Protein Engineering

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Engineering Cell Surfaces for Orthogonal Selectability**

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The selective addition of markers to expressed proteins has become a standard procedure in molecular biology. The most frequently used markers are fluorescent proteins and epitope tags.[1,2] Fluorescent markers such as the green fluorescent protein (GFP) allow confirmation that the protein to which it is appended is expressed, and enables a study of the subcellular localization of the protein by fluorescent microscopy[3] as well as the isolation of cells expressing the protein by fluorescent cell sorting.[4] Similarly, when protein tagging is used, either an antigen for which a companion antibody is available or a peptide that has affinity for, for example, nickel,[5] is appended to a protein. This tag is used to study the localization of a given protein in cells, identify the molecule after analytical procedures, or isolate it by affinity chromatography.[5,6] Thus, at the level of individual proteins, the experimenter has a choice of methods to study the expression patterns of the marked protein and even isolate the cells expressing it by cell sorting. Importantly, in all these methods one assumes that the marker does not alter the properties of the protein to which it is appended. Remarkably, what seems to be missing from the armamentarium of molecular biology is a robust genetic method to mark the surface of the cells themselves, such that certain cells can be easily and simply isolated from a population where they may be a minor component. Amongst other uses, such a method could be important for enriching for transformants, especially when the transformation frequency is low and would facilitate combinatorial antibody selections of phage that bind to cell surfaces where, as for transformants, the target cell can be a minor component of an otherwise large population. Also, such a method would allow for the rapid affinity-based isolation of plasma membranes for biochemical studies. Here we present a system for the affinity selection of cells and plasma membranes which is based on the expression of the chitin-binding domain of the enzyme chitinase on the cell surface.

The enzymes chitinase and cellulase, which hydrolyze chitin and cellulose, respectively, are the two most abundant insoluble polymers on earth. These enzymes are unusual because, in addition to the canonical enzyme format, where the active site has affinity for the substrate, they each have an appended small domain that binds to their insoluble substrates (Figure 1 A,B). In the case of the chitin-binding domain, this small appendage has nearly covalent binding energy for the substrate,[7] which is a readily abundant and cheap insoluble polymer of *N*-acetylglucosamine. These binding domains are termed ChBD and CBD for the chitin and cellulose binding domains, respectively. The ChBD has been widely used in the immobilization of antibodies[8] and other proteins.[9] If the ChBD or CBD could be expressed on the eukaryotic cell surface, the cells would have high affinity for insoluble polymers that are not present in animal cells.

Our general strategy was to construct vectors that expressed the ChBD linked to enhanced green fluorescent protein (EGFP) on the cell surface (Figure 1 C). One vector (BH1) was constructed based on the pBudCE4.1 vector (Invitrogen). The ChBD–EGFP cassette was inserted into the cloning site under the control of the cytomegalovirus (CMV) promoter. The second cloning site, which is under the control of the EF-1α promoter, can be used to express another gene of interest (Figure 1 D). The other vector (BH2) was constructed based on the pCEP4 vector (Invitrogen). The CMV-ChBD–EGFP and EF-1α promoter cassettes from BH1 were inserted before the OriP of pCEP4 (Figure 1 D). The construct is anchored to the cell membrane using the PDGF gene product, which is a protein that spans the plasma membrane. This membrane-spanning domain sits between the ChBD protein that is expressed on the outer surface of the cell and the EGFP protein that is present on the cytoplasmic side of the membrane (Figure 1 C). Since the EGFP protein is fluorescent, it allows for the rapid determination of the efficiency of a given transformation.

To determine whether the transformation with the plasmid encoding the ChBD was efficient and was expressed on the cell surface in a functional form, the transformed cells were tested for their ability to both express GFP and bind to chitin beads. EGFP alone and EGFP linked to the ChBD were efficiently expressed (Figure 2 A). The ChBD expression was confined to the cell surface (Figure 2 B). When the cells expressing the ChBD domain on their cell surface were mixed with chitin beads for 30 minutes, the cells bound to the beads, whereas cells that only expressed EGFP did not (Figure 3 A). In this experiment, the surface of the chitin beads appears to be saturated with cells that express the ChBD (arrows). The absolute number of cells binding to the beads is a function of the ratio of beads to cells. In the pictured experiment, excess cells were used solely for ease of visualization. After washing the beads, cells expressing the ChBD–EGFP-encoded constructs retained their affinity for the chitin beads (Figure 3 B). To determine the enrichment power of the method, different ratios of transformed to nontransformed cells were studied to determine the ability to recover rare

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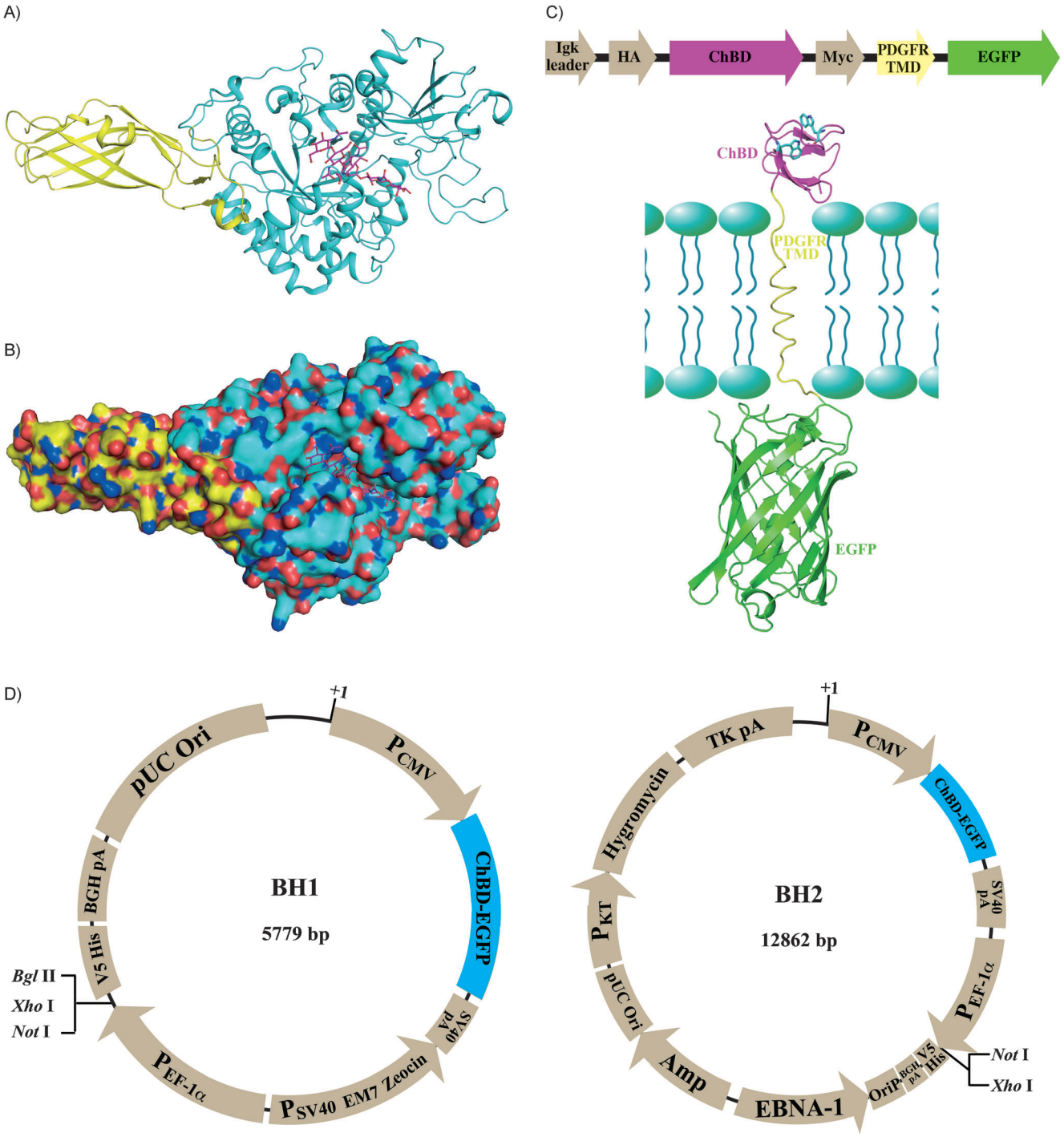

Figure 1. Structure of ChBD in *Vibrio harveyi* chitinase A. Ribbon diagrams (A) and electrostatic surface (B) of the *Vibrio harveyi* chitinase A in complex with chitooligosaccharides (PDB ID: 3B9A).[12] The N-terminal ChBD is in yellow; the catalytic and the insertion domains are in cyan. The chitin oligosaccharides bound in the catalytic cleft are shown in ball-and-stick models in magenta. Images were prepared using PyMol software.[13] C) Schematic diagrams of the ChBD–EGFP construct. The construct used the IgK leader, that is the murine Ig κ-chain V-J2-C signal peptide, and included a gene encoding the hemagglutinin A (HA) epitope before the N terminus of the ChBD. The ChBD is from *Bacillus circulans* WL-12;[14] PDGFR TMD is the platelet-derived growth factor receptor transmembrane domain; EGFP is the enhanced green fluorescent protein. A gene encoding the Myc epitope is located between the ChBD and the transmembrane domain. D) Schematic diagrams of BH1 vector (left), and BH2, an Epstein Barr virus based vector (right).

cells. Transformed cells could be recovered when they represented only 0.5% of the population. Usually, a higher percentage of transformed cells are present after transformations. Thus, the method should be general and in many cases avoid the, often long, post-transformation enrichment procedures such as antibiotic selections.

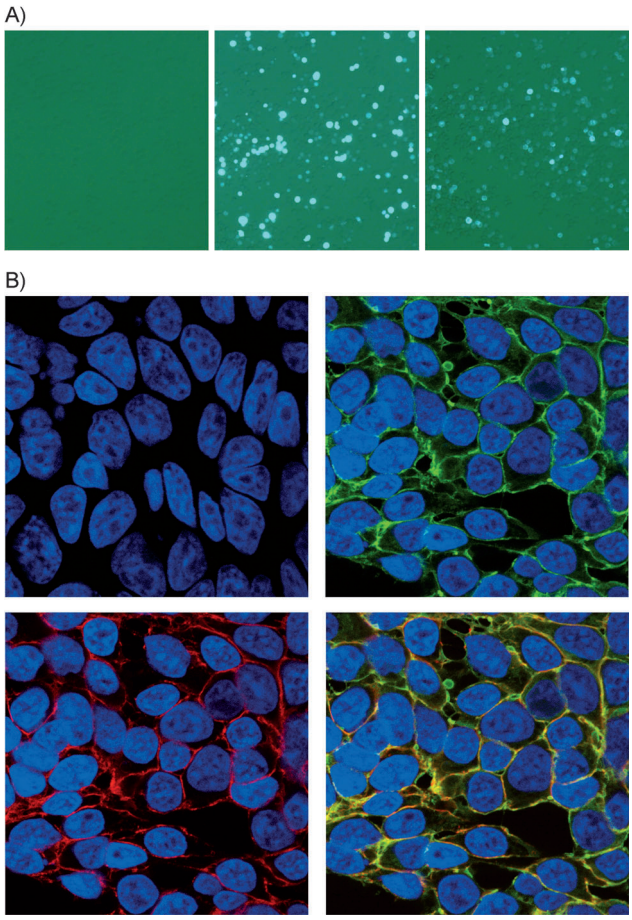

Figure 2. Cell-surface expression of ChBD–EGFP in BH1-transfected HEK 293T cells. A) expression of EGFP in mock (left), pBudCE4.1-EGFP (middle), and BH1 (right) transfected HEK 293T cells. B) Confocal images showing that no ChBD–EGFP is expressed in mock-transfected HEK 293T cells (top left), while ChBD is uniformly expressed in BH1-transfected HEK 293T cells (top right). The ChBD colocalizes with wheat germ agglutinin, which marks the external surface of the plasma membrane (bottom left and bottom right).

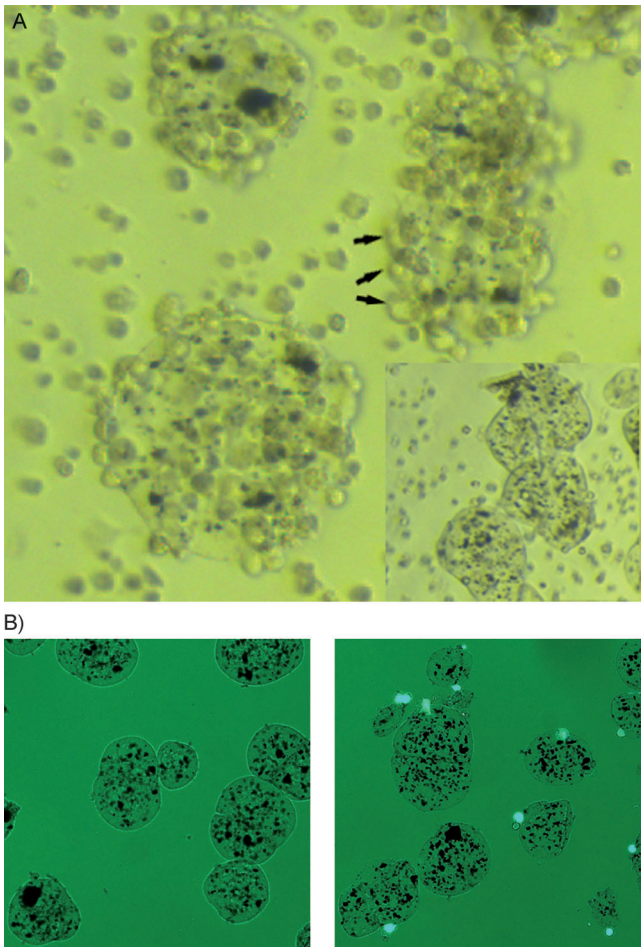

Figure 3. Cell-surface expression of ChBD–EGFP in BH1-transfected HEK 293T cells allows attachment of cells to chitin-coated beads. A) The cells expressing ChBD–EGFP bound to the beads, whereas cells that only expressed EGFP did not (insert). B) After selection, non-ChBD–EGFP-expressing cells did not bind to chitin-coated beads (left), while ChBD–EGFP-expressing cells could specifically bind to the chitin-coated beads (right).

It was essential to determine if the cells remained viable after they bound to the chitin beads. To show this, cells were bound to the chitin beads and then the complex was thoroughly washed and placed back into culture. The cells that bound initially were easily visible (Figure 4 arrows). New growth of cells was evident after 4 days in culture. Thus, the cells that bound to the beads were capable of replication, which qualitatively appeared unimpeded. After the beads were saturated with cells, the newly replicated cells were no longer attached to the beads. Thus, a culture could be established from the viable progeny of cells that were originally attached to the beads.

In addition to selection of intact cells that express the ChBD, the method should be useful for the purification of plasma membranes for biochemical studies. To test this possibility, HEK 293T cells that expressed the ChBD on their cell surface were sonicated and the membrane fragments were collected on chitin beads. Gel electrophoresis and Western blotting studies showed that this procedure afforded highly purified membrane preparations (Figure 5). β-Actin was used as a control to show that there is no contamination in the membranes that were isolated on the basis of their affinity for chitin-coated beads. Actin is present in large amounts in the cell cytoplasm, and if the purification were not stringent this protein would show up as a contaminant in the purified membranes.

Herein we have presented a new method in which the eukaryotic cell surface is engineered for selectability. Importantly, the binding functionality that is used does not have a counterpart in eukaryotes, thereby endowing the modified cell surface with a property that is orthogonal to animal cells. One might think that selections using antibodies to cell-surface proteins or the avidin–biotin system would be alternatives to the method presented here. While antibodies might be an alternative, they generally do not have the affinity of the ChBD and are much more expensive to use. The avidin–biotin system is not an alternative because avidin is a tetramer and biotin is an essential vitamin, with the ligand already present in all cells. Thus, even if the avidin tetramer

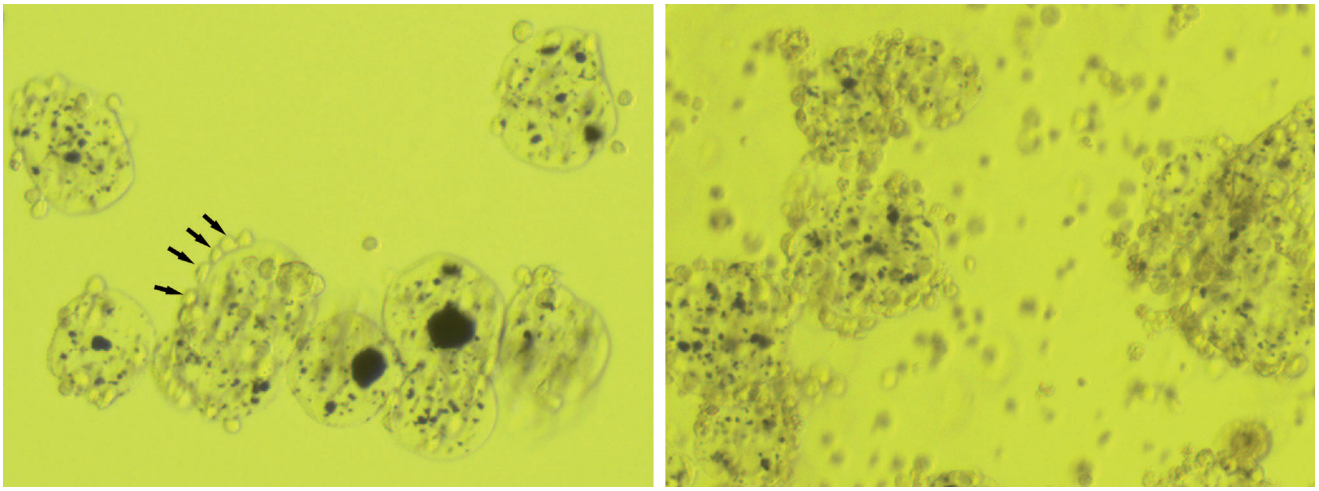

Figure 4. Cell viability after binding to chitin beads. After binding to the chitin beads, non-ChBD–EGFP-expressing cells were washed away, leaving only ChBD–EGFP-expressing cells bound to the beads (left). After washing the beads, the bound cells were placed back into tissue culture. The cells continued to grow and their progeny remained bound to the chitin beads until the beads became saturated, after which the cells grew independently (right).

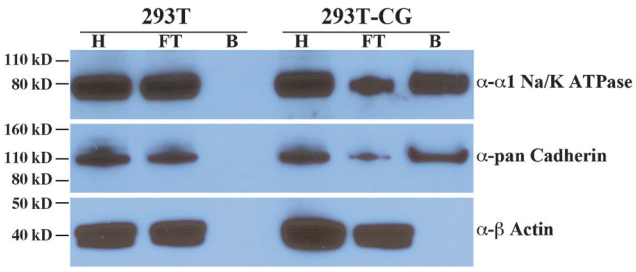

Figure 5. Cell-membrane purification facilitated by the ChBD. Cell-membrane fragments from ChBD–EGFP-transfected HEK 293T cells (293T-CG) were studied. Antibodies against two cell-membrane marker proteins, α1 sodium/potassium ATPase and pan cadherin were used to mark membrane fractions attached to chitin beads (B), the total cellular homogenate (H), and flow-through fraction (FT). As a control, cell-membrane fragments from mock-transfected HEK 293T cells (293T) were studied. An antibody against β actin was used to monitor the efficiency of the membrane purification.

could be expressed in a functional form on the cell surface, it would already be saturated with ligand. Indeed, the avidin–biotin system has been tried and failed in eukaryotic cells.[10] Another alternative to the method presented here, is fluorescence-activated cell sorting, which is time honored and generally robust, but it is a method that requires expensive instrumentation as opposed to the "bench-top" procedure described herein.

We expect this method to find wide use not only for the selection of cells but also for isolating cells in vitro and in vivo in any situation where one wishes to trace the fate and ultimately recover a minor population of cells. Toward this end, we are currently generating transgenic mouse strains where the ChBD is either constitutively expressed in all cells or inducible in certain cell lineages. If this is successful, it should allow long-term studies on cell fates, where the likely immunogenicity of the ChBD would otherwise be a problem in cell-transfer experiments. Thus, cells that constitutively express the ChBD can be followed in other mouse strains where expression of the ChBD is repressed by, for example, tetracycline inactivation of a sensitive tetracycline-dependent transactivator.[11]

Experimental Section

Cell culture: Human embryonic kidney HEK 293T cells were purchased from ATCC, and maintained in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO) supplemented with 10% fetal calf serum (GIBCO) and non-essential amino acids (GIBCO) at 37 °C in a humidified CO_2 incubator. HEK 293T cells were transfected with X-tremeGENE 9 DNA transfection reagent (Roche). Human B lymphocyte Wil2-NS cells were purchased from ATCC, and maintained in RPMI-1640 (ATCC) supplemented with 10% fetal calf serum (GIBCO) at 37 °C in a humidified CO_2 incubator. Wil2-NS cells were transfected by electroporation.

Plasmid construction: ChBD–EGFP was cloned using overlapping polymerase chain reactions (PCRs). First, the fragment extending from the IgK leader to the PDGFR TMD was obtained using the following primers:

CMV-CBD-5: ACGCGTCGACATGGAGACAGACACACTCCTGCTATG

CMV-CBD-A: ATGGAGACAGACACACTCCTGCTATGGGTACTGCTG-CTCTGGGTTCCAGGTTCCACTGGTGACTATCCATATGATGTTCCAGAT-TATGCTG

CMV-CBD-B: GCTGTGTTGACCTGCCAAGCGGATACACCAGGATTT-GTCGTGGCCGGCTGGGCCAAGGCTCCAGCATAATCTGGAACATCATA-TGGATAGTC

CMV-CBD-C: GCTTGGCAGGTCAACACAGCTTATACTGCGGGACAA-TTGGTCACATATAACGGCAAGACGTATAAATGTTTGCAGCCTCACACAT-CATTGGCAG

CMV-CBD-D: GAGATGAGCTTCTGTTCGAGGCCTCGGGGGCCTTGA-AGCTGCCACAAGGCAGGAACGTTGGATGGTTCCCATCCTGCCAATGA-TGTGTGAGGCTG

CMV-CBD-E: GGCCTCGAACAGAAGCTCATCTCAGAAGAGGATCT-GAATGCTGTGGGCCAGGACACGCAGGAGGTCATCGTGGTGCCACACT-CCTTGCCCTTTAAGG

CMV-CBD-F: CATGATGAGGATGATAAGGGAGATGATGGTGAGCAC-CACCAGGGCCAGGATGGCTGAGATCACCACCACCTTAAAGGGCAAGGAG-TGTGGC

CMV-CBD-3: CGCGGATCCCTAACGTGGCTTCTTCTGCCAAAGCATGATGAGGATGATAAGGGAGATGATG

Second, the EGFP fragment was amplified from the pEGFP (Clontech) using the following primers:

EF-GFP-5: CCGTTTCGAACCAGACGTCTGGGCCGCCACCATGGTGAGCAAGGGCGAGGAG

EF-GFP-3: CCGTTTCGAACTCCAGCATGCTGGTCTTGTACAGCTCGTCCATGCCGAG

To obtain pBudCE4.1-EGFP, this fragment was inserted into *Bst*B I sites of pBudCE4.1.

Third, ChBD and the EGFP genes were fused using the following primers:

CG-5: ACGCGTCGACATGGAGACAGACACACTCCTGCTATGGGTAC

CG-CP-F: ATGGAGACAGACACACTCCTGCTATGGGTAC

CG-CP-R: GCTCCTCGCCCTTGCTCACCATGCTGCCTCCTGCAGCGGCCGCTCCGGAACGTGGCTTCTTCTGCCAAAGCATG

CG-EGFP-F: ATGGTGAGCAAGGGCGAGGAGC

CG-EGFP-R: CTTGTACAGCTCGTCCATGCCGAGAG

CG-3: CGCGGATCCCTACTTGTACAGCTCGTCCATGCCGAGAG

To obtain BH1, the ChBD–EGFP fusion was inserted into *Sal* I and *Bam*H I sites of pBudCE4.1 (Invitrogen). Then, the *Not*I site in EGFP was deleted using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent) with the primer CGTTCCGGAGCAGCCGCTGCAGG. To obtain BH2, ChBD–EGFP and the CMV promoter were amplified from BH1 and inserted into *Bsr*G I and *Bam*H I sites of pCEP4 (Invitrogen). Then, the EF-1α cassette was amplified from BH1 and inserted into the *Xba* I and *Pci* I sites of pCEP4.

Immunofluorescence using confocal microscopy: BH1 and mock transfected HEK 293T cells growing on collagen-coated coverslips were fixed in 10% formaldehyde before staining. The cell membranes were stained with 5 $\mu g\,mL^{-1}$ AlexaFluor 555 conjugated wheat germ agglutinin (Invitrogen). The nuclei were stained with 5 $\mu g\,mL^{-1}$ Hoechst 34580 (Invitrogen). Confocal fluorescent images were obtained using a Bio-Rad (Zeiss) Radiance 2100 Rainbow laser scanning confocal microscope attached to a Nikon TE2000-U microscope with infinity corrected optics with a 60× objective. After sequential excitation, blue, green, and red fluorescent images of the same cell were collected. Images were analyzed with ImageJ software. The term colocalization refers to the coincidence of green and red fluorescence, as measured by the confocal microscope.

Cell binding: BH1 or pBudCE4.1-EGFP transfected Wil2-NS cells were mixed with 200-fold nontransfected cells and then incubated with magnetic chitin-coated beads (New England Biolabs) for 1 h. Then, the beads were separated with a magnet and washed with phosphate-buffered saline (PBS). An aliquot of beads was transferred to slides and observed by fluorescence microscopy. To visualize the beads and the cells at the same time and also show the localization of cells relative to the beads, bright light was used to visualize the beads.

Cell-membrane purification: BH1 or mock-transfected HEK 293T cells were harvested, washed with cold PBS, and then sonicated in PBS on ice. The homogenate was incubated with magnetic chitin-coated beads. The beads were separated with a magnet and the unbound fraction (flow-through) was collected. The beads were washed thoroughly with PBS, and resuspended in PBS. The homogenate, flow-through, and bead fractions were treated with SDS-PAGE loading buffer, and then loaded for Western blotting. Antibodies against α1 sodium/potassium ATPase (ab7671, Abcam) and pan cadherin (ab22744, Abcam) were used together with secondary HRP-conjugated goat-anti-mouse IgG (1030–05, Southern Biotech). HRP-conjugated antibody against β actin was purchased from Cell Signaling (5125).

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