The C-terminal unique region of desmoglein 2 inhibits its internalization via tail–tail interactions

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Desmosomal cadherins, desmogleins (Dsgs) and desmocollins, make up the adhesive core of intercellular junctions called desmosomes. A critical determinant of epithelial adhesive strength is the level and organization of desmosomal cadherins on the cell surface. The Dsg subclass of desmosomal cadherins contains a C-terminal unique region (Dsg unique region [DUR]) with unknown function. In this paper, we show that the DUR of Dsg2 stabilized Dsg2 at the cell surface by inhibiting its internalization and promoted strong intercellular adhesion. DUR also facilitated Dsg tail–tail interactions. Forced dimerization of a Dsg2 tail lacking the DUR led to decreased internalization, supporting the conclusion that these two functions of the DUR are mechanistically linked. We also show that a Dsg2 mutant, V977fsX1006, identified in arrhythmogenic right ventricular cardiomyopathy patients, led to a loss of Dsg2 tail self-association and underwent rapid endocytosis in cardiac muscle cells. Our observations illustrate a new mechanism desmosomal cadherins use to control their surface levels, a key factor in determining their adhesion and signaling roles.

Introduction

Adherens junctions and desmosomes are essential for mediating intercellular adhesion in epithelial and cardiac tissues and, in addition, provide positional and signaling cues that regulate cell proliferation, polarity, migration, and differentiation (Schock and Perrimon, 2002; Green and Simpson, 2007; Niessen et al., 2011). The assembly and disassembly of cell–cell junctions is carefully choreographed during epithelial morphogenesis and remodeling (Niessen et al., 2011). Altering junction stability or assembly state through loss of function, mutation, or posttranslational modification can lead to inherited disorders, blistering diseases, and cancer (Holthöfer et al., 2007; Simpson and Green, 2007; Thomason et al., 2010; Amagai and Stanley, 2012; Brooke et al., 2012).

The adhesive core of adherens junctions and desmosomes comprises members of the cadherin superfamily—classical cadherins (e.g., E-cadherin) in adherens junctions and desmosomal cadherins (desmogleins [Dsgs] and desmocollins) in desmosomes (Green and Gaudry, 2000; Pokutta and Weis, 2007). In both cases, adhesive interactions are mediated by trans-interactions between the N-terminal cadherin ectodomains on the surface of neighboring cells. The C-terminal tails are embedded in a cytoplasmic plaque consisting of armadillo proteins, cytoskeletal adaptors, and their associated cytoskeletal connections. Although adherens junctions organize and regulate the assembly state of cortical actin, desmosomes provide integrity to tissues by anchoring intermediate filaments to sites of desmosomal adhesion.

The extracellular repeats that define the cadherin superfamily are fairly well conserved in classical and desmosomal cadherins, but the domain structure of the cytoplasmic tails exhibits unique features (Hulpiau and van Roy, 2009). The membrane proximal regions in both cases contain regions that associate with armadillo gene family members. More distally, Dsgs contain an extended C-terminal unique region (Dsg unique region [DUR]) with unknown function (Koch et al., 1990). This region can be divided into a linker region, a series of repeats each
Reepithelialization, and cell renewal in the epidermis), and endocytosis is a key regulator of this process. Some efforts have been made to determine how Dsg is endocytosed in the presence of various environmental stimuli: autoantibodies, kinase inhibitors, and calcium depletion (Holm et al., 1993; Delva et al., 2008; Klessner et al., 2009; Jolly et al., 2010; Jennings et al., 2011). Engagement of transmembrane receptors with internalization machinery is frequently dictated by the presence of specific sequences in their cytoplasmic tails (Bonifacino and Traub, 2003), but the contribution of Dsg cytoplasmic sequences to regulation of endocytosis is poorly understood. Among the four Dsg isoforms, Dsg2 is the first to be synthesized during consisting of 29 ± 4 residues, and a terminal domain (Fig. 1A). Electron microscopy showed that the predominant form of DUR is a monomer, consisting of a globular head attached to a thin tail. Dimers and oligomers were also observed but less frequently (Rutman et al., 1994). Another biophysical study demonstrated that the DUR is intrinsically disordered with an inducible structure (Kami et al., 2009). The potential modulatory roles conferred by the DUR on Dsg or desmosomes and how the DUR exerts these functions are unknown.

Cell–cell adhesion plays important roles in many cellular functions. Cell adhesive structures undergo dynamic changes during tissue remodeling (e.g., embryonic development, wound reepithelialization, and cell renewal in the epidermis), and endocytosis is a key regulator of this process. Some efforts have been made to determine how Dsg is endocytosed in the presence of various environmental stimuli: autoantibodies, kinase inhibitors, and calcium depletion (Holm et al., 1993; Delva et al., 2008; Klessner et al., 2009; Jolly et al., 2010; Jennings et al., 2011). Engagement of transmembrane receptors with internalization machinery is frequently dictated by the presence of specific sequences in their cytoplasmic tails (Bonifacino and Traub, 2003), but the contribution of Dsg cytoplasmic sequences to regulation of endocytosis is poorly understood. Among the four Dsg isoforms, Dsg2 is the first to be synthesized during

![Figure 1](image-url). DUR is required for strong cell–cell adhesion. (A) Schematic representation of Dsg2 and Dsg2 mutants. P, precursor sequence; EC, extracellular cadherin repeat; EA, extracellular anchoring domain; TM, transmembrane domain; IA, intracellular anchoring domain; ICS, intracellular cadherin-like sequence; L, linker domain; RUD, repeat unit domain; TD, terminal domain. (B, a) SCC68 or SCC9 cells expressing Dsg2.ICS-GFP or Dsg2.FL-GFP were transfected with 20 nM siRNA oligos targeting endogenous Dsg2 (Dsg2) or a nonspecific sequence (control [Ctl]). 48 h after transfection, cell lysates were collected and immunoblotted with the indicated antibodies. The epitope for 4B2 is in the C terminus of Dsg2 tail and therefore recognizes endogenous Dsg2 and Dsg2.FL-GFP but not Dsg2.ICS-GFP. (b) SCC68 cells stably expressing Dsg2.ICS-GFP or Dsg2.FL-GFP were transfected with 20 nM siRNA oligos targeting endogenous Dsg2 (Dsg2) or a nonspecific sequence (control KD). 24 h after transfection, cells were collected and immunoblotted with the indicated antibodies. (c) SCC68 or SCC9 cells expressing Dsg2.ICS-GFP or Dsg2.FL-GFP were transfected with 20 nM siRNA oligos targeting endogenous Dsg2 (Dsg2) or a nonspecific sequence (control KD). 24 h after transfection, cells were stained for DP (red) and imaged using an N-SIM system. Bar, 1 µm. (C) Mutant expressing SCC68 cells grown in medium with 0.09 mM Ca2+ were lysed in 0.5% Triton X-100 buffer. Supernatants (S) were collected after centrifugation. To the remaining pellets (P), USB equal to the volume of the supernatant was added, and pellets were solubilized. Equal amount of supernatant and pellet samples from each mutant were blotted for FLAG. Similar results were obtained for cells in 1.0 mM Ca2+:...
development (Fleming et al., 1991), the most prevalently expressed in desmosomes of adult tissues, and has the longest DUR (Schäfer et al., 1994). Here, we address the contribution of the Dsg2 DUR sequence to intercellular adhesive strength and how DUR regulates Dsg2’s stability and endocytic behavior. Through analysis of a panel of Dsg2 mutants, we conclude that the DUR strengthens cell–cell adhesion and stabilizes Dsg2 at the cell surface by inhibiting its internalization. We also show that the DUR can mediate Dsg2 tail self-interactions and that forced dimerization of Dsg2 lacking the DUR is sufficient to attenuate internalization. Furthermore, we demonstrate that V977fsX1006, a Dsg2 DUR mutant identified in arrhythmogenic right ventricular cardiomyopathy (ARVC) patients, loses Dsg2 tail–tail interactions, and undergoes rapid endocytosis in cardiac muscle cells. These results reveal a new role for the DUR and a possible mechanism for regulating the level and organization of Dsg2 at the cell surface during development and homeostasis of adult tissues.

**Results**

**Dsg2 DUR is required for strong cell–cell adhesion**

To investigate the contribution of the DUR to cell–cell adhesion and Dsg2 dynamics, we generated a series of mutants with progressive deletions of previously designated domains within the cytoplasmic tail of Dsg2 (Fig. 1A; Schäfer et al., 1994). Full-length (FL) Dsg2 and Dsg2 mutants were each tagged with a FLAG and/or a GFP epitope at the C terminus and designated by the name of the last remaining domain. Squamous cell carcinoma cell lines (SCCC68 and SCC9) were retrovirally transduced to express each Dsg2 construct (Fig. S1). The GFP-tagged mutant-expressing cells were then sorted by flow cytometry before being used for further experiments. A dispase assay was performed to compare the mechanical integrity of epithelial cell sheets expressing Dsg2.ICS-GFP– and Dsg2.FL-GFP (Fig. 1B, b). In this assay, cells were grown in high calcium medium until confluent. The cell sheet was then released from the substrate by specifically disrupting matrix adhesions by enzymatic digestion with dispase (Hudson et al., 2004). The lifted cell sheet was then subjected to mechanical shear stress, and the number of cell sheet fragments generated was used as an inverse indicator of cell–cell adhesive strength. To rule out the possible contribution of endogenous Dsg2 in this system, we knocked down endogenous Dsg2 using siRNA oligonucleotides (oligos) targeting the 3′ UTR of Dsg2 and used nonspecific siRNA oligos as a control before assaying adhesive strength (Fig. 1B, a). As shown in Fig. 1B, regardless of the status of endogenous Dsg2, Dsg2.ICS-GFP–expressing cells produced two to three times more fragments under mechanical stress than Dsg2.FL-GFP–expressing cells, suggesting the DUR is required to facilitate strong cell–cell adhesion.

One possible explanation for the observed decrease in intercellular adhesion is that without the DUR, Dsg2.ICS-GFP is unable to assemble into desmosomes. To test whether the DUR is required to target Dsg2 to junctions, we stained the GFP mutant-expressing cells for desmoplakin (DP), a component of the desmosome plaque. The immunofluorescence signals for DP and GFP were then captured and reconstructed using Nikon structured illumination microscopy (N-SIM), which produces two times the resolution of conventional optical microscopes. All desmosomes exhibit a bilaterally symmetrical structure comprising closely opposed plasma membranes containing Dsgs and desmocollins, sandwiched by two cytoplasmic plaques made of DP and its associated proteins. By N-SIM, we detected a DP-Dsg-DP arrangement along the plasma membrane only under conditions supporting desmosome assembly. Using this “railroad” pattern as an indicator, we demonstrate that both Dsg2.ICS-GFP and Dsg2.FL-GFP assemble into desmosomes regardless of the status of the endogenous Dsg2 (Fig. 1B, c). These results suggested that the DUR is not required for Dsg2 to incorporate into desmosomes.

Even though both GFP mutants were observed to incorporate into desmosomes, the reduced adhesive strength exhibited by cell sheets expressing Dsg2.ICS-GFP suggested differences in the structure or stability of the Dsg2-containing adhesive complexes. To begin to test this idea, the Triton X-100 solubility of the Dsg2 mutants was tested (Fig. 1C). Sequential deletion of distal regions of Dsg2 resulted in a progressive shift into the Triton X-100–insoluble pool, raising the possibility that the DUR regulates Dsg2 stabilization through strengthening association with detergent-insoluble cytoskeletal or membrane elements. The DUR decreased Dsg2 Triton X-100 insolubility even in low calcium medium, suggesting that association with the intermediate filament cytoskeleton is unlikely to completely account for these differences.

**Dsg2 DUR inhibits Dsg2 internalization**

The aforementioned observations suggested that Dsg2 mutants lacking the DUR form less stable structures than FL Dsg2 and prompted us to ask whether these mutants are also more likely to be subjected to endocytic turnover. To investigate whether the DUR regulates Dsg2 endocytosis, proteins on the surface of SCC68 cells were biotinylated, and the internalized pool of Dsg2 was tracked over time as described in the Materials and methods (Fig. 2B). Among the Dsg2 mutants, Dsg2.IA was internalized most efficiently. Dsg2.IA was the second best internalized construct, suggesting there may be a “signal” residing in the ICS domain that promotes internalization. Interestingly, mutants containing the DUR region (Dsg2.RUDI, Dsg2.RUDII, Dsg2.RUDV, and Dsg2.FL) were also internalized less efficiently than the Dsg2.ICS (Fig. 2B and C). These results suggest that the DUR negatively regulates Dsg2 internalization. The same effect of the DUR on Dsg2 internalization was also observed in SCC9 cells (Fig. 2G), suggesting that the inhibitory function of the DUR is cell type independent.

Because the region consisting of the linker and the first repeat unit domain was sufficient to exhibit an inhibitory effect, we used Dsg2.ICS and Dsg2.RUDI to further investigate the impact of this region on Dsg2 stability. A biotinylation assay with higher resolution time course was conducted (Fig. 2D). The difference in internalization efficiency between Dsg2.ICS and Dsg2.RUDI was observed as short at 2 min after the 37°C incubation. This suggests that the difference in the cytoplasmic/surface ratio is likely caused by inhibition of internalization rather
Dsg2 DUR inhibits Dsg2 internalization independently of Dsg2 ectodomain engagement

Dsg ectodomains engage in homophilic and/or heterophilic interactions in the extracellular space (Chitaev and Troyanovsky, 1997; Nie et al., 2011). We used two approaches to examine the potential contribution of the Dsg2 ectodomain on DUR-dependent inhibition of Dsg2 internalization. Calcium binding is critical for ectodomain conformation and cadherin interactions (Watt et al., 1984; Syed et al., 2002), and cells are unable to assemble desmosomes in low extracellular calcium. Here in the first approach, we cultured and analyzed Dsg2 mutant-expressing SCC68 cells in medium containing 0.09 mM (low), 0.25 mM (medium), or 1.0 mM (normal) calcium (Fig. 2 F). Although the internalization rate of all the mutants tested decreased with increasing calcium concentration, the presence of the DUR sequence in Dsg2.RUDI conferred an inhibitory role on internalization compared with Dsg2.ICS independent of ectodomain engagement and under conditions that do not support desmosome assembly.

In the second approach, we fused variable lengths of the Dsg2 cytoplasmic tail with the nonadhesive IL2R (interleukin 2 receptor)-α chain ectodomain and transmembrane domain (Fig. 3 A). The resulting chimeras were stably expressed in SCC68 cells via retroviral transduction (Fig. 3 B), and their internalization efficiency was analyzed. Adding IA-ICS of Dsg2 to the IL2R ecto- and transmembrane domain did not alter the internalization rate (Fig. S2 A). However, addition of the region downstream of IA-ICS led to reduced internalization—IL2R:RUDI and IL2R:FL were internalized much less efficiently than IL2R:ICS, confirming the inhibitory role of DUR (Fig. 3 C).

Taking advantage of the absence of endogenous IL2R-α in SCC68 cells, we performed a fluorescence-based antibody internalization assay using the chimera-expressing cells. In this assay, the IL2R chimeras on the cell surface were labeled using an IL2R antibody at 4°C, and internalization was allowed to proceed by raising the temperature to 37°C (Xiao et al., 2005). Because the internalized pool is protected from biochemical removal of remaining surface-labeled antibody, this assay allows
Lipid raft–mediated endocytosis encompasses various pathways characterized by their dependency on regulators including dynamin, caveolin-1 (Cav1), and flotillin (Doherty and McMahon, 2009). Dynamin is a GTPase involved in vesicle fission from the plasma membrane. Next, we tested whether Dsg2 internalization is dynamin dependent. Endogenous Dsg2 exhibited enhanced border localization in response to dynasore, a dynamin inhibitor (Fig. S2 C). Similar behaviors were observed for Dsg2.ICS, Dsg2.RUDI, Dsg2.FL, and their counterpart IL2R chimeras (Fig. 4 B), suggesting that Dsg2 internalization depends on dynamin regardless of the status of the DUR.

Cav1 is a scaffolding component of caveolae, a type of lipid raft. Dsg2 has been shown to bind to Cav1 (Brennan et al., 2012), but whether and how this interaction impacts Dsg2 internalization are unclear. We knocked down Cav1 with siRNA and tested the internalization of either endogenous Dsg2 or Dsg2 mutants (Dsg2.ICS, Dsg2.RUDI, IL2R:ICS, and IL2R:RUDI) by biotinylation or antibody internalization assays (Fig. S2, D–F). Knocking down Cav1 caused little change in the ratio of cytoplasmic/surface intensity per cell, suggesting either Cav1 is not required for Dsg2 internalization or Dsg2 internalizes through multiple pathways that can compensate for the loss of Cav1. In either case, DUR does not play a determining role.

Other potential endocytosis regulators, flotillin-1 and clathrin, were tested using siRNA-mediated silencing, and neither stood out as a major sole determining factor in regulating Dsg2 internalization (Fig. S3). Collectively, these data support the idea that with or without the DUR, Dsg2 internalizes through a cholesterol-sensitive, dynamin-dependent pathway. Because of the complexity of the endocytic routing pathways, we cannot rule out the possibility that some fraction of Dsg2 is routed in a DUR-dependent manner by mechanisms not examined here.

DUR mediates intermolecular interactions of the Dsg2 tail

Another possible explanation for the DUR inhibitory effect is that the conformation of the Dsg2 tail depends on the DUR.

Dsg2 constructs with or without the DUR share endocytic pathways

The data presented so far demonstrate that the Dsg2 DUR controls the kinetics of Dsg2 internalization. It is possible that the difference in kinetics may be caused by the different internalization pathways taken by Dsg2 in the presence or absence of DUR. To address this, we compared the responses of Dsg2 constructs to various inhibitors of endocytosis. It is reported that Dsg2 resides in lipid rafts (Brennan et al., 2012), which are cholesterol-enriched hubs for signaling and endocytosis events (Simons and Gerl, 2010). Removal of cholesterol from SCC68 cells by brief methyl-β-cyclodextrin treatment led to reduced internalization of endogenous Dsg2 (Fig. S2 B), suggesting that the integrity of lipid raft structure plays a positive role in Dsg2 internalization. The sensitivity to cholesterol removal was also exhibited by Dsg2.ICS and Dsg2.RUDI (Fig. 4 A), which indicates that the internalization of both Dsg2.ICS and Dsg2.RUDI are cholesterol dependent.

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DUR may provide or eliminate interaction sites for binding partners that are important for Dsg2 internalization. To examine the complex profile of Dsg2 mutants, sucrose gradient fractionation was performed. Because of the poor solubility of Dsg2 in conventional buffers, protein complexes in Dsg2 mutant-expressing cells were preserved by cross-linking, solubilized in urea buffer, and subjected to ultracentrifugation (Fig. 5 A). Both IL2R and IL2R:ICS were distributed evenly across the right half of the fraction spectrum (6–11). In contrast, IL2R:FL distribution was concentrated in fractions 9 and 10. The same difference in fraction distribution was observed between Dsg2.ICS and Dsg2.FL, consistent with the idea that DUR plays a role in controlling the distribution of Dsg2 in different subcellular complexes. The shift between Dsg2.ICS and Dsg2.RUDI was not as dramatic as the shift between Dsg2.ICS and Dsg2.FL, raising the possibility that the impact of multiple repeat unit domains is additive.

A previous study reported that sequences downstream of the ICS contribute to plakoglobin (Pg) association with the related Dsg1 molecule (Choi et al., 2009). To determine whether differences in Pg binding that could contribute to Dsg2 dynamic behavior are conferred by the DUR, we compared the amount of Pg that coimmunoprecipitated with FL and mutant Dsg2 molecules. As shown in Fig. S4 A, both the Dsg2 mutants and IL2R chimeras harboring the DUR sequences coprecipitated with more Pg than those containing just the IA or ICS domains. This correlated with a trend toward decreased total Pg levels in cells expressing the shorter mutants (Fig. S1 A). To test whether increased Pg binding contributes to DUR-mediated inhibition of internalization, we generated a triple alanine Dsg2 mutant (Pg binding deficient [PGde]; L828A, F832A, and L835A) on the Dsg2.FL (wild type [WT]) backbone, which largely abrogated Pg binding (Fig. S4 B). However, PGde did not exhibit any difference in its ability to internalize compared with WT (Fig. S4 B). Furthermore, RNAi-mediated silencing of Pg or silencing of the associated armadillo proteins PKP2 or PKP3 did not measurably alter the size of the internalized pool of Dsg2 using the cell surface biotinylation assay (unpublished data). Together, these results suggest that binding to Pg is not a determining factor in Dsg2 internalization.

Another potential factor controlling complex formation and protein stability is the oligomeric status of the core protein. Some evidence suggests that desmosomal cadherins form trans- and cis-interactions with each other through their ectodomains (Syed et al., 2002; He et al., 2003; Al-Amoudi et al., 2007). Little is known about the interaction of the cytoplasmic tail of cadherins within the desmosomal plaque. To test whether DUR assists in Dsg2 tail–tail interactions, we used the in situ proximity ligation assay (PLA) to test for evidence of DUR-dependent interactions of the Dsg2 tail (Fig. 5 B). This technique visualizes two proteins in close proximity (0–40 nm) by detecting an antibody-based fluorescence signal produced by the rolling cycle amplification reaction (Söderberg et al., 2006). Because an antibody for detecting homodimerization of Dsg2 mutants was not available, we asked whether endogenous Dsg2 dimerizes with Dsg2.ICS or Dsg2.RUDI (Fig. 5 B, a). Although Dsg2.ICS and Dsg2.RUDI were expressed at similar levels and in a similar percentage of cells in the population, the PLA signal produced in Dsg2.RUDI-expressing cells was significantly greater than that generated by the Dsg2.ICS-expressing cells (Fig. 5 B, b). This is consistent with the hypothesis that DUR facilitates a Dsg2 tail–tail interaction.

A yeast CytoTrap two-hybrid system was used to further confirm the DUR-mediated interaction. We observed that DUR, but not IA-ICS, interacted with DUR, suggesting there is no intramolecular interaction within the Dsg2 tail, but there is a DUR-mediated intermolecular interaction between Dsg2 tails (Fig. 5 C). Further definition of the minimal region required for this interaction was not possible because some of the smaller fragments of DUR were not compatible with the CytoTrap system and tended to grow as temperature-sensitive revertants. Nevertheless, collectively, the data support the idea that sequences in the DUR mediate intermolecular Dsg2 tail interactions.

**Dsg2 tail-tail interaction negatively regulates internalization**

So far, we demonstrated two novel functions for the DUR: inhibition of Dsg2 internalization and mediation of Dsg2 tail–tail interactions. To investigate whether these two events
A mutation in DUR associated with human disease disrupts the Dsg2 tail-tail interaction and leads to rapid endocytosis in cardiac cells

ARVC is an autosomal-dominant inherited disease, characterized by arrhythmias and progressive fibrofatty replacement of the myocardium. In ~50% of cases, known mutations in desmosomal genes, including Dsg2, have been associated with ARVC, but the mechanisms by which these mutations contribute to disease pathogenesis are poorly understood (Syrris et al., 2007; Bhuiyan et al., 2009; Christensen et al., 2010; Tan et al., 2010; Bauce et al., 2011).

Here, three pathogenic mutations in the Dsg2 DUR region (Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy Genetic Variants Database) were generated and examined for their ability to mediate tail–tail interactions. A mutation in DUR associated with human disease disrupts the Dsg2 tail-tail interaction and leads to rapid endocytosis in cardiac cells.

A regulated homodimerization system was designed based on the human FKBP (FK506-binding protein) 12 and its ligand (ARIAD Pharmaceuticals). One copy of a variant of FK506-binding protein (FKBP36v) followed by a HA epitope tag was fused to the C terminus of Dsg2. The fusion protein (Dsg2.ICS-Fv1E) was then expressed in SCC68 cells. Dimerization of Dsg2.ICS-Fv1E was induced by treating cells with AP20187, a cell-permeable bivalent compound (Fig. 6 A). The biotinylation assay was conducted to compare the internalization efficiency of Dsg2.ICS-Fv1E in the presence of AP20187 or ethanol (control). A decrease in internalization was observed when cells were treated with AP20187, supporting the idea that Dsg2 tail–tail interaction inhibits its endocytic internalization (Fig. 6 B).
Discussion
The desmosomal cadherins contain specialized structural features that set them apart from classical cadherins. How these motifs contribute to their adhesive functions or recently described roles in cell proliferation, apoptosis, differentiation, and virus infection (Eshkind et al., 2002; Brennan et al., 2007; Nava et al., 2007; Getsios et al., 2009; Wang et al., 2011) is not well understood. Here, we interrogate the function of the DUR, which was first identified in Dsg1 ~20 yr ago (Koch et al., 1990). We show here that the DUR strengthens cell–cell adhesion, mediates cis-interactions between Dsg2 tails, and provides a brake on cadherin internalization (Fig. 8).

The total amount and organization of cell surface cadherins determines the adhesive strength they can provide. Regulation of cadherin trafficking through exo- and endocytosis is a critical determinant controlling cadherin functions, especially in dynamic, developing tissues and during tissue remodeling, including cancer invasion (Bryant and Stow, 2004; Green et al., 2010; Neirasova et al., 2011). Here, we investigated the endocytosis of Dsg2 in epithelial cancer cell lines and demonstrate that the unique C-terminal region, or DUR, inhibits internalization. This inhibitory role is at least in part an intrinsic attribute of the DUR that does not depend on the presence of adhesive ectodomain interactions or desmosomes, as it occurs even in low calcium media that does not support assembly of intercellular junctions. Lipid raft and dynamin were reported to be involved in the endocytosis of some classical cadherins (Akhtar and Hotchin, 2001; Paterson et al., 2003; Taulet et al., 2009). Dsg2 shows the same dependence on these two factors. Interestingly, independent of DUR status, all Dsg2 constructs tested share the same endocytic features as endogenous Dsg2: they all internalize through a cholesterol- and dynamin-dependent pathway. This suggests that DUR is not rerouting Dsg2 but is in some way inhibiting entry into common pathways.

One way to achieve this inhibition would be to sequester Dsg2 in a complex that has less access to endocytic machinery. Consistent with this idea, the presence of the DUR region alters Dsg2 complex profiles. The DUR may control the formation of Dsg2 complexes by affecting its (a) posttranslational modifications, (b) binding partners, or (c) oligomeric status. Although these outcomes are not mutually exclusive, we show here using a combination of yeast two-hybrid analysis and in situ proximity ligation that the DUR can mediate Dsg2 tail–tail interaction. It has been suggested that the disordered, flexible structure of DUR may provide an interaction surface for other binding partners or itself (Rutman et al., 1994; Kami et al., 2009). Our studies provide direct evidence that such interactions between Dsg2 tails occurs in cells, although we were unable to determine the exact oligomeric status (cis-dimer or higher order of oligomers) of Dsg2. Although the DUR was not absolutely required for targeting Dsg2 to desmosomes, DUR-dependent tail–tail interactions may promote clustering with other components of the membrane scaffolding and possibly strengthen association with the intermediate filament network. In the present work, we showed that induced dimerization of Dsg2 tails missing the DUR is sufficient to inhibit its internalization. However, it is likely that in the context of FL Dsg2, other DUR functions also contribute to Dsg2 dynamic behavior. Our work suggests that tail–tail interaction is one mechanism the DUR utilizes to inhibit Dsg2 internalization.

interactions based on yeast CytoTrap assay (Fig. 7A). Human Dsg2.FL (WT) and the V977fsX1016 mutant were transiently expressed in mouse HL-1 cardiac cells (Claycomb et al., 1998). Although the expression level of the V977fsX1006 was lower than the Dsg2.FL (WT) control, other desmosomal protein levels were not affected (Fig. 7B). By immunofluorescence, V977fsX1006 showed prominent perinuclear staining but little cell membrane staining, suggesting impaired delivery and/or increased internalization of the mutant protein (Fig. 7C). To test whether alterations in internalization behavior contribute to this difference in distribution, we conducted an antibody internalization assay. The ratio of the internalized to cell surface fluorescence was over twofold greater for V977fsX1006, suggesting that it is more efficiently internalized from the cell surface (Fig. 7D). These data raise the possibility that increased turnover of Dsg2, possibly leading to impaired stability and function of the cardiac intercalated disc, could contribute to ARVC disease pathogenesis.
Our data raise the possibility that modulation or disruption of the DUR-mediated tail–tail interaction could target Dsg2 for internalization during normal tissue remodeling or disease pathogenesis. Consistent with this hypothesis, a pathogenic ARVC variant unable to mediate Dsg2 tail–tail interactions was observed to undergo rapid endocytosis in cardiac HL-1 cells. The abnormal behavior of this variant could destabilize cardiac intercalated discs and possibly contribute to the observed arrhythmias and sudden death that can occur in patients with this poorly understood disorder.

Another possible modulator of DUR function is proteolytic cleavage. Dsg1 is cleaved by caspase-3 at D888 in RUDII in response to UV-induced apoptosis (Dusek et al., 2006). In addition, a prominent 130-kD Dsg2 form containing the ectodomain, but lacking the repeat unit domain or Dsg terminal domain was detected in human colonic mucosa (Kolegraff et al., 2011). In either case, a DUR-lacking Dsg may behave differently from FL Dsg because of altered clustering and internalization behavior.

Our observations demonstrate for the first time that the Dsg2 DUR mediates intermolecular interactions between the Dsg2 cytoplasmic domains and links this property to inhibition of Dsg2 internalization. This discovery could have important implications for both normal tissue remodeling and homeostasis as well as disease pathogenesis, including cancer and inherited diseases of the desmosome.

Materials and methods

Cell culture
The human-derived squamous cell carcinoma cell lines SCC68 and SCC9 were gifts from J. Rheinwald (Harvard Medical School, Boston, MA). SCC68 cells were maintained in keratinocyte serum-free media (Invitrogen) supplemented with 0.09 mM Ca²⁺, 0.2% bovine pituitary extract, and 0.3 ng/ml EGF. SCC9 cells were maintained in DMEM/F-12 media supplemented with 10% FBS. HL-1 cells were a gift from W. Claycomb (Louisiana State University School of Medicine, New Orleans, LA). HL-1 cells were grown in Claycomb medium containing 10% FBS, penicillin/streptomycin at 100 U/ml and 100 µg/ml, respectively, 0.1 mM norepinephrine, and 2 mM L-glutamine. For HL-1 cells, cell culture dishes/cover slips were precoated with a solution of 0.5% fibronectin and 0.2% gelatin from 3 h to overnight at 37°C.

Generation of cDNA constructs
To generate the truncated Dsg2 constructs, PCR amplifications were performed on Dsg2-Myc (p685; available from GenBank under accession no.
Confluent cells were rinsed with PBS twice and then incubated with 2.4 U/mL dispase in PBS at 37°C for 10–20 times until the cell sheets broke apart into fragments. The fragments were then transferred into a 35-mm dish and imaged with a dissecting scope (MZ6; Leica) and MetaVue imaging software (Molecular Devices).

**Biotinylation assay**

Three P60 dishes were prepared for each construct to determine the total surface labeling, internalized pool of Dsg2 at 30 min, and a control for stripping efficiency (0 min). All three coverslips were incubated at 4°C for 1 h with a specific antibody (anti-DUR antibody at 1:100; 6DB at 1:200) in either keratinocyte serum-free medium containing 3% BSA and 20 mM Heps (for SCC68 cells) or Claycomb medium with 20 mM Hepes (for HL-1 cells). Unbound antibody was removed by washing with cold PBS three times. Surface-labeled and 0-min coverslips were kept at 4°C, whereas the 30-min coverslip was transferred to 37°C for 30 min in prewarmed culture media. After returning to 4°C, the 30-min coverslip was washed with PBS three times. To strip the residual from the cell surface, the 30- and 0-min coverslips were washed with acid solution (0.5 mM NaCl and 0.5 mM acetic acid) four times for 15 min and then with PBS three times for 10 min. All coverslips were then fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked with 2% normal goat serum for 30 min at 4°C, and then incubated at 4°C overnight. After that, the beads were washed with RIPA buffer four times and analyzed by quantitative western blot analysis. The DUR inhibiting Dsg2 internalization by promoting Dsg2 tail-tail interaction, which stabilizes Dsg2 at the cell surface by inhibiting its internalization. When DUR-DUR interaction is disrupted (e.g., by mutation or deletion of the DUR), Dsg2 is more rapidly internalized from the cell surface. Dsg2 undergoes cholesterol- and dynamin-dependent endocytosis, independent of the status of DUR. The released cell sheets were transferred to a polyallomer tube (Beckman Coulter) by layering different concentrations of sucrose solutions (prepared in 0.5x USB; 1.5 mL of 15% sucrose solution was layered at the bottom, and 0.9 mL of 13, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.5 mL of 10%, 5%, and 2.5% sucrose solutions).
and 4% sucrose solutions were successively layered upon one another with ~800 µl cell lysate placed on the very top. The sucrose gradient was then centrifuged in a swinging bucket rotor (SW 40 Ti; Beckman Coulter) at 34,400 rpm [mean relative centrifugal force of 150,000 g; acceleration profile of 1 and deceleration profile of 0] for 18 h at 20°C. A total of 11 or 12 0.9-1.0 ml fractions were collected from the bottom of the tube. Fractions were reduced by incubating samples with 5% β-mercaptoethanol at 37°C for 1 h before performing immunoblotting.

In situ PLA

The experiment was performed using a CytoTrap vector kit (Agilent Technologies) and procedures were executed following the manufacturer’s recom-

tion reagent red. Fluorescence signals were visualized, and images were taken using a microscope (DSM; Leica) fitted with a 40x oil objective (Plan Fluorat, NA 1.0). Signal intensity was quantified using MetaMorph 7.0 imaging software.

Immunoprecipitation and immunofluorescence

The FLAG immunoprecipitation was performed by lysing cells in RIPA buffer (10 mM Tris, pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 5 mM EDTA, and 2 mM EGTA) supplemented with EDTA-free protease inhibitor (Roche). Lysates were centrifuged at 30,000 rpm at 4°C for 30 min. Supernatants were incubated with 30-40 µl anti-FLAG M2 affinity gel (Sigma-Aldrich) at 4°C for 2 h. The immune complexes were then washed with rebuffering Laemml buffer at 95°C and further analyzed by Western blotting.

Immunofluorescence was performed as previously described (Klessner et al., 2009). Samples were mounted in Gelvatol medium and imaged at room temperature. Wide-field images were taken using a microscope (DMR; 40 or 63× objective lens, NA 1.0, Plan Fluorat and NA 1.32, Plan-Apochromat, respectively) and a digital camera (ORCA-100 model C4742-95; Hamamatsu Photonics). The images were then processed using MetaMorph software. Subdiffraction resolution images were taken using a structured illumination super-resolution microscope (N-SIM; Nikon; Gustafsson, 2000). For N-SIM analysis, the samples were illuminated with spatially high-frequency patterned excitation light [100x objective lens, NA 1.49; TiE N-SIM microscope [Nikon] and iXon X3 897 camera [Andor Technology]]. The moiré patterns were produced and analytically processed (Elements version 4 software; Nikon) to reconstruct the subresolution structures of the samples.

Antibodies and inhibitors

The following antibodies were used in this study: anti-FLAG (anti-FLAG; Sigma-Aldrich), 608 [anti-Dsg2; gift from J. Wahl, III, University of Nebraska Medical Center, Omaha, NE], anti-IL2Rα (anti-IL2Rα; Santa Cruz Biotechnology, Inc.), HECD1 (anti–E-cadherin; Abcam), C2206 (anti–β-catenin; C2206; Sigma-Aldrich), Ab12 (anti–EGF receptor; Thermo Fisher Scientific), E7 (anti–β-tubulin; University of Iowa Developmental Studies Hybridoma Bank), 1407 (anti-PI3; Aves Laboratories), P114, anti-DF (Angst et al., 1990), GAPDH (anti-GAPDH; G9545; Sigma-Aldrich), 1903 (anti-Dsg3; gift from J. Stanley, University of Pennsylvania, Philadelphia, PA), 6013 (antiFu2, Progen Biotechnik), C4v1 (anti-C4v1, BD), 3F10 (anti-HA; Santa Cruz Biotechnology, Inc.), HA.11 clone 16b12 (anti-HA; Covance), 7G6 and 23E3 (anti-Dsc2 and anti-Pkp3, respectively; provided by F. van Roy, University of Ghent, Ghent, Belgium), 4B2, and the anti-Dsg2 cytodomain (Dusek et al., 2006).

Peroxidase-conjugated secondary antibodies used for Western blot analysis included goat anti–mouse, goat anti–rabbit, and goat anti–chicken (Rockland). Fluorophore-conjugated secondary antibodies used for immuno-

fluorescence included goat anti–mouse and goat anti–rabbit (Alexa Fluor; Invitrogen), Cylcoatrimide (EMD), dyesore (Sigma-Aldrich), methyl–
β-cyclodextrin (Sigma-Aldrich), diethylhexyl-(succinimidyl-propionate), and AP20187 [ARIAD Pharmaceuticals] were used at concentrations of 10 µg/ml, 80 µM, 5 mM, 20 µg/ml, and 10 nM, respectively.

Online supplemental material

Fig. S1 shows that Dsg2 mutants can be delivered to cell–cell borders, and the expression level of most of the junctional proteins remains unchanged. Fig. S2 shows that cholesterol and dynamin promote the internalization of endogenous Dsg2, whereas Cav1 has no effect on the internalization of either endogenous Dsg2 or Dsg2 mutants. Fig. S3 shows that the internalization of endogenous Dsg2 remains unchanged when the function of flotil-

lin-1 and clathrin heavy chain is disrupted. Fig. S4 shows that DUR promotes Pg association with Dsg2, but disruption of the Pg–Dsg2 interaction has no effect on Dsg2 internalization. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.2011202105/DC1.

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