Supporting Information

Evidence for a multi-site ligand binding and stretching of filamin by integrin and migfilin

Sujay Subbayya Ithychanda and Jun Qin*
Department of Molecular Cardiology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195

Materials: All restriction enzymes and reagents for molecular biology were from New England Biolabs, Fermentas and Sigma Chemical Company. Full-length human filamin A construct from Dr. Fumihiko Nakamura of Harvard Medical School was used as a template to amplify the 19-21(2045-2329aa) repeat. The Filamin A_{var-1} 19-21 mutant was generated using the Stratagene mutagenesis kit. All the constructs were cloned into pGSTparallel vector (*1*) for over-expression in *E. coli* BL21(DE3) and authenticity conformed by DNA sequencing. Cultures were induced at $0D_{600}$ with 0.3mM IPTG at room temperature for 16 hours. Induced *E. coli* was lysed in 50mM Tris pH 8.0, 10% glycerol, 10mM $MgCl_2$, 1mM DTT, protease inhibitors, DNAase and lysozyme. Lysate was passed through glutathione sepharose (GE Healthcare), washed with 50mM Tris pH 8.0, 1mM DTT and eluted with 10mM glutathione in 50mM Tris pH 8.0, 1mM DTT. Pure fractions were cleaved with TEV protease in 50mM Tris pH 8.0, 1mM DTT and 0.5mM EDTA. Cleaved GST tag was removed by passing through Glutathione sepharose. A final size exclusion chromatographic step was performed to obtain greater than 95% pure protein as judged by SDS-PAGE. Peptides were synthesized in the Biotechnology core of the Lerner Research Institute and confirmed by mass spectrometry.

Methods:
All NMR and ITC measurements were performed in 25mM Sodium phosphate buffer, pH 6.4, 1mM DTT and 5mM NaCl at 30^{o}C. ^{1}H-^{15}N HSQC experiments were performed using a cryo-cooled Bruker 600 MHz spectrometer at the Cleveland Clinic. Isothermal calorimetry measurements were carried out using Microcal VP_{itc200} instrument. Peptides were weighed carefully and dissolved in the buffer assuming a purity of 80%. Protein was estimated by recording absorbance at A_{280}. The syringe concentration of the peptide was 2mM and the protein concentration in the cell was 70µM. 1µl of the peptide ligand was injected every 4 minutes. A blank run were the peptide was injected into buffer without protein was performed to estimate the heat of dilution of the peptide. The average heat of dilution from this blank run was subtracted from the experiment to generate the final thermal profile. The data fitted to one site (n=1, fixed) and two site (n=2, fixed) binding models resulted in K_d values of 153±23µM and 66±16µM respectively. The data that we report in the main body of the report was obtained with a free floating stoichiometry and it matches well with a two site binding mode. The data was also fitted to a sequential model with comparable affinities.

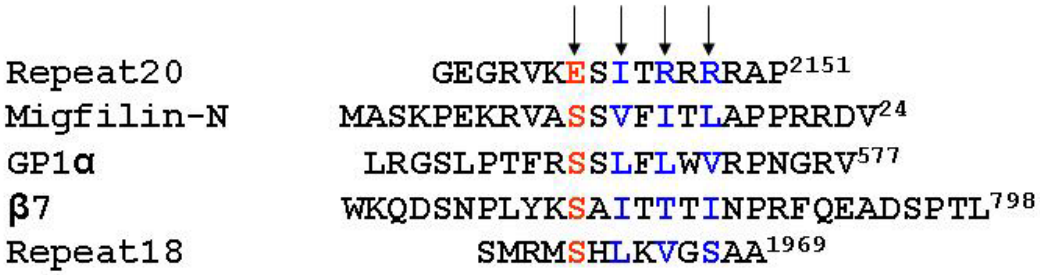

Figure S1: Comparison of the structurally characterized filamin ligands (Gp1bα, Migfilin-N and β7 integrin) with intra-filamin residues causing auto-inhibition. The alternate hydrophobic residues (in blue) are missing in repeat 18 and 20. Note that in most of the ligands, two prolines flank the binding sequence, a feature absent in repeat 18 and 20 binding segments. The last amino acid of the sequence is numbered as in the mature proteins.

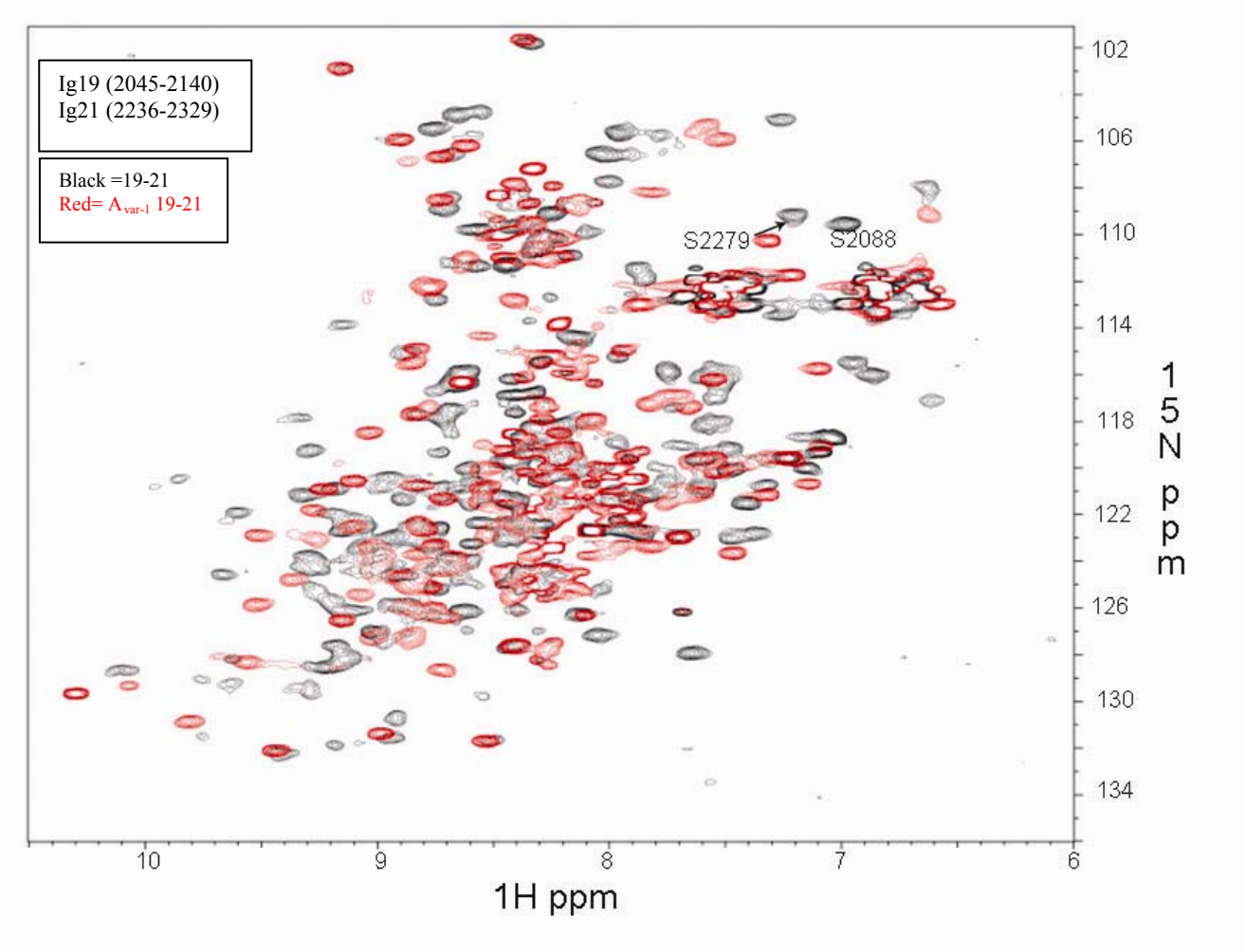

Figure S2: HSQC comparison of filamin A 19-21 and filamin A_{var-1} 19-21 filamin A shows substantial differences. Filamin A_{var-1} 19-21 protein has greater than 100 resonances missing.

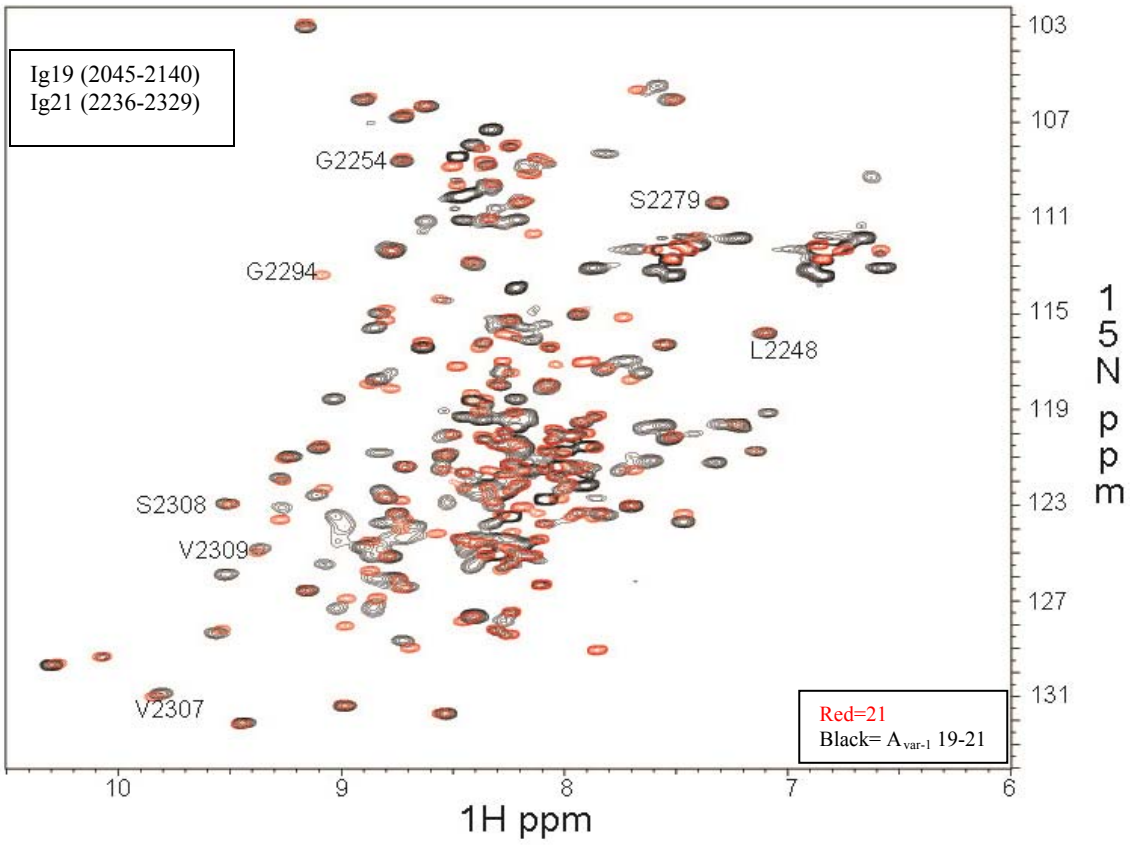

Figure S3: HSQC spectra of filamin A_{var-1} 19-21overlays well with that of isolated filamin A 21. There are a few peaks that are missing or slightly misaligned. To a large extent the two spectra look similar though the filamin A_{var-1} 19-21 protein has 150 more amino acids

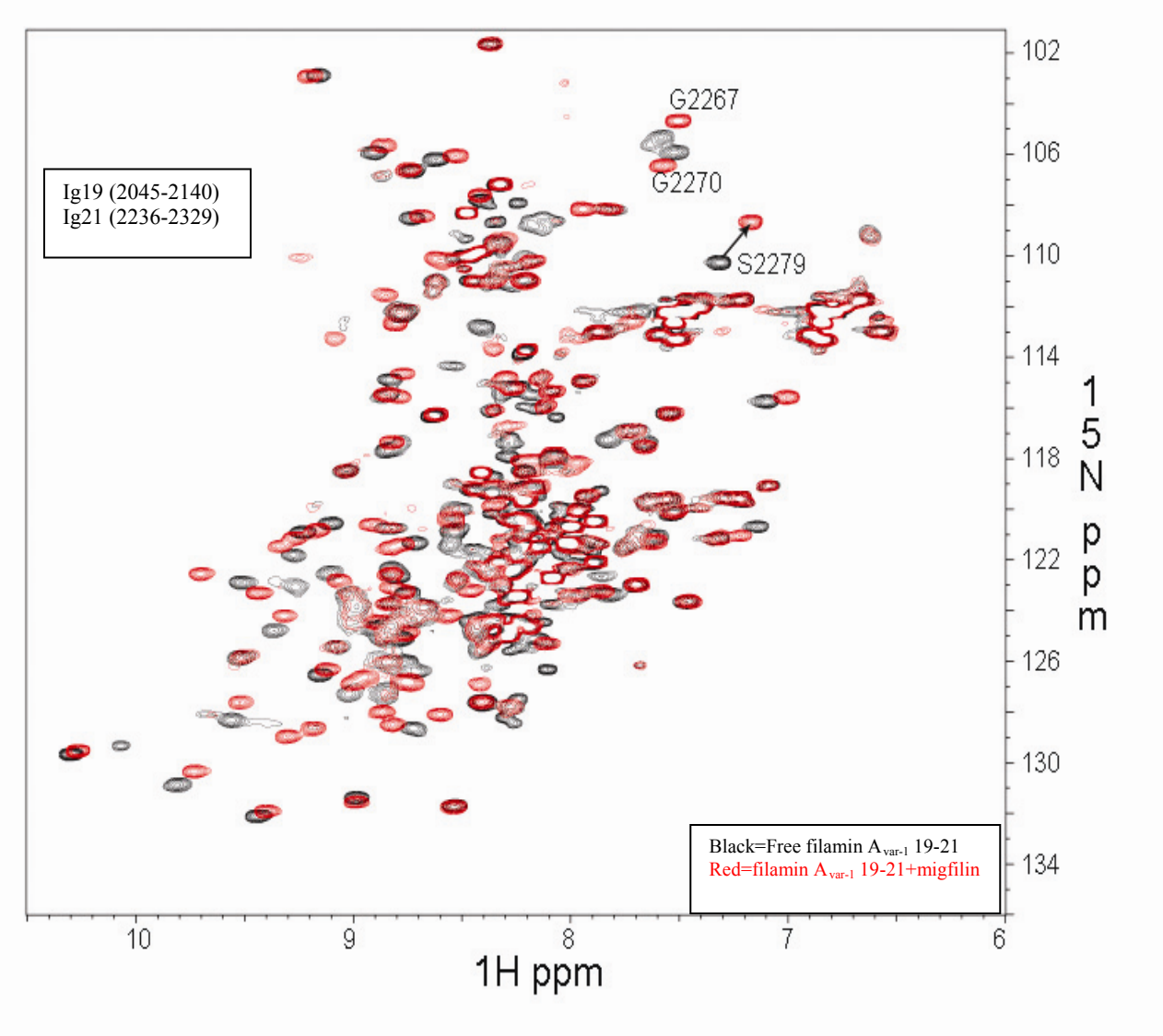

Figure S4: HSQC spectra of free and migfilin bound filamin A_{var-1} 19-21. Though repeat 19 is unfolded in this protein the repeat 21 remains capable to bind migfilin robustly.

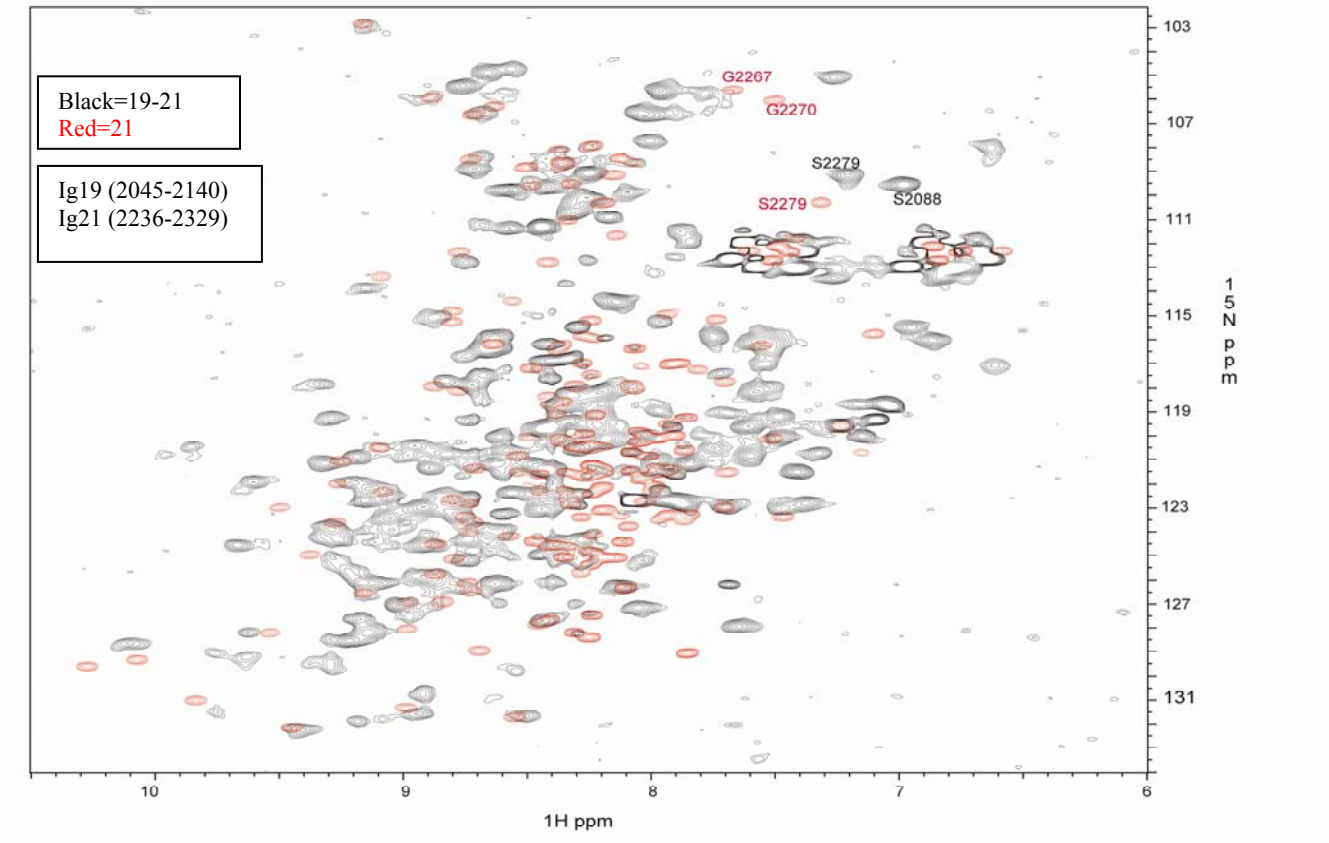

Figure S5: HSQC spectra of filamin A 19-21 and filamin A 21do not match at all unlike filamin A_{var-1} 19-21 and 21. This is evidence that repeat 21 adopts a different conformation in 19-21.

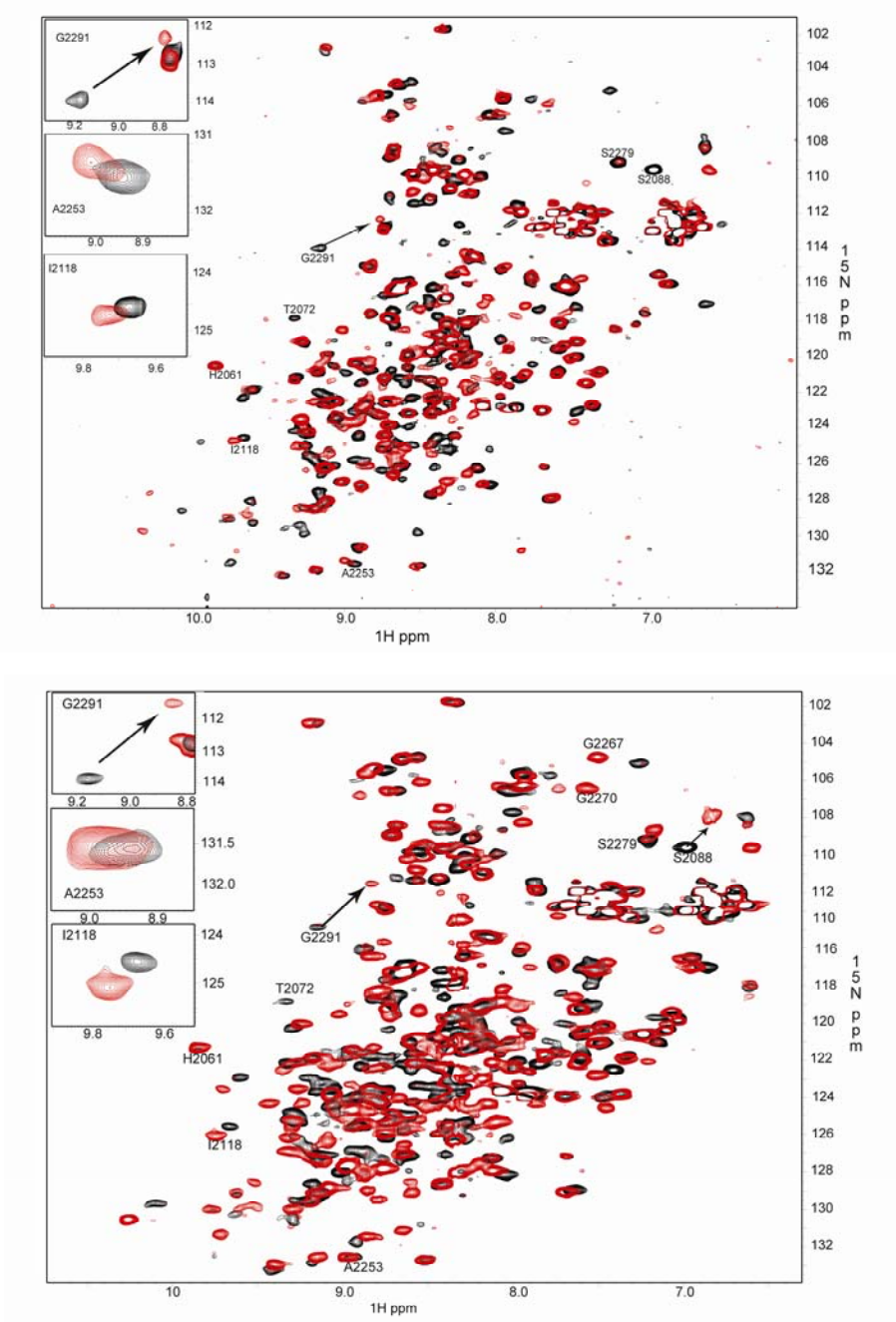

Figure S6: (A) HSQC spectrum of repeat 19-21 (free, black) and bound to integrin β7 peptide (red). The spectra were recorded at 600MHz and the protein-peptide ratio is 1:4. Peaks corresponding to 19 and 21 are perturbed and/or broadened, indicating simultaneous occupation of both 19 and 21. (B) HSQC spectrum of repeat 19-21 (in black is the free form) and bound to migfilin peptide (in red). Chemical shift changes from both repeat 19 and 21 are clearly marked. Appearance of glycines 2267 and 2270 from repeat 21 bound migfilin is a signature for repeat 21 occupation. A few well identified peaks from either repeat are highlighted in the inset in both A and B.