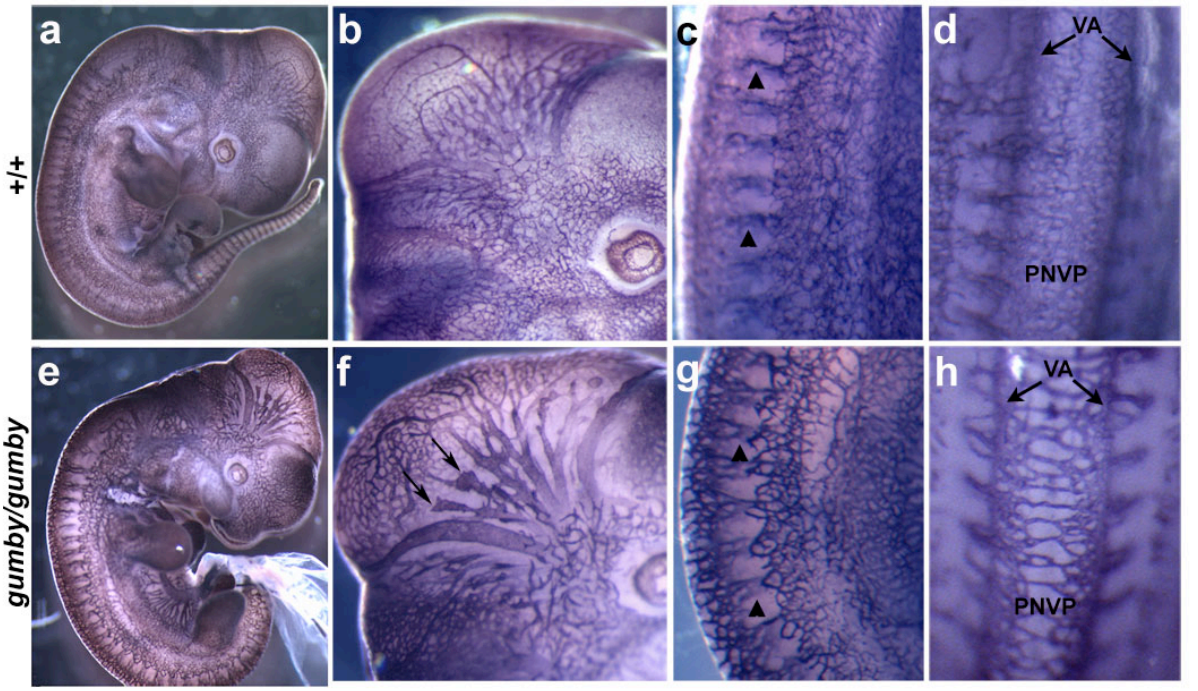
Supplementary material for:

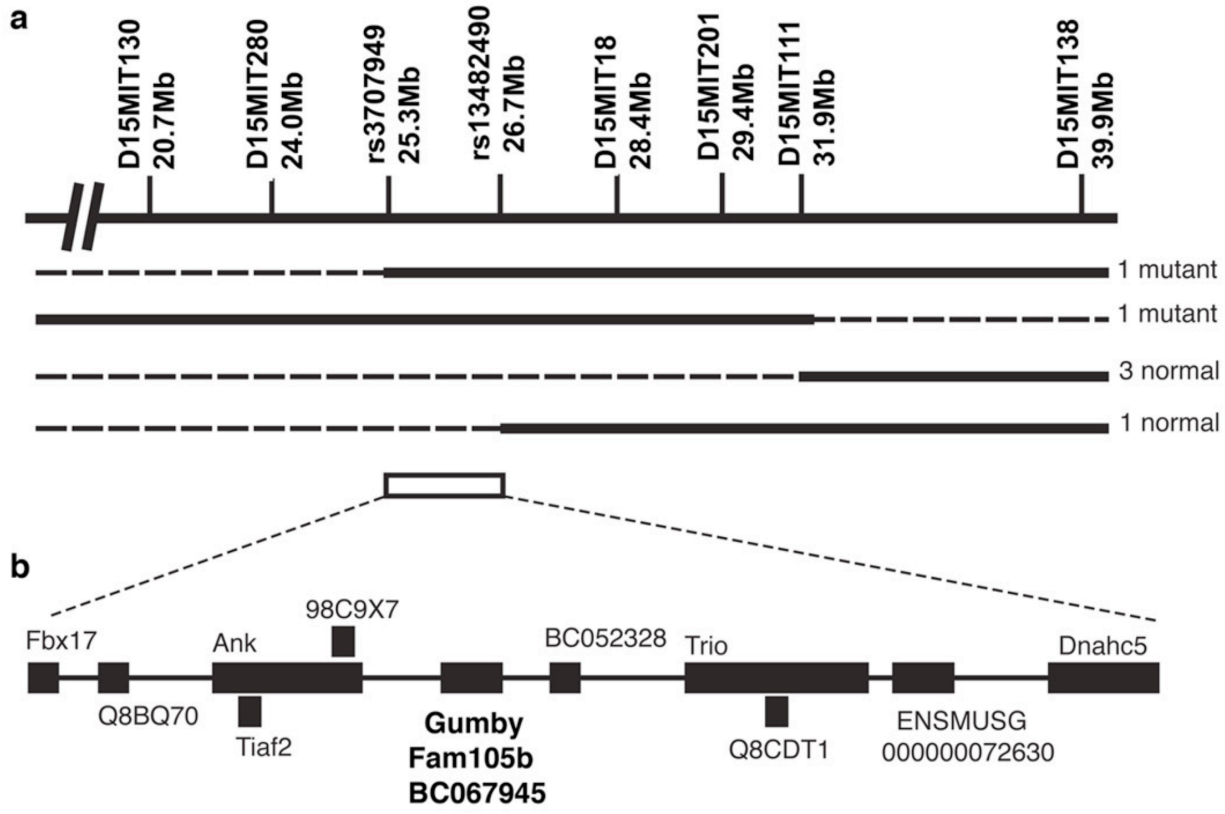
The linear ubiquitin-specific deubiquitinase Gumby/Fam105b regulates angiogenesis

Elena Rivkin, Stephanie M. Almeida, Derek F. Ceccarelli, Yu-Chi Juang, Teresa A. MacLean, Tharan Srikumar, Hao Huang, Wade H. Dunham, Ryutaro Fukumura, Gang Xie, Yoichi Gondo, Brian Raught, Anne-Claude Gingras, Frank Sicheri and Sabine P. Cordes

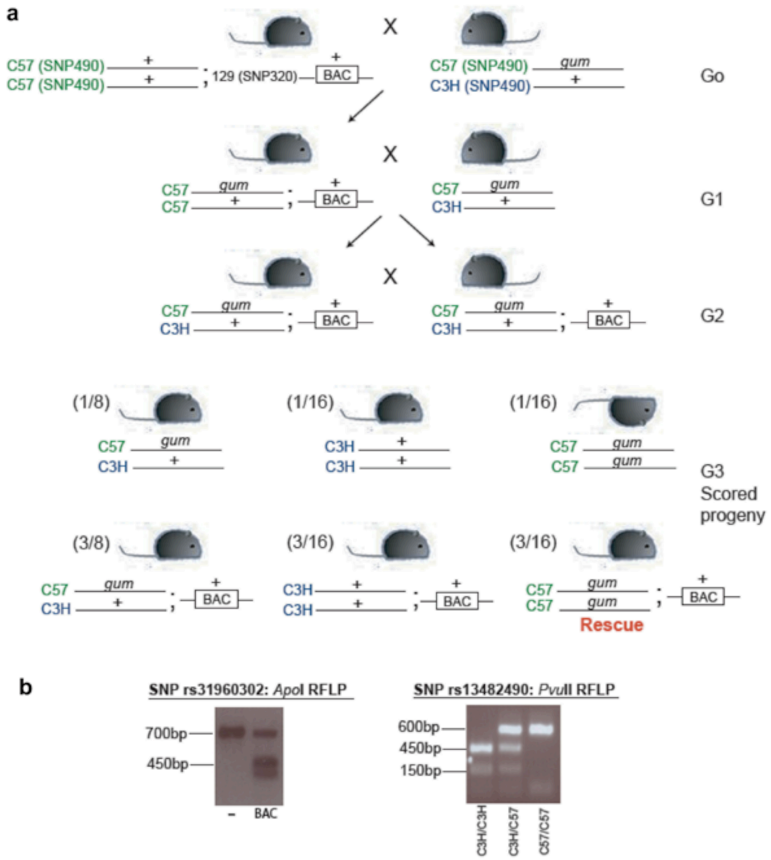
Supplementary Figures



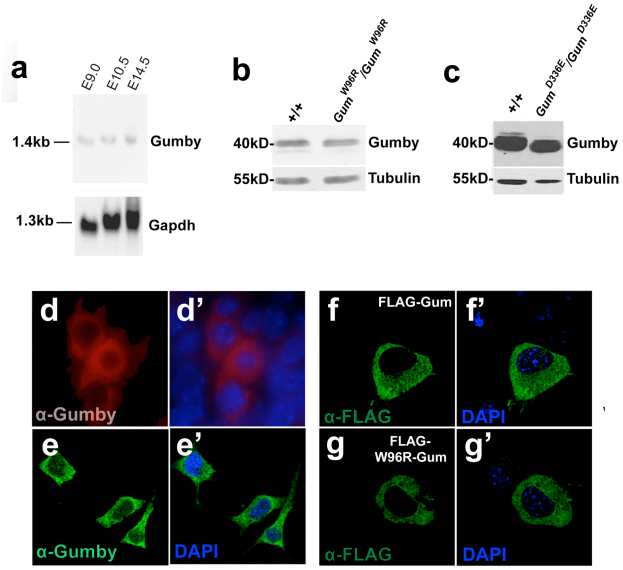
Supplementary Figure 1. Branching of vessels is impaired in *gumby/gumby* embryos.
Whole mount immunohistochemistry with anit-PECAM-1 antibody reveals that the complex branching observed in **a-d,** +/+ embryos is reduced in **e-h,** *gumby*/*gumby* embryos at E11.0. Cranial vessels of **e, f,** *gumby/gumby* embryos have thick primary branches with many 'blebs' (indicated with arrows in **f**), where secondary branches should form. **c, g,** Lateral and **d, h,** dorsal views of the trunk for each embryo. The perineural vascular plexus (PNVP) is less intricate in **h**, mutants than **d**, +/+ embryos. VA, vertebral artery.



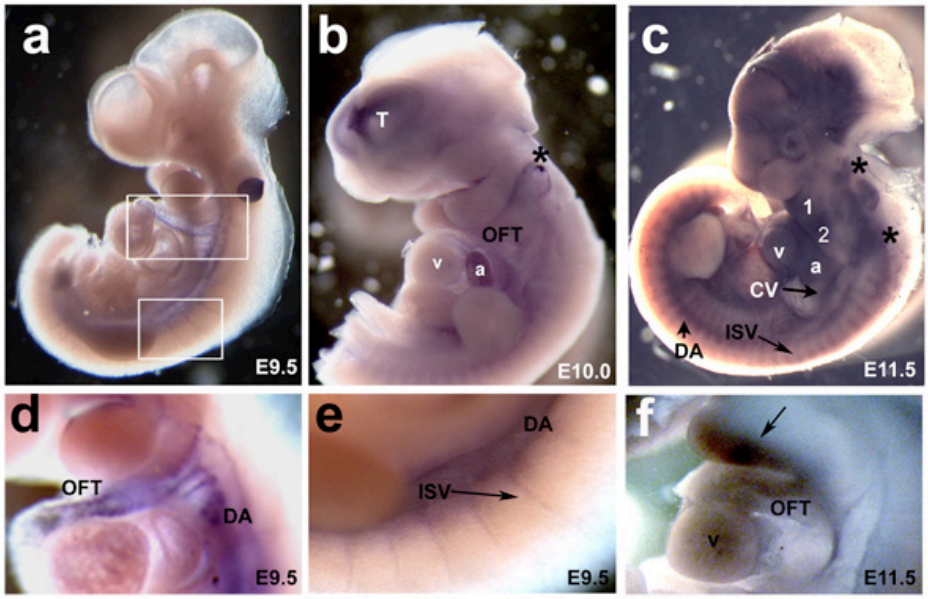
Supplementary Figure 2. Genetic fine mapping of the *gumby* mutation.
a, The 1.4Mb *gumby* critical interval and markers used to refine the *gumby* critical interval are shown. Informative recombinant animals are indicated. The solid line indicates C57BL6/J genomic DNA on which *gumby* (*Gum*^{W96R}) arose. The dashed lines indicate C3H/HeJ genomic DNA. **b**, Eleven genes map to the *gumby* critical interval.



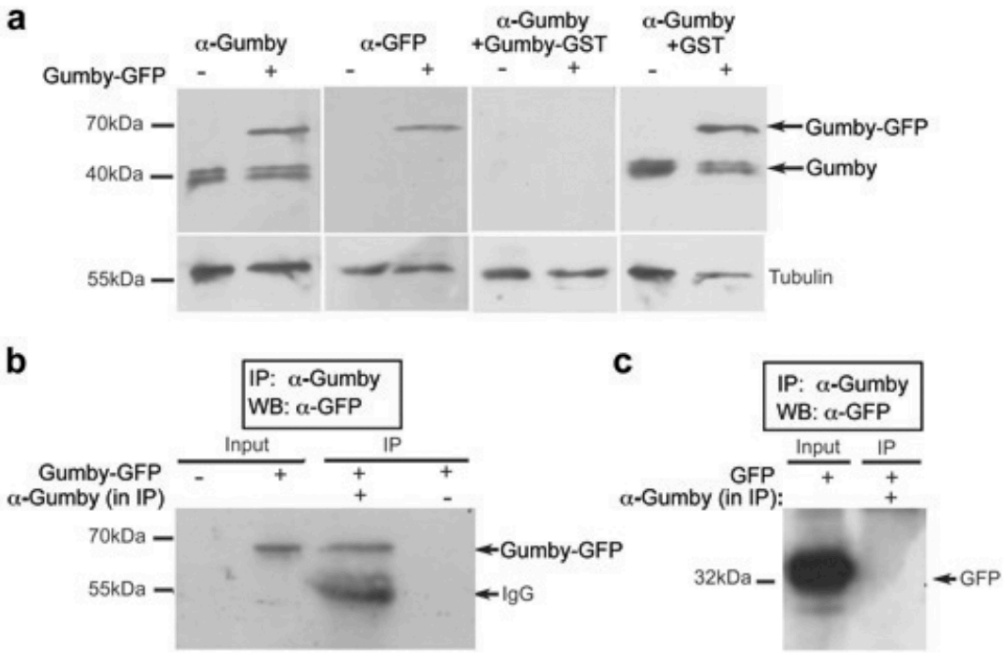
Supplementary Figure 3. BAC rescue breeding strategy.
a, Outline of breeding strategy used to test for BAC transgenic rescue of the *gumby* phenotype. For BAC Lines, two generation crosses were required to generate *gumby*/+ mice carrying the BAC transgene, with the wild-type 'T' allele carried on the C3H strain and mutant 'A' allele on the C57 strain (G2:C57 ^{gumby}/C3H^{+};BAC). Presence of the BAC was determined by SNP rs31960302 (SNP320) that distinguishes between BAC 129Sv/J (129) and C57/C3H strains. The genotype at the *gumby* locus was inferred from SNP rs13482490 (green/blue). Progeny from G2 intercross at >E13.5 that are viable and have a C57/C57;BAC genotype indicates rescue. **b,** PCR assays used to genotype for rescue. To determine presence of BAC transgene, PCR products were digested with *Apo*I to score the RFLP corresponding to SNP rs31960302. To infer the genotype at the *Gumby* locus, PCR products were digested with *Pvu*II to score the RFLP corresponding to SNP rs13482490.



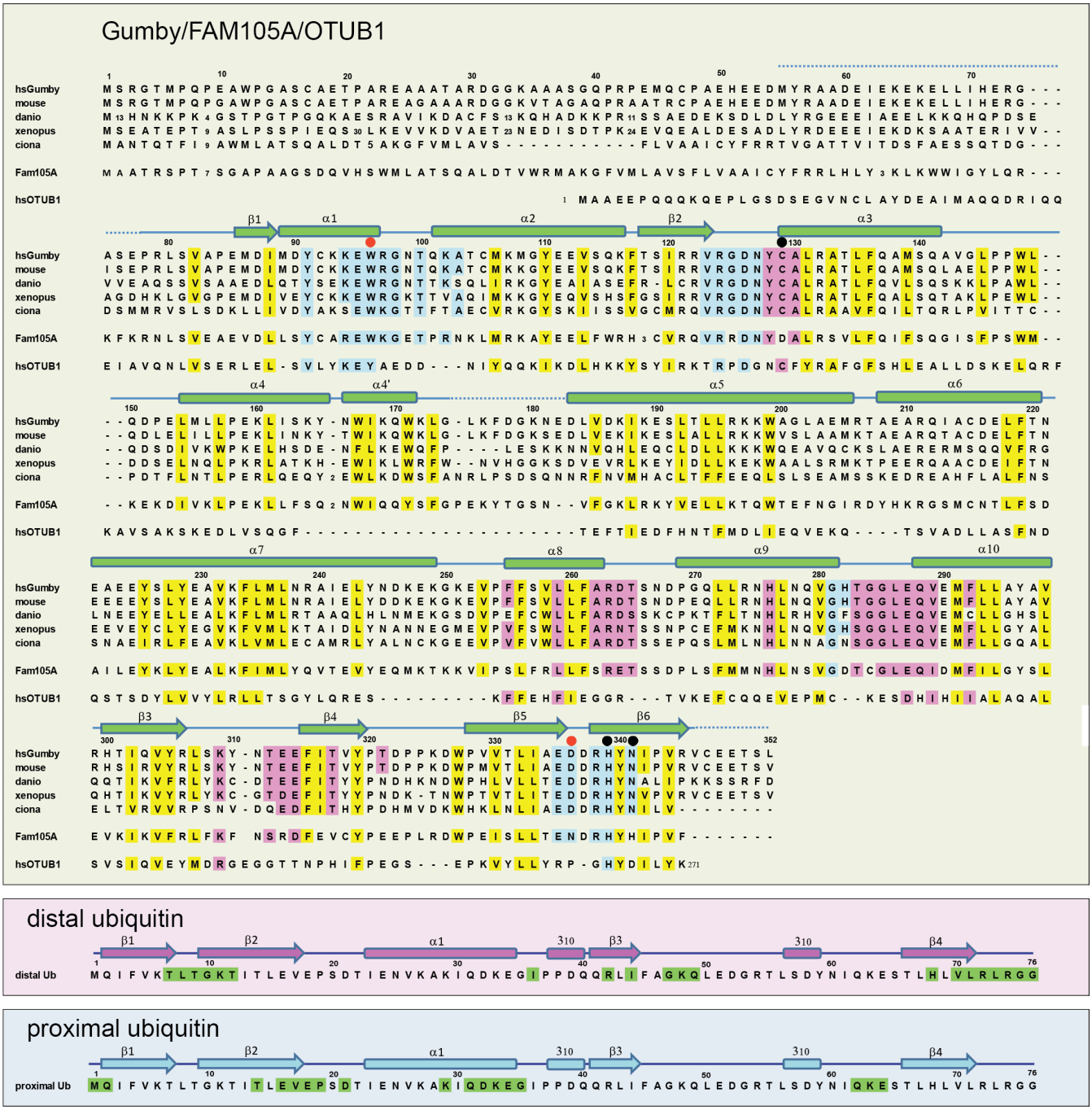
Supplementary Figure 4. Gumby protein levels and cytoplasmic localization are not compromised by *Gumby* mutations.
a, A single *Gumby* transcript of ~1.4Kb was detected in Northern blot analyses of total RNA from E9.5-14.5 embryos. **b, c**, Immunoblot analysis of lysates from E12.5 **b,** *gumby/gumby* (Gum^{W96R}/Gum^{W96R}) embryos and **c.** Gum^{D336E}/Gum^{D336E} embryos and their +/+ littermates using an anti-Gumby antibody detected a close doublet at ~40kDa**.** **d**, **d'**, **e**, **e'**, Immunofluorescence with anti-Gumby antibody (red in **d**, **d'**, green in **e, e'**) detected endogenous Gumby protein localized in the cytoplasm in; **d**, **d'**, human umbilical vein endothelial cells (HUVEC) and **e**, **e'**, Neuro2A cells. **f**, **f'**, **g**, **g'**, In transiently transfected Neuro2A cells, anti-FLAG antibody (*green*) detected cytoplasmic distribution of; **f**,**f'** FLAG-tagged wildtype (FLAG-Gum) and **g**,**g'** FLAG-tagged $Gumby^{W96R}$ (FLAG-W96R-Gum).



Supplementary Figure 5. Whole-mount RNA *in situ* hybridization analyses of *Gumby* expression. **a**, At E9.5 *Gumby* is expressed in the vasculature, including the dorsal aorta, intersomitic vessels, and outflow tract (OFT). At **b**, E10.0 and **c**, E11.5, *Gumby* is expressed in the heart, first and second branchial arches (1, 2), and neural crest cells (asterisks in **b**, **c**). **d**, **e**, Magnified views of **a**. **d**, expression in the OFT at E9.5, and **e**, in the intersomitic vessels (ISV) and dorsal aorta (DA). **f**, magnified view of **c** showing expression in the distal branchial arches (arrow in **f**), OFT, and heart at E11.5. a, atrium; CV, cardinal vein; t, telencephalon; v, ventricle.

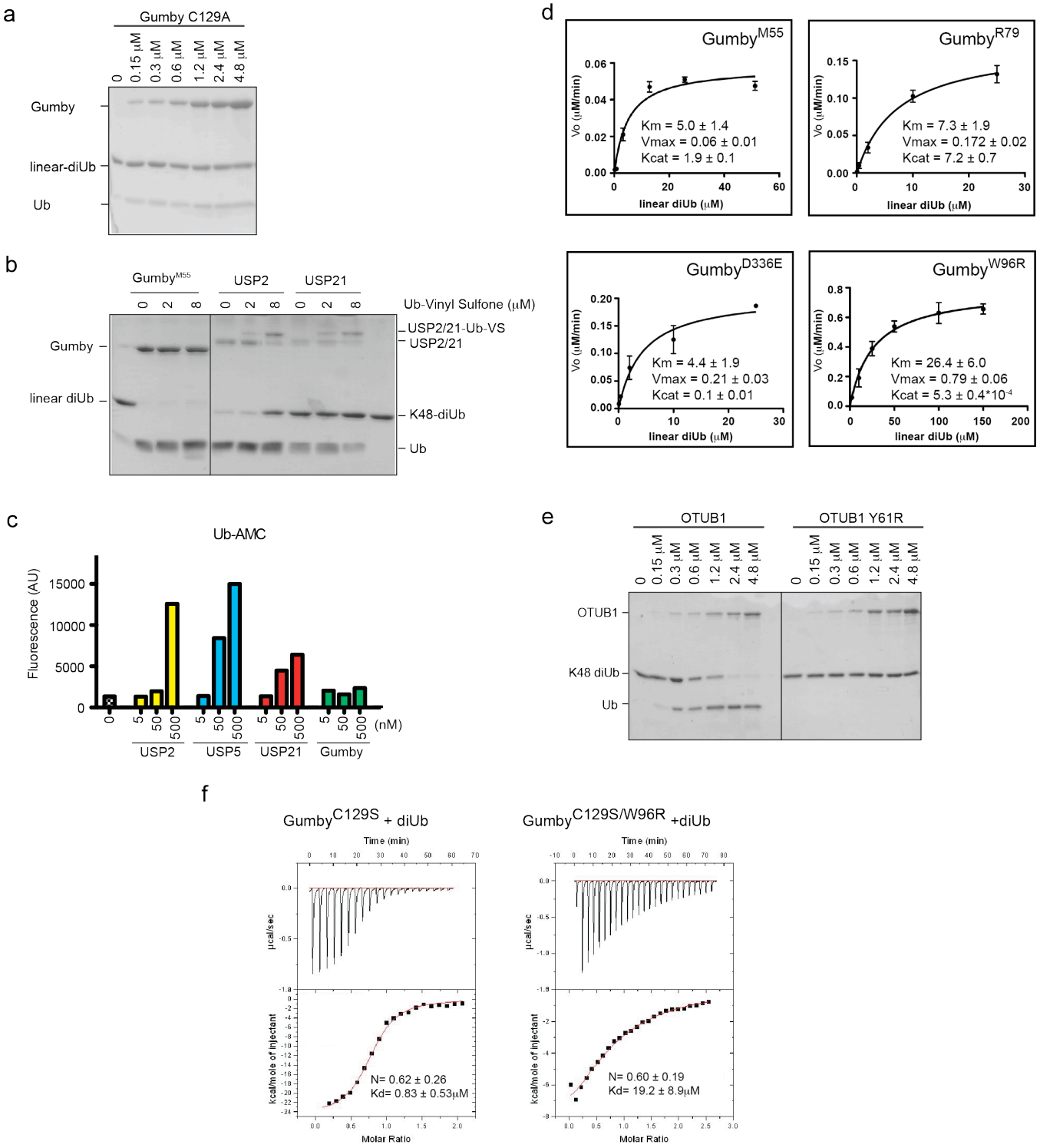


Supplementary Figure 6. Specificity of Gumby antibody.
a, To test antibody specificity, Neuro2A cells were transiently transfected with Gumby-GFP fusion constructs (+), and lysates of these cells were run with untransfected controls (-) for immunoblot analysis. Lysates were blotted for comparison with anti-Gumby (α-Gumby) and anti-GFP (α-GFP) antibodies to evaluate the ability of Gumby antibody to recognize Gumby-GFP fusion protein. The Gumby antibody recognizes the 70kDa Gumby-GFP fusion protein and a doublet at ~40kDa, matching the predicted size of endogenous Gumby protein. To test antibody specificity, Gumby antibody was preabsorbed prior to hybridization with either Gumby-GST or GST alone. Preabsorbing with Gumby-GST, but not GST alone, completely eliminated the signal corresponding to endogenous and transfected proteins. α-Tubulin was used as a loading control. **b,** Gumby-GFP proteins were immunoprecipitated with α-Gumby from Gumby-GFP transfected (+) Neuro2A cells and blotted with α-GFP. **c,** Control immunoprecipitation of GFP-expressing cell lysates with α-Gumby antibody did not immunoprecipitate GFP.



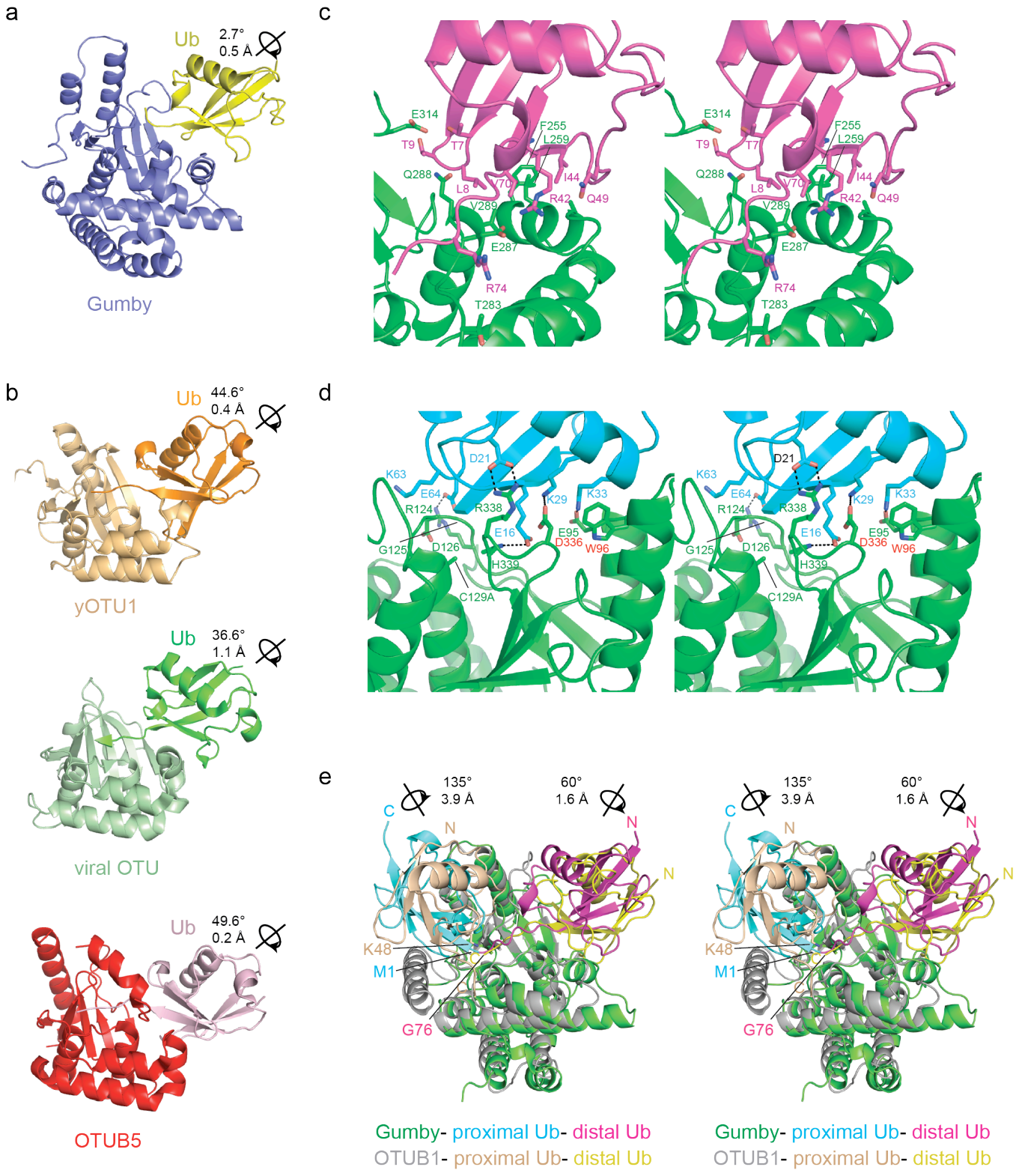
Supplementary Figure 7. Structure based sequence alignment of Gumby and OTUB1 deubiquitinating enzymes.
The amino acid sequence of Gumby from human (hsGumby: NP 612357.4), mouse (NP_001013814.2) danio (CAQ14933.1), xenopus (XP_002933167.1) and ciona (XP_002128617.1) were aligned with the human Fam105A (NP_061891) and OTUB1 sequences (hsOTUB1: NP_060140) by Clustal and manual curation based on superimposed crystal structures. Secondary structure and residue numbers of Gumby are indicated. Conserved hydrophobic residues within Gumby, Fam105A and OTUB1 proteins are coloured yellow. Residues in the Gumby crystal structures that interact with the proximal and distal

ubiquitins are coloured cyan and pink respectively. Catalytic triad residues are indicated with black circles and Gumby W96R and D336E positions by red circles. Residues in distal and proximal ubiquitin that contact Gumby are indicated in green. The Gumby-like protein FAM105A displays 41% sequence identity with Gumby. While the projected distal and proximal ubiquitin binding surfaces are highly similar (24/45 residues are identical), FAM105A lacks the requisite active site cysteine and asparagine residues to support catalysis and does not bind or cleave linear di-ubiquitin substrate (data not shown).



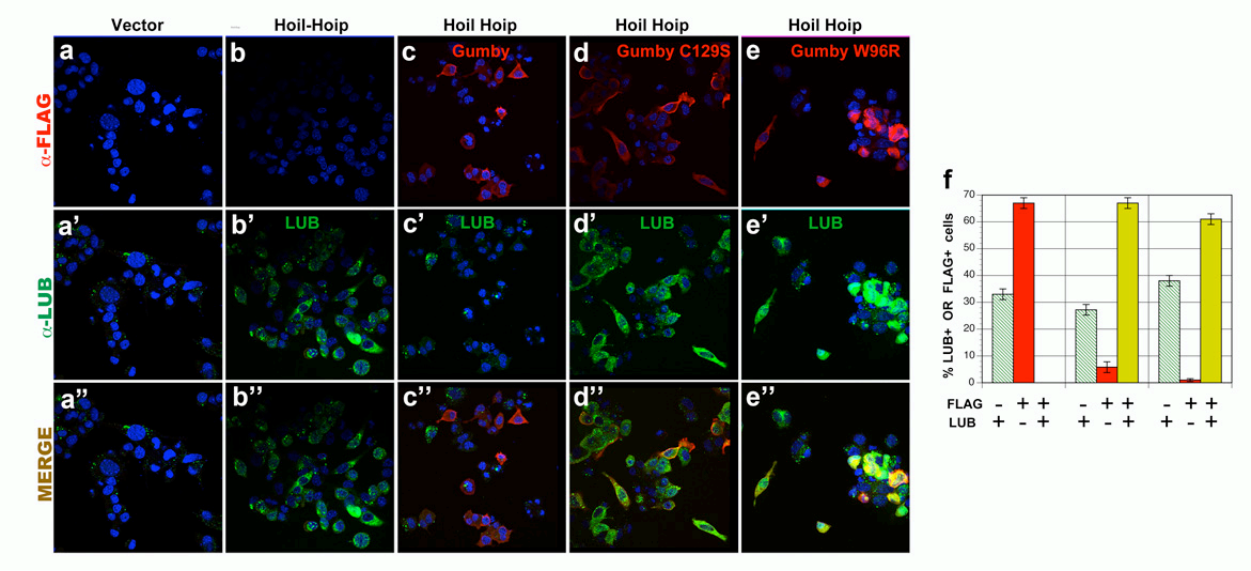
Supplementary Figure 8. Biochemical and biophysical analysis of Gumby.
a, Enzyme titration of GumbyC129A at the indicated concentrations against fixed linear di-ubiquitin substrate. **b,** Comparison of the sensitivity and reactivity of Gumby, USP2 and USP21, towards increasing concentrations of the suicide inhibitor ubiquitin-vinyl sulfone. **c,** Analysis of the relative ability of Gumby, USP2, USP5 and USP21 to cleave the model DUB substrate Ub-AMC. **d,** Michaelis-Menten plots of linear di-ubiquitin cleavage by GumbyM55, GumbyR79, GumbyD336E and GumbyW96R. Initial velocities (V_0) +/- s.e.m. for each substrate concentration were averages from

experiments performed in duplicate. **e,** Enzyme titration of OTUB1 and the Y61R mutant at the indicated concentrations against fixed linear di-ubiquitin substrate. **f,** Isothermal titration calorimetry analysis of linear di-ubiquitin binding to GumbyM55 and to the penotypic GumbyM55 W96R mutant. Gumby proteins were tested in a C129S mutant background to abolish DUB cleavage of the di-ubiquitin substrate. K_d and N values represent the mean +/- s.e.m. from three or more experiments. Representative binding isotherms and model fitting shown.

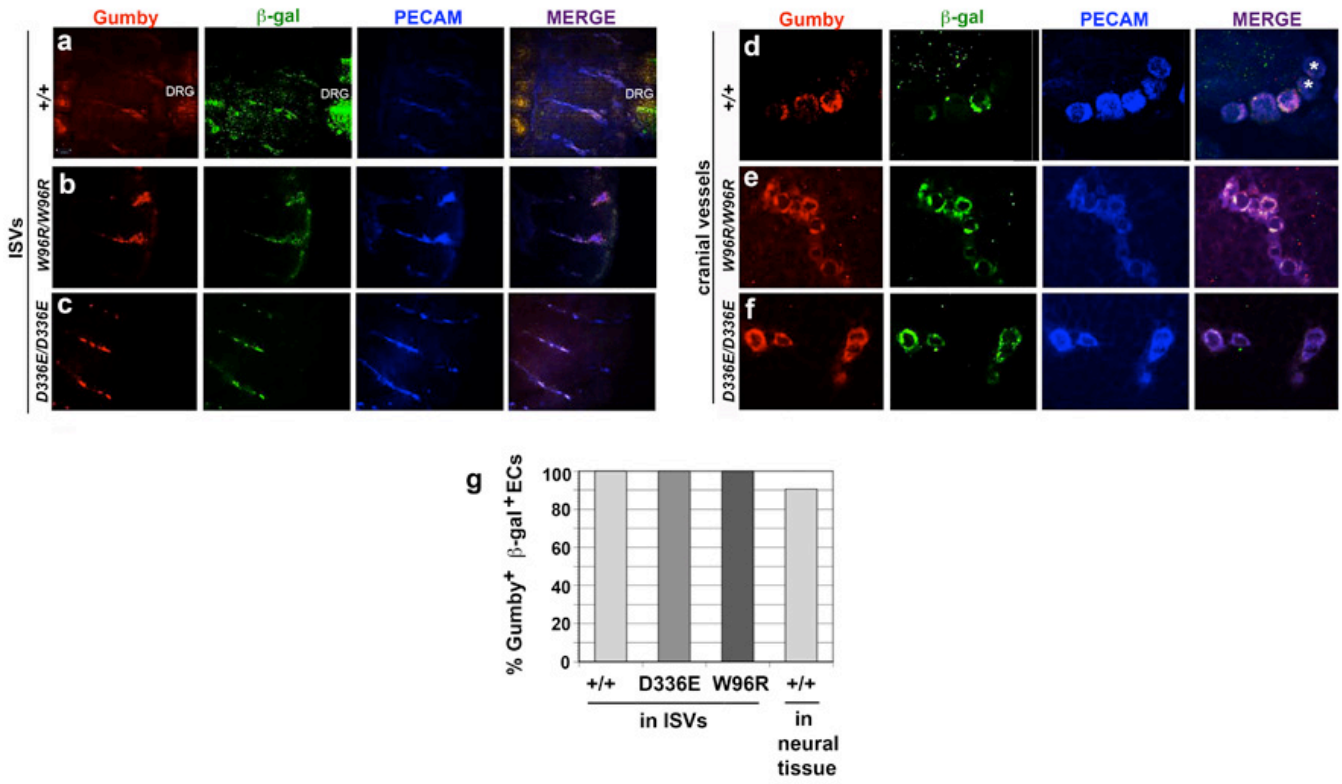


Supplementary Figure 9. Structural analysis of Gumby-ubiquitin complexes.
a, Ribbons representation of the Gumby-mono ubiquitin complex, highlighting differences in rotation and center of mass position of the distally bound ubiquitin relative to the Gumby-linear di-ubiquitin

complex. OTU domains were superimposed and differences in the ubiquitin position were calculated using Dyndom3D [48]. **b**, Structures of yeast OTU1, viral OTU and OTUB5 ubiquitin complexes (PDB 3BY4, 3PRP, and 3TMP) highting differences in rotation and center of mass poistions of ubiquitin relative to the Gumby-mono ubiquitin complex. **c**, Stereo view of the distal ubiquitin-Gumby binding interface. Colouring is as in Fig. 3d. Select residues involved in the interaction are shown as sticks. **d**, Stereo view of proximal ubiquitin-Gumby binding interface. Colouring is as in Fig. 3d. Select residues involved in the interaction are shown as sticks. Gumby mutants tested in this study are labeled in red. **e**, Stereo comparison of Gumby and OTUB1 bound to their preferred di-ubiquitin substrates. Rotational and center of mass differences are indicated for each ubiquitin moiety. Superpositions were generated using the OTU domain coordinates.



Supplementary Figure 10. Catalytically active Gumby reduces linear ubiquitin in transfected Neuro2A (N2A) cells.
Immunofluorescent staining with anti-linear ubiquitin specific (green) and anti-FLAG (red) antibodies detects linear ubiquitin (green) in N2A cells co-transfected with HOIL and HOIP. Appreciable levels of linear ubiquitin are not detected in cells expressing **a**, FLAG control vector or **c,** FLAG-Gumby (red). However, high levels of linear ubiquitin can be detected in most cells expressing **b**, HA-HOIL-1 and myc-HOIP alone or in the presence of **d**, FLAG-Gumby^{C129S} or **e**, FLAG- Gumby^{W96R}. **f,** Quantification of percentage of cells with linear ubiquitin expressing various FLAG-Gumby constructs. (n=3) Data are presented as means +/- s.e.m.



Supplementary Figure 11. Gumby is localized to canonical Wnt responsive endothelial cells (ECs) Immunofluorescence shows co-localization of Gumby (red), β-galactosidase (β-gal; green) and PECAM-1 (blue) in E10.5 embryonic sagital sections through the trunks of **a**, +/+;*TOPGAL/+*, **b,** Gum^{W96R}/Gum^{W96R};*TOPGAL/+* and **c,** Gum^{D336E}/Gum^{D336E};*TOPGAL/+* embryos and, at higher magnification, ECs in hindbrains of **d**, +/+;*TOPGAL/+*, **e**, Gum^{W96R}/Gum^{W96R};*TOPGAL/+* and **f**, Gum^{D336E}/Gum^{D336E};*TOPGAL/+* embryos. asterisks in **d**, mark ECs without Gumby and β-gal. All experiemnts shown here were performed at signal saturation for β-gal immunofluorescence. **g**, Quantification of percentage of ECs that co-express Gumby and β-gal in intersomitic vessels (ISVs) of normal and mutant;TOPGAL/+ embryos and in 394 ECs within neural tissue of +/+; TOPGAL/+ embryos.

Table 1 Data collection and refinement statistics

	Gumby^{M55}SeMet	Gumby^{M55}-C129S + Ub	Gumby^{R79}-C129A + linear diUb
Data collection			
Space group	$P2_12_12_1$	$P2_1$	$P2_12_12$
Cell dimensions			
a, *b*, *c* (Å)	43.3, 71.8, 94.2	84.2, 43.8, 113.9	183.6, 186.1, 219.7
α, β, γ (°)	90.0, 90.0, 90.0	90.0, 108.3, 90.0	90.0, 90.0, 90.0
Resolution (Å)	40-1.6	25-2.4	50-2.8
R_{sym} or R_{merge}	6.1 (58.4)	16.1 (61.9)	13.2 (84.8)
$I/\sigma I$	36.6 (3.1)	13.9 (2.2)	14.7 (1.8)
Completeness (%)	99.6 (100)	96.7 (76.1)	99.9 (99.9)
Redundancy	3.8 (3.7)	5.8 (3.8)	7.4 (7.7)
Refinement			
Resolution (Å)	40-1.6	25-2.4	50-2.8
No. reflections	37444	31314	173695
R_{work}/ R_{free}	16.7/19.8	21.7/26.7	22.4/25.4
No. atoms			
Protein	2088	5334	40096
Ligand/ion			
Water	222	162	0
B-factors			
Protein	14.6	25.0	91.8
Ligand/ion	31.7	53.5	
Water	25.2	27.8	-
R.m.s deviations			
Bond lengths (Å)	0.03	0.017	0.004
Bond angles (°)	2.21	1.64	0.98

*Each data set was derived from a single protein crystal.
*Highest resolution shell is shown in parenthesis.

Experiment	Bait	Hit Gene Name	Hit Score	Peptide Number	Unique peptide Number	Coverage
GUMBY_ΔPBM1	GUMBY	GUMBY	995	67	18	58.8
	GUMBY	**HOIP**	747	23	11	21
	GUMBY	DNM1L	145	3	3	9
	GUMBY	DPYSL2	108	3	2	11
GUMBY_ΔPBM2	GUMBY	GUMBY	425	11	8	36.1
	GUMBY	**HOIP**	313	6	5	10.5
	GUMBY	DPYSL2	203	4	4	11.5
	GUMBY	DNM1L	162	3	3	7.2
GUMBY_C105X1	GUMBY	GUMBY	394	93	7	20.7
	GUMBY	**HOIP**	532	14	7	14.1
GUMBY_C105X2	GUMBY	GUMBY	432	50	7	20.7
	GUMBY	**HOIP**	345	7	5	11.2
GUMBY_C105X3	GUMBY	GUMBY	274	31	5	18.8
	GUMBY	**HOIP**	457	15	6	12.4
GUMBY_C105X4	GUMBY	GUMBY	486	80	8	21
	GUMBY	**HOIP**	526	18	8	15.3

Supplementary Table 2: Results from the affinity purification coupled to mass spectrometry (AP-MS) experiment. Two independent biological replicates for FLAG-GUMBY_ΔPBM and four for FLAG-GUMBY_C105X were analyzed as described in the Supplementary Methods. Eight negative control runs (from cells expressing only the FLAG tag) were analyzed in parallel. All data was subjected to SAINT analysis: proteins with an AvgP $\geq$ 0.9 were selected as potential interactors. To further ensure that only high quality interactions were reported, only those interactors detected with $\leq$10% frequency across a database consisting of >2000 FLAG AP-MS experiments were listed. Lastly, proteins detected with 2 spectra or less in any replicate were removed. The "Hit Gene Name" is the official Gene Symbol as per NCBI Entrez Gene, the "hit score" is the protein Mascot score. "Peptide number" represents spectral counts, and "Unique peptide number" is the number of unique peptides (as per Mascot's definition of uniqueness). "Coverage" is the percentage of the amino acid sequence of the protein, which was sequenced here.

Supplementary Table 3. Primers used in these studies

(i) Primers used for mapping the *gumby* mutation

Marker	5'→3' Primer sequence	C57 specific allele or amplicon (bp)	C3H specific allele or amplicon (bp)
D15Mit130	F:CATATTTTGCAATTTTTAGTAATAGGC	185	197
	R:CAACACAGAAATAAAAGTGAGAGAGG		
D15Mit252	F:CTTCAAACATGTTATCATTGTCACA	124	132
	R:CTTCTGTATTCACAGGTGCTCG		
D15Mit280	F:TCTCTCTTCCCTCTCTATCTCGC	150	148
	R:TCTCTCTTCCCTCTCTATCTCGC		
rs13482490	F:GGGATGATTGCTGGAAGACTGATGG	A allele	G allele; *Pvu*II RFLP
	R:TGACTGTTCCTGTCTCCCTGG		
D15Mit18	F:GGGCTAATTGATAAATGATTAGTGC	134	138
	R:CCCAATTCAGGGTTTCTAACC		
D15Mit201	F:TTTTGGAGTCTTTCAGTTTTCTCC	101	95
	R:TTGAGTGGTATAATTTTGATTTACACA		
D15Mit111	F:GTTTCAGAAGGCAATGTCTGG	167	189
	R:GCTCAGTGCTAATCTCTGACTCC		
D15Mit138	F:TTCAATTCCCTTTTGTCAAATG	149	127
	R:CAAGACCCTAGATTCAGTCTACCC		

(ii) Primers used for sequencing the genes that map to the *gumby* critical interval

Gene	5'→3' Primer sequence	Region sequenced	Amplicon (bp)
ENSMUSG00000726630		**Genomic**	
	F:GAGGCGGGCTCGTCCGCCGC	Exon 1, Exon 2	357
	R:GTGGATCGCAGAGCCCCGAT		
Q8CDT1 (ENSMUSG00000052811)		**Genomic**	
	F:ACGTCGTCCAGCAGTTCCTTCTG	**Exon 1**	985
	R:TAAGTTCTCGTGTTATGAAACTTG		
BC052328		**Genomic**	
	F:GCGCTCCCATCGCTGAGCTT	Exon 1	499
	R:ACCGACCAGCTTGTCCACGC		
	F:CTCACTTGCTGTCCAGGTGA	Exon 8 (first half)	1080
	R:GAATTGTAATAAACATCTGAT		
	F:CAGAAGACATTGATTAAAGAG	Exon 8 (last half)	1105
	R:CAGTCAAAAGCTTTATGAGTTC		
		cDNA	
	F:GCATGGAGGCGCCGAGGAGCGC	Exons 2-5	399
	R:CGAAACTGTACTGCTGGATCCAG		
	F:CTTCAGCCAAGGCCTCTCCTTC	Exons 6-7	541
	R:TGCAAAGTCTCTGGAGTTAAAC		
Gumby/Fam105b		**Genomic**	
	F:AGGTAAGTACTACCCGGCATC	Exon 1	711
	R:TCTTTCCCAAATTCCTAGCGCTT		
	F:GAGGAGCCATTCAGGAGAGT	Exon 2	377
	R:AGACTGGATGTATGAGAGGAC		
	F:CCTAGGAAGGCAGTGTCTC	Exon 3	340

	R:CCTTATCTTTGCAGTCTGTTTCC		
	F:GGAGCACTGTTGCAGATGCT	Exon 4	450
	R:TCAGGCAACATGAGCAGAAAGG		
	F:GGACTCCTGTAGATTTGAACC	Exon 5	598
	R:AGTTCCCTGTCATGCACCTAC		
	F:TGGCATGTTGCTTACGGTTCC	Exon 6	505
	R:TCAGGAAGTGCTGGACCAGA		
	F:GGAGACTGTTTGCAGCTAAG	Exon 7	815
	R:CAAAAGCAGATGAGGCTATC		
Q8C9X7		**Genomic**	
	F:GAACCAGACCCTAAATTCCAAC	Exon 1	702
	R:GTGGAGAGAGGCAACGATCCAA		
Tiaf1		**Genomic**	
	F:CTTCCTTCTTCTTCTTTTGTG	Exon 1	657
	R:TCTCCTTAATGAGAATCACTG		
Q8BQ79		**Genomic**	
	F:GGCGCTTGTGGAACAACTGA	Exon 1	626
	R:AAGCCCCCAGCTCCTTCTAT		

(iii) Primers used in transgenic rescue experiments

BAC rescue			
Marker	**5'→3' Primer sequence**	**C57& C3H specific allele (genomic)**	**129SvJ specific allele (BAC)**
rs31960302	F:TTAGGGCTTGGCTAGAGCT	G allele	A allele: *Apo*I RFLP
	R:CCGTTCTTTGTCTTTCTCTCC		
BAC-1	F:GCCCGTTGTACTTTGTTCCC (genomic)		
	R:CAGCTGTCCCACACATCAAG (vector)		
BAC-2	F:CCTCCTAAGGTTTCACAAATGC (genomic)		
	R:CTTAATTAAGGATCGATCCGGCG (vector).		

(iv) Primers used for cloning

Plasmid	Primers used for cloning	Vector used for cloning
Gumby- GFP (N-terminal)	F:GGGGACAAGTTTGTACAAAAAAGCAGGC(AttB)TACCGTGC TGCAGATGAAATAG	pDONR201→ pDEST53 (Invitrogen)
	R:GGGGACCACTTTGTACAAGAAAGCTGGG(AttB)TTCTTGGC AGTCACATGTGTG	
GFP – Gumby (C-terminal)	F:GGGGACAAGTTTGTACAAAAAAGCAGGC(AttB)TACCCCAC AGCCGAGGTCAA	pDONR201→pDEST47 (Invitrogen)
	R:GGGGACCACTTTGTACAAGAAAGCTGGG(AttB)TCCACACT GGTCTCCTCACACA	
MBP- Gumby	F:GGAGGAATTC(EcoRI)TACCGTGCTGCAGATGA	pMAL-C2 vector (NEB)
	R:TGTGTCTAGA(XbaI)TCACACACTGGTCTCCTCAC	
GST- Gumby	F:GAGGCCATGGG(NcoI)CCGTGCTGCAGATGA	pGEX-HTa (Pharmacia)
	R:CGACTAGT(SpeI)CTCACACACTGGTCTCCTCAC	
FLAG- Gumby^{M55}	F:GCGTTTAAAC(PmeI)TACCGTGCTGCAGATGAAATAG	3xFLAGpcDNA3.1
	R: CTCGAgcggccgc(NotI)caTCACACACTGGTCTCCTCACACAC	
FLAG-Gumby^{M1}	F: ATGAAggcgcgcc(AscI)aAGTCGGGGGACTATGCCCC	3XFLAGpcDNA3.1
	R: CTCGAgcggccgc(NotI)caTCATAGACTGGTCTCCTCACACAC	
FLAG-Gumby$^{\Delta PBM}$	R: CTCGAgcggccgc(NotI)caTCATGCAGCGGCCGCCTCACACACT CTGACGGGG	3XFLAGpcDNA3.1
FLAG-Gumby^{C105X}	R: CTCGAgcggccgc(NotI)caGCCCATTTTCATtcaCGTTGCTTTCT	3XFLAGpcDNA3.1

(v) Primers used to genotype Gum^{W96R} and Gum^{D336E} mice

Mouse mutant	5'→3' Primer sequence
Gum^{W96R}	**Forward outer** 5'-ATTAAGAGAGCACTCTGGTTTATAGAGG-3'; **Reverse outer** 5'-TTAGAACACCAATATTTACAGCATTGTC-3' **T allele** 5'-GCTTTCTGAGTATTTCCTCTGCA-3' **A allele** 5'-GATATCATGGACTACTGCAAAAAAGTAA-3'.
Gum^{D336E}	**D336 Forward inner** 5'-CCAATGGTGACCCTCATAGCTGAGAAT-3' **E336 Reverse inner** 5'-CTGACAGGGATGTTATAGTGCCGACCT-3' **D336 Forward outer** 5'-AGAACACTCATTTGCTTTTCCTTCCCAG-3' **D336 Reverse outer** 5'-GAGAGTTCTCCCAGATTGTCCTTGGAGA-3'