A WAVE2–Arp2/3 actin nucleator apparatus supports junctional tension at the epithelial zonula adherens

Suzie Verma, Siew Ping Han, Magdalene Michael, Guillermo A. Gomez, Zhe Yang, Rohan D. Teasdale, Aparna Ratheesh, Eva M. Kovacs, Radiya G. Ali, and Alpha S. Yap

Division of Molecular Cell Biology, Institute for Molecular Bioscience, and School for Biomedical Sciences, University of Queensland, St. Lucia, Brisbane 4072, Australia

ABSTRACT The epithelial zonula adherens (ZA) is a specialized adhesive junction where actin dynamics and myosin-driven contractility coincide. The junctional cytoskeleton is enriched in myosin II, which generates contractile force to support junctional tension. It is also enriched in dynamic actin filaments, which are replenished by ongoing actin assembly. In this study we sought to pursue the relationship between actin assembly and junctional contractility. We demonstrate that WAVE2–Arp2/3 is a major nucleator of actin assembly at the ZA and likely acts in response to junctional Rac signaling. Furthermore, WAVE2–Arp2/3 is necessary for junctional integrity and contractile tension at the ZA. Manoeuvers that disrupt the function of either WAVE2 or Arp2/3 reduced junctional tension and compromised the ability of cells to buffer side-to-side forces acting on the ZA. WAVE2–Arp2/3 disruption depleted junctions of both myosin IIA and IIB, suggesting that dynamic actin assembly may support junctional tension by facilitating the local recruitment of myosin.

INTRODUCTION The epithelial zonula adherens (ZA) is a specialized adhesive junction implicated in tissue integrity and apical contractility (Sawyer et al., 2010). It is characterized by the local accumulation of E-cadherin in a ring-like zone at the apical–lateral interface between cells (Tepass and Hartenstein, 1994; Muller and Wieschaus, 1996; Otani et al., 2006; Kovacs et al., 2011). This lies contiguous to concentrations of F-actin that have been reported to organize into dense bundles (Hirokawa et al., 1983; Tepass and Hartenstein, 1994). The ZA is a site where adhesion is coordinated with contractile forces, which combine to generate tension in the junction (Fernandez-Gonzalez et al., 2009; Ratheesh et al., 2012). Consistent with this, the apical actin ring is enriched in myosin II (Ivanov et al., 2005; Smutny et al., 2010), the major contractile tension generator within cells, and disruption of myosin II or its upstream activators perturbs ZA integrity (Smutny et al., 2010; Ratheesh et al., 2012). Junctional tension in the ZA may contribute to morphogenesis of epithelial sheets (Loveless and Hardin, 2012), orient cells within tissues (Fernandez-Gonzalez et al., 2009), and allow the ZA to resist side-to-side (orthogonal) forces (Smutny et al., 2011).

Myosin II generates contractile force by binding to, and sliding, actin filaments (Vicente-Manzanares et al., 2009). Self-assembly of individual myosins into antiparallel minifilaments allows plus end-directed motor activity to slide filaments in networks toward one another (Verkhovsky et al., 1995; Clark et al., 2007). This also implies that properties of their interacting actin filaments can influence myosin II–driven contractility. Indeed, actin filament geometry and polarity have recently been demonstrated to affect contractility (Reymann et al., 2012). An important potential determinant of actin network geometry is the molecular mechanism responsible for mediating nucleation, the rate-limiting step in actin assembly (Michelot and Drubin, 2011). Thus the Arp2/3 complex nucleates the assembly of branched actin networks, whereas formin-based nucleation generates unbranched filaments. Of interest, Arp2/3 activity appears to limit the action of myosin in generating retrograde actin flow (Yang et al., 2012). Furthermore, type II myosins in yeast tend to segregate toward formin-derived linear actin filament arrays.

The epithelial zonula adherens (ZA) is a specialized adhesive junction where actin assembly and myosin-driven contractility coincide. The junctional cytoskeleton is enriched in myosin II, which generates contractile force to support junctional tension. It is also enriched in dynamic actin filaments, which are replenished by ongoing actin assembly. In this study we sought to pursue the relationship between actin assembly and junctional contractility. We demonstrate that WAVE2–Arp2/3 is a major nucleator of actin assembly at the ZA and likely acts in response to junctional Rac signaling. Furthermore, WAVE2–Arp2/3 is necessary for junctional integrity and contractile tension at the ZA. Manoeuvers that disrupt the function of either WAVE2 or Arp2/3 reduced junctional tension and compromised the ability of cells to buffer side-to-side forces acting on the ZA. WAVE2–Arp2/3 disruption depleted junctions of both myosin IIA and IIB, suggesting that dynamic actin assembly may support junctional tension by facilitating the local recruitment of myosin.
and potentially comprise bundled filaments (Hirokawa et al., 1983; Tepass and Hartenstein, 1994). In addition, a cortical pool was seen at the junctional membrane that was spatially distinguishable from Tepass and Hartenstein, 1994). Therefore, the role of WAVE in junctional actin regulation as the basis for testing the effect of Arp2/3-mediated nucleation upon force generation at the ZA.

RESULTS

WAVE2 is a component of the zonula adherens

We performed our studies in Caco-2 colon epithelial cells, a cell line that forms simple polarized monolayers with prominent ZAs at their cell–cell junctions. Quantitative real-time PCR revealed that WAVE2 was the predominant form expressed in these cells; only trace WAVE1 signals were identified, and WAVE3 was essentially undetectable (Figure 1A). The expression of WAVE2 protein in Caco-2 cells was confirmed by immunoblotting, which showed a single polypeptide band that was substantially reduced by small interfering RNA (siRNA), confirming the specificity of the antibody (Figure 1B).

Confocal immunofluorescence microscopy demonstrated clear junctional staining for WAVE2, which coaccumulated with E-cadherin at the apical ring of the ZA (Figure 1C). WAVE2 staining was largely abolished by siRNA, supporting the specificity of the ZA staining pattern (Supplemental Figure S1A), which was further corroborated by localization of WAVE2-mCherry with E-cadherin at apical cell–cell contacts (Supplemental Figure S1B).

Taken together, these findings identify WAVE2 as a component of the epithelial ZA. Consistent with this, we found that E-cadherin and WAVE2 coimmunoprecipitate (Figure 1D). Furthermore, E-cadherin was necessary for WAVE2 to localize to junctions, as WAVE2 staining at cell–cell contacts was substantially reduced when E-cadherin was depleted by siRNA (Figure 1, E and F). Note that E-cadherin RNA interference (RNAi) cells remained in contact with one another, as ZO-1 staining revealed the persistence of tight junctions, albeit with altered morphology. Overall these observations suggest that WAVE2 is recruited to the ZA in response to E-cadherin adhesion.

Rac GTPase signaling regulates junctional WAVE2

WASP/WAVE proteins can be regulated by the Rho-family GTPases Rac and Cdc42. In particular, WAVE has commonly been identified as a downstream target of Rac (Steffen et al., 2004; Insall and Mackesky, 2009; Padrick and Rosen, 2010), and both Rac and Cdc42 can mediate E-cadherin signaling (Kim et al., 2000; Kraemer et al., 2007). Indeed both Rac (Figure 2A) and Cdc42 (Figure 2B) coaccumulated with WAVE2 at the ZA. We then expressed dominant-negative (DN) mutants of Rac (N17) and Cdc42 (N17) in Caco-2 cells and
WAVE2 regulates junctional actin assembly

On close inspection, WAVE2 was seen to stain at the junctional cortex (Figure 3A), its peak fluorescence typically lying between the apical actin rings (Figure 3B). This corresponds to the principal site of barbed-end labeling (Kovacs et al., 2011). Furthermore, WAVE2 colocalized with the Arp3 component of the Arp2/3 complex (Figure 3C). To assess actin nucleation at the ZA, we briefly incubated saponin-permeabilized cells with Alexa 594–labeled G-actin used at a concentration that preferentially labels the actin filament barbed ends (Kovacs et al., 2011). Cells were costained with phalloidin to examine their effect on junctional WAVE2. DN-Rac, but not DN-Cdc42, significantly reduced WAVE2 localization at the ZA (Figure 2, C–E), evidence that WAVE2 might localize to the ZA in response to Rac activation being mediated by intermediary proteins, notably the multimeric WAVE regulatory complex (WRC; Steffen et al., 2004; Padrick and Rosen, 2010). Indeed, both Sra1 and Nap1, components of the WRC, localized to junctions (Figure 2, F and G), suggesting that junctional WAVE2 might act as part of the WRC.

Both barbed-end labeling and steady-state F-actin pools at the junctional cortex and in apical rings were largely abolished, replaced by residual puncta of F-actin that also stained for WAVE2 and Arp2/3 (Figure 4A). After removal of latrunculin, puncta were progressively replaced by a more diffuse phalloidin pattern. With time, the junctional region was repopulated by F-actin, initially appearing loosely packed at the cortex and eventually organizing to form apical actin rings. WAVE2 was also recruited to junctions, first in puncta and then distributing throughout an apical junctional ring (Figure 4A). However, the F-actin content was consistently reduced in the WAVE2 KD cells (Figure 4B,C), as was the rate of initial recovery after removal of latrunculin (unpublished data). Moreover, F-actin filaments at the junctions remained disorganized in the WAVE2 KD cells, even 60 min after latrunculin washout. This further supported the notion that WAVE2 contributes to junctional actin assembly.

WAVE2 supports ZA integrity and junctional dynamics of E-cadherin

WAVE2 depletion dramatically altered the integrity of the zonula adherens. E-cadherin staining intensity was reduced, and the ring-like organization of apical E-cadherin seen in control cells appeared fragmented in WAVE2 KD cells, being often interrupted by discontinuities (Figure 5, A and B). This alteration in E-cadherin staining at the ZA was not due to an overall change in cadherin expression: surface levels of E-cadherin were similar in the two cell lines. In control cells the majority of cellular cadherin was accessible to

FIGURE 2: Rac signaling regulates junctional WAVE2. (A, B) Rac and Cdc42 coaccumulate with WAVE2 at the zonula adherens. Caco-2 cells were immunostained for WAVE2 and either Rac (A) or Cdc42 (B) and imaged by confocal microscopy. (C–E) Junctional WAVE2 requires Rac signaling: Caco-2 cells transiently expressing dominant-negative (DN) Rac or DN Cdc42 mutants were immunostained for WAVE2 and the epitope tag (not shown). Asterisks mark cells positive for DN-Rac or DN-Cdc42 expression, and arrows indicate contacts between transgene-expressing cells. Junctional WAVE2 was reduced at contacts between cells expressing DN-Rac but not at contacts between cells expressing DN-Cdc42 (D, E; n = 30 contacts from three independent experiments each; data are means ± SEM, normalized to control. ****p < 0.0001; n.s. not significant; AU, arbitrary units). (F, G) WAVE regulatory complex components are junctional proteins. Confluent Caco-2 monolayers were costained for E-cadherin and either Sra1 (F) or Nap1 (G) and imaged at the ZA. Scale bars, 10 μm.
surface trypsinization in the absence of Ca$^{2+}$, and this was not
might depend on WAVE2 function. (Figure 5F). This suggested that a mobile pool of surface E-cadherin
and WAVE2 siRNA cells. Representative images are shown in F and quantitation in G. Data are
incorporation of Alexa 594 G-actin and phalloidin staining, respectively, in control, Arp3 siRNA, 
perturbed in Arp3 and WAVE2 RNAi cells. Barbed ends and junctional F-actin were labeled by
contacts from three independent experiments). (F, G) Junctional actin nucleation and F-actin are 
junctional Arp3 quantitated by line scan analysis in control and WAVE2 KD cells in (E; n = 40 contacts from three independent experiments). (F, G) Junctional actin nucleation and F-actin are 
perturbed in Arp3 and WAVE2 RNAi cells. Barbed ends and junctional F-actin were labeled by
incorporation of Alexa 594 G-actin and phalloidin staining, respectively, in control, Arp3 siRNA, 
and WAVE2 siRNA cells. Representative images are shown in F and quantitation in G. Data are 
n = 24 for control and n = 24 for Arp3 siRNA; n = 15 contacts for control and n = 13 contacts for 
WAVE2 siRNA from three independent experiments. ***p < 0.001; ****p < 0.0001; data are 
means ± SEM, normalized to control; AU, arbitrary units. Scale bars, 10 μm.

In contrast to the alteration in E-cadherin
staining, ZO-1 remained detectable at apical 
junctions, and the fluorescence intensity of ZO-1 was unchanged in WAVE KD cells (Figure 5G). Morphologically, however, ZO-1 
staining was more irregular and less linear in the WAVE KD cells than in controls, an ap-
pearance that was confirmed by quantit-
jung junctional linearity (Figure 5H; Otani et al., 2006). Overall this implies that WAVE2 has a relatively selective effect to support 
ZAs integrity, with more subtle morphologi-
effects on tight junctions.

WAVE2 and Arp2/3 are necessary 
for junctional tension in the zonula 
adehrers

The linear morphology of tight junctions is
often interpreted as an index of apical junc-
tional tension (Otani et al., 2006). Thus the 
alter ed linearity in ZO-1 staining suggested 
that WAVE2 depletion might reduce apical 
junctional tension. We also observed that E-
cadherin–GFP at apical junctions shows 
increased side-to-side (orthogonal) move-
ments when myosin II is depleted (Smutny et al., 2011) and, by implication, junctional 
tension is reduced (Ratheesh et al., 2012). 
Accordingly, we then examined the effect of 
WAVE2 depletion on apical E-cadherin–GFP 
movement in time-lapse movies (Figure 6, 
A–C, and Supplemental Movies S1 and S2).
Apical junctions in control cells, reflecting 
the ZAs, were relatively stable, and kymo-
graphs showed minimal orthogonal fluctua-
tions in the x-y-plane. In contrast, WAVE2 KD 
cells displayed much more dramatic side-to-
side movements, as evident in a comparison 
of kymographs of junctional movement 
(Figure 6A). We characterized these further 
by Fourier analysis (Smutny et al., 2011), 
which revealed increased power in both lower- and higher-frequency peaks, consis-
tent with increased movement (Figure 6B). 
Earlier we found that changes in low-fre-
quency power in these Fourier analyses re-
flected slow, unidirectional movement of 
the junctions from one side to the other 
(Smutny et al., 2011). Consistent with this,
we found that overall translocation of junctions, measured by the 
slope of the kymographs, was increased in WAVE KD cells (Figure 6C). Thus WAVE2 contributes to buffering apical junctions from 
side-to-side movements.

Then we directly tested the effect of WAVE2 on junctional ten-
sion by using a femtosecond laser to cut junctions (Fernandez-Gon-
zalez et al., 2009) and measuring the instantaneous rate of recoil of 
their vertices as an index of junctional tension (Ratheesh et al., 2012; 
Figure 6D). Control junctions displayed rapid recoil of their vertices 
when junctional integrity was cut by the laser nanoscissors. In con-
trast, both instantaneous rate of recoil (indicating tension) and the 
degree of recoil were reduced by WAVE2 KD (Figure 6, E and F). 
This suggested that WAVE2-mediated actin assembly might

FIGURE 3: WAVE2 regulates junctional actin assembly. (A, B) WAVE2 localized at the ZA 
between apical F-actin rings. WAVE2 and F-actin were visualized by confocal 
immunofluorescence imaging (A) and the fluorescence profiles measured by line scan analysis of 
20 contacts (B). A representative line scan is marked by a white bar in the merged image in A. 
(C) Coaccumulation of WAVE2 and Arp3 at the ZA imaged by confocal immunofluorescence 
microscopy. (D, E) Junctional Arp3 is depleted in WAVE2 RNAi cells. Control and WAVE2 siRNA 
cells were immunostained for WAVE2 and Arp3. Costaining in WAVE2 KD cells is shown in D and 
junctional Arp3 quantitated by line scan analysis in control and WAVE2 KD cells in (E; n = 40 
contacts from three independent experiments). (F, G) Junctional actin nucleation and F-actin are 
perturbed in Arp3 and WAVE2 RNAi cells. Barbed ends and junctional F-actin were labeled by
incorporation of Alexa 594 G-actin and phalloidin staining, respectively, in control, Arp3 siRNA, 
and WAVE2 siRNA cells. Representative images are shown in F and quantitation in G. Data are 
n = 24 for control and n = 24 for Arp3 siRNA; n = 15 contacts for control and n = 13 contacts for 
WAVE2 siRNA from three independent experiments. ***p < 0.001; ****p < 0.0001; data are 
means ± SEM, normalized to control; AU, arbitrary units. Scale bars, 10 μm.

4604 | S. Verma et al. 
Molecular Biology of the Cell
cytoskeleton contains a high proportion of dynamic actin filaments, and its maintenance requires ongoing actin assembly (Kovacs et al., 2011; Mangold et al., 2011). This raises the question of whether actin assembly affects contractility and junctional tension at the ZA. Our current findings lead to three conclusions. First, the WAVE2–Arp3 apparatus is a major nucleator of actin assembly at the ZA. Second, this nucleator complex, and by implication the actin networks that it generates, is necessary for junctional tension. Finally, the WAVE2–Arp2/3 complex supports junctional tension by facilitating the recruitment of myosin II to the ZA. Together these suggest that a close relationship exists between Arp2/3-mediated actin assembly and myosin-driven contractility at the ZA.

WAVE2–Arp2/3 is a major nucleator of actin assembly at the ZA
The high rates of actin filament turnover at cadherin junctions strongly imply that ongoing assembly is necessary to maintain its steady-state F-actin content. Arp2/3 is one potential nucleator for junctional actin assembly, as it localizes to the ZA (Helwani et al., 2004; Verma et al., 2004; Kovacs et al., 2011), possibly through a direct or indirect interaction with the E-cadherin molecular complex (Kovacs et al., 2002). We now find that Arp3 RNAi reduces both actin nucleation and steady-state junctional F-actin content. Taken together with earlier experiments using drug (Kovacs et al., 2011) or dominant-negative (Verma et al., 2004) inhibitors, these establish Arp2/3 as a major contributor to actin filament homeostasis at these junctions. This is consistent with the recent demonstration that Arp2/3 was necessary for the junctional cytoskeleton to reassemble after latrunculin treatment (Tang and Brieher, 2012). These data do not exclude contributions from other nucleators, notably formins (Kobielak et al., 2004; Carramusa et al., 2007), but suggest that alternative nucleators cannot fully compensate when the activity of Arp2/3 is inhibited, either acutely or over more prolonged periods.

Our data further identify WAVE2 as a key part of this junctional actin nucleator apparatus. Arp2/3 is intrinsically inactive, and it is stimulated in response to cell signaling by intermediary proteins, notably the WASP/WAVE family (Padrick and Rosen, 2010). The Caco-2 cells used in our experiments express both N-WASP (Kovacs et al., 2004; Verma et al., 2004) and WAVE2, with little, if any, mRNA for other WAVE proteins detectable. Although N-WASP supports the junctional actin cytoskeleton, it appears to act at a postnucleation step to stabilize filaments (Kovacs et al., 2011). In contrast, WAVE2 depletion mimicked the effects of Arp3 RNAi, substantially reducing junctional nucleation and steady-state junctional F-actin content. This confirms and extends earlier work that identified WAVE2 as a regulator of junctional actin integrity in MDCK cells but did not assess actin nucleation directly (Yamazaki et al., 2007). Furthermore, WAVE2 depletion reduced junctional Arp2/3, strengthening the notion that these proteins function together as a nucleator apparatus at the ZA. WAVE2 localization required E-cadherin, and association between

WAVE2 and Arp2/3 support junctional myosin II
Apical junctional tension is generally believed to be driven by myosin II–based contractility. Of the three myosin II proteins found in mammalian cells, myosin IIA and IIB localize to the ZAs of Caco-2 cells (Figure 7A), as they also do in MCF-7 cells (Smutyň et al., 2010). In contrast, junctional accumulation of both myosin IIA and IIB was significantly reduced in WAVE2 KD cells, accompanied by an increase in myosin staining on basal stress fibers (Figure 7, A and B, and Supplemental Figure S3). This suggested that dynamic actin assembly was necessary to support junctional accumulation of both these myosins. Consistent with this, junctional myosin IIA and IIB were also reduced in Arp3 siRNA cells (Figure 7C). In both experiments junctional myosin IIA was reduced to a greater extent than was junctional myosin IIB.

DISCUSSION
The zonula adherens appears to be a specialized junction where cell–cell adhesion is coupled to actomyosin to generate contractile tension (Sawyer et al., 2010; Ratheesh et al., 2012). However, the ZA...
Dynamic actin assembly regulates myosin II recruitment

We further suggest that WAVE2–Arp2/3 promotes junctional tension by facilitating the recruitment of myosin II to the junction. This notion was prompted by the observation that myosin IIA and myosin IIB, which both contribute to junctional tension (Ratheesh et al., 2012; Gomez and Yap, unpublished data), were reduced at junctions in WAVE2 or Arp3 KD cells, although total cellular levels of both proteins were unchanged (unpublished data). These findings add to the matrix of factors that influence the subcellular localization of myosin II. Known regulatory influences include signals such as the Rho-ROCK pathway and Rap1, which promote the accumulation of myosin IIA and myosin IIB at the ZA, respectively (Smutny et al., 2010; McLachlan and Yap, 2011; Ratheesh et al., 2012). We propose that local actin regulation collaborates with these signaling pathways to promote the robust recruitment of myosin II to the ZA. Similarly, actin network geometry and organization can affect the binding of other myosins (Nagy et al., 2008; Brawley and Rock, 2009).

This raises the interesting question of whether filament branching by Arp2/3 substantively constrains myosin II recruitment or contractility. It is plausible to predict that branched actin networks would limit myosin II action at the ZA, based on evidence that Arp2/3 constrains myosin II–based cortical flow (Yang et al., 2012) and that myosins can be excluded from Arp2/3-nucleated networks (Weinberg and Drubin, 2012). Our findings argue, however, that Arp2/3 ultimately promotes myosin II action at the ZA. One possibility is that network reorganization, potentially including debranching, occurs after nucleation to promote optimal myosin binding and contractility. The existence of reorganization might be inferred from earlier evidence that the apical actin rings of the ZA contain actin sarcomeres, however, actin filaments undergo turnover that can be promoted by increased contractility (Skwarek-Maruszewska et al., 2009), implying that mechanisms must exist to sustain contractility despite filament turnover. In our experience, actin assembly and contractile tension both occur at the epithelial ZA (Verma et al., 2004; Kovacs et al., 2011; Ratheesh et al., 2012), and our present findings demonstrate that Arp2/3 and WAVE2 are necessary to support junctional tension. Thus junctional tension, measured using laser nanoscissors, was substantially decreased by either Arp3 or WAVE2 RNAi. Similarly, WAVE2 depletion altered the normal linear morphology of apical junctions and decreased the ability of cells to buffer side-to-side movement at the junctions, both features consistent with decreased junctional tension (Otani et al., 2006; Smutny et al., 2011). Overall we conclude that ongoing actin assembly is necessary for tension to be generated at the ZA.

FIGURE 5: WAVE2 regulates E-cadherin organization and dynamics at the zonula adherens. (A, B) WAVE2 KD reduces apical concentration of E-cadherin into the ZA. Representative confocal immunofluorescence images of E-cadherin in control and WAVE2 KD cells (A) and quantitation by line scan analysis (B; n = 20 contacts from three independent experiments). (C) Total and surface E-cadherin (E-cad) levels in control and WAVE2 KD cells measured by sensitivity to digestion by extracellular trypsin in the presence or absence of Ca2+. Results are representative of three independent experiments. Ctrl, no trypsin added; +Ca, trypsin added in media containing 2 mM Ca2+; –Ca, trypsin added in calcium-free media. GAPDH (GDH) was used as a loading control. (D–F) FRAP of junctional E-cadherin–GFP expressed in E-cadherin shRNA cells (n = 6). Half-life of recovery (E) and mobile fraction (F) were reduced in WAVE2 KD cells. (G) Junctional linearity was reduced in WAVE2 KD cells, as revealed by immunostaining for ZO-1 and quantitated (H; linearity index) as described in Materials and Methods. Data are means ± SEM, normalized to controls; n = 29 for control and n = 33 for WAVE2 KD from three independent experiments; AU, arbitrary units; ***p < 0.001, ****p < 0.0001. Scale bars, 10 μm.

The two proteins was detectable by communoprecipitation analysis. This biochemical interaction would allow actin assembly to be focused on cadherin adhesions. WAVE2 at the ZA appears to act downstream of Rac signaling and it is likely to respond as part of the wave regulatory complex. This emphasizes the central role that Rac plays in dynamic actin assembly at cadherin junctions (Braga et al., 1997; Kraemer et al., 2007), as it does at other subcellular locations (Steffen et al., 2004; Padrick and Rosen, 2010).

WAVE2–Arp2/3 supports junctional tension at the ZA

The relationship between actin filament dynamics and contractility is complex. Arp2/3 can functionally constrain myosin II function (Yang et al., 2012), suggesting that actin assembly may antagonize contractility; indeed, specialized contractile apparatuses, such as the sarcomeres of skeletal and cardiac muscle, are distinguished by apparently stable actin filament populations (Ono, 2010). Even in the two proteins was detectable by communoprecipitation analysis. This biochemical interaction would allow actin assembly to be focused on cadherin adhesions. WAVE2 at the ZA appears to act downstream of Rac signaling and it is likely to respond as part of the wave regulatory complex. This emphasizes the central role that Rac plays in dynamic actin assembly at cadherin junctions (Braga et al., 1997; Kraemer et al., 2007), as it does at other subcellular locations (Steffen et al., 2004; Padrick and Rosen, 2010).

WAVE2–Arp2/3 supports junctional tension at the ZA

The relationship between actin filament dynamics and contractility is complex. Arp2/3 can functionally constrain myosin II function (Yang et al., 2012), suggesting that actin assembly may antagonize contractility; indeed, specialized contractile apparatuses, such as the sarcomeres of skeletal and cardiac muscle, are distinguished by apparently stable actin filament populations (Ono, 2010). Even in the two proteins was detectable by communoprecipitation analysis. This biochemical interaction would allow actin assembly to be focused on cadherin adhesions. WAVE2 at the ZA appears to act downstream of Rac signaling and it is likely to respond as part of the wave regulatory complex. This emphasizes the central role that Rac plays in dynamic actin assembly at cadherin junctions (Braga et al., 1997; Kraemer et al., 2007), as it does at other subcellular locations (Steffen et al., 2004; Padrick and Rosen, 2010).

WAVE2–Arp2/3 supports junctional tension at the ZA

The relationship between actin filament dynamics and contractility is complex. Arp2/3 can functionally constrain myosin II function (Yang et al., 2012), suggesting that actin assembly may antagonize contractility; indeed, specialized contractile apparatuses, such as the sarcomeres of skeletal and cardiac muscle, are distinguished by apparently stable actin filament populations (Ono, 2010). Even in the two proteins was detectable by communoprecipitation analysis. This biochemical interaction would allow actin assembly to be focused on cadherin adhesions. WAVE2 at the ZA appears to act downstream of Rac signaling and it is likely to respond as part of the wave regulatory complex. This emphasizes the central role that Rac plays in dynamic actin assembly at cadherin junctions (Braga et al., 1997; Kraemer et al., 2007), as it does at other subcellular locations (Steffen et al., 2004; Padrick and Rosen, 2010).
bundles (Hirokawa et al., 1983; Tepass and Hartenstein, 1994). Given that the actin rings in our cells were also depleted when the WAVE2–Arp2/3 apparatus was perturbed, we hypothesize that filaments in the rings might originally have been nucleated by Arp2/3 and subsequently remodeled to generate bundles. A cortical pool of filaments can be identified at the membrane (Zhang et al., 2005), separable from the prominent apical cables (Kovacs et al., 2011). It is tempting to hypothesize that these cortical filaments are the nascent products of Arp2/3 nucleation, which may serve to limit myosin II incorporation at the cortex itself. Subsequent filament reorganization may then generate bundles and allow myosin II incorporation. Testing this hypothesis will require ultrastructural characterization of actin organization in the apical rings in our cell systems, as well as analysis of postnucleation regulators, work that is ongoing in our laboratory.

Overall our findings identify WAVE2–Arp2/3 as necessary for myosin II recruitment and tension generation at the zonula adherens. We hasten to add, however, that not all the junctional effects of WAVE2–Arp2/3 can be explained by the recruitment of myosin II. Minimally, the contractile activity of myosins is generated by filament sliding. It is likely that loss of junctional filaments also contributed to the reduced junctional tension seen in WAVE2–
Arp3-depleted cells. Nor are the junctional effects of WAVE2–Arp2/3 wholly attributable to the effect of junctional tension. Of note, we found that the unidirectional movement of the ZA from one side to another was more pronounced in WAVE2 KD cells, although myosin IIB depletion (which would reduce tension) decreased the rate of unidirectional movement (Smutny et al., 2011). Instead, WAVE2-nucleated filaments may buffer junctional translocation by themselves acting as resistance elements and/or by anchoring other potential resistance elements, such as myosin VI (Maddugoda et al., 2007). We add that, although we favor the notion that these orthogonal junctional movements reflect the mechanical effect of the junctional cytoskeleton, it remains possible that they are subject to forces generated elsewhere in the cell that are transmitted to the junctions.

Finally, it is noteworthy that E-cadherin mobility appeared to be decreased in FRAP studies. We interpret these FRAP analyses to reflect molecular movement or mobility of cadherin (within the membrane or by turnover) in contrast to the larger-scale movement of the whole ZA. Indeed, we tracked the sites of fluorescence recovery to ensure that we allowed for any junctional movement. Our findings thus suggest that WAVE2 supports a dynamic pool of cadherin at the ZA, which contrasts with the stabilization of cadherin mobility by myosin IIA (Ratheesh et al., 2012). Arp2/3 has been implicated in clathrin-mediated endocytosis (Weinberg and Drubin, 2012), but whether turnover accounts for the effect of WAVE2 on E-cadherin dynamics remains to be examined.

In any case, our present findings emphasize that the junctional cytoskeleton is an integrated system in which filament dynamics is coordinated with motor action. These also involve an overlaid network of signals, including Rac, Rho, and Rap1 GTPases and Src kinase signaling, which have all been implicated in regulating different aspects of the junctional cytoskeleton (Braga et al., 1997; Kraemer et al., 2007; McLachlan and Yap, 2011). A deeper characterization of the junctional cytoskeleton will then entail understanding how these signals and effectors are integrated into a robust cellular system.

MATERIALS AND METHODS

Cell culture and transfections

Caco-2 cells were cultured in RPMI complete growth medium and grown at 37°C in 5% CO2. Cells were transfected with Lipofectamine 2000 or Lipofectamine RNAiMAX (both from Invitrogen, Carlsbad, CA) according to manufacturer’s instructions for plasmids or siRNAs, respectively.

Antibodies and immunoprecipitation

Primary antibodies used in this study were as follows: rabbit polyclonal against WAVE2 (1:50 for immunofluorescence [IF], 1:2000 for Western blot [WB]; Cell Signaling Technology, Beverly, MA), rabbit polyclonal against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:2000 WB; R&D, Minneapolis, MN), mouse monoclonal HEC-D1 against ectodomain of E-cadherin (1:50; 1:100 WB; kind gift from P. Wheelock, University of Nebraska, Omaha, NE, with the permission of M. Takeichi), mouse monoclonal against ZO-1 (1:100 IF; Invitrogen), mouse monoclonal against Rac1 (1:500 IF; Upstate Biotechnologies, Millipore, Billerica, MA), mouse monoclonal against Arp3 (1:50 IF; Sigma-Aldrich), rabbit polyclonal against myosin IIA (1:600 IF; Abcam, Cambridge, MA), rabbit polyclonal against myosin IIB (1:400 IF; Sigma-Aldrich), mouse monoclonal against Arp3 (1:50 IF; Sigma-Aldrich), mouse monoclonal against myosin IIB CMII26 (1:50 IF; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), and rabbit polyclonal against myosin IIB (1:100 IF; Sigma-Aldrich). Secondary antibodies were species-specific antibodies conjugated with Alexa Fluor 488, 594, or 647 (Invitrogen) for immunofluorescence or with horseradish peroxidase (Bio-Rad, Hercules, CA) for immunoblotting. F-actin was stained using Alexa 488- or Alexa 594-phalloidin (1:500; Invitrogen).

Cells were lysed in 1 ml of lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 50 mM sodium fluoride, 2 mM sodium vanadate, 0.1% bovine serum albumin, and complete protease inhibitors [Roche Applied Science, Indianapolis, IN]). Immunoprecipitations were performed using ~1 mg of total protein, to which 2 μg of antibody and 20 μl of packed slurry of protein A–Sepharose beads (GE Healthcare, Piscataway, NJ) were added. Protein complexes were dissociated from beads, and samples were run on SDS–PAGE gels.

Real-time PCR

Total RNA was extracted from Caco-2 cell line using RNeasy Mini Kit (Qiagen, Valencia, CA) and was reverse transcribed using a SuperScript III First Strand Synthesis System (Life Technologies) as per the
manufacturer’s instructions. cDNA samples were assayed by quantita-
tive real-time PCR for Wasf1, Wasf2, and Wasf3 (WAVE1, WAVE2,
and WAVE3, respectively) gene expression using inventoried TaqMan
gene expression assays (Applied Biosystems, Foster City, CA; assay ID: Hs01591751_m1: Wasf1; Hs00819075_gH: Wasf2;
Hs00903488_m1: Wasf3). Relative expression of Wasf1, Wasf2,
and Wasf3 was normalized to housekeeping gene HPRT1 expres-
sion (assay ID: Hs01003267_m1) using a comparative method
(2−ΔΔCt) according to the ABI relative quantitation guide.

**Immunofluorescence microscopy and image analysis**
For Rac and Cdc42 immunostaining, cells were fixed in 10% tricho-
roacetic acid for 10 min. For all other stainings, cells were fixed with
4% paraformaldehyde in cytoskeletal stabilization buffer (10 mM
1,4-piperazinediethanesulfonic acid at pH 6.8, 100 mM KCl, 300 mM sucrose, 2 mM ethylene glycol tetraacetic acid (EGTA), and
2 mM MgCl2) on ice for 20 min. Fixed coverslips were then permea-
bilized with 0.25% Triton X-100 in phosphate-buffered saline for
10 min at room temperature. Confocal images were acquired on a
Zeiss LSM710 microscope (Carl Zeiss, Jena, Germany) and processed
using ImageJ (National Institutes of Health, Bethesda, MD) and
Photoshop (Adobe, San Jose, CA).

Quantitative analysis of staining intensity at contacts was per-
formed in ImageJ with the line scan function, as previously de-
scribed (Smutny et al., 2010). Briefly, a line of length 40 pixels was
selected centered on and perpendicular to a contact. The PlotPro-
file feature was used to record the pixel intensities along the se-
lected line, and the data were imported into Prism 5 (GraphPad
Software, La Jolla, CA). Pixel intensity was corrected for back-
ground, and a Gaussian curve was fitted to each intensity profile,
from which the mean and SD of the maximum heights were calcu-
lated. For the latrunculin assay, a region of interest spanning each
contact was drawn using the 10-pixel-wide ImageJ freehand line
tool. The mean phalloidin intensity for each region of interest was
then measured.

**Assays**
The latrunculin washout assay was a modification of a recently de-
scribed protocol (Tang and Brieher, 2012). Caco-2 cells were trans-
fected with control or WAVE2 siRNA oligos, plated onto glass cov-
erslips, and grown to confluency. Coverslips were then treated with
1 μM latrunculin A (Sigma-Aldrich) for 2 h. Cells were rinsed twice
and incubated with cell growth media for 0–60 min, after which they
were processed for immunostaining as described.

Barbed ends were labeled by G-actin incorporation (Kovacs
et al., 2011). Confluent monolayers of Caco-2 cells were permeabi-
lized with saponin for 7 min in the presence of 0.45 μM Alexa
594–tagged G-actin to favor barbed-end incorporation. Cells were
then fixed in 4% paraformaldehyde in cytoskeletal stabilization buf-
fer containing 2% Triton X-100 and Alexa 488-phalloidin (1:500).
Surface E-cadherin was measured by sensitivity to surface trypsin
digestion (Verma et al., 2004). Briefly, lysates were prepared from
monolayers that were untreated (control) or trypsinized in the pres-
ence of 2 mM CaCl2 (Ca+) or in the absence of 2 mM CaCl2 and
presence of 2 mM EGTA (−Ca). Samples were run on SDS–PAGE
gels and probed with antibodies directed against the ectodomain of
human E-cadherin (HECD-1) or GAPDH as a loading control.

**Live-cell imaging**

**Analysis of contact movements.** Cells were grown on glass-
bottomed dishes (In Vitro Scientific, Sunnyvale, CA), and live-cell
imaging was performed on a spinning-disk confocal system
(Ultra-View; PerkinElmer, Waltham, MA) mounted on an IX81
Olympus microscope (Olympus, Tokyo, Japan) with ×60 and
×100/1.40 numerical aperture Plan Apochromat objectives and an
Orca-1 ER camera (Hamamatsu, Hamamatsu, Japan) driven by
MetaMorph imaging software, version 7 (Molecular Devices,
Sunnyvale, CA). Kymographs were generated using the Multiple
Kymograph function in ImageJ. Contact movement in E-cadherin-
GFP–expressing cells was quantitated as previously described
(Smutny et al., 2011). Briefly, movies were processed by drawing a
line perpendicular to a contact, and the position along the line at
which the maximum intensity occurred was recorded for each
time frame using an ImageJ script. The translational component
was obtained by calculating the slope of the best-fit line to the
position of maxima over time. The oscillatory component was
then obtained by subtracting the translational component from
the positional data and then decomposing the result into its
frequency components by fast Fourier transform.

**FRAP experiments.** FRAP experiments were performed with a
Zeiss LSM510 microscope (Carl Zeiss, Jena, Germany) with a heated
stage maintained at 37°C. Images were acquired using a 100×
objective/1.4 numerical aperture oil Plan Apochromat immersion
lens, and E-cadherin–GFP at contacts was photobleached using a
constant region of interest (ROI) using a 488-nm laser with its
transmission set to 100%. Recovery in the photobleached area was
tracked from frame to frame to correct for movement of the overall
junction. Fluorescence intensity profiles were corrected for
acquisition photobleaching and normalized to prebleach values as
previously described (Kovacs et al., 2011). Normalized FRAP curves
were fitted to a recovery curve using Prism to calculate half-times of
recovery and plateau levels (Ratheesh et al., 2012).

**Laser nanoscissors.** Localized ablation of a small region of cell–cell
contacts in E-cadherin–expressing cells was performed as previously
described (Ratheesh et al., 2012). Briefly, experiments were per-
formed on a Zeiss LSM S10 confocal microscope at 37°C. Images
were acquired using a 63× objective, 1.4 numerical aperture oil
Plan Apochromat immersion lens at 1.5x digital magnification and
pinhole adjusted to 3 Airy units to obtain a 2-μm optical section.
For ablation, a Ti:sapphire laser (Chameleon Ultra; Coherent
Scientific, Santa Clara, CA) tuned to 790 nm was used to ablate cell
contacts labeled with E-cadherin–GFP. A constant ROI was marked
for each experiment and ablated with 30 iterations of the 790-nm
laser with 50% transmission. GFP fluorescence was determined
before (three frames) and after (four frames) ablation with an interval
of 15 s per frame, using a 488-nm laser for excitation and a 500–
to 550-nm emission filter. Image analysis was performed using ImageJ.
The distance, d, between vertices that defined the ablated contact
was measured as a function of time, t. Next the average distance
before the ablation step, d(0), was subtracted from the distance
values, and the resulting values were plotted and fitted to the
following equation:

\[
d(t) = d(0) + \text{plateau} \cdot (1 - e^{-kt})
\]

where \(k\) is the rate constant. The instantaneous recoil (rate of recoil
at \(t = 0\) s) was determined as

\[\text{Instantaneous recoil} = \text{plateau} \cdot k\]

Average instantaneous recoil was determined for 30–50 contacts
in three independent experiments and then normalized to the value
observed in control conditions.
ACKNOWLEDGMENTS
We thank our colleagues for gifts of reagents and all the members of our lab for their thoughtful suggestions and support. This work was supported by the National Health and Medical Research Council of Australia (63137), the Australian Research Council (DP120104667), the Human Frontiers Science Program, and the Kids Cancer Project of the Oncology Children’s Foundation. A.S.Y. (631383) and R.D.T. (511042) are Research Fellows of the National Health and Medical Research Council (Australia). Confocal imaging was performed at the Institute for Molecular Bioscience/Australian Cancer Research Foundation Cancer Biology Imaging Facility, established with the generous support of the Australian Cancer Research Foundation.

REFERENCES

Molecular Biology of the Cell