FITC-labelled cholera toxin B (CTxB) subunit (Sigma) was used in the absence of FCS. When required, washed cells were stained with the specific second step reagent.

For intracellular staining, fixed cells were labelled with anti-pTα antibodies and permeabilized in PBS with 0.15% Triton X-100 for 5 min followed by staining with anti-$p56^{lck}$ antibodies. Cells were depleted of membrane cholesterol by treatment with 10 mM methyl-β-cyclodextrin (Sigma) in Dulbecco's modified Eagle's medium supplemented with 0.4% fatty-acid-free bovine serum albumin, 1 μg ml^{-1} transferrin, 8.1 μg ml^{-1} monothioglycerol and 20 mM HEPES pH 7.4 (lipid-free medium) for 1 h at 37 °C. Confocal microscopy was done on a LSM 510 Zeiss confocal microscope. For quantitative colocalization analysis, we used a Zeiss inverted microscope (Axiovert 35) as conventional microscopy is more sensitive than confocal microscopy for this analysis[9]. Images were acquired and processed with a CCD camera (Photometrics) cooled to 10 °C and coupled to the IPLAB Spectrum Imaging software. The spatial distribution of two labelled molecules was analysed on recorded images using the Co-localization Image Analysis program from the SPIMAC (Spectral Imaging on Macintosh) software as described[9].

Subcellular fractionations, biosynthetic labelling and western blotting

Cells were lysed in Triton X-100 as described[12], mixed with 1 ml 80% sucrose, and overlaid with two phases of 2 ml 30% sucrose and 1 ml 5% sucrose, respectively. Samples were centrifuged at 200,000*g* at 4 °C for 14–16 h, and 0.4 ml fractions were collected and numbered from the top of the gradient, precipitated in trichloroacetic acid (TCA), resolved by SDS–PAGE in reducing 8–15% gradient gels, transferred to nitrocellulose membranes and blotted with the indicated antibodies and specific secondary reagents. For analysis of CD3ε phosphorylation, raft fractions (from 3 to 5) and loading zone fractions (from 9 to 12) were immunoprecipitated with anti-CD3ε monoclonal antibody followed by Protein A Sepharose. Anti-CD3ε immunoprecipitates were resolved by SDS–PAGE in non-reducing 8–15% gradient gels, transferred to nitrocellulose membranes and sequentially blotted with anti-phosphotyrosine mAb 4G10 and goat anti-CD3ε Ig.

Biosynthetic labelling of endogenous palmitate was carried out by incubation of cells in the presence of ^{3}H-palmitate (0.5 mCi) for 4 h at 37 °C in lipid-free medium without monothioglycerol. Cells were lysed in 1% Triton X-100, 0.2% saponin, and lysates were immunoprecipitated with either anti-TCRβ or anti-Lck monoclonal antibody coupled to Protein A Sepharose. After separation by SDS–PAGE in non-reducing conditions, samples were transferred to nitrocellulose membranes, which were used for autoradiography with BioMax TranScreen intensifying system (Kodak). For membrane and cytosol separation, either untreated cells or cells treated with 10 mM MβCD or 10 μM PP2 (Calbiochem) for 15 min at 37 °C, were lysed in hypotonic buffer (20 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM $MgCl_2$, 0.5 mM DTT and protease inhibitors) and disrupted by homogenization on ice with a Dounce homogenizer. Salt concentration was adjusted to 150 mM NaCl, and intact cells, nuclei and cytoskeleton were pelleted by two centifugations at 5,000 r.p.m. for 5 min in microcentrifuge at 4 °C. The supernatant was centrifuged at 100,000*g* for 1 h and membrane pellets solubilized in 0.5% Triton X-100 for immunoprecipitation with anti-ZAP-70 Ig. The supernatant corresponding to cytosol was equilibrated to 0.5% Triton X-100 and immunoprecipitated with anti-ZAP-70 Ig.

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Distinct β-catenins mediate adhesion and signalling functions in *C. elegans*

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In flies and vertebrates, Armadillo/β-catenin forms a complex with Tcf/Lef-1 transcription factors, serving as an essential co-activator to mediate Wnt signalling. It also associates with cadherins to mediate adhesion. In *Caenorhabditis elegans*, three putative β-catenin homologues have been identified: WRM-1, BAR-1 and HMP-2. WRM-1 and the Tcf homologue POP-1 mediate Wnt signalling by a mechanism that has challenged current views of the Wnt pathway[1–3]. Here we show that BAR-1 is the only β-catenin homologue that interacts directly with POP-1. BAR-1 mediates Wnt signalling by forming a BAR-1/POP-1 bipartite transcription factor that activates expression of Wnt target genes such as the Hox gene *mab-5*. HMP-2 is the only β-catenin homologue that interacts with the single cadherin of *C. elegans*, HMR-1. We conclude that a canonical Wnt pathway exists in *C. elegans*. Furthermore, our analysis shows that the functions of *C. elegans* β-catenins in adhesion and in signalling are performed by separate proteins.

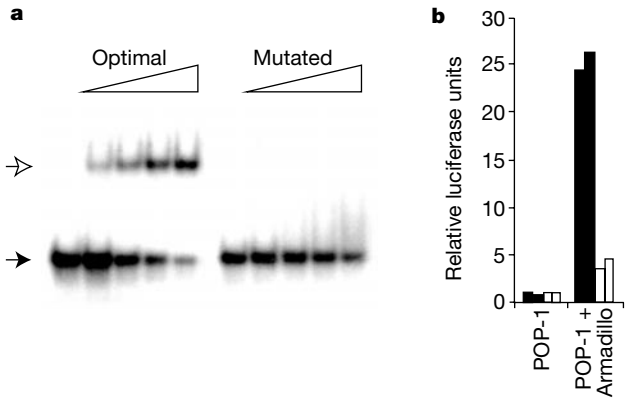
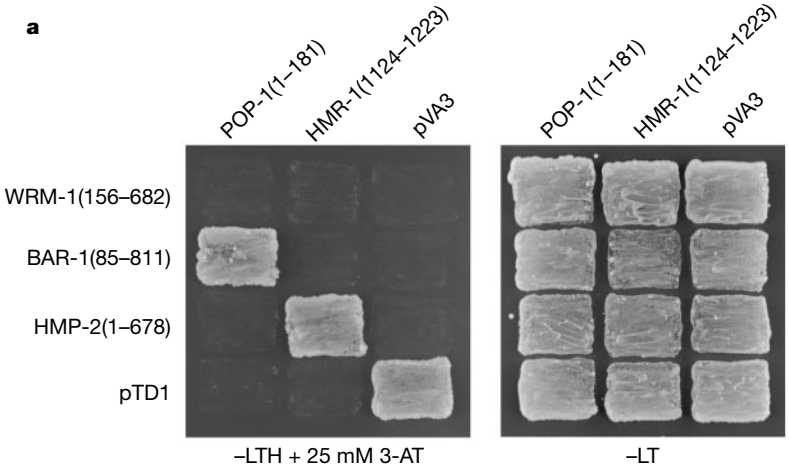


Figure 1 POP-1 is a functional Tcf homologue. **a**, Gel retardation analysis was performed with recombinant POP-1(180–280) containing the HMG DNA-binding domain. The POP-1 HMG domain binds the optimal Tcf target sequence GATCAAAG, but not the mutated sequence GATCCCCG. White arrow, shifted band; black arrow, free probe. **b**, POP-1 and Armadillo activate a Tcf reporter gene. Expression constructs for POP-1 and Armadillo were transfected into IIAI.6 cells with the TOP luciferase reporter[18] which contains optimal Tcf-binding sites (black bars), or the negative control FOP luciferase which contains mutated Tcf-binding sites (white bars).



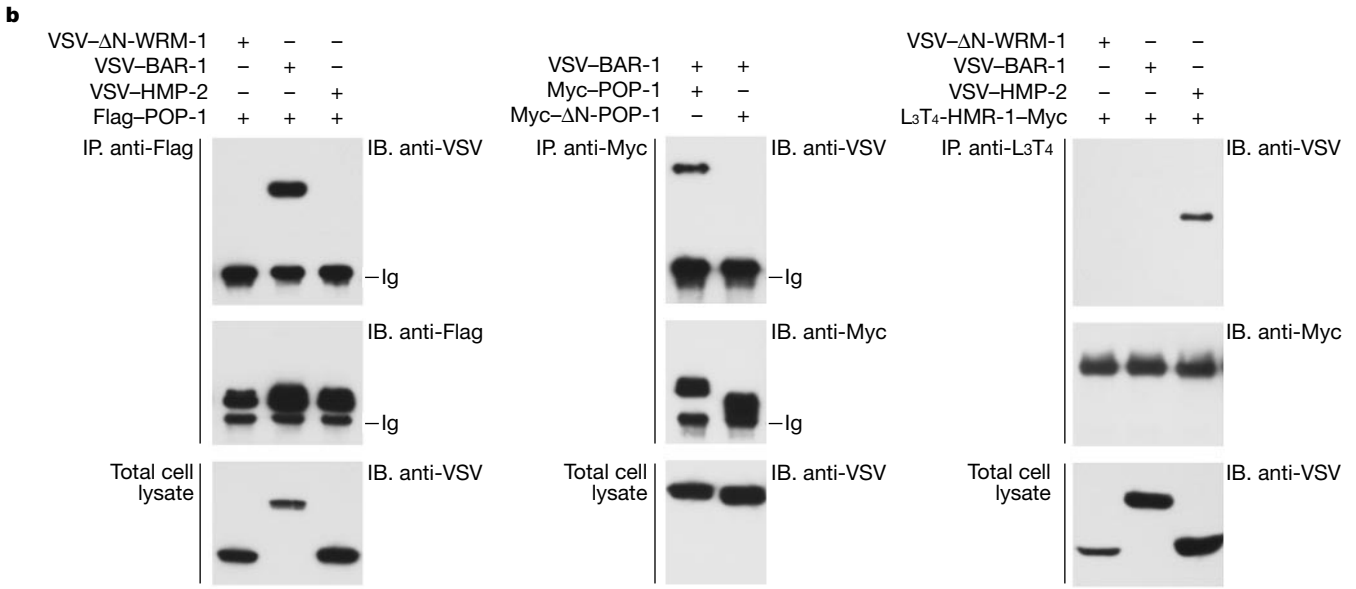


Figure 2 Physical interactions between the three *C. elegans* β-catenins and POP-1 and HMR-1. **a**, Yeast two-hybrid assay showing the specific interactions between POP-1 and BAR-1 and HMR-1 and HMP-2. The WRM-1–Gal4BD fusion does not interact with POP-1 or HMR-1, but does interact with other proteins (data not shown). **b**, Expression vectors were transfected into 293T cells as indicated. VSV-tagged BAR-1 is specifically co-immunoprecipitated (IP) with Flag-tagged POP-1, as is shown in the anti-VSV immunoblot (IB) (left). This interaction requires the N terminus of POP-1 (middle). Only VSV-tagged HMP-2 is precipitated with the intracellular β-catenin interaction domain of HMR-1 fused to L3T4.

In flies and vertebrates, Armadillo/β-catenin has a dual function in adhesion and Wg/Wnt signalling. Indeed, Armadillo was first identified for its role in Wg signalling, whereas β-catenin and the related protein Plakoglobin were initially discovered as adhesion molecules[4,5]. In *C. elegans*, three highly divergent β-catenin homologues have been identified[6]. Of these, HMP-2 is most similar to vertebrate and fly β-catenin[7]. Mutations in *hmp-2* cause defects in hypodermal enclosure and body elongation, indicating that it may be involved in cellular adhesion. Indeed, HMP-2 colocalizes with the cadherin HMR-1 and the α-catenin HMP-1 in adherens junctions[7]. BAR-1 shows a slightly lower overall similarity to β-catenin and is required for several patterning events during larval development[8,9]. The third homologue, WRM-1, shows the lowest similarity to β-catenin[1]. WRM-1 is involved in a divergent Wnt pathway, where it opposes rather than mediates signalling by the Tcf-like transcription factor POP-1 (ref. 1). Instead of binding POP-1 directly, WRM-1 binds and activates a kinase, LIT-1, which in turn phosphorylates POP-1 (refs 2, 3). The activities of WRM-1 and LIT-1 ensure that POP-1, which probably functions as a repressor in this pathway[10–12], is asymmetrically distributed between the daughter cells of many embryonic and larval cell divisions, allowing the specification of different cell fates.

POP-1 contains a single Tcf-like HMG-box DNA-binding domain[10] and an acidic region within its amino terminus that has limited sequence similarity to the β-catenin interaction domain of other Tcf members[13]. The HMG domain is highly conserved within the Tcf family, with 92% sequence identity between, for example, human TCF-1 and *Drosophila* dTcf/Pangolin[13]. Despite the fact that POP-1 and TCF-1 share only 54% sequence identity within the HMG-box, we show that POP-1 recognizes the Tcf consensus site. A bacterially expressed POP-1 fragment containing the HMG domain specifically binds the optimal Tcf target site, but not a sequence that lacks the core Tcf target motif (Fig. 1a). Tcf transcription factors bind Armadillo/β-catenin to activate transcription of target genes[13–17]. In transient transfections using a previously established β-catenin–Tcf reporter gene assay[13,18], we found that co-expression of POP-1 and Armadillo specifically activated transcription of the Tcf reporter, whereas POP-1 alone did not (Fig. 1b). We conclude that POP-1 is a functional Tcf homologue.

Tcf transcription factors interact with Armadillo/β-catenin by binding to Armadillo repeats 3 to 8 (ref. 13). To determine which of the three *C. elegans* β-catenins binds directly to POP-1, we analysed possible interactions using a yeast two-hybrid assay. POP-1 was fused to the Gal4 DNA-binding domain, and fragments of each *C. elegans* β-catenin homologue containing the 12 Arm repeats were fused with the Gal4 activation domain. A direct physical interaction was only observed between POP-1 and BAR-1 (Fig. 2a). To investigate the specific interaction between POP-1 and BAR-1, we co-expressed Flag-tagged POP-1 with either VSV-tagged ΔN-WRM-1, BAR-1 or HMP-2 in vertebrate cells, and asked which of the three β-catenin homologues immunoprecipitated with POP-1. In this experiment, we used a truncated WRM-1 (ΔN-WRM-1) that lacks the N-terminal LIT-1/Nlk interaction domain[2], but retains the Arm repeat region. As is shown in Fig. 2b, only BAR-1 was precipitated with POP-1. We found that the interaction between POP-1 and BAR-1 requires the acidic region at the N terminus of POP-1. Removal of the first 44 amino acids of POP-1 (ΔN-POP-1) resulted in the loss of BAR-1 binding (Fig. 2b). These data show that POP-1 specifically interacts with BAR-1.

Next, we tested whether the association between POP-1 and BAR-1 results in the transcriptional activation of a Tcf target gene. Only co-expression of POP-1 and BAR-1 resulted in significant activation of a Tcf reporter gene, whereas co-expression of POP-1 with WRM-1 or HMP-2 had no effect (Fig. 3a). As expected, the transcriptional activation of the Tcf reporter required the BAR-1 interaction domain of POP-1. Thus, co-expression of ΔN-POP-1 with BAR-1 did not activate the Tcf reporter (Fig. 3a). These data indicate that POP-1 and BAR-1 may function in Wnt target gene activation analogously to Tcf and β-catenin in flies and vertebrates.

To investigate this further, we tested whether POP-1 and BAR-1 are required for the expression of the putative Wnt target gene, *mab-5*. The Hox gene *mab-5* controls the direction of migration of the neuroblast QL and its daughter cells (denoted collectively as QL.d) during larval development[19,20]. In wild-type animals, the QL daughter cells migrate to positions that are posterior to their point of origin. In *mab-5* loss-of-function mutants, the QL.d cells migrate in the opposite, anterior direction. The expression of *mab-5* in the QL lineage is regulated by a putative Wnt pathway. Mutations in *egl-20/Wnt*[9,21], *lin-17/Fz*[21,22] and *bar-1* (refs 8, 9) all result in anterior migration of the QL.d cells and produce a loss of *mab-5* expression in the QL lineage.

The only existing *pop-1* mutation, *pop-1(zu189)*, produces a maternal-effect lethal phenotype[10] and has no zygotic effect on QL.d migration. Therefore, we could not directly investigate the function of *pop-1* in this pathway by using this mutation. We have previously used overexpression of truncated Tcf mutants that lack the β-catenin interaction domain to produce potent dominant-negative mutants[13,15]. As is shown in Fig. 3b, overexpression of ΔN-POP-1 strongly inhibited Tcf reporter activation by full-length POP-1 and BAR-1. We generated stable transgenic lines that inducibly express ΔN-POP-1 from a heat-shock promoter. The effect of ΔN-POP-1 expression on QL.d migration was assessed by scoring the final positions of the two QL.pa daughter cells, which can be easily recognized, relative to the positions of the anterior and posterior daughters of the six seam cells, V1 to V6 (ref. 21) (Fig. 4a). Whereas the QL.pa daughter cells were found at a normal position (near V5.a) in non-heat-shocked animals, a 1-h heat shock at hatching resulted in the anterior migration of these cells, with >80% of QL.pa daughters localizing anterior to V2.p. This migration defect is similar to the phenotype of *egl-20*/Wnt and *mab-5* null mutants[21], and indicates that overexpression of ΔN-POP-1 may abrogate the expression of *mab-5*. To verify this, we used a *mab-5::lacZ* reporter construct that mimics the expression

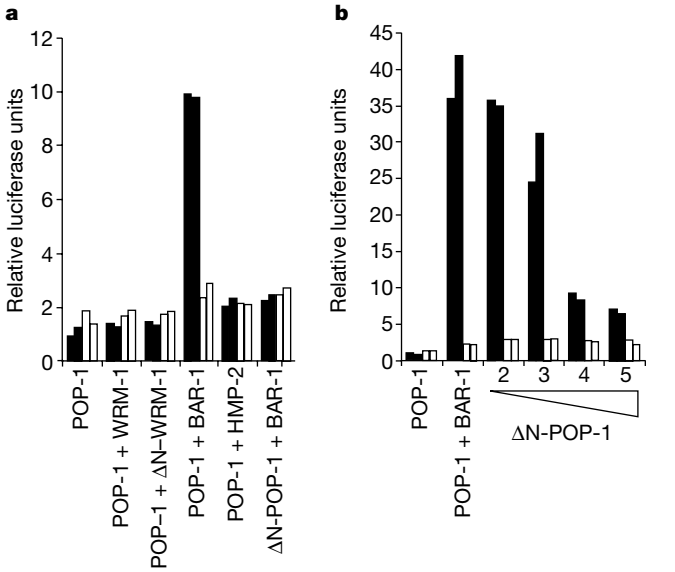


Figure 3 POP-1 and BAR-1 activate transcription of a Tcf reporter gene. **a**, Expression constructs were transfected into IIAI.6 cells with the TOP reporter (black bars) or the negative control FOP (white bars) as indicated. Only POP-1 and BAR-1 activate the Tcf reporter. The BAR-1 interaction domain of POP-1 is required for this activity (ΔN-POP-1). **b**, Overexpression of ΔN-POP-1 inhibits activation of the Tcf reporter by full-length POP-1 and BAR-1. ΔN-POP-1 was transfected at increasing concentrations (2–5 μg) with full-length POP-1 and BAR-1.

of the endogenous *mab-5* gene[20]. We found that 26/26 non-heat-shocked animals expressed the *mab-5::lacZ* reporter in the QL daughter cells, whereas 34/34 heat-shocked animals did not (Fig. 4b), indicating that overexpression of ΔN-POP-1 inhibits expression of *mab-5*. Consistent with these observations, RNA-mediated interference of *pop-1* zygotic function (see Methods) also resulted in the anterior migration of the QL.d cells and the loss of *mab-5* expression in the QL lineage (M.H., in preparation). We found that the other two β-catenin homologues, WRM-1 and HMP-2, are not required for QL.d migration. RNA-mediated interference of either *hmp-2* ($n = 21$) or *wrm-1* zygotic functions ($n = 33$), did not result in QL.d migration defects. We conclude that POP-1 and BAR-1 function in a canonical Wnt pathway that regulates the expression of target genes such as *mab-5*.

As discussed above, vertebrate and fly Armadillo/β-catenin also functions as an adhesion molecule by interacting with the cytoplasmic tail of cadherins. A single classical cadherin, HMR-1, has been identified in *C. elegans*[7] and extensive database searches of the now virtually complete genome sequence have revealed no other candidates. We investigated which of the three *C. elegans* β-catenins bind HMR-1. The β-catenin interaction domain of HMR-1 (ref. 7) was fused to the Gal4 DNA-binding domain (BD) and tested for direct physical interaction with the three *C. elegans* β-catenins in a yeast two-hybrid assay. HMR-1 interacted only with HMP-2 (Fig. 2a). To investigate this further, we fused the extracellular part of mouse CD4 (L3T4) with the transmembrane and intracellular domains of HMR-1 to localize HMR-1 at the plasma membrane. We co-expressed the HMR-1 fusion protein with VSV-tagged ΔN-WRM-1, BAR-1 or HMP-2 in vertebrate cells and investigated which of the *C. elegans* β-catenin homologues interacted with the intracellular domain of HMR-1. Only HMP-2 immunoprecipitated

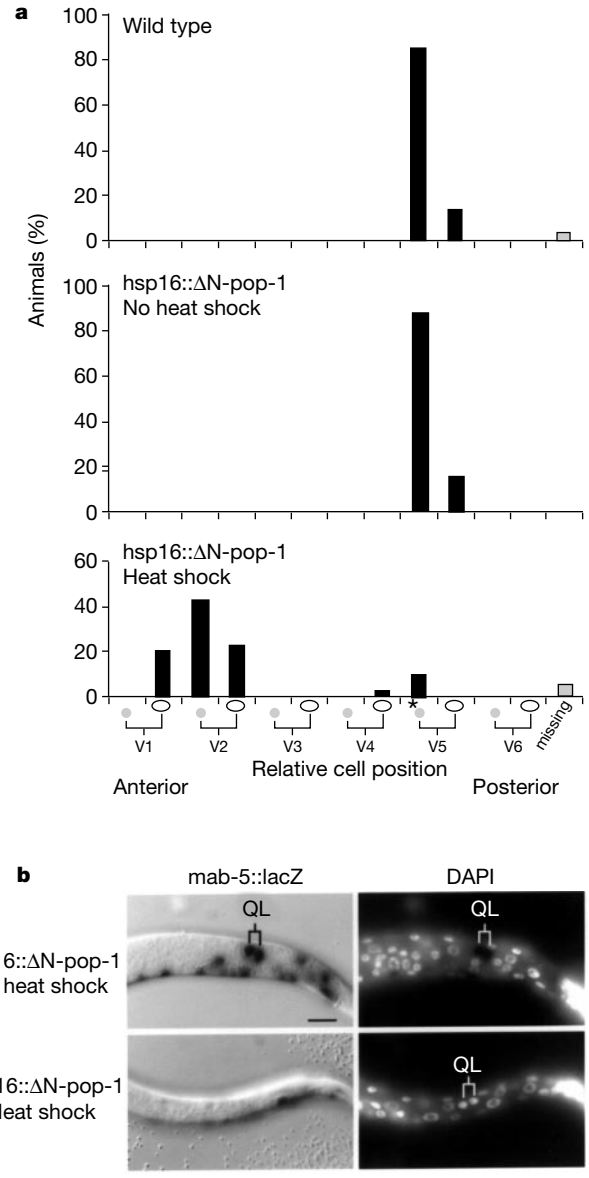


Figure 4 Expression of ΔN-POP-1 inhibits *mab-5* expression. **a**, Expression of ΔN-POP-1 caused anterior migration of the QL.d cells. Final positions of the QL.pa daughters were scored[21] in wild-type ($n = 32$), non-heat-shocked ($n = 29$) and heat-shocked ($n = 45$) animals containing *huIs4*. Asterisk above V5.a marks the birth place of QL. Black bars indicate the proportion of cells at each position. The grey bar indicates the proportion of missing cells. **b**, Expression of ΔN-POP-1 blocked the expression of a *mab-5::lacZ* reporter construct in the QL.d cells. Whole-mount larvae, 3–5 h after hatching, were fixed and stained for β-galactosidase expression[20] and the DNA stain DAPI.

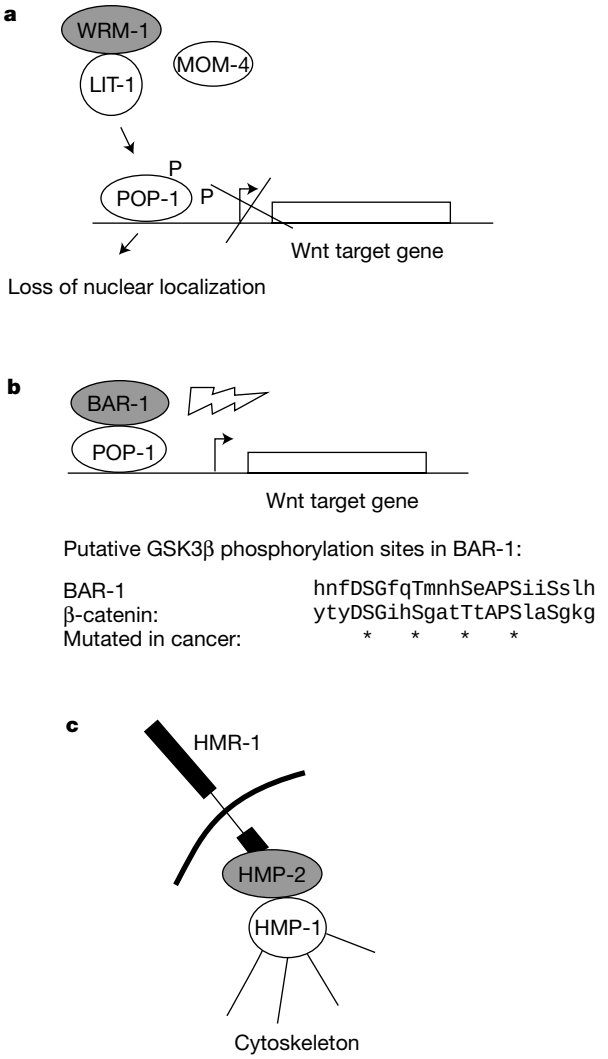


Figure 5 The signalling and adhesion functions of Armadillo/β-catenin are distributed over three different β-catenin homologues in *C. elegans*. **a**, WRM-1 acts in a divergent Wnt pathway. WRM-1 binds to and activates the kinase LIT-1, which in turn phosphorylates POP-1. Phosphorylation of POP-1 may result in its loss of nuclear localization[2,24]. **b**, BAR-1 is part of a canonical Wnt pathway. BAR-1 and POP-1 have a function in Wnt target gene activation that is analogous to the function of β-catenin and Tcf in flies and vertebrates. BAR-1 is the only *C. elegans* β-catenin that harbours a set of putative GSK3β phosphorylation sites in its N-terminal sequence. **c**, HMP-2 is the only *C. elegans* β-catenin that interacts with the cadherin HMR-1.

with HMR-1 (Fig. 2b), again showing a specific interaction between these two proteins. Thus the cadherin HMR-1 interacts specifically with HMP-2, but not with WRM-1 or BAR-1.

We propose that the signalling and adhesion functions of Armadillo/β-catenin have been distributed between separate β-catenin homologues in *C. elegans* (Fig. 5). Two β-catenins function in Wnt signalling. WRM-1 is part of a divergent Wnt pathway that, in collaboration with a mitogen-activated protein (MAP) kinase pathway, mediates the asymmetric distribution of POP-1 between daughter cells of many anterior/posterior cell divisions[1–3,11,12,23] (reviewed in ref. 24). POP-1 probably acts as a repressor in this pathway[10–12] and differences in POP-1 expression levels between daughter cells may allow the specification of different fates. We show that BAR-1 is part of a Wnt pathway that is similar to that in flies and vertebrates. BAR-1 can directly associate with POP-1 to activate a Tcf reporter. In addition, we show that BAR-1 and POP-1 are required for the expression of the endogenous Wnt target gene *mab-5*. Of the three *C. elegans* β-catenins, only BAR-1 contains a set of four conserved GSK3β phosphorylation sites[8] (Fig. 5b). These sites are essential for the regulation of β-catenin by the Wnt pathway and are frequently mutated in cancers[25,26]. *C. elegans* contains a single putative APC homologue, APR-1[1]. In vertebrates, APC forms a complex with GSK3β, Axin and β-catenin to downregulate β-catenin levels in the absence of Wnt signalling[4,5]. We find that none of the *C. elegans* β-catenins bind APR-1 in a yeast two-hybrid assay (data not shown). Together with the observation that the *C. elegans* genome does not contain a clear Axin homologue[6], this indicates that BAR-1 may be regulated differently.

HMP-2 is the only *C. elegans* β-catenin that interacts with the single classical cadherin, HMR-1. This interaction agrees with the *hmp-2* mutant phenotype and with the colocalization of HMP-2 with HMR-1 and the α-catenin HMP-1 in adherens junctions[7]. We conclude that HMP-2 functions specifically in adhesion (Fig. 5c). Vertebrates express a second β-catenin-like protein, Plakoglobin, which is part of the desmosomal adhesion complex. It is unclear whether Plakoglobin functions specifically in adhesion or also has a role in Wnt signalling[27]. The functions of Armadillo and β-catenin in Wnt signalling and adhesion are physically separable[28,29]. It is unclear whether Armadillo/β-catenin in adherens junctions may directly or indirectly affect the cytoplasmic signalling pool. Mutations in cadherins have been identified in many epithelial tumours. An attractive explanation for the oncogenic potential of cadherin mutations is the release of β-catenin from adherens junctions, which in turn can interact with Tcf transcription factors to activate the expression of Wnt target genes[30]. Our data indicate that, at least in *C. elegans*, the adhesion and signalling pools of β-catenin are separate entities. □

Methods

Cloning of complementary DNAs

We cloned full-length cDNA of *pop-1* (GenBank accession no. U37532), *bar-1* (AF063646), *wrm-1* (AF013951) and *hmp-2* (AF016853) and parts of the *hmr-1* (AF016854) cDNA by polymerase chain reaction on total *C. elegans* cDNA, and checked them by sequencing. The cDNAs were cloned in-frame with N-terminal Flag, Myc or VSV tags in pCDNA3 (Invitrogen). ΔN-WRM-1 has an N-terminal truncation of the first 134 amino acids. A *hmr-1* fragment encoding amino-acids 904–1223 was cloned in-frame with the extracellular part of L3T4 and a carboxy-terminal Myc-tag.

Gel-shift experiments

A POP-1(180–280) fragment containing the HMG-box DNA-binding domain was purified as a His-tagged protein. Gel shifts were performed as described[18].

Interaction studies

We performed two-hybrid experiments as in ref. 15. pVA3 encodes a murine p53–Gal4 binding domain fusion in pGBT9, and pTD1 contains SV40 large T in pGADGH (Clontech). Expression vectors were transfected into 293T cells using FuGENE transfection reagent (Boehringer Mannheim). Cells were lysed in Triton X-100 lysis buffer[26] and immunoprecipitations were performed with anti-Flag M2 (Sigma), anti-Myc (9E10), anti-VSV-G (P5D4) or anti-L3T4 (GK1.5) (Pharmingen) and protein A/G beads (Santa Cruz).

Luciferase reporter assays

Luciferase reporter assays were performed as described[13,18]. Briefly, we transfected 2×10^6 IIAI.6 cells with 1 μg of the luciferase reporter pTKTOP or the negative control pTKFOP, 1 μg of a POP-1 expression construct, 5 or 10 μg of the different β-catenin expression constructs and 50 ng of the internal transfection control pTKRenilla. Luciferase assays were as recommended by the manufacturer (Promega). Luciferase measurements were normalized for transfection efficiency using the renilla control.

Transgenic animals, RNA-mediated interference and scoring QL.d cell migrations

We cloned a truncated *pop-1* fragment encoding ΔN-POP-1(45–438) into the heat-shock promotor vector pPD49.78 and injected it with the *rol-6(su1006)* marker plasmid pRF4. Animals containing the integrated transgene *huIs4* were heat-shocked at hatching for 1 h at 33 °C. RNA-mediated interference (RNAi) was performed as described[1]. To determine the zygotic phenotype of maternal effect lethal genes such as *pop-1* and *wrm-1*, we injected double-stranded RNA into an RNAi resistant mutant and analysed the phenotypes of the cross-progeny (M.H., in preparation). The QL.pa daughter cells were scored in the late L1 stage as described[21]. *mab-5* expression was assayed using the *mab-5::lacZ* reporter transgene *muIs2* (ref. 20).

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Genomic analysis of metastasis reveals an essential role for RhoC

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The most damaging change during cancer progression is the switch from a locally growing tumour to a metastatic killer. This switch is believed to involve numerous alterations that allow tumour cells to complete the complex series of events needed for metastasis[1]. Relatively few genes have been implicated in these events[2–5]. Here we use an *in vivo* selection scheme to select highly metastatic melanoma cells. By analysing these cells on DNA arrays, we define a pattern of gene expression that correlates with progression to a metastatic phenotype. In particular, we show enhanced expression of several genes involved in extracellular matrix assembly and of a second set of genes that regulate, either directly or indirectly, the actin-based cytoskeleton. One of these, the small GTPase RhoC, enhances metastasis when overexpressed, whereas a dominant-negative Rho inhibits metastasis. Analysis of the phenotype of cells expressing dominant-negative Rho or RhoC indicates that RhoC is important in tumour cell invasion. The genomic approach allows us to identify families of genes involved in a process, not just single genes, and can indicate which molecular and cellular events might be important in complex biological processes such as metastasis.

To provide insight into the pattern of gene expression that allows tumours to metastasize, we compared the gene expression profile of melanoma variants with low or high metastatic potential. As shown in Fig. 1, the system involves the *in vivo* selection of highly metastatic melanoma cells from a population of poorly metastatic tumour cells[6]. When nude mice were injected intravenously with amelanotic human A375P tumour cells, relatively few pulmonary metastases were observed (Fig. 2a). When these rare metastases were dissected free from the lungs and the cells grown in tissue culture, however, the resulting cells showed enhanced metastatic capacity, confirming that highly metastatic cells can be selected from a heterogeneous population of poorly metastatic tumour cells[7]. Furthermore, if successive metastases (designated M1 and M2) were isolated, expanded in tissue culture, and re-introduced into host mice as shown in Fig. 1, significantly more pulmonary metastases were observed (Fig. 2b). When mouse B16F0 melanoma cells were subjected to this same *in vivo* selection scheme, highly metastatic pulmonary tumours (designated F1, F2 and F3) were isolated, as previously described for this cell line[6]. When the poorly metastatic A375P or B16F0 and the *in vivo*-selected metastatic A375 or B16 cells were grown as subcutaneous tumours, there was no observable difference in tumour size (see Supplementary Information), indicating that we had selected for a difference in metastatic, but not tumorigenic, properties of the melanomas. These results support the hypothesis that specific gene products can regulate metastasis without altering the growth properties of a tumour[8]. Therefore, we sought to identify metastasis-specific genes using a functional genomics approach.

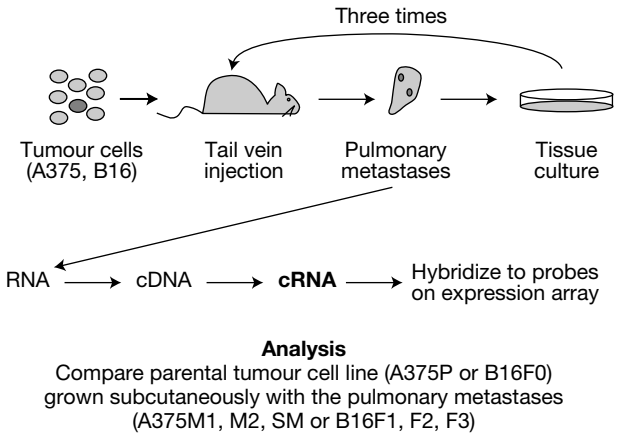

Figure 1 *In vivo* selection scheme. Poorly metastatic melanoma cell lines (human A375P or mouse B16F0) were injected intravenously into the tail veins of host mice and pulmonary metastases were isolated. Either these metastases were minced and grown in tissue culture (to be injected into additional host mice) or RNA was extracted to prepare the labelled cRNA used to hybridize to the oligonucleotide arrays. The procedure to select for highly metastatic tumour cells was repeated two (A375) or three (B16) times. A375SM cells were previously derived in a similar manner[11].

RNAs extracted from these pulmonary metastases and from the parental A375P and B16F0 lines grown as subcutaneous tumours were used to prepare complementary RNAs (cRNAs), which were hybridized to oligonucleotide microarrays (human: 7,070 genes; mouse: 6,347 genes, with around 50% overlap in the genes represented) to determine the array of differentially expressed genes (Fig. 1). The entire data set is available at our web site at http://www.genome.wi.mit.edu/MPR and in Supplementary Information. Table 1 lists those genes expressed at consistently higher levels in pulmonary metastases derived from the A375P line (M1, M2 and SM) and the mouse B16F0 line (F1, F2 and F3). To ensure that the enhanced expression of these genes in the pulmonary metastases was not due solely to the influence of the microenvironment in which the metastatic cells were growing, we also grew metastatic A375SM cells subcutaneously and compared their expression profile with that of subcutaneous A375P tumours. We found that 15 of the 16 genes continued to show enhanced expression when metastatic A375 cells were grown as a subcutaneous tumour (see Supplementary Information), indicating that the expression of these genes is intrinsic to the metastatic cells. Note, however, that the tumour microenvironment may help to regulate the absolute level of gene expression.

As the set of genes represented on the human and mouse arrays partially overlapped, some signals appeared in both species (Table 1). Three genes, fibronectin, RhoC and thymosin β4, were expressed at higher levels (≥2.5-fold) in all three metastases selected from both the human A375 and mouse B16 cell lines. Enhanced expression of these three genes in the pulmonary metastases was

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