A genome-wide screen identifies conserved protein hubs required for cadherin-mediated cell–cell adhesion

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Cadherins and associated catenins provide an important structural interface between neighboring cells, the actin cytoskeleton, and intracellular signaling pathways in a variety of cell types throughout the Metazoa. However, the full inventory of the proteins and pathways required for cadherin-mediated adhesion has not been established. To this end, we completed a genome-wide (~14,000 genes) ribonucleic acid interference (RNAi) screen that targeted Ca^{2+}-dependent adhesion in DE-cadherin–expressing *Drosophila melanogaster* S2 cells in suspension culture. This novel screen eliminated Ca^{2+}-independent cell–cell adhesion, integrin-based adhesion, cell spreading, and cell migration. We identified 17 interconnected regulatory hubs, based on protein functions and protein–protein interactions that regulate the levels of the core cadherin–catenin complex and coordinate cadherin-mediated cell–cell adhesion. Representative proteins from these hubs were analyzed further in *Drosophila* oogenesis, using targeted germline RNAi, and adhesion was analyzed in Madin–Darby canine kidney mammalian epithelial cell–cell adhesion. These experiments reveal roles for a diversity of cellular pathways that are required for cadherin function in Metazoa, including cytoskeleton organization, cell–substrate interactions, and nuclear and cytoplasmic signaling.

Complete screen data

Primary screen:
http://jcb-dataviewer.rupress.org/jcb/browse/7555/S202/

Secondary screen:
http://jcb-dataviewer.rupress.org/jcb/browse/7555/S252/

Introduction

Central to the structure and function of many tissues are epithelial monolayers (Bryant and Mostov, 2008), which are organized by cell adhesion to the ECM and cell–cell junctions that include the tight junction, desmosomes, and the adherens junction (AJ; Nelson, 2009). Together, cell–cell junctions coordinate cell recognition and sorting, cell signaling, and the generation of functional cell polarity, which are essential for metazoan development and tissue organization (Harris and Tepass, 2010; Niessen et al., 2011).

The AJ provides the primary linkage between epithelial cells and contains members of the cadherin superfamily of transmembrane Ca^{2+}-dependent cell–cell adhesion proteins (Brasch et al., 2012). The cytoplasmic domain of cadherins interacts with β-catenin, p120-catenin, and the actin regulator, α-catenin, which are thought to coordinate cytoskeleton remodeling, protein trafficking, and signal transduction in response to cell–cell adhesion (Hartsock and Nelson, 2008).

Although the organization of other cell–cell junctions diverges in metazoans, the AJ is largely conserved, highlighting its central role in animal biology. For example, the amino acid sequence homology between mammalian and *Drosophila melanogaster* classical cadherin cytoplasmic domain, β-catenin, and α-catenin are 37.2/62.0%, 67.8/83.3%, and 62.0/86.0% (percent identity/percent similarity), respectively (Tepass et al., 2001; Hartsock and Nelson, 2008). This structural and functional

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Abbreviations used in this paper: AJ, adherens junction; WAVE, Wiskott–Aldrich syndrome protein family verprolin homologous protein.

THE JOURNAL OF CELL BIOLOGY

conservation means that insights about AJ function in simple model organisms can be directly translated to more complex mammalian systems.

AJs are fundamental to multicellularity, which complicates loss-of-function analysis in genetically tractable organisms. AJs are also intimately linked with other cell–cell junctions and downstream pathways, making them difficult to isolate. Thus, identifying proteins and pathways that are specific to cadherin-mediated cell–cell adhesion is challenging (Franke, 2009), and relatively few AJ-specific proteins have been characterized (see Discussion).

RNAi screens provide a method of analyzing cadherin-based adhesion proteins and pathways outside of a multicellular organism. A previous study using limited siRNA libraries in migrating mammalian cells did not distinguish specific roles of proteins/pathways involved in cadherin-mediated adhesion and other cell adhesion and migration processes (Simpson et al., 2008). *Drosophila* S2 cells have emerged as a powerful tool to dissect diverse, evolutionarily conserved cellular processes by allowing access to the entire *Drosophila* genome while minimizing the redundancy that resulted from early genome duplication in mammals (Goshima et al., 2007). *Drosophila* S2 cells, which are derived from phagocytic hematopoietic cells, do not express DE-cadherin and do not form Ca^{2+}-dependent cell aggregates (Oda et al., 1994).

To investigate proteins and pathways specific for AJ function, we established a *Drosophila* S2 cell adhesion assay that restricted analysis to Ca^{2+}-dependent, cadherin-mediated cell–cell adhesion, and the exclusion of other adhesion processes; this heterologous system provides a way of defining important regulatory hubs and pathways specifically involved in cadherin-mediated cell–cell adhesion. We completed a genome-wide (∼14,000 genes) RNAi screen and then analyzed proteins in both *Drosophila* oogenesis and mammalian MDCK cells to test the evolutionary conservation of protein functions. We identified 17 interconnected regulatory hubs comprising ∼400 proteins that include unexpected pathways and unknown proteins, some of which overlap with cell migration pathways, which are required to coordinate cadherin-mediated cell–cell adhesion.

Results

S2 cells expressing DE-cadherin fully recapitulate cadherin-mediated adhesion

We generated an S2 cell line that stably expressed DE-cadherin (DECAD-S2) and was able to form small cadherin-dependent cell aggregates in suspension culture. By concentrating cells in the center of the suspension by gently swirling, the cells formed macroscopic Ca^{2+}-dependent cell aggregates within 10–15 min (Fig. 1 A). This system provides a robust cell–cell adhesion assay that is inducible, Ca^{2+}- and cadherin-dependent, and independent of cell–substrate (ECM) adhesion, cell spreading, and migration.

To test whether core cytoplasmic proteins of the cadherin adhesion complex were present upon DE-cadherin expression in DECAD-S2 cells, we analyzed the levels of β-catenin, α-catenin, and p120-catenin. Levels of all catenins were increased

(Fig. 1 B), and all catenins localized to sites of cell–cell contact (Fig. 1 D) with actin (Fig. 1 E) in DECAD-S2 cells. Similar effects have been reported in mammalian fibroblasts expressing ectopic E-cadherin (Ozawa et al., 1989; Nagafuchi et al., 1991). The Ca^{2+}-independent cell–cell adhesion protein echinoid (the *Drosophila* nectin-like protein; Fig. 1 C; Wei et al., 2005) is also found at the AJ in some cell types, but RT-PCR of S2 and DECAD-S2 cell RNA did not detect echinoid expression; this is in agreement with the absence of Ca^{2+}-independent adhesion in DECAD-S2 cells (Fig. 1 A). Collectively, these results show that expression of DE-cadherin in DECAD-S2 cells resulted in up-regulation of proteins of the core cadherin–catenin adhesion complex and induction of Ca^{2+}-dependent cell–cell adhesion.

DECAD-S2 cells, similar to S2 cells, are susceptible to RNAi, although an additional trypsinization step is required before treatment to break up cell clusters and increase exposure of cells to RNAi (Fig. 1 F). Treatment with dsRNAs that targeted each of the core cadherin–catenin adhesion proteins (DE-cadherin, β-catenin, and α-catenin) in DECAD-S2 cells inhibited the formation of Ca^{2+}-dependent cell aggregation (Fig. 1 G). In contrast, p120-catenin knockdown had little or no effect on DECAD-S2 cell aggregation (Fig. 1 G) consistent with previous results showing that p120-catenin is not essential for cell–cell adhesion in *Drosophila* (Myster et al., 2003). Rho1 was not up-regulated in DECAD-S2 cells (Fig. 1 B), and Rho1 knockdown did not affect DECAD-S2 aggregation (Fig. 1 G), indicating that it is not involved in early stages of cadherin-mediated cell–cell adhesion in these cells. Together, these data show that cadherin-mediated adhesion is functional in DECAD-S2 cells and requires core proteins of the cadherin complex, β-catenin, and α-catenin and that RNAi knockdown can distinguish between proteins required for cell–cell adhesion and those that are not.

A whole genome-wide screen for cadherin-mediated cell–cell adhesion in DECAD-S2 cells

The assay for a genome-wide RNAi screen for loss of DE-cadherin–mediated, Ca^{2+}-dependent cell–cell adhesion in DECAD-S2 cells was optimized in a 96-well format. The surface of the wells was pacified with the surfactant Pluronic F127 to block nonspecific cell binding to the surface of the well. Trypsinized cells were treated with RNAi for 5 d under conditions to minimize cell–cell contacts (low cell density and no swirling). To form cell aggregates, 96-well plates were briefly shaken to suspend cells and then swirled for 20 min to induce cell–cell contacts (see Materials and methods), and finally given another short, gentle shake to disrupt any weak cell–cell associations (Fig. 2 A). Hoechst-stained cells were automatically imaged and scored. A gradient of adhesion defects was observed, but computational analysis was unable to detect more intermediate phenotypes (unpublished data). Therefore, three different images for each RNAi were scored by eye between 0 (strong aggregation) and 4 (no aggregation; Fig. 2 B). All wells averaging a score of >1.5 were considered positive and processed for a second screen using coded RNAi that allowed double-blind quantification of cell–cell adhesion.
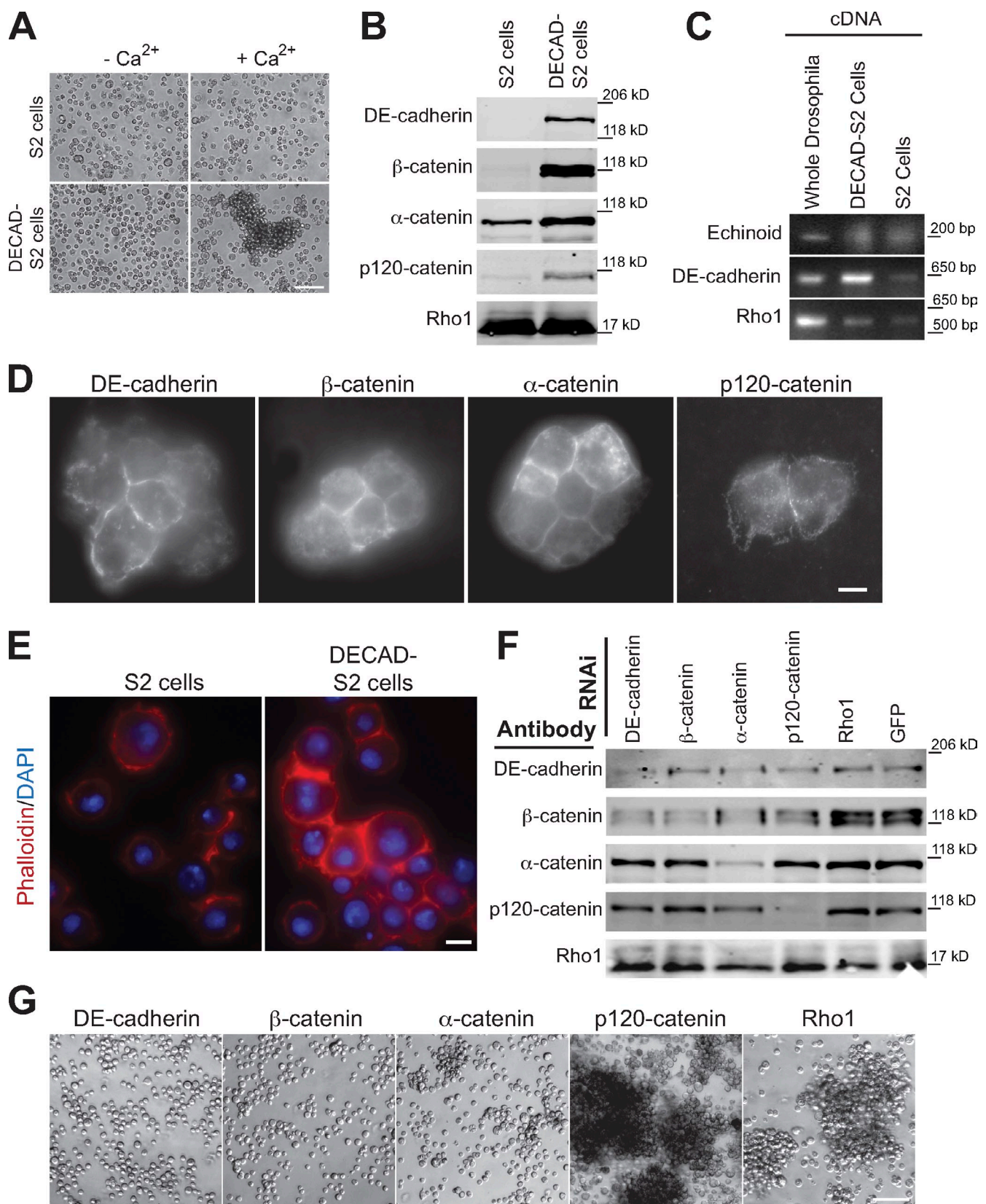
Figure 1. **Properties of DECAD-S2 cells.** (A) Bright-field microscopy of S2 or DECAD-S2 cells in Schneider's media or Hank's buffer with and without Ca^{2+}. (B) Western blots of the indicated proteins in whole-cell lysates of S2 or DECAD-S2 cells. Each lane was loaded with protein from 2.5×10^5 cells. (C) RT-PCR of the indicated genes from cDNA collected from whole *Drosophila* adults, S2 cells, or DECAD-S2 cells. (D) Immunofluorescence of the indicated proteins in DECAD-S2 cells. (E) Rhodamine phalloidin staining of S2 and DECAD-S2 cells. (F) Western blot analysis of the indicated proteins in different RNAi treatments of DECAD-S2 cells. Each lane was loaded with protein from 2.5×10^5 cells. (B, C, and F) Hashes indicate molecular mass standards. (G) Bright-field microscopy of RNAi-treated cells after plates swirling to induce aggregate formation. Bars: (A and G) 100 µm; (D and E) 2 µm.
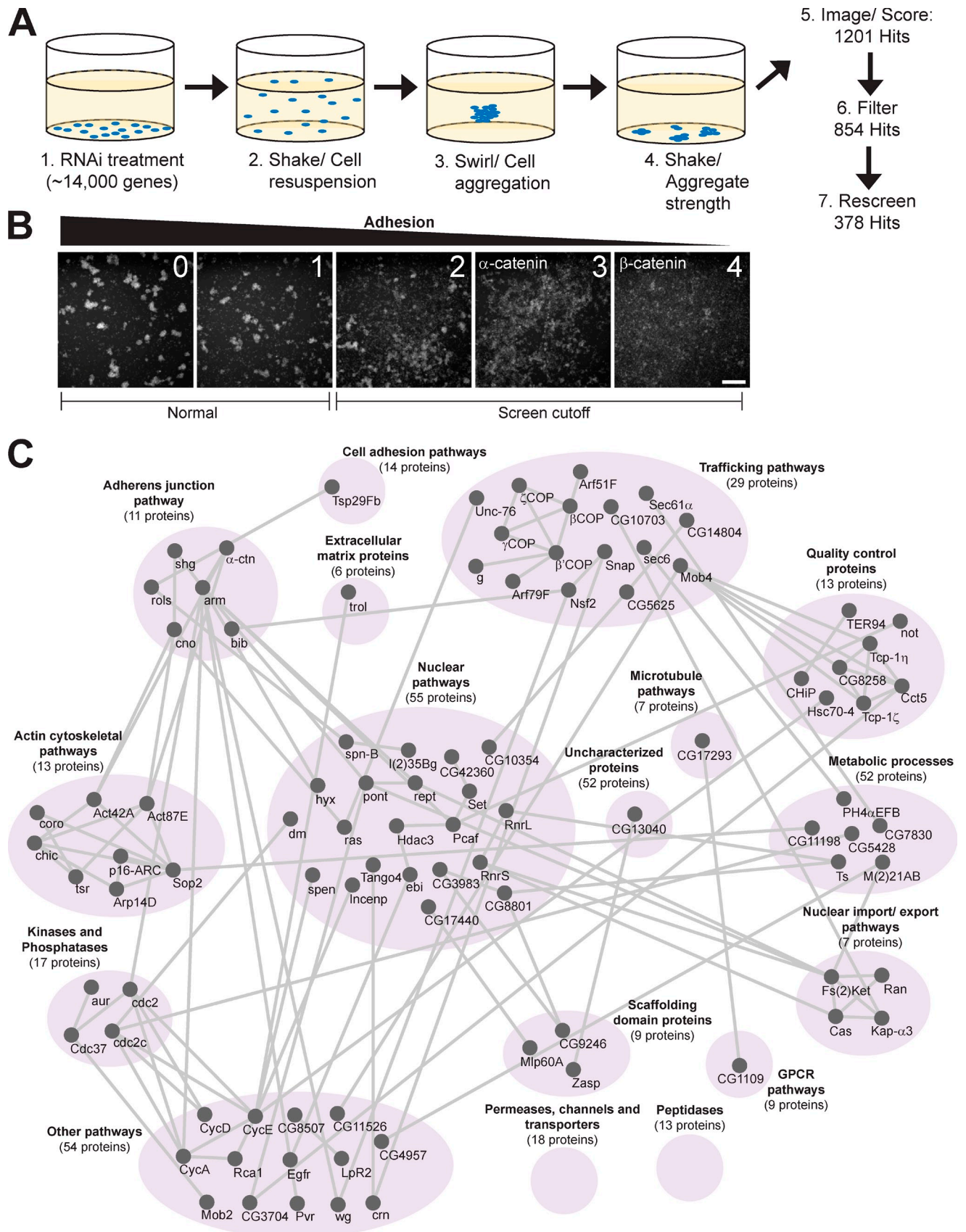
Figure 2. **A genome-wide screen for cadherin-mediated adhesion.** (A) Schematic for the RNAi screen in DECAD-S2 cells and summary of screen results. (B) Automated images from the screen showing well images ranging from strong aggregation to no aggregation. Bar, 50 µm. (C) Identified regulatory hubs. Experimental interactions for hits were identified with STRING, and results were visualized in Cytoscape software.

The initial screen yielded 1,201 RNAi's that produced defective cell–cell aggregation in DECAD-S2 cells. Unmasking the identity of the RNAi's revealed that the hits included DE-cadherin, β-catenin, and α-catenin, confirming the reliability of the screen. 347 of the initial hits were involved in protein expression (transcription, translation, splicing, ribosome assembly, and other housekeeping pathways; Table S1). Failure of those cells to aggregate may be caused by loss of generic pathways involved in protein expression and were not pursued further. Of the remaining 854 positive hits, 803 were rescreened in duplicate with RNAi's to regions of the genes that were different from those targeted by RNAi in the first screen. (Two genes were present in duplicate among the 854 positive hits, and 49 genes were not pursued because the database indicated that the genes were considered obsolete or because it was not possible to design nonoverlapping RNAi's.) This second screen yielded 378 hits with a score of >1.5 (Table S2). From this list, ∼100 had a cell aggregation score similar to that of cells with a knockdown of either DE-cadherin (4.0), β-catenin (4.0), or α-catenin (2.67; Table S2).

A BLAST (Basic Local Alignment Search Tool) search against the human genome was performed for the 378 proteins to identify the closest homologues, and the proteins were grouped into hubs based on known protein functions from previous experiments (Fig. 2 C and Table S2). In addition, STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) analysis identified all known physical interactions between these proteins (Fig. 2 C). From this analysis, we identified 17 regulatory hubs that comprised proteins involved in the core AJ complex, other cell adhesion pathways, regulation of actin cytoskeleton assembly and dynamics, microtubule organization, protein trafficking, protein quality control, kinases/phosphatases, ECM proteins, scaffolding protein complexes, metabolic processes, G protein–coupled receptor pathways, nuclear import/export, nuclear pathways, permeases and transporters, proteases, and others (see Discussion; Fig. 2 C).

Defects in AJ core complex expression in RNAi-treated DECAD-S2 cells

RNAi-induced cell–cell adhesion defects could be caused by decreased levels of the core cadherin adhesion complex. Therefore, we analyzed the protein levels of DE-cadherin, β-catenin, and α-catenin in the top 100 hits, which covered a range of proteins in different hubs (Fig. 3, A–C; and Table S3). This analysis revealed three main categories of effects of RNAi-mediated knockdown: (1) The largest group of proteins had decreased levels of either DE-cadherin or β-catenin (<0.50-fold), indicating a role in regulating the stability of cadherin adhesion complex. (2) Levels of DE-cadherin and catenins were similar to control levels, indicating a role downstream of the regulation of protein levels or protein interactions in the core cadherin–catenin complex. (3) α-Catenin levels were increased (>1.25-fold), indicating a role in controlling the level of α-catenin independently of cadherin and β-catenin. Thus, the control of core cadherin–catenin levels is a critical process in cell–cell adhesion and one that is regulated by a diverse number of regulatory hubs.

Drosophila oocyte position defects in germline mutants from proteins identified in the DECAD-S2 RNAi screen

We sought to validate the physiological relevance of the proteins identified in the DECAD-S2 cell screen in an in vivo multicellular system in which cadherin function is strictly required for a developmental process. In the Drosophila germarium, DE-cadherin is enriched on the surface of both the oocyte and posterior follicle cells, and cadherin–cadherin interactions are required for correct oocyte positioning during oogenesis (Fig. 4 A; Godt and Tepass, 1998). For this analysis, we chose 40 proteins out of the top 100 that were also representative of the 17 regulatory hubs (Table S2).
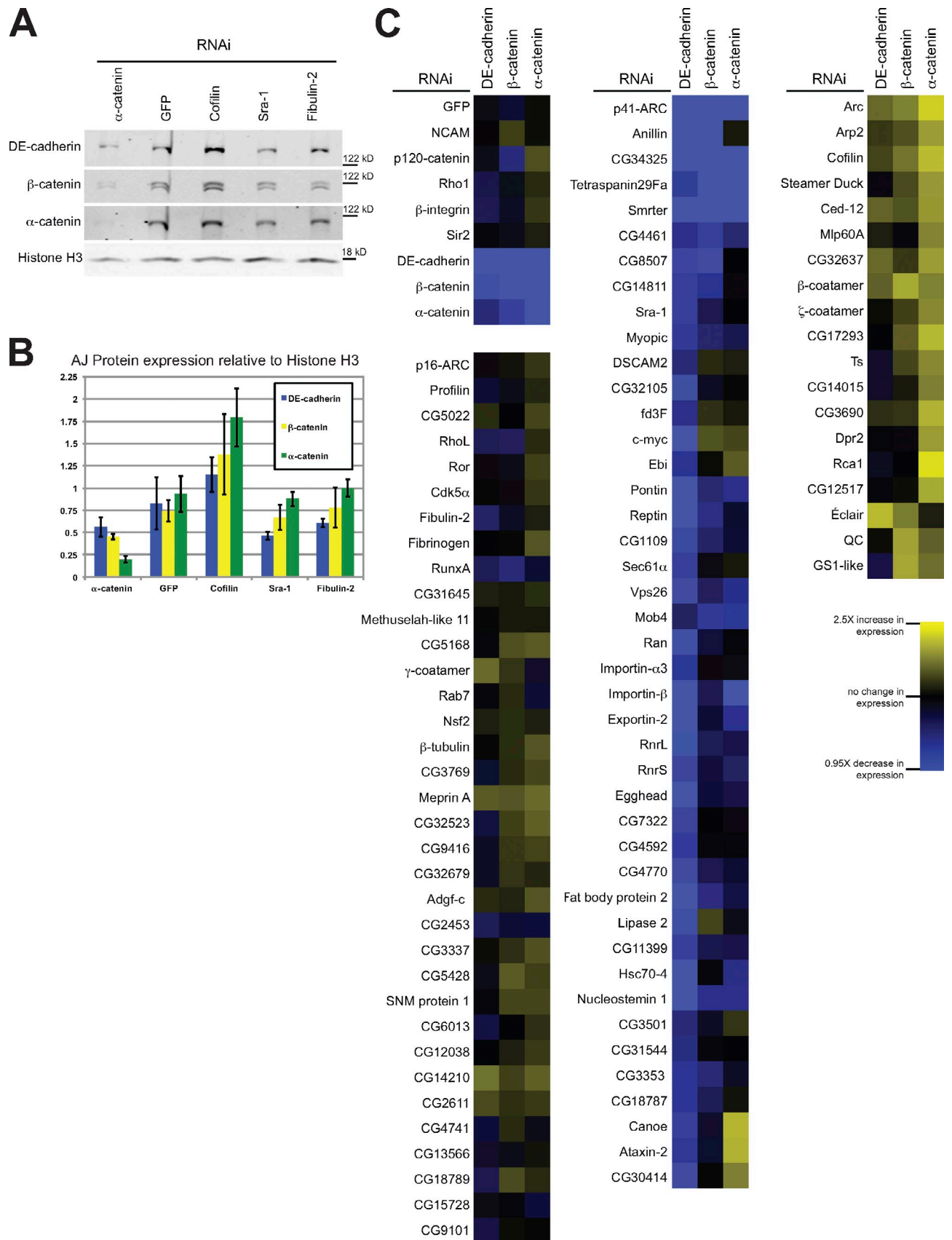
Expression of each RNAi was driven in the germline using Drosophila TRiP (Transgenic RNAi Project) RNAi lines. Ovaries were collected for each RNAi line, and oocyte position in the germarium was determined by staining for Orb, which localizes to the oocyte (Lantz et al., 1994), and F-actin (phalloidin), which outlines each cell. Approximately 100 oocytes (stages 1–5) were analyzed for each TRiP RNAi line, and each was scored based on the position of the oocyte from posterior to anterior of the germarium (Fig. 4 B; Godt and Tepass, 1998).

18 TRiP RNAi lines produced viable ovaries, and the majority of them had clear defects in oocyte positioning compared with the wild-type (Fig. 4 C). These mutants included several hubs: the core AJ complex, regulators of the actin cytoskeleton, scaffolding proteins, ECM proteins, and nuclear pathways. These results confirm the importance of the proteins identified in the DECAD-S2 screen for DE-cadherin–dependent cell–cell adhesion and tissue organization in vivo. A common theme to these mutants was the inclusion of proteins that regulate the actin cytoskeleton, indicating long-range effects of cadherin functions in the global positioning of the oocyte in the multicellular organization of the germarium. Similar to DE-cadherin and Armadillo (β-catenin) germline mutants (Godt and Tepass, 1998), other oocyte defects were also present in some of the TRiP RNAi lines, including misshapen cells, the presence of two oocytes, and decreased germ cell number (unpublished data), which were not scored. This result indicates that these proteins either play additional roles independently of cadherin functions or that there are additional cadherin functions in the germ cell niche and germ cell differentiation (González-Reyes, 2003).

A large portion of the genes analyzed had global defects on germarium organization, which made scoring oocyte positioning defects ambiguous (Fig. 4 C); these phenotypes included sterility, shrunken ovaries, and gross ovary/germarium morphological defects. Several of these mutants disrupt nuclear or cytoplasmic trafficking pathways that could impinge on many processes involved in the localization and function of the cadherin–catenin complex in the oocyte. It remains unclear what step of DE-cadherin–mediated adhesion is affected by proteins identified with oocyte position defects.

E-cadherin–mediated adhesion defects in MDCK cells from hits identified in the DECAD-S2 RNAi screen

To test whether proteins important for cadherin cell–cell adhesion identified in DECAD-S2 cells and Drosophila germarium

Figure 3. **Cadherin–catenin levels in DECAD-S2 cell knockdowns.** (A) Western blot showing DE-cadherin, β-catenin, and α-catenin expression in different knockdowns. Hashes indicate molecular mass standards. (B) Quantification of three Western blots showing percent expression of the indicated proteins relative to histone H3. Error bars show SEMs for three independent experiments. (C) DE-cadherin, β-catenin, and α-catenin protein levels were calculated relative to histone H3 expression and averaged across three samples, and the deviation in expression from GFP controls was imaged in TreeView.
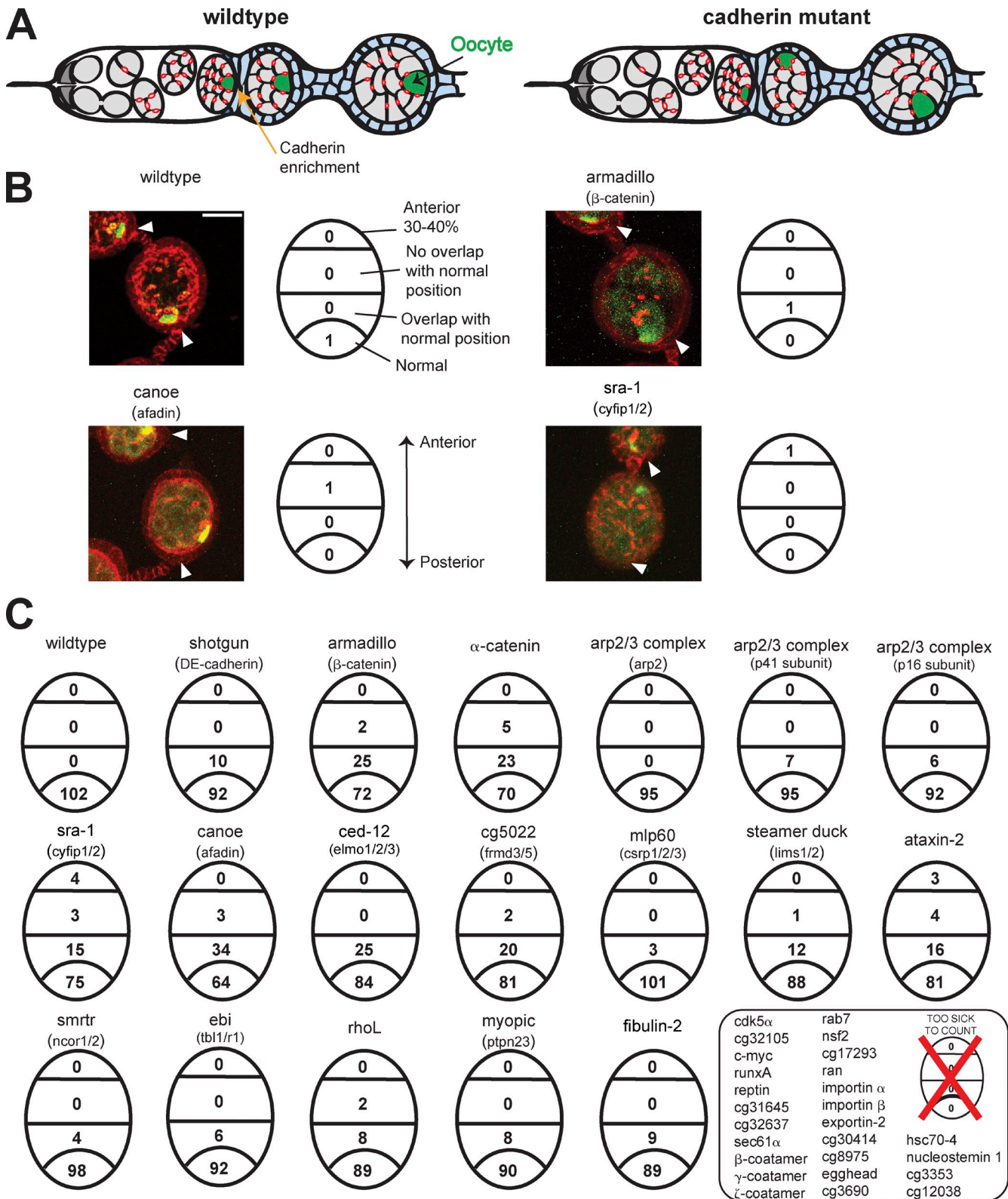
Figure 4. **Oocyte placement defects in germline knockdowns.** (A) Schematic of germarium showing oocyte placement in wild-type and mutant flies. (B) Examples of different oocyte defects and explanation of scoring. Arrowheads indicate posterior location of the egg chamber. Bar, 25 µm. (C) Scores for the indicated germline knockdowns. The box indicates mutants that could not be scored.

Table 1. **List of genes, homologies, and results of mammalian proteins**

Name (*Drosophila*)	Name (mammals)	Function	Regulatory hub	Oocyte position defect (>5% defective)	Hanging drop assay defective
Canoe	AFADIN	AJ component/actin-binding protein	AJ	Yes	Yes
Ataxin-2	ATXN2	Aggregation-prone protein, stress granules, and endocytosis	Other	Yes	Yes
Sra-1	CYFIP1/2	Rac binding/WAVE complex	Actin	Yes	Yes
Steamer duck	LIMS1/2	LIM domain protein and focal adhesions	Adhesion pathways	N/A	No
Mlp60A	CSRP1/2	LIM domain protein and muscle function	Scaffolding domains	No	No
Ced-12	ELMO1/2/3	RacGEF (DOCK180)	Adhesion pathways	Yes	Yes
CG5022	FRMD3/5	FERM domain	Scaffolding domains	Yes	No
ebi	TBL1/R1	Wnt signaling, DNA binding, and transcription	Nuclear	Yes	Yes
Smrter	NCOR1/2	DNA binding and transcription	Nuclear	No	No

N/A, not available.

were conserved in mammals, we screened for defects in cell–cell adhesion in mammalian MDCK epithelial cells. 20 proteins with mammalian orthologues were chosen that were broadly representative of the hubs identified from the DECAD-S2 cell screen (Table 1). RT-PCR showed that each gene was expressed in MDCK cells (Fig. 5 A). For each candidate, MDCK cells were treated with siRNAs and analyzed using a hanging drop assay. Similar to the DECAD-S2 cell adhesion assay, the MDCK hanging drop assay depends solely on Ca^{2+}-dependent cell–cell adhesion in suspension culture, in the absence of cell–substrate (ECM) adhesion and cell migration (Fig. 5 B; Benjamin et al., 2010).

Six siRNAs targeting cyfip1, cyfip2, afadin, atxn2, elmo2, and tbl1r had a clear defect in cell–cell adhesion (Fig. 5 C and Fig. S1 A). Proteins that did not affect cell–cell adhesion in MDCK cells may not have been efficiently depleted, or there was a lack of conservation between species; these proteins were not pursued further. Expression levels of each protein affecting MDCK cell–cell adhesion were analyzed by Western blotting and showed that cyfip1, cyfip2, afadin, atxn2, elmo2, and tbl1r levels were decreased by >80% (Fig. 6). For cases in which multiple orthologues were expressed, we used paired siRNA knockdowns specific to each orthologue. The effect of RNAi-mediated knockdown of both cyfip1 and cyfip2 was stronger than the knockdown of either alone, indicating that both proteins likely have a shared or redundant function in cell–cell adhesion (Fig. S1 B). Dual knockdown of both tbl1 and tbl1r had a slightly stronger defect than tbl1r alone, suggesting that tbl1 has some overlapping or redundant functions with tbl1r in cadherin-mediated adhesion (Fig. S1 B). RNAi knockdown of both elmo1 and elmo3 also blocked cell–cell adhesion, suggesting a potential role in cell–cell adhesion. The interrelationship between the three elmo orthologues in cell–cell adhesion was not pursued further (Fig. S1 B).

To determine how cadherin-mediated adhesion was disrupted in RNAi-treated MDCK cells, we analyzed the levels of proteins in the cadherin–catenin complex. Knockdown of afadin, elmo2, tbl1r, and cyfip2 resulted in a ∼50% reduction in levels of proteins of the core cadherin–catenin complex (Fig. 7, A and B). This suggests that expression and function of these

proteins are essential to maintain the normal level and stability of the cadherin–catenin complex. Notably afadin/canoe, cyfip/sra-1, and tbl1/ebi had decreased levels of DE-cadherin in DECAD-S2 cells (Fig. 3 C). However, elmo/ced-12 and ataxin-2 did not affect cadherin–catenin protein levels (Fig. 3 C), indicating that mechanisms different from those regulating protein stability may be important.

We next investigated the localization of E-cadherin and the targeted protein in each knockdown and identified two categories: (1) afadin and cyfip1/2 that localized to cell–cell contacts and (2) tbl1r, elmo2, and ataxin-2 that localized elsewhere in the cell. Afadin localized to sites of cell–cell adhesion in agreement with a previous study (Ikeda et al., 1999), and its knockdown reduced the amount of E-cadherin at sites of cell–cell contacts (Fig. 7 C) as shown also by Western blotting (Fig. 7, A and B). Cyfip1 and cyfip2, two components of the Wiskott–Aldrich syndrome protein family verprolin homologous protein (WAVE) complex, were also localized at cell–cell contacts and in the cytoplasm, consistent with the known localization of other members of the WAVE complex (Yamazaki et al., 2007). Knockdowns of both cyfip1 and cyfip2 reduced E-cadherin localization at cell–cell contacts, although the effect of cyfip2 knockdown alone appeared stronger (Fig. 7). Based on their colocalization with cadherin, afadin and cyfip1/2 likely affect cadherin–catenin levels and cell–cell adhesion directly.

Tblr1 localized in both the cytoplasm and nucleus, as expected from its known activities (Li and Wang, 2008). In tblr1 knockdowns, E-cadherin at cell–cell contacts was reduced, and there was increased staining of E-cadherin in cytoplasmic foci (Fig. 7). Elmo-2 localized to discrete puncta in the cytoplasm, and knockdown of elmo-2 resulted in a dramatic reorganization of E-cadherin from cell–cell contacts into intracellular, vesicle-like puncta (Fig. 7). Ataxin-2 labeling was mainly cytoplasmic and was notably absent from cell–cell contacts, and knockdown of ataxin-2 had no obvious effect on E-cadherin staining at cell–cell contacts (Fig. 7). That these proteins localize to intracellular compartments but still affected cell–cell adhesion suggests that they function at steps other than cadherin organization at cell–cell junctions, such as protein trafficking or turnover.
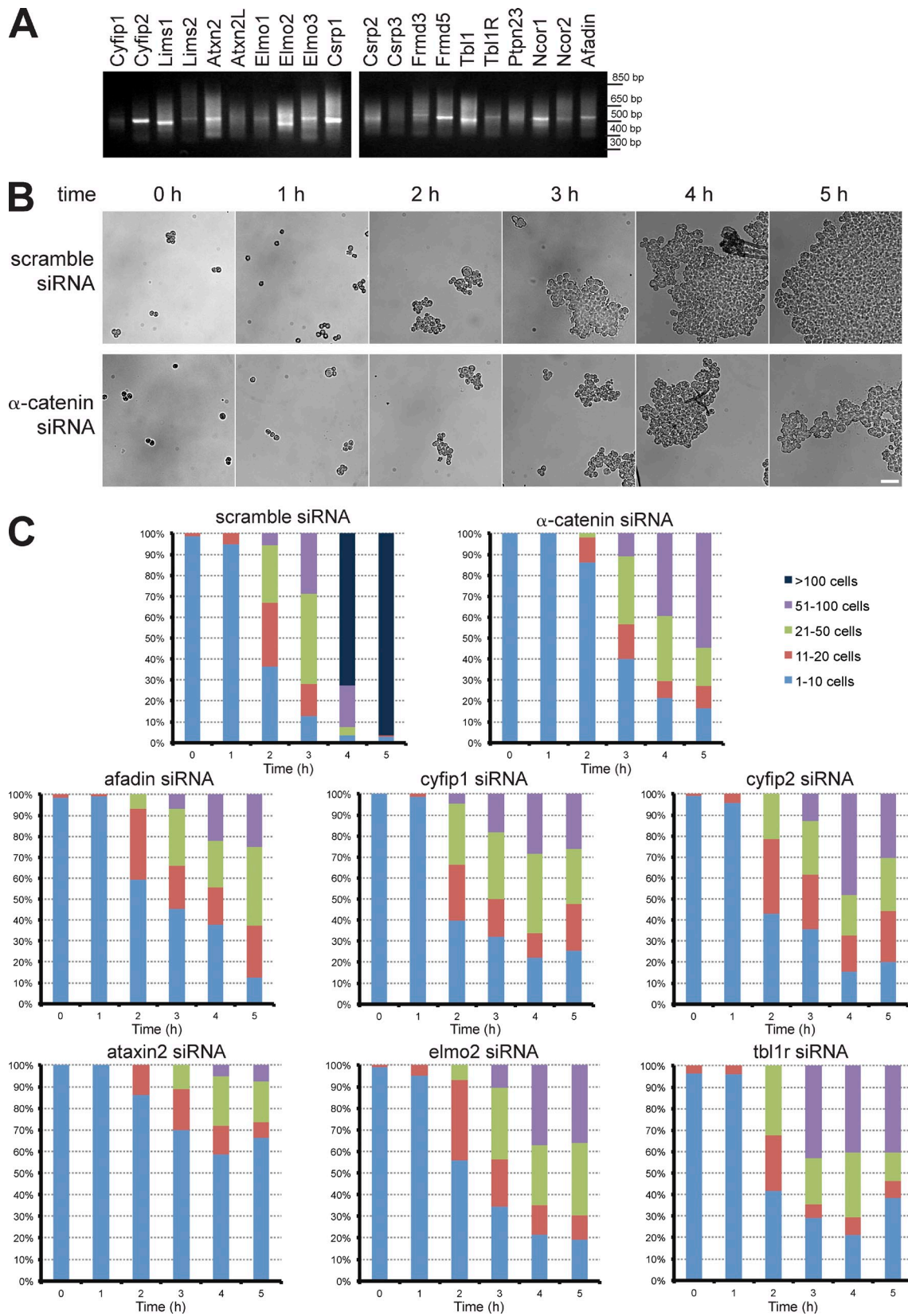
Figure 5.   **Defects in hanging drop assay using MDCK cells.** (A) RT-PCR of the indicated genes showing expression in MDCK cells. Hashes indicate molecular mass standards. (B) Bright-field images showing aggregation of MDCK cells at different time points during the hanging drop assay for scramble and α-catenin siRNA–treated cells. Bar, 50 μm.(C) Quantification of hanging drop assay for the indicated siRNA in which the cells were binned into cluster classes: 1–10, 11–20, 21–50, 51–100, or >100 cells. For each time point, the percentage of cells in each category is shown. The data shown are from a single representative experiment out of three repeats in which at each time point ∼5 × 10⁴ cells were analyzed.
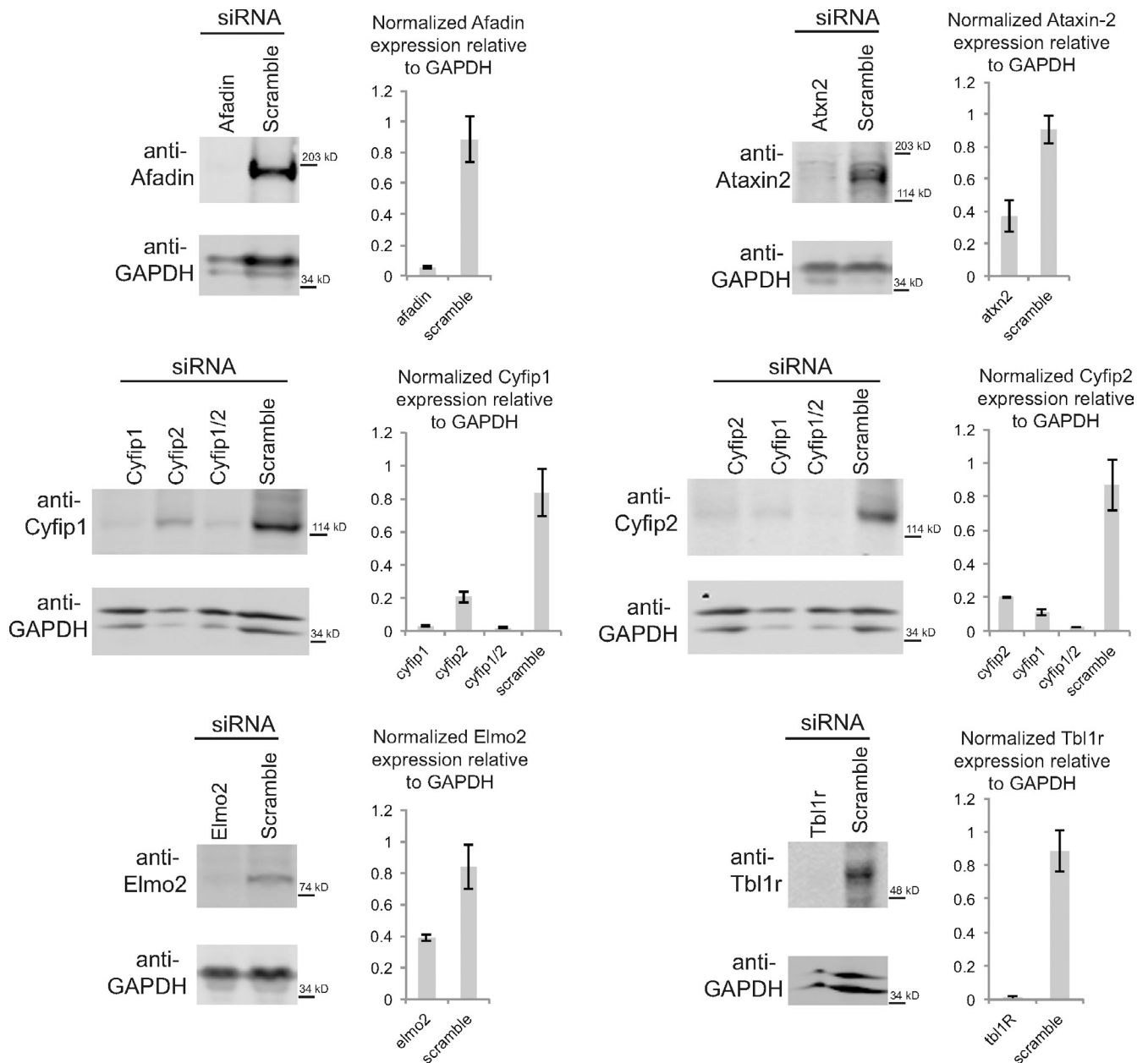
Figure 6. **Efficiency of siRNA in MDCK cells.** For each indicated gene, a Western blot showing siRNA construct and scramble effects on expression and quantification from three experiments. Hashes indicate molecular mass standards. Error bars show SEMs for three independent experiments.

Discussion

Our study is the first successful genome-wide RNAi screen for defects specific to the cadherin-mediated adhesion pathway. We established a robust assay in suspension culture that specifically excluded Ca²⁺-independent cell–cell adhesion, integrin–substrate/ECM adhesion, and cell spreading and migration pathways. This screen is distinct from others that used a limited RNA library in an assay that required cell migration pathways (Simpson et al., 2008), data mining of the literature (Zaidel-Bar, 2013), or a suppression screen of a weak α-catenin mutant in *Caenorhabditis elegans* (Lynch et al., 2012). In total, the screen identified ∼400 proteins, many of which were unexpected. These proteins clustered into 17 regulatory hubs, which revealed

the involvement of surprisingly diverse cellular pathways from the nucleus to the plasma membrane that are integrated to drive cadherin-mediated cell–cell contact formation. We focused on analyzing representative components of these hubs. Similar hubs were also identified in a screen that rescued an α-catenin mutant in *C. elegans*, including cell junction proteins, cytoskeletal proteins, ECM proteins, protein trafficking, and metabolism (Lynch et al., 2012). That there are some similarities in the products of these different screens further supports the general usefulness and specificity of the screen in S2 cells.

Expression of DE-cadherin in *Drosophila* S2 cells fully engaged the Ca²⁺-dependent cadherin adhesion pathway (Fig. 1) and led to the up-regulation of protein levels of the core cadherin–catenin complex. Although p120-catenin is required for
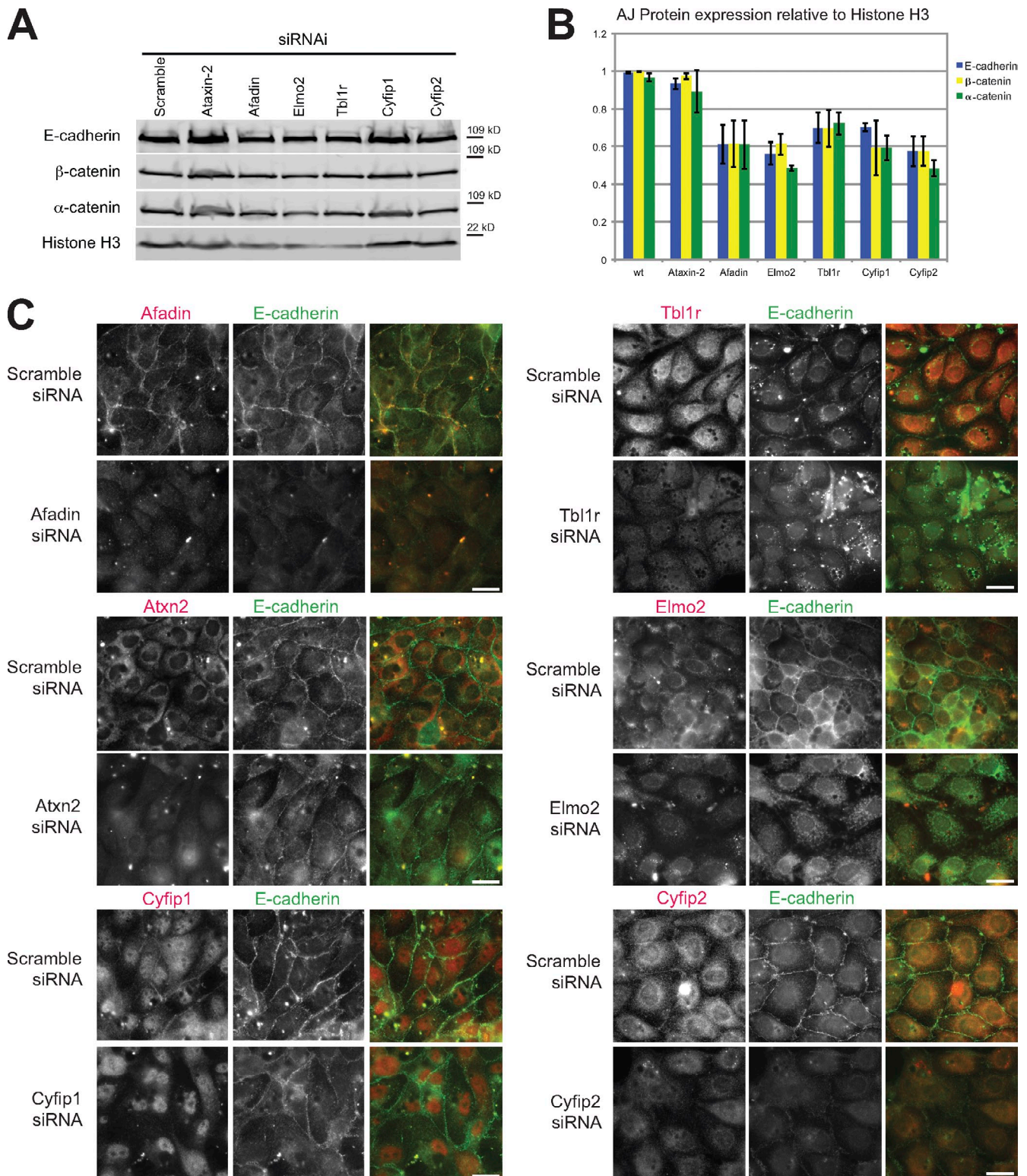
Figure 7. **Cadherin–catenin effects in siRNA-treated MDCK cells.** (A) Western blots for the indicated siRNA-treated cells. Hashes indicate molecular mass standards. (B) Quantification of three Western blots showing percent expression of the indicated proteins relative to histone H3. wt, wild type. Error bars show SEMs for three independent experiments. (C) Immunofluorescence for the indicated siRNA-treated cells. Bars, 25 µm.

cell–cell adhesion in mammalian cells but not in *Drosophila* S2 cells (Fig. 1), we found that p120-catenin protein level increased upon DE-cadherin expression, perhaps as a result of protein stabilization by binding DE-cadherin similar to

the other catenins. Similar to DECAD-S2 cells, mammalian L cells expressing E-cadherin up-regulated core complex proteins (Ozawa et al., 1989; Nagafuchi et al., 1991). Thus, there is a conserved mechanism involving stabilization of constitutively

expressed β-catenin and α-catenin by binding to ectopic DE-cadherin. Significantly, over a third of the top 100 proteins identified in the DECAD-S2 cell screen induced a reduction in the levels of proteins in the core cadherin–catenin complex (Fig. 3). These results indicate that stable accumulation of the core cadherin complex at the plasma membrane is a major mechanism for regulating cell–cell adhesion and that there is surprising complexity in the diversity of regulatory hubs involved. Comparing proteins identified in apparently nonpolarized DE-cadherin–expressing S2 cells with more traditional cell adhesion systems may be challenging. To address this concern, we analyzed a subset of hub proteins in both *Drosophila* oogenesis and mammalian MDCK epithelial cells. Collectively, these approaches highlight the value of the screen.

A regulatory hub containing several known AJ proteins was identified, but several other AJ-associated proteins were not identified, including vinculin or an EPLIN-related LIM domain–containing protein (Abe and Takeichi, 2008; Twiss et al., 2012). This may be because of nonfunctional RNAi in the initial screen, differences between organism requirements similar to the case with p120-catenin (Myster et al., 2003), or a role for these proteins later in the AJ pathway during compaction or organization of an epithelial monolayer, which were not assayed in this screen. Our screen identified canoe/afadin (Mandai et al., 1997), which is thought to primarily function by binding the nectin class of Ca^{2+}-independent cell–cell adhesion proteins at the AJ (Takai et al., 2008). However, expression of the *Drosophila* nectin-like protein echinoid was not detected in DECAD-S2 cells (Fig. 1 C), indicating that canoe/afadin may function through a link to the cadherin complex. Indeed, afadin binds α-catenin (Pokutta et al., 2002) and p120-catenin (Sato et al., 2006) or binds indirectly to the cadherin complex through links to the actin cytoskeleton (Mandai et al., 1997). In vivo, canoe/afadin also functions in Rap1 signaling (Boettner and Van Aelst, 2009) and during later events involving cell–cell adhesion, including apical constriction (Sawyer et al., 2009, 2011).

Actin networks drive membrane protrusions that initiate cell–cell contact and form the contractile ring that underlies the AJ (Yonemura, 2011). The screen uncovered an actin hub, which included components of the Arp2/3 complex, profilin, and cofilin, all of which are fundamental to the regulation of actin dynamics (Pollard et al., 2000). Cyfip/sra-1 has a conserved role in cell–cell adhesion (Figs. 3, 5, and 7) and is a component of the WAVE complex, a potent Arp2/3 activator (Pollitt and Insall, 2009). The WAVE complex is phosphorylated by src and binds Rac1-GTP via cyfip/sra-1 to relieve autoinhibition of the WAVE complex (Ibarra et al., 2005; Ardern et al., 2006). Significantly, cyfip knockout mice also have defects in cell–cell adhesion (Silva et al., 2009). Cyfip1/2 localized to MDCK cell–cell contacts (Fig. 7), similar to other WAVE components WAVE1, Nap-1, and Abi (Yamazaki et al., 2007; Ryu et al., 2009). Cyfip siRNA in MDCK cells showed that both cyfip1 and cyfip2 are required for Ca^{2+}-mediated cell–cell adhesion. Our results indicate that cyfip1/2 knockdowns destabilized the cadherin–catenin complex, which agrees with observations that RNAi knockdowns of other WAVE complex proteins (WAVE1/2 and Abi) also reduced cadherin levels at mature cell–cell contacts

(Ryu et al., 2009). The Rac–WAVE pathway has been well studied in cell migration pathways, and another study has linked activation of the Rac–WAVE complex in cell–cell adhesion and organization of the actin cytoskeleton (Verma et al., 2012).

The DECAD-S2 cell screen specifically targeted cadherin-mediated contact formation and excluded cell migration and cell–substrate adhesion pathways. Nevertheless, many of the proteins that affect cadherin-mediated adhesion are also involved in other cell adhesion or cell migration pathways (e.g., ced-12/elmo [Lu and Ravichandran, 2006], sra-1/cyfip [Takenawa and Suetsugu, 2007], steamer duck/LIMS [Wickström et al., 2010], and fibulin-2 [de Vega et al., 2009]). A previous study may have overlooked this category of proteins because their assay involved not only cell–cell adhesion but also cell spreading and migration on ECM (Simpson et al., 2008). We found that ced-12/elmo2 knockdown caused defects in cadherin-mediated processes in *Drosophila* and MDCK cells, indicating a conserved role. Elmo in conjunction with DOCK180 acts as a RacGEF downstream of Rho signaling at integrin-based adhesions (Lu and Ravichandran, 2006; Côté and Vuori, 2007), but our results demonstrate a novel requirement for elmo2 in efficient cadherin accumulation at cell–cell contacts. Interestingly, the elmo/ced-12 homologue in *C. elegans* is essential for Rab5-mediated endocytic recycling (Sun et al., 2012). Because we detected a decrease in cadherin–catenin protein levels in elmo2/ced-12 RNAi-treated MDCK cells (Fig. 3 C), elmo2/ced-12 may also be involved in cadherin turnover or recycling.

One of the largest hubs identified by the screen comprised nuclear proteins (Fig. 2). The changes in protein expression levels between S2 cells and DECAD-S2 cells (Fig. 1 B) indicate significant changes in protein levels upon DE-cadherin expression, and proteins involved in nuclear pathways may be involved in this process. The nuclear hub also indicates a potential novel link between β-catenin, Wnt signaling, and cadherin-mediated cell–cell adhesion. Reptin and pontin, two chromatin-remodeling AAA+ ATPases implicated in the negative regulation of β-catenin–mediated transcription (Bauer et al., 2000; Gallant, 2007), were identified in the screen (Table S2). Furthermore, the screen identified the NCoR–HDAC3–TBL1 nuclear corepressor complex, which regulates gene transcription (Table S2; Yoon et al., 2003), and tbl1 and tbl1r, which are required for β-catenin–mediated transcription in response to Wnt signaling (Li and Wang, 2008). Unexpectedly, we found that tbl1r RNAi induced cell–cell adhesion defects in *Drosophila* and MDCK cells, and tbl1r RNAi reduced DE-cadherin levels and caused DE-cadherin mislocalization. Thus, nuclear activities of reptin/pontin and NCoR–HDAC3–TBL1 corepressors may play essential roles in controlling the expression of protein pathways in response to DE-cadherin expression and cadherin-mediated adhesion.

In summary, we have completed the first genome-wide screen for proteins and pathways specifically involved in cadherin-mediated cell–cell adhesion. We identified ∼400 proteins required for cadherin-mediated early cell–cell adhesion that were assigned to 17 regulatory hubs based on protein functions and interactions. Although some of the proteins have been characterized in cell–cell adhesion, the majority of identified proteins function in pathways not previously linked to cell–cell

adhesion, including elmo, tbl1r, and atxn2, and ~12% are proteins with unknown functions. Our subsequent analysis of representative components revealed conserved functions of these regulatory hubs in diverse cellular functions in *Drosophila* and mammalian epithelial cells. This database should provide a rich resource to explore novel proteins and pathways and their involvement in cadherin-mediated cell–cell adhesion.

Materials and methods

Cell culture and stable cell lines
Drosophila S2U cells were maintained in Schneider's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), penicillin, streptomycin, and kanamycin (Goshima et al., 2007). Full-length DE-cadherin was cloned into pAc5.1/V5–His B (Invitrogen) and cotransfected with blasticidin and hygromycin plasmids in S2U cells to generate stable expression lines (Millar et al., 1994). For RNAi treatment, DE-cadherin–expressing S2 cells were treated with 625 µg/ml trypsin for 20 min at 25°C and then resuspended in serum-free Schneider's medium. 1 µg RNA was incubated with 6×10^5 cells for 30 min in serum-free Schneider's medium followed by a 5-d recovery in Schneider's medium supplemented with 20% heat-inactivated fetal bovine serum (Goshima et al., 2007). RNAi-treated cells were maintained in plasticware coated with a 10% Pluronic 127 (Sigma-Aldrich) solution to minimize cell–substrate interactions. MDCK G type II cells were grown in DMEM with 1 g/liter sodium bicarbonate, 10% fetal bovine serum (Atlas Biologicals), penicillin, streptomycin, and kanamycin. For MDCK cells, two rounds of 10 µg siRNA was transfected (Lipofectamine 2000) for 18-h periods, and cells were analyzed after a 24-h recovery.

RNAi design and synthesis
The initial genome-wide screen used the V2 RNAi library (Thermo Fisher Scientific; Goshima et al., 2007). Nonoverlapping RNAi's that were 100–800 bp in length and minimized repeated sequences were generated for rescreening DECAD-S2 cells (Goshima et al., 2007). Canine siRNA oligonucleotides were designed by Thermo Fisher Scientific (Table S4).

Screening process and scoring
After 5 d of RNAi treatment, 1 µg/ml Hoechst 33342 (Thermo Fisher Scientific) was added to each well. 96-well plates were shaken for 5 min at 800 rpm on a vortex mixer (MixMate; Eppendorf). 96-well plates were then placed on a platform that rotated around a 3-cm radius (D. Proffit, Molecular and Cellular Physiology–Stanford Institute for Neuro-Innovation and Translational Neuroscience Engineering Shop, Stanford School of Medicine, Stanford, CA) for 20 min at 250 rpm and were next shaken for 30 s at 650 rpm on a MixMate. Plates were imaged using an automated microscope (ImageXpress Micro; Molecular Devices) with a 4× objective. Images were acquired with MetaMorph (Molecular Devices) and a camera (CoolSNAP HQ; Photometrics). For scoring, three images/well were ranked by eye based on aggregation (0 = strong aggregation; 4 = no aggregation). A score of >1.5 was considered defective aggregation.

Immunofluorescence, phalloidin staining, and Western blotting
For S2 cell immunofluorescence, the cover glass was incubated for 10 min with 0.5 mg/ml concanavalin A (Sigma-Aldrich) and washed with water, and S2 cells were allowed to settle and adhere for 20–30 min (D'Ambrosio and Vale, 2010). Cells were then fixed in 10% methanol (−20°C) and labeled with antibodies specific for DE-cadherin (DCAD2; T. Uemura, Kyoto University, Sakyo-ku, Kyoto, Japan; Developmental Studies Hybridoma Bank), Armadillo (N2 7A1; E. Wieschaus, Princeton University, Princeton, NJ; Developmental Studies Hybridoma Bank), α-catenin (DCAT-1; M. Takeichi, RIKEN Center for Developmental Biology, Chuo-ku, Kobe, Japan; Developmental Studies Hybridoma Bank), p120-catenin (p1B2; S. Parkhurst, Fred Hutchinson Cancer Research Center, Seattle, WA; Developmental Studies Hybridoma Bank), or Rho1 (p1D9; S. Parkhurst; Developmental Studies Hybridoma Bank). S2 cells were prepared similarly for rhodamine phalloidin (Invitrogen) staining but fixed in 3.8% PFA. MDCK cells were adhered to collagen-coated cover glass and fixed in methanol (except for cyfip2 labeling, which involved fixation in 3.8% PFA). Antibodies used in immunofluorescence experiments were E-cadherin (Decma; Sigma-Aldrich), l-afadin (A0349; Sigma-Aldrich), ataxin-2 (611378; BD), cyfip1 (AB6046; EMD Millipore), cyfip2 (ab79716; Abcam), elmo2 (ab2240; Abcam), and tblr1 (ab24550; Abcam). Samples were imaged

with a 40×/1.3 NA EC Plan Neofluar lens (MDCK cells; Carl Zeiss) or 100×/1.4 NA Plan Apochromat lens (S2 cells; Carl Zeiss). Images were acquired with AxioVision LE64 software (Carl Zeiss) and a camera (Axio-Cam MRm; Carl Zeiss). Western blotting was performed as described previously (Benjamin et al., 2010). All lysates were made in 4× Laemmli buffer. All aforementioned antibodies were used at 1:1,000, histone H3 (ab1791; Abcam) was used at 1:5,000, and GAPDH was used at 1:2,000 (ab8245; Abcam). Quantification of blots was performed using ImageJ (National Institutes of Health).

RT-PCR
Total RNA of MDCK cells, S2 cells, DECAD-S2 cells, or whole *Drosophila* was extracted using the TRIZOL reagent (Invitrogen). 1 µg RNA was used to generate cDNA with an RT-PCR kit (PrimeScript; Takara Bio Inc.). For each gene, two oligonucleotide pairs (Table S4) were used to analyze expression after 18, 21, 24, 27, and 30 cycles of PCR.

Oogenesis experiments
Drosophila TRiP lines were obtained from the Bloomington Stock Center (Table S4). TRiP lines or a GFP control was crossed with a germline driver (Table S4). All experiments were performed at 30°C. *Drosophila* ovaries were dissected in PBS and sheared through a cutoff pipette tip, fixed in 4% PFA (Electron Microscopy Sciences) for 30 min at 4°C, incubated in heptane for 10 min, washed in PBS, and stained (O'Reilly et al., 2008). Orb (orb 4H8 and orb 6H4; P. Schedl, Princeton University, Princeton, NJ; Developmental Studies Hybridoma Bank) was used for oocyte staining, and rhodamine phalloidin was used for actin staining. Cells were imaged with 20×, 0.5 NA EC Plan Neofluar lens on a laser-scanning confocal system (LSM 510 Meta; Carl Zeiss). 50-µm-thick confocal stacks were collected for each germarium and connected egg chambers. Maximal intensity z stacks were generated for each stack and scored by eye for posterior oocyte localization.

Hanging drop assay
The assay was performed as previously described (Benjamin et al., 2010). MDCK cells were plated at low density, and cells were trypsinized, centrifuged, and resuspended at 2.5×10^5 cells/ml. 20-µl drops of cell suspensions were placed on 35-mm culture dish lids. At each time point, drops were triturated 10 times through a 20-µl pipette, and 4 µl of 16% PFA was added. The entire sample was mounted on a slide, observed at 10×, and scored by eye. Representative images were collected (Axiovert 200M; Carl Zeiss). For each siRNA, the experiment was repeated twice.

Online supplemental material
Fig. S1 shows hanging drop results for siRNA constructs that had no effect on cell aggregation and combinatorial siRNA hanging drop experiments. Table S1 contains all proteins identified in the primary screen. Table S2 indicates the proteins identified in the secondary screen. Table S3 lists protein expression levels used to generate Fig. 3 C. Table S4 contains siRNA sequences, RT-PCR primers, and fly strains used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201306082/DC1. Additional data are available in the JCB DataViewer at http://dx.doi.org/10.1083/jcb.201306082.dv.

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