



Supplementary Materials for  
**Plant “helper” immune receptors are Ca<sup>2+</sup>-permeable  
nonselective cation channels**

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**Other Supplementary Material for this manuscript includes the following:**

(available at [science.sciencemag.org/cgi/content/full/science.abg7917/DC1](https://science.sciencemag.org/cgi/content/full/science.abg7917/DC1))

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## Materials and Methods

### Recombinant protein cloning, expression, and purification:

cDNA of *AtNRG1.1* CC 1-124 (WT, 2K/E and 7K(R)/E) was codon optimized for recombinant protein expression in *E. coli* and subcloned into the pET24a plasmid (Genescript) with a N-terminal (His)<sub>6</sub> tag and C-terminal Strep II tag (WSHPQFEK). All proteins were produced in *E. coli* BL21 (DE3) cells using the autoinduction method (46) and purified to homogeneity using nickel affinity chromatography followed by size-exclusion chromatography. The selenomethionine (SeMet) incorporated form of 7K(R)/E was produced using SelenoMet<sup>TM</sup> medium from Molecular Dimensions and 0.1 mM isopropyl-1-thio-D-galactopyranoside (IPTG) for protein induction. Briefly, *E. coli* cells were grown at 37°C until the OD<sub>600</sub> reached 0.6. The temperature was then decreased to 18°C with protein induction for 18 hours. Cells were pelleted by centrifugation at 6000 x g at 4°C for 30 minutes. All cell pellets were resuspended in 5 mL of the lysis buffer (50 mM HEPES pH 8.0, 300 mM NaCl and 2 mM DTT) per gram of cell paste. Phenylmethanesulfonylfluoride (PMSF; 1 mM final concentration), lysozyme, and DNase were added to the cell resuspension immediately prior to lysing. The resuspended cells were lysed using sonication and then centrifuged at 41, 211 x g for 20 minutes to remove cell debris. The supernatant was loaded onto a nickel HisTrap 5 mL column (GE Healthcare) pre-equilibrated with 20 mL of the wash buffer (50 mM HEPES pH 8.0, 300 mM NaCl, 30 mM imidazole) at a rate of 3 mL/min. The column was then washed with 100 mL of wash buffer and the bound protein was eluted with elution buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 500 mM imidazole). Elution fractions were monitored via A<sub>280</sub>, elution peak pooled, and further purified using a S200 HiLoad 16/600 column pre-equilibrated with the gel-filtration buffer (10 mM HEPES pH 7.5, 150 mM NaCl and 1mM DTT). The A<sub>280</sub> elution peak fractions were confirmed by SDS-PAGE, pooled, and the proteins were concentrated to a final concentration of 10 mg/mL. The concentrated proteins were flash-frozen as 30 µL aliquots in liquid nitrogen and stored at -80°C.

Protein crystallization, data collection, and structure determination:

All proteins were crystallized using the sitting drop vapor diffusion technique. A protein solution consisting of ~10 mg/mL 7K(R)/E native or 7K(R)/E SeMet was mixed at a 1:1 ratio with a precipitant solution composed of 0.1 M MES (pH 5.5) and 1 M potassium sodium tartrate.

5 7K(R)/E crystals formed within 3 days. Similarly, crystals of 2K/E were produced by mixing a 1:1 ratio of ~10 mg/mL protein solution with a precipitant solution consisting of 0.1 M HEPES: NaOH (pH 7.5) and 0.3 M magnesium formate. 2K/E crystals formed within 2 days.

10 Protein crystals of 7K(R)/E native were transferred directly into a mother liquor consisting of the well solution supplemented with ethylene glycol to a final concentration of 20% (v/v). Likewise, 7K(R)/E SeMet and 2K/E crystals were transferred into a mother liquor consisting of the well solution supplemented with glycerol to a final concentration of 20% (v/v). Once transferred, crystals were immediately flash cooled in liquid nitrogen in preparation for x-ray diffraction data collection.

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X-ray diffraction data for the 7K(R)/E SeMet construct (M1, M45, M58, and M98) were collected from a single crystal at the 23-IDD beamline using a wavelength tuned to 0.979 Å. Diffraction images were processed with the HKL2000 suite (47) and the structure was solved using the single-wavelength anomalous diffraction (SAD) method through the *Autosol* pipeline within the *PHENIX* suite. An initial model was built using the *Autobuild* pipeline within the *PHENIX* suite (48). X-ray diffraction data for native crystals of 7K(R)/E were collected at the 23-IDD beamline to a higher resolution than that of the 7K(R)/E SeMet crystals. The native 7K(R)/E diffraction images were processed using the HKL2000 suite and the structure was subsequently solved by molecular replacement using PHASER (49) with the 7K(R)/E SeMet build as a search model. X-ray diffraction data for 2K/E were collected at the 23-IDD beamline and the structure was solved by molecular replacement using PHASER with 7K(R)/E as the search model. All models were extended by several rounds of manual model building with COOT (50). Successive refinements were performed with Phenix.refine or REFMAC within the

25

CCP4 suite (48, 51). Structural analyses were carried out using DALI (52) and PyMOL (http://www.pymol.org). Figures were generated using PyMOL. Structures of 7K(R)/E native and 2K/E native are available in the PDB as accessions 7L7V and 7L7W, respectively.

DNA constructs and vectors used:

5 The *NRG1.1* CDS (wildtype and all analyzed mutants) were synthesized into a pUC57/Kan plasmid (Genescript) including the gateway compatible recombination sites (attR1/attR2). LR reactions (Gateway Cloning Technology, Life Technologies; Carlsbad, USA) were performed to introduce CDS's into the estradiol-inducible destination vector pMDC7 with a C-terminal YFP-HA CDS (53), or the 35s-driven destination vector pGWB641 (54). The clones of NRG1.1 1-10 180,  $\Delta$ N16 and structure-derived mutants (all C-terminal GFP-fusions) were constructed in pICSL86922 (35S promoter and TMV omega enhancer) using Golden Gate cloning (55).

Bacterial strains and growth conditions.

*Agrobacterium tumefaciens* strain GV3101/pMP90 were grown in LB media at 28°C. Antibiotic concentrations used (in g/mL) Kanamycin 100, Gentamycin 50 and Spectinomycin 100, 15 Rifampicin 100. Constructs were transformed into *Agrobacterium tumefaciens* strain GV3101/pMP90 and used for transient expression in *Nicotiana benthamiana*. NRG1.1 WT and variants were transiently expressed in *Nicotiana benthamiana*.

Determination of NRG1.1 localization using confocal imaging:

Briefly, *Agrobacterium tumefaciens* strains were grown overnight at 28°C in LB media 20 containing the appropriate antibiotics. The overnight cultures were centrifuged for 8 min at 8,500 rpm and the pellets were resuspended in induction buffer (10 mM MgCl<sub>2</sub>, 10 mM MES pH 5.6, 150  $\mu$ M acetosyringone). The OD<sub>600</sub> was adjusted to 0.05 (35S::P19) and 0.3 for NRG1.1 constructs and the specific subcellular marker constructs (35S::BRI1-mRFP – a gift from Klaus Harter, UBQ10::VMA12-RFP – a gift from Karen Schuhmacher – and 35S::PLC2-CFP). 25 Samples were mixed as indicated. *Agrobacteria* mixtures were infiltrated into young leaves of 4-6 weeks old *N. benthamiana* WT plants using a 1-ml needleless syringe. The *N. benthamiana* plants were grown on soil under 12h light / 12h dark cycles (24°C/22°C, 65% humidity). Induction was done 24 hours post infiltration using either 20  $\mu$ M  $\beta$ -estradiol (Sigma-Aldrich; St.

Louis, USA) and 0.001% [v/v] Silwet L-77 by spraying. Leaves were imaged for protein localization between 6 h- 24 h post induction with the confocal laser scanning microscope LSM880 from Zeiss (Oberkochen, Germany), using C-apochromat 40x or 63x/1.2 W Korr FCS M27 water-immersion objectives and the ZENblack software. YFP was excited using a 514 nm laser collecting emission between 516-556 nm; RFP was excited using a 561 nm laser with an emission spectrum of 597-633 nm, CFP was excited with a 458 nm laser and the emission spectrum was 463-513 nm. Focal plane images were processed with the ZENblue software (Zeiss) for adjustment of brightness and contrast. Maximum Z-projection images were processed with ImageJ/Fiji (56).

#### UV-light imaging of cell death phenotypes:

Briefly, *A. tumefaciens* strains were infiltrated into *N. benthamiana* leaves as described above. The OD<sub>600</sub> was adjusted to 0.1 (35S::P19) and 0.8 for NRG1.1 and ADR1 constructs. For Ca<sup>2+</sup> inhibitor treatments, 2 mM LaCl<sub>3</sub> or GdCl<sub>3</sub> was added to the infiltration media prior to infiltration. Induction was done 24 hours post infiltration using 100 μM β-estradiol (Sigma-Aldrich; St. Louis, USA) and 0.002% [v/v] Silwet L-77 by spraying. 1-2 days post induction the leaves were placed under UV lamps (B-100AP, UVP) and photographed using a digital camera with a yellow filter.

#### In planta semi-quantification of Ca<sup>2+</sup> influx:

Briefly, *A. tumefaciens* strains were infiltrated into transgenic GCaMP3 *N. benthamiana* (38) leaves and protein expression induced as described above. Leaf discs (0.5 cm diameter) were placed on 200 uL ddH<sub>2</sub>O in Nunclon 96 Flat Bottom Black plates (Thermo-Fisher) and equilibrated for 1 h in room temperature. After equilibration, GCaMP3 fluorescence was recorded using TECAN Infinite M200 Pro plate-reader, using excitation at 470 nm (7 nm bandwidth) and emission detection at 525nm (20nm bandwidth) with 20 μs integration time and 5 ms settle time. Absolute fluorescence values for each experiment were normalized to the untreated control value as F/F<sub>UT</sub> (where F was the measured fluorescence at a given time point and F<sub>UT</sub> was the averaged measurement for water-equilibrated uninfiltrated samples at each time points). Plotted values are averages of six replicate leaf discs, with the SE represented by error

bars. A similar protocol was used for semi-quantification of  $\text{Ca}^{2+}$  influx in Arabidopsis GCaMP6 plants(57). Plants were infiltrated with 10mM  $\text{MgCl}_2$  with or without various *Pf0-1* strains at  $\text{OD}_{600\text{nm}} = 0.2$ . After 30 min of drying, 0.5cm leaf discs were gathered and equilibrated on water for 30 min. Fluorescence was recorded every 10 min for 800 min. Blank values (water only) were subtracted to sample values and calcium influx was expressed as  $F/F_0$ , where F was the measured fluorescence at a given time point and  $F_0$  was the averaged measurement for  $\text{MgCl}_2$ -infiltrated samples at this time points.

#### Ion leakage measurements:

Briefly, *A. tumefaciens* strains were infiltrated into *N. benthamiana* leaves and protein expression induced as described above. 12 leaf discs (0.5 cm diameter) from 3 independent plants were collected into clear tubes with 20 ml of distilled water and incubated at room temperature under continuous light (three replicates per sample). Changes in conductivity were measured at the indicated time points with an Orion Model 130 (Thermo-Fisher).

#### Human cell transfection:

Human cervical cancer cells (HeLa) or HEK293 were cultivated in DMEM medium containing 10% fetal bovine serum and 1% penicillin / streptomycin in a  $\text{CO}_2$  incubator at 37C. Cells were grown in 12-well plates overnight to 70-90% confluence and co-transfected with the “sleeping beauty” pCMV(CAT)T7-SB100X vector containing the sleeping beauty transposase and the pSBtet-Pur vectors - a gift from Long Ping V. Tse and Bill Marzluff - containing the synthesized, human codon optimized, NRG1.1 constructs (Genescript) at a 1:10 ratio respectively.

GASDERMIN-D L192D N-terminal pore-forming domain (aa 1-275), a potent inducer of cell death, and the loss of function GASDERMIN-D L192D DN5 were used as controls (24, 58, 59).

All constructs were YFP-tagged at the C-terminus. Briefly, for each co-transfection, 25 $\mu\text{L}$  of Opti-MEM medium was mixed with 0.75 $\mu\text{L}$  of Lipofectamine 3000 (Invitrogen) in one tube while 1 $\mu\text{g}$  of the plasmid mix was added to 25 $\mu\text{L}$  of Opti-MEM medium and 1 $\mu\text{L}$  of P3000 reagent in another tube. Solutions were mixed together and incubated at room temperature for 15 minutes before being added to the cell culture. After 2 days, puromycin 1 $\mu\text{g}\cdot\text{mL}^{-1}$  (Sigma) was added to the cultures to select transfected cells.

Protein assays:

*Agrobacterium tumefaciens* strains were infiltrated into *N. benthamiana* leaves as described above. The OD<sub>600</sub> was adjusted to 0.1 (35S::P19) and 0.8 for NRG1.1 constructs. Induction was done 24 hours post infiltration using 100 μM β-estradiol (Sigma-Aldrich; St. Louis, USA) and 0.002% [v/v] Silwet L-77 by spraying. Tissues for protein assays were collected 6 h post induction.

Total protein extract was prepared by adding total protein buffer [100 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA pH 8.0, 0.1% SDS, 10 mM dithiothreitol (DTT) and 1x Sigma plant protease inhibitor cocktail] to the homogenized tissue at a ratio of 5 uL per mg (FW) tissue. Lysate was cleared by centrifuge at 20,000 x g for 30 min at 4°C.

For microsomal fractionation, sucrose buffer [20mM Tris (pH 8), 0.33M sucrose, 1mM EDTA, 5mM DTT and 1x Sigma plant protease inhibitor cocktail] was added to the homogenized tissue at a ratio of 5 uL per mg (FW) tissue. The extract was centrifuged at 2,000 x g for 5 minutes at 4°C to remove debris. The resulting supernatant was transferred to a new tube and designated as total protein. Cytoplasmic fraction was prepared by harvesting the supernatant after spinning the total protein fraction at 20,000 x g for 1 h at 4°C. The total membrane fraction was prepared from the resulting pellet by resuspending in 200 uL of buffer B (Minute<sup>TM</sup> plasma membrane protein isolation kit, Invent Biotechnologies) and spinning at 7,800 x g for 15 min at 4°C. The pellet was designated as total membrane fraction. The supernatant was transferred to 2 mL Eppendorf tube and mixed with 1.6 mL cold PBS buffer mixed by vortexing and spun at 16,000 x g for 45 min at 4°C to pellet the plasma membrane fraction.

For blue native polyacrylamide gel electrophoresis, modified GTEN buffer (60) [10% glycerol, 100 mM Tris-Cl pH7.5, 1mM EDTA, 150mM NaCl, 5mM DTT, 0.5% n-dodecyl-B-D-maltoside (DDM) and 1x Sigma plant protease inhibitor cocktail] was added to the homogenized tissue at a ratio of 5 uL per mg (FW) tissue. Lysate was cleared by centrifuge at 20,000 x g for 30 min at 4°C. The supernatant was transferred to a new tube and mixed with 4x NativePAGE Sample buffer (Invitrogen).

For SDS-PAGE, proteins were mixed with 4x SDS-PAGE loading buffer and heated for 5 min at 95°C and resolved in 10% SDS-PAGE gels. Separated proteins were transferred to nitrocellulose membrane (GE Healthcare). For BN-PAGE, proteins were resolved in 3-12% and NativePAGE™ (Invitrogen) gels, respectively, then transferred to PVDF membrane using  
5 iBlot™ 2 gel transfer device according to the instructions from the manufacturer. Membranes were blocked with 5% non-fat milk TBST and probed with primary anti-GFP (Roche, Cat. #: 11814460001, 1:1000) and secondary HRP-conjugated anti-mouse antibody (R&D Systems, Cat. #: HAF007, 1:5000).

10 Cell death observation in human cells:

After 2 passages, cells were seeded on 8 well chamber slides (Nunc Lab-Tek 155411), and protein expression was induced by addition of doxycycline 1µg.mL<sup>-1</sup> (PHR1145, Sigma). 6 hours after doxycycline addition, the cells incubated in incubation buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl<sub>2</sub>, pH 7.4) containing 1µg.mL<sup>-1</sup> propidium iodide (PI) for 5 min and  
15 observed under a confocal microscope LSM710 AxioObserver (Zeiss), using the Plan-Apochromat 10X/0.45 M27 objective. YFP was excited using a 514 nm laser collecting emission between 520-559 nm; PI was excited using a 561 nm laser with an emission spectrum of 590-627 nm. Percentage of cell death was determined as the number of PI-positive cells amongst YFP positive cells.

20 Plasma membrane pore quantification with scanning electron microscopy:

Transfected cells were seeded on glass 12mm glass slides and grown overnight to 70-90% confluence. 6 hours after doxycycline treatment, the media was removed and cells were washed in PBS and fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate at room temperature for 30  
25 minutes and stored for several days at 4°C. To better preserve the membrane structure, decrease shrinkage artefacts and reduce the metal coating thickness, the cells were post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 15 minutes followed by subsequent treatment with 2% tannic acid in water for 15 minutes and 1% osmium tetroxide in water for 15 minutes (61). The coverslips were rinsed in deionized water prior to gradual dehydration with  
30 ethanol (30%, 50%, 75%, 100%, 100%) and critical point dried using carbon dioxide as the transitional solvent (Samdri-795 critical point dryer, Tousimis Research Corporation, Rockville,



MD). Coverslips were mounted on 13mm aluminum stubs using double-sided carbon adhesive and sputter coated with 3 nm of gold-palladium alloy (60 Au:40 Pd, Cressington Sputter Coater 208HR, model 8000-220, Ted Pella, Redding, CA). Images were taken using a Zeiss Supra 25 FESEM operating at 5 kV, using the InLens detector, 30µm aperture, and ~5 mm working distance (Carl Zeiss SMT Inc., Peabody, MA). Plasma membrane pores were observed at 200,000 X magnification. Two micrographs were taken per cell and at least 11 cells were observed per cell line. Experiment was performed twice with similar results.

#### Imaging of $[Ca^{2+}]_i$ in HeLa cells:

Transfected HeLa cells were seeded on eight-well chambered coverglasses (Nunc Lab-tek 155411) and grown overnight in a CO<sub>2</sub> incubator at 37°C. 6 to 8h post induction with 1 µg mL<sup>-1</sup> doxycycline, a Fura-2-based Ca<sup>2+</sup> imaging assay was performed as previously described (29). Cells were loaded with the Ca<sup>2+</sup> sensitive dye Fura-2AM (5 µM; Sigma) and incubated in a low [CaCl<sub>2</sub>] standard buffer containing 130 mM NaCl, 3 mM KCl, 0.6 mM MgCl<sub>2</sub>, 10 mM glucose, 10 mM HEPES, pH7.4 (adjusted with NaOH), and 0.1 mM CaCl<sub>2</sub> for 30 min. A 7.5 mM CaCl<sub>2</sub> standard buffer was added with a peristaltic pump (Dynamax RP-1, Rainin) to adjust final [CaCl<sub>2</sub>] to 2.5 mM. Fura-2 fluorescence imaging was performed using the Zeiss Axiovert 200 microscope equipped with two filter wheels (Lambda 10-3; Sutter Instruments) and a CMOS camera (Prime 95B sCMOS; Roper Scientific). Excitation was at 340 nm and 380 nm, and emission images at 510 nm were collected using the MetaFluor Fluorescence Ratio Imaging Software (Molecular Devices). Ratiometric ( $F_{340\text{ nm}}/F_{380\text{ nm}}$ ) image data from 10 cells per well were analyzed. Experiments were carried out at room temperature (22–24°C). Delta ratio was found by calculating the difference of the average fluorescence ratio from 10 cells from 6 time points after switching to high [CaCl<sub>2</sub>] and those same cells at 6 time points before switching to high [CaCl<sub>2</sub>].

#### Imaging of $[Cl^-]_i$ in HeLa cells:

Transfected HeLa cells were seeded on eight-well chambered borosilicate cover glasses (Nunc Lab-tek 155411) and grown overnight. At 6 to 8h post induction with 1 µg mL<sup>-1</sup> doxycycline, cells were subjected to a 6-Methoxy-N-ethylquinolinium iodide (MEQ) -based Cl<sup>-</sup> assay as previously described (33). DihydroMEQ was prepared freshly from MEQ. 5 mg of MEQ were

dissolved in 100  $\mu$ L of distilled water. The MEQ solution was flushed with nitrogen and reduced to dihydroMEQ by addition of 32  $\mu$ mol of sodium borohydride with continuous purging with nitrogen. DihydroMEQ was extracted with chloroform, dried with anhydrous sodium sulfate and the organic extracts were evaporated under a vacuum. DihydroMEQ was dissolved in DMSO and added to a low chloride buffer (5 mM KCl, 20 mM HEPES, 90 mM mannitol) at a final concentration of approximately 50  $\mu$ M. Cells were incubated for 5-10 minutes in the low chloride, dihydroMEQ containing buffer, rinsed and incubated 15 minutes in 200  $\mu$ L of low chloride buffer before confocal observation. Basal MEQ fluorescence was imaged for 5 min before addition of 100  $\mu$ L of 200 mM NaCl. Data was analyzed using FiJi (56). The MEQ fluorescence of 12 cells was followed up in each experiment. Experiments were carried out at room temperature.

#### Mammalian cell electrophysiology:

HEK293 cells transfected with NRG1.1 DV or NRG1.1 DV D3N E14Q were seeded on glass coverslip (12mm borosilicate, #1 thickness, Fisher scientific 12-545-80P), and ion channel activities were analyzed using the patch clamp electrophysiological technique as previously described (28, 29, 62). Whole-cell currents were recorded only on YFP-positive cells 4 to 8h after doxycycline induction. Pipettes (Kimax 51) were pulled in 2 to 3 steps using the P-1000 pipette puller and heat-polished to obtain 1–5 M $\Omega$  resistance in the standard pipette solution (140 mM CsCl, 5 mM EGTA and 10 mM HEPES, pH 7.4 (adjusted with CsOH). Liquid junction potentials were measured as previously described and subtracted to the pipette voltage. Calcium and magnesium bath solutions consisted of 112 mM CaCl<sub>2</sub> or MgCl<sub>2</sub>, 10mM glucose, and 10 mM HEPES (pH 7.4, adjusted with CaOH or MgOH). TEA-Cl bath solution consisted of 224 mM TEA-Cl, 10 mM glucose and 10 mM Tris (pH 7.4 adjusted with HCl). TEA-Cl pipette solution contained 20 mM TEA-Cl, 5 mM EGTA and 10 mM Tris (pH 7.4, adjusted with HCl). Osmolality of all solutions were adjusted to 290 mOsm with sorbitol. Whole-cell voltage-clamp experiments were performed using the Axopatch 200B patch-clamp amplifier (Molecular Devices), and data were acquired using Digidata 1322A digitizer and the pClamp software. The whole-cell currents were recorded at room temperature with the holding potential at -50 mV and the voltage ramp ranging from +50 to -200 mV in 1.9 s. No leak subtraction was performed. Permeability ratios for divalent cations to Cs<sup>+</sup> ( $P_X/P_{Cs}$ ) were calculated as follows:

5  $P_X/P_{Cs} = [Cs^+]_i \exp(E_{rev}F/RT)(1+\exp(E_{rev}F/RT))/4[X^{2+}]_o$  where  $E_{rev}$  is the reversal potential (as measured from voltage ramp experiments),  $F$  is the Faraday's constant,  $R$  is the universal gas constant, and  $T$  is the absolute temperature. Brackets indicate ionic activities, and ion activity coefficients were 0.75 for  $Cs^+$  and 0.25 for  $Ca^{2+}$  and  $Mg^{2+}$  (28).

**Table S1 | Crystallography data collection and refinement statistics**

	2K/E native	7K(R)/E native
PDB code	7L7W	7L7V
Wavelength (Å)	1.03316	1.03317
Resolution range (Å)	40.00 – 2.55 (2.59 – 2.55)	40.00 – 2.95 (3.00 – 2.95)
Space group	R 3 :H	P 2 <sub>1</sub> 2 2 <sub>1</sub>
a, b, c (Å)	114.257, 114.257, 59.855	84.332, 89.656, 149.398
$\alpha, \beta, \gamma$ (°)	90, 90, 120	90, 90, 90
Total reflections	78,469	289,192
Unique reflections	9,220 (338)	24,727 (1,218)
Multiplicity	8.5 (3.9)	11.7 (8.5)
Completeness (%)	96.2 (72.7)	99.8 (100.0)
I/ $\sigma$ (I)	27.2 (2.6)	18.4 (1.0)
Wilson B factor (Å <sup>2</sup> )	57	54
R <sub>merge</sub> (%)	10.5 (90.8)	13.0 (168.7)
R <sub>meas</sub> (%)	11.1 (101.4)	13.5 (179.3)
R <sub>pim</sub> (%)	3.6 (43.7)	3.9 (59.4)
CC <sub>1/2</sub>	0.995 (0.692)	0.996 (0.506)
CC*	0.999 (0.904)	0.999 (0.820)
Refinement statistics		
Resolution (Å)	38.13 – 2.55 (2.64 – 2.55)	39.61 – 2.95 (3.03 – 2.95)
Reflections used in refinement	9,144 (658)	23,168 (1,670)
R <sub>cryst</sub>	0.193 (0.314)	0.219 (0.340)
R <sub>free</sub>	0.233 (0.419)	0.240 (0.362)
Number of non-hydrogen atoms	1,005	5,239
macromolecules	996	5,239
solvent	7	0
Protein residues	118	651
RMS bonds (Å)	0.0085	0.013
RMS angles (°)	1.096	1.628
Ramachandran Favored (%)	94.8	93.7
Ramachandran Allowed (%)	5.2	6.2
Ramachandran Outliers (%)	0	0.2
Rotamer outliers (%)	0	5.7
Clashscore	6.40	9.50

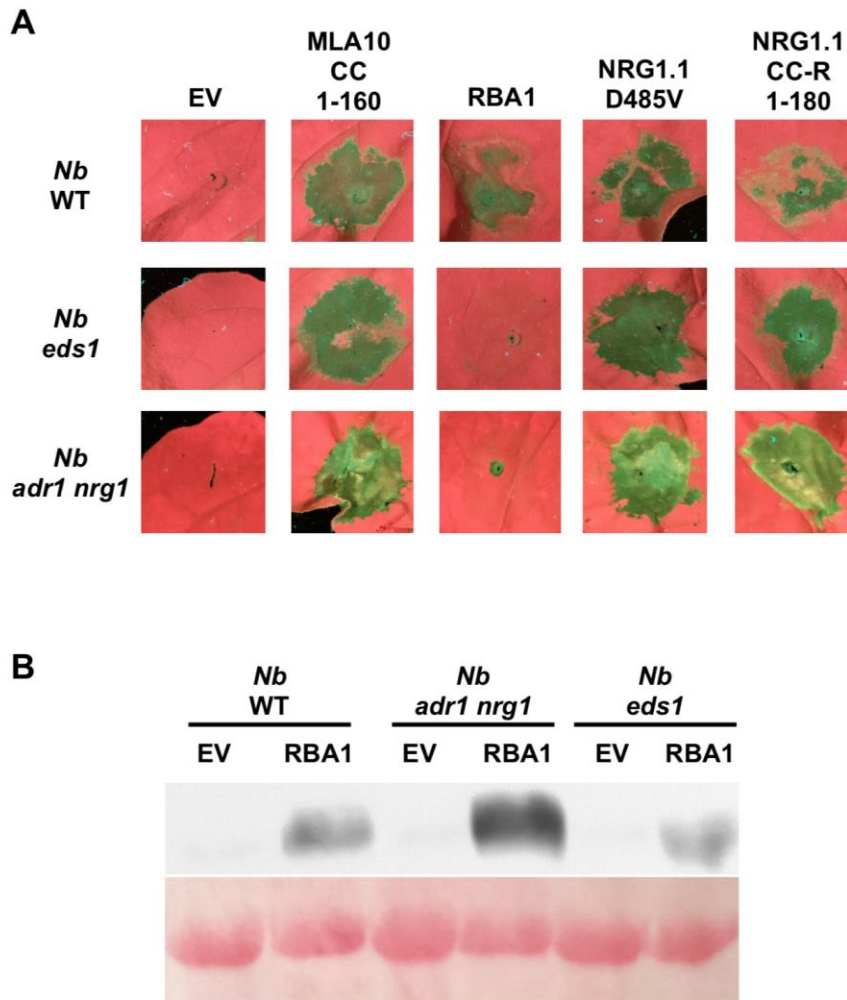
Average B-factor ( $\text{\AA}^2$ )	73	94
macromolecules ( $\text{\AA}^2$ )	73	94
solvent ( $\text{\AA}^2$ )	58	N/A

**Table S2: Proteins structurally similar to AtNRG1.1 2K/E, based on program Dali**

PDB ID	Z-score	RMSD (Å)	Description
6j5v	10.0	2.3	Arabidopsis CC-NB-LRR ZAR1
4m70	9.4	2.4	Potato CC-NB-LRR Rx
4btf	9.3	2.4	Mouse MLKL
2ncg	8.8	2.8	Wheat CC-NB-LRR Sr33
6e7e	8.5	2.7	Inclusion membrane protein A
3rip	8.5	3.2	Gamma-tubulin complex component 4
3ay5	8.4	2.8	Cyclin-D1-binding protein 1
3zni	8.1	2.7	E3 Ubiquitin-protein Ligase CBL-B
1r0d	8.0	3.0	Huntingtin interacting protein 12
5y2g	7.9	3.5	Maltose-binding Periplasmic protein, protein B

**Table S4: List of oligos used in this study**

<b>Oligos for cloning NRG1.1_1-180:GFP in pCSL86922</b>	
<b>Name</b>	<b>Sequence</b>
OF298_ATNRG1_7_1-180-GSAForward	taGGTCTCtAatgaacgattgggctagttgggaata
OF299_ATNRG1_8_1-180-GSARreverse	taGGTCTCtAGCAGATCCcttctttaactccccaatggcca
OF304_GSAGFPForward	taGGTCTCtTGCTGGATCTGCTatggtgagcaaggcgagg
OF305_GSAGFPReverse	taGGTCTCtaagcctactgtacagctcgtccatgcc
OF477_NRG1_17-180_F	taGGTCTCtAATGTTTTCCAAGCTCCTGAAAGTTGTGATTGA
<b>Oligos for ADR1 N-terminal negative charge mutations</b>	
<b>Name</b>	<b>Sequence</b>
ADR1_D6Ncttc69F	tataggtctc <b>gcttc</b> ATGGCTTCGTTTCATA <b>aAT</b> CTTTTTCGCCGGC <b>GAC</b> ATCACGAC
ADR1_D11Ncttc69F	tataggtctc <b>gcttc</b> ATGGCTTCGTTTCATAG <b>AT</b> CTTTTTCGCCGGC <b>CaAC</b> ATCACGACGCAAC TCTTAAAGCTGCT
ADR1_D611CTTC69F	tataggtctc <b>gcttc</b> ATGGCTTCGTTTCATA <b>aAT</b> CTTTTTCGCCGGC <b>CaAC</b> ATCACGACGCAAC TCTTAAAGCTGCT
ADR1a_GTCA69-R	tataggtctcat <b>GACC</b> GTGACACAGTCAAAAACAAAACCTTA
ADR1b_GTCA69-F	tataggtctc <b>ggtca</b> CCGAATTTTGAGAATTTGGAGTCTTGTATA
ADR1b_GGAC69-R	tataggtctc <b>agttcc</b> CTCAAACATCGTGTTGCGTCACAAATACA
ADR1c_GGAC69-F	tataggtctca <b>GGAC</b> CTAGCCCTTCATATGTCCAATCGTG
ADR1_CACC69-R	tataggtctc <b>aggtg</b> ATCGTCAAGCCAATCCACGGTGAAG



**Fig. S1. Auto-active AtNRG1.1 D485V triggers cell death independently of EDS1 and NRG1/ADR1 in *Nb*.**

- 5 (A) UV-image of *Nicotiana benthamiana* (*Nb*) leaves of indicated genotypes *Nb*, *Nb eds1* or *Nb adr1 nrg1* infiltrated with Agrobacterium harboring indicated Myc-tagged constructs to observe cell death phenotype 3 days post infiltration. A 180 aa N-terminal fragment of NRG1.1 is a control for cell death inductions (63). (B) Myc-tagged RBA1 proteins extracted from harvested leaf tissues 26 hours post infiltration were resolved by SDS-PAGE and blotted for Myc.



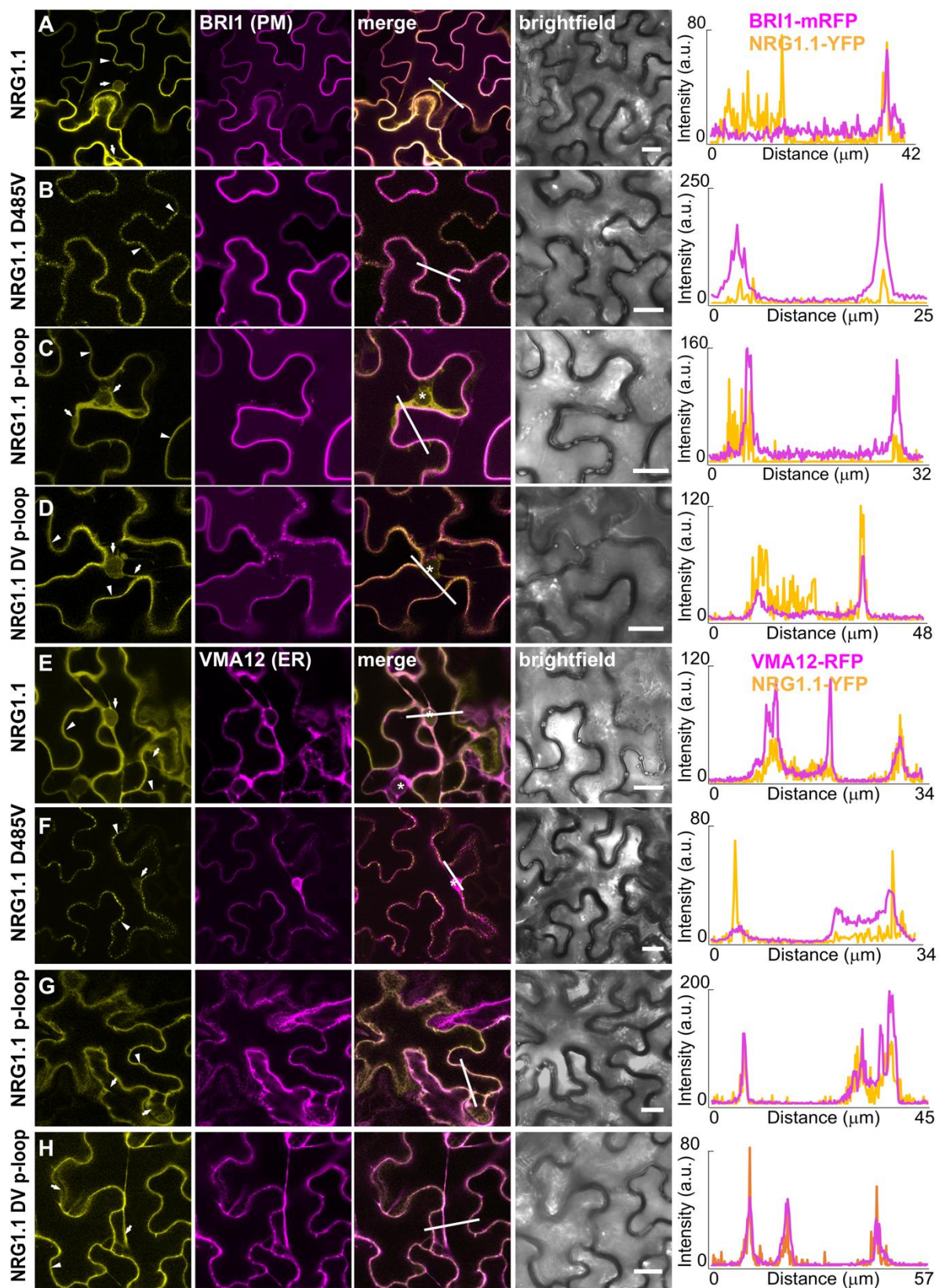


Fig. S2. Active NRG1.1 localizes to the plasma membrane. Continued.

**Fig. S2. Active NRG1.1 localizes to the plasma membrane.**

**A** to **D** are representative images showing the (co-)localization of wildtype NRG1.1 (**A**), NRG1.1 D485V (**B**), NRG1.1 p-loop (**C**) and NRG1.1 DV p-loop (**D**) with the plasma membrane (PM) localized BRI1-mRFP. **E** to **H** are representative images showing the (co-) localization of wildtype NRG1.1 (**E**), NRG1.1 D485V (**F**), NRG1.1 p-loop (**G**) and NRG1.1 DV p-loop (**H**) with the endoplasmic reticulum (ER) localized VMA12-RFP. Notably, active NRG1.1 D485V colocalizes poorly with VMA12 (ER) and strongly with BRI1 (PM) whereas the WT, inactive p-loop and DV p-loop colocalize strongly with the ER marker VMA12 and partially with the PM marker BRI1. Indicated proteins were transiently expressed in 4-6 week-old wildtype *Nicotiana benthamiana* plants. Expression of the indicated NRG1.1 constructs was induced 24 hours post infiltration with 20uM  $\beta$ -estradiol + 0.001% [v/v] Silwet L-77 and confocal imaging was performed 6 to 24 hours post induction. Arrowheads in **A** to **H** point to plasma membrane and/or punctate structure localization and arrows indicate endoplasmic reticulum localization. Lines in the merged images indicate the path of the intensity profile shown on the right and are pointing from left to right or up to down. Scale bars = 20  $\mu$ m.

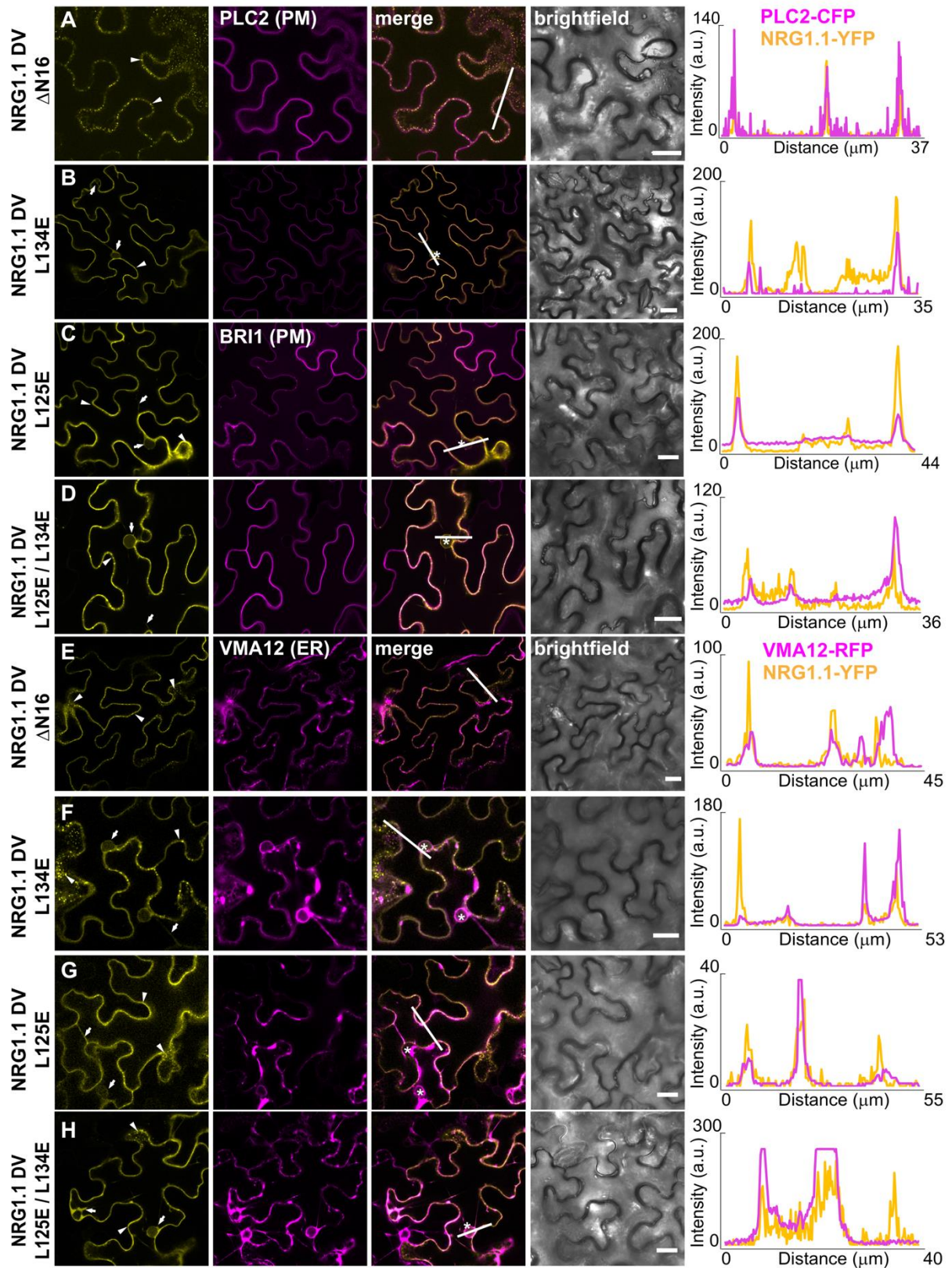


Fig. S3. Impact of structure-derived mutations on NRG1.1 localization. Continued.

**Fig. S3. Impact of structure-derived mutations on NRG1.1 localization.**

**A to D** are representative images showing the (co-) localization of NRG1.1 DV  $\Delta$ N16 (**A**), NRG1.1 DV L134E (**B**), NRG1.1 DV L125E (**C**) and NRG1.1 DV L125E/L134E (**D**) with the plasma membrane (PM) localized BRI1-mRFP or PLC2-CFP, as indicated. **E to H** are representative images showing the (co-)localization of NRG1.1 DV  $\Delta$ N16 (**E**), NRG1.1 DV L134E (**F**), NRG1.1 DV L125E (**G**) and NRG1.1 DV L125E/L134E (**H**) with the endoplasmic reticulum (ER) localized AtVMA12-RFP. Notably, NRG1.1 DV  $\Delta$ N16 and NRG1.1 DV L125E colocalize strongly with the PM marker PLC2 or BRI1 and less so with the ER marker VMA12, whereas NRG1.1 DV L134E and NRG1.1 DV L134E/L125E colocalize strongly with the ER marker VMA12 and less so with the PM markers PLC2 or BRI1. Indicated proteins were transiently expressed in 4-6 week-old wildtype *Nicotiana benthamiana* plants. Expression of indicated NRG1.1 constructs was induced 24 hours post infiltration with 20uM  $\beta$ -estradiol + 0.001% [v/v] Silwet L-77 and confocal imaging was performed 6 to 24 hours post induction. Arrowheads in **A to H** point to plasma membrane and/or punctate structure localization and arrows indicate endoplasmic reticulum localization. Lines in the merged images indicate the path of the intensity profile shown on the right and are pointing from left to right or up to down. Scale bars = 20  $\mu$ m.

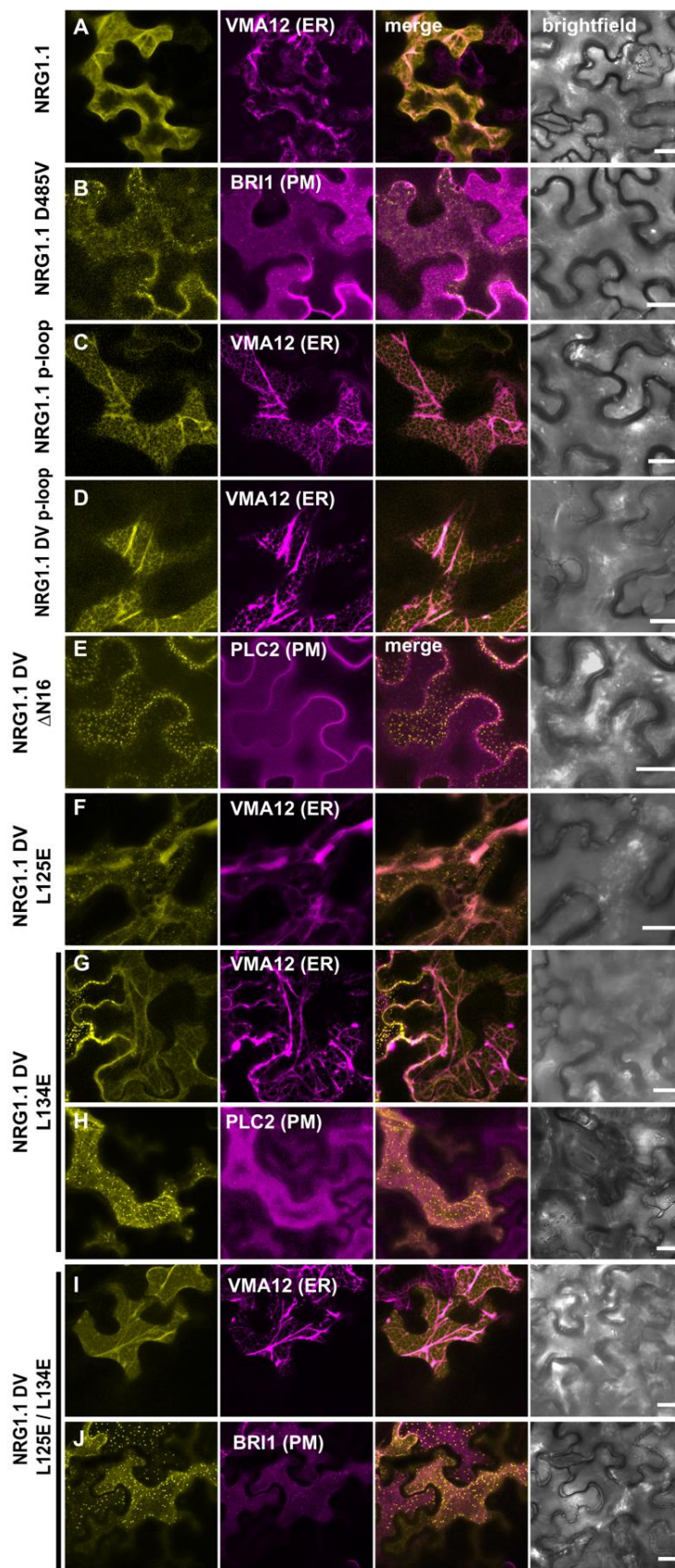


Fig. S4. Surface view of NRG1.1 WT and variants. Continued.

**Fig. S4. Surface view of NRG1.1 WT and variants.**

**A to J** are representative surface view images showing the (co-) localization of wildtype NRG1.1 (**A**), NRG1.1 D485V (**B**), NRG1.1 p-loop (**C**) and NRG1.1 DV p-loop (**D**), NRG1.1 DV  $\Delta$ N16 (**E**), NRG1.1 DV L125E (**F**), NRG1.1 DV L134E (**G, H**) and NRG1.1 DV L125E/L134E (**I, J**) with either the plasma membrane (PM) localized AtBRI1-mRFP or AtPLC2-CFP or the endoplasmic reticulum (ER) localized AtVMA12-RFP. Importantly, PM localization is not uniform. PM localized NRG1.1 DV, NRG1.1 DV  $\Delta$ N16 and NRG1.1 DV L134E were found in puncta at the PM whereas inactive NRG1.1 WT, p-loop and DV p-loop were associated with characteristic ER strands in some examples, NRG1.1 L134E and NRG1.1 DV L134E/L125E, to a much lesser extent NRG1.1 L125E, exhibited puncta at the PM which may reflect some residual PM localization (see also Fig 2C). Indicated proteins were transiently expressed in 4-6 week-old wildtype *Nicotiana benthamiana* plants. Expression of indicated NRG1.1 constructs was induced 24 hours post infiltration with 20uM  $\beta$ -estradiol + 0.001% [v/v] Silwet L-77 and confocal imaging was performed 6 to 24 hours post induction. Surface view images were constructed with the ImageJ software by combining 7-15 Z-stacks of the surface of individual cells into a maximum projection image. Note: For both the NRG1.1 DV L134E and NRG1.1 DV L125E/L134E mutants, we observed cells showing both strong ER localization (**G** and **I**) and the punctate localization pattern at the PM (**H** and **J**). Scale bars = 20  $\mu$ m.

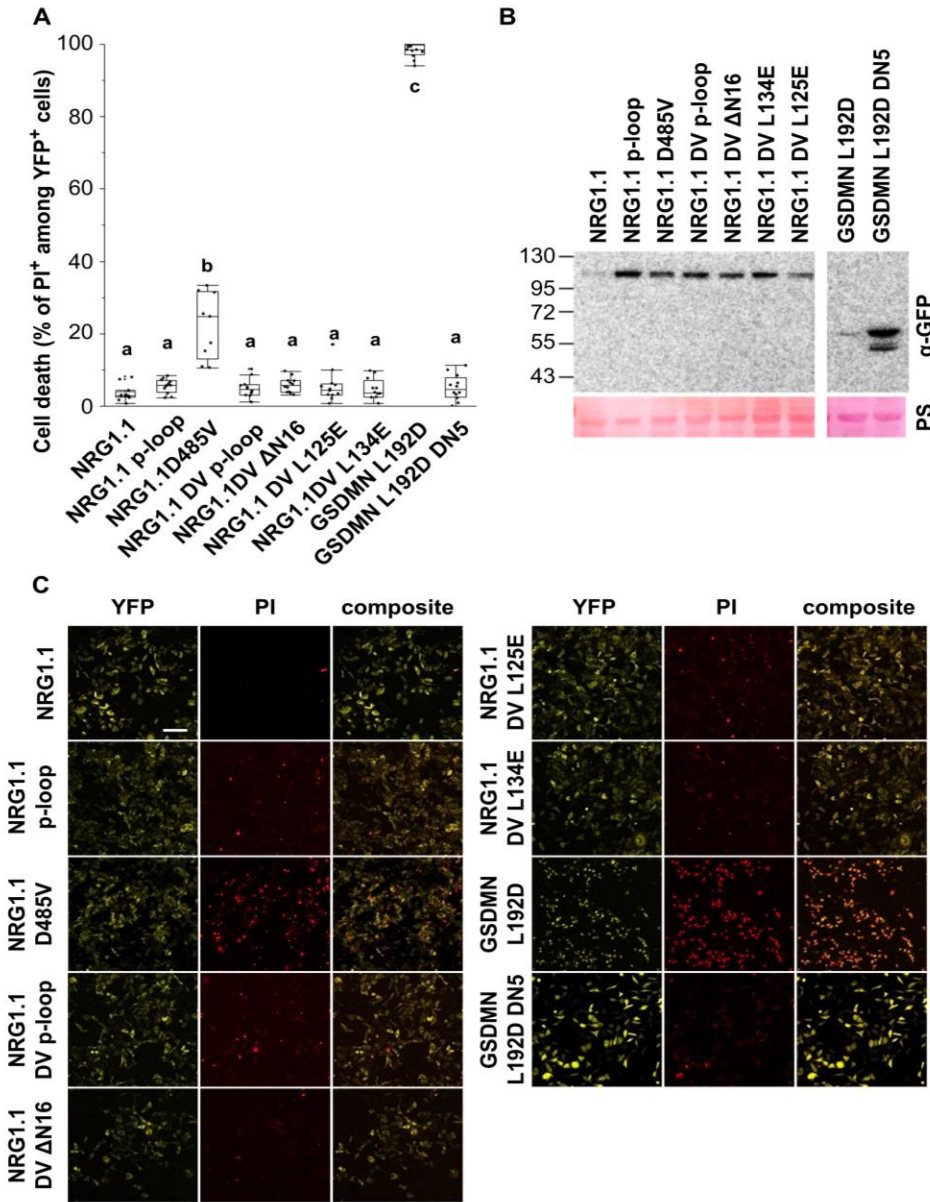


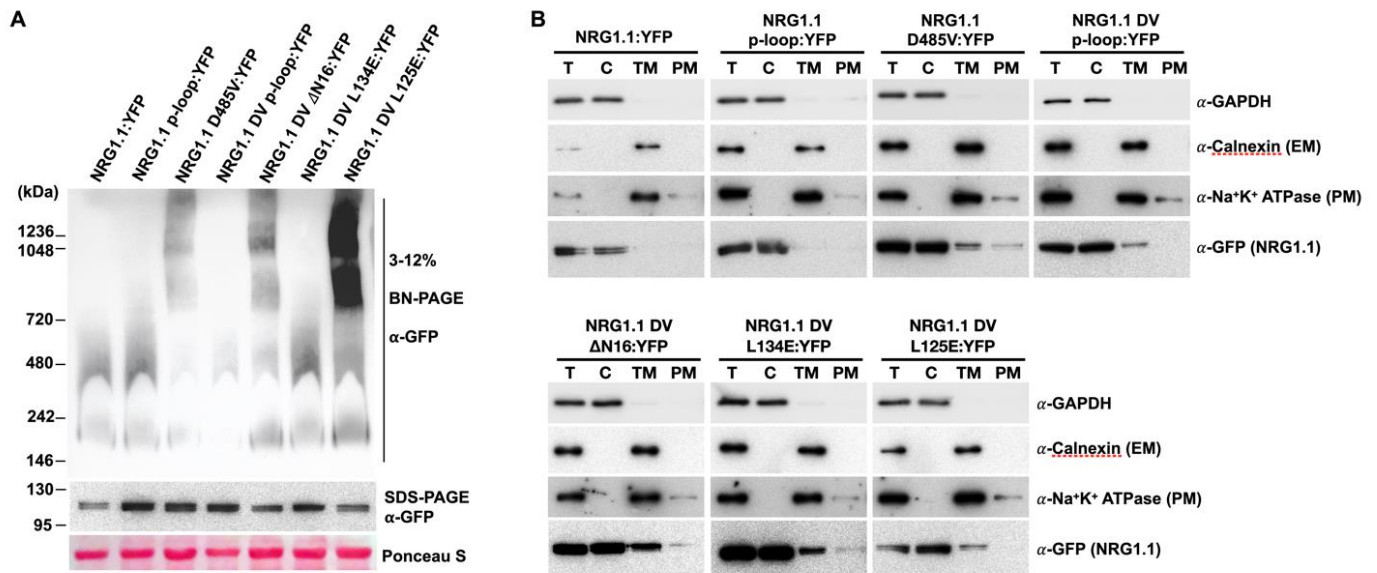
Fig. S5. NRG1.1 cell death induction function is conserved in HeLa cells. Continued.

**Fig. S5. NRG1.1 cell death induction function is conserved in HeLa cells.**

**A** Cell death percentage observed 6 hours post-induction (hpi) of protein expression with  $1\mu\text{g.ml}^{-1}$  doxycycline. Cells were stained with propidium iodide (PI) and observed with a confocal microscope. The cell death is calculated as the percentage of  $\text{PI}^+$  cells among the  $\text{YFP}^+$  cells.

5 Each data point is the average of counts from at least 3 different confocal images. The data presented here summarize 3 independent experiments. Letters represent statistical difference (ANOVA with post-hoc Tukey HSD test,  $p\text{-value}<0.0001$ ). NRG1.1 cell death function can be recapitulated in HeLa cells. **B** Western blot confirming protein accumulation at 6hpi with doxycycline. Ponceau S stands for Ponceau Staining. **C** Representative confocal fluorescence  
10 images used for cell death quantification in **A**. Scale bar is  $100\mu\text{m}$ .

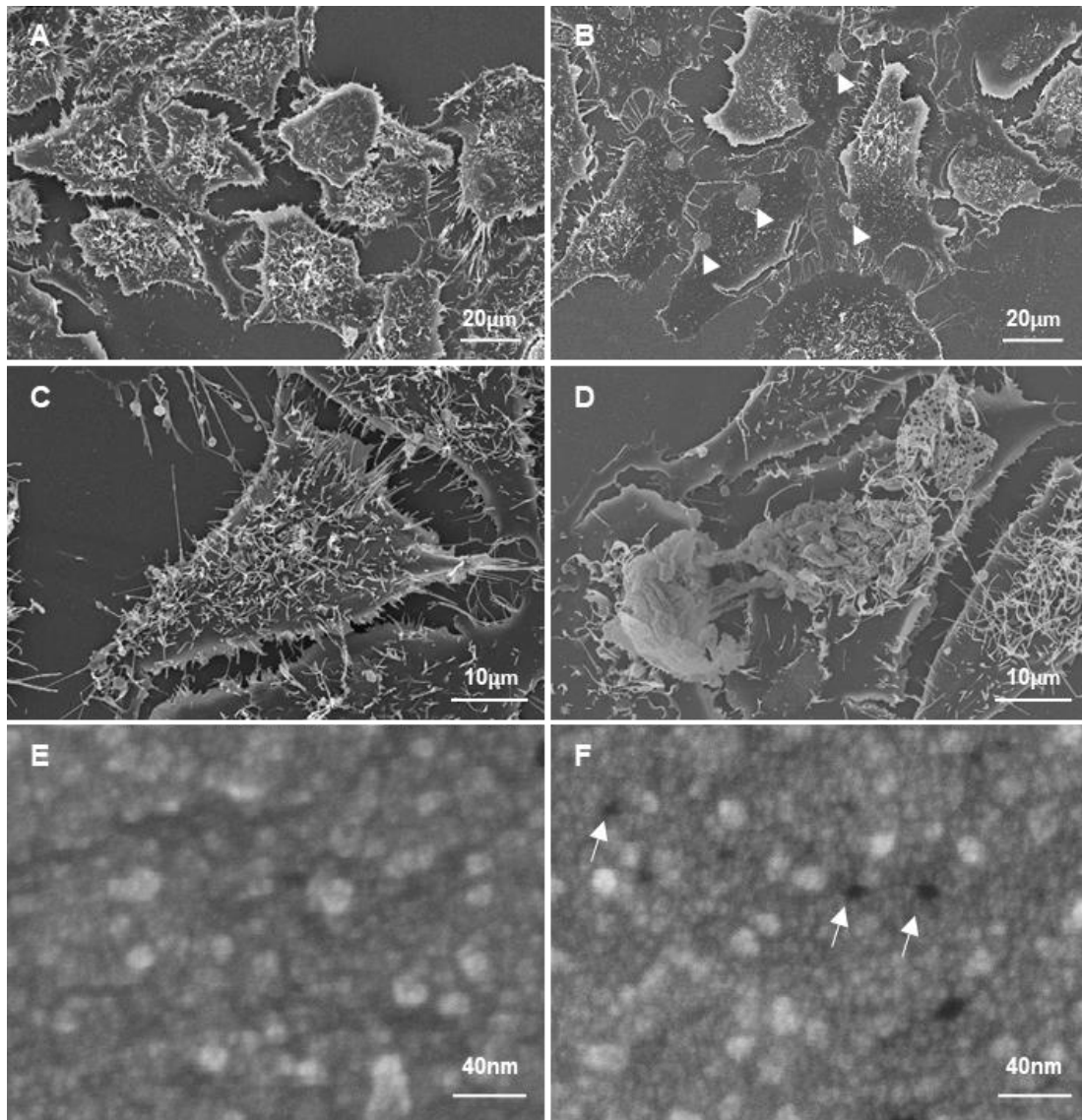




**Fig. S6. Active NRG1.1 oligomerizes and localizes to the plasma membrane in HeLa cells.**

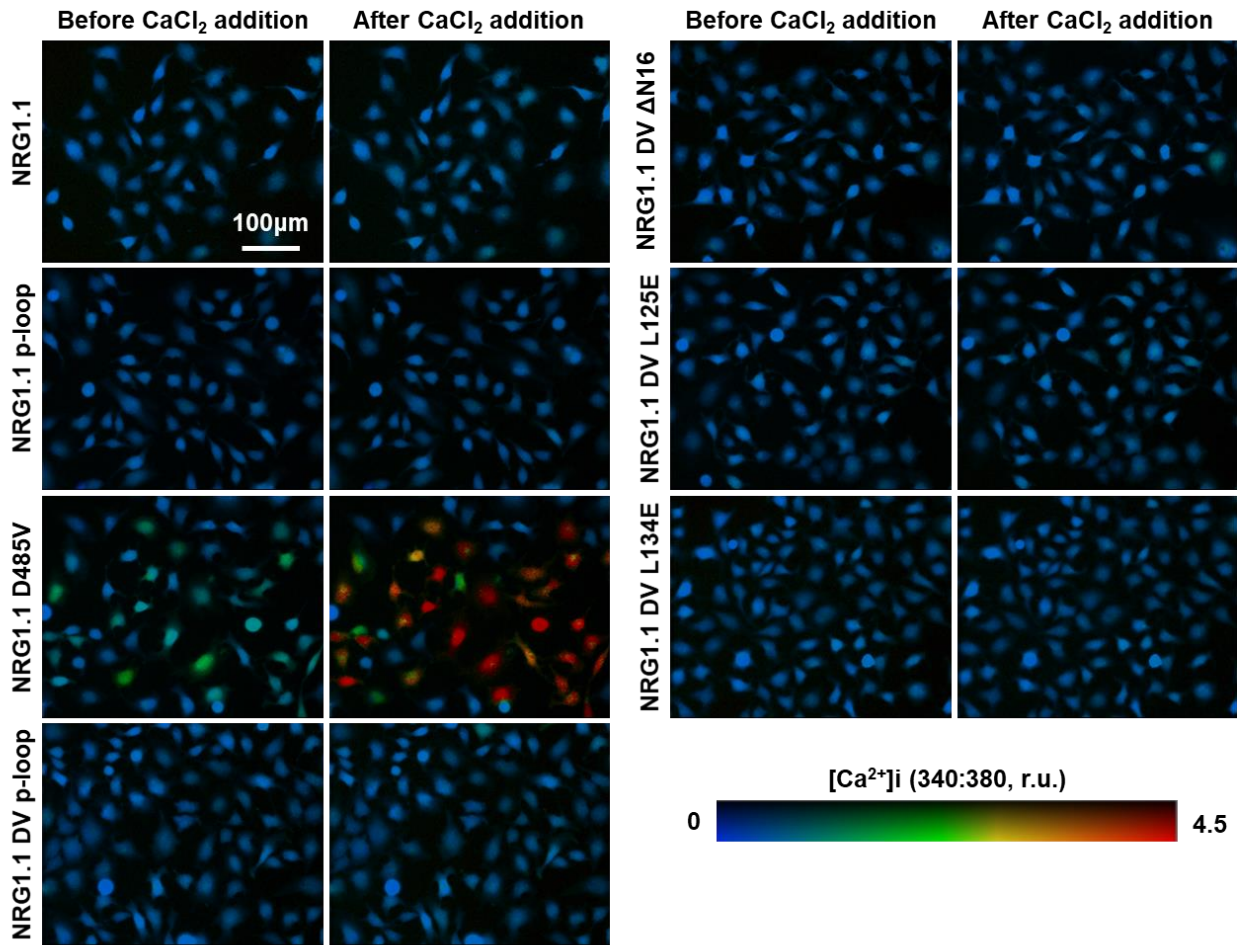
5 A NRG1.1 DV forms high molecular weight oligomers in HeLa cells. Duplicate samples were analyzed by either SDS-PAGE (monomers) or BN-PAGE (oligomers). YFP tagged proteins were detected with anti-GFP antibody. Ponceau S stain is shown as loading control. **B** NRG1.1

10 localizes to the plasma membrane in HeLa cells. HeLa cells expressing indicated constructs were fractionated to Total proteins (T), cytosolic (C), total membrane (TM) and plasma membrane (PM) fractions and verified by marker proteins: Cytosol, GAPDH; endoplasmic reticulum membrane (EM), Calnexin; PM, Na<sup>+</sup>K<sup>+</sup>ATPase. NRG1.1 proteins were detected by anti-GFP antibody.



**Fig. S7. Morphology of NRG1.1 D485V expressing cells.**

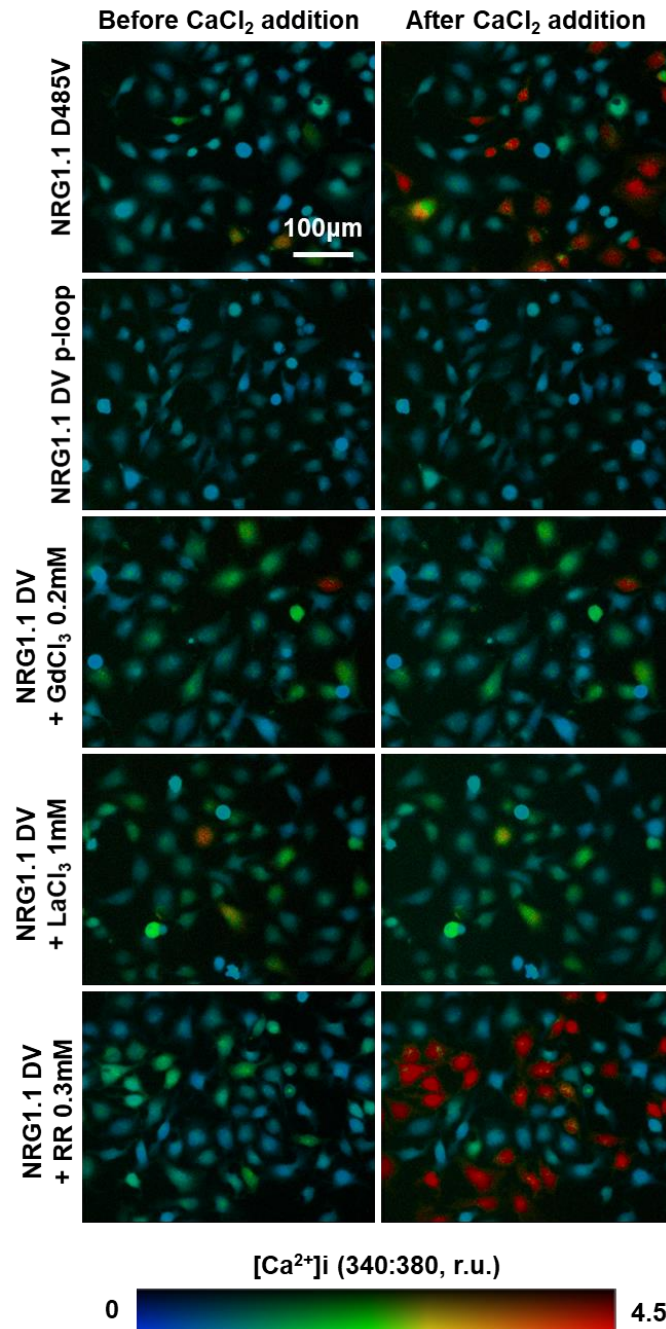
Representative scanning electron microscopy pictures of HeLa cells 6 hours post doxycycline  
 5 induction of inactive NRG1.1 D485V p-loop (**A**, **C** and **E**) or active NRG1.1 D485V (**B**, **D** and  
**F**). HeLa cells expressing the active NRG1.1 D485V (**B**) seem to have lost plasma membrane  
 integrity and consequently appear flat with fewer microvilli compared to cells expressing the  
 inactive NRG1.1 D485V p-loop (**A**). White arrowheads indicate large holes in the plasma  
 membrane visible in many NRG1.1 D485V expressing cells. NRG1.1 D485V expressing cells  
 10 occasionally showed “bubbling” reminiscent of pyroptosis (**D**). Expression of active NRG1.1  
 D485V was associated with a large number of plasma membrane pores (**F**) compared to the  
 inactive control (**E**). White arrows indicate some plasma membrane pores.



**Fig. S8. Fura-2-based quantification of intracellular [Ca<sup>2+</sup>]<sub>i</sub> in HeLa cells expressing NRG1.1 and variants.**

Representative fluorescence microscopy images of HeLa cells expressing NRG1.1 WT or variant, as indicated, before or 2 minutes after 2.5mM CaCl<sub>2</sub> addition. Intracellular [Ca<sup>2+</sup>]<sub>i</sub> was calculated from Fura-2 emission ratios (340:380 nm) and scaled using a pseudo-color bar. The ratios were quantified in Fig 3K.

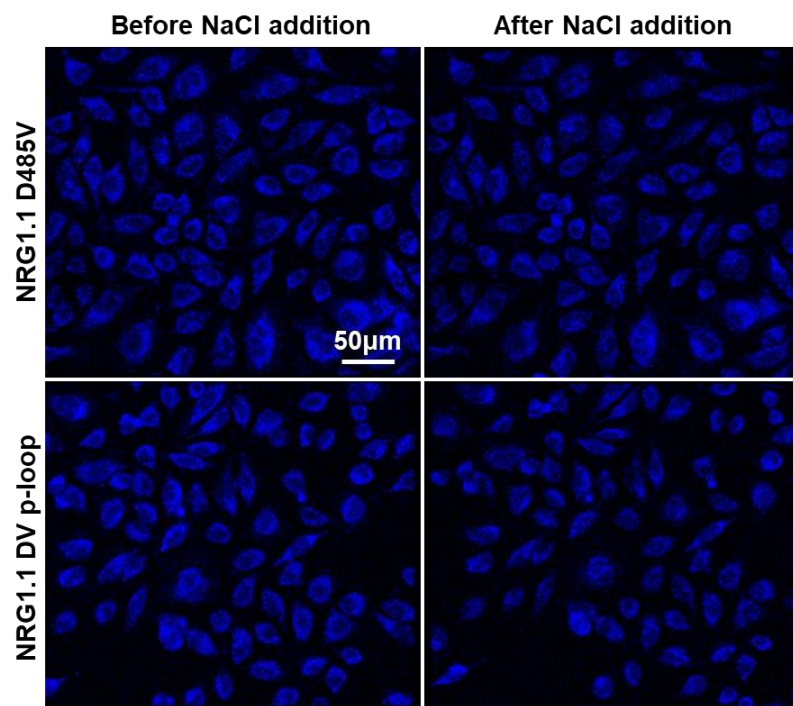
5



**Fig. S9. Fura-2-based quantification of Calcium channel blockers impact on intracellular [Ca<sup>2+</sup>]<sub>i</sub> in NRG1.1 D485V-expressing HeLa cells.**

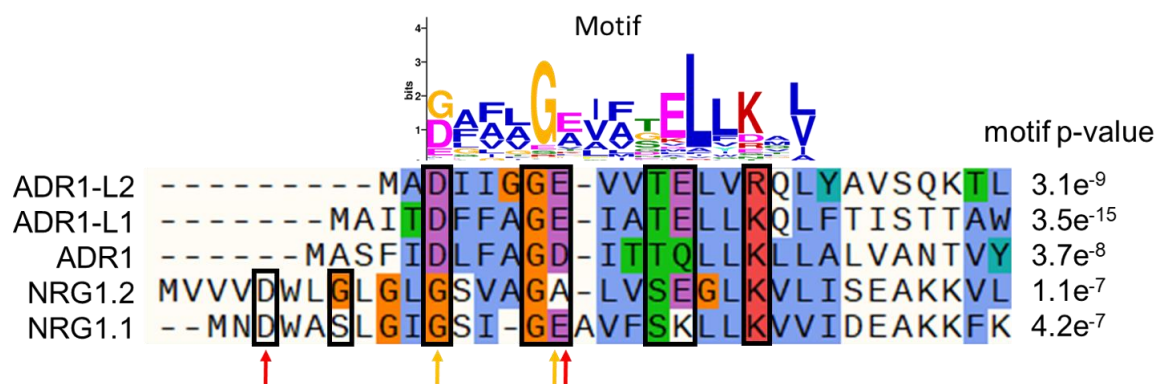
Representative fluorescence microscopy images of HeLa cells expressing active NRG1.1 D485V in the presence of typical calcium channel blockers, before or 2 minutes after 2.5 mM CaCl<sub>2</sub> addition. Intracellular [Ca<sup>2+</sup>]<sub>i</sub> was calculated from Fura-2 emission ratios (340:380 nm) and scaled using a pseudo-color bar. The ratios were quantified in Fig3 L.

5



**Fig. S10. MEQ-based quantification of intracellular  $[Cl^-]$  in NRG1.1 D485V and NRG1.1 DV p-loop-expressing HeLa cells.**

Representative fluorescence microscopy images of HeLa cells expressing active NRG1.1 D485V or suppressed NRG1.1 DV p-loop, before or 2 minutes after 200 mM NaCl addition. Intracellular  $[Cl^-]$  was visualized from MEQ fluorescence quenching (emission at 440nm). The signal was quantified in Fig3 M.



5 **Fig. S11. The RNL N-terminal motif aligned to Arabidopsis ADR1s and NRG1s N-termini.**  
*De novo* motif discovery algorithm (MEME, meme-suite.org, (64)) run on 334 RNL N-terminal  
 sequences (pfam.xfam.org; Table S3) allowed the identification of a conserved pattern of  
 charged, polar or glycines residues (black boxes) separated by 2 to 3 hydrophobic residues  
 (Motif E-value = 2.8e-1139, 331 occurrences). The motif is partially degenerated in NRG1.1 and  
 10 NRG1.2 with K19 (NRG1.1) and A17 (NRG1.2) replacing E residues. Interestingly, NRG1.1  
 and NRG1.2 possess an N-terminal extension comprising D-G/S residues that seem to follow the  
 same spacing pattern. Red and orange arrows indicate the residues that were mutated in NRG1.1  
 and ADR1 respectively.

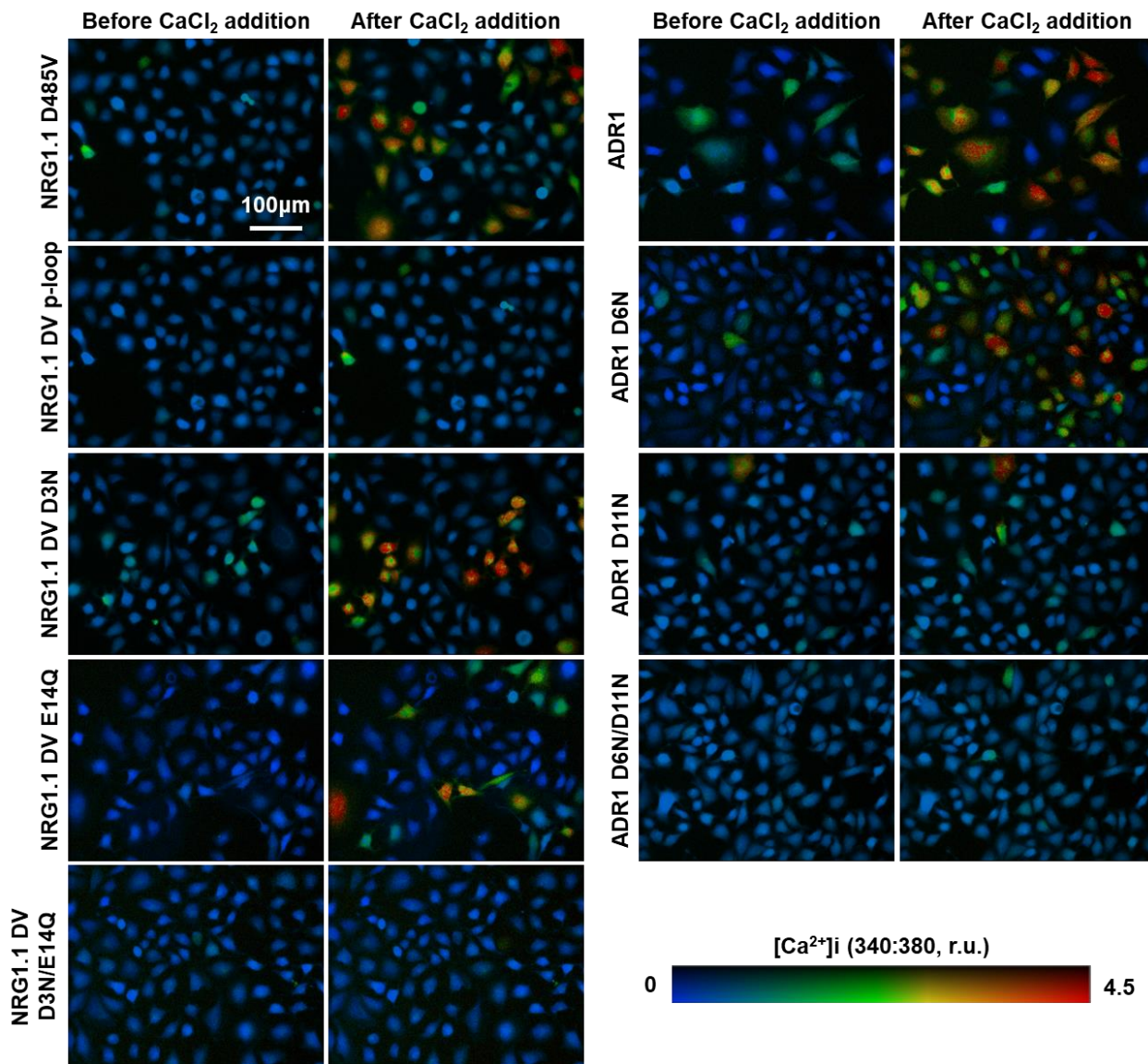
E.value	Protein name	
	Top Motif	DAFLGE - IFT ELLKAL
2.80E-09	AtADR1-L1.1	DFFAGE - IATELLKQL
2.80E-09	AtADR1-L1.2	DFFAGE - IATELLKQL
9.40E-09	NbS00036756g0009.1	DFFAGE - IATELLKHL
3.70E-06	Solyc04g079420	DFFAGE - ITTELIKYL
0.0016	Solyc02g090380	GAALGP - VFDQLLKAV
0.0025	AtADR1-L2	DII GGE - VVT ELVRQL
0.0066	NbNRG1	GAALGP - VFDILLKAV
0.028	AtADR1	DLFAGD - ITTQLLKLL
0.089	AtNRG1.1	GSI - GEAVFSKLLKVV
0.34	AtNRG1.2	GSVAGA - LVSEGLKVL
0.69	HORVU7Hr1G116000.1	SSVVG T - VVT KLHKMI
0.97	HORVU3Hr1G109680.6	DFV TTT - VASHLWNPV
1.1	HORVU5Hr1G001020.2	DFGLSR - VISESH TQV
1.2	NbS00012490g0007.1	EIVLGG - VTRDLWKHI
2.3	HORVU5Hr1G078100.19	GWVASP - IISELYKKA
6.1	Os10g33440.2	DAGVEE - VAE EYYDEL
6.4	Bv4_091520_ohgj.t1	DNL PGD - VCWSIFKQL
6.4	HORVU5Hr1G085920.2	SAFLRS - VMGKLFQVL
6.4	HORVU5Hr1G085940.2	SAFLRS - VMGKLFQVL
7.5	Os10g07400.1	DDARDE - IVSKILRAA
7.5	Os10g07400.2	DDARDE - IVSKILRAA
9.4	Solyc11g069620	DAFLSS - AFDVLI DRL
9.8	Bv3_063870_jjky.t1	EFVFGF - IVSLLQEL
9.9	Solyc10g055050	TRIIPE - VTKELYKPL
9.9	NbS00016761g0012.1	GAF LSS - VLQVLF DRL

RNLs  
CNLs/CNS

**Fig. S12. The RNL N-terminal motif is not shared by CNLs.**

The RNL N-terminal motif was used to scan (MAST, meme-suite.org, (65)) a database comprising 988 putative CC-NLRs and CC<sub>R</sub>-NLRs from six representative plant species (Arabidopsis, sugar beet, tomato, N. benthamiana, rice and barley) from (34). The top 10 motif-containing protein were all RNLs and CNLs exhibited only a very degenerated version of the motif.

5

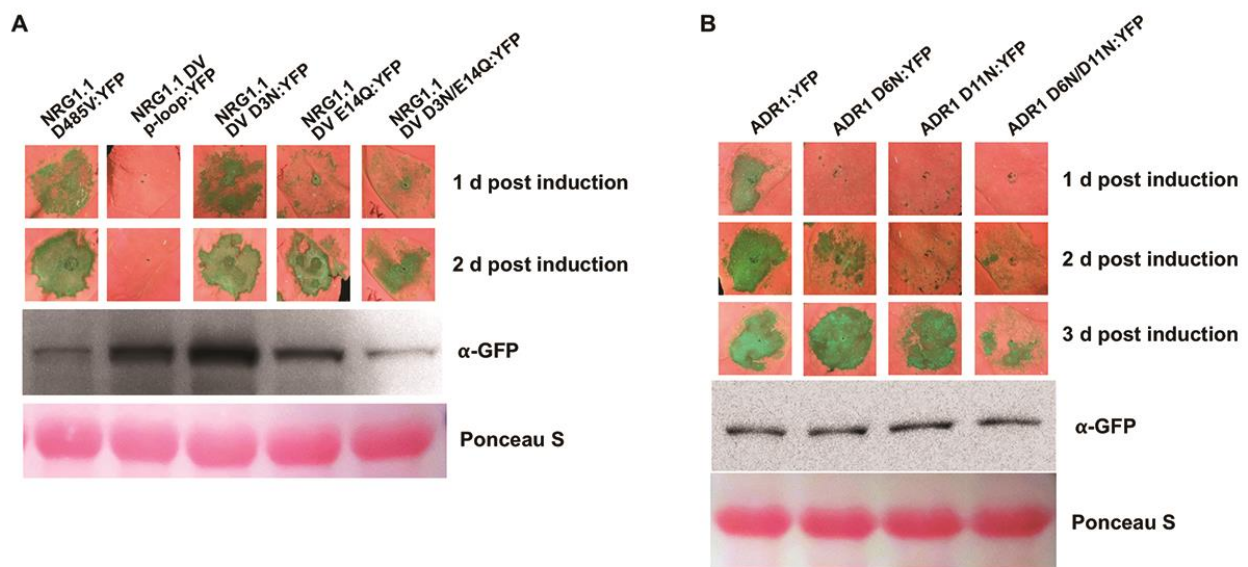


**Fig. S13. Impact of mutation in the N-terminal RNL motif on intracellular [Ca<sup>2+</sup>] in NRG1.1 D485V and ADR1-expressing HeLa cells, as visualized with Fura-2.**

Representative fluorescence microscopy images of HeLa cells expressing active NRG1.1

- 5 D485V, ADR1 or variants, before or 2 minutes after 2.5mM CaCl<sub>2</sub> addition. Intracellular [Ca<sup>2+</sup>] was calculated from Fura-2 emission ratios (340:380 nm) and scaled using a pseudo-color bar. The ratios were quantified in Fig4 H and J.



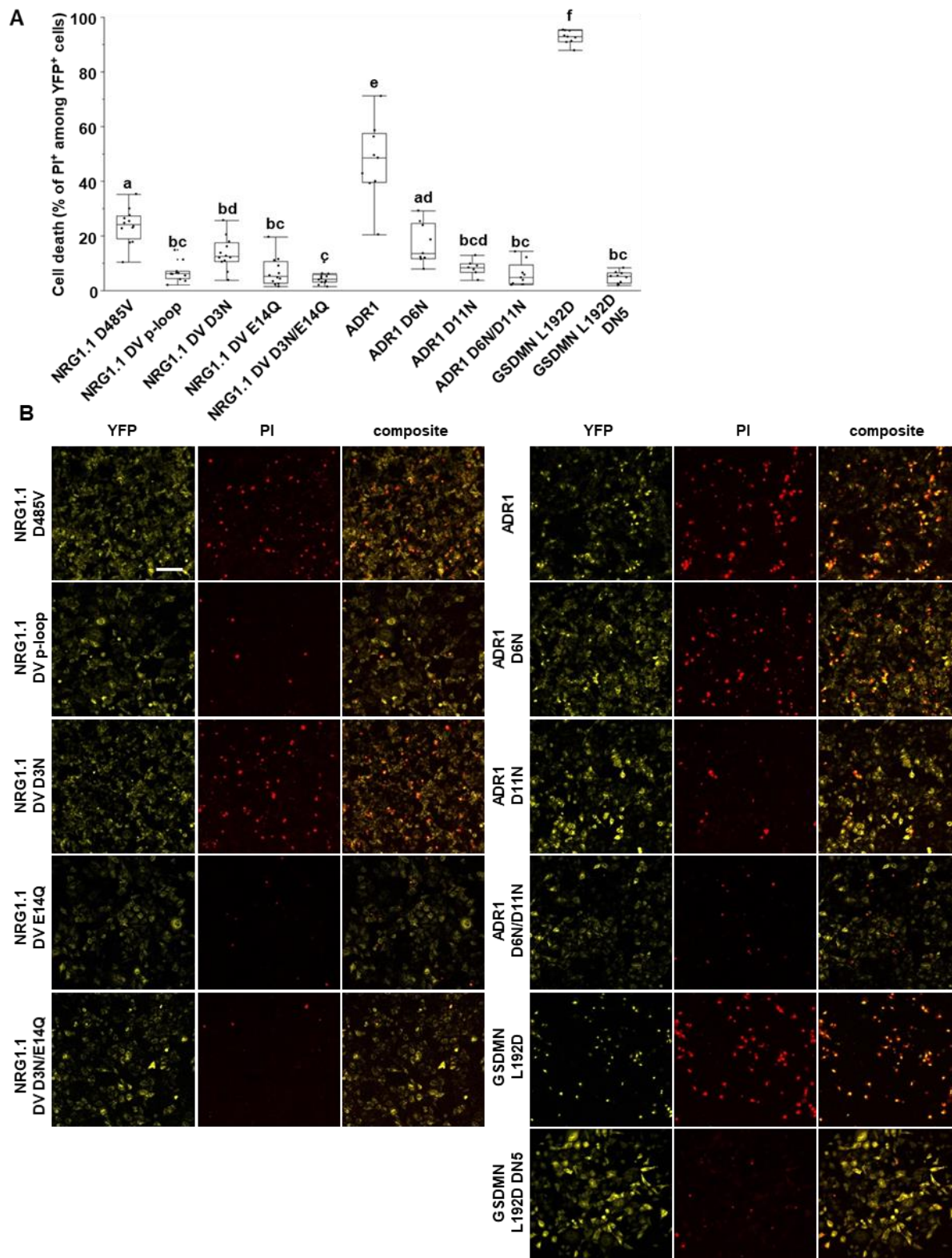


**Fig. S14. Mutation of N-terminal negatively charged residues in NRG1.1 and ADR1 delays cell death in *Nicotiana benthamiana***

5

UV-image of *Nb* leaves infiltrated with *Agrobacterium* harboring indicated constructs to observe cell death phenotypes. YFP tagged NRG1.1 and ADR1 proteins extracted from harvested tissues 6 hours post induction were resolved by SDS-PAGE and blotted with anti-GFP antibody.

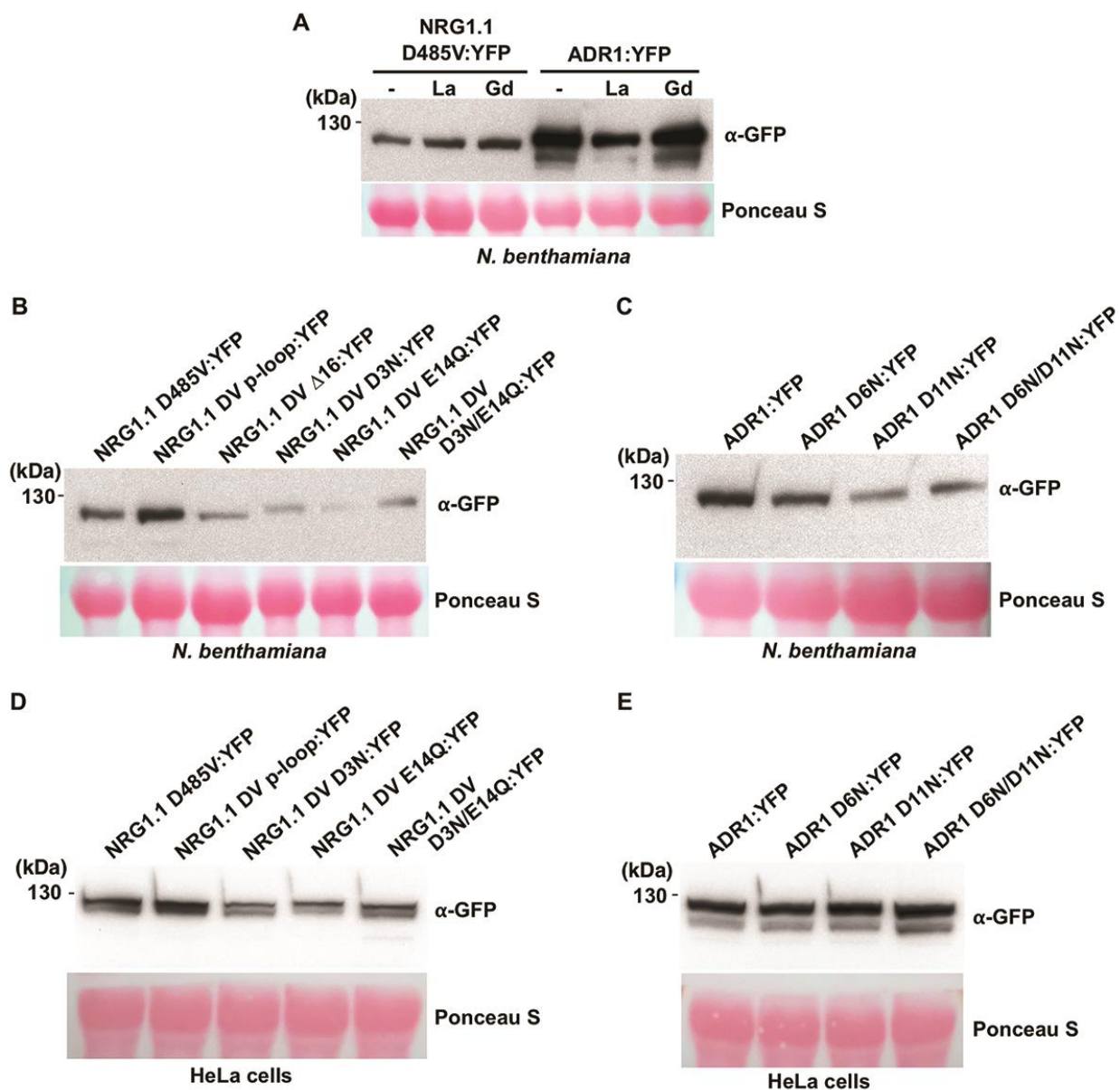
10



**Fig. S15. Negatively charged residues in the conserved RNL N-terminal motif are required for cell death in HeLa cells. Continued.**

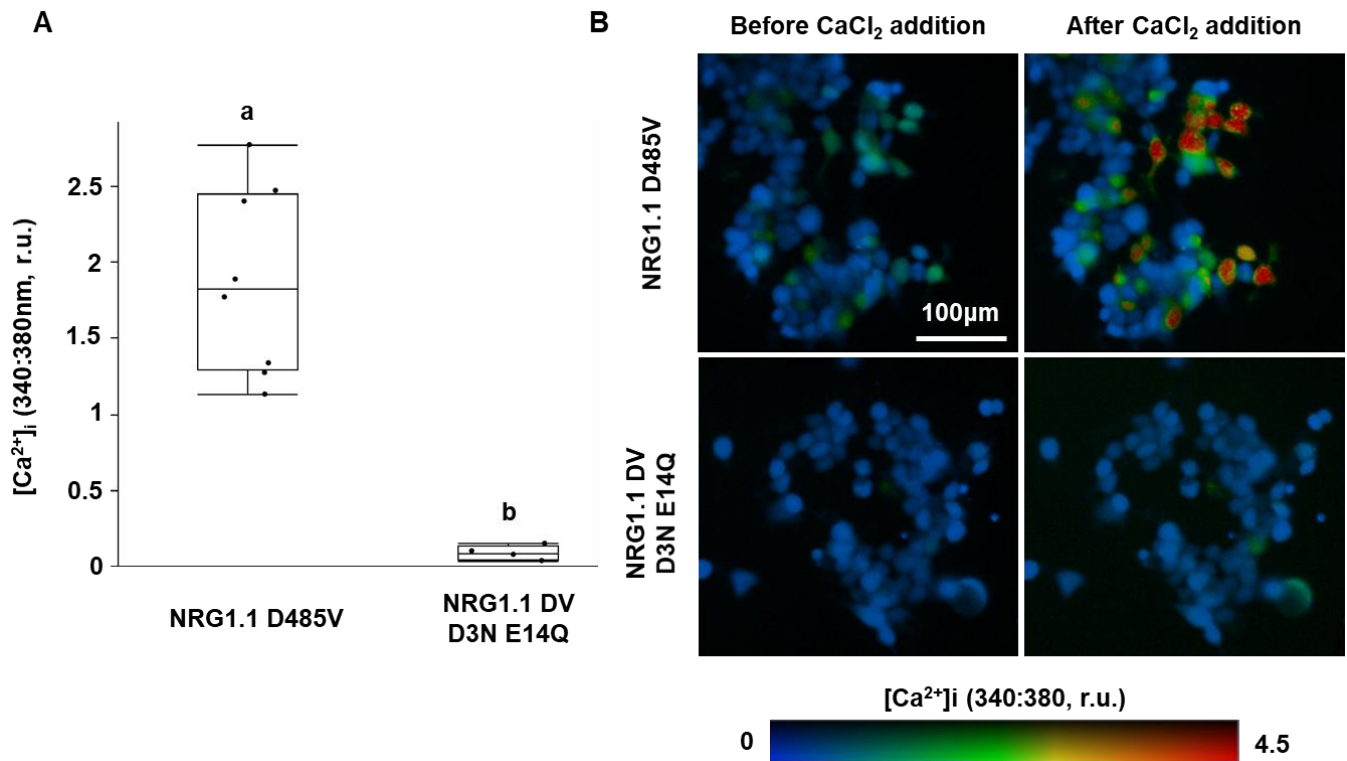
**Fig. S15. Negatively charged residues in the conserved RNL N-terminal motif are required for cell death in HeLa cells.**

5 **A** Cell death percentage observed 6 hours post-induction (hpi) of protein expression with 1  $\mu\text{g.ml}^{-1}$  doxycycline. Cells were stained with propidium iodide (PI) and observed with a confocal microscope. The cell death is the percentage of PI<sup>+</sup> cells among the YFP<sup>+</sup> cells. The data presented here comes from 3 independent experiments. Letters represent statistical difference (ANOVA with post-hoc Tukey HSD test, p-value<0.05). **B** Representative fluorescence images used for cell death quantification in A. Scale bar is 100 $\mu\text{m}$ .



**Fig. S16. RNL proteins are correctly expressed in *N. benthamiana* and HeLa cells.**

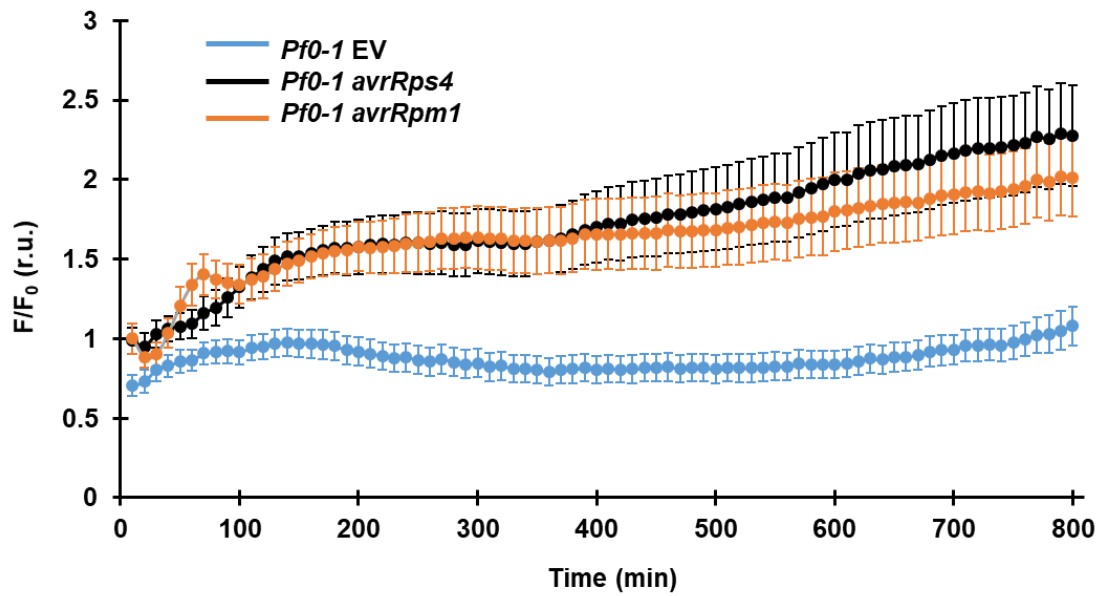
5 Total proteins extracted from samples of Fig. 4B (A), Fig. 4C and 4D (B), Fig. 4E and 4F (C), Fig. 4G and 4H (D), and Fig. 4I and 4J (E) were resolved in 8% SDS-PAGE gel and transferred to nitrocellulose membranes. The RNL proteins were detected by immunoblotting the membranes with anti-GFP antibody.



**Fig. S17. NRG1.1 D485V triggers cytoplasmic  $Ca^{2+}$  influx in HEK293 cells.**

(A) Intracellular  $[Ca^{2+}]_i$  in HEK293 cells expressing active NRG1.1 D485V or NRG1.1 DV D3N E14Q loss of function mutant, before or 2 minutes after 2.5mM  $CaCl_2$  addition. Intracellular  $[Ca^{2+}]_i$  was calculated from Fura-2 emission ratios (340:380 nm) and scaled using a pseudo-color bar. Letters represent statistical difference (ANOVA with post-hoc Tukey HSD test,  $p < 0.05$ ).

(B) Representative fluorescence microscopy images of HEK293 cells in (A).



**Fig. S18. Effector triggered RNL-dependent RPS4 TNL activity is associated with elevated cytoplasmic  $[Ca^{2+}]$ .**

Representative time course measurement of intracellular  $[Ca^{2+}]$ . *Arabidopsis* plants carrying the intracellular  $Ca^{2+}$  sensor GCaMP6 (57) were infiltrated with *Pseudomonas fluorescens* 0-1 (*Pf0-1*, a *Pseudomonas* strain naturally devoid of type 3 secretion system that was engineered to express a *P. syringae* type 3 secretion system and deliver effectors (66)), carrying empty vector (EV), *avrRps4* or *avrRpm1* effector genes, as indicated. Error bars represent s.e.m (N = 16). The experiment was performed three times with similar results.

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