# Tagged Small Molecule Library Approach for Facilitated Chemical Genetics

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**ABSTRACT**

Chemical genetics is a powerful method which utilizes small molecule regulators to reveal the molecular basis of diverse biological processes. However, the current chemical genetic approach sometimes meets a serious bottleneck during the process of target identification. One faces difficulty in conjugating the active compound to an affinity matrix without losing or reducing its activity that leads to laborious structure–activity relationship (SAR) studies. To facilitate this process, we have developed a tagged triazine library containing a built-in linker that provides a straightforward transition from phenotypic screening to target identification. A strategy for constructinig a tagged library and applications with a streamlined target identification and subsequent mechanistic study are discussed in this Account.

## Introduction

Since the sequencing of the human genome, the correlation of individual genes for their gene products or functions has become a major challenge. Classical genetics, often termed "forward genetics", is one of the approaches for solving this challenge. First, a random mutagenesis (either mutagen-inducing agents or genetic knockout) is performed on an organism to cause a variety of mutants. A particular mutant with an interesting physical characteristic (phenotype) is selected from the genetic pool. The related gene mutation (genotype) responsible for the observed phenotype is then identified to explain its function, a study from phenotype to genotype.[1–3] Later, reverse genetics emerged with the development of molecular biology techniques, which progress from genotype to phenotype.[4] A specific gene of interest that one would like to study is first selected. The direct manipulation (modification or deletion) of this gene brings about some changes in an organism via which the selected gene's function will be inferred.[3–5]

Chemical genetics is operating in the same context as genetics, but with the use of chemical tools (small molecules) that act as the effective biological regulators in place of genetic mutations.[4,6] In forward chemical genetics (Figure 1), a set of compounds are collected or synthesized, analogous to mutagens in forward genetics.[7] This set of compounds is then screened with an organism in identifying a specific chemical that causes a certain phenotypic change, mostly by the activation or inhibition of a protein binder. Such a target is identified and investigated further for the connection of the observed phenotype to the function of the target protein.[4] In reverse chemical genetics (Figure 1), a protein of interest is first selected, instead of a gene in reverse genetics. An effective chemical (an activator or an inhibitor) for this protein is identified from the screening with a large collection of compounds. This chemical is then introduced into the cell or the organism to study the resulting changes that implicate the protein function.[4,8]

Despite the similarity in strategies, chemical genetics show several advantages over classical genetics. A genetic mutation in classical genetics is permanent; thus, its function is completely altered.[9] This complete alteration reduces the chances of studying some of the genes essential to survival or development, since their permanent activation or inhibition would be lethal, causing the death of the organism.[4,10] In addition, it is technically difficult to control the genetic mutation of a complex organism such as a mammal, due to the large physical size, large diploid genome, and slow reproduction rate.[11,12] On the other hand, chemical genetics utilizes the small molecule regulator that can switch the biological effect on and off at will, thus inducing the conditional effect.[3] This allows for the use of less critical doses of the compound in overcoming the limitations in classical genetics. In addition, the small molecule regulator allows for relatively easy and flexible manipulations of the complex organism. Its effect is somewhat hastened by perturbing the existing protein binders, which allows for a real-time study.[8,11] However, classical genetics retains its unique advantages in that a genetic knockout is highly specific, which may be challenging for a small molecule regulator in chemical genetics.[4]

## Bottleneck in Chemical Genetics

Although chemical genetics is a powerful method of searching for small molecule regulators of diverse biological processes, it sometimes encounters a serious bottleneck, the identification of a target protein.[8,13] The most common method in target identification is the biochemical approach that uses the affinity matrix to pull down a hidden target. Once a hit compound is chosen, it is normally modified to add a linker in a proper position

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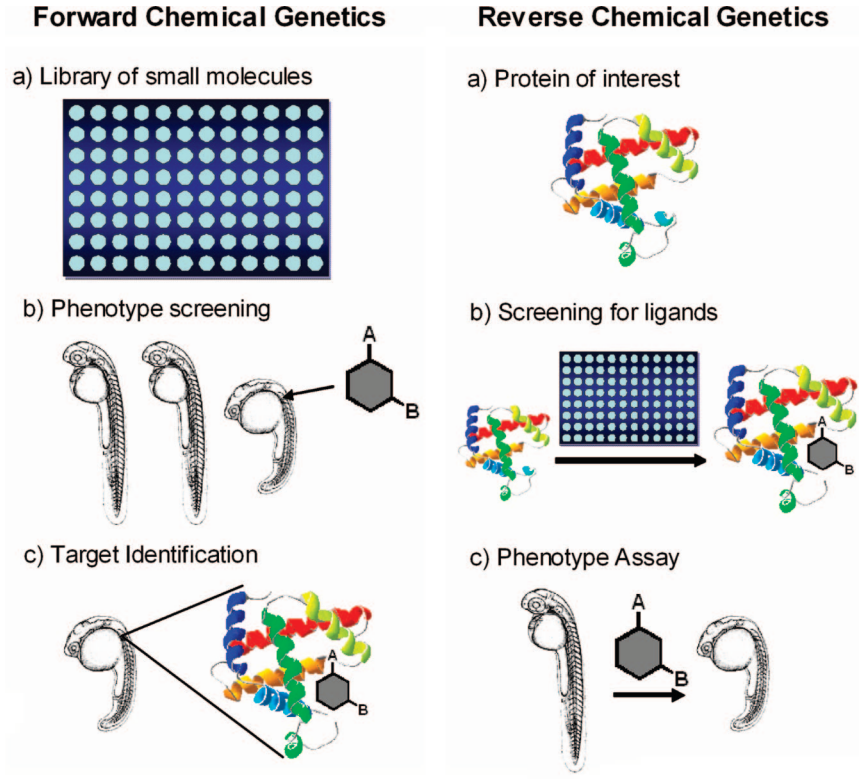



**FIGURE 1.** Flowchart of forward and reverse chemical genetics.

and conjugated to a solid-phase resin such as agarose gels. The solid resin is then exposed to cellular extracts, and unbound proteins are washed out. The subsequent SDS gel and mass analysis of a bound protein reveals the identity of the target protein. Despite its successful applications to several natural products, including trapoxin and its analogue K-trap,[6] this common method shows two underlying drawbacks in the procedure. First, the active compound should be conjugated to a solid resin to fish out a target protein, necessitating modification of the active compound. To incorporate a chemical linker into an active compound, a thorough structure–activity relationship (SAR) study is normally required to find the adequate position; this is time-consuming and laborious, and sometimes impossible to find without the loss of its activity. Second, since the binding event between a hit compound and a target is the major trigger to pull down the potential target, the successful fishing comes with a high affinity for a target.[13] However, most of the hits from chemical genetic screenings are moderately potent with a low micromolar affinity.[13] The modification of hits with the attachment of a linker may further reduce the binding affinity toward a target. Therefore, it is not surprising that this affinity matrix method leads to failures in some cases.

## Tagged Library Approach for Solution

Many methods for overcoming the problems related to target identification have been developed. Several modifications of biochemical approaches are developed which include chimeric ligands for improving the ligand binding affinity,[14,15] a modified assay procedure for reducing the level of nonspecific protein binding,[16] and a photoinducible linker for covalent linking to a target in vivo.[17] A yeast three-hybrid system is one of the alternative target identification methods with an affinity-based selection.[18,19] The genetic phenocopying,[20] the genetic–genomic analysis that screens for mutations that produce drug resistance or sensitivity,[21] and hypothesis-driven experimentation with knockout or mutant strains are effective genetic–genomic approaches.[22] Furthermore, the combined chemical and genetic analysis overcomes the limitation of the sole affinity-based biochemical method.[23] A gene expression profiling with cDNA microarrays[24] and the proteomic approach with protein microarrays[22,25] are additional effective approaches to target identification.

Alternatively, a tagged approach to facilitating the chemical genetics process has been devised.[26] A tag is literally any moiety that can be added to the compounds for additional functions. In the design of libraries, some functional tags will be integrated into a library scaffold for subsequent applications such as identification, purification, and visualization. For example, peptide nucleic acids (PNA) have been used as a tag to identify a hit compound from the split and pool synthetic library with the specific PNA sequence encoded during a library synthesis.[27] In a mRNA display, mRNA conceptually tags all the peptide library members so that the attached peptide can be identified from reverse transcriptase and PCR.[28] Furthermore, click chemistry-based tags have been used in studies of activity-based protein profiling (ABPP) for visualization of bound proteins.[29]

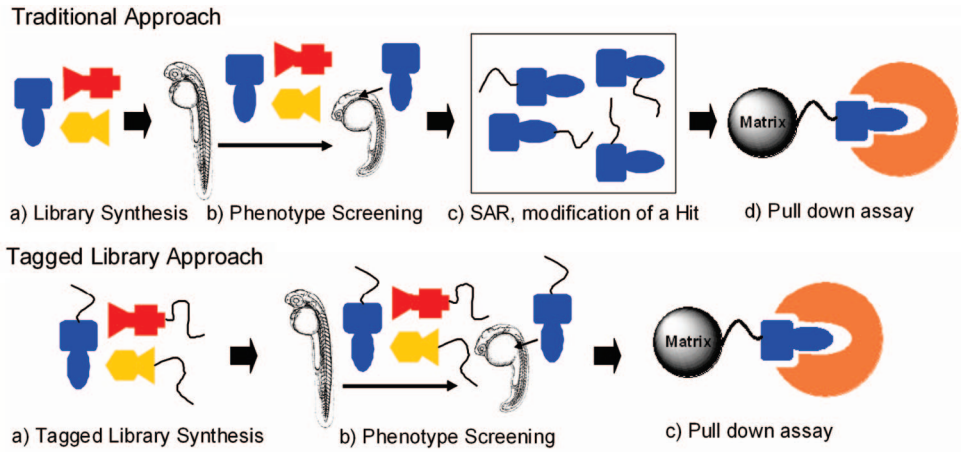



**FIGURE 2.** Outline of the traditional approach and the tagged library approach in chemical genetics.

In a similar context, we have used a tagged library approach for the purpose of target protein identification. All small molecules in the library were tagged with a length of linker during synthetic steps. A selected hit compound from the phenotypic screening contains the intrinsic linker that is necessary for the following pulldown experiment (Figure 2). The incorporation of a linker in the beginning stage removes or reduces the need for subsequent modifications of the hit compound and thus expedites the process of target identification. In our library, this tag was a triethylene glycol (TG)-based linker with a terminal amine functionality that can be utilized for immobilization on solid resin. This pre-attached linker may interfere with the biological activity or contribute to the binding interaction toward a target. However, this long and flexible alkyl ether functionality in the linker is less likely to be critical for the binding event, and the major binding interaction will come from the compound scaffold. Furthermore, a linker effect can be analyzed by simply removing it after the selection of hit compound, since the removal of the existing linker is much easier than the addition of a new linker. We have applied this tagged library approach to various model systems. This strategy demonstrated two significant advantages: (i) the expedited transition from a phenotypic screening to target identification without extensive modification of hits and (ii) the successful pulldown of targets with multiple compounds that are moderately (micromolar) potent. In this Account, a strategy for constructing a tagged library and applications with a streamlined target identification and subsequent mechanistic study are discussed.

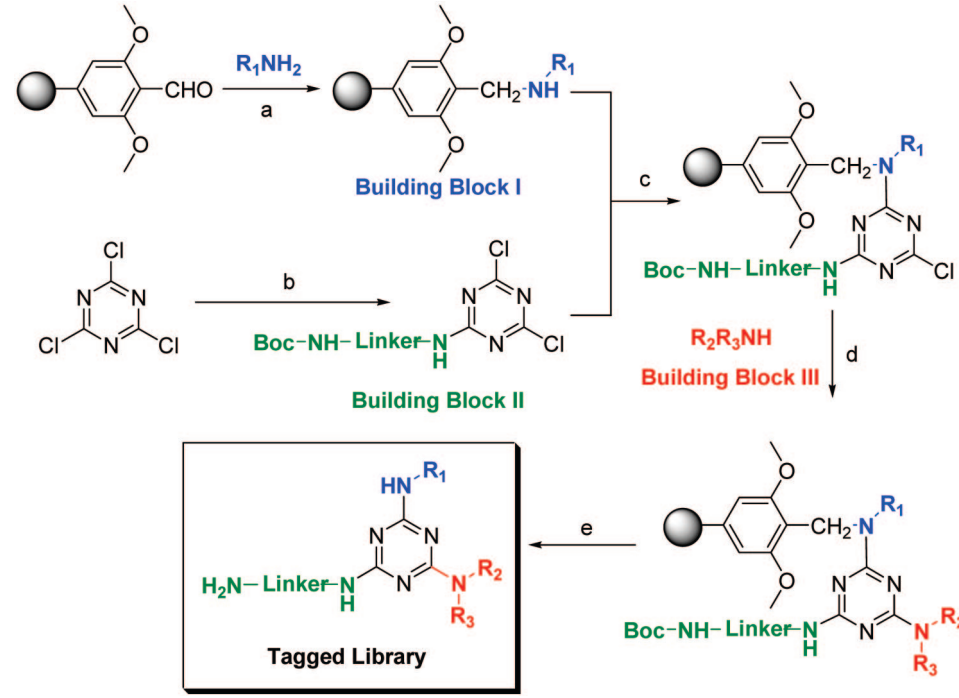
## Construction of a Tagged Library

Our initial efforts were aimed at constructing the compound library. The selection of the scaffold for the library design is the first, crucial step for further application.[30] Two things need to be considered in selecting the scaffold. First, chemical structures that are effective and sufficient for perturbing the biological system are preferred. It is hard to predict the optimal structure since no such general rules exist so far.[3,11] However, it is commonly expected that a chemical scaffold with a high degree of similarity to existing biologically active ligands would yield high chances of binding capability.[30] Second, from a chemistry point of view, it is a high priority to select a chemical scaffold that allows easy manipulations for the library generation.[31] Our choice of library was based on the triazine scaffold. It shares a high degree of structural similarity with purine and pyrimidine, which are prevalent for various protein ligands in the cellular context and developed for many enzyme substrates or cofactors.[31] Furthermore, the triazine scaffold has three symmetrical modification sites that allow for highly diversified members with each modification. Thus, we have explored the general synthetic procedure of a trisubstituted triazine for library generation. Although several triazine libraries were reported initially,[32,33] we sought to expand triazine structures in a divergent manner on the solid resin and reduce the remaining impurity so that the final products can be readily utilized for biological screening without further purification.

Initially, an orthogonal solid-phase method for synthesizing a triazine-based combinatorial library from which anti-tubulin triazine derivatives, tubulyzine, were identified by our group was developed.[34] With the same strategy, a tagged library was constructed with the expansion of the library size on the triazine scaffold. Three building blocks were separately prepared and assembled through three orthogonal stepwise reactions (Scheme 1). For the first building blocks, various amines were directly attached on PAL aldehyde resin by the reductive amination reaction. A series of TG-containing linkers were coupled with triazine trichloride in solution separately for the second building blocks and loaded on the resin. The third building blocks were introduced on the last chlorine position of triazine with a large diversity of commercially available amines as building blocks. The subsequent acidic cleavage afforded the highly pure 1536 triazine derivatives ready for use in biological screenings.

During the phenotypic screenings of a tagged triazine library with several model organisms, we came to suspect that the exposure of N-terminal free amine on a tag may induce the general toxicity of the compounds, and the resulting positive charge would be less favorable for cell

**Scheme 1. Synthetic Scheme of the Tagged Triazine Library with Three Stepwise Orthogonal Reactions[a]**




[a] (a) $R_1NH_2$, AcOH, THF, 1 h, then $NaB(OAc)_3H$, 12 h; (b) a series of Boc-NH-linker-$NH_2$, THF, 0 °C, 1 h; (c) building blocks I and II, DIEA, THF, 60 °C, 3 h; (d) $R_2R_3NH$, DIEA, 1:1 NMP/*n*-BuOH mixture, 120 °C, 3 h; (e) 10% TFA, DCM, 30 min.

penetration. Thus, a slight modification was made for construction of the library. A Boc group in the second building block was replaced with a benzoyl functionality that remained even after the final acidic cleavage step, to improve the general bioavailability of the compounds with higher hydrophobicities and to reduce general toxicity.[35]

## Small Molecule Suppressor on Eye and Brain Development of Zebrafish

Many model organisms have been developed over decades in the field of genetics and drug discovery, including bacteria, yeast, *Drosophila melanogaster* (fruit fly), *Caenorhabditis elegans*, *Dania rerio* (zebrafish), *Xenopus* oocytes and/or extracts, and other animal models.[36] Chemical genetics takes advantage of these model systems with the same principle. The zebrafish is one of the promising whole organism models and confers several advantages. As a vertebrate, the zebrafish develops highly evolved structures with discrete organs such as brain, sensory organs, heart, and muscles. Development of these structural organs is clearly visible through their completely transparent skin that allows for observations of dynamic developmental processes.[37] In addition, they are small and reproduce very easily from an egg to an embryo stage in fewer than 3 days, which is favorable for a large library screening. Excellent reviews of the chemical genetics study of zebrafish are available.[38,39]

The synthesized highly pure tagged triazine compounds were screened for morphological changes in a zebrafish (*D. rerio*) embryo in 96 wells using a high-magnification microscope.[40] This phenotypic screening found one compound (active at 50 $\mu$M) that significantly suppressed the development of eyes and brain (Figure 3c). To probe the importance of a TG tag with respect to activity, the active compound was modified by removing a TG tag (encephalazine, Figure 3b). This showed that a tag-free compound (active at 2.5 $\mu$M) is even more potent than the original structure, suggesting that this TG tag is not important for the activity. Along with a structurally similar but inactive compound as a negative control, the hit compound was readily immobilized on agarose beads using the built-in linker (Figure 3a). The subsequent pulldown experiment identified two strong bands on the SDS gel with the hit compound-immobilized bead, not with a negative control. The specificity of protein bindings was confirmed by a competition assay with a free hit compound (encephalazine) against an affinity matrix. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis suggested four ribosomal subunit proteins (S5, S13, S18, and L28) as target proteins, which were previously reported to be involved in brain and eye development by a genetic mutation.[40] Therefore, this example demonstrated the power of a tagged library through a facilitated transition of the hit compound discovery to the target identification without the need for a cumbersome SAR study.

## Small Molecule Regulators of Pigmentation of Melanocytes

A cell-based phenotypic screening is one of the alternatives for chemical genetics studies. This cell-based screening has been extensively applied for the study of neurobiology,[41] platelets,[42] cytoskeleton,[43] stem cells,[44] and orphan diseases.[45] In particular, we have used melano-

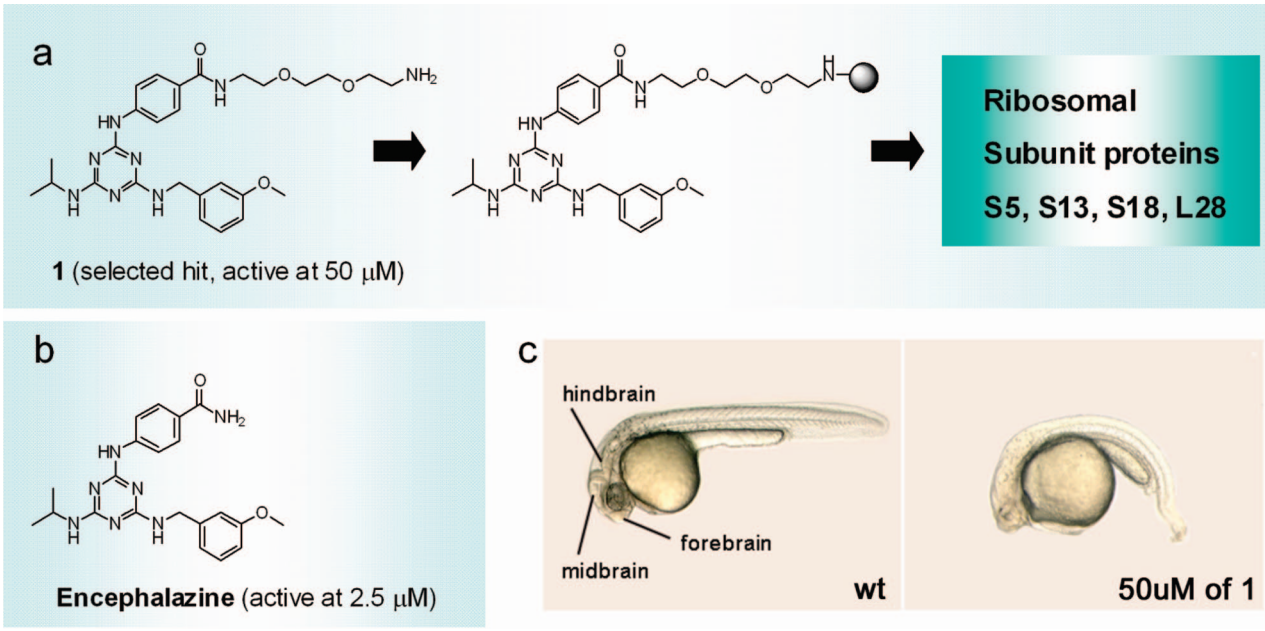



**FIGURE 3.** Small molecule suppressor (**1**) of the brain and eye development of a zebrafish embryo found with its target. (a) The active hit was readily loaded on an affinity matrix using a built-in TG linker and identified targets. (b) The removal of the linker exhibited a more potent effect. (c) A phenotypic change in the embryo (left, wild type; right, 50 $\mu$M **1**).

cytes to identify small molecule regulators involved in pigmentation.[46–48] These cells produce melanin in the skin, eyes, and hair and control the level of melanogenesis. Small molecules that promote pigmentation have the potential to reduce the incidence of UV-induced skin damage and carcinogenesis.[47] On the other hand, depigmenting agents are important for the prevention of skin diseases such as melasma and post-inflammatory melanoderma that results from the increased level of melanin.[48] In addition, albinism is a form of hypopigmentary disorder and is characterized by a serious lack of pigment in the eyes, skin, and hair in various species. A mutation (albino type 1) or mistrafficking (albino type 2) of tyrosinase, the rate-limiting enzyme in melanogenesis, has been reported to cause this symptom.[49]

Initially, we screened a tagged triazine library with albino type 2 murine melanocyte to identify a potential small molecule that rescues albinism.[46] Six compounds (including **MPD11**, 3–4-fold increase in the level of pigmentation at 3 $\mu$M, in Figure 4a) were found to induce enhanced pigmentation with an increase in melanin level when detected by the absorbance-based melanin assay. With affinity matrices of six hit compounds along with three inactive compounds as negative controls, the pulldown experiment suggested a mitochondrial F1F0-ATP synthase as a target protein. The competition assay of hit compounds against affinity matrices confirmed the specific binding to a mitochondrial F1F0-ATP synthase. Furthermore, the cellular distribution of tyrosinase was monitored with antibody immunostaining which demonstrated that active triazine compounds induced the pigmentation by correcting a tyrosinase trafficking into cytoplasmic distribution.

With the similar melanin detection assay, a normal melanocyte was screened with a tagged triazine library to identify the pigmentation inducer.[47] One potent pigmentation inducer, melanogenin (423% increase in the level of pigmentation above untreated one at 5 $\mu$M) (Figure 4b), was identified and found to increase the level of pigmentation through upregulation of the level and activity of tyrosinase. The following pulldown experiment identified a 32 kDa protein "prohibitin" as a target. Incubation of cell lysates with free melanogenin and melanogenin-immobilzed beads confirmed the specific binding of melanogenin to prohibitin. However, the implication of prohibitin in the pigmentation pathway was not known previously. Therefore, the siRNA technology was utilized to further determine the functional role of prohibitin. When the prohibitin gene was silenced by siRNA, the pigmentation enhancement by melanogenin was significantly attenuated, confirming that prohibitin is the functional target of melanogenin.

Along with pigmentation inducers, depigmenting agents were sought by screening a tagged triazine library in a normal melanocyte.[48] Four compounds (including **TGD10**, decreased to <40% of an untreated one at 10 $\mu$M, in Figure 4c) were identified as significantly inhibiting the melanin synthesis in melanocytes. A tyrosinase enzymatic assay of these triazine compounds showed that they reduced the level of pigmentation by acting as competitive inhibitors at the L-DOPA binding site. The built-in linker on each hit compound allowed the pulldown experiment to confirm a target protein from the cellular context and showed that these triazine compounds are specifically binding to tyrosinase.

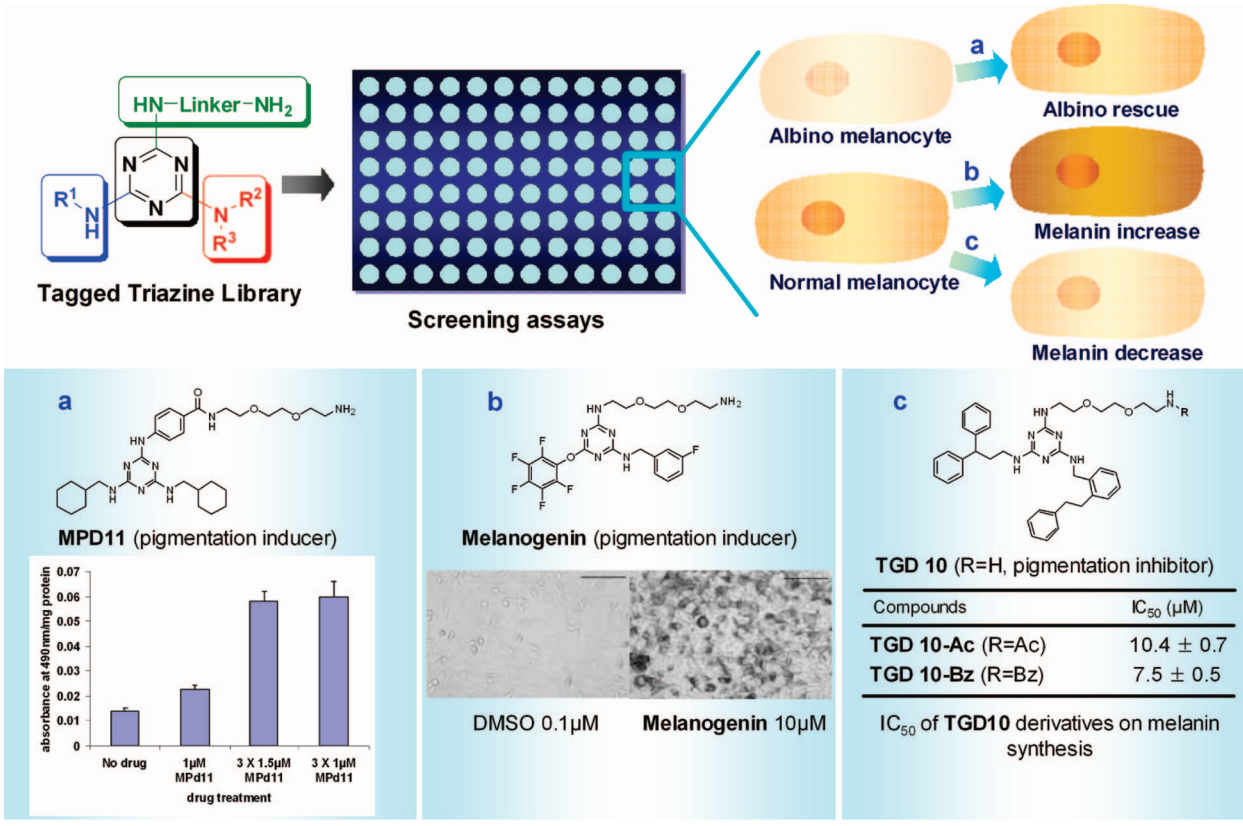



**FIGURE 4.** Pigmentation inducers and suppressors are found by screenings of the tagged triazine library in albino or normal melanocytes.

## Insulin Mimetic Small Molecule Discovered in *C. elegans*

*C. elegans* is being used more frequently as a model system in chemical genetics.[4] It is the first multicellular organism to have its genome completely sequenced, and the RNA interference (RNAi) was first developed on this organism. It is small and transparent and produces many progeny with a short life cycle that allows for high-throughput screening and visual observation of developmental processes. We selected a mutant form of *C. elegans* (*daf-2* mutant) as a model system that develops the growth arrest status (dauer form) (Figure 5a).[35] Since DAF-2 signaling in *C. elegans* is analogous to the insulin signaling pathway in mammalian systems,[35] a chemical rescuer of the *daf-2* mutant to the normal growth can be a potential drug candidate for the treatment of diabetes.

We screened a tagged triazine library for dauer escapes of *daf-2* mutants by soaking them in a solution of triazine compounds in a 96-well plate. This phenotypic screening identified one compound, GAPDS, that induced the normal growth of the *daf-2* mutant (Figure 5a). To confirm the relevance of this activity in *C. elegans* to a mammalian system, 3T3-L1 pre-adipocytes were tested for glucose uptake and exhibited a dose-dependent increase upon GAPDS treatment. A further immunostaining study showed that the level of PtdIns(3,4,5)$P_3$, a known stimulator for glucose uptake, was increased by GAPDS treatment (Figure 5a), demonstrating that GAPDS is a small molecule stimulator for the insulin signaling pathway.

A biochemical pulldown experiment suggested 11 putative binding partners from the GAPDS-conjugated affinity matrix. To dissect the functional target protein, each putative binder gene was tested with RNAi knockdown experiments (Figure 5b). RNAi of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) showed dauer escapes, as did GAPDS, implying the inhibitory effect of GAPDS on GAPDH function. The cross-linking study of GAPDH showed that tetrameric GAPDH was disassembled into monomers upon GAPDS treatment. This unique segregation of GAPDH was found to suppress the hydrolysis of PtdIns(3,4,5)$P_3$ and PtdIns(4,5)$P_2$ and therefore stimulates glucose uptake. Thus, this study has successfully demonstrated the novel function of GAPDH with respect to the insulin signaling pathway, which suggests GAPDH as a new target for diabetes treatment.

## Small Molecule Microarrays with a Tagged Library

With their introduction in 1999 and following pioneering works by the Schreiber group,[50,51] small molecule microarrays allowed for a high-throughput screening with a miniaturized format and developed as a highly efficient tool for reverse chemical genetics.[4,25] With the development of the relevant technologies, a small molecule microarray has demonstrated its power, by which thousands of small molecules are immobilized on a glass slide and simultaneously screened with a protein of interest that finally leads to the discovery of novel small molecule

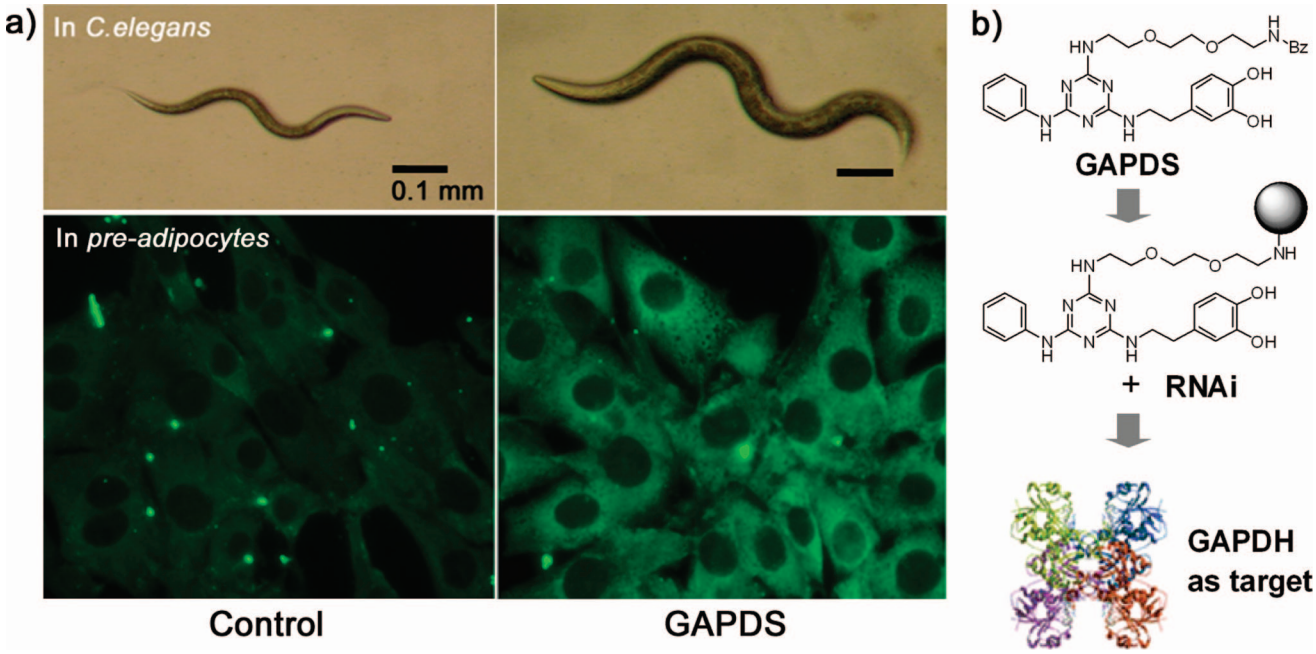



**FIGURE 5.** Discovery of new GAPDH function. (a) GAPDS (100 $\mu$M) rescues a *daf-2* mutant in *C. elegans* (dauer form, top left) to normal growth (top right). In the immunostaining study of mammalian cells, GAPDS (10 $\mu$M, 1 min) exhibited enhanced glucose uptake via the 2.2-fold increase in the level of PtIns(3,4,5)$P_3$ (bottom left, control; bottom right, GAPDS-treated). (b) The pulldown experiment with the GAPDS-conjugated affinity matrix and RNAi tests identified GAPDH as a target protein.

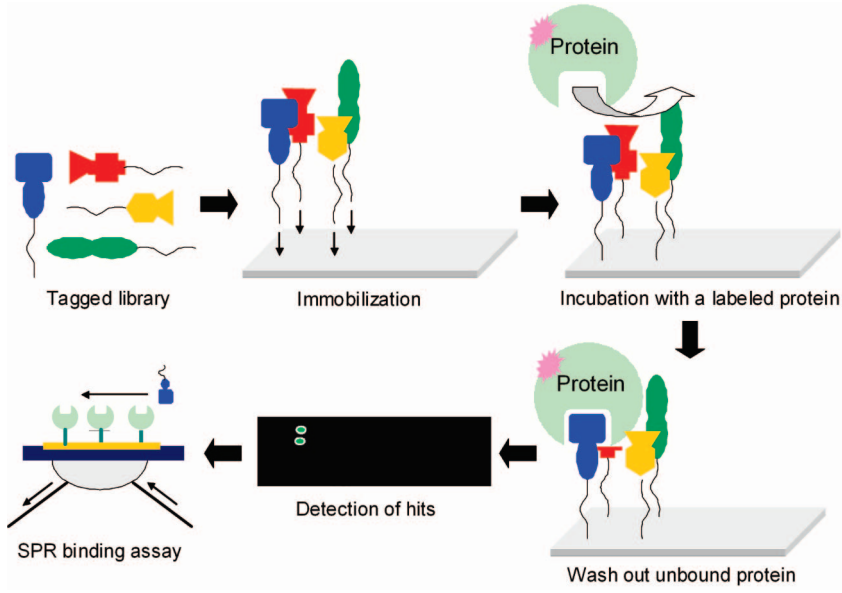



**FIGURE 6.** Advantageous immobilization of the tagged library with intrinsic tags on small molecule microarrays.

regulators.[52] However, a limitation via which all the small molecules must contain some functional group that can react selectively with an exposed functional group on the glass surface by special attachment reactions or methods exists.[52]

Given that all of our triazine derivatives contain the built-in TG tag, we took advantage of a tagged library for small molecule microarrays. In total, 5376 spots corresponding to 2688 triazine library compounds were directly printed on the chemically activated glass slide.[53] This TG linker provides not only a covalent bonding site but also a sufficient space between the compounds and the microarray surface that gives the conformational flexibility of the triazine compounds. A fluorescently labeled human immunoglobulin G (IgG) was screened on this microarray, and three specific binding compounds were identified. These selected hit compounds were further tested for their binding affinity with surface resonance plasma (SPR) and may indicate a future application for the isolation and purification of IgG (Figure 6).

## Conclusion

Chemical genetics is a powerful approach that identifies the small molecule regulators involved in complex bio-

logical phenomena. In particular, target identification and mechanistic study is a core step in dissecting the phenomena. Thus, determining the biological events that occur in the phenotype that is being studied often requires enormous time and effort. We have introduced a tagged library approach as one of the solutions to expediting this process. A triazine scaffold was highly successful for the introduction of the intrinsic tag with the diversification of library members. This tagged triazine library was screened on a series of model system, and it identified several small molecule regulators: a developmental suppressor in zebrafish embryos, pigmentation inducers and inhibitors in melanocyte, and an insulin-mimetic small molecule in *C. elegans*. The selected hit compounds with the built-in linker were easily utilized for the isolation of their target proteins. The successful target identification provided a clear breakthrough in revealing the mechanism for the observed phenotype. Thus, a tagged library approach has significantly contributed to the facilitation of chemical genetics. We anticipate that diverse scaffolds of a tagged library will be used in the field of chemical genetics in the near future.

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