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A split luciferase-based reporter for detection of a cellular macromolecular complex

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Short title: Split luciferase for snRNP detection

ABSTRACT

The spliceosome is a highly dynamic macromolecular ribonucleoprotein (RNP) machine that catalyzes pre-mRNA splicing by assembling U1, U2, U4, U5, and U6 small nuclear (sn)RNPs. To process large numbers of introns with a limited number of snRNPs, synthesis and recycling of snRNPs must be maintained within an appropriate range to avoid their shortage. However, the mechanism that maintains cellular snRNP levels is unknown. Molecules that modulate cellular snRNP levels may help to define this mechanism but are not available. Therefore, the goal of the present study was to develop a reporter for snRNP levels using split luciferase based on proteomic analysis of snRNPs. We constructed an expression library of a luciferase fragment fused to core components of U5 snRNP and used it to isolate pre-mRNA processing factor 6 (PRPF6) and small nuclear ribonucleoprotein 40kDa (U5-40K) that specifically reconstitute luciferase activity in the U5 snRNP complex. We show here that this reporter detects the effects of small molecules on the levels of the U5 snRNP-reporter protein complex. Our approach provides an alternative assay to discover small molecules targeting a macromolecular complex when the structure of the complex is not precisely identified.

Keywords: snRNP; spliceosome; split luciferase

Splicing of pre-mRNA removes an intron from a pre-mRNA and ligates the 5' and 3' exons in a two-step reaction catalyzed by the macromolecular RNA-protein spliceosome complex. The spliceosome assembles on pre-mRNA by stepwise recruitment of five small nuclear ribonucleoprotein (snRNP) subunits called U1, U2, U4, U5, and U6 that comprise specific snRNAs and proteins. After a splicing reaction is completed, the spliceosome is disassembled and snRNPs are recycled [1; 2].

Because U4, U5, and U6 snRNPs are recruited onto pre-mRNA as the U4/U6.U5 tri-snRNP and U5 snRNP join U4/U6 di-snRNP to form tri-snRNP, the cell maintains five different pools of U4, U5, and U6 snRNPs. These include U4, U5, U6 “mono” snRNPs, U4/U6 di-snRNP, and U4/U6.U5 tri-snRNP, although mono snRNPs are further divided into different classes depending on the nature of associated factors[1; 2; 3]. To process large numbers of introns using a limited number of snRNPs, synthesis and recycling of snRNPs must be maintained at an appropriate rate. Cell division reduces the number of snRNPs in daughter cells by approximately 50%. Therefore, the cell must generate snRNPs to the parental level during each cell cycle, otherwise this will finally trigger a shortage of snRNPs.

To avoid such a disaster, the cell employs a regulatory network to maintain snRNP levels. Indeed, when p110 levels, a recycling factor of U4/U6 di-snRNP, are knocked down in zebrafish, a feedback mechanism likely upregulates transcription of splicing factors that are functionally related to U4/U6.U5 tri-snRNP and probably compensate for the defect [4]. Moreover, depletion of core

components of snRNPs in mammalian cells often causes cell cycle arrest [5; 6; 7], which suggests a link between the levels of the splicing machinery and the cell cycle. However, detailed knowledge of the components of the regulatory network and the mechanism that enables cells to detect cellular snRNP levels are unknown.

Moreover, previous studies show that the expression of snRNA and protein components of snRNP is regulated during development in a tissue-specific manner [8], suggesting that the cell maintains adequate pools of snRNP by modulating the rate of snRNP synthesis or recycling. However, it is unknown whether the differences in cellular snRNP levels among different cell types are physiologically relevant.

Although molecules that modulate cellular snRNP levels would help to answer these questions, such molecules are unknown, likely because of the lack of assays to identify them. Various splicing inhibitors and modulators have been discovered serendipitously or through the use of rational approaches [9; 10; 11], and some have been identified using high-throughput screening (HTS) [12; 13; 14]. The screening strategy relies on detecting the completion of a splicing reaction. However, these assays do not detect cellular snRNP levels. To overcome this problem, we aimed to develop an assay to identify small molecules from a chemical library using HTS. We report here a reporter that detects indirectly the cellular levels of a specific snRNP.

Split luciferase provides the basis for a method originally developed to detect direct protein-protein interactions that occur in the presence of a small molecule [15]. For this purpose, luciferase was split into N- and C- terminal domains and fused to sensor domains. Reconstitution of luciferase activity depends on direct or indirect interaction of the sensor domains. This simple principle is useful for various purposes and can detect the presence of specific DNA or RNA sequences through tertiary interaction using a DNA or RNA-binding domain as a sensor domain [16; 17]. A three-hybrid system consisting of three proteins has been used to screen for protein kinase inhibitors [18]. However, there is no report of the use of split luciferase to detect the formation of a macromolecular complex such as snRNP, likely because there is no rational approach to find or design reporter proteins that reconstitute luciferase activity when specifically incorporated into a target complex.

A macromolecular complex involves many protein-protein interactions, and in the case of snRNPs, the components of snRNPs and their interactions are known [19; 20; 21]. This information allowed us to construct a luciferase fragment-fusion gene library comprising U5 snRNP factors. We screened the library to discover a gene set that encodes products that detect the U5 snRNP and reconstitutes luciferase activity. Here we also present evidence suggesting that the reporter proteins are incorporated into the U5 snRNP and only the mono U5 snRNP reconstitutes luciferase activity. We used our

reporter to test the effects of drugs on cellular U5 snRNP levels and found that treatment with the proteasome inhibitor MG132 significantly reduced reporter activity. Moreover, inhibition by MG132 reflected the change of the U5 snRNP-reporter complex levels. These results suggest that our reporter assay is a promising method to identify small molecules that affect U5 snRNP and may facilitate developing a reporter that detects small molecules that target other macromolecular complexes.

Materials and methods

Plasmids

Vectors to express split-luciferase reporters were constructed by inserting a luciferase gene-fragment and a flexible linker region into pcDNA3 (Life Technologies). (Fig. 1) N- and C-terminal fragments of the firefly luciferase included amino acid residues 1–398 and 394–550, respectively, to avoid high signal/background ratios [22]. The conservative mutations T591>C and T576>C were introduced to ablate *EcoRI* and *MfeI* sites in the firefly luciferase gene to enable the use of these enzymes to construct the plasmids. Splicing factor cDNA clones were obtained from the I.M.A.G.E. Consortium (www.imageconsortium.org), except for hDib1, which was synthesized by reverse transcription (RT)-PCR templated by total RNA isolated from mammary ductal carcinoma T47D cells. Restriction enzyme sites were added to 5' and 3' ends of the coding region using PCR. Cloned cDNAs lack a

translation termination (STOP) codon, because the same cDNA clone can be cloned into four different plasmids in which the luciferase fragments are oriented in four different orientations. When a reporter contains snRNP proteins joined to the C-terminus of a luciferase fragment, a STOP codon in the multiple-cloning site that also exists in the reporter is used. Reporter plasmids containing PRPF6 differ slightly from common vectors that employ other sets of cloning sites (see Supplementary Fig. 1) To establish stable transfectants of split-luciferase reporter genes, another plasmid called “pSplit2-Osp” was constructed. This construct uses pEXPR-IBA105 (IBA, Gottingen) to attach the One-STrEP tag in place of the FLAG tag at the N-terminus of a reporter-fusion protein, and the PCR-amplified PRPF6 *EcoRI-Sall* fragment is cloned into the *EcoRI/XhoI* sites (also see Supplementary Fig. 1). Sequences of PCR amplified clones were confirmed by dideoxy sequencing. Vectors constructed in the present work will be deposited in Addgene after acceptance of this manuscript. I.M.A.G.E. clone number and sequences of oligonucleotide primers used for construction were as follows: for Human *TXNL4A(Dib1)*, hDib1-5': 5'-GCCATGTCGTACATGCTCCC-3'; hDib1-3': 5'-CTCAGTAGCGGTACTTGGTG-3'; hDib1-5'*EcoRI*-ATG: 5'-CGAATTCATGTCGTACATGC-3'; hDib1-3'-*XhoI*: 5'-GCTCGAGGTAGCGGTACTTG-3', for Human *PRPF6* (MGC:1852 IMAGE:2988165), PRPF6-5'*EcoRI*-ATG: 5'-GGGAATTCATGAACAAGAAGAAG-3'; PRPF6-3'+stop-*Sall*: 5'-GGGTCGACTCAGAAGGTGTTCTTG-3'; PRPF6-3'-*KpnI*:

5'-GGGGTACCGAAGGTGTTCTTGATG-3';

PRPF6-3'-*Sall*:

5'-GGGTCGACGAAGGTGTTCTTGATG-3', for Mouse *PRPF8* (MGC:118730 IMAGE:30945329),

PRPF8-5'-*MfeI*-ATG:

5'-CCAATTGATGGCCGAGTGTTTC-3';

PRPF8-3'-*XhoI*:

5'-CCTCGAGGGCATAGAGGTCCTC-3'; for Mouse *EFTUD2* (MGC:65664 IMAGE:6810127),

EFTUD2-5'-*EcoRI*-ATG:

5'-GGAATTCATGGATACTGACTTG-3';

EFTUD2-3'-*XhoI*:

5'-GCTCGAGCATGGGATAATTGAG-3', for Human *snRNP40*(U5-40K) (MGC:1910

IMAGE:3504573), 40K-*MfeI*-5' ATG: 5'-CCAATTGATGATAGAACAGC-3'; 40K-3'-*XhoI*:

5'-CCTCGAGCTGAATCTCTCCC-3'

Cell culture and transfection

The 293T cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum. For transient reporter expression, 293T cells were transfected with the reporter plasmids using Lipofectamine 2000 according to the manufacturer's instructions. To obtain stable transfectants, 293T cells were transfected with pBabe-Hyglo to select for resistance to 400 µg/ml of hygromycin B. Approximately two weeks after transfection, hygromycin B-resistant cells were selected and the luminescence-positive clones were identified.

Split-luciferase assay

The 293T cell clones expressing reporter plasmids were cultured on 6-well plates and lysed in 100 μ L of SL buffer (20 mM Hepes-KOH (pH 7.9), 150 mM NaCl, 1.5 mM $MgCl_2$, and 0.25% NP-40), after centrifugation at 11,100g for a minute 20 μ L of supernatants were mixed with 100 μ L of luciferase substrate solution (TOYO Ink), and luminescence was immediately detected using a Lumat LB9507 (Berthold Technologies). To test effects of small compounds, cells stably transfected with reporter plasmids were seeded onto a 48-well plate in 500 μ L of medium the day before the assay. To add small compounds, the medium was removed and 250 μ L of medium containing compounds at various concentrations (see Table 1) were added to wells. After one or six hours the cells were lysed in 50 μ L of SLT buffer (20 mM Hepes-KOH (pH 7.9), 150 mM NaCl, 1.5 mM $MgCl_2$, and 0.5% Triton-X100), and 20 μ L of crude extract was used to measure luciferase activity as described above. To screen inhibitors from a small set of small compounds, the concentrations of compounds were set according to “SOGAIZAI KATSUYOU HANDBOOK (Yodo-sya, 2006; Japanese)”. For statistical analysis, Dunnett’s test was used to compare the effects of individual compounds with those of the vehicle (DMSO) control.

***In vitro* protein synthesis**

To prepare reporter proteins *in vitro*, a reticulocyte lysate of a TnT® Quick Coupled Transcription/Translation System (Promega, Madison, WI) was used according to a manufacture's instruction, and 1 or 2 μ L of reticulocyte lysate containing synthesized proteins were mixed, incubated on ice for 15 min, and split-luciferase activity was measured using the same protocol described above for cell extracts. All proteins synthesized *in vitro* were used for the assay immediately after translation.

Western blotting and antibodies

To determine the expression levels of reporter proteins and endogenous snRNP factors, proteins extracted using SL buffer were separated on a 12% polyacrylamide gel and subjected to western blotting. Each antibody was further reacted with an IRDye 800 (Rockland, Philadelphia, PA, USA) or an Alexa Fluor 680-conjugated secondary antibody (Molecular Probes, Eugene, OR, USA) and visualized using an infrared imaging system (Odyssey, LI-COR, Lincoln, NE, USA). Antibodies and their dilutions used were as follows: mouse monoclonal anti-FLAG (1:1000, M2, Sigma-Aldrich, St. Louis, MO, USA), mouse monoclonal anti-PRPF8 (1:1000, Molecular Probes), rabbit polyclonal anti-PRPF6 (1:1000, Santa Cruz Biotechnology, Inc.), rat monoclonal anti-PRPF3 (1:1000, ([23])), rat anti-PRPF6 serum (1:1000, this study), and rat monoclonal anti-U5-40K/SNRNP40/WDR55 (1:1000, this study). The rat anti-PRPF6 and anti-U5-40K antibodies were raised according to a published

method [24]. Briefly, an emulsion containing 100 µg of recombinant human PRPF6 (1-398 aa) or 1 mg of a synthetic peptide representing the C-terminus of U5-40K protein (peptide sequence: SINEVAFHPDEPII) in Freund's complete adjuvant was injected into Wistar rats. After two weeks, sera and lymph node cells were collected, and the cells were fused to the mouse myeloma cell line X63/Ag8-653, and clones secreting specific antibodies against U5-40K were selected. 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 number 08-0468).

Glycerol gradient analysis

Whole-cell extracts for split luciferase assay was loaded onto 10–40% glycerol gradients in SL buffer without NP-40. Nuclear extracts were prepared using a previously described method [25], and the extracts were also analyzed using the same protocol. Samples were centrifuged in 11 mL gradients at 4°C for 15 h at 17,000*g* in an SW41 rotor. Fractions (550 µL) were collected from the top of the gradient and acetone precipitated. Precipitates were suspended in 100 µL of Laemmli buffer, and 10 µL of sample was used for western blotting. For northern blotting, RNA was extracted from 500 µL of fractions using ISOGEN-LS according to the manufacture's instruction. RNA pellets were suspended

in 20 μ L of distilled water and a 5 μ L sample was applied to a 10% polyacrylamide (mono:bis; 20:1) denaturing gel containing 7M urea. Separated RNA was electroblotted onto a Hybond-N+ Nylon membrane (GE Healthcare) and hybridized with biotinylated oligo-DNA probes (probe sequences are listed in Supplementary materials) using PerfectHyb solution (TOYOBO). Hybridized probes were detected using avidin-conjugated Alexa 680 (Life Technologies) and visualized using the Odyssey infrared imaging system. The sequences of probes used for hybridization were as follows: U4: 5'-GCCAATGCCGACTATATTGCAAGTCGTCA-3'; U5: 5'-GGTTAAGACTCAGAGTTGTTCTCTCCAC-3'; U6: 5'-GGAACGCTTCACGAATTTGCGTGTCATCC-3'.

Affinity purification and MS analysis

Affinity purification of the reporter complex by One-STrEP-tag was performed according to the manufacturer's instruction. Briefly, whole-cell extract from the stable transfectant of reporter plasmids were mixed with Strep-Tactin sepharose® (IBA) in 500 μ L of SL buffer. Mixed samples were rotated for an hour at 4°C. After extensive washing with SL buffer, proteins absorbed to the Sepharose were eluted in the SL buffer containing biotin. The eluted complex was next mixed with anti-FLAG® M2 affinity gel (Sigma-Aldrich) in 500 μ L of SL buffer and rotated again for an hour at 4°C. After

washing, purified complexes were eluted with SL buffer by adding FLAG® peptide, and eluates were loaded onto 10% SDS-PAGE gels. Protein bands were visualized using a Silver Stain MS kit (WAKO) and digested with In-Gel Tryptic Digestion Kit (Thermo). Digested proteins were eluted and analyzed by MALDI-TOF-MS UltraFlexII (BRUKER) to identify proteins in each band.

Coimmunoprecipitation and pull-down assays

Cultures of 293T cells stably expressing U5-Luc reporters were grown in 10-cm dishes, and proteins were prepared as described above. Proteins were immunoprecipitated with an agarose-conjugated anti-FLAG antibody (M2, Sigma) or bound to Strep-Tactin Sepharose (ABI). Proteins immunoprecipitated by anti-Flag or proteins bound to Strep-Tactin Sepharose were analyzed using western blotting to detect coprecipitated proteins. To determine the components in the reporter-protein complex, the SDS gel were silver-stained. Bands were excised, and the proteins in each band were extracted and analyzed by mass spectrometry. These results are shown in Supplementary Fig. 2

Results and Discussion

To identify molecules in a library that alters the cellular snRNP level, detection of snRNPs using a small-scale assay is critical. Protein components of snRNPs interact specifically within each snRNP

[19]. Thus, snRNP-specific protein-protein interaction (PPI) level may reflect the snRNP level. We applied the luciferase fragment complementation assay [15] to detect such snRNP-specific PPIs (Fig. 1). The split-luciferase system has three advantages as follows: First, luciferase fragment complementation is reversible, so that the assay should detect an increase or decrease of PPI. Second, the sensitivity of the luciferase assay is high enough to permit the use of 96-well plates. Finally the assay gives high signal-background ratio because no enzymatic activity exists without reconstitution of the luciferase activity.

To develop an assay, we first constructed expression vectors encoding snRNP proteins fused to luciferase N-terminal or C-terminal fragments in four different orientations, which we call NO, ON, CO, and OC (Fig. 2). The O indicates the position of the inserted snRNP factors in the reporter. To test which pair of reporter proteins reconstitute luciferase activity, we prepared pairs of reporter plasmids as indicated (Fig. 3) and used them to transfect 293T cells. We tested 32 combinations of reporters and found that two gene pairs, “NO-PRPF6 and CO-Dib1” and “NO-PRPF6 and OC-U5-40K” reconstituted luciferase activity (Fig. 3). Interestingly, when the luciferase fragments were fused at a different position of the same gene, the proteins did not luminesce. Thus, reconstitution of the luciferase activity only occurs when the luciferase fragments are positioned in a specific orientation, suggesting that reconstitution of luciferase activity depends on formation of a specific PPI and not on

nonspecific aggregation. Because PRPF6 and hDib1 interact directly [19], we determined whether luciferase activity was regenerated similarly by NO-PRPF6 and OC-U5-40K. When these reporter proteins were synthesized *in vitro* and incubated together (supplemental Fig. 2) the reaction mixture generated strong luminescence, in contrast to PRPF6 and U5-40K. Because PRPF8 interacts with PRPF6 and U5-40K [21], we added recombinant PRPF8; however, luciferase activity was not detected, suggesting that reconstitution of luciferase activity in 293T cells required other factors.

Therefore, we fractionated a whole-cell extract using glycerol gradient sedimentation and determined the luciferase activity of each fraction. (Fig. 4A) Western blotting of each fraction showed that most of the reporter proteins were distributed in fractions 2–7 (Fig. 4A); however, the peak of luciferase activity was detected in fraction 12, which contained PRPF8, a core component of U5 snRNP. In contrast, for PRPF6 and hDib1 reporter proteins, the lighter fractions from 3–5 (Fig. 4A) were active, and fraction 4, lacking PRPF8, generated the peak luciferase activity. A very small peak was detected in fraction 12. Thus, PRPF6 and hDib1 reporter proteins reconstitute luciferase activity by direct binding to each other, and the incorporation of reporter proteins into the snRNP is not required. These findings are consistent those of another study [19] and our *in vitro* experiment.

Next, we determined whether the complex yielding the luciferase activity generated by PRPF6 and U5-40K reporter proteins involved formation of U5 snRNP. Glycerol gradient fractions of a nuclear

extract prepared from 293T cells expressing PRPF6 and U5-40K reporter proteins were analyzed. The position of each snRNP was determined by Northern blotting of snRNA, and PRPF8 and PRPF3 were used as markers for U5 snRNP and U4/U6 snRNP, respectively. PRPF6 and U5-40K reporter proteins in fraction 14, which contains PRPF8 and U5 snRNA but not U4/U6 snRNP, generated luciferase activity (Fig. 4B). Western blotting showed that both reporter proteins were present in fractions containing U5 snRNP (fractions 13–15) and U4/U6.U5 tri-snRNP (fractions 17 and 18). However, luciferase activity was undetectable in the latter. The structure of luciferase fragments may not be suitable for reconstituting the activity in tri-snRNP. For example, deletion of the N-terminus of PRPF6 and the phosphorylation sites within inhibits the growth of *Saccharomyces pombe* [26], suggesting that proteins binding at the N-terminus of PRPF6 exist. These proteins may interfere the reconstitution of luciferase activity in tri-snRNP because a larger fragment of luciferase was fused to the N-terminus of PRPF6. The result suggests that the PRPF6 and U5-40K reporter proteins reconstitute luciferase activity only in the free U5 snRNP-like complex.

To further validate the identity of the complex that generated luminescence, we established a cell line stably expressing PRPF6 and U5-40K reporter proteins (Supplementary Fig. 3A) and purified the reporter protein complex using two-step affinity purification, because each reporter protein contained different affinity tags. The purified complex retained luciferase activity (data not shown), suggesting

that it maintained its active conformation. The proteins that had been copurified with the reporter proteins were identified as PRPF8, SNRNP200, EFTUD2, and PRPF6 reporter fusion proteins. Components of U4/U6 snRNP and tri-snRNP-specific proteins were not detected (Supplementary Fig. 4). From the results of density gradient analysis, we concluded that PRPF6 and U5-40K reporter proteins reconstituted the luciferase activity in the free U5 snRNP.

To test whether the reporter assay detects changes in the U5 snRNP levels, we first checked the stability of luciferase activity among stable clones expressing reporters for four hours after cyclohexamide treatment to inhibit protein synthesis (Supplementary Fig. 3B). Cells that express high and stable levels of a target reduce the frequency of false positives at screening for small molecule inhibitors. We treated the cell line with actinomycin D to inhibit transcription so as to deplete pre-mRNAs, resulting in the accumulation of excess amounts of tri-snRNP and free U5 snRNP. Alternatively, inhibition of transcription by actinomycin D can inhibit the formation of the tri-snRNP formation at the coiled body, because active transcription is required to maintain U6 snRNA levels [27; 28] and to form this nuclear substructure [29; 30]. In either case, we predicted that actinomycin D should increase reporter activity. Indeed, actinomycin D treatment induced an approximate 2-fold increase over the control value (Fig. 5A), and no change was detected in the reporter protein expression.

We next tested nineteen inhibitors (listed in Table I), none of which increased luciferase activity to the same extent as actinomycin D (Fig. 5B). In particular, treating cells with DMSO for six hours increased reporter activity by a factor of approximately 1.3. The split luciferase activity decreased gradually in the order of extract preparation. To avoid the effects of systematic errors on the identification of active compounds, we focused on time-dependency of these compounds' effects as well as fold-change of reporter activity (Fig. 5). Five compounds were identified as the inhibitors satisfying both criteria. Interestingly, treatment with either trichostatin A or nocodazole reduced reporter activity; however, the reduction was reversed after six hours by a mechanism that remains to be determined. Because the histone deacetylase inhibitor trichostatin A [31] inhibits *in vitro* splicing at a high concentration (~1 mM) [32], this may have affected reporter activity. However, the concentration of trichostatin A used here was much lower than the *in vitro* study. A more likely explanation is that transcription from the CMV promoter used to drive the reporter gene expression is upregulated and caused increased reporter activity.

MG132 was the strongest inhibitor of luciferase activity after incubation for six hours. It is difficult to explain this result, because a proteasome inhibitor induces the accumulation of proteins that would be otherwise degraded by the proteasome, and a simple prediction for the effect of MG132 treatment is no change or a slight increase of luciferase activity caused by accumulation of the reporter

proteins.

To determine whether the reduction of reporter activity by MG132 treatment reflected the reduction of the level of the U5-snRNP reporter complex, we performed glycerol gradient sedimentation analysis. (Fig. 6A) These experiments show that MG132 treatment decreased luciferase activity in the peak fraction and that actinomycin D increased luciferase activity in fractions eluting around the peak fraction. However, neither MG132 nor actinomycin D changed the distribution and peak of luciferase activity compared with those in DMSO-treated cells. These findings suggest that the changes in reporter activity induced by these drugs were not caused by formation of an aberrant complex.

Because it was possible that MG132 directly affected luciferase activity, we assessed the physical interactions of OC-U5-40K with another reporter protein, NO-PRPF6, as well as other snRNP proteins. FLAG-tagged OC-U5-40K was immunoprecipitated by an anti-FLAG antibody, and the coprecipitated proteins were analyzed by western blotting. In the extracts prepared from cells treated with MG132, the extent of coprecipitation of the NO-PRPF6 reporter protein were reduced to 50% of the control level, which is consistent with the reduction of luciferase activity. Further, when we determined whether endogenous PRPF6, PRPF3, and PRPF8 coprecipitated with OC-U5-40K, there were no changes in the coprecipitation efficiency of the proteins detected. These results suggest that MG132

treatment affects the levels of the free U5 snRNP reporter complex, likely through the change in efficiency of incorporation or stability of the NO-PRPF6 reporter protein in the U5 snRNP complex that occurs by an unknown mechanism. In the MG132-treated cell extract, the extent of coprecipitation of the NO-PRPF6 reporter protein was reduced to 50% of the control level, which is consistent with the reduction of luciferase activity (Fig. 6B). Further, we determined whether endogenous PRPF6, PRPF3 and PRPF8 coprecipitated with OC-U5-40K and no changes were shown in coprecipitation efficiency of all proteins detected. These results suggest that MG132 treatment affects the levels of the free U5 snRNP reporter complex probably through the change of efficiency of incorporation or stability of the NO-PRPF6 reporter protein in the U5 snRNP complex by unknown mechanisms.

In conclusion, we show here that the split-luciferase system detected the U5 snRNP macromolecular complex, and that the reporter activity reflected the change of the cellular levels of the U5 snRNP reporter complex caused by treatment with actinomycin D and MG132.

However, there are some limitations to this approach. For example, to apply this method to other complexes, a significant issue is finding a functional reporter-gene pair. Because there is no rational strategy to design a functional reporter protein, it is necessary to prepare a library of expression vectors for the luciferase fragment-fusion proteins and to screen the library, which would be

time-consuming. However, this may be advantageous, because random screening of a gene pair that reconstituted reporter activity clearly shows that a precise understanding of the structure of the complex is not necessary. Only knowledge of the complex's components is required.

There are specific problems with the reporter system described here. For example, two U5 snRNP components were employed (PRPF6 and U5-40K). Because PRPF6 is required to form tri-snRNP by acting as an adaptor between U4/U6 and U5 snRNP, reporter activity can be affected when the U5 snRNP level is changed or when the efficiency of incorporation of NO-PRPF6 into U5 snRNP is changed. Further, the fusion of a large N-terminal fragment of luciferase may interfere with the normal function of PRPF6, which would alter the turnover rates between the native U5 snRNP and the U5 snRNP-reporter protein complex. Therefore, the reporter is useful for identifying conditions that could change the U5 snRNP levels; however, it is necessary to confirm U5 snRNP levels by other methods that detect the level of the complex. Despite such a limitation, the sensitivity and a small-scale format of our assay are suitable for HTS. It will be interesting to use our reporter to screen a library of small molecules to discover those that affect cellular U5 snRNP levels.

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Figure legends

Fig. 1. Strategy to detect snRNP formation using split luciferase. The products encoded by a gene pair fused to the N- or C-terminal domains of luciferase should not directly bind each other, although both are components of a specific snRNP. This insures that reconstitution of luciferase activity occurs only when both reporter fusion proteins are incorporated into the same snRNP complex, and the activity reflects the cellular snRNP level.

Fig. 2 Schematic structure of split-luciferase reporters for detecting snRNP. Sequences encoding a target protein represented as “prpX” are cloned into the *EcoRI* and *XhoI* sites in all four reporter constructs, resulting in the fusion of prpX with luciferase N- or C-terminal domains in each indicated orientation. The target protein and luciferase are separated by the peptide sequence GTGGGGSGGGGS.

Fig. 3 Screening of gene pairs for split-luciferase activity. The split-luciferase activity of 293T cells

transiently transfected with reporter plasmids (as indicated) were compared. PRPF6;hDib1 and PRPF6;U5-40K were identified as the gene pair that reconstituted luciferase activity.

Fig. 4 Distribution of the luciferase activity in a glycerol gradient. (A) Whole cell extracts transiently expressing the indicated split-luciferase reporters were applied to the 10-40% glycerol gradient and the ratio of the luciferase activity in each fraction to total activity is plotted. The snRNP components were detected by western blotting of each fraction. Exogenous reporter-fusion PRPF6 and U5-40K constructs are indicated as NO-PRPF6 and OC-U5-40K. (B) The distribution of luciferase activity in the nuclear extract of a stable transfectant of NO-PRPF6 and OC-U5-40K was analyzed same as shown in (A). In addition to proteins in snRNP, U4, U5, and U6 snRNA were also detected by northern blotting. Biotin-labeled hybridization probes were used, and the hybridized membrane was reacted with streptavidin-conjugated Alexa Fluor 680 to detect snRNA.

Fig. 5 (A) Cells stably transfected with the U5 snRNP reporter were treated with actinomycin D, and the expression levels of reporter proteins were detected using western blotting. ($n = 3$, $**P < 0.01$ unpaired Student t test, two-tailed). (B) The stable transfectant was treated with known inhibitors for one or six hours, and the luciferase activity was determined. The histogram represents the mean of

three independent experiments performed in duplicate. The error bar shows the standard error of the mean ($n = 3$, * $P < 0.05$, ** $P < 0.01$ versus DMSO). Numbers below the histogram indicate the ratio of time-dependent changes of luciferase activity, and SDs are shown in brackets ($^{\#}P < 0.05$, $^{##}P < 0.01$ versus DMSO).

Fig. 6 (A) Whole cell extracts prepared from MG132- or actinomycin D-treated 293T-U5-luc cells were fractionated through a glycerol gradient. The luciferase activity in each fraction was measured and plotted in the upper panel. The distribution of OC-U5-40K is shown below. (B) The OC-U5-40K reporter protein was immunoprecipitated by anti-FLAG antibody, and bound proteins were analyzed using western blotting.

Supplementary Fig. 1 Schematic structure of split-luciferase reporters containing PRPF6.

Supplementary Fig. 2 (A) To confirm protein expression of in vitro-synthesized proteins used in (B), synthesized proteins were separated using SDS-PAGE and detected by western blotting. (A) Reporter proteins that were positive in transient transfection assays were synthesized in vitro and mixed to determine their ability to reconstitute luciferase activity in vitro.

Supplementary Fig. 3 (A) Hygromycin-resistant stable transfectants of the reporter plasmids were selected, and analyzed for luciferase activity. (B) Luciferase activity and reporter-protein expression (NO-PRPF6 and OC-U5-40K) of four clones were determined after cyclohexamide treatment.

Supplementary Fig. 4 Proteins in the affinity-purified reporter complex were resolved using SDS-PAGE and detected by silver staining. OC-U5-40K (lane 1) was bound to StrepTactin Sepharose using the One-STrEP tag, and NO-PRPF6 was immunoprecipitated by an anti-FLAG antibody (lane 2) from the whole cell extract prepared from cells stably transfected with the reporter transfectant expression vector. The protein obtained using the sequential two-step affinity purification using both anti-FLAG antibody and StrepTactin Sepharose is shown in lane 3.

Compounds	Final concentration
ActinomycinD	1 μ M
DRB	10 μ M
Camptothecin	2 μ M
TrichostatinA	1 μ g/ml
LeptomycinB	1 nM
MG132	20 μ M
Nocodazole	1 μ g/ml
Mimosine	5 μ M
D4476	2 μ M
LY83583	0.5 μ M
SO22536	2 μ M
KN-93	0.1 μ M
STO-609	100 μ g/ml
Roscovitine	28.2 μ M
PD98059	20 μ M
SB203580	1 μ M
K252a	1 μ M
JIP-1 peptide	0.51 μ M
Akt1/2 inhibitor	2.1 μ M

Table1

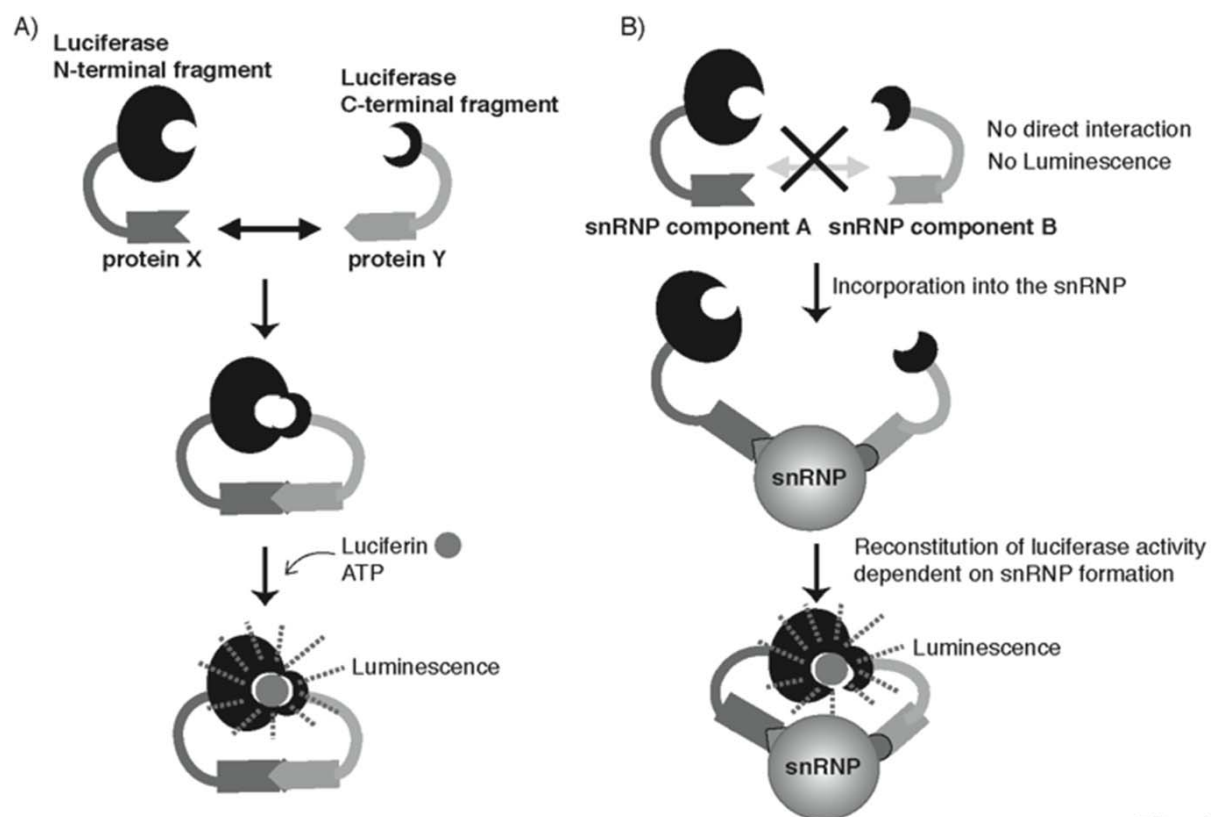


Fig. 1

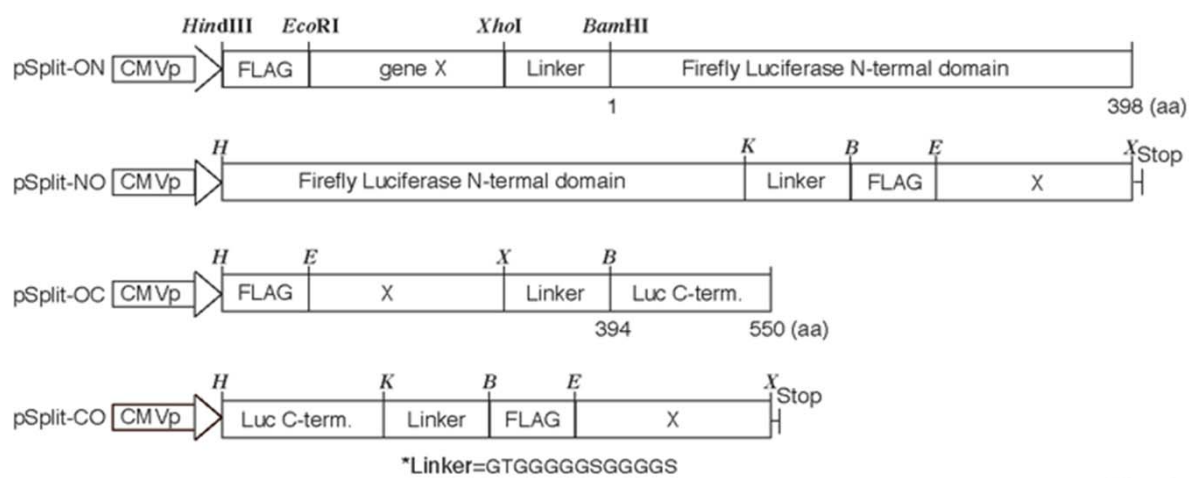


Fig. 2

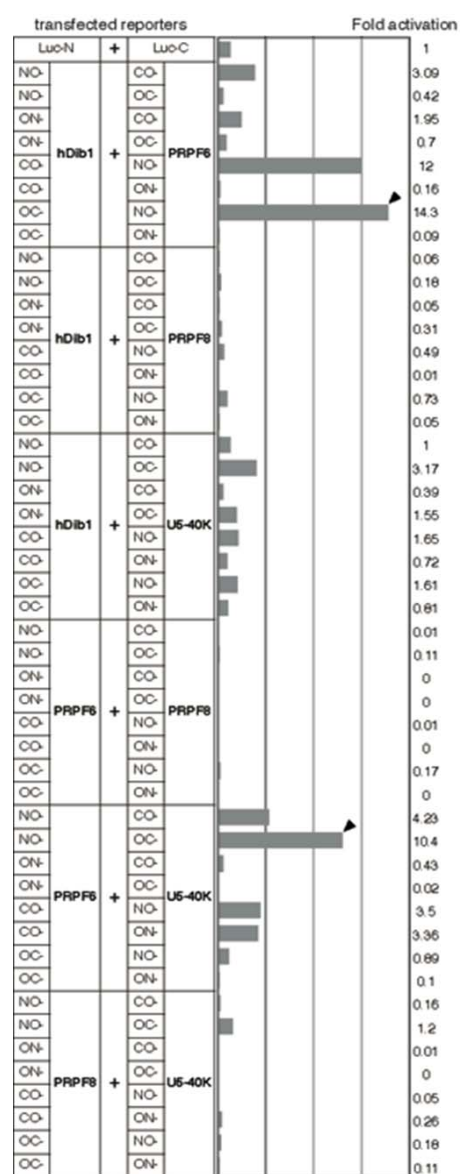


Fig. 3

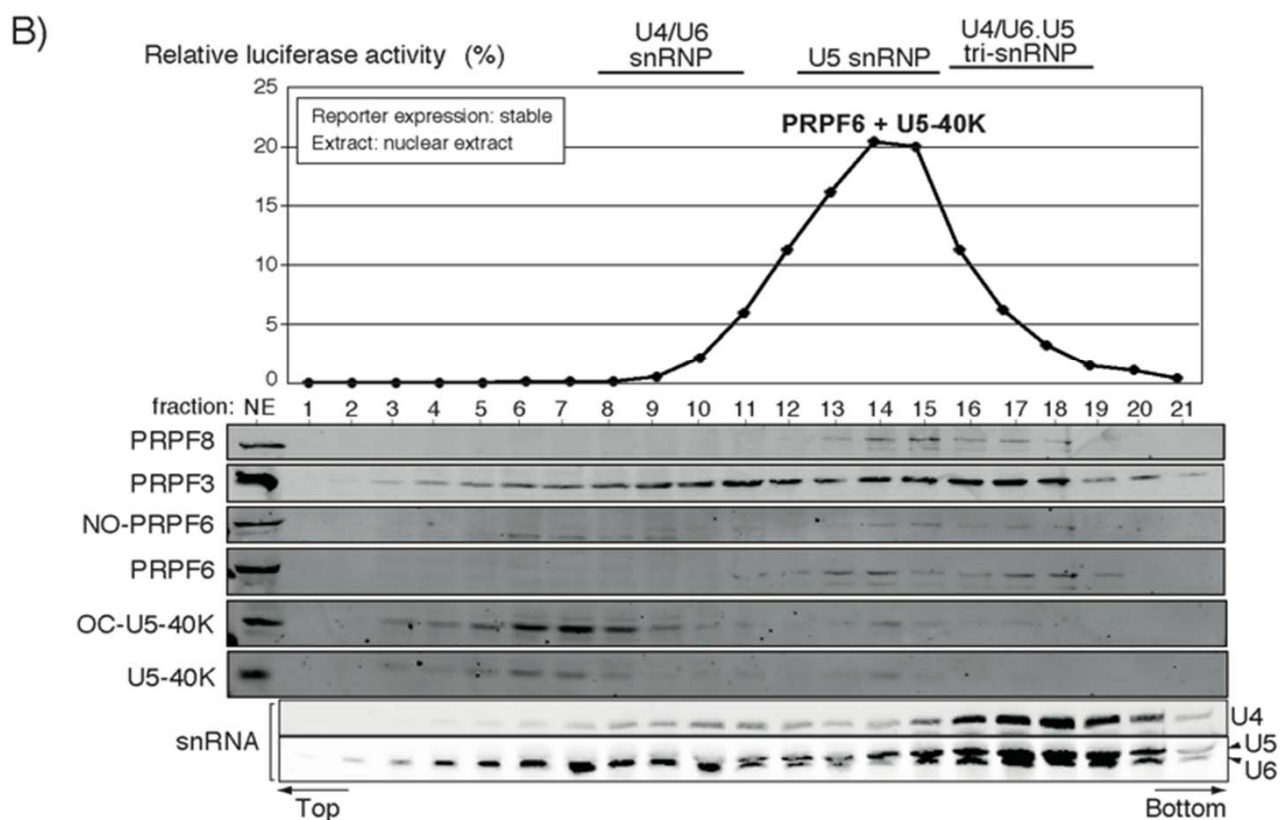
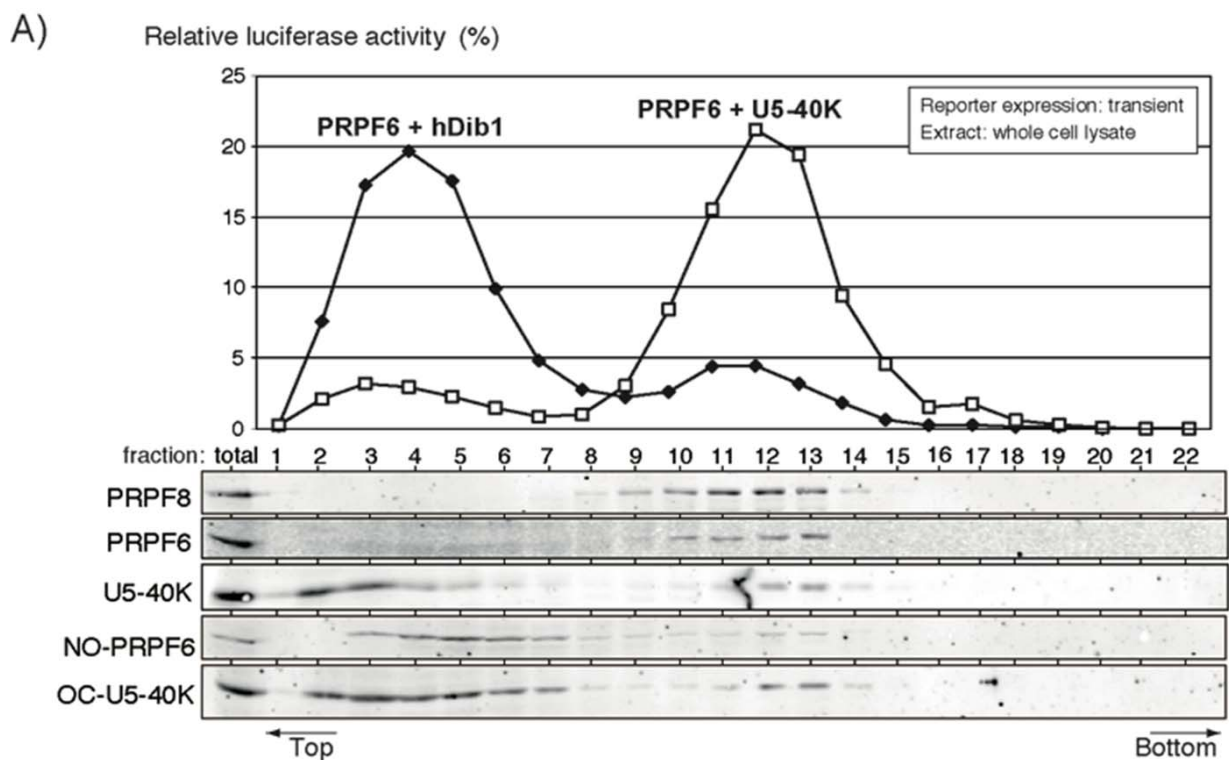


Fig. 4

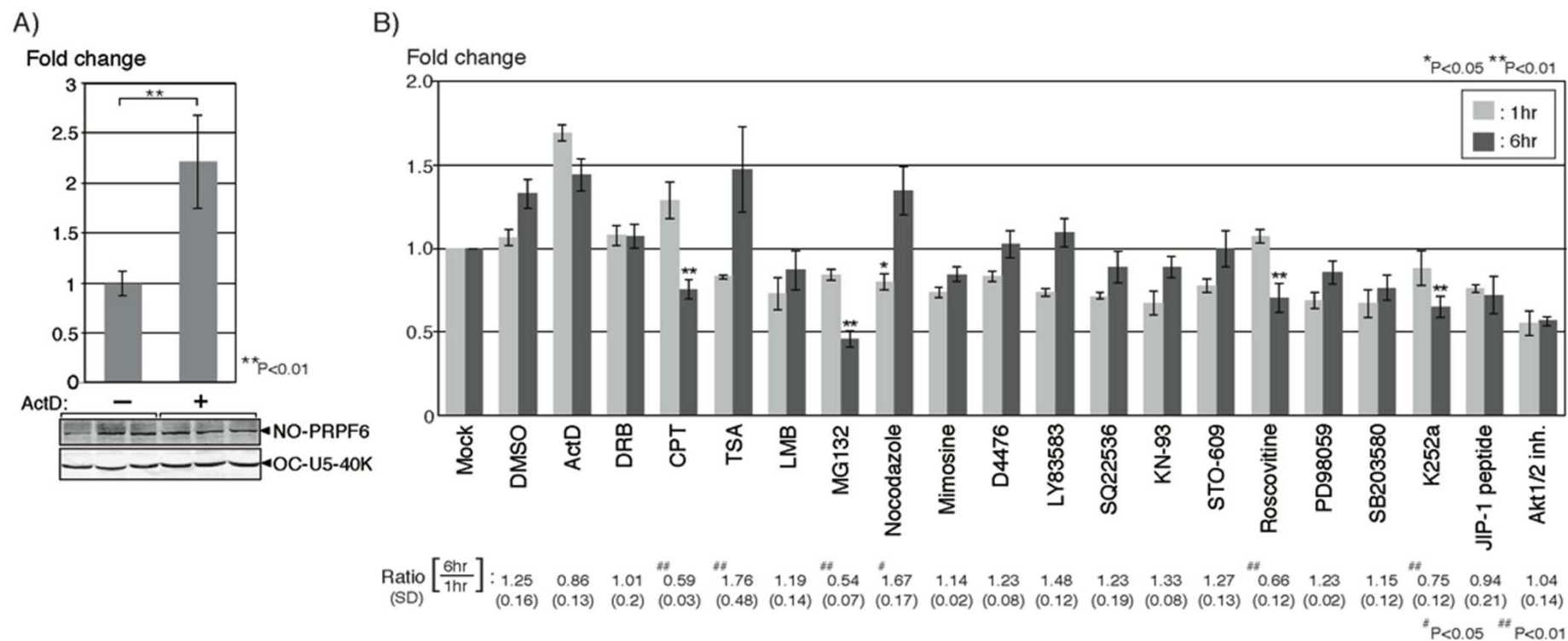
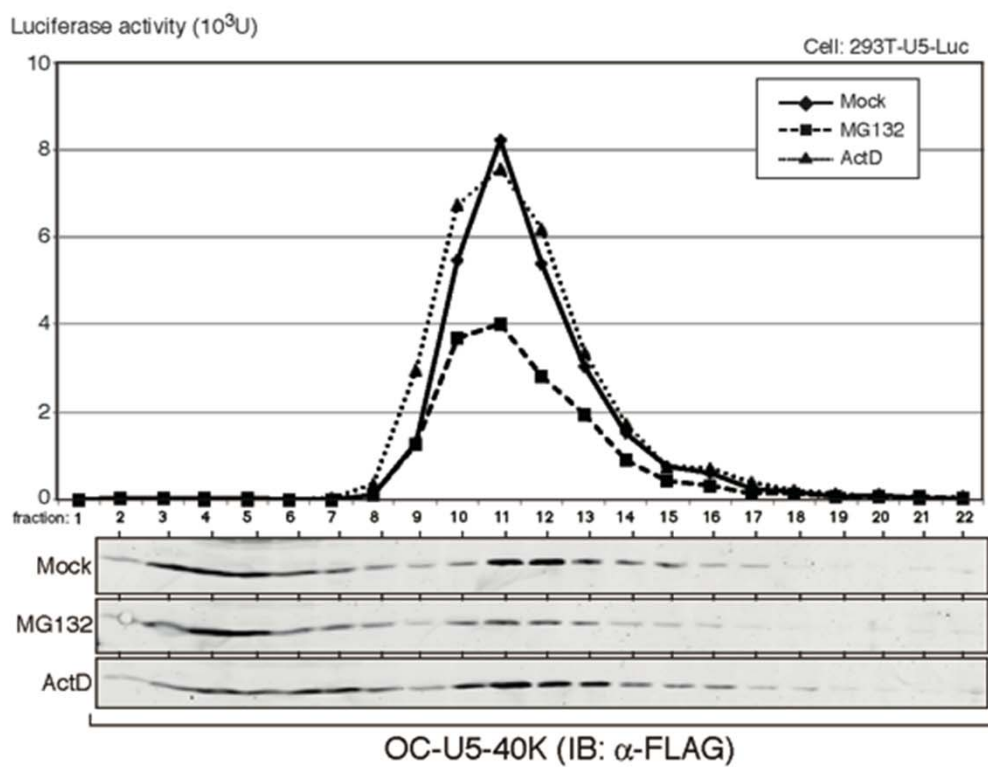


Fig. 5

A)



B)

