Beyond structure: mechanism and dynamics of intercellular adhesion

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Abstract
This review summarizes findings from multiple complementary quantitative investigations of adhesion by classical cadherins. The systems investigated range from single molecules to cells, and the approaches used quantify the kinetics, energetics and mechanical strengths of cadherin bonds. The cumulative results demonstrate that cadherins adhere via a multistage binding mechanism that involves multiple extracellular domains. In kinetic measurements of cell adhesion, cell pairs first form a low-probability-binding state with fast kinetics. This is followed by a lag and a slow transition to a second, high-probability, binding state. This two-stage process is independent of the cytoplasmic domain. Studies with domain-deletion mutants demonstrate that the N-terminal domains are required for the first, fast, weak binding. However, the full-ectodomain and EC3 (extracellular repeat 3), in particular, are required to form the second, high-probability, binding state, which is characterized by slow dissociation kinetics and much stronger adhesive bonds. Together, these different studies reveal a more complex multistage binding mechanism than was predicted by structural models.

Introduction
Determining how cadherins form cell–cell junctions is central to understanding their role in morphogenesis and in disease. The challenge is to determine how these proteins form intercellular junctions, and to determine the structural basis of junction assembly/disassembly and of intercellular mechanics.

Cadherins are transmembrane proteins, and their extracellular region embeds the adhesive function [1]. The extracellular segment folds into five cadherin-type EC (extracellular repeat) domains, which are numbered 1–5 from the N-terminal domain (EC1–EC5) [1]. Structures of cadherin fragments [2] and interpretations of electron tomography images of desmosomal cadherins [3] suggest that cadherins adhere through a simple mechanism that only involves the mutual binding of Trp2 from the N-terminal EC domain (EC1) of one cadherin into a hydrophobic pocket on the EC1 domain of the opposed protein. This simple ‘stand dimer’ mechanism should be easily validated by a variety of quantitative biophysical approaches.

Structures are, however, only one form of physical evidence for molecular mechanisms. One cannot see bond energies, kinetics or, in the case of adhesion, bond strengths. In cases where multiple interactions may contribute to protein function, a single structure represents only one of several possible relevant states. Conformational changes, multiple binding states and co-operativity can further elaborate adhesion mechanisms. Except for the simplest mechanisms, validating adhesion models requires multiple evaluations of the protein function. Proposed functional models are thus substantiated, not only by structures, but also by quantitative biophysical measurements that are capable of testing postulated mechanisms.

In the present article, I describe results of three different quantitative biophysical approaches used to quantify the kinetics, energetics, mechanical strengths and binding mechanisms of homophilic cadherin adhesion. These complementary approaches enabled the quantitative testing of existing models of cadherin adhesion and provided evidence for alternative mechanisms. The systems studied range from single molecules to cells. The findings described here demonstrate that cadherin adhesion involves multiple binding states, which exhibit different kinetics and mechanical strengths. Furthermore, these different states require multiple extracellular domains, but initial binding events do not depend on the cytoplasmic domain.

Cell adhesion kinetics exhibit a two-stage binding mechanism that requires multiple EC domains

MP (micropipette) measurements of cell-binding kinetics
The MP manipulation technique measures binding between single cell pairs bearing complementary receptors and ligands (Figure 1a) [4]. Typically, one of the cells is an RBC (red blood cell), although this is not required. Two cells are aspirated into opposite MPs and brought into contact for a defined period. Upon separation, intercellular adhesion
Figure 1 | Experimental configurations of the MP manipulation technique and BFP

(a) CHO cell and modified RBC used in the MP measurements. The CHO cell expressing wild-type C-cadherin (C-CHO) (left) is aspirated into a glass pipette. The RBC (right) is modified with immobilized C-cadherin EC1-EC5-Fc. (b) Cartoon illustrating the protein configurations on the opposing cells. The wild-type C-cadherin on the C-CHO (top) faces the Fc-tagged cadherin ectodomain bound to the RBC surface (bottom) via an anti-(human Fc) monoclonal antibody (mAB). The antibody is covalently bound to the RBC surface. (c) In the BFP, a cadherin-modified microbead is bound to the surface of an RBC, which is aspirated into the pipette. The opposite MP holds a second bead modified with cadherin fragments. (d) Illustration of the cadherin orientations on the two beads.

causes the RBC to distort. The cell recoils to its unperturbed shape at bond failure.

MP measurements quantify cell adhesion and adhesion kinetics [5]. Kinetic measurements quantify the cell-binding probability as a function of the contact time. The binding probability \( P_a \) is the number of detected binding events, \( n_{adhesion} \), divided by the total number, \( N_{total} \), of cell–cell contacts, \( P_a = n_{adhesion}/N_{total} \). It should be emphasized that these MP measurements quantify binding probabilities rather than adhesion. From time-dependent-binding-probability curves, one can determine the kinetic rates and two-dimensional affinities of intercellular receptor–ligand bonds [5]. This approach was used to study the binding kinetics of Fcγ receptors, selectins, integrins and CD8 with their respective receptors [5–12].

We used the MP technique to investigate the kinetics of intercellular adhesion mediated by *Xenopus* C-cadherin [13]. We measured binding kinetics between CHO (Chinese-hamster ovary) cells expressing wild-type cadherin and RBCs modified with immobilized C-cadherin ectodomains. Epitope-tagged cadherin ectodomains were immobilized to the RBC surface via monoclonal antibodies, which were bound covalently to the RBC surface. Figure 1(b) shows the protein architecture and configurations in the measurements.

**Cadherin-binding kinetics are biphasic [13]**

Kinetic measurements tested current models for cadherin binding and determined rate constants, when feasible, for the kinetic processes [13]. In the strand-exchange model, cadherins bind by the reciprocal docking of Trp residues into hydrophobic sites on the adjacent protein [2]. We tested this model by comparing measured cell-adhesion kinetics with the kinetic behaviour predicted for this mechanism. The thus predicted time-dependent-binding probability for the strand-exchange model is a single exponential rise to a limiting plateau as in Figure 2(a).

In contrast with the predicted behaviour, the measured time course between CHO cells expressing wild-type cadherin and RBCs modified with Fe-conjugated CEC1–5 (C-cadherin EC1–EC5) is biphasic, i.e. there are two different kinetic stages (Figure 2b) [13]. Within the first 2 s, the binding probability increases rapidly to the first plateau at 0.4–0.5, i.e. 40–50% of the cell contacts resulted in binding. This low-probability-binding state is followed by a 2–5 s lag and a transition to a second plateau at a higher binding probability. Control measurements conducted in EDTA or with blocking antibody reduced the binding probability to 0.1 (Figure 2b).

These results reveal two cadherin-binding states that form on different timescales. To determine the underlying
mechanism, we first identified the structural regions required for the different kinetic steps in the binding curves. Kinetic measurements were taken with the soluble ectodomains alone (lacking the transmembrane and cytoplasmic domains) and with domain deletion mutants ΔEC3 and ΔEC345, which lack EC3 and EC3–EC5 respectively. These constructs were characterized previously in cell-adhesion assays [14].

Biphasic kinetics are independent of the cytoplasmic domain [13]
We first addressed the impact of the cytoplasmic domain on the kinetic profiles. The binding-probability time courses measured between two RBCs modified similarly with the soluble Fc-tagged ectodomains similarly exhibited the two-stage binding curves. The biphasic kinetics are therefore independent of the cytoplasmic domain. This is an important finding because it is often assumed that the recombinant cadherin ectodomain is a model for cadherin adhesion at the cell surface. This assumption had never been demonstrated directly. This comparison of the soluble ectodomain and wild-type cadherin shows that the ectodomains determine initial intercellular binding.

EC1–EC2 domains facilitate the first, fast, step, but the lag and transition to the high-probability-binding state requires EC3 [13]. Binding probability curves measured between CHO cells expressing wild-type C-cadherin and RBCs modified with the deletion mutants ΔEC3 or ΔEC345 only exhibited the fast rise to a plateau at low binding probability (Figure 2c). There was no lag or transition to a higher-probability-binding state. Furthermore, since both mutants exhibit the first, fast, step, and since both contain EC1–EC2, this fast binding step is attributed to the EC1–EC2 domains. However, the lag and transition to the high-probability-binding state require EC3.

These cell-binding kinetics reveal a multistage binding process that requires multiple EC domains, but not the cytoplasmic region. The first, fast, step maps to the EC1–EC2 region and probably involves binding via Trp exchange. Fragments with EC1–EC2 domains, but lacking the EC3 segment, only exhibit a fast, low-probability, binding state. The EC3 domain is required for the subsequent transition to the higher-probability-binding state.

Single cadherin bonds exhibit multiple states with a hierarchy of strengths and kinetic rates
The MP studies lack molecular details and they do not quantify the strengths of the different kinetic states. Measurements of forces to rupture single non-covalent bonds can quantify the strengths and intrinsic dissociation rates of the bonds [15,16]. One can also determine the number of bound states between molecular pairs [17].

Single-bond-rupture measurements are widely used to investigate molecular interactions, and the studies demonstrating their ability to elucidate details of receptor–ligand binding are too numerous to cite here. Cadherin adhesion was similarly investigated at the single-molecule level, in order to probe more deeply into the binding mechanism.

Principles of single-bond-rupture measurements: relating force to bond chemistry
To rupture single non-covalent bonds, one typically increases the force on the bond at a constant rate, \( r \), until the bond fails at the ‘rupture force’. At the single-molecule level,
bond-rupture events are stochastic. The measured forces are therefore not single unique values, but follow a probability distribution determined by the bond properties and the loading rate $r_l$ [16]. The measured distribution of rupture forces is related to the intrinsic bond dissociation rate $k_{\text{off}}$ and a parameter $x_f$, which is related to the width of the activation barrier [16]. For a molecular bond with a single energy barrier, histograms of rupture forces exhibit a single peak with a maximum at the most probable rupture force $F_{\text{mp}}$. According to one model [16], $F_{\text{mp}}$ is related to the bond parameters and loading rate by:

$$F_{\text{mp}} = \frac{k_B T}{x_f} \ln \frac{k_B T}{x_f} \times r_l$$

where $k_B$ is Boltzmann’s constant and $T$ is the absolute temperature. With this relationship, one determines $x_f$ and $k_{\text{off}}$, by plotting $F_{\text{mp}}$ against $\ln (r_l)$. For simple bonds, the plot will be linear with a slope proportional to $1/x_f$ and an intercept determined by $k_{\text{off}}$ [16].

**Cadherins form multiple adhesive states with different kinetics [18,19]**

Here I describe BFP (biomembrane force probe) measurements of the rupture of individual homophilic cadherin bonds [20]. In the BFP setup (Figure 1c), an RBC is aspirated into one pipette. A microbead modified with immobilized cadherins is ‘glued’ biochemically on to the RBC. The opposed MP holds a second bead coated with the complementary cadherin (Figure 1d). The two beads are brought into contact, and then pulled apart at a loading rate $r_l$. Bond failure is detected when the RBC recoils back to its unperturbed shape. The magnitude of the rupture force is determined by analysing the distortion of the RBC at the point of rupture. $F_{\text{mp}}$ is then determined from peaks of rupture-force histograms measured at loading rates $r_l$ from 10 to $10^6$ pN/s.

Independent studies were conducted with mouse E-cadherin and with *Xenopus* C-cadherin [18,19]. The first measurements were taken with Fc-tagged EC1–EC2 fragments covalently coupled to the microbeads. The EC1–EC2 fragment contains Trp2 and its complementary binding site. It is also the segment associated with the first, fast, binding step in the cell-adhesion kinetics (Figure 2b). The bond-rupture measurements showed that these EC1–EC2 fragments form weak bonds with fast unbinding kinetics. The force histogram measured with C-cadherin EC1–EC2 fragments at a loading rate $r_l$ of $69 \pm 5$ pN/s exhibits a major peak (Figure 3a) with a maximum at $\sim 32$ pN. The most probable rupture force (peak maximum) was measured at loading rates $r_l$ ranging from 10 to $10^5$ pN/s. The plot of $F_{\text{mp}}$ against $\ln (r_l)$ was linear (Figure 3b), and the x-intercept returned a value of $0.019 \pm 0.004$ s$^{-1}$ for $k_{\text{off}}$ for the bond [18]. The force distributions obtained with the EC1–EC2 fragments of E-cadherin and C-cadherin were somewhat broader than predicted for the rupture of a single bond. Further analysis of the histogram showed that it comprises the major peak (green curve in Figure 3a) and a second ‘hidden’ peak due to a bond with a higher dissociation rate (orange curve in Figure 3a). Perret et al. [19] developed a method for identifying hidden bound states under a broad peak. This approach uses different force-time sequences to rupture the bonds. Alternatively, Bayas et al. [18] used bond lifetime measurements to determine the number of different adhesive states and their kinetic parameters. This information is also obtained from statistical analyses of the force histograms [17]. These approaches confirmed that the C-cadherin EC1–EC2 fragment forms two weak bonds with high dissociation rates of 3.9 and 0.019 s$^{-1}$. Results obtained with E-cadherin are similar [19].

In contrast with the relatively simple force distributions measured with the EC1–EC2 fragments, the histograms measured with the full-length cadherin ectodomains are more complex (Figure 3c). The distribution is much broader and the dominant peak (blue curve in Figure 3c) is at a higher force. The plot of $F_{\text{mp}}$ against $\log (r_l)$ for this prominent peak was also linear (Figure 3d). The x-intercept returned a $k_{\text{off}}$ value of $(3.9 \pm 0.7) \times 10^{-1}$ s$^{-1}$. The full ectodomain forms at least one stronger bond with a much lower dissociation rate than for either of the EC1–EC2 bonds [18].

Detailed analyses of the broad histograms measured with E-cadherin and C-cadherin showed that the EC1–EC5 fragments of both proteins exhibit four different bonds, which differ in their strengths and dissociation rates [18,19]. In Figure 3(c), the probability distributions superimposed on the histogram were calculated with the determined parameters for the four C-cadherin bonds. Comparison of the different bound states [21,22] also verified that the four bonds are independent and are not due to multiple simultaneous cross-links between the two beads [18,19]. These results demonstrate that the full-length ectodomains form the same weak fast bonds measured with EC1–EC2 fragments, but they also form two additional stronger bonds with dissociation rates that are more than two orders of magnitude lower than those of the weak EC1–EC2 bonds.

These single-bond-rupture studies show remarkable parallels to the MP manipulation measurements. The cadherins exhibit multiple adhesive states. The EC1–EC2 domains form bonds with fast association/dissociation kinetics. The EC1–EC2 bonds are weak, and the full ectodomain is required for both the strongest adhesion and the high-probability-binding state. On the basis of these similarities, one cannot help but speculate that the high-probability-binding state and the strong-adhesive states are closely related, if not the same.

There is a further parallel between the results from these different techniques. If we assume that the high-probability-binding state and the strong cadherin bond are the same, then we would predict that the relative populations of weak and strong bonds in the histograms would change with protein–protein contact time. The peak amplitudes of the weak fast-forming EC1–EC2 bonds would be high initially. With increasing contact time, the relative population of strong bonds would increase while the population of weak bonds decreases. Perret et al. [19] demonstrated this shift in the bond populations with protein contact time. At 0.1 s, the amplitudes of the low-rupture force and high-rupture force peaks were roughly comparable. However, at 3 s, the relative
population of the strong bonds increased and the population of weak states decreased substantially. We observe similar trends in AFM (atomic force microscopy) measurements of E-cadherin and N-cadherin bonds (Q. Shi, Y.-H. Chien and D. Leckband, unpublished work). Thus, at both the single-molecule and cell levels, cadherins undergo a time-dependent transition between adhesive states. The first state exhibits fast kinetics and requires EC1–EC2, but the second, slow-forming, adhesive state requires additional EC domains.

**Surface force measurements of distance-dependent interaction potentials between cadherin monolayers**

The MP and BFP measurements quantified the dynamics and mechanical strengths of cadherin bonds. They did not, however, identify the extracellular domain interactions that are responsible for the different bound states at either the cell or single-protein levels. Both BFP and MP measurements are ‘blind’ in that they lack spatial details concerning the complex dimensions or the impact of different domains on complex formation.

**SFA (surface force apparatus) quantifies the distance-dependence of intersurface potentials**

The SFA quantifies the interaction energy between two surfaces in liquid (or air) as a function of the separation distance [23]. A distinguishing feature of this instrument is that, unlike the MP, BFP or AFM, it quantifies the absolute distance between two opposed surfaces with ± 1 Å (1 Å = 0.1 nm) resolution [23,24]. In addition, the SFA quantifies the normalized

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**Figure 3** | Single-bond-rupture measurements with CEC12–Fc (ΔEC345) and CEC1–5–Fc

(a) Force histogram of measurements between CEC12–Fc fragments at an $r_f$ of 68 pN/s. The gold and green curves are the distributions calculated with the experimentally determined parameters for each bond contributing to the histogram. (b) Most probable rupture force, $F_{\text{mp}}$, for the peak indicated by the arrow in (a) against ln ($r_f$). (c) Force histogram of measurements of CEC1–5–Fc fragments at an $r_f$ of 69 pN/s. The gold, green, blue and red curves are the distributions calculated with the parameters determined for the four cadherin bonds. The orange and green peaks correspond to the two bonds measured with CEC12. (d) Most probable rupture force, $F_{\text{mp}}$, for the peak indicated by the arrow in (c) against ln ($r_f$).
force between two macroscopic surfaces. This normalized force is directly proportional to the energy/area between the surfaces [25]. The force-normalized sensitivity enables measurements of molecular interactions with energies of the order of 0.59 kcal/mol (1 kcal = 4.184 kJ) at 25°C [23,26].

SFA measurements of both biological and non-biological materials quantify adhesion energies and the ranges and magnitudes of intersurface forces at the molecular level. They also revealed details of the molecular architectures of materials between the surfaces. Notable examples include measurements of ordered water adjacent to surfaces. SFA measurements thus detected eight to ten water layers adjacent to mica sheets. The layers had a periodicity of 3 Å, which is the diameter of a water molecule [27,28]. The distance resolution enabled the visualization of hemi-fusion between bilayer membranes [29]. Studies of biomolecules documented the impact of flexible ligand (biotin) tethering on the range, magnitude and dynamics of adhesion to membrane-bound streptavidin [30,31]. Measurements between bilayers displaying complementary receptors and ligands quantified the ranges and magnitudes of forces between streptavidin and biotin [32,33], cytochrome b₅ and cytochrome c [34], anti-fluorescein antibody and fluorescein [35], and the adhesion proteins CD2, CD48 and CD58 [36,37]. In the latter cases, the molecules adhered at intermembrane distances that agreed quantitatively with the crystallographic dimensions of the proteins and/or protein complexes. A more recent study investigated SNARE (soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor) proteins [38].

**C-cadherin ectodomains adhere at three distinct intermembrane distances**

The normalized force or energy/area as a function of the bilayer separation was measured between oriented cadherin monolayers immobilized on supported lipid bilayers. Fc-tagged C-cadherin ectodomains (CEC1–5–Fc) were immobilized to His₆-tagged Protein A, which was bound to supported bilayers containing NTA (nitrilotriacetate)-functionalized lipids (Figure 4a) [39].

Figure 4(b) shows the normalized force compared with distance between the membranes displaying cadherin ectodomains. Forces are measured during approach (decreasing D) and separation. If the proteins adhere, the force drops below zero. The lowest force in the curve is the magnitude of adhesion. At this point, the bonds break under the applied force and the surfaces snap out of contact. The membrane distance, D, at the minimum indicates the maximum extension of the bound molecules under tension, at bond rupture.

During approach, the oriented cadherin monolayers repel (F > 0) at D < 55 nm. During separation, the cadherins adhere at three distinct membrane gap distances. The strongest adhesion at D = 39 nm is measured when the bilayers are brought close enough so that the ectodomains fully overlap.

We compared the position of this bond with the extended...
length of the cadherin ectodomain, accounting for the dimensions of the anchoring layers (Fc tag, Protein A fragment and NTA headgroup). At 39 nm, the ectodomains would overlap fully in an antiparallel configuration [39]. One can also probe for adhesion at different membrane separations, by controlling the membrane separation distance (extent of protein overlap) before pull off. This is the same approach used to demonstrate water structuring near surfaces [27]. We thus identified two additional bonds at 38 and 53 nm. Adhesion at 53 nm is consistent with EC1–EC1 binding. Adhesion at the other distances implicates additional domains in binding, in agreement with the MP and BFP studies [13,18,19]. The strongest adhesion is at 39 nm, and the weakest adhesion is at 53 nm. This multistage binding mechanism was demonstrated with three different classical cadherins for both homophilic and heterophilic cadherin adhesion [40].

Domain-deletion mutants identified structural regions responsible for these three bonds. Here, SFA measurements provided spatial information lacking in the other measurements. We studied six different deletion mutants [39], but Table 1 summarizes the main findings. First, all fragments containing EC1–EC3 formed three spatially separated adhesive bonds with the order of bond strength as exhibited by the full ectodomain. Secondly, removing EC3 (∆EC3) eliminated the strongest adhesive bonds, consistent with the MP results. The ∆EC3 mutants still adhered via the outer EC1–EC2 domains. EC1–EC2 fragments bound to each other and to the full ectodomain. Thirdly, the EC3–EC5 (∆EC12) fragment adhered to both the full-length ectodomain and a second EC3–EC5 fragment at 38 and 39 nm respectively. The adhesion energy was low, but, within experimental error, binding was at the same distance as the strongest bond between the full ectodomains. Taken together, these measurements show that cadherins form multiple bound states. The strongest adhesion requires EC3, and the EC1 domains form a weaker bond at larger membrane separations. Although the SFA quantifies different parameters associated with cadherin adhesion, these findings are consistent with both the cell-adhesion kinetics and the single-bond-rupture studies.

### Concluding remarks

These different, but complementary, measurements reveal a more complex mechanism than predicted by a single N-terminal strand exchange. All three approaches show that cadherins form multiple kinetic and adhesive states that require different EC domains. The results confirm that EC1–EC2 segments alone form mechanically weak bonds with low adhesion energies and high kinetic rates.

In a clear departure from the conventional cadherin adhesion model, all three approaches described in this review reveal additional adhesive and kinetic states that require the full ectodomain or EC3 specifically. These findings support a mechanism in which cadherin first forms fast weak EC1–EC2 bonds, but then transitions to a second state characterized by high binding probability, slow dissociation kinetics and strong adhesion. In cell-binding kinetics, the high-probability-binding state requires EC3, as does the strongest bond measured with the SFA. The strongest bond between single cadherins similarly requires the full ectodomain.

Several challenges remain. A current aim is to elucidate the mechanism underlying the two-stage binding kinetics and its relationship to the different adhesive and kinetic states identified by force measurements. A further question concerns the role of Trp2. The apparent loss in cell adhesion due to the W2A substitution is often cited in support of a model in which adhesion involves only the Trp2 exchange. However, surface force [41], MP manipulation [13] and antibody-binding [42] studies suggest that Trp2 may allosterically modulate global cadherin activity. Although the adhesion mechanism described here is more complex than predicted by structural models, the combination of structural data and quantitative measurements of protein function provide a more complete picture of how these complex proteins mediate cell adhesion. A comprehensive model of cadherin adhesion at the cell surface must address these different features of cadherin binding.

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Table 1 | Summary of intermembrane distances of adhesive bonds between cadherin-domain-deletion mutants

<table>
<thead>
<tr>
<th>Cadherin fragment</th>
<th>First minimum (nm)</th>
<th>Second minimum (nm)</th>
<th>Third minimum (nm)</th>
</tr>
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<tbody>
<tr>
<td>EC1–EC5</td>
<td>38.2 ± 0.6</td>
<td>44.5 ± 1.0</td>
<td>53.1 ± 0.8</td>
</tr>
<tr>
<td>EC1–EC3 (∆EC45)</td>
<td>21.1 ± 0.9 (Δ = 17.1 nm)</td>
<td>26.9 ± 0.2 (Δ = 17.6)</td>
<td>34.9 ± 0.5 (Δ = 18.2)</td>
</tr>
<tr>
<td>EC1–EC2 (∆EC35)</td>
<td>–</td>
<td>–</td>
<td>27.1 ± 0.9 (Δ = 26.0)</td>
</tr>
<tr>
<td>EC3–EC5 (∆EC12)</td>
<td>38.6 ± 0.4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EC1–EC2–EC4–EC5 (∆EC3)</td>
<td>–</td>
<td>–</td>
<td>42.4 ± 0.8 (Δ = 10.7)</td>
</tr>
</tbody>
</table>

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References


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