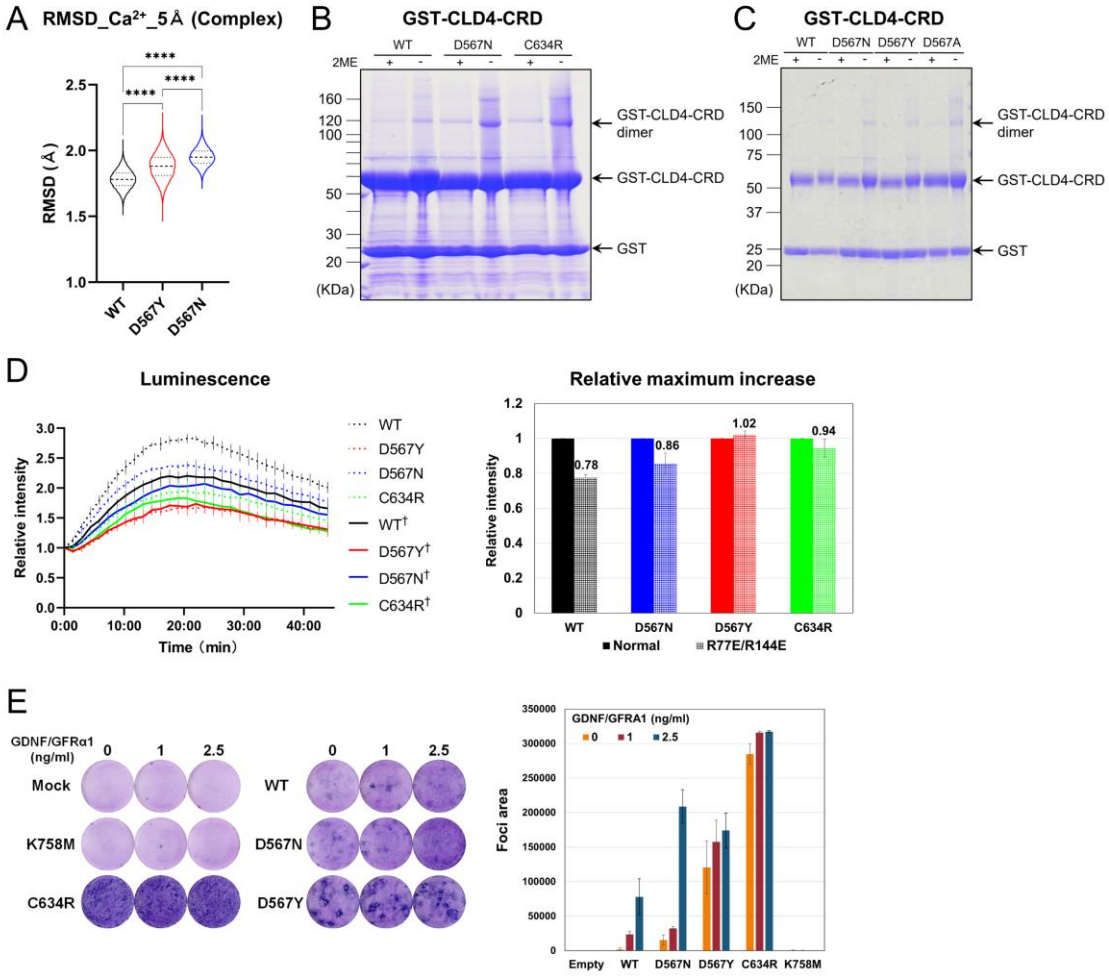
Supplementary Figure S3



Supplementary Figure S3. Illegitimate intermolecular disulfide bond formation induced by CaLM mutations

A, Violin plot showing the RMSD of CRD residues located within 5 Å from the Ca^{2+} ion, estimated from MD simulation of the RET-GDNF-GFRα1 extracellular complex. MD simulation was performed in triplicate for wild-type (WT) or each RET mutant, and structures extracted from the 0.5–1 μs trajectories every 20 ps were subjected to calculation. The black dashed line represents the median values. Each dotted line represents the interval between the end of the first quartile and the beginning of the fourth quartile. Asterisks represent

significantly different distributions (****: P < 0.0001; one-way ANOVA with Tukey's multiple comparisons test).

B, CBB staining of purified GST-tagged RET CLD4-CRD of WT or D567N and C634R mutants expressed in Sf21 cells. Purification was performed under reducing or non-reducing conditions, i.e., presence or absence of 2-ME.

C, CBB staining of purified GST-tagged RET CLD4-CRD of D567Y and D567A mutants expressed in Sf21 cells in the presence of calcium and under reducing and non-reducing conditions, i.e., presence or absence of 2-ME.

D, NanoBiT assay to examine in-cell protein-protein interactions. U2OS cells were transfected with plasmids expressing RET-ECD C-terminally fused to LgBiT or SmBiT. At 24 h post-transfection, cells were incubated with or without GDNF and GFRα1 at a final concentration of 7.4 μg/mL, and then with Nano-Glo Live Cell substrate. Left, RET dimerization was evaluated by the increase in luminescence after addition of GDNF and GFRα1 for 44 min. The signal is reported as a ratio to the "untreated" control condition (without ligand), which was set to 1. The solid and dotted lines represent measurements for WT, D567Y, D567N, and C634R mutants with (indicated by †) or without the R77E/R144E mutation, respectively. Points represent the mean ± SD. Right, relative maximum increases in luminescence of WT and mutant RET (without R77E/R144E mutations) compared with those with the R77E/R144E mutations. Columns represent the mean ± SD.

E, Transforming ability of CaLM mutants. *Left*, NIH3T3 focus formation. The transforming ability of lentiviruses to express CaLM mutants was examined using empty virus infection and lentiviruses expressing the C634R (CCM) mutant. Transformation was examined in the absence and presence of GDNF/GFRα1 (1.0 or 2.5 ng/mL). *Right*, transformation ability of each mutant. The total transformation foci area measured with the ImageJ program was calculated using the values for empty virus infection as the background. Columns represent the mean ± SD of three independent experiments.