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Capicua DNA-binding sites are general response elements for RTK signaling in *Drosophila*

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SUMMARY

RTK/Ras/MAPK signaling pathways play key functions in metazoan development, but how they control expression of downstream genes is not well understood. In *Drosophila*, it is generally assumed that most transcriptional responses to RTK signal activation depend on binding of Ets-family proteins to specific cis-acting sites in target enhancers. Here, we show that several *Drosophila* RTK pathways control expression of downstream genes through common octameric elements that are binding sites for the HMG-box factor Capicua, a transcriptional repressor that is downregulated by RTK signaling in different contexts. We show that Torso RTK-dependent regulation of terminal gap gene expression in the early embryo critically depends on Capicua octameric sites, and that binding of Capicua to these sites is essential for recruitment of the Groucho co-repressor to the *huckebein* enhancer in vivo. We then show that subsequent activation of the EGFR RTK pathway in the neuroectodermal region of the embryo controls dorsal-ventral gene expression by downregulating the Capicua protein, and that this control also depends on Capicua octameric motifs. Thus, a similar mechanism of RTK regulation operates during subdivision of the anterior-posterior and dorsal-ventral embryonic axes. We also find that identical DNA octamers mediate Capicua-dependent regulation of another EGFR target in the developing wing. Remarkably, a simple combination of activator-binding sites and Capicua motifs is sufficient to establish complex patterns of gene expression in response to both Torso and EGFR activation in different tissues. We conclude that Capicua octamers are general response elements for RTK signaling in *Drosophila*.

KEY WORDS: Capicua, *Drosophila*, RTK signaling

INTRODUCTION

Receptor tyrosine kinase (RTK) signaling pathways control a broad spectrum of developmental decisions, including cell proliferation, differentiation, morphogenesis and survival (Schlessinger, 2000; Simon, 2000). Many RTK pathways signal through the conserved Ras/MAPK cassette, which then leads to phosphorylation of nuclear transcription factors and other cellular proteins. At the transcriptional level, RTK signals induce a wide variety of target gene responses in different contexts, but the molecular mechanisms underlying these responses are not well understood. In *Drosophila*, *in vivo* validated RTK effectors include the Ets factors Pointed and Yan (Simon, 2000; Tootle and Rebay, 2005), the HMG-box repressor Capicua (Cic) (Jiménez et al., 2000; Goff et al., 2001; Roch et al., 2002; Astigarraga et al., 2007; Tseng et al., 2007) and the Groucho (Gro) co-repressor (Hasson et al., 2005; Cinnamon et al., 2008; Cinnamon and Paroush, 2008; Jennings and Ish-Horowicz, 2008). Consequently, the analysis of these effectors can provide general insights into the regulatory mechanisms by which RTK signals control gene expression and development.

The *Drosophila* Torso RTK pathway represents an excellent model of transcriptional regulation in response to RTK activation (Furriols and Casanova, 2003). In this system, localized activation of the Torso receptor at each pole (termini) of the early blastoderm embryo controls the specification of terminal body structures by inducing the expression of two zygotic gap genes: *tailless* (*tll*) and *huckebein* (*hkb*) (Pignoni et al., 1990; Brönner and Jäckle, 1991). This induction involves a mechanism of derepression: both genes are normally repressed in medial regions of the embryo and the Torso signal relieves this repression at the poles (Liaw et al., 1995; Paroush et al., 1997; Jiménez et al., 2000). Repression of *tll* and *hkb* requires several nuclear factors, including Cic and Gro, which are both downregulated by the Torso signal (Paroush et al., 1997; Häder et al., 2000; Jiménez et al., 2000; Goff et al., 2001; Astigarraga et al., 2007; Cinnamon et al., 2008). Thus, loss of Cic or Gro function causes derepression of *tll* and *hkb* in medial regions of the embryo, which then leads to repression of central gap genes such as *knirps* (*kni*) and *Krüppel* (*Kr*) (Paroush et al., 1997; Jiménez et al., 2000; Goff et al., 2001; Löhr et al., 2009) (see Fig. S1 in the supplementary material). Conversely, mutations that render Cic or Gro insensitive to MAPK phosphorylation cause inappropriate repression of *tll* and *hkb* at the poles (Astigarraga et al., 2007; Cinnamon et al., 2008). Additionally, various studies have implicated other factors, such as GAGA/Trx-like, Dorsal, Retained (Retn; also known as Dead-ringer) or Tramtrack, in *tll* and/or *hkb* regulation (Liaw et al., 1995; Häder et al., 2000; Chen et al., 2002).

It is currently assumed that terminal gap genes contain complex enhancer regions that are bound by several, perhaps redundantly acting, transcription factors. However, how these activities converge to regulate Torso-dependent expression of *tll* or *hkb* is not

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understood. For example, analysis of a *hkb* enhancer indicated a role of Dorsal, Retn and Gro in Torso-mediated regulation of this enhancer (Häder et al., 2000). Cic is also required for *hkb* repression, but it has not yet been possible to demonstrate direct binding of Cic to *hkb* cis-regulatory regions (Jiménez et al., 2000). Recently, a DNA-binding motif for the Cic protein has been identified in humans (Kawamura-Saito et al., 2006), and it has been noted that this motif resembles a short regulatory element in the *tll* upstream region, the *torso response element* (*tor-RE*), which restricts *tll* expression to the posterior pole of the embryo (Liaw et al., 1995; Löhr et al., 2009). Consequently, it is possible that Cic represses *hkb* expression by binding to *tor-RE*-like elements, thus contributing to Torso-dependent regulation of this target.

Here, we report that *tor-RE*-like octameric sequences present in the *hkb* enhancer region function as binding sites for Cic and play a central role in the response of this target to Torso regulation. We also show that these Cic-binding motifs are essential for recruitment of the Gro co-repressor to *hkb* enhancer sequences in vivo. We then show that similar elements control the restricted expression of the *intermediate neuroblasts defective* (*ind*) gene in the neuroectodermal region of the embryo. This regulation occurs downstream of the EGFR RTK signaling pathway, indicating that Cic-binding sites function downstream of different RTK signals. Identical sites mediate Cic-dependent regulation of another EGFR target, *argos*, in the developing wing. Using synthetic enhancer constructs, we find that Cic octamers are sufficient to provide the regulatory information necessary to translate RTK signaling inputs into precise transcriptional responses in different tissues. We conclude that Cic octameric sites are general response elements for RTK signaling in *Drosophila*.

MATERIALS AND METHODS

DNA constructs

A GST-Cic^{HMG} expression construct was generated by amplifying a fragment encoding the *Drosophila* Cic HMG-box region (corresponding to residues 481-580) with primers *hmg1* (5′ AAT GAA TTC CCG CAG CTG GGC AGC 3′) and *hmg2* (5′ TAT CCC GGG TCC GCT CGC CTT TCC 3′), and subcloning the resulting fragment into *pGEX-6P-2*. This construct (*pGEX-6P-2-Cic^{HMG}*) is structurally equivalent to the *pGEX6P-2-Cic-HMG* construct from human Cic made by Kawamura-Saito et al. (Kawamura-Saito et al., 2006).

To generate *hkb$^{0.4}$-lacZ*, the *hkb$^{0.4}$* fragment was amplified using primers *hkb1* (5′ AAT GAA TTC ACG TTC GCT GGC CGA G 3′) and *hkb2* (5′ GAA GGA TCC ATA AAA CGC GGT CCG 3′), digested with *Eco*RI and *Bam*HI, and subcloned in *Eco*RI/*Bam*HI-digested *pCaSpeR-hs43-lacZ*. *hkb$^{0.4mut}$-lacZ* was made similarly but using a *pUC57-hkb$^{0.4mut}$* plasmid template in which the two TGAATGAA sites had been mutated to CACACGCA by recombinant PCR.

hb-lacZ was generated by amplifying a 270 bp *hb* enhancer with primers *hb1* (5′ ATG AAT TCG CTA GCT GCC TAC TCC 3′) and *hb2* (5′ AAT GCG GCC GCA CGC GTC AAG GGA 3′) and digesting the resulting product with *Eco*RI and *Not*I for cloning into *pCaSpeR-hs43-lacZ*. *hbC-lacZ* was made by inserting two TGAATGAA sites as *Not*I-*Spe*I and *Spe*I-*Bam*HI adaptors downstream of the *hb* sequence.

Bcd-lacZ was made by amplifying a synthetic array of four Bcd-binding sites separated by scrambled spacers (Hanes et al., 1994), digesting the PCR product with *Eco*RI and *Bam*HI, and subcloning the resulting fragment in *pCaSpeR-hs43-lacZ*. To generate *CBcdC-lacZ*, we first joined a 45 bp module from *hkb$^{0.4}$* containing two TGAATGAA sites with the above Bcd-binding site fragment using recombinant PCR. This fragment was subcloned upstream of a second copy of the above 45 bp element to create a *CBcdC* module, which was then inserted as an *Eco*RI-*Bam*HI fragment into *pCaSpeR-hs43-lacZ*. *CBcdC^{TRE}-lacZ* and *CBcdC^{mut}-lacZ* were made similarly, using versions of the *hkb* 45 bp module mutated to TCAATGAA or CACACGCA, respectively.

ind$^{0.5}$-lacZ was created by amplifying the *ind$^{0.5}$* fragment with primers *ind1* (5′ AAT GAA TTC AAA CGT TTT GTT ATA ATC 3′) and *ind2* (5′ GAA GGA TCC GGA AGA CAC TTC ATG 3′), and subcloning the resulting fragment in *pUC57*. The 0.5 kb *ind$^{0.5}$* fragment was then recovered by digesting the *pUC57-ind$^{0.5}$* plasmid with *Bam*HI and (partially) with *Eco*RI, and ligated to *Eco*RI/*Bam*HI-digested *pCaSpeR-hs43-lacZ*. *ind$^{0.5mut}$-lacZ* was made similarly using a *pUC57-ind$^{0.5mut}$* plasmid template in which the TGAATGAA sites had been mutated to CACACGCA by recombinant PCR.

argos$^{1.0}$-lacZ was generated using the *argos$^{1.0}$* enhancer fragment amplified with primers *argos1* (5′ ATG AAT TCG AGA TGA AAG TTT ATA G 3′) and *argos2* (5′ CAT TTT CAC ACC TGA CTG CAG 3′), and subcloning the resulting fragment in T-overhang *pUC57*. *argos$^{1.0}$* was then recovered as an *Eco*RI-*Bam*HI fragment and subcloned into *pC4PLZ*. *argos$^{1.0mut}$-lacZ* was made similarly using the corresponding *argos$^{1.0mut}$* fragment carrying mutated Cic sites (CACACGCA).

CUASC-lacZ was made by first joining five tandem Gal4-binding sites to the 45 bp module from *hkb$^{0.4}$* containing two Cic sites. This fragment was then inserted upstream of a second copy of the Cic-site module to create a *CUASC* enhancer, which was then subcloned as an *Eco*RI-*Bam*HI fragment in *pC4PLZ*.

Protein expression and EMSA experiments

GST-HMG-box fusion proteins were expressed and purified as described previously (Paroush et al., 1994). In vitro binding assays were carried out as described by Kawamura-Saito et al. (Kawamura-Saito et al., 2006). Briefly, incubations were performed in a 15 μl volume containing 0.1-0.2 μg of GST-HMG-box protein, 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 6% glycerol, 0.5% Triton-X100, 10 μg BSA, 1-2,5 μg poly(dI-dC) and 1 μg of single-stranded DNA. After 15 minutes of preincubation at 4°C, ~0.05 pmol of ^{32}P-labeled DNA probe was added and the incubation was continued for another 45 minutes at the same temperature. Reactions were resolved on 5% nondenaturing polyacrylamide gels at 4°C in 0.5× TBE.

Drosophila stocks

The *cic^{1}*, *cic^{2}*, *cic^{fetE11}* and *tor^{4021}* alleles have been described before (Jiménez et al., 2000; Goff et al., 2001; Roch et al., 2002; Klinger et al., 1988). *cic$^{\Delta C2}$* embryos were obtained from transheterozygous females carrying two different *cic$^{\Delta C2}$* insertions (Astigarraga et al., 2007). Embryos devoid of maternal *gro* activity were obtained using the *gro^{MB36}* allele (Jennings et al., 2008) in combination with the *ovo^{D}*-FLP-FRT system (Chou et al., 1993). Embryos lacking maternal *Ras* function, alone or in combination with *cic*, were generated similarly using the *Ras$^{\Delta C40b}$* and *cic^{Q474X}* alleles (Tseng et al., 2007). *dorsal* (*dl*) mutant embryos were derived from *dl^{1}/dl^{4}* mothers (FlyBase). Other transgenic insertions and mutants used were *cic-HA* construct (Astigarraga et al., 2007), *argos^{w11}* (Freeman et al., 1992), the *rho^{ve} vn^{1}* combination (Diaz-Benjumea and García-Bellido, 1990), *UAS-cic* (Lam et al., 2006) and *UAS-λtop* (Queenan et al., 1997). Transgenic lines were obtained by standard P-element transformation and several independent lines were analyzed for each reporter construct.

Embryo and wing disc analyses

Embryos were fixed in 4% formaldehyde-PBS-heptane for 20 minutes. In situ hybridizations were carried out using digoxigenin-UTP labeled antisense RNA probes, and anti-digoxygenin antibodies conjugated to alkaline phosphatase (Roche). Immunostainings were performed using the following primary antibodies: anti-dpErk (Cell Signaling; 1:50 dilution), anti-HA (12CA5, Roche; 1:400 dilution) and anti-β-galactosidase (40-1a, Developmental Studies Hybridoma Bank; 1:250 dilution). Signals were detected using secondary fluorochrome-conjugated antibodies (Molecular Probes). Embryos were mounted in Permount (in situ hybridizations) or Fluoromount-G (immunostainings). Wing discs were fixed in 4% paraformaldehyde-PBS for 20 minutes, processed for immunostaining using anti-HA and anti-β-galactosidase (anti-β-Gal) antibodies, and mounted in Fluoromount-G.

Chromatin immunoprecipitation assays

ChIP assays were performed using staged embryo collections from homozygous lines containing the *hkb*$^{0.4}$*-lacZ* or *hkb*$^{0.4mut}$*-lacZ* transgenes. Embryos were dechorionated in 100% bleach and subsequently fixed for 20 minutes in 10 ml crosslinking buffer (3% formaldehyde, 50 mM HEPES [pH 7.6], 1 mM EDTA, 0.5 mM EGTA, 100 mM NaCl) and 30 ml heptane. Crosslinking was stopped with 125 mM glycine. Crosslinked chromatin was sheared by sonication to an average size of 500 bp and immunoprecipitated using anti-Gro antibodies (two different rabbit polyclonal antisera raised against the N-terminal region of the protein). Control experiments using pre-immune serum or no antibody resulted in signals below 0.05% of input. Immunoprecipitated complexes were sequentially washed with low salt buffer [50 mM HEPES (pH 7.9), 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 140 mM NaCl, 0.1% deoxycholate], high salt buffer [50 mM HEPES (pH 7.9), 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 500 mM NaCl, 0.1% deoxycholate], LiCl buffer [20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 250 mM LiCl, 0.5% deoxycholate, 0.5% NP-40] and TE. The chromatin was eluted with TE containing 1% SDS and 0.1 M $NaHCO_3$, and cross-linking was reversed by incubating at 65°C overnight. The resulting DNA was purified by chloroform extraction and ethanol precipitation, and quantified by qPCR using the FastStart SYBR Green Master Mix (Roche) on an Opticon Monitor 2 system (Bio-Rad). Three to five independent biological replicates (in which independent embryo collections were subjected to separate crosslinking and IP before separate qPCR) were analyzed for each amplicon. As reported in other Gro ChIP studies (Martinez and Arnosti, 2008), these replicates produced some variability reflected in the standard deviation (s.d.) of the data. Nevertheless, the results were highly consistent over multiple experiments using the two anti-Gro antibodies. A *P*-value was calculated by comparing *hkb*$^{0.4}$ with *hkb*$^{0.4mut}$ for all data points from amplicons within *hkb*$^{0.4}$ (amplicons C-F) using a two-tailed *t*-test.

RESULTS

Cic represses *hkb* expression via TGAATGAA octamers

The human Cic protein binds the octameric sequence TGAATG(G/A)A (Kawamura-Saito et al., 2006). This element exhibits a single-nucleotide mismatch when compared with the core sequence of the *tor-RE*, TGCTCAATGAA (Liaw et al., 1995; Löhr et al., 2009). We assayed the ability of human and *Drosophila* Cic to bind to TGAATGAA and TCAATGAA sequences in gel-shift assays and observed similar interactions with both sites, indicating that T(G/C)AATGAA motifs are recognized by *Drosophila* Cic in vitro (see Fig. S2 in the supplementary material). We have also analyzed the role of Cic in repression of *tll* via the *tor-RE*. Using transgenes that contain *tll* enhancer sequences (Liaw et al., 1995), we provide evidence that Cic represses *tll* by binding to the *tor-RE*, and that this repression is inhibited by Torso signaling at the posterior pole (see Fig. S3 in the supplementary material).

We then searched for T(G/C)AATGAA motifs in the *hkb* upstream region and identified several TGAATGAA elements that are well conserved among *Drosophila* species (Fig. 1A; data not shown). Two such conserved sites are included in the *hkb* enhancer region identified by Häder et al. (Häder et al., 2000). To test whether Cic represses *hkb* through these motifs, we first defined a minimal *hkb* enhancer fragment that accurately reproduces the endogenous *hkb* pattern (Fig. 1A; data not shown). This 0.4 kb enhancer (designated *hkb*$^{0.4}$) directs highly restricted expression at both poles of the embryo (Fig. 1B). This pattern depends on Cic repression because it expands in *cic*^{1} embryos lacking maternal *cic* function (Fig. 1C) (Jiménez et al., 2000). Conversely, *cic*$^{\Delta C2}$ embryos expressing a Cic derivative insensitive to Torso-mediated downregulation (Astigarraga et al., 2007) show diminished *hkb*$^{0.4}$*-lacZ* expression at both poles (Fig. 1D). Mutagenesis of the two

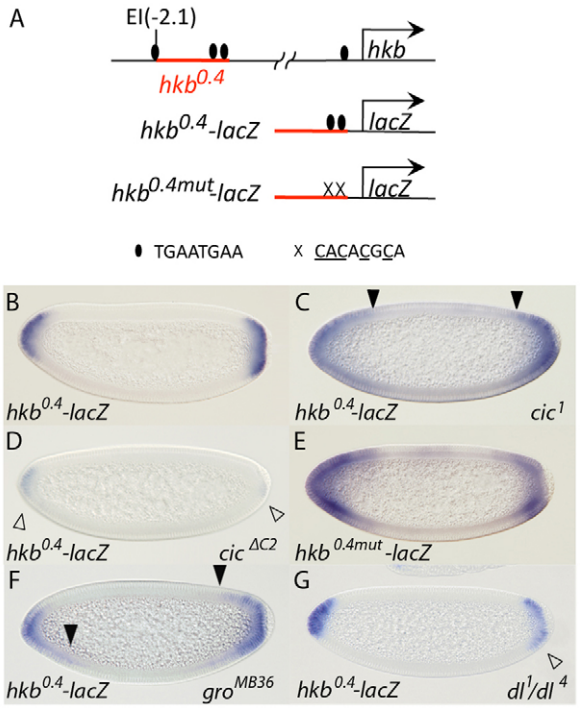

Fig. 1. Torso signaling regulates *hkb* expression via TGAATGAA repressor elements. (**A**) The *hkb* locus depicting the *hkb*$^{0.4}$ enhancer (red line). EI, *Eco*RI restriction site located 2.1 kb upstream of the transcription start site. The structure of *lacZ* reporters is shown below. (**B-G**) mRNA expression patterns of *hkb*$^{0.4}$*-lacZ* (B-D,F,G) and *hkb*$^{0.4mut}$*-lacZ* (E) in otherwise wild-type (B,E), *cic*^{1} (C), *cic*$^{\Delta C2}$ (D), *gro*^{MB36} (F) and *dl*^{1}*/dl*^{4} (G) embryos. Closed arrowheads in C and F indicate derepressed *hkb*$^{0.4}$*-lacZ* expression in *cic*^{1} and *gro*^{MB36} embryos. Open arrowheads in D and G indicate reduced *hkb*$^{0.4}$*-lacZ* expression in *cic*$^{\Delta C2}$ and *dl*^{1}*/dl*^{4} embryos.

TGAATGAA sites in *hkb*$^{0.4}$*-lacZ* causes expanded reporter expression that resembles the pattern of *hkb*$^{0.4}$*-lacZ* in *cic*^{1} embryos (Fig. 1E). We conclude that regulation of *hkb* expression requires direct binding of Cic to conserved TGAATGAA cis-acting octamers.

For comparison, we also analyzed *hkb*$^{0.4}$*-lacZ* expression in embryos devoid of maternal Gro function. Gro activity is essential for restricting *tll* and *hkb* expression to the embryonic poles, although the mechanism of Gro action in this context remains uncertain (Paroush et al., 1997; Jiménez et al., 2000; Häder et al., 2000; Cinnamon et al., 2008) (see below). As shown in Fig. 1F, there is significant *hkb*$^{0.4}$*-lacZ* derepression in *gro*^{MB36} mutant embryos, similar to the effect seen in *cic*^{1} embryos. Thus, both Cic and Gro play similar roles in repressing the *hkb*$^{0.4}$ enhancer. By contrast, embryos lacking Dorsal activity, another maternal regulator which functions as both an activator and repressor and is implicated in *hkb* regulation (Häder et al., 2000; Hong et al., 2008), displayed reduced *hkb*$^{0.4}$*-lacZ* expression at the posterior pole (Fig. 1G), indicating that Dorsal is required for activating *hkb* expression in posterior regions (see below).

Cic repressor sites are sufficient to mediate Torso-dependent regulation

Although binding of Cic to *hkb*$^{0.4}$ is essential for repressing this enhancer, the response to Torso regulation might involve additional factors bound to the enhancer. To address this issue, we asked

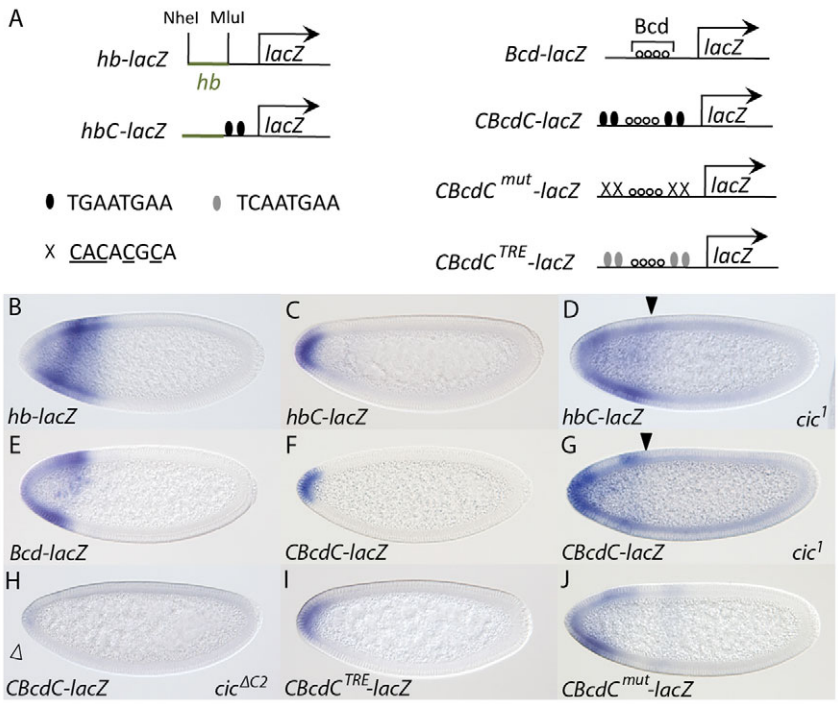

Fig. 2. Cic-binding motifs confer Torso-dependent regulation to synthetic enhancers. (**A**) Diagram of *lacZ* reporters containing Bcd-activating sequences and T(G/C)AATGAA sites. The 270 bp *hb* enhancer (delimited by *Nhe*I and *Mlu*I restriction sites) is indicated in green. (**B-J**) mRNA expression patterns of *hb-lacZ* (B), *hbC-lacZ* (C,D), *Bcd-lacZ* (E), *CBcdC-lacZ* (F-H), *CBcdC^{TRE}-lacZ* (I) and *CBcdC^{mut}-lacZ* (J) in otherwise wild-type (B,C,E,F,I,J), *cic^{1}* (D,G) and *cic^{ΔC2}* (H) embryos. Closed arrowheads in D and G indicate expanded *hbC-lacZ* and *CBcdC-lacZ* expression in *cic^{1}* embryos. The open arrowhead in H indicates residual *CBcdC-lacZ* expression at the anterior pole.

whether Cic octamers are sufficient for Torso-dependent regulation of synthetic enhancers. We first tested whether Cic-binding sites (TGAATGAA) linked to a heterologous enhancer would make it responsive to Torso regulation. We selected a 270 bp promoter fragment from the *hunchback* (*hb*) gene, which normally drives intense staining in the anterior third of the embryo (construct *hb-lacZ*; Fig. 2A,B) (Struhl et al., 1989). Linking the same fragment to a single pair of Cic-binding motifs (construct *hbC-lacZ*) caused restricted expression from ~91 to 100% embryo length (EL; 0% being the posterior tip of the embryo; Fig. 2C). This pattern resembles the anterior domain of *hkb* expression and precisely corresponds to the area of Cic downregulation by the Torso pathway (Jiménez et al., 2000; Kim et al., 2010). Furthermore, this pattern depends on Cic because it expands posteriorly in *cic^{1}* embryos (Fig. 2D). Thus, the addition of Cic repressor sites confers Torso-dependent expression to the *hb* enhancer.

The *hb* enhancer is activated by the anteriorly expressed Bicoid (Bcd) factor (Struhl et al., 1989; Driever and Nüsslein-Volhard, 1989). Therefore, we tested whether a simple combination of Bcd and Cic sites would also respond to Torso regulation. A construct containing four multimerized Bcd sites drive anterior expression from ~73 to 100% EL (construct *Bcd-lacZ*; Fig. 2A,E). By contrast, a transgene in which the Bcd sites are flanked by two Cic sites on either side is expressed in a restricted pattern from 92 to 100% EL (construct *CBcdC-lacZ*; Fig. 2A,F). In *cic^{1}* embryos, *CBcdC-lacZ* expression expands posteriorly up to ~74% EL (Fig. 2G), whereas it almost disappears in *cic^{ΔC2}* embryos (Fig. 2H). A similar construct containing TCAATGAA sites corresponding to the *torRE* (*CBcdC^{TRE}-lacZ*) also showed highly restricted expression in the Torso signaling domain (Fig. 2I). Finally, mutation of the four Cic sites to CACACGCA caused derepressed reporter expression similar to the *Bcd-lacZ* pattern (construct *CBcdC^{mut}-lacZ*; Fig. 2J). These results indicate that Cic repressor sites combined with Bcd activator sequences are sufficient to provide a direct highly localized readout of Torso signaling activity at the anterior pole.

Cic-binding motifs are required for recruitment of Gro to the *hkb* enhancer

Because the Gro co-repressor does not bind DNA, it is believed to be recruited to terminal enhancers by one or more DNA-bound repressors (Paroush et al., 1997; Häder et al., 2000; Jiménez et al., 2000; Cinnamon et al., 2008; Jennings and Ish-Horowicz, 2008). We and others have proposed different mechanisms by which Gro could interact with terminal repressors such as Dorsal, Retn or Cic to silence *tll* and *hkb* expression (Häder et al., 2000; Jiménez et al., 2000). Given that *hkb$^{0.4}$-lacZ* expression depends on both Gro activity and intact Cic regulatory sites (Fig. 1), we analyzed whether such sites are required for recruitment of Gro to the *hkb$^{0.4}$* enhancer. To this end, we first monitored association of Gro to the *hkb$^{0.4}$-lacZ* transgene by chromatin immunoprecipitation (ChIP) assays using anti-Gro antibodies and qPCR. These experiments were performed using staged embryo collections (90-180 minutes after egg laying) carrying two copies of *hkb$^{0.4}$-lacZ*. We designed a set of amplicons that span the *hkb$^{0.4}$* enhancer and the flanking sequences present in the reporter construct (Fig. 3A). Some of these amplicons (A, B, G, H and I) are specific for the reporter and do not amplify endogenous genomic sequences, whereas amplicons C-F potentially amplify both the homozygous transgenic and endogenous *hkb$^{0.4}$* enhancers. As shown in Fig. 3B, we found association of Gro with most of the intact *hkb$^{0.4}$* enhancer (blue bars for amplicons C-F), but not with regions flanking the enhancer (amplicons A, B, G and I), although a small peak is observed at the transcriptional start site (amplicon H). Interestingly, within the enhancer, Gro levels were somewhat higher upstream of the Cic sites (amplicons D and E). This upstream region includes binding sites for Dorsal and Retn (Fig. 3A), two factors that have been implicated in *hkb* regulation and are known to bind Gro directly (Dubnicoff et al., 1997; Valentine et al., 1998; Häder et al., 2000).

We then used the same approach to assay binding of Gro to the *hkb$^{0.4mut}$* enhancer containing mutant Cic sites. In this case, Gro was detected at significantly lower levels compared with the wild-

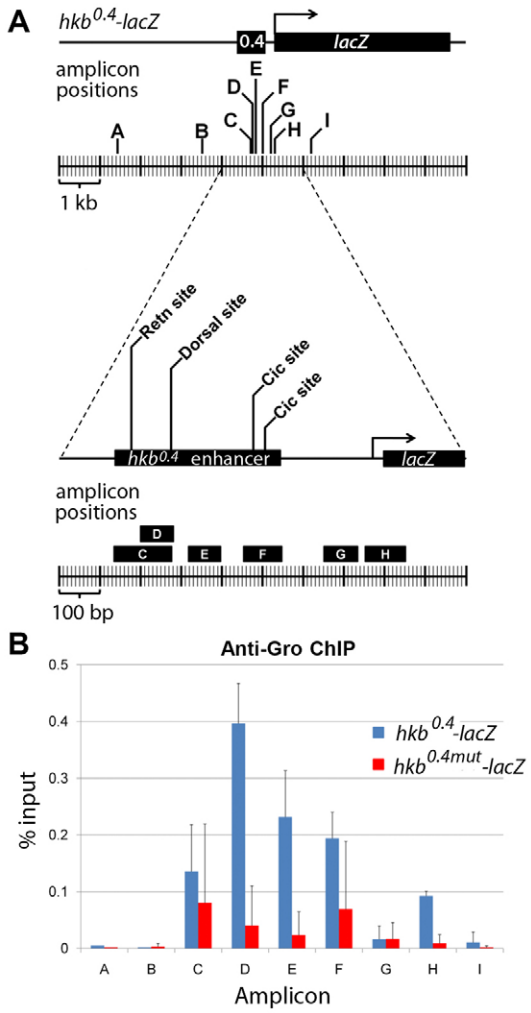

Fig. 3. Association of Gro with the *hkb*$^{0.4}$ enhancer requires intact Cic regulatory sites. (**A**) The *hkb*$^{0.4}$-*lacZ* transgene contains the 0.4 kb *hkb* enhancer, which includes two Cic-binding sites, a Dorsal-binding site and a Retn-binding site upstream of the *lacZ* reporter. The positions of amplicons A-I are shown relative to *hkb*$^{0.4}$-*lacZ*. (**B**) Crosslinked chromatin was isolated from embryos carrying the *hkb*$^{0.4}$-*lacZ* (blue bars) or the *hkb*$^{0.4mut}$-*lacZ* (red bars) transgenes. Anti-Gro ChIP was assayed by qPCR using amplicons A-I. Each bar represents the average (±s.d.) of three to five independent biological replicates. Background levels resulting from pre-immune ChIP controls were subtracted out of all signals.

type enhancer (red bars in Fig. 3B). This decrease is observed throughout the enhancer, including the region upstream of the mutant Cic sites. Averaging across the four amplicons within the enhancer, we find that mutagenesis of the Cic sites reduces Gro association with the enhancer by 4.5-fold (P<0.01). As amplicons C-F should detect signals derived from both transgenic and endogenous enhancers, the larger than twofold decrease observed for the mutant sample suggest a higher efficiency of Gro immunoprecipitation from transgenic versus endogenous *hkb*$^{0.4}$ sequences, perhaps owing to a more open conformation of the transgene. Similar data were obtained using two different anti-Gro antibodies (data not shown). Taken together, these results indicate that binding of Cic to specific sites in *hkb*$^{0.4}$ is essential for recruitment of Gro to this enhancer.

Cic represses *ind* expression downstream of EGFR signaling

We searched for potential Cic-binding sites in genes that might be targets of other RTK signaling pathways. One gene identified in these analyses, *ind*, functions as a target of the EGFR RTK pathway in the neuroectodermal region of the embryo (Skeath, 1998; Weiss et al., 1998; von Ohlen and Doe, 2000; Hong et al., 2008). *ind* expression begins at mid stage 5, forming sharp four- or five-cell wide longitudinal stripes on either side of the embryo (Weiss et al., 1998) (Fig. 4C). This pattern requires activator inputs from the Dorsal morphogen, as well as from the EGFR pathway, which is active in lateral domains overlapping the *ind* stripes (Skeath, 1998; von Ohlen and Doe, 2000; Hong et al., 2008) (Fig. 4B,D). The *ind* stripes are limited ventrally by the Ventral neuroblasts defective (Vnd) repressor expressed in the ventral neuroectoderm, and dorsally by the dorsal limit of the EGFR signaling domain (Weiss et al., 1998; von Ohlen and Doe, 2000). Previous analyses of *ind* regulation have identified a repressor element (the *A-box* motif) that controls the dorsal limit of *ind* expression through an unknown factor (Stathopoulos and Levine, 2005). We noted that the *A-box* sequence (WTTCATTCATRA) matches the complementary sequence of the Cic-binding motif. This, together with the requirement of EGFR signaling for *ind* expression, prompted us to study the role of Cic in *ind* regulation.

We reasoned that activation of *ind* expression by the EGFR pathway could involve downregulation of Cic protein in the lateral ectoderm. Indeed, EGFR activity in the neuroectoderm (as visualized by immunostaining against double-phosphorylated Erk/MAPK) precisely correlates with a sharp decline in Cic protein levels in this region (Fig. 4B). This suggests that EGFR signaling controls the dorsal limit of *ind* expression by defining a corresponding limit of Cic downregulation. We then monitored *ind* expression in *cic*^{1} embryos and observed altered *ind* stripes that appeared both dorsally expanded at the anterior and retracted from the posterior (Fig. 4E). In embryos derived from *cic*^{1}/*cic*^{2} females, which contain even lower Cic activity (Roch et al., 2002), the *ind* stripes are shorter and further expanded towards the dorsal side, resulting in eight- to 10-cell wide staining at late stage 5 (Fig. 4F). Thus, Cic has two effects on *ind* expression: it defines the dorsal limit of the *ind* stripes and maintains their expression in the abdominal region. The latter effect is reminiscent of the indirect positive role of Cic on *kni* and *Kr* expression (see Fig. S1 in the supplementary material), and it could thus reflect repression of *ind* by the terminal gap genes extending from the posterior pole in the absence of Cic. Consistent with this idea, we fail to detect *ind* expression in *torso*^{4021} gain-of-function mutant embryos where the terminal gap genes are severely derepressed (Fig. 4G) (Klinger et al., 1988; Brönner and Jäckle, 1991).

To test whether Cic represses *ind* through *A-box* elements, we generated a *lacZ* reporter driven by a 0.5 kb *ind* enhancer fragment encompassing two *A-box* motifs and a Dorsal-binding site (Fig. 4A) (Stathopoulos and Levine, 2005). This reporter (*ind*$^{0.5}$-*lacZ*) forms weak, discontinuous lateral stripes of expression in late blastoderm embryos (Fig. 4H). As in the case of endogenous *ind* stripes, the *ind*$^{0.5}$-*lacZ* stripes are abolished in embryos lacking EGFR/Ras signaling activity (derived from females carrying *Ras*$^{\Delta C40b}$ germ-line clones; Fig. 4D,I). In *cic*^{1} embryos, the *ind*$^{0.5}$-*lacZ* stripes are more uniform and expand by three to five cell diameters dorsally (Fig. 4J), whereas *cic*^{1}/*cic*^{2} embryos show ectopic expression almost up to the dorsal midline (Fig. 4K). Additionally, this expanded *ind*$^{0.5}$-*lacZ* expression persists in embryos derived from *Ras*$^{\Delta C40b}$ *cic*^{Q474X} double mutant germline

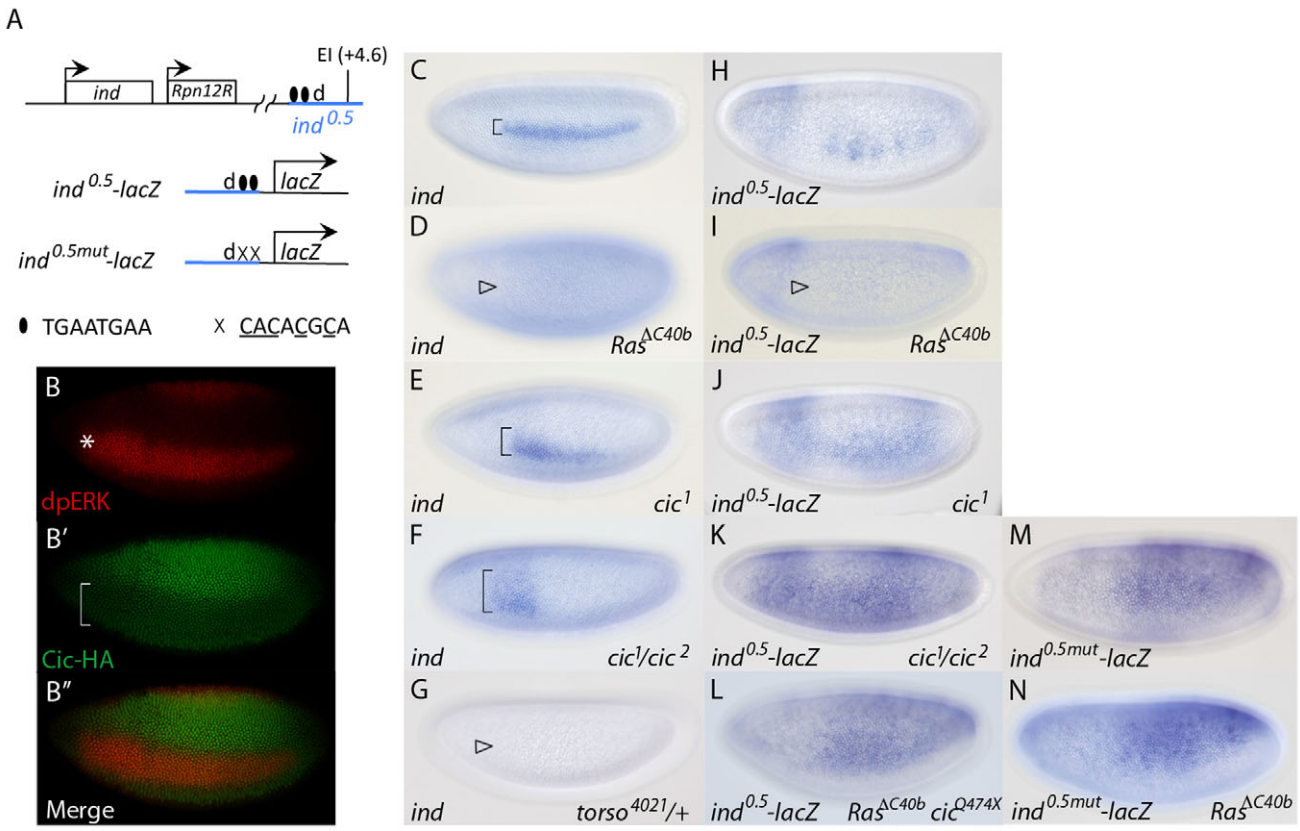

Fig. 4. EGFR induces *ind* expression by relieving Cic repression. (**A**) The *ind* locus showing the neighboring *Rpn12R* gene (predicted to encode a component of the proteasome) and the *ind*$^{0.5}$ enhancer present in the 3′-flanking region (blue line). EI, *Eco*RI site present 4.6 kb downstream of the *ind* transcription start site. d, Dorsal-binding site (GGGAAATTCCC). *lacZ* reporters driven by *ind*$^{0.5}$ enhancer sequences are also shown. (**B-B″**) Stage 5 *cic-HA; cic*^{1} embryo stained with anti-dpERK (red, B) and anti-HA (green, B′) antibodies; the merged image is shown in B″. EGFR activation in the lateral neuroectoderm (asterisk in B) produces a corresponding downregulation of Cic levels in ventrolateral regions (bracket in B′). (**C-N**) *ind* (C-G), *ind*$^{0.5}$*-lacZ* (H-L) and *ind*$^{0.5mut}$*-lacZ* (M,N) mRNA expression patterns in wild-type (C,H,M), *Ras*$^{\Delta C40b}$ (D,I,N), *cic*^{1} (E,J), *cic*^{1}/*cic*^{2} (F,K), *Ras*$^{\Delta C40b}$ *cic*^{Q474X} (L) and *tor*^{4021}/+ (G) embryos. All images are lateral surface views of mid- to late-stage 5 embryos. Brackets in C,E,F indicate the maximal width of *ind* stripes. Open arrowheads in D,G,I indicate loss of *ind* and *ind*$^{0.5}$*-lacZ* expression in *Ras*$^{\Delta C40b}$ and *tor*^{4021} backgrounds.

clones (Fig. 4L), implying that EGFR/Ras signaling normally induces *ind*$^{0.5}$*-lacZ* expression by downregulating Cic. Finally, mutation of the two *A-box* motifs in *ind*$^{0.5}$ caused derepression throughout lateral and dorsal regions of the embryo (Fig. 4M), and this pattern was unaffected in embryos lacking EGFR/Ras activity (Fig. 4N), consistent with removal of Cic being sufficient for EGFR-dependent induction of the *ind*$^{0.5}$ enhancer. Together, these results indicate that the *A-box* motifs in *ind* are binding sites for Cic protein that respond to EGFR regulation via Cic derepression.

Cic octamers mediate EGFR-dependent regulation during wing development

Previous analyses showed that Cic behaves as a repressor of *argos* expression in the wing imaginal disc (Roch et al., 2002). *argos* is an EGFR signaling target that encodes a feedback inhibitor of this pathway (Freeman et al., 1992; Golembo et al., 1996). During wing development, EGFR activity defines the position of wing veins and leads to downregulation of Cic in presumptive vein cells, particularly in two rows of cells running along the future wing margin and in prospective veins L3, L4 and L5 (Fig. 5A) (Roch et al., 2002). This pattern of Cic downregulation is markedly complementary to the expression of *argos*, as visualized with the *argos*^{w11} enhancer trap reporter (compare Fig. 5A with 5B) (Gabay et al., 1997). Reduced Cic function in *cic*^{2}/*cic*^{fetE11} discs causes *argos*^{w11} derepression in intervein cells at levels similar to those of endogenous wing margin and L5 stripes (Fig. 5C; see also Fig. S4 in the supplementary material) (Roch et al., 2002). This ectopic expression is weaker than in stripes L3 and L4, suggesting that these stripes are subject, at least in part, to Cic-independent regulation, an idea supported by the relatively normal development of the L3-L4 intervein region in *cic* mutant adults (Fig. S4 in the supplementary material). We also analyzed *argos*^{w11} expression in discs lacking both Cic and EGFR signaling activities, using the *cic*^{2}/*cic*^{fetE11} background in combination with *rhomboid* (*rho*) and *vein* (*vn*) alleles that eliminate EGFR signaling in the wing disc (Martín-Blanco et al., 1999). This caused generalized *argos*^{w11} expression throughout the wing pouch without enhancement in stripes L3 and L4 (Fig. 5D), suggesting that EGFR signaling induces *argos*^{w11} expression in prospective veins by relieving Cic repression, and that an additional EGFR-dependent input reinforces this expression in stripes L3 and L4.

To investigate whether Cic represses *argos* directly through Cic octameric sites, we first identified several conserved TGAATG(G/A)A motifs within the first intron of *argos* (Fig. 5E; data not shown). Next, we selected a 1.0 kb intron fragment containing five such sites (four TGAATGAA and one

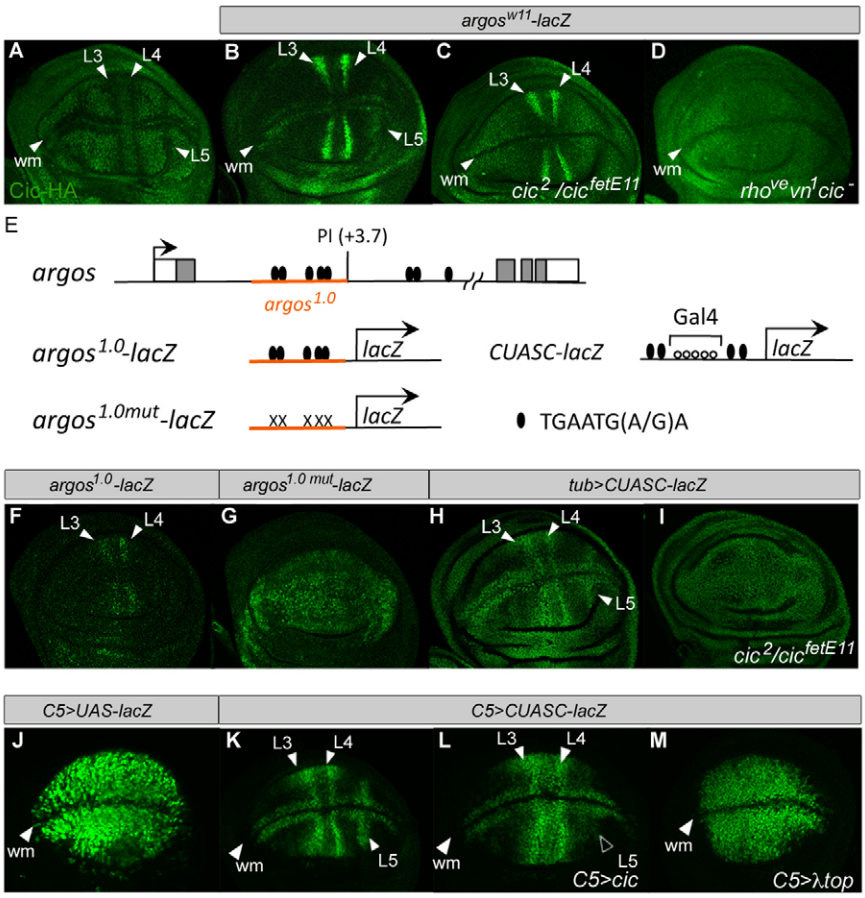

Fig. 5. EGFR signaling regulates *argos* expression through Cic octamers. (**A**) Staining of *cic-HA* third instar wing disc using anti-HA antibody; arrowheads indicate the stripes of Cic downregulation in response to EGFR signaling. wm, wing margin. (**B-D**) Anti-β-Gal staining of $argos^{w11}$ expression in otherwise wild-type (B), cic^{2}/cic^{fetE11} (C) and rho^{ve} vn^{1} cic^{2}/rho^{ve} vn^{1} cic^{fetE11} (D) wing discs. (**E**) Diagram of the *argos* locus indicating the $argos^{1.0}$ enhancer (orange); exons are depicted by boxes and coding sequences are shown in gray. PI, *PstI* site present 3.7 kb downstream of the transcription start site. The structure of *lacZ* reporters is shown below. (**F**,**G**) β-Gal expression patterns of $argos^{1.0}$-*lacZ* (F) and $argos^{1.0mut}$-*lacZ* (G) reporters in wing discs. (**H**,**I**) Anti-β-Gal staining of *tubulin-Gal4/CUASC-lacZ* imaginal discs from otherwise wild-type (H) or cic^{2}/cic^{fetE11} (I) larvae. (**J**) β-Gal expression in *UAS-lacZ/+; C5-Gal4/+* imaginal disc. (**K-M**) β-Gal expression patterns resulting from *C5-Gal4*-directed activation of *CUASC-lacZ* in imaginal discs from otherwise wild-type (K), *UAS-cic* (L) or *UAS-λtop* (M) larvae. β-Gal expression is lost in prospective L5 vein cells after Cic overexpression (open arrowhead in L).

TGAATGGA motifs) and other conserved sequences. When placed upstream of a *lacZ* reporter, this fragment (designated $argos^{1.0}$) directs restricted expression in presumptive veins L3 and L4 (Fig. 5E,F), indicating that it mediates partial aspects of *argos* regulation. By contrast, the same fragment carrying mutated Cic sites drives widespread expression in the wing pouch and peripheral regions of the disc (Fig. 5E,G; data not shown). Thus, conserved Cic-binding sites in *argos* restrict its expression to prospective wing vein cells of the disc.

To test whether Cic-binding sites are sufficient to mediate EGFR-dependent regulation in the wing, we assayed an artificial enhancer containing five GAL4-binding sites flanked on either side by two tandem TGAATGAA motifs (construct *CUASC-lacZ*; Fig. 5E). Indeed, inducing ubiquitous GAL4 expression under the control of the *tubulin* or *hsp-70* promoters leads to localized activation of the *CUASC* enhancer in prospective veins (Fig. 5H and data not shown). This restricted pattern depends on Cic, as it becomes significantly derepressed in *cic* mutant discs (Fig. 5I). We also monitored *CUASC-lacZ* expression driven by the *C5-GAL4* line, which is active in the presumptive wing pouch (Fig. 5J) (Yeh et al., 1995). As shown in Fig. 5K, *C5-GAL4* activates *CUASC-lacZ* expression only in presumptive vein cells of the wing pouch. Co-expression of *CUASC-lacZ* and Cic [using an *UAS-cic* construct (Lam et al., 2006)] with the same driver resulted in loss of *lacZ* expression in presumptive vein L5 (Fig. 5L), which correlated with loss of vein L5 in adult wings (Fig. S4 in the supplementary material). Conversely, co-expressing *CUASC-lacZ* together with *UAS-λtop*, which encodes a constitutively active form of EGFR (Queenan et al., 1997), caused severe *lacZ* derepression throughout the presumptive wing pouch (Fig. 5M; see also Fig. S4 in the supplementary material). This pattern recapitulates *C5-GAL4*-mediated activation of the standard *UAS* enhancer lacking Cic sites (Fig. 5J), and is therefore consistent with generalized downregulation of Cic in the *C5-GAL4>UAS-λtop* background. Thus, our results indicate that EGFR signaling controls *argos* expression through octameric Cic sites, and that such sites are sufficient to define a complex pattern of EGFR-mediated activation in the developing wing.

DISCUSSION

RTK signaling pathways play key functions in metazoan development, but the molecular mechanisms underlying RTK-initiated responses are not well understood. Until recently, it was generally assumed that Pointed and Yan were the only nuclear effectors of all RTK pathways in the fly [see, for example, Simon (Simon, 2000)]. However, several studies have identified the Cic repressor as an important sensor of some of these pathways (Jiménez et al., 2000; Goff et al., 2001; Astigarraga et al., 2007; Tseng et al., 2007). Here, we have shown that Cic regulatory functions downstream of Torso and EGFR signals depend on common TGAATGAA DNA octamers and that, at least in certain assays, these octamers are sufficient to induce localized RTK responses in vivo.

Our results show that regulation of *hkb* expression in response to Torso signaling crucially depends on conserved TGAATGAA elements recognized by Cic (Fig. 1). We also find that these elements combined with Bcd activator sequences are sufficient to establish localized reporter expression in the anterior pole of the

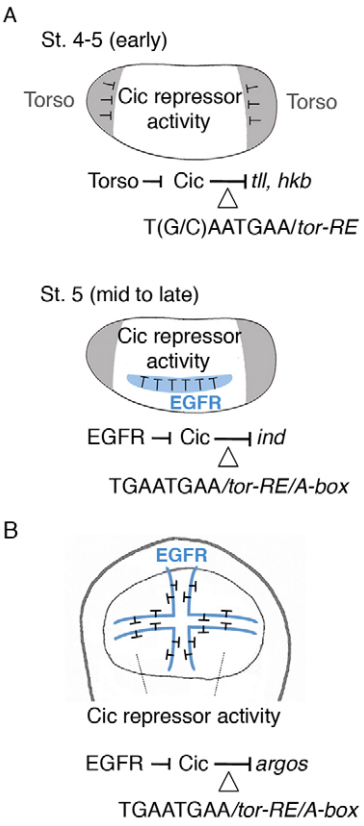

Fig. 6. Cic regulatory elements mediate Torso and EGFR responses. (**A**) Sequential activation of the Torso (gray) and EGFR (blue) RTK pathways downregulates Cic along the AP and DV embryonic axes. Both pathways relieve Cic repression mediated by common cis-regulatory elements. Developmental stages (St.) are indicated. (**B**) EGFR signaling (blue) induces *argos* expression via Cic sites; activation of the pathway in vein cells leads to downregulation of Cic repressor activity, thereby derepressing *argos* transcription.

embryo (Fig. 2). It thus appears that binding of Cic to specific sites in *hkb* is the key step for delimiting *hkb* expression in response to Torso activation. Therefore, although we cannot rule out that other (possibly redundant) Torso-dependent factors contribute to *hkb* regulation, we propose that this regulation largely depends on broadly distributed activators such as Bcd, Dorsal and Lilliputian (Reuter and Leptin, 1994; Häder et al., 2000; Tang et al., 2001) (Fig. 1G), and localized Cic repression.

We also find that association of Gro to the *hkb* enhancer requires the presence of intact Cic octamers in the enhancer. How does this association occur? Although Cic and Gro proteins interact in vitro, we have not yet demonstrated a direct correlation between such binding and Cic repressor activity in vivo (Jiménez et al., 2000; Astigarraga et al., 2007) (C.N. and G.J., unpublished). Our finding that Gro associates with sequences containing Dorsal and Retn sites is consistent with a role of these factors in recruiting Gro to the *hkb* enhancer, possibly through cooperative interactions with Cic. However, mutations in *dorsal* or *retn* do not cause clear derepression of $hkb^{0.4}$-*lacZ* or *hkb* expression (Fig. 1G) (Häder et al., 2000). It is also possible that local recruitment of Gro by Cic results in subsequent spreading of the co-repressor along the entire $hkb^{0.4}$ enhancer, a mechanism that may involve oligomerization of Gro and binding to hypoacetylated histones (Courey and Jia, 2001; Song et al., 2004; Martinez and Arnosti, 2008).

Our results indicate that patterning of the dorsal-ventral (DV) embryonic axis requires a mechanism of EGFR-mediated derepression that is similar to the role of Torso signaling in the anterior-posterior (AP) terminal system. In both cases, a local source of RTK activation downregulates the Cic repressor, thus inducing expression of Cic targets in restricted patterns (Fig. 6A). During DV patterning, the Dorsal morphogen activates the expression of several targets in ventral and lateral regions of the embryo, and it is believed that decreasing amounts of Dorsal protein help establish the dorsal limits of those expression domains. However, Dorsal nuclear levels appear rather uniform across the *ind* expression domain (Kanodia et al., 2009; Liberman et al., 2009), indicating that other mechanisms define the dorsal limit of *ind* expression. Indeed, previous studies have shown that EGFR signaling plays a key role in setting this border (Weiss et al., 1998; von Ohlen and Doe, 2000), and suggested the existence of unknown repressors restricting *ind* expression in dorsal regions (Stathopoulos and Levine, 2005). Our results indicate that these two events are linked through a mechanism of EGFR-mediated downregulation of Cic repressor activity.

During wing vein specification, there is a precise correlation between EGFR/MAPK signaling, Cic downregulation and *argos* transcription in prospective wing vein cells (Fig. 5A,B) (Gabay et al., 1997). Furthermore, our data show that Cic represses *argos* directly (Fig. 5E-G), and that Cic octamers alone are sufficient to interpret the EGFR activation signal to produce an *argos*-like response (Fig. 5H-M, Fig. 6B). However, the *CUASC-lacZ* reporter does not recapitulate all aspects of *argos* transcription, because only endogenous *argos* shows elevated expression in presumptive veins L3 and L4. This difference probably depends on localized determinants that regulate gene expression in the L3-L4 region (Blair, 2007), and do not affect *CUASC-lacZ*. Still, *argos* regulation during wing development appears largely dependent on EGFR-mediated downregulation of Cic as well as on positive input(s) by localized or ubiquitous activators, which may include the Osa/Eyelid factor (Terriente-Félix and de Celis, 2009). In addition, both loss- and gain-of-function experiments show strong correlation between Cic-dependent activity through TGAATGAA elements and differentiation of wing veins in the adult (Fig. 5 and see Fig. S4 in the supplementary material) (Goff et al., 2001; Roch et al., 2002), suggesting that Cic is an important sensor of EGFR signaling in this system. Cic probably controls additional EGFR targets involved in wing vein specification and other EGFR-regulated processes such as cell proliferation in imaginal discs (Tseng et al., 2007). Future studies will probably reveal new roles of Cic and its binding sites downstream of RTK signaling cascades.

In summary, Cic regulates multiple RTK signaling responses by binding to conserved octameric sites in target enhancers, indicating that conservation between these RTK pathways extends to specific response elements in cis-regulatory regions. Notably, these octamers are sufficient to translate RTK signaling inputs into localized transcriptional responses in different tissues: RTK signals produce complementary gradients (Torso) or boundaries (EGFR) of Cic downregulation that are then translated into complementary patterns of target gene expression through relief of Cic repression. This mechanism represents a particular case of ‘default repression’, a general strategy of developmental control whereby target genes induced by signaling pathways are maintained repressed in the absence of signaling (Barolo and Posaknony, 2002). For example, a similar derepression switch occurs during TGF-β/Dpp-mediated induction of *optomotor-blind* transcription via relief of Brinker repression (Sivasankaran et al., 2000; Barolo and Posaknony, 2002).

Finally, the human Cic protein binds octameric sequences related to those characterized here (Kawamura-Saito et al., 2006). The best-characterized targets of Cic in human cells are ETS genes of the *pea3* family (Kawamura-Saito et al., 2006), which are known to respond to FGF RTK activation in different vertebrate systems (e.g. Roehl and Nüsslein-Volhard, 2001; Raible and Brand, 2001). Therefore, it will be interesting to ascertain whether Cic octamers also mediate RTK responses in those systems.

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Competing interests statement
The authors declare no competing financial interests.

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