

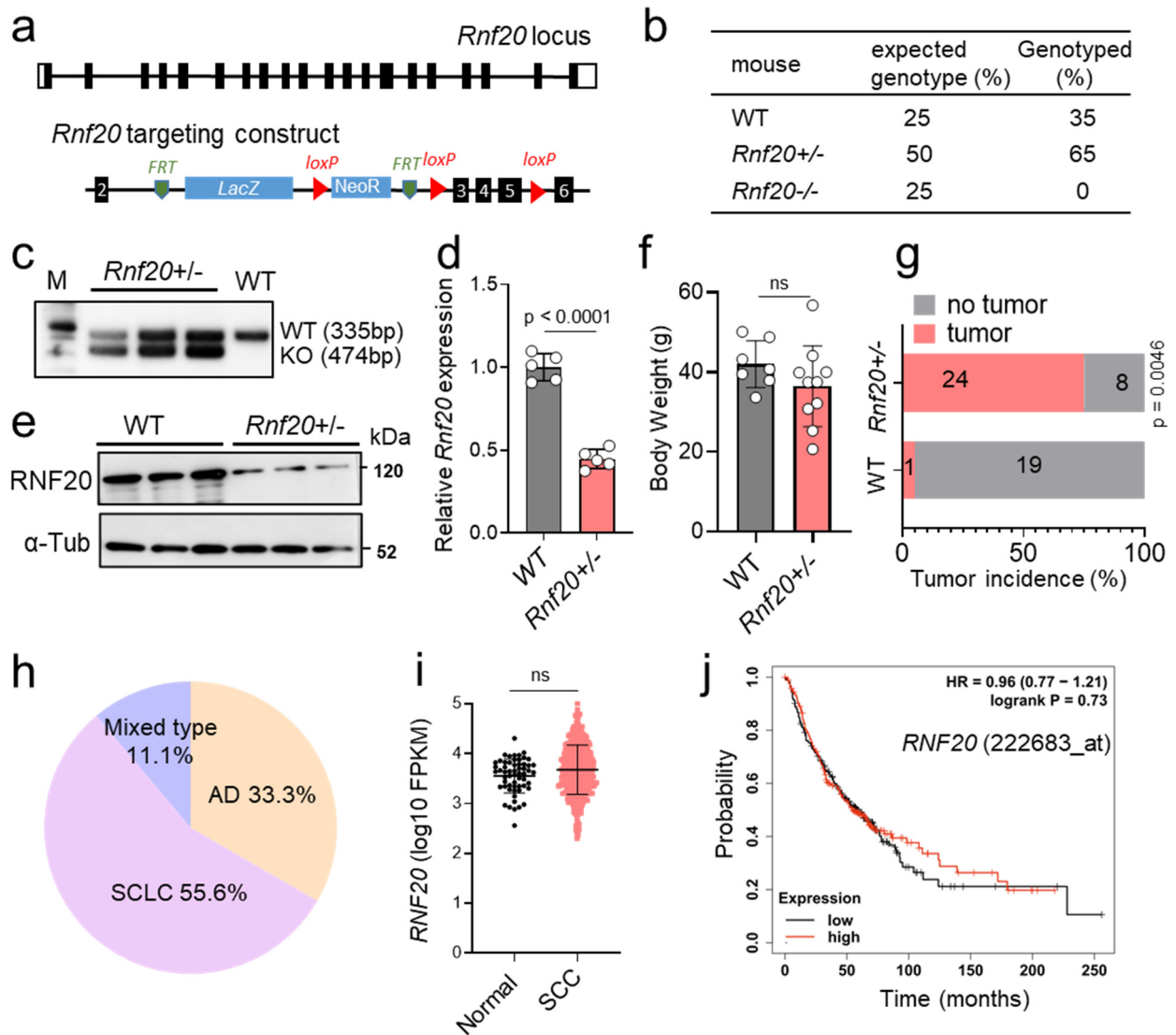
## Supplementary Information

for

### **RNF20 links the DNA damage response and metabolic rewiring in lung cancer through HIF1 $\alpha$**

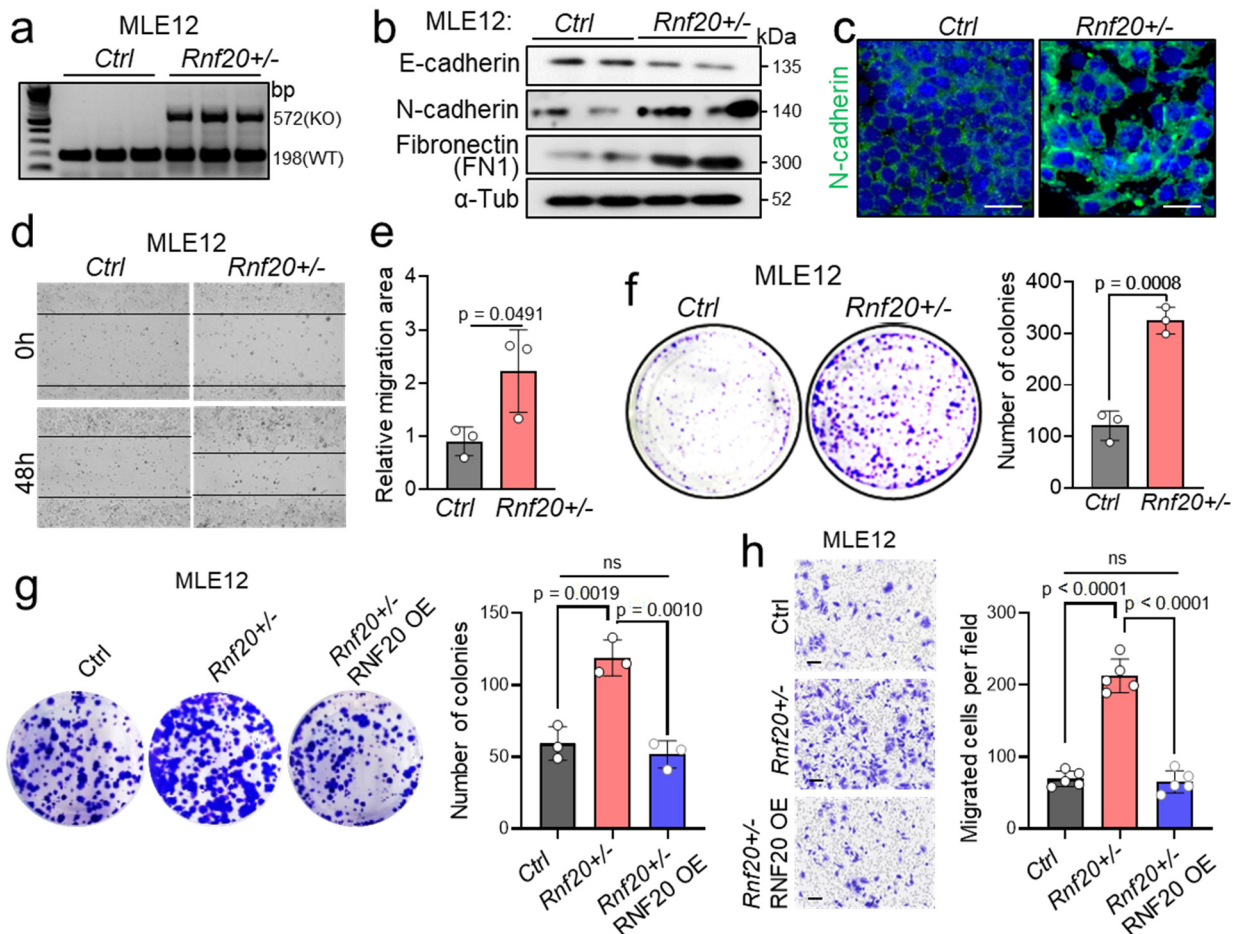
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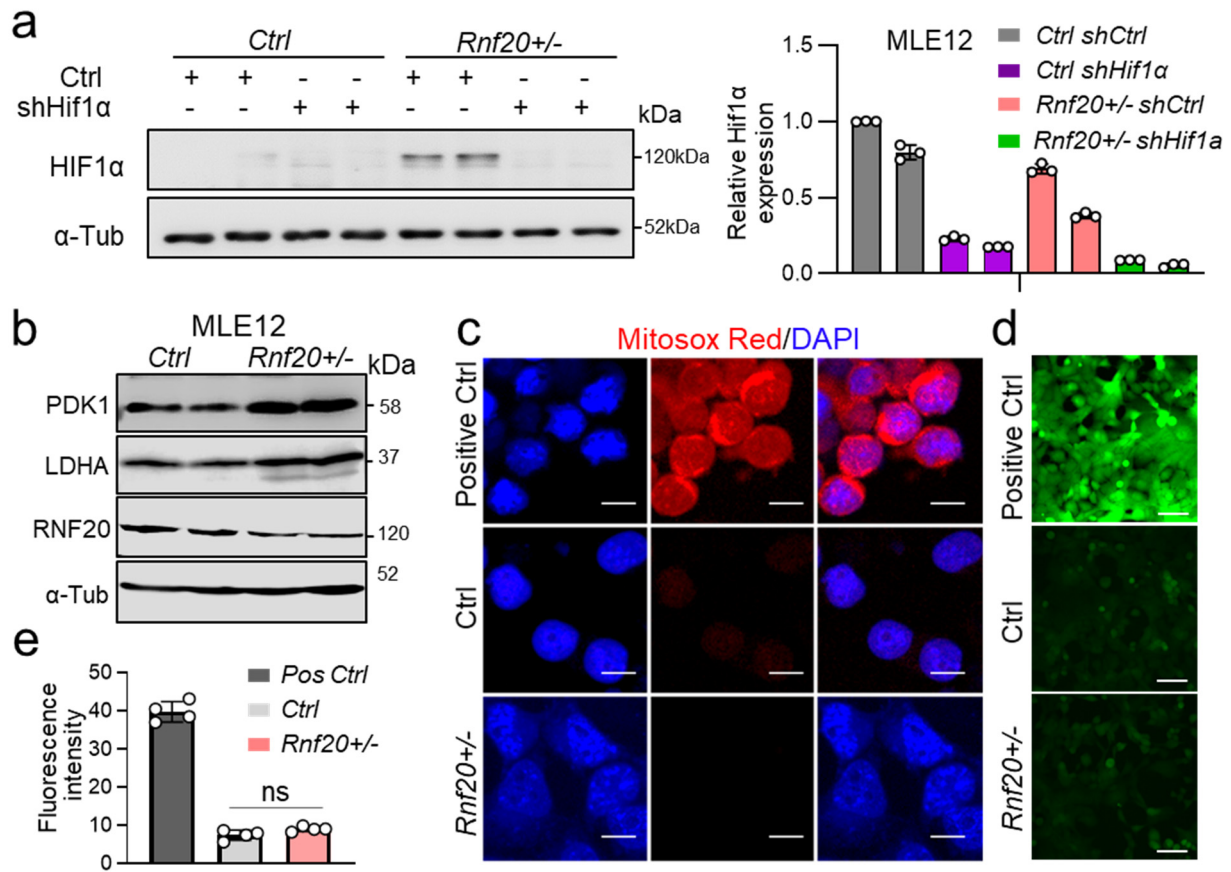
**Supplementary Fig. 1. *Rnf20* loss in lung cancer.** **a** Schematic diagram of the *Rnf20* genomic locus and the *Rnf20* targeting construct<sup>33</sup>. **b** Percentage distribution of expected genotypes compared to the actual genotypes of mice obtained after breeding. **c** PCR genotyping results showing distinct bands for wild-type (WT) and knockout (KO) alleles. **d** mRNA expression of *Rnf20* in lungs of control (WT) and *Rnf20*<sup>+/-</sup> mice (n = 5). **e** WB analysis of protein extracts from lungs of WT and *Rnf20*<sup>+/-</sup> mice using an anti-RNF20 antibody. **f** Body weight of WT (n = 7) and *Rnf20*<sup>+/-</sup> (n = 10) mice at 1 year of age. **g** Bar plot presenting the number of WT and *Rnf20*<sup>+/-</sup> mice with or without tumors. **h** Pie chart showing the percentage of AD, SCLC or mixed type tumors (with characteristics of both AD and SCLC) in *Rnf20*<sup>+/-</sup> tumor-bearing mice. **i** Normalized expression of *RNF20* in normal lung (n = 58) and lung squamous cell carcinoma (SCC) (n = 502) tissues in TCGA datasets. FPKM stands for

fragments per kilobase of exon per million mapped fragments. **j** Overall survival (Kaplan-Meier plot) of lung squamous cell carcinoma patients<sup>34</sup> expressing high vs low levels of *RNF20*. For pairwise comparisons, statistical analysis was performed using two-tailed Student's t-test (**d**, **f**, **i**). Data are shown as mean  $\pm$  SEM. ns, non-significant. 'n' indicates biological replicates. Source data are provided as a Source Data file.



**Supplementary Fig. 2. *Rnf20* haploinsufficiency induces cell migration and anchorage-independent growth.** **a** PCR genotyping results showing distinct bands for WT and *Rnf20* KO alleles in control and *Rnf20*<sup>+/-</sup> MLE12 cells. **b** Western blot analysis of total protein extracts of control and *Rnf20*<sup>+/-</sup> MLE12 cells using E-cadherin, N-cadherin and fibronectin antibodies. α-Tubulin served as a loading control. **c** Immunostaining for N-cadherin of control and *Rnf20*<sup>+/-</sup> MLE12 cells. Scale bars, 20 μm. **d, e** Representative images of wound gap closure (**d**) and quantification of the relative migration area (**e**) in a scratch wound healing assay with control and *Rnf20*<sup>+/-</sup> MLE12 cells (n = 3). Scale bars, 20 μm. **f** Clonogenic assay performed with control and *Rnf20*<sup>+/-</sup> MLE12 cells. Representative images are shown on the left, quantification of the number of colonies is shown on the right (n = 3). **g** Clonogenic assay performed with control, *Rnf20*<sup>+/-</sup> and RNF20 overexpressing (OE) *Rnf20*<sup>+/-</sup> MLE12 cells. Representative images are shown on the left, quantification of the number of colonies is shown on the right (n = 3). **h** Boyden chamber migration assay with control, *Rnf20*<sup>+/-</sup> and RNF20 overexpressing (OE) *Rnf20*<sup>+/-</sup> MLE12 cells (left) and quantification of the number of migrated cells per field

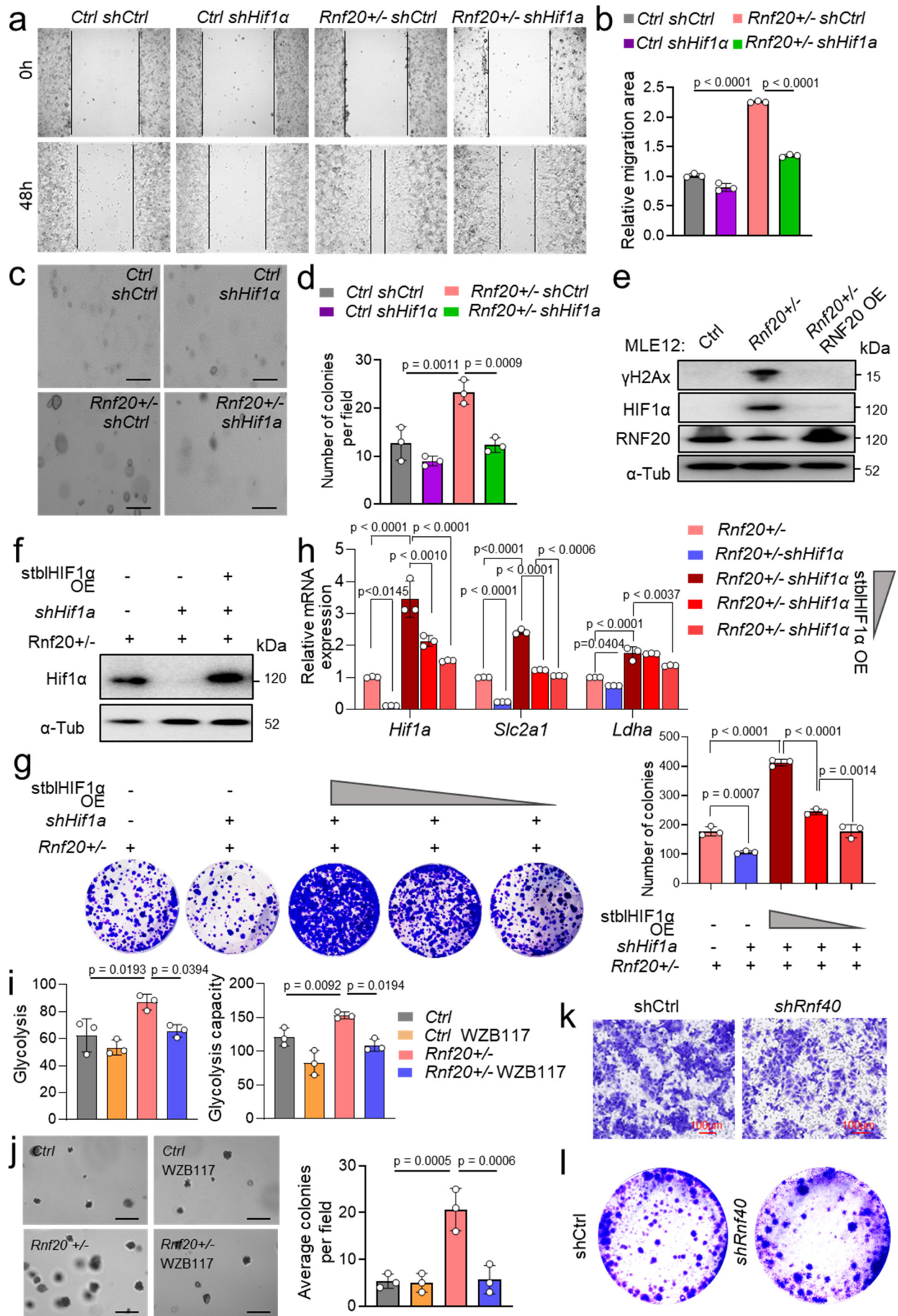
(right) (n = 5). Statistical analysis in **(e, f)** was performed using a two-tailed Student's t-test. Multiple comparisons in **(g, h)** were performed using one-way ANOVA with Turkey's multiple comparisons test. Data are shown as mean  $\pm$  SEM. 'n' indicates biological replicates. Source data are provided as a Source Data file.



**Supplementary Fig. 3. Functional and molecular alterations upon *Rnf20* loss of function.**

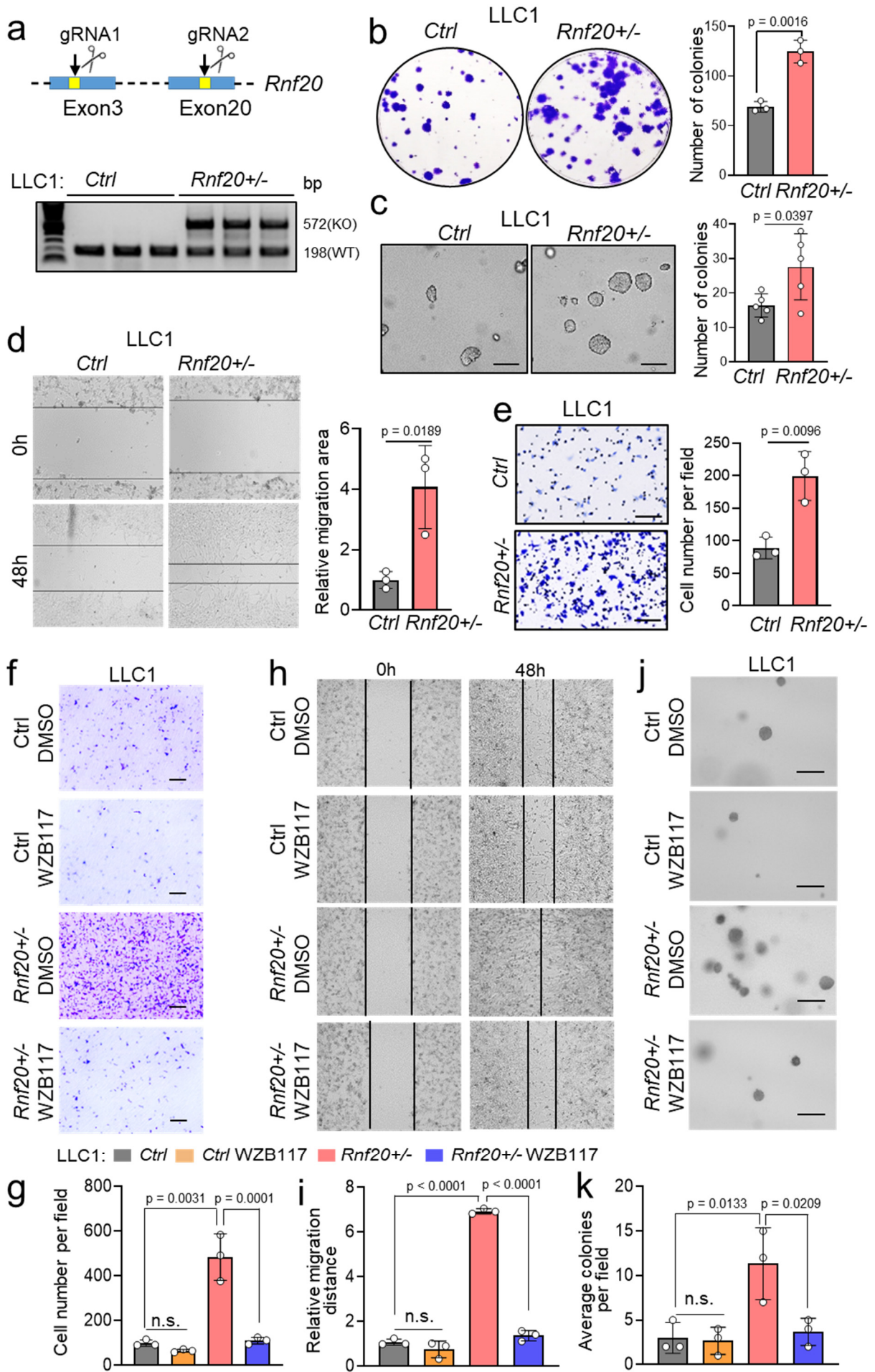
**a** Western blot analysis of total protein extracts of control and *Rnf20*<sup>+/-</sup> MLE12 cells overexpressing either control shRNA or shRNA against *Hif1a* using HIF1α antibody (left panel) and qPCR analysis of *Hif1a* mRNA expression (right panel). **b** Western blot analysis of total protein extracts of control and *Rnf20*<sup>+/-</sup> MLE12 cells using PDK1, LDHA and RNF20 antibodies. **c** Mitochondrial superoxide production in control and *Rnf20*<sup>+/-</sup> MLE12 cells measured with the mitochondrial superoxide indicator MitoSOX. Scale bars, 20 μm. **d, e** Total Reactive Oxygen Species (ROS) levels in control and *Rnf20*<sup>+/-</sup> MLE12 cells. Images of cells stained with the total Reactive Oxygen Species (ROS) Assay 488 nm kit (**d**) and fluorescence intensity measurements (**e**). Scale bars (**d**), 100 μm. Statistical analysis in (**e**) was performed using a two-tailed Student's t-test. Data are shown as mean ± SEM. ns, non-significant. 'n' indicates biological replicates. Source data are provided as a Source Data file.



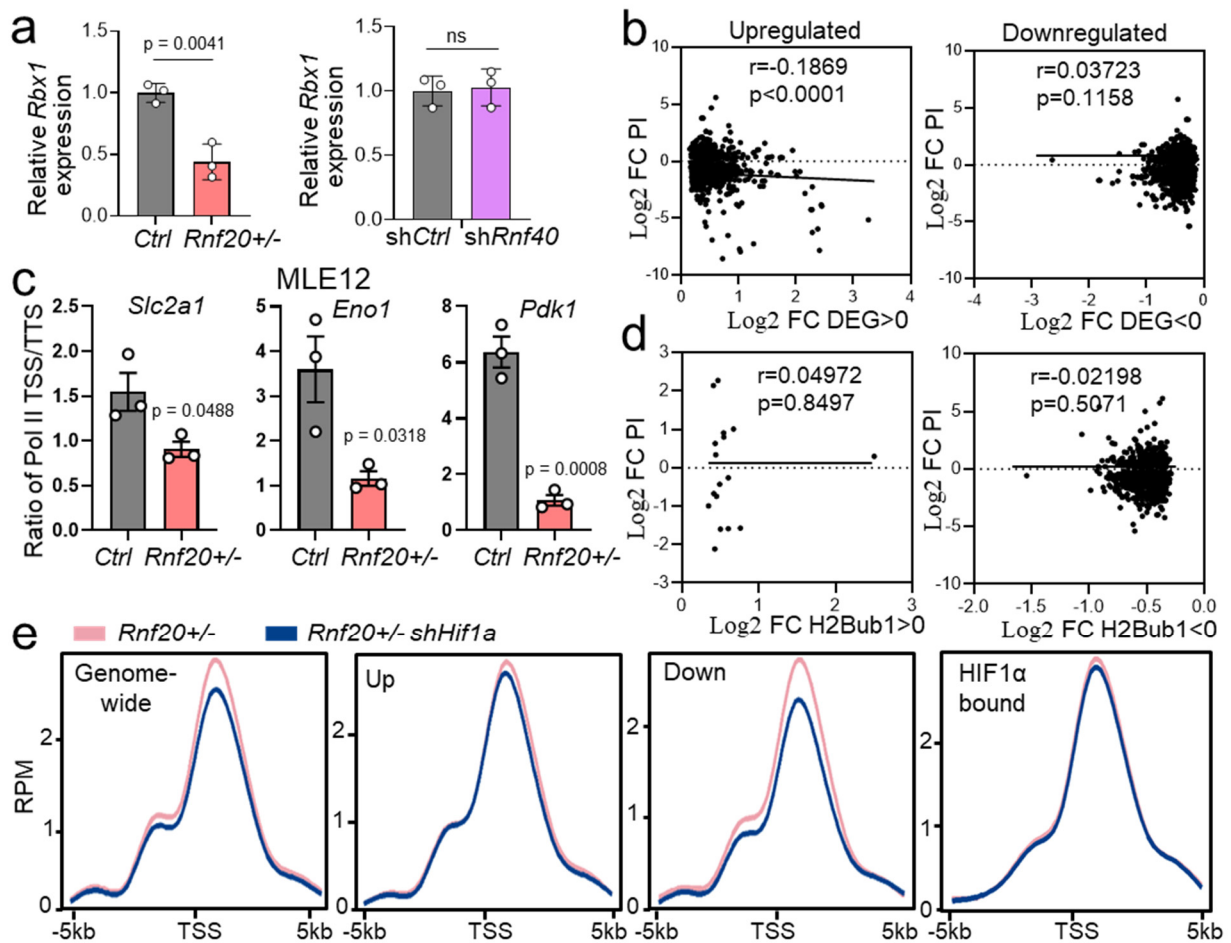


**Supplementary Fig. 4. *Hif1a* silencing or inhibition of glucose uptake rescues the increased growth and migration of *Rnf20*<sup>+/-</sup> lung epithelial cells.** **a, b** Representative images of wound gap closure (**a**) and quantification of the relative migration area (**b**) in a scratch wound healing assay with control and *Rnf20*<sup>+/-</sup> MLE12 cells overexpressing either control shRNA or shRNA against *Hif1a* (n = 3). **c, d** Soft agar assay to determine the anchorage-independent growth and quantification of the number of colonies (**d**, n = 3). Scale bars, 200  $\mu$ m. **e** Western blot analysis of  $\gamma$ H2Ax, HIF1 $\alpha$  and RNF20 in total cell lysates from control, *Rnf20*<sup>+/-</sup> and RNF20 OE *Rnf20*<sup>+/-</sup> MLE12 cells. **f** Western blot analysis of HIF1 $\alpha$  in total cell lysates from *Rnf20*<sup>+/-</sup> MLE12 cells, or *Rnf20*<sup>+/-</sup> cells expressing shRNA against *Hif1a* either alone or together with a constitutively active (non-degradable) human HIF1 $\alpha$  overexpression construct (stbHIF1 $\alpha$  OE). **g** Clonogenic assay performed with *Rnf20*<sup>+/-</sup> MLE12 cells, or *Rnf20*<sup>+/-</sup> cells expressing shRNA against *Hif1a* alone or together with varying amounts of stbHIF1 $\alpha$  OE construct. Representative images are shown on the left, quantification of the number of colonies is shown on the right (n = 3). **h** qPCR analysis of glycolysis and hypoxia-related genes in *Rnf20*<sup>+/-</sup> MLE12 cells, or *Rnf20*<sup>+/-</sup> cells expressing shRNA against *Hif1a*, either alone or in combination with varying amounts of stbHIF1 $\alpha$  OE construct (n = 3). **i** Relative glycolysis (left) and maximum glycolytic capacity (right) after addition of glucose and oligomycin on control and *Rnf20*<sup>+/-</sup> MLE12 cells treated either with DMSO or with WZB117 measured by Seahorse XF Glycolysis Stress Test Kit. **j** Soft agar assay to determine the anchorage-independent growth of control and *Rnf20*<sup>+/-</sup> MLE12 cells treated either with DMSO or with WZB117 (left) and quantification of the number of colonies (right) (n = 3). Scale bars, 200  $\mu$ m. **k** Representative images of Boyden chamber-based migration assay with control or *Rnf40*-mediated knockdown MLE12 cells. Scale bars, 100  $\mu$ m. **l** Representative images of clonogenic assay performed with control or *Rnf40*-knockdown MLE12 cells. Multiple comparisons were performed using one-way ANOVA with Šídák's (**b, d, i, j**) or Tukey's (**g, h**) multiple comparisons test. Data are shown as mean  $\pm$  SEM. 'n' indicates biological replicates. Source data are provided as a Source Data file.



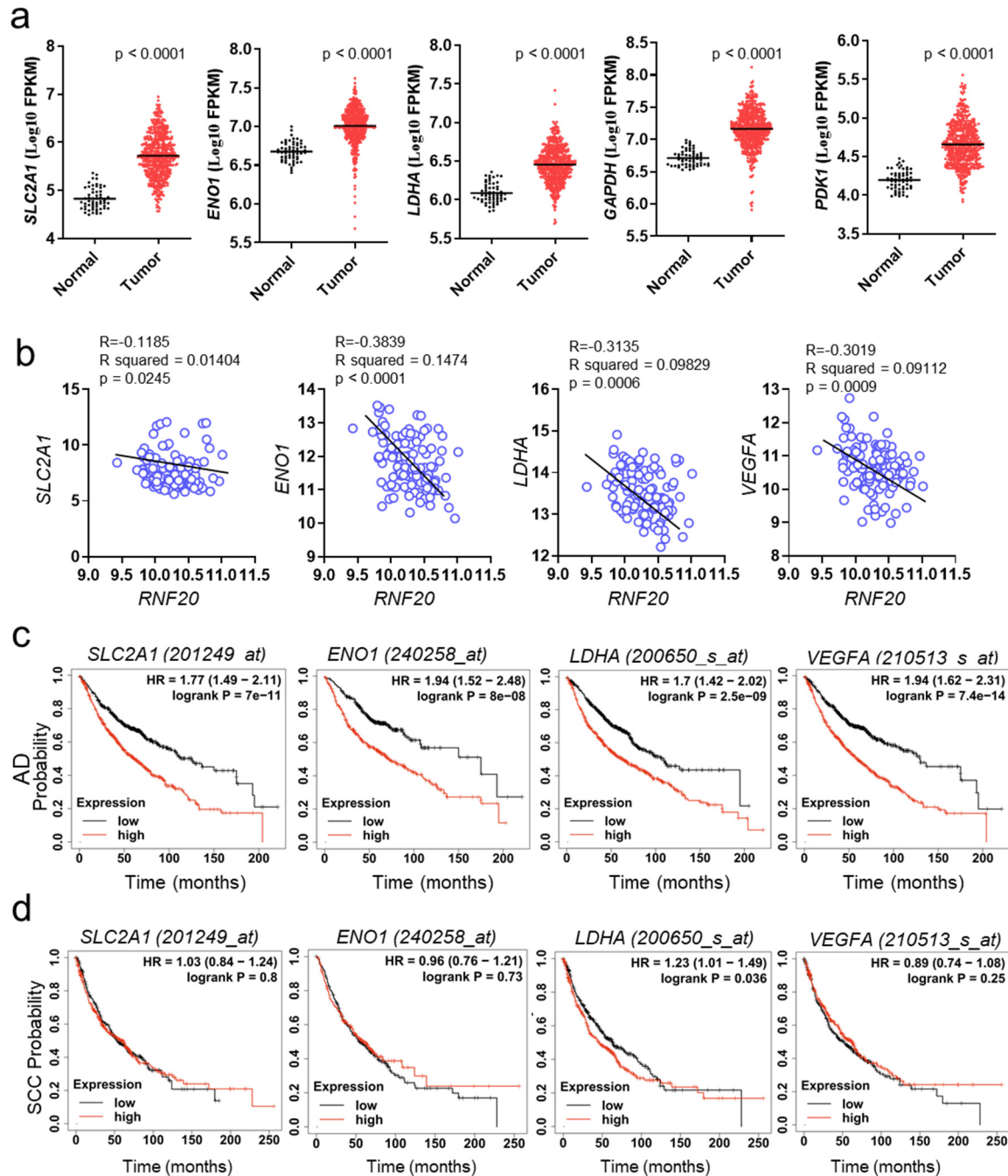


**Supplementary Fig. 5. *Hif1a* silencing or inhibition of glucose uptake rescues the increased growth and migration of *Rnf20*<sup>+/-</sup> LLC1 lung cancer cells.** **a** Schematic representation of the strategy used to generate *Rnf20*<sup>+/-</sup> LLC1 cells (top), and PCR genotyping results showing distinct bands for WT and *Rnf20* KO alleles. The scissors icon is created in BioRender; Lityagina, O. (2025) <https://BioRender.com/s41f438>. **b** Representative images of a clonogenic assay with control and *Rnf20*<sup>+/-</sup> LLC1 cells (left) and quantification of the number of colonies in the soft agar (right, n = 3). **c** Representative images of a soft agar assay to determine the anchorage-independent growth of control and *Rnf20*<sup>+/-</sup> LLC1 cells (left) and quantification of the number of colonies in the soft agar (right, n = 5). Scale bars, 200  $\mu$ m. **d** Representative images of wound gap closure (left) and quantification of the relative migration area (right) in a scratch wound healing assay with control and *Rnf20*<sup>+/-</sup> LLC1 cells (n = 3). **e** Boyden chamber migration assay with control and *Rnf20*<sup>+/-</sup> LLC1 cells showing representative images (left) and quantification of the number of migrated cells per field (right). n = 3. Scale bars, 150  $\mu$ m. **f, g** Representative images of Boyden chamber-based migration assay with control and *Rnf20*<sup>+/-</sup> LLC1 cells treated either with DMSO or with WZB117 (**f**) and quantification on the migrated cells per field (**g**, n = 3). Scale bars, 100  $\mu$ m. **h, i** Representative images of scratch wound healing assay with control and *Rnf20*<sup>+/-</sup> LLC1 cells treated either with DMSO or with WZB117 (**h**), and quantification of the relative migration area (**i**) (n = 3). **j, k** Soft agar assay to determine the anchorage-independent growth of control and *Rnf20*<sup>+/-</sup> LLC1 cells treated either with DMSO or with WZB117 (**j**) and quantification of the number of colonies (n = 3) (**k**). Scale bars, 200  $\mu$ m. Statistical analysis in (**b, c, d, e**) was performed using a two-tailed Student's t-test. Multiple comparisons in (**g, i, k**) were performed using one-way ANOVA with Turkey's multiple comparisons test. Data are shown as mean  $\pm$  SEM. 'n' indicates biological replicates. Source data are provided as a Source Data file.



**Supplementary Fig. 6. RNF20- and HIF1α-dependent transcriptional and H3K4me3 changes.** **a** Relative *Rbx1* expression in control versus *Rnf20*<sup>+/-</sup> MLE12 cells (left), or in control and *Rnf40*-mediated knockdown MLE12 cells (right, n = 3). **b** Pearson correlation (r) for the Log<sub>2</sub> FC of PI and Log<sub>2</sub> FC of upregulated (left) or downregulated (right) genes in *Rnf20*<sup>+/-</sup> versus control MLE12 cells. **c** Ratio of the Pol II enrichment at the TSS and TTS of *Slc2a1*, *Eno1* and *Pdk1* in control and *Rnf20*<sup>+/-</sup> MLE12 cells determined by Pol II Chip-qPCR (n = 3). TSS, Transcription Start Site; TTS, Transcription Termination Site. **d** Pearson correlation (r) for the Log<sub>2</sub> FC of PI and Log<sub>2</sub> FC of genes showing increased (left) or decreased (right) H2Bub1 levels in *Rnf20*<sup>+/-</sup> versus control MLE12 cells. **e** Average genome-wide H3K4me3 ChIP-seq signal, as well as H3K4me3 ChIP-seq signal at genes that are upregulated or downregulated upon RNF20 loss or bound by HIF1α in *Rnf20*<sup>+/-</sup> MLE12 cells, or *Rnf20*<sup>+/-</sup> MLE12 cells after shRNA mediated *Hif1a* silencing. Statistical analysis in (a, c)

was performed using a two-tailed Student's t-test. Data are shown as mean  $\pm$  SEM. ns, non-significant. 'n' indicates biological replicates. Source data are provided as a Source Data file.



**Supplementary Fig. 7 Expression levels of metabolic genes in lung AD and lack of correlation between the expression of glycolytic enzymes and survival of SCC patients.**

**a** Normalized expression of the metabolic enzymes *ENO1*, *PDK1*, *LDHA*, *SLC2A1* and *GAPDH* in normal lung (n = 59) and adenocarcinoma (n = 532) tissues in the TCGA datasets.

**b** Pearson correlation (r) for the mRNA expression levels of *RNF20* and genes involved in glycolysis (*SLC2A1*, *ENO1*, *LDHA*) or the hypoxic response (*VEGFA*) in lung AD cancer datasets (GSE19188, GSE27262). **c** Overall survival (Kaplan-Meier plot) of lung

adenocarcinoma patients<sup>34</sup> expressing high vs low levels of the RNF20/HIF1 $\alpha$ -dependent genes, *SLC2A1*, *ENO1*, *LDHA* and *VEGFA*. **d** Overall survival (Kaplan-Meier plot) of lung squamous carcinoma (SCC) patients expressing high vs low levels of *SLC2A1*, *ENO1*, *LDHA* and *VEGFA*.