Microtubule inhibitors enhance DNA transfection efficiency through autophagy receptor p62/SQSTM1

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Short title: Tubulin inhibitors enhance transfection efficiency
Abstract

Ectopic gene expression is an indispensable tool in biology and medicine. However, it is often limited by the low efficiency of DNA transfection. It is known that depletion of p62/SQSTM1 enhances DNA transfection efficiency by preventing the degradation of transfected DNA. Therefore, p62 is a potential target of drugs to increase transfection efficiency. To identify drugs that enhance transfection efficiency, a non-biased high-throughput screening was applied to over 4,000 compounds from the Osaka University compound library, and their p62-dependency was evaluated. The top-scoring drugs were mostly microtubule inhibitors, such as colchicine and vinblastine, and all of them showed positive effects only in the presence of p62. To understand the mechanisms, the time of p62-dependent ubiquitination was examined using polystyrene beads that were introduced into cells as materials that mimicked transfected DNA. The microtubule inhibitors caused a delay in the ubiquitination. Furthermore, the level of phosphorylated p62 at S405, which is required for ubiquitination during autophagosome formation, markedly decreased in the drug-treated cells. These results suggest that microtubule inhibitors inhibit p62-dependent autophagosome formation. Our findings provide new insights into the mechanisms of DNA transfection and also provide a solution to increase DNA transfection efficiency.

Keywords: high-throughput screening; autophagy; LC3; p62; phosphorylation; ubiquitination; gene delivery

Abbreviations: ATG, autophagy-related gene; CK2, Casein kinase 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; HDAC6,
histone deacetylase 6; KO, knockout; LC3, microtubule-associated protein light chain 3; MEF, murine embryonic fibroblast; NF-κB, nuclear factor-kappa B; TBK1, TANK-binding kinase 1; ULK1, Unc-51 like autophagy activating kinase1; WIPI1, WD repeat domain phosphoinositide-interacting protein 1
**Introduction**

Gene delivery is one of the most important steps for gene therapy and genetic modification in basic science. Gene therapy has great potential in clinical medicine, and this concept has become well established in therapeutic approaches. In basic science, DNA transfection is a powerful tool that enables the study of gene functions and their products in cells.

In the gene delivery process, endocytosis is a crucial pathway for the regulation of cellular uptake of plasmid DNA\(^1\)\(^-\)\(^3\). Importantly, endocytosis can promote the induction of selective autophagy, also called xenophagy\(^4\). Generally, autophagy is a cytosolic bulk degradation pathway for recycling biomolecules through nonspecific degradation of proteins and organelles under nutrient starvation conditions. In contrast, selective autophagy plays an important defensive role against cellular infection by pathogens, as part of a starvation-independent autophagic defense system\(^5\)\(^-\)\(^7\). The conjugation of ubiquitin (Ub) to target pathogens is an initial and important process in selective autophagy. Ubiquitination assists in the recruitment of autophagy receptor proteins, including p62/sequestosome-1 (p62/SQSTM1), against pathogens or transfected DNA\(^4\)\(^,\)\(^8\)\(^-\)\(^11\). Therefore, suppression of the autophagy pathway can be a target for drugs to increase transfection efficiency.

We have reported that the depletion of p62/SQSTM1 protein (hereafter designated p62) greatly increases the efficiency of DNA transfection in cultured cells\(^11\). To monitor the behavior of the transfected DNA, we developed an experimental system using DNA-conjugating beads that mimic the transfected DNA. In this system, DNA-conjugated beads together with pHrodo, a fluorescent marker for endosome rupture, are incorporated into cells and their intracellular dynamics are analyzed in a live cell\(^11\).
Using this system, we demonstrated that the transfected DNA is incorporated into cells through endocytosis, released into the cytosol from the endosomes, and entrapped via autophagy in a p62-dependent manner. Furthermore, we demonstrated that the recruitment of Ub around the transfected material is significantly delayed in p62 gene-knockout murine embryonic fibroblast (p62KO-MEF) cells compared with that in normal MEF cells\textsuperscript{12}. Additionally, the phosphorylation of S405 (human S403) of p62 is a crucial step for the recruitment of Ub to the target site of transfected materials that mimic ectopic DNA\textsuperscript{11}. Hence, phosphorylation of S405 of p62 is an essential step for transfection-induced selective autophagy\textsuperscript{12}. Since p62 plays an important role in gene delivery through initial ubiquitination of the transfected DNA, inhibition of p62 may increase transfection efficiency. The aim of this study was to identify a small chemical compound that blocks the initial step of p62-dependent selective autophagy to enhance DNA transfection efficiency in mammalian cells.

\textbf{Results}

\textit{High-throughput screening for drugs enhancing transfection efficiency}

To identify potential compounds that can enhance transfection efficiency, high-throughput screening based on a luciferase assay was performed on MEF cells using an automated workstation. MEF cells were seeded in 384-well plates and incubated for 18 h with each compound from the Osaka University compound library (4,400 compounds)\textsuperscript{13}, at a final concentration of 10 \( \mu \)M. As a negative control, DMSO (at a final concentration of 1\%) was used instead of the compounds. The cells were transfected with the pCMV-Luc plasmid and incubated for 28 h; luciferase gene expression was driven by the cytomegalovirus immediate early (CMV-IE) promoter in
this plasmid. After cell viability assay, a luciferase reporter assay was carried out (Fig. 1A). Among the 4,400 compounds tested, 160 had severe effects on cell viability and were therefore removed from further analysis. For the remaining 4,240 compounds, cell viability in the presence of each compound was more than 96%. The transfection efficiency of the cells treated with each compound is plotted in Fig. 1B, the luciferase activity was normalized to cell viability. In this first screening, out of the 4,240 tested compounds, we identified 87 compounds that increased luciferase activity compared with the negative control (approximately 2.1% positive hit rate). The cutoff value used for selection was the mean value of the DMSO control + 4 × standard deviation (SD) (mean 0.347, SD = 0.242).

The activity of these 87 compounds was further analyzed via a second screening using MEF cells cultured in 96-well plates and incubated for 16 h with each of these compounds at a concentration of 1 μM. The luciferase reporter assay showed that the transfection efficiency increased in the presence of each of these compounds, with a range of approximately 2- to 260-fold that of the control, DMSO (Fig. 2A). This result indicates that all the 87 compounds exhibit a transfection-enhancing activity (Table S1). Among these, 14 were microtubule inhibitors. Notably, the top 10 compounds were all microtubule inhibitors, including colchicine and vinblastine (Fig. 2B).

Microtubule inhibitors enhance gene transfection efficiency

To further evaluate the effects of these compounds on DNA transfection efficiency, we selected two well-used microtubule inhibitors: colchicine and vinblastine. These were ranked second and third, respectively, in the 2nd screening (Fig. 2A). Firstly, we
examined the dose-dependency of these inhibitors in MEF cells (Fig. 2C). Cells were treated with colchicine or vinblastine at various concentrations, and transfected with the pCMV-Luc DNA plasmid. The luciferase activity was then measured. The transfection efficiency increased in a chemical dose-dependent manner in the MEF cells (Fig. 2C). Statistical analysis showed that the EC50 of colchicine and vinblastine was 239.1 and 26.29 nM, respectively, in the MEF cells (Fig. 2C). Furthermore, we evaluated the transfection efficiency by measuring the fluorescence level of the GFP expressed in the MEF cells. There were greater numbers of GFP-positive cells among the MEF cells treated with colchicine or vinblastine than among the DMSO-treated control cells (Fig. 2D). These results suggest that treatment with colchicine and vinblastine can enhance transfection efficiency.

It has been reported that depolymerization of microtubules activates the transcription factor, NF-κB, and induces NF-κB-dependent gene expression\(^ {14}\). Therefore, it is possible that treatment with microtubule inhibitors may induce the activation of gene expression through NF-κB activation. In fact, the CMV promoter, which is used in the pCMV-Luc plasmid, possesses an NF-κB binding site\(^ {14, 15}\); therefore, this can affect luciferase gene expression via promoter activation. To test this possibility, we established a cell line (MEF-LUC cells) with a CMV-driven luciferase plasmid integrated into the genome. These cells were treated with colchicine or vinblastine for 16 h, and the levels of luciferase activity were measured in the presence and absence of colchicine or vinblastine (Fig. S1). Treatment with colchicine showed only a slight increase in luciferase gene expression, and its fold-increase was much lower than that of DNA transfection (Fig. S1; compare with Fig. 2C). Treatment with vinblastine also showed a similar result, with almost no increase in luciferase
expression. These results suggest that colchicine and vinblastine affect transfection efficiency, but not by enhancing promoter activity.

**Transfection enhancement occurs in the presence of autophagy receptor p62**

Since p62 acts as an inhibitory factor for DNA transfection\(^1\), the transfection-enhancing activity of tested compounds may be deduced by the invalidation of the p62-dependent autophagic pathway. Based on this hypothesis, we examined the activity of the 87 compounds at a concentration of 1 μM in p62KO-MEF cells under the same condition as in Fig. 2A (Fig. 3A). All of them exhibited no or little transfection-enhancing activity in p62KO-MEF cells (Fig. 3A). As microtubule inhibitors (compounds 1-10 in Figs. 2A, 2B and 3A) seemed to show slight increase of transfection enhancing activity, we further tested colchicine and vinblastine for transfection-enhancing activity at various concentrations from 15.6 to 2000 nM (Figs. 3B and 3C), and found that these two microtubule inhibitors did not show transfection-enhancing activity at any of the concentrations tested in those cells (Fig. 3B and 3C).

This result suggests that microtubule inhibitors function in a p62-dependent autophagic pathway. This is consistent with the recruitment of microtubule-associated protein 1A/1B-light chain 3 (LC3; also called ATG8), a marker protein for autophagosome, to transfected DNA, which occurs in a p62-dependent manner\(^1\).

**p62-dependent ubiquitination is delayed by microtubule inhibitors**

To understand the molecular mechanisms of the p62-dependent enhancement of transfection efficiency by colchicine and vinblastine, we employed an experimental method using polystyrene beads that had been developed to monitor the behavior of the
transfected DNA\textsuperscript{16} (Fig. 4A). In this method, the beads are incorporated into cells with transfection reagents via endocytosis and enter the cytosol after rupture of the endosomal membrane. The beads that appeared in the cytosol were targeted for autophagy, similar to the transfected DNA\textsuperscript{16,17} (Fig. 4A). To monitor them in living cells, the beads were pre-conjugated with pHrodo dye, which emits fluorescence under acidic pH conditions, such as in the acidic endosome, but not in the cytosol. This dye, therefore, serves as a marker of endosome membrane rupture, as described previously\textsuperscript{16}. The pHrodo-conjugated beads were incorporated into MEF cells expressing a GFP-fused Ub protein (GFP-Ub MEF cells)\textsuperscript{12}, and the assembly of GFP-Ub around the beads was observed in a living cell using time-lapse fluorescence microscopy (Fig. 4B). In the control DMSO-treated cells, the time for Ub recruitment to the beads was approximately 3–4 min (Fig. 4B, middle panel) after pHrodo fluorescence disappeared (Fig. 4B, upper panel). In contrast, the time for GFP-signal accumulation was 9–10 min in 500 nM colchicine-treated MEF cells (Fig. 4C, middle panel), which was longer than that in the control cells (Fig. 4B). Statistical analysis was performed to determine the timing of GFP-signal accumulation around the beads after the loss of pHrodo signals in cells expressing GFP-Ub with or without the inhibitor (Fig. 4D). It showed that, in the DMSO-treated cells, the time for Ub recruitment to the beads was ~4 min (median) after pHrodo fluorescence disappeared (mean and SD, 4.167 ± 1.88 min, \( n = 24 \) beads; lane 1 in Fig. 4D). Moreover, the timing of GFP-signal accumulation was ~6 min (median) in 100 nM colchicine-treated MEF cells (mean and SD: 8.286 ± 8.36 min, \( n = 28 \) beads: lane 2 in Fig. 4D), ~11 min (median) in 500 nM colchicine-treated MEF cells (mean and SD: 11.32 ± 11.30 min, \( n = 22 \) beads: lane 3 in Fig. 4D), and ~6 min (median) in the vinblastine-treated MEF cells (mean and SD: 6.48 ± 3.81 min, \( n = 31 \) beads; lane 4...
Additional results show that the GFP-Ub signals did not accumulate within 60 min in colchicine- or vinblastine-treated GFP-Ub MEF cells (these beads were not counted and were not included in the n; upper column in Fig. 4D). These results show that GFP-Ub accumulation to the beads is significantly delayed in the colchicine- or vinblastine-treated MEF cells, and also suggest that intact microtubules are important for the recruitment of Ub to the target sites.

The active form of p62 is suppressed by microtubule inhibitors

It has been reported that the phosphorylated form of p62 at the amino acid residue S405 (p62 S405) is required for Ub recruitment in the process of selective autophagy. Therefore, Ub recruitment can be delayed by a decrease in the level of phosphorylated p62 at S405. To test this idea, we performed Western blot analysis to evaluate total p62 protein levels and p62 S405 phosphorylation levels (Fig. 5). Before DNA transfection (0 h), the p62 S405 phosphorylation levels were very low. However, after transfection (24 h), these levels greatly increased in DMSO-treated MEF cells, although the p62 levels remained unchanged. This suggests that DNA transfection induces an increase in p62 S405 phosphorylation. In the MEF cells treated with colchicine or vinblastine, however, the levels of p62 S405 phosphorylation decreased. This suggests that microtubule inhibitors inhibit Ub recruitment by decreasing the level of phosphorylated p62 S405, which is required for Ub recruitment in selective autophagy. This implies that microtubule inhibitors are responsible for the delay in Ub recruitment and therefore increase transfection efficiency in MEF cells.

Discussion
Microtubule structure/function and transfection efficiency

In this study, we used a non-biased, high-throughput screening approach to identify small chemical compounds that increase DNA transfection efficiency. The top 10 compounds were all microtubule inhibitors. Therefore, inhibition of the microtubule structure or function seems to trigger an increase in transfection efficiency. This finding is consistent with previous reports on cultured vascular smooth muscle cells and CV-1 cells, in which microtubule inhibitors, such as colchicine, vinblastine, vincristine, nocodazole, and podophyllotoxin, increased the transfection efficiency. Interestingly, some reports showed that microtubule-polymerizing agents, such as paclitaxel, also increased the transfection efficiency in COS-7 and A549 cells. The polymerizing agent, docetaxel, a chemical compound closely related to paclitaxel, was included in our screening results for the top 10 compounds (Fig. 2B, Rank 7). Moreover, tubulin deacetylation inhibitors, such as HDAC6 inhibitors, also increased the transfection efficiency in A549 cells, TC7 cells, and mesenchymal stem cells. This is likely because acetylated tubulin can stabilize microtubule structures, which in turn implies that microtubule structure stabilization can also increase gene transfection efficiency. Taken together, these findings strongly suggest that inhibition of the intact structure or dynamic nature of microtubules is important for increasing the efficiency of gene transfection.

Microtubule inhibitors affect selective autophagy pathways

Our results showed that treatment of cells with colchicine or vinblastine increased transfection efficiency in a p62-dependent manner. However, it is difficult to attribute these observations to one particular cause. Since microtubules are major contributors to
the trafficking of several components in the endosomal/lysosomal pathway, it is logical
to propose that these inhibitors may block the autophagy pathway. The role of
microtubules in autophagosome formation appears to be different based on the culture
medium conditions, e.g., vegetatively growing medium (basal) or starvation medium
(inducible) conditions. Several studies have used microtubule inhibitors under basal
conditions to show that microtubules do not participate in autophagosome formation. However, under inducible conditions, disassembling the microtubules with these
inhibitors prevented autophagosome formation, suggesting that the role of microtubules
is crucial in this step. Previous studies have shown that transfected DNA also
induces the selective autophagy pathway; hence, microtubule inhibitors may affect
transfection-induced autophagosome formation. The detailed mechanisms of the
inhibition of autophagosome formation following microtubule inhibitor treatment are
not clear; however, several autophagy factors are associated with microtubules,
including ATG8 (LC3), ATG1 (ULK1), ATG6 (Beclin1), ATG18 (WIPI1), and p62.
LC3 has long been thought to be involved in the regulation of the assembly and
disassembly of microtubules. Furthermore, ATG18-positive pre-autophagosomal
structures can move along microtubules, and this movement is highly sensitive to
microtubule inhibitor treatment. These data suggest that microtubules contribute to the
sequestration, recruitment, and movement of autophagy factors for the formation of the
inducible autophagosome. Therefore, microtubule inhibitors may block the inducible
autophagy pathway, resulting in a decrease in the likelihood of DNA degradation and an
increase in the transfection efficiency.

Microtubule inhibitors decrease the level of phosphorylated p62
The ubiquitination of endosome membrane proteins surrounding exogenous material, such as transfected DNA, is the initial step in inducible selective autophagy\textsuperscript{11,32,33}. In our study, treatment with colchicine or vinblastine delayed the recruitment of Ub proteins. This delay in recruitment is also caused by depletion of p62 or the mutation of the phosphorylation site at S405 of p62 (human S403)\textsuperscript{12}. Since p62 has been reported as one of the microtubule-associated factors\textsuperscript{27}, treatment with a microtubule inhibitor may affect p62-mediated Ub recruitment. Specifically, our results showed that p62 S405 phosphorylation was significantly impaired by treatment with colchicine or vinblastine after transfection. p62 S405 is phosphorylated by kinases such as CK2 and TBK1\textsuperscript{34,35}. These kinases are also associated with tubulin\textsuperscript{36-39}. After transfection, the recruitment of these kinases may be affected by treatment with microtubule inhibitors. Further studies are necessary to elucidate the detailed mechanisms by which these microtubule inhibitors can affect the phosphorylation levels of p62.

In this study, a non-biased high-throughput screening method demonstrated that microtubule inhibitors enhanced transfection efficiency in a p62-dependent manner. This indicates that p62 function is associated with microtubule structure, and this function is critical for the control of transfection efficiency.

**Materials and Methods**

**Plasmids**

The pGL4.50 [luc2/CMV/Hygro] vector (NCBI Accession: EU921840.1), which encodes the luciferase reporter gene luc2, was used as a luciferase expression vector and named pCMV-Luc (E1310; Promega, Madison, WI, USA). The GFP expression
plasmid pCMX-AFAP was prepared as previously described. pBABE-puro was purchased from Addgene (1764; Addgene, Cambridge, MA, USA).

To create the PB-CMV-LUC-Zeo vector, we first constructed the PB-EF1-MCS-IRES-Zeo vector. To insert the zeocin resistance gene DNA sequence, two DNA fragments (#1 and #2) were amplified as follows: the cloning site with Kozak sequence fragment #1 was amplified from the PB-EF1-MCS-IRES-Neo vector (PB533A-2; System Biosciences, Palo Alto, CA, USA) using PCR and the following primers: 5′-

CTGAAGGATGCCAGGATGTACCCCTATTGT-3′ and 5′-

TCCGGACGCCATGGTTGTTG-3′. The zeocin cord fragment #2 was amplified from the pcDNA3.1/Zeo(+) vector (V86020; Thermo Fisher Scientific, Yokohama, Japan) using PCR and the following primers: 5′-

ACAACCATGGCGTCGGGAAATGGCCAAGTTGACCAGTGCCGTTCC-3′ and 5′-

TCCAGAGGTTGATTGTCGACTCAGTCCTGCTCCTCGGCCACGAA-3′.

Following digestion with KpnI and SalI, fragments #1 and #2 were inserted into the PB-EF1-MCS-IRES-Neo vector using the In-Fusion HD Cloning Kit (639648; Takara Bio Inc. Kusatsu, Japan). This resulted in the PB-EF1-MCS-IRES-Zeo vector.

Next, the human cytomegalovirus immediate early enhancer and promoter (CMV-IE) DNA sequence (#3) was amplified from the pGL4.50 [luc2/CMV/Hygro] vector using PCR and the following primers: 5′-

GGGGATACGGGGAAAAGGCCTCGTTACATAACTTACGGTAAATG-3′ and 5′-

GAATTCGCTAGCTCTAGAAGCTCTGCTTATATAGACCTCCCACC-3′. The luciferase DNA sequence (#4) was amplified from the pGL4.50 [luc2/CMV/Hygro] vector using PCR and the following primers: 5′-

TCTAGAGCTAGCGAATTCAAGAGATGCCCAAAAACATTAAGAA-3′
Following digestion with EcoRI and StuI, fragments #3 and #4 were inserted into the PB-EF1-MCS-IRES-Zeo vector using the In-Fusion HD Cloning Kit. This resulted in the PB-CMV-LUC-Zeo vector.

**Cell Strains**

p62KO-MEF cells (p62⁻/⁻ cells) and their parental MEF cells were kindly provided by Dr. Tetsuro Ishii (University of Tsukuba)⁴¹. MEF cells stably expressing GFP-Ub were generated as previously described¹². Briefly, to obtain MEF cells or p62KO-MEF cells stably expressing luciferase, MEF cells or p62KO-MEF cells were transfected with the PB-CMV-LUC-Zeo plasmid and cultured in the presence of 100 μg/mL Zeocin (R25501, Thermo Fisher Scientific), and then single clones (MEF-LUC cells) were selected. Each stable clone was examined for luciferase protein expression using a luciferase reporter gene assay.

**Cell Culture**

All cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (D6429; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum in the presence of 5% CO₂ at 37°C.

**High-Throughput Screening for enhancer compounds**

MEF cells were seeded at a density of 0.8 × 10³ cells per well (384-well microplate, 781091; Greiner Bio-One, Tokyo, Japan) using a Multidrop COMBI (Thermo Fisher Scientific), and incubated in culture medium for 6 h. The cells were treated with 1%
DMSO (negative control) or the screening compounds (10 μM each) using Fluent780® Automation Workstation (Tecan Japan, Kawasaki, Japan) with a 96-channel head adapter and Tecan sterile tips (30048824; Tecan Japan). After 16 h, the cells were transfected with 25 ng of pCMV-Luc plasmid using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer’s protocol. The cells were then incubated for 28 h, followed by measurement of cell viability with the RealTime-Glo™ MT Cell Viability Assay (E9713; Promega) using the GloMax® Discover Microplate Reader (Promega). Luciferase activity was measured with the ONE-Glo™ Luciferase Assay System (E6120; Promega) using the GloMax® Discover Microplate Reader.

Luciferase Assays

Cells were seeded at a density of 0.45 × 10^4 cells per well (96-Well Assay Plate; 3603 Corning, Corning, NY, USA) and incubated in culture medium for 6 h. The cells were treated with 1% DMSO (negative control) or the screening compounds (0.1–10 μM each). After 16 h, the cells were transfected with 100 ng of pCMV-Luc plasmid using Lipofectamine 2000 according to the manufacturer’s protocol. The cells were then incubated for 28 h, followed by measurement of cell viability with the RealTime-Glo™ MT Cell Viability Assay to normalize cell number using the GloMax™ 96 Microplate Luminometer (Promega). Luciferase activity was measured with the ONE-Glo™ Luciferase Assay System using the GloMax™ 96 Microplate Luminometer. The mean EC50 values and standard deviations were determined from three independent experiments.

Preparation of pHrodo-conjugated Beads
pHrodo-conjugated beads were prepared as previously described. Briefly, Dynabeads M-270 Streptavidin (DB65306; Thermo Fisher Scientific) were washed three times with phosphate buffered saline (PBS) and resuspended in 100 mM sodium bicarbonate buffer (pH 8.5) to an appropriate concentration (typically a 1:10 or 1:20 dilution). pHrodo-succinimidyl ester (P36600; Thermo Fisher Scientific) was then added to the bead suspension and incubated in sodium bicarbonate buffer for 1 h at room temperature (about 26°C). After the conjugation reaction, the beads were washed with sodium bicarbonate buffer and suspended in PBS.

Incorporation of Beads into Living Cells

Beads were incorporated into cells as previously described. One day before incorporating the beads, GFP-Ub MEF cells were seeded onto 35-mm glass-bottom culture dishes (P35G-1.5-10-C; MatTek, Ashland, MA, USA) at a density of 1.5 × 10^5 cells/dish in culture medium. Transfection-reagent-coated beads were prepared by mixing pHrodo-conjugated beads with Effectene transfection reagent (301425; Qiagen, Tokyo, Japan) according to the manufacturer instructions, except that the bead suspension was used instead of DNA solution. The resulting bead mixture (~10 μL) was mixed with 90 μL of the culture medium and added to the cells by replacing the medium. After incubation for 1 h at 37°C in a CO2 incubator, the cells were washed twice with fresh growth medium to remove unattached beads and then further incubated for the time indicated in each experiment.

Time-Lapse Imaging
Cells were treated with 100 ng/mL Hoechst33342 (B2261; Sigma-Aldrich) for 15 min to stain chromosomes, as previously described\(^{42}\). After replacing the culture medium with fresh medium not containing phenol red, time-lapse observation was performed using an oil-immersion objective lens (UApo40/NA1.35; Olympus, Tokyo, Japan) on a DeltaVision microscope system (GE Healthcare Life Sciences Japan, Tokyo, Japan) placed in a temperature-controlled room (37°C), as previously described\(^{42}\). Images were obtained every minute for ~60 min.

**Western Blot Analysis**

Western blot analysis was performed as previously described\(^ {12}\). Briefly, cell lysates were prepared in a lysis buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 x Phosphatase Inhibitor Cocktail Solution II (160-24371; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and 1 x protease inhibitor cocktail (Nacalai tesque Inc., Kyoto, Japan)]. The lysates were subjected to electrophoresis on NuPAGE 4% to 12% Bis-Tris gels (NP0321; Thermo Fisher Scientific). Proteins were transferred to polyvinylidene fluoride membranes and probed using anti-p62(SQSTM1) (PM045; MBL, Nagoya, Japan), anti-Phospho-SQSTM1/p62(Ser403) (D8D6T; Cell Signaling Technology, Danvers, MA, USA), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (14C10; Cell Signaling Technology) antibodies, and secondary antibody conjugated to horseradish peroxidase (NA9340V; GE Healthcare Life Sciences). Protein bands were stained with ImmunoStar Zeta (295-72404; FUJIFILM Wako Pure Chemical Corporation) and detected by chemiluminescence using a ChemiDoc MP imaging system (Bio-Rad, Tokyo, Japan).
**Statistical Analysis**

The $p$-values were obtained by performing Kruskal-Wallis tests using GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA, USA).

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**Author contributions**

MT, HO, KW, TK, CM, KN, BL and AT performed the experiments. MT, HO, AT, YH, and TH designed the experiments. All authors analyzed and discussed the data, and MT, HO, YH, and TH wrote the manuscript.

**Conflict of Interest**
All authors declare that: (i) no support, financial or otherwise, has been received from any organization that may have an interest in the submitted work; and (ii) there are no other relationships or activities that could appear to have influenced the submitted work.
Reference


its target DNA. Biochemical and biophysical research communications 320: 218-225.


Figure Legends

Fig. 1. High-throughput screening for drugs enhancing transfection efficiency.

(A) Schematic diagram of high-throughput screening system using luciferase reporter gene assay. A cell viability assay was performed using the RealTime-Glo™ MT Cell Viability Assay system. (B) MEF cells were treated overnight with 10 μM of each compound from the Osaka University compound library. DMSO was used for negative controls. Luciferase activity and cell viability were measured, and the results are plotted. Red broken lines represent the mean + 4 × SD of all negative control assay points.

Fig. 2. Second screening of compounds selected in high-throughput screening.

(A) MEF cells were treated for 16 h with 1 μM each of the top 87 compounds identified in the primary screen. DMSO was used for negative controls. The relative fold change in the luciferase activity of each compound is plotted. The activity of DMSO-treated cells is set as 1. The second and third columns are colchicine and vinblastine as indicated. (B) List of top 10 potential compounds for enhancement of transfection efficiency. (C) MEF cells were treated with the indicated concentrations of colchicine (left graph) or vinblastine (right graph). The cells were transfected with the pCMV-Luc plasmid and incubated for 24 h, after which reporter gene assays were performed. Luciferase activity was normalized to cell viability. Black thick bars and thin lines indicate the mean and SD, respectively, of at least three independent experimental results. (D) MEF cells (a, d), 250 nM colchicine-treated MEF cells (b, e), and 50 nM...
vinblastine-treated MEF cells (c, f) were examined using fluorescence microscopy 24 h after transfection of a GFP-expressing plasmid (upper panels; GFP); the lower panels represent the corresponding bright-field (BF) images of the upper panels. Scale bar = 250 μm.

Fig. 3. Colchicine and vinblastine treated MEF cells do not enhance transfection efficiency in the absence of p62.

(A) p62KO-MEF cells were treated for 16 h with 1 μM each of the top 87 compounds identified in the primary screen. DMSO was used for negative controls. The relative fold change in the luciferase activity of each compound is plotted. The activity of DMSO-treated cells is set as 1. The right two columns are reproduced from Fig. 2A for comparison. (B, C) p62KO-MEF cells were treated with the indicated concentrations of (B) colchicine or (C) vinblastine. These stable cell lines were incubated for 43 h, after which reporter gene assays were performed. Luciferase activity was normalized to cell viability. Each value is indicated as the mean ± SD of at least three independent experimental results.

Fig 4. Colchicine and vinblastine treated MEF cells affect the timing of ubiquitination.

(A) Schematic diagram of experimental system using beads incorporated into cells to monitor behavior of transfected materials. (B, C) Time-lapse images of pHrodo and
GFP-Ub fluorescence around a single pHrodo bead in MEF cells. Images were obtained every minute for approximately 60 min. The panels show representative images of pHrodo and GFP-Ub fluorescence in (B) GFP-Ub MEF cells treated with DMSO as a control and (C) GFP-Ub MEF cells treated with 500 nM colchicine. Scale bar = 2 μm.

(D) Statistical analysis was performed for the timing of GFP-signal accumulation around the beads after the loss of pHrodo signals in the GFP-Ub MEF cells. The results are plotted as follows: mock control treated with DMSO (lane 1), 100 nM colchicine-treated MEF cells (lane 2), 500 nM colchicine-treated MEF cells (lane 3), and 100 nM vinblastine-treated MEF cells (lane 4). The median values were 4 min for GFP-Ub (n = 24 beads), 6 min for 100 nM colchicine (n = 28 beads), 6 min for 500 nM colchicine (n = 22 beads), and 6 min for 100 nM vinblastine (n = 31 beads). Three independent experiments were performed for each lane and the total bead number is indicated as n.

Statistical differences (p < 0.0001) were determined using the Kruskal–Wallis test. Error bars indicate 95% confidence intervals.

**Fig. 5. Colchicine and vinblastine treated MEF cells decrease the level of phosphorylated p62 at S405.**

Western blot analysis was performed for total p62 and S405-phosphorylated p62 in MEF cells under the indicated conditions. GAPDH was used as a loading control.
Fig. 1

A

cell compounds DNA transfecion cell viability assay Luciferase assay

4hr 18hr 28hr

B

![Graph showing luciferase/cell viability against compounds.]

Luciferase/cell viability [unit]

0 1000 2000 3000 4000

mean+4SD

compounds
Fig. 2

A

Fold luciferase activity (R.U.)

compounds

1. PODOFIOX: microtubule, antineoplastic, antimitotic agent
2. COLCHICINE: microtubule, antimitotic, antigout agent
3. VINBLASTINE SULFATE: microtubule, antineoplastic, spindle poison
4. DEOXYSSAPPANONE B 7,4'-DIMETHYL ETHER: microtubule
5. VINCRISTINE SULFATE: microtubule, antineoplastic
6. PICROPODOPHYLLIN ACETATE: microtubule, IGF-1R, antineoplastic
7. DOCETAXEL: microtubule, antineoplastic
8. FENBENDAZOLE: microtubule, anthelmintic, antinematodal drug
9. FLUBENDAZOLE: microtubule, anthelmintic, antinematodal drug
10. PICROPODOPHYLLIN: microtubule, IGF-1R, antineoplastic

B

No | chemical name | target | function | PubChem CID
---|----------------|--------|----------|-------------
1  | PODOFIOX      | microtubule | antineoplastic, antimitotic agent | 10607
2  | COLCHICINE    | microtubule | antimitotic, antigout agent | 6167
3  | VINBLASTINE SULFATE | microtubule | antineoplastic, spindle poison | 241902
4  | DEOXYSSAPPANONE B 7,4'-DIMETHYL ETHER | microtubule | | 4026888
5  | VINCRISTINE SULFATE | microtubule | antineoplastic | 429332
6  | PICROPODOPHYLLIN ACETATE | microtubule, IGF-1R | antineoplastic | 233299
7  | DOCETAXEL | microtubule | antineoplastic | 148124
8  | FENBENDAZOLE | microtubule | anthelmintic, antinematodal drug | 3334
9  | FLUBENDAZOLE | microtubule | anthelmintic, antinematodal drug | 35802
10 | PICROPODOPHYLLIN | microtubule, IGF-1R | antineoplastic | 72435

D

MEF control [DMSO] | MEF colchicine [250 nM] | MEF vinblastine [50 nM]

GFP

BF

Scale bars: 100 μm
Fig. 3

**A**

Fold Luciferase activity (R.L.U) vs. compounds for p62KO-MEF and MEF.

**B**

Fold Luciferase activity (R.L.U) vs. colchicine [nM] for p62KO-MEF.

**C**

Fold Luciferase activity (R.L.U) vs. vinblastine [nM] for p62KO-MEF.
Fig. 4

A

GFP-Ub wt MEF cell

GFP-Ub

colchicine or vinblastine

pHrodo-conjugated beads

acidic endosome

raptured acidic endosome

GFP-Ub

B

GFP-Ub MEF, DMSO

[min] -2 0 2 4 6 8 10 12 14 16

pHrodo

GFP-Ub

merge

C

GFP-Ub MEF, colchicine 500nM

[min] -2 0 2 4 6 8 10 12 14 16

pHrodo

GFP-Ub

merge

D

GFP-Ub accumulation time [min]

inhibitor treatment

mock 100nM colchicine 500nM colchicine 100nM vinblastine

over 60 min
Fig. 5

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<tr>
<td>Colchicine</td>
<td>500nM</td>
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PhosphoS405-p62  
p62               
GAPDH
| Rank | Structure | Name                      | Formula                          | Fold activity in MEF | Fold activity in p62KO-MEF |
|------|-----------|---------------------------|                                 |                     |                          |
| 1    | ![PODOFILOX](image) | PODOFILOX | C_{22}H_{22}O_{8} | 297.24              | 4.46                    |
| 2    | ![COLCHICINE](image) | COLCHICINE | C_{22}H_{25}N_{6}O_{6} | 294.20              | 4.94                    |
| 3    | ![VINBLASTINE SULFATE](image) | VINBLASTINE SULFATE | C_{46}H_{58}N_{4}O_{9}.H_{2}O_{4}S_{2} | 263.07              | 6.90                    |
| 4    | ![DEOXYSAPPANONE B 7,4'-DIMETHYL ETHER](image) | DEOXYSAPPANONE B 7,4'-DIMETHYL ETHER | C_{18}H_{18}O_{5} | 54.43                | 4.72                    |
| 5    | ![VINCRISTINE SULFATE](image) | VINCRISTINE SULFATE | C_{46}H_{56}N_{4}O_{10}.H_{2}O_{4}S_{2} | 46.77              | 0.66                    |
| 6    | ![PICROPODOPHYLIN ACETATE](image) | PICROPODOPHYLIN ACETATE | C_{24}H_{24}O_{9} | 35.46                | 2.19                    |
| 7    | ![DOCETAXEL](image) | DOCETAXEL | C_{43}H_{53}N_{14}.3H_{2}O | 33.78              | 9.43                    |
| 8    | ![FENBENDAZOLE](image) | FENBENDAZOLE | C_{15}H_{13}N_{3}O_{5} | 27.14              | 7.83                    |
| 9    | ![FLUBENDAZOLE](image) | FLUBENDAZOLE | C_{17}H_{13}F_{2}N_{2}O_{3} | 21.35              | 4.53                    |
| 10   | ![OXIBENDAZOLE](image) | OXIBENDAZOLE | C_{18}H_{21}Cl_{2}F_{2}N_{2}O_{2} | 8.77               | 1.09                    |
| 11   | ![C18 H17 N O2 S](image) | | | 6.80 | 1.80 | 
| 12   | ![C15 H26 N4 O5 S2](image) | | | 6.19 | 1.70 | 
| 13   | ![OXIBENDAZOLE](image) | | | 4.64 | 0.85 | 
| 14   | ![DEOXYSAPPANONE B 7,3'-DIMETHYL ETHER ACETATE](image) | | | 4.50 | 2.07 | 
| 15   | ![PHENETHYL CAFFEATE (CAPE)](image) | | | 4.44 | 1.63 | 

Table S1. Identification of 87 potential compounds for enhancement of transfection efficiency.
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<th></th>
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<th>Formula</th>
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Comparison of 87 compounds derived from the Osaka University compound library, showing their chemical structure, name, formula, and activity in 1 μM of a 96-well luciferase assay in MEF cells and p62KO-MEF cells.
Fig. S1. Colchicine and vinblastine treatments do not affect the promoter activity of the luciferase gene.

(A, B) MEF cells stably expressing luciferase (MEF-LUC) were treated with the indicated concentrations of (A) colchicine or (B) vinblastine. After incubation for 43 h, the luciferase activity of the cells was measured and normalized to cell viability. Each value is indicated as the mean ± SD of at least three independent experimental results.