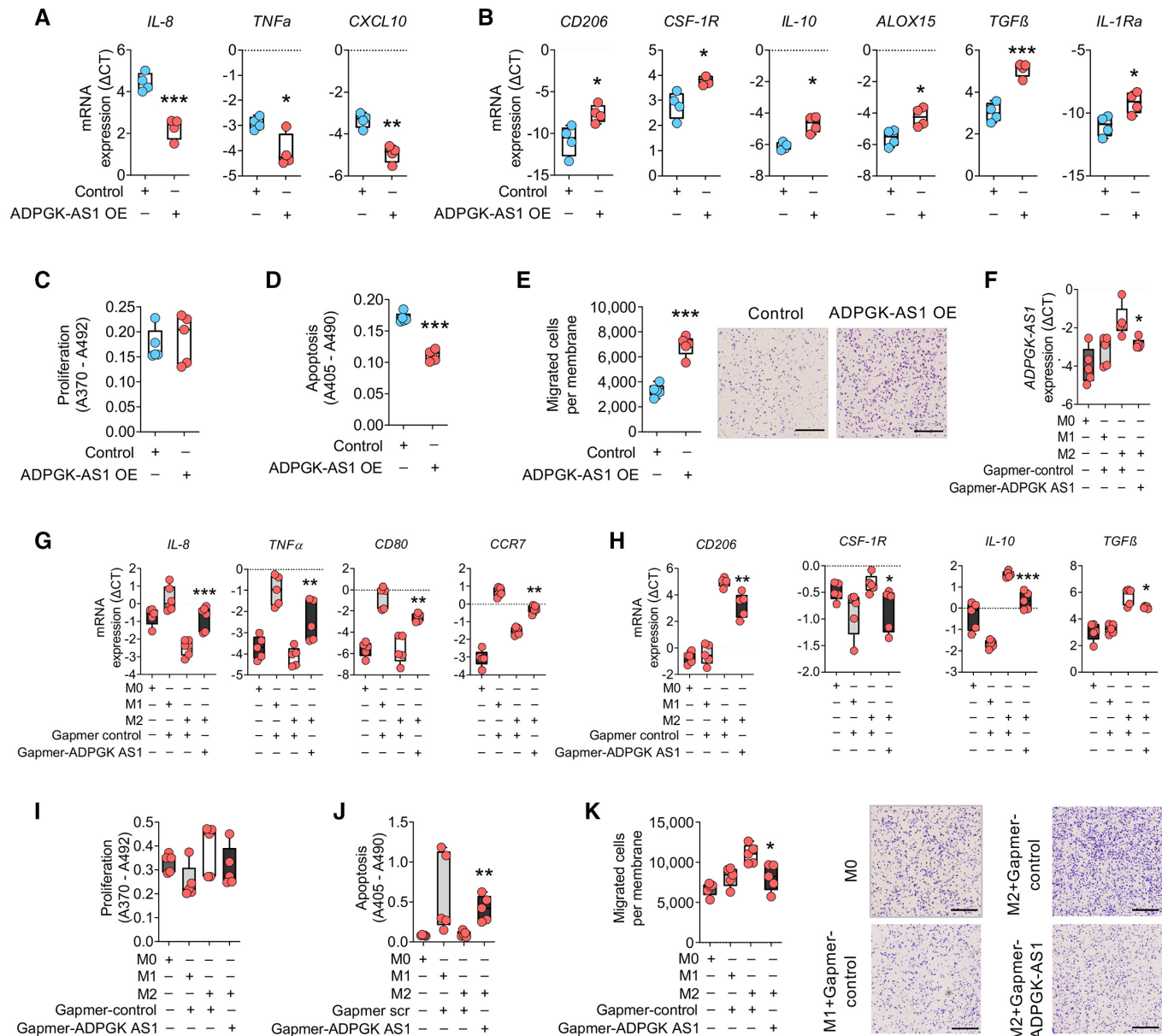


Figure 6. ADPGK-AS1 regulates macrophage activation state and influences tumor-cell apoptosis and migration.

- A, B mRNA expression analysis (Δ CT) of M1 (*TNF α* , *IL-8*, *CXCL10*) and M2 (*CSF-1R*, *CD206*, *IL-10*, *ALOX15*, *TGF β* , *IL-1Ra*) macrophage marker genes in THP1 control and ADPGK-AS1 OE cells. *n* = 4 independent experiments. **P* $\leq$ 0.05, ****P* $\leq$ 0.001 compared with control.
- C Proliferation of A549 cells treated with conditioned medium (CM) of THP1 control and ADPGK-AS1 OE macrophages. *n* = 5 independent experiments.
- D Apoptosis of A549 cells treated with CM from THP1 control and ADPGK-AS1 OE macrophages. *n* = 5 independent experiments. ****P* $\leq$ 0.001 compared with control.
- E Migration (right panel) of A549 cells treated with CM from THP1 control or ADPGK-AS1 OE macrophages. Representative membrane images are shown in the left panel. *n* = 5 independent experiments. ****P* $\leq$ 0.001 compared with control. Scale bar = 400 μ m.
- F RNA expression (Δ CT) analysis of *ADPGK-AS1* in PBMC-derived M1-like (M1) and M2-like (M2) macrophages transfected with antisense LNA GapmeRs specific for *ADPGK-AS1* or a negative control. *n* = 5 biological replicates. **P* $\leq$ 0.05 compared with M2 control.
- G, H mRNA expression analysis (Δ CT) of M1 (*TNF α* , *IL-8*, *CD80*, *CCR7*) and M2 (*CSF-1R*, *CD206*, *IL-10*, *TGF β*) macrophage marker genes in primary M1-like (M1) and M2-like (M2) macrophages transfected with antisense LNA GapmeRs specific for *ADPGK-AS1* or negative control. *n* = 5 biological replicates, **P* $\leq$ 0.05, ***P* $\leq$ 0.01, compared to M2 control.
- I Proliferation of tumor cells (A549) treated with CM of PBMC-derived M1-like (M1) and M2-like (M2) macrophages transfected with antisense LNA GapmeRs specific against *ADPGK-AS1* or a negative control. *n* = 5.
- J Apoptosis of tumor cells (A549) treated with CM from PBMC-derived M1-like (M1) or M2-like (M2) macrophages transfected with antisense LNA GapmeRs specific for *ADPGK-AS1* or a negative control. *n* = 5 biological replicates. ***P* $\leq$ 0.01, compared with M2 control.
- K Migration (right panel) of tumor cells (A549) treated with CM from PBMC-derived M1-like (M1) or M2-like (M2) macrophages transfected with antisense LNA GapmeRs specific for *ADPGK-AS1* or a negative control. Representative membrane images are shown in the left panel. *n* = 5 biological replicates. **P* $\leq$ 0.05, compared with M2 control. Scale bar = 400 μ m.

ADPGK-AS1 knockdown mice showed intense staining for cleaved caspase 3, whereas M2-like control tumors had relatively low levels of this marker. LDH activity, which is also often used as a clinical marker for malignant transformation in patients with cancer (Forkasiewicz *et al*, 2020), was substantially higher in tumors from

M2-like control coinjected animals compared with the tumors of the *ADPGK-AS1* knockdown group (Fig 7E). This result highlighted the tumor-promoting function of M2-like macrophages. RNA isolation and expression analysis of these tumors revealed decreased expression of *ADPGK-AS1* in the M2-like *ADPGK-AS1* KD group (Fig 7F).

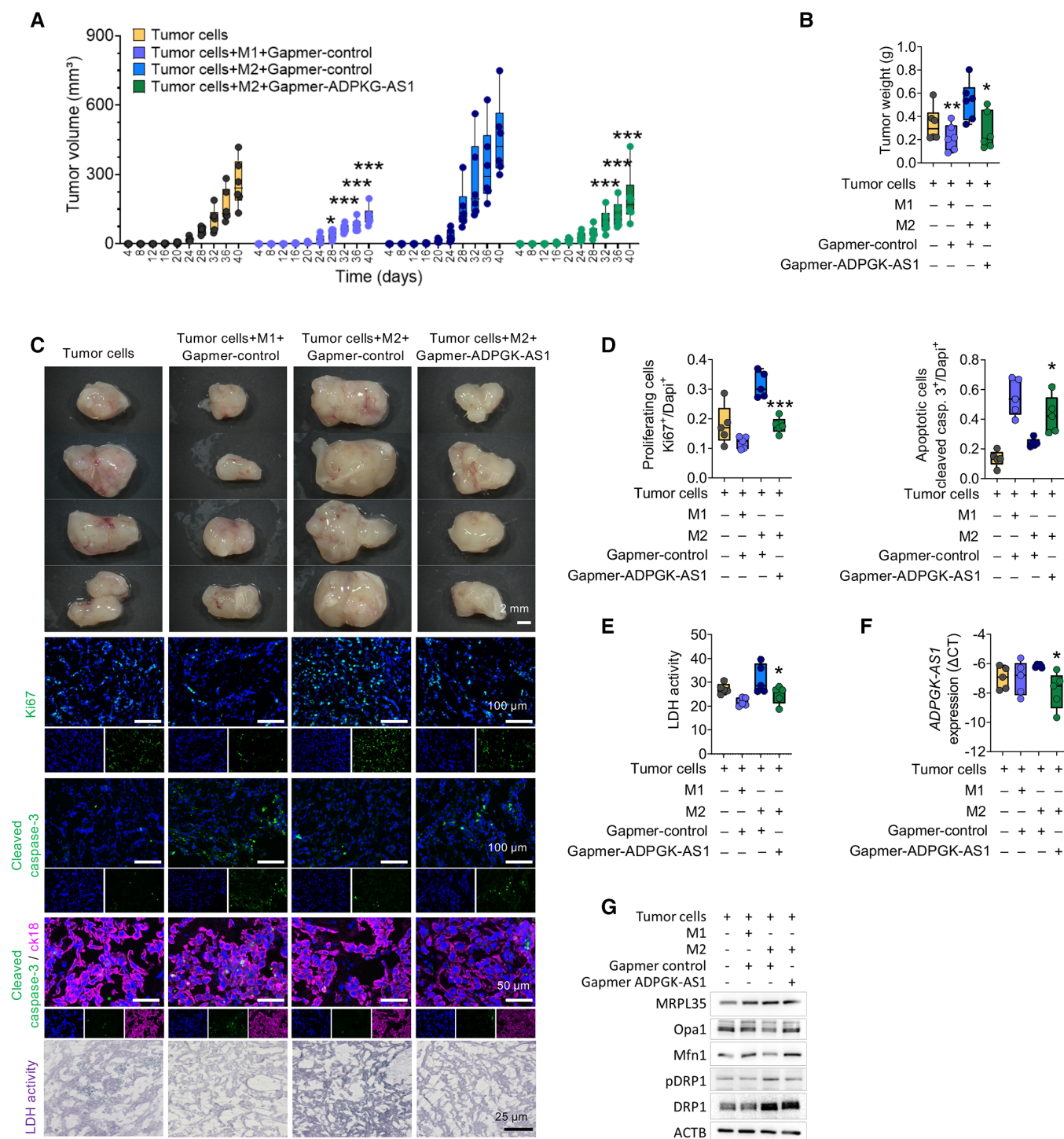


Figure 7.

Figure 7. ADPGK-AS1 knockdown inhibits tumor growth *in vivo*.

- A Time-dependent tumor growth (size, mm³) analysis within 40 days after subcutaneous coinjection with M1-like or M2-like macrophages transfected with antisense LNA GapmeRs specific for *ADPGK-AS1* or a negative control together with A549 tumor cells or A549 cells alone as a control. Each dot represents an animal. $n = 6$ biological replicates. *** $P \leq 0.001$ compared with the group of tumor cells coinjected with M2 control macrophages.
- B Tumor weight at 40 days after coinjection with M1-like (M1) or M2-like (M2) macrophages transfected with antisense LNA GapmeRs specific for *ADPGK-AS1* or a negative control together with A549 tumor cells or A549 cells alone as a control. Each dot represents a mouse. $n = 6$ biological replicates. * $P \leq 0.05$, compared with tumor cells.
- C Representative brightfield images of tumors (scale bar = 2 mm) and fluorescence images (Ki67, cleaved caspase 3, scale bar = 100 μ m; cleaved caspase 3 (green) and cytokeratin 18 (magenta), scale bar = 50 μ m) and LDH enzyme activity (scale bar = 25 μ m) after coinjection with M1-like (M1) or M2-like (M2) macrophages transfected with antisense LNA GapmeRs specific for *ADPGK-AS1* or negative control (ctrl) together with A549 tumor cells alone as a control.
- D Quantification of Ki67⁺ cells and cleaved caspase 3⁺ of coinjected tumors. $n = 5$ biological replicates. ** $P \leq 0.01$, *** $P \leq 0.001$ compared with M2 control.
- E Quantification of LDH enzyme activity in coinjected tumors. $n = 5$ biological replicates. * $P \leq 0.05$ compared with M2 control.
- F RNA expression analysis of *ADPGK-AS1* in coinjected tumor tissues. $n = 5$ biological replicates. * $P \leq 0.05$, compared with M2 control.
- G Representative immunoblot of the mitochondrial-related proteins MRPL35, Opa1, Mfn1, pDRP1, and DRP1 about β -actin in isolated tumor tissue. $n = 3$ biological replicates.

Source data are available online for this figure.

Analysis of the *ADPGK-AS1* interacting partner MRPL35 revealed higher levels of MRPL35 in tumors derived from coinjected A549 cancer cells and M2-like control macrophages, but reduced MRPL35 levels for the M1 control as well as the M2-like *ADPGK-AS1* knockdown group (Fig 7G). Overall, these results demonstrated that increased *ADPGK-AS1* expression in TAMs correlated with increased tumor growth and progression *in vivo*.

ADPGK-AS1* influences human lung tumor progression *ex vivo

Next, *ADPGK-AS1* was analyzed via an *ex vivo* method, namely using precision-cut lung slices (PCLS). We extended this method to human tumor PCLS (tPCLS), which allowed us to culture and directly treat the preserved human lung cancer tissue (Fig 8A and B). For this approach, we performed antisense LNA GapmeR-mediated KD of *ADPGK-AS1* or added *ADPGK-AS1* OE macrophages to tPCLS. We also stained for markers of proliferation (EdU) and apoptosis (TUNEL) together with cytokeratin and DAPI. The tPCLS were then analyzed with confocal imaging (Fig 8C and D). EdU/DAPI and TUNEL/DAPI ratios revealed reduced cell proliferation and increased apoptosis in *ADPGK-AS1* KD tPCLS compared with cells in the negative control slices. Conversely, the presence of *ADPGK-AS1* OE macrophages led to increased proliferation and decreased apoptosis (Fig 8E). Notably, we were able to detect successful downregulation versus overexpression of *ADPGK-AS1* and MRPL35 on RNA expression in the tPCLS tissue (Fig 8F). Furthermore, multispectral flow cytometry analysis of the immune cell infiltrates of the tPCLS after *ADPGK-AS1* knockdown or overexpression (Appendix Fig S5A) revealed an increase of M1-like macrophages after *ADPGK-AS1* knockdown and a decrease after *ADPGK-AS1* OE (Fig 8G). In contrast, the numbers of M2-like macrophages decreased after knockdown, whereas they increased after *ADPGK-AS1* OE. Notably, there was no significant change in the abundance of other immune cell populations such as CD8⁺, CD4⁺, Treg cells, B cells, monocytes, or neutrophils (Appendix Fig S5B). In addition, these results could be reproduced with healthy PCLS with the addition of GFP-labeled A549 cells and culture in macrophage CM, i.e., to mimic lung tumor growth (Fig EV4A–D). Furthermore, we ruled out the possibility that the observed effects were due to the effect of *ADPGK-AS1* on cancer cells by using *in vitro* proliferation, apoptosis, and migration assays for A549 cells transfected with antisense LNA GapmeRs (Fig EV5A–D). Additionally, we repeated the human

PCLS experiment with A549-GFP cells and again carried out a knockdown of *ADPGK-AS1*; however, there was no effect (Fig EV5E). Similarly, no change in functional assays or human PCLS experiments was seen with A549 *ADPGK-AS1* OE cells (Fig EV5F–J). In summary, these results demonstrated that high expression of *ADPGK-AS1* in TAMs could drive the M2-like macrophage phenotype and contribute to lung tumor growth, whereas *ADPGK-AS1* knockdown could enhance the M1-like macrophage phenotype and reduce lung tumor progression *ex vivo*.

Discussion

In the present study, we provide strong evidence that the lncRNA *ADPGK-AS1* (i) is associated with macrophage mitochondria and interacts with specific mitochondrial ribosomal proteins; (ii) regulates mitochondrial metabolism, energy production, and signaling; and (iii) determines the phenotypic M1-like versus M2-like state. We also demonstrate that high levels of lncRNA *ADPGK-AS1* in TAMs promote lung tumor growth, and knockdown of lncRNA *ADPGK-AS1* in TAMs suppresses lung tumor growth as demonstrated in *in vitro*, *ex vivo*, and *in vivo* studies. TAMs have been known to be crucial players during tumor development and progression. Several studies have shown that TAM density correlates with poor prognosis in lung cancer patients (Ruffell & Coussens, 2015; Schmall *et al*, 2015; Zheng *et al*, 2020). Macrophages can dynamically adapt their phenotype to either a more pro-inflammatory and anti-tumorigenic (M1-like) or an anti-inflammatory tumor-promoting (M2-like) phenotype. This plasticity is orchestrated by various cytokines, chemokines, and metabolites within the TME (Ley, 2017; Li *et al*, 2020; Sarode *et al*, 2020a). Understanding the molecular mechanisms underlying this plasticity is important for the development of new immunotherapeutic strategies. LncRNAs can influence different cellular processes through various signaling pathways, and many lncRNAs, such as *Metastasis-Related Lung Adenocarcinoma Transcript 1* (*MALAT1*), are known to be commonly deregulated in various cancer types (Ji *et al*, 2003; Yang *et al*, 2015; Jin *et al*, 2017; Wu *et al*, 2018). In the present study, we analyzed macrophage phenotype-associated regulation of lncRNA transcripts and were able to identify lncRNA *ADPGK-AS1* as being strongly upregulated in tumor-promoting M2-like macrophages. *ADPGK-AS1* has been previously described to be highly expressed in several cancer types and cell lines, such as colon

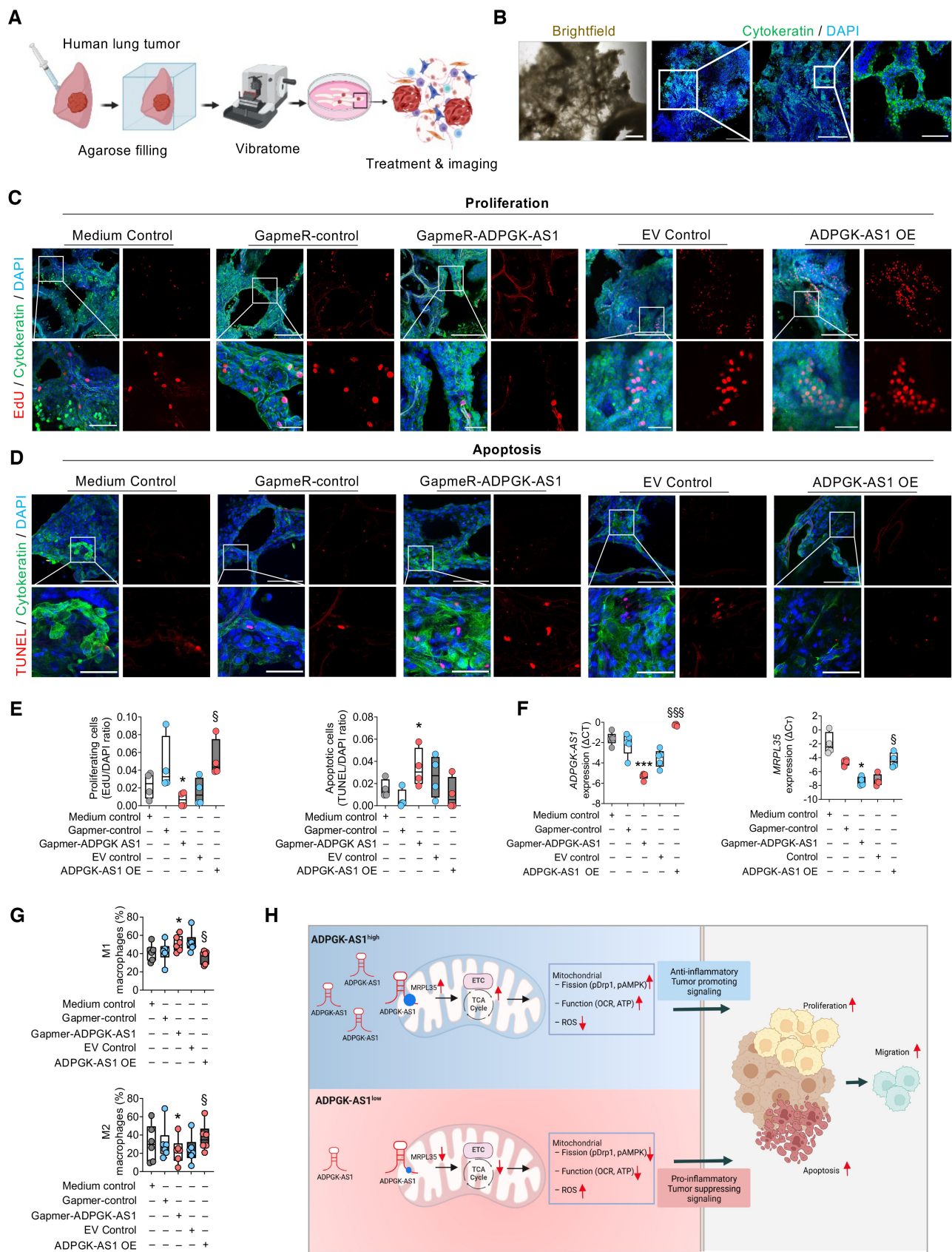


Figure 8.

Figure 8. ADPGK-AS1 influences human lung tumor progression ex vivo.

- A Schematic overview of the preparation of precision-cut lung slices (PCLS) from a human tumor-bearing lung lobe.
- B Representative brightfield and fluorescence images of tumor PCLS stained with cytokeratin (green) to visualize the tumor area. Nuclei were stained with DAPI (blue). Scale bar (left to right) = 500 μ m, 1 mm, 500, and 100 μ m.
- C, D Representative fluorescence images of PCLS with proliferative (EdU⁺) and apoptotic (TUNEL⁺) cells (red) and nuclear dye (DAPI⁺, blue) in the tumor area (cytokeratin⁺, green). Scale bar = 250 μ m (c, upper panel), 150 μ m (d, upper panel), and 50 μ m (c and d lower panel, magnification).
- E Quantification of proliferative (EdU⁺) and apoptotic (TUNEL⁺) cells (red) about total cell number (DAPI⁺, blue) in the tumor area (Cytokeratin⁺, green). $n = 4$ independent experiments. * $P \leq 0.05$, *** $P \leq 0.001$ compared with control GapmeR, §§ $P \leq 0.001$ compared with empty vector (EV) control.
- F RNA expression analysis of *ADPGK-AS1* and *MRPL35* in tumor PCLS treated with antisense LNA GapmeRs specific for *ADPGK-AS1* or a negative control or seeded with THP1 control or *ADPGK-AS1* OE cells. $n = 5$ independent experiments. * $P \leq 0.05$, *** $P \leq 0.001$ compared with control GapmeR, § $P \leq 0.05$, §§ $P \leq 0.001$ compared with EV control.
- G Multispectral flow cytometry analysis of M1-like and M2-like macrophage populations in tumor PCLS treated with antisense LNA GapmeRs specific for *ADPGK-AS1* or a negative control or seeded with THP1 control or *ADPGK-AS1* OE cells. $n = 6$ biological replicates. * $P \leq 0.05$ compared with the GapmeR negative control, § $P \leq 0.05$ compared with the EV control.
- H Proposed mechanism: M2-like macrophages express high levels of *ADPGK-AS1* with translocation of the transcript into the mitochondria, leading to an increased abundance of TCA cycle metabolites, increased mitochondrial activity, and induction of mitochondrial fission, thereby enhancing the number of mitochondria in these macrophages. Subsequently, macrophages exhibit increased anti-inflammatory and tumor-promoting signaling, leading to enhanced lung tumor cell proliferation and migration. Knockdown of *ADPGK-AS1* in macrophages has the reverse effect, with an increase in pro-inflammatory signaling and upregulated ROS, leading to lung tumor cell apoptosis. These results suggest the potential for using *ADPGK-AS1* as a therapeutic target in lung cancer via the regulation of the TAM phenotype.

cancer, pancreatic cancer, and breast cancer (Song *et al*, 2018; Yang *et al*, 2019; Jiang & Wang, 2020). Mechanistically, *ADPGK-AS1* has been suggested to act as an RNA sponge for microRNAs such as miR-525 and miR-3,196 in the cytoplasm, leading to reduced tumor-cell migration, e.g., via regulating the epithelial-to-mesenchymal transition.

Importantly, our data provides the first evidence that *ADPGK-AS1* in TAMs and M2-like macrophages is enriched in mitochondria and modulates mitochondrial metabolism, dynamics, and phenotype, thereby impacting tumor progression. Several cytoplasmic lncRNAs have been reported to influence mitochondrial metabolism. For example, the cytoplasmic lncRNAs (*Cerox1*, *Tug1*, and *Uca1*) have been proposed to regulate mitochondrial bioenergetics and functions by indirectly influencing mitochondrial-related genes or miRNAs (Long *et al*, 2016; Li *et al*, 2017; Sirey *et al*, 2019; Wang *et al*, 2022). In addition, the cytoplasmic lncRNA *stem and progenitor enrichment required for hematopoietic differentiation* (*Spehd*) was shown to be required for hematopoiesis and effective differentiation of myeloid progenitors with functional oxidative phosphorylation (Delas *et al*, 2019). Also, lncRNA *Survival Associated Mitochondrial Melanoma Specific Oncogenic Non-Coding RNA* (*SAMMSON*) was shown to interact with p32, thereby regulating mitochondrial homeostasis and metabolism, especially in cancer cells (Leucci *et al*, 2016). Few studies, however, have posited intra-mitochondrial functions for nuclear-encoded lncRNAs with a hitherto unresolved import mechanism. Zhao *et al* (2019) discovered that *MALAT1* is normally enriched in the nucleus but is also found in mitochondria collected from HepG2 cells. *MALAT1*-deficient HepG2 cells produced less ATP and impaired cell invasion capacity, suggesting a role of this lncRNA in mitochondrial metabolism. To date, few nuclear-encoded mitochondria-associated lncRNAs have been reported to be upregulated in cancer cell lines (Long *et al*, 2016; Li *et al*, 2017; Sirey *et al*, 2019; Gambi *et al*, 2022; Wang *et al*, 2022). In addition, the existence and functional relevance of these lncRNAs in macrophages are completely unknown. To the best of our knowledge, *ADPGK-AS1* is one of the few lncRNAs that has been shown to translocate to mitochondria in macrophages in the context of tumors, as demonstrated by the results from our

FISH, RNA pulldown, RNA immunoprecipitation (RIP), subcellular fractionation, and colocalization studies.

Mitochondria contain a small genome that is transcribed and further translated via mitochondrial ribosomal proteins (MRPs) (Taanman, 1999). MRPs were recently shown to be involved in the translation of mitochondrial genes, regulation of oxidative phosphorylation, and induction of apoptosis (Huang *et al*, 2020).

Interestingly, we found that *ADPGK-AS1* OE in macrophages resulted in increased numbers of mitochondria and hence a greater abundance of mitochondrial DNA as well as an increased abundance of MRPs and components of the electron transport chain (ETC), such as cytochrome C. MRPL35, which we identified as an interaction partner of *ADPGK-AS1*, was previously shown to play a key role in coordinating the synthesis and assembly of cytochrome C in yeast, thereby directly regulating the rate of oxidative phosphorylation (Box *et al*, 2017). Consistent with these findings, elevated MRPL35 and cytochrome C levels in our *ADPGK-AS1* OE macrophages were associated with increased mitochondrial respiration. Deregulation of MRPs can lead to mitochondrial metabolic disorders or cellular dysfunction, and several MRPs are associated with various cancer types. Notably, MRPL35 was reported to be a prognostic marker for colorectal cancer (Sotgia *et al*, 2017; Zhang *et al*, 2019). Interestingly, our results show that MRPL35 was found to be highly expressed in the TAMs of lung cancer patients, all of which were M2-like TAMs. These data, together with the knowledge of upregulated expression of *ADPGK-AS1* in TAMs, suggest an important role for MRPL35 and *ADPGK-AS1* in TAMs. In addition, high MRPL35 levels in TAMs of lung cancer patients were correlated with shorter disease-free and overall survival, thus suggesting a central role for *ADPGK-AS1* in the plasticity and mitochondrial function of TAMs via MRPL35 with an impact on lung cancer biology.

It has recently become clear that mitochondria are highly mobile organelles that have an impact on the metabolism, division, differentiation, and death of cells. Interestingly, both the mitochondrial fusion markers mitofusin 1 (Mfn1) and optic atrophy 1 (Opa1) (El-Hattab *et al*, 2018; Altieri, 2019; Xie *et al*, 2020) were found to be downregulated in cells with high *ADPGK-AS1* expression. Further, mitochondrial fission-associated proteins Drp1 and AMPK (Herzig &

Shaw, 2018) become activated in cells overexpressing *ADPGK-AS1*. Mitochondrial dynamics are very important in quality control, that is, regulation of mitophagy and maintenance of metabolic homeostasis. Although mitochondrial dynamics are known to be connected with their physiological functions, the dynamics still need to be fully understood in the context of the immune response (Wai & Langer, 2016; Gao et al, 2017). Notably, we found that high *ADPGK-AS1* expression upregulated TCA cycle activity and increased mitochondrial membrane potential.

Thus, *ADPGK-AS1* alters macrophage metabolism toward enhanced oxidative phosphorylation (OXPHOS) linked with the anti-inflammatory tumor-promoting M2-like phenotype (Kelly & O'Neill, 2015). Additionally, elevated ROS levels in macrophages are known to be important in host defense against pathogens and are usually upregulated in M1-like macrophages (Tan et al, 2016; Deng et al, 2019). Thus, the downregulation of ROS noted upon *ADPGK-AS1* overexpression further fits into the switch toward an M2-like phenotype.

Unexpectedly, the mitochondrial ROS reduction in *ADPGK-AS1* OE macrophages is concomitant with mitochondrial fission or fragmentation, which suggests that ROS production can be independent of mitochondrial morphology under *ADPGK-AS1* overexpressing conditions. In support of this idea, it has been previously shown that increasing mitochondrial mass is not correlated with ROS production under inflammatory conditions (Widdrington et al, 2018). Along the same line, Yu et al (2006) showed that increased ROS production under high glucose conditions did not lead to mitochondrial fragmentation. Considering the functional role of MRPs in the mitochondrial translation-morphology axis (Cheong et al, 2020), the increase in M1 marker genes along with the downregulation of M2 marker genes after treatment with puromycin in *ADPGK-AS1* OE macrophages suggests that the mitochondrial translational machinery may be involved in the M2-like macrophage/TAM phenotype as well as in mitochondrial fission driven by *ADPGK-AS1* OE in macrophages. However, we cannot rule out the possibility that *ADPGK-AS1*-associated cytoplasmic translational machinery has an impact on macrophage markers because puromycin can also inhibit translational machinery in the cytoplasmic compartment. Further studies are needed to elucidate the cytoplasmic functions of *ADPGK-AS1* in macrophages and other immune cells.

Notably, the phenotype of increased mitochondrial membrane potential, higher oxygen consumption rate, and decreased ROS in *ADPGK-AS1* OE macrophages could be rescued by either *ADPGK-AS1* or MRPL35 knockdown, further confirming that this function of *ADPGK-AS1* might be executed through MRPL35. In addition, increased ROS, as seen by MRPL35 knockdown, has already been shown in colorectal cancer cells (Sotgia et al, 2017; Zhang et al, 2019). Further, the cause-and-effect relationship between mitochondrial morphology, OCR, and ROS seems to be cell-specific, as it has been shown that embryonic stem cells have a higher basal OCR than trophoblast stem cells, despite their similar mitochondrial morphology (Choi et al, 2020). However, exactly how MRPs regulate the interplay between mitochondrial morphology, energy, and ROS production still needs to be further elucidated, especially in immune cells.

Furthermore, we wanted to know if this regulation definitely plays a role in the context of lung cancer-associated macrophages. Therefore, we used coculture experiments to facilitate crosstalk

between macrophages and lung cancer cells to generate TAMs. These experiments revealed that *ADPGK-AS1* was upregulated in the TAMs; and its expression is correlated with elevated expression of M2-like macrophage markers and enhanced concentrations of the TCA metabolites fumarate, malate, and citrate. “Teaching” macrophages adjacent to tumor cells to acquire an M2-like tumor-promoting phenotype thus reflects the macrophage changes induced by *ADPGK-AS1* overexpression. The central role of *ADPGK-AS1* in driving an M2-like macrophage/TAM phenotype with an impact on cancer biology is further supported by a series of *in vitro*, *in vivo*, and *ex vivo* experiments. In artificially polarized macrophages, high expression of *ADPGK-AS1* was shown to enhance M2-like macrophage activation, which was linked with decreased apoptosis and increased migration in tumor-cell functional assays. Downregulation of *ADPGK-AS1* showed the complete opposite effect, that is, increased expression of inflammatory M1-like markers while M2-like marker genes were downregulated. Furthermore, the inhibition of *ADPGK-AS1* in M2-like macrophages induced tumor-cell apoptosis and reduced tumor-cell migration. Due to the lack of a mouse homolog, we used highly immunosuppressed NSG mice to develop a humanized *in vivo* tumor model for further investigation of the biological significance of *ADPGK-AS1* in the cancer context. Indeed, the knockdown of *ADPGK-AS1* in M2-like macrophages in these studies significantly attenuated tumor growth by induction of apoptosis and reduction of tumor size. Additionally, the interaction partner MRPL35 was reduced in tumors that developed under the influence of macrophages with an *ADPGK-AS1* knockdown. Finally, we aimed to characterize the therapeutic potential of *ADPGK-AS1* blockade in a human tissue system. To this end, we established the *ex vivo* method of tumor PCLS, which enabled us to directly culture and treat human lung cancer tissue/cells. Here, inhibition of *ADPGK-AS1* again led to reduced tumor progression with an increase in tumor-cell apoptosis and decreased proliferation, together with a switch of macrophage markers toward a M1-like phenotypic state. In addition, by investigating the immune cell profile, we were able to detect a phenotypic switch in macrophages, whereas no other immune cells were affected, suggesting a specific influence of *ADPGK-AS1* on macrophages. Macrophages have emerged as promising targets in cancer immunotherapies because macrophages account for up to 50% of infiltrating immune cells in the TME (Duan & Luo, 2021).

In conclusion, our findings strongly support the notion that *ADPGK-AS1* possesses a crucial regulatory role in determining the TAM phenotype in the lung cancer microenvironment. Therapeutically targeting this lncRNA, for example, through antisense oligonucleotides, may thus represent a novel approach for the treatment of several pathologies associated with macrophage deregulation, in particular lung cancer.

Materials and Methods

Cell lines

Human cell lines A549, BEAS-2B, H1650, HEK293-T, and THP1 were purchased from the American Type Culture Collection (ATCC, Manassas, USA) with cell-culture conditions per ATCC guidelines. A549 and HEK293-T cells were cultured in Dulbecco's modified

Eagle's medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (P/S). H1650 cells were cultured in RPMI 1640 medium, supplemented with 10% FCS and 1% P/S. THP1 cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 1% P/S, and 5% HEPES. Media and supplements were purchased from Gibco (Texas, USA). All cell lines were tested for mycoplasma using the LookOut® Mycoplasma PCR Detection kit (Merck, Darmstadt, Germany).

Generation macrophages

Primary human macrophages were generated starting with PBMCs isolated from the buffy-coat fraction of blood samples obtained from the blood bank of the Universities Giessen and Marburg Lung Center (UGMLC) through Ficoll density gradient centrifugation as previously described (Schmall *et al*, 2015; Sarode *et al*, 2020b). PBMCs were seeded on 6-well plates (Sarstedt, Nümbrecht, Germany) and coverslips (Neuvitro, #H-18-1.5-PDL). The PBMCs were cultured for 1 h in RPMI 1640 medium supplemented with 1% P/S. Afterward, nonadherent cells were removed, and the remaining PBMCs were cultured in macrophage medium (RPMI 1640 medium with 2% human serum, and 1% P/S) for 7 days to allow monocyte-to-macrophage differentiation. The density of macrophages was roughly 1×10^5 cells per well in six-well plates.

Regarding THP1 monocytes, we treated THP1 cells with 10 ng/ml of phorbol 12-myristate-12 acetate (PMA) for 24 h and changed to a PMA-free medium while allowing them to differentiate into macrophages for another 24 h. Further, we activated and polarized macrophages to the M1 phenotype by stimulating M0 macrophages with LPS (100 ng/ml, Sigma) and Interferon γ (IFN γ) (100 U/ml, Roche, Basel, Switzerland). PBMC-derived macrophages were activated to the M2 phenotype by stimulating M0 macrophages with IL-4 (20 ng/ml; VWR, Radnor, USA) for 24 h, while THP1 macrophages were activated to M2 by stimulation of M0 macrophages with IL-4 and IL-13 (20 ng/ml) for 24 h.

Coculture

Macrophages were cocultured with A549 cells using a transwell system with a 6-well layout (Sarstedt, Nümbrecht, Germany). First, 5×10^5 A549 cells were seeded into transwells with a pore size of 0.4 μ m and allowed to attach to the membrane. After 20 min, transwell inserts were placed in the 6-well plates containing macrophages, with subsequent incubation for 24 h. Further, A549 and macrophages were seeded separately as controls. After incubation, the CM was harvested, centrifuged, and stored at -80°C . Additionally, each cell type was harvested separately for RNA or protein isolation.

Transfection with siRNA and antisense LNA GapmeRs

Macrophages or A549 cells were transfected with different siRNAs and/or antisense LNA GapmeRs using the HiPerFect Transfection Reagent (Qiagen, Hilden, Germany) in an Opti-MEM serum-free medium. Silencer Select siRNA and negative controls were obtained from ThermoFisher Scientific (#4392420, ADPGK-AS1 ID; n513852; #4390824 IDH ID: s7119, negative control #4390843; #4392421, MRPL35 ID s27942), and antisense LNA GapmeRs (#339511,

ADPGK-AS1 #LG00304486-DDA (CAGATAGCAGGTGACA), negative control #339516) were obtained from Qiagen (Hilden, Germany). Transfected cells were cultured in the appropriate medium for 24 h.

Stable transfection

Stable overexpression of *ADPGK-AS1* and stable overexpression in THP1 cells was achieved using lentiviral vectors (pLV-puro-CMV) containing cDNA for a specific lncRNA that was purchased from VectorBuilder (Chicago, USA). An empty vector was used as a negative control. Lentiviral particles were produced by cotransfection of HEK293T cells with a lentiviral plasmid (psPAX2; Addgene, Watertown, USA) and packaging plasmid (pMD2.G; Addgene, Watertown, USA) using TurboFect transfection reagent (Fermentas, Waltham, USA). The medium containing the viral particles was collected after 48–72 h of transfection. THP1 cells were cultured in 6-well plates and transduced with lentivirus in the presence of 6 μ g/ml polybrene (Merck, Darmstadt, Germany). Thereafter, 24 h after transfection, the medium was replaced with a fresh medium containing puromycin (Gibco, Texas, USA).

Proliferation and apoptosis assay

Tumor cells (A549 or H1650) were seeded at 5,000 cells/well in a 96-well plate and incubated in the appropriate growth medium for 24 h, followed by serum starvation for 24 h. After starvation, the cells were incubated for another 24 h in with different CM, such as M0 – M2 macrophages, THP1 control, and *ADPGK-AS1* OE macrophages, for another 24 h. The following day, proliferation was assessed using a bromodeoxyuridine (BrdU) cell proliferation assay kit, and apoptosis was assessed using an ELISA-based cell death detection kit (both from Roche, Basel, Switzerland), according to the manufacturer's protocol.

Migration assay

The migratory capacity of tumor cells (A549 and H1650) was quantified using a Boyden Chamber transwell assay or wound healing assay. For the transwell assay, we seeded 5×10^4 cells/100 μ l medium in the upper part of cell culture inserts with a 0.8 μ m pore size (BD Biosciences, San Jose, USA), and the inserts were placed in a 24-well companion plate containing 700 μ l per well of CM, such as M0, M1, M2, and THP1 control as well as *ADPGK-AS1* OE macrophages. A549 cells were incubated for 6 h and H1650 cells overnight at 37°C . The transwell inserts were subsequently washed with 1x phosphate-buffered saline (PBS). Inserts were wiped from the inside to remove nonmigrated cells and placed in methanol for fixation, followed by crystal violet staining for 10 min. After washing with distilled water, each membrane was mounted on a slide with Pertex (Meditex GmbH, Burgdorf, Switzerland). Slides were scanned using the NanoZoomer 2.0-HT digital slide scanner C9600 (Hamamatsu Photonics, Japan). The number of migrated cells was quantified using ImageJ software (National Institutes of Health, Bethesda, USA), as previously described by (Schmall *et al*, 2015). For the wound healing assay, 30,000 A549 cells per well were seeded in 96-well image lock plates (Essen Bioscience, Hertfordshire, UK). On the next day, wells were scratched using a wound maker (Sartorius, Göttingen, Germany) and cells were treated with 100 μ l/well CM.

Wound healing was imaged and analyzed using the IncuCyte® SX5 (Sartorius, Göttingen, Germany) with the respective software.

RNA isolation and cDNA synthesis, for quantitative PCR

Total RNA was extracted from cell pellets by lysing in either in TRIzol or QIAzol (Qiagen, Hilden, Germany), followed by purification using the RNeasy Mini kit (Qiagen, Hilden, Germany). The RNA was then reverse transcribed to yield complementary DNA (cDNA) using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Waltham, USA) according to the manufacturer's instructions. Also, quantitative PCR (qPCR) was performed using the SYBR Green PCR Master Mix and the StepOne real-time PCR System (Applied Biosystems, Waltham, USA). Intron-spanning human primers were designed using sequence information obtained from the National Center for Biotechnology Information (NCBI) database, and purchased from Sigma-Aldrich (Taufkirchen, Germany). Expression was determined using the ΔCT method. The CT-values were normalized to the housekeeping gene encoding hypoxanthine-guanine phosphoribosyl transferase (HPRT1) using the equation $\Delta\text{CT} = \text{CT}_{\text{reference}} - \text{CT}_{\text{target}}$. The primer sequences used in this study are shown in AppendixTable S2.

Western blotting

Cells (THP1 control, *ADPGK-AS1* OE macrophages, and PBMC-derived macrophages) were lysed in RIPA lysis buffer containing protease and phosphatase inhibitors. Lysates were then cleared via 15 min of centrifugation. Proteins in lysates were separated using 10–15% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules, USA). Each membrane was then blocked with 5% milk in Tris-buffered saline and Tween 20 (TBS-T) for 1 h, followed by incubation with a primary antibody overnight at 4°C on a rotating platform. After washing with TBS-T, each membrane was incubated with a secondary antibody conjugated with horseradish peroxidase. Bound protein-antibody conjugates were detected using an enhanced chemiluminescence detection system (Biozym, Germany). The details of the used antibodies are shown in AppendixTable S3.

Cloning

An empty vector control for *ADPGK-AS1* OE was produced by digesting the plasmid pLV-CMV-*ADPGK-AS1* plasmid (#VB181004-1001qb, VectorBuilder, Chicago, USA) with NotI and PacI to cut out the *ADPGK-AS1* cDNA sequence. The empty plasmid backbone was incubated with T4 DNA polymerase (NEB, Frankfurt Main, Germany) to generate blunt ends, then phosphorylated using the T4 polynucleotide kinase (PNK, NEB, Frankfurt Main, Germany), and ligated with T4 DNA ligase (NEB, Frankfurt Main, Germany). For cloning of the *ADPGK-AS1* cDNA sequence into vector pcDNA 3.1+, PCR was carried out with primers flanking the *ADPGK-AS1* cDNA sequence containing restriction sites for Acc65I and NotI (forward primer: ATATTAGGTACCAAAAGTACAAAGAAAGGAGGTAGTGTC, Reverse Primer: ATTTGCGCGCCGCTTTTACACTTGTTTCATTTTTA) using plasmid pLV-CMV-*ADPGK-AS1* as a template. The purified PCR product (insert), as well as plasmid pcDNA 3.1+ cleaved with Acc65I and NotI (vector), were ligated with a molar ratio of vector to insert of 1:2–1:5 using the T4 DNA ligase (NEB, Frankfurt Main,

Germany). For the luciferase assay, the ADPGK promotor region was amplified by PCR using genomic DNA as a template and primers designed to flank the promotor region, containing restriction sites for XhoI and HindIII (forward primer: ATATTACTC GAGTGGCTCAGTCCCCTCTGGGTGCCA, reverse primer: ATTTAA GCTTCTAGCCCGCGCCTCTTCCGGGCTC). The pGL3 basic plasmid (Promega, Madison, USA) was cleaved with XhoI and HindIII (NEB, Frankfurt Main, Germany), and the purified PCR product (insert) was ligated into the restricted pGL3 plasmid (vector) as described. Generated plasmids were transformed in competent *E. coli* (10-beta, NEB, Frankfurt Main, Germany) and purified using the plasmid mini or maxi kit (Qiagen, Hilden, Germany).

Luciferase assay

Macrophages were cotransfected in six-well plates using a firefly luciferase construct containing the ADPGK promotor and a *Renilla* luciferase construct. After cotransfection, cells were incubated for 24 h. Luciferase activities were quantified with the dual-luciferase reporter assay system (Promega, Madison, USA), according to the manufacturer's protocol, by using a luminescence plate reader (Tecan infinite M200 PRO, Tecan Group, Männedorf, Switzerland). The ratio of the Firefly luciferase signal to the *Renilla* luciferase signal was calculated as previously described by (Sarode et al, 2020b).

Immunofluorescence staining

For immunofluorescence staining, we seeded 200,000 THP1-derived macrophages on cover slips, using frozen tissue sections and fresh precision-cut lung slices (PCLS). Tissue sections were dried at room temperature (RT), then fixed with acetone or methanol at –20°C for 15 min. Cells were washed with PBS and subsequently fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature, whereas PCLS were fixed with 1% paraformaldehyde (PFA) at 4°C overnight. After fixation, slides, cells, and tissues were permeabilized with 0.3% Triton X-100/PBS (cells and slides for 10 min, PCLS for 30 min), and then blocked in 5% bovine serum albumin for 1 h. The samples were washed with 1x PBS three times for 5 min each and incubated with primary antibodies at 4°C overnight. Samples were then washed with 1x PBS for 5 min and incubated with a fluorochrome-conjugated secondary antibody for 1 h at room temperature. The samples were washed again with 1x PBS for 5 min and stained the nuclei with 4',6-diamidino-2-phenylindole (DAPI) for 15 min at room temperature. Subsequently, each sample was mounted with either DAKO tissue mounting medium (Agilent, CA, USA) or ProLong Glass mounting medium (Invitrogen, Waltham, USA). Staining was visualized via either wide-field microscopy (Keyence, Osaka, Japan) or confocal microscopy (Zeiss LSM 710, Leica SP8). Details of the antibodies used in this study are shown in AppendixTable S3.

Fluorescence in situ hybridization (FISH)

RNA FISH was used to visualize the subcellular localization of RNA transcripts within a cell. For this purpose, custom LNA detection probes for *ADPGK-AS1* (Qiagen, #339500, LCD0165475-BKP), *MALAT1* (Qiagen, #339500, LCD0161992-BKP), *β -actin* (Exiqon, #300500, 247370-1), and a nonbinding probe (Qiagen, #339500, LCD0172658-BKP) were designed and modified at the 5'-end with

the red fluorescence dye TYE-665. Macrophages were grown on coverslips and before staining, rinsed with 1x PBS and fixed in 4% PFA for 7 min at room temperature. The fixative was removed by washing three times with 1x PBS for 5 min. Cells were permeabilization was then performed in 0.5% Triton X-100, supplemented with RNase inhibitor (Applied Biosystems, Waltham, USA), on ice for 10 min. Subsequently, coverslips were washed with 1x PBS for 5 min and rinsed once with 2X SSC buffer (Gibco, Texas, USA). The hybridization mixture containing the fluorescence probe was heated to 80°C for 90 s before use. Hybridization was subsequently performed overnight at 37°C in a humid chamber (Slide Moat, Boekel Scientific, Netherlands). After incubation, cells were washed four times with 2X SSC buffer with 50% Formamide for 20 min at 37°C. Coverslips were then mounted in mounting medium containing DAPI. Slides were stored at 4°C or –20°C for long-term storage. Fluorescence signals were analyzed, and representative images were taken with a ZEISS Imager Z1 or Leica SP8 confocal microscope.

RNA pulldown

For RNA pulldown, RNAs encoded in pcDNA3.1+ plasmids were linearized with ScaI (single cutter not present in the *ADPGK-AS1* cDNA sequence) before *in vitro* transcription. This was done using the HiScribe T7 quick, high-yield RNA synthesis kit (New England Biolabs, Frankfurt, Germany). Transcribed RNA was isolated and biotinylated using a 3'-end biotin labeling Kit (Pierce, Waltham, USA) according to the manufacturer's instructions. RNA was again isolated, and 3 µg of biotinylated RNA was resuspended in the RNA folding buffer to be heated up to 90°C for 2 min. Samples were then incubated on ice for 3 min and then for 25 min at room temperature to allow the correct folding of the RNA. RNA pulldown was performed by incubating biotinylated RNA in macrophage whole cell lysate in a head-over-tail rotator at 4°C overnight. The formed RNA-protein complexes were crosslinked via exposure to UV light for 90 s. Streptavidin beads were equilibrated with dilution buffer, and then they were added to the samples for further incubation for 1 h at 4°C in a head-over-tail rotator for pulldown of the crosslinked RNA-protein complexes. The beads were washed four times for 10 min each in dilution buffer at 4°C. The final washing step was carried out in dilution buffer without the addition of detergents or proteinase inhibitors, and beads were transferred into a fresh tube. Samples were centrifuged and the supernatants discarded, and beads were frozen at –80°C for subsequent mass spectrometry. For controls, either 1.5x SDS loading buffer was added to the beads to elute proteins for western blot analysis or TRIzol/QIAzol (Qiagen, Hilden, Germany) was added for RNA isolation and expression analysis via qPCR. The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol *et al.*, 2019) partner repository with the dataset identifier PXD030605. (Username: reviewer_pxd030605@ebi.ac.uk, Password: 6RQg2uMc). A list of the top 50 detected proteins in the pulldown is shown in AppendixTable S1.

Subcellular fractionation

Macrophages were grown on 10-cm plates and harvested by scraping and centrifugation before the experiment. For subcellular

fractionation to obtain cytoplasmic and nuclear fractions, cells were washed with cold 1x PBS and centrifuged shortly at high speed. Each cell pellet was resuspended in a weak lysis buffer (10 mM HEPES pH 7.9 with 10 mM KCl, 0.1 mM EDTA, and 0.1 mM EGTA), and incubated on ice for 15 min. After the addition of 10% Nonidet P-40 (NP-40), each sample was briefly mixed vigorously and centrifuged for 10 min at 1,699 g. The supernatant containing the cytoplasmic fraction was transferred into a new tube and lysed in TRIzol. The pellet containing the nuclei was washed twice with buffer A and then also lysed in a half volume TRIzol that was used for a cytoplasmic fraction. For fractionation of the cytoplasm and mitochondrial fraction, the mitochondrial isolation Kit (Abcam, #ab110170) was performed using a 2.0-ml dounce homogenizer (VWR, Radnor, USA) according to the manufacturer's instructions. For subsequent qPCRs, the same volume of the fractions was used. The calculation of the respective percentage of total gene expression was done by summarizing the calculated relative expression in both compartments. Several control genes were used to verify successful fractionation.

Seahorse analysis

To measure oxygen consumption rate (OCR), a 96-well Seahorse XF cell culture plate (Agilent, Santa Clara, USA) was seeded with 80,000 THP1 cells per well in RPMI medium containing PMA. After 24 h, the medium was replaced with fresh medium without PMA and again incubated for 24 h. Afterwards, OCR was analyzed using the Agilent Seahorse XF Analyzer.

ATP determination

For the determination of ATP production, the ATP Determination Kit (ThermoFisher Scientific, Waltham, USA) was used according to the manufacturer's instructions. Further, 1×10^6 THP1 cells were seeded into 6-well plates and differentiated into macrophages through PMA treatment. A total of 90 µl of THP1 macrophage whole cell extract was mixed with a 10 µl reaction solution containing D-luciferin and firefly luciferase. Subsequently, luminescence was measured using a fluorescence plate reader (Tecan Infinite M200 PRO).

Mitochondrial membrane potential

THP1 cells 1×10^6 per well were seeded into 6-well plates and differentiated into macrophages before the analysis of mitochondrial membrane potential using the JC-1 mitochondrial membrane potential assay Kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. Cells were collected, washed in 1x dilution buffer, and resuspended in JC-1 working solution. After 30 min of incubation at 37°C in the dark, cells were washed with 1x dilution buffer and resuspended in 1x supplemented dilution buffer. A total of 200,000 THP1 cells per well were seeded into a dark 96-well plate and analyzed with a fluorescent plate reader (Tecan Infinite M200 PRO). Excitation and emission wavelengths were 535 ± 17.5 nm and 530 ± 15 nm, respectively. Afterward, cells were transferred into a transparent well plate that was shortly centrifuged, and JC-1 staining was analyzed with a fluorescence microscope (Keyence, Osaka, Japan).

MitoSOXROS staining

Reactive oxygen species (ROS) formation in THP1 macrophages was determined using the MitSOX™ red mitochondrial superoxide indicator (Invitrogen, Waltham, USA) according to the manufacturer's instructions. THP1 macrophages were grown on cover slips and treated with a 5 μ M MitoSOX reagent working solution for 10 min at 37°C. Subsequently, coverslips were washed three times with warm 1x PBS and imaged using a fluorescence microscope (Keyence, Osaka, Japan).

Enzyme activity assays

Enzyme activity assays and staining of tissue sections were carried out as described by (Miller *et al*, 2017). Specific buffers were prepared as follows: For the LDH or IDH assays, the buffer was 0.1 Tris-maleate buffer pH 7.5; for the SDH activity assay, it was 0.1 M Tris-HCl buffer pH 8.0. In the enzyme-specific buffers, 10% polyvinyl alcohol was dissolved at 60°C with stirring until the mixture was clear. Enzyme-specific assay media were prepared (including negative control reactions) with the addition of 200 mM sodium oxamate (LDH inhibition), 100 mM oxaloacetic acid (IDH inhibition), and 250 mM malonic acid (for SDH inhibition). An assay medium containing the enzyme-specific substrates was applied to cover the whole tissue section or cell slide. Enzyme reactions were carried out at RT for around 15 min or until high staining was visible, and stopped by the removal of the incubation medium and washing with PBS. Tissue sections or cell slides were either mounted directly or used following antibody staining.

Metabolome measurement via LC–MS/MS

Cell samples were lysed in ice-cold 85% methanol (10 μ l/mg) with two freeze-thaw cycles. Medium samples were directly processed. The homogenate or culture medium was centrifuged (15,000 g, 5 min, 4°C). An equal volume of supernatant was collected, an isotope-labeled internal standard mix was added, and the samples were evaporated to dryness in a Concentrator Plus (Eppendorf, Wesseling-Berzdorf, Germany). Samples were reconstituted in 50 μ l of water +0.5% formic acid, transferred to autosampler vials, and subsequently analyzed via liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). Negative ionization ESI-LC MS/MS was performed on an Agilent 1290 Infinity LC system (Agilent, Waldbronn, Germany) coupled to a QTrap 5500 mass spectrometer (Sciex, Darmstadt, Germany). Ion source parameters were as follows: CUR 30 psi, CAD medium, Ion Spray Voltage - 4,500 V, TEM 400°C, GS1 45 psi, GS2 25 psi. TCA metabolites were identified with authentic standards and/or via retention time, elution order from the column, and 1–2 transitions. For quantification, specific MRM transitions for every compound were normalized to appropriate isotope-labeled internal standards. Reversed-phase LC separation was performed by using a Waters Acquity UPLC HSS T3 column (150 mm $\times$ 2.1 mm, 1.8 μ m). The column oven temperature was 40°C, and the autosampler was 6°C. Injection volume was 2.5 μ l. Solvent A consisted of water containing 0.5% formic acid, and solvent B consisted of 100% methanol containing 0.5% formic acid. Compounds were eluted with a flow rate of 0.4 ml/min and with the following 10 min gradient: 2% B

for 1.5 min, a 3 min gradient to 100% B, and a cleaning and equilibration step.

Preparation of precision-cut lung slices (PCLS)

All the tissues were obtained from the Department of Thoracic Surgery at the University Clinics of Giessen and Marburg (UKGM) and provided by the UGMLC Giessen Biobank. The study protocol for tissue donation was approved by the ethics committee (“Ethik Kommission am Fachbereich Humanmedizin der Justus Liebig Universität Giessen”) of the University Hospital Giessen (Giessen, Germany) in accordance with national law and with “Good Clinical Practice/International Conference on Harmonization” guidelines. Written informed consent was obtained from each patient or the patient's next of kin (reference AZ 58/15).

Lobes of the human lung (healthy or tumor-bearing) were filled through an open bronchus with 2.5% low melting agarose (Sigma, Missouri, USA) in PBS: DMEM/F12 medium 1:1 (v/v) containing 1% penicillin–streptomycin. After inflation of the whole lobe, the entry opening was closed, and the lobe was placed on ice to cool down and solidify the agarose. The filled lung lobe was cut into smaller pieces and embedded in 4% agarose, then cut into 300–400 μ m thick sections using a VT1200S vibratome (Leica, Wetzlar, Germany). Tumor PCLS was transfected with antisense LNA GapmeRs as previously described and seeded with THP1 cells. On healthy PCLS, A549-GFP cells were seeded, and allowed to settle down on the tissue. After 30 min, macrophage CM was added and incubated for 24 h. Tumors and healthy PCLS were fixed in 1% PFA at 4°C overnight before IF staining. The detection of proliferating cells was done using the Click-IT™ EdU cell proliferation Kit (Invitrogen, Waltham, USA), while the detection of apoptotic cells was done using In-situ cell death detection Kit TMR rot (Roche, Basel, Switzerland), according to the manufacturer's instructions. Stained PCLS were imaged using the Leica SP8 confocal microscope. For flow cytometry, PCLS were dissociated into a single cell suspension by using the MACS tumor dissociation kit and gentleMACS dissociator (Milteny Biotec, Bergisch Gladbach, Germany).

Animal experiment

All mice were maintained under specific pathogen-free conditions and handled according to the guidelines of the European Union Commission on Laboratory Animals. Female, 16 weeks old, *NOD.Cg-Prkdc^{scid}Il2rgtm1Wjl/SzJ* (NSG) mice were obtained from Jackson Laboratory (Bar Harbor, USA). All the animal experiments were performed at the Max Planck Institute for Heart and Lung Research (Bad Nauheim, Germany) and were approved by local authorities (Regierungspräsidium Darmstadt, Hessen, Germany, Proposal No. B2/1225). NSG mice were subcutaneously coinjected with 1×10^6 A549 cells and 1×10^6 polarized and transfected macrophages in a 200 μ l total volume. On day 40, mice were euthanized and the tumors harvested as previously described (Schmall *et al*, 2015).

Hematoxylin and eosin staining

Frozen sections on slides were thawed for 10 min on RT and then fixed for 10 min in acetone or methanol at –20°C. After additional

10 min at RT, slides were washed for 5 min in distilled water and then stained with hematoxylin (Invitrogen, Waltham, USA). Slides were washed and placed in 96% ethanol for 1 min before staining with an eosin solution (Richard-Allan Scientific, Kalamazoo, USA) for 4 min. Slides were then rinsed with water and sequentially dehydrated through immersion in 96% ethanol, 99.8% ethanol, and xylol. They were subsequently mounted with coverslips using Entellan (Sigma-Aldrich, Taufkirchen, Germany) and mounting medium (Merck Millipore, Darmstadt, Germany).

Coding probability and *in vitro* translation

To estimate the coding potential of a lncRNA, cDNA sequences were obtained using the NCBI database and analyzed using the coding potential calculator on the EMBnet website (<https://www.embnet.org/wp/resources/coding-potential-calculator/>) as well as a protein-coding assessment tool (<https://wlc.b.oit.uci.edu/cpat/>). Plasmids containing the cDNA of the respective lncRNA were used for *in vitro* transcription and translation. The Promega TnT quick-coupled transcription/translation system and transcend nonradioactive translation detection system were used for *in vitro* transcription and translation of each full-length lncRNA from the T7 promoter of a pcDNA3.1+ plasmid, per the manufacturer's instructions. Following the completion of the transcription/translation reaction, 1 µl of the reaction product was added to 15 µl of SDS sample buffer, heated for 2 min to 100°C to denature the proteins, and then loaded onto a 4–12% 1-mm 15-W NuPage SDS–polyacrylamide gel (Life Technologies). Protein products labeled with the biotinylated transcend tRNA were detected with streptavidin antibodies and western blue reagents per the manufacturer's instructions. The luciferase T7 control DNA plasmid supplied with the TnT quick-coupled transcription/translation system was used as a positive control.

RNA-seq analysis

RNA was isolated from M1 and M2 polarized PBMC-derived macrophages using the miRNeasy micro kit (Qiagen, Hilden, Germany). This was combined with on-column DNase digestion (DNase Set, Qiagen) to avoid genomic DNA contamination. RNA integrity was analyzed with a BioAnalyzer 2100 (Agilent, Santa Clara, USA) and a LabChip GX Touch 24 (PerkinElmer). We used total RNA (2 µg) for Truseq Stranded mRNA Library library preparation following the low sample protocol, and sequencing was performed with the NextSeq500 instrument (Illumina) using v2 chemistry with a 2 × 75-bp single-end setup.

The resulting raw reads were assessed for quality, adapter content, and duplication rates with FastQC (Andrews S. 2010, FastQC: a quality control tool for high-throughput sequence data, available online at: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Trimmomatic version 0.39 was employed to trim reads having a quality value less than Q15 with a window of 5 nucleotides (Bolger et al, Trimmomatic: a flexible trimmer for Illumina sequence data). Only reads longer than 15 nucleotides were cleared for further analyses. Trimmed and filtered reads were aligned versus the Ensembl human genome version hg38 (GRCh38) using STAR 2.7.9a with the parameters “--outFilterMismatchNoverLmax 0.1 --outFilterMatchNmin 20 --alignIntronMax 200000” (Dobin et al, STAR: ultrafast universal RNA-seq aligner). The number of reads

aligning to genes was counted with the featureCounts 2.0.2 tool from the Subread package (Liao et al, featureCounts: an efficient general-purpose program for assigning sequence reads to genomic features). Only reads mapping at least partially inside exons were admitted and aggregated per gene. Reads overlapping multiple genes or aligning to multiple regions were excluded. A combined raw count matrix was produced and batch corrected per donor (batch 1: *_1, batch 2: *_2, batch 3: *_3) using CountClust (Dey et al, 2017), Visualizing the structure of RNA-seq expression data using the grade of membership models, PLoS Genetics). The batch-corrected matrix was used for differential expression analysis using DESeq2 version 1.30.1 (Love et al, Moderated estimation of fold change and dispersion for RNA-Seq data with DESeq2). Differentially expressed genes (DEG) of M2/M1 were analyzed at a log2 fold change of > 0.59 or < −0.59 and a Benjamini-Hochberg adjusted *P*-value of < 0.05. The Ensembl annotation was enriched with UniProt data (release 08.03.2018) based on Ensembl gene identifiers (Activities at the Universal Protein Resource (UniProt)).

5'/3' rapid amplification of cDNA ends (RACE)

The SMARTer® RACE 5'/3' kit (Takara Bio, Kusatsu, Japan) was used to identify the transcript variant and 5' and 3' end of the *ADPGK-AS1* cDNA according to the manufacturer's protocol. First, the total RNA of PBMC-derived M2-like macrophages was used to synthesize cDNA for 5'- or 3'-RACE with the specific provided 5'- or 3'-Primers. Next, the cDNA was diluted and used as a template for the RACE PCR using universal primers as well as primers specific for the *ADPGK-AS1* sequence containing a sequence for the in-fusion cloning into the pRACE vector (5'-Primer: GATTACGCCAAGCTTGGGCACACAGATAGCAGGTGACAGA, 3'-Primer: GATTACGCCAAGCTTCAGGGTCTTGATGAAGGGAGGGGT). PCR products were loaded on an agarose gel, and fitting bands were cut and purified by NucleoSpin Gel and the PCR Clean-Up Kit. Purified PCR products were used for in-fusion cloning into a linearized pRACE vector, which was subsequently transformed into the provided Stellar Competent bacteria. Transformed cells were plated on an LB plate containing 100 µg/ml ampicillin. On the next day, individual clones were picked from the plate, and plasmids were isolated with a GenElute™ Plasmid Miniprep-kit (Merck, Darmstadt, Germany). To determine the presence of the RACE insert, plasmids were tested by restriction digestion with EcoRI and HindIII (NEB, Massachusetts, USA) and subsequently sent for sanger sequencing (Eurofins Genomics, Ebersberg, Germany).

RNA immunoprecipitation (RIP)

PBMC-derived macrophages were grown on 6-cm dishes and polarized to an M2-like phenotype by stimulation with IL-4 (20 ng/ml) for 24 h. Macrophages were washed once with 1× cold PBS and crosslinked with 1% formaldehyde for 5 min. The reaction was quenched with glycine (125 mM) on ice for 5 min. Cells were scraped in 1× PBS and collected by centrifugation at 1,000 rpm at 4°C for 5 min. Cells were lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% Sodium deoxycholate, proteinase inhibitors), and 10% was saved as input. Four micrograms of anti-MRPL35, anti-MRPL15, or anti-IgG antibodies were coupled with 50 µl of Protein A Dynabeads (ThermoFisher

Scientific, Waltham, USA) in lysis buffer and washed once with high salt buffer (1 M NaCl) and twice with lysis buffer. The antibody-coupled beads were added to the cell lysate and rotated for 1 h at 4°C. Beads were collected by centrifugation (1,000 rpm, 2 min) and washed three times with high salt buffer (4°C, 10 min, rotator) and then washed twice with buffer PNK (350 mM Tris-HCl pH 6.5, 50 mM MgCl₂, 5 mM DTT). For elution, all wash buffer was removed, and RNA isolation was performed with QIAzol (Qiagen, Hilden, Germany) as described under RNA isolation.

Multiplex immunofluorescence staining

Tissue microarrays (TMAs) were constructed from paraffin blocks of selected lung specimens. The Ethical Committee of the University Hospital Munich, Lungbiobank Heidelberg, and the Biobank Platform of the German Center for Lung Research in Germany approved the collection and analysis of all samples (AZ 58/15) in accordance with the national laws and the Good Clinical Practice/International Conference on Harmonization guidelines. Informed consent was obtained from all patients (Weigert *et al*, 2022). TMA lung cancer tissue sections were stained with Opal 7-Color Automation IHC kits (Akoya Bioscience) in the BOND-RX Multiplex IHC Stainer (Leica). Each section was put through 6 sequential rounds of staining, which included blocking in 5% BSA followed by incubation with primary antibodies as follows, MRP-L35 (Invitrogen, PA5-38964, 1:100), MRP-L15 (G Biosciences, ITT2848, 1:200), TNF α (Abcam, ab6671, 1:500), pCK (DAKO, ab7753, 1:200), CD206 (Cell Signaling, 91992, 1:250), CD68 (DAKO, M087601-2, 1:100), corresponding secondary HRP-conjugated antibodies, and Opal fluorophores as described (Strack *et al*, 2020; Zheng *et al*, 2020). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) contained in the Opal 7-Color Automation IHC Kits, and slides were mounted with Fluoromount-G (Southern Biotech). Imaging was performed with the PhenoImager HT (Akoya Bioscience), and images were analyzed using the phenotyping application of the inForm software V2.5 (Akoya Bioscience).

Multispectral flow cytometry

Single-cell suspensions of tPCLS were generated as described above. Single-cell suspensions were blocked with FcR blocking reagent (Miltenyi Biotec) in 0.5% PBS-BSA for 20 min, stained with fluorochrome-conjugated antibodies, and analyzed on a FACS-Symphony A5SE flow cytometer (BD Biosciences). Live single cells were identified by FSC and SSC characteristics. The data were analyzed using FlowJo V10 (TreeStar). All antibodies and secondary reagents were titrated to determine optimal concentrations. CompBeads (BD) were used for single-color compensation to create multicolor compensation matrices. For gating, fluorescence minus one control was used. The instrument calibration was controlled daily using Cytometer Setup and Tracking Beads (BD Biosciences). The following antibodies were used: CD3-BUV805 (#612896, BD Biosciences), CD4-BB630 (#562316, BD Biosciences), CD8-BV650 (#743067, BD Biosciences), CD14-PerCP-Cy5.5 (#561116, BD Biosciences), CD15-BUV805 (#742057, BD Biosciences), CD16-BV650 (#563692, BD Biosciences), CD19-APC-H7 (#560252, BD Biosciences), CD25-PE-Cy7 (#557741, BD Biosciences), CD33-BV510 (#563257, BD Biosciences), CD45-AF700 (#368514, BD Biosciences), CD80-BV711 (#740801, BD

Biosciences), CD206-PE/Cy7 (#321124, BioLegend), CD326-FITC (#324203, BioLegend), HLA-DR-APC/Fire750 (#307658, BioLegend), MerTK-BV421 (#367603, BioLegend).

Analysis of mitochondrial protein synthesis

THP1 control or *ADPGK-AS1* OE macrophages (1×10^6) were grown in 6-well plates and treated with O-propargyl-puromycin (OP-puro, Jena Bioscience, Jena, Germany) as previously described (D'Andrea *et al*, 2016; Delaunay *et al*, 2022). Cells were incubated with 50 μ M OP-puro for 1 h and 200 nM MitoTracker (red, ThermoFisher Scientific, Waltham, USA) was added 30 min before cell collection. Cells were rinsed with 1x PBS, scraped, and collected by centrifugation (1,000 rpm, 5 min). Mitochondrial isolation was carried out with the mitochondrial isolation kit (Abcam, #ab110170) according to the manufacturers' instructions. The extracted organelles were fixed with 1% PFA for 15 min on ice in the dark. After fixation, samples were washed in PBS and permeabilized in PBS supplemented with 3% FCS and 0.1% saponin (Sigma-Aldrich) for 5 min at RT. To conjugate OP-puro to a fluorochrome, an azide-alkyne cycloaddition was performed for 30 min at room temperature in the dark. For this, the Click-iT Cell Reaction Buffer Kit (ThermoFisher Scientific) and 5 μ M of Alexa Fluor 488-Azide (ThermoFisher Scientific) were used. To remove Excel reagents and reduce the background signal, the cells were washed twice with PBS supplemented with 3% FCS and 0.1% saponin. Finally, all samples were mounted on a slide, and the fluorescence signal was imaged with a confocal microscope (Leica SP8).

Transmission electron microscopy

The TEM preparation was performed as described in (Madel *et al*, 2014). Sixty nanometer ultra-thin sections were obtained using a Leica UC7 and collected on copper grids. After post-staining with uranyl acetate and lead citrate, the ultra-thin sections were examined with a JEM-1400 Plus transmission electron microscope (Jeol, Japan), operated at an accelerating voltage of 120 kV. Digital images were recorded with an EM-14800Ruby Digital CCD camera unit.

Statistical analysis

All data were analyzed using Prism 6.0 and 9.0 (GraphPad Software), and statistical comparisons between the two groups were made with the unpaired sample student's *t*-test. Comparisons of more than two groups utilized the one-way analysis of variance followed by Tukey's *post hoc* test. Data were represented as mean $\pm$ SEM.

Data availability

The RNA-seq data were deposited in the Gene Expression Omnibus archive (accession number GSE195440: <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE195440>). The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol *et al*, 2019) partner repository with the dataset identifier PXD030605 (<http://www.ebi.ac.uk/pride/archive/projects/PXD030605>).

Expanded View for this article is available [online](#).

Acknowledgments

The authors thank Yanina Knepper, Jeanette Knepper, Dr. Rajender Nandigama, Dr. Sascha Seidel, Margarete Mijatovic, Dr. Sebastian Konzok, and the Imaging Platform of the MPI for Heart and Lung Research for excellent technical assistance. Work in the laboratories of RS was supported by the Max Planck Society, the Institute for Lung Health (ILH), and the German Center for Lung Research (DZL). BMBF (KMU-innovative-22: miRTumorProst): 031B0768C, Deutsche Forschungsgemeinschaft (DFG, German Research Foundation): SA 1923/7-1, SFB1213 (Project A10N*); the Excellence Cluster Cardio-Pulmonary Institute (EXC 2026: Cardio Pulmonary Institute (CPI), project 390649896), and the State of Hesse (LOEWE iCANx, Project A6 and Area C). Work in the laboratories of SSP was supported by the Max Planck Society, the German Center for Lung Research (DZL), European Research Council (ERC) Consolidator Grant (#866051 to SSP), Deutsche Forschungsgemeinschaft (DFG, German Research Foundation): SFB1213 (Project A01 and A05); the Excellence Cluster Cardio-Pulmonary Institute (EXC 2026: Cardio Pulmonary Institute (CPI), project 390649896); and the State of Hesse (LOEWE iCANx, Project B4, B5, and Area C). Work in the laboratories of MSL and IW was supported by the DFG Transregio TRR267 (Projekt-ID 403584255 – TRR 267, TP A04, TP Z02). Open Access funding enabled and organized by Projekt DEAL. Open Access funding enabled and organized by Projekt DEAL.

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Disclosure and competing interests statement

The authors declare that they have no conflict of interest.

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Expanded View Figures

Figure EV1. Selection and characterization of *ADPGK-AS1*.

- A Schematic overview of criteria for choosing appropriate deregulated lncRNA candidates based on RNA-seq data for M1-like and M2-like macrophages.
- B Exon/intron structure of the three annotated transcript variants of *ADPGK-AS1* (lower panel) with RNA sequencing coverage (sashimi plot) from our sequencing of M2 macrophages of the same genomic location (upper panel), split by read per strand (m2_f=plus strand, m2_r=minus strand).
- C 5' and 3' sequences of *ADPGK-AS1* identified by 5'/3' RACE are compared with the sequence of *ADPGK-AS1* transcript variant 1.
- D Coding probability and coding potential score using the cDNA-sequence of *ADPGK-AS1*, with β -actin as an example of a protein-coding sequence and MALAT1 as an example of a noncoding transcript.
- E Transcription and subsequent translation of *ADPGK-AS1* to exclude the coding function of proteins > 10 kDa. The asterisk denotes the encoded protein in the positive control (PC) and negative control (NC).
- F RNA expression analysis of *ADPGK-AS1* in different cell lines and macrophage phenotypes. $n = 6$. Data was represented as mean $\pm$ SEM, $**P \leq 0.01$ compared to healthy cell line B2B.
- G RNA expression of *ADPGK-AS1* in an Illumina Seq dataset (Source: UCSC genome browser, July 2021).
- H, I RNA expression analysis of *ADPGK-AS1* in PBMC-derived (left panel) and THP1-derived macrophages (right panel), either left untreated (M0) or activated to the M1-like phenotype by stimulation with IFN γ and LPS or to the M2-like phenotype by stimulation with IL-4. $n = 4$. (PBMC-derived macrophages) and $n = 6$ (THP1-derived macrophages) independent experiments, mean $\pm$ SEM, student's unpaired t -test. $*P \leq 0.05$, $***P \leq 0.001$ compared with M0.
- J RNA-pulldown validation of *ADPGK-AS1* using RNA isolated from streptavidin beads of *ADPGK-AS1* or control samples. $n = 4$ independent experiments.
- K Representative immunoblot of MRPL15, MRPL35 localization after subcellular fractionation of macrophages into cytoplasmic and mitochondrial fractions. β -actin served as the cytoplasmic control, succinate dehydrogenase (SDHA) as the mitochondrial control, ERp72 and protein disulfide isomerase (PDI) as endoplasmic reticulum (ER) controls, and Lamin A/C as the nuclear control. $n = 3$ independent experiments.

Source data are available online for this figure.

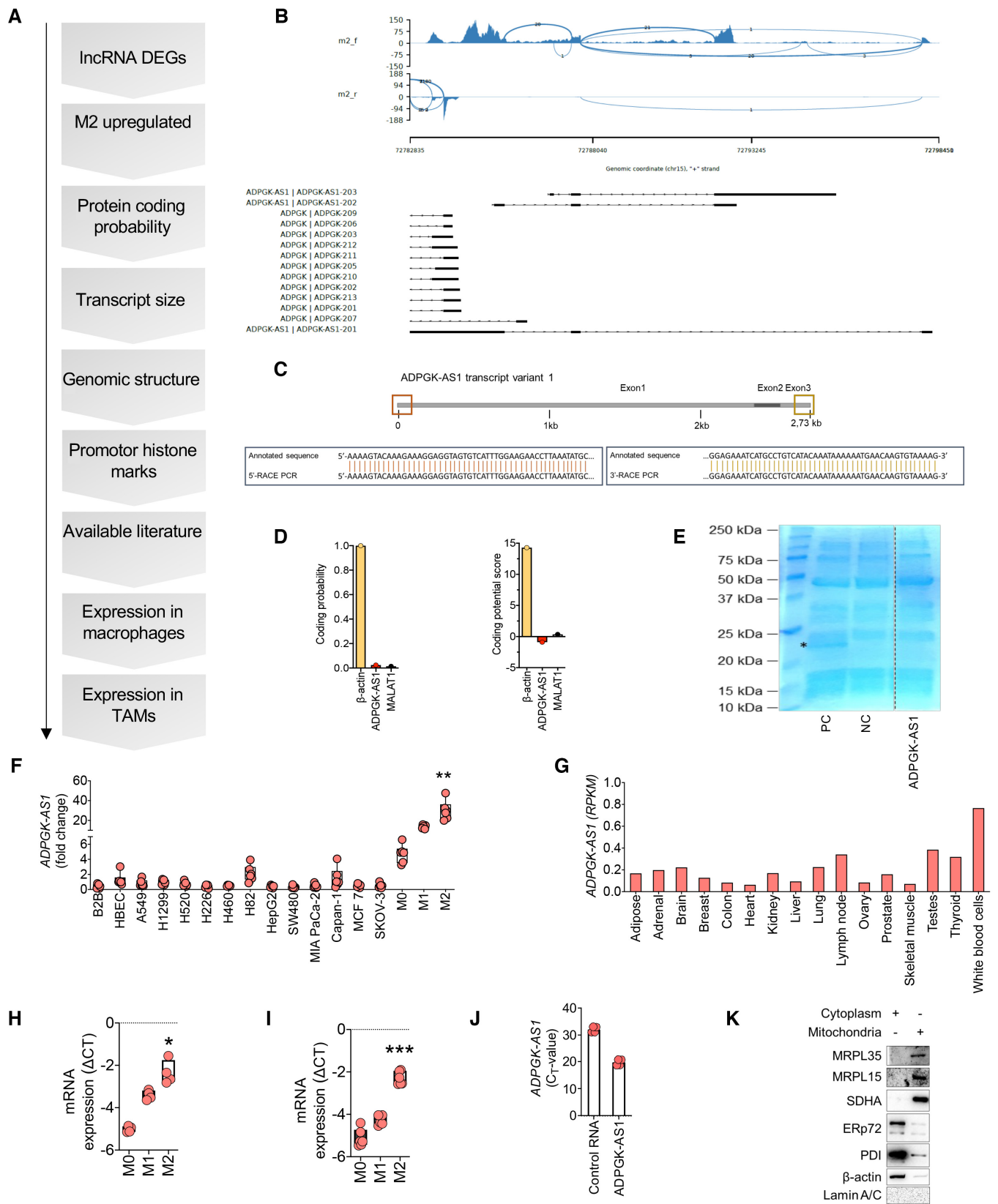


Figure EV1.

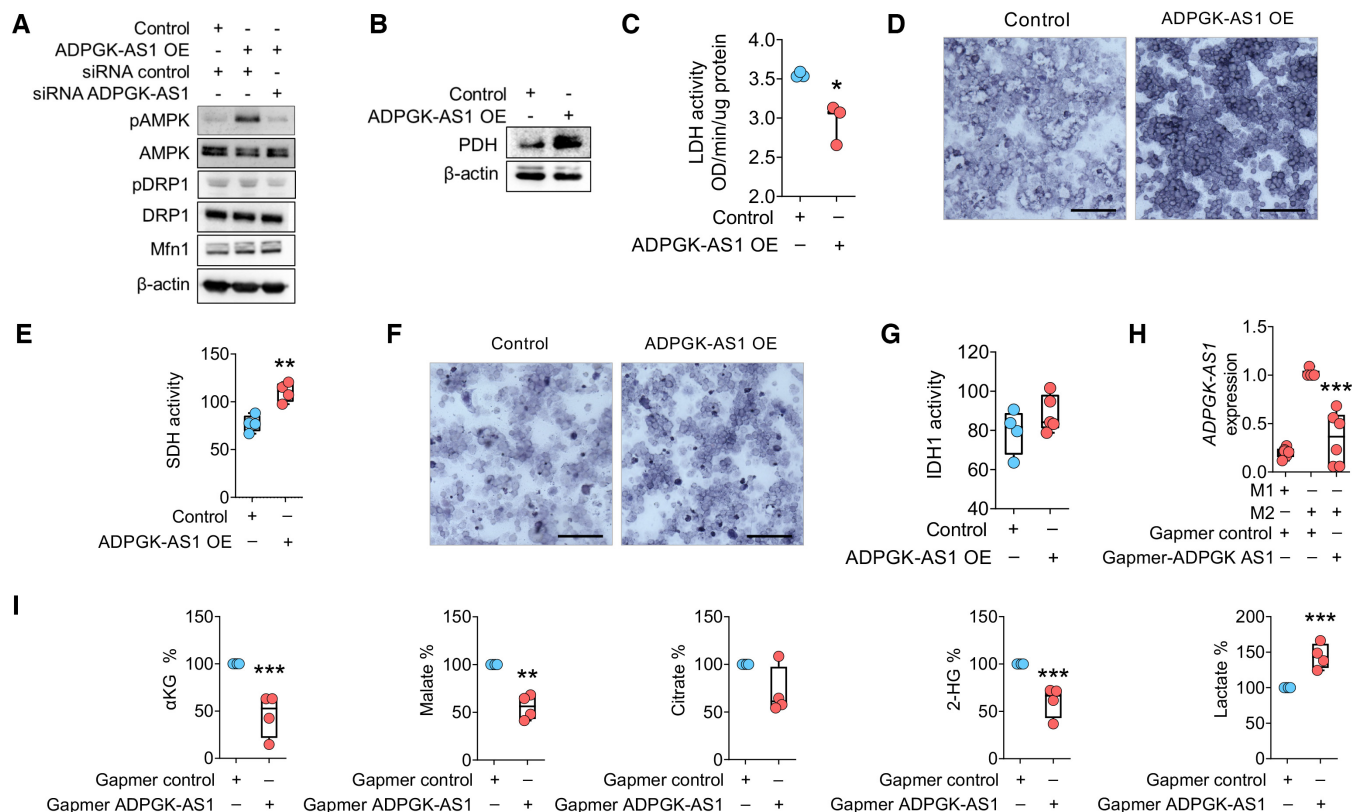


Figure EV2. ADPGK-AS1 enhances the mitochondrial respiratory chain and stimulates the activities of TCA cycle enzymes.

- A** Representative immunoblot of mitochondrial fission (DRP1, pDRP1, AMPK, pAMPK) and fusion (Mfn1) marker markers and β-actin in THP1 control and ADPGK-AS1 OE macrophages transfected with a siRNA against ADPGK-AS1 or a negative control. $n = 3$ independent experiments.
- B** Representative immunoblot of pyruvate dehydrogenase (PDH) and β-actin in THP1 control and ADPGK-AS1 OE macrophages. $n = 3$ independent experiments.
- C** LDH enzyme activity in ADPGK-AS1 OE macrophages compared to control cells. $n = 3$ independent experiments, mean $\pm$ SEM, student's unpaired t -test, $*P \leq 0.05$, compared with control.
- D–G** Enzymatic activity by staining and quantification of succinate dehydrogenase (SDH) and isocitrate dehydrogenase (IDH1) in THP1 control and ADPGK-AS1 OE macrophages. scale bar = 100 μ m $n = 4$ independent experiments, mean $\pm$ SEM, student's unpaired t -test, $**P \leq 0.01$, compared with control.
- H** ADPGK-AS1 expression in PBMC-derived macrophages polarized to the M1-like (M1, +LPS/IFN γ for 24 h), M2-like (M2, +IL-4 for 24 h) or M0 (unstimulated) state and then transfected with antisense LNA GapmeRs against ADPGK-AS1 or a negative control. $n = 6$ biological replicates, mean $\pm$ SEM, one-way ANOVA, $***P \leq 0.001$, compared with M2 control.
- I** LC-MS/MS analysis of deregulated metabolites of PBMC-derived macrophages polarized to the M2-like state (M2, +IL-4 for 24 h) and then transfected with antisense LNA GapmeRs against ADPGK-AS1 or a negative control. Results represent the percentage of the ratio of lysate to supernatant. $n = 4$ biological replicates, mean $\pm$ SEM, student's unpaired t -test, $**P \leq 0.01$, $***P \leq 0.001$ compared with the GapmeR control.

Source data are available online for this figure.

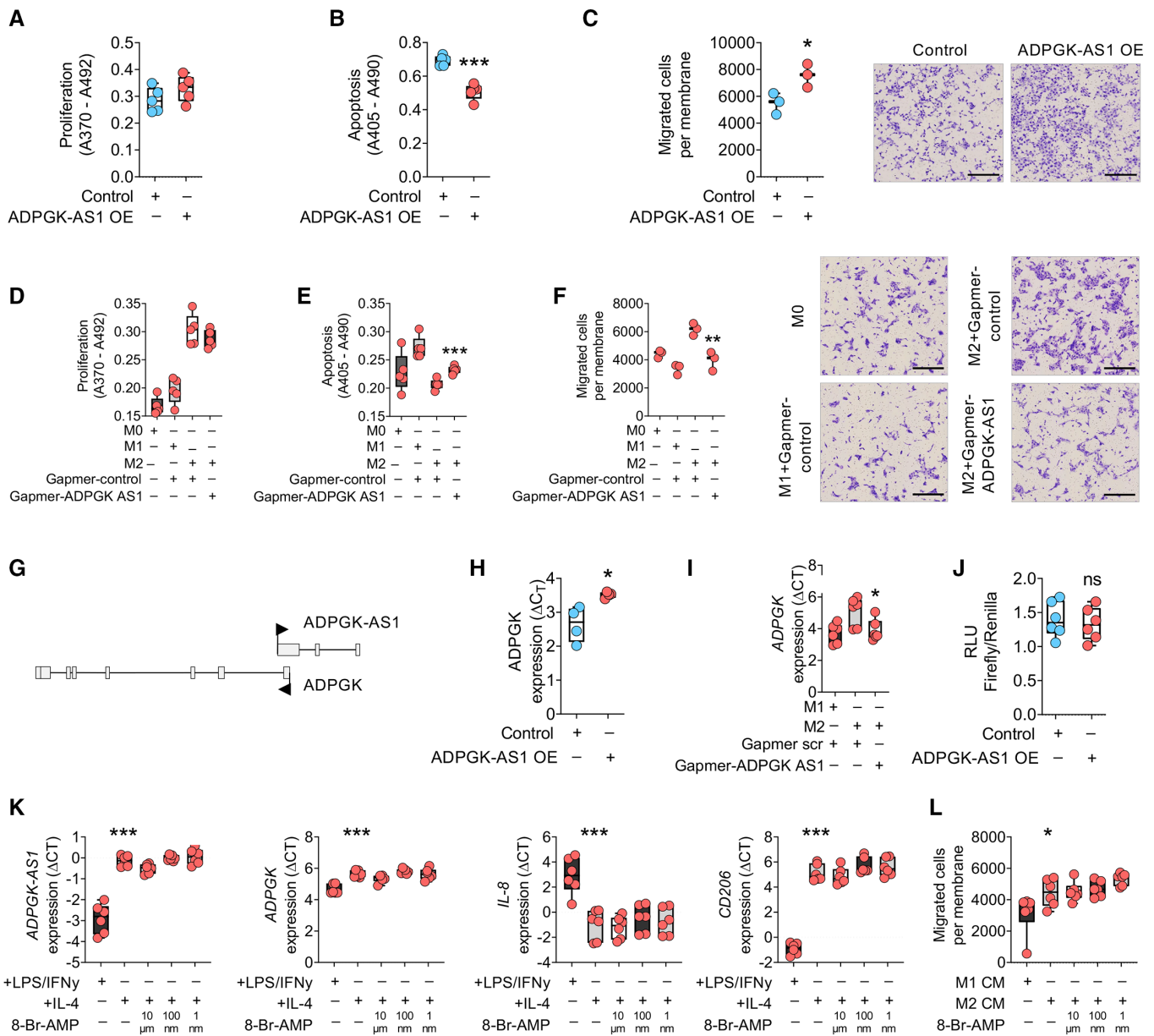


Figure EV3. ADPGK-AS1 has antitumor activity in vitro, and its function is not regulated via ADPGK.

A–C Proliferation ($n = 5$), apoptosis ($n = 5$), and migration ($n = 3$) (left panel) with representative membrane images (right panel) of H1650 cells treated with CM from THP1 control or ADPGK-AS1 OE macrophages. The n values represent independent experiments, mean $\pm$ SEM, student's unpaired t -test, * $P \leq 0.05$, *** $P \leq 0.001$, compared with control. Scale bar = 400 μ m.

D–F Proliferation ($n = 5$), apoptosis ($n = 5$), and migration ($n = 3$) (left panel) with representative membrane images (right panel) of H1650 cells treated with CM from primary M1-like (M1) or M2-like (M2) macrophages transfected with antisense LNA Gapmer-AS1 or a negative control. The n values represent biological replicates, mean $\pm$ SEM, one-way ANOVA, ** $P \leq 0.01$, *** $P \leq 0.001$, compared with M2 control. Scale bar = 400 μ m.

G Schematic diagram of the genomic region of the ADPGK and ADPGK-AS1 genes.

H mRNA expression analysis of ADPGK in THP1 control and ADPGK-AS1 OE macrophages. $n = 4$ technical replicates, mean $\pm$ SEM, student's unpaired t -test, * $P \leq 0.05$, compared with control.

I Expression of ADPGK mRNA in primary M1-like (M1) and M2-like (M2) macrophages transfected with antisense LNA Gapmer-AS1 or a negative control. $n = 6$ technical replicates, mean $\pm$ SEM, one-way ANOVA, * $P \leq 0.05$, compared with M2 control.

J Luciferase-based assay for analyzing activation of the ADPGK promoter in THP1 control and ADPGK-AS1 OE cells. $n = 6$ technical replicates, mean $\pm$ SEM, student's unpaired t -test.

K RNA expression of ADPGK-AS1, ADPGK, and macrophage activation markers IL-8 and CD206 in macrophages treated with different concentrations of ADPGK inhibitor 8-Br-AMP. $n = 6$ technical replicates, mean $\pm$ SEM, one-way ANOVA, * $P \leq 0.05$, compared with M2 control.

L Migration of tumor cells (A549) treated with CM of M1-like or M2-like macrophages treated with 8-Br-AMP. $n = 6$ technical replicates, mean $\pm$ SEM, one-way ANOVA, * $P \leq 0.05$, compared with M1.

Figure EV4. ADPGK-AS1 influences lung cancer cell proliferation and apoptosis ex vivo in normal lung PCLS.

- A Schematic overview and experimental design of precision-cut lung slices (PCLS) using healthy (nontumor) lung lobes and subsequent seeding with A549-GFP cells.
- B Representative images of healthy PCLS (red, autofluorescence) with A549-GFP (green), apoptotic cells (TUNEL⁺, magenta), and proliferative cells (EdU⁺, cyan). Scale bar = 500 μ m (brightfield images), 50 μ m (fluorescence images on the right).
- C, D Quantification of GFP⁺ tumor cells (A549, $n = 6$), EdU⁺ (proliferative, $n = 5$), and TUNEL⁺ (apoptotic, $n = 4$) cells on healthy PCLS treated with CM (medium control, M0, M1, or M2 macrophages transfected with antisense LNA GapmeRs specific for *ADPGK-AS1*) or negative control, THP1 control (EV), and *ADPGK-AS1* overexpressing (OE) macrophages. The n values represent biological replicates, mean $\pm$ SEM, one-way ANOVA, * $P \leq 0.05$, *** $P \leq 0.001$, compared with M2 GapmeR-control, §§§ $P \leq 0.001$ compared with EV control.

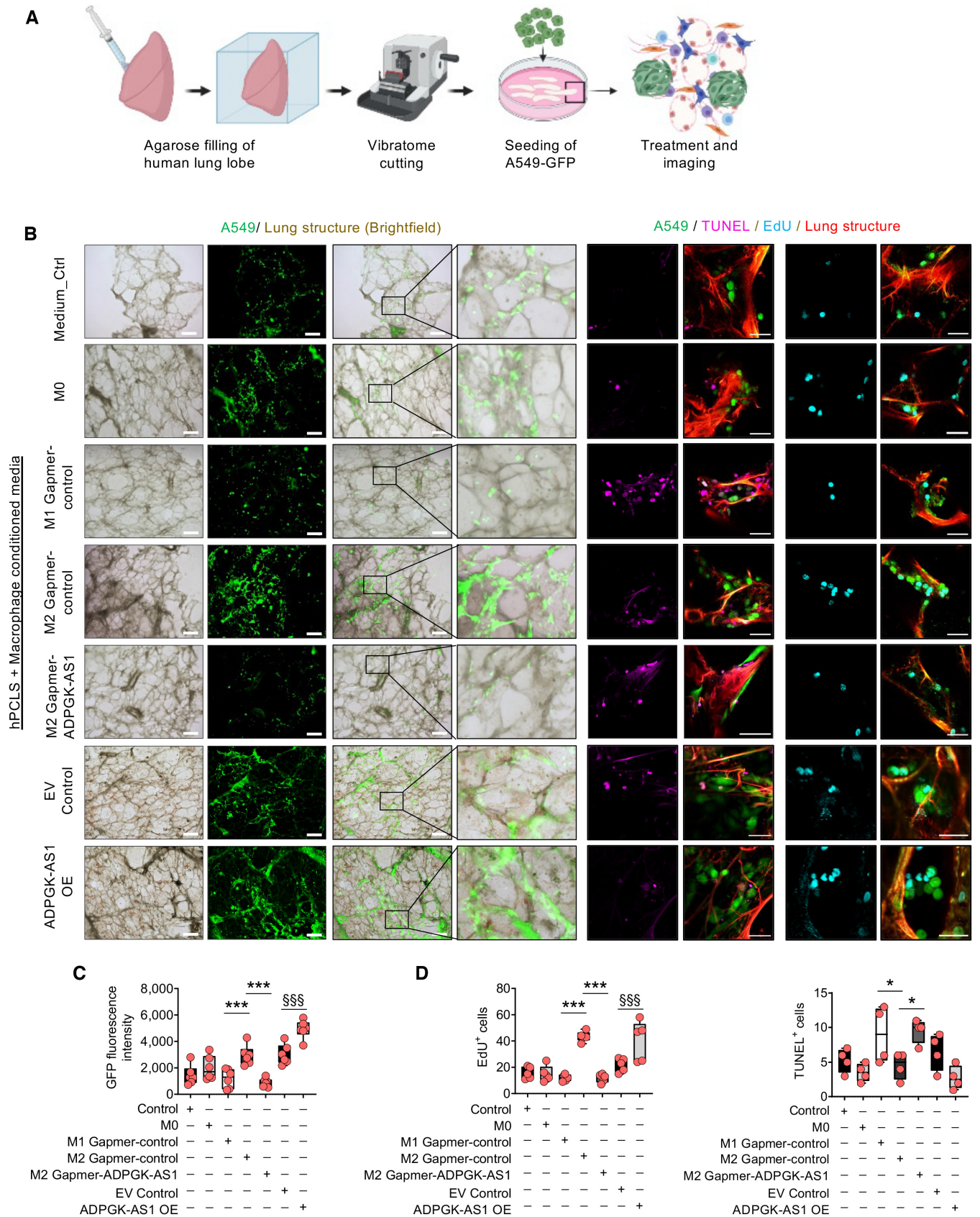


Figure EV4.

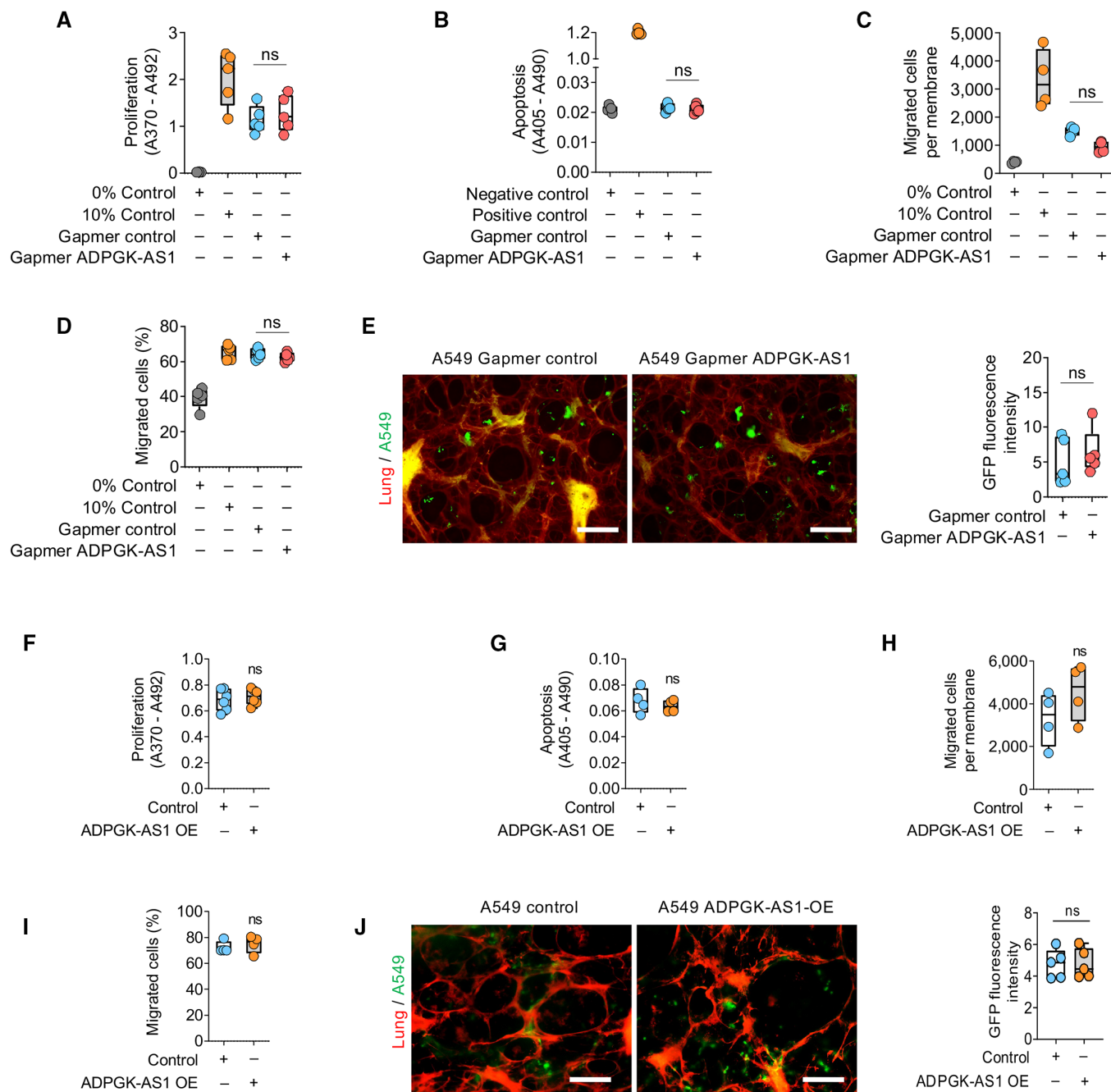


Figure EV5. ADPGK-AS1 knockdown has no effect on A549 cells.

A–D Proliferation ($n = 5$), apoptosis ($n = 5$), migration (transwell, $n = 4$), and wound healing ($n = 5$) assays of tumor cells (A549) transfected with antisense LNA GapmeRs against *ADPGK-AS1* or a negative control. The n values represent independent experiments, mean $\pm$ SEM, one-way ANOVA. ns, nonsignificant.

E Human healthy PCLS seeded with A549-GFP cells transfected with antisense LNA GapmeRs against *ADPGK-AS1* or a negative control with quantification of A549-GFP-positive cells after 48 h. $n = 5$ independent experiments, mean $\pm$ SEM, student's unpaired t -test. ns, nonsignificant, scale bar = 500 μ m.

F–I Proliferation ($n = 6$), apoptosis ($n = 4$), migration (transwell, $n = 4$), and wound healing ($n = 4$) assays of tumor cells (A549) control and *ADPGK-AS1* OE cells. The n values represent independent experiments, mean $\pm$ SEM, one-way ANOVA. ns, nonsignificant.

J Human healthy PCLS seeded with A549-GFP transfected with the *ADPGK-AS1* OE plasmid or an empty vector control with quantification of A549-GFP positive cells after 48 h. $n = 5$ independent experiments, mean $\pm$ SEM, student's unpaired t -test. ns, nonsignificant scale bar = 500 μ m.

Long non-coding RNA *ADPGK-AS1* regulates macrophage mitochondrial and phenotypic state – impact on lung cancer

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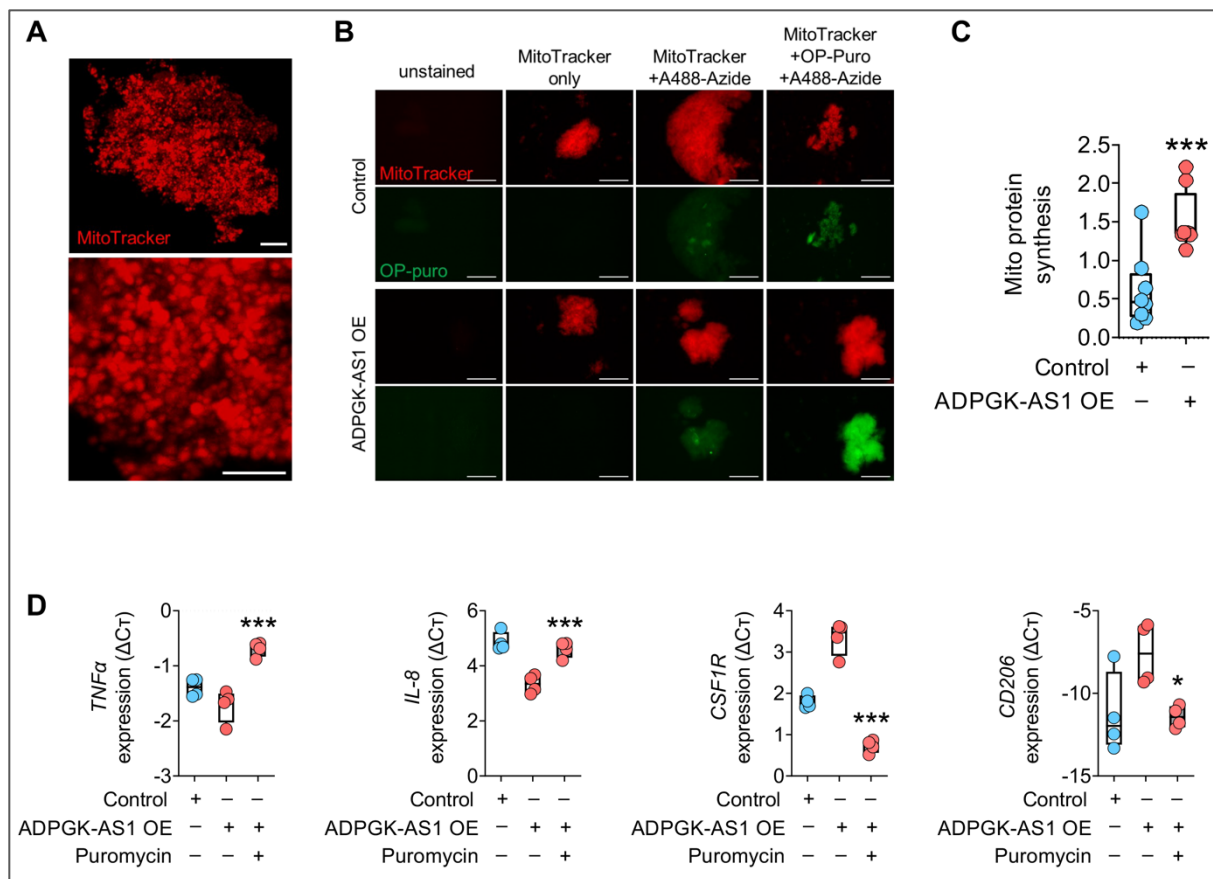
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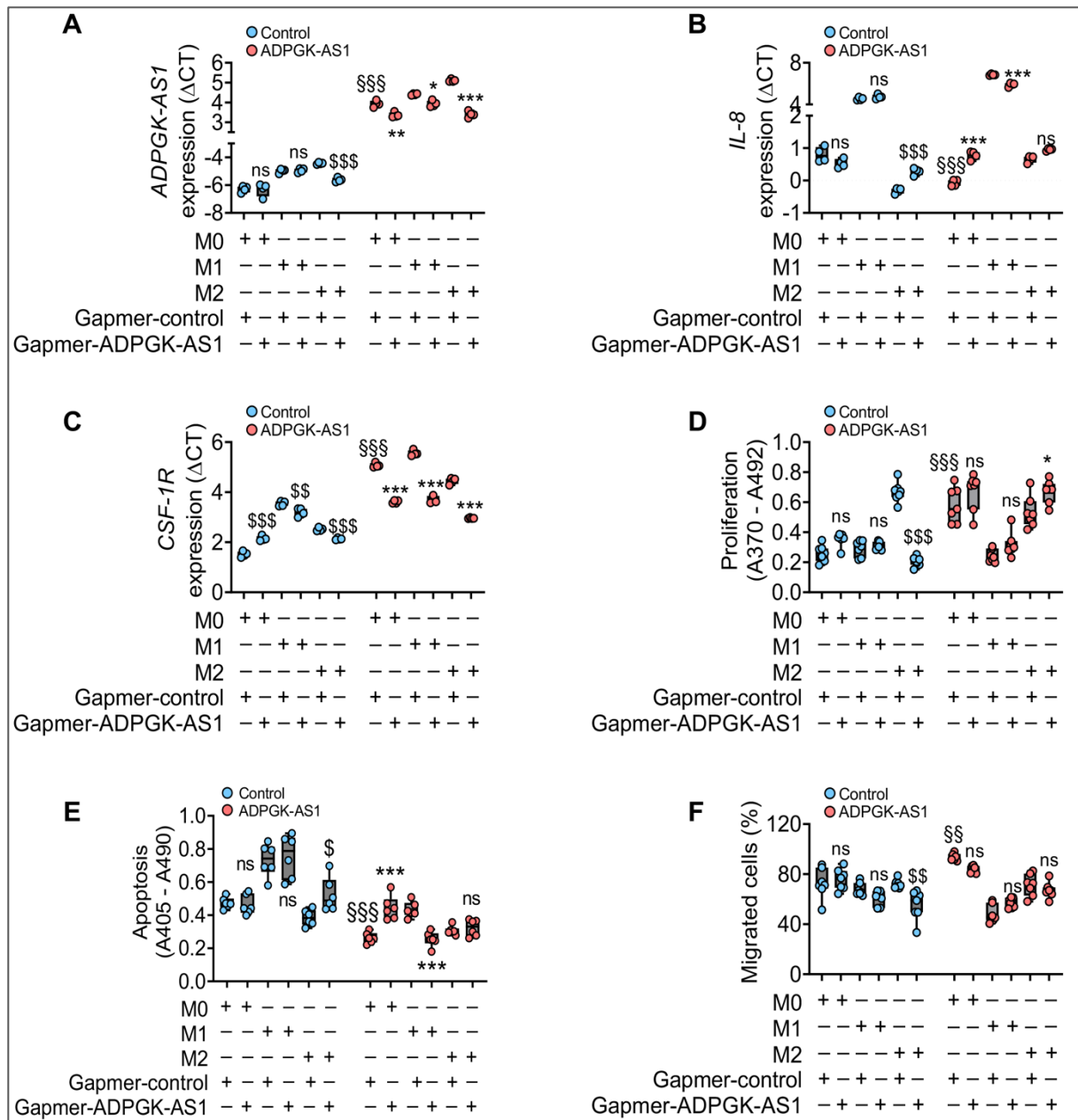
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Appendix Fig. S1: Modulation of *ADPGK-AS1* expression alters mitochondrial translation efficiency

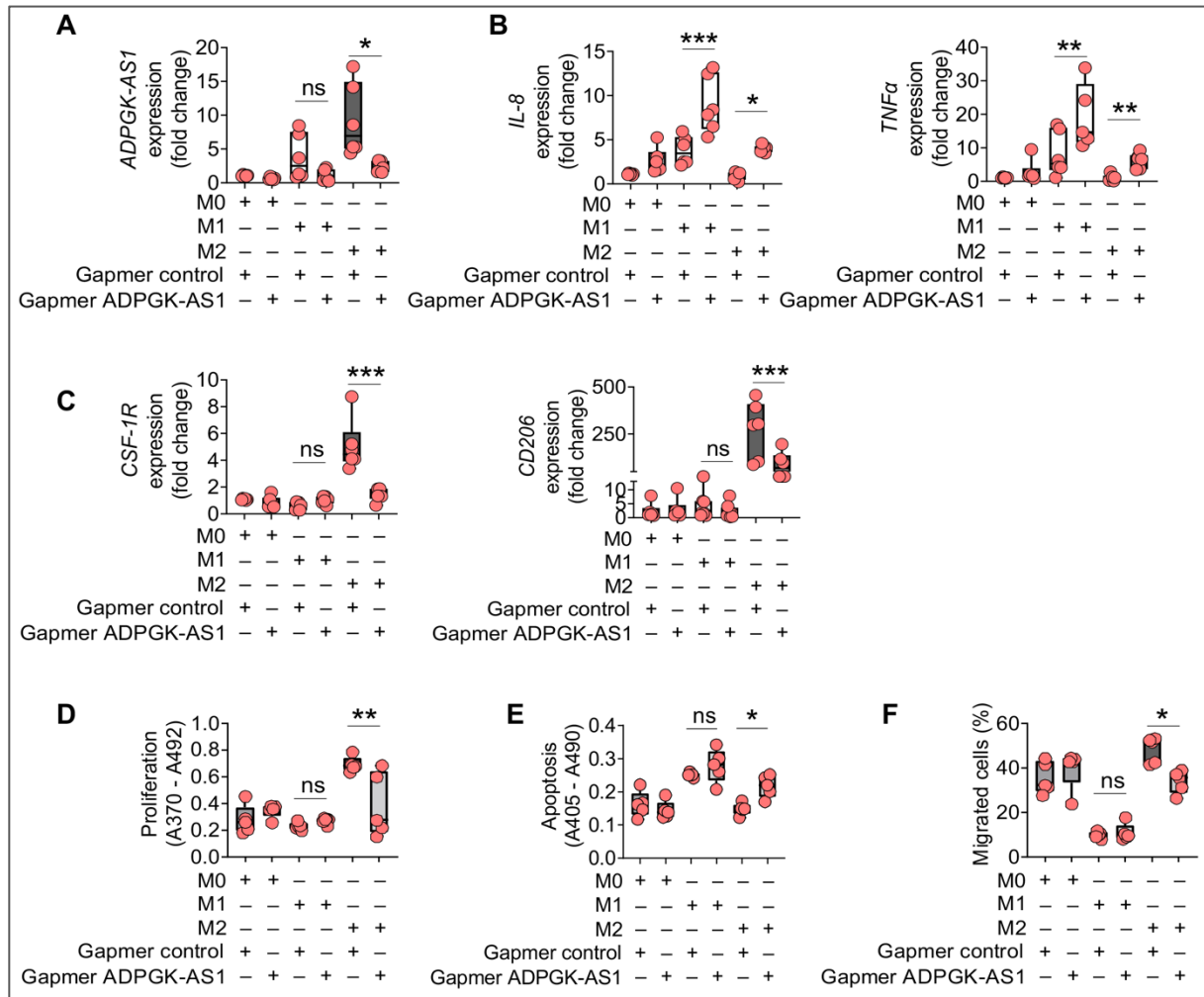
- (A) Representative confocal images of isolated mitochondria from THP1 macrophages stained with MitoTracker. Scale bar = 5 μ m (upper panel), 3 μ m (lower panel).
- (B) Isolated mitochondria (red) of THP1 control and *ADPGK-AS1* OE macrophages treated with O-propargyl-puromycin (OP-Puro) and coupled with Alexa-488-azide (green). Scale bar = 100 μ m.
- (C) Quantification of OP-Puro-conjugated Alexa Fluor-488 fluorescence signal in isolated mitochondria of THP1 control and *ADPGK-AS1* OE macrophages. n = 8 images, mean $\pm$ SEM, student's unpaired t-test, ***p $\leq$ 0.001 compared with control.
- (D) mRNA expression analysis (Δ CT) of M1 (*TNF α* , *IL-8*) and M2 (*CSF-1R*, *CD206*) macrophage marker genes in THP1 control and *ADPGK-AS1* OE cells treated with 1 μ g puromycin for 24 hours. n = 4 independent experiments. *p $\leq$ 0.05, ***p $\leq$ 0.001 compared with untreated *ADPGK-AS1* OE.



Appendix Fig. S2: Full characterization of the effect of an *ADPGK-AS1* OE in macrophages on the M0, M1 or M2 phenotype.

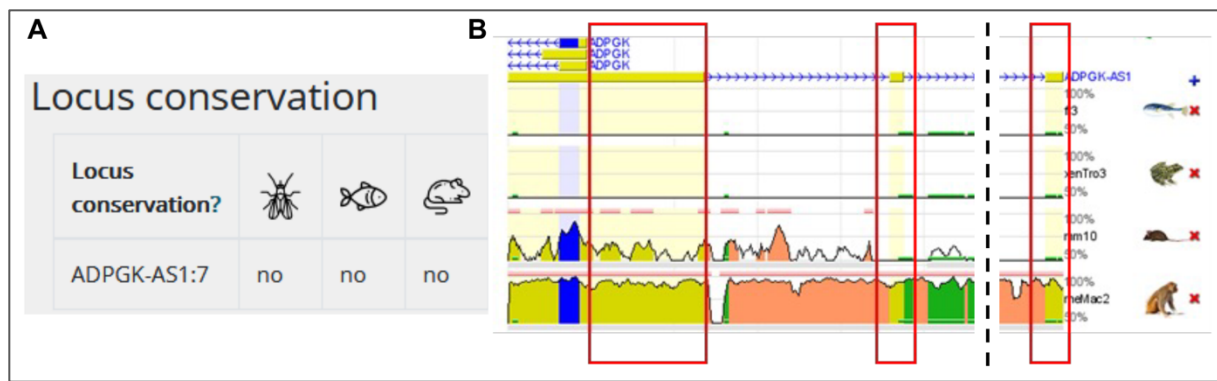
- (A – C) Analysis mRNA expression (Δ CT) of *ADPGK-AS1*, M1 (*IL-8*), and M2 (*CSF-1R*) macrophage marker genes in THP1 control and *ADPGK-AS1* OE cells activated toward the M1-like (M1, +LPS/IFN γ for 24 hours), M2-like (M2, +IL-4 for 24 hours) or M0 (unstimulated) state and then transfected with antisense LNA GapmeRs against *ADPGK-AS1* or negative control. n = 4 independent experiments, mean $\pm$ SEM, two-way ANOVA \$\$\$p $\leq$ 0.001 compared with control M0 GapmeR-control; \$\$p $\leq$ 0.01, \$\$\$p $\leq$ 0.001 compared with GapmeR control, *p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001 compared with *ADPGK-AS1* OE GapmeR control. ns = non-significant.
- (D – F) Proliferation, apoptosis, and migration (wound healing) of tumor cells (A549) treated with CM from THP1 control or *ADPGK-AS1* OE cells activated toward the M1-like (M1, +LPS/IFN γ for 24 hours), M2-like (M2, +IL-4 for 24 hours), or M0 (unstimulated) state and then transfected with antisense LNA GapmeRs against *ADPGK-AS1* or negative control. n = 8 independent experiments, mean $\pm$ SEM, two-way ANOVA. \$\$\$p $\leq$ 0.01, \$\$\$p $\leq$ 0.001 compared with control M0 GapmeR-control; \$p $\leq$ 0.05, \$\$p $\leq$ 0.01, \$\$\$p $\leq$ 0.001 compared with GapmeR control, *p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001 compared with *ADPGK-AS1* OE GapmeR control. ns = non-significant.

≤ 0.01 , $$$$p \leq 0.001$ compared with control, $*p \leq 0.05$, $***p \leq 0.001$ compared with *ADPGK-AS1* OE GapmeR control. ns= non-significant.



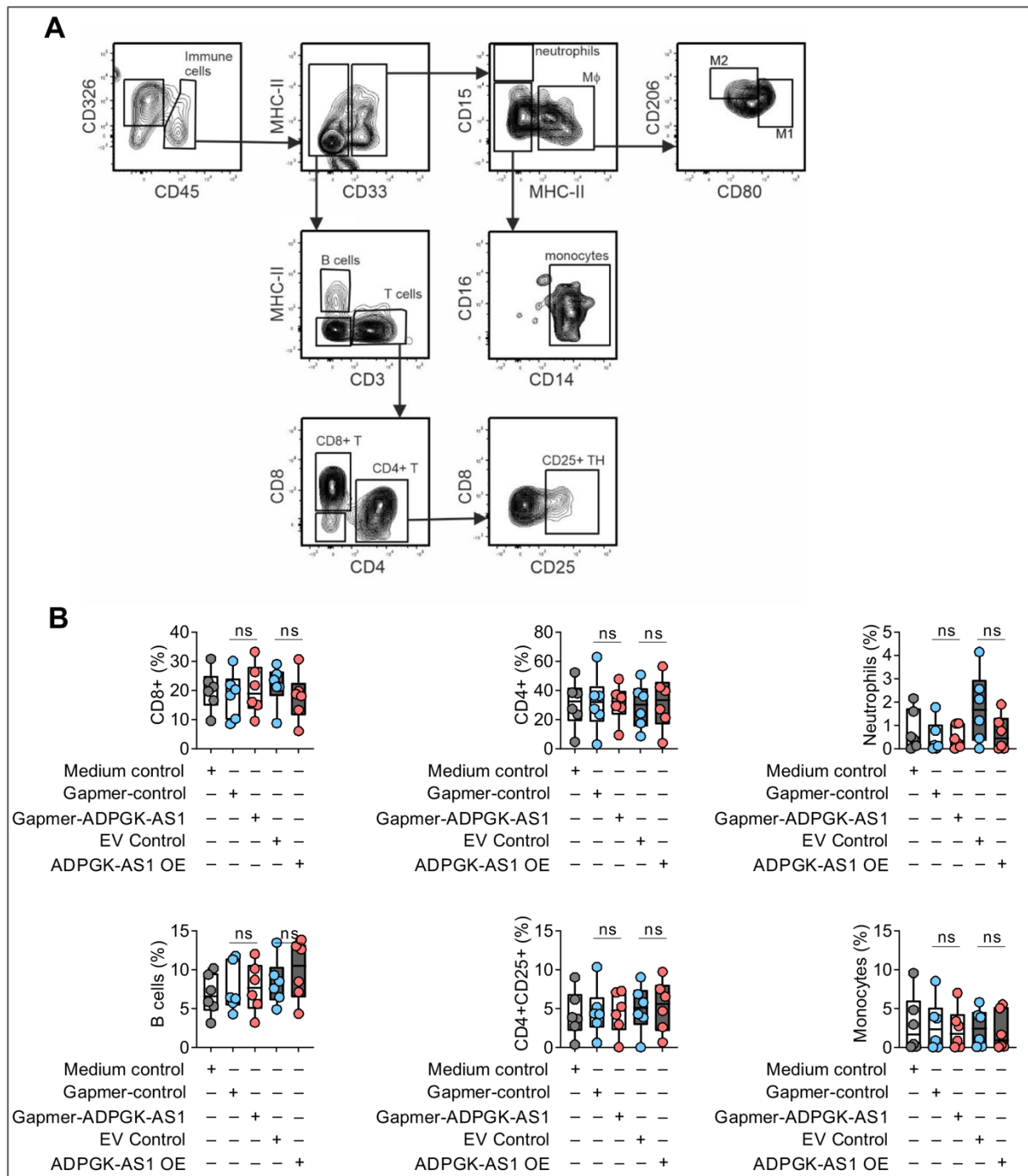
Appendix Fig. S3: Phenotypic switch in macrophages after *ADPGK-AS1* knockdown is only observed in M2-like macrophages

- (A–C) mRNA expression analysis (fold change) of *ADPGK-AS1*, M1 (*IL-8*, *TNFα*), and M2 (*CSF-1R*, *CD206*) macrophage marker genes in primary M0, M1-like (M1, +LPS/IFN γ for 24 hours), and M2-like (M2, +IL-4 for 24 hours) macrophages transfected with antisense LNA GapmeRs specific for *ADPGK-AS1* or negative control. n = 6 biological replicates, mean $\pm$ SEM, one-way ANOVA. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, compared with GapmeR control. ns = non-significant.
- (D–F) Proliferation, apoptosis, and migration (wound healing) of tumor cells(A549) treated with CM from PBMC-derived M0, M1-like (M1, +LPS/IFN γ for 24 hours), and M2-like (M2, +IL-4 for 24 hours) macrophages transfected with antisense LNA GapmeRs specific for *ADPGK-AS1* or negative control. n = 5 biological replicates, mean $\pm$ SEM, one-way ANOVA, $*p \leq 0.05$, $**p \leq 0.01$, compared with GapmeR control. ns = non-significant.



Appendix Fig. S4: *ADPGK-AS1* is not conserved in other species.

- (A) Locus conservation of the human *ADPGK-AS1* gene in *Drosophila melanogaster*, zebrafish, and mouse assessed by Incipedia.org based on conservation of flanking protein-coding genes.
- (B) Analysis of evolutionarily conserved regions of the human *ADPGK-AS1* gene by sequence alignment with the respective gene locus in zebrafish, *Xenopus laevis*, mouse, and rhesus macaque using the ECR browser. Red boxes mark exon regions. The dashed black line marks the partially removed intron region.



Appendix Fig S5: Immune cell profile of tumor PCLS

- (A) Gating strategy for multispectral flow cytometry analysis of tumor PCLS treated with antisense LNA GapmeRs specific for *ADPGK-AS1* or a negative control or seeded with THP1 control or *ADPGK-AS1* OE cells.
- (B) Immune-cell populations (CD8+, CD4+, neutrophils, B cells, CD4+CD25+ T cells, and monocytes) identified in tumor PCLS treated with antisense LNA GapmeRs specific for *ADPGK-AS1* or a negative control or seeded with THP1 control or *ADPGK-AS1* OE cells. $n = 6$ biological replicates, mean $\pm$ SEM, one-way ANOVA. ns = non-significant.

Protein groups	p-value <i>ADPGK-AS1</i>/Control	Fold enriched <i>ADPGK-AS1</i>/Control
MRPL35	0.001698391	3.26518147
MRPL15	0.00782195	2.272417539
SEC61B	0.110728621	3.078797338
SRPRB	0.089140126	2.852936577
DNAJB11	0.104962252	2.800138351
VAC14	0.118470833	2.532993194
IGHG1;IGHG3	0.172987798	2.418595666
FUS	0.114057828	2.289699077
OAS2	0.153088634	2.223886509
ACTB	0.364124424	2.215637111
AP1B1;AP2B1	0.169909726	2.204897311
REXO4	0.186607407	2.142099372
LTV1	0.28918152	2.096932206
GRN	0.23820752	2.041384144
CEBPB	0.197276557	2.013587947
SPTBN1	0.375564614	2.008035797
COPE	0.308653329	1.987516006
RPL37A	0.100540938	1.954015767
ACOT13	0.18561266	1.910812606
TUBB8	0.294876276	1.908031563
WDFY4	0.286511635	1.876144711
GTPBP1	0.288872015	1.781569344
CC2D1A	0.114722996	1.761886354
MRPL23	0.158199636	1.723541352
PSMD4	0.224326138	1.714345423
CDC42	0.265853086	1.694655905
RPL30	0.164336624	1.674511265
LLPH	0.364131556	1.665473077
HSP90AB2P	0.402542704	1.664050171
TBC1D2	0.539288603	1.657335433
MRPS33	0.297251736	1.640105194
C8orf33	0.119193296	1.636151664
SF3B2	0.512606358	1.601721109
AKAP13	0.250715242	1.592607955
GAS7	0.254700851	1.58900909
ARHGEF7	0.177510085	1.578289733
RPL11	0.44615179	1.563750119
GLTSCR2	0.478860038	1.555589095
DOCK2	0.413178314	1.555475301
MGAT1	0.406781183	1.545122083
DDX54	0.273907624	1.538062464
RTTN	0.647305423	1.536701345
DNAJC2	0.175115488	1.52553092
PRR14L	0.477885667	1.523730924

SMARCA5	0.227836702	1.511233897
GBP5	0.537799117	1.509322724
HIST2H3A	0.232912029	1.501588347
TBXAS1	0.186215232	1.490658053
ALG2	0.421315035	1.486155441
YWHAQ	0.243540534	1.48032521
HSPA6;HSPA7	0.441296921	1.452233235

Appendix Table S1: TOP50 enriched proteins identified by mass spectrometry after RNA pulldown. Identified peptides matches to a protein group. Only if unique peptides are identified, the peptide can be assigned to a single protein.

Name	Forward primer 5' → 3'	Reverse primer 5' → 3'
47S rRNA	GCTGACACGCTGTCCTCTG	ACGCGCGAGAGAACAGCAG
α-Tubulin	AGATGCTGCCAATAACTATG	CTTGCCATAATCAACTGAGAG
ADPGK	TCATCTTCTCTCACGACCTCTC	AGCTCCCTGTAGTCATACTGG
ADPGK-AS1-1 ADPGK-AS1-2	CCAACTCCTTTCCTAGCCCG TGCTGACTGTCCAGCGATCAT T	TGTGACGTAGCGCTTGTGT TCCACCCTTTAGCCTTGTGTGA
ALOX15	CTTCCAAGCTTATAATTCCCCA C	GATTCCTTCCACATACCGATAG
CCR7	GCTGGTGGTGGCTCTCCTT	GTAATCGTCCGTGACCTCATCT T
CD206	TTCGGACACCCATCGGAATTT	CACAAGCGCTGCGTGGAT
CD80	AGGGAACATCACCATCCAAG	TGCCAGTAGATGCGAGTTTG
CSF-1R	GAGAGCTATGAGGGCAACAG	TCCGAGGGTCTTACCAAAT
CXCL10	GCACCATGAATCAAAGTCC	GGTAGTCCTTGAATGCCACT
HPRT1	TGACACTGGCAAAACAAT	GGTCCTTTTACCAGCAA
IL-1Ra	CTATCAGGCCCTCCCCATGGC	CAACTAGTTGGTTGTTCTCTCC
IL-8	ACAGCAGAGCACACAAGCTTC	CAGGAAGGCTGCCAAGAG
IL-10	GAGGCTACGGCGCTGTCA	TCCACGCCTTGCTCTTG
KB-1991G8.1-1 KB-1991G8.1-2	TTGGCAGGGGTGAAATGACT AGTCTGCAGGCAGATAGGCT	CCAGTGTCTATCACAGCTCG GCTCAGGTCAAATGCCAGTC
LINC01800-1 LINC01800-2	GTGTCTGCAGCCCACAGTAA CCCCAGGGTCATGCAAATCA	GCCACCTGTCTGATGGTCTT CCAACAAGGACCCTGCCTAT
mtND1	GCTCTCACCATCGCTCTTCT	CCGATCAGGGCGTAGTTTGA
mtND2	AGCACCACGACCCTACTACT	TGGTGGGGATGATGAGGCTA
mtND4	ACAAGCTCCATCTGCCTACG	GCTTCAGGGGGTTTGGATGA
mtATP6	ACCACAAGGCACACCTACAC	TATTGCTAGGGTGGCGCTTC
mtATP8	ACTACCACCTACCTCCCTCAC	GGATTGTGGGGGCAATGAATG
mtCO1	CTTTTCACCGTAGGTGGCCT	AGTGGAAAGTGGGCTACAACG
mtCO2	CCGTCTGAACTATCCTGCCC	GAGGGATCGTTGACCTCGTC
mtCyB	ACCCCTAGGAATCACCTCC	GCCTAGGAGGTCTGGTGAGA
PCNA	TTTTCTGTCAACAAATTTGTAC CTC	CTGCATTTAGAGTCAAGACCCT TT
P53	CAGCACATGACGGAGGTTGT	TCATCCAAATACTCCACACGC
RP11-184M15.1-1 RP11-184M15.1-2	CATCCAGGACATGCCAGCTA GGCATGGACTGGATCTGACAA	GCGGGATTGTCTGGTTTCAA GCAGTGTGTTATGGGGCGAT
RP4-644L1.2-1 RP4-644L1.2-	GAAATGGCACTGAACAGCCG AAGGTCCTTCGAGTGTTGGC	TCACCTCTGCCCCGTTAGAGA GTGCCCTTGGGGAGCTATTT
RP11-333E1.1-1 RP11-333E1.1-2	CAATGACAAAGCAGTCGTACC A CACAGACGAGTTTGCCGTGA	GTCTACAGACGACACTTCTCT TGGATCTGTCTCTGCATGGG
TGFβ	GCGTGCGGCAGCTGTACATT	GGGCCAGGACCTTGCTGTACT
TNFα	GAGGCCAAGCCCTGGTATG	CGGCCGATTGATCTCAGC

Appendix Table S2: Human primer sequences.

Name	Host	Source	Catalog No.	Application	Dilution
β-actin	Mouse	Abcam	ab8227	WB	1:5000
MRPL15	Rabbit	GBiosciences	ITT2848	WB	1:1000
MRPL35	Rabbit	Invitrogen	PA5-38964	WB	1:1000
TOM20	Rabbit	Abcam	ab186734	WB, IF	1:1000, 1:300
SDHA	Rabbit	Cell signaling	5839	WB	1:1000
αb-crystallin	Rabbit	Abcam	ab13497	WB	1:1000
PCG-1	Rabbit	Abcam	ab72230	WB	1:1000
Cytochrome C	Mouse	Invitrogen,	33-8200	WB	1:500
Mfn1	Rabbit	Cell signaling	14739	WB	1:1000
Opa1	Rabbit	Cell signaling	80471	WB	1:1000
Drp1	Rabbit	Cell signaling	8570	WB	1:1000
pDRP1	Rabbit	Invivogen	PA5-64821	WB	1:1000
PDH	Rabbit	Cell signaling	2784	WB	1:1000
AMPKα	Rabbit	Cell signaling	2532	WB	1:1000
pAMPKα (Thr172)	Rabbit	Cell signaling	2535	WB	1:500
Ki67	Rabbit	Abcam		IF	1:250
Cyclin D1	Rabbit	Cell signaling	2978	WB	1:300
Cleaved caspase 3	Rabbit	Cell signaling	9664	WB, IF	1:500, 1:200
Cytokeratin 18	Rabbit	Abcam	ab181597	IF	1:250
Lamin A/C	Mouse	Santa Cruz	sc-376248	WB	1:1000
Anti-mouse IgG HRP-linked	Anti-Mouse	Promega	W4018	WB	1:5000
Anti-rabbit IgG HRP-linked	Anti-Rabbit	Promega	W4028	WB	1:5000
Anti-rabbit IgG Alexa-Fluor-488-linked	Anti-Rabbit	Life technologies	A11008	IF	1:5000

Appendix Table S3: Details of the antibodies used in this study