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Note

Development of a Cell-Based Assay Using a Split-Luciferase Reporter for Compound Screening

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Recently, the finding of recurrent mutations in the spliceosome components in cancer has indicated that the spliceosome is a potential target for cancer therapy. However, the number of small molecules known to affect the cellular spliceosome is currently limited probably because of the lack of a robust cell-based approach to identify small molecules that target the spliceosome. We have previously reported the development of a genetic reporter to detect the cellular levels of small nuclear ribonucleoproteins (snRNPs), which are subunits of the spliceosome, using a split luciferase. However, the original protocol was designed for small scale experiments and was not suitable for compound screening. Here, we found that the use of cell lysis buffer used in blue native polyacrylamide gel electrophoresis (BN-PAGE) dramatically improved the sensitivity and the robustness of the assay. Improved assay conditions were used to discover a small molecule that altered the reporter activity. Our method may be used with other cellular macromolecular complexes and may assist in the discovery of small bioactive molecules.

Key words split luciferase, compound screening, spliceosome, small nuclear ribonucleoprotein (snRNP)

INTRODUCTION

Because many mutations have been found in the spliceosome components in cancer and genetic diseases, small molecules that can alter the function and/or level of small nuclear ribonucleoprotein (snRNP) may be potential therapeutics.[1] However, the number of such compounds that are effective in cells is still limited, perhaps because of a lack of methodological diversity in cell-based assays targeting the spliceosome.[2,3] We have previously reported a split luciferase reporter that could be used to detect the cellular macromolecular RNA–protein complex called snRNP,[4] the building block of the spliceosome. Spliceosomal snRNPs are divided into five classes based on the type of snRNAs involved: U1, U2, U4, U5, and U6 snRNPs.[5–7] Our reporter expresses Prp6 and U5-40K, which are protein components of the U5 snRNP, as a fusion gene with split luciferase fragments.[8] To apply our reporter to compound screening, we needed to improve our assay for use with an automated system because automated assays need to be performed on a smaller scale, compared with the original method, which only yielded weak and unstable luciferase activity. Here, we describe the development of an improved method to perform compound screening using the split luciferase reporter. The use of buffer system for blue native polyacrylamide gel electrophoresis (BN-PAGE) dramatically improved the sensitivity and the robustness of the assay. Our method may be used to discover small bioactive molecules that target other cellular macromolecular complexes.

MATERIALS AND METHODS

Cell Culture The 293T cells and its derivative reporter cell line[4] were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum.

Cell-Based Split Luciferase Assay for Small Number of Samples The 293T reporter cell line was cultured on six-well plates and lysed in 100 μL of split luciferase (SL) buffer containing 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES)–KOH (pH 7.9), 150 mM NaCl, 1.5 mM $MgCl_2$, and 0.25% NP-40, and 20 μL of lysates was mixed with 100 μL of luciferase substrate solution using a PicaGene Luminescence Kit (TOYO B-NET, Tokyo, Japan). The emitted luminescence was immediately detected using a Lumat LB9507 (Berthold Technologies, Bad Wildbad, Germany).

Screening Using the Cell-Based Split Luciferase Assay Stable 293T reporter cells were manually plated at 6×10^5 cells in 200 μL of media in white-wall 96-well plates (3912, Corning, Corning, NY, U.S.A.) and the plates were placed into a CO_2 incubator overnight without overlapping. When starting the assay, the plates containing library compounds were set in an HTS machine HORNET-HTS (WAKO, Osaka, Japan), and the machine added media to the compounds. The reporter cell plates were transferred to HORNET-HTS and 150 μL of media was removed from each well, followed by addition of 50 μL of compound-media mixtures. After incubation for 4 h, 50 μL of media was removed and 50 μL of BN buffer [20 mM HEPES–KOH (pH 7.4), 20 mM KCL, 0.5 M 6-aminohexanoic acid, 0.5% Triton-X100, and 10% glycerol] was added to the well, followed by gentle pipetting. In the buffer used to prepare the cell extract for blue native PAGE, sodium chloride replaced the 6-aminohexanoic acid in the BN buffer.[9] Steady-Glo luciferase substrate solution (100 μL, E2550, Promega, Madison, WI, U.S.A.) was added to the well and then the machine transferred the plates to the microplate reader immediately and the luminescence was measured.

To check the quality of the assay in each plate, Z' values

$$Z' = 1 - (3SD_B + 3SD_C)/(\text{Mean}_c - \text{Mean}_B)$$

signal-to-background ratio (S/B)

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$$S/B = \mathrm{Mean}_c/\mathrm{Mean}_B$$

and Coefficient of Variation (CV) values

$$CV = 100(SD_c/\mathrm{Mean}_c)$$

were determined (S.D.: standard deviation, B: background wells and C: control (DMSO-treated) wells). A mixture of puromycin and MG132, which decreases the reporter activity, was used as a positive control. To confirm the reproducibility, positive compounds obtained from the first screening were further tested using the same protocol, and compounds that passed the reproducibility test twice were defined as positive compounds.

Glycerol Gradient Analysis A whole cell extract was prepared from the 293T reporter cell line using SL buffer and 500 μL of the extract was loaded onto a 10–40% glycerol gradient in SL buffer. Samples were centrifuged in 11-mL gradients at 4 °C for 15 h at 37000 rpm in an SW41 rotor. Fractions were collected from the top of the gradient manually and aliquots (20 μL) were used to detect the luminescence as described for the split luciferase assay of the whole cell extract. The proteins in a half of a fraction were precipitated by adding two volumes of cold acetone and the precipitates were resuspended in 100 μL of Laemmli buffer. Aliquots of 10 μL of the suspended samples were used for Western blotting.

Western Blotting and Antibodies Proteins extracted by SL buffer or the proteins precipitated from the glycerol gradient fractions were separated on sodium dodecyl sulfate (SDS)-PAGE and subjected to Western blotting with the respective antibodies. Each antibody was further reacted with an IRDye 800- (Rockland, U.S.A.) or Alexa Fluor 680-conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and visualized by an infrared imaging system Odyssey (LI-COR, Lincoln, NE, U.S.A.). The antibodies used were mouse monoclonal anti-FLAG (1 : 1000, M2, Sigma-Aldrich), mouse monoclonal anti-Prp8 (1 : 1000, 2834c1a, Abcam), rat polyclonal anti-Prp6 serum, rat monoclonal anti-Prp3[10] (1 : 1000), and rat monoclonal anti-U5-40K[4] (1 : 1000) antibodies. The rat anti-Prp6 was obtained by immunizing rats with recombinant human Prp6 (1–398 aa) in adjuvant and the serum was collected after 2 weeks. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the Committee for Animal Research at Hokkaido University (Permit No. 08–0468).

Statistical Analysis Statistical analyses were performed using the Mann–Whitney U test. Data are presented as means with S.D.

RESULTS AND DISCUSSION

In our reporter system, the reporter proteins are not able to interact with each other directly, as illustrated in Fig. 1, but are positioned close together when incorporated into the U5 snRNP. In the U5 snRNP, the luciferase fragments of the reporter proteins reconstitute to form the active luciferase enzyme depending on their proximity and configuration; therefore, the luminescence reflects the presence of U5 snRNP containing the two reporter proteins. This set of reporter proteins only reconstitutes to result in luciferase activity in free U5 snRNP, not in the U4/U6.U5 tri-snRNP, which is a fully assembled precursor complex, or in further assembled spliceosome complexes. Therefore, it is predicted that inhibition of transcription that reduces the demand for the spliceosome may cause an increase in reporter activity through the accumulation of free U5 snRNP. The inhibition of transcription by actinomycin D caused a rapid increase in reporter activity in 1 h.[4] In addition, the reporter activity was decreased in the presence of MG132, accompanied with a decrease in the co-precipitation of the reporter-fusion U5-40K protein for both endogenous and the reporter-fusion Prp6, proteins.[4] Although the precise mechanism of the mode of action of MG132 is still unclear, our pilot experiments showed that the effect of MG132 was enhanced by simultaneous

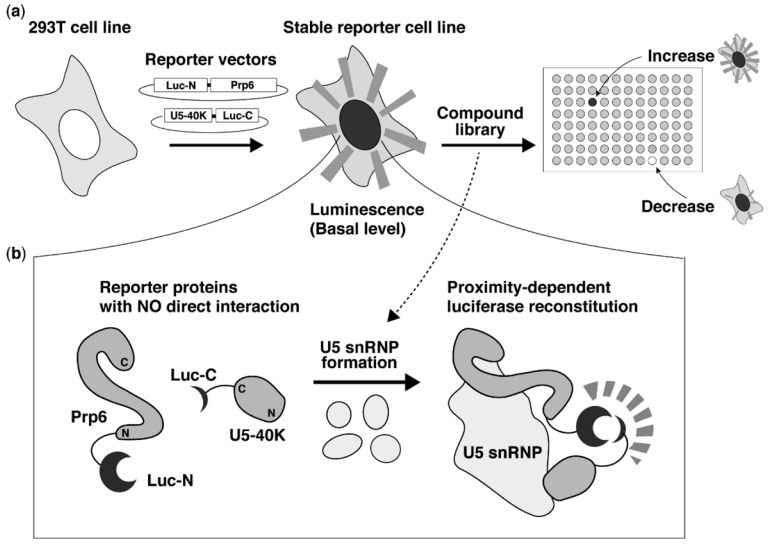

Fig. 1. Schematic Representation of the Reporter System

(a) A stable cell-line expressing the U5 snRNP reporter plasmids was theoretically able to respond to both decreases and increases in the content of the U5 snRNP reporter complex. (b) The N-terminal fragment of luciferase was fused to the *N*-terminus of human Prp6 protein, and the C-terminal fragment of luciferase was fused to the *C*-terminus of human U5-40K protein. These two fusion proteins constitute the U5 snRNP reporter. These proteins cannot reconstitute luciferase by themselves; however, when they are incorporated into the U5 snRNP both the luciferase fragments are positioned in close proximity, which enables the reconstitution of the luciferase, resulting in luminescence being observed.

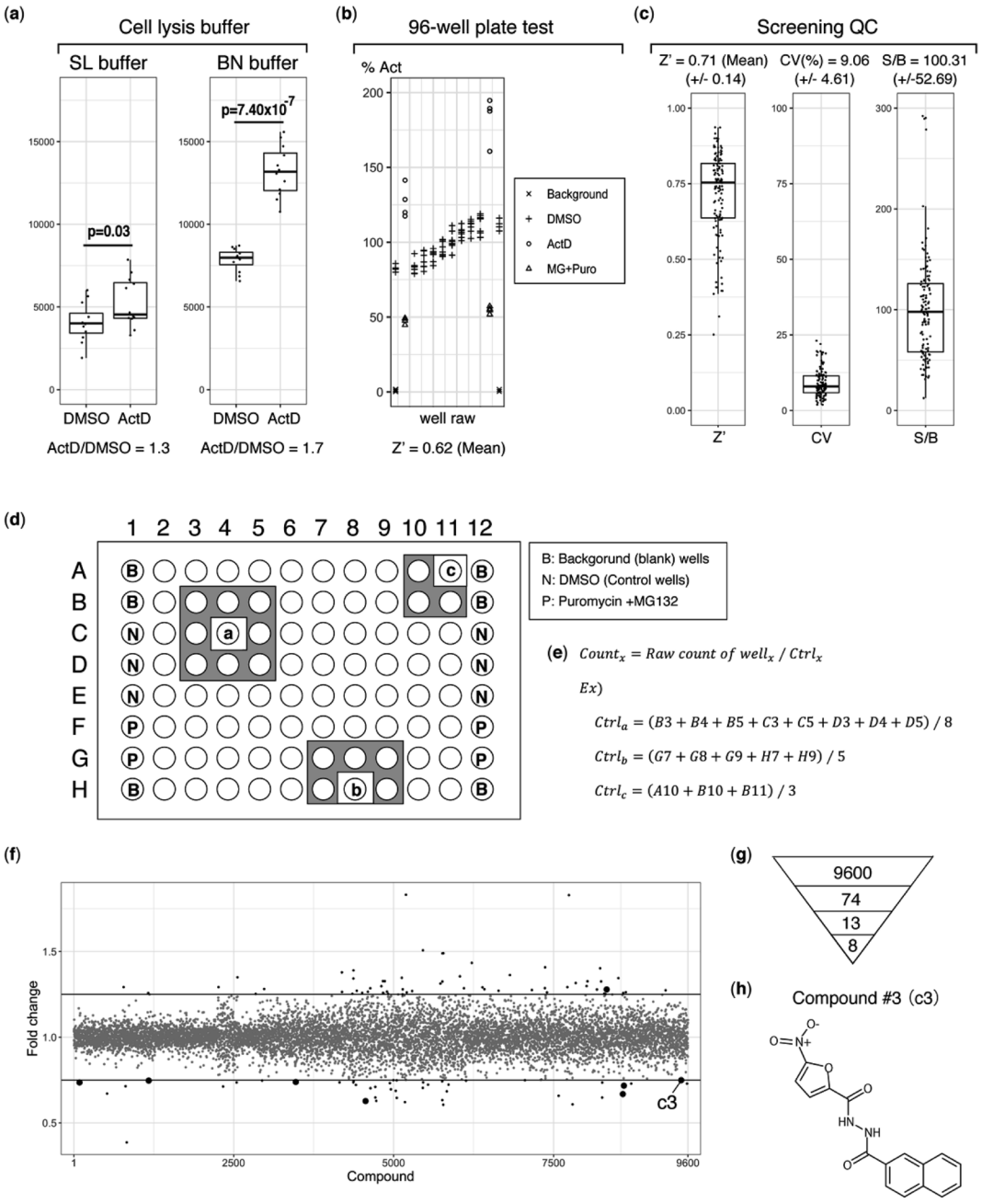

Fig. 2. (a) The Preparation of the Whole Cell Lysate Using BN Buffer Improved the Response to the Treatment with Actinomycin D

The Mann–Whitney *U* test was used to determine differences in the detection sensitivity between the two cell-lysis buffers. (b) Investigation of the quality of the assay performed using an automated program with the HTS machine. Z′ score was calculated comparing DMSO-treated wells to background wells. (c) The Z′, S/B, and CV values obtained in our screening (120 plates) were plotted to visualize the quality of the screening. (d) Schematic representation of the well placement in a plate and the method used to derive the values for each compound. To calculate the fold change upon treatment with each compound, it was necessary to determine the value of the control. In our assay, control wells positioned on the edges of the plate were not suitable because there were differences in the distribution of luciferase activity within the plate. Thus, we considered the average values of the surrounding wells as a virtual control for a well. Notably, the number of wells used to calculate a virtual control was different depending on the position of the well in the plate. (e) The formulae used to calculate the control values are shown at the right for wells "a," "b," and "c." (f) The plotted results of the first screening. Compounds showing reporter activity that was increased or decreased by over 25% from the mean were subjected to a second assay using the same protocol. Larger circles indicate hit compounds and the compound c3 is indicated by the letter. (g) The numbers of compounds that remained after each step in the repeated assays. (h) Structure of the hit compound #3 (c3).

treatment with puromycin, which allowed the use of this pair of compounds as a control to evaluate the decrease in reporter activity. Thus, we could investigate whether the assay format was applicable for use in multi-well plates using actinomycin D and the mixture of MG132 and puromycin as controls.

The reconstituted luciferase activity obtained using our original method was heavily variable when it was performed in a 96-well plate format, which made the assay difficult to apply for compound screening (Fig. 2a). In addition, the response to actinomycin D decreased in the 96-well plate format, which indicated that the sensitivity of the assay might not be sufficient to find hit compounds. Fortunately, we have investigated visualizing the reporter protein complex using BN-PAGE, in which the protein complex is separated in the native condition, and found that the cell lysate prepared using the BN buffer for BN-PAGE increased the reconstituted luciferase activity (Fig. 2a). The buffer contained 6-aminohexanoic acid at a high concentration to increase protein solubilization[9] and using this buffer

further improved the response to actinomycin D treatment in the assay (Fig. 2a). This increased response allowed the application of our reporter to assays in the 96-well plate format. Although Z′ score and the response to the positive controls were reproducible well, occasionally, there were differences in the distribution of luciferase activity within the plate (Fig. 2b), which interfered with the calculation of fold change using the control wells placed at the right and left edges of the plates. To solve this problem, we assumed that most of the wells around each well showed no significant change. Using this assumption, we calculated the fold change for each well using the averaged activity of the surrounding wells as a hypothetic control (Figs. 2d, e). Because only 1% of compounds altered the reporter activity, our standardizing method could be used to reduce the variation between experiments (Fig. 2c).

Using the improved protocol, we screened a compound library consisting of 9600 unidentified compounds. Because of the working speed of the HTS machine to process 96-well plates with pipetting and mixing, 960 compounds in 12 plates were tested in a day. Several hit compounds were identified in the first screening (Fig. 2f), and the compounds were further tested using the same protocol twice to check the reproducibility. Eight compounds were shown to alter the reporter activity (Fig. 2g). Among them, compound c3 was further assessed because c3 had not been reported in other screenings using the same compound library (Fig. 2h). Importantly, only 74 compounds were obtained as hits in our first trial, which was less than expected. This unexpected low false-positive rate allowed the use of the surrounding wells as a virtual control for screening the compound library.

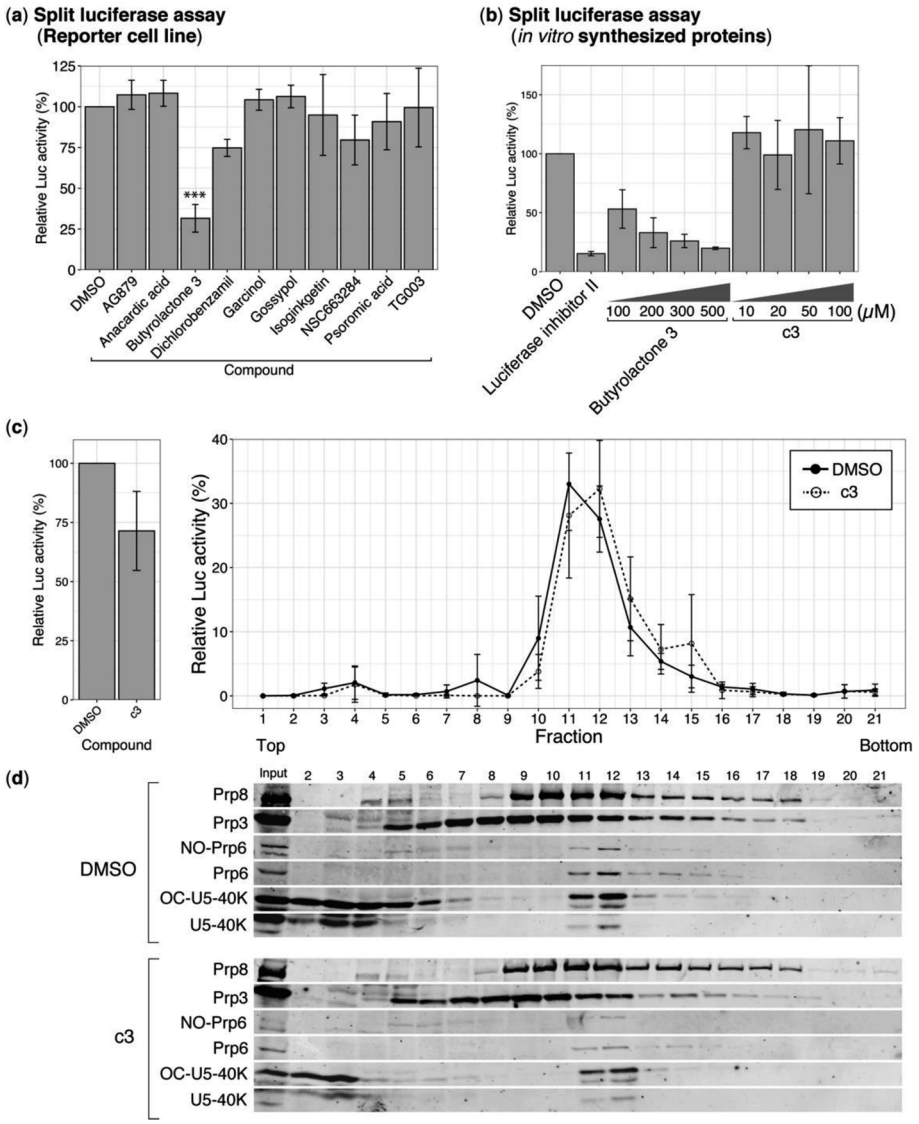

Fig. 3. (a) The Effect of Known Splicing Inhibitors in the U5 snRNP Reporter Assay

Cells were treated with the compounds indicated below the histogram, for 4 h, and the reporter activity was measured manually. The experiments were independently repeated three times in duplicate and the means are indicated with the S.D. as error bars. The Tukey HSD *post-hoc* test was used to detect significant differences between compounds (***$p < 0.001$). (b) Effect of the compounds on the luciferase activity reconstituted *in vitro*. The reticulocyte lysate system was used to synthesize the split luciferase reporter proteins (NO-Prp3 and CO-Prp4) that reconstitute luciferase *via* a direct interaction. The lysate containing the *in vitro* synthesized proteins was mixed with compounds at the concentrations indicated. Luciferase inhibitor II was used as a positive control of the inhibitory effect. The histograms represent the means of three technical replicates with the S.D. as error bars. (c, d) The whole cell extract was loaded onto a 10–40% glycerol gradient and centrifuged for 16 h. Fractions were obtained manually from the top of the tube and the distribution of the luciferase activity was plotted by calculating the ratio of each fraction against the sum of all fractions (%). (e) The proteins were collected from the remaining fractions and analyzed by Western blotting. The experiments were independently repeated three times and the points in the curve show the mean and S.D. calculated from all experiments.

Known compounds that affect the spliceosome assembly were also evaluated in our reporter system. Because our reporter detects free U5 snRNP, compounds that target the assembly steps in which U5 snRNP is involved were selected. Among the tested compounds, only butyrolactone 3 significantly reduced the reporter activity; however, there is the possibility that butyrolactone 3 generally inhibited the reconstituted split luciferase activity. To confirm this possibility, c3 and butyrolactone 3 were added to an *in vitro* split luciferase assay in which the components were reconstituted by a reporter pair of Prpf3 and Prpf4 (in preparation). The results showed that treatment with butyrolactone 3 caused general inhibition of the split luciferase activity. In contrast, c3 showed no effect on the *in vitro* split luciferase activity, which excluded the possibility that c3 affected the enzymatic activity of luciferase. Consistent with this result, normal luciferase activity exhibited in cultured cells was not largely affected by c3, although longer exposure (24 h) or higher concentration (25 μM) reduced the luciferase activity to about a half (data not shown).

Then, to examine if any compositional or configurational changes occurred within the U5 snRNP in the presence of c3, we investigated the distribution of the luciferase activity in glycerol gradient sedimentation (Fig. 3). While the total amount of the luciferase activity was reduced (Fig. 3a), the position of the peak fraction was not changed (Fig. 3b). This result suggested that the c3-induced reduction in the luciferase activity may have been triggered by a decrease in the content of the U5 snRNP reporter complex rather than by changes in the composition or configuration of the reporter complex. Although the mechanism by which c3 affected the reporter activity remains elusive, these results indicated that our screening system using the split luciferase reporter was applicable for the identification of active compounds at a cellular level.

Interestingly, c3 has been reported to show an inhibitory effect on thioredoxin reductase.[11] SMN, which is a chaperone protein of the snRNP assembly, has been reported to be oxidized under conditions of oxidative stress, causing a reduction in the chaperone activity.[12] Thus, disturbance of the redox state by c3-mediated inhibition of the thioredoxin recycling system may inhibit the assembly of the U5 snRNP. Alternatively, the folding of luciferase may be an indirect target of c3 because the firefly luciferase protein contains four cysteines that may form disulfide bonds.[13–15] Our results excluded the possibility that c3 directly inhibited the enzymatic activity of luciferase; however, it is still possible that c3-mediated perturbation of the cellular redox state indirectly affected the reconstitution of the split luciferase. Further studies are required to understand why c3 was obtained as a hit compound in our screening to pave the way for the use of the split luciferase assay as an alternative cell-based approach to screen compound libraries. In addition to somatic mutations of U2 snRNP proteins in myelodysplastic syndrome and leukemia, overexpression or amplification of core component genes of U5 snRNP are found in some solid tumors, suggesting that aberrancy in U5 snRNP contributes to tumorigenesis.[16] If c3 truly can reduce cellular U5 snRNP levels, elucidation of the mode of action of c3 may provide a potential therapeutic strategy against cancer by the alteration of U5 snRNP.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials This article contains supplementary materials.

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