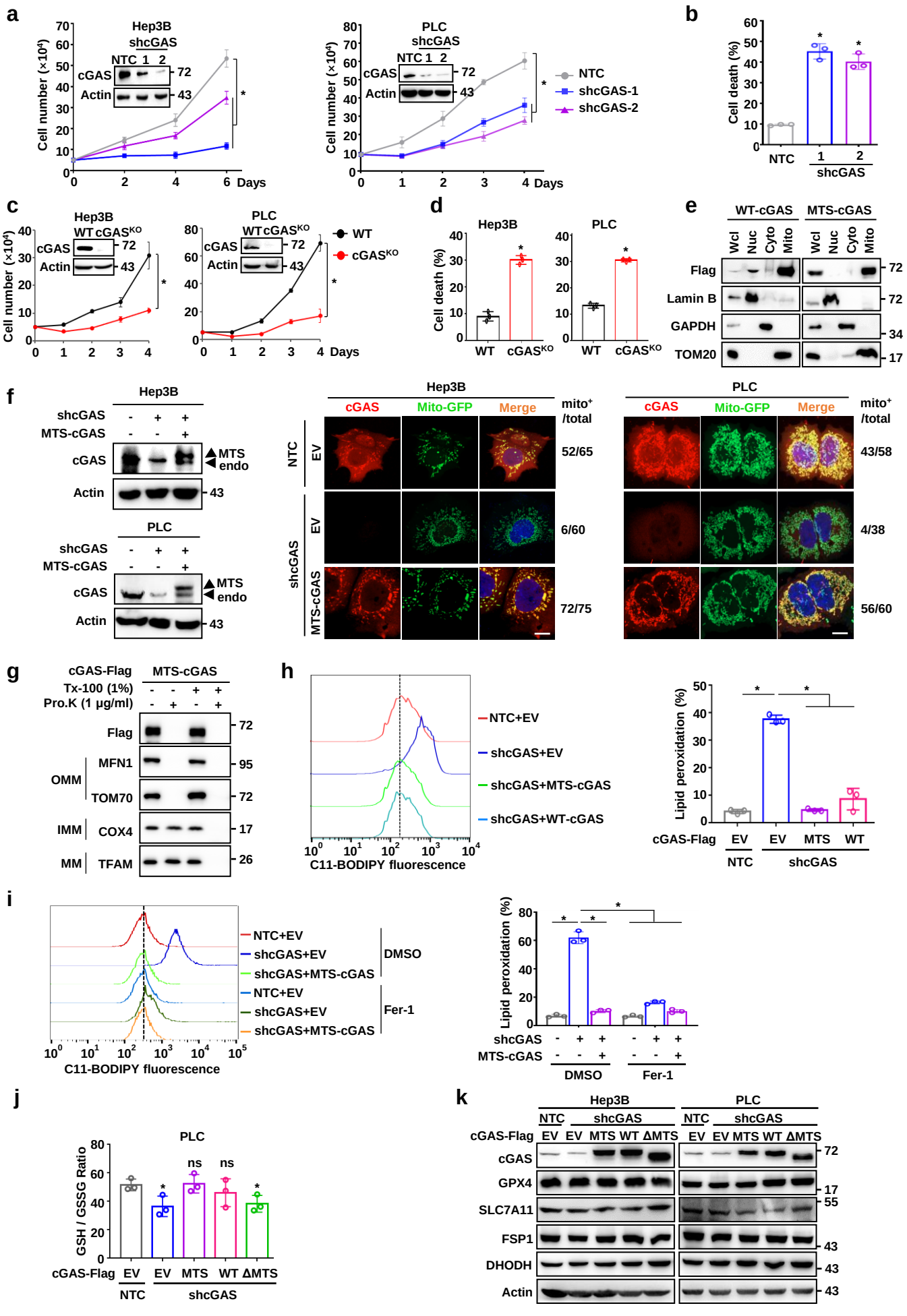
a
Hep3B
shcGAS
NTC 1 2
cGAS
Actin
72
43
Cell number (×10⁴)
Days
PLC
NTC
shcGAS-1
shcGAS-2
b
Cell death (%)
NTC
shcGAS
c
Hep3B
WT cGAS^KO
PLC
WT
cGAS^KO
d
Hep3B
PLC
WT
cGAS^KO
e
WT-cGAS
MTS-cGAS
Wcl
Nuc
Cyto
Mito
Flag
Lamin B
GAPDH
TOM20
34
17
f
Hep3B
shcGAS
MTS-cGAS
cGAS
MTS
endo
Actin
PLC
cGAS
Mito-GFP
Merge
mito⁺ /total
NTC
EV
shcGAS
MTS-cGAS
52/65
6/60
72/75
43/58
4/38
56/60
g
cGAS-Flag
MTS-cGAS
Tx-100 (1%)
Pro.K (1 μg/ml)
Flag
OMM
MFN1
TOM70
IMM
COX4
MM
TFAM
95
26
h
NTC+EV
shcGAS+EV
shcGAS+MTS-cGAS
shcGAS+WT-cGAS
C11-BODIPY fluorescence
Lipid peroxidation (%)
cGAS-Flag
EV
MTS
WT
i
DMSO
Fer-1
shcGAS
MTS-cGAS
j
PLC
GSH / GSSG Ratio
ns
ΔMTS
k
Hep3B
PLC
NTC
shcGAS
cGAS-Flag
EV
MTS
WT
ΔMTS
cGAS
GPX4
SLC7A11
FSP1
DHODH
Actin
55

Supplementary information, Fig S4 Mitochondrial localized cGAS inhibits ferroptosis

a, Cell growth analysis of HCC cells with cGAS knockdown (Hep3B cells in the left and PLC cells in the right). **b**, Cell death analysis of Hep3B cells with cGAS knockdown. **c**, Cell growth analysis of HCC cells with cGAS knockout (Hep3B cells in the left and PLC cells in the right). **d**, Cell death analysis of HCC cells with cGAS knockout (Hep3B cells in the left and PLC cells in the right). **e**, Western blotting analysis of the protein levels of cGAS in subcellular fractions including nucleus, cytosol and mitochondria in Hep3B cells overexpressing wild type cGAS ($cGAS^{WT}$) or cGAS fused with MTS ($cGAS^{MTS}$). Fractionation fidelity was verified by detection of Lamin B in the nuclear fraction, GAPDH in the cytosolic fraction and TOM20 in the mitochondrial fraction. **f**, Representative immunofluorescence images stained for cGAS in Hep3B (middle) and PLC (right) cells with endogenous cGAS knockdown and exogenous MTS-cGAS overexpression. The mitochondria were marked by Mito-GFP. Values shown in the right are the numbers of cells displaying mitochondrial cGAS relative to the total numbers of cells examined. The knockdown and overexpression efficiency were analyzed by Western blotting analysis (left). Scale bars, 20 μm. **g**, Proteinase K protection assays were performed in the presence of the permeabilizing agent Triton X-100 on purified mitochondria isolated from Hep3B cells overexpressing cGAS fused with MTS (MTS-cGAS). Extent of digestion was determined by blotting for key intra-mitochondrial proteins (MFN1, TOM70, COX4, TFAM). OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; MM, mitochondrial matrix. **h**, Lipid peroxidation analysis of Hep3B cells with endogenous cGAS knockdown and overexpressing wild type cGAS ($cGAS^{WT}$) or cGAS fused with MTS ($cGAS^{MTS}$). Representative histogram data and statistical results are shown. **i**, Lipid peroxidation analysis of PLC cells with endogenous cGAS knockdown and exogenous MTS-cGAS overexpression treated with Fer-1. Representative histogram data and statistical results are shown. **j**, Measurement of GSH/GSSG ratio in PLC cells with indicated genotypes. **k**, Western blotting analysis of the protein levels of master regulators in ferroptosis defense system in Hep3B cells and PLC cells with indicated

genotypes. β-Actin served as the loading control. For (**a**) to (**d**) and (**i**) to (**j**), the data are presented as the mean ± SD of three independent experiments. *p* value was calculated by two-tailed unpaired Student's *t-test* (**b, d**, **h, i, j**), or one-way analysis of variance (ANOVA) (**a,c**). *, $p \leq 0.05$; ns, not significant.