Supplemental Materials

Molecular Biology of the Cell

Fontenele et al.

Supplementary Methods

Mass Spectrometry Sample Preparation

Following SDS-PAGE and Coomassie staining, bands corresponding to the region of the gel immunoreactive to Cactus-eGFP were excised and diced into 1 mm^3 cubes. Gel pieces were destained with ammonium bicarbonate buffer, washed extensively, subjected to DTT thiol reduction, iodoacetamide alkylation, overnight trypsin digestion, and peptide elution, by the method of (Shevchenko et al., 2006). Peptides were desalted using StageTip micro-scale reversed-phase chromatography (Rappsilber et al., 2003), then subjected to reversed-phase nano-LC-MS and MS/MS performed on a nano-flow capillary high pressure HPLC system (Nano Ultra 2D Plus, Eksigent, Dublin, CA) coupled to an LTQ-Orbitrap XL hybrid mass spectrometer (ThermoFisher Scientific, San Jose, CA) outfitted with a Triversa NanoMate ion source robot (Advion, Ithaca, NY). Sample concentration and washing was accomplished online using a trapping capillary column (150 µm x ca. 40 mm, packed with 3 µm, 100 Å Magic AQ C18 resin, Michrom, Auburn, CA) at a flow rate of 4 µl/min for 4 min, while separation was achieved using an analytical capillary column (75 µm x ca. 15 cm, packed with 1.7 µm 100 Å BEH C18 resin, Waters, Milford, MA), under a linear gradient of A and B solutions (solution A: 3% acetonitrile/ 0.1% formic acid/0.1% acetic acid; solution B: 97% acetonitrile/0.1% formic acid/0.1% acetic acid) over 180 min at a flow rate of approximately 400 nl/ min. Nano electrospray ionization was carried out using the NanoMate ion source at 1.74 kV, with the LTQ heated capillary set to 200 °C. Full-scan mass spectra were acquired in the Orbitrap in positive-ion mode over the m/z range of 335–1800 at a resolution of 100,000. MS/MS spectra were simultaneously acquired using the LTQ for the seven most abundant multiply charged species in the full-scan spectrum having signal intensities of >1000 NL. Dynamic exclusion was set such that MS/MS was acquired only once for each species over a period of 120 s.

Mass Spectrometry Data Analysis

Resultant LC-MS/MS data were subjected to preprocessing into peaklist files (mgf) using ProteomeDiscoverer (v. 1.3, ThermoFisher). These files were searched against either the UniProt *Drosophila melanogaster* database, or a custom database consisting of the cactus-eGFP fusion

protein sequence, using the Mascot search engine (v. 2.2.7, Matrix Science, London, UK.), allowing for trypsin, semi-trypsin, or no-enzyme cleavage specificity, methionine oxidation and *N*-terminal protein acetylation as variable modifications, and carbamidomethylation of cysteines a fixed modification. Alternatively, error tolerant modification searching was employed. Aggregate peptide spectral match results were imported into Scaffold software (Proteome Software, Portland, OR) for consolidation according to the PeptideProphet and ProteinProphet parsimony algorithms (ISB, Seattle WA) and were filtered to a 95% peptide confidence level, which corresponded to an estimated false discovery rate of ≤ 0.01%.

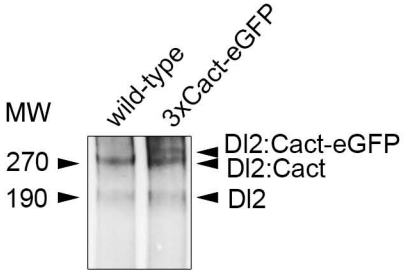
References:

Rappsilber, J., Ishihama, Y. and Mann, M. (2003). Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Analytical chemistry* **75**, 663–70.

Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V and Mann, M. (2006). In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nature protocols* **1**, 2856–60.

Suppl. Figure 01

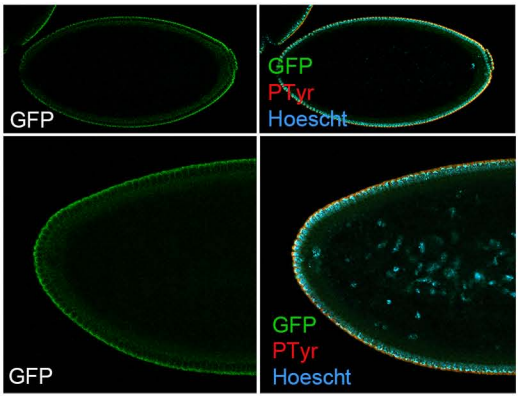
A



B

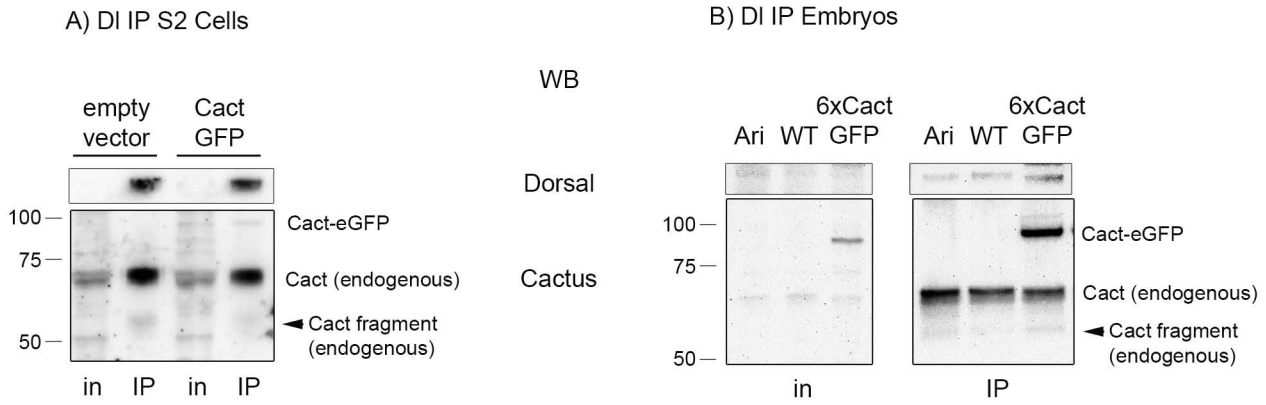
Maternal genotype	Embryonic lethality
cact[A2]/+	30%
cact[A2]/cacteGFP # 3	17%
cact[A2]/ cacteGFP # 7	10%
cact[A2]/2x cacteGFP	6%

C



Supplementary Figure 1. Cactus-eGFP constructs are functional.
(A) Embryo samples analysed in non-reducing gel and anti-Dl western blot showing two high molecular weight complexes, of 270 kDa and 190 kDa, corresponding to 2Dl:1Cact and 2Dl, respectively. A third complex is seen in embryos expressing Cact-eGFP, corresponding to 2Dl:1Cact-eGFP. (B) Embryos from mothers expressing Cact-eGFP partially recover the 30% lethality of loss-of-function *cact* alleles. (C) Cact-eGFP is distributed along the embryo DV axis in the same patern reported for endogenous Cact. Cactus in green, phosphotyrosine in red and nuclei blue.

Suppl. Figure 02



Supplementary Figure 2. Cactus fragments are produced endogenously and interact with Dorsal.
Co-immunoprecipitation with Dl of full-length Cact and a Cact fragment that may harbor an N-terminal deletion similar to that observed for Cact-eGFP. Immunoprecipitation was performed on S2 cells transfected with an empty pAcPA vector or with pAc-*cact-eGFP* (A) or in embryos maternally expressing a *CalpA* RNAi construct (Ari), wild-type embryos (WT),or embryos expressing 6 copies of pT-*cact-eGFP* under the control of the maternal GAL4 promoter (6x*cact-eGFP*) (B). Western blots were probed with antibodies against Dl and Cact. in=input, IP=immunoprecipitate.

Suppl. Figure 03

A

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MPSPTKAAEAATKATATSDCSCSAASVEQRAPSNAANPSSSLATSGKIGG   50
KTQDQTAAINKQKEFAVPNETSDSGFISGPQSSQIFSEEIVPDSEEQDKD  100
QQESAPQKEQPVVLDSGIIDEEEDQEEQEKEEEHQDTTTATATADSMRLKHS 150
ADTGIPQWTVESHLVSRGEQLNNLGQSSSTQITGRSKVQSSTASTANANP  200
SGSGATSSAPPSSINIMNAWEQFYQQNDDGDTPLHLACISGSVDVVAALI  250
RMAPHPCLLNIQNDVAQTPLHLAALTAQPNIMRILLLAGAEPTVRDRHGN  300
TALHLSCIAGEKQCVRALTEKFGATEIHEAHRQYGHRSNDKAVSSLSYAC  350
LPADLEIRNYDGERCVHLAAEAGHIDILRILVSHGADINAREGKSGRTPL  400
HIAIEGCNEDLANFLLDECEKLNLETATYAGLTAYQFACIMNKSRMQNIL  450
EKRGAETVTPPDSDYDSSDIEDLDDTKMYDRFGDPRYFVSYNGGNPMTVA  500
VSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTT  550
GKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFF  600
KDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNV  650
YIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHY  700
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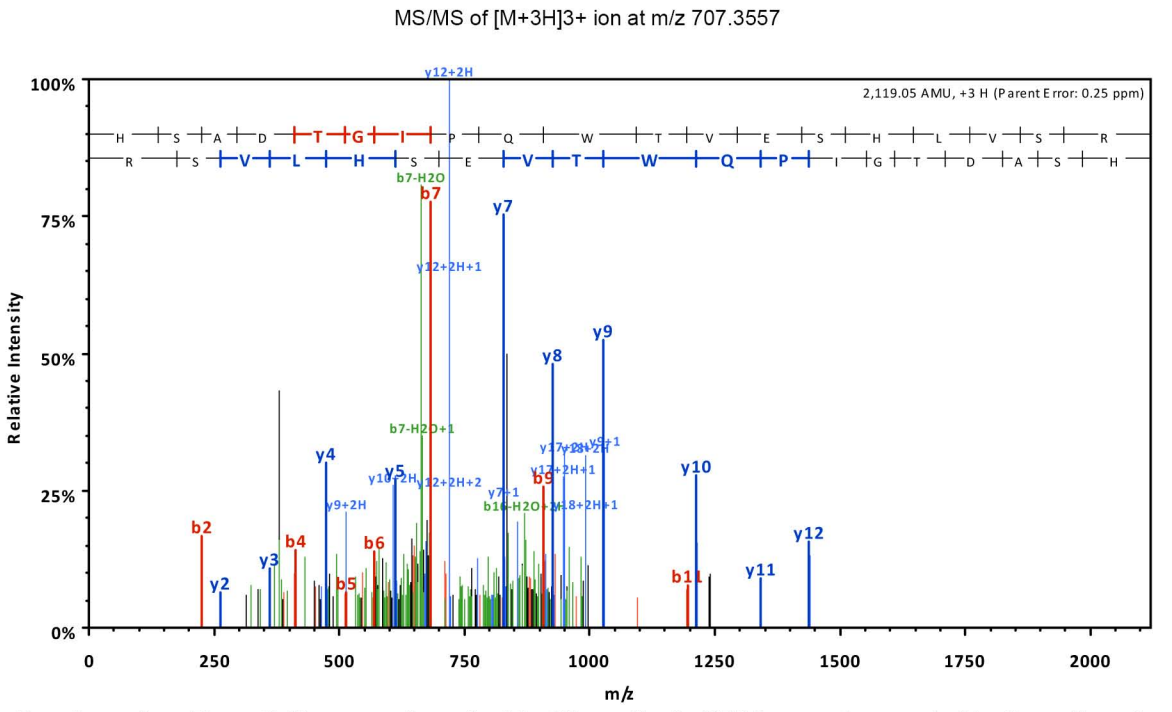
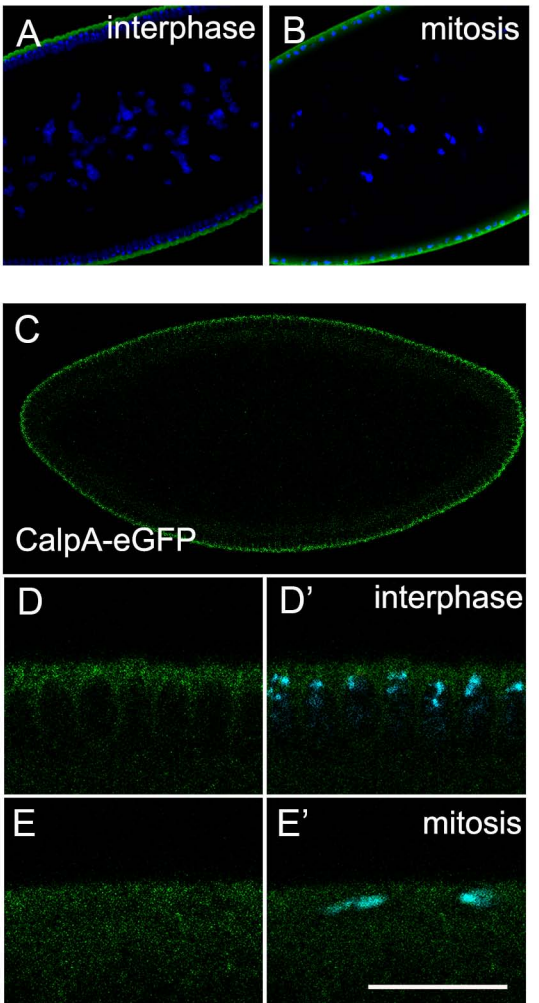
B



Supplementary Figure 3. Mass spectrometry identifies a Cact-eGFP fragment generated by the action of CalpA. Cact-eGFP was subjected to the action of CalpA in S2 cells as in Figure 3C. A GFP-positive band in the 64-65 kDa region was excised from gels and analyzed by mass spectrometry. (A) Full-length Cact-eGFP protein sequence. Colored amino acids represent peptide sequences covered by MS/MS matches to the Cactus-eGFP sequence. Methionin in red represents the initial site used in *cact*[E10] mutants and CactE10-eGFP transgene. Underlined are eGFP sequences. (B) N-terminal-most peptide matched with high confidence was 149-167 (^{149}HSADTGIPQWTVESHLVSR$_{167}$), irrespective of whether trypsin cleavage or no enzyme specificity was used for the search. No sequences N-terminal to amino acid residue 149 were detected, consistent with N-terminal truncation of the fusion protein.

Suppl. Figure 04



Supplementary Figure 4. Calpain A distribution is dynamic.

(A,B) Endogenous Calpain A is localized beneath the plasma membrane and apical to the nuclei during interphase (A). During mitosis (B) CalpA shows a diffuse cytoplasmic distribution. (C,D,E) This pattern is replicated by CalpA-eGFP. CalpA-eGFP is evenly distributed throughout the embryo (C). High magnification of a dorsal region of blastoderm stage embryos during interphase (D) and mitosis (E). All panels CalpA in green, nuclei in blue. Dorsal side is up, anterior left. Bar = 20 μm.