

RESEARCH REPORT

A novel function for the I κ B inhibitor Cactus in promoting Dorsal nuclear localization and activity in the *Drosophila* embryo

Maira Arruda Cardoso^{1,2}, Marcio Fontenele^{1,3}, Bomyi Lim⁴, Paulo Mascarello Bisch², Stanislav Y. Shvartsman⁴ and Helena Marcolla Araujo^{1,2,*}

ABSTRACT

The evolutionarily conserved Toll signaling pathway controls innate immunity across phyla and embryonic patterning in insects. In the *Drosophila* embryo, Toll is required to establish gene expression domains along the dorsal-ventral axis. Pathway activation induces degradation of the I κ B inhibitor Cactus, resulting in a ventral-to-dorsal nuclear gradient of the NF κ B effector Dorsal. Here, we investigate how *cactus* modulates Toll signals through its effects on the Dorsal gradient and on Dorsal target genes. Quantitative analysis using a series of loss- and gain-of-function conditions shows that the ventral and lateral aspects of the Dorsal gradient can behave differently with respect to Cactus fluctuations. In lateral and dorsal embryo domains, loss of Cactus allows more Dorsal to translocate to the nucleus. Unexpectedly, *cactus* loss-of-function alleles decrease Dorsal nuclear localization ventrally, where Toll signals are high. Overexpression analysis suggests that this ability of Cactus to enhance Toll stems from the mobilization of a free Cactus pool induced by the Calpain A protease. These results indicate that Cactus acts to bolster Dorsal activation, in addition to its role as a NF κ B inhibitor, ensuring a correct response to Toll signals.

KEY WORDS: NF κ B, I κ B, Toll, Cactus, Dorsal-ventral patterning, *Drosophila*

INTRODUCTION

The evolutionarily conserved Toll receptor pathway is implicated in the control of development, proliferation and immunity. Toll signals are modulated at many levels, a characteristic that facilitates an assortment of possible outcomes (Mitchell et al., 2016). To understand Toll pathway architecture it is necessary to define quantitatively how each pathway element contributes to the final response. Inhibitor of NF κ B (I κ B) proteins comprise the Toll responsive complex together with NF κ B effectors. Therefore, they are central elements of the Toll pathway that require careful analysis of their effects.

Drosophila is a unique system in which to study how I κ B proteins tune Toll responses, as disturbances in I κ B function can be analyzed concomitantly across a range of Toll activation levels. During embryogenesis, ventral-lateral activation of maternal Toll receptors

leads to a ventral-to-dorsal nuclear gradient of the NF κ B/c-Rel protein Dorsal (Dl). Inside the nucleus, the differential affinity of Dl-target genes for Dl enables the spatial control of gene expression along the dorsal-ventral (DV) axis (Rushlow and Shvartsman, 2012). High Toll signals lead to ventral mesodermal gene expression, intermediate signals induce lateral neuroectodermal genes, whereas low signals allow dorsal ectodermal gene expression. Toll signals are transduced through mobilization of adaptor proteins, protein kinase activation, and ultimately phosphorylation and proteasomal degradation of the sole *Drosophila* I κ B protein Cactus (Cact). Cact degradation then exposes nuclear localization sequences in Dl and nuclear translocation ensues (Stein and Stevens, 2014).

The amount of nuclear Dorsal (nDl) is used as readout for the level of Toll pathway activation. Quantitative analysis of nDl in fixed and live embryos has shown that the Dl gradient is visible from early blastoderm cycle 9, increasing in amplitude with time until mitotic cycle 14 (Kanodia et al., 2009; Liberman et al., 2009; Reeves et al., 2012). Although the shape and dynamics of the Dl gradient have been described under wild-type conditions, how *cactus* (*cact*) disturbances alter these characteristics has not been investigated. Using quantitative analysis we show that, under specific circumstances, loss of *cact* flattens the nDl gradient, implying that *cact* is able to augment Dl nuclear accumulation in addition to its widely established role in inhibiting Toll signals.

RESULTS AND DISCUSSION

Cact augments responses to high Toll signals

In order to identify how Cact tunes NF κ B responses, we investigated the effects of reducing *cact* on Dl nuclear localization and expression of Dl target genes using quantitative fluorescent immunolabeling and *in situ* hybridization. It has previously been reported that the nDl gradient expands and the ventral *twist* (*twi*) expression domain widens in embryos generated from mothers carrying *cact* loss-of-function germline clones (Roth et al., 1991). This effect results from depriving the embryo of Cact protein, which releases Dl inhibition in the cytoplasm. Smaller reductions in *cact* lead to milder effects along the DV axis, as revealed by the embryonic cuticle pattern, gastrulation movements and colorimetric *in situ* hybridization (Govind et al., 1993; Isoda and Nusslein-Volhard, 1994; Roth et al., 1991).

To quantify the effect of reducing *cact*, we progressively decreased maternal Cact activity using a series of loss-of-function allelic combinations (Fig. 1 and Figs S1 and S2). One dose of a strong *cact* loss-of-function allele induces 30% embryo lethality, despite only a mild decrease in the number of amnioserosa cells (*cact*[A2]/+ mothers) (Govind et al., 1993) and no detectable alteration in nDl (Fig. 1B,E,F,I,J and Table S1). Further decreasing *cact* function increases nDl in regions of the embryo that receive intermediate or low Toll signals (Fig. 1B-D): in *cact*[A2]/*cact*[011],

¹Instituto de Ciências Biomédicas, Federal University of Rio de Janeiro, Rio de Janeiro, RJ 21941-902, Brazil. ²Instituto de Biofísica Carlos Chagas Filho, Federal University of Rio de Janeiro, Rio de Janeiro, RJ 21941-902, Brazil. ³Institute of Molecular Entomology, Brazil. ⁴Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ 08544, USA.

*Author for correspondence (haraújo@histo.ufrj.br)

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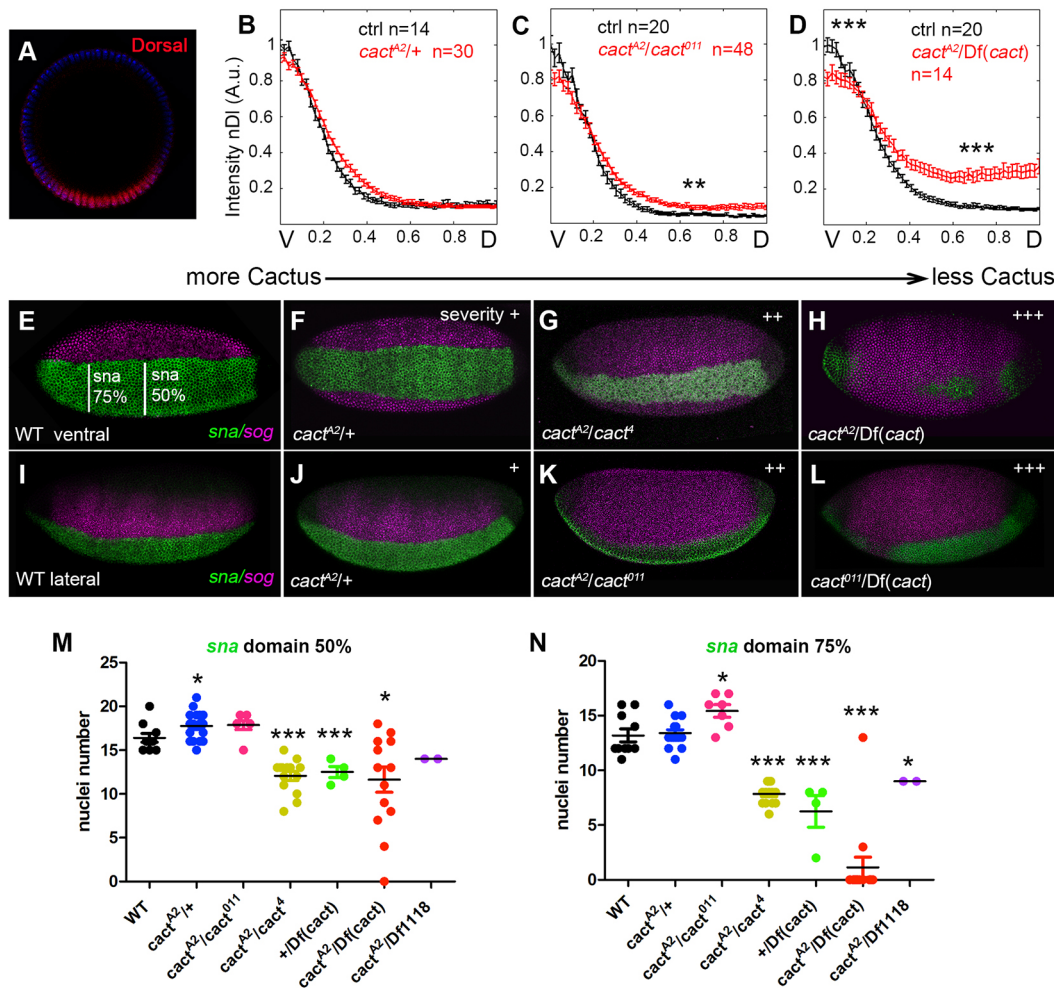


Fig. 1. Cact inhibits Toll in lateral regions and enhances Toll signals in ventral regions of the embryo. (A) Optical section showing a control DI gradient. (B–D) Nuclear DI was extracted, measured and plotted as half gradients for control embryos (black) or embryos from *cact*[A2]/+ (B), *cact*[A2]/*cact*[011] (C) or *cact*[A2]/*Df(cact)* (D) mothers (red). The y axis represents nDI fluorescence intensity along the ventral-to-dorsal embryonic axis (x axis). Data are mean±s.e.m. (E–L) *In situ* hybridization for *sna* (green) in the mesoderm and *sog* (pink) in the lateral neuroectoderm of embryos with maternal *cact* loss-of-function alleles. In embryos from *cact*[011]/+ or *cact* [A2]/+, the ventral and lateral territories are similar to wild type (F,J). Stronger allelic combinations lead to dorsal expansion of lateral *sog* and ventral *sna* reduction in the prospective mesoderm (G,H,K,L). Anterior is leftwards and posterior is rightwards in E–L; dorsal is upwards in G–L. Plus signs in upper right corner indicate the severity of the allelic *cact* combination (+++>+>+), defined by loss of activity and protein levels (Fig. S1). (M,N) The *sna* domain width measured at 50% and 75% egg length. Data are mean±s.e.m. Statistically significant differences were determined using Student's *t*-test (****P*≤0.001, **P*≤0.05).

intermediate (lateral) nDI levels expand to dorsal regions of the embryo (Fig. 1C). These results are in agreement with the embryonic phenotype reported for *cact*[H8] homozygotes (Roth et al., 1991), showing loss of dorsal *zen* gene expression and ventralized cuticles (Table S1 and Fig. S2G). Surprisingly, stronger loss-of-function allelic combinations, as in *cact*[A2]/*Df(cact)* (Fig. 1D), *cact*[A2]/*cact*[4] and *cact*[A2]/*Df(2L)III18* (Fig. S2A–C,E) have the same effect on lateral and dorsal territories, but additionally reduce nDI in the ventral domain. As these three genotypes alter *cact* function, their antagonistic effects on ventral and lateral nDI are likely due to *cact*.

The changes in nDI shown above are reflected in the pattern of DI target genes. The lateral domain of *short gastrulation* (*sog*) expression, a gene that requires intermediate levels of nDI for activation (Stathopoulos and Levine, 2002), expands ventrally and dorsally in several *cact* loss-of-function combinations (Fig. 1G,H,K,L). Dorsal *sog* expansion results from activation by increased nDI, while ventral *sog* expansion probably results from a decrease in nDI

and the narrower domain of the Snail repressor. Consistent with this interpretation and with the ventral decrease in nDI, the ventral prospective mesodermal domains of *snail* (*sna*) and *twi* expression, genes that require high levels of nDI for activation (Ip et al., 1991; Papatsenko and Levine, 2005), reduce as Cact activity drops (Fig. 1G,H,L–N, Fig. S2B,C,E and S3B). Therefore, we find that Cact performs an additional function to enhance Toll signals, which is distinct from its established function in inhibiting DI nuclear translocation.

Next, we investigated whether this positive effect depends on DI, taking into consideration that the deficiency used in one of the allelic combinations displaying this effect deletes *dl* in addition to *cact* [*Df(cact)*]. In embryos laid by *dl*[−] heterozygous mothers, nDI decreases ventrally and the *sna* domain narrows compared with wild type (Fig. 2A,C) (Fontenele et al., 2013; Kanodia et al., 2009). We reasoned that decreasing *dl* should sensitize the embryo to Cact reductions if the positive role of Cact depends on DI. In agreement with this hypothesis, a mild reduction in Cact activity attained in

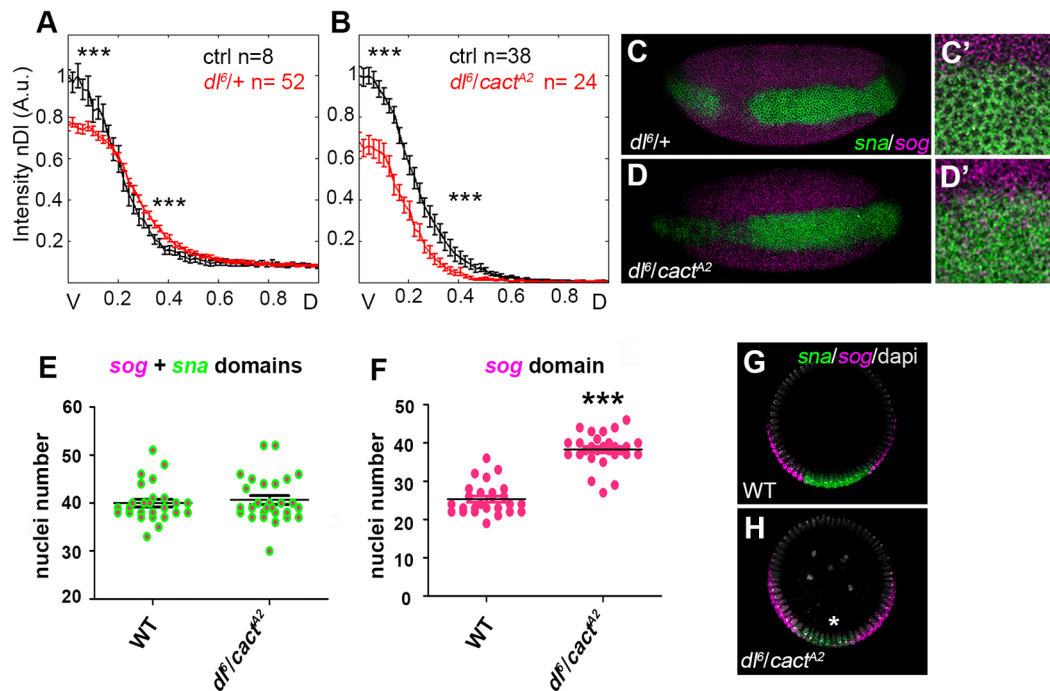


Fig. 2. The ability of Cact to enhance Toll is sensitive to the amount of Dorsal. (A,B) nDI gradients for control embryos (black) or embryos from *dl[6]/+* (A) or *dl[6]/cact^{A2}* (B) mothers (red). The nDI gradient decreases by reducing maternal *dl* and *cact*, compared with control embryos stained concomitantly. Data are mean \pm s.e.m. (C–D') *In situ* hybridization for *sna* (green) and *sog* (pink) in *dl[6]/+* (C,C') or *dl[6]/cact^{A2}* (D,D') whole mounts. (C'D') Higher magnifications of the ventral region showing abutting ventral *sna* and lateral *sog* domains (C') versus stochastic *sna* and *sog* in the ventral territory (D'). (E–H) Quantification of *sog*+*sna* domains (E) and *sog* domains alone (F) obtained from wild-type (G) or *dl[6]/cact^{A2}* (H) transverse sections. Coincident *sog*+*sna* expression is seen in ventral regions in H, unlike wild type or *dl[6]/+*. *sog* domain size measured in E,F includes both left and right sides of the embryo because in *dl[6]/cact^{A2}* this domain is frequently continuous. Data are mean \pm s.e.m. Statistically significant differences were determined using Student's *t*-test (****P*≤0.001).

dl[6]/cact^{A2} significantly decreases nDI in the ventral and lateral domains of the embryo (Fig. 2B). This effect is not observed in *cact^{A2}/+* (Fig. 1B). In addition, in *dl[6]/cact^{A2}* the lateral *sog* domain expands into the ventral, high Toll activity domain (Fig. 2C–H). Under this condition the ventral *sna* domain is stochastic, enabling ventral *sog* expression in nuclei devoid of the Sna repressor, in agreement with the nDI gradient format (Fig. 2B). However, the total *sog* plus *sna* domain is similar to wild type. In *dl[6]/cact⁴* (Fig. S6F), we see a reduction in the *sna* domain compared with *dl[6]/+*, strengthening the hypothesis that the positive role of Cact depends on DI.

Disturbing the balance between full-length and N-terminal-deleted Cact enhances Toll

Next, as loss of Cact can result in a decrease in nDI, we tested whether altering Cact levels by overexpression results in a corresponding increase in nDI and DI target genes. Two pathways control Cact levels and activity: Cact is phosphorylated at N-terminal serine residues, ubiquitinated and degraded through the proteasome in response to Toll pathway activation (Bergmann et al., 1996; Fernandez et al., 2001; Hecht and Anderson, 1993; Reach et al., 1996; Shelton and Wasserman, 1993). Cact is also subject to C-terminal CKII kinase phosphorylation through a Toll-independent pathway (Belvin et al., 1995; Liu et al., 1997; Packman et al., 1997), priming Cact for cleavage by the Calpain A (CalpA) protease (Fontenele et al., 2009). N-terminal truncated Cact, hereafter referred to as Cact[E10], is not degraded in response to the Toll pathway. Interestingly, CalpA activity generates Cact[E10], and possibly releases a second full-length free Cact molecule (Fontenele et al., 2013).

To test the effect of increasing Cact activity on the nDI gradient we used gain-of-function alleles and transgenic overexpression lines. The *cact[E10]* and *cact[BQ]* mutants were characterized as gain-of-function alleles that produce N-terminal-deleted proteins that were unresponsive to Toll (Bergmann et al., 1996; Roth, 2001), but that retain their ability to bind DI (Fontenele et al., 2013). In trans to *cact* loss-of-function alleles, these mutant proteins inhibit Toll pathway activity along the DV axis: the severity of the gain-of-function phenotype increases with severity of the loss-of-function allele (Bergmann et al., 1996; Govind et al., 1993; Roth et al., 1991). In *cact[E10]/cact^{A2}*, the ventral *sna* domain decreases, consistent with Toll pathway inhibition due to *cact[E10]*. Surprisingly, no inhibition is seen dorsally as the lateral *sog* domain remains dorsally expanded, compared with *cact^{A2}/Df(cact)* (Fig. 3A,B and Fig. 1H). These DI-target effects are in agreement with nDI gradient alterations (Fig. 3C). Nonetheless, *cact[E10]* and stronger *cact* loss-of-function allelic combinations are lethal, impairing further analysis of a positive effect exerted in the presence of *cact[E10]*.

To investigate the effects of altering Cact and Cact[E10] activity, we expressed GFP-tagged Cact chimeric proteins in oocyte and early embryos using a maternal promoter [α tub67C, referred to as CaM (Fernandez et al., 2001)]. In all genetic backgrounds tested, *cact-eGFP* expression either had no effect or decreased nDI levels and the size of DI-target expression territories, conforming to Cact inhibitory function (Fig. 3D–K and Fig. S4). Importantly, when expressed in a *cact^{A2}/cact[011]* loss-of-function background, *cact-eGFP* recovers the nDI gradient and reduces lateral *sog* to a wild-type pattern (Fig. S4A,F,I, compare with Fig. 1C), confirming that CaM>*cact-eGFP* produces functional Cact that binds to and inhibits DI and responds to Toll signals (Fontenele et al., 2013).

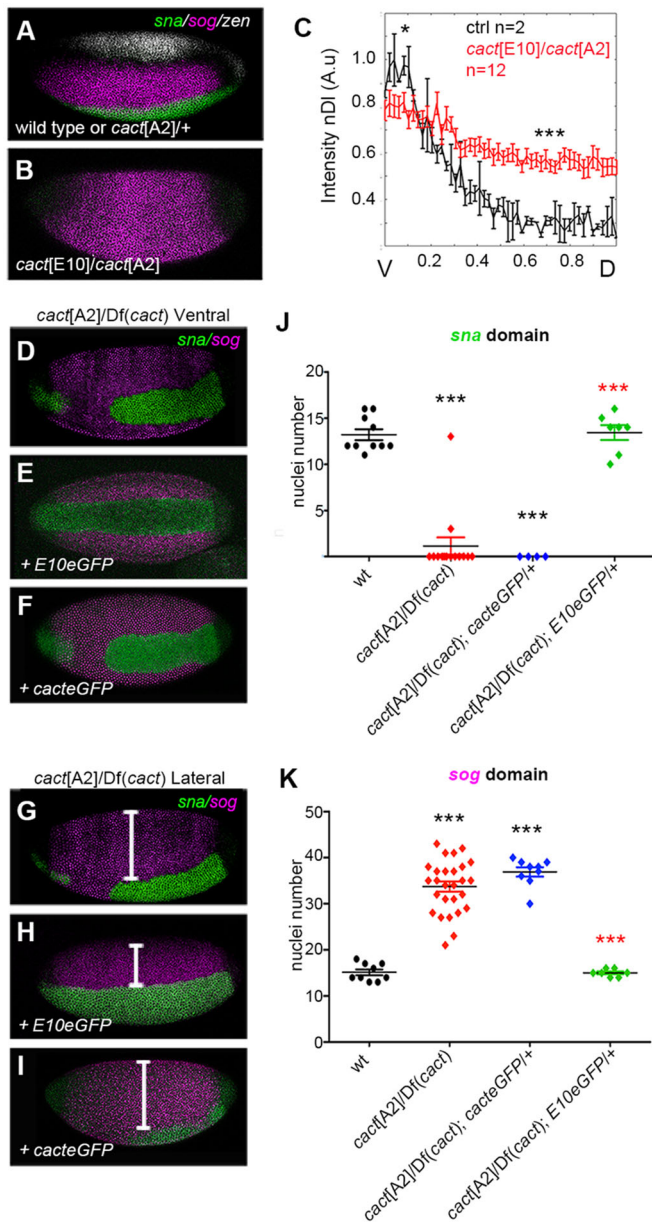


Fig. 3. *cact*[E10] overexpression enhances Toll. (A,B) *In situ* hybridization for *sna*, *sog* and *zen* in wild type (A) and *cact*[E10]/*cact*[A2] (B) showing complete expansion of lateral *sog*. (C) nDI gradients for control embryos (black) or embryos from *cact*[E10]/*cact*[A2] mothers (red). Data are mean \pm s.e.m. (D–I) *In situ* hybridization for *sna* and *sog* in *cact*[A2]/*Df(cact)* (D,G), *cact*[A2]/*Df(cact)*; *cact*E10-eGFP/+ (E,H) and *cact*[A2]/*Df(cact)*; *cact*eGFP/+ (F,I). (J,K) Quantification of *sna* (J) and *sog* (K); bars in G–I) domains at 75% egg length. Full *sog* expansion dorsally is defined when the domain is greater than 25 cells and no dorsal *zen* expression is observed. Data are mean \pm s.e.m. Statistically significant differences were determined using Student's *t*-test (* $P \leq 0.05$, *** $P \leq 0.001$).

Conversely, *cact*[E10]-eGFP expression increases the ventral *sna* and decreases the lateral *sog* domains that were modified in *cact*[A2]/*Df(cact)* (Fig. 3D,E,G,H,I,K). Therefore, in this genetic background Toll signals enhance ventrally but are inhibited laterally compared with *cact*[A2]/*Df(cact)*. This result is consistent with the nDI gradient that reverts to an almost wild-type format (Fig. S5) and akin to the opposing effects shown in *cact* loss-of-function assays (Fig. 1 and Fig. S2 and S3). Importantly, *cact*[E10]-eGFP has no effect on DI target genes when expressed in the less severe

cact[A2]/*cact*[011] background (Fig. S4). Thus, the effects of Cact[E10]-eGFP depend on the severity of the *cact* loss-of-function background, similar to the behavior displayed by endogenous *cact* [E10]. This characteristic may stem from differences in functionality of mutant versus wild-type Cact protein, such as different DI-binding affinities or interactions with other elements of the Toll-dependent and -independent pathways.

The ability of Cact[E10] to alter Toll signals is strongest in the presence of limited DI, a condition that also reduces endogenous Cact levels (Whalen and Steward 1993; Bergmann et al., 1996). The lateral *sog* domain expands ventrally in *cact*[E10]/*dl*[6] (Fig. S6G–I) due to loss of inhibition by Sna, although no effect is observed in *cact*[E10]/+. Likewise, *cact*[E10]-eGFP has no effect on the nDI gradient in the presence of wild-type DI levels (Fig. S4C) but produces an inhibitory effect comparable with Cact-eGFP in a *dl*-heterozygous background (Fig. S6A,B compare with Fig. 2A). Collectively, the results described above indicate the existence of a delicate balance between levels of full-length Cact, truncated Cact (Cact[E10]) and DI for proper Toll signaling events.

The Toll-independent pathway may implement the positive function of Cact

The phenotypes presented here resulting from *cact* loss-of-function and overexpression alleles show that *cact* is able to enhance Toll signals. Loss of *cact* function results in loss of this positive activity. *cact*[E10]eGFP expression recovers the loss of this positive function, suggesting it acts on the same mechanism that is impaired in specific *cact* allelic combinations. This interpretation is strengthened by the observation that under the loss-of-function and in the *cact*[E10] gain-of-function conditions, the effects are strongest by reducing *cact* and/or *dl*. However, these results raise several questions: by what mechanism does Cact augment Toll signals and what process does *cact*[E10] modify to recover the loss of this positive effect?

First and foremost, endogenous Cact[E10] is a product of the Toll-independent pathway. We have shown that the CalpA protease targets exclusively DI-free Cact. Our data suggest that CalpA generates Cact[E10] and releases a full-length Cact molecule. Conversely, Cact[E10] inhibits CalpA activity, forming a regulatory loop (Fontenele et al., 2013). Mathematical modeling pointed out the importance of I κ B proteins in modulating Toll signals in vertebrates and in *Drosophila* sp. (Ambrosi et al., 2014; Kearns and Hoffmann, 2009; O'Dea et al., 2007). In particular, it was shown that free I κ B is an important regulatory target for the control of Toll signals (Konrath et al., 2014). In *Drosophila*, Cact partitions between free and NF κ B-bound complexes. DI-bound Cact corresponds to the Toll-responsive complex (1Cact:2DI), whereas DI-free Cact (2Cact) is a target of the Toll-independent pathway (Bergmann et al., 1996; Liu et al., 1997). Taking into account that CalpA enhances Toll signals and produces Cact[E10] from DI-free Cact, the simplest interpretation of our results is that the positive function attributed to Cact stems from the Toll-independent pathway. Conforming to this hypothesis, CalpA activity is extremely sensitive to DI and Cact levels, as it is reduced in *dl*[−] and *cact*[−] (Fontenele et al., 2009, 2013), reminiscent of the positive Cact effects here described.

Notably, vertebrate calpains target I κ B proteins, and the pathways that control free and NF κ B-bound Cact and I κ B α are well conserved (Han et al., 1999; Li et al., 2010; Pando and Verma, 2000; Schaecher et al., 2004; Shen et al., 2001; Shumway et al., 1999). Therefore, our findings of a positive function for Cact may have important implications for the control of vertebrate Toll signals.

Based on the arguments above, we propose a model in which free Cact (unbound to DI) is modified by the action of CalpA to enhance Toll signals in the embryo by replenishing Cact:2DI complexes (Fig. 4). However, other mechanisms involving Cact and Cact[E10] could potentially enhance Toll. Appropriate Toll responses depend on pre-signaling complex mobilization (Marek and Kagan, 2012; Sun et al., 2004), endocytosis (Huang et al., 2010; Lund et al., 2010) and DNA-bound NF κ B nuclear resident time (Mitchell et al., 2016; O'Connell and Reeves, 2015). Thus, mechanisms involving Cact and Cact[E10] that modify these functions may positively impact Toll signals and explain our results (Fig. 4B). Furthermore, it was recently proposed that Cact functions by a shuttling mechanism to concentrate DI ventrally (Carrell et al., 2016 preprint), akin to the shuttling mechanism exerted by the BMP inhibitor Sog to concentrate BMPs dorsally (Mizutani et al., 2005; Shimmi et al., 2005; Umulis et al., 2006). Although further research is required to understand how Cact enhances nDI levels in the embryo and consequently DI-target gene expression, we have clearly shown that Cact exerts a positive effect on DI nuclear uptake, that this effect is strongest when DI levels are limiting, and that Cact[E10] modifies an essential process responsible for generating this positive effect.

Therefore, we have uncovered a novel function for the Cact inhibitor to enhance Toll responses in the *Drosophila* embryo.

MATERIALS AND METHODS

Fly stocks and genetic crosses

Lines used in this study were: loss-of-function *cact*[A2] and *cact*[011], generously provided by Steve Wasserman; and *dl*[6] and *cact*[4], obtained from the Bloomington Indiana Stock Center. *Df*(2L)*cact*[255] and *Df*(2L)III18 were used as *cact* deficiencies. *Df*(2L)*cact*[255] deletes both *dl* and *cact*, whereas *Df*(2L)III18 does not delete *dl*. As *Df*(2L)III18 is not viable in several allelic combinations *Df*(2L)*cact*[255] was used in most panels unless stated otherwise and is referred to as *Df*(*cact*). Maternal overexpression lines were *CaM>cact-eGFP* and *CaM>cact*[E10]-*eGFP*, which have been described previously (Fontenele et al., 2013). All embryos were collected from mothers of the respective genotypes crossed with wild-type Canton S males.

Immunoblotting

Bleach dechorionated 30 min- to 1 h 30 min-old embryos of the appropriate genotypes were homogenized in lysis buffer (1 embryo/ μ l) and prepared for SDS-PAGE as described previously (Fontenele et al., 2009). Endogenous Cact was detected with monoclonal antiserum from Developmental Studies

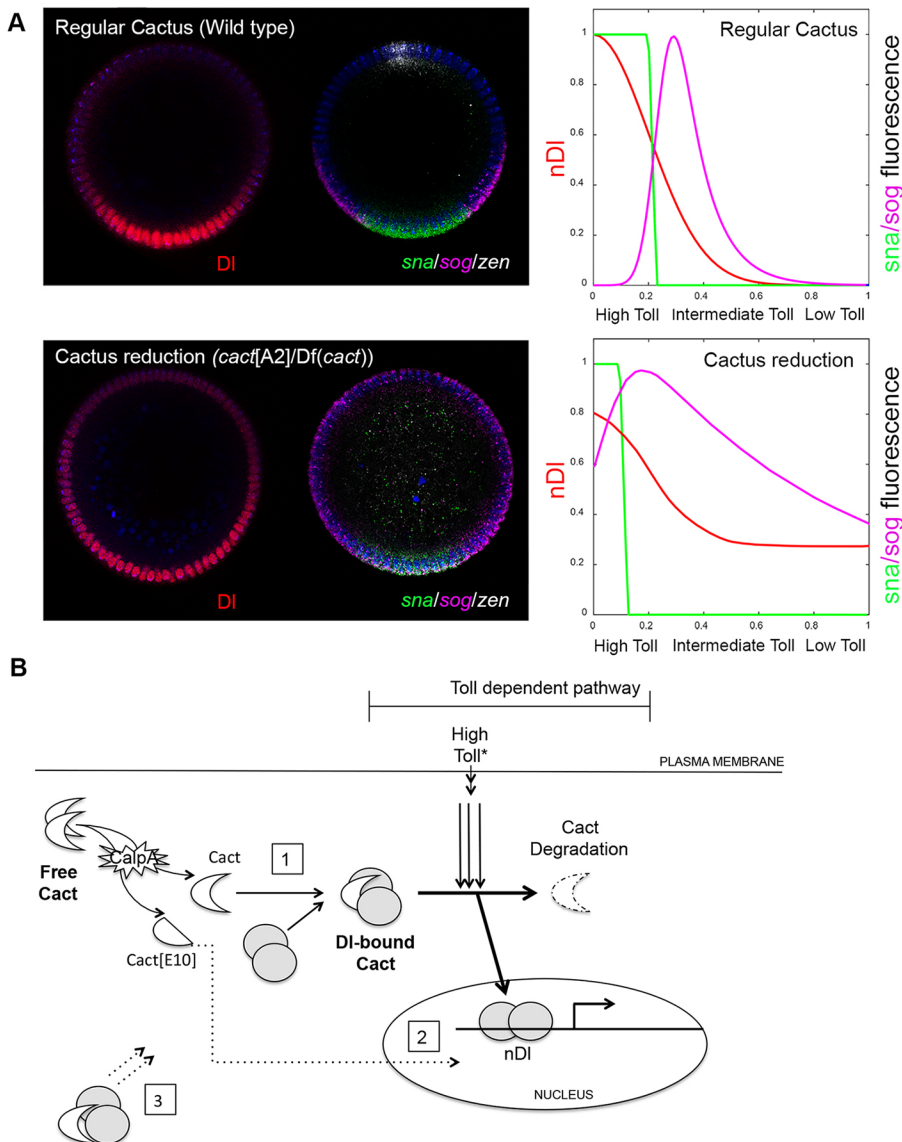


Fig. 4. Model for positive Cact function. (A) nDI (red) and DI targets (ventral *sna*, green; lateral *sog*, pink; dorsal *zen*, white) in embryo optical cross-sections and graphical representation. Decreasing *cact* function (Cact reduction) results in ventral domain reduction (loss of positive function) and expansion of the lateral territory (loss of inhibitory function). (B) Three possible mechanisms for positive Cact function, based on deploying a Toll-independent pathway: (1) CalpA activity releases Cact[E10] and free full-length Cact, replenishing DI:Cact signaling complexes that respond to Toll; (2) nuclear Cact[E10], generated by CalpA, increases DI resident time at promoters; and (3) a flux of free Cact[E10] or free full-length Cact increases DI diffusion from dorsal to ventral regions of the embryo over time.

Hybridoma Bank (DSHB, anti-Cact, 1:500). Anti- α -Tubulin was used as loading control (DM1 α , 1:3000, Sigma). Western blot quantitative data were generated by measuring band intensity relative to tubulin, from direct luminescence using a ImageQuant LAS 4000 (GE Healthcare).

Immunohistochemistry and fluorescent *in situ* hybridization

For nDI gradient visualization, mutant and control Histone-GFP embryos were mixed, fixed and processed concomitantly as described previously (Fontenele et al., 2013). Primary antisera used were monoclonal anti-DI (7A4, 1:100, DSHB) and anti-GFP (NB600308, 1:1000, Novus Biologicals, to detect control gradients). DI target genes were visualized by *in situ* hybridization as described previously (Fontenele et al., 2009).

Quantitative analysis

Images of the nDI gradient and DI target genes were collected from mid-stage 14 embryos, as defined by the amount of membrane invagination around nuclei. DI gradient quantification was as described previously (Kanodia et al., 2009) using Matlab, collected at 85% egg length using a microfluidic device to orient embryos for end-on imaging (Chung et al., 2011). For upright imaging, a Nikon 60 \times Plan-Apo oil objective was used, and images were collected at the focal plane \sim 90 μ m from the embryo anterior pole. For the overall effect on DI target genes, embryos were imaged laterally. All genotypes were processed and analyzed in parallel; thus, the same wild-type control is shown in graphs. Images were acquired with a Nikon A1 or a Leica LSM confocal microscope.

Statistical analysis

Student's *t*-test was performed for all experiments. Results are displayed as mean \pm s.e.m. The level of significance is shown in each figure ($***P\leq 0.001$, $**P\leq 0.01$, $*P\leq 0.05$).

Acknowledgements

We thank Trudi Schubach and Attilio Pane for critical reading of the manuscript, and are greatly indebted to Siegfried Roth for making available essential information on *cact* mutant data. We are grateful to the anonymous reviewers for helpful suggestions to improve the manuscript. Monoclonal antibodies anti-DI and anti-Cact, originally developed by Ruth Steward, were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242, USA. Stocks obtained from the Bloomington *Drosophila* Stock Center (supported by NIH P40OD018537) were used in this study.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: P.M.B., S.Y.S., H.M.A.; Validation: M.A.C., M.F., B.L.; Formal analysis: M.A.C., M.F., B.L.; Investigation: M.A.C., M.F.; Resources: P.M.B., S.Y.S.; Writing - original draft: M.A.C., H.M.A.; Writing - review & editing: M.A.C., P.M.B., S.Y.S., H.M.A.; Visualization: M.F., H.M.A.; Supervision: P.M.B., S.Y.S., H.M.A.; Project administration: H.M.A.; Funding acquisition: H.M.A.

Funding

This research was funded by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil, 477157/2013-0 to H.M.A.). S.Y.S. was supported by the National Institutes of Health (NIH) (R01 GM107103). M.A.C. was a recipient of Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/Brazil) local and overseas fellowships. Deposited in PMC for release after 12 months.

Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.145557.supplemental>

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