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# Overexpressed Gai1 exerts pro-tumorigenic activity in nasopharyngeal carcinoma

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The current study tested the expression and potential functions of Gai1 in nasopharyngeal carcinoma (NPC). The Cancer Genome Atlas (TCGA) database results demonstrate that *Gai1* transcripts' number in NPC tissues is significantly higher than that in the normal nasal epithelial tissues. Its overexpression correlates with poor survival in certain NPC patients. Moreover, Gai1 is significantly upregulated in NPC tissues of local primary patients and in different primary human NPC cells. Whereas its expression is relatively low in cancer-surrounding normal tissues and in primary nasal epithelial cells. Genetic silencing (via shRNA strategy) or knockout (via CRISPR-sgRNA method) of Gai1 substantially suppressed viability, proliferation, cell cycle progression, and migration in primary NPC cells, causing significant caspase-apoptosis activation. Contrarily, ectopic Gai1 expression exerted pro-tumorigenic activity and strengthened cell proliferation and migration in primary NPC cells. Gai1 is important for Akt-mTOR activation in NPC cells. Akt-S6K phosphorylation was downregulated after Gai1 shRNA or KO in primary NPC cells, but strengthened following Gai1 overexpression. In Gai1-silenced primary NPC cells, a S473D constitutively-active mutant Akt1 (caAkt1) restored Akt-S6K phosphorylation and ameliorated Gai1 shRNA-induced proliferation inhibition, migration reduction and apoptosis. Bioinformatics analyses proposed zinc finger protein 384 (ZNF384) as a potential transcription factor of *Gai1*. In primary NPC cells, ZNF384 shRNA or knockout (via CRISPR-sgRNA method) decreased *Gai1* mRNA and protein expression, whereas ZNF384 overexpression upregulated it. Importantly, there was an increased binding between ZNF384 protein and the *Gai1* promoter in human NPC tissues and different NPC cells. In vivo studies showed that intratumoral injection of Gai1-shRNA-expressing adeno-associated virus (AAV) impeded subcutaneous NPC xenograft growth in nude mice. Gai1 downregulation, Akt-mTOR inactivation, and apoptosis induction were detected in Gai1-silenced NPC xenograft tissues. Gai1 KO also effectively inhibited the growth of NPC xenografts in nude mice. Together, overexpressed Gai1 exerts pro-tumorigenic activity in NPC possibly by promoting Akt-mTOR activation.

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## INTRODUCTION

Nasopharyngeal carcinoma (NPC), a common malignancy in nasopharynx epithelia, demonstrates diverse etiopathology and histopathology [1, 2]. NPC is one of most malignant cancers, causing over 72,000 death each year globally [2–4]. Its incidence is still rising and can exceed two cases per ten thousand people in certain regions (i.e. South East Asia) [5–7]. Epstein-Barr virus (EBV) infection is one primary risk factor of NPC [2–4] and cigarette smoking/exposure is another important factor [2–4]. NPC has three histology types, including squamous cell carcinoma (SCC), non-keratinizing carcinoma and undifferentiated carcinoma [5–7]. The current standard therapies for NPC include radiotherapy plus conventional chemotherapy, molecularly-targeted therapy and immunotherapy, which have increased overall survival of a large proportion of NPC patients [1, 2]. Yet, for the NPC patients with recurrent or metastatic carcinomas, the prognosis is still poor and survival is often short. Thus, there are urgent needs to explore new oncotargets and to develop corresponding therapeutics for NPC [8].

Gai family proteins [9] have three primary members including Gai1, Gai2, and Gai3 [10–12]. Gai proteins bind ligand-activated G-protein coupled receptors (GPCR) [10–12]. Thereafter, activated Gai proteins will inhibit adenylyl cyclase (AC) and deplete cyclic AMP (cAMP) [10–12]. Studies have also reported the unconventional role of Gai proteins (in particular Gai1 and Gai3) in signaling for non-GPCR receptors [13–21]. Gai1 and Gai3 can associate with ligand-activated receptor tyrosine kinases (RTKs), including EGFR (epidermal growth factor receptor) [22], VEGFR2 (vascular endothelial growth factor receptor 2) [18], BDNF receptor TrkB [19], SCF receptor c-Kit [14], required for downstream Akt-mTOR and Erk cascades activation. Several non-RTK receptors, including Netrin-1 receptor CD146, R-spondin3 (RSPO3) receptor LGR4 [13], IL-4 receptor IL-4R [17] and LPS receptor TLR4, also required Gai1 and Gai3 to transduce downstream signalings.

Recent studies have proposed a possible pro-tumorigenic role of Gai1 in cancer cells. Liu et al. reported that Gai1 expression is elevated in human glioma tissues possibly owing to the

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downregulation of an anti-Gai1 microRNA, miR-200a [20]. Contrarily, overexpression miR-200a or genetic silencing of Gai1 potently inhibited glioma cell growth in vitro and in vivo [20]. Overexpression of YME1L, a mitochondrial protein, enhanced transcription and expression of Gai1, thereby promoting glioma cell growth in vitro and intracranial glioma xenograft growth in mice [23]. Lv *et al.*, discovered that upregulation of Gai1 was important for gastric cancer growth [24]. PINK1-AS, a novel long non-coding RNA, promoted Gai1 expression and gastric cancer cell growth possibly by sponging miR-200a [24]. The current study tested the expression and potential functions of Gai1 in NPC and explored the possible underlying mechanisms.

## MATERIALS AND METHODS

### Reagents

Fluorescence dyes, including TUNEL, DAPI, EdU, and propidium iodide (PI) were provided by Thermo-Fisher Invitrogen (Suzhou, China). All the antibodies were provided by Dr. Cao at Soochow University [20]. Other reagents were reported in our previous study [25].

### Human tissues

As reported early [25], NPC tumor tissues and matched adjacent normal nasopharynx epithelial tissues were obtained from twenty ( $n = 20$ ) primary NPC patients. All patients were administrated at the First affiliated Hospital of Soochow University (Suzhou, China) and each provided written-informed consent. Tissues were obtained freshly at the time of surgery and were immediately stored in liquid nitrogen. The protocols of testing human tissues were approved by the Ethics Committee of Soochow University, according to the principles of the Declaration of Helsinki.

### Immunohistochemistry (IHC) staining

Briefly, paraffin-embedded tissue sections were baked at 60 °C for 2 h. After dewaxing and hydration, the tissue slices were covered with citric acid buffer for 15 min at 95 °C. Next, 3% hydrogen peroxide was added, followed by incubation with the primary antibody at 4 °C overnight. Slices were then re-warmed and washed. Slices were incubated with biotin-labeled sheep anti-rabbit IgG for 1.5 h, washed and incubated with the horseradish-labeled streptavidin. Then, tissue slices were stained with diaminobenzidine and were re-dyed with Hematoxylin for 5 min, covered with 1% hydrochloric acid alcohol (75%) solution and rinsed. Finally, slices were dehydrated, sealed, and scanned.

### Tissue immunofluorescence staining

Briefly, paraffin-embedded tissue sections were baked at 60 °C for 2 h. After dewaxing and hydration, the tissue slices were covered with citric acid buffer for 15 min at 95 °C and were washed with PBS for three times. Goat serum was used to block the tissue slices for 20 min at 37 °C. The tissue slices were thereafter incubated with the described TUNEL and DAPI fluorescence dyes, washed and visualized under a confocal microscope (ZEISS).

### Cells

The detailed protocols for culturing primary human NPC cells were described early [25, 26]. The primary cells, pNPC-1, pNPC-2, pNPC-3, and pNPC-4, were derived from four different patients. The primary human nasal epithelial cells (HNEpC) from two donors (pHNEpC-1 and pHNEpC-2) were provided by Dr. Chen at Jiangsu University [26] and were reported in our previous study [25]. All the primary human cells were verified routinely. The protocols were approved by the Ethics Committee of Soochow University and were in accordance with Declaration of Helsinki. All patients provided written-informed consent.

### shRNA-induced gene silencing

The lentivirus encoding two different shRNA sequences against human Gai1 (*GNAI1*), Gai1-shRNA-s1 or Gai1-shRNA-s2 (with non-overlapping sequences), were provided from Dr. Cao at Soochow University [13–17, 20]. The zinc finger protein 384 (ZNF384) shRNA (sq-3)-expressing lentivirus was provided by Dr. Cao as well [27]. The lentivirus-packed ZNF460 shRNA was designed and verified by Genechem (Shanghai, China). The shRNA-expression virus (at MOI = 11.6) was directly added to cultured NPC cells or epithelial cells (in polybrene-containing complete medium at 70%

confluence). Control cells were infected with lentivirus encoding scramble control non-sense shRNA ("shC"). Forty-eight hours after virus infection, cells were back to complete medium and puromycin was added to select stable cell colonies. The selection process took 5–6 passages and expression of Gai1 in the stable cells was always verified at mRNA and protein levels. For in vivo animal studies, Gai1-shRNA-s2 sequence was inserted into the adeno-associated virus (aav) construct (as reported previously [25]) and aav was generated afterwards.

### Gene overexpression

The lentivirus encoding Gai1-expressing construct ("oeGai1") was provided by Dr. Cao [13–17, 20]. The lentivirus encoding ZNF384-expressing GV369 construct ("oeZNF384") was provided by Genechem (Shanghai, China). NPC cells or normal epithelial cells, cultured in polybrene-containing complete medium at 70% confluence, were infected with the lentivirus (at MOI = 10). As the control treatment, cells were infected with lentivirus encoding the empty vector ("Vec"). Forty-eight hours after virus infection, cells were back to complete medium and puromycin was added to select stable cells. The selection process took 5–6 passages. Overexpression of targeted genes was verified by at both mRNA and protein levels.

### CRISPR-Cas9-induced gene knockout (KO)

The primary NPC cells were first infected with the lentivirus encoding the Cas9-expressing construct (from Dr. Cao), and stable cells formed after selection using puromycin. The Cas9-expressing NPC cells were then infected with lentivirus with the CRISPR-Gai1-KO construct [containing small guide RNA (sgRNA) against human *GNAI1*] (from Dr. Cao [17]) and stable cells were established after six passages of puromycin selection. The stable cells were then distributed into 96-well plates and Gai1 KO was verified using PCR. At last, the single stable Gai1 KO primary NPC cells were formed and these cells were named as "Gai1 KO" cells. ZNF384 KO in NPC cells was through the same procedure using the verified sgRNA from Genechem. For the control treatment, the Cas9-expressing primary human NPC cells were infected with the CRISPR-KO control construct ("Cas9-C").

### Akt1 mutation

The lentiviral particles containing the S473D constitutively active mutant Akt1 (caAkt1) were provided by Dr. Chen [28] and were added to cultured NPC cells. Stable caAkt1-expressing cells were established with selection by puromycin. Control cells were infected with lentiviral particles with empty vector ("Vector").

### siRNA

Verified siRNAs against different transcription factors (ZNF460, ZNF384, EWSR1-FLI1, ZNF680, and Elf5) were provided by Genechem (Shanghai, China) and each (at the concentration of 200 nM) was individually transfected to NPC cells using Lipofectamine 3000. The transfection was repeated once at 24 h and stopped at 48 h. At least 70% reduction of targeted mRNA was achieved by each utilized siRNA. The control cells were transfected with non-sense control siRNA (siC).

### Chromatin immunoprecipitation (ChIP) assay

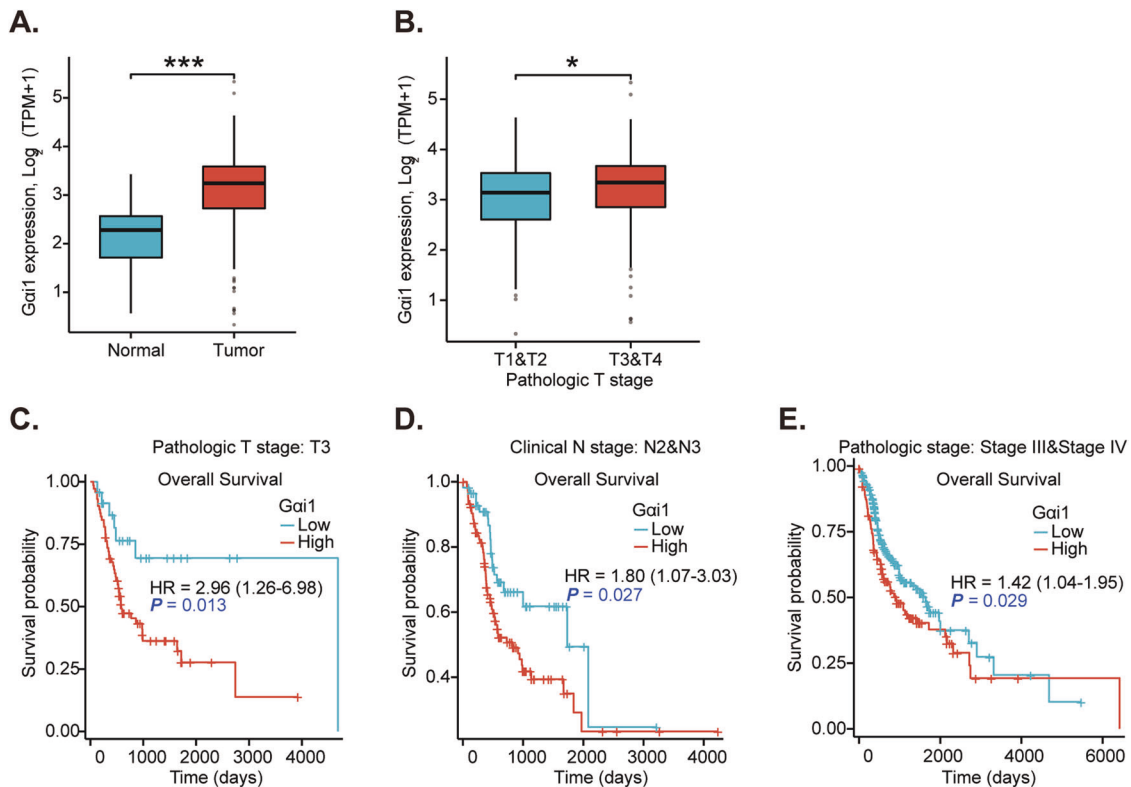
As described [29], fresh tissue lysates or total cellular lysates were homogenized using a homogenizer [30] and were diluted in ChIP dilution buffer (from Dr. Cao [29]). Lysates were further immunoprecipitated with an anti-ZNF384 antibody and ZNF384-associated DNA was eluted by protein A/G agarose (Santa Cruz Biotech) containing NaCl. The proposed *Gai1* promoter in the JASPAR database was thereafter tested by quantitative PCR (qPCR).

### Other assays

Western blotting, quantitative real-time PCR (qRT-PCR), cell viability CCK-8 assay, nuclear EdU staining of cell proliferation, "Transwell" in vitro migration, the Caspase-3 activity assay, JC-1 assaying of mitochondrial depolarization, Histone DNA ELISA and nuclear TUNEL staining of cell apoptosis were described in detail in our previous study [25].

### Xenograft studies

The nude mice were reported previously [25]. At six million cells per mouse, the primary human NPC cells, pNPC-1, were subcutaneously (*s.c.*) injected into the flanks of the nude mice and pNPC-1 xenografts were established after three weeks. The xenograft-bearing mice were then



**Fig. 1** *Gai1* overexpression in NPC tissues. The Cancer Genome Atlas (TCGA) database shows *Gai1* mRNA transcripts in NPC tissues (“Tumor”) and normal nasopharynx epithelial tissues (“Normal”) (A). Subgroup analyses of *Gai1* mRNA expression in NPC tissues with the described pathologic stages (B). The Kaplan–Meier Survival analyses of *Gai1*-low and *Gai1*-high NPC patients with the described pathological grades (C–E). “TPM” stands for “Transcripts Per Million”; “HR” stands for “Hazard Ratio”. The numerical values were mean  $\pm$  standard deviation (SD). \* $P < 0.05$ . \*\*\* $P < 0.01$ .

assigned into two groups randomly with ten mice in each group. The mice were then subject to intratumoral injection of the designated adeno-associated virus (aav). Virus was injected twice (48 h between intervals). The protocols were approved by the Ethics Committee and Institute Animal Ethics Review Board of Soochow University.

### Statistical analyses

The numerical data in this study were distributed normally and were always shown as mean  $\pm$  standard deviation (SD). The Student’s t-test (Excel 2013) was utilized when comparing two groups. Otherwise, one-way ANOVA plus a Scheffe’ and Tukey Test (SPSS 23.0) were utilized for multiple groups’ comparison.  $P < 0.05$  indicates significant difference.

## RESULTS

### *Gai1* overexpression in NPC tissues

We first searched The Cancer Genome Atlas (TCGA) database and *Gai1* (*GNAI1*) expression data in NPC tissues was retrieved. As shown, the number of *Gai1* mRNA transcripts in NPC tissues (“Tumor”) was significantly higher than that in normal nasopharynx epithelial tissues (“Normal”) (Fig. 1A). The subgroup analyses further demonstrated that *Gai1* overexpression in human NPC tissues correlated with higher T-stages (Fig. 1B). Moreover, *Gai1* overexpression in NPC tumor tissues was correlated with poor overall survival of a large portion of NPC patients, including patients with T3-stage NPCs (Fig. 1C), N2-N3 NPCs (Fig. 1D) and pathological stage III-IV NPCs (Fig. 1E).

### *Gai1* upregulation in NPC tissues of local patients and in different primary NPC cells

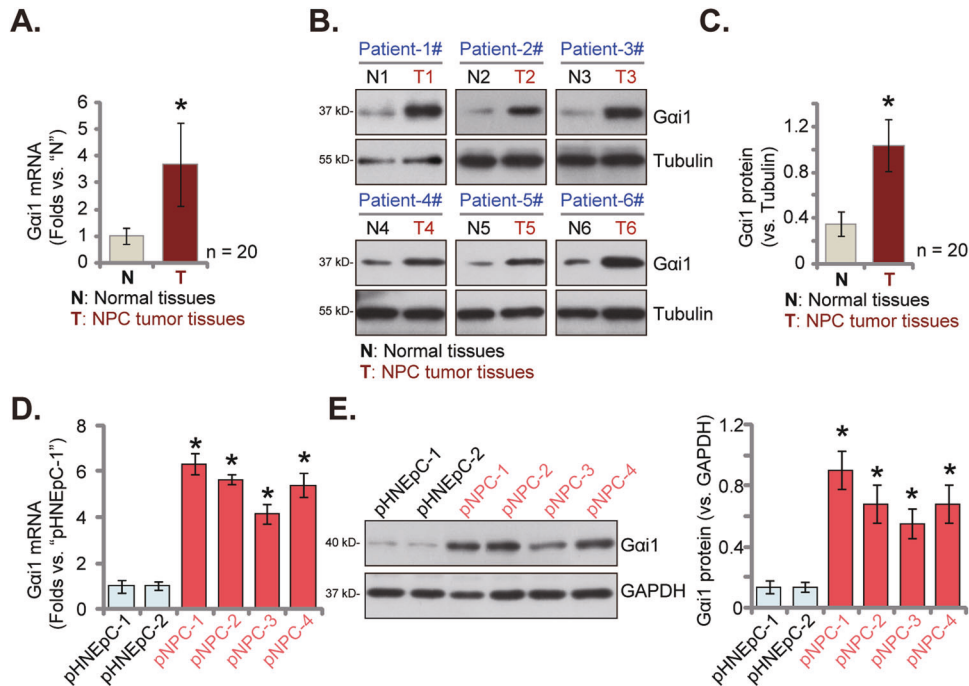
Next we tested expression of *Gai1* in NPC tissues of locally-administrated patients. We obtained NPC tumor tissues (“T”) and matched adjacent normal nasopharynx epithelial tissues (“N”)

from twenty ( $n = 20$ ) primary NPC patients. All patients were administrated at authors’ institution [25]. As shown, *Gai1* mRNA expression in tumor tissues was over three folds higher than that in adjacent normal tissues (Fig. 2A). Western blotting assay data in Fig. 2B confirmed upregulation of *Gai1* protein in NPC tumor tissues of six representative patients (“Patient-1#” to “Patient-6#”). The quantified results of all twenty sets of blotting data demonstrated that *Gai1* protein upregulation in NPC tissues was significant ( $P < 0.05$  vs. “N” tissues) (Fig. 2C).

We also tested *Gai1* expression in different NPC cells. Using the previously-described method [25], primary human NPC cells that were derived from four different patients (“Patient-1#” to “Patient-4#”), namely “pNPC-1”, “pNPC-2”, “pNPC-3” and “pNPC-4#”, were obtained. As shown, mRNA (Fig. 2D) and protein (Fig. 2E) expression of *Gai1* was significantly upregulated in all primary NPC cells. Yet in the primary human nasal epithelial cells (HNEpC) that were derived from two donors, “pHNEpC-1” and “pHNEpC-2” (as reported previously [25]), *Gai1* mRNA and protein expression was relatively low (Fig. 2D and E). These results showed *Gai1* upregulation in NPC tissues of local patients and in different primary human NPC cells.

### *Gai1* silencing inhibits viability, proliferation, cell cycle progression, and migration in primary NPC cells

To understand the possible function role of *Gai1* in NPC cells, the shRNA strategy was utilized to knockdown *Gai1*. Specifically, to the pNPC-1 primary NPC cells (reported previously [25]) *Gai1* shRNA-packed lentivirus was added. Stable cells were thereafter established after puromycin treatment. Two different shRNAs against *Gai1* [20], *Gai1*-shRNA-s1 and *Gai1*-shRNA-s2 (with non-overlapping sequences), were utilized and each resulted in substantial silencing of *Gai1* mRNA (Fig. 3A) and protein



**Fig. 2** **Gai1 upregulation in NPC tissues of local patients and in different primary NPC cells.** *Gai1* mRNA (A) and protein (B, C) expression in the described NPC tumor tissues ("T";  $n = 20$ ) and matched adjacent normal nasopharynx epithelial tissues ("N";  $n = 20$ ) was shown, with results quantified. *Gai1* mRNA (D) and protein (E) expression in the described primary human NPC cells and primary human nasal epithelial cells ("pHNEpC-1" and "pHNEpC-2") was shown. The numerical values were mean  $\pm$  standard deviation (SD,  $n = 5$ ). Scale Bar = 100  $\mu\text{m}$ . \*  $P < 0.05$  vs. "N" tissues or "pHNEpC-1" cells.

(Fig. 3B). Whereas expression of *Gai2* and *Gai3*, two other *Gai* family members, was unchanged (Fig. 3A, B). Knockdown of *Gai1* by the targeted shRNAs impeded pNPC-1 cell proliferation and decreased nuclear EdU incorporation (Fig. 3C). Cell viability (CCK-8 OD) was also decreased in *Gai1*-silenced pNPC-1 primary cells (Fig. 3D). Moreover, *Gai1* silencing in pNPC-1 cells disrupted cell cycle progression (Fig. 3E), as it increased G1 phase cell percentage while decreasing S phase cell percentage (Fig. 3E). In addition, the applied *Gai1* shRNAs largely inhibited the mobility of pNPC-1 cells. The number of migrated pNPC-1 cells (Fig. 3F) was significantly decreased after *Gai1* shRNA. The scramble non-sense control shRNA ("shC"), expectably, failed to significantly alter *Gai1/2/3* expression (Fig. 3A, B) and pNPC-1 cell functions (Fig. 3C–F).

To study whether *Gai1* exert similar activity in other primary NPC cells, the *Gai1*-shRNA-s2-expressing lentivirus was added to primary human NPC cells that were derived from other primary patients, including pNPC-2, pNPC-3, and pNPC-4 (see Fig. 2). Puromycin was again employed to select stable cells. As shown, *Gai1* mRNA expression was downregulated in the primary NPC cells with *Gai1*-shRNA-s2 (Fig. 3G). Similar to the functional results in pNPC-1 cells, knocking down of *Gai1* by *Gai1*-shRNA-s2 decreased cell viability (Fig. 3H), suppressed cell proliferation (EdU incorporation, Fig. 3I) and inhibited cell migration (Fig. 3J) in these primary NPC cells.

We also tested the potential effect of *Gai1* silencing in nasal epithelial cells. To this aim, pHNEpC-1 and pHNEpC-2 cells, the primary nasal epithelial cells from two healthy donors (see Fig. 2), were infected with *Gai1*-shRNA-s2-expressing lentivirus and were then treated with puromycin to establish stable cells. The applied shRNA resulted in substantial *Gai1* mRNA silencing (Fig. 3K). Unlike the functional consequence in NPC cells, shRNA-induced silencing of *Gai1* failed to inhibit viability (CCK-8 OD, Fig. 3L) and proliferation (EdU incorporation, Fig. 3M) in the HNEpCs.

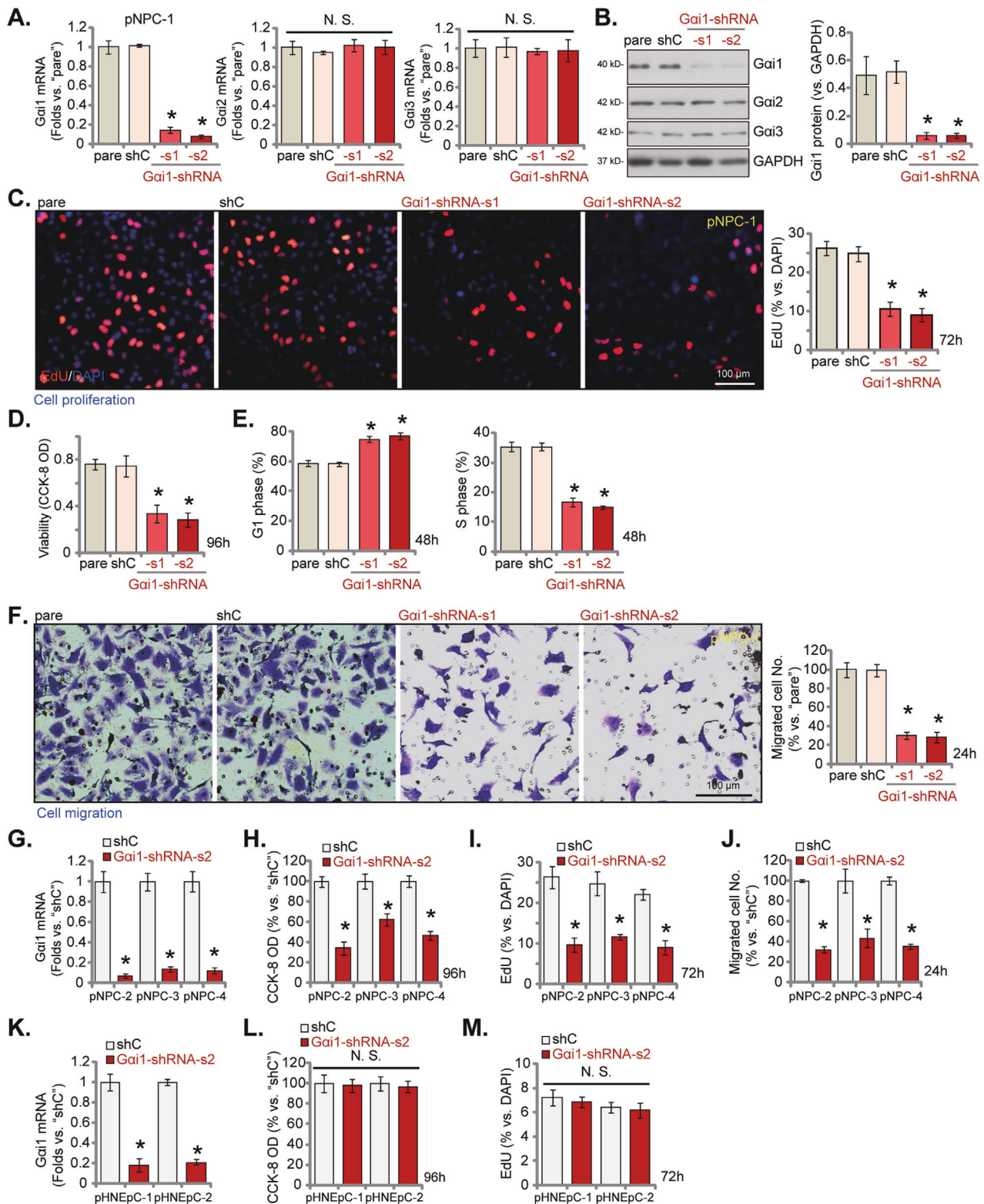
### **Gai1 silencing provokes apoptosis in primary NPC cells**

*Gai1* knockdown by viral shRNA potently inhibited cell viability, proliferation, cell cycle progression, and migration in primary NPC cells, we next tested its effect on cell apoptosis. The Caspase-3 activity was significantly increased in pNPC-1 cells with *Gai1*-shRNA-s1/2 (Fig. 4A). shRNA-induced *Gai1* knockdown also caused cleavages of Caspase-3, PARP (-), and Caspase-9 in pNPC-1 cells (Fig. 4B). The histone-bound DNA contents, one key indicator of apoptosis activation, were increased in *Gai1*-shRNA-expressing pNPC-1 cells (Fig. 4C). Moreover, with *Gai1* silencing JC-1 green fluorescence monomers were accumulated in pNPC-1 cells, supporting mitochondrial depolarization (Fig. 4D). Results further showed that the number of nuclei with positive TUNEL staining was significantly increased in *Gai1*-shRNA-expressing pNPC-1 cells (Fig. 4E). These results together confirmed that *Gai1* silencing provoked pNPC-1 cell apoptosis. The shC control treatment, as expected, failed to induce Caspase-3 activation in pNPC-1 cells (Fig. 4A–E).

In other primary human NPC cells, pNPC-2, pNPC-3, and pNPC-4, stable *Gai1* knockdown by *Gai1*-shRNA-s2-expressing lentivirus (see Fig. 3) also provoked Caspase-3 activation (Fig. 4F) and increased the number of nuclei with positive TUNEL staining (Fig. 4G). On the contrary, in pHNEpC-1 and pHNEpC-2 primary epithelial cells, *Gai1* silencing by the *Gai1*-shRNA-s2 treatment (see Fig. 3) failed to induce significant Caspase-3-apoptosis activation (Fig. 4H and I).

### **Gai1 knockout results in anti-cancer activity in primary NPC cells**

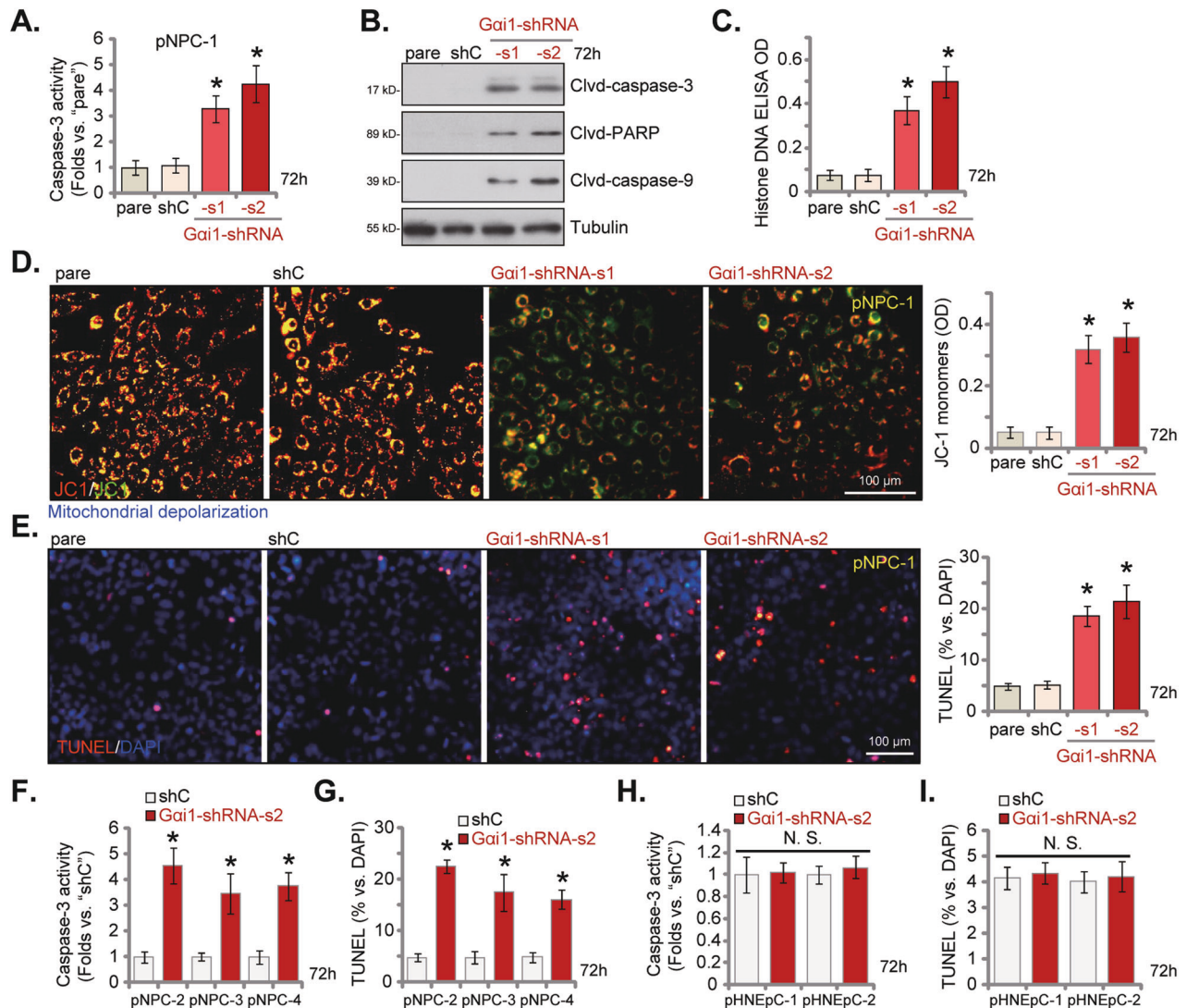
To exclude the possible off-target effect of the applied shRNAs and to completely knockout (KO) *Gai1*, the CRISPR/Cas9 strategy was employed. Specifically, to the Cas-9-expressing pNPC-1 cells, the CRISPR-*Gai1*-KO construct-expressing lentivirus was added. Single stable cells were then formed via puromycin selection and *Gai1* KO screening and these cells were named as *Gai1*-KO cells. As shown, the protein expression of *Gai1* was depleted in *Gai1*-



KO pNPC-1 cells (Fig. 5A, B). Whereas its expression was intact in pNPC-1 cells with CRISPR/Cas9 control construct ("Cas9-C") (Fig. 5A, B). Expression of Gai2 and Gai3 was not altered in Gai1-KO pNPC-1 cells (Fig. 5A, B). With Gai1 KO, pNPC-1 cell proliferation (EdU incorporation in nuclei, Fig. 5C) was substantially inhibited. The "Transwell" assay results further showed that Gai1 KO

dramatically suppressed pNPC-1 in vitro cell migration (Fig. 5D). In addition, CRISPR/Cas9-caused Gai1 KO provoked apoptosis activation in pNPC-1 cells (Fig. 5E), as the TUNEL-stained nuclei ratio was significantly augmented (Fig. 5E). Therefore, Gai1 KO resulted in profound anti-cancer activity in pNPC-1 cells, further supporting a key role of Gai1 in NPC cell progression.

**Fig. 3 Gai1 silencing inhibits viability, proliferation, cell cycle progression and migration in primary NPC cells.** pNPC-1 cells with the designated *Gai1* shRNA (*Gai1*-shRNA-s1 and *Gai1*-shRNA-s2, representing two different sequences) or scramble non-sense control shRNA ("shC"), were cultured and expression of *Gai1*/2/3 (both mRNA and protein) was tested (A,B). The exact same number of the above cells were cultivated for designated hours, cellular functions, including cell proliferation (EdU-incorporated nuclei percentage, C), viability (CCK-8 OD, D), cell cycle progression (E) and in vitro cell migration ("Transwell" assays, F) were measured, with results quantified. Other stable primary NPC cells (pNPC-2, pNPC-3 and pNPC-4, derived from three primary patients) or the primary human nasal epithelial cells (pHNEpC-1 and pHNEpC-2, derived from two donors), expressing shC or *Gai1*-shRNA-s2 were formed, with *Gai1* mRNA expression tested (G, K). The exact same number of the above cells were cultivated for designated hours, cell viability (H, L), proliferation (I, M) and migration (J) were examined, with results quantified. The numerical values were mean  $\pm$  standard deviation (SD,  $n = 5$ ). "pare" indicates the parental control cells. \*  $P < 0.05$  vs. "shC" cells. "N. S." stands for non-statistical difference ( $P > 0.05$ ). Experiments in this figure were repeated five times, with similar results obtained. Scale Bar = 100  $\mu$ m.

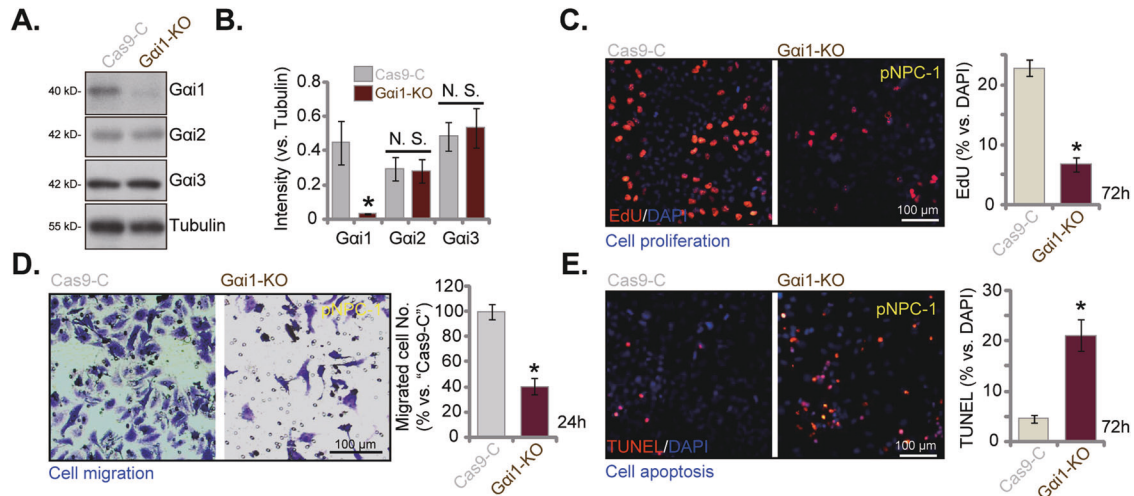


**Fig. 4 Gai1 silencing provokes apoptosis in primary NPC cells.** pNPC-1 cells with the designated *Gai1* shRNA (*Gai1*-shRNA-s1 and *Gai1*-shRNA-s2, representing two different sequences) or scramble non-sense control shRNA ("shC") were cultivated for 72 h, Caspase-3 activity (A), cleavages of apoptosis-related proteins (B), Histone DNA contents (C) and mitochondrial depolarization (accumulation of JC-1 green fluorescence monomers, D) were tested. Cell apoptosis was measured via calculating TUNEL-incorporated nuclei percentage (E); Other primary NPC cells (pNPC-2, pNPC-3 and pNPC-4) or the primary human nasal epithelial cells (pHNEpC-1 and pHNEpC-2), expressing shC or *Gai1*-shRNA-s2, were formed and cultivated for 72 h, the Caspase-3 activity (F, H) and apoptosis (TUNEL staining assays, G, I) were tested. The numerical values were mean  $\pm$  standard deviation (SD,  $n = 5$ ). "pare" indicates the parental control cells. \*  $P < 0.05$  vs. "shC" cells. "N. S." stands for non-statistical difference ( $P > 0.05$ ). Experiments in this figure were repeated five times, with similar results obtained. Scale Bar = 100  $\mu$ m.

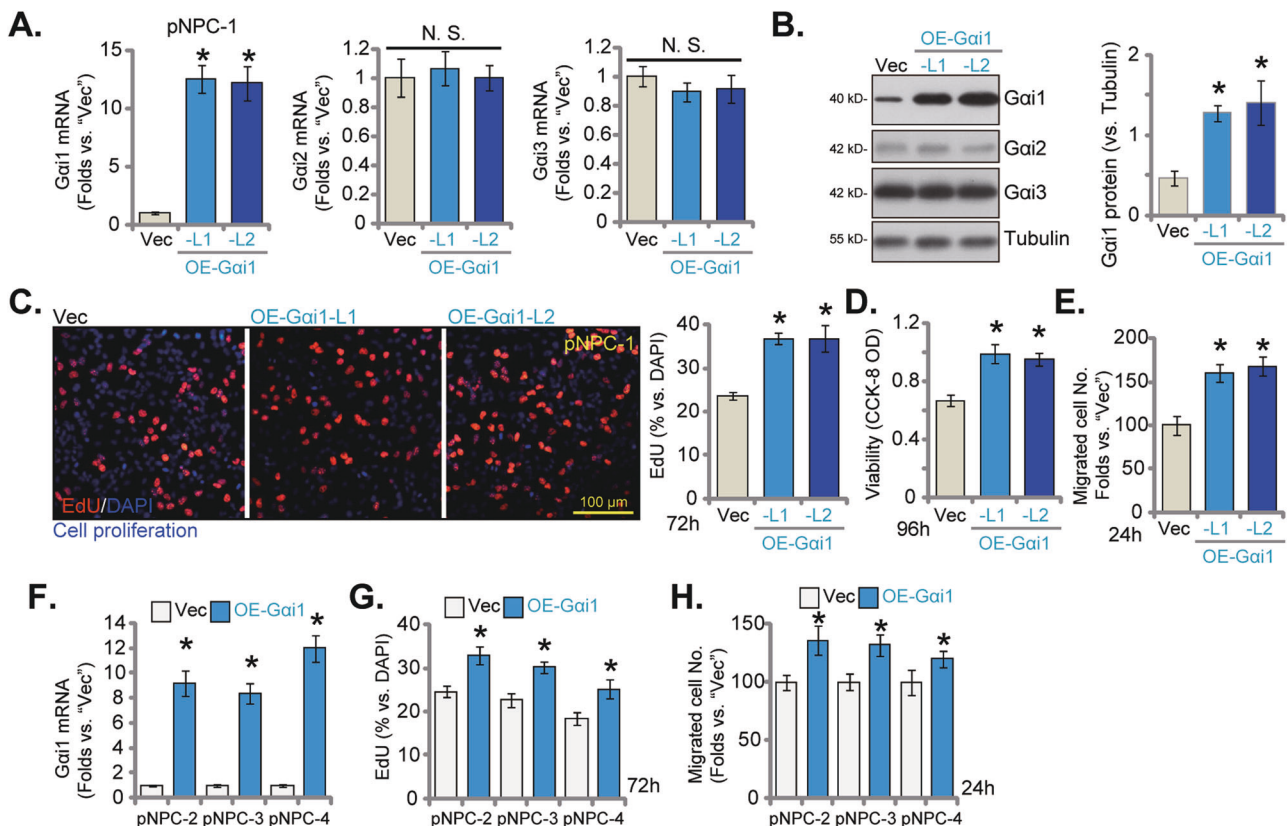
#### Ectopic *Gai1* overexpression causes cancer-promoting activity in primary NPC cells

The results above clearly showed that *Gai1* knockdown or KO resulted in significant anti-cancer activity in primary human NPC

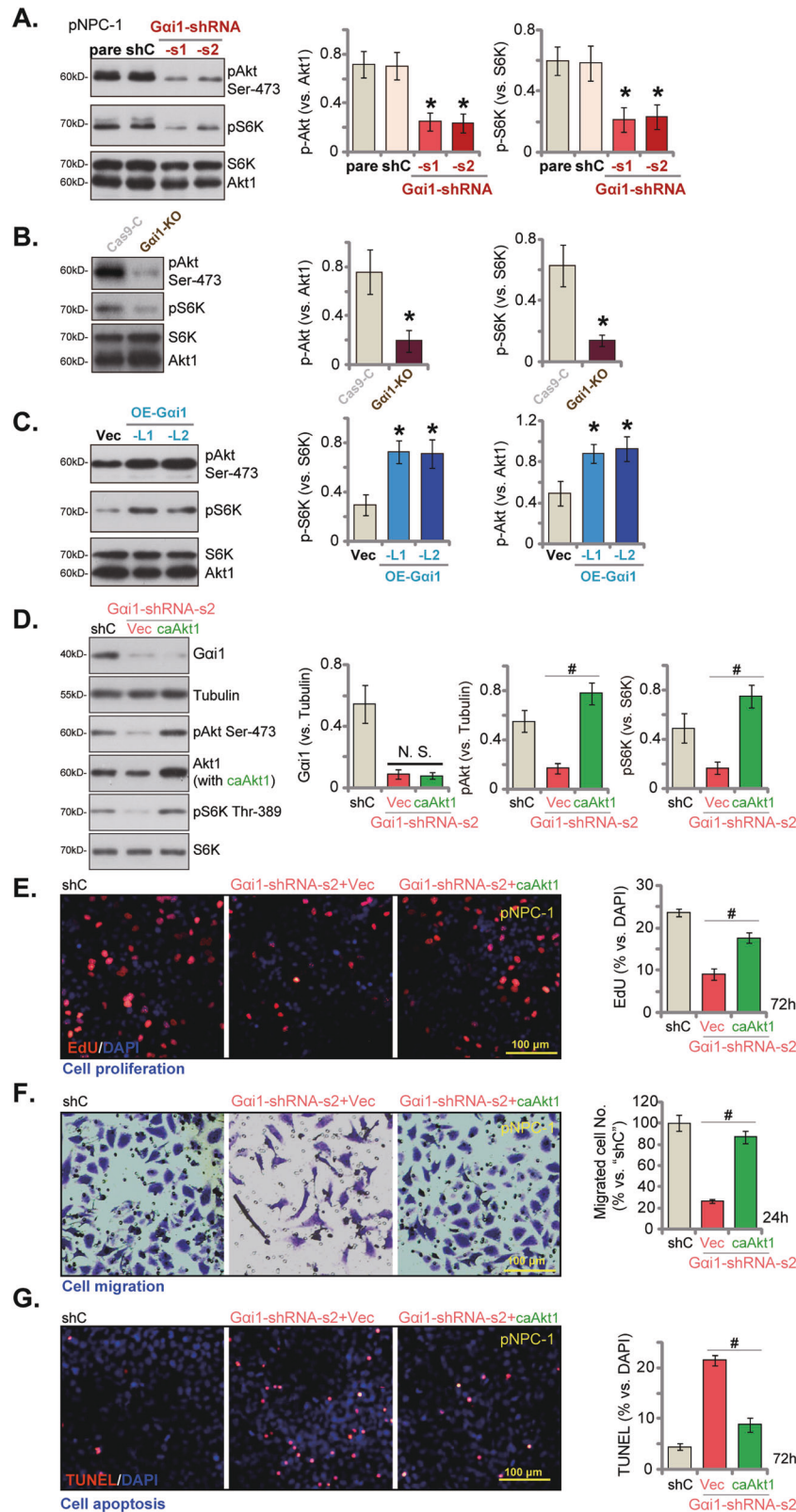
cells. We next hypothesized that ectopic *Gai1* overexpression might exert opposite functions and could promote NPC cell growth. Therefore, the lentivirus with the *Gai1*-expressing construct ("OE-*Gai1*", from Dr. Cao [13–16, 20]) were added to pNPC-1 cells. After



**Fig. 5** **Gai1 KO results in anti-cancer activity in primary human NPC cells.** pNPC-1 cells with the Cas9-expressing construct plus the CRISPR-Gai1-KO construct ("Gai1-KO") were established. Control cells were with the Cas9-expressing construct plus the CRISPR-KO control construct ("Cas9-C"). Expression of Gai1/2/3 protein was tested, with results quantified (A, B). The exact same number of above cells were cultivated for designated hours, cell proliferation (EdU-incorporated nuclei percentage, C), in vitro cell migration ("Transwell" assays, D) as well as cell apoptosis (TUNEL assays, E) were tested, with results quantified. The numerical values were mean  $\pm$  standard deviation (SD,  $n = 5$ ). \* $P < 0.05$  vs. "Cas9-C" cells. "N. S." stands for non-statistical difference ( $P > 0.05$ ). Experiments in this figure were repeated five times, with similar results obtained. Scale Bar = 100  $\mu$ m.



**Fig. 6** **Ectopic Gai1 overexpression causes cancer-promoting activity in primary NPC cells.** pNPC-1 cells with the Gai1-expressing construct (OE-Gai1-L1 and OE-Gai1-L2, representing two stable cell selections) or the empty vector ("Vec") were formed and expression of Gai1/2/3 (both mRNA and protein) was tested (A, B). The exact same number of the above cells were cultivated for designated hours, cellular functions, including cell proliferation (EdU-incorporated nuclei percentage, C) and viability (CCK-8 OD, D), and in vitro cell migration ("Transwell" assays, E) were measured, with results quantified. Other primary NPC cells (pNPC-2, pNPC-3 and pNPC-4) with the Gai1-expressing construct (OE-Gai1) or the empty vector ("Vec") were formed as well, and expression of Gai1 mRNA measured (F); Cells were cultivated for indicated times, cell proliferation (G) and migration (H) were tested similarly, with results quantified. The numerical values were mean  $\pm$  standard deviation (SD,  $n = 5$ ). \* $P < 0.05$  vs. "Vec" cells. "N. S." stands for non-statistical difference ( $P > 0.05$ ). Experiments in this figure were repeated five times, with similar results obtained. Scale Bar = 100  $\mu$ m.



treatment with puromycin, two stable cell selections, OE-*Gai1*-L1 and OE-*Gai1*-L2, were formed. As compared to control pNPC-1 cells with empty vector ("Vec"), *Gai1* mRNA (Fig. 6A) and protein (Fig. 6B) expression was remarkably increased in OE-*Gai1* cells, whereas expression of *Gai2* and *Gai3* was unchanged (Fig. 6A, B). With *Gai1* overexpression, pNPC-1 cell proliferation was enhanced and the Edu

nuclei percentage was significantly increased (Fig. 6C). Moreover, OE-*Gai1* increased CCK-8 viability in pNPC-1 cells (Fig. 6D). Ectopic *Gai1* overexpression also accelerated pNPC-1 in vitro cell migration (Fig. 6E). Next the lentivirus with the *Gai1*-expressing construct ("OE-*Gai1*") was also added to other primary NPC cells (pNPC-2, pNPC-3 and pNPC-4), and stable cells formed after puromycin selection. *Gai1*



**Fig. 7 Gai1 is important for Akt-mTOR activation in NPC cells.** Stable pNPC-1 cells with the designated *Gai1* shRNA (*Gai1*-shRNA-s1 and *Gai1*-shRNA-s2, representing two different sequences), scramble non-sense control shRNA ("shC"), the Cas9-expressing construct plus the CRISPR-*Gai1*-KO construct ("*Gai1*-KO"), the CRISPR-KO control construct ("Cas9-C"), the *Gai1*-expressing construct (OE-*Gai1*-L1 and OE-*Gai1*-L2, representing two stable cell selections) or the empty vector ("Vec") were established and expression of listed proteins was shown (A–C). *Gai1*-shRNA-s2-expressing pNPC-1 cells were further stably transduced with a S473D constitutively-active mutant Akt1 (caAkt1) or the empty vector ("Vec"), expression of listed proteins was shown (D). These cells were further cultivated for designated hours, cell proliferation, migration and apoptosis were examined via EdU-nuclei staining (E), "Transwell" (F) and TUNEL-nuclei (G) assays, respectively. The numerical values were mean  $\pm$  standard deviation (SD,  $n = 5$ ). "pare" indicates the parental control cells. \* $P < 0.05$  vs. "shC"/"Cas9-C"/"Vec" cells. # $P < 0.05$ . "N. S." stands for non-statistical difference ( $P > 0.05$ ). Experiments in this figure were repeated five times, with similar results obtained. Scale Bar = 100  $\mu$ m.

mRNA levels were remarkably increased in these primary NPC cells (Fig. 6F). Importantly, *Gai1* overexpression enhanced proliferation by facilitating nuclear EdU incorporation in the primary NPC cells (Fig. 6G). Moreover, the in vitro cell migration was accelerated following *Gai1* overexpression in pNPC-2/-3/-4 primary cells (Fig. 6H).

### Gai1 is important for Akt-mTOR cascade activation in NPC cells

*Gai1* was shown to associate with both RTKs [16, 18, 19, 21, 22] and non-RTK receptors [13, 15], mediating downstream Akt-mTOR cascade activation. Considering the contribution of Akt-mTOR overactivation in the tumorigenesis and progression of NPC [4, 31, 32], we therefore explored the possible role of *Gai1* on Akt-mTOR activation in NPC cells. In pNPC-1 cells, *Gai1* silencing, by *Gai1*-shRNA-s1 and *Gai1*-shRNA-s2, decreased phosphorylation of Akt (Ser-473) and S6K (Thr-389) (Fig. 7A). Total Akt1 and S6K protein expression was unchanged (Fig. 7A). Moreover, CRISPR-sgRNA-induced KO of *Gai1* significantly inhibited Akt-mTOR activation (Akt-S6K phosphorylation) in pNPC-1 cells (Fig. 7B), without affecting total Akt1 and S6K expression (Fig. 7B). On the contrary, in *Gai1*-overexpressed pNPC-1 cells (OE-*Gai1*-L1 and OE-*Gai1*-L2, see Fig. 6), Akt-S6K phosphorylation was augmented (Fig. 7C). These results supported that *Gai1* is indeed important for Akt-mTOR activation in NPC cells.

To understand the relationship between *Gai1*-driven NPC cell growth and Akt-mTOR cascade activation, a S473D constitutively-active mutant Akt1 (caAkt1) was stably transduced to *Gai1*-silenced pNPC-1 cells (with "*Gai1*-shRNA-s2", see Fig. 3). As shown, caAkt1 completely restored Akt-S6K phosphorylation without affect *Gai1* protein expression in pNPC-1 cells (Fig. 7D). In pNPC-1 cells caAkt1 largely inhibited *Gai1* silencing-caused proliferation inhibition (Fig. 7E), migration reduction (Fig. 7F) and apoptosis (Fig. 7G). These results supported that mediating Akt-mTOR cascade activation should be a primary mechanism of *Gai1*-driven NPC cell growth.

### ZNF384 is an important transcription factor of Gai1 in NPC cells

Considering that both mRNA and protein expression of *Gai1* was increased in NPC tissues and cells (Figs. 1, 2), we hypothesized that there could be a transcriptional mechanism of *Gai1* upregulation in NPC tissues. Few studies reported the definite transcription factors of *Gai1* in human cells, we therefore searched JASPAR transcription factor database [33]. Five transcription factors with the highest possible binding affinity to *Gai1* were identified, including ZNF460, ZNF384, EWSR1-FLI1, ZNF680 and Elf5 (Fig. 8A). We next designed siRNAs targeting each of the five different transcription factors. These siRNAs were individually transfected to pNPC-1 cells and their efficiency on *Gai1* mRNA expression was analyzed. As shown, only ZNF460 siRNA and ZNF384 siRNA resulted in significant *Gai1* silencing in pNPC-1 cells (Fig. 8B). Silencing of other transcription factors was ineffective on *Gai1* expression in pNPC-1 cells (Fig. 8B). ZNF384 siRNA was more potent than ZNF460 siRNA in down-regulating *Gai1* in pNPC-1 cells (Fig. 8B).

Next, pNPC-1 cells were infected with ZNF384 shRNA-expressing lentivirus [27] and stable cells, shZNF384, were formed after puromycin selection. Alternatively, the Cas-9-expressing pNPC-1 cells were stably transduced with a lentiviral CRISPR-ZNF384-KO construct to establish ZNF384 KO cells ("koZNF384").

As compared to the control pNPC-1 cells with scramble control shRNA plus CRISPR/Cas9 control construct ("shC+Cas9-C"), ZNF384 mRNA (Fig. 8C) and protein (Fig. 8D) expression was substantially decreased in both shZNF384 and koZNF384 pNPC-1 cells. Importantly, *Gai1* mRNA (Fig. 8C) and protein (Fig. 8D) expression was downregulated as well after ZNF384 depletion in pNPC-1 cells. These results further supported that ZNF384 could be an important transcription factor of *Gai1* in NPC cells.

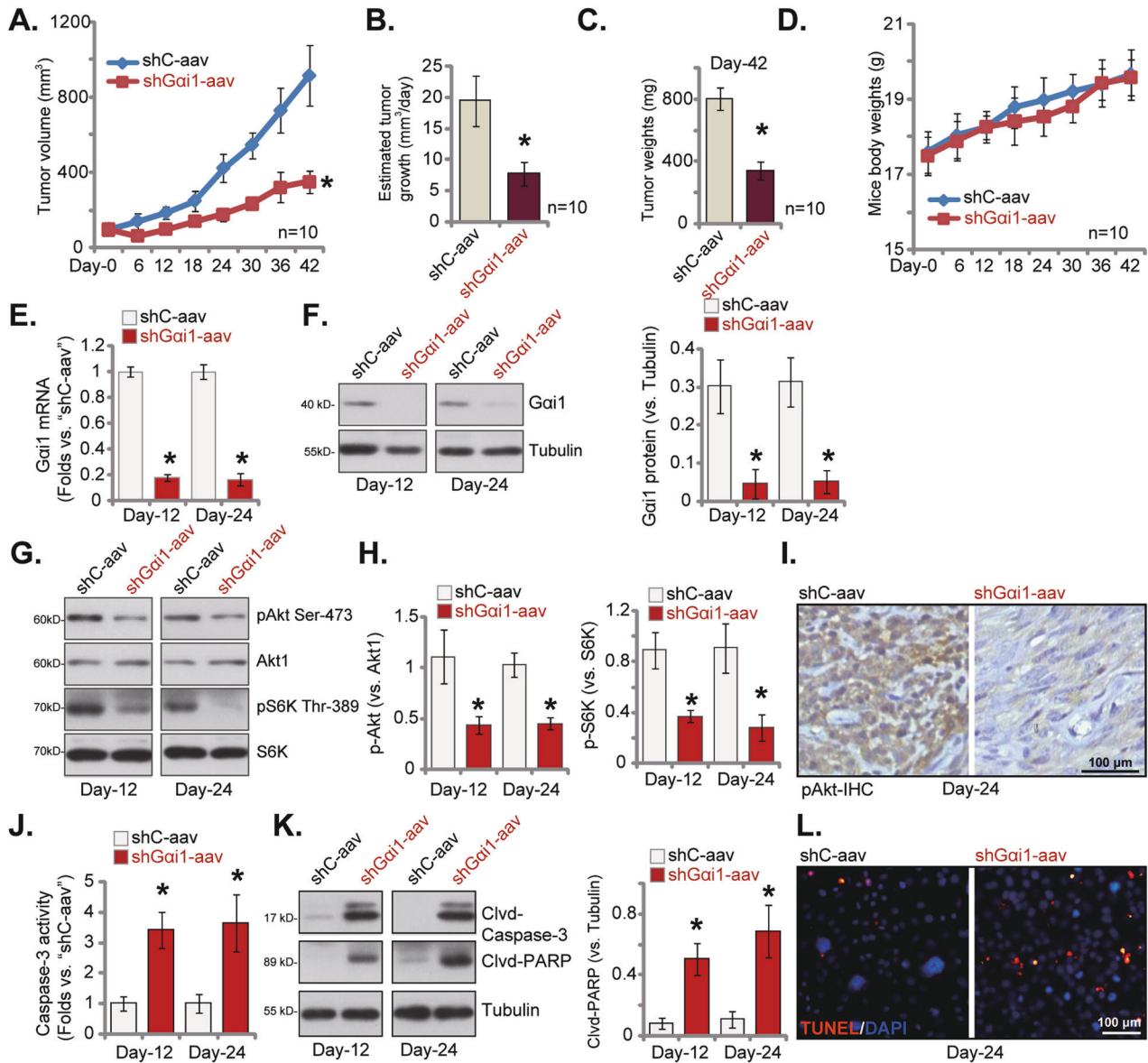
To further support our hypothesis, the lentivirus encoding the ZNF384-expressing construct was added to pNPC-1 cells. Stable cells, "oeZNF384", were formed following puromycin-mediated selection. These cells showed substantial ZNF384 mRNA (Fig. 8E) and protein (Fig. 8F) upregulation. With ZNF384 overexpression, *Gai1* mRNA (Fig. 8E) and protein (Fig. 8F) expression was upregulated as well. Importantly, ChIP assay results showed that the binding between ZNF384 protein and the proposed *Gai1* promoter region (in the JASPAR database) was significantly increased in NPC tissues of local patients (Fig. 8G). Moreover, the binding between the two was significantly increased in different primary NPC cells (pNPC-1, pNPC-2, pNPC-3, and pNPC-4) (Fig. 8H). ZNF384-*Gai1* promoter binding affinity was relatively low in normal nasopharynx epithelial tissues ("N") (Fig. 8G) and also in HNEpC cells (Fig. 8H). These results supported that the increased binding between the ZNF384 and the *Gai1* promoter region could be a primary mechanism of *Gai1* upregulation in NPC.

Stable silencing of ZNF460, using the same lentiviral shRNA strategy, led to moderate (less than 50%) but significant *Gai1* protein downregulation (Fig. 8I). Notably, the downregulation of *Gai1* induced by ZNF384 shRNA/KO (Fig. 8D) was significantly more robust compared to ZNF460 shRNA (see Fig. 8I). Importantly, both ZNF384 and ZNF460 protein expression exhibited an increase in NPC tumor tissues ("T") (Fig. 8J), while their expression remained relatively low in adjacent normal nasopharynx epithelial tissues ("N") (Fig. 8J).

### Gai1 depletion inhibits NPC xenograft growth in nude mice

The potential role of *Gai1* on NPC cell growth in vivo was explored. As described previously [25], pNPC-1 primary cells, at six million cells of each mouse, were injected s.c. to flanks of nude mice [25]. Thereafter, pNPC-1 xenografts were established after three weeks and volume of each xenograft was close to 100 mm<sup>3</sup> ("Day-0"). Next, pNPC-1 xenograft-bearing nude mice were randomly assigned into two groups. For the treatment group, aav-packed *Gai1*-shRNA-s2 ("sh*Gai1*-aav") was intratumorally injected. The control group mice were intratumorally injected with aav-packed scramble control shRNA ("shC-aav"). Virus injection was repeated after 48 h ("Day-2") and every six days tumor volumes were recorded. As shown, sh*Gai1*-aav injection impeded the growth of pNPC-1 xenografts in nude mice and volumes were significantly lower (Fig. 9A). Using the described method [25], estimated daily pNPC-1 xenograft growth, in mm<sup>3</sup> per day, was calculated (Fig. 9B). Results showed that sh*Gai1*-aav injection substantially suppressed pNPC-1 xenograft growth (Fig. 9B). The animal experiments were terminated at Day-42, and all pNPC-1 xenografts were isolated and weighted. sh*Gai1*-aav group pNPC-1 xenografts were significantly lighter and smaller than shC-aav group ones (Fig. 9C). The animal body weights, on the other hand, were indifferent between the mice (Fig. 9D). These results





**Fig. 9** Gai1 depletion inhibits NPC xenograft growth in nude mice. pNPC-1 xenograft-bearing nude mice were intratumorally injected Gai1-shRNA-s2-expressing adeno-associated virus ("shGai1-aav") or scramble control shRNA adeno-associated virus ("shC-aav"), the volumes of pNPC-1 xenografts (A) and animal body weights (D) were recorded every six days. The estimated daily pNPC-1 xenograft growth was calculated (B); At day-42, all pNPC-1 xenografts were isolated and weights (C). The tissue lysates of the described pNPC-1 xenografts were obtained and expression of the described mRNAs and proteins was tested (E, F, G, H, and K), with Caspase-3 activity tested as well (J). Alternatively, the described pNPC-1 xenograft slices were subjected to IHC staining p-Akt (Ser-473) (I), or subjected to immuno-fluorescence staining of TUNEL/DAPI (L). Values were mean  $\pm$  standard deviation (SD). In (A–D), ten mice were in each group ( $n = 10$ ). For (E–L), five random tissue pieces in each xenograft were tested ( $n = 5$ ). \* $P < 0.05$  vs. "shC-aav" group. Scale bar = 100  $\mu$ m.

supported that shGai1-aav injection inhibited NPC xenograft growth in nude mice.

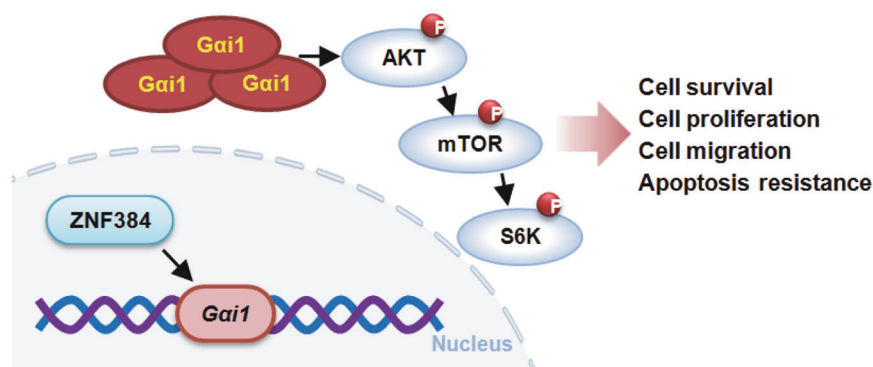
On the 12th day (Day-12) and the 24th day (Day-24) after initial virus injection, one pNPC-1 xenograft was isolated from each group. Four total pNPC-1 xenografts were obtained. Part of the xenografts were cut into pieces and were homogenized. As shown, mRNA (Fig. 9E) and protein (Fig. 9F) expression of Gai1 was remarkably downregulated in pNPC-1 xenograft tissues with Gai1-shRNA virus injection. Moreover, Akt-S6K1 phosphorylation was decreased in Gai1-silenced pNPC-1 xenograft tissues (Fig. 9G, H). IHC assay results further supported Akt inhibition in the pNPC-1 xenograft tissues (Fig. 9I).

Further analyzing xenograft tissues showed that the Caspase-3 activity was augmented in shGai1-aav-injected pNPC-1 xenograft

tissues (Fig. 9J), where levels of cleaved-Caspase-3 and cleaved-PARP were increased (Fig. 9K). Moreover, tissue slide immunofluorescence results further supported apoptosis activation in Gai1-silenced pNPC-1 xenografts, as the TUNEL-positive apoptotic nuclei percentage was augmented (Fig. 9L). Together these signaling results showed that shGai1-aav injection resulted in Gai1 silencing, Akt-mTOR inhibition and apoptosis in pNPC-1 xenografts.

To further corroborate the role of Gai1 in NPC cell growth in vivo, we conducted subcutaneous injections of Gai1-KO pNPC-1 cells and control Cas9-C pNPC-1 cells into the flanks of nude mice. After an eight-week period, we isolated and measured the resulting xenografts. Gai1-KO pNPC1 xenografts exhibited significantly smaller sizes and lower weights compared to the Cas9-C pNPC1 xenografts (Fig. S2A, B). The body weights of the mice in

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**Fig. 10** The proposed signaling cartoon of this study. The increased binding between the transcription factor ZNF384 and the *Gai1* promoter causes *Gai1* overexpression, which exerts pro-tumorigenic activity in nasopharyngeal carcinoma (NPC) possibly by promoting Akt-mTOR activation.

the two groups were not significantly different (Fig. S2C). In addition, *Gai1* protein expression was substantially reduced in *Gai1*-KO pNPC1 xenografts (Fig. S2D), while the expression levels of *Gai2* and *Gai3* remained unchanged (Fig. S2D). These findings provide strong support for the notion that *Gai1* KO effectively inhibited the growth of pNPC1 xenografts in nude mice.

### DISCUSSION

NPC is considered as a poor-prognosis malignancy, mainly due to the lack of characteristic clinical symptoms [1, 8, 9]. The failure of current treatment methods is also one main reason for patients' poor survival [1, 8, 9]. To improve NPC patients' prognosis, exploring novel signaling targets is urgently needed and an in-depth understanding of molecular events associated with pathogenesis and progression of the cancer is critical [1, 8, 9]. Discovering novel therapeutic targets is also essential for developing effective targeted therapies [1, 8, 9].

The results of the present study supported a key pro-cancerous role of *Gai1* in NPC. TCGA results demonstrate that *Gai1* transcripts' number in NPC tissues is significantly higher than that in the nasal epithelial tissues. Its overexpression correlates with poor survival of a large number of NPC patients. Moreover, *Gai1* mRNA and protein expression is significantly elevated in NPC tissues of local patients and in primary NPC cells. Whereas its expression is relatively low in cancer-surrounding normal tissues and in nasal epithelial cells. Genetic silencing or KO of *Gai1* substantially suppressed viability, proliferation, cell cycle arrest, and migration, and induced apoptosis activation in primary NPC cells. Contrarily, ectopic *Gai1* expression in primary NPC cells exerted pro-tumorigenic activity and augmented cell proliferation and migration. In vivo studies showed that intratumoral injection of *Gai1*-shRNA-expressing aav impeded subcutaneous pNPC-1 xenograft growth in nude mice. *Gai1* KO also effectively inhibited the growth of pNPC1 xenografts in nude mice. Therefore, over-expressed *Gai1* is a novel and promising therapeutic target of NPC.

Recent studies have proposed a pivotal role of *Gai1* in mediating Akt-mTOR cascade activation by a number of different stimuli [13–22, 24, 34, 35]. In gastric cancer tissues, *Gai1* immunoprecipitated with multiple RTKs (EGFR, PDGFR $\alpha$  and FGFR) as well as the adapter protein Gab1, mediating downstream Akt-mTOR activation [20]. *Gai1*, along with *Gai3*, associated with RTKs in human glioma tissues, essential for the downstream Akt-mTOR activation [16, 20, 23]. *Gai1* was also required for Akt-mTOR activation by different non-RTK receptors, including Netrin-1-stimulated CD146 [15], interleukin-4 (IL-4)-activated IL-4R $\alpha$  [17], LPS-stimulated Toll-like receptor 4 (TLR4) [35] and RSPO3-activated leucine-rich repeat G protein-coupled receptor 4 (LGR4) [13].

Here we found that *Gai1* is important for Akt-mTOR activation in NPC cells. Akt-S6K phosphorylation was downregulated with *Gai1* shRNA or KO in primary NPC cells, but was augmented after *Gai1* overexpression. In *Gai1*-silenced primary NPC cells, caAkt1 restored Akt-S6K phosphorylation and largely ameliorated *Gai1* shRNA-induced proliferation inhibition, migration reduction and apoptosis. Akt-mTOR inactivation was also detected in *Gai1*-silenced NPC-1 xenograft tissues. Thus, mediating Akt-mTOR activation could be a primary mechanism of *Gai1*-driven NPC cell growth in vitro and in vivo (see Fig. 10).

Studies have explored a possible cancer-promoting activity of the transcription factor ZNF384 in different cancers. Meng et al. showed that ZNF384 is upregulated in breast cancer and is important for cancer cell metastasis [36]. ZNF384 is possible transcription factor for the histone acetyltransferase HBO1, the latter promoted osteosarcoma cell growth in vitro and in vivo [27]. Yan et al. demonstrated that ZNF384 overexpression promoted colorectal cancer metastasis by upregulating MMP2 [37].

One important discovery of this study is that ZNF384 could be a primary transcription factor of *Gai1* in NPC cells (see Fig. 10). In primary NPC cells, ZNF384 shRNA or KO decreased *Gai1* mRNA and protein expression, whereas ZNF384 overexpression upregulated *Gai1* expression. Importantly, there was an increased binding between ZNF384 protein and the proposed *Gai1* promoter in both human NPC tissues and cells. Increased binding between the two could be the primary mechanism of *Gai1* upregulation in NPC (see Fig. 10).

### CONCLUSION

Overexpressed *Gai1* exerts pro-tumorigenic activity in NPC possibly by promoting Akt-mTOR activation.

### DATA AVAILABILITY

All data are available upon request.

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## AUTHOR CONTRIBUTIONS

All listed authors conceived, designed, and supervised the study, performed the experiments and the statistical analysis. All authors drafted the article and revised it critically for important intellectual content, and with final approval of the version submitted to the journal.

## COMPETING INTERESTS

All authors listed in this study declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The authors declare no potential conflicts of interest.

## ETHICS STATEMENT

Experiments were carried out according to Ethics Committee of Soochow University, in according to the principles of Declaration of Helsinki.

## ADDITIONAL INFORMATION

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