

# Effects of Ligand Mobility on Ligand–Receptor Interactions Driving Integrin-Mediated Cell Adhesion

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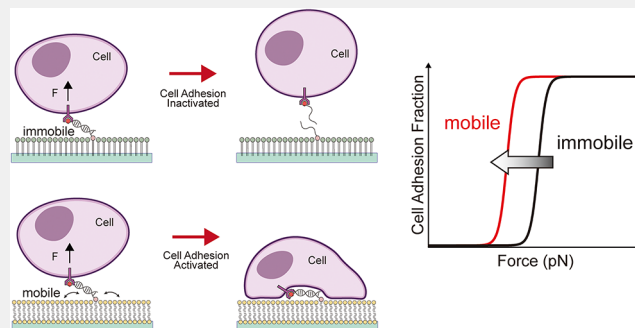
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**ABSTRACT:** Ligand–receptor interactions that drive cell adhesion occur within dynamic membrane environments, where ligand mobility can influence force transmission and downstream signaling. Despite its potential significance, the mechanical role of ligand mobility in regulating the activation threshold for cell adhesion remains poorly understood. Here, we quantified how ligand mobility modulates the apparent adhesion threshold for integrin-mediated cell adhesion using DNA-based tension gauge tethers. By directly comparing mobile ligands presented on supported lipid bilayers with immobilized ligands on rigid substrates, we demonstrated that mobile ligands exhibited apparent adhesion thresholds consistently lower than those of immobile ligands across multiple cell types. Controlled modulation of membrane fluidity further revealed that the ligand diffusion constants are inversely proportional to apparent adhesion thresholds. These results highlight the critical role of ligand mobility in ligand–receptor interactions and advance our understanding of how membrane physical properties regulate cellular signaling.

**KEYWORDS:** Ligand–receptor interactions, Ligand mobility, Cell adhesion, Integrin, cRGDfK, Tension gauge tether



Mechanotransduction, the process by which cells sense and respond to mechanical cues from neighboring cells or their environment called extracellular matrix (ECM), is essential for regulating a wide range of cellular activities such as cell adhesion, migration, proliferation, differentiation, apoptosis, and immune activation.<sup>1–6</sup> At the molecular level, the typical pathway of mechanotransduction is initiated through ligand–receptor interactions that transmit mechanical forces across the cell membrane and convert them into biochemical signaling.<sup>7</sup> And abnormal mechanotransduction is responsible for various diseases including muscular dystrophies and cancers.<sup>8</sup> Quantifying the forces involved in the ligand–receptor interactions is therefore critical for understanding how cells interpret and respond to mechanical information, as well as mechanotransduction-associated disease pathways.

Cell adhesion is mediated by the interaction of integrin receptor with the ECM where RGD (Arg–Gly–Asp)-containing ligands are located.<sup>9–11</sup> The magnitude of force transmitted through integrin–RGD interactions contributes to the activation of adhesion signaling, making integrin-mediated adhesion a well-established model for studying mechanotransduction. Numerous experimental methods have been developed to quantify these interactions at the single-molecule level, enabling direct measurement of piconewton-level forces characteristic of individual ligand–receptor interaction.<sup>12–15</sup> Among these methods, molecular tension force microscopy (MTFM)-based techniques<sup>13</sup> and tension gauge tether (TGT) assays<sup>14</sup> have emerged as two major platforms for quantifying

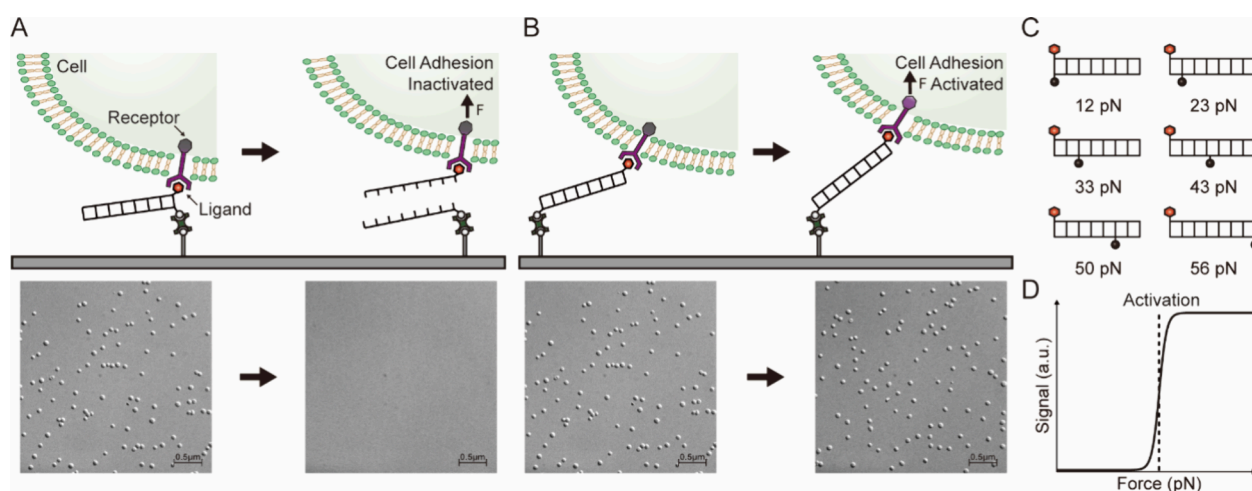
piconewton-scale ligand–receptor interaction forces, due to their high-throughput piconewton force measurement capability.

MTFM-based methods enable direct visualization of forces exerted by integrins using reversible molecular springs such as hairpin DNAs, often implemented on supported lipid bilayers (SLBs),<sup>16</sup> where ligands are laterally mobile and presented in a membrane-like environment. In contrast, the TGT assays provide threshold-based force measurements using double-stranded DNA (dsDNA) tethers that rupture irreversibly at defined force thresholds but have been predominantly applied to immobilized ligands on rigid substrates such as poly(ethylene glycol) (PEG)-coated glass. Recent studies have refined TGT-based approaches by demonstrating that TGT rupture depends on tension-dependent lifetimes, allowing them to function as both force threshold and duration sensors.<sup>17,18</sup> DNA-based tension probes have evolved to measure force duration and loading rates, providing deeper mechanistic insight into ligand–receptor interactions.<sup>19</sup> For example, such approaches can be applied to distinguish

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**Figure 1. Schematic overview of TGT-based quantification of ligand–receptor interactions inducing cell adhesion.** (A) Representative images of cells seeded on substrates functionalized with low-rupture-force TGT sensors before and after washing, showing removal of nonadherent cells. (B) Representative images of cells seeded on substrate functionalized with high-rupture-force TGT sensors before and after washing, showing stable cell adhesion. (C) Schematic of TGT with tunable rupture forces. All TGTs share the same DNA sequence, while the rupture force is controlled by the relative positions of the biotin anchor (black sphere) and the ligand (cRGDfK, red hexagon). (D) Cell adhesion activation exhibits a sharp apparent adhesion threshold, resulting in a sigmoidal dependence of cell attachment on ligand–receptor interaction force.

between slip bonds and catch bonds, which is important for understanding how mechanical forces regulate cellular adhesion and downstream signaling responses. However, despite these advances, most previous studies employed force sensors under a single, fixed ligand environment, either mobile or immobile, without directly comparing ligand–receptor interaction forces across different ligand mobility conditions within the same measurement framework. This makes it difficult to systematically investigate the effect of ligand mobility on ligand–receptor interactions by simply comparing results across independent studies.

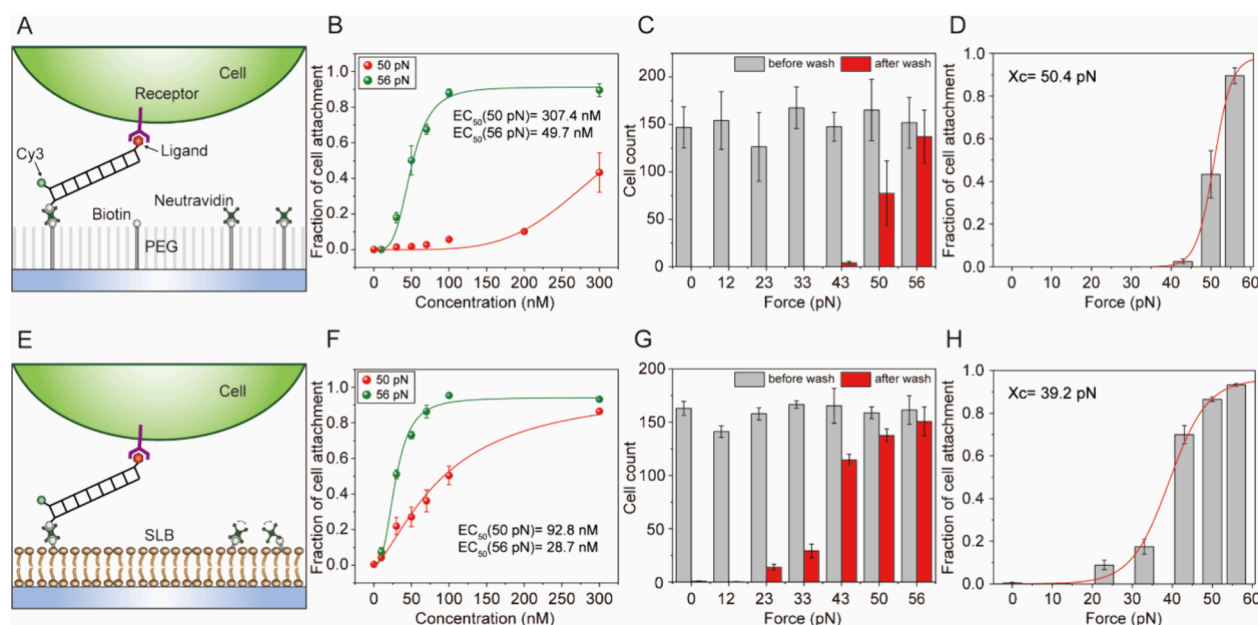
Here, we address this limitation by applying TGT assays to both rigid substrates and SLBs within a unified experimental platform. The cRGDfK ligands were functionalized to TGT sensors, and integrin-mediated cell attachment was assessed after incubating human cells on TGT-functionalized surfaces. Ligand mobility on SLBs was systematically modulated by varying cholesterol content in SLBs, which regulates the membrane fluidity and dynamics.<sup>20</sup> Mobile ligands reduced the apparent adhesion thresholds across multiple cell types despite differences in cell-specific activation behaviors. The apparent adhesion thresholds exhibited an inverse linear correlation with the diffusion coefficient of the ligands. Overall, this work provides key insights into the role of ligand mobility in regulating ligand–receptor interactions, contributing to a deeper understanding of cell adhesion and mechanotransduction.

To investigate how ligand mobility affects ligand–receptor interaction, we examined the interaction between cellular integrin receptors and cRGDfK, a well-established ligand that activates integrin-mediated cell adhesion.<sup>21</sup> To quantify the mechanical forces involved in these interactions, we employed TGT force sensors, which are dsDNA constructs engineered to rupture at defined critical force thresholds. When the force transmitted through the ligand–receptor interaction exceeds the TGT rupture force, the DNA duplex irreversibly dissociates, terminating force transmission and preventing the activation of cell adhesion (Figure 1A). Under these conditions, cells failed to maintain mechanical contact with

the substrate, resulting in cell detachment during the washing step (Figure 1A). In contrast, when the transmitted force is below the TGT rupture force, the TGT duplex remains intact, allowing sustained force transmission that leads to the activation of integrin-mediated adhesion pathways (Figure 1B). Consequently, cells adhere to the substrate and remain attached after being washed (Figure 1B).

Each TGT is a dsDNA tether conjugated with a biotin moiety for surface immobilization and a ligand for receptor engagement. The relative positions of the biotin anchor and the ligand define the force application geometry, enabling precise tuning of the rupture force without changing the DNA sequence or length. While TGTs provide well-defined rupture forces determined by sequence and pulling geometry, the effective rupture probability can be influenced by other factors such as surface tether architecture, temperature, and the local molecular environment. These parameters were kept constant across all experimental conditions, minimizing systematic variations and enabling the reliable interpretation of relative differences in apparent adhesion thresholds. We employed TGT constructs with rupture forces ranging from 12 pN to 56 pN, which was designed and validated in a previous study.<sup>14</sup> Ligands positioned closest to the biotin anchor experience an unzipping geometry and exhibit the lowest rupture force (12 pN), whereas ligands positioned furthest away undergo a shearing geometry with the highest rupture force (56 pN).<sup>14,22</sup> Intermediate ligand positions correspond to partial shearing modes with rupture forces spanning intermediate values (Figure 1C and Table S1). Cell attachment fractions were measured across TGTs with rupture forces ranging from 12 to 56 pN, and the apparent adhesion threshold was then determined by fitting the cell attachment fractions to a sigmoidal function and calculating the force corresponding to the half-maximum fraction (Figure 1D).

We first compared two substrate types, a PEG-coated surface and an SLB composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) using HeLa (human cervical carcinoma) cells. On a PEG-coated surface, TGTs are immobilized at fixed positions via biotin-neutravidin linkage, as the PEG layer is



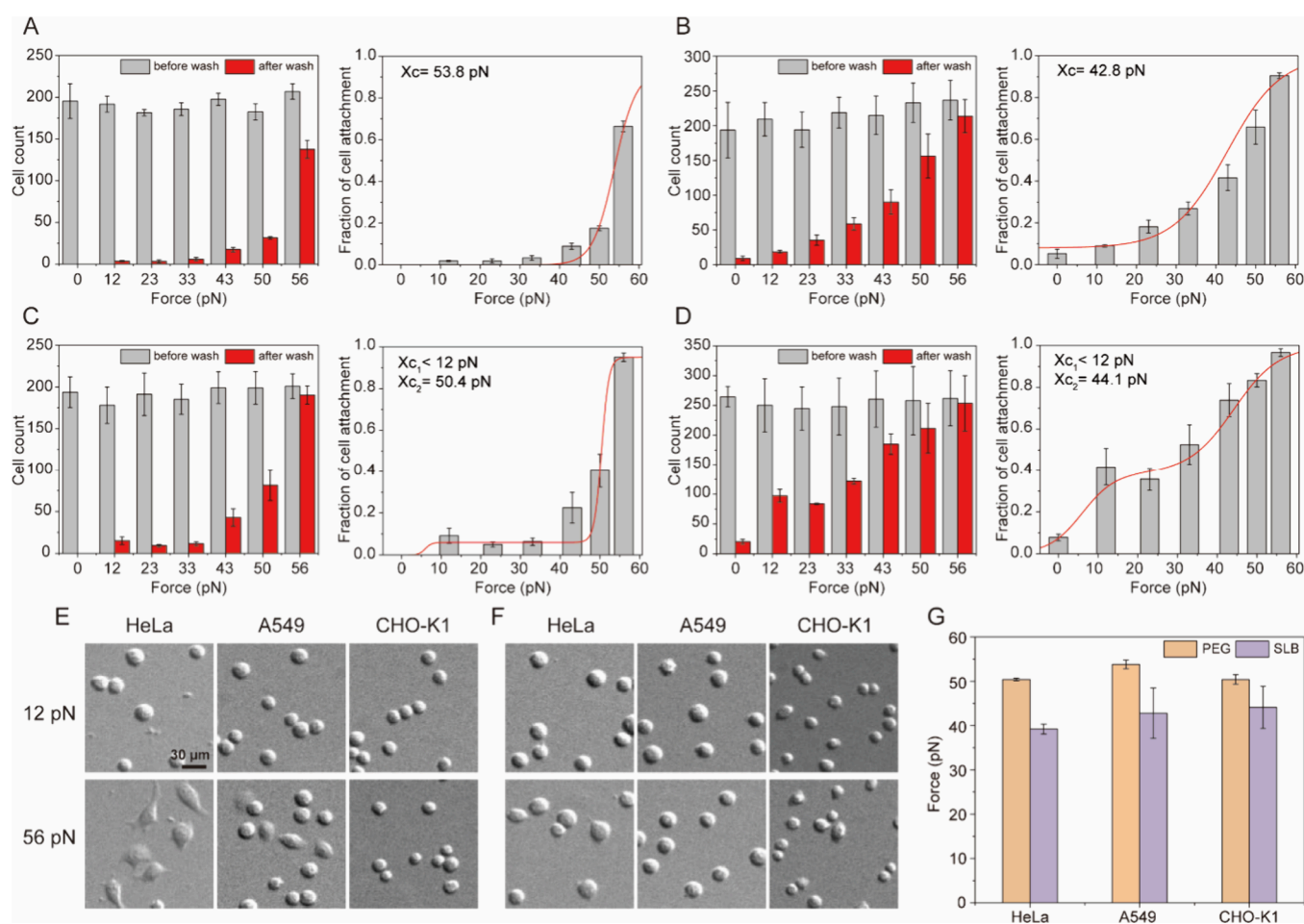
**Figure 2. Substrate-dependent activation of cell adhesion.** (A, E) Schematic illustrations of cell adhesion mediated by ligand–receptor interactions on (A) PEG-coated and (E) DOPC-coated substrates. (B, F) Fraction of attached cells on (B) PEG-coated and (F) SLB-coated substrates with varying concentrations of ligands, using TGT sensors with rupture forces of 50 pN (red line) and 56 pN (green line). (C, G) Average numbers of cells before (gray bars) and after (red bars) washing on (C) PEG-coated and (G) SLB-coated substrates using 300 nM TGT sensors with different rupture forces. (D, H) Fraction of cell attachment as a function of TGT sensor rupture force on (D) PEG-coated and (H) SLB-coated substrates. The rupture force corresponding to half-maximal attachment ( $X_c$ ) was 50.4 pN on PEG and 39.2 pN on SLB substrates.

covalently attached to the substrate and does not allow lateral diffusion. In contrast, on DOPC SLBs, biotinylated phospholipids diffuse freely within the lipid bilayer, enabling lateral diffusion of the attached TGTs and ligands (Figure 2A, 2E). We measured the fraction of attached cells with increasing ligand concentrations using TGT sensors with rupture forces of 50 and 56 pN (TGT-50pN and TGT-56pN) over a concentration range up to 300 nM, on both PEG- and DOPC-coated substrates (Figures 2B, 2F, and S1). The resulting cell attachment curves were fitted using the Hill equation, producing higher Hill coefficients for ligands on PEG-coated surfaces (4.5 and 3.9 for TGT-50pN and TGT-56pN, respectively) compared to those on DOPC SLBs (1.5 and 2.8, respectively). This behavior likely reflects collective mechanical activation of cell adhesion rather than molecular cooperativity, consistent with previous studies showing that force-dependent clustering and cytoskeletal reinforcement promote the engagement of multiple-receptor interactions at the cellular level.<sup>23</sup> On mobile SLBs, lateral diffusion of ligands allows cells to recruit ligands beneath adhesion sites, thereby compensating for lower bulk ligand density and producing a more gradual increase in adhesion with ligand concentration. Consistent with this interpretation, the EC<sub>50</sub> values on PEG substrates (307.4 nM and 49.7 nM) were substantially higher than those on DOPC SLBs (92.8 nM and 28.7 nM), supporting that higher ligand densities are required to activate cell adhesion when ligands are immobilized. Because maximum adhesion was reached at ligand concentrations above 100 nM for TGT-56 pN on both substrates, a concentration of 300 nM TGTs was used for subsequent experiments to ensure full cell attachment at the highest rupture force tested (Figures 2B, 2F, and S1).

Next, we quantified the cell attachment fraction using a series of TGT sensors with rupture force of 12, 23, 33, 43, 50, and 56 pN on both PEG and SLB substrates using HeLa cells

(Figures 2C, 2D, 2G, 2H, and S2). The cell attachment fraction as a function of rupture force was fitted by using a Boltzmann sigmoidal fit, from which the half-maximal value was defined as the apparent adhesion threshold. Interestingly, ligands presented on SLBs exhibited an apparent adhesion threshold (39.2 pN) lower than those on PEG substrates (50.4 pN), suggesting that the ligand environment significantly affects the force threshold required to activate stable cell adhesion. This reduced apparent adhesion threshold observed on SLBs may arise from lateral ligand mobility, which can facilitate force redistribution during ligand–receptor engagement and thereby reduce the activation force required for stable adhesion. Alternatively, the ligand mobility may modify the temporal profile of force transmission at individual bonds. For example, dynamic ligand rearrangement could shorten the duration of the force applied to each bond, slow the loading rate, or distribute forces more efficiently across multiple parallel interactions. Under these conditions, low-force TGTs would be less likely to rupture, leading to a shift in the postwash retention curve even without a substantial change in peak force. Our results on PEG substrate are consistent with previous studies utilizing the same experimental platform.<sup>14</sup> However, reported activation forces required for cell adhesion vary widely in the literature, likely due to differences in ligand mobility, integrin constructs, and cell types.<sup>14,24–26</sup> Therefore, directly comparing these studies to isolate the effect of ligand mobility is challenging. We addressed this ambiguity by measuring the force thresholds for cell adhesion on both immobile (PEG) and mobile (SLB) substrates using the same experimental framework, demonstrating that ligand mobility reduces the force threshold required for cell adhesion in HeLa cells.

To further test whether the reduced apparent adhesion threshold on mobile SLBs is conserved across different cell types, we extended our analysis to A549 (human lung

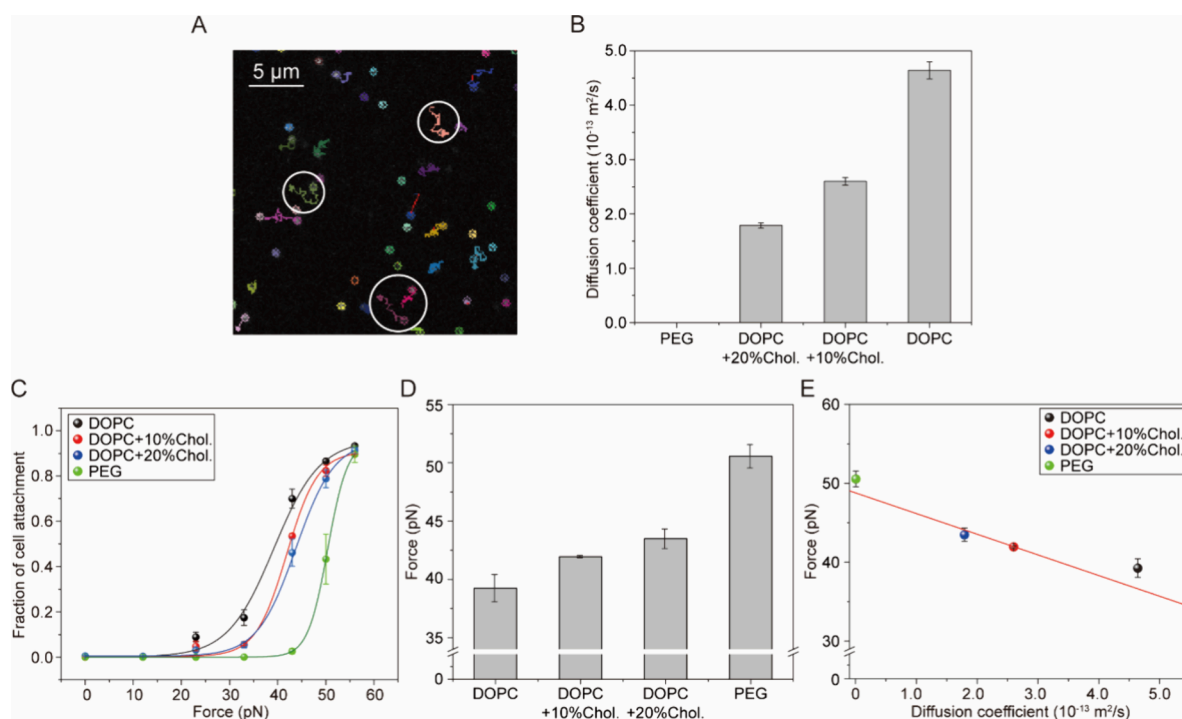


**Figure 3. Apparent adhesion thresholds across different cell types.** (A–D) Quantification of apparent adhesion threshold on PEG-coated and SLB-coated substrates using A549 and CHO-K1 cells. For each condition, the left panels show the average number of cells before and after washing, and the right panels show the fraction of attached cells as a function of TGT rupture force. (A) A549 on PEG. (B) A549 on SLB. (C) CHO-K1 on PEG. (D) CHO-K1 on SLB. CHO-K1 cells exhibited a two-step activation behavior, with a lower apparent adhesion threshold ( $X_{c1} < 12$  pN) and a higher apparent adhesion threshold ( $X_{c2}$ ) of 50.4 pN on PEG and 44.1 pN on SLB substrates. (E, F) Representative DIC images of cells cultured for 1 h on each substrate functionalized with TGT force sensors of 12 pN (top) and 56 pN (bottom). (E) PEG-coated and (F) SLB-coated substrates. In each panel, images from left to right correspond to HeLa, A549, and CHO-K1 cells. (G) Apparent adhesion thresholds ( $X_c$  values) obtained from sigmoidal fitting for each cell type and substrate condition. SLB substrates show lower apparent adhesion thresholds than PEG substrates across all cell types.

carcinoma) and CHO-K1 (nontransformed Chinese hamster ovary) cells. A549 cells exhibited a sigmoidal increase in cell attachment fraction with increasing TGT rupture force, similar to HeLa cells, with a lower activation threshold force on mobile SLBs compared to PEG-coated substrates (Figures 3A, 3B, and S3). On PEG surfaces, A549 cells showed negligible attachment at rupture forces  $\leq 33$  pN, followed by a sharp increase in adhesion at higher-force TGTs, producing an apparent adhesion threshold of 53.8 pN. In contrast, the apparent adhesion threshold on SLBs was reduced to 42.8 pN, demonstrating that ligand mobility lowers the force threshold required for adhesion activation (Figure 3A, 3B). Unlike HeLa and A549 cells, CHO-K1 cells displayed a two-step sigmoidal response, characterized by a low apparent adhesion threshold below 12 pN on both PEG and SLB substrates and a high apparent adhesion threshold at 50.4 pN on PEG and 44.1 pN on SLBs (Figures 3C, 3D, and S4). While CHO-K1 cells exhibit a distinct multistep activation mechanism, the high apparent adhesion threshold was again lower on mobile SLBs than on PEG substrates. Moreover, the two-step activation behavior appeared more clearly on SLBs, suggesting that ligand

mobility enhances the manifestation of the low-force adhesion pathway. Despite differences in adhesion mechanisms across cell types, the apparent adhesion threshold was consistently reduced on mobile SLBs compared with immobilized PEG-coated surfaces. Representative differential interference contrast (DIC) images of HeLa, A549, and CHO-K1 cells on substrates functionalized with TGT-12pN and TGT-56pN sensors are displayed in Figure 3E and 3F, illustrating more extended and stable cell attachment on PEG-coated surfaces. Figure 3G summarizes the apparent adhesion thresholds across all cell types and substrate conditions, suggesting that ligand mobility consistently lowers the force threshold for adhesion activation.

Our measurement of apparent adhesion thresholds near  $\sim 40$  pN is consistent with previously reported values for RGD-integrin-mediated focal adhesion reinforcement and contractility.<sup>14</sup> Interestingly, the additional lower apparent adhesion threshold observed in CHO-K1 cells indicates a distinct mechanical commitment threshold for adhesion stabilization. This difference may reflect variations in integrin utilization or early force-loading dynamics. CHO-K1 cells may initiate



**Figure 4. Ligand mobility modulates the apparent adhesion threshold.** (A) Representative trajectories of individual Cy3 dye-labeled TGT force sensors on SLB substrates. (B) Diffusion coefficients analyzed from MSD analysis for TGT force sensor on DOPC, DOPC containing 10% cholesterol and 20% cholesterol, and PEG-coated surfaces. Ligands on PEG surfaces exhibited minimal mobility, whereas ligands on DOPC SLBs show the highest mobility, which decreases with increasing cholesterol content. (C) Cell attachment fraction as a function of TGT rupture force on DOPC, DOPC + 10% cholesterol, DOPC + 20% cholesterol, and PEG surfaces. Solid lines indicate Boltzmann sigmoidal fits used to determine the apparent adhesion threshold. (D) Average apparent adhesion thresholds on each substrate, analyzed from the fits in (C), showing higher apparent adhesion thresholds on PEG compared to SLB substrates. (E) Correlation curve between ligand diffusion coefficient and apparent adhesion thresholds, demonstrating that increased ligand mobility is associated with lower apparent adhesion thresholds.

adhesion through low-force nascent contacts while retaining the capacity to develop mature focal adhesions at higher forces. In comparison, HeLa and A549 cells appear to require greater mechanical resistance to drive focal adhesion maturation and reinforcement.<sup>27,28</sup> This interpretation is consistent with previous studies showing that carcinoma cells with higher metastatic potential often exhibit increased actomyosin contractility and generate larger traction stresses compared with nontransformed or less invasive cells.<sup>29,30</sup> And the relatively large difference in apparent adhesion threshold between mobile and immobile ligands observed in carcinoma cells may reflect their mechanical adaptability,<sup>31,32</sup> which enables dynamic regulation of force generation and adhesion reinforcement in response to the mechanical environment, thereby increasing sensitivity to ligand mobility. Thus, the observed differences likely reflect cell-type-specific mechanical loading behaviors in addition to potential differences in integrin subtype engagement.

To further investigate the effect of ligand mobility on the apparent adhesion threshold, we systematically modulated the ligand mobility by varying cholesterol content in DOPC SLBs, as cholesterol is known to reduce membrane fluidity for DOPC.<sup>20</sup> SLBs were prepared using DOPC containing 0%, 10%, or 20% cholesterol, and ligand mobility was quantified using Cy3-labeled TGT sensors. Diffusion coefficients were extracted from mean square displacement analysis by tracking individual TGT sensors using total internal reflection fluorescence (TIRF) microscopy.<sup>33</sup> The representative TIRF images and corresponding diffusion trajectories are shown in Figure 4A. The measured diffusion coefficients were  $4.6 \times$

$10^{-13} \text{ m}^2/\text{s}$  for DOPC,  $2.6 \times 10^{-13} \text{ m}^2/\text{s}$  for DOPC with 10% Cholesterol, and  $1.8 \times 10^{-13} \text{ m}^2/\text{s}$  for DOPC with 20% Cholesterol, confirming a progressive reduction in ligand mobility with increasing cholesterol content in SLBs (Figures 4B, S5). As expected, ligands on PEG-coated surfaces exhibited minimal mobility, as they were supposed to fix at defined positions (Figure 4B). The diffusion constant of DOPC in SLBs has been previously reported to be  $\sim 1\text{--}3 \times 10^{-12} \text{ m}^2/\text{s}$  for DOPC SLBs by fluorescence correlation spectroscopy or fluorescence recovery after photobleaching, which is at least 2 times higher than the value obtained in our study.<sup>34,35</sup> This discrepancy likely arises because we tracked bulkier DOPC-conjugated TGT sensors rather than free DOPC molecules. Then, we quantified the cell attachment fraction using HeLa cells on these four substrates with different ligand mobilities and determined the corresponding apparent adhesion thresholds. The apparent adhesion threshold increased from 39.2 pN on DOPC SLBs to 41.9 pN on DOPC+10% Cholesterol, 43.8 pN on DOPC+20% Cholesterol, and 50.4 pN on PEG substrates (Figure 4C, 4D). The apparent adhesion threshold exhibited an inverse linear correlation with the ligand diffusion coefficient, demonstrating that reduced ligand mobility is associated with higher force thresholds for cell adhesion activation (Figure 4E). These results provide direct quantitative evidence that ligand mobility modulates the mechanical threshold required for integrin-mediated cell adhesion. Reduced mobility may constrain force redistribution and ligand recruitment at adhesion sites, thereby elevating the effective force required for stable cell adhesion, or alternatively

alter force transmission dynamics, leading to differences in rupture probability.

In summary, we systematically quantified how ligand mobility regulates the mechanical threshold for integrin-mediated cell adhesion using TGT sensors. By comparing immobilized ligands on PEG substrates with mobile ligands on SLBs, we demonstrate that ligand mobility consistently lowers the apparent adhesion threshold required for stable cell adhesion across multiple cell types including HeLa, A549, and CHO-K1 cells. This trend was conserved despite cell-type-specific adhesion mechanisms, indicating that ligand mobility is a general physical determinant of adhesion force thresholds. Furthermore, controlled modulation of ligand mobilities revealed an inverse correlation between the ligand diffusion coefficient and apparent adhesion threshold. Taken together, these findings establish ligand mobility as key regulators of ligand–receptor interactions, providing insight into how the membrane environment modulates cellular mechanotransduction at the molecular level.

## ■ ASSOCIATED CONTENT

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/cbmi.6c00057>.

Materials and Methods, Table S1 and Figures S1–S5 (PDF)

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### Notes

The authors declare no competing financial interest.

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