The EMBO Journal (2011) 30, 1444–1458 | © 2011 European Molecular Biology Organization | All Rights Reserved 0261-4189/11
www.embojournal.org

THE EMBO JOURNAL

Jerky/Earthbound facilitates cell-specific Wnt/Wingless signalling by modulating β-catenin–TCF activity

Hassina Benchabane[1], Nan Xin[1], Ai Tian[1], Brian P Hafler[2], Kerrie Nguyen[1], Ayah Ahmed[1] and Yashi Ahmed[1,*]

[1]Department of Genetics and the Norris Cotton Cancer Center, Dartmouth Medical School, Hanover, NH, USA and [2]Department of Molecular Biology, Princeton University, Princeton, NJ, USA

Wnt/Wingless signal transduction directs fundamental developmental processes, and upon hyperactivation triggers colorectal adenoma/carcinoma formation. Responses to Wnt stimulation are cell specific and diverse; yet, how cell context modulates Wnt signalling outcome remains obscure. In a Drosophila genetic screen for components that promote Wingless signalling, we identified Earthbound 1 (Ebd1), a novel member in a protein family containing Centromere Binding Protein B (CENPB)-type DNA binding domains. Ebd1 is expressed in only a subset of Wingless responsive cell types, and is required for only a limited number of Wingless-dependent processes. In addition, Ebd1 shares sequence similarity and can be functionally replaced with the human CENPB domain protein Jerky, previously implicated in juvenile myoclonic epilepsy development. Both Jerky and Ebd1 interact directly with the Wnt/Wingless pathway transcriptional co-activators β-catenin/Armadillo and T-cell factor (TCF). In colon carcinoma cells, Jerky facilitates Wnt signalling by promoting association of β-catenin with TCF and recruitment of β-catenin to chromatin. These findings indicate that tissue-restricted transcriptional co-activators facilitate cell-specific Wnt/Wingless signalling responses by modulating β-catenin–TCF activity.

The EMBO Journal (2011) **30,** 1444–1458. doi:10.1038/emboj.2011.67; Published online 11 March 2011
Subject Categories: signal transduction; development
Keywords: β-catenin; cell context; signal transduction; TCF; Wnt

Introduction

The evolutionarily conserved secreted ligands Wnt/Wingless activate a signal transduction pathway that governs fundamental aspects of metazoan development and is misregulated in several human diseases, including the majority of colorectal carcinomas (Clevers, 2006; MacDonald *et al*, 2009). The Wnt signalling cascade elicits distinct responses that control cell proliferation, fate specification, differentiation, and apoptosis. The majority of Wnt target genes are cell specific, and this specificity depends on numerous contextual factors, including developmental history, as well as strength, timing, and duration of Wnt stimulation (Logan and Nusse, 2004). For example, during mammalian skeletal muscle regeneration, early exposure of myogenic precursor cells to Wnt induces their specification as fibroblasts, whereas later exposure signals a switch from myoblast proliferation to myocyte differentiation (Brack *et al*, 2007, 2008). The observation that the same signal and signalling pathway can elicit many different outcomes underlies two long-standing questions: how is specificity achieved in Wnt signalling response and how does cell identity influence this choice? Despite the intense effort that has informed our current understanding of Wnt signal transduction, the molecular mechanisms that determine how context-specific responses are elicited upon Wnt stimulation remain largely unknown.

The half-life of β-catenin/Armadillo (Arm), an essential transcriptional co-activator, is the primary determinant of Wnt pathway activity (Huang and He, 2008). β-Catenin is targeted for proteasomal degradation by a complex that includes Adenomatous polyposis coli (APC), Axin, and glycogen synthase kinase-3 (GSK3). Wnt stimulation inhibits the activity of this complex, resulting in β-catenin stabilization and nuclear translocation. Nuclear β-catenin interacts with the sequence-specific DNA-binding proteins TCF/LEF (T-cell factor/lymphoid enhancer factor) to activate Wnt target gene transcription (Arce *et al*, 2006). Widely utilized factors that modify chromatin or function in the basal transcription machinery are recruited by β-catenin–TCF to Wnt target gene enhancers, as are the Wnt pathway transcriptional co-factors Pygopus and BCL9/Legless (Mosimann *et al*, 2009).

As the β-catenin–TCF complex is expressed ubiquitously, cell-specific Wnt target gene activation is likely facilitated by other factors; yet, genetic evidence for such factors has been scarce. Here, we describe a genetic screen in Drosophila for components that promote Wingless signalling, and identify a novel nuclear factor, Earthbound 1 (Ebd1), which belongs to a protein family containing Centromere Binding Protein B (CENPB)-type DNA binding domains. Ebd1 is expressed in a restricted subset of Wingless responsive cells and is required for only a limited number of signalling responses. Ebd1 associates with Armadillo/β-catenin and TCF. Two other Drosophila CENPB domain proteins have partially redundant roles with Ebd1. In addition, the human CENPB domain protein Jerky, previously implicated in development of certain forms of juvenile epilepsy, functions as an Ebd1 homologue. Jerky replaces Ebd activity when expressed in flies, promotes Wnt signalling in colon carcinoma cells, stabilizes the β-catenin–TCF complex, and facilitates recruitment of β-catenin to chromatin. These studies provide evidence that tissue-restricted transcriptional co-activators facilitate

*Corresponding author. Department of Genetics, Dartmouth Medical School, Remsen 7400, Hanover NH 03755, USA. Tel.: +1 603 650 1027; Fax: +1 603 650 1188; E-mail: yfa@dartmouth.edu

Received: 13 September 2010; accepted: 10 February 2011; published online: 11 March 2011

cell-specific Wnt signalling responses by modulating β-catenin–TCF activity.

Results

Identification of Ebd1 in a genetic screen for factors that promote Wingless signalling

A gradient of Wingless activity patterns the Drosophila pupal retina, with the highest Wingless levels inducing apoptosis of all photoreceptors at the retinal periphery (Tomlinson, 2003; Lin *et al*, 2004). Our previous work revealed that loss of Adenomatous polyposis coli 1 (Apc1) results in aberrant activation of Wingless signalling in all retinal photoreceptors, thereby inducing their ectopic apoptosis (Figure 1A and B; Ahmed *et al*, 1998; Benchabane *et al*, 2008). This *Apc1* mutant eye phenotype is a sensitive indicator of Wingless pathway activity, as reduction of only two-fold in gene dosage of *arm* or *TCF* is sufficient for suppression of the apoptosis (Ahmed *et al*, 1998; Benchabane *et al*, 2008). Therefore, to identify components that promote Wingless signalling, we performed a genetic modifier screen in which we searched for suppressors of *Apc1* mutant retinal apoptosis. Among the suppressors we identified is a lethal allele of *legless,* which encodes a co-activator in the Armadillo–TCF transcription complex (Kramps *et al*, 2002), validating our approach. We also isolated an allele of a locus we named *earthbound 1* (*ebd1*). Partial suppression of *Apc1* mutant apoptosis is observed in $ebd1^{QF1}$ heterozygotes, whereas nearly complete suppression is present in $ebd1^{QF1}$ homozygotes (Figure 1C). We mapped *ebd1* to the novel gene *CG3371* at cytological position 61C (Supplementary Figure S1A–D). Two large deletions in this region, *Df(3L)4136* and *Df(3L)27-3*, which when

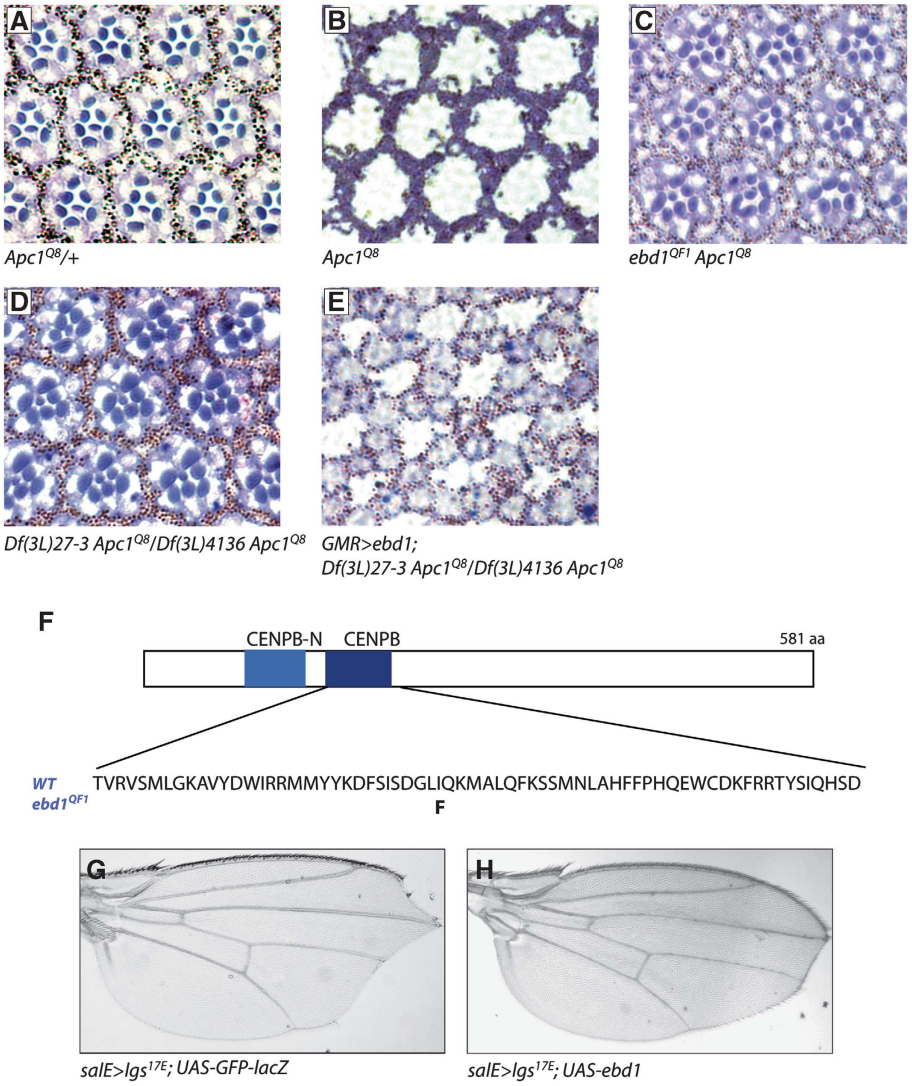

Figure 1 Ebd1 promotes Wingless signalling. (**A–E**) Cross-sections through adult retinas. (**A**) As in wild-type, eight photoreceptors, seven of which are seen in the plane of focus, are present in $Apc1^{Q8}$ heterozygous flies. Each group of photoreceptor cells is surrounded by a pigment cell lattice, identified by small, dark pigment granules. In *Apc1* homozygous mutants, photoreceptors undergo apoptosis (**B**), which is suppressed in $ebd1^{QF1}$ $Apc1^{Q8}$ double mutants (**C**), and in flies transheterozygous for deficiencies *Df(3L)4136* and *Df(3L)27-3* (**D**). (**E**) Expression of *UAS-ebd1* under the control of the eye-specific enhancer *GMR-Gal4* restores apoptosis. (**F**) Schematic representation of Ebd1 with its two putative CENPB-type DNA binding domains, CENPB-N and CENPB (blue). The amino-acid sequence of the second CENPB domain and isoleucine to phenylalanine substitution found in $Ebd1^{QF1}$ are shown. (**G**, **H**) Adult wings in which dominant-negative Lgs^{17E} is expressed under control of the *spalt major enhancer (salE)* are shown. Wings are oriented with proximal left and anterior up. Expression of Lgs^{17E} attenuates Wingless signalling, causing wing notches (**G**), which are rescued by co-expression of Ebd1 (**H**).

transheterozygous eliminate 11 genes including *ebd1*, prevent apoptosis in the *Apc1* mutant (Figure 1D; Supplementary Figure S1A), as does the P element *EY01876*, which is inserted in the *ebd1* 5′ untranslated region (Supplementary Figure S1C–E). Two smaller deletions, *Df(3L)5* and $ebd1^{240}$, which when transheterozygous eliminate only *ebd1*, confirmed the suppressed apoptosis (Supplementary Figure S1E and F). Finally, a transgene encoding only Ebd1, expressed in the retina using UAS/Gal4 (Fischer *et al*, 1988; Brand and Perrimon, 1993), restores apoptosis, confirming identification of the correct gene (Figure 1E).

Ebd1 is a member of a family of proteins containing CENPB-type DNA binding domains (Schultz *et al*, 1998; Letunic *et al*, 2006), named after the sequence-specific DNA-binding protein CENPB (Masumoto *et al*, 1989). Proteins containing CENPB domains are found from yeast to humans and function in diverse processes, including centromere assembly and transcriptional activation (Mojzita and Hohmann, 2006; Okada *et al*, 2007). Many CENPB domain proteins, including Ebd1, contain two tandem homeodomain-like helix-turn-helix domains, termed CENPB-N and CENPB, that are important for DNA binding (Pluta *et al*, 1992; Murakami *et al*, 1996). The *QF1* mutation is located in the second CENPB domain of Ebd1 (Figure 1F), and results in an isoleucine to phenylalanine substitution at position 188, a highly conserved residue.

Ebd1 promotes Wingless signalling

To determine whether Ebd1 promotes Wingless signal transduction or solely promotes photoreceptor apoptosis, we examined the effects of Ebd1 on several other Wingless-dependent processes. A dominant-negative Legless protein, Lgs^{17E}, which is impaired in interaction with Armadillo, attenuates Wingless signalling when expressed in the wing primordium, causing a fully penetrant notched wing phenotype (Figure 1G; Kramps *et al*, 2002; Mosimann *et al*, 2006). Co-expression of Ebd1 rescues this phenotype in all wings analysed, indicating restoration of Wingless transduction (Figure 1H), whereas co-expression of GFP or β-galactosidase has no effect (Figure 1G). We also examined two other phenotypes resulting from ectopic Wingless signalling in the *Apc1* mutant retina: aberrant photoreceptor fate specification, as indicated by ectopic *homothorax* expression (Supplementary Figure S2A–C; Benchabane *et al*, 2008), and aberrant shortening in photoreceptor length (Supplementary Figure S2H and I; Benchabane *et al*, 2008). Inactivation of *ebd1* partially prevents ectopic *homothorax* expression (Supplementary Figure S2D–G) and completely eliminates shortened photoreceptors (Supplementary Figure S2J and K). In addition, ectopic Wingless signalling resulting from *Apc2* inactivation in retinal neurons (Benchabane *et al*, 2008) is prevented by elimination of Ebd1 (Supplementary Figure S2L–N). These data indicate that Ebd1 facilitates Wingless signal transduction.

Ebd1 is expressed in only a subset of Wingless-dependent cell types

To identify cells and developmental stages in which Ebd1 functions, we generated an Ebd1 polyclonal antiserum and immunostained embryonic, larval, and pupal tissues. During embryogenesis, Ebd1 is expressed in neurons, myoblasts and myofibres, glial cells, and nephrocytes (garland cells) (Figure 2A–H; Supplementary Figure S3). In larvae and pupae, Ebd1 expression persists in these same cell types, and is also present in salivary glands (Figure 2I–L; Supplementary Figure S4 and data not shown). We observed a similar tissue-specific pattern with a reporter gene activated by two independently derived *ebd1-GAL4* lines (Figure 2M–O and data not shown). Together, these data reveal cell type-specific Ebd1 expression that is restricted primarily to myocytes, neurons, and glial cells. In contrast with the ubiquitous expression of core Wingless pathway components, Ebd1 is notably absent in ectodermally derived epithelial cells.

Ebd1 is required for indirect flight muscle development, but not for the majority of Wingless-dependent processes

Inactivation of core Wingless pathway components causes widespread aberrant cell fate specification throughout development and characteristic external morphological defects in many adult tissues (Nusslein-Volhard and Wieschaus, 1980; Wieschaus and Riggleman, 1987; Baker, 1988a, b). By contrast, *ebd1* null mutants are viable and display a largely normal exterior morphology, suggesting that defects resulting from *ebd1* loss are restricted to specific tissues or developmental stages. In addition, *ebd1* null mutants have no observable defects in the specification or migration of embryonic RP2 motor neurons (Supplementary Figure S5; Supplementary Table 1), two developmental processes dependent on Wingless signalling (Chu-LaGraff and Doe, 1993; Bhat, 2007). However, we observed that some *ebd1* mutant adults have abnormal wing posture and are unable to fly, and examined their flight musculature to identify the cause. The bulk of adult thoracic muscles are made up of two groups of indirect flight muscles: dorsal longitudinal muscles (DLMs) and dorso-ventral muscles (DVMs) (Figure 3A). Each thoracic hemisegment contains six DLMs and three sets of DVMs. All females and a third of *ebd1* mutant males lack one or more DLMs, with an increased size in remaining DLMs (Figure 3B; Supplementary Table 2). In addition, most *ebd1* mutants have reduced number of DVMs (data not shown).

In addition to Ebd1, the *Drosophila melanogaster* genome encodes four other proteins with CENPB domains (Supplementary Figure S6A). We isolated deletions in each of these genes; even upon simultaneous inactivation of all five CENPB domain proteins, viable flies with no external patterning defects are observed (data not shown). However, we examined ectopic expression of two of these CENPB proteins, encoded by *CG12972* and *CG13895*, and found that they prevent attenuated Wingless signalling resulting from dominant-negative Legless, indicating that they may also promote Wingless transduction in a limited subset of cells (Supplementary Figure S6B and C).

To determine whether there exists functional redundancy between Ebd1 and other CENPB family members, we examined flight muscles in mutants with deletions in these genes. A DLM loss phenotype is also present in 83% of *CG12972/earthbound 2* (*ebd2*) mutant females, but in no males (Figure 3C; Supplementary Table 2). Further, the severity of the *ebd1* mutant muscle loss phenotype, with respect to both penetrance and expressivity, increases upon simultaneous inactivation of *ebd2*. All *ebd1 ebd2* double mutants display DLM loss (Figure 3D; Supplementary Table 2) and loss of flight activity (data not shown). The muscle loss is rescued by

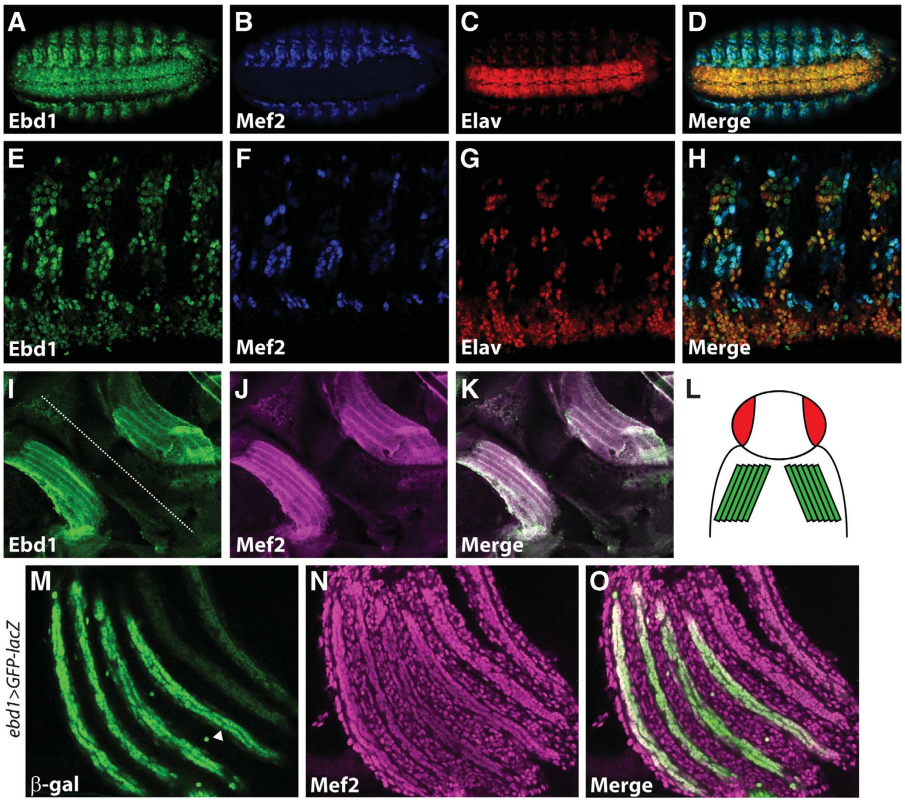

Figure 2 Ebd1 is expressed in neurons and muscles. (**A**–**H**) Confocal images of late embryo stained for Ebd1 (green; **A**, **E**). Myoblasts and myofibres are marked with α-Mef2 (blue; **B**, **F**), and neurons with α-Elav (red; **C**, **G**). Higher magnification is shown in (**E**–**H**) and merged images in (**D**) and (**H**). (**I**–**K**) Confocal images of immunostained pupal thorax, 24 h after puparium formation (APF). Pupa is stained for Ebd1 (green; **I**) and Mef2 (magenta; **J**). The dashed line indicates the midline (**I**). A merged image is shown in (**K**). Ebd1 and Mef2 are expressed in dorsal longitudinal muscle (DLM) fibres and myoblasts. (**L**) Schematic representation of pupa indicating the position of DLMs (green). (**M**–**O**) Confocal images of immunostained DLMs, 22 h APF in which β-galactosidase is expressed under control of the *ebd1* enhancer. Pupa is stained for β-gal (green; **M**) and Mef2 (magenta; **N**). Merged image is shown in (**O**). β-galactosidase is expressed in all six DLM fibres and some myoblasts (arrowhead).

expressing an *ebd1* transgene under control of the *ebd1-Gal4* driver (Figure 3E). Functional redundancy also exists between Ebd1, Ebd2, and the CENPB domain protein encoded by *CG13895* in promoting ectopic Wingless signalling in *Apc1* mutant retinal neurons (Supplementary Figure S6E and F). Together, these data reveal that some redundancy exists in Ebd function, and also confirm the tissue- and stage-specific requirement for Ebd proteins.

Attenuated Wingless signalling and Ebd1 loss result in similar flight muscle defects that are reversed upon Ebd1 overexpression

Previous studies revealed that Wingless expression in a subset of larval wing ectodermal cells promotes proliferation and maintains cell fate in overlying myoblasts, which contribute to adult flight muscle (Sudarsan *et al*, 2001). Indeed, we find that disruption of Wingless transduction in indirect flight muscles, by expression of dominant-negative TCF ($dTCF^{\Delta N}$; van de Wetering *et al*, 1997) in *ebd1*-expressing cells, results in 'held-up' wings, severe reduction in indirect flight muscle size (Figure 3F and I), and loss of flight activity (Figure 3H and K). Similarly, overexpression of the negative regulator Axin in wing disc-associated myoblasts or *ebd1*-expressing cells results in partial to complete loss of indirect flight muscles (Supplementary Figure S7A–C). Most convincingly, reduction in Wingless levels, as found in hypomorphic *wingless* mutants, also results in indirect flight muscle defects (Supplementary Figure S7D and E). Loss of flight muscles and flight activity caused by $dTCF^{\Delta N}$ is rescued by co-expression of a constitutively active Armadillo protein, Arm^{S10} (Pai *et al*, 1997) or Pygopus, whereas expression of GFP or β-galactosidase has no effect (Figure 3F–H and data not shown). Thus, although dominant-negative TCF is markedly reduced in ability to associate with Armadillo (van de Wetering *et al*, 1997), high levels of Armadillo generated by Arm^{S10} expression appear sufficient to promote an Arm^{S10}–$dTCF^{\Delta N}$ interaction, either directly or indirectly, or to promote formation of an Arm^{S10}–endogenous TCF complex that competes effectively for chromatin association. Together, these results indicate that Wingless transduction is required in *ebd1*-expressing myofibres for flight muscle development. In addition, the muscle loss phenotypes observed upon Wingless pathway attenuation are more severe than those observed upon loss of Ebd1 and Ebd2, or even upon simultaneous loss of all five CENPB proteins, indicating that some Wingless-dependent flight muscle development proceeds normally in the absence of Ebd family proteins.

To examine whether Ebd1 can promote Wingless signalling during flight muscle development, we co-expressed Ebd1 and $dTCF^{\Delta N}$ with the *ebd1-GAL4* driver. Upon co-expression of Ebd1, flight muscles appear wild type, and flight activity is restored in all flies examined (Figure 3I–K). We ruled out the possibility that this rescue results from transcriptional repression of the *ebd1-GAL4* driver by Ebd1 (Supplementary Figure

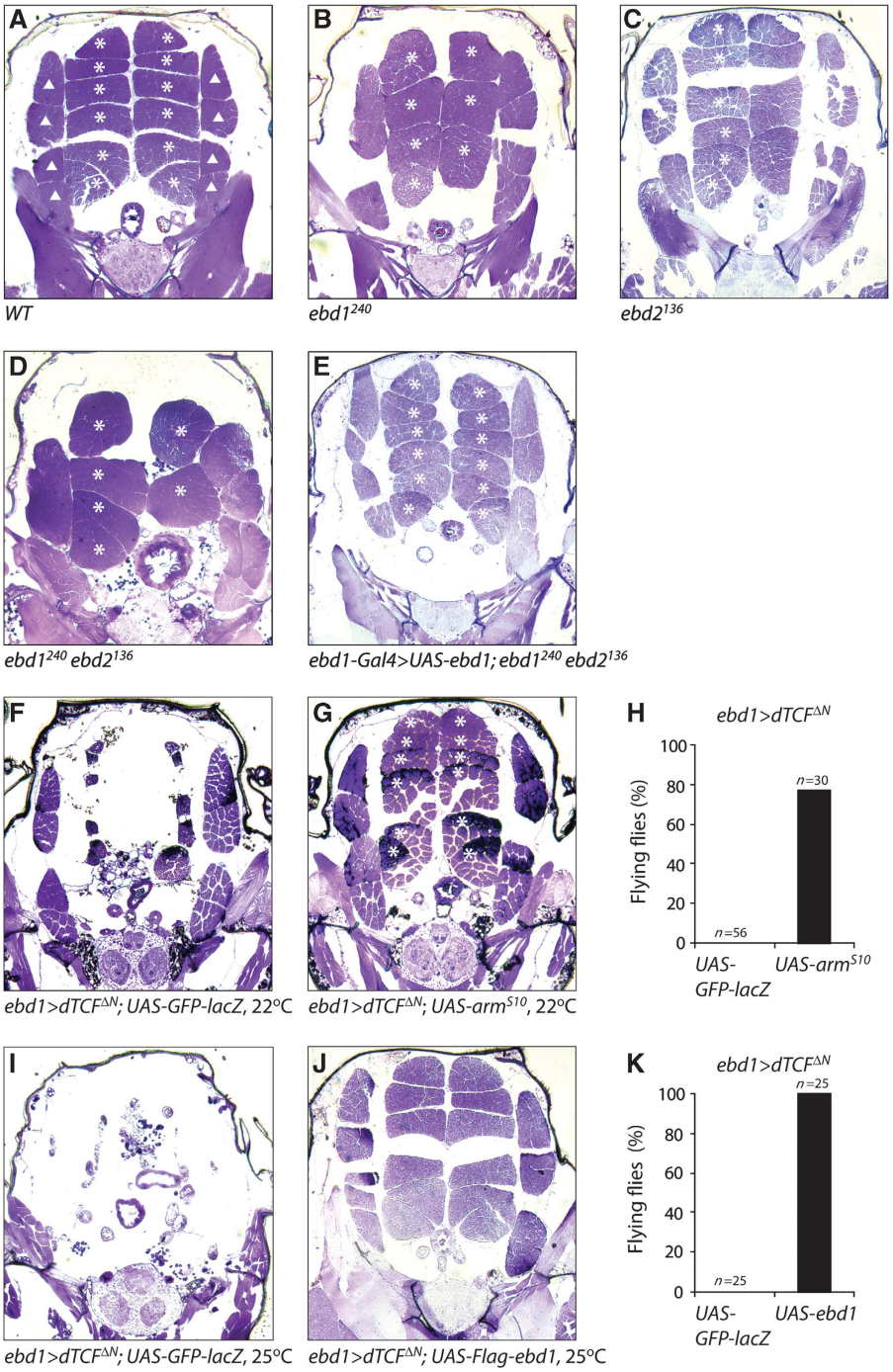

Figure 3 *ebd1*, *ebd2*, and Wingless signalling mutants lack a subset of indirect flight muscles. (**A**–**G**, **I**, **J**) Transverse section of adult thoraces. (**A**–**E**) Dorso-ventral muscles (DVMs, ▲) and dorsal longitudinal muscles (DLMs, *) are indicated. Wild-type (**A**), homozygous $ebd1^{240}$ (**B**), $ebd2^{136}$ (**C**), and $ebd1^{240}$ $ebd2^{136}$ double mutants (**D**). (**E**) Expressing an *ebd1* transgene rescues muscle loss in $ebd1^{240}ebd2^{136}$ double mutants. (**F**–**K**) Attenuation of Wingless signalling in *ebd1*-expressing cells reduces the size and number of indirect flight muscles, resulting in loss of flight activity. This is rescued by expression of Arm^{S10} or Ebd1. (**F**–**H**) Adult females expressing dominant-negative $dTCF^{\Delta N}$ protein, under control of the *ebd1* enhancer were raised at 25°C until larval third instar and then 22°C until eclosion. Co-expression of GFP and β-galactosidase (**F**) or Arm^{S10} (**G**) is shown. DLMs (*) are indicated in (**G**). (**H**) Flight assay of $ebd1>dTCF^{\Delta N}$; *UAS-GFP-lacZ* and $ebd1>dTCF^{\Delta N}$; $UAS\text{-}arm^{S10}$ adult flies. Number of flies examined (*n*) is indicated. (**I**–**K**) Adult $ebd1>dTCF^{\Delta N}$ males raised at 25°C; co-expression of GFP and β-galactosidase (**I**) or Ebd1 (**J**) is shown. (**K**) Flight assay of *ebd1-Gal4>UAS-*$dTCF^{\Delta N}$; *UAS-GFP-lacZ* and *ebd1-Gal4>UAS-*$dTCF^{\Delta N}$; *UAS-ebd1* adult flies.

S8A). The similarity between some muscle defects in *ebd1* mutants and by inhibition of Wingless signalling in *ebd1*-expressing cells, as well as the ability of Ebd1 to rescue this attenuated Wingless signalling, suggest that *ebd1* mutant muscle loss results from disruption of Wingless transduction.

Ebd1 acts downstream of the Apc/Axin/GSK3 destruction complex

To determine at what level in the Wingless pathway Ebd1 functions *in vivo*, we used the Apc/Axin/GSK3 destruction complex as a reference point in genetic epistasis. Ectopic

Wingless signalling in retinal neurons resulting from inactivation of either Apc1 or Apc2 is prevented by Ebd1 loss (Figure 1; Supplementary Figures S1 and S2), indicating that Ebd1 acts downstream of the destruction complex. This conclusion is supported by the observation that Ebd1 loss prevents ectopic Wingless signalling resulting from expression of constitutively active Armadillo (Arm^{S10}), which cannot be targeted for degradation by the destruction complex. Wild-type photoreceptors extend the entire length of the retina, from lens to base (Wolff and Ready, 1993). By contrast, ectopic Wingless signalling resulting from Arm^{S10} shortens photoreceptor length; photoreceptors are detected apically, but absent basally (Figure 4A and B; Benchabane *et al*, 2008). Ebd1 inactivation prevents this morphological defect (Figure 4C and D). These results indicate that Ebd1 promotes Wingless signalling downstream of Apc/Axin/GSK3.

To rule out the possibility that promotion of Wingless signalling by Ebd1 is due to a general effect on transcription, or a specific effect on the transcription of Wingless pathway components, we analysed expression of *Glycerol-3-phosphate dehydrogenase*, *dTCF*, *Arm*, and *Pygo* in flies lacking *ebd1* and *ebd2*. The expression level of these genes is not reduced in *ebd1 ebd2* double mutants, indicating that Ebd does not promote Wingless signalling through an indirect effect on transcription (Supplementary Figure S8B–E).

Human Jerky shares sequence similarity with Ebd1 and can replace Ebd1 function

We next sought to determine whether there exist any human homologues of Ebd1. The human genome encodes 17 proteins containing at least one CENPB domain, and 13 proteins that contain two tandem CENPB domains, including CENPB, TIGGER transposable element derived 1 (TIGD1) through TIGD7, and Jerky (JRK/JH8) (SMART analysis; Letunic *et al*, 2006; Schultz *et al*, 1998). We examined several human CENPB domain proteins to determine whether they are able to activate the Wnt signalling transcriptional reporter TOPFLASH (Korinek *et al*, 1997) in the Wnt responsive cell line HEK293T. Human Jerky increases TOPFLASH reporter activity by two- to three-fold in response to Wnt3a addition, whereas CENPB, TIGD1, TIGD2, and TIGD4 do not (Figure 5A and B and data not shown). By contrast, Jerky does not activate FOPFLASH, a control reporter containing mutated TCF-binding sites, indicating specificity of the Jerky-mediated TCF reporter activation (Figure 5B).

To determine whether human Jerky is functionally homologous to Ebd1, we expressed Jerky in flies under control of the *ebd1* promoter, and examined the ability of Jerky to rescue flight muscle defects in *ebd1 ebd2* double mutants. Whereas *ebd1 ebd2* mutants display muscle loss defects with 100% penetrance (Figure 3D; Supplementary Table 2), expression of Jerky rescues this phenotype, but expression of GFP or β-galactosidase does not (Figure 5C and data not shown). Thus, human Jerky can compensate for Ebd1 and Ebd2 loss, suggesting that Jerky is a human homologue of Ebd.

Endogenous human Jerky promotes Wnt transduction in colon carcinoma cells

In support of our genetic studies, human Jerky was identified as a positive modulator of β-catenin–TCF-mediated transcription in a high-throughput RNA interference (RNAi) screen in colon carcinoma cells (Major *et al*, 2008); this work revealed that Jerky facilitates activation of a TCF reporter, as well as several endogenous β-catenin–TCF target genes. We independently confirmed these conclusions and performed a number of additional controls for specificity. Indeed, we find that Jerky is expressed in the *APC* mutant colon carcinoma cell line DLD-1 (Figure 5D), and that Jerky-specific small interfering RNAs (siRNAs) reduce levels of endogenous and

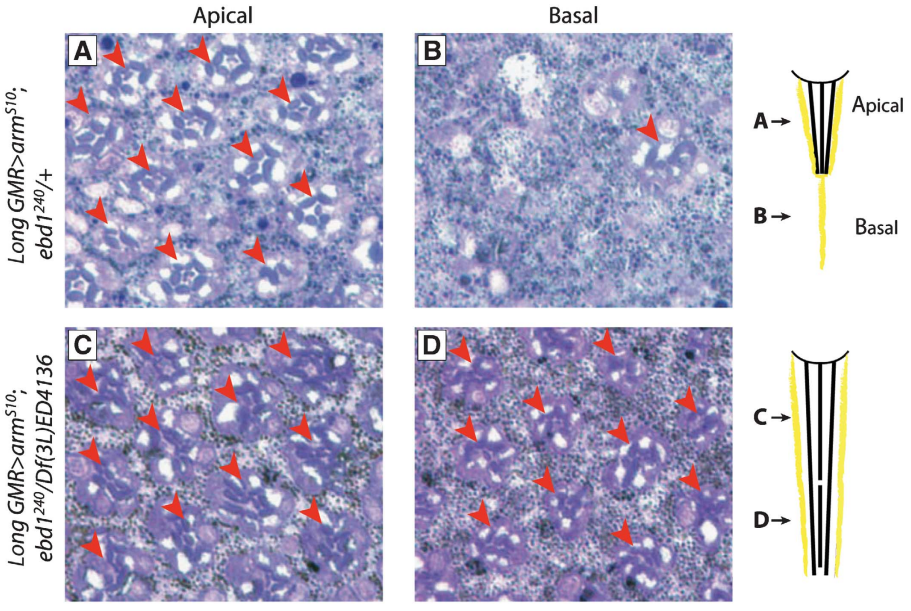

Figure 4 *ebd1* functions downstream of Axin/Apc. (**A**–**D**) Cross-section through adult retinas expressing Arm^{S10} under control of the eye-specific *long GMR* enhancer is shown. Schematic representations of transverse sections through retina with photoreceptors (black) and pigment cells (yellow) are shown on right. Locations of cross-sections presented in (**A**–**D**) are indicated. (**A**, **B**) Photoreceptors (arrowheads) are present at apical (**A**), but not basal (**B**) levels of arm^{S10} retina with two wild-type copies of *ebd1* (data not shown), and in arm^{S10} flies heterozygous for $ebd1^{240}$. (**C**, **D**) Complete loss of *ebd1* prevents the shortening of photoreceptors, which are now present at apical (**C**) and basal (**D**) levels.

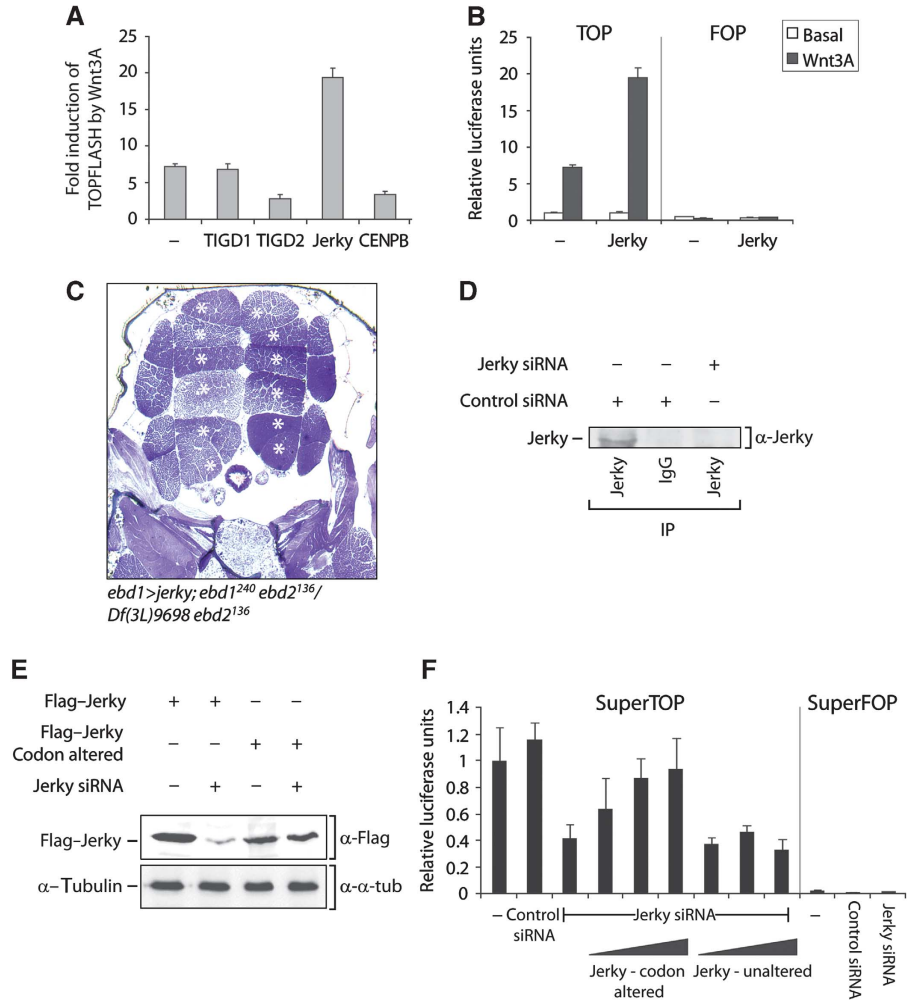

Figure 5 The human CENPB domain protein Jerky promotes Wnt signalling. (**A**, **B**) Jerky overexpression enhances Wnt3A-dependent transcriptional activation in HEK293T cells, as measured using the TOPFLASH luciferase reporter. (**A**) Results are expressed as ratio of Wnt3A dependent to basal induction of TOPFLASH. Human Jerky enhances Wnt3A-dependent activation of TOPFLASH, whereas TIGD1, TIGD2, and CENPB do not. (**B**) Basal and Wnt3A-dependent induction of TOPFLASH by Jerky are shown. Jerky has no effect on the control reporter FOPFLASH. (**C**) Jerky expression rescues muscle defects in *ebd1 ebd2* double mutants. Transverse adult thoracic section showing 12 DLMs (*) with wild-type morphology when *jerky* is expressed in *ebd1 ebd2* double mutants. (**D**) *jerky* siRNAs reduce Jerky levels in DLD-1 cells. Lysates of cells transfected with *jerky* siRNA or control siRNA were immunoprecipitated with Jerky or control IgG antibodies and immunoblotted with α-Jerky. (**E**) *jerky* siRNA reduces expression of ectopic Jerky in DLD-1 cells. Cells were transfected with siRNA and *FLAG-jerky* cDNA containing endogenous sequence or silent point mutations in the siRNA targeted region. Lysates were immunoblotted with α-Flag or anti-α-tubulin. The expression of Jerky encoded by unmodified cDNA is reduced upon siRNA addition, whereas expression of Jerky encoded by mutant cDNA, containing silent point mutations in the region targeted by siRNA, is not affected. (**F**) siRNA-mediated knockdown of *jerky* reduces SuperTOPFlash luciferase activity in DLD-1 human colonic carcinoma cells, whereas a control siRNA does not. SuperTOPFlash activity in cells transfected with *jerky* siRNA is rescued with *jerky* cDNA containing silent point mutations in the region targeted by siRNA. Jerky encoded by an unmodified cDNA does not rescue SuperTOPFlash. siRNAs have no effect on the control reporter SuperFOPflash.

transfected Jerky in DLD-1 cells (Figure 5D and E), whereas scrambled control siRNAs have no effect (Figure 5D). siRNA-mediated knockdown of Jerky in DLD-1 cells reduces activity of the Wnt transcriptional reporter SuperTOPFlash, whereas scrambled siRNAs (control siRNAs) have no effect (Figure 5F). By contrast, *jerky* siRNAs have no effect on the control reporter SuperFOPFlash (Figure 5F). Further, co-transfection of *jerky* cDNA containing silent mutations in the region targeted by the siRNAs ('codon-altered Jerky'), and thus refractory to siRNA-mediated knockdown (Figure 5E), prevents the RNAi-mediated decrease in SuperTOPFlash reporter activity, whereas co-transfection of *jerky* cDNA with endogenous sequences does not (Figure 5F), confirming *jerky* siRNA specificity and ruling out off-target effects. Coupled with previous findings (Major *et al*, 2008), these data indicate that endogenous human Jerky promotes Wnt signalling in *APC* mutant colon carcinoma cells.

Jerky and Ebd1 associate with components of the β-catenin–TCF transcription complex

As Ebd1 is a nuclear protein that promotes Wingless signalling downstream of the destruction complex, we hypothesized that Ebd1 and Jerky might function in the β-catenin–Lef/TCF–BCL9–Pygopus transcription complex. To test this possibility, we expressed FLAG–Jerky and Myc–β-catenin, HA–Pygo2, or BCL9 in HEK293T cells. Co-immunoprecipitation revealed that Jerky associates with β-catenin, Pygopus, and Lef1, but not BCL9 (Figure 6A–C; Supplementary Figure S10A). Importantly, we confirmed these interactions with endogenous Jerky. Interaction of endogenous human Jerky

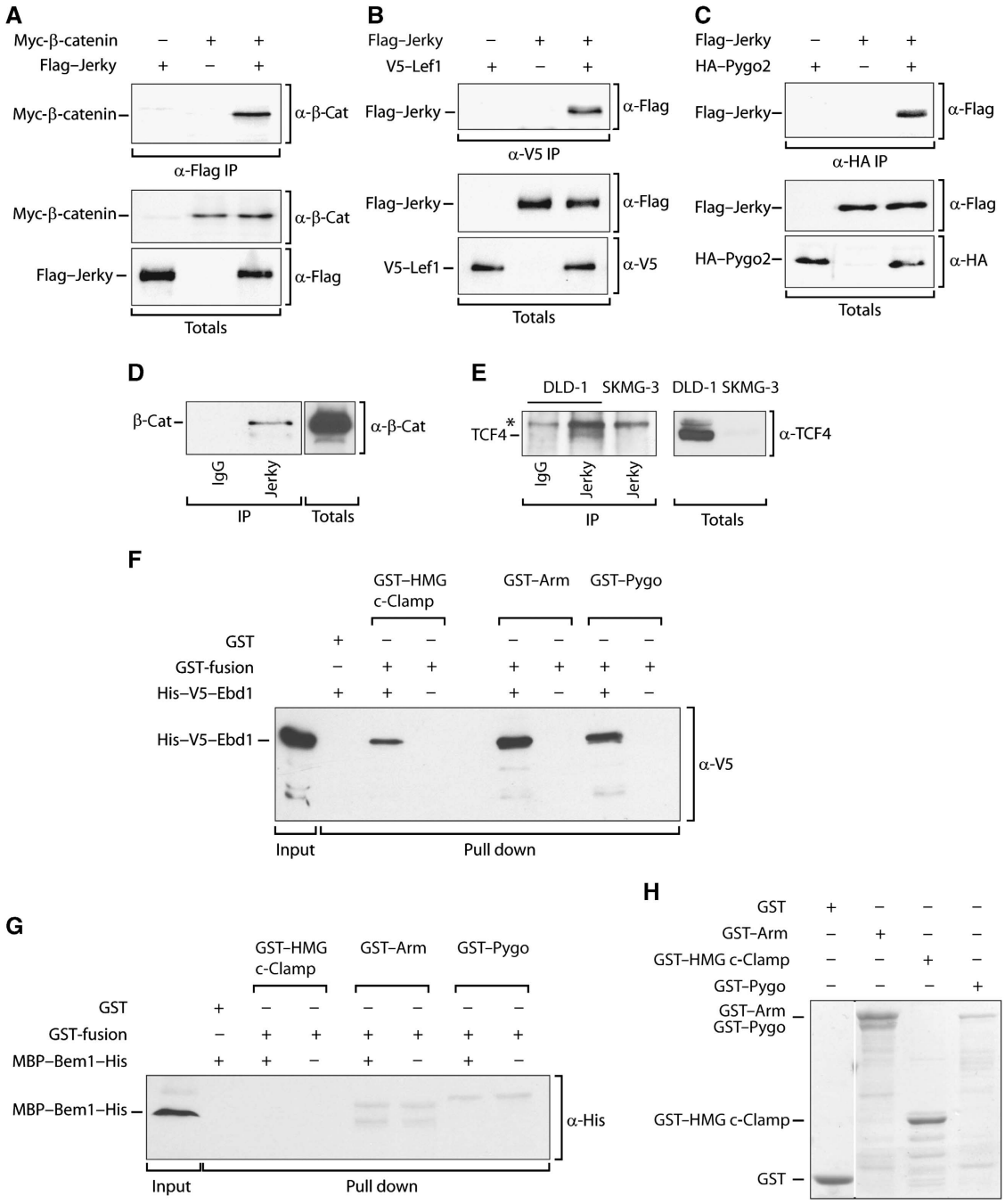

Figure 6 Jerky and Ebd1 interact with β-catenin/Armadillo, TCF/Lef1, and Pygopus. (**A–C**) HEK293T cells. (**A**) Jerky interacts with β-catenin. Lysates from cells transfected with FLAG–Jerky and Myc–β-catenin were immunoprecipitated with α-FLAG and immunoblotted with α-β-catenin. (**B**) Jerky interacts with Lef1. Lysates from cells transfected with FLAG–Jerky and V5–Lef1 were immunoprecipitated with α-V5 and immunoblotted with α-Flag. (**C**) Jerky interacts with Pygopus 2 (Pygo2). Lysates from cells transfected with FLAG–Jerky and HA–Pygo2 were immunoprecipitated with α-HA and immunoblotted with α-FLAG. (**D**) Endogenous Jerky interacts with β-catenin. DLD-1 cell lysates were immunoprecipitated with Jerky or control IgG antibodies and immunoblotted with anti-β-catenin. (**E**) Endogenous Jerky interacts with TCF4. DLD-1 or SKMG-3 cell lysates were immunoprecipitated with Jerky or control IgG antibodies, and immunoblotted with α-TCF4. The position of a non-specific band is indicated by (*). The TCF4-specific band is not observed in SKMG-3 immunoprecipitates. (**F–H**) Ebd1 interacts directly with dTCF, Arm, and Pygo. Bacterially expressed His–V5-tagged Ebd1 (**F**) or MBP–Bem1–His (**G**, used as negative control) was purified and incubated with bacterially expressed and purified GST, GST–HMG–C-clamp, GST–Arm, or GST–Pygo. Proteins bound to the GST fusion proteins were detected by immunoblotting with anti-V5 (**F**) or anti-His (**G**) antibodies. (**H**) Coomassie staining of GST fusion proteins.

with β-catenin and TCF4 was examined in DLD-1 colon carcinoma cell lysates, and as a control for specificity, SKMG-3 glioblastoma cell lysates, in which TCF4 is not expressed (Figure 6D and E). Jerky is immunoprecipitated from DLD-1 lysates with Jerky antiserum, but not an IgG control. Immunoblotting of proteins co-precipitating with Jerky revealed that Jerky interacts with β-catenin and TCF4 (Figure 6D and E). By contrast, β-catenin and TCF4 are not detected in IgG control immunoprecipitates, and TCF4 is not detected in Jerky antiserum immunoprecipitates from SKMG-3 control lysates (Figure 6E). As additional controls for specificity, we examined TIGD4 and CENPB, two human

CENPB proteins that do not activate the TCF reporter TOPFLASH, and detected no interaction with β-catenin, Lef1, or Pygopus (Supplementary Figure S10E–J). These studies indicate that endogenous Jerky associates with β-catenin and TCF, and likely functions in the β-catenin–TCF complex.

To test whether Ebd1 also associates with components of the Armadillo–TCF–Legless–Pygopus complex, we expressed FLAG–Ebd1 and HA–Armadillo, HA–Pygopus, V5–Legless, or Myc–dTCF in HEK293T cells. Immunoprecipitation with anti-FLAG antibodies revealed that Ebd1 associates with Armadillo, Pygopus, and dTCF, but not Legless (Supplementary Figures S10C and S11A–C). These results parallel the interactions of Jerky with β-catenin, TCF4, and Pygopus, but not BCL9. We confirmed these results using glutathione-S-transferase (GST) interaction assays. Armadillo and Pygopus bind to GST–Ebd1 (Supplementary Figure S11D and E), and Ebd1 binds to a GST fusion protein containing the HMG and C-clamp domains of dTCF (Chang *et al*, 2008b) (Supplementary Figure S11F). Moreover, bacterially produced His–Ebd1, but not an unrelated His-tagged protein (His–Bem1) (Xu and Wickner, 2006), associates with GST–Arm, GST–Pygo, and GST–HMG–C-clamp, suggesting that these protein interactions are direct (Figure 6F–H).

Separable binding sites mediate β-catenin–Lef1–Jerky interaction

To test the model that β-catenin, Jerky, and Lef1 form a complex, and do not compete for binding to each other, we identified regions in TCF/LEF and Jerky important for their association by expressing Flag-tagged Lef1 fragments and V5–Jerky in HEK293T cells. Co-immunoprecipitation experiments indicate that the amino-terminal half of the Lef1 HMG domain is sufficient for interaction with Jerky (Supplementary Figure S12A and B), paralleling the interaction between Ebd1 and the HMG domain of Drosophila TCF (Supplementary Figure S11F). Thus, the binding site for Jerky at the Lef1 carboxy-terminus is distinct from the binding site for β-catenin at the Lef1 amino-terminus (Arce *et al*, 2006).

To map domains in Jerky mediating association with β-catenin and Lef1, we performed *in vitro* pull-down experiments with bacterially expressed GST–Jerky fragments (Liu *et al*, 2003). β-catenin and Lef1 interact with full-length GST–Jerky, but not Jerky fragments lacking the amino-terminus (Supplementary Figure S12C and D). Deletions of the Jerky carboxy-terminus do not disrupt Lef1 binding, but markedly attenuate β-catenin binding. Thus, the first 118 amino acids of Jerky, encompassing both CENPB domains, are sufficient for Lef1 binding. By contrast, association of β-catenin with Jerky is dependent on both amino-terminal and carboxy-terminal regions in Jerky. These data indicate that β-catenin, Lef1, and Jerky likely do not compete for binding, as the β-catenin and Lef1-binding sites in Jerky are partially separable, and the β-catenin and Jerky-binding sites in Lef1 are completely separable, supporting the model that Lef1/TCF, Jerky, and β-catenin can form a complex.

The QF1 mutation in the Ebd1 CENPB domain disrupts Ebd1 intracellular localization, interaction with Wingless pathway components, and activity

The *ebd1*^{QF1} mutant isolated in our genetic screen contains an isoleucine to phenylalanine substitution at a highly conserved residue of the CENPB domain. To examine how this mutation disrupts Ebd1 function, we analysed the subcellular localization of epitope-tagged wild-type Ebd1 and Ebd1^{QF1} proteins. When ectopically expressed in the embryonic ectoderm, Ebd1 is primarily nuclear (Supplementary Figure S13B–E), whereas Ebd1^{QF1} is present at higher levels in the cytoplasm (Supplementary Figure S13F–I), indicating that the isoleucine to phenylalanine substitution results in aberrant cytoplasmic retention or nuclear export. We confirmed this intracellular localization with endogenous embryonic Ebd1 and Ebd1^{QF1} proteins (Figure 7A–F).

We also investigated whether interaction of Ebd1 with Wingless pathway components is disrupted by the *QF1* mutation. Pull-down experiments with bacterially expressed GST-Ebd1 and GST-Ebd1^{QF1} revealed a marked reduction in the ability of Ebd1^{QF1}, as compared with Ebd1, to interact with Arm, dTCF, and Pygo (Figure 7G–J). Thus, alteration of a single residue in the second CENPB domain disrupts Ebd1 nuclear localization as well as its ability to interact with Wingless pathway components, likely explaining the impaired function of Ebd1^{QF1}.

Human Jerky acts as a bridge that promotes association of β-catenin with Lef1 and recruitment of β-catenin to chromatin

The ability of Jerky to interact with Lef1 and β-catenin raised the possibility that Jerky could act as a bridge to promote or stabilize association of Lef1 with β-catenin. To test this hypothesis, we examined whether Jerky enhances association of β-catenin with Lef1. We expressed Flag–Lef1 and a β-catenin–luciferase fusion protein in HEK293T cells in the presence or absence of Jerky. The association of β-catenin–luciferase with FLAG–Lef1 in anti-FLAG immunoprecipitates was detected by measuring light emission (Barrios-Rodiles *et al*, 2005; Miller *et al*, 2009) (Figure 8A–C). The level of β-catenin–luciferase bound to Lef1 increases significantly in the presence of Jerky, suggesting that Jerky facilitates β-catenin–Lef1 association.

To determine whether the Jerky-enhanced interaction of β-catenin with Lef1 promotes recruitment of β-catenin to chromatin, we performed subcellular fractionation of HEK293T cell lysates (Figure 8D). Nuclear extracts were fractionated into chromatin-bound protein and soluble nuclear protein pools (Mendez and Stillman, 2000). The effective separation of chromatin-bound and nuclear soluble fractions was confirmed by analysing the distribution of histone H3 and the transcription factor Ets1 (Figure 8D). Jerky results in a Lef1-dependent increase in levels of chromatin-associated β-catenin. In addition, the level of chromatin-associated Lef1 does not decrease in the presence of Jerky, suggesting that the Jerky–Lef1 association does not disrupt interaction of Lef1 with its cognate DNA-binding sites. These data indicate that Jerky enhances recruitment of β-catenin to chromatin, and coupled with the protein association studies, provide evidence for a chromatin-associated Lef1–Jerky–β-catenin complex.

The Lef1 dependence of Jerky's ability to promote the recruitment of β-catenin to chromatin suggests that Jerky can act as a bridge that promotes association of β-catenin and Lef1 in a manner independent of the known direct interaction between these two proteins. To determine whether Jerky can mediate a β-catenin–Lef1 interaction even when direct

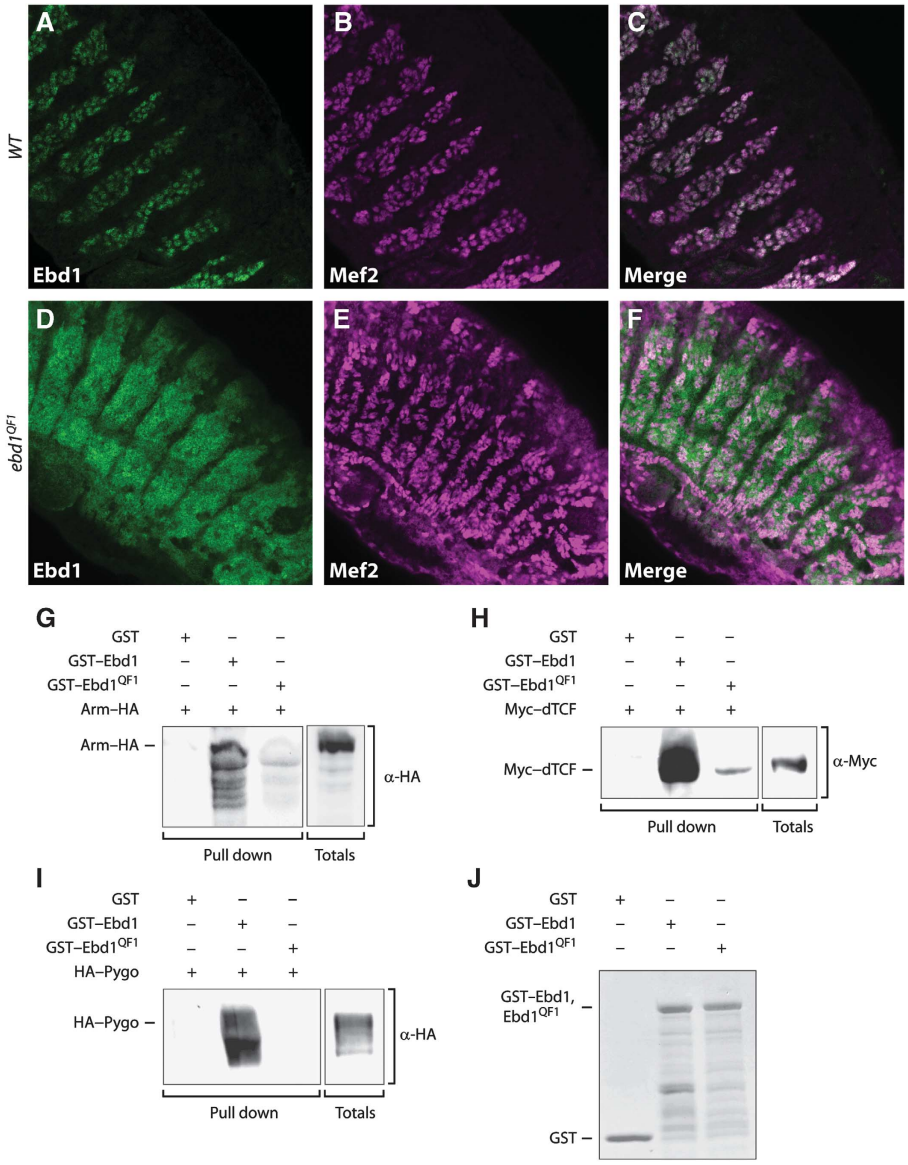

Figure 7 The *QF1* mutation in the CENPB domain results in mislocalization of Ebd1 and decreased association of Ebd1 with Arm, dTCF, and Pygo. (**A**–**F**) Confocal images of late wild-type (**A**–**C**) and *ebd1*^{QF1} mutant embryo (**D**–**F**) showing Ebd1 expression (green; **A**, **D**) in myoblasts. The nucleus is demarcated by Mef2 staining (magenta; **B**, **E**). Merged images are shown in (**C**) and (**F**). (**G**–**J**) Bacterially expressed GST–Ebd1, GST–Ebd1^{QF1}, or the GST control were purified and incubated with lysates from HEK293T cells transfected with HA-tagged Arm (**G**), Myc-tagged dTCF (**H**), or HA-tagged Pygo (**I**). Proteins bound to the GST fusion proteins were detected by immunoblotting with anti-HA (**G**, **I**) or anti-Myc (**H**) antibodies. (**J**) Coomassie staining of GST fusion proteins.

interaction between Lef1 and β-catenin is disrupted, we expressed FLAG–Lef1 protein lacking the amino-terminal region required for β-catenin association, but retaining ability to associate with Jerky (FLAG–Lef1-ΔN), along with V5–Jerky, in HEK293T cells. FLAG–Lef1-ΔN/V5–Jerky complexes were immunoprecipitated using anti-FLAG antibodies and subsequently incubated with HEK293T cell lysates expressing Myc–β-catenin. Myc–β-catenin interacts with immunoprecipitated complexes containing both Lef1-ΔN and Jerky but not with those containing only Lef1-ΔN, indicating that Jerky can mediate an interaction between β-catenin and Lef1-ΔN (Figure 8E). These findings indicate that Jerky can indeed form a bridge between β-catenin and Lef1, and promotes or stabilizes their association independently of the direct β-catenin–Lef1 interaction.

Human Jerky promotes Wnt signalling in vivo by facilitating β-catenin–TCF complex formation

Thus, even when the Lef1 β-catenin binding site is disrupted, Jerky retains ability to promote interaction of β-catenin with Lef1. Therefore, we hypothesized that in certain *in vivo* contexts, Jerky may facilitate β-catenin–TCF transcriptional activity by bridging β-catenin and TCF and stabilizing their interaction. To test this hypothesis, we examined whether Jerky rescues flight muscle defects resulting from dTCF$^{\Delta N}$, which contains a deletion of the Armadillo/β-catenin-binding site. Indeed, whereas overexpression of dTCF$^{\Delta N}$, a dominant-negative variant of dTCF which contains a deletion of the Armadillo/β-catenin-binding site, results in severe loss of indirect flight muscles, this phenotype is almost completely prevented upon co-expression of Jerky in the same cells

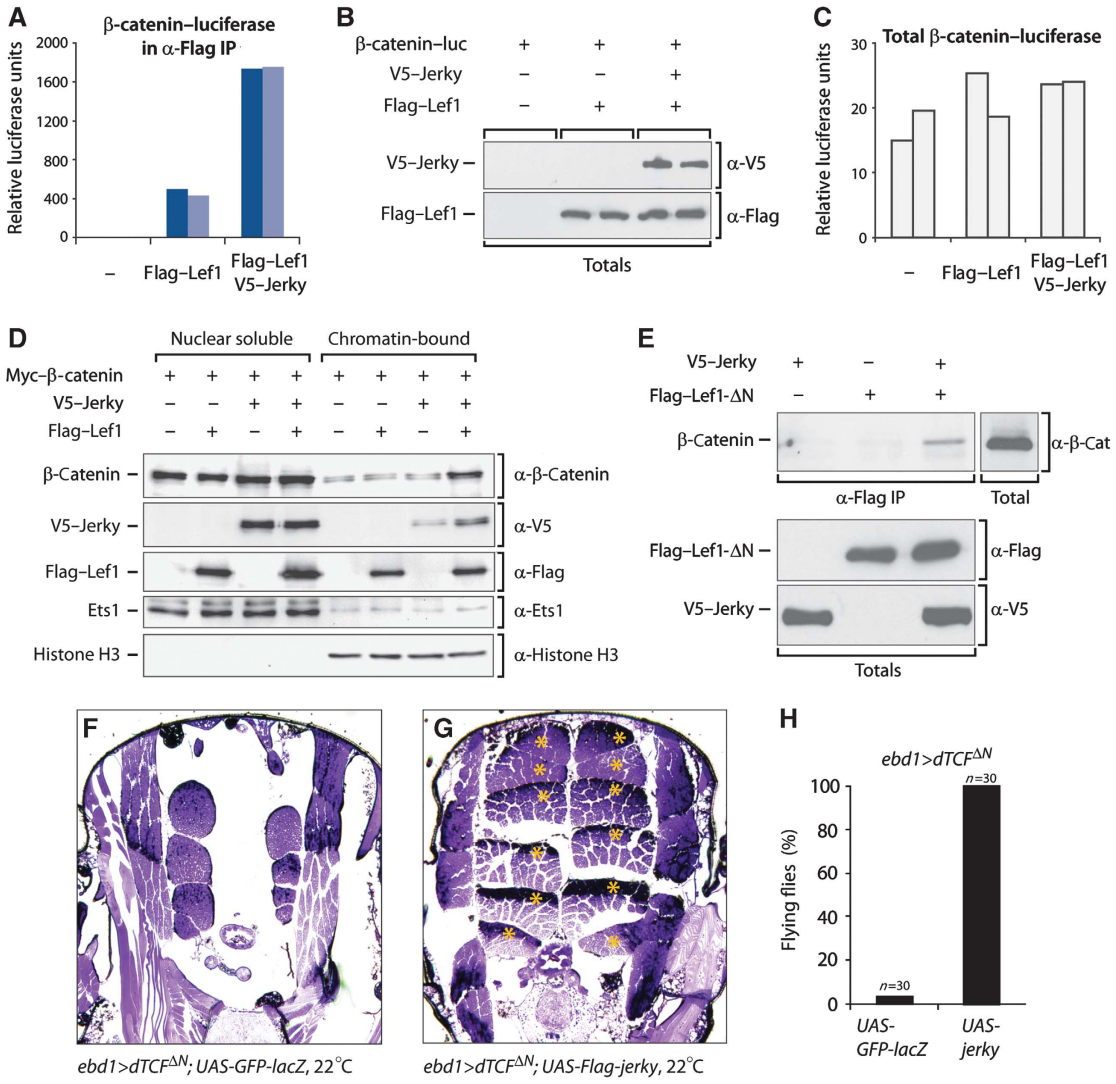

Figure 8 Jerky facilitates interaction of β-catenin with Lef1 and association of β-catenin with chromatin. (**A**–**C**) Jerky expression increases association between Lef1 and a β-catenin–luciferase fusion protein. Lysates from HEK293T cells transfected with β-catenin–luciferase and FLAG–Lef1, in the presence or absence of V5–Jerky, were immunoprecipitated with α-FLAG. β-catenin in immunoprecipitates was detected by measuring luciferase activity (**A**). Interaction of β-catenin with FLAG-tagged Lef1 increased significantly in the presence of Jerky. All transfections were performed in duplicate, as represented by the light and dark blue bars in (**A**), and the dual samples in (**B**) and (**C**). (**B**) Immunoblotting of total lysates with α-FLAG or α-V5. (**C**) β-Catenin–luciferase levels in total cell lysates, as assayed by measuring luciferase activity. To obtain comparable β-catenin levels, half of the β-catenin encoding plasmid was transfected in cells co-transfected with V5–Jerky. (**D**) Jerky increases association of β-catenin with chromatin in a Lef1-dependent manner. HEK293T cells transfected with Myc–β-catenin, FLAG–Lef1 and V5–Jerky were fractionated into nuclear soluble and chromatin-bound fractions. Normalized aliquots of each extract were analysed by immunoblotting. (**E**) Jerky mediates an interaction between β-catenin and Flag–Lef1-ΔN. The first 33 amino acids in Lef1, required for direct interaction with β-catenin, are eliminated in Flag–Lef1-ΔN. Lysates from HEK293T cells transfected with FLAG–Lef1-ΔN and V5–Jerky were immunoprecipitated with α-FLAG. Protein G-bound immunoprecipitated complexes were then incubated with cell lysates expressing Myc–β-catenin. β-Catenin interacting with the Protein G-bound complexes was detected by immunoblotting. Immunoblotting of aliquots of total lysates is shown. (**F**–**H**) Expression of human *jerky* in Drosophila rescues muscle loss in *ebd1>dTCF*$^{\Delta N}$ adults. Transverse thoracic sections of adult females raised at 22°C are shown in (**F**) and (**G**). (**F**) DLMs are absent or reduced in size in *ebd1>dTCF*$^{\Delta N}$ flies. (**G**) All 12 DLMs (*) are of normal size when *jerky* is co-expressed. (**H**) Expression of *jerky* rescues flight defects in *ebd1>dTCF*$^{\Delta N}$ flies. Number of flies analysed is indicated (*n*).

(Figure 8F–H). All DVMs and DLMs are recovered and flight activity is restored in all flies examined. These data support the model that Jerky promotes Wnt signalling by acting as a bridge to facilitate interaction between β-catenin and TCF. Furthermore, overexpression of Ebd1 also rescues the *dTCF*$^{\Delta N}$ muscle loss phenotype (Figure 3I–K), suggesting that Jerky and Ebd1 promote Wnt signalling in an analogous manner, and providing further evidence that Jerky functions as an Ebd1 homologue. Together, our data indicate that Jerky and Ebd1 facilitate Wnt/Wingless signalling by promoting β-catenin–TCF-dependent transcription.

Discussion

Restricted Ebd1 expression facilitates cell-specific Wingless signalling responses

How does cell identity influence transcriptional activation of Wnt/Wingless target genes? Starting with an unbiased

genetic screen for components that facilitate Wingless signalling, we have discovered an unanticipated role for the novel Drosophila CENPB domain protein Ebd1 in promoting cell-specific responses to Wingless signalling. Ebd1 is required for Wingless transduction in a restricted developmental context, and is expressed in only a subset of cells in which Wingless transduction is active, indicating that Ebd1 functions as a context-specific facilitator, and not as a general component in the Wingless pathway. Indeed, the absence of Ebd1 expression in many tissues is likely critical for proper development, as our attempts to ectopically express Ebd1 using several tissue-specific promoters resulted in cell and/or organismal lethality (HB and YA, unpublished data). Further, within Ebd1-expressing cells, some Wingless-dependent developmental processes proceed normally upon Ebd loss, suggesting that only a subset of Wingless target genes is dependent on Ebd. Specifically, flight muscle loss resulting from Wingless pathway inactivation is more severe than that resulting from Ebd loss, even upon simultaneous elimination of all five Drosophila CENPB proteins. Together, our data provide genetic evidence that cell-specific responses to Wingless signalling are mediated in part by tissue-specific co-factors that modulate activity of the Armadillo/TCF transcription complex.

Ebd is a cell-specific adaptor for Armadillo and TCF

Our *in vivo* epistasis experiments support the model that Ebd1 functions at the level of the Armadillo–TCF transcriptional complex, and our *in vitro* data indicate that Jerky and Ebd1 contribute to Wnt/Wingless target genes activation by enhancing β-catenin–TCF complex formation and β-catenin recruitment to chromatin. Thus, Jerky and Ebd likely serve an adaptor function similar to that previously documented for human transducin β-like protein 1 (TBL-1) (Li and Wang, 2008). However, by contrast with TBL-1, which promotes activation of all Wnt target genes analysed, only certain Wingless-dependent developmental events (and by extension, associated target genes) require Ebd. How does an Arm–TCF adaptor protein such as Ebd1 promote the transcription of only a subset of Wingless target genes? Our data are consistent with two distinct models. Ebd may act solely as an Arm–TCF adaptor, such that Wingless target genes requiring relatively high Arm–TCF complex levels may be dependent on Ebd for activation, whereas activation of target genes requiring lower Arm–TCF levels may be Ebd independent. Alternatively, given the presence of its CENPB DNA binding domains, Ebd1 may provide two functions—to act as an Armadillo–TCF adaptor and to also associate with cognate sites in enhancer elements. Thus, Ebd1 DNA-binding specificity may also contribute directly to Wingless target gene selection. The existence of cell-specific factors such as Ebd that modulate Arm–TCF activity was presaged by previous studies, which indicated that binding of DNA by TCF is a regulated process; not all TCF-binding site clusters bind TCF *in vivo*, and TCF binding at a particular locus varies between cell types, suggesting that optimal TCF binding to cognate sites likely requires recruitment by cell-specific transcription co-factors (Parker *et al*, 2008).

Regulation of cell-specific Wnt target gene transcription

Taken together with previous studies, our findings reveal that modulation of Wnt/Wingless responses is facilitated by several distinct modes of transcriptional regulation. For example, recruitment of β-catenin to chromatin can occur in a TCF-independent manner, through association of β-catenin with sequence-specific DNA-binding co-activators that extend the range of Wnt/β-catenin target genes (Kioussi *et al*, 2002; Olson *et al*, 2006; see also MacDonald *et al*, 2009; Notani *et al*, 2010). In addition, cooperative signalling between the Wnt/Wingless pathway and other signal transduction pathways also modulates target gene specificity. For instance, molecular cross-talk at the transcriptional level between the mammalian Wnt pathway and the TGFβ or JNK pathway is mediated by interaction of Lef-1 with Smads and TCF-4 with c-Jun, respectively (Labbe *et al*, 2000; Nishita *et al*, 2000; Nateri *et al*, 2005). Signal transduction pathway cross-talk promotes Wingless target gene activation in Drosophila also (Riese *et al*, 1997; Halfon *et al*, 2000). Further, some modulators of Wingless pathway activity, such as Lines and Split ends/Spenito, do not associate with Armadillo or TCF directly, but instead act downstream or in parallel with Armadillo–TCF to specify cell fate (Hatini *et al*, 2000, 2005; Lin *et al*, 2003; Chang *et al*, 2008a). Our findings reveal that context-dependent responses to Wnt/Wingless stimulation are also facilitated by cell-specific co-factors that interact with both β-catenin and TCF to enhance their activity.

Human Jerky facilitates assembly of the β-catenin–TCF complex and recruitment of β-catenin to chromatin

The human CENPB domain protein Jerky shares functional homology with Ebd1. Mouse Jerky was originally identified as a neuronally enriched protein (Liu *et al*, 2002) in a murine epilepsy model; heterozygous inactivation of *jerky* results in recurrent generalized seizures (Toth *et al*, 1995), whereas homozygous *jerky* inactivation also results in growth and fertility defects (Donovan *et al*, 1997). The subsequent mapping of human Jerky (JH8/JRK) to chromosome 8p24 revealed linkage to a susceptibility locus for juvenile myoclonic epilepsy and childhood absence epilepsy (Zara *et al*, 1995; Morita *et al*, 1998; Moore *et al*, 2001). Recently, a genome-wide RNA interference screen identified human Jerky as an activator of a TCF-dependent reporter as well as endogenous Wnt/β-catenin target genes in colon carcinoma cells (Major *et al*, 2008). Jerky was found to facilitate the activation of a subset, but not all endogenous Wnt/β-catenin target genes examined, paralleling our *in vivo* analysis of Ebd function. Our findings indicate that Jerky facilitates Wnt pathway-dependent transcription by stabilizing the β-catenin–TCF complex and promoting recruitment of β-catenin to chromatin. Our findings also raise the possibility that attenuation of Wnt transduction underlies generalized seizures, as well as growth and fertility defects in mice with reduced Jerky activity, and may contribute to development of certain types of childhood epilepsy.

Materials and methods

Fly stocks

ebd1^{QF1} was isolated in an ethyl methanesulphonate mutagenesis screen (Lewis and Bacher, 1968) for dominant suppressors of photoreceptor apoptosis in the *Apc1*^{Q8} mutant. Other fly lines used are described in Supplementary data.

Crosses were performed at 25°C, unless otherwise indicated.

Plasmids
A detailed description of plasmids is provided in Supplementary data.

Immunohistochemistry and histology
To generate Ebd1 antiserum, full-length His–Ebd1 protein was expressed in bacteria, purified in denaturing conditions, and used as an immunogen in rabbits and guinea pigs. The Ebd1 antiserum was presorbed against 0–4 h wild-type embryos and used at 1:2000 for staining pupal muscles, and 1:4000 for larval brain, imaginal discs, and embryos. Other antibodies, suppliers, and fixation conditions are described in Supplementary data.

Flight assay
Adult flies, at least 2 days post-eclosion, were placed in small groups on a benchtop, and assessed for ability to fly away.

Cell lines and transfection
HEK 293T, DLD-1, and SKMG-3 cells were cultured in Dulbecco's modified medium supplemented with 10% fetal bovine serum. HEK293T cells were transiently transfected using calcium phosphate-DNA precipitation (Hayashi *et al*, 1997) and DLD-1 cells using Lipofectamine 2000 (Invitrogen).

Luciferase assay
HEK293T cells were transiently transfected with TOPFLASH or FOPFLASH firefly luciferase reporter plasmids, and DLD-1 cells with SuperTOPFlash or SuperFOPFlash plasmids. The *CMV-Renilla* reporter was used as an internal control. (For detailed description, see Supplementary data.) Cells were lysed 48 h post-transfection in Passive Lysis Buffer (Promega) and luciferase activity measured using the Dual-Luciferase Reporter Assay System (Promega). Luciferase values were normalized by dividing firefly luciferase values by *Renilla* values. Transfections were performed in triplicate.

Immunoprecipitation, immunoblotting, and subcellular fractionation
HEK293T cells were lysed 48 h post-transfection in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) containing phosphatase and protease inhibitors (25 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 0.01 mg/ml Pepstatin A). HEK293T lysates were fractionated using the Subcellular protein fractionation kit (Thermo Scientific). Fly embryos were dechorinated in 50% bleach, washed in 0.7% NaCl, 0.3% Triton X-100, and homogenized in lysis buffer containing 0.5 mM DTT using a Dounce homogenizer. Antibodies used for immunoprecipitation and immunoblotting are described in Supplementary data. Immunoprecipitates were collected using Protein-A/G PLUS-Agarose beads (Santa Cruz Biotechnology) and separated by SDS–PAGE. The Luciferase Assay System (Promega) was used for light detection of luciferase–β-catenin (Barrios-Rodiles *et al*, 2005).

GST interaction assays
For GST interaction assays, GST–Ebd1 and GST–Ebd1^{QF1} proteins were purified using Glutathione Sepharose 4 Fast Flow beads (GE Healthcare) and incubated with cell lysates from transfected HEK293T cells at 4°C for 4 h. The beads were washed in 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, and associated proteins were detected by immunoblotting with anti-HA or anti-Myc antibody.

To assess direct protein interactions, His–V5–Ebd1 or MBP–Bem1–His were purified from bacterial lysates using TALON metal affinity resins (Clontech). His-fusion proteins were eluted off the resin using 150 mM imidazole and incubated with GST–HMG–C-clamp, GST–Arm or GST–Pygo bound to Glutathione Sepharose 4 Fast Flow beads. After 3 h incubation at 4°C, the beads were washed as described above, and the associated His-fusion proteins detected with anti-V5 or anti-His antibodies.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We thank E Wieschaus for generous help with the mutagenesis screen; A Mehra and K Nguyen for suggesting the name Earthbound; C Pikielny, G Deshpande, S Ogden, A Mehra, and V Ambros for thoughtful comments on the manuscript; K Basler, C Desplan, M Bienz, M de la Roche, R Mann, M Freeman, L Shashidhara, G Struhl, L Attisano, J Wrana, E Verheyen, P Fisher, B Paterson, J DiRenzo, P Ernst, S Tevosian, R DasGupta, R Moon, K Cadigan, W Wicker, H Xu, M Toth, J Fernandes, A Lavanway, H Higgs, M Israel, P Kuzontkoski, K Siago, Drosdel, Kyoto stock center, Bloomington Drosophila Stock Center, and Developmental Studies Hybridoma Bank for reagents, equipment, or technical advice; V Marlar, J Baird, and E Hughes for technical support. This work was supported by Emerald Foundation, Norris Cotton Cancer Center, General Motors Cancer Research Foundation, American Cancer Society (IRG-82-003-21), National Cancer Institute (KO8CA078532 and RO1CA105038), Howard Hughes Medical Institute, through an award to Dartmouth Medical School (76200-560801) and National Science Foundation (DBI-1039423 for purchase of a Nikon A1RSi confocal). FlyBase provided important information used in this work.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Ahmed Y, Hayashi S, Levine A, Wieschaus E (1998) Regulation of armadillo by a Drosophila APC inhibits neuronal apoptosis during retinal development. *Cell* **93:** 1171–1182

Arce L, Yokoyama NN, Waterman ML (2006) Diversity of LEF/TCF action in development and disease. *Oncogene* **25:** 7492–7504

Baker NE (1988a) Embryonic and imaginal requirements for wingless, a segment-polarity gene in Drosophila. *Dev Biol* **125:** 96–108

Baker NE (1988b) Transcription of the segment-polarity gene wingless in the imaginal discs of Drosophila, and the phenotype of a pupal-lethal wg mutation. *Development* **102:** 489–497

Barrios-Rodiles M, Brown KR, Ozdamar B, Bose R, Liu Z, Donovan RS, Shinjo F, Liu Y, Dembowy J, Taylor IW, Luga V, Przulj N, Robinson M, Suzuki H, Hayashizaki Y, Jurisica I, Wrana JL (2005) High-throughput mapping of a dynamic signaling network in mammalian cells. *Science* **307:** 1621–1625

Benchabane H, Hughes EG, Takacs CM, Baird JR, Ahmed Y (2008) Adenomatous polyposis coli is present near the minimal level required for accurate graded responses to the Wingless morphogen. *Development* **135:** 963–971

Bhat KM (2007) Wingless activity in the precursor cells specifies neuronal migratory behavior in the Drosophila nerve cord. *Dev Biol* **311:** 613–622

Brack AS, Conboy IM, Conboy MJ, Shen J, Rando TA (2008) A temporal switch from notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. *Cell Stem Cell* **2:** 50–59

Brack AS, Conboy MJ, Roy S, Lee M, Kuo CJ, Keller C, Rando TA (2007) Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. *Science* **317:** 807–810

Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118:** 401–415

Chang JL, Lin HV, Blauwkamp TA, Cadigan KM (2008a) Spenito and Split ends act redundantly to promote Wingless signaling. *Dev Biol* **314:** 100–111

Chang MV, Chang JL, Gangopadhyay A, Shearer A, Cadigan KM (2008b) Activation of wingless targets requires bipartite recognition of DNA by TCF. *Curr Biol* **18:** 1877–1881

Chu-LaGraff Q, Doe CQ (1993) Neuroblast specification and formation regulated by wingless in the Drosophila CNS. *Science* **261:** 1594–1597

Clevers H (2006) Wnt/beta-catenin signaling in development and disease. *Cell* **127:** 469–480

Donovan GP, Harden C, Gal J, Ho L, Sibille E, Trifiletti R, Gudas LJ, Toth M (1997) Sensitivity to jerky gene dosage underlies epileptic seizures in mice. *J Neurosci* **17:** 4562–4569

Fischer JA, Giniger E, Maniatis T, Ptashne M (1988) GAL4 activates transcription in Drosophila. *Nature* **332:** 853–856

Halfon MS, Carmena A, Gisselbrecht S, Sackerson CM, Jimenez F, Baylies MK, Michelson AM (2000) Ras pathway specificity is determined by the integration of multiple signal-activated and tissue-restricted transcription factors. *Cell* **103:** 63–74

Hatini V, Bokor P, Goto-Mandeville R, DiNardo S (2000) Tissue- and stage-specific modulation of Wingless signaling by the segment polarity gene lines. *Genes Dev* **14:** 1364–1376

Hatini V, Green RB, Lengyel JA, Bray SJ, Dinardo S (2005) The Drumstick/Lines/Bowl regulatory pathway links antagonistic Hedgehog and Wingless signaling inputs to epidermal cell differentiation. *Genes Dev* **19:** 709–718

Hayashi S, Rubinfeld B, Souza B, Polakis P, Wieschaus E, Levine AJ (1997) A Drosophila homolog of the tumor suppressor gene adenomatous polyposis coli down-regulates beta-catenin but its zygotic expression is not essential for the regulation of Armadillo. *Proc Natl Acad Sci USA* **94:** 242–247

Huang H, He X (2008) Wnt/beta-catenin signaling: new (and old) players and new insights. *Curr Opin Cell Biol* **20:** 119–125

Kioussi C, Briata P, Baek SH, Rose DW, Hamblet NS, Herman T, Ohgi KA, Lin C, Gleiberman A, Wang J, Brault V, Ruiz-Lozano P, Nguyen HD, Kemler R, Glass CK, Wynshaw-Boris A, Rosenfeld MG (2002) Identification of a Wnt/Dvl/beta-Catenin –> Pitx2 pathway mediating cell-type-specific proliferation during development. *Cell* **111:** 673–685

Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, Vogelstein B, Clevers H (1997) Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science* **275:** 1784–1787

Kramps T, Peter O, Brunner E, Nellen D, Froesch B, Chatterjee S, Murone M, Zullig S, Basler K (2002) Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex. *Cell* **109:** 47–60

Labbe E, Letamendia A, Attisano L (2000) Association of Smads with lymphoid enhancer binding factor 1/T cell-specific factor mediates cooperative signaling by the transforming growth factor-beta and wnt pathways. *Proc Natl Acad Sci USA* **97:** 8358–8363

Letunic I, Copley RR, Pils B, Pinkert S, Schultz J, Bork P (2006) SMART 5: domains in the context of genomes and networks. *Nucleic Acids Res* **34** (Database issue)**:** D257–D260

Lewis EB, Bacher F (1968) Method for feeding ethyl-methane sulfonate (EMS) to Drosophila males. *Dros Inform Serv* **43:** 193

Li J, Wang CY (2008) TBL1-TBLR1 and beta-catenin recruit each other to Wnt target-gene promoter for transcription activation and oncogenesis. *Nat Cell Biol* **10:** 160–169

Lin HV, Doroquez DB, Cho S, Chen F, Rebay I, Cadigan KM (2003) Splits ends is a tissue/promoter specific regulator of Wingless signaling. *Development* **130:** 3125–3135

Lin HV, Rogulja A, Cadigan KM (2004) Wingless eliminates ommatidia from the edge of the developing eye through activation of apoptosis. *Development* **131:** 2409–2418

Liu W, Seto J, Donovan G, Toth M (2002) Jerky, a protein deficient in a mouse epilepsy model, is associated with translationally inactive mRNA in neurons. *J Neurosci* **22:** 176–182

Liu W, Seto J, Sibille E, Toth M (2003) The RNA binding domain of Jerky consists of tandemly arranged helix-turn-helix/homeodomain-like motifs and binds specific sets of mRNAs. *Mol Cell Biol* **23:** 4083–4093

Logan CY, Nusse R (2004) The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* **20:** 781–810

MacDonald BT, Tamai K, He X (2009) Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* **17:** 9–26

Major MB, Roberts BS, Berndt JD, Marine S, Anastas J, Chung N, Ferrer M, Yi X, Stoick-Cooper CL, von Haller PD, Kategaya L, Chien A, Angers S, MacCoss M, Cleary MA, Arthur WT, Moon RT (2008) New regulators of Wnt/beta-catenin signaling revealed by integrative molecular screening. *Sci Signal* **1:** ra12

Masumoto H, Masukata H, Muro Y, Nozaki N, Okazaki T (1989) A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromeric satellite. *J Cell Biol* **109:** 1963–1973

Mendez J, Stillman B (2000) Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. *Mol Cell Biol* **20:** 8602–8612

Miller BW, Lau G, Grouios C, Mollica E, Barrios-Rodiles M, Liu Y, Datti A, Morris Q, Wrana JL, Attisano L (2009) Application of an integrated physical and functional screening approach to identify inhibitors of the Wnt pathway. *Mol Syst Biol* **5:** 315

Mojzita D, Hohmann S (2006) Pdc2 coordinates expression of the THI regulon in the yeast Saccharomyces cerevisiae. *Mol Genet Genomics* **276:** 147–161

Moore T, Hecquet S, McLellann A, Ville D, Grid D, Picard F, Moulard B, Asherson P, Makoff AJ, McCormick D, Nashef L, Froguel P, Arzimanoglou A, LeGuern E, Bailleul B (2001) Polymorphism analysis of JRK/JH8, the human homologue of mouse jerky, and description of a rare mutation in a case of CAE evolving to JME. *Epilepsy Res* **46:** 157–167

Morita R, Miyazaki E, Fong CY, Chen XN, Korenberg JR, Delgado-Escueta AV, Yamakawa K (1998) JH8, a gene highly homologous to the mouse jerky gene, maps to the region for childhood absence epilepsy on 8q24. *Biochem Biophys Res Commun* **248:** 307–314

Mosimann C, Hausmann G, Basler K (2006) Parafibromin/Hyrax activates Wnt/Wg target gene transcription by direct association with beta-catenin/Armadillo. *Cell* **125:** 327–341

Mosimann C, Hausmann G, Basler K (2009) Beta-catenin hits chromatin: regulation of Wnt target gene activation. *Nat Rev Mol Cell Biol* **10:** 276–286

Murakami Y, Huberman JA, Hurwitz J (1996) Identification, purification, and molecular cloning of autonomously replicating sequence-binding protein 1 from fission yeast Schizosaccharomyces pombe. *Proc Natl Acad Sci USA* **93:** 502–507

Nateri AS, Spencer-Dene B, Behrens A (2005) Interaction of phosphorylated c-Jun with TCF4 regulates intestinal cancer development. *Nature* **437:** 281–285

Nishita M, Hashimoto MK, Ogata S, Laurent MN, Ueno N, Shibuya H, Cho KW (2000) Interaction between Wnt and TGF-beta signalling pathways during formation of Spemann's organizer. *Nature* **403:** 781–785

Notani D, Gottimukkala KP, Jayani RS, Limaye AS, Damle MV, Mehta S, Purbey PK, Joseph J, Galande S (2010) Global regulator SATB1 recruits beta-catenin and regulates T(H)2 differentiation in Wnt-dependent manner. *PLoS Biol* **8:** e1000296

Nusslein-Volhard C, Wieschaus E (1980) Mutations affecting segment number and polarity in Drosophila. *Nature* **287:** 795–801

Okada T, Ohzeki J, Nakano M, Yoda K, Brinkley WR, Larionov V, Masumoto H (2007) CENP-B controls centromere formation depending on the chromatin context. *Cell* **131:** 1287–1300

Olson LE, Tollkuhn J, Scafoglio C, Krones A, Zhang J, Ohgi KA, Wu W, Taketo MM, Kemler R, Grosschedl R, Rose D, Li X, Rosenfeld MG (2006) Homeodomain-mediated beta-catenin-dependent switching events dictate cell-lineage determination. *Cell* **125:** 593–605

Pai LM, Orsulic S, Bejsovec A, Peifer M (1997) Negative regulation of Armadillo, a Wingless effector in Drosophila. *Development* **124:** 2255–2266

Parker DS, Ni YY, Chang JL, Li J, Cadigan KM (2008) Wingless signaling induces widespread chromatin remodeling of target loci. *Mol Cell Biol* **28:** 1815–1828

Pluta AF, Saitoh N, Goldberg I, Earnshaw WC (1992) Identification of a subdomain of CENP-B that is necessary and sufficient for localization to the human centromere. *J Cell Biol* **116:** 1081–1093

Riese J, Yu X, Munnerlyn A, Eresh S, Hsu SC, Grosschedl R, Bienz M (1997) LEF-1, a nuclear factor coordinating signaling inputs from wingless and decapentaplegic. *Cell* **88:** 777–787

Schultz J, Milpetz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: identification of signaling domains. *Proc Natl Acad Sci USA* **95:** 5857–5864

Sudarsan V, Anant S, Guptan P, VijayRaghavan K, Skaer H (2001) Myoblast diversification and ectodermal signaling in Drosophila. *Dev Cell* **1:** 829–839

Tomlinson A (2003) Patterning the peripheral retina of the fly: decoding a gradient. *Dev Cell* **5:** 799–809

Toth M, Grimsby J, Buzsaki G, Donovan GP (1995) Epileptic seizures caused by inactivation of a novel gene, jerky, related to centromere binding protein-B in transgenic mice. *Nat Genet* **11:** 71–75

van de Wetering M, Cavallo R, Dooijes D, van Beest M, van Es J, Loureiro J, Ypma A, Hursh D, Jones T, Bejsovec A, Peifer M, Mortin M, Clevers H (1997) Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. *Cell* **88:** 789–799

Wieschaus E, Riggleman R (1987) Autonomous requirements for the segment polarity gene armadillo during Drosophila embryogenesis. *Cell* **49:** 177–184

Wolff T, Ready DF (1993) Pattern formation in the drosophila retina. In *The Development of Drosophila Melanogaster*, Bate M, Martinez Arias A (eds), Vol. II, pp 1277–1325. Cold Spring: Cold Spring Harbor Laboratory Press

Xu H, Wickner W (2006) Bem1p is a positive regulator of the homotypic fusion of yeast vacuoles. *J Biol Chem* **281:** 27158–27166

Zara F, Bianchi A, Avanzini G, Di Donato S, Castellotti B, Patel PI, Pandolfo M (1995) Mapping of genes predisposing to idiopathic generalized epilepsy. *Hum Mol Genet* **4:** 1201–1207

