Cardoso et al. Supplementary data

Supplementary Table I

cactus **maternal genotypes studied and their effect on cuticle and on embryo viability**.

maternal genotype	embryo viability	cuticle phenotype	severity
cact[011]/+	100% viable	NA	0
cact[A2]/+	70%	WT (Fontenele 2013)	0
Df(2L)*cact*255/+	-	WT or V4	0
cact[011]/ *cact*[011]	-	V3 (Roth 1991)	+
cact[A2]/ *cact*[A2]*	-	V2 (Roth 1991)	+++
cact[A2]/ *cact*[011]	0% (n>500)	V3 or V2	++
cact[A2]/ *cact*[4]**	0% (n>500)	V2	+++
cact[011]/ Df(2L)*cact*255	0% (n>500)	V3 or V2	++
cact[A2]/ Df(2L)*cact*255	0% (n=525)	V2 or V1	+++
cact[A2]/ Df(2L)III18	0% (n>500)	-	+++
cact[A2]/ *dl*[6]	55% (n=800)	WT or V4	NA
cact[4]/ *dl*[6]	79% (n=138)	-	NA
dl[6]/+	97%	WT (Araujo and Bier, 2000)	NA
cact[E10]/ *cact*[A2]	0% (n>500)	L2-D1 (Roth 1991)	NA

Cuticle phenotypes are as in Roth et al, 1991, where ventralization increases V4<V3<V2<V1, and corresponds to expansion of ventro-lateral cuticle rich in denticles and progressive loss of dorso-lateral and dorsal structures.

Severity is defined as the degree of phenotypic strength due to loss of Cact activity, based on the ensemble of effects on cuticle, protein levels and viability.

* With current *cact*[A2] strains obtained either from the stock center or directly from researchers we were unable to generate homozygous *cact*[A2] adults.

**cact[A2]/cact[4] is semilethal, but cact[4]/Df(2L)III18 is lethal, thus this maternal genotype is not presented in the table.

NA not applicable

Supplementary Figure 1
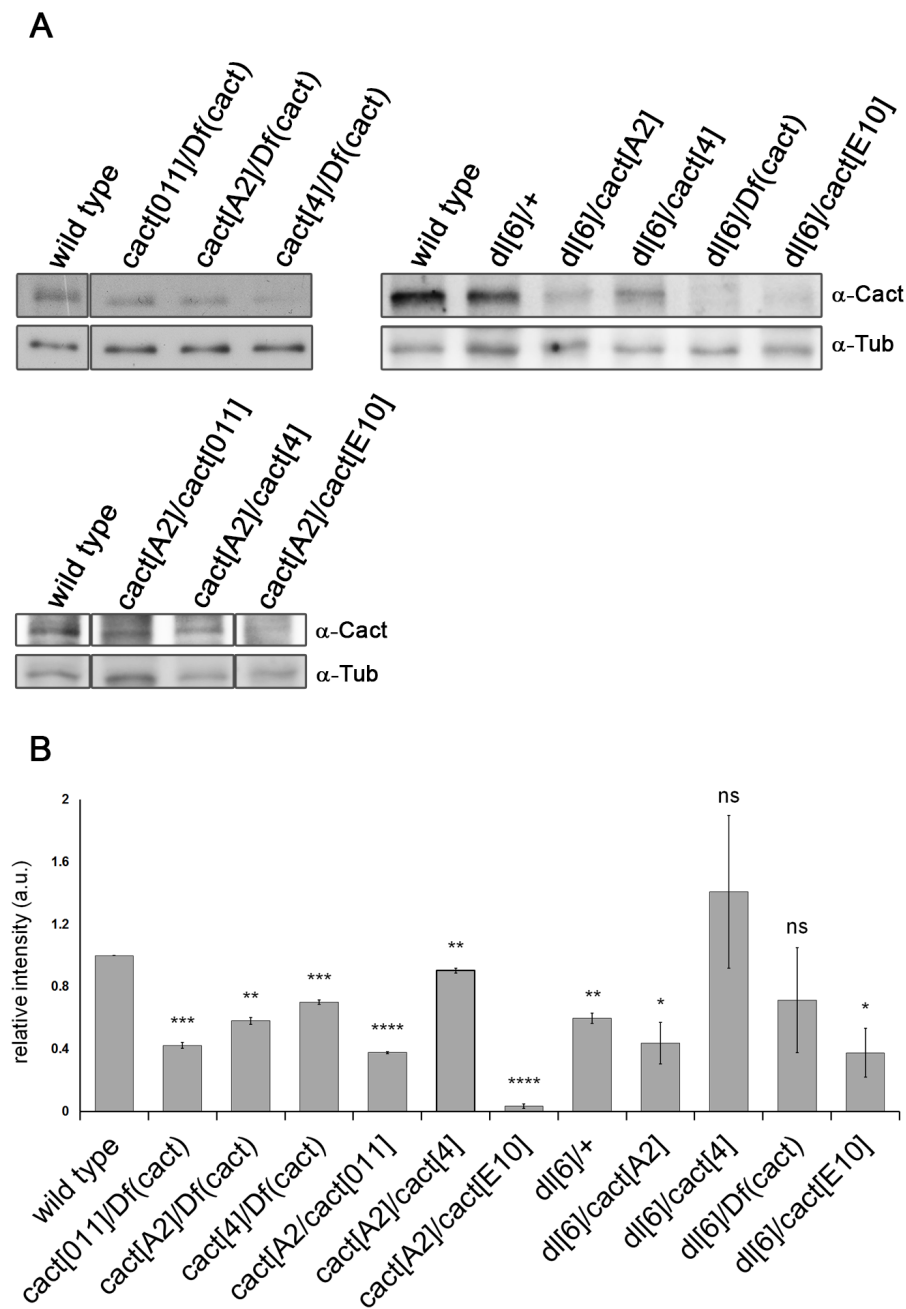
Cactus protein levels in 30min-1h30min ol d em bryos, re sulting from
maternal loss-of-function allelic combinations. Three representative western
blots (A) and quantification (B) of fullb length Cact protein levels in the
different heteroallelic combinations with *dorsal* and *cact* alleles as well as
deficiencies used

in this study. Three different samples were prepared and analyzed for each genotype. Normalized Cactus levels were calculated using the Tubulin loading control. Cact levels in single heterozygotes for the different alleles are not shown since they result in no significant alteration in Cact protein levels. Lower Mw Cact[E10] is not shown in the blots, but is present in amounts comparable to wild type Cact levels in *cact*[A2]/*cact*[E10] and *dl*[6]/*cact*[E10]. Statistically significant differences based on Student's t-test an d di splayed as mean±s.e.m. (****P≤0.0001***P≤0.001, **P≤0.01, *P≤0.05).

Supplementary Figure 2
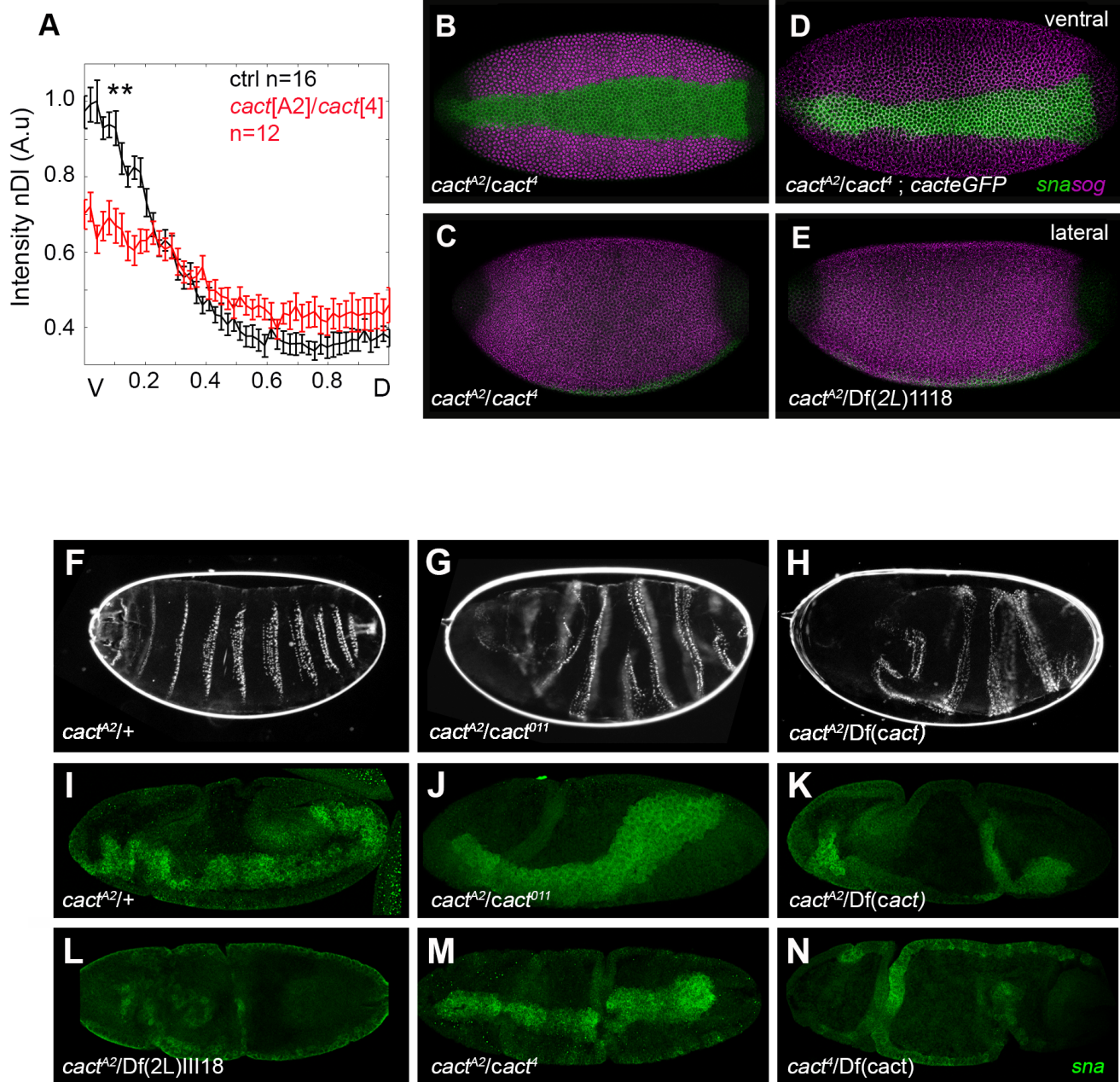
Loss of *cact* activity reduces nDl in ventral regions of the embryo. In (A) embryos from *cact*[A2]/*cact*[4] (red) mothers. The amount of nuclear Dl decreases in ventral regions of the embryo and increases in lateral regions,

respective to control embryos (black). B-E) *in situ* hybridization for *sna* and *sog* in *cact*[A2]/*cact*[4] (B,C), *cact*[A2]/*cact*[4]; *cact-eGFP*/+ (D) and *cact*[A2]/Df(2L)III18 (E) embryos shows that loss of *cact* reduces ventral *sna* and widens lateral *sog*. *cacteGFP* overexpression produces no effect on *cact*[A2]/*cact*[4] *sog* and *sna* domains. Fb H) Cuticle pattern for *cact*[A2]/+ showing a wild type phenotype, (F), *cact*[A2]/*cact*[011] with a characteristic V2 ventralized pattern (G), and *cact*[A2]/Df(cact) ventralized cuticles that are slightly elongated (H). Ib N) *in situ* hybridization for *sna* or *twi* (K), in gastrulation stage embryos. The internal mesoderm layer is clearly seen in *cact*[A2]/+ (I) and *cact*[A2]/*cact*[011] (J), reduced in *cact*[A2]/Df(cact) (K), and sometimes almost absent in *cact*[A2]/Df(2L)III18 (L), *cact*[A2]/*cact*[4] (M) and *cact*[4]/Df(cact) (N). The gastrulation pattern is also abnormal in Jb N. Ventral views in B,D,F,L,M, lateral views in C,E,I,J,K,N. Anterior is left and posterior is right in all panels.

Supplementary Figure 3
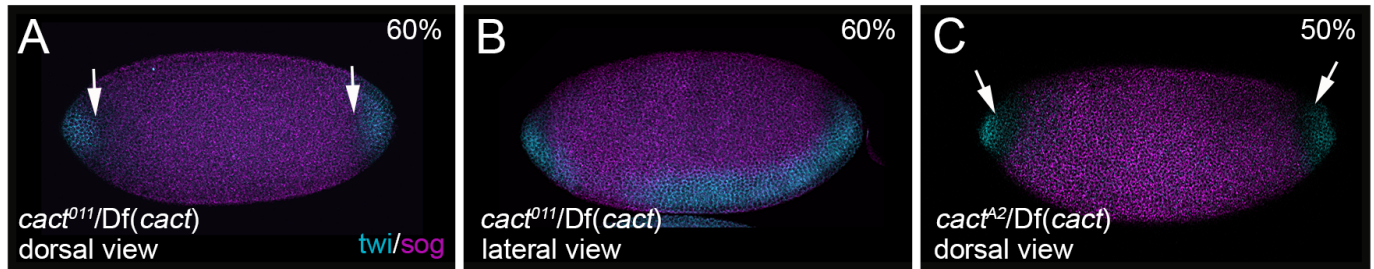
cact loss of-function mutants alter the ventral domain of *twist* expression. It has been reported that in embryos from homozygous mothers carrying a viable loss-of-function *cact* allele, the ventral *twist* domain expands dorsally at the anterior and posterior tips of the embryo (Roth et al, 1991). Accordingly, we observe a small anterior-posterior expansion of *twist* in *cact*[011]/Df(*cact*) (A) in addition to a loss of ventral *twist* expression (B). Contrarily, in embryos from *cact*[A2]/Df(*cact*) mothers, no anterior-posterior expansion is observed (C). A,C are dorsal views, B is a ventral-lateral view. Anterior is left, posterior to the right.

Supplementary Figure 4
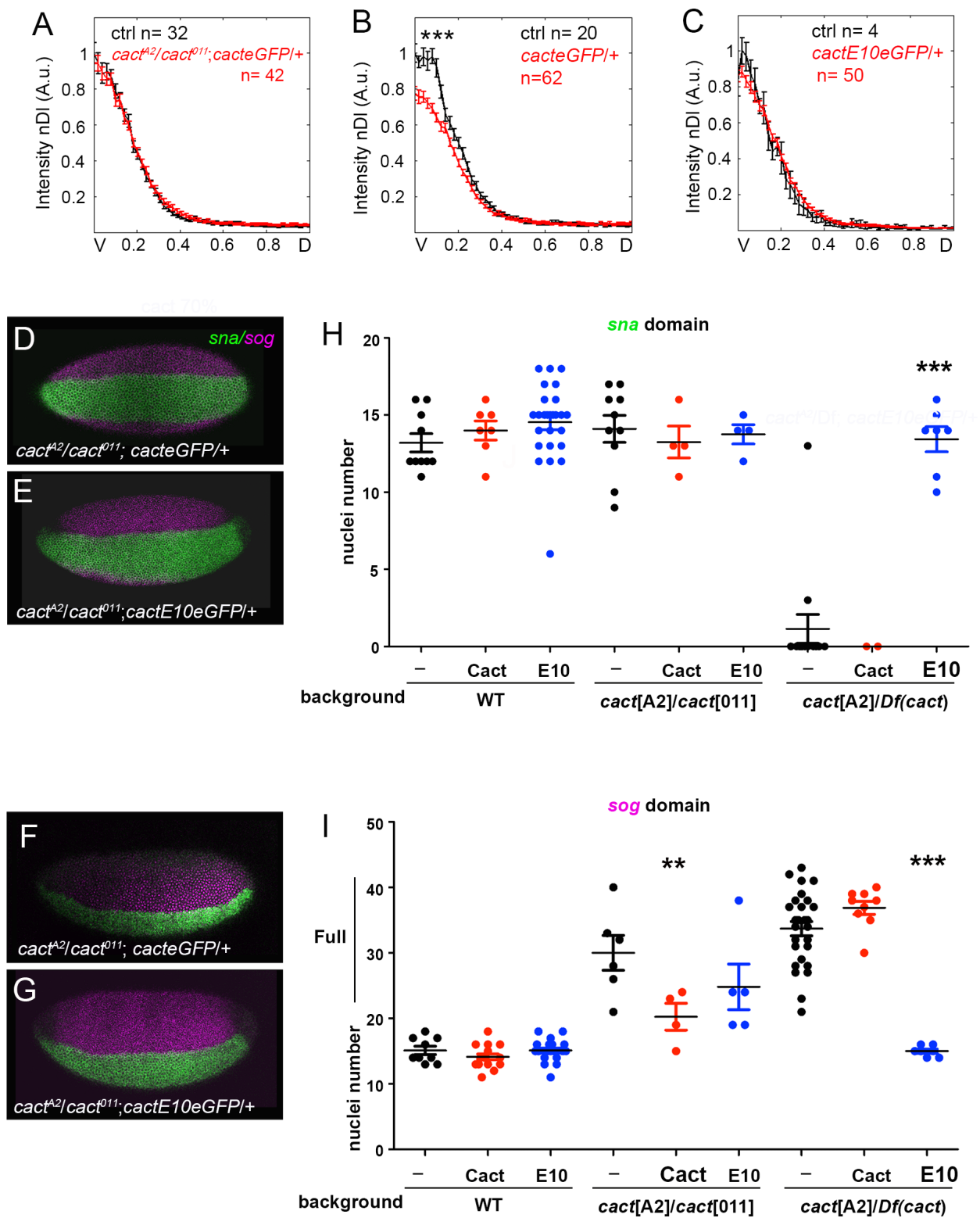
Full-length Cact and truncated Cact[E10] overexpression produce qualitatively different effects on the DV axis. A-C) Nuclear Dl gradients for control embryos (black) or embryos from *cact[A2]/cact[011]; cact-eGFP/+* (A), *cact-eGFP/+* (B) or *cactE10-eGFP/+* (C) mothers (red). When endogenous Cact is reduced (A), Cact-eGFP recovers nDl to a wild type pattern (compare to Fig 1C). Overexpression of full-length Cact-eGFP slightly decreases nDl in a wild type background (B), while Cact[E10]-eGFP has no effect (C). D-G) *in situ* hybridization for *sna* and *sog* in *cact*[A2]/*cact*[011]; *cact-eGFP*/+ (D,F); *cact*[A2]/*cact*[011]; *cactE10-eGFP*/+ (E,G) embryos and (H,I) quantification of *sna* (H) and *sog* (I) in these different genotypes, plus *cact*[A2]/Df(*cact)*; *cact-eGFP*/+ and *cact*[A2]/Df(*cact*); *cactE10-eGFP*/+ from Fig 3 for direct comparison. The size of the *sog* domain is defined as "full" expansion when it expands completely to the dorsal side and no dorsal *zen* expression is observed. Differences are statistically significant based on Student's t-test and displayed as mean±s.e.m. (***P<0.001, **P<0.01). Interestingly, Cact-eGFP is able to generate CactE10-eGFP but does not enhance nDl levels ventrally in *cact*[A2]/Df(*cact*), unlike CactE10-eGFP. This is possibly a matter of stoichiometry between Cact-eGFP and CactE10-eGFP. By inducing *cact-eGFP* expression we increase both full length Cact-eGFP and CactE10-eGFP, generated by the action of CalpA. By expressing *cactE10-eGFP* we skew this proportion towards a greater ratio of E10/WT forms.

Supplementary Figure 5
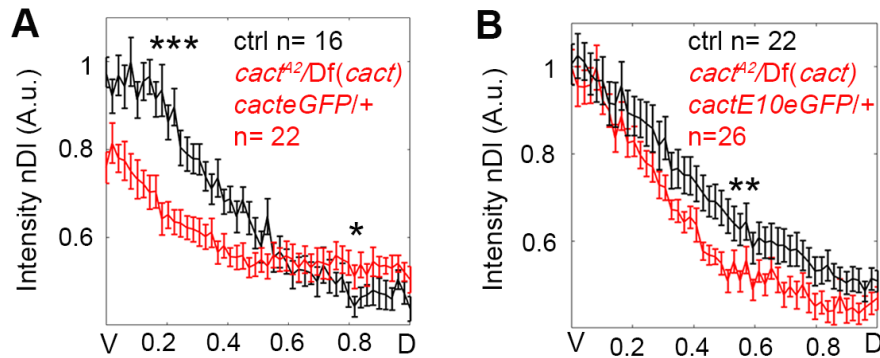
Cact[E10] exerts positive and inhibitory effects on the nDl gradient when endogenous wild type *cact* is reduced. nDl gradients resulting from overexpression of Cact-eGFP (A) or Cact[E10]-eGFP (B) in a *cact*[A2]/Df(*cact*) background (compare to Fig 1D) shows that Cact-eGFP does not alter the *cact*[A2]/Df(*cact*) nDl gradient in ventral regions, while Cact[E10] almost recovers the wild type nDl pattern, consistent with *sna* and *sog* expression domains shown in Fig 3. Control gradients in black in both graphs.

Supplementary Figure 6
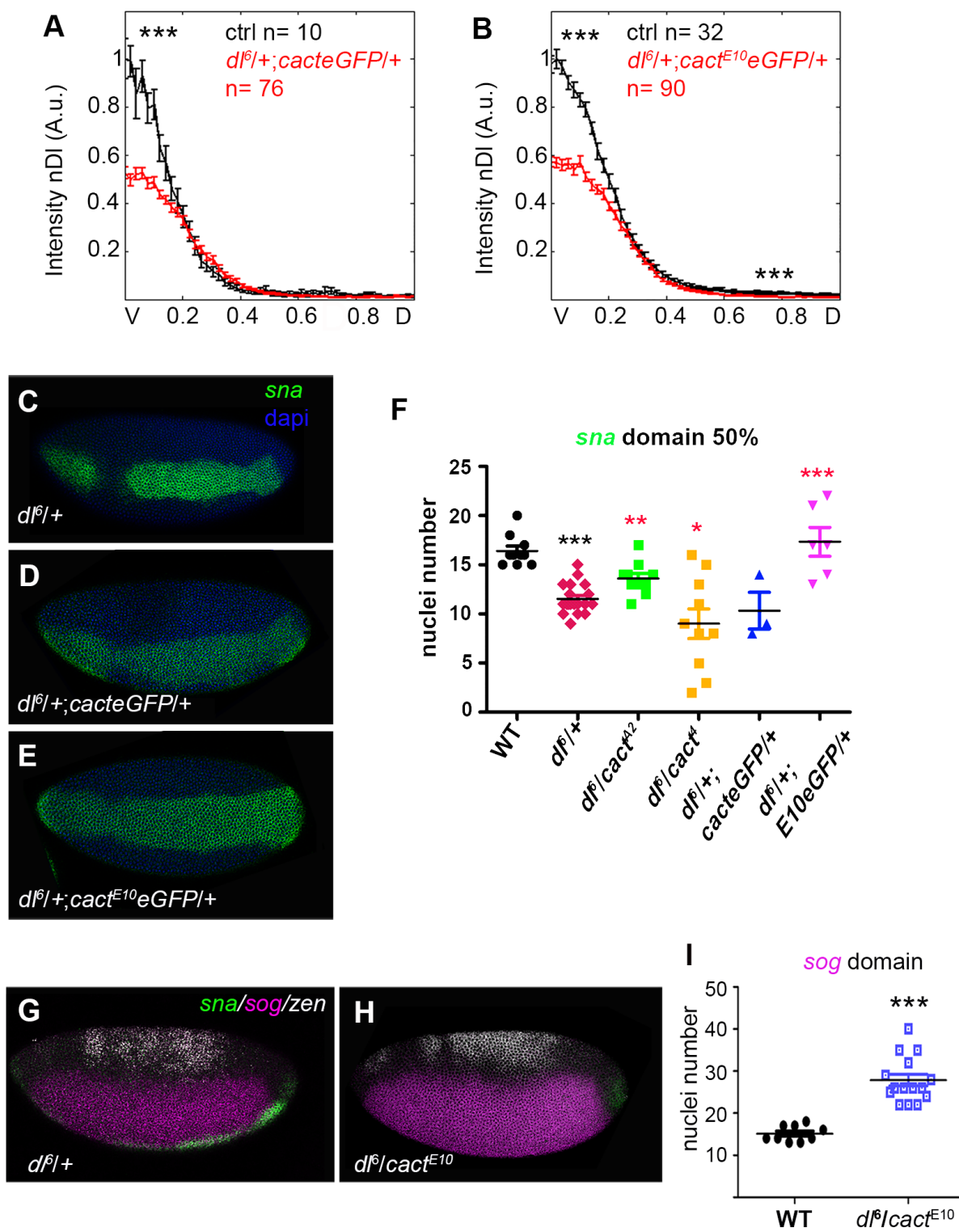
Cact[E10] exerts a strong effect on the nDl gradient when the amount of Dl is reduced. A,B) nDl gradients resulting from overexpression of Cact-eGFP (A) or Cact[E10]-eGFP (B) in a *dl-* heterozygous background. Control gradients in black in both graphs. Cb E) *in situ* hybridization for the ventral gene *sna* reveals that Cact[E10]b eGFP recovers the loss of the ventral *sna* domain observed in *dl*[6]/+ (E). This positive effect is also seen by quantification of the *sna* domain (F). Note that the *sna* domain in *dl*[6]/*cact*[A2] is stochastic, coinciding with *sog* expression as shown in Fig 2. Gb I) *in situ* hybridization for ventral *sna,* lateral *sog* and dorsal *zen* shows that the *cact*[E10] allele expands *sog* both ventrally and dorsally (H) in relation to *dl[6]*/+ (G), as quantified in (I), where the *sog* domain in *dl[6]*/+ is equivalent to wild type. Therefore, *cact*[E10] exerts a dual effect to enhance and to inhibit Toll responses. Differences are statistically significant based on Student's tb test and displayed as mean±s.e.m. (***P<0.001, **P<0.01).