

Multiple post-translational modifications regulate E-cadherin transport during apoptosis

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Summary

E-cadherin is synthesized as a precursor and then undergoes cleavage by proprotein convertases. This processing is essential for E-cadherin maturation and cell adhesion. Loss of cell adhesion causes detachment-induced apoptosis, which is called anoikis. Anoikis can be inhibited despite loss of cell–matrix interactions by preserving E-cadherin-mediated cell–cell adhesion. Conversely, acute loss of E-cadherin sensitizes cells to apoptosis by unknown post-translational mechanisms. After treatment of breast cancer cells with drugs, we found that two independent modifications of E-cadherin inhibit its cell surface transport. First, O-linked β -N-acetylglucosamine (O-GlcNAc) modification of the cytoplasmic domain retains E-cadherin in the endoplasmic reticulum. Second, incomplete processing by proprotein convertases arrests E-cadherin transport late in the secretory pathway. We demonstrated these E-cadherin modifications (detected by specific lectins and antibodies) do not affect binding to α -catenin, β -catenin or γ -catenin. However, binding of E-cadherin to Type I gamma phosphatidylinositol phosphate kinase (PIPKI γ), a protein required for recruitment of E-cadherin to adhesion sites, was blocked by O-GlcNAc glycosylation (O-GlcNAcylation). Consequently, E-cadherin trafficking to the plasma membrane was inhibited. However, deletion mutants that cannot be O-GlcNAcylated continued to bind PIPKI γ , trafficked to the cell surface and delayed apoptosis, confirming the biological significance of the modifications and PIPKI γ binding. Thus, O-GlcNAcylation of E-cadherin accelerates apoptosis. Furthermore, cell-stress-induced inactivation of proprotein convertases, inhibited E-cadherin maturation, further exacerbating apoptosis. The modifications of E-cadherin by O-GlcNAcylation and lack of pro-region processing represent novel mechanisms for rapid regulation of cell surface transport of E-cadherin in response to intoxication.

Key words: E-cadherin, Modifications, O-linked β -N-acetylglucosamine, Transport, Type I gamma phosphatidylinositol phosphate kinase

Introduction

Epithelial cell–cell adhesion determines tissue organization, and loss of cell adhesion activates anoikis (Yap et al., 2007; Grossmann, 2002). An important component of this process is E-cadherin, a single-membrane-spanning protein with a conserved cytoplasmic domain and divergent extracellular regions composed of five cadherin domains separated by interdomain Ca^{2+} binding sites (Gumbiner, 2000). Cadherins mediate cell–cell adhesion through interactions between N-terminal cadherin domains on opposing cell surfaces and by organization of the actin cytoskeleton through α -catenin (Nelson, 2008).

Assembly of E-cadherin-based adherens junctions (AJs) is obligatory for establishment of polarized epithelia. Rab11 (Lock and Stow, 2005), p120-catenin (Peifer and Yap, 2003), tyrosine phosphorylation (Behrens et al., 1993) and ubiquitylation (D'Souza-Schorey, 2005) control the trafficking and assembly of E-cadherin in mammalian cells. Recent studies have shown that type I gamma phosphatidylinositol phosphate kinase (PIPKI γ) acts as a signaling scaffold that links an adaptor protein complex to E-cadherin (Ling et al., 2002; Ling et al., 2007). The depletion of PIPKI γ or disruption of PIPKI γ binding to either E-cadherin or adaptor protein complexes results in defects in E-cadherin transport and blocks adherens junction formation (Ling et al., 2007).

E-cadherin is synthesized as a 140 kDa precursor that undergoes cleavage by proprotein convertase family proteins associated with the trans-Golgi network (TGN) that remove an N-terminal pro-region (Posthaus et al., 1998; Beavon, 2000). Furin, a member of the proprotein convertase family, efficiently cleaves the E-cadherin precursor (Posthaus et al., 1998). This processing is essential for correct folding of the extracellular portion of the mature molecule and is required to mediate the homotypic calcium-dependent binding of E-cadherin extracellular domains (Beavon, 2000; Ozawa and Kemler, 1990).

We previously reported that the cytoplasmic domain of E-cadherin is modified by the addition of O-linked β -N-acetylglucosamine (O-GlcNAc) after treatment of cells with thapsigargin (TG), an irreversible inhibitor of the sarcoplasmic or endoplasmic reticulum calcium ATPase (Zhu et al., 2001; Lytton et al., 1991; Denmeade and Isaacs, 2005). In TG-treated cells, the apparent molecular weight of E-cadherin increased by 20 kDa, a molecular mass change that is unlikely to be due solely to the addition of O-GlcNAc to serine and threonine residues (Zhu et al., 2001; Vosseller et al., 2001). The nature and functional significance of this other modification are unknown.

We present evidence here that cytoplasmic O-GlcNAcylation blocks exit of E-cadherin precursors from the ER and interferes with PIPKI γ binding. We identify the second modification as loss

of pro-region processing that presumably results from inhibition of proprotein convertases and show that this is regulated separately from O-GlcNAcylation. Loss of plasma membrane localization of E-cadherin accelerates the induction of apoptosis, because prevention of O-GlcNAcylation by deletion mutagenesis restored PIPKI γ binding, E-cadherin trafficking, and attenuated apoptosis. Our data suggest that in response to some agonists, both independent post-translational modifications contribute to the rapid induction of apoptosis by inhibiting the assembly of E-cadherin complexes on the plasma membrane.

Results

E-cadherin is independently modified by O-GlcNAcylation and pro-region retention

We previously reported that in the human breast cancer cell line MCF-7 and Madin-Darby Canine Kidney (MDCK) epithelial cells, exposure to TG, an agent that causes ER stress by depleting intraluminal Ca²⁺, resulted in the addition of O-GlcNAc to the

cytoplasmic domain of E-cadherin. O-GlcNAcylation was assayed as increased binding of the modified E-cadherin to the GlcNAc-binding lectin wheat germ agglutinin (WGA) that was abolished by treatment with β -hexosaminidase (Zhu et al., 2001). This O-GlcNAcylation pattern is not bound by two monoclonal antibodies (110.6 and RL2) that recognize specific patterns of O-GlcNAcylation (data not shown). Furthermore, in TG-treated cells, there is a 20 kDa change in electrophoretic mobility of E-cadherin (Fig. 1A, lane 3) suggesting that additional modification(s) might occur when cells are treated with TG (Zhu et al., 2001).

To determine whether E-cadherin modifications are unique to TG-treated cells, we tested a variety of other agents that cause cell stress, including tunicamycin (TN), dithiothreitol (DTT), brefeldin A (BFA) and ceramide (CER). The addition of TG, BFA and CER, but not TN, resulted in the appearance of 140 kDa E-cadherin (Fig. 1B, asterisk), indicating that this modification is common but not an obligate result of ER

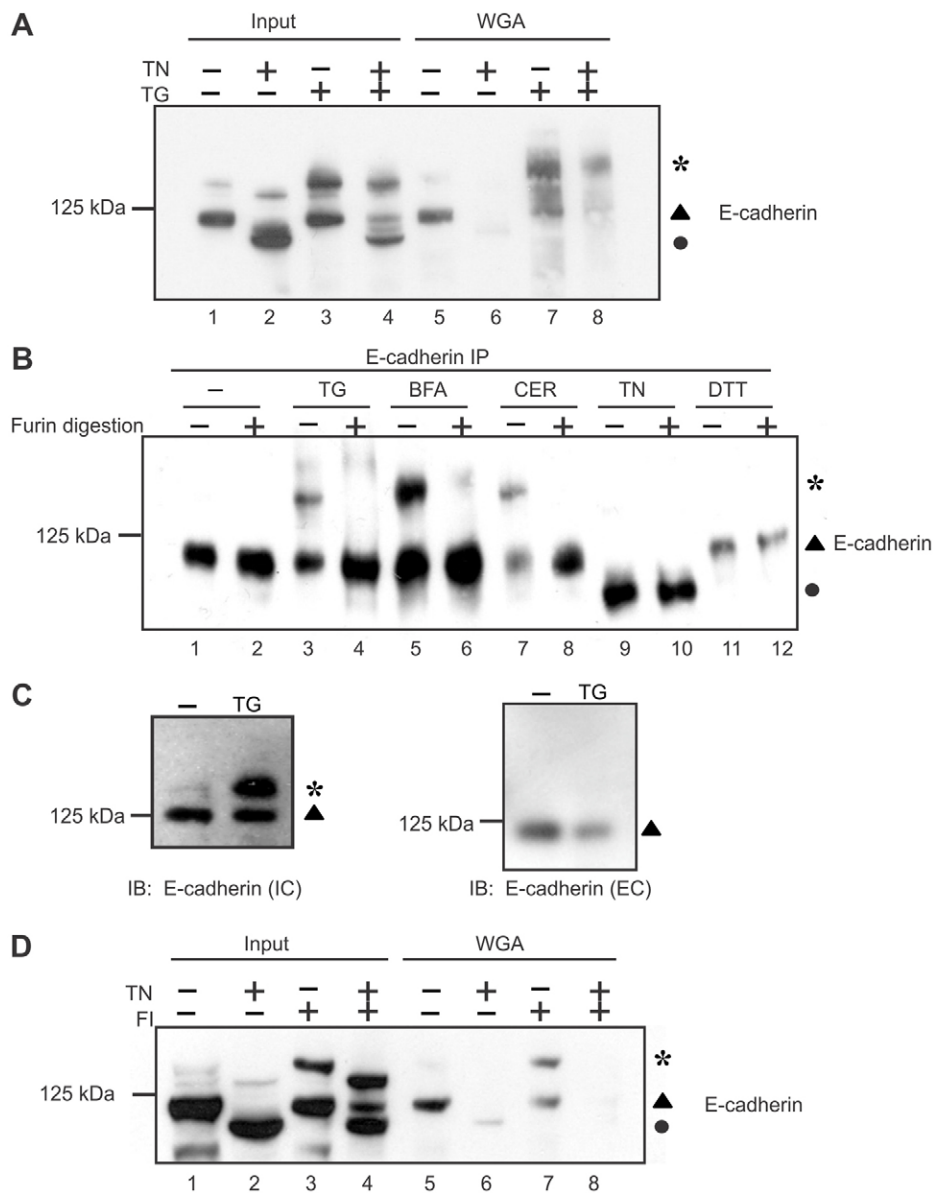


Fig. 1. E-cadherin undergoes two independent post-translational modifications in stressed cells. (A) Lysates prepared from MCF-7 cells treated with DMSO control (-), 10 μ g/ml TN, 400 nM TG, or both for 24 hours were analyzed by E-cadherin immunoblotting directly or after WGA binding. In all blots ▲ indicates mature E-cadherin, * indicates modified E-cadherin and ● indicates E-cadherin with N-glycosylation prevented by TN. (B) Lysates prepared from MCF-7 cells 400 nM TG for 24 hours, 10 μ g/ml BFA for 24 hours, 100 μ M CER for 8 hours, 10 μ g/ml TN for 48 hours, 10 mM DTT for 6 hours respectively were immunoprecipitated (IP) with E-cadherin antibody, subjected to furin digestion, then TCA precipitated and analyzed by immunoblotting for E-cadherin. These drug treatment conditions apply to all figures unless otherwise specified. (C) MCF-7 cells without or with TG treatment were analyzed by immunoblotting with an E-cadherin antibody raised against an epitope in the cytoplasmic domain (IC; unless otherwise specified, all subsequent E-cadherin immunoblots were probed with this antibody) or E-cadherin antibody SHE78-7 (EC), which recognizes an epitope in the extracellular domain of mature E-cadherin. (D) Lysates prepared from MCF-7 cells treated with DMSO control (-), TN, 200 μ M furin inhibitor (FI) or both for 24 hours were immunoblotted for E-cadherin either directly (Input) or after WGA binding (WGA). FI treatment condition applies to all figures.

stress or apoptosis (supplementary material Fig. S1A). O-GlcNAcylation of E-cadherin was examined by binding to WGA a lectin that binds with high affinity to O-GlcNAc, but with low affinity also binds to N-linked glycans (Natsuka et al., 2005). To permit unambiguous identification of O-GlcNAc-modified proteins, N-linked glycan formation was inhibited by treating the cells with TN before the glycoproteins were isolated by binding to WGA agarose. As expected, treatment with TN prevented N-linked glycosylation of E-cadherin and therefore decreased the molecular mass of the mature protein (Fig. 1A, lane 2, •). Although TN treatment abolished the binding of 120 kDa E-cadherin (Fig. 1A, lane 2, ▲) to WGA, a substantial fraction of 140 kDa E-cadherin still bound to WGA (Fig. 1A, lane 7 and 8, *), indicating that either 140 kDa E-cadherin is a preferential substrate for O-GlcNAcylation or that O-GlcNAcylation leads to the increase in apparent molecular mass. Treatment of cells with CER yielded the 140 kDa E-cadherin without O-GlcNAcylation, confirming that most of the apparent increase in molecular mass is due to a second independent post-translational modification. Exposure to BFA gave rise to both modifications, as did exposure to TG (supplementary material Fig. S1D,E).

The apparent molecular mass of the modified E-cadherin (140 kDa) is similar to the molecular mass of an E-cadherin precursor that contains the pro-region normally removed by proprotein convertases in the trans-Golgi. Therefore, we probed immunoblots with a monoclonal antibody (SHE78-7, EC) that recognizes an epitope created by processing E-cadherin to the mature form (Laur et al., 2002). Because EC bound the 120 kDa but did not bind to 140 kDa form of E-cadherin induced by both TG (Fig. 1C) and BFA (supplementary material Fig. S1E), we conclude that the latter does not contain the epitope characteristic of the mature protein. Furthermore, after immunoprecipitation (IP) of the 140 kDa E-cadherin induced by TG, BFA and CER, incubation with the proprotein convertase furin yielded a product with the same ~120 kDa electrophoretic mobility as mature E-cadherin (Fig. 1B). Therefore, we conclude that the 140 kDa form of E-cadherin is the pro form of the protein. Because CER does not lead to O-GlcNAcylation of E-cadherin, the common effect of these three agents is to inhibit the activity of a furin-like protease in cells.

To determine the relationship between inhibition of E-cadherin pro-region cleavage and O-GlcNAcylation, MCF-7 cells were treated with furin inhibitor (FI) and E-cadherin alterations and the cellular response were analyzed (Hallenberger et al., 1992). In contrast to TG, FI did not cause ER stress or apoptosis (supplementary material Fig. S2A, top two panels), but it inhibited E-cadherin processing in cells, because the 140 kDa form of E-cadherin was evident (Fig. 1D, lane 3). As expected for N-glycosylated proteins, both forms of E-cadherin bound to WGA (Fig. 1D, lane 7). However, in FI-treated cells, binding of

both species to WGA was abrogated by TN treatment (Fig. 1D, lane 8), indicating that the 140 kDa E-cadherin that resulted from furin inhibition was not O-GlcNAcylation. Thus retention of the E-cadherin pro-region is regulated independently of O-GlcNAcylation.

O-GlcNAcylation prevents E-cadherin trafficking by inhibiting export from the ER

We examined the effect of the separate modifications on E-cadherin trafficking by exploiting the differential sensitivity of N-linked glycosylation to N-glycosidase F (PNGase F) and Endoglycosidase H (Endo H) digestion (Maley et al., 1989). Endo H removes the mannose-rich sugars added in the ER, but does not hydrolyze complex type N-glycosylation that occurs in the cis to medial Golgi cisternae. PNGase F hydrolyzes both types of N-glycosylation (Maley et al., 1989). Thus Endo H resistance with PNGase F sensitivity reflects export from the ER and transit through the cis-Golgi, whereas sensitivity to both glycosidases indicates retention in the ER (Spellman et al., 1990).

As expected, after treatment with either TG or FI, both the processed and pro-region forms of E-cadherin were sensitive to PNGase F (supplementary material Fig. S2B). This indicates that all of the E-cadherin species were N-glycosylated. In addition, the bulk of the E-cadherin from untreated cells (a control for authentic transport to the plasma membrane) was resistant to Endo H digestion but sensitive to PNGase F, indicative of efficient export from the ER (Fig. 2, lanes 1–3). In FI-treated cells, pro-E-cadherin was also resistant to Endo H digestion and sensitive to PNGase F digestion, indicating that this protein also exits the ER (Fig. 2, lanes 4–6) even though it does not transit to the cell surface (Ozawa and Kemler, 1990) (supplementary material Fig. S2A, bottom panel, lane 5). Thus, retention of the pro-region is not sufficient to abolish export from the ER as expected, because the pro-region is normally removed later in the secretory pathway. By contrast, in lysates from TG-treated cells, either Endo H or PNGase F digestion reduced the apparent molecular mass of the 140 kDa modified E-cadherin bands similarly (Fig. 2, lanes 7–9). Taken together with our previous report showing that TG treatment does not cause a general block in protein exit from the ER (Zhu et al., 2001), these data indicate that TG-induced O-GlcNAcylation selectively prevents pro-E-cadherin from exiting from the ER.

The polyserine (pSer) region is required for O-GlcNAcylation of E-cadherin

On cytoplasmic and nuclear proteins, as well as the cytoplasmic domains of some transmembrane proteins, O-GlcNAc can be attached through the hydroxyl group of serines or threonines (Wells and Hart, 2003). Studies on mutants of these target proteins that cannot be glycosylated have increased our

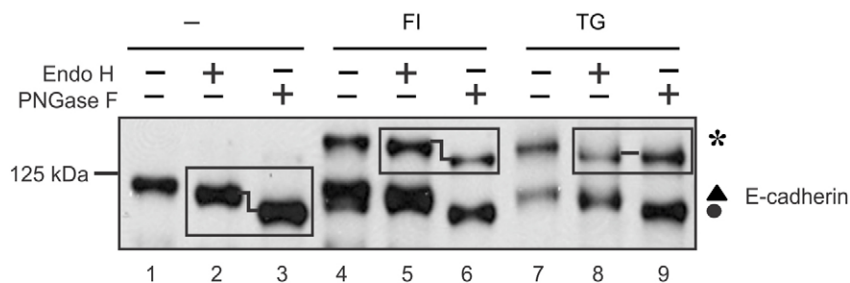


Fig. 2. E-cadherin O-GlcNAcylation inhibits its trafficking to cell surface by preventing E-cadherin exit from the ER. Lysates from MCF-7 cells treated with DMSO control (-), FI or TG were digested with Endo H or PNGase F and analyzed by immunoblotting for E-cadherin. The increased mobility after PNGase F digestion in untreated or FI-treated cells is indicated with a stepped line (lanes 2,3,5,6).

understanding of the biological function of O-GlcNAcylation (Comer and Hart, 2000). To map the region(s) of E-cadherin that are subject to O-GlcNAcylation, we generated a hemagglutinin (HA)-tagged E-cadherin with a deletion of the pSer region located in the cytoplasmic domain (deletion of amino acids 838–853, E-cad Δ pSer) because this sequence is a potential candidate for the multiple O-GlcNAcylation sites required to result in binding to WGA (Fig. 3A) (Vosseller et al., 2001). HA-tagged constructs of wild-type E-cadherin (E-cad WT) and a mutant with the entire cytoplasmic domain deleted (deletion of amino acids 728–882, E-cad Δ cyto) served as controls that can and cannot be O-GlcNAcylated, respectively.

To assess O-GlcNAcylation and pro-region retention, MCF-7 and MDCK cell clones stably expressing the proteins (supplementary material Fig. S3) were exposed to TG and/or TN and whole cell lysates or WGA precipitates were analyzed by immunoblotting as above (Fig. 3B). In response to TG treatment, the HA-tagged proteins migrated as higher molecular mass species consistent with pro-region retention, as seen with the endogenous E-cadherin (Fig. 3B, *). A fraction of the higher molecular mass E-cad WT protein was also O-GlcNAcylated in response to TG and TN and therefore bound to WGA (Fig. 3B, top panel lane 8). By contrast, no detectable E-cad Δ pSer bound to WGA after TG and TN treatment (Fig. 3B, bottom panel, lane 8) despite the fact that most of this protein also retained the pro-region (Fig. 3B, bottom panel, lane 4). The most likely explanation for loss of O-GlcNAcylation of E-cad Δ pSer is that the deleted region contains the serines that are modified. Alternatively, deletion of the pSer region might affect the folding and accessibility to O-GlcNAcylation at another site.

O-GlcNAc-modified E-cadherin does not bind PIPKI γ

The PIPKI γ -interacting region on E-cadherin overlaps with the pSer region required for O-GlcNAcylation (Ling et al., 2007). To determine whether deletion of the pSer region from E-cadherin interfered with PIPKI γ binding to E-cadherin, we examined complex formation in HEK293 cells co-transfected with plasmids encoding FLAG-tagged PIPKI γ and HA-tagged E-cad Δ pSer or HA-tagged E-cad WT (Fig. 4A, top panel and second panel). Immunoprecipitation with antibody against the HA tag followed by immunoblotting for FLAG on PIPKI γ revealed that PIPKI γ bound to E-cad Δ pSer and E-cad WT similarly (Fig. 4A, third panel).

To investigate the effect of O-GlcNAc modification of E-cadherin on the interaction between PIPKI γ and E-cadherin, MCF-7 cells stably expressing FLAG-tagged PIPKI γ were treated with TN and TG as above. In MCF-7 cells, the interaction between PIPKI γ and E-cadherin was not affected by TN (inhibited N-glycosylation) but was greatly decreased in response to TG (stimulated O-GlcNAcylation) (Fig. 4B, top panel, lanes 4–6). More importantly, precipitation with WGA revealed that PIPKI γ does not bind to O-GlcNAc modified E-cadherin (Fig. 4B, top panel, lane 10). Overexpression of PIPKI γ did not inhibit O-GlcNAcylation of E-cadherin because it still bound to WGA (Fig. 4B, middle panel, lane 10). Because O-GlcNAcylation disrupts the interaction between E-cadherin and PIPKI γ , we examined whether O-GlcNAcylation affects the interaction between E-cadherin and α -catenin as a ‘bridge’ to the cytoskeleton (Drees et al., 2005). We noted that α -catenin bound to total E-cadherin in the absence or presence of TG (Fig. 4B, bottom panel, lanes 4–6) and to O-GlcNAcylated E-cadherin as indicated by immunoblotting after WGA–Sepharose precipitation

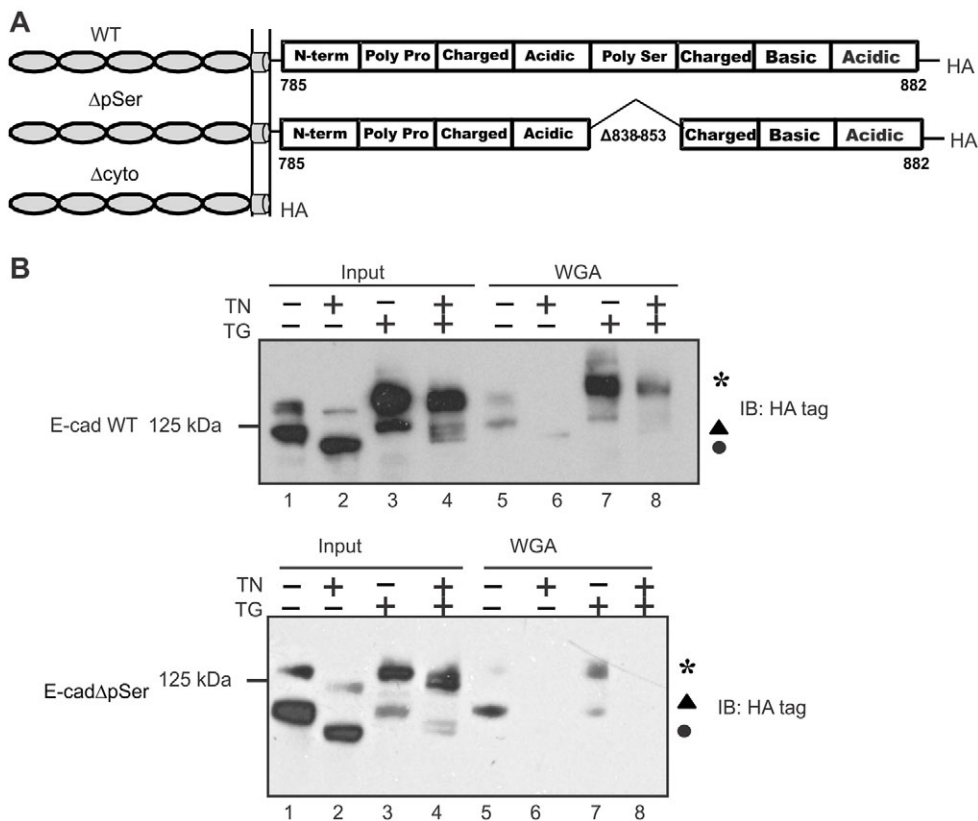


Fig. 3. The polyserine region in the E-cadherin cytoplasmic domain is required for TG-induced E-cadherin O-GlcNAcylation. (A) Defined regions of wild type E-cadherin cytoplasmic domain were demonstrated previously. The corresponding diagram for HA tagged E-cad WT and E-cad Δ pSer are indicated. (B) MCF-7 cells expressing HA tagged E-cad WT (top) and E-cad Δ pSer (bottom) were treated with DMSO control (–), TN, TG or both for 24 hours. Cell lysates were analyzed by immunoblotting for HA tag (Input) or by WGA binding followed by immunoblotting.

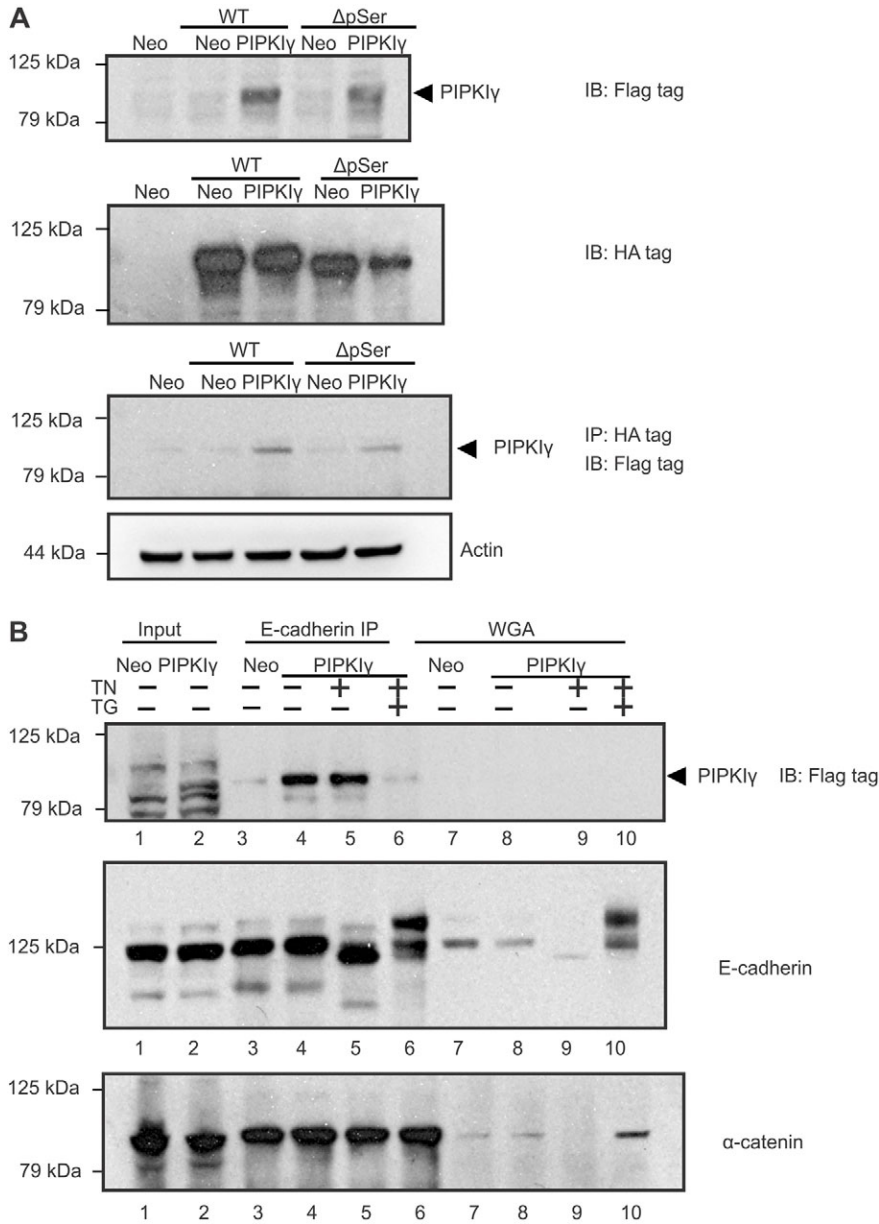


Fig. 4. The E-cadherin–PIPK1 γ interaction is intact after pSer region deletion but is disrupted by O-GlcNAcylation. (A) HEK293 cells were cotransfected with either HA tag empty vector (Neo), HA tagged E-cad WT or E-cad Δ pSer and either FLAG tag control vector (Neo) or FLAG-tagged PIPK1 γ . The expression of HA-tagged E-cadherin and FLAG-tagged PIPK1 γ was analyzed by immunoblotting for FLAG tag (top panel) and HA tag (second panel). The interaction between PIPK1 γ and E-cad WT or E-cad Δ pSer was analyzed by immunoprecipitation with anti-HA tag antibody and immunoblotting for the FLAG tag (third panel). Actin blot is shown as a loading control (bottom panel). (B) MCF-7 cells transfected with PIPK1 γ or control vector (Neo) were treated with DMSO control (–), TN, or TN plus TG for 24 hours. Total cell lysates (Input, lanes 1,2), immunoprecipitates with E-cadherin antibody (lanes 3–6) or WGA pull-down (lanes 7–10) were immunoblotted for FLAG tag (top), E-cadherin (middle) or α -catenin (bottom).

(Fig. 4B, bottom panel, lanes 9,10). We reported previously that treatment with TG did not affect binding of β -catenin or γ -catenin to E-cadherin (Zhu et al., 2001). Furthermore, deletion of the pSer region did not affect the E-cadherin– β -catenin interaction or E-cadherin– γ -catenin interaction (supplementary material Fig. S4), even though phosphorylation on serines in this region of E-cadherin has been reported to enhance the β -catenin binding affinity (Stappert and Kemler, 1994; Lickert et al., 2000). Taken together, these data suggest that O-GlcNAcylation selectively inhibits E-cadherin binding to PIPK1 γ .

Prevention of O-GlcNAcylation maintains transport of E-cadherin to the plasma membrane during cell stress

Because PIPK1 γ is essential for trafficking of E-cadherin to the plasma membrane, we examined the effect of the deletion mutants on the cell surface expression of E-cadherin after TG exposure. We detected surface proteins by biotinylation,

streptavidin precipitation and subsequent immunoblotting. After exposure of the cells to TG for 24 hours, HA-tagged full-length E-cadherin was not detected at the cell surface (Fig. 5A, compare lanes 1 and 2), suggesting a total block in cell surface transport. Under the same conditions, both E-cad Δ cyto and E-cad Δ pSer transport was reduced by TG treatment but the mature forms of the proteins continued to be detected on the plasma membrane (Fig. 5A). The decrease in intensity of the bands corresponding to E-cad Δ cyto and E-cad Δ pSer after treatment with TG is probably due to retention of the unprocessed (pro-region containing) forms of the proteins at a post-Golgi location in the secretory pathway as we noted for endogenous E-cadherin (Fig. 2) and consistent with our previous observation that transport of unprocessed form is arrested in the late secretory pathway (Zhu et al., 2001).

We also noted a separate mechanism whereby the E-cadherin mutants increased surface expression of E-cadherin. By using a monoclonal antibody against the cytoplasmic domain that does

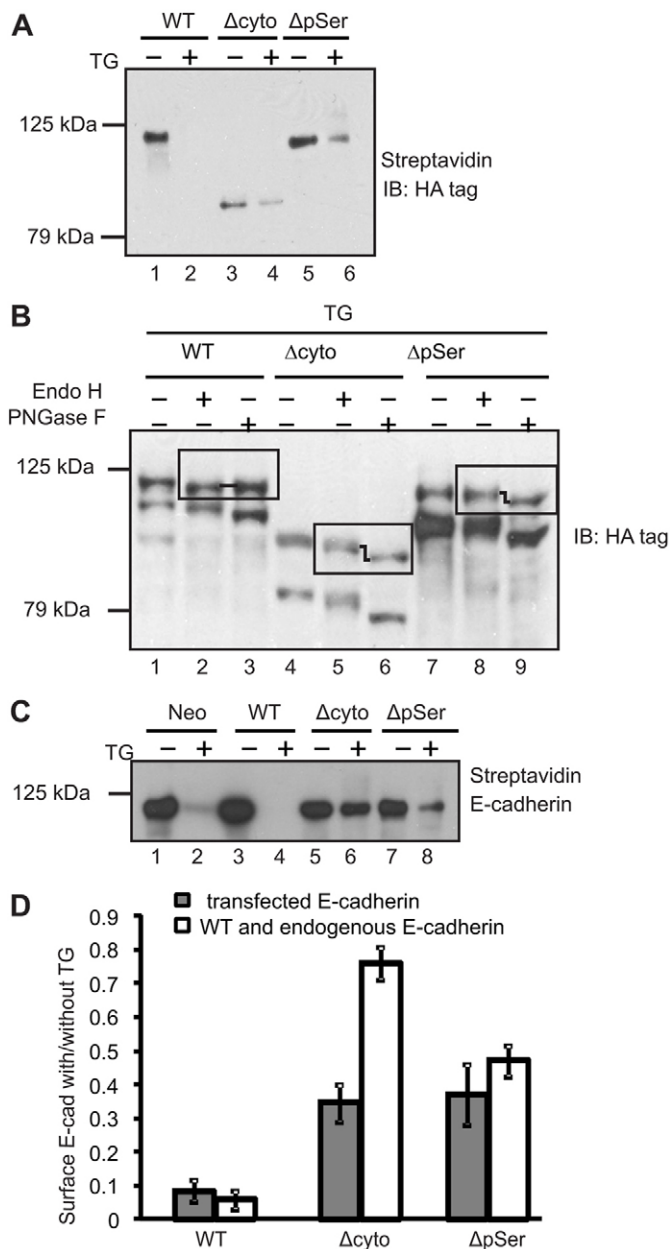


Fig. 5. Prevention of E-cadherin O-GlcNAcylation maintains both endogenous and transfected E-cadherin targeting to the plasma membrane. (A) The expression of transfected E-cadherin on the cell surface in MCF-7 cells expressing HA-tagged E-cad WT, E-cadΔcyto or E-cadΔpSer treated with DMSO control (-) or TG for 24 hours were examined by surface biotinylation and immunoblotting for HA tag. (B) Lysates from MCF-7 cells expressing HA-tagged E-cad WT, E-cadΔcyto and E-cadΔpSer treated with TG for 24 hours were digested with Endo H or PNGase F and analyzed by immunoblotting for the HA tag. (C) Cell surface E-cadherin expression in MCF-7 cells expressing HA tagged E-cad WT, E-cadΔcyto or E-cadΔpSer treated with DMSO control (-) or TG for 24 hours were examined by surface biotinylation and immunoblotting for E-cadherin with the antibody (IC) that recognizes cytoplasmic domain on endogenous E-cadherin and E-cad WT. (D) The relative proportion of transfected or endogenous E-cadherin remaining on cell membrane after TG treatment for 24 hours. The ratio was calculated based on the measurements of the intensity of the relevant bands (supplementary material Fig. S5C) shown in the immunoblots in A,C.

not recognize either deletion mutant (supplementary material Fig. S5A), any cell surface E-cadherin detected in biotin-labeled cells using this antibody represents endogenous or transfected wild-type protein. Remarkably, in TG-treated cells, expression of either E-cadΔcyto or E-cadΔpSer, but not WT, increased the amount of endogenous mature E-cadherin on the cell surface (Fig. 5C, compare lane 4 with lanes 6 and 8). Quantification of multiple blots revealed that this effect of increased cell surface expression of endogenous E-cadherin is particularly pronounced in cells expressing E-cadΔcyto (supplementary material Fig. S5C). Thus transfection of the non-glycosylatable mutants attenuated the decrease in total cell surface E-cadherin after TG treatment by two mechanisms: increased transport of the mutants themselves (Fig. 5D, dark bars), and by facilitating the transport or stabilization of endogenous E-cadherin 'in trans' (Fig. 5D, open bars).

To confirm these results, we also measured the amount of cell surface protein by immunofluorescence staining of non-permeabilized MCF-7 cells stably expressing either E-cad WT or E-cadΔpSer. E-cadherin was labeled with an antibody directed against the extracellular domain (EC) (detecting both WT and mutant E-cadherin equally) and visualized using a fluorescein-labeled secondary antibody (supplementary material Fig. S6A). Cell number was estimated by staining with Syto63 dye. The rate at which the green to red ratio decreases after TG treatment was used to estimate the rate at which E-cadherin disappears from the cell surface. Loss of cell surface E-cadherin was significantly greater in cells expressing exogenous HA-tagged E-cad WT compared with that observed in cells expressing E-cadΔpSer (supplementary material Fig. S6B, $P=0.047$ by two-way ANOVA), which is consistent with that observed with the biotin labeling and immunoblotting results summarized above. By comparison, BFA and CER, which cause O-glycosylation and/or pro-region retention, also prevent E-cadherin transport to the cell surface (supplementary material Fig. S7). This is not an invariant feature of cells undergoing programmed cell death because it is not observed in MCF-7 cells treated with TNF α .

To examine potential differences in exit from the ER during cell surface trafficking of E-cadherin and the deletion mutants, the sensitivity of N-linked glycosylation to PNGase F and Endo H digestion was determined. In untreated cells, most exogenous E-cad WT, E-cadΔcyto and E-cadΔpSer was PNGase F sensitive and Endo H resistant (supplementary material Fig. S5B), demonstrating that they exited the ER and proceeded to the Golgi. By contrast, after treatment with TG, the 140 kDa form of E-cad WT was sensitive to Endo H digestion, consistent with O-GlcNAcylation serving as an ER retention signal and thereby preventing processing of E-cadherin in the Golgi (Fig. 5B, lanes 1–3). However, the forms of both E-cadΔcyto and E-cadΔpSer containing the pro-region were Endo H resistant but PNGase F sensitive (Fig. 5B, lanes 4–6 and 7–9, respectively), indicating that the pro-region forms of the deletion mutants exited the ER. Thus O-GlcNAcylation, not pro-region retention, prevents trafficking from the ER to the Golgi.

Prevention of E-cadherin O-GlcNAcylation maintains E-cadherin function and delays apoptosis

To investigate the effect of O-GlcNAcylation of E-cadherin on cell death, MCF-7 cells expressing E-cad WT, E-cadΔpSer or E-cadΔcyto were treated with TG, and apoptosis was assessed by Poly(ADP-ribose) polymerase (PARP) cleavage and Annexin V

staining. PARP cleavage was delayed in cells expressing E-cad Δ cyto or E-cad Δ pSer at a time point at which PARP cleavage was almost complete and much of the cleaved product was further degraded in control cells (Fig. 6A). Delayed cell death in cells expressing the mutant forms of E-cadherin that were not O-GlcNAcylation substrates was confirmed by two-color flow cytometric analysis measuring Annexin-V-FITC and propidium iodide (PI) staining (Fig. 6B). In these experiments, TG treatment reduced the fraction of viable cells expressing E-cad WT to 38.7% (Annexin V⁻/PI⁻) whereas at the same time point 70.2% and 82.7% of the cells expressing E-cad Δ cyto or E-cad Δ pSer were viable, respectively (Fig. 6B). Thus, expression of either E-cad Δ cyto or E-cad Δ pSer substantially reduced TG-induced cell death. To confirm that these changes are not cell line or species specific, we examined the non-transformed, canine renal epithelial cell line MDCK expressing HA-tagged human E-cad WT and E-cad Δ cyto (supplementary material Fig. S3C). Consistent with the results in MCF-7 cells after treatment with TG, less PARP cleavage was observed for MDCK cells expressing human E-cad Δ cyto than for cells expressing E-cad WT.

To link the modification of E-cadherin with the effect on cell survival, we directly measured the adhesive activity of E-cad WT and E-cad Δ pSer in the presence or absence of TG treatment (Fig. 6C). After shaking in an incubator, the percentage of floating cells was 17% and 37% at 16 and 24 hours of TG treatment, respectively, in cells expressing WT E-cad, but only

5% and 11% in E-cad Δ pSer-expressing cells. Thus prevention of O-glycosylation maintains the cell adhesion function of E-cadherin. Furthermore, F-actin formation and cell polarity were maintained by preventing O-glycosylation during TG treatment (supplementary material Fig. S8).

Discussion

As a crucial component of epithelial junctional complex, E-cadherin is known to be post-translationally modified by phosphorylation and N-glycosylation (Liwosz et al., 2006; Gumbiner, 2005; Jeanes et al., 2008). As shown here, E-cadherin can also be modified by O-GlcNAcylation (Zhu et al., 2001) and retention of the pro-region. The latter effect is probably due to the inhibition of a luminal furin-like protease. Recent reports indicated that O-GlcNAc transferase (OGT) also has intrinsic proteolytic activity (Capotosti et al., 2011). Both enzymatic functions are required sequentially to activate the mitotic regulator, HCF-1. In our case, selected cellular stressors activate the transferase activity of OGT while repressing the activity of a separate protease, and these effects occur in distinct cellular compartments (cytoplasm and ER lumen, respectively). Both of these independently regulated changes have a profound effect on E-cadherin trafficking and on the susceptibility of cells to apoptosis.

O-GlcNAcylation of E-cadherin inhibits both the binding of PIPKI γ to E-cadherin (Fig. 4) and trafficking is blocked in the ER. As a result, the N-linked glycans on the extracellular domain

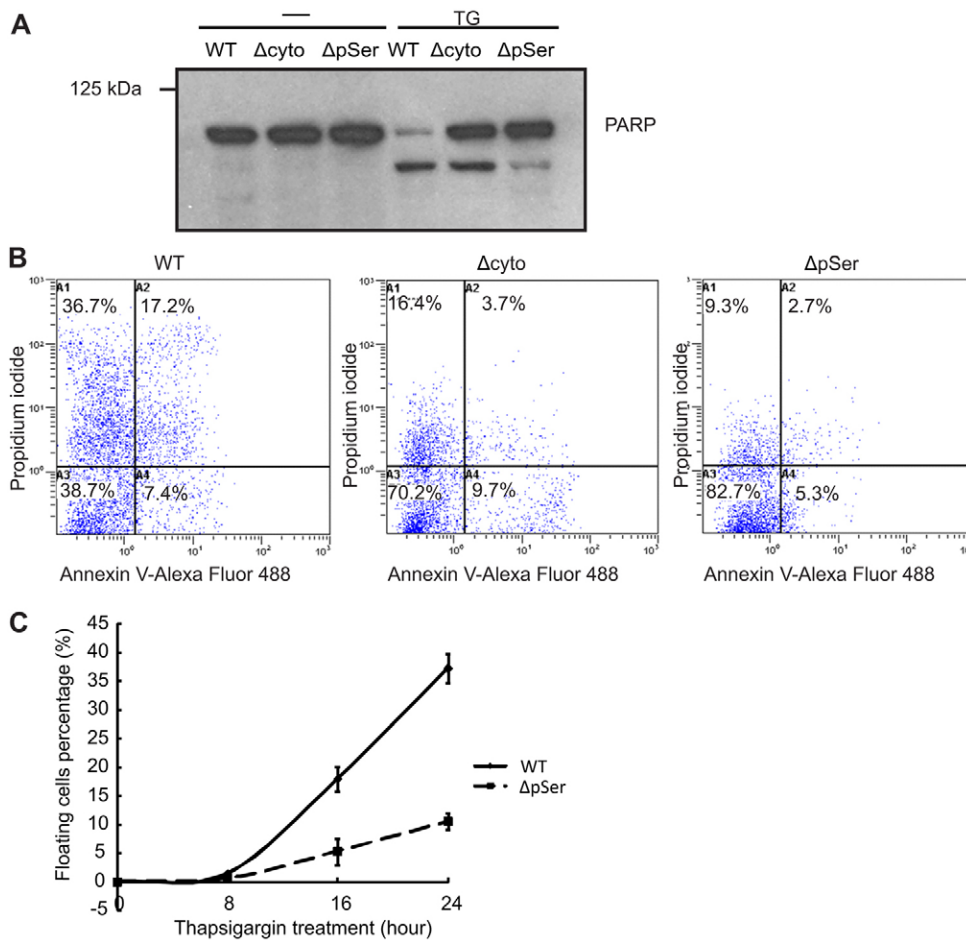


Fig. 6. Prevention of E-cadherin O-GlcNAcylation maintains E-cadherin function and protects cells from stress-induced apoptosis. (A) Lysates from MCF-7 cells expressing HA tagged E-cad WT, E-cad Δ cyto and E-cad Δ pSer that were either treated with DMSO control (-) or TG for 24 hours were analyzed for PARP cleavage by immunoblotting. (B) MCF-7 cells expressing HA-tagged E-cad WT, E-cad Δ cyto or E-cad Δ pSer were treated with TG for 24 hours, then stained with Annexin V coupled to Alexa Fluor 488 and PI and analyzed by flow cytometry. Data are shown for a single experiment representative of three independent experiments. (C) MCF-7 cells expressing E-cad WT and E-cad Δ pSer were treated with TG. After shaking at 500 r.p.m. on the Eppendorf Thermomixer inside the tissue culture incubator, non-adherent cells were counted at 0, 8, 16 and 24 hours.

of E-cadherin remain sensitive Endo H whereas the pro-region, which is normally removed by furin-like proprotein convertases in the TGN, is retained. Retention of E-cadherin in the ER reduced the total amount of the protein at the cell surface (Figs 2, 5) and exacerbated apoptosis. Consistent with this interpretation, expression of mutant E-cadherin which lacks the pSer region that we identified as necessary for O-GlcNAcylation (Fig. 3) prevented apoptosis induced by drugs that increased O-GlcNAcylation (Fig. 6). A schematic overview of these effects is shown in Fig. 7.

The O-GlcNAc modification has been found to regulate a number of cellular functions (Gambetta et al., 2009; Kreppel and Hart, 1999). However, the regulatory effects of O-GlcNAcylation on E-cadherin trafficking and PIPKI γ interaction have not been reported previously. Thus, our data represent the first report that O-glycosylation prevents the binding of a kinase to the glycosylated substrate – this is the opposite of the effect that O-glycosylation of keratin has on the binding of Akt (Ku et al., 2010). Moreover, little is understood about how O-GlcNAcylation is regulated in cells. Although we observed increased O-GlcNAcylation during certain forms of cell stress, it is likely that O-GlcNAcylation of E-cadherin also occurs in response to less drastic stimuli and therefore, contributes to other forms of regulation of intracellular adhesion. The E-cadherin–PIPKI γ –AP1B interaction serves as a foundational signal for exocytic targeting and basolateral sorting of E-cadherin (Ling et al., 2007). Furthermore, recent reports indicate that the association of increased PIPKI γ expression with invasiveness and poor prognosis in breast cancer. This effect might be mediated by PIPKI γ decreasing the binding of E-cadherin to

β -catenin, which in turn can become transcriptionally competent and activate cell proliferation and metastatic pathways (Sun et al., 2010). However, the simplest interpretation of our data demonstrating that O-GlcNAc modification disrupts PIPKI γ binding to E-cadherin (Fig. 4B) and prevents cell surface transport is that these events are interconnected. The fact that E-cad Δ pSer was not O-GlcNAc modified (Fig. 3B), continued to bind PIPKI γ (Fig. 4A) and transited to the plasma membrane (Fig. 5) strongly suggests that inhibition of E-cadherin transport in response to TG is due to O-GlcNAcylation of E-cadherin rather than some other effect of TG such as loss of ER Ca $^{2+}$. This also explains why during TG treatment, E-cad Δ pSer continued to target to the plasma membrane where it delayed apoptosis (Figs 5, 6). Although the results of the adhesion assay time course (Fig. 6C) and the measurement of apoptosis (Fig. 5, Fig. 6A,B) are not directly comparable, they suggest that loss of adhesion precedes Annexin V staining. However, it is difficult to assign a specific sequence because apoptosis is a cell-autonomous process, and clearly more work will be required to fully understand the kinetics of these events at the single-cell level.

Adhesion-dependent survival is a fundamental aspect of cell behavior (Gilmore et al., 2009). It is not clear why expression of mutants lacking the pSer region or the entire cytoplasmic domain of E-cadherin delayed apoptosis in response to stimuli that trigger O-GlcNAcylation of wild-type E-cadherin. However, both mutants continued to transit to the cell surface in drug-treated cells. It is possible that increased adhesion for these mutants is sufficient to delay apoptosis in MCF-7 cells. However, we have also noted that expression of the mutants allows more of the

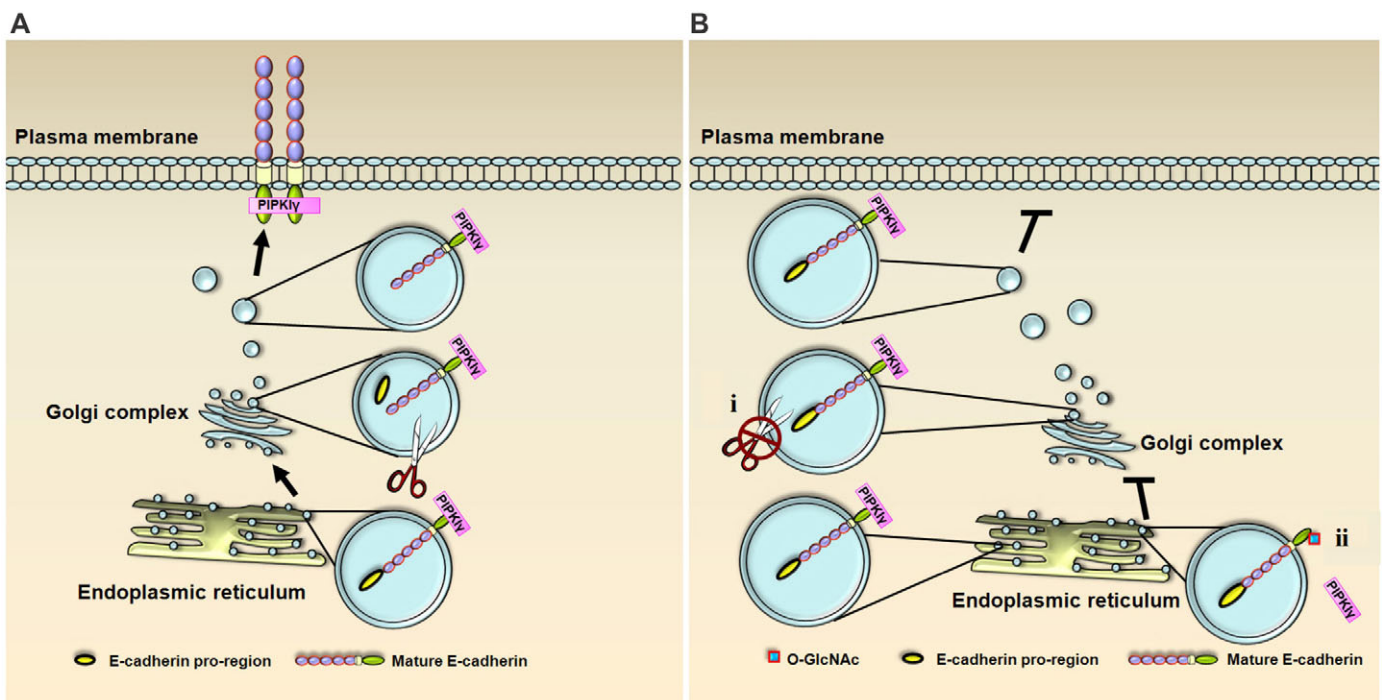


Fig. 7. Cellular stress induces multiple modifications of E-cadherin that prevent trafficking to the plasma membrane. (A) The E-cadherin trafficking pathway in healthy cells. (B) Cellular stress disrupts E-cadherin transport to the plasma membrane through two independent mechanisms: (i) inhibition of furin cleavage of the pro-region from E-cadherin in the TGN; pro-E-cadherin is not transported to the plasma membrane. (ii) O-GlcNAcylation of the cytoplasmic domain of E-cadherin prevents the binding of PIPKI γ , and the O-GlcNAcyated E-cadherin is retained in the endoplasmic reticulum. In this simplified diagram, signal peptide cleavage, other post-translational modifications and binding partners for E-cadherin are omitted.

processed endogenous WT E-cadherin to assemble, or remain on the plasma membrane. In the latter case, the survival signal might be mediated by signaling through the intact cytoplasmic tail of endogenous WT E-cadherin (possibly in mixed complexes), or by a combination of both increased adhesion and E-cadherin signaling mechanisms.

The endocytic recycling of cell surface E-cadherin is susceptible to oncogenic dysregulation that is mediated by multiple proteins, including the Cbl and Nedd4 ubiquitin ligases, which represent potential targets for novel cancer therapies (Mosesson et al., 2008). We have shown here that the initial trafficking and assembly of the plasma membrane E-cadherin complex is also tightly regulated, and recent work has indicated that OGT is a good target for pharmaceutical intervention (Gloster et al., 2011). Therefore, further dissection and manipulation of the regulatory mechanisms of O-GlcNAcylation in E-cadherin adhesion in a context of stress have important implications for diverse disease processes such as apoptosis, development and metastasis.

Materials and Methods

Cell culture and reagents

Human breast cancer cells MCF-7 were grown in α -minimal essential medium (α -MEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific, Logan, UT). MDCK cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% FBS. Thapsigargin (Invitrogen), Brefeldin A (Sigma-Aldrich, St Louis, MO), Tunicamycin (Sigma), Furin Inhibitor Decanoyl-Arg-Val-Lys-Arg-chloromethylketone (BACHEM, Torrance, CA), and C2-ceramide (Sigma) were dissolved in dimethylsulfoxide (DMSO) whereas DTT (Sigma) was dissolved in dH₂O and then they were diluted into culture medium to the indicated concentrations.

Constructs

cDNA for human full-length E-cadherin (E-cad WT) hEcad/pcDNA3 was provided by Cara J. Gottardi (Northwestern University, Chicago, IL). E-cadherin cytoplasmic domain truncated mutant (E-cad Δ cyto) was amplified by PCR using 5' (5'-ACTCGAGCGGCCGCATGGGCCCTTGGAGCCGAGC-3') and 3' (5'-GCTGACTCTAGACTATCAAAGAGCGTAATCTGGAACATCGTATGGGTA-CATAAGAAACAGCAAGAGCAG-3') oligonucleotide primers between nucleotides 125 and 2317 as defined as previously (Gottardi et al., 2001). cDNA encoding E-cad Δ pSer was provided by Patrick J. Casey (Duke University, Durham, NC). All these three cDNAs were subcloned into pRc/CMV vectors with a coding sequence for the HA tag reengineered on the C-terminal.

Stable expression of wild type (WT) E-cadherin and E-cadherin variants in MCF-7 cells

Plasmids encoding E-cad WT as well as E-cad Δ cyto and E-cad Δ pSer were transfected into MCF-7 cells or MDCK cells with ExGen 500 (Fermentas, Glen Burnie, MD) and colonies were selected in G418 (500 μ g/ml). Stable clones were first isolated with cloning cylinders, expanded, and screened for protein expression by western analysis. Cell clones expressing E-cad WT and E-cadherin mutants were screened by immunoblotting using HA tag antibody and high expression clones were picked for further studies.

Antibodies

The antibodies, including mouse monoclonal antibody (mAb) against E-cadherin cytoplasmic domain (IC) (BD Biosciences, San Diego, CA) and p120-catenin (BD Biosciences), mouse mAb against E-cadherin extracellular domain (EC) SHE78-7 (Invitrogen), mouse mAb against HA tag (Covance Research, Princeton, NJ), mouse mAb against KDEL (Enzo Life Sciences, Plymouth Meeting, PA), mouse mAb against PARP (Enzo Life Sciences, Plymouth Meeting, PA) and β -actin (Santa Cruz Biotechnology, Santa Cruz, CA) were used for immunoblots. WGA-agarose was obtained from Sigma. For immunoprecipitation, mouse anti-E-cadherin cytoplasmic domain (BD Biosciences) and mouse mAb against HA tag (Covance Research) were used. For immunofluorescence, mouse mAb against E-cadherin extracellular domain SHE78-7 (EC) was used.

Surface biotinylation assay

To biotin label cell surface proteins, EZ-Link biotin (Thermo Scientific, Waltham, MA) dissolved in phosphate-buffered saline (PBS) at 0.5 mg/ml was added to cells for 30 minutes as specified. Then cells were lysed and 30 μ l of streptavidin-conjugated paramagnetic particles (Promega, Madison, WI) was added to 100 μ l

biotinylated cell lysates, mixed for 6 hours at 4°C and washed three times with cell lysis buffer. The beads were collected using a paramagnetic isolator and proteins released by incubation in SDS loading buffer at 100°C for 10 minutes.

PNGase F and Endo H digestion

After WGA binding or E-cadherin and HA tag immunoprecipitation, beads were washed with 1% Triton X-100 buffer three times and then were systematically digested with PNGase F (New England BioLabs, Ipswich, MA) or Endo H (New England BioLabs, Ipswich, MA) at 37°C for 1 hour according to the manufacturer's recommendations.

TCA precipitation

100% (w/v) trichloroacetic acid (TCA) was added to the digestion products to a final concentration of 20%. Then samples were vortexed and kept on ice for 15 minutes before centrifuge. The pellets were washed with 50% ethyl ether anhydrous and 50% ethanol. Then pellets were dried in SpeedVac System and dissolved in SDS loading buffer at 100°C for 10 minutes. The samples were analyzed by immunoblotting.

Immunoprecipitation and immunoblotting

For immunoprecipitation, 1×10^7 cells were lysed in 1 ml of cell lysis buffer (10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl) for 15 minutes on ice, and passed through a 26 gauge needle four times. Cellular debris and nuclei were removed by centrifugation at 15,871 g for 10 minutes in a microcentrifuge. Protein concentration was determined for the supernatant using BCA assay (Thermo Scientific, Waltham, MA), and 100 μ g of total protein from each sample was immunoprecipitated with 1 μ g of antibody against E-cadherin or HA tag. GammaBind G-Sepharose (GE Healthcare, Piscataway, NJ) was added, and samples were mixed by rotation for 4 hours at 4°C. For binding with WGA-agarose beads (Sigma), 100 μ g of total protein was incubated with beads, samples mixed by rotation. Then the beads were pelleted and washed three times before the addition of 10 μ l of SDS loading buffer to the beads and being boiled at 100°C for 10 minutes for immunoblotting. Proteins were separated on 8% denaturing SDS-PAGE gels and transferred to PVDF membrane. The membrane was incubated with various primary antibodies diluted 1:1000. The membrane was then incubated with the corresponding secondary antibody coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:10,000. Labeled proteins were visualized with Enhanced Chemiluminescence (Perkin Elmer, Waltham, MA).

Immunofluorescence

MCF-7 cells were seeded on coverslips and grown as described (Zhu et al., 2001). Non-permeabilized cells were stained with cell-permeable dye Syto-63 (Invitrogen) as an internal standard for cell number or volume at 37°C for 30 minutes, washed twice in PBS, fixed with 4% paraformaldehyde and then incubated with E-cadherin antibody directed against the external domain SHE78-7 (EC, dilution, 1:500) at 37°C for 1 hour, washed and incubated for 1 hour with fluorescent secondary antibody donkey anti-mouse IgG-fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch Laboratories) at 1:30 and observed by confocal microscope. For ZO-1 staining, cells were pre-extracted with 0.2% Triton X-100 in 100 mM KCl, 3 mM MgCl₂, 1 mM CaCl₂, 200 mM sucrose, and 10 mM HEPES (pH 7.1) for 2 minutes on ice. Then the cells were fixed with 4% paraformaldehyde for 30 minutes, washed twice in PBS for 5 minutes and incubated with 1% bovine serum albumin in PBS with 0.5% Triton X-100 for 30 minutes at room temperature. Cells were incubated at room temperature with 5 μ g/ml anti-ZO-1 antibody (Invitrogen, Camarillo, CA) for 1 hour. To visualize the nuclei, cells were stained with 5 μ M Draq5 (Biostatus, Shephed, UK) for 30 minutes at room temperature.

Phalloidin staining

To stain F-actin, MCF-7 cells were fixed with 4% paraformaldehyde for 30 minutes, washed twice in PBS for 5 minutes and incubated with 1% BSA in PBS with 0.5% Triton X-100 for 30 minutes at room temperature. Then cells were incubated with Alexa-Fluor-488-conjugated phalloidin [1:100 in 1% BSA (Invitrogen)] for 30 minutes at room temperature and imaged on an Opera High Content Screening System (Evotec, Hamburg, Germany).

Confocal microscopy

Fluorescence images were taken at room temperature using a laser-scanning confocal microscope (Leica TCS SP5, Buffalo Grove, IL) with 63 \times plan apochromat glycerin immersion objective (1.3 NA) and steady state photomultiplier tubes (PMTs, Leica Microsystems, Buffalo Grove, IL) to detect and digitize the image. The coverslips were mounted with mounting medium (Sigma). The excitation wavelength for FITC was 488 nm with emission collected at 530 \pm 15 nm and the excitation wavelength of Syto63 was 633 nm with emission collected at 673 \pm 15 nm. A complete set of images were recorded using the Leica TCS SP5 software by setting and fixing the imaging parameters on the brightest

samples to ensure no saturation. Images were analyzed using Acapella software (Acapella Studio 2.0.0.7388, Perkin Elmer, Waltham, MA). The intensity of E-cadherin staining was normalized against the Syto63 intensity to correct for changes in cell shape.

Image analysis and quantification

MCF-7 cells transfected with plasmids encoding Neo (control vector), E-cad WT and E-cad Δ PSer were treated with TG for 0, 5, 10, 15, 20, 25 hours, and then were fixed without permeabilization of cell membranes. E-cadherin on plasma membrane was stained by FITC (green) and cytoplasmic dye Syto63 (red) was applied as an internal control. Four cell images were collected for each sample using a deconvolution microscope all with the same exposure time. Acapella software was used to capture cellular matter in the green/red channels (for a green-red image, the same matter was used). The total green intensity and red intensity were collected in the cell area for each image. For each image we calculated: Ratio=Total green intensity/Total red intensity, with replicates and plotted averages for each time period. Because Syto63 stain (red) should be constant for each cell, we plotted the average value of red per cellular matter pixel for each time period.

Measurement of cell death

PARP cleavage was quantified by immunoblotting using PARP C-2-10 mAb (Enzo Life Sciences, Plymouth Meeting, PA) as described previously (Kimura et al., 2003). Cell survival was also quantified using Annexin-V–Alexa-Fluor-488 and PI staining. This assay identifies viable cells (Annexin-V⁻ PI⁻), apoptotic cells (Annexin-V⁺ PI⁻) and necrotic cells (Annexin-V⁺ PI⁺) (Vermes et al., 1995). MCF-7 cells were washed with cold PBS twice and then resuspended in 1× binding buffer (0.01 M HEPES, pH 7.4, 0.14 M NaCl, 2.5 mM CaCl₂) at a concentration of ~1×10⁶ cells/ml. Annexin-V–Alexa-Fluor-488 (Invitrogen) and PI (Sigma) were added before incubation on ice for 15 minutes. Cells were analyzed by Flow Cytometry (Beckman Coulter Epics Altra, Brea, CA).

Cell adhesion assay

To assess cell adhesion, we used an assay that measures loss of attachment to a cell culture dish with lateral shaking. MCF-7 cells expressing E-cad WT and E-cad Δ PSer were seeded at the density of 5×10⁶ per 10 cm dish. Cells were treated with TG, DMSO (negative control) and 5 mM EDTA (positive control). Then cells were shaken at 500 r.p.m. on Thermomixer 5436 (Eppendorf, Hamburg, Germany) inside a tissue culture incubator. Floating cells were counted over time on a hemocytometer at time points of 0, 8, 16, 24 hours after TG treatment on a Telaval 3 Inverted Microscope (Jena, Belfountain, ON, Canada). As the exposure to 5 mM EDTA for 30 minutes at 37°C causes detachment of cells by abrogating all calcium-dependent E-cadherin function, the percentage of floating cells at each time point was calculated by the floating cell count in the TG-treated dish divided by the number of floating cells in the EDTA-treated dish.

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References

- Beavon, I. R. (2000). The E-cadherin-catenin complex in tumour metastasis: structure, function and regulation. *Eur. J. Cancer* **36**, 1607-1620.
- Behrens, J., Vakaet, L., Friis, R., Winterhager, E., Van Roy, F., Mareel, M. M. and Birchmeier, W. (1993). Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/beta-catenin complex in cells transfected with a temperature-sensitive v-SRC gene. *J. Cell Biol.* **120**, 757-766.
- Capotosti, F., Guernier, S., Lammers, F., Waridel, P., Cai, Y., Jin, J., Conaway, J. W., Conaway, R. C. and Herr, W. (2011). O-GlcNAc transferase catalyzes site-specific proteolysis of HCF-1. *Cell* **144**, 376-388.
- Comer, F. I. and Hart, G. W. (2000). O-Glycosylation of nuclear and cytosolic proteins. Dynamic interplay between O-GlcNAc and O-phosphate. *J. Biol. Chem.* **275**, 29179-29182.
- Denmeade, S. R. and Isaacs, J. T. (2005). The SERCA pump as a therapeutic target: making a “smart bomb” for prostate cancer. *Cancer Biol. Ther.* **4**, 14-22.
- Drees, F., Pokutta, S., Yamada, S., Nelson, W. J. and Weis, W. I. (2005). Alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. *Cell* **123**, 903-915.
- D'Souza-Schorey, C. (2005). Disassembling adherens junctions: breaking up is hard to do. *Trends Cell Biol.* **15**, 19-26.
- Gambetta, M. C., Oktaba, K. and Müller, J. (2009). Essential role of the glycosyltransferase sxc/Ogt in polycomb repression. *Science* **325**, 93-96.
- Gilmore, A. P., Owens, T. W., Foster, F. M. and Lindsay, J. (2009). How adhesion signals reach a mitochondrial conclusion-ECM regulation of apoptosis. *Curr. Opin. Cell Biol.* **21**, 654-661.
- Gloster, T. M., Zandberg, W. F., Heinonen, J. E., Shen, D. L., Deng, L. and Vocadlo, D. J. (2011). Hijacking a biosynthetic pathway yields a glycosyltransferase inhibitor within cells. *Nat. Chem. Biol.* **7**, 174-181.
- Gottardi, C. J., Wong, E. and Gumbiner, B. M. (2001). E-cadherin suppresses cellular transformation by inhibiting beta-catenin signaling in an adhesion-independent manner. *J. Cell Biol.* **153**, 1049-1060.
- Grossmann, J. (2002). Molecular mechanisms of “detachment-induced apoptosis–Anoikis”. *Apoptosis* **7**, 247-260.
- Gumbiner, B. M. (2000). Regulation of cadherin adhesive activity. *J. Cell Biol.* **148**, 399-404.
- Gumbiner, B. M. (2005). Regulation of cadherin-mediated adhesion in morphogenesis. *Nat. Rev. Mol. Cell Biol.* **6**, 622-634.
- Hallenberger, S., Bosch, V., Angliker, H., Shaw, E., Klenk, H. D. and Garten, W. (1992). Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. *Nature* **360**, 358-361.
- Jeanes, A., Gottardi, C. J. and Yap, A. S. (2008). Cadherins and cancer: how does cadherin dysfunction promote tumor progression? *Oncogene* **27**, 6920-6929.
- Kimura, M., Haisa, M., Uetsuka, H., Takaoka, M., Ohkawa, T., Kawashima, R., Yamatsuji, T., Gunduz, M., Kaneda, Y., Tanaka, N. et al. (2003). TNF combined with IFN-alpha accelerates NF-kappaB-mediated apoptosis through enhancement of Fas expression in colon cancer cells. *Cell Death Differ.* **10**, 718-728.
- Kreppel, L. K. and Hart, G. W. (1999). Regulation of a cytosolic and nuclear O-GlcNAc transferase. Role of the tetratricopeptide repeats. *J. Biol. Chem.* **274**, 32015-32022.
- Ku, N. O., Toivola, D. M., Strnad, P. and Omary, M. B. (2010). Cytoskeletal keratin glycosylation protects epithelial tissue from injury. *Nat. Cell Biol.* **12**, 876-885.
- Laur, O. Y., Klingelhöfer, J., Troyanovsky, R. B. and Troyanovsky, S. M. (2002). Both the dimerization and immunochemical properties of E-cadherin EC1 domain depend on Trp(156) residue. *Arch. Biochem. Biophys.* **400**, 141-147.
- Lickert, H., Bauer, A., Kemler, R. and Stappert, J. (2000). Casein kinase II phosphorylation of E-cadherin increases E-cadherin/beta-catenin interaction and strengthens cell-cell adhesion. *J. Biol. Chem.* **275**, 5090-5095.
- Ling, K., Doughman, R. L., Firestone, A. J., Bunce, M. W. and Anderson, R. A. (2002). Type I gamma phosphatidylinositol phosphate kinase targets and regulates focal adhesions. *Nature* **420**, 89-93.
- Ling, K., Bairstow, S. F., Carbonara, C., Turbin, D. A., Huntsman, D. G. and Anderson, R. A. (2007). Type I gamma phosphatidylinositol phosphate kinase modulates adherens junction and E-cadherin trafficking via a direct interaction with mu 1B adaptin. *J. Cell Biol.* **176**, 343-353.
- Liwoz, A., Lei, T. and Kukuruzinska, M. A. (2006). N-glycosylation affects the molecular organization and stability of E-cadherin junctions. *J. Biol. Chem.* **281**, 23138-23149.
- Lock, J. G. and Stow, J. L. (2005). Rab11 in recycling endosomes regulates the sorting and basolateral transport of E-cadherin. *Mol. Biol. Cell* **16**, 1744-1755.
- Lytton, J., Westlin, M. and Hanley, M. R. (1991). Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. *J. Biol. Chem.* **266**, 17067-17071.
- Maley, F., Trimble, R. B., Tarentino, A. L. and Plummer, T. H., Jr. (1989). Characterization of glycoproteins and their associated oligosaccharides through the use of endoglycosidases. *Anal. Biochem.* **180**, 195-204.
- Mosesson, Y., Mills, G. B. and Yarden, Y. (2008). Derailed endocytosis: an emerging feature of cancer. *Nat. Rev. Cancer* **8**, 835-850.
- Natsuka, S., Kawaguchi, M., Wada, Y., Ichikawa, A., Ikura, K. and Hase, S. (2005). Characterization of wheat germ agglutinin ligand on soluble glycoproteins in *Caenorhabditis elegans*. *J. Biochem.* **138**, 209-213.
- Nelson, W. J. (2008). Regulation of cell-cell adhesion by the cadherin-catenin complex. *Biochem. Soc. Trans.* **36**, 149-155.
- Ozawa, M. and Kemler, R. (1990). Correct proteolytic cleavage is required for the cell adhesive function of uvomorulin. *J. Cell Biol.* **111**, 1645-1650.
- Ozawa, M. and Kemler, R. (1998). The membrane-proximal region of the E-cadherin cytoplasmic domain prevents dimerization and negatively regulates adhesion activity. *J. Cell Biol.* **142**, 1605-1613.
- Peifer, M. and Yap, A. S. (2003). Traffic control: p120-catenin acts as a gatekeeper to control the fate of classical cadherins in mammalian cells. *J. Cell Biol.* **163**, 437-440.
- Posthaus, H., Dubois, C. M., Laprise, M. H., Grondin, F., Suter, M. M. and Müller, E. (1998). Proprotein cleavage of E-cadherin by furin in baculovirus over-expression system: potential role of other convertases in mammalian cells. *FEBS Lett.* **438**, 306-310.
- Spellman, M. W. (1990). Carbohydrate characterization of recombinant glycoproteins of pharmaceutical interest. *Anal. Chem.* **62**, 1714-1722.

- Stappert, J. and Kemler, R.** (1994). A short core region of E-cadherin is essential for catenin binding and is highly phosphorylated. *Cell Adhes. Commun.* **2**, 319-327.
- Sun, Y., Turbin, D. A., Ling, K., Thapa, N., Leung, S., Huntsman, D. G. and Anderson, R. A.** (2010). Type I gamma phosphatidylinositol phosphate kinase modulates invasion and proliferation and its expression correlates with poor prognosis in breast cancer. *Breast Cancer Res.* **12**, R6.
- Vermes, I., Haanen, C., Steffens-Nakken, H. and Reutelingsperger, C.** (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods* **184**, 39-51.
- Vosseller, K., Wells, L. and Hart, G. W.** (2001). Nucleocytoplasmic O-GlcNAcylation: O-GlcNAc and functional proteomics. *Biochimie* **83**, 575-581.
- Wells, L. and Hart, G. W.** (2003). O-GlcNAc turns twenty: functional implications for post-translational modification of nuclear and cytosolic proteins with a sugar. *FEBS Lett.* **546**, 154-158.
- Yap, A. S., Crampton, M. S. and Hardin, J.** (2007). Making and breaking contacts: the cellular biology of cadherin regulation. *Curr. Opin. Cell Biol.* **19**, 508-514.
- Zhu, W., Leber, B. and Andrews, D. W.** (2001). Cytoplasmic O-GlcNAcylation prevents cell surface transport of E-cadherin during apoptosis. *EMBO J.* **20**, 5999-6007.