Dual expression recombinase based (DERB) single vector system for high throughput screening and verification of protein interactions in living cells

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ABSTRACT

Identification of novel protein interactions and their mediators is fundamental in understanding cellular processes and is necessary for protein-targeted therapy. Evidently high throughput formatting of these applications in living cells would be beneficial, however no adequate system exists. We present a novel platform technology for the high throughput screening and verification of protein interactions in living cells. The platform's series of Dual Expression Recombinase Based (DERB) destiny vectors individually encode two sets of recombinase recognizable sequences for inserting the protein open reading frame (ORF) of interest, two sets of promoters and reporter tags in frame with the ORFs for detecting interactions. Introduction into living cells (prokaryotic and eukaryotic) enables the detection of protein interactions by fluorescence resonance energy transfer (FRET) or bimolecular fluorescence complementation (BiFC). The DERB platform shows advantages over current commercialized systems by introducing recombinase based cloning and compatible accepting vectors validated through proof-of-principle experiments and the identification of an unknown interaction.

INTRODUCTION

The completion of the human genome sequencing project has presented the opportunity and challenge of identifying a staggering number of gene products, proteins, their interactions and modifiers. In addition to the 35,000 predicted proteins in the human genome, mutation and alternative splicing contribute further to this astounding number and to the quantity of possible cellular interactions[1]. Accordingly, the targeted therapy of a single protein can affect processes outside of the original intent which would be avoided with the introduction of a large-scale pre-screening system to identify interaction partners. The integration of a technical development into protein therapy will provide significant improvements to current research methods and aid in the prevention and treatment of disease[2,3].

The most familiar method for screening protein interactions *in/ex vivo* is the yeast two-hybrid assay. It is prevalent despite its complicated, time consuming protocol and requirement for the results to be confirmed in additional living cell systems[4]. Alternative *in/ex vivo* approaches include FRET[5-7] and BiFC[8,9], however the plasmids for analysis require individual construction which reduces the screening efficiency[5-10]. Integration of these approaches into efficient high throughput detection has thus far not been successful.

In order to overcome these limitations, we have developed a platform that supports high throughput screening and verification of protein interactions in living cells. We have engineered a DERB (Dual Expression Recombinase Based) vector platform to facilitate highly efficient cloning/subcloning of potential interaction partners into a final expression vector. The DERB vectors encode the sequences of two separate sets of

promoters, reporter tags for FRET or BiFC detection, and recombinase recognizable sites for the insertion of ORF of the proteins of interest and selection securities for efficient cloning. Introducing a protein couplet into a single destiny vector through two-step recombination eliminates the requirements of multiple step restriction-purification-ligation subcloning and co-transfection. Additionally, introducing the vectors into living cells, both prokaryotic and eukaryotic, enables the examination of the proteins of interest utilizing generic laboratory equipment including but not limited to the plate reader, flow cytometer, fluorescent microscope and/or confocal microscope.

To appreciate the potential of the DERB platform and demonstrate its relevance in hypothesis-driven research we applied it to detect protein interactions in the adiponectin signaling pathway. This signal transduction pathway consisting of recently identified receptors and down stream constituents is becoming an important area of research and development due to its role in public health issues including diabetes mellitus[11], obesity and cancer[12,13]. However, limited knowledge of the adiponectin receptor interaction partners restricts the development of therapeutic reagents for relief from the associated diseases. The identification of a novel receptor interaction in the pathway is attributed to the DERB technology. The potential of this method extends to include the automated *ex vivo* screening of therapeutics to modulate essential interactions, such as the adiponectin receptor interaction identified. The platform ultimately supplies an innovative method for the screening and confirmation of protein interactions in living cells, which can be effectively integrated into a high throughput configuration facilitating the process of screening for modulators that regulate protein-protein interactions.

RESULTS

Efficient integration of desired protein ORFs

The proteins of interest present in individual donor vectors were sequentially introduced into the recombinase recognizable loci of a DERB destiny vector (**Fig. 1ai**). Two independent recombination reactions brought the destiny vector to its dual protein expression vector formation and enabled protein interaction detection through FRET or BiFC after introducing into the cells. The initial LR Clonase recombination integrated ORF1 between the Att sites of the destiny vector while the subsequent CRE recombinase recombination inserted ORF2 into the LoxP site. The LR Clonase mediated reaction product was introduced into *Escherichia coli (E. coli)* DH5α for negative selection of the recombination byproduct and unsuccessful insertion constructs. The *ccdB* gene was either introduced into the reaction byproduct plasmid, devoid of ORF1, or remained in the destiny vector and prevented growth of both plasmids in DH5α. Confirmation of the Att recombination with ORF1 was achieved through polymerase chain reaction (PCR) with primers specific to the ORF1 sequence and Att insert boundary (**Fig. 1aii**). The subsequent reaction mixture of the CRE recombination was directly transformed into *E. coli* DH5α whereby successful insertions manifested in large concentric clones and unsuccessful clones succumbed to chloramphenicol and sucrose. Examination of the putative dual expression clones with electrophoresis identified effective ORF2 introductions evident by the increased plasmid size, proportional to the length of insertion, attained above the size without insertion (**Fig. 1aiii**). Collectively the *SucB* and antibiotic selection securities prevented the growth of clones with unsuccessful recombination vectors and provided the foundation of the platform's cloning efficiency **(Supplementary DERB Selection Strategy)**. Finalized dual expression vectors were introduced into prokaryotic (*E. coli* BL21(DE3)) or eukaryotic (HeLa) cells dependent upon the specified promoter of the destiny vector utilized. Induction of the system translated the two proteins individually fused to either the Yc Yn (**Fig. 1bi**) or ECFP EYFP (**Fig. 1bii**) set of tags for BiFC or FRET protein interaction detection, respectively.

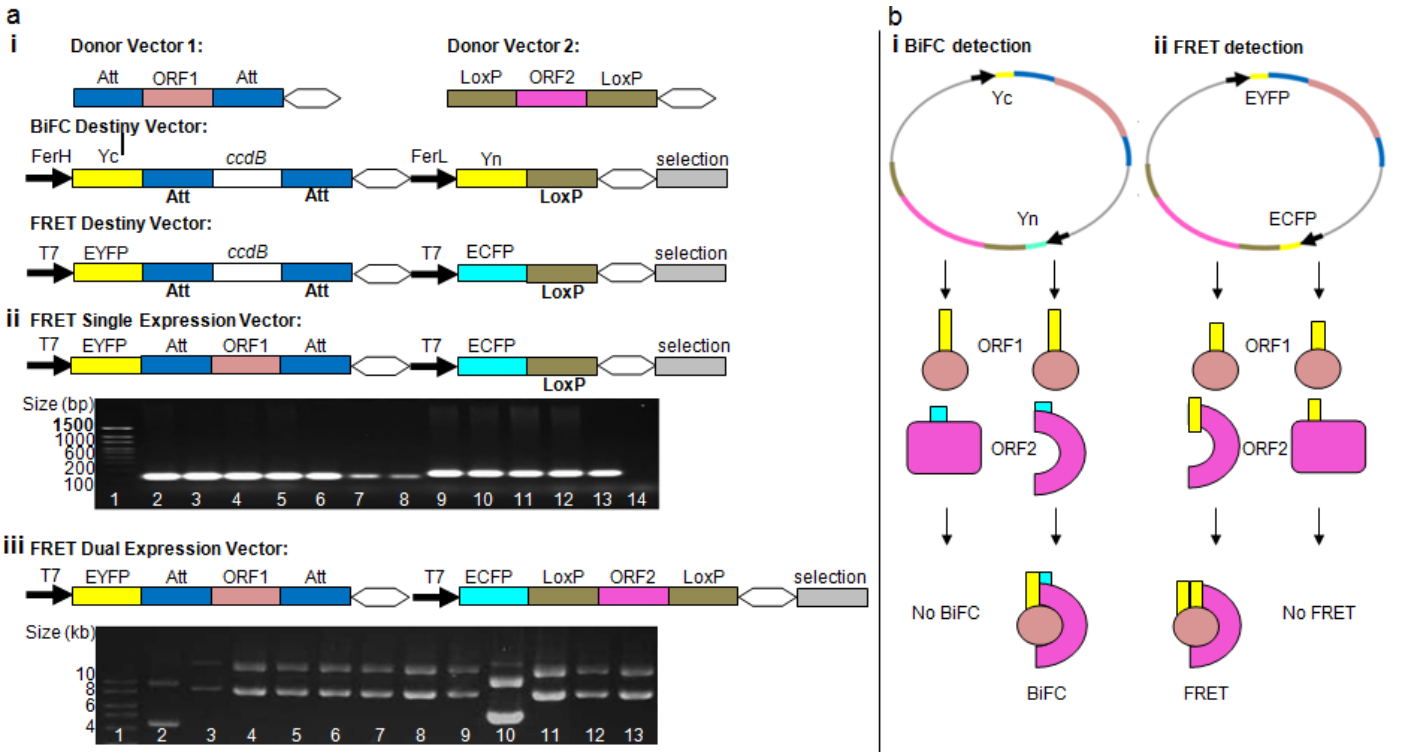

Figure 1 | Schematic of the two recombination reactions between two donor vectors and DERB destiny vector generating a dual expression vector for immediate protein-protein interaction detection. (**a**)(**i**) Insertion of the ORFs 1 and 2 of interest from donor vectors with Att and LoxP loci respectively into a destiny vector designated by its promoter presence and interaction detection (BiFC or FRET). (**ii**) ORF1 introduction into a T7 and FRET destiny vector was mediated with LR Clonase. PCR confirmed the successful single expression vectors by forward primer, specific to the ORF1 sequence, and reverse primer, binding specific to the Att insert boundary, to permit fragment amplification. Insertion was successful in clones present in lanes 2-13 which contrasted the no clonase, no insertion negative control vector in lane 14. (**iii**) Successful insertions performed ORF2 introduction from the LoxP donor vector with CRE recombinase. Electrophoresis examination of the plasmid isolated from picked clones revealed successful ORF2 presence in lanes 3-9 and 11-13 by the evident 3kb size increase above the no recombinase control in lane 2and 10 without insertion. Methodology of the platform construction is presented in **Supplementary Methods 1**. (**b**) Dual expression vectors were introduced into the desired cell model and induced to express the proteins of interest (ORF1 and ORF2) fused to the (**i**) Yc Yn or (**ii**) ECFP EYFP set of reporter tags. Positive interaction partners reconstituted fluorescence through BiFC or generated FRET whereas non-interacting proteins did not generate a signal.

Innate ability for protein interaction detection

Standardized expression vector controls were developed for validation of FRET and BiFC results in all destiny vector derivative lineages. The p-ECFPEYFP expression type vectors expressed the ECFPEYFP fusion protein and demonstrated a positive FRET signal. Conversely to the fusion, ECFP and EYFP or Yc and Yn were individually expressed from the p-ECFP-EYFP or p-Yc-Yn derivatives and demonstrated a negative interaction. The reported interaction partners of APPL1-ADIPOR1, APPL1-ADIPOR2[11] and homopolymer VIM were additionally integrated into a FRET and BiFC positive control collection while VIM and TUBA1B contributed to the negative interaction controls. Vimentin is well known to form polymers while there is no direct interaction between vimentin and tubulin structure[14]. Consideration of the differences in post-

translational modification between prokaryotic and eukaryotic cells prompted twin line analysis of the platform and was mediated through the derivatives and associated controls aforementioned. Plate reader examination established a p-ECFP-EYFP background fluorescence level below the p-ECFPEYFP fusion FRET intensity (FRETN). Constructs p-ECFPAPPL1-EYFPADIPOR1 and p-ECFPAPPL1-EYFPADIPOR2 displayed positive interaction FRETN mirroring the fusion value (**Fig. 2a**). Ultimately every control in the system whether expressed in a prokaryotic or eukaryotic line attained parallel FRETN behavior, enabling examination in either cell line. Confocal microscope examination of pFer-ECFPAPPL1-EYFPADIPOR1 and pFer-ECFPAPPL1-EYFPADIPOR2 further validated results (**Fig. 2b**).

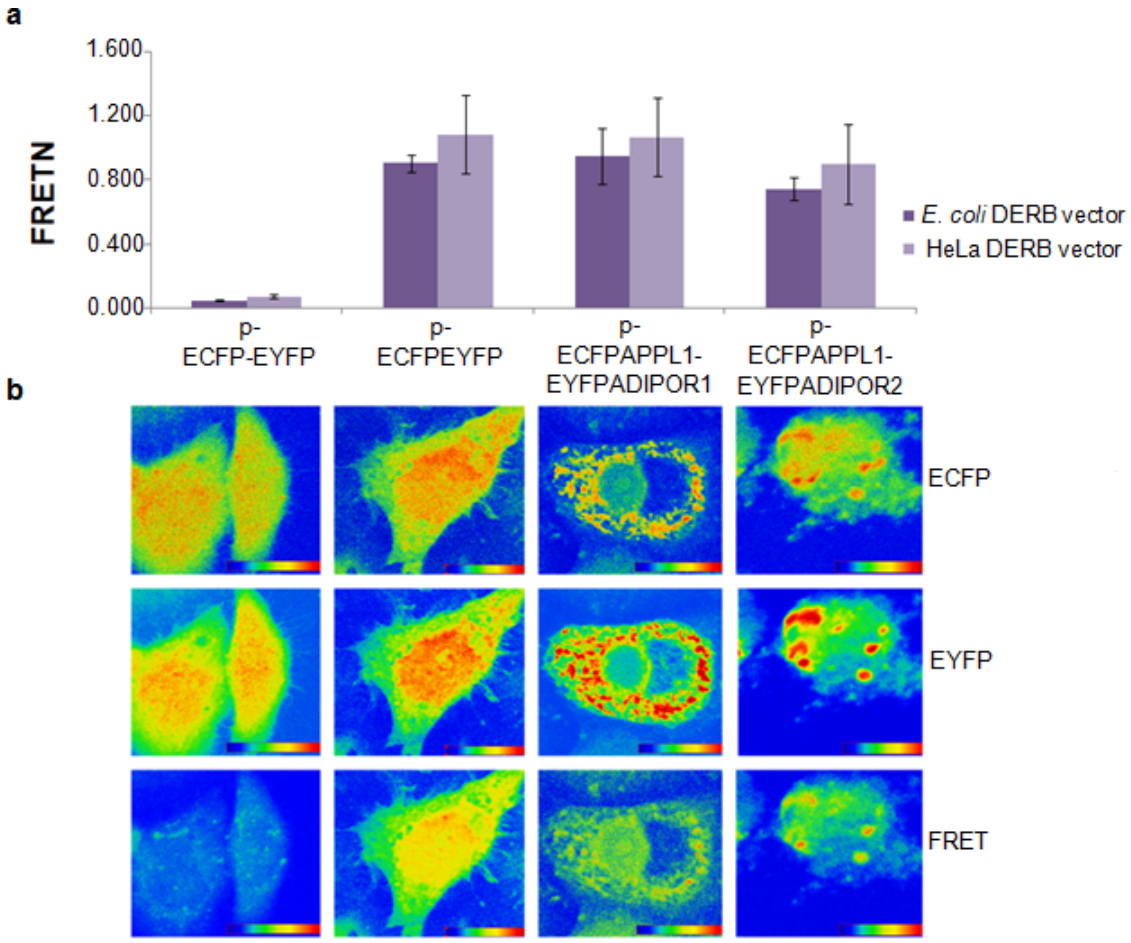

Figure 2 | FRET detection of protein interactions validated with the use of both plate reader and confocal microscope analysis (**a**) The 96-well formatted plate reader detected similar FRETN from protein couplets encoded in both prokaryotic (pT7) and eukaryotic (pFer) DERB vector derivatives. Unspecific p-ECFP-EYFP established background was surpassed by the p-ECFPEYFP fusion in the prokaryotic (p=0.0014) and eukaryotic (p=0.0005) cells. The APPL1 and ADIPOR1 in *E. coli* (p=0.011) and HeLa cells (p=0.0005) as well as the pT7-ECFPAPPL1-EYFPADIPOR2 (p=0.003) and pFer-ECFPAPPL1-EYFPADIPOR2 (p=0.00005) FRETN values all constituted positive interaction. (**b**) Confocal visualization of eukaryotic (HeLa) cells of ECFP (top row), EYFP (middle row) and FRET (bottom row) fluorescence distribution ensured dual protein presence. The pFer-ECFPAPPL1-EYFPADIPOR1 and pFer-ECFPAPPL1-EYFPADIPOR2 transfected cells surpassed the background FRETN fluorescence validating interaction detection.

BiFC protein interaction detection

An alternative destiny vector lineage was engineered to integrate two proteins of interest through the aforementioned two-step recombination and translate the proteins fused to divisions of EYFP. The C-terminal (Yc) and N-terminal (Yn) parts generated no fluorescence individually however fluoresced when brought into close proximity of each other, and thus its fusion with interacting proteins generated fluorescence[8,9]. HeLa cells were transfected with BiFC dual expression vectors and screened for EYFP fluorescence with both the 96-well plate reader and flow cytometer while fluorescent microscope analysis provided visual validation of the interactions. Expression vector pFer-YcVIM-YnVIM transfected cells generated substantial EYFP emission above the negligible pFer-YcVIM-YnTUBA1B background in all analyzers. The plate reader confirmed previous FRET results by detecting positive interactions in cells expressing APPL1 with ADIPOR1 or ADIPOR2 (data not shown). Flow cytometry further validated positive interactions by detecting EYFP fluorescent cell populations in pFer-YcAPPL1-YnADIPOR1 transfected HeLa cells (**Fig. 3a**). Consecutive confirmation with fluorescent microscopy demonstrated interactions among pFer-YcVIM-YnVIM, pFer-YcAPPL1-YnADIPOR1 protein partners (**Fig. 3b**).

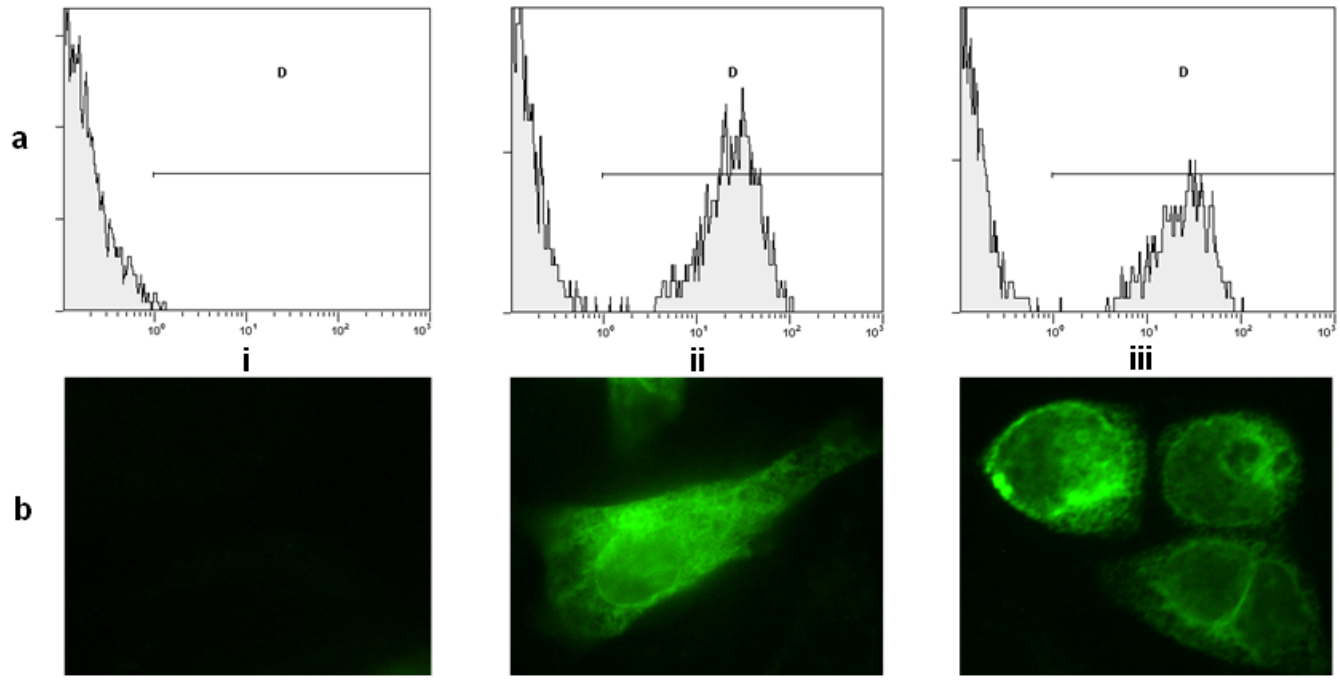

Figure 3 | Protein interactions detected in the DERB platform with BiFC were validated by flow cytometer (top) and fluorescent microscope (bottom) analysis. (**a**) The absence of EYFP fluorescence was in (**i**) pFer-YcVIM-YnTUBA1B whereas positive cell populations were prominent with (**ii**) pFer-YcVIM-YnVIM and (**iii**) pFer-YcAPPL1-YnADIPOR1. (**b**) The fluorescent microscope background level was established with the (**i**) pFer-YcVIM-YnTUBA1B and was surpassed by (**ii**) pFer-YcVIM-YnVIM and (**iii**) pFer-YcAPPL1-YnADIPOR1 transfected HeLa cells.

Protein interaction analysis validated with immunoprecipitation

Destiny vector pFer-ECFPAPPL1-EYFPADIPOR1 HeLa cell lysate was incubated with anti-ADIPOR1 followed by protein G beads to form bead- anti-ADIPOR1-ADIPOR1 complexes. Collection of the complexes and proteins associated to ADIPOR1 were western blotted with anti-APPL1 and HRP conjugated secondary antibody. Bands revealed APPL1 presence within the ADIPOR1 collection and confirmed interaction (**Fig. 4**).

Figure 4 | Immunoprecipitation confirmed DERB platform protein interaction analysis. (**a**) APPL1 presence in the pFer-ECFPAPPL1-EYFPADIPOR1 transfected HeLa cell lysate was identified prior (**-**) to and following (**+**) immunoprecipitation of ADIPOR1. (**b**) Anti-actin control identified the presence of actin prior (-) to but not following (+) immunoprecipitation of identical ADIPOR1 complexes confirming the APPL1 and ADIPOR1 protein interaction.

Novel interaction attributed to DERB platform

Consistent validation of the DERB platform with standardized controls prompted investigation beyond known interacting partners. Standardized control derivatives pECFP-EYFP, p-ECFPVIM-EYFPTUBA1B, p-YcVIM-YnTUBA1B, p-ECFPEYFP, p-ECFPVIM-EYFPVIM and p-YcVIM-YnVIM established result consistency throughout the various applications. FRET investigation with pT7-ECFPADIPOR1-EYFPADIPOR1 resulted in the identification of a novel receptor interaction (**Fig. 5a**). Investigation of pFer-YcADIPOR1-YnADIPOR1 with a 96-well plate reader and flow cytometer confirmed results identified with the FRET prokaryotic construct (**Fig. 5b**). Further affirmation of the protein interaction through fluorescent microscopy with the pFer-YcADIPOR1-YnADIPOR1 expression vector supported all previous results. Validity was furthered through the western blotting of the pFer-YcADIPOR1-YnADIPOR1 construct which revealed unbiased detection and the conclusion of the ADIPOR1- ADIPOR1interaction (**Fig. 5c**).

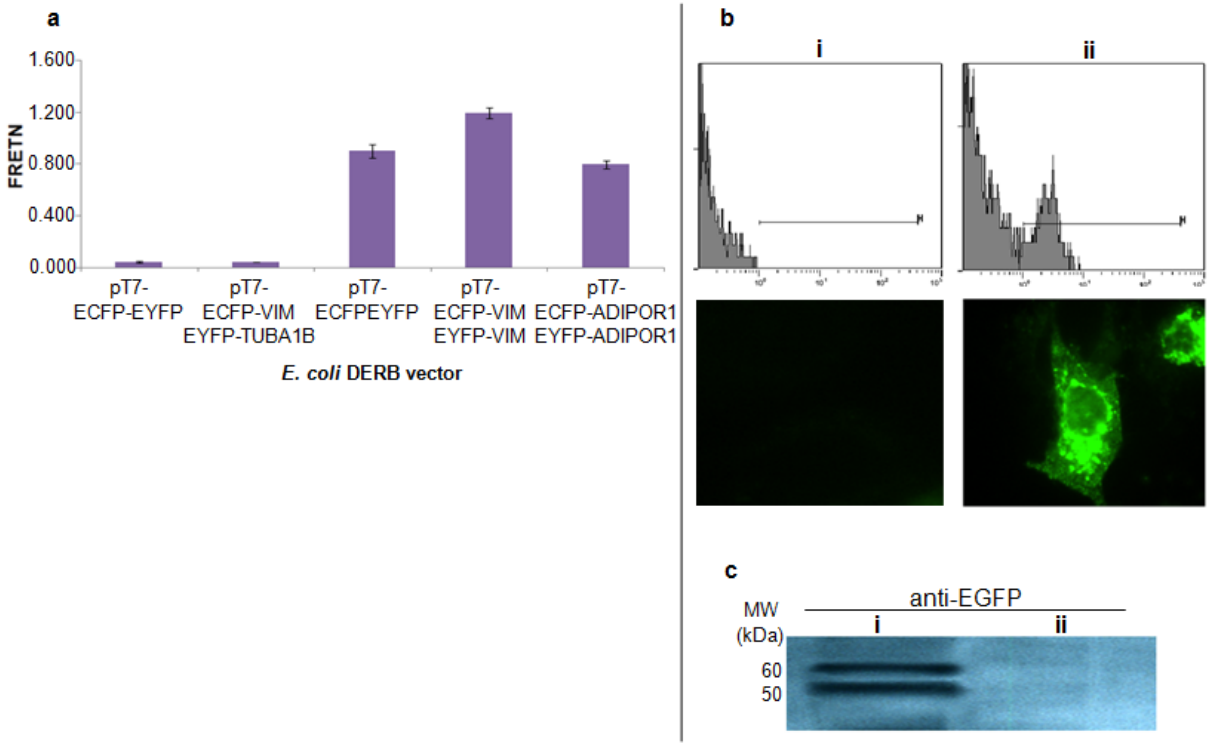

Figure 5 | DERB platform verification of a novel protein interaction between ADIPOR1 and ADIPOR1. (**a**) Prokaryotic construct pT7-ECFPADIPOR1-EYFPADIPOR1 transformed into *E. coli* BL21(DE3) exhibited substantial FRETN above pT7-ECFP-EYFP and pT7-ECFPVIM-EYFPTUBA1B. (**b**) BiFC in eukaryotic (HeLa) cells utilized (**i**) pFer-ECFPVIM-EYFPTUBA1B presented no obvious fluorescence in the flow cytometer analysis (top) and no fluorescence was visualized with the fluorescent microscope (bottom). (**ii**) pFer-YcADIPOR1-YnADIPOR1 showed a positive EYFP population (top) and obvious reconstitution of EYFP under the microscope (bottom) thus supporting the putative ADIPOR1-ADIPOR1 interaction. (**c**) Western blotting showed HeLa cells transfected with pFer-YcADIPOR1-YnADIPOR1 expressed YcADIPOR1 and YnADIPOR1 proteins with expected sizes 52.7 and 61.6 kDa respectively. Differential sizes of an identical ORF was attributed to the unequal division of the fused Yc and Yn parts and enabled western blot quantification of the proteins. (**i**) Substantial band intensity was presented after development with anti-EGFP polyclonal antibody (cross-reacted with EYFP) and exposed comparable protein levels for interaction detection. (**ii**) Mock transfected HeLa cell lysate was negative for the presence of Yc or Yn and revealed antibody specificity.

DISCUSSION

Modern research in the field of protein interactions in living cells is restricted in practice by the limited number of known proteins and/or interaction partners[15-17]. Consequently, there is an emerging demand for the high throughput screening (HTS) of protein interactions and modifiers to ultimately benefit the development of protein-based therapeutics with minimal side effects[18]. HTS integration of this procedure into living cells is dependent upon the degree of automation at two fundamental stages; the cloning/subcloning of protein ORFs and detection of protein interactions.

Our DERB platform provides an efficient subcloning approach for the HTS and verification of novel protein interactions with its ORF contained donor vectors and line of dual-expression destiny vectors. Companies including Open Biosystems, Clontech and Invitrogen offer donor vector libraries with various protein ORFs that are compatible for straight insertion into all DERB destiny vectors. Should the ORF of interest not be commercially available, production of either a LoxP or Att donor vector is easily performed with a single PCR reaction with the Clontech in-fusion PCR cloning kit or Invitrogen attB-PCR cloning system, respectively. Production of all DERB expression vectors with recombinase based reactions abolishes the time constraint associated with ORF subcloning, restrictive enzyme digestion, DNA purification and ligation procedures. Efficiency in the DERB platform extends to enable the direct heat shock transformation of *E. coli* with recombination reaction mixtures in a multi-well format. This ability permits high throughput selection including *ccdB* replacement and antibiotic resistance acquirement enabling the generation of only clones containing the desired insert. The LR Clonase insertion of ORF1 into the Att loci of the DERB vector removes the *ccdB* construct from the resultant single expression vector and attaches it to the recombination byproduct to prevent its growth in *E. coli* DH5α (**Fig. 1aii**). Subsequent CRE mediated recombination introduces ORF2-CmR into a DERB single expression vector forming a dual expressing vector, which renders its resistance to chloramphenicol and sucrose. The donor vector sensitivity to sucrose (SucB) and the single expression DERB vector deficient of CmR gene collectively prevent the proliferation of clones with unsuccessful recombination plasmids (**Fig. 1aiii**). The aforementioned selection strategy provides highly efficient dual expression vector generation with rare exceptions, thus rendering extensive clone selection from those generated as a redundant practice (See **Supplementary: DERB Selection Strategy** for details). That, coupled to the ability of immediate introduction into prokaryotic or eukaryotic cells, demonstrates the capability and efficiency of the platform for enabling high throughput formatting of protein interaction examination.

Translating the proteins of interest fused to either the ECFP EYFP or Yc Yn set of fluorescent tags provides a direct path for the FRET or BiFC detection of protein interactions. Expression of the ORF partners is controlled by the dual presence of an identical (pT7, CMV) or similar (modified FerH, FerL) promoter, which is responsible for the induction of both fusion proteins, and ensures their simultaneous expression at substantial levels (**Fig. 5c**). The well cited FRET donor-sensitized acceptor fluorescence three-channel method[19,20] for the detection of protein interactions enables the use of numerous generic analyzers thus increasing the accessibility of the DERB technology. In the conformational experiments utilizing confocal microscopy, images of the controls and experimental groups were acquired with an identical laser intensity and PMT value. FRETN was obtained through the equation of Xia[21,22] which provided measurements with a standard error of less than 7%[19]. Though confocal microscope analysis is reliable for FRETN measurement, it is difficult to be integrated into a high throughput protocol. As such, it is best to utilize confocal microscopy as a final confirmation to complement the limitations of the individual analyzers with the strengths of the alternatives. Automation of the plate reader and/or flow cytometry BiFC analysis to determine protein interaction, however, is highly probable. The collection of EYFP fluorescence intensity and the determination of background threshold would enable immediate interaction detection. Introducing BiFC investigation into the multi-well plate reader and/or flow cytometry complements the time consuming limitations of confocal microscopy analysis and renders BiFC practice highly applicable to high throughput formatting. Furthermore, the scrutiny of quantifying channel cross-talk and ECFP-EYFP fluorescence equalization is removed, attracting greater efficiency to its detection.

Companies including InVivogen and Novagen provide products used for the subcloning of two ORFs of interest into a single expression plasmid. Their technology mediates vector construction through the classical cloning methodology, which prevents formatting for high throughput screening. Gateway cloning platforms, offered by Invitrogen, introduced destiny vectors with recombination sites to facilitate recombinase insertion of multiple ORFs[23]. Despite its recombination advantage, Invitrogen destiny vectors do not innately encode a reporter fragment at the recombination loci and require the individual attachment of markers to donor vectors for protein interaction determination. Furthermore, aforementioned commercially available donor vector libraries compatible for direct insertion into Invitrogen cannot be used to detect interactions in the living cells. Regardless, the widely used single expression vectors with an ORF fused reporter fragment require co-transfection, need selection of double transfected cells from the single transfected and non-transfected, questioning its efficiency. Complications extend to the unequal expression of proteins in the cells co-transfected by single expression vectors as the ratio of different vectors integrated into individual cells cannot be controlled, which creates bias amongst the reporter tags, leading to pseudo-negative results. In contrast, the

DERB platform technology has been engineered with consideration to the shortfalls of the gateway cloning and hence mediates insertion into recombination sites with an in-frame fused reporter fragment through two recombinase-mediated reactions with commercially available donor vectors. High throughput transfection initiates the expression of both tagged proteins at similar levels, ensuring straightforward interaction detection which was validated by our proof-of-principle experiments.

The platform's unique ability to test a series of proteins in search of an interaction partner(s) with a known protein confirmed the interaction between ADIPOR1 and ADIPOR1. Western blotting confirmed the simultaneous expression of the Yn or Yc tagged ADIPOR1 proteins after the single transfection of living cells, establishing the interaction and reliability of the system. The adiponectin receptor signal transduction system is essential in many illnesses including diabetes, obesity and cancer, which are important public health issues. The interaction between adiponectin receptor 1 is likely significant to the activation and subsequent signal transduction as dimerization or polymerization of receptors is well known to be important for signaling functions[24]. Our platform provides a simple tool for the screening and verification of receptor interaction partners.

A possible concern regarding the workability of the destiny vectors relates to their large size[25]. It has been reported that about 300kb of an insert sequence can be cloned or subcloned into common vectors[26]. The size of our destiny vectors without ORFs is approximately 7-8kbs, while the final dual expression vectors with two ORFs is extended to 11-13kb. There was no noticeable difficulty in the cloning and subcloning procedures with these vector clones. Strong signal of protein interaction in our system implied the expression and interaction of the tagged proteins were not obviously altered by the present size range of the plasmid vectors.

In summary, the dual expression recombinase based (DERB) vector platform provides a technique for rapid, efficient and faultless cloning/subcloning of two ORFs in-frame with detection tags. Resulting vectors can express both tagged proteins by a single transformation/transfection reaction. This system permits detection within 24hrs of transformation. Its selective culture strategy ensures that practically all the *E. coli* cells grown in the selective media express substantial amounts of two tagged proteins from the dual expression DERB vector, minimizing pseudo-negative output during screening. FRET or BiFC signal detection with a plate reader and/or a flow cytometer guarantees high specificity of the signal in prokaryotic and/or eukaryotic cells during screening and/or verification while providing the option of high throughput application. Thus, the DERB presents an alternative and unique method for the high throughput identification of protein interactions which overcomes the limitations associated with existing technologies (**Supplementary Comparison of Technologies**). While the present paper described only two detection methodologies (FRET, BIFC), we are

undertaking construction of new detection platforms including enzyme fragment complementation[27] to broad the applications/capability of the DERB technology. The platform is further being used to screen for modulators of protein interactions by means of applying modulator candidates to the culture medium, an application not possible with current commercially available products or techniques. Given the interest of academic and commercial laboratories in the investigation of protein function and the development/screening of novel drugs, the DERB platform possesses universal appeal and tremendous financial value.

METHODS

DERB vector compilation. Commercially obtained plasmids pDNR, pLP-ECFP, pEYFP, pEYFP-Tub (Clontech) and pDEST, pT-Rex-DEST30, pRSET-A, pENTR221-ADIPOR1, pENTR221-ADIPOR2 (Invitrogen) were directly applied to the creation and examination of the DERB platform. FRET destiny vectors comprised of the ECFP EYFP set of tags were introduced into prokaryotic (**Supplementary Table 1**) and eukaryotic (**Supplementary Table 2**) cells whereas BiFC destiny vectors with the Yc Yn tag set were introduced into eukaryotic cells (**Supplementary Table 3**). The sources of other entry vector or other vectors with the ORF of interest used are listed in **Supplementary Table 4.**

Cell culture and transfection. *E. coli* DB3.1 (Invitrogen), and DH5α (New England Biolabs) served host for prokaryotic and eukaryotic DERB vector generation. *E. coli* BL21(DE3) expressed DERB vector proteins after induction with IPTG (1~2mM) for 6-16h at 18-25°C for interaction analysis. HeLa (ATCC, CCL-2) cells were grown in MEMα (Invitrogen) media supplemented with 10% FCS, 10U/ml penicillin, 100μg/ml streptomycin, non-essential amino acid and L-glutamine (Invitrogen). Mammalian cells were seeded onto glass cover slips in multi-well culture plates and transfected with DERB vectors through Lipofectamine-2000 (Invitrogen), PEI (MBI, Fermentas) or CaPi (Promega) methods 24 h after seeding.

Statistical analysis. Experimental data was subjected to a two-tailed paired sample t-test analysis. Standard deviation (STDV) was calculated from at least three triplicates per experiment for the plate reader examination of FRETN and EYFP. Mock transfected HeLa and *E. coli* cells presented the zero expression level for the plate reader, flow cytometer, confocal microscope and fluorescent microscope. Supplementary controls, specific to the experiment, ensured reliability of the experimental group data and were only considered statistically significant if p values were less than 0.05.

Additional Methods. Description of the sample preparations for immunoprecipitation and western blot in addition to FRET and BiFC analysis with the confocal microscope, plate reader, flow cytometer and fluorescent microscope are presented in **Supplementary Methods 2.** Please refer to **Supplementary Methods 3** for MIMX information (The minimum information required for reporting a molecular interaction experiment)[28].

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AUTHOR CONTRIBUTIONS

J.P.L devised the primary design of the DERB platform, which in cooperation with L.K.B. performed the experiments under the supervision and guidance of J.H.P. Manuscript preparation was in collaboration with all authors.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interest. Please refer to **Supplementary** for details.

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Supplementary

COMPETING INTERESTS STATEMENT

Declaration: J.P.L. and J.H.P. are named as inventors on US patent applications entitled " **Method for Detecting Molecular Interactions** " which were filed on **July 19, 2007 (**Serial No. **60/929,962).**

Supplementary: DERB vector clone selection strategy (Prokaryotic expression example)

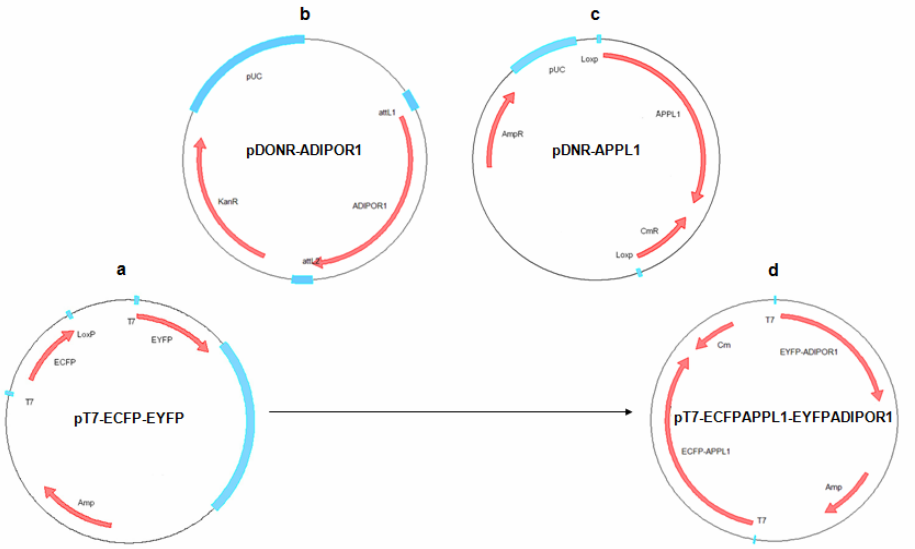
Name	Vector	Selective ORFs	Detail
First recombination reaction (LR clonase mediated):			
Donor Vector1	pENTR-ORF1 (or pDONR-ORF1)	$Kana^R$ or Spn^R	Resist to kanamycin or Resist to Spectinomycin
Destiny Vector1	pT7-ECFP-EYFP	CmR, ccdB	Resist to chloramphenicol Only grow in *E. coli* with gryA462 allele (e.g. DB3.1)
Single Expression Vector	pT7-ECFP- EYFPORF1	Amp^R	Resist to ampicillin
Recombination 1 by-product	pENTR-ccdB	Cm^R Cm^R, ccdB	Resist to chloramphenicol Only grow in DB3.1
Culture Medium: LB-Ampicillin	Host E. Coli: DH5a:		
Selection strategy: *E. coli* with donor vector 1 does not grow in ampicillin media Destiny vector 1, or recombination 1 by-product cannot amplify in E. coli strain without gryA462 allele (DB3.1)			
Second recombination reaction (Cre Recombinase mediated):			
Donor Vector2	pDNR-ORF2 (Or pDNR-Dual-ORF2)	Amp^R Cm^R, SucB	Resist to ampicillin Resist to chloramphenicol Sensitive to sucrose
Destiny Vector2 (i.e. Single Expression Vector)	pT7-ECFP- EYFP-ORF1	Amp^R	Resist to ampicillin
Recombination 2 by-product	pDNR-SucB,	Amp^R SucB	Resist to ampicillin Sensitive to sucrose
Double Expression Vector	pT7-ECFPORF2- EYFPORF1	Amp^R Cm^R,	Resist to ampicillin Resist to chloramphenicol Resist to sucrose
Culture Medium: LB- chloramphenicol-sucrose	Host *E. coli*: DH5α:		
Selection strategy: *E. coli* with donor vector 2 or recombination 2 by-product does not grow in media with sucrose *E. coli* with Destiny vector 2 does not grow in media with chloramphenicol			

Supplementary: Comparison of DERB and yeast 2-hybrid system for detecting protein interactions

	DERB Technology	Yeast 2-hybrid System
Library construction:		
Interaction partners	Known ORFs library	Bait to unknown library
Method of construction	Recombinase based	Classic method
High throughput option	Yes	No
Detect of interactions:		
Host cells	Bacteria Mammalian cells Others	Yeast
One round of screen time	1-3days	1-3months
Interaction Pair	Known partners	Bait known, Prey unknown
Result processing	Known directly	Need further processing
Confirmation of Interactions:		
Further subcloning and/or Sequencing for Identify interaction partner	No need	Need 1-3 months
Further confirmation of interaction In other in/ex vivo systems	No	Need 1-3 months
Total time needed: After the system is set up	1 week	6-12 months
Reliability of the result:	High	Low (Pseudo positive/negative)
Further development Screen modulators of interaction	Easy	Difficult

Supplementary Methods 1

Methodical compilation of DERB expression vectors. Subcloning of the desired ORF couplet into the DERB vector is demonstrated, as an example, through the generation of pT7-ECFPAPPL1-EYFPADIPOR1 (**Supplementary Fig. 1**). The adiponectin receptor 1 (ADIPOR1) ORF from donor vector pDONR-ADIPOR1 was inserted between the Att recombinase recognizable sites in the pT7-ECFP-EYFP destiny vector through LR Clonase mediated reaction. ORF2 for protein APPL1 from donor vector pDNR-APPL1 was inserted into the LoxP location of the successful ADIPOR1 inserted single expression vectors by CRE recombinase. The reaction product was immediately used to transform *E. coli* DH5a to identify viable dual expression vectors. Derivatives of the dual expression vector, pT7-ECFPAPPL1-EYFPADIPOR1, were produced to facilitate analysis on various readers. The derivatives contained identical protein constituents but differed in promoters, pCMV-ECFPAPPL1-EYFPADIPOR1 and pFer-ECFPAPPL1-EYFPADIPOR1, or reporter tags, pFer-YcAPPL1-YnADIPOR1.



Supplementary Figure 1 | Dual expression vector generation from two recombination reactions with donor vectors. (**a**) Prokaryotic plasmid derivative comprised of two sets of promoters, recombinase sites and ECFP EYFP fluorescent tags ensured translation of tagged proteins. Donor vector contained proteins (**b**) ADIPOR1 and (**c**) APPL1 were inserted into the respective recombinase recognizable, LoxP and Att, sites and generated (**d**) pT7-ECFPAPPL1-EYFPADIPOR1 dual expression vector. Other elements like antibiotic resistance gene, chemical resistance or sensitive gene are not shown.

Supplementary Table 1 | DERB platform derivatives for FRET examination in prokaryotic cells

Plasmid	Promoter	Proteins Expressed
pT7-ECFP-EYFP	T7	ECFP, EYFP , two proteins
pT7-ECFPEYFP	T7	ECFPEYFP, one fusion protein
pT7-ECFPVIM-EYFPVIM	T7	ECFPVimentin, EYFPVimentin, two tagged proteins
pT7-ECFPVIM-EYFPTUBA1B	T7	ECFPVimentin, EYFPαTubulin1B, two tagged proteins
pT7-ECFPAPPL1-EYFPADIPOR1	T7	ECFPAPPL1, EYFPAdiponectin Receptor 1, two tagged proteins
pT7-ECFPAPPL1-EYFPADIPOR2	T7	ECFPAPPL1, EYFPAdiponectin Receptor 2, two tagged proteins
pT7-ECFPADIPOR1-EYFPADIPOR1	T7	ECFPAdiponectin Receptor 1, EYFPAdiponectin Receptor 1,

Supplementary Table 2 | DERB platform derivatives for FRET examination in eukaryotic cell lines

Plasmid	Promoter	Proteins Expressed
pCMV-ECFP-EYFP	CMV	ECFP, EYFP, two proteins
pCMV-ECFPEYFP	CMV	ECFPEYFP, one fusion protein
pCMV-ECFPVIM-EYFPVIM	CMV	ECFPVimentin, EYFPVimentin, two tagged proteins
pCMV-ECFPVIM-EYFPTUBA1B	CMV	ECFPVimentin, EYFPαTubulin1B, two tagged proteins
pCMV-ECFPAPPL1-EYFPADIPOR1	CMV	ECFPAPPL1, EYFPAdiponectin Receptor 1, two tagged proteins
pCMV-ECFPAPPL1-EYFPADIPOR2	CMV	ECFPAPPL1, EYFPAdiponectin Receptor 2, two tagged proteins
pFer-ECFP-EYFP	FerH, FerL	ECFP, EYFP, two proteins
pFer-ECFPEYFP	FerH, FerL	ECFPEYFP, one fusion protein
pFer-EYFPVIM-EYFPVIM	FerH, FerL	ECFPVimentin, EYFPVimentin, two tagged proteins
pFer-ECFPVIM-EYFPTUBA1B	FerH, FerL	ECFPVimentin, EYFPαTubulin1B, two tagged proteins
pFer-ECFPAPPL1-EYFPADIPOR1	FerH, FerL	ECFPAPPL1, EYFPAdiponectin Receptor 1, two tagged proteins
pFer-ECFPAPPL1-EYFPADIPOR2	FerH, FerL	ECFPAPPL1, EYFPAdiponectin Receptor 2, two tagged proteins
pFer-ECFPADIPOR1-EYFPADIPOR1	FerH, FerL	ECFPAdiponectin Receptor 1, EYFPAdiponectin Receptor 1,

Abbreviations: CMV Cytomegalovirus; FerH Human Ferritin heavy chain, FerL Human Ferritin light chain; 5'UTR replaced by the mouse and chimpanzeeEF1α with addition of SV40 and CMV enhancers.

Supplementary Table 3 | DERB platform derivatives for BiFC examination in eukaryotic cell lines

Plasmid	Promoter	Proteins Expressed
pFer-Yc-Yn	FerH, FerL	Yc, Yn
pFer-YcVIM-YnVIM	FerH, FerL	Yc-Vimentin, Yn-Vimentin, two tagged proteins
pFer-YcVIM-YnTUBA1B	FerH, FerL	Yc-Vimentin, Yn-αTubulin1B, two tagged proteins
pFer-YcAPPL1-YnADIPOR1	FerH, FerL	Yc-APPL1, Yn-Adiponectin Receptor 1, two tagged proteins
pFer-YcAPPL1-YnADIPOR2	FerH, FerL	Yc-APPL1, Yn-Adiponectin Receptor 2, two tagged proteins
pFer-YcADIPOR1-YnADIPOR1	FerH, FerL	Yc-Adiponectin Receptor 1, Yn-Adiponectin Receptor 1

Abbreviations: Yc EYFP C-terminal division, Yn EYFP N-terminal division;
FerH and FerL: same as **Supplementary Table 2**.

Supplementary Table 4 | Sources of other plasmids

Plasmid	Proteins Expressed	Source	Host organism	UniGene No.	UniProt No.
pDNR-TUBA1B	α tubulin1B	In Lab	*Homo sapiens*	NM006082	P68366
pDNR-VIM	Vimentin	In Lab	*Homo sapiens*	NM03380	P08670
pENTR-Vim	Vimentin	In Lab	*Homo sapiens*	NM03380	P08670
pENTR- TUBA1B	α tubulin1B	In Lab	*Homo sapiens*	NM006082	P68366
pDNR-APPL1	APPL1	In Lab	*Homo sapiens*	NM012096	Q9UKG1
pECFP-EYFP	ECFP-EYFP fusion Protein	Dr. He LSGift			
pCDNA-APPL1	APPL1	Dr. Dong LQ Gift	*Homo sapiens*	NM012096	Q9UKG1
pENTR- ADIPOR1	Adiponectin Receptor1	Invitrogen	*Homo sapiens*	NM015999	Q96A54
pENTR- ADIPOR2	Adiponectin Receptor2	Invitrogen	*Homo sapiens*	AK025085	Q86V24
pENTR-APPL1	APPL1	OpenBiosystems	*Homo sapiens*	NM012096	Q9UKG1

Supplementary Methods 2

Immunoprecipitation. HeLa cells transfected with pFer-ECFPAPPL1-EYFPADIPOR1 were sonicated and centrifuged for 10 min at 10,000 g at 4°C. Supernatants were collected as cell lysate and stored at -80°C. Cell lysate (200 μg) was mixed with 1 μg of anti-ADIPOR1 (Abcam) and incubated overnight at 4°C to form antigen-antibody complexes. Protein G Sepharose 4 Fast Flow beads (GE Healthcare) were prepared according to manufacture directions in a 1:1 lysate buffer to bead ratio. Antigen-antibody complexes were added into the 1:1 beads-lysate buffer preparation for 2 h, enabling antigen-antibody-Protein G beads complex formation. Beads were recovered through centrifugation following by resuspension and rinses with lysis buffer. Loading buffer was added to the beads sample followed by 5 min 100°C incubation, and centrifugation to collect the supernatant as finalized IP sample for western blotting.

Western blotting. IP samples were loaded into a 10% SDS-PAGE gel and transferred onto nitrocellulose membranes with a wet transfer module (Bio-Rad) according to manufacturer's instruction. The membrane was placed into blocking buffer for 1 h and blotted with the primary antibody specific to the experiment (anti-APPL1 for aforementioned IP sample) overnight at 4°C. The protein partner in the dual expression vector secondary antibody, conjugated to HRP, was incubated for 1 h at room temperature. After washing with TBST, the blot was reacted with chemiluminescence HRP substrate solution (Pierce) and exposed to X-ray film.

Similar translation of both proteins in the dual expression vector was verified through HeLa cells transfected with pFer-YcADIPOR1-YnADIPOR1. The lysate from the cells prepared after 46 h from transfection was loaded into the PAGE gel and transferred onto a nitrocellulose membrane. After blocking with 5% skimmed milk, goat anti-GFP antibody (Rockland Inc.) and HRP conjugated rabbit anti-goat antibody were applied to the membrane. Chemiluminescent reagent was applied for detection of the HRP signal.

Confocal microscope FRET analysis. Confocal microscope LSM510 (Carl Zeiss) operated with a 40mW argon laser and accompanying LSM software. Donor-sensitized acceptor fluorescence three-channel method[3,4] was established for the collection of ECFP, EYFP and FRET images at a 60x water immersion objective. ECFP channel was excited at 458 nm and collected emission with a 475-525 nm BP filter. The EYFP channel was excited at 514 nm and collected signal image through a 530 nm LP filter. FRET was analyzed through 458 nm excitation and 530 nm emissions.

FRET efficiency quantification. ECFP values were equalized to the median of all ECFP emission values for the replicates of the identical sample. The correction factor which brought each ECFP value to the median was applied to the individual replicate EYFP and FRET emission. Quantification of FRETN was performed according to the cited equation of Xia[5,6], $FRETN=\{I_{FRET}-(I_{ECFP}*\alpha)-(I_{EYFP}*\beta)\}/(I_{ECFP}*I_{EYFP})$, whereby α and β were the percentage of ECFP or the EYFP bleed through to FRET channel emission respectively.

Plate reader optimization. The SPECTRAmax GEMINI XS dual-scanning microplate spectrofluorometer (Molecular Devices Corporation) collected FRET emission from DERB expression vectors transformed *E. coli* BL21(DE3) or transfected HeLa cells. Cellular density in 96-well plates was equalized at OD600 prior to FRET analysis. The spectrofluorometer was set according to manufacturing guidelines and literature[7,8]. ECFP was excited at 458nm and collected at 480nm with cutoff at 475nm whereas EYFP excitation wavelength was 514nm, emission 540nm and cutoff 530nm. FRET signal was collected with excitation at 458nm, emission at 540nm cutoff at 530nm.

The CytoFluo Series 4000 plate reader (PerSpective Biosystems) collected BiFC emissions. Cells were equalized in 96-well plates with OD600 through the respective function on the plate reader. Excitation of EYFP was programmed to 485/20 nm with emission collection at 530/25 nm.

Flow Cytometer BiFC detection. Data collection from the flow cytometer (Beckman Coulter) was processed through algorithms of the Beckman Coulter Expo32 ADC/XL4 software. HeLa cells transfected with DERB vector (pFer-YcVIM-YnTUBA1B) expressing non-interacting tagged proteins Yc-Vimentin, Yn-αTubulin1B established the fluorescence background level. HeLa cells were counterstained with PI. FL1 and FL3 channels were used for detecting EYFP BiFC reconstitution and PI fluorescence respectively.

EYFP BiFC reconstitution visualization with fluorescent microscopy. The DM IRB Deconvolution Microscope (Leica) and Peligo EX Q image CCD camera (Lual Electronic Products Ltd) were used to collect images of the live and fixed cultured cells. Open Lab (Leica) software was used for image overlay, storage and export of BiFC visual representations.

Supplementary Methods 3

MIMX information: The MIMIX checklist

- Essential administrative information:
  - Contact email: jianping.lu@hrcc.on.ca; Jehonathan.Pinthus@hrcc.on.ca
  - Publication title: Dual expression recombinase based (DERB) single vector system for high throughput screening and verification of protein interaction in living cells
  - First author: JianPing Lu
  - Publication identifier: Nature Methods
- Experiment parameters:
  - Host system
    The host organism: *Homo sapiens (Taxonomy ID:* 9606), HeLa cells (ATCC, CCL-2)
    Escherichia coli (*Taxonomy ID:* 562), DB3.1, DH5α
  - Interaction detection method
    Root term MI:0001
    MI:0019 coimmunoprecipitation,
    MI:0113 western blot.
    MI:0055 fluorescent resonance energy transfer
    MI:0809 bimolecular fluorescence complementation
    MI:0416 fluorescence microscopy
    MI:0663 confocal microscopy
  - Participant identification method
    The method by which the interaction participants were determined
    Root term MI:0002
    MI:0078 nucleotide sequence
    MI:0113 western blot.
  - Participant list. Each molecule should be characterized by:
    - Database
      Root term: MI:0444
      UniProt MI:0486
    - Accession number Q96A54
    - Version number: NA
    - Name Adiponectin Receptor 1
    - The species of origin for the molecule identified by NCBI taxonomy identifier: 9606
    - Biological role (The biological role of the molecule in the interaction)
      Root term: MI:0500
      MI:0499 unspecified role
    - Experimental role
      The experimental role of the molecule in the interaction
      MI:0503 self

SUPPLEMENTARY REFERENCES

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