Development of intensiometric indicators for visualizing N-cadherin interaction across cells

N-cadherin (NCad) is a classical cadherin that mediates cell–cell interactions in a Ca^{2+}-dependent manner. NCad participates in various biological processes, from ontogenesis to higher brain functions, though the visualization of NCad interactions in living cells remains limited. Here, we present intensiometric NCad interaction indicators, named INCIDERs, that utilize dimerization-dependent fluorescent proteins. INCIDERs successfully visualize reversible NCad interactions across cells. Compared to FRET-based indicators, INCIDERs have a ~70-fold higher signal contrast, enabling clear identification of NCad interactions. In primary neuronal cells, NCad interactions are visualized between closely apposed processes. Furthermore, visualization of NCad interaction at cell adhesion sites in dense cell populations is achieved by two-photon microscopy. INCIDERs are useful tools in the spatiotemporal investigation of NCad interactions across cells; future research should evaluate the potential of INCIDERs in mapping complex three-dimensional architectures in multi-cellular systems.
In multicellular organisms, cell adhesion plays an essential role in cell sorting, the formation and maintenance of tissues and organs, and synaptic transmission. In cell adhesion, the adhesive molecules govern the interactions across the cells. Neural cadherin (N-cadherin, hereafter NCad) is a cell adhesion molecule that belongs to the classical cadherin family and mediates Ca2+-dependent cell adhesions. NCad contributes to a broad range of biological processes, including ontogenesis, cell migration, neurite outgrowth, and synaptic potentiation, though, due to a lack of appropriate detection techniques, it remains unclear when and where NCad interactions occur. To date, only one NCad interaction indicator has been developed. The indicator exploits a Förster resonance energy transfer (FRET) mechanism between two fluorescent proteins, which is widely used in the development of fluorescent indicators of various cellular events. Using the FRET-based NCad interaction indicator, Kim et al. investigated the Ca2+-responsive dynamics of NCad interactions across cells. However, FRET-based indicators sometimes encounter a hurdle to their broad application due to their low signal contrast that causes difficulty in detecting FRET signals.

Intensiometric indicators are considered effective alternatives for detecting protein conformation changes and protein–protein interactions via an alteration in fluorescence intensity. For the visualization of cell–cell interactions, split-GFP technique can be used. GRASP, mGRASP, SynView, and eGRASP have been developed based on functional complementation between split superfolder GFP fragments (named GFP1-10 and GFP11). Applications in brain studies have achieved partial visualization of connectomes with a high signal contrast. A recently developed GRAPHIC system, utilizing the fragments GFP1-7 and GFP8-11, successfully visualized cell–cell interactions and neuronal connections with comparatively high signal intensity. However, visualization of cell–cell interactions using this technology is inhibited by some limitations. For example, the emergence of fluorescence from the reconstituted split components by cell–cell interactions requires a time delay derived from the maturation of chromophores, and the irreversibility of the process makes it impossible to monitor the dissociation of cell–cell interactions.

As a technique to visualize protein–protein interactions, a dimerization-dependent fluorescent protein (ddFP) has recently been adopted for various genetically encoded indicators to monitor apoptosis, Ca2+ levels, kinase activity, phosphatidylinositol 4,5-bisphosphate (PIP2) levels, and small GTPase activity. A ddFP consists of two fluorescent protein–monomers, namely ddFP-A and ddFP-B that has and lacks chromophores, respectively. The chromophore in ddFP-A, which is quenched in the monomeric state, emits bright fluorescence when forming a heterodimer with ddFP-B. ddFP was originally developed as a red variant ddRFP from dTomato and followed by a green variant ddGFP and a yellow variant ddYPFP. These color variants give applicability on multicolor imaging and combination with optogenetic tools. Unlike split-GFP technique, ddFPs do not require a time delay after protein–protein interactions and are reversible, which allows for real-time monitoring of transitions between interactions.

In this study, we evaluate the efficacy and practicality of INCIDERs: fluorescent indicators for the detection of NCad interactions. INCIDERs with reversible properties overcome the limitations of split-GFP-based indicators and were successfully applied in the monitoring of the association and dissociation of NCad interactions in living cells. We successfully visualized NCad interactions in primary neurons and spheroids consisting of multi-layered cells.

Results
Design of ddFP-based NCad interaction indicators (INCIDERs). To generate intensityometric NCad interaction indicators, we referred to the FRET-based indicator for NCad interaction. This indicator contains a cyan fluorescent protein (Cerulean) and a yellow fluorescent protein (Venus) as the FRET donor and acceptor, respectively; each fluorescent protein is inserted into the respective NCad molecules. Their insertion site (the 311th residue) at a second extracellular cadherin (EC) domain was near the interface of intercellular NCad interaction, and the fluorescent protein insertion did not impair the localization or function of NCad. By replacing the fluorescent proteins with ddGFP-A and ddFP-B, respectively, we generated ddGFP-A-inserted NCad (NCad-GA) and ddFP-B-inserted NCad (NCad-B) (Fig. 1a). We used two variants of ddFP-B with different affinities to ddGFP-A, ddFP-B1 and ddFP-B3 (Kd = 3 μM and 40 μM, respectively). The indicators composed of the combinations of NCad-GA/NCad-B and NCad-GA/NCad-B3 were denominated as INCIDER (Indicator for N-Cadherin Interaction upon Dimerization)-1 and INCIDER-3, respectively. To evaluate heterodimer formation and the associated fluorescence, we transiently co-expressed the INCIDERs in HEK293T cells. While some artificial fluorescent puncta, which are considered autofluorescence were observed in the cells individually expressing components of the INCIDER (NCad-GA, NCad-B, or NCad-B3), there was no fluorescence at their cell–cell interfaces. On the other hand, cells co-expressing INCIDER-1 and INCIDER-3 fluoresced at the cell adhesion site and intracellular space, as did the fluorescence localization of Venus-inserted NCad (NCad-V), indicating heterodimer formation of INCIDERs in intra- and intercellular spaces (Supplementary Fig. 1).

Detection of intercellular NCad interaction by INCIDERs. Since INCIDERs are based on the co-expression of two components in the same cell, intracellular and intercellular NCad interactions become indistinguishable. To specifically monitor intercellular NCad interactions, we co-cultured cells expressing only one of the INCIDER components, i.e., NCad-GA or NCad-B. Cells expressing NCad-GA and NCad-B were distinguished by the nuclear localized fluorescent marker proteins mCherry and miRFP670, respectively. Fluorescence was observed at the cell adhesion sites between mCherry-positive and miRFP670-positive cells (Fig. 1b), which verified the detection of intercellular NCad interactions by INCIDERs. In a comparison of fluorescence intensity between INCIDER-1 and INCIDER-3, INCIDER-1 had a slightly higher signal (Fig. 1c), which was expected given the higher heterodimer affinity between ddGFP-A and ddFP-B. INCIDER fluorescence signals were positively correlated with their expression levels, as estimated by their expression marker fluorescent proteins (Supplementary Fig. 2a, b).

It is possible that the heterodimerization between ddGFP-A and ddFP-B enhances association of cell–cell interactions. To address the possibility, we examined the adhesive functions of INCIDERs using K562 cells that lack endogenous adhesion proteins. While K562 cells that were not transfected with exogenous NCad constructs were observed as separated cells, those expressing exogenous NCad constructs formed cell aggregates (Supplementary Fig. 3a). Compared with cells expressing non-tagged NCad, the size of cell aggregates expressing ddFP-based NCad (INCI-DER) and FRET-based NCad indicators (NCad-FRET) tended to be smaller. However, INCIDER and NCad-FRET induced the formation of cell aggregates to the same extent (Supplementary Fig. 3b). This result shows that the insertion of fluorescent proteins into NCad slightly weakens the adhesive function, but heterodimerization of ddGFP-A and ddFP-B does not significantly enhance association of cell–cell interactions.

To examine the importance of the ddGFP-insertion site for the detection of NCad interactions, we generated NCad with ddGFP-A and ddFP-B...
inserted at a site distant to the intercellular NCad interaction interface, referred to as NCad-GA$_{\text{prox}}$, NCad-B1$_{\text{prox}}$, and NCad-B3$_{\text{prox}}$ (Supplementary Fig. 4a). As in the previous report, NCad in which Venus was inserted at the same site (NCad-V$_{\text{prox}}$) localized at the cell adhesion sites, indicating that the insertion of a fluorescent protein at this site does not impair NCad localization (Supplementary Fig. 4b). Fluorescence of HEK293T cells co-expressing NCad-GA$_{\text{prox}}$ and NCad-B$_{\text{prox}}$ (NCad-B1$_{\text{prox}}$ or NCad-B3$_{\text{prox}}$) indicated heterodimer formation between ddGFP-A and ddFP-B predominantly in the intracellular space (Supplementary Fig. 4b). Cells individually expressing NCad-GA$_{\text{prox}}$ or NCad-B$_{\text{prox}}$ were co-cultured to evaluate the efficiency of NCad-GA$_{\text{prox}}$/NCad-B$_{\text{prox}}$ for visualization of intercellular NCad interactions. Compared to INCIDERs, little fluorescence was observed at the cell adhesion sites between NCad-GA$_{\text{prox}}$-expressing cells and NCad-B$_{\text{prox}}$-expressing cells (Supplementary Fig. 4c). These results demonstrate the importance of the insertion site for heterodimerization of ddGFP-A and ddFP-B across cells.

Visualization of intercellular NCad interaction by co-expressed INCIDER components. INCIDERs are supposed to form not only a trans-interaction across the opposed plasma membranes of different cells, but also a cis-interaction on the individual membrane. Cells co-expressing INCIDER components fluoresced at cell adhesion sites (Supplementary Fig. 1). This prompted us to examine, in a co-culture experiment, how trans- and cis-interactions contribute to the fluorescence signal (Fig. 2a). We prepared two groups of cells: (i) cells co-expressing NCad, lacking a fluorescent protein, with an expression marker EBFP2-NLS and (ii) cells co-expressing INCIDER components (NCad-GA and NCad-B) with an expression marker mCherry-NLS. If fluorescence is detected between EBFP2-positive and mCherry-positive cells (B–R), it indicates that INCIDERs fluoresce with cis-interactions. On the other hand, if fluorescence is detected only between mCherry-positive cells (R–R), the fluorescence of INCIDERs would be solely derived from trans-interactions. We observed fluorescence of INCIDERs at R–R (Fig. 2b, open arrowheads), but not at B–R (Fig. 2b, closed arrowheads). While NCad-V showed low contrast of Venus signals between R–R and B–R (1.37), those of INCIDER-1 and -3 signals between R–R and B–R were high (5.31 and 7.72, respectively) (Fig. 2c). These results indicate that co-expressed INCIDER components predominantly visualize trans-interactions.

Confirmation of signal specificity for NCad interactions. Since ddGFP-A and ddFP-B have an affinity for heterodimer formation, INCIDERs might generate signals only via the self-interaction of ddGFP-A and ddFP-B regardless of NCad interactions. To address this issue, we evaluated the activity of a mutant NCad that can form neither trans- nor cis-interactions. We introduced W2A/R14E and V81D/V174D mutations to eliminate trans- and cis-interactions, respectively. To exclude the effect of endogenous NCad, we used L cells that lack endogenous cadherins and established stable cell lines of fluorescent protein-inserted wild-type NCad (NCadWT-V, NCadWT-GA, NCadWT-B1, and NCadWT-B3) and fluorescent protein-inserted mutant NCad (NCadmut-V, NCadmut-GA, NCadmut-B1, and NCadmut-B3). We confirmed, by western blot analysis using an anti-NCad antibody, that NCad was detected as two bands (upper and lower bands corresponding to unprocessed and processed NCad, respectively) and expression levels of total NCad varied between cell lines to some extent (Fig. 3a, b). In the stable cell line, NCadmut-V was localized at the plasma membrane and cell–cell interface, similar...
to NCad^{WT-V} (Fig. 3c). We co-cultured L cells stably expressing NCad^{mut-GA} and NCad^{mut-B}, respectively. Although ddGFP signals were detected at the cell adhesion sites between NCad^{WT-GA}-expressing and NCad^{WT-B}-expressing cells, significantly fewer signals were detected at the cell–cell interfaces between NCad^{mut-GA}-expressing and NCad^{mut-B}-expressing cells (Fig. 3c, d). It is possible that a difference in membrane localization efficiency between NCad^{WT} and NCad^{mut} contributed to a difference...
To address this possibility, we performed surface staining of NCad WT-V-expressing cells and NCadmut-V-expressing cells and estimated the efficiency by calculating the ratio of immunoreactive signals using anti-GFP antibody to Venus signals. We found that NCad mut-V localized at the plasma membrane with approximately 60% efficiency of NCad WT-V (Supplementary Fig. 5a, b). This result implies that poorer ddGFP signals from NCadmut-GA and NCadmut-B were partially contributed to by poorer membrane localization. Since a difference in membrane localization was not so high compared with ddGFP signals, we concluded that INCIDERs give fluorescence dependent on the NCad-mediated cell–cell interaction.

We further performed the co-culture in the combination of NCadWT/NCadmut. Both NCadWT-GA/NCadmut-B1 pair and NCadWT-GA/NCadmut-B3 pair (INCIDER-3), NCadmut-GA/NCadmut-B1 pair, and NCadmut-GA/NCadmut-B3 pair. Scale bar, 20 μm. d Wild-type (WT) and mutant (mut) INCIDERs’ fluorescent signals at cell–cell contact sites were quantified and compared. Data are presented as lower quartile (lower whisker), median (center line), and upper quartile (upper whisker). Significant differences were analyzed by Mann-Whitney U test. ****p < 0.0001. 166 (WT of INCIDER-1), 156 (mut of INCIDER-1), 139 (WT of INCIDER-3), and 227 (mut of INCIDER-3) cell–cell contact sites from three independent experiments were analyzed.
NCadmut-GA/NCadWT-B1 pair showed ddGFP signals that were lower than the NCadWT-GA/NCadWT-B1 pair (Supplementary Fig. 5c, d). This result raises a possibility that NCadWT weakly interacts with NCadmut. However, further analysis is required to reach a conclusion as we did not examine the interaction between NCadWT and NCadmut.

Time-lapse imaging of reversible NCad interaction. To assess the detectability of reversible intercellular NCad interactions, we performed time-lapse imaging immediately after seeding cells expressing individual INCIDER components. During the formation of cell–cell contact, the INCIDER fluorescence signal increased at the cell adhesion sites. After the addition of EGTA to the culture medium (for the chelation of extracellular Ca\(^{2+}\) that is essential for intercellular NCad interaction\(^9\)), the INCIDER signal gradually decreased over time (Fig. 4a). INCIDER-1 and INCIDER-3 showed similar time trajectories related to fluorescence intensity and there was no significant difference between them, regardless of the differences in affinity to ddGFP-A. Signal intensity reached 50% ~50 min after the addition of EGTA (Fig. 4b, c). We further examined whether INCIDERs can monitor a re-association of NCad. INCIDER signals that declined with EGTA treatment were increased with the washout of EGTA again (Fig. 4d–f). These results indicate that INCIDERs show the appropriate availability for monitoring the transition between association and dissociation of NCad interactions.

Comparison between INCIDERs and conventional indicators for cell–cell interactions. To determine relative performance, we compared the signal contrast between FRET-based NCad indicator (Ncad-FRET) and INCIDERs. We individually expressed and subsequently co-cultured Cerulean-inserted NCad (C-Ncad) and Venus-inserted NCad (V-Ncad) in COS7 cells. FRET was confirmed at the cell adhesion sites as a YFP/CFP ratio (Fig. 5a). We measured the YFP/CFP ratio at the cell adhesion sites and non-cell adhesion surfaces of cells expressing C-Ncad. The signal contrast for FRET was calculated by dividing the YFP/CFP ratio at cell adhesion sites by that at non-cell adhesion sites (Fig. 5c).
INCIDERs also visualized NCad interactions at the COS7 cell adhesion sites (Fig. 5b). The signal contrast for INCIDERs was calculated by dividing the fluorescence intensity at the cell adhesion sites by that at the non-cell adhesion sites of NCad-GA expressing cells. The signal contrast of INCIDERs was ~70-times higher than that of Ncad-FRET (Fig. 5c). INCIDERs enabled the clear identification of NCad interactions across cells without post-hoc image processing.

Next, we compared the performance between INCIDERs and a split-GFP-based indicator for cell–cell interactions (mGRASP24). mGRASP showed green fluorescence at the cell adhesion sites similar to INCIDERs and its signal was more than twice as high as that of INCIDERs (Fig. 5d, e). Split-GFP-based indicators for cell–cell interactions are potentially unable to monitor the dissociation of cell–cell interactions 25,28,30. To evaluate the relative dissociation property, we examined the effect of EGTA treatment on pre-formed cell–cell interactions. We first co-cultured HEK293T cells individually expressing INCIDERs or mGRASP components for 6 h and then further cultured them in the presence or absence of 10 mM EGTA for 2 h. The INCIDER signals were diminished by incubation with EGTA while mGRASP showed signals regardless of EGTA treatment (Fig. 5f, g). This result shows that INCIDERs can monitor the dissociation of cell–cell interactions unlike split-GFP-based indicators.
Efficacy of INCIDER visualization of NCad interactions in different specimens. Finally, we assessed the performance and application potential of INCIDERs across a range of samples. NCad interaction in immature neurons is involved in the determination of neuronal polarity and neurite growth and maintenance. To examine the detectability of INCIDERs in neurons, we expressed individual INCIDER components in primary cultured cortical neurons and assessed fluorescence after 2 days in vitro (DIV). As expected, the INCIDER fluorescence signal was predominantly observed at the contact sites of neuronal processes (Fig. 6a, Supplementary Fig. 6). The signal was specifically detected between closely apposed processes of an NCad-GA-expressing neuron and an NCad-B-expressing neuron (Fig. 6b). By contrast, a process of an NCad-GA-expressing neuron was not detected when co-cultured with an NCad-B1-expressing neuron (Fig. 6b).
neuron that was not attached to an NCad-B-expressing neuron showed little fluorescence (Fig. 6b). We also applied NCad-FRET to neurons. However, FRET signal was not detected well at the contact sites between C-Ncad-expressing neurons and V-Ncad-expressing neurons, which was possibly due to the low signal contrast of NCad-FRET. (Supplementary Fig. 7).

We examined the functionality of INCIDERs in multi-layered spheroids expressing INCIDER components. Although NCad stable cell lines formed spheroids, the parental L cells failed to form spheroids (Supplementary Fig. 8). Using a two-photon microscope, we confirmed that INCIDER fluorescence signals were detected in spheroids formed by NCad-GA-expressing cells and NCad-B-expressing cells (Fig. 6c, d). These results suggest that INCIDERs can be applied to a range of specimens with different physical characteristics.

**Discussion**

Here, we developed ddFP-based indicators for NCad interactions by inserting ddFPs in NCad. We showed that INCIDER signals were dependent on NCad interactions, not the self-interaction of ddGFP-A and ddFP-B (Fig. 3). However, INCIDER should generate the signal via the ddGFP-A/ddFP-B heterodimerization. This notion raises an interpretation that NCad interacting property of INCIDER leads to the formation of a ddGFP-A/ddFP-B heterodimer. Therefore, this ddGFP-A/ddFP-B heterodimerization possibly affects cell–cell interactions. Cell aggregation assay using K562 cells showed that INCIDERs induced cell aggregation to the same extent as Ncad-FRET, indicating that the heterodimerization of ddGFP-A/ddFP-B does not significantly induce cell–cell interactions (Supplementary Fig. 3). However, EGTA treatment experiments showed that time scale of fluorescence decay of INCIDERs upon EGTA treatment was in the order of minute-to-hour (Fig. 4a–c) although that of NCad-FRET has been reported to be in the order of seconds. This means that the heterodimerization of ddGFP-A/ddFP-B possibly affects the dissociation of NCad interactions. While INCIDERs are reversible unlike split-GFP-based indicators (Fig. 5f, g), it is noticeable that the relatively slow decay is a limitation of INCIDERs. The development of ddFP-B mutants with lower affinity to ddGFP-A may solve this limitation.

We used ddFP-B1 and ddFP-B3, respectively, to prepare ddFPB1 and ddFP-B3, 3 µM and 40 µM, respectively. Since the 𝐾d of the homodimerization of NCad is approximately 25 µM, NCad-B3 would be less likely to interfere with NCad interactions. However, there was no significant difference in time-dependent fluorescence intensity between INCIDER-1 and INCIDER-3 (Fig. 4b, c). Fluorescence intensity was significantly higher in INCIDER-1 compared to INCIDER-3 (Fig. 1c). Thus, INCIDER-1 seems to be the most suitable indicator for monitoring NCad interactions, though further experimental comparisons between these INCIDERs are required to comprehensively determine their respective advantages.

INCIDERs function not only as indicators, but also adhesion molecules that promote cell–cell interactions by maintaining intercellular distance. This property can disturb endogenous NCad functions or confer a gain-of-function by overexpression of interactions. To promote the accuracy of assays, the expression level should be adjusted by, for example, establishing stable cell lines or knock-in cells. The co-expression of INCIDER components (NCad-GA and NCad-B) predominantly visualized a trans-interface of NCad interactions at the cell–cell junctions (Fig. 2), independent of whether two adjacent cells express different components of INCIDER. Therefore, the effective monitoring of NCad interactions could be achieved by using knock-in cells or knock-in mice expressing NCad-GA and NCad-B heterozygously.

Split-GFP-based indicators for cell–cell interactions, such as m- or eGRASP and SynView, have aided our understanding of connectomes by direct visualization of neural connections. However, their irreversibility does not allow for real-time monitoring of connectome changes, which the ddFP-based technique in the current study could achieve with an appropriate level of fluorescence.

In summary, we developed intensiometric NCad interaction indicators, INCIDERs, utilizing ddGFP that can reversibly visualize NCad interactions across cells. The INCIDERs can be applied to a wide range of specimens and could provide useful insights regarding the spatiotemporal dynamics of NCad interactions in various biological processes, including organogenesis and higher brain functions.

**Methods**

**Procedural construction.** All expression plasmids were subcloned into a pCAGGSi vector. To generate NCad-V, the PCR product encoding mVen, flanked by a BamHI and Nhel site, was inserted at amino acid position 311 of mouse NCad with a C-terminal HA tag. For the construction of NCad-GA, NCad-B1, and NCad-B3, we amplified ddGFP-A, ddFP-B1, and ddFP-B3, flanked by a BamHI and Nhel site, from the synthesized gene fragments (Integrated DNA Technologies). In order to construct NCad-V, we inserted mVen, flanked by Nhel sites, at amino acid position 714 of mouse NCad by overlapping PCR. NCad-GAprox, NCad-B1prox, and NCad-B3prox were generated by inserting ddGFP-A, ddFP-B1, and ddFP-B3, flanked by Nhel sites, into NCad-Vprox that the mVen was excised by Nhel, respectively. To distinguish cells expressing NCad-GA and NCad-B in the coculture, marker fluorescent proteins mCherry and miRFP670 were tagged with a nuclear localization signal (NLS) and co-expressed bicistronically via a self-cleavable P2A peptide. To generate NCad-GA with P2a-mCherry-NLS, NCad-B1 with P2a-miRFP670-NLS, and NCad-B3 with P2a-miRFP670-NLS, the PCR products encoding P2a-mCherry-NLS and P2a-miRFP670-NLS were inserted between the P2A and a Nhel site. The Agel site is inserted to the HA tag and the stop codon of NCad-GA, NCad-B1, and NCad-B3. The NotI site is derived from pCAGGSi. mCherry-NLS and EBFP2-NLS were constructed by subcloning the PCR products into the pCAGGSi vector. In order to generate mutant NCad, we introduced four mutations by site-directed mutagenesis in combination with overlapping PCR. To establish stable cell lines, retrovirus plasmids were subcloned into pCXpuro and pCXbxs vectors. EBFP2-NLS and mCherry-NLS were subcloned into the pCX4bxs vector. Fluorescent protein-inserted NCad constructs were subcloned into the pCXpuro vector. For the construction of FRET-based NCad interaction indicators, C-Ncad and V-Ncad, we inserted Cerulean and Venus, flanked by transposon-derived extra amino acids, at amino acid position 311 of mouse NCad by overlapping PCR according to a previous study. PaavCAG-post-mGRASP-2A-dTomato (Addgene plasmid #34912) and paavCAG-pre-mGRASP-mCerulean (Addgene plasmid #34910) were gifted by Jinho Kim. To construct post-mGRASP-2A-mCherry-NLS and pre-mGRASP-2A-EBFP2-NLS, NCad-GAprox, NCad-B1prox, and NCad-B3prox were inserted between the P2A and a Nhel site. The NotI site is derived from pCAGGSi. mCherry-NLS and EBFP2-NLS were inserted into EcorI/Agel-digested NCad-GA-2A-mCherry-NLS and NCad-B1-2A-EBFP2-NLS plasmids, respectively.

**Animals.** Animal experimentation was performed according to the Institutional Guidelines on Animal Experimentation at Keio University. Pregnant ICR mice were purchased from Japan SLC (Japan) and housed under a 12 h light/12 h dark cycle in a temperature-controlled room. The day of vaginal plug detection was considered as embryonic day 0 (E0).

**Cell culture, plasmid transfection, and retroviral infection.** HEK293T cells (RIKEN BRC), L cells (ATCC), and CO57 cells (RIKEN BRC) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (FBS, Bioset) at 37°C in humidified air containing 5% CO₂. K562 (RIKEN BRC) cells were maintained in Iscove’s modified Dulbecco’s medium (IMDM, Thermo Fisher Scientific) supplemented with 10% (v/v) FBS. An etoposide murine leukemia virus-packaging cell line, Platinum-E (PLAT-E)9, was purchased from Dr. Hideki Shibata (Nagoya University) and maintained in Dulbecco’s medium supplemented with 10% (v/v) FBS, 1 µg/mL of puromycin (InviBioGen), and 10 µg/mL of blasticidin (InviBioGen). HEK293T cells were transfected using polyethylenimine MAX (Cosmo Bio). COS7 cells were transfected using Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific). K562 cells were electroporated using an electroporator (NEPA21: NEPAGENE). Cells stably expressing NCadWT-V and NCadmut-V were established as follows: PLaV2hE cells were transfected with pCXpuro-NCadWT-V or pCXpuro-NCadmut-V by FuGENE6 (Roche Diagnostics) according to the manufacturer’s protocol. After 48 h,
the cleared culture media were supplemented with 8 μg/mL of polybrene (Nakalai Tesque) and used for infection. More than 24 h after infection, L cells were selected with 10 μg/mL of puromycin. To establish L cells respectively expressing wild-type NCad-GA, NCad-B1, and NCad-B3 by using PLAT-E cells transfected with pCX4puro-NCad-GA, pCX4puro-NCad-B1, and pCX4puro-NCad-B3, respectively. Consequently, they were selected with 5 μg/mL of puromycin and 10 μg/mL of blasticidin.

For primary culture of cortical neurons with NCad-GA or NCad-B1, plasmids encoding NCad-GA-P2A-mCherry or NCad-B1-P2A-EBFP2 was introduced into the mouse embryonic cortical cells by in utero electroporation as previously described. Three days later, electroporated areas in the cortices were dissected and dissociated with papain (Nakalai Tesque). mCherry or BFP positive cells were sorted from the dissociated cells by fluorescence activated cell sorting (FACS) (MoFlo XDP, Beckman). After sorting, an equal number of cells were mixed in phenol red-free DMEM/F12 (Thermo Fisher Scientific) with B-27 supplement (Thermo Fisher Scientific) and cultured in Neurobasal Medium (Thermo Fisher Scientific) supplemented (1% (v/v) FBS. After 3 d, spheroids were placed on glass-bottom dishes and incubated on ice for 30 min. After centrifugation at 18,000 g for 10 min at 4 °C, the cleared cell lysates were subjected to SDS-PAGE, followed by western blot using a rabbit polyclonal anti-human NCad antibody (×200 dilution, Code No. 598, MBL) for 1 h at room temperature. The cells were then washed with PBS containing 1% (v/v) BSA and incubated with an Alexa Fluor633-conjugated goat antibody against rabbit IgG (×1000 dilution, Cat# A21070, Thermo Fisher Scientific) for 1 h at room temperature. After washing with PBS containing 1% (v/v) BSA three times and rinsing with PBS, the coverslips were mounted on microscopic slides with Prolong Glass Antifade Mountant (Thermo Fisher Scientific).

For neuronal cells at 2 DIV, cultured dishes were mounted in 40% O2 incubator chamber equipped with a ×25, 1.05 NA water-immersion objective lens XLPLN25XWMP2 (Olympus). Excitation wavelengths for EBFP2, INDICER, Venus, mCherry, and mRFP670 were 405, 488, 488, 543, and 633 nm, respectively. In subsequent cell imaging experiments, cells transiently or stably expressing indicated proteins were imaged on glass-bottomed dishes coated with Cellstrat Type IC (Nitta gelatin). After 24 to 48 h, the cells were observed using a confocal microscope.

For FREt ratio imaging, Cerulean was excited with a 405-nm laser. Donor and acceptor emissions were collected at 460–500 nm and 515–615 nm, respectively. An intensity display mode (IMD) image was created using MetaMorph software (Molecular Devices).

For neuronal cells at 2 DIV, cultured dishes were mounted in a 40% O2 incubator chamber fitted onto a TCS SP8 laser scanning confocal microscope (Leica Microsystems). Images were acquired using the confocal microscope with a PL APO 40×/1.30 NA oil-immersion objective lens (UApo/340 40×/1.35) or a ×60 1.35 NA oil-immersion objective (UPLSAPO60XO) (Olympus). Excitation wavelengths for EBFP2, INDICER, Venus, mCherry, and mRFP670 were 405, 488, 488, 543, and 633 nm, respectively. In subsequent cell imaging experiments, cells transiently or stably expressing indicated proteins were imaged on glass-bottomed dishes coated with Cellstrat Type IC (Nitta gelatin). After 24 to 48 h, the cells were observed using a confocal microscope.

Cell imaging. For the co-culture experiment (Figs. 1b 5d, f and Supplementary Fig. 4c), HEK293T cells were individually transfected with expression plasmids and cultured for 24 h. The cells were then washed with PBS containing 1 mM EDTA and mixed in phenol red free DMEM/F12 (Thermo Fisher Scientific) supplemented with 10% FBS. Cells were co-cultured in a 15 mL conical tube under slow rotation at room temperature for 8 h and seeded on a glass-bottomed dish coated with 0.1% (v/v) PEI (P3143, Sigma-Aldrich). Fluorescence and DIC images were acquired using an Olympus FV-1000 laser scanning confocal microscope with an IX81 microscope equipped with a ×40 1.35 numerical aperture (NA) oil-immersion objective lens (U/Apo/340 40×/1.35) or a ×60 1.35 NA oil-immersion objective (Olympus) (UPLSAPO60XO). Excitation wavelengths for K562 cells were placed on a glass-bottomed dish coated with poly-D-lysine (Sigma-Aldrich) and cultured in Neurobasal Medium (Thermo Fisher Scientific) with B-27 supplement (Thermo Fisher Scientific) and L-glutamine (Sigma-Aldrich).

For primary culture of cortical neurons with NCad-GA or NCad-B1, plasmids encoding C-Ncad or V-Ncad, cortices from embryonic day 10 (E10) were dissociated and dissociated with papain (Nakalai Tesque). mCherry or BFP positive cells were sorted from the dissociated cells by fluorescence activated cell sorting (FACS) (MoFlo XDP, Beckman). After sorting, an equal number of cells were mixed and plated onto a glass bottom dish (MatTek) coated with poly-D-lysine (Sigma-Aldrich) and cultured in Neurobasal Medium (Thermo Fisher Scientific) with B-27 supplement (Thermo Fisher Scientific) and L-glutamine (Sigma-Aldrich). For neuronal cells at 2 DIV, cultured dishes were mounted in a 40% O2 incubator chamber equipped with a ×25, 1.05 NA water-immersion objective lens XLPLN25XWMP2 (Olympus) and a TiSapphire laser Mai-Tai DeepSee eEPH (Spectra-Physics). Excitation wavelengths for INDICER and mCherry were 930 and 1,040 nm, respectively. We were unable to detect the EBFP2 signal due to the available microscopic optical setting.

Fluorescence images were analyzed using ImageJ. Merged images were synthesized from fluorescence images without modification and DIC images by ImageJ through "Merge Channels" function.

Western blot analysis. Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 1% Triton X-100) supplemented with Protease Inhibitor Cocktail (purification of Histidine-tagged proteins (Sigma-Aldrich) and incubated on ice for 30 min. After centrifugation at 18,000 g for 10 min at 4 °C, the cleared cell lysates were subjected to SDS-PAGE, followed by western blot using a rabbit polyclonal anti-human NCad antibody (×200 dilution, Cat# M142, Takara Bio) and a mouse monoclonal anti-GAPDH antibody (×1000 dilution, clone 6C5, sc-32233, Santa Cruz Biotechnology).

Data availability. The data and materials supporting this research are available from the authors on reasonable request. Plasmid constructs encoding NCad-V (Addgene ID, 191665), NCad-GA (Addgene ID, 191666), NCad-B1 (Addgene ID, 191667), and NCad-B3 (Addgene ID, 191668) are available through Addgene. The source data underlying Figs. 1c, 2c, 3b, d, 4b, c, e, 5c, e, g, 6d, 52a, 52b, 55b, and 55d are provided as Supplementary Data. Uncropped images of western blots in Fig. 5a are provided in Supplementary Fig. 9.

Received: 21 December 2021; Accepted: 22 September 2022; Published online: 07 October 2022

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