CHMP2A regulates tumor sensitivity to natural killer cell-mediated cytotoxicity

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Natural killer (NK) cells are known to mediate killing of various cancer types, but tumor cells can develop resistance mechanisms to escape NK cell-mediated killing. Here, we use a “two cell type” whole genome CRISPR-Cas9 screening system to discover key regulators of tumor sensitivity and resistance to NK cell-mediated cytolysis in human glioblastoma stem cells (GSC). We identify CHMP2A as a regulator of GSC resistance to NK cell-mediated cytotoxicity and we confirm these findings in a head and neck squamous cells carcinoma (HNSCC) model. We show that deletion of CHMP2A activates NF-κB in tumor cells to mediate increased chemokine secretion that promotes NK cell migration towards tumor cells. In the HNSCC model we demonstrate that CHMP2A mediates tumor resistance to NK cells via secretion of extracellular vesicles (EVs) that express MICA/B and TRAIL. These secreted ligands induce apoptosis of NK cells to inhibit their antitumor activity. To confirm these in vitro studies, we demonstrate that deletion of CHMP2A in CAL27 HNSCC cells leads to increased NK cell-mediated killing in a xenograft immunodeficient mouse model. These findings illustrate a mechanism of tumor immune escape through EVs secretion and identify inhibition of CHMP2A and related targets as opportunities to improve NK cell-mediated immunotherapy.
Natur al killer (NK) cells play a key role in tumor-immune surveillance owing to their ability to identify and kill both hematological malignancies and solid tumors, including control of metastatic disease\(^1\)-\(^3\). Genomic studies demonstrated multiple and diverse genetic alterations in cancer\(^4\) with some mutations that can induce resistance\(^5\) or increase sensitivity to immune cells\(^6\). Although many NK cell-activating and inhibitory ligands have been identified\(^7\), these do not fully explain the mechanisms that may make tumor cells sensitive or resistant to NK cell-mediated activity. Recently, “two cell type” (TCT) CRISPR-Cas9 screens have been used to mimic the mutational multiplicity of tumors to identify novel mechanisms that regulate NK cell or T-cell-mediated antitumor activity\(^8\)-\(^10\).

Glioblastoma multiforme (GBM) is a heterogeneous brain tumor with a complex mutational pattern and an intricate tumor microenvironment (TME)\(^1\). Intradatum heterogeneity in GBM is sustained by glioblastoma stem cells (GSCs) that drive resistance to therapy\(^1\). Therefore, GSCs provide an important cell population to identify mechanisms of resistance and previously unidentified targets for NK cell-based immunotherapy. Here, we utilized a TCT-screening approach to identify key genes that increased or reduced the sensitivity of GSC to NK cell-mediated cytotoxicity. Since head and neck squamous cells carcinoma (HNSCC) is among the most highly immune-infiltrated cancer types with a high degree of infiltration of NK cells, which correlates significantly with patient’s survival\(^12\),\(^13\), we subsequently validated these findings on this model.

Extracellular vesicles (EVs) are emerging as a key component in the biogenesis of GBM and HNSCC, promoting migration, invasion, persistence, and contribute to the modification of the TME to support tumor progression\(^14\)-\(^19\). Indeed, EVs have been proposed as biomarker for the diagnosis of GBM\(^20\)-\(^22\) and more recently Hoshino and colleagues\(^23\) have proposed to use EVs as biomarkers to identify multiple types of cancers. Ligands for NK cell-activating receptors are often upregulated on tumor cells or during infection\(^24\), and the loss of these ligands reduces NK cell recognition and killing. MHC Class I Polypeptide-Related Sequence A/B (MICA/B) are ligands expressed on tumor cells for the NK-activating receptor NK group 2 member D (NKG2D)\(^24\); however, some tumors including leukemia, prostate cancer, melanoma, breast, lung, ovarian, and colon carcinomas frequently downregulate or shed these ligands to limit the cytotoxicity of NK cells\(^5\),\(^25\)-\(^27\). NKG2D ligands can be secreted on the surface of EVs, impairing NK cells' functions\(^26\)-\(^30\). Indeed, EVs-bearing MICA/B can act as decoys and inhibit NK cells\(^26\). Therefore, blocking the shedding of NKG2D ligands can increase NK cells' antitumor activity\(^31\).

Tumor-derived EVs carrying tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) can induce apoptosis in NK cells and CD8\(^+\) T lymphocytes\(^32\)-\(^35\).

Chemokines can promote the recruitment of NK cells to the TME to regulate NK cell-mediated antitumor activity\(^36\). CXCL10 increases NK cell migration\(^37\), and secretion of CXCL10 has been correlated with reduced tumor growth in xenograft models of lymphoma, squamous cell carcinoma, and adenocarcinoma of the lung\(^38\). In a model of GBM-expressing CXCL12, Muller et al.\(^39\) modified epidermal growth factor receptor (EGFR)-III expressing chimeric antigen receptor (CAR) NK cells to overexpress CXCR4, showing improved migration and a decrease in tumor volume.

Here, we identify chromatin-modifying protein/charged multivesicular body protein (CHMP2A) as a target for increasing GSC and HNSCC cell sensitivity to NK cell-mediated killing. CHMP2A is a subunit of the endosomal sorting complexes required for transport III (ESCRT-III), a molecular complex involved in the formation of multivesicular bodies\(^40\) and EVs biogenesis\(^41\),\(^42\). We demonstrate the loss of CHMP2A increases GSC and HNSCC cell sensitivity to NK cell-mediated killing in vitro, as well as in vivo using an HNSCC xenograft model. CHMP2A-knockout (KO) tumor cells secrete more CXCL10 and CXCL12, increasing NK cell migration in vitro. Moreover, since we observe that tumor-derived EVs can inhibit NK cell activity through MICA/B and TRAIL, deletion of CHMP2A limits the secretion of EVs by the tumor cells leading to enhanced NK cell-mediated cytotoxicity. Together, these studies demonstrate that CHMP2A-mediated activity provides a target whose ablation or inactivation can increase NK cell-mediated antitumor activity.

**Results**

Genome-wide CRISPR screening of GSC identifies key genes regulating NK cell activity. We performed a TCT genome-wide CRISPR screen in four GSC lines to identify perturbations that modulate NK cells-mediated killing activity (Fig. 1a). GSC 1517, 387, CW468, and D456 were lentivirally transduced to express the Brunello short-guide RNA (sgRNA) library and Cas9 endonuclease. Nine days post infection, cells were either cultured without effectors or challenged with peripheral blood NK cells at a 2:1 E:T ratio for 24-h. We compared changes in sgRNA abundance between the challenged and unchallenged GSC ranking the genes using MAgeCK\(^43\) and the top 25 hits that increased sensitivity or resistance to NK cells killing were ranked by RIGER using the Kolmogorov–Smirnov algorithm (Fig. 1b). The screen revealed that in NK cell-sensitive GSC, target genes were enriched in the ER-phagosome pathway, antigen presentation, cellular localization, and regulation of innate immune response (Fig. 1c).

Cells lacking the proteins encoded from the major histocompatibility gene complex (HLA) are a natural target of NK cells and, as expected, the KO of genes involved in antigen presentation like HLA-A, HLA-B, HLA-C, HLA-E, as well as for TAP1 and TAP2 increased GSC sensitivity to NK cells (Fig. 1d). We observed an expected stratification of some NK cell-activating ligands (MICA, MICB, ULBP1, ULBP2, ULBP3, FAS, TRAIL-R1, TRAIL-R2, and ICAM1) that increased GSC resistance once targeted with sgRNAs (Fig. 1d). The whole-genome CRISPR screen revealed CHMP2A as top hit that increased the sensitivity of GSC to NK cell-mediated cytotoxicity (Fig. 1b). CHMP2A is a subunit of the ESCRT-III protein machinery and involved in EVs secretion\(^41\),\(^42\), a pathway used by tumor cells to impair NK cells function\(^26\)-\(^29\) and therefore a potential target to improve NK cell-mediated antitumor activity, which we wanted to explore more thoroughly.
significant increase in NK cell-mediated cytotoxicity (Fig. 2c) in CHMP2A-KO cells. The KO of CHMP2A did not significantly impair the proliferation and viability of Cal27 cells (Supplemental Fig. 1c). Next, to confirm the increased sensitivity to NK cells we observed was indeed due to CHMP2A-KO, we overexpressed CHMP2A in Cal27 where CHMP2A had been previously knocked out (Fig. 2d). Rescuing CHMP2A expression restored resistance to NK cell-mediated killing, demonstrating that CHMP2A has a role in the resistance of Cal27 cells to NK cell-mediated killing (Fig. 2e). Although tumor cells can modulate their sensitivity to NK cells, we did not see any significant change in the expression of NK cell-activating or inhibitory ligands in Cal27 CHMP2A-WT (Supplemental Fig. 2a) and CHMP2A-KO cells (Supplemental Fig. 2b). Subsequently, we investigated The Cancer Genome Atlas (TCGA) database for GBM and HNSCC to evaluate a possible correlation between low CHMP2A expression and increased overall survival (Supplemental Fig. 3). We observed that low CHMP2A is associated with slightly increased (but not statistically significant) survival in GBM (Supplemental Fig. 3a), while the survival benefit of low CHMP2A expression is statistically significant in HNSCC (Supplemental Fig. 3b).

CHMP2A increases NK cell migration via secretion of CXCL10 and CXCL12. To investigate potential mechanisms by which CHMP2A regulates NK cell-mediated killing, we analyzed the transcriptome of GSC cells by RNA sequencing. In 387 and CW468 cells, we found substantial differences in the expression of genes related to the tumor-immune system interaction, as well as membrane trafficking and endocytosis (Fig. 3). The most upregulated genes included cytokines, chemokines, and genes expressed during the innate immune response. Since tumor cells can modify their microenvironment and reduce the migration and antitumor activity of effector cells, including NK cells, we were interested to identify CXCL10 and CXCL12 as increased in expression in the KO cells. We measured CXCL10 and CXCL12 produced by CW468 and CAL27-WT and CHMP2A-KO and found higher levels of CXCL10 and CXCL12 were secreted in the media when CHMP2A was deleted (Fig. 4a, b). Next, we assessed the migration of NK cells using CHMP2A-KO or WT cells as chemoattractants. An increase in NK cell migration was observed when NK cells were co-cultured with CW468 CHMP2A-KO and Cal27 CHMP2A-KO compared with WT cells ($p < 0.01$) (Fig. 4c). Interestingly we observed the upregulation of NF-κB P65 in Cal27 CHMP2A-KO
**Fig. 2** CHMP2A-KO increases GSC and HNSCC cell lines sensitivity to NK cell-mediated cytotoxicity. 

**a** 4 hour cytotoxicity assay to determine the effect of CHMP2A in GSC resistance to NK cell-mediated cytotoxicity. Two independent sgRNAs were used (sg#2, sg#3). **b** Immunoblot analysis of WT and KO GSC lines KO for CHMP2A using two sgRNAs (sg#2, sg#3). GAPDH serves as a loading control. **c** 4 hour cytotoxicity assay to determine the effect of CHMP2A in HNSCC-resistance to NK cell-mediated cytotoxicity. **d** Western blot analysis showing the rescue of KO phenotype. Here we show Cal27 cells expressing WT levels of CHMP2A, no CHMP2A (KO), or complemented CHMP2A (KO+ overexpressed CHMP2A). HEK293 CHMP2A overexpression lysate (OL) was used as a control. The overexpressed CHMP2A is fused with DDK and MYC tags explaining the 4 kDa increase in molecular weight. For this western blot, GAPDH was used as a loading control. **e** 4 hour cytotoxicity assay to determine the effect of CHMP2A in Cal27 cells expressing WT levels of CHMP2A, no CHMP2A (KO), or complemented CHMP2A (KO+ overexpressed CHMP2A). The error bars represent standard error from the mean (±SEM) across n = 3 replicates. Statistical analysis was performed by one-tailed paired Student t test. Representative of n = 3 independent experiments.
by immunoblotting (Fig. 4d). It has been previously reported that NF-κB P65 activation can increase CXCL10 secretion46,47, therefore we used a reporter plasmid to further analyze NF-κB activity.

We measured a three-fold increase in NFkB activity in Cal27 CHMP2A-KO compared with Cal27 CHMP2A-WT (Fig. 4e). Since ESCRT-III has been described to be a negative regulator of constitutive NF-κB activity by preventing the accumulation and the activation of NF-κB-inducing receptors48, further studies could elucidate the possible role of CHMP2A in regulating NF-κB signaling and the secretion of CXCL10.

**CHMP2A regulates tumor cell production of EVs.** We next evaluated the activity of NK cells when co-cultured in conditioned media from CW468 KO or WT cells culture. CW468-WT conditioned media reduced NK cell-mediated killing of CW468-WT compared with CW468 CHMP2A-KO-conditioned media cells (Supplemental Fig. 4a, b). NK cells also demonstrated less killing of Cal27-WT and CHMP2A-KO when cultured with WT conditioned media (Supplemental Fig. 4c, d). Since EVs can impair NK cell activity28,29,32–34, we tested if CHMP2A-KO could influence EV production from tumor cells by nanoparticle tracking49 (Fig. 5a, b and Supplemental Fig. 5a, b). We demonstrated that Cal27 CHMP2A-KO conditioned media had 59.5% fewer EVs than Cal27-WT (p < 0.0001) (Fig. 5c). In addition, we found EVs from KO cells were significantly bigger (p < 0.001) with an average size of 246.40 nm (SEM ± 19.70 nm) compared with 162.40 nm (± 25.90 nm) for Cal27-WT cells (Fig. 5d). The analysis on the GSC line CW468 showed comparable results.
Cal27-WT-derived EVs induce apoptosis in NK cells, limiting their antitumor activity. To further investigate the effect of EVs on NK cells, we analyzed EVs from WT and CHMP2A-KO Cal27 cells by flow cytometry for the expression of MICA/B, TRAIL, FasL, and ULBPs. Although no differences in expression of these markers were observed between Cal27-WT and KO-derived EVs (Fig. 6a), we found EVs expressed NKG2D ligands MICA/B, and TRAIL. Notably, previous studies demonstrate EVs containing MICA/B can inhibit NK cell activity\textsuperscript{28,29} and EVs-bearing TRAIL can induce apoptosis in NK cells\textsuperscript{32–34}. Consequently, since NK cells express NKG2D and TRAIL receptors (TRAIL-R1 and TRAIL-R2) (Supplemental Fig. 6a), we hypothesized Cal27-derived EVs could reduce NK cell viability. NK cells were incubated with Cal27-derived EVs and assessed viability through the activation of the Caspase-3–7 (Casp3–7) pathway. EVs derived from Cal27 cells significantly increased NK cell death after 3 hour of treatment compared with untreated NK cells (Fig. 6b). Subsequently, we incubated NK cells with EVs for 24-h, supplemented with MICA/B or TRAIL blocking antibodies that led to significantly less cell death than NK cells incubated with EVs only or EVs incubated with the matching isotype control antibodies (Fig. 6c) (in Supplemental Fig. 6b we report the data from the first 5 hours as compared with the data displayed in Fig. 6b). These results demonstrate that Cal27-derived EVs-bearing MICA/B and TRAIL can induce apoptosis in NK cells. Next, to determine whether the EVs were increasing the resistance of Cal27-KO cells to NK cells we co-cultured NK cells with Cal27 CHMP2A-KO cells and EVs. Cal27 CHMP2A-KO showed significantly reduced
sensitivity to NK cell killing when incubated together with EVs ($p < 0.01$), comparable with WT levels (Fig. 6d). Similarly, to Cal27, EVs significantly increased the viability of CW468 reducing the killing activity of NK cells (Supplemental Fig. 5e). Treatment with MICA/B and TRAIL blocking antibodies significantly reduced the inhibiting effect of EVs when NK cells were co-cultured with KO Cal27 cells plus EVs (Fig. 6e). A combination of the two antibodies further improved the killing of the NK cells (Fig. 6e). In addition, we demonstrated these EVs reduced NK cell-mediated killing of K562 cells, an NK cell-sensitive cell line, and this inhibition was rescued by blocking MICA/B or TRAIL (Supplemental Fig. 6c). As shown in Fig. 6a, EVs secreted by tumor cells are composed of small (SEV) and large (LEV) EV. We separated through a gradient of
ultracentrifugation LEV from SEV and co-cultured with NK cells for four hours. Interestingly, NK cells significantly increased the number of apoptotic cells. NK cells did not. A combination of SEV and LEV enhanced apoptosis in NK cells (Supplemental Fig. 6d). To further confirm the role of SEV in impairing NK cell-mediated killing, we performed a killing assay coculturing Cal27 CHMP2A-KO cells with SEV, LEV, or a combination of the two (Supplemental Fig. 6e). SEV reduced the killing of NK cells confirming their role in inhibiting NK cell killing of Cal27. Together these results demonstrate that Cal27 cells utilize EVs as a mechanism to increase resistance to NK cell-mediated cytotoxicity through the induction of NK cell death.

**Discussion**

NK cells are a key part of the innate immune system and play an important role in controlling the development of malignancies. However, solid tumors are generally less sensitive than hematological malignancies to NK cell-mediated killing, at least in part owing to the immune inhibitory properties of the TME. Frequently, the tumor cells halt immune cells migration or inhibit their cytolytic functions, reducing the efficacy of these treatments.

CRISPR-Cas9 screens have been used recently to discover new protein targets or druggable molecular pathways in tumor cells and combined with effector cells in TCT screenings. In their study, Pech et al. used an NK cell-based TCT to identify DDB1-and-Cul4-associated factor 15 (DCAF15) as a novel regulator of NK cell-mediated killing. Notably, this work used the NK2 cell line as effector cells and the highly NK cell-sensitive erythroleukemia K562 cell line as the target cell population. It is possible that these findings using an aneuploid NK cell line and the very NK cell-sensitive K562 cells do not fully reflect the diversity of mechanisms that NK cells use to engage and kill tumor cells, especially solid tumors that are typically more resistant to NK cell activity. More recently a whole-genome CRISPR screen has been combined with profiling relative inhibition simultaneously in mixtures (PRISM) approach to study the response versus the resistance of several solid tumor cell lines to NK cells. B7-H6 upregulation was found to be an important gene associated with marked NK cell sensitivity while HLA-E KO was associated with increased sensitivity to NK cell cytotoxicity. Although not present in the top 20 hits, we also observed HLA-E as an important negative regulator of response to NK cells in our CRISPR screen (Fig. 1d), altogether with other MHC class I genes and genes involved in surface expression of MHC I molecules.

Using a CRISPR-Cas9 TCT screen we identified CHMP2A, a component of the ESCRT-III complex, as a regulator of GSC and HNSCC cell sensitivity to NK cell-mediated cytotoxicity. We observed increased migration of NK cells towards CHMP2A KO tumors that showed increased secretion of CXCL10 and CXCL12, chemokines involved in NK cell migration. Our findings are also supported by a bioinformatic analysis of the TCGA database for GBM and HNSCC describing an inverse correlation between CHMP2A expression and the NK cell survival.
signature in tumors. That is, there is higher NK cell infiltration of tumors that have lower CHMP2A expression.

Notably, the polyprotein ESCRT-III that contains CHMP2A as a subunit is involved in the last phase of cell membrane deformation and fission, a process required for EVs budding. Although some studies have suggested that the ESCRT-III complex can mediate tumor cell resistance to killing, more precise mechanisms have been unclear. EVs have been characterized to impair the activation of immune cells within tumors. In patients with HNSCC, EVs are associated with the suppression of the antitumor effect of NK cells by binding to NKG2D and TRAIL-R to induce apoptosis on NK cells. By CHMP2A KO we reduce EVs secretion from tumor cells decreasing their inhibitory function. Tumor cells CHMP2A KO also secrete CXCL10 and CXCL12, chemokines that increase NK cell migration culminating in enhanced NK cell-mediated cytotoxicity.

Fig. 7 CHMP2A-KO HNSCC xenograft model shows increased sensitivity to NK cells. a Schematic of in vivo treatment. Non-obese diabetic/severe combined immunodeficiency/γc−/− (NSG) mice were inoculated subcutaneously with 6 × 10⁶ cells (Cal27-WT or KO) and injected i.v. with 1 × 10⁷ NK cells. NK cells were supported by injections IL-15 daily and IL-2 every other day for 1 week. Tumor volume was monitored every 2–3 days. b Tumor volume progression over 21 days shown as mean ±SEM of n = 5 mice per treatment. Statistical analysis was performed by two-way ANOVA and Bonferroni’s post hoc multiple comparison test (ns, not significant). c Representative flow cytometry dot plots showing increased NK cell infiltration in Cal27 CHMP2A KO tumors. The gating strategy has been defined by combining NK cells and a cell suspension from the untreated tumor. Tumor samples without NK cell treatment were mixed with 2 × 10⁴ human NK cells, stained with anti-human CD45 and anti-human CD56 antibodies or isotype controls, and analyzed. Cells double positive for (human) hCD45 and hCD56 were gated and the gate was used to quantify the number of infiltrating NK cells in Cal27-WT and CHMP2A KO tumors. The graph shows the average NK cell infiltration for all mice in each group (n = 4 mice per group). Error bars represent ±SEM across n = 4 technical replicates and an unpaired one-tailed Student’s t test was performed to determine statistical significance. d Cartoon describing the proposed mechanism of CHMP2A KO in tumor cells. Tumor cells secrete EVs-bearing ligands like MICA/B and TRAIL, which suppress the antitumor effect of NK cells by binding to NKG2D and TRAIL-R to induce apoptosis on NK cells. By CHMP2A KO we reduce EVs secretion from tumor cells decreasing their inhibitory function. Tumor cells CHMP2A KO also secrete CXCL10 and CXCL12, chemokines that increase NK cell migration culminating in enhanced NK cell-mediated cytotoxicity (the cartoon has been created with Biorender.com).
lymphocytes, (including NK cells), and with disease stage and activity. In GBM, EVs are involved in inducing immune-tolerance through the activation of myeloid-derived suppressor cells. More in general EVs are considered to play a relevant role in creating an immune-suppressive microenvironment in GBM and HNSCC. Moreover, it has been recently described that gain of function TP53 can be transferred from tumor cells to normal fibroblasts. Here, we observed a correlation between reducing EVs secretion in GSC and HNSCC cells and the increased sensitivity to NK cell killing both in vitro and in vivo. Focusing on the mechanisms by which EVs promote tumor resistance, EVs carrying NKG2D ligands were shown to induce a downregulation of cell surface NKG2D in both NK and T cells, or act as decoys. Blocking the shedding of NKG2D ligands can increase NK cell-antitumor activity. Nevertheless, not only MICA/B are involved in this resistance mechanism, and recently Sharma and colleagues showed EVs expressing TRAIL mediated killing, including inducing apoptosis in NK cells. These findings suggest CHMP2A and more widely ESCRT-III being part of the key immune-suppressive mechanism that involves secretion of tumor EVs that can inhibit NK cell-mediated killing, including inducing apoptosis in NK cells. Therefore, tumor-derived EVs may not only be used as a reliable biomarker to characterize tumors, but also considered to better define the immune escape mechanisms used by different tumors and studied to develop new strategies to overcome tumor resistance to immune cells.

In conclusion, we have used a TCT CRISPR screen to discover a key gene that regulates tumor sensitivity to NK cells. This study provides an explanation of a mechanism that contributes to resistance in GBM and HNSCC to NK cell-mediated killing. Our data demonstrate CHMP2A and tumor secreted EVs can be responsible for inducing apoptosis in NK cells, thus limiting their cytotoxic potential. The evaluation of the expression of CHMP2A and characterization of tumor secreted EVs in patients may explain some mechanisms of immune escape and speed the development of new drugs that can target these immune-suppressive processes.

Methods

All studies comply with local and national ethical guidelines. Animal studies were approved by the University of California, San Diego Institutional Animal Care and Use Committee and followed the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. All studies with biological agents were approved by the University of California, San Diego Institutional Biosafety Committee.

Lentiviral transduction on GSCs. The sgRNA library and single-targeted sgRNA lentiviral plasmids for GSC transduction were purchased from Addgene (#52961). sgRNA#1—AGAGCCGCAAGGAGCTACTG, sgRNA#2—CAAATCGGC GCATACGGCC, sgRNA#3—TCGAATGGCAAAGCAGCTAGA, sgRNA#4—TC TCTAGTTCGTGCCTGCG. HEK 293FT (4 × 10^6) were seeded the day before transfection in a 100 mm^2 culture dish. 24 h later cells were co-transfected with sgRNA-Cas9 lentivector (5 µg) or shRNA plasmids (Sigma), PAX3 (5 µg) (Addgene), and pMD2.G (1.5 µg) (Addgene) using LipoD293 (SignaGen Laboratories) following manufacturer’s protocol. Lentiviral supernatant was collected 48 h post-transfection, filtered 0.45 µm, and concentrated using Lentivirus Concentration Solution (Takara Bio). Recipient cells once reached 40–50% confluence, were infected and incubated at 37 °C ON with viral supernatant containing 10 µg/ml polybrene (EMD Millipore) and replaced 24 h later with fresh media. After 48 h, transduced cells were cultured in fresh media containing 1 µg/ml puromycin (EMD Millipore) for 3–5 days. Single clone selection was performed in Cal27 mock and KO cells seeding cells at limiting dilution after drug selection. Single clones were identified by sequencing PCR products containing amplified DNA nearby the sgRNA binding site. sgRNA#3—TCTAGTGGCAAAGCAGCTAGA has been used to generate Cal27-KO cells.

CRISPR-Cas9 screen analysis. FASTQ files were trimmed to 20 bp CRISPR guide sequences using BBduk from the BBDMap (B. Bushnell) toolkit and quality control using qbiovInformatics (FASTX). CRISPRCas9seq v29 was used to search for RPA2 reads in the library and processed into counts using the MAGECK “count” function. Genes were then filtered such that the sum of normalized counts >10. Hits were ranked by RIGER using the Kolmogorov–Smirnov algorithm.

RNA-seq analysis. RNA-seq analysis was performed as previously described. Briefly, the total RNA was isolated from GSCs. RNA integrity was assessed before co-culture and GSCs after monoculture for 24 h.

Cytokine production was measured using Cytoscape Reactome FI plugin (RRID:SCR_003032). Genes upregulated with a knockout at log2 FC > 1 and FDR < 0.05 and genes downregulated with a knockdown at log2 FC < −1 and FDR < 0.05, plus the target gene were input into Reactome FI, and all genes with at least one edge were included in the network plot. Pathway enrichment was performed on this network of genes using the Reactome FI enrichment option. Box plots for genes from selected pathways were generated using RNA-seq TPM data. KEGG pathway visualizations were generated using the R/Bioconductor package pathview (https://www.bioconductor.org/packages/release/bioc/html/pathview.html) for selected pathways.

Cell culture. GSC lines 387, CW468, D456, and 1571 were cultured in phenol red-free Neurobasal media plus B27 supplement (Invitrogen), supplemented with 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 10 ng/ml basic fibroblast growth factor, 1 mg/ml epidermal growth factor (R&D Systems). Cal27, Detroit568 and UMSCC17B were cultured in Dulbecco’s Modified Eagle Medium (DMEM)-F12 1:1 (Gibco), 10% fetal bovine serum (FBS), supplemented with 2 mM L-glutamine (Invitrogen), 0.1 mM MEM non-essential amino acids (NEAA) (Invitrogen) (HNSCC complete media). Cal27-WT and KO cells were cultured in serum-free media (SFM) Defined Keratinocyte SFM (Invitrogen) supplemented with 2 mM L-glutamine (Invitrogen), 0.1 mM NEAA (Invitrogen) and 2 ng/ml EGF (R&D Systems) (Cal27 SFM). HEK 293FT cells were cultured in DMEM (Invitrogen), 10% FBS supplemented by 0.1 mM NEAA, 2 mM L-glutamine, 1 mM MEM Sodium Pyruvate, 1% PenStrep and 500 µg/ml Geneticin (Invitrogen).

Generation of CHMP2A KO cell lines using lentiviral vectors. Four sgRNA targeting CHMP2A were chosen targeting the whole-genome CRISPR library (Addgene #73179) and cloned into the sgRNA-Cas9 lentivector (Addgene #52961). sgRNA#1—AGAGCCGCAAGGAGCTACTG, sgRNA#2—CAAATCGGC GCATACGGCC, sgRNA#3—TCGAATGGCAAAGCAGCTAGA, sgRNA#4—TC TCTAGTTCGTGCCTGCG. HEK 293FT (4 × 10^6) were seeded the day before transfection in a 100 mm^2 culture dish. 24 h later cells were co-transfected with sgRNA-Cas9 lentivector (5 µg) or shRNA plasmids (Sigma), pPAX3 (5 µg) (Addgene), and pMD2.G (1.5 µg) (Addgene) using LipoD293 (Sigma) following manufacturer’s protocol. Lentiviral supernatant was collected 48 h post-transfection, filtered 0.45 µm, and concentrated using Lentivirus Concentration Solution (Takara Bio). Recipient cells once reached 40–50% confluence, were infected and incubated at 37 °C ON with viral supernatant containing 10 µg/ml polybrene (EMD Millipore) and replaced 24 h later with fresh media. After 48 h, transduced cells were cultured in fresh media containing 1 µg/ml puromycin (EMD Millipore) for 3–5 days. Single clone selection was performed in Cal27 mock and KO cells seeding cells at limiting dilution after drug selection. Single clones were identified by sequencing PCR products containing amplified DNA nearby the sgRNA binding site. sgRNA#3—TCTAGTGGCAAAGCAGCTAGA has been used to generate Cal27-KO cells.

Isolation and expansion of NK cells. Peripheral blood mononuclear cells (PBMC) were isolated through density gradient centrifugation from an apheresis product (obtained from the San Diego Blood Bank Center) and NK cells were sorted using EasySep Human NK Cell Enrichment Kit (StemCell Technologies) which depletes CD3^+ and CD19^+ cells. The use of PBMC from anonymized donors was approved by the Committee on the Use of Human Subjects in Research at the University of California, San Diego. Peripheral blood mononuclear cells were cultured in RPMI 1640 (Invitrogen), 10% heat-inactivated FBS, 2 mM L-glutamine, 1% PenStrep and supplemented with 50 IU/mL of IL-2 every three days or media change. NK cells...
were co-cultured once a week with irradiated K562-mbll-21-4-IgB1 artificial antigen-presenting cells kindly provided by Dr. Dean A. Lee ( Nationwide Children’s Hospital) for expansion1,1.

**Generation of CHMP2A overexpressing Cal27.** In all, 8 × 10⁵ cells Cal27-KO were transfected with CHMP2A (NM_014453) Human Tagged ORF Clone (Origene) via nucleofection using Amaxa 2D (Lonza) and Cell Line Nucleofector Kit V (Lonza) following manufacturer’s protocol. After nucleofection cells were seeded in six-well plates and 24 h later media was changed and supplemented with Geneticin (1 µg/ml) (Invitrogen) for selection. CHMP2A overexpression was assessed by immunoblotting.

**Immunoblotting.** Immunoblotting was performed according to Invitrogen protocols for Mini Gel Tank and Blot2 dry system. In brief, cell lysates were prepared to incubate cells with RIPA buffer (Invitrogen) containing Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). EVs lysates were prepared by incubating cells with 50 µl of RIPA buffer (Invitrogen) containing Halt Protease Inhibitor Cocktail (Invitrogen) and sonicated for 10 min. In all, 25 µl of EVs lysate was loaded on the gel. Proteins were separated using NuPAGE Bis-Tris 4–12% gels (Invitrogen) and transferred to nitrocellulose membranes using the iBlot dry method. Membranes were blocked for 40 min in 4% milk and incubated with the primary antibodies in 4% milk 1.5 h at room temperature for anti-CHMP2A antibody or ON at 4 °C for all the other antibodies used. After incubating the membrane with the appropriate secondary antibody conjugated to horseradish peroxidase, protein levels were detected using Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore). CHMP2A overexpression lysate (OL control used in Fig. 2c) was purchased from Origene, CHMP2A Antibody, rabbit polyclonal, Proteintech, cat. no. MA5-15738. CD9, clone (D8O1A) Rabbit mAb, Cell Signaling, cat. no. 13174S (1:1000).

**Tipifarnib treatment.** Cal27-WT and CHMP2A KO cells were treated with tipi-farnib as follows: prior to performing NK cells killing assays, cells were trypsinized and harvested by centrifugation at 400 × g, pellet resuspended, cells seeded at 1 × 10⁶ cells/ml in HNSCC compete for media and treated with tipifarnib 1 µM or DMSO as a control for 72 h. Prior to collection and analysis of cell-derived EVs, Cal27 cells were trypsinized and harvested by centrifugation at 400 × g, cells washed in 5 µl of 0.22 µm filtered sterile Dulbecco’s phosphate-buffered saline (DPBS) and harvested by centrifugation. Pellet was resuspended in Cal27 SFM, 5 × 10⁶ cells plated in 15 ml of Cal27 SFM in a T75 flask and treated with tipifarnib 1 µM or DMSO as control for 72 h.

**Antibodies used.** The following antibodies were used for flow cytometry (all anti-human unless indicated): PE-Cy7 anti-human CD56 mlgG1, BioLegend, clone HCDC56, cat. n. 318318 (1:100)
APC anti-human CD45, mlgG1, BD Biosciences, clone HI30, cat. n. 555485 (1:50)
APC anti-human MICA/MICB Antibody, Biologend, cat. n. 320907 (1:100)
Human TRAIL-R2/TNFRSF10B PE-conjugated Antibody - R&D, cat. n. FAB6311P (1:100)
PE anti-human ULBP2/6, R&D, Clone: 165903, cat. n. FAB12898P (1:100)
Human ULBP3 Alexa Fluor® 647-conjugated Antibody, R&D, cat. n. FAB1517B (1:100)
PE anti-hCD178 (FasL), eBioscience, clone. 12-9919-42 (1:100)
PE Mouse Anti-Human CD112, BD Biosciences, clone. n. 551057 (1:50)
PE Mouse Anti-Human HLA-ABC, BD Biosciences, clone. n. 557349 (1:50)
PE anti-CD155 antibody, Biologend, cat. n. 337609 (1:100)
PE anti-HLA-E antibody, Biologend, cat. n. 342603 (1:100)
APC anti-human CD274 (R7-H1, PD-L1) Antibody [Clone: 29E.2A3], Biologend, cat. n. 329707 (1:100)
APC anti-human CD276 (B7-H3) Antibody [Clone: MIH42], Biologend, cat. n. 351003 (1:100)
PE anti-human CD262 (DR5, TRAIL-R2) Antibody [Clone: DR2-4 (7-8)], Biologend, cat. n. 307405 (1:100)
APC anti-human CD261 (DR4, TRAIL-R1) Antibody [Clone: DR1], Biologend, cat. n. 307207 (1:100)
APC Mouse Anti-Human CD314 (NKGD2), BD Biosciences, cat. n. 558071 (1:100)

**Flow cytometry NK cells killing assay.** Target cells were pre-stained with CellTrace Violet (Thermo Fisher Scientific) at a final concentration of 5 mM in PBS for 15 min at 37 °C. Cells were incubated in complete culture medium containing FBS for 5 min and harvested by centrifugation. Cells were resuspended in culture media prior to being mixed with NK cells at the indicated effector to target (E:T) ratios. When performing EVs or anti-MICA/B and anti-TRAIL blocking treatments, reagents or EVs were added to the specific wells in culture media plus an Fc Receptor Binding Inhibitor (Thermo Fisher Scientific), 20 µl/test. Co-cultures were incubated at 37 °C and after 3.5 h CellEvent Caspase-3/7 Green Detection Reagent (Thermo Fisher Scientific) was added for an additional 30 min of culture for a total incubation time of 4h. SYTOX AADvanced dead cell stain solution (Thermo Fisher Scientific) was added after the final 5 min of staining. Cells were then analyzed by flow cytometry. NK cells killing was calculated by subtracting the background of untreated target cells from all the other samples of the same experimental group. Experiments were performed with three independent biological triplicates. To perform the killing assay described in Supplemental Fig. 4, CW688 WT and KO cells were cultured for three days, and media was collected after 5 min centrifugation at 400 × g to remove dead cells. Target cells and NK cells were co-cultured on conditioned media and the killing assay was performed and analyzed as described above.

**Flow cytometry Caspase-3/7 apoptosis assay.** NK cells were pre-stained with CellTrace Violet (Thermo Fisher Scientific) at a final concentration of 5 mM in PBS for 15 min at 37 °C. Cells were incubated in complete culture medium containing FBS for 5 min and harvested by centrifugation. NK cells were resuspended in media containing CellEvent Caspase-3/7 Green Detection Reagent and SYTOX AADvanced dead cell stain solution and treated with Cal27-derived EVs and incubated for 4 h. Treated cells and the corresponding untreated controls were collected every hour by flow cytometry. Experiments were performed with three independent biological triplicates.

**Incucyte Caspase-3/7 apoptosis assay.** NK cells were pre-stained with CellTrace Far Red (Thermo Fisher Scientific) at a final concentration of 5 mM in PBS for 15 min at 37 °C. Cells were incubated in complete culture medium containing FBS for 5 min and harvested by centrifugation. NK cells were resuspended in media containing Incucyte Caspase-3/7 Green Apoptosis Assay Reagent (Essen Bioscience) diluted by a factor of 1000 and 1 × 10⁴ NK were seeded in a 96 well plate previously coated with Pol-o-Lysine (Sigma-Aldrich) ON at 37 °C. Depending on treatments combinations Cal27-derived EVs, anti-MICA/B, anti-TRAIL, mouse IgG1, and IgG2a isotypes were mixed in culturing media and added to NK cells. Cells were centrifuged for 5 min at 400 × g to let NK cells adhere to the coated well monitored on the Incucyte ZOOM to acquire images every 1 h for 24 h. Experiments were performed with 3 independent biological triplicates. The apoptosis was quantified by analyzing the overlay of Caspase-3/7 (green) within the red labeled NK cells.

**Transwell migration assay.** WT and KO cells were seeded at 1 × 10⁵ cell/well in a 24 well plate in 600 µl SFM. Two days later 1 × 10⁵ NK cells were added in 200 µl SFM to the upper chamber (5 µm pore size), and the plates were incubated for 3 h at 37 °C. Cells in 300 µl of media in the lower chamber were incubated with NKG2D antibody or mouse IgG1 to discriminate NK cells from any floating tumor cells and after 30 min NK cells number was determined by flow cytometry counting cells in 200 µl of media using a NovoCyte flow cytometer. Data are presented as a number of NK cells calculated in 600 µl of media. The typical NK cells marker CD56 (NCAM) was not used to detect NK cells because expressed on GSC (data not shown).

**Chemokine analysis.** CW688 and Cal27 cells line supernatants were generated by culturing 1 × 10⁵ cells/ml in 24 well plates in SFM for 48 h. Dead cells were removed by 5 min centrifugation at 400 × g and the supernatants were analyzed for CXCL10 and CXCL12 using IP-10 (CXCL10) Human ELISA Kit (Innvitrogen) and ELISA, Human CXCL12/SDF-1 DuoSet (R&D Systems) according to the manufacturer’s protocols on Infinite 200Pro (Tecan). Experiments were performed in duplicate and repeated three times.

**Fluorescence-activated cell sorting (FACS).** All the flow cytometry-based assays were performed on a NovoCyte (ACEA Biosciences) and data were analyzed using NovoExpress software. Cal27 cells were trypsinized and centrifuged at 400 × g for 5 min while NK cells resuspended in media and centrifuged at 400 × g for 5 min. Cells were resuspended in DPBS + 2% FBS (flow buffer) and stained with trypan blue; cell counting using an inverted microscope, and 1 × 10⁶ cells were dispersed per sample. Cells were incubated on ice in the dark for 20 min in a flow buffer containing the antibodies of interest. Stained cells were centrifuged at 400 × g for 5 min and washed flow buffer two times. Finally, cells were resuspended in 300 µl of flow buffer containing SYTOX Blue Dead Cell Stain (Life Technologies) diluted by a factor of 1000 and analyzed by FACS.
fiery luciferase activity of Cal27 CHMP2A-WT was set as 1. Experiments were performed in triplicates and repeated two times.

**EVs harvesting and tracking analysis.** At day 1, WT or CHMP2A-KO cells were harvested according to the cell type, pellet resuspended, and cells washed in 5 ml of 0.22 μm filtered sterile DPBS and harvested by centrifugation. Cal27 and CW468 were resuspended in zSEM and 5 × 10^6 cells plated in 15 ml of SFM (Neurobasal media for GSC or Cal27 SFM for Cal27) in T75 flasks and cultured at 37°C for 3 days. At day 3, EVs were collected following the manufacturer's protocol. In brief, conditioned media was centrifuged at 3000 × g for 15 min to remove dead cells and apoptotic bodies. 1.5 ExoQuick ULTRA EVs Isolation Kit for Tissue Culture Media (SBI System Biosciences) was added to collected media, gently mixed, and incubated at 4°C ON. The following day the supernatant was centrifuged at 3000 × g for 10 min to remove the supernatant and EVs were resuspended in 0.22 μm filtered sterile DPBS ±SEM and 5 × 10^6 cells plated in 15 ml of SFM. For EVs tracking analysis, EVs were resuspended in 1 ml of 0.22 μm filtered sterile DPBS and analyzed on a NanoSight LM10.

**SEV and LEV separation through ultracentrifugation.** At day 1 Cal27-WT cells were harvested, pellet resuspended, and cells washed in 5 ml of 0.22 μm filtered sterile DPBS and harvested by centrifugation. Cal27 were resuspended in zSEM and 5 × 10^6 cells plated in 15 ml of SFM in T75 flasks and cultured at 37°C for 3 days. At day 3, media was collected and centrifuged at 2000 × g for 10 min to remove dead cells. The pellet was discarded, and the supernatant ultracentrifuged at 10,000 × g for 30 min. After centrifugation, the supernatant was collected, and the pellet that contains LEV was resuspended in 0.22 μm filtered sterile DPBS. The supernatant was further ultracentrifuged at 100,000 × g for 2 h to collect SEV. After ultracentrifugation, the supernatant was gently removed and the pellet of SEV resuspended in 0.22 μm filtered sterile DPBS.

**Flow cytometry analysis of EVs.** On Day 1, freshly isolated Cal27-derived EVs collected as described in the paragraph above were stained using PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich) following a slightly modified manufacturer’s protocol. EVs were resuspended in Diluent C, mixed with PKH26, and incubated for 5 min at RT. PKH26 was quenched with 2 ml of 0.22 μm filtered sterile DPBS + 10% BSA bringing the volume up to 8.5 ml in SFM. ExoQuick ULTRA EVs Isolation Kit solution was added at 1:5 ratio, gently mixed, and incubated at 4°C ON. On day 2 EVs were harvested by centrifugation at 3000 × g for 10 min and resuspended in 0.22 μm filtered sterile DPBS ± 1% BSA (EVs flow buffer) and distributed in 100 μl volume. EVs were incubated as single staining with MICA/B, TRAIL, ULBP3, ULBP2/5/6, Fas, antibodies, and matching isotypes for 30 min on ice and diluted with 200 μl of EVs flow buffer. Samples were analyzed by flow cytometry gating the red fluorescent particles and measuring specific antigen expression. Due to the nanometer size of the EVs, we previously observed unspecific signals derived from non-EVs particles within the antibody's solutions. To overcome the problem, besides staining EVs with PKH26 we designed the experiment including control samples for single antibodies only, EVs flow buffer, EVs flow buffer + PKH26, and unstained EVs, to remove all the unspecific signals.

**Transmission electron microscopy (TEM).** Isolated EVs were incubated on formvar/carbon-coated 100-mesh copper grids for 10 min, washed with water, and stained with 2% uranyl acetate aqueous solution for 1 min. Grids were viewed using Transmission electron microscopy (TEM) provided with this paper. The findings of this study have been deposited in GEO with the accession code GSE175863. The RNA sequencing data that support the findings of this study have been deposited in GEO with the accession code GSE175862. The RNA sequencing data that support the findings of this study have been deposited in GEO with the accession code GSE175862.

**TGA HNSCC and GBM data sets analysis.** Gene expression data from HNSCC and GBM tumors were obtained from TCGA. The gene sets representing NK cells were generated by performing differential expression analysis on the gene expression dataset from Novosilov et al. (2011) ( GEO dataset accession GSE24759) containing mRNA profiles for 38 distinct purified populations of human hematopoietic cells, and then taking the top 20 genes overexpressed and top 20 genes underexpressed in NK cells, compared to all other cell types. The gene set of overexpressed genes was named “NK_20_up” and the underexpressed one was named “NK_20_down”. Single sample GSEA (sgGSEA) was performed on the bulk TCGA tumor expression data with both gene sets to assess the degree of up and down enrichment of each HNSCC and GBM TCGA sample. A final NK_20 enrichment score was calculated by subtracting the NK_20_down sgGSEA score from the NK_20_up sgGSEA for each HNSCC and GBM TCGA sample. This NK_20 score was then correlated with the profile of CHMP2A mRNA expression in the HNSCC and GBM TCGA samples (Supplemental Fig. 7) using the mutual information between those two profiles (Information Coefficient A^2). Survival analysis was also performed on the HNSCC and GBM TCGA datasets by dividing patients into a CHMP2A “high” and “low” expression median expression of CHMP2A (Supplemental Fig. 3). Correlation of CHMP2A with overall survival in HNSCC and GBM TCGA samples was analyzed using the Kaplan-Meier method and statistical significance was assessed using the standard Mantel–Cox log-rank test.
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**Acknowledgements**

We thank Dr. J. Silvio Gutkind (Moores Cancer Center, University of California San Diego, La Jolla, CA) for providing the HNSCC cell lines. This project was supported by NIH grants: U24CA220341 (PT), U01CA217885 (DSK and PT), U24CA194107 (PT), P30CA023100 (DSK and PT), R01DE026870 (PT), U01DE028227 (PT), CA217066 (BCP), R35CA197718 (JNR), and an award from the State of California P30CA023100 (DSK and PT), R01DE026870 (PT), U01DE028227 (PT), CA217066 (PT).

**Author contributions**

Q.X. and D.B. conceived and designed the study. D.B., Q.X., B.C.P., L.C., X.L., and J.Y. performed the experiments. D.B., Q.X., and B.C.P. developed the methodology. D.S.K., J.N.R., and P.T. provided resources. B.C.P. performed the screening analysis. T.V.P. performed the TCGA data set analysis. D.B.and C.Z. performed EVs preparation and analysis. P.A. performed electron microscopy. D.S.K. and J.N.R. supervised the work. D.B. and D.S.K. wrote the original draft of the manuscript. D.S.K., J.N.R., D.B., M.C., L.C., C.Z., B.C.P., Q.X., P.T., W.K., and H.Z. reviewed and edited the manuscript.

**Competing interests**

D.S.K. is a co-founder and advisor to Shoreline Biosciences and has an equity interest in the company. D.S.K. also consults for Qihan Biotech and VisiCELL Medical for which he receives income and/or equity. Studies in this work are not related to the work of those companies. The terms of these arrangements have been reviewed and approved by the University of California, San Diego in accordance with its conflict-of-interest policies. The remaining authors declare no competing interests.

**Additional information**

**Supplementary information**

The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-29469-0.

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**Peer review information**

Nature Communications thanks Marco Herold and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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