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Albumin-conjugated drug is irresistible by single gene mutation of endocytic system: verification by genome-wide CRISPR-Cas9 loss-of-function screens

Fang Yuan[1], Mengnan Sun[1], Huiqin Liu[1], Feng Qian*[1]

[1] School of Pharmaceutical Sciences, Beijing Advanced Innovation Center for Structural Biology, and Key Laboratory of Bioorganic Phosphorus Chemistry & Chemical Biology (Ministry of Education), Tsinghua University, Beijing 100084, China.

* Correspondence to:

Feng Qian (Email: qianfeng@tsinghua.edu.cn)

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Abstract

Albumin-conjugated drugs attain KRAS mutant cancer targeting through KRAS-enhanced macropinocytosis and intensified lysosomal degradation due to reduced neonatal Fc receptor (FcRn) expression. The cytosolic delivery of active payloads relies on endocytosis and subsequent intracellular processing of albumin delivery vehicles, wherein complex regulatory mechanisms and molecular machineries are closely involved. Despite the obvious merit of KRAS targeting, could such an endocytic process involving extra molecular regulators also bring about extra vulnerabilities to albumin-conjugated drugs, particularly, unexpected drug resistance? To assess such risks, here we performed an unbiased drug resistance mechanism comparison in pancreatic cancer, between free triptolide (TP, a potent cytotoxin) and albumin-conjugated TP, using genome-wide CRISPR-Cas9 loss-of-function screens. GTF2H5, a subunit of GTF2H transcription factor complex, was the only hit identified regardless of forms of TP treatment. With drug efficacy tests on GTF2H5 knockout clones, we further concluded that GTF2H5 deficiency conferred drug resistance primarily due to the pharmacological mechanism of action (MoA) of TP. In addition, molecules previously considered to be able to affect endocytosis and intracellular processing were not enriched during the screening with albumin-conjugated TP. With the aid of genome-wide CRISPR-Cas9 loss-of-function screens, we conclude that the pharmacological resistance of the active payload, rather than any potential loss-of-function mutations in endocytic molecular machineries, is the solely crucial drug resistance mechanism of albumin-conjugated drugs.

Keywords

Drug resistance, albumin-conjugated drug, CRISPR-Cas9 screen, triptolide, pancreatic cancer

Abbreviations

clustered regularly interspaced short palindromic repeats, CRISPR; CRISPR-associated protein 9, Cas9; knockout, KO; wild type, WT; kirsten rat sarcoma, KRAS; pancreatic ductal adenocarcinoma, PDAC; non-small-cell lung cancer, NSCLC; neonatal Fc receptor, FcRn; triptolide, TP; mechanism of action, MoA; RNA interference, RNAi; genome-wide CRISPR-Cas9 knockout, GeCKO; single guide RNA, sgRNA; model-based analysis of genome-wide CRISPR-Cas9 knockout, MAGeCK; 4-dimethylaminopyridine, DMAP; 1-hydroxybenzotriazole,

HOBT; 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide, EDC; 5-(N-ethyl-N-isopropyl)-amiloride, EIPA; false discovery rate, FDR; fold change, FC; half maximal inhibitory concentration, IC_{50}; fluorescence-activated cell sorting, FACS; matrix-assisted laser desorption-ionization time of flight mass spectrometry, MALDI-TOF MS; general transcription factor 2H, GTF2H; general transcription factor 2H subunit 5, GTF2H5

1. Introduction

Oncogenic KRAS is one of the most frequent mutations in human cancer. With a mutation incidence as high as 90-95%, KARS activating mutations in pancreatic ductal adenocarcinoma (PDAC) are considered to transform cells with strong oncogene addictions at the beginning of disease progression, and efficacious inhibitions of such driver molecule may be of great value in clinical treatment[1]. Pharmacological inhibitors for KRAS oncoprotein are generally challenging, mostly due to its shallow binding pocket and high functional affinity which is historically considered to be undruggable[2]. One recent breakthrough is direct targeting of $KRAS^{G12C}$, a mutant allele of oncogenic KRAS, which has a cysteine residue and thus provides inhibitor covalently binding opportunity. $KRAS^{G12C}$ inhibitors have been demonstrated with expected efficacy in several pre-clinical models of NSCLC and colon cancer[3]. However, for PDAC, $KRAS^{G12C}$ only represents one of many mutation versions of oncogenic KRAS (mutation rate <2% in PDAC), and for the remaining alleles, especially $KRAS^{G12D}$, $KRAS^{G12V}$, and $KRAS^{G12R}$ with a larger patient population and worse prognosis, direct targeting remains challenging[4].

Previously, we demonstrated that albumin conjugation was a viable oncogenic KRAS targeted drug delivery strategy[5]. In non-transformed endothelial cells, the albumin-conjugated drugs undergo clathrin-mediated endocytosis, where FcRn-mediated recycling exocytosis minimizes the intracellular release of active payloads thus the cytotoxicity. In comparison, enhanced macropinocytosis and lysosomal degradation of albumin-conjugated drugs occur in KRAS mutant cancer cells, and produce significantly increased intracellular drug release. Benefiting from these differential intracellular processing mechanisms dictated by KRAS genotype, albumin-conjugated drugs attain targeted drug actions in KRAS mutant PDAC and substantially enlarged the therapeutic window of payloads by ~10 times.

Distinct from free drugs, the cytosolic delivery of albumin-conjugated drugs relies heavily on the endocytosis and intracellular trafficking within the targeted cells, wherein complex regulatory mechanisms and molecular machineries are involved, as suggested by studies such as genome-wide RNA interference (RNAi) screens[6]. It has been acknowledged that perturbations of endocytic regulators could divert the delivery route of a drug that is conjugated to or encapsulated in its delivery vehicle, yet it remains unknown that to what degree such perturbations could affect the overall pharmacological efficacy of such drug delivery systems. Could these engineered drug

delivery systems, such as albumin-conjugated drugs, encounter extra drug resistance vulnerabilities due to the aforementioned complexity? This requires a conclusive answer to avoid any future application risks.

TP is a diterpenoid triepoxide extracted from *Tripterygium wilfordii* Hook F., and is one of the most potent cytotoxins towards PDAC with much higher activity compared to common chemotherapeutic agents including gemcitabine, 5-fluorouracil, nab-paclitaxel, irinotecan and platinum[7,8]. TP attracted considerable interest as an anti-cancer drug candidate, while its translation may be hampered by severe toxicities on multiple organs[9]. We believe that TP could be able to offer unique clinical benefits for PDAC, if its narrow therapeutic window could be widened by drug delivery strategies, such as albumin conjugation. Here, we exploited the unbiased genome-wide CRISPR-Cas9 loss-of-function screening, a powerful and unprecedented genetic tool with prior-proved feasibility and robustness, to identify any possible genes that could critically affect the efficacy of free TP and albumin-conjugated TP[10]. The established genome-wide single guide RNA (sgRNA) library includes 123,411 sgRNAs targeting 19,050 genes, and 4-6 guides per target gene on average were designed to further minimize off-target potentials and gain high signal-to-noise interpretations[11].

2. Methods and materials

2.1. Materials

Reagents were purchased from commercial sources: Triptolide (Chengdu Purifa Technology Development Co., Ltd, China), bovine serum albumin (Amresco, USA), succinic anhydride (J&K Scientific, China), triethylamine (Macklin, China), 4-dimethylaminopyridine (Macklin, China), 1-hydroxybenzotriazole (Macklin, China), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (Macklin, China).

2.2. Cell line

Pancreatic cancer cell line MIA PaCa-2 was originally obtained from American Type Culture Collection and maintained in lab regularly. Cell culture of MIA PaCa-2 and its derived knockout clones was based on DMEM (Gibco) medium supplemented with 10% fetal bovine serum (FBS) (Gemini, # 900108) and 1% penicillin and streptomycin (Gibco).

2.3. Synthesis and characterization of albumin-conjugated TP

The synthesis of triptolide-succinate (TPS) and albumin-conjugated TP was based on a previously reported synthetic method[12]. Briefly, TP (200 mg, 0.54 mM) and succinic anhydride (300 mg, 3 mM) were dissolved into 5 mL chloroform, then 0.5 mL triethylamine (TEA) and 4-dimethylaminopyridine (DMAP, 122 mg, 1 mM) were added. The reaction solution was stirred at room temperature overnight under nitrogen protection. The progress was detected by thin layer chromatography with chloroform/methanol (20:1, v/v) to ensure esterification of all TP. After the reaction, chloroform was evaporated, and the remaining solids were washed with water and subsequently dried under vacuum. To purify TPS, a silica gel system eluting with chloroform/methanol (20:1, v/v) was used. 205.4 mg (80.0%) faint yellow product (TPS) was finally purified and the structure was characterized by 1H NMR (400 MHz) and MALDI-TOF MS. BSA (100 mg, 5mg/mL) was dissolved in 0.1 M borate buffer. TPS (0.2 mM) together with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC, 100 mg) and 1-hydroxybenzotriazole (HOBT, 50 mg) were additionally dissolved in 2 mL acetonitrile and added dropwise to the protein solution. Subsequently the solution was stirred for 24 h in refrigerator. Insoluble products, uncoupled TPS and others were removed by centrifuging and passing through size exclusion chromatography twice (GE Healthcare, Superdex 200 5/150 GL # 28906561). The protein eluent was collected in distilled water for molecular weight identification using a MALDI-TOF MS, and the TP:albumin molar ratio in conjugates was calculated based on the molecular weight difference between the conjugate and albumin.

For tetramethyl rhodamine (TMR) labeling, 2 mg TMR was dissolved into 100 μL DMSO and added dropwise into the conjugate solution (100mg conjugates in PBS, pH 7.4). The mixture was stirred at room temperature for 3-4 h. To remove uncoupled TMR, size exclusion chromatography (GE Healthcare, Superdex 200 5/150 GL # 28906561) was engaged for purification.

2.4. Drug release study of the albumin-conjugated TP

Release kinetics of TP from the albumin conjugates was determined by measuring the content of free TP in a drug release study, using DMEM as the release medium to simulate the extracellular environment. Briefly, 10 mg of albumin-conjugated TP was dissolved in 1 mL DMEM medium at 37 ºC, and 100 μL of sample was collected at different time points and added into 500 μL methanol/acetonitrile (3:1). To completely extract the released TP and precipitate

albumin, sample mixtures were vortexed and then centrifuged at 12,000 × g for 10 min at 4 ºC. The TP concentration of the supernatant was determined using a HPLC system (Prominence-i9 LC-2030, Shimadzu) with a C18 column and a mobile phase of water/acetonitrile (25:75) at flow rate of 1mL/min, and detection was performed at 218 nm using a PDA detector.

2.5. Endocytosis study of albumin-conjugated TP

Macropinocytosis inhibitor, 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), was used to confirm the cell entry mechanism of albumin-conjugated TP. Cells were pre-treated with 50 μM EIPA for 30 min and then 1 mg/mL TMR-labeled conjugates were added and incubated for 2-4 h. At the end of incubation time, cells were rinsed with PBS and collected for cytometer detection (PE channel, BD LSRFortessa, BD Bioscience) measuring TMR fluorescent intensity.

2.6. Plasmids and library screening

The principal of CRISPR-Cas9 mediated knockout is that endonuclease Cas9 can be guided to a specific genomic target by sgRNAs and generate double strand break and loss-of-function mutagenesis. In this library screening, Genome-wide CRISPR-Cas9 Knockout (GeCKO) sgRNA pooled library (Addgene # 1000000049), FUCas9-Cherry, were used to express sgRNAs and Cas9 protein as suggested[9]. Plasmids were firstly packaged into lentiviral particles with RSV, VSV and pMDL packaging plasmids using FuGENE6 transfection reagent and 293 T cell. For each screen, cherry fluorescence was confirmed before the infection to ensure expression of the Cas9 component. The sgRNA coverage was set ~100, indicating that more than 100 cells received any single sgRNA. Cells were infected with library lentivirus at a low multiplicity of infection (MOI, normally ~0.1-0.3 to ensure for each cell only one guide enters) with spinfection (2000 rpm, 1 h) and incubated overnight. The medium was aspirated 17-24 h post infection and replaced with puromycin containing medium selecting for sgRNA transfected cells (selection pressure: 0.75 μg/mL puromycin, 5 days). After selection, sgRNA transfected cells were collected and underwent drug selection at lethal doses for either albumin-conjugated TP (albumin-TP) or free TP. Under the lethal dose selection pressure, cells without resistant mutagenesis could not survive, and only cells whose loss-of-function mutations protected them from drug induced cell killing would be enriched during the screening. The final enriched drug resistant cells were further collected, and their genome DNAs were extracted and subjected to deep sequencing for sgRNAs inside. From the comparison of sgRNA distribution between the experimental and control groups, the enriched

sgRNAs were isolated and corresponding genes that conferred drug resistance phenotype were identified. The overall schematic illustration of the screening strategy is shown in Figure 1.

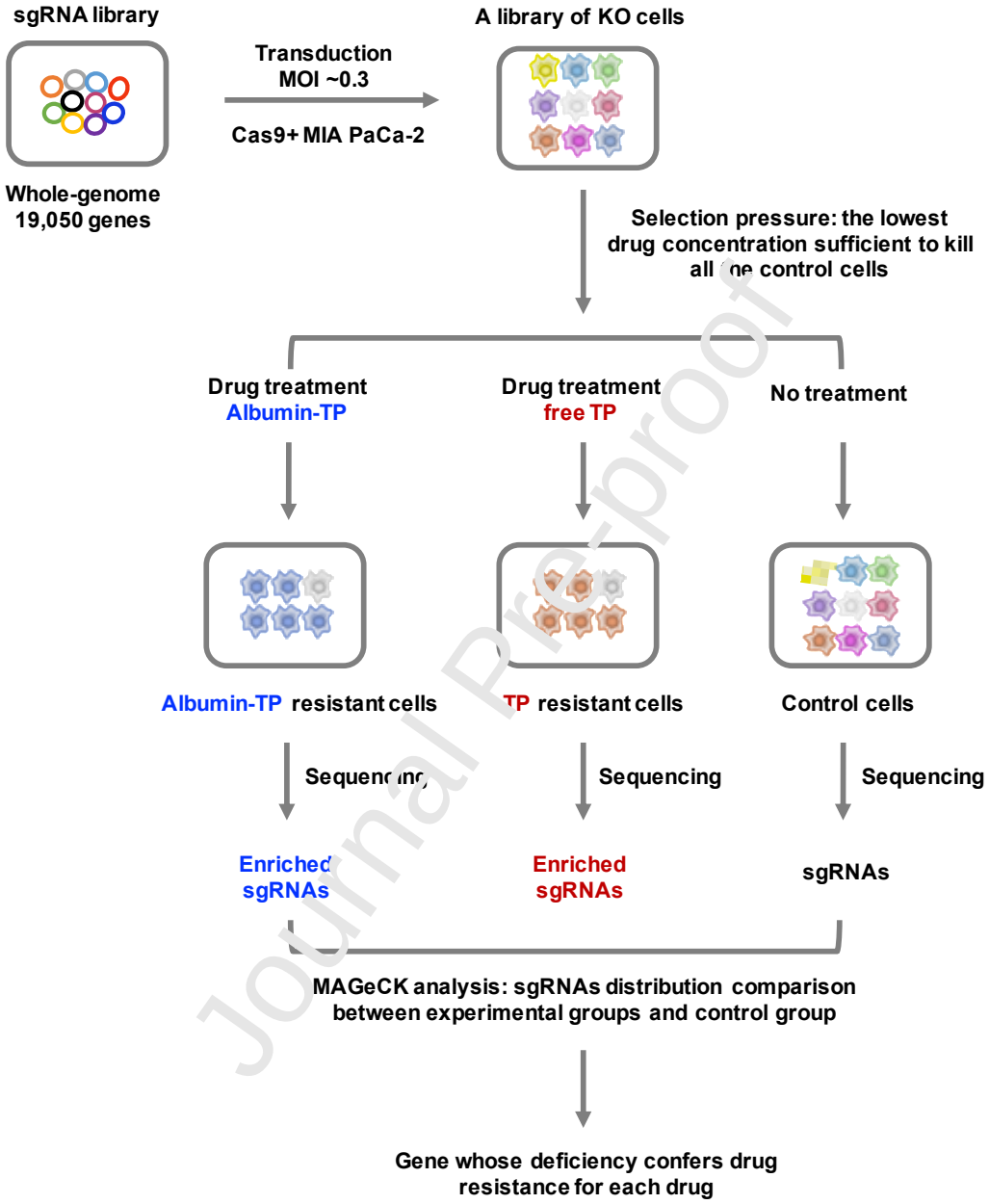

Figure 1, Screening design and workflow. Transducing Cas9 expressing MIA PaCa-2 cells with the unbiased genome-wide CRISPR-Cas9 sgRNA library, and a library of knockout (KO) cells was consequently generated. The KO cells were then divided into different groups, and one of them stayed as control group while the others underwent drug selection with either albumin-conjugated TP (albumin-TP) or free TP. After the drug selection, sgRNAs from drug resistant cells were amplified for each group and subjected to deep sequencing. Through MAGeCK analysis method, the enriched sgRNAs, sgRNAs distribution changes between experimental group and control group, and eventually, the gene whose deficiency confers drug resistance, would be identified.

2.7. Deep sequencing, MAGeCK analysis, and hit identification criteria

Genome DNA (gDNA) from enriched drug resistant cells was collected with GeneJET genomic DNA extraction kit (Thermo K0712) and corresponding amplicon library was generated with one-step PCR (35 cycles of reaction). Model-based Analysis of Genome-wide CRISPR-Cas9 knockout (MAGeCK) screening analysis method, a well-established algorithm, was adapted[13]. Amplicon samples were sent for deep sequencing (Hiseq-PE150 from Novogene Genetics, China). The obtained sequencing data were further analyzed with MAGeCK software (version 0.5.7) and visualized figures were generated directly. For hit identification criteria, the identified hit should demonstrate consistency among multiple sgRNAs designed for it, as well as a much lower p value and false discovery rate (FDR) value compared to the rest[14].

2.8. Generation of GTF2H5 knockout clones

Fgh1t-UTG vector encoding inducible sgRNA and aforementioned FUCas9-mcherry were applied to generate loss-of-function clones[15]. The guide sequence was selected from GeCKO library (GTF2H5|2|+(4 rv) UID: HGLib_21533 sequence: GTACAGCAGAAACTGCTTCA), and 1 μg/mL doxycycline was latter used to induce sgRNA expression. After the transduction, cells were selected by fluorescence-activated cell sorting (FACS, GFP positive, mCherry positive, BD Influx high-speed cell sorter, BD Bioscience) and sorted into single clones. Due to the lack of suitable antibodies, deep sequence was utilized for confirmation of induced mutations around target region. Primers used for PCR amplicon are: forward ACAAGCTGTCTTACAATCATGTG; reverse CAGGGCATTGGACTCATC.

2.9. Selection pressure, cell viability, and clonogenic assay

Before screening, dose-response curves of drugs were performed to determine an optimized selection pressure, i.e., the minimal dose that can kill all the control cells. For both free TP and albumin-conjugated TP, a titration of concerned drug was tested for drug effects on cell viability and clonogenic ability, from 1 nM to 100 μM for both drugs.

For cell viability assay, 1000-3000 cells were seeded into 96 well plates, and once attached, cells were then treated with respective drugs for 36-48 h. The culture medium was aspirated and replaced with fresh one without drug for further cell viability observation. CellTiter Glo (Promega) was used to assess cell viability according to the product protocol, and corresponding drug

response curves and IC_{50} values were calculated by Graph Pad Prism software (version 6.0). For clonogenic assay, 100 cells were originally seeded in 6-well plate, and then underwent drug treatment for 36-48 h. After the treatment, medium was aspirated and replaced with fresh one. Cells were then cultured for 7-14 days, and the colonies formed were stained with crystal violet and quantified by Image J automatic particle counting.

2.10. Statistical Analysis

The statistical significance of CRISPR screening candidate genes was calculated by MAGeCK algorithm (version 0.5.7)[13]. Log2 fold change > 1 and false discovery rate < 10% were set as thresholds for significantly enriched genes. In the line graphs, data are presented as mean and standard deviation (S.D.).

3. Results

3.1. Synthesis and characterization of albumin-TP conjugates

The synthesis schematic of albumin-conjugated TP (albumin-TP) is illustrated in Figure 2. TP was firstly coupled with succinic anhydride to generate triptolide succinate (TPS) intermediate (Figure 2A), and the structure of TPS was confirmed by both MALDI-TOF MS (Figure 2C) and 1H NMR (Supplementary Figure 1). TPS was then conjugated to BSA through the formation of amide bonds with amine groups of lysine residues of albumin (Figure 2B), and the average molecular weight of the purified conjugates demonstrated a clear shift from albumin at 66.35 KD to albumin-TP at 66.96 KD (Figure 2D), based on which the average molar ratio of TP:albumin in conjugates was determined to be ~1.4:1.

The albumin-TP conjugates displayed a hydrodynamic diameter below 10 nm (Figure 2E). We further examined the drug release profile of albumin-TP in DMEM cell culture media, and observed that less than 4% of TP was released while more than 96% albumin-TP remained conjugated up to 48 h, indicating negligible extracellular free drug during the time course of screening study, which is undesired and could complicate or negate the screening result of intact albumin-TP conjugates (Figure 2F).

As reported and previously confirmed[5,16], KRAS enhanced macropinocytosis is a major endocytosis mechanism for albumin, which could be validated by selective inhibition of macropinosome formation using EIPA. To confirm such mechanism remains for albumin-TP

conjugates, internalization of TMR-labeled albumin-TP conjugates by KRAS mutant cells was quantified after EIPA blockage and a ~50% attenuation was observed, confirming strong macropinocytosis dependence (Figure 2G). Once internalized through macropinocytosis, albumin-drug conjugates undergo lysosomal degradation and the therapeutic payloads are subsequently released to exert their pharmacological effects[5]. As expected, both albumin-TP and free TP are highly cytotoxic towards MIA PaCa-2 cells (Figure 2H), while the relatively reduced potency of albumin-TP (IC_{50} ~100 nM) is likely caused by the kinetics of intracellular TP release throughout the dynamic endocytic transport process of albumin-TP.

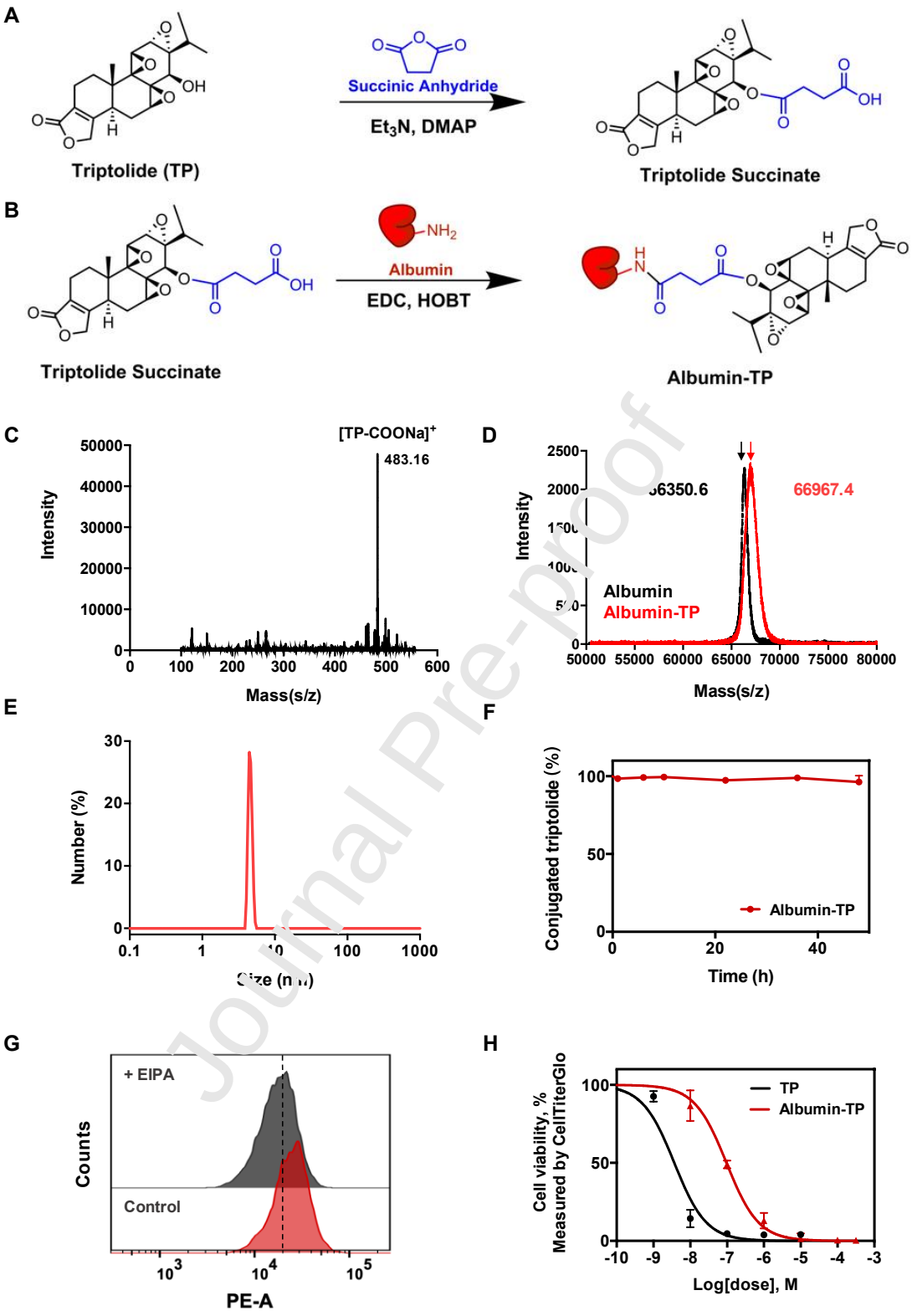

Figure 2, Preparation and characterization of albumin-TP conjugates. (A-B) Synthesis of triptolide succinate and further conjugation with albumin through the formation of amide bonds. (C-D) MS spectra of TPS (C), albumin and albumin-TP (D). (E-F) Size distribution (E) and drug release kinetics (F) of albumin-TP in DMEM cell culture medium without FBS addition, pH 7.4. (G) Decreased internalization of albumin-TP into MIA PaCa-2 cells by EIPA blockage, upper panel: intracellular fluorescent intensity of TMR-labeled conjugates in the presence of EIPA,

lower panel: intracellular fluorescent intensity of TMR-labeled conjugates without EIPA. (H) Toxicity of free TP and albumin-TP towards MIA PaCa-2 cell viability, measured by CellTiter Glo. Error bars represent S.D.

3.2. Enrichments of GTF2H5 loss-of-function cells in both albumin-TP and free TP screen panels

The loss-of-function screens were performed using IC_{95} concentration of albumin-TP and free TP. Based on the MAGeCK analysis of sgRNA distribution, we observed that GTF2H5 loss-of-function cells were robustly enriched in albumin-TP screen panel with a p value of 2.63E-07, a false discovery rate of 0.00495 and a calculated enrichment fold change value of 326 (Figure 3A and 3B). GTF2H5 ranked 1st by MAGeCK robust ranking aggregation (RRA) algorithm, an algorithm that can prioritize genes enriched in the drug resistant clones compared to control clones (Figure 3C).

Genes ranking top 10 by the algorithm were analyzed. GTF2H4 and GTF2H2C, ranking 3rd and 4th, are molecules also from GTF2H family, working within the same complex with GTF2H5 and may therefore be true hits, though obviously not as strong as GTF2H5. ARMC5, ranking 2nd, was also enriched in one replicate of the free TP screening, but not exclusively in albumin-TP screening, thus was not considered to be associated with the albumin conjugation delivery system. For other top 10 gene candidates (Figure 3D), enrichment was obtained when merely 1 out of 6 total guides was applied (Figure 3E-3M). Along with skeptical and far-outnumbered FDR values (Table 1), an accidental outcome instead of a true hit appears to be highly likely.

GTF2H5 loss-of-function mutation was enriched not only in albumin-TP screen panel but also in free TP screen panel. Under free TP selection, GTF2H5, stood out robustly with a p value of 3.62E-07 (Figure 4A), a false discovery rate of 4.95E-03, a calculated enrichment fold change value of 124 (Figure 4B). All its 6 independent guides were enriched after TP screening, and GTF2H5 ranked 1st by the positive selection RRA score, justifying it as a true hit (Figure 4C).

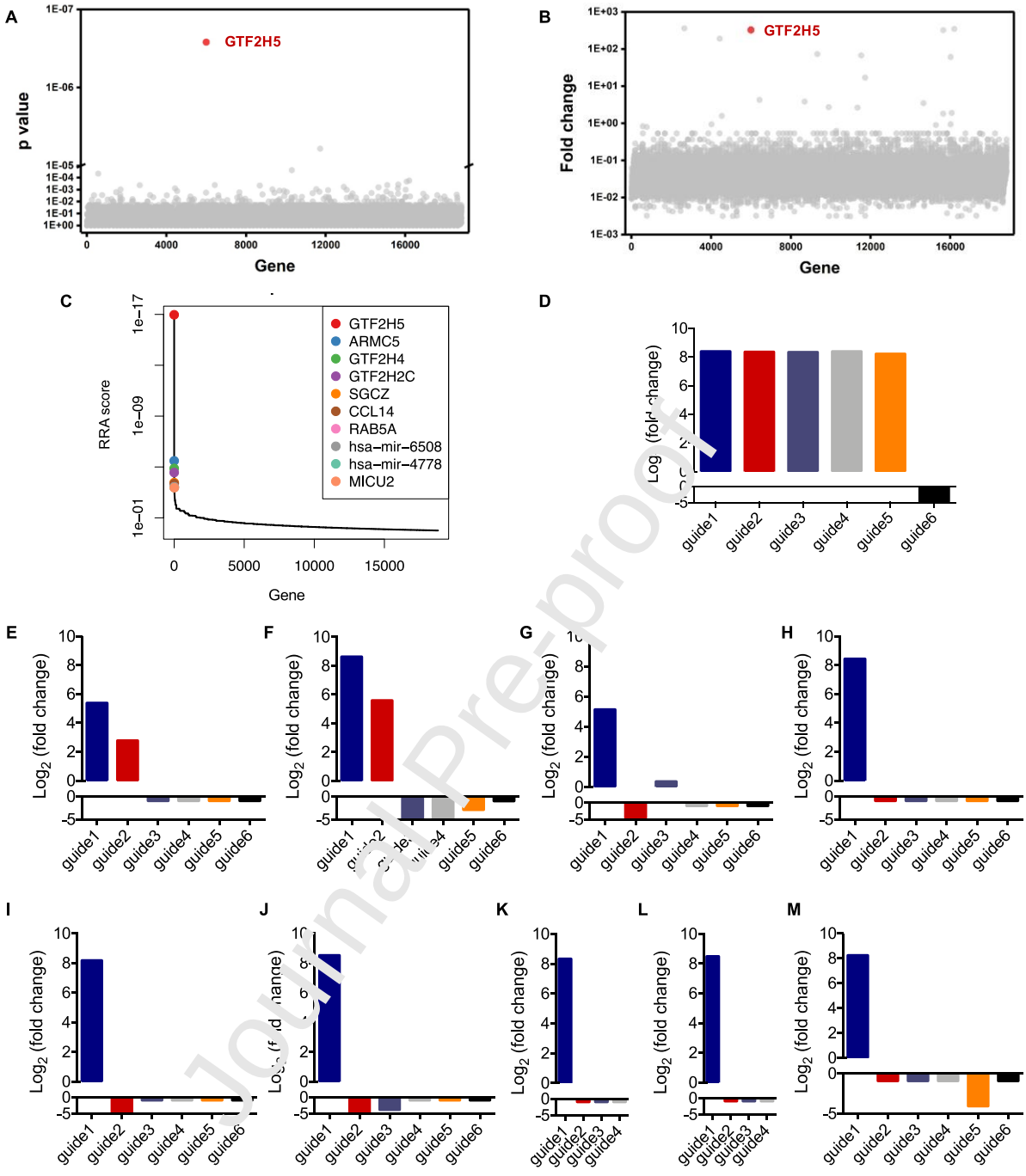

Figure 3, Identification of GTF2H5 deficiency conferring albumin-TP resistance. (A) Identification of top candidate gene GTF2H5 by MAGeCK p value analysis after albumin-TP selection. GTF2H5 is labeled as a large red dot. (B) Scatterplot showing enrichment of GTF2H5 loss-of-function mutation after albumin-TP selection. Fold change for each gene represents its frequency from albumin-TP resistant cells versus its frequency from control cells. GTF2H5 is labeled as a large red dot. (C) Gene ranking by robust ranking aggregation (RRA) score from MAGeCK analysis under albumin-TP treatment showing GTF2H5 outperformed others. Ranking top 10 genes are shown in the plot. (D) Enrichment consistency among 6 guides designed for GTF2H5 after albumin-TP selection. 5 out of 6 guides showed enrichment under the selection. (E-M), Inconsistent guides distribution for other RRA score top 10 genes: (E) ARMC5, (F)

GTF2H4, (G) GTF2H2C, (H) SGCZ, (I) CCL14, (J) RAB5A, (K) has-mir-6508, (L) has-mir-4778, (M) MICU2.

Rank	Gene	Number of enriched sgRNAs	FDR
1	GTF2H5	5	4.95E-03
2	ARMC5	2	0.056931
3	GTF2H4	2	0.146865
4	GTF2H2C	2	0.216584
5	SGCZ	1	0.654455
6	CCL14	1	0.659241
7	RAB5A	1	0.751397
8	hsa-mir-6508	1	0.751397
9	hsa-mir-4778	1	0.751397
10	MICU2	1	0.751397

Table 1, Top 10 genes ranked by RRA score in albumin-TP screen panel. Number of enriched guides and false discovery rate (FDR) value of respective genes are denoted.

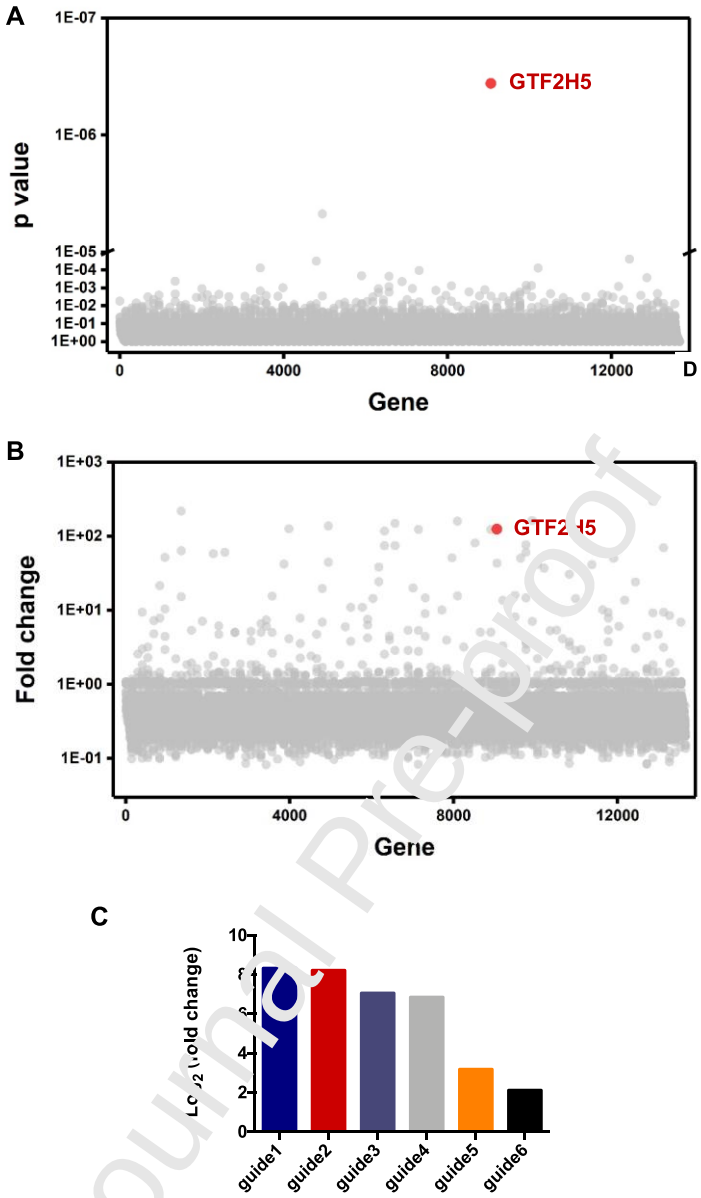

Figure 4, Identification of GTF2H5 deficiency conferring TP resistance. (A) Identification of top candidate gene GTF2H5 by MAGeCK p value analysis after free TP selection. GTF2H5 is labeled as a large red dot. (B) Scatterplot showing enrichment of GTF2H5 loss-of-function mutation after free TP selection. Fold change for each gene represents its frequency from free TP resistant cells versus its frequency from control cells. GTF2H5 is labeled as a large red dot. (C) Consistent enrichment among multiple guides designed for GTF2H5 after free TP selection. all 6 guides showed enrichment.

3.3. Genes governing endocytosis or intracellular trafficking were not enriched in the albumin-TP screen panel

A total of 2 genes emerged in the albumin-TP screen panel, with the threshold of FDR < 10%. However, neither of them was related with endocytosis or intracellular vesicle processing.

Previously known macropinocytosis regulators such as CDC42, RAC1, PI3K and PAK1[17], and molecules involved in lysosome functions including TFE3, IPO8, MITF, and LAMP2[18], were not enriched after albumin-TP selection, implying loss-of-function of any of them could not rescue cells from albumin-TP mediated cell killing (Table 2).

Gene	Number of enriched sgRNAs	Log_2 FC	FDR	p value
CDC42	0	-4.93	0.995217	0.84418
RAC1	0	-4.59	0.991163	0.56318
PI3K	0	-4.5645	0.9942	0.75334
PAK1	0	-4.33	0.962701	0.39302
TFE3	0	-5.23	0.936214	0.17464
IPO8	0	-4.2042	0.962701	0.39302
MITF	0	-5.33	0.9942	0.79629
LAMP2	0	-5.45	0.987627	0.61232

Table 2, Status of genes relevant to macropinocytosis and lysosome function in albumin-TP screen panel. Number of enriched guides, log_2 fold change (log_2 FC), false discovery rate (FDR), and p value of respective genes are denoted.

3.4. Validation of GTF2H5 deficiency conferring TP resistance

TP has been reported to be a covalent inhibitor of XPB, a subunit of GTF2H transcription factor complex, and is able to inhibit its RNA polymerase II-mediated transcription and therefore exert anti-proliferation effect[19]. Given GTF2H5 as a subunit of GTF2H, the molecular target of TP, we hypothesized that GTF2H5 deficiency resulted in the pharmacological resistance of TP itself but not resistance of albumin delivery strategy, and led to the drug resistance to albumin-conjugated TP. To validate the assumption, we re-generated GTF2H5 knockout MIA PaCa-2 clones using the CRISPR-Cas9 system. Exploited sgRNA sequence was adapted from GeCKO library, with a puromycin selection marker. We generated and sorted the edited cells. Clone 4 and clone 8, 2 derived clones with validated indel mutations around the CRISPR-Cas9 editing site, were used for further drug efficacy tests (Supplementary Figure 2). We found that the

derived GTF2H5 deficient clones showed strong resistance to TP cytotoxicity, under both clonogenic assay (Figure 5A, Supplementary Table 1) and cell viability assay (Figure 5B). Similarly, the albumin-conjugated TP was unable to bypass GTF2H5 deficiency, and displayed further efficacy reduction due to their reliance on TP drug release to achieve therapeutic effect (Figure 5C).

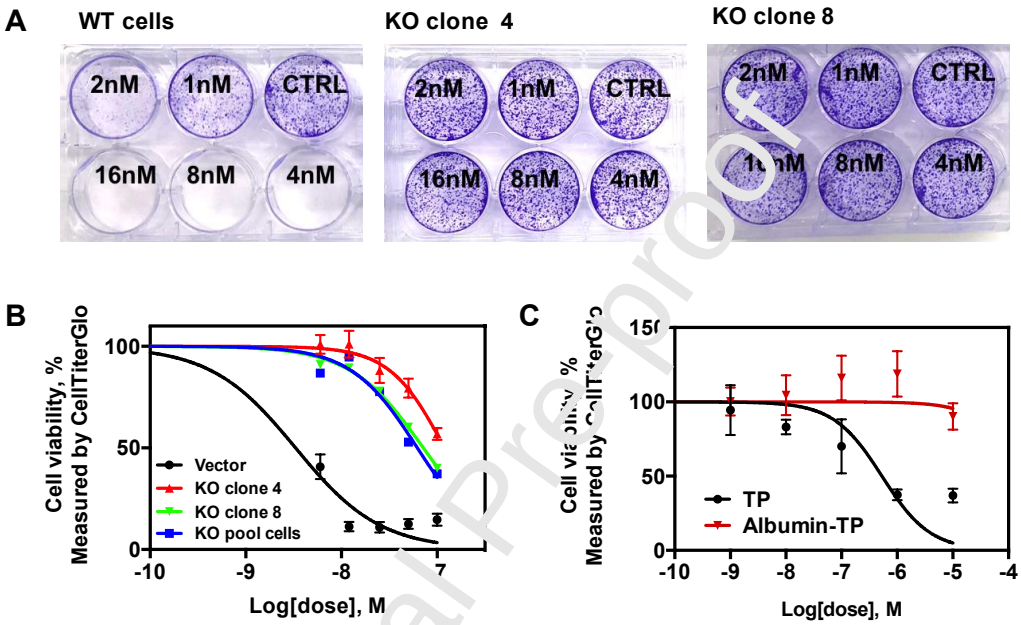

Figure 5, Validation of GTF2H5, whose deficiency impairs drug effect of free TP and therefore albumin-TP. (A-B) Deficiency of GTF2H5 resulted in reduced TP cytotoxicity with lower ability to inhibit cell clonogenesis (A) and cell viability (B). (C) Simultaneously insensitization of cells towards albumin-TP under the GTF2H5 deficiency. Wild type (WT). Knock out (KO). Error bars represent S.D.

4. Discussion

Unlike free drugs, drugs delivered by advanced drug delivery systems, such as protein conjugations, nanoparticle encapsulations, etc., rely heavily on the endocytic and intracellular processing within the targeted cells, and activate payloads become available to their sites of action only upon the completion of such steps and released from their carriers[20]. A recent study showed that endocytosis engaging active transport mechanisms might also be responsible for intra-tumor transport of nanoparticles, challenging the traditional passive transport theory[21]. An efficient endocytic transport attains desired pharmacological effects; however, a complex process controlled by various molecular machineries could sometimes be disrupted by genetic

perturbations. Panarella et al. reported an RNAi-based high-throughput screen with microscopy strategy on fluorescence labeled polystyrene nanoparticles in HeLa cells, and found that out of a library of 408 genes, myosin and a few Rab proteins dominated endocytosis and intracellular traffic of their polystyrene nanoparticles[22]. Also, Sahay et al conducted a small molecule-based library screen combining automated confocal microscopy, and identified pathways related to NPC1, V-ATPase, mTOR, cathepsins, and ionic balance, affected the cellular entry and trafficking of their siRNA loading lipid nanoparticles[23].

Here we aimed to investigate whether any single gene deficiency could affect macropinocytosis, intracellular trafficking or lysosomal degradation to the extent of jeopardizing the efficacy of albumin-conjugated drugs. Direct microscopic observation of fluorescence labeled particles remains as the major state-of-the-art characterization strategy to understand interactions between drug carriers and cells. Despite its undisputable contributions to this field, this method does not provide a systemic and mechanistic evaluation at the molecular level. In the current study, we presented a genome-wide CRIPSR-Cas9 loss-of-function screening method to reveal the direct and unambiguous connections between gene and the pharmacological efficacy of drug delivery systems, and demonstrated that it could serve as a complementary research tool to provide otherwise unreachable molecular insights. Obviously, this methodology could be similarly applied to understand the biological interactions between other drug delivery systems with different size or chemistry, and any other types of cancerous or non-cancerous cells. Similar strategies used in this research to optimize the screening conditions, i.e., the determination of drug sensitive cells, the selection of screening pressure to provide a clear-cut phenotype signal, etc., could also be applied in future other studies.

With a low infection MOI, most cells receive only one sgRNA, and mutagenesis happens around one gene locus per cell. A library of knockout cells wherein each cell harbored one single gene mutation was therefore generated. Considering the selection pressure was set as the drug concentration sufficient to kill the majority of control cells, only genes of crucial impact rather than partial or even marginal influence would be identified. During the screening with albumin-TP, two replicates were conducted but only TP related resistance mechanism was identified, while none of the endocytosis or intracellular trafficking related genes was enriched. However, we do believe that there are genes whose knockout confer partial endocytic resistance, but not to the

critical point to create sufficient drug resistance and rescue the cells from IC_{95} selection pressure. Therefore, the albumin-conjugated drug is irresistible by any single gene mutation of the endocytic system, and such interpretation is consistent with previous knowledge that a regulatory network rather than one single gene underpinning cellular and subcellular processes like macropinocytosis and lysosomal activities[6,24,25]. Owing to the regulatory network, compensation mechanisms, using alternative endocytosis routes or alternative macropinocytosis mediators, could still support albumin cellular and subcellular transport and metabolism under tough conditions. It would certainly be interesting to carry out future studies on tumor cells suffering genetic chaos with the existence of multiple gene knockouts, to reveal whether and to what degree a combination of different gene perturbations could insensitize cells towards albumin-conjugated drugs or other drug delivery systems.

It must be noted that, there could be other causes to our screen finding. One could be, there do exist genes whose knockout could substantially negate the endocytosis or intracellular metabolism capabilities of albumin-conjugated drugs, thus confer substantial drug resistance; however, knockout of any of these genes causes reduction of cell viability or clonal expansion ability and subsequently the screen selection progress would eliminate cells like this and apparently no enrichment would be observed. For example, KRAS, an activator for macropinocytosis and also an essential gene, did appear to following this pattern and did not display enrichment after the screens. Several other genes related with macropinocytosis may not be enriched after the screens either: Yao et al reported that knockdown of SDC1, which encode the macropinocytosis mediator protein syndecan 1, would lead to decreased PDAC genesis and maintenance both *in vitro* and *in vivo*[26]; Jayashankar et al observed that a mutant variant of actin capping protein regulator CARMIL1, which aided a specific impairment of macropinocytosis in breast cancer cells, could also inhibit retard tumor growth[27]. As being demonstrated again recently, macropinocytosis inhibition could be indeed lethal for pancreatic cancer cells [28], therefore, any un-identified genetic targets essential for macropinocytosis could be missed by this screen method. The fact that none of the macropinocytosis related genes were enriched in this genome-wide CRIPSR-Cas9 loss-of-function screen might have further suggested the critical role of macropinocytosis during the proliferation of PDAC.

On the other hand, the revealed hit, GTF2H5, encodes a protein named p8 or TTDA. p8 itself

has been previously reported to have a critical role in GTF2H complex repair activity, where it interacts with XPB, the major molecular target of TP[19], and stimulates XPB ATPase activity[29]. The additional significance of p8 includes its role as a stabilizer for GTF2H complex so as to maintain its intracellular concentration[30]. The loss-of-function of p8 via some unknown mechanisms could have hindered the binding between TP and XPB, and induced resistance against TP. The exact molecular mechanism could become a tempting pharmacological research topic to be explored further.

5. Conclusion

Using a genome-wide CRISPR-Cas9 loss-of-function screening method, we hereby comprehensively assessed the risk of drug resistance against albumin-conjugated drugs under genetic perturbation of any single gene. Albumin-conjugated TP and the free TP, were screened for their resistance vulnerabilities. GTF2H5 deficiency, a mechanism that belongs to the resistance mechanism of TP itself, was mutually identified in both screen panels, while no endocytosis or intracellular processing related genes stood out in the panel of albumin-conjugated TP. Our findings indicate that, comparing to any single gene deficiency along the endocytosis and intracellular trafficking pathway, pharmacological resistance of the payload remains the solely crucial drug resistance vulnerability for albumin-conjugated drugs, and the whole-genome CRISPR-Cas9 loss-of-function screening offers an unique tool to pinpoint crucial genes governing the efficacy of drug delivery systems.

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Author contribution

F.Q. conceived the idea of exploiting CRISPR-Cas9 screening method to investigate the intracellular deposition of drug delivery systems. F.Q. and F.Y designed the research and F.Y. performed the experiments with library screening, MAGeCK analysis and preliminary validation.

M.S. prepared drug conjugates. F.Y. and F.Q. organized the manuscript, and H.L. assisted with the discussion.

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Supplementary data

Attached below.

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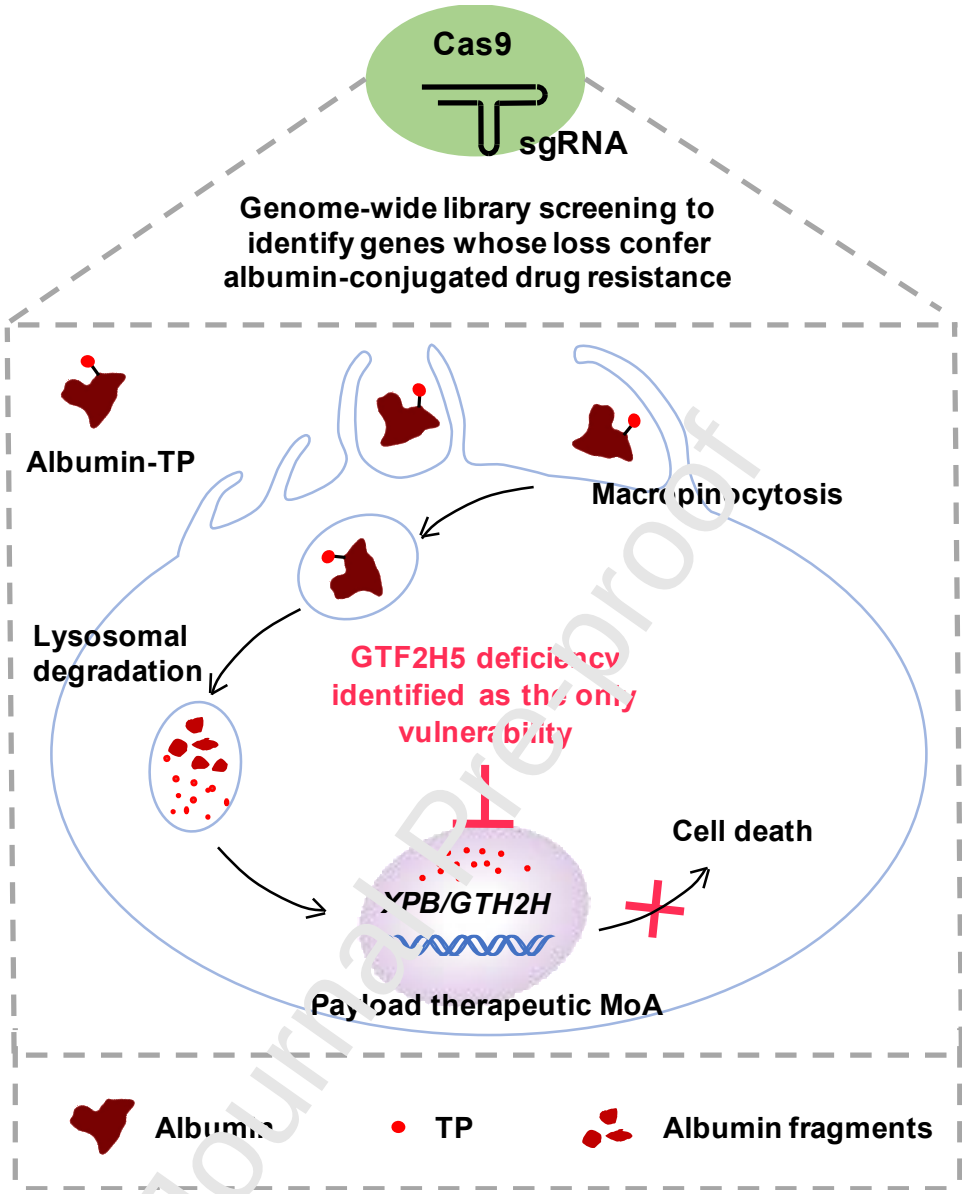
Graphic Abstract



Highlights

- In KRAS mutant pancreatic cancer cells, albumin-TP conjugates rely on macropinocytosis and lysosomal degradation to achieve cellular entry and intracellular release of TP, thus to produce pharmacological effects and cause cell death.
- With the pooled genome-wide CRISPR-Cas9 loss-of-function screens, GTF2H5 deficiency, a resistance mechanism of the payload cytotoxic agent TP, is identified as the only drug resistance vulnerability.
- Mutations of endocytic genes are not enriched in albumin-TP drug resistant cells.

Author contribution

F.Q. conceived the idea of exploiting CRISPR-Cas9 screening method to investigate the intracellular deposition of drug delivery systems. F.Q. and F.Y designed the research and F.Y. performed the experiments with library screening, MAGeCK analysis and preliminary validation. M.S. prepared drug conjugates. F.Y. and F.Q. organized the manuscript, and H.L. assisted with the discussion.