Effective cell-free drug screening protocol for protein-protein interaction

Shaked Ashkenazi[1], Alexander Plotnikov[2], Anat Bahat[1] and Rivka Dikstein[1]*

Running title: HTS protocol for protein-protein interaction drugs

Key words: High throughput drug screen; HTS, protein-protein interaction; NF-κB; p65; RelA

[1]Department of Biomolecular Sciences, The Weizmann Institute of Science, Rehovot 76100, Israel.

[2]The Nancy and Stephen Grand Israel National Center for Personalized Medicine, The Weizmann Institute of Science, Rehovot 76100, Israel

*Corresponding author: Tel: 972-8-9342117; E-mail: rivka.dikstein@weizmann.ac.il

Abstract

Specific protein-protein interaction (PPI) is an essential feature of many cellular processes however, targeting these interactions by small molecules is highly challenging due to the nature of the interaction interface. Thus screening for PPI inhibitors requires enormous number of compounds. Here we describe a simple and improved protocol designed for a search of direct PPI inhibitors. We engineered a bacterial expression system for the split Renilla luciferase (RL) complementation assay that monitors PPI. This enables production of large quantities of the RL fusion proteins in a simple and cost effective manner that is suitable for very large screens. Subsequently, inhibitory compounds are analyzed in a similar complementation assay in living cultured mammalian cells to select for those that can penetrate cells. We applied this method to NF-κB, a family of dimeric transcription factors that plays central roles in immune responses, cell survival and aging, and its dysregulation is linked to many pathological states. This strategy led to the identification of several direct NF-κB inhibitors. As the described protocol is very straightforward and robust it may be suitable for many pairs of interacting proteins.

Introduction

Specific protein-protein interactions (PPI) are essential for all cellular functions, among them signaling pathways and gene expression[1–6] but they are rarely targeted by drugs. For example, transcription factors were considered “un-druggable” for a long time despite the pivotal role they play in determining the outcome of different cellular events. However, recent studies have demonstrated that direct targeting of transcription factors is indeed possible, partly thorough interfering with oligomerization, which is typical to many transcription factors[7–11]. As transcription factors are highly specific in their activity, drugs developed this way are likely to be more specific and to produce less side effects compared with strategies that interfere with the pleiotropic signaling pathways[9,11]. Given that PPI involves multiple contact sites, identifying small molecules that can disrupt these contacts effectively is challenging[6]. One solution is to perform large screens of small molecule libraries. Such screens require large quantities of the target proteins that are difficult to achieve using mammalian cell based assays.

In this work we present an improved multi-step protocol for a high throughput drug screen, aimed to identify inhibitors of PPI, utilizing the split Renilla luciferase (RL) complementation assay. This protocol has several major advantages: it is based on a cell-free assay that uses bacterially expressed recombinant proteins. As such it is simple, rapid and yields large quantities of target proteins suitable for high throughput screens (HTS), and therefore it is cost effective. In addition, it enables focusing on direct PPI inhibitors without the involvement of other cellular aspects that can indirectly affect the target proteins. As the protocol involves an additional step of a low-throughput assay in living mammalian cells, it selects for those compounds that can penetrate cells.

We successfully applied this protocol to NF-κB, which requires dimerization for proper DNA binding and trans-activation [1,12–18]. NF-κB plays key roles in regulation of the immune

system, inflammation and stress response. Its deregulation is linked to a variety of pathological states such as chronic inflammation, autoimmune diseases and cancer, which makes it a prominent target for drug development[18–22]. We identified several direct NF-κB dimerization inhibitors, one of which is Withaferin A[8]. While this protocol was developed for NF-κB it is likely to be suitable for many pairs of interacting proteins.

Materials and methods

Plasmid Construction

To construct the mammalian expression plasmids of the split-RL, we used the RSV-Renilla (pRL-null, Promega) as a backbone for all constructs applying the RF-cloning procedure[23] in several steps: first, we introduced the linker GGTGGCGGAGGGAGC, corresponding to amino acids GGGGS between position 687 and 688 of the RL nucleotide sequence (primer: 5'- CCGTTAGTAAAAGGTGGTGGTGGCGGAGGGAGCAAACCTGACGT TGTACAA -3' and its reverse complement sequence as a reverse primer). Second, we removed either the C-terminus of the protein, leaving the N-terminus (1-229) and the linker or removing the N-terminus and leaving a newly introduced ATG, the linker and the C-terminus of the protein (230-311) as suggested by Jiang et al. [24] . Primers: for removing the C-terminus 5'- GGTGGCGGAGGGAGCTAATTCTAGAGCGGCCGCTTCGAGCAG-3' and its reverse complement; for removing the N-terminus 5'-GACTCACTATAGGCTAGCCACCATGGGTGGCGGAGGGAGC-3' and its reverse complement. Third, we added p65 (1-320) to create the fusion proteins p65-linker-C-RL and N-RL-linker-p65 (primers: 5'-CTCACTATAGGCTAGCCACCATGGACGAACTGTTCCCCCTCATC-3', 5'-CACCGCTCCCTCCGCCACCTCCGCTGAAAGGACTCTTCTTC-3' and 5'-GGTGGCGGAGGGAGCATGGACGAACTGTTCCCCCTC-3', 5'-GAAGCGGCCGCTCTAGAATTATCCGCTGAAAGGACTCTTCTTC-3' respectively. In order to construct the two additional fusion proteins (p65-linker-N-RL and C-RL-linker-p65, Fig. 1A) we used the existing constructs and replaced the N-RL with C-RL and vice versa along with appropriate start and stop codons (primers: 5'-CTCACTATAGGCTAGCCACCATGAAACCTGACGTTGTACAAATT-3', 5'-GCCACCGCTCCCTCCGCCACCTTGTTCATTTTTGAGAACTCGCTC-3' and 5'-AGCGGTGGCGGAGGGAGCAAAATGACTTCGAAAGTTTATGAT-3', 5'-

CTCGAAGCGGCCGCTCTAGAATTAACCACCTTTTACTAACGGGAT-3'). When using two different proteins all 8 pairs should be evaluated accordingly. After validating the constructs and choosing the best pair, we removed positions 299-320 of p65, which include the intrinsic nuclear localization signal in order to avoid nuclear accumulation. This was only performed on N-RL-p65 and C-RL-p65 that together gave the best RL signal (primers: 5- GACGATCGTCACCGGATTGAGTAATTCTAGAGCGGCCGCTTCGAG-3' and its reverse complement). Next, we added a 6xHis tag to the C-terminus of the fusion protein N-RL-p65 and a flag tag to the C-terminus of C-RL-p65. To introduce the 6xHis tag we used an internal forward primer from p65 5'-TCTATGAGGCTGAGCTCTG-3' and the reverse primer which included the 6xHis sequence 5'-GCCGCTCTAGAATTAGTGGTGATGGTGATGATGCTCAATCCGGTGACG-3'. Primers for the addition of a flag tag – 5'- ACGATCGTCACCGGATTGAGGATTACAAGGATGACGAC-3' and 5'- GAAGCGGCCGCTCTAGAATTACTTATCGTCGTCATCCTT-3'. The luminescence activity was measured in order to make sure that the tags do not interfere with the interaction (Fig. 1C).

For the bacterial expression plasmid we used the RSV-N-RL-p65 and RSV-C-RL-p65 as template to transferred them into pRSF-duet (Merck-Novagen). N-RL-p65 was cloned downstream to the internal 6xHis tag of the plasmid (primers: 5'-CATCACCATCATCACCACAGCCAGGATCCGATGACTTCGAAAGTTTATGATC -3', 5'-TCGACTTAAGCATTATGCGGCCGCAAGCTTTTACTCAATCCGGTGACGATCG -3'). C-RL-p65 was cloned together with the newly introduced flag tag (primers: 5'-ATTAGTTAAGTATAAGAAGGAGATATACATATGAAACCTGACGTTGTACAAATTG-3', 5'-CGCAGCAGCGGTTTCTTTACCAGACTCGAGTTACTTATCGTCGTCATCCTTGTAATC-3'). The NF-κB dependent A20-Luciferase was previously described[25].

Protein expression and analysis

Transformed BL-21 bacteria were grown in 37° up to O.D ~ 0.6, after which 1mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added. Cells were incubated in 37°C for 3 hours and then harvested in a buffer containing 20mM Tris pH 8, 150mM NaCl, 10% glycerol, 0.5% NP-40, 2mM EDTA, 1% cocktail of protease inhibitor (Sigma-Aldrich) and 1% PMSF. Cell suspension was sonicated for 12 cycles of 45 seconds followed by 60 seconds interval at high intensity and then centrifuged at 13,000 rpm for 30 minutes. The supernatant was then analyzed for luminescence activity and Western blot with anti-p65 antibodies (Sc-8008, Santa Cruz Biotechnology). The amount of the RL-p65 proteins in the crude lysate was estimated to be 0.1 µg/ml according to quantitative western blot with purified p65 as standard. For the drug screen we used ~0.1 ng of RL-p65 per well.

Cells, transfections and extract preparations

HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Transfection into these cells and A20-luciferase reporter gene analysis were done as previously described[25]. Whole-cell extract was prepared using Reporter Lysis Buffer® (Promega).

HTS protocol

1536 well plate (Nunc) were pre-plated with DMSO (vehicle control) and about 45000 compounds in a final concentration of 10µM using Echo® 550 Liquid Handler (Sunnyvale, CA, USA). 5µl of the bacterial lysate containing split-RL-p65 proteins (diluted 1:10 in phosphate buffer containing 80mM K_2HPO_4 and 20mM KH_2PO_4) were added using GNF system (San Diego, CA, USA). The lysates were spun down and incubate for 15 minutes at room temperature in the dark. Subsequently, 5µl of Coelenterazine (CTZ, Gold Biosciences, 5µg/ml) in phosphate buffer was added using the GNF liquid handler and

plates were incubated for 12 minutes at RT. Luminescence signals were detected by luminescence module of PheraStar FS plate reader (BMG Labtech, Ortenberg, Germany). Compounds that reduced the luminescence activity by 30% were selected for a dose response assay of 0.3μM, 1μM, 3μM, 10μM and 30μM. For this assay we used the same split-RL-p65 lysate along with bacterial lysate expressing recombinant full-length RL that served as a control for compounds that inhibit the RL enzymatic activity rather than p65 dimerization.

For the live cells split-RL assay HEK293T cells were transfected with 350ng/ml of each of the split-RL plasmid. Twenty-four hours later cells were washed once with PBS (Sigma-Aldrich) and then detached by trypsin B (0.25%, Biological Industries), followed by centrifugation and resuspension of the cells in PBS. 384 well plates (Greiner) were pre-plated with the selected compounds to a final concentration of 0.9μM, 1.8μM, 3.75μM, 7.5μM, 15μM, 30μM and 60μM. 8000 cells (diluted in phosphate buffer) were distributed into each well. Cells were incubated with gentle shaking for 1 hour at room temperature, after which 20μl of CTZ were added for an additional 15 minutes incubation in the dark and then luminescence was measured. Trypan Blue viability assay (Sigma-Aldrich) was performed at the end of experiment. We found that that more than 95% of cells were alive.

The analysis of the luminescence data for all the above-described assays was performed using GeneData software (Basel, Switzerland).

Results and discussion

Design and preparation of the split-Renilla system

One of the approaches to study interactions between a pair of known proteins is by complementation assays. These assays employ a fusion between target proteins and fragments of a reporter gene: when the two target proteins associate the two fragments of the reporter become adjacent to each which can result in reconstitution of their activity and production of a measurable signal[3,8,24,26,27]. This method can be used in high throughput screens aimed to discover new inhibitors of specific protein-protein interactions[6,27–30]. The present protocol is based on the split-Renilla complementation system, which is a highly sensitive and quantitative method for recording protein-protein interaction. Renilla luciferase (RL) is an enzyme that oxidizes coelenterazine (CTZ) to produce luminescence. In this system the RL enzyme is divided into N- and C-terminal fragments (1-229 and 230-311, respectively) and fused to target proteins along with a flexible linker[24]. Association of the target proteins has the potential to bring the two RL fragments in close proximity, which can restore the enzymatic activity. The innovative aspect of this protocol is the integration of two screens: (i) the use of a cell-free split-RL assay with recombinant target proteins that are produced in large amounts, as required for HTS, in a simple, fast and cost effective manner; (ii) a subsequent live-cell-based assay to select for those direct PPI inhibitors that can also penetrate mammalian cells.

The first stage of the protocol is the construction of the split-RL pair. As it is difficult to predict the best spatial organization of the interacting proteins that allows the reconstitution of the RL activity, it is necessary to clone each of the target protein next to the two split-RL fragments either at the N- or the C-terminal sides (8 plasmids). We applied this method to NF-κB transcription factor in which its activity is dependent on the formation of dimers between NF-κB family members[16,31,32]. Their dimerization is mediated by a highly

conserved Rel homology region (RHR) that is susceptible to mutations in two separate domains[8]. To simplify the procedure we focused on p65 homodimers and tested all four combinations of pairs in transfected HEK293T cells (Fig 1A and Fig 1B). To verify the specificity of the interaction between the p65 proteins we compared the luminescence achieved by split-RL-p65 pairs to pairs containing only the RL counterparts ("empty"). We also analyzed pairs that contain one RL-p65 fusion protein and one "empty" RL protein. The latter pairs consistently yielded lower levels of luminescence than the "empty" pair most likely since the RL-p65 halves formed dimers, making them less available for random interactions. This phenomenon is not expected to be a major issue when studying a complex of two different proteins. Of the four possible pair combinations of RL-p65 fusion proteins only two pairs resulted in a signal that is significantly above the negative controls, i.e. N-RL-p65 / C-RL-p65 and N-RL-p65 / p65-C-RL. Removal of the nuclear localization signal (NLS) of p65 in order to avoid nuclear accumulation resulted in substantially improved signal (Fig. 1C). Addition of His Flag tags to the RL-p65 pairs did not compromise the luminescent signal (Fig. 1C). A significant decrease in luminescence signal was observed when each p65 monomer bears the point mutation E211A that is known to be deleterious for dimerization[8,15]. The utilization of mammalian expression system for the initial analysis is slightly faster than expressing all these plasmids in bacteria and is also useful in the second part of the screen.

Following the identification of the best split-RL dimerization pair, the next step is to create a single bacterial expression plasmid (pRSFDuet, Merck-Novagen) for both N-RL-p65 and C-RL-p65 fusion proteins each with a different tag (Fig. 2A). This is a suitable system for equivalent expression levels, detection of the target proteins by western blot and easy purification if needed. The expression of the two fusion proteins was even, as validated by western blot using anti-p65 antibody (Fig. 2B). The bacterial cells were lysed in a buffer that is suitable for the RL enzymatic activity and luminescence was measured

(Fig. 2C). As the luminescence signal obtained with the crude bacterial lysate was very high, no further purification for enrichment was required. This straightforward strategy enabled a simple production of a large quantity of the RL fusion proteins from a relatively small amount of bacterial cell culture (50-1000 ml) that was sufficient for a large drug screen.

For successful screening it is important to determine in advance that the interaction between the target proteins is dynamic and can be interrupted. For the split-RL-p65 we conducted a competition with a recombinant fragment of p65 N-terminus (1-298, Fig. 2A). First, we estimated the relative amount of p65 in the lysate and then added increasing amount of p65 to split-RL-p65 lysate which (Fig. 2B and Fig. 2C). The lysates were incubated for 15 minutes before the addition of CTZ substrate and further incubated for 12 minutes in the dark. We observed a concentration dependent reduction of the luminescence activity, indicating that the free p65 effectively competes for the split-RL-p65 dimer. This result confirms that p65 dimerization has the potential to be disrupted. We also prepared bacterial cells that express full-length RL for calibrating the detection system and as a negative control (Fig. 2A).

Assay development

To apply the split-RL for a large screen it is possible to use 384 or 1536 well plates. The assay should be calibrated according the size of the well. For the RL-p65 pair the volume for 384 and 1536 well plate was set to be 40µl and 10µl, respectively, where the ratio of the lysate to the substrate was determined to be 1:1.

From our experience it is important to optimize the dynamics of the RL light production activity for different RL fusion pairs. Following the addition of the CTZ substrate the signal should be monitored every 1 minute for 30 minutes in the dark. For RL-p65 pair, the light intensity is the highest within the first 1 minute of the reaction and drops gradually

over the course of the next 30 minutes (Fig. 2D). Considering the time required for reading an entire plate in our setting (4 minutes) we chose to perform the reading of the screen 12 minutes after the addition of the CTZ, in which the reduction in the light activity was moderate. We set the dispensing and reading in same direction (row wise, left to right); however, some decrease of luminescence signal was indeed observed in the right bottom part of the plate. It was taken into account in the data analysis, where we normalized the data in each row to its own control.

HTS protocol

For the split-RL-p65 pair we used 1536 well plates (Nunc) which were pre-plated with DMSO as a vehicle control and ~45,000 compounds to a final concentration of 10μM. However, it is may be better to use higher concentrations of the drug up to 50μM. 5μl of diluted split-RL-p65 lysate were added to appropriated wells. The plates were spun down and incubated for 15 minutes at RT in the dark. 5μl of CTZ in phosphate buffer (5μg/ml) was added and incubated for 12 minutes at RT in the dark following determination of the luminescence activity.

Hits validation

Following data analysis and normalization we selected 380 compounds that reduced the luminescence by >30% for subsequent analysis. When the screen is done with higher concentration of the compounds the number of luminescence inhibitory compounds is expected to rise and the threshold for compound selection should be adjusted. To filter out compounds that affect the RL enzymatic activity the next step is to compare the effect of those small molecules on RL-p65 pair and on full-length RL in 5 concentrations – 0.3μM, 1μM, 3μM, 10μM and 30μM in order to establish a dose-response link. Most of the small molecules (366) were found to inhibit both, which suggests that they inhibit the production

of luminescence, yet, 14 molecules specifically inhibited the split-RL-p65 and were chosen for further analysis. The chemical structures and the IC-50 results of 3 representative compounds are presented in Fig. 3A.

Mammalian cells contain many elements that could influence the ability and the extent by which the identified small molecules can affect the target proteins (i. e. interacting proteins, intracellular processing of the compound and cell permeability). To identify the compounds that can act in the context of mammalian cells we added an additional live-cell screening step. Sub-confluent HEK293T cells were transiently transfected with plasmids containing RL-p65 pair. Twenty-four hours later cells were washed once with PBS and then detached gently using trypsin, which was then replaced with PBS. We assured their viability using trypan blue staining and then determined that 8000 cells/well in a 384 well plate provide adequately measurable luminescence. We distributed 20µl of cells in each well, which was pre-plated in advance with increasing concentrations of the lead compounds to create final concentrations of 0.9µM, 1.8µM, 3.75µM, 7.5µM, 15µM, 30µM and 60µM. Cells were incubated for 1 hour at room temperature and then 20µL of CTZ were added for an additional 15 minutes incubation. Luminescence was measured and IC-50 was determined (Fig. 3B). To validate that the luminescence signal originates from cells, the light activity of the supernatant was also determined and found to be insignificant compared to the signal derived from the cells. As can be seen in Fig. 3B, only 2 out of the 3 split-RL-p65 inhibitors shown in Fig. 3A could also inhibit the cellular activity, suggesting that one of these compounds most likely could not penetrate cells.

To further test the ability of the hits to inhibit p65 activity we determined their inhibitory effect on its transcriptional activity. Sub-confluent HEK293T cells were co-transfected with a firefly luciferase reporter gene driven by the promoter of the NF-κB responsive gene A20 together with expression plasmid of full-length p65. The small

molecules or vehicle (DMSO) were added to the cells six hours after transfection. Cells were lysed 24 hours later and the activity the A20-driven reporter gene was determined (Fig. 3C). The data revealed that only one molecule could inhibit NF-κB activity in a dose dependent manner. This molecule, Withaferin A (WFA) was further validated in additional assays as a direct binder of p65 and an inhibitor of dimerization of p65-p65 homodimer and p65-p50 heterodimer[8]. The one molecule that could not penetrate the cell had no effects on the expression of the reporter gene as expected (Fig. 3B and C). One molecule that inhibited the cellular split-RL-p65 (Fig. 3B, middle panel) had no effect of the reporter gene (Fig. 3C, middle panel). This can be explained either by lack of accessibility to the full-length p65 or that its inhibitory activity is targeted to the split-RL and not p65.

In summary we present here a sequence of steps, schematically shown in Fig. 4, for an upgraded usage of the split-RL system for identification of true PPI inhibitors with high probability. While it is possible to use other reporter genes to search for PPI inhibitors, the remarkable sensitivity of the Renilla activity relative to other reporters such as firefly luciferase, GFP, beta-gal and others, makes it superior for cost-effective large screens. The use of recombinant target proteins in crude bacterial lysate simplifies the method although purification is possible if needed. Furthermore, it enables generation of high quantities of proteins that are suitable for large screens necessary for PPI targets. Most importantly this protocol allows the identification of direct inhibitors with a defined mechanism of action which is difficult to achieve with phenotypic screens. The subsequent screening steps, which are done in living cells, discriminate between true and false hits obtained in the bacterial lysate. The activity of the identified lead compounds should be validated using direct binding experiments as well as appropriate biological and functional studies (Fig. 4). This screening protocol can be easily adapted to any pair of interacting proteins in mammalian cells.

Acknowledgements

This work was supported by grants from the Minerva Foundation (#712278), Israel Science Foundation (#1168/13) and Yeda's CEO Fund (KYY2015-795). R.D. is the incumbent of the Ruth and Leonard Simon Chair of Cancer Research.

References

1. Vallabhapurapu, S. & Karin, M. Regulation and function of NF-kappaB transcription factors in the immune system. *Annu. Rev. Immunol.* **27,** 693–733 (2009).
2. Hirtreiter, A. *et al.* Spt4/5 stimulates transcription elongation through the RNA polymerase clamp coiled-coil motif. *Nucleic Acids Res.* **38,** 4040–51 (2010).
3. Gilad, Y., Shiloh, R., Ber, Y., Bialik, S. & Kimchi, A. Discovering protein-protein interactions within the programmed cell death network using a protein-fragment complementation screen. *Cell Rep.* **8,** 909–921 (2014).
4. Csizmok, V., Follis, A. V., Kriwacki, R. W. & Forman-Kay, J. D. Dynamic Protein Interaction Networks and New Structural Paradigms in Signaling. *Chem. Rev.* **116,** 6424–6462 (2016).
5. Zhang, X., Wang, Y., Wang, J. & Sun, F. Protein-protein interactions among signaling pathways may become new therapeutic targets in liver cancer (Review). *Oncol. Rep.* **35,** 625–638 (2016).
6. Wehr, M. C. & Rossner, M. J. Split protein biosensor assays in molecular pharmacological studies. *Drug Discov. Today* **21,** 415–429 (2016).
7. Hagenbuchner, J. & Ausserlechner, M. J. Targeting transcription factors by small compounds — Current strategies and future implications. *Biochem. Pharmacol.* **107,** 1–13 (2016).
8. Ashkenazi, S., Plotnikov, A., Bahat, A., Ben-zeev, E. & Warszawski, S. A Novel Allosteric Mechanism of NF- □ B Dimerization and DNA Binding Targeted by an Anti-Inflammatory Drug. **36,** 1237–1247 (2016).
9. Emery, J. G., Ohlstein, E. H., Jaye, M., Emery, J. G. & Jaye, M. Therapeutic modulation of transcription factor activity. **22,** 233–240 (2001).
10. Suarez-alvarez, B. *et al.* Inhibition of Bromodomain and Extraterminal Domain Family Proteins Ameliorates Experimental Renal Damage. 1–16 (2016).

doi:10.1681/ASN.2015080910

11. Kostas A. Papavassiliou, A. G. P. Transcription Factor Drug Targets. **5,** 1–5 (2016).
12. Hayden, M. S. & Ghosh, S. Shared principles in NF-kappaB signaling. *Cell* **132,** 344–62 (2008).
13. Tsui, R. *et al.* IκBβ enhances the generation of the low-affinity NFκB/RelA homodimer. *Nat. Commun.* **6,** 7068 (2015).
14. Huxford, T. *et al.* Solvent Exposed Non-contacting Amino Acids Play a Critical Role in NF-κB/IκBα Complex Formation. *J. Mol. Biol.* **324,** 587–597 (2002).
15. Huxford, T., Huang, D.-B., Malek, S. & Ghosh, G. The Crystal Structure of the IκBα/NF-κB Complex Reveals Mechanisms of NF-κB Inactivation. *Cell* **95,** 759–770 (1998).
16. Huxford, T. & Ghosh, G. A Structural Guide to Proteins of the NF- B Signaling Module. *Cold Spring Harb. Perspect. Biol.* **1,** a000075–a000075 (2009).
17. Grivennikov, S. I., Greten, F. R. & Karin, M. Immunity, inflammation, and cancer. *Cell* **140,** 883–99 (2010).
18. Ben-Neriah, Y. & Karin, M. Inflammation meets cancer, with NF-κB as the matchmaker. *Nature immunology* **12,** 715–23 (2011).
19. Mantovani, A. *et al.* Cancer-related inflammation. *Nature* **454,** 436–44 (2008).
20. Colotta, F., Allavena, P., Sica, A., Garlanda, C. & Mantovani, A. Cancer-related inflammation, the seventh hallmark of cancer: Links to genetic instability. *Carcinogenesis* **30,** 1073–1081 (2009).
21. Nagel, D., Vincendeau, M., Eitelhuber, a C. & Krappmann, D. Mechanisms and consequences of constitutive NF-κB activation in B-cell lymphoid malignancies. *Oncogene* **33,** 1–11 (2014).
22. Hanahan, D. & Weinberg, R. a. Hallmarks of cancer: The next generation. *Cell* **144,** 646–674 (2011).

23. Unger, T., Jacobovitch, Y., Dantes, A., Bernheim, R. & Peleg, Y. Applications of the Restriction Free (RF) cloning procedure for molecular manipulations and protein expression. *J. Struct. Biol.* **172,** 34–44 (2010).

24. Jiang, Y. *et al.* Split Renilla luciferase protein fragment-assisted complementation (SRL-PFAC) to characterize Hsp90-Cdc37 complex and identify critical residues in protein/protein interactions. *J. Biol. Chem.* **285,** 21023–36 (2010).

25. Amir-Zilberstein, L. *et al.* Differential regulation of NF-kappaB by elongation factors is determined by core promoter type. *Mol. Cell. Biol.* **27,** 5246–59 (2007).

26. Shekhawat, S. S. & Ghosh, I. Split-protein systems: beyond binary protein-protein interactions. *Curr. Opin. Chem. Biol.* **15,** 789–97 (2011).

27. HATTORI, M. & OZAWA, T. Split Luciferase Complementation for Analysis of Intracellular Signaling. *Anal. Sci.* **30,** 539–544 (2014).

28. Hattori, M. & Ozawa, T. High-throughput live cell imaging and analysis for temporal reaction of G protein-coupled receptor based on split luciferase fragment complementation. *Anal. Sci.* **31,** 327–30 (2015).

29. Leng, W. *et al.* Novel Bioluminescent Activatable Reporter for Src Tyrosine Kinase Activity in Living Mice. *Theranostics* **6,** 594 (2016).

30. Chan, J. D. *et al.* A Miniaturized Screen of a Schistosoma mansoni Serotonergic G Protein-Coupled Receptor Identifies Novel Classes of Parasite-Selective Inhibitors. *PLOS Pathog.* **12,** e1005651 (2016).

31. Hoffmann, a, Natoli, G. & Ghosh, G. Transcriptional regulation via the NF-kappaB signaling module. *Oncogene* **25,** 6706–16 (2006).

32. Ghosh, S. & Hayden, M. S. Celebrating 25 years of NF-κB research. *Immunol. Rev.* **246,** 5–13 (2012).

Figure legends

Figure 1: The split-RL complementation assay of p65 homodimer. **A**. A scheme of the four possible fusion protein orientations. **B**. HEK293T cells were co-transfected with pairs of all possible RL-p65 fusion proteins or with the "empty" RL counterparts. Cells were harvested 24 hours later and luminescence was evaluated and normalized to the luminescence obtained by the two "empty" RL fragments. **C**. HEK293T cells were co-transfected with either WT or E211A RL-p65 (1-298) fusion proteins (with His and flag tags) without the nuclear localization signal or with the "empty" RL counterparts. Cells were harvested 24 hours later and luminescence was evaluated and normalized to the luminescence obtained by the two "empty" RL fragments. Asterisk denotes statistically significant difference $p<0.05$.

Figure 2: Calibrations of the recombinant RL-p65 fusion proteins for the HTS. **A**. A scheme of the constructs that were used in the subsequent experiments. The first describes the expression plasmid of the split-RL-p65 pair; the second is the competitor p65 and the third is the full-length RL enzyme. **B**. Bacterial cells transformed with either the parental plasmid (ctrl), the split-RL pair and the competitor p65 were induced for their expression and analyzed by western blot using p65 specific antibody. **C**. A competition of the split-RL-p65 complementation assay with increasing amounts of recombinant p65. **D**. A kinetic analysis of the luminescence signal after the addition of the CTZ substrate. CTZ was added to recombinant RL-p65 and luminescence was measured every minute for the duration of 30 minutes. Each time point is the average of 32 repeats. The asterisks in B and C sections denote statistically significant difference, * $p<0.05$ and ** $p<0.01$.

Figure 3: Analysis of RL-p65 inhibitors identified by the HTS. **A**. The chemical structure and the dose-response results of 3 lead compounds from the libraries of Analyticon,

Enamine and Maybridge, which were found in the high-throughput screen. Recombinant RL-p65 fusion proteins or full-length RL were incubated with increasing concentrations of the compounds (0.3μM, 1μM, 3μM, 10μM and 30μM) for 15 minutes. CTZ was added and luminescence was read 12 minutes later. The blue and the red lines represent the results of the split-RL-p65 and the full-length RL, respectively. **B**. Live-cell dose-response split-RL-p65 assay. HEK293T cells were co-transfected with plasmids containing RL-p65 fusion proteins. Twenty-four hours later cells were detached and resuspended in PBS. Viability was evaluated and then 8000 live cells were added to each well in 384 well-plate and incubated with increasing concentrations of the lead compounds for 1 hour, followed by the addition of CTZ and luminescence measurement. The graph in each panel corresponds to the molecule in the same panel in Fig. 3A. **C.** The effect of the lead compound on p65 transcriptional activity. Sub-confluent HEK293T cells were co-transfected with a reporter gene driven by the promoter of the NF-κB responsive gene A20, together with an additional reporter gene which served as an internal control for transfection efficiency along with wild-type p65. The lead compounds were added to the cell cultures 6 hours after transfection. Cells were harvested 24 hours after transfection and inhibition of the A20 reporter gene was evaluated. The asterisks denote statistically significant difference, * $p<0.05$ and ** $p<0.01$.

Figure 4: A schematic representation of the various steps of the screening protocol for PPI inhibitors.