Supplementary data

Figure S1

A three-step schematic representation of SBED-mediated cross-linking used to determine non-competitive binding of CaM and moesin FERM to the tail of L-selectin.

Sulfo-N-hydroxysuccinimidyl-2-(6-[biotinamido]-2-(p-azido benzamido)-hexanoamido) ethyl-1,3'-dithioproprionate, or SBED, is a heterobifunctional chemical crosslinker. It possesses an amine-reactive NHS-ester group at one end and a UV-activatable aryl azide group (N_3) at the other end. In between the two reactive groups is a thiol-cleavable bond, which can be reduced using dithiolthreitol (DTT) or beta-mercaptoethanol. A biotin group ("B") is attached between the thiol-cleavable bond and the aryl azide group. It is the transfer of biotin from the "bait" (i.e. the L-selectin tail) to the "prey" (i.e. moesin or CaM) that is monitored in this experiment. Labelling of WT L-selectin tail peptide with SBED was achieved by following the manufacturer's instructions. The SBED-labelled L-selectin tail was subsequently separated from free SBED by gel filtration, using a 5 ml 1,800 dalton molecular weight cut-off desalting column (Pierce). Approximately 3.6 μM SBED-labelled peptide was mixed with equal amounts (4.6 μM) of CaM and/or moesin FERM domain, and left in the dark to incubate for 60 min. UV cross-linking was performed using a Stratalinker (Stratagene) for 30 min at room temperature. Note that due to the photo-activatable aryl group, all procedures prior to UV cross-linking were performed under red light in a dark room. The reaction mixture was then stopped with 2x Laemmli protein loading buffer, which was subsequently boiled and resolved on 4-12% NuPAGE polyacrylamide gels (Invitrogen). Gels were then prepared for transfer to PVDF membrane, and streptavidin-HRP was used to detect transfer of biotin from the tail of L-selectin to either CaM or moesin FERM.

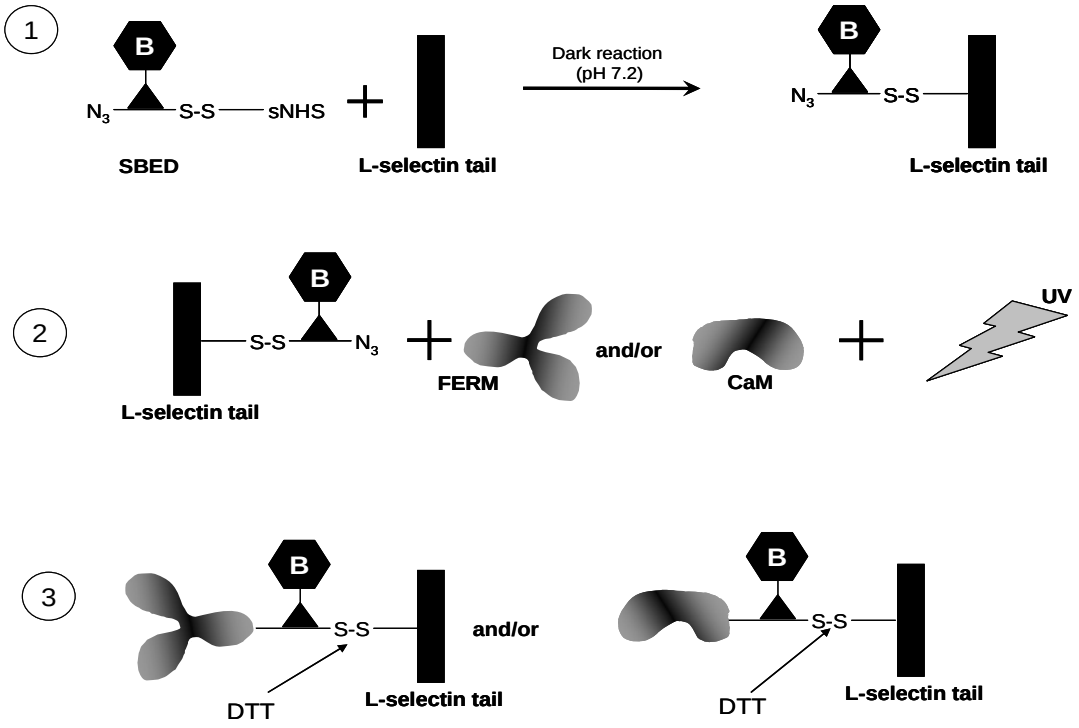

Figure S2

A breakdown of the hydrogen bonds predicted to exist between CaM, L-selectin and moesin

After a dynamics simulation (using AMBER99, YASARA and YAMBER2 software) the molecular complex was inspected for the presence of hydrogen bonds (H-bonds). These are summarised in the tables below. The modelled complex is provided as a supplementary pdb file.

bbO = backbone oxygen atom; bbN = backbone nitrogen atom.

L-selectin residue		FERM residue
R357	→	N251 + D252
bbO, S364	→	N247 + bbN, I248
K365	→	bbO, R246 + bbO, K258
N369	→	H288 + bbO, S243

L-selectin residue		CaM Residue
R356	→	D131 + bbO, D131
R357	→	E127
bbN, L358	→	bbO, E127
K359	→	bbO, G134 + bbO, I100
K360	→	E140
K362	→	E114 + bbO, E114
bbN, R366	→	bbO, L112

CaM residue		FERM Residue
E83	→	N62 + K63
E84	→	N62 + K60 + K63
bbO, K148	→	K278
bbO, M144	→	K278
E114	→	R246
bbO, G113	→	R246

Figure S3

Confocal microscopy reveals co-localisation of moesin, L-selectin and CaM in Cos-7 cells. Cos-7 cells were transiently transfected with C-terminally HA-tagged (constitutively active) full-length moesin, WT L-selectin (untagged) and CaM-GFP. DREG 56 labelling was performed on live cells. Excess antibody was washed off and subsequently labelled with Cy5-conjugated secondary antibody. Cells were then fixed in 4% PFA and permeabilised with 0.1% NP40. Rabbit anti-HA antibody, followed by Alexa fluor 555-conjugated goat anti-rabbit secondary antibody, was used to detect moesin. TD moesin = constitutively active (where threonine at position 558 is pseudophosphorylated by adding aspartate (D)). Bar = 10 μM

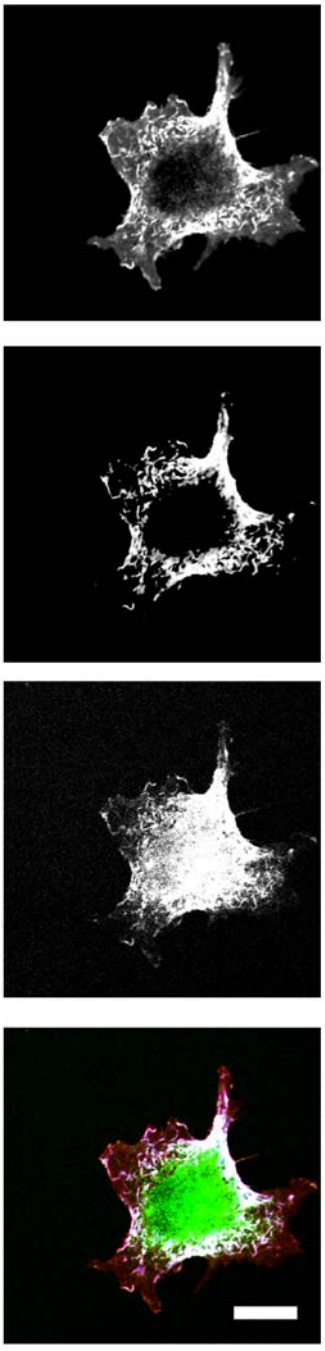

Figure S4

Clustering of CD44 does not induce CaM/ERM interaction. Cos-7 cells were transiently transfected with WT CD44, ezrin FERM-GFP and CaM-mCherry. Clustering of CD44 was achieved using murine anti-CD44 monoclonal antibody, E1/2, followed by cross-linking with a Cy5-conjugated donkey anti-mouse secondary antibody. Our preliminary experiment demonstrated that although CD44 is an ezrin-binding protein, its clustering could not induce CaM/ezrin interaction. This result suggests that CaM/ezrin FERM interaction is driven specifically via clustering of L-selectin. Images show the GFP multiphoton intensity image and (where appropriate) corresponding widefield CCD camera image of the mCherry. Lifetime is shown as a pseudocolour scale of blue (high lifetime) to red (low lifetime=FRET).