DNAJB4 molecular chaperone distinguishes WT from mutant E-cadherin, determining their fate in vitro and in vivo

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E-cadherin (Ecad) is a well-known invasion suppressor and its loss of expression is common in invasive carcinomas. Germline Ecad mutations are the only known genetic cause of hereditary diffuse gastric cancer (HDGC), demonstrating the causative role of Ecad impairment in gastric cancer. HDGC-associated Ecad missense mutations can lead to folding defects and premature proteasome-dependent endoplasmic reticulum-associated degradation (ERAD), but the molecular determinants for this fate were unidentified. Using a Drosophila-based genetic screen, we found that Drosophila DnaJ-1 interacts with wild type (WT) and mutant human Ecad in vivo. DnaJ (Hsp40) homolog, subfamily B, member 4 (DNAJB4), the human homolog of DnaJ-1, influences Ecad localization and stability even in the absence of Ecad endogenous promoter, suggesting a post-transcriptional level of regulation. Increased expression of DNAJB4 leads to stabilization of WT Ecad in the plasma membrane, while it induces premature degradation of unfolded HDGC mutants in the proteasome. The interaction between DNAJB4 and Ecad is direct, and is increased in the context of the unfolded mutant E757K, especially when proteasome degradation is inhibited, suggesting that DNAJB4 is a molecular mediator of ERAD. Post-translational regulation of native Ecad by DNAJB4 molecular chaperone is sufficient to influence cell adhesion in vitro. Using a chick embryo chorioallantoic membrane assay with gastric cancer derived cells, we demonstrate that DNAJB4 stimulates the anti-invasive function of WT Ecad in vivo. Additionally, the expression of DNAJB4 and Ecad is concomitantly decreased in human gastric carcinomas. Altogether, we demonstrate that DNAJB4 is a sensor of Ecad structural features that might contribute to gastric cancer progression.

INTRODUCTION

E-cadherin (Ecad) is the main component of Adherens Junctions in the epithelia and acts as the master regulator of cell–cell adhesion. In cancer, it has been extensively described as a tumor suppressor since it suppresses cell invasion, and its loss is a frequent characteristic of invasive carcinomas (1). We have previously shown that Ecad expression can be efficiently regulated by post-translational mechanisms, such as endoplasmic reticulum-associated degradation (ERAD) (2) or trafficking deregulation (3) leading to cancer progression. It is now clear that post-translation regulation of Ecad may account for its loss in invasive carcinomas (4,5). In hereditary diffuse gastric cancer (HDGC), where germline CDH1 (the gene encoding Ecad) mutations are found in 30% of the cases, missense mutations are found in 30% of the cases, missense mutations lead to partial or complete loss of Ecad protein expression, frequently without affecting the RNA levels (6). In this clinical setting, approximately half of the missense mutations described

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in the literature lead to Ecad structural destabilization, a criterion associated with loss of function and cancer development (7). Accordingly, the identification of the molecular partners with a role in Ecad structural recognition and regulation assume significant biological relevance.

Molecular chaperones are the main regulators of protein homeostasis and their function include the capacity to regulate protein folding, aggregation and degradation (8). DNAJ proteins belong to the HSP40 family and are known to transfer substrates to Hsp70 chaperones, stimulating their ATPase domain and conferring specificity to this family of chaperones (9). Humans have 41 different DNAJ encoding genes, among which type B proteins, that contain an extreme N-terminal J-domain, a glycine/phenylalanine-rich region, a cysteine-rich region, and a variable C-terminal domain. DNAJB1 (classical Hsp40) is heat inducible and cooperates with HSPA1A (Hsp70) and HSPA8 (Hsc70) in luciferase refolding in vitro and in vivo (10,11). Interestingly, it has recently been proposed that Hsp40 proteins can be associated to the Endoplasmic Reticulum and act in misfolding recognition (12). Although there is a high homology between DNAJB1 and DnaJ (Hsp40) homolog, subfamily B, member 4 (DNAJB4), the latter is not as significantly induced by heat shock, and is thought to act as a housekeeping HSP40/DNAJ (13). It has been demonstrated that DNAJB4 has little impact in refolding and aggregation suppression (14), but its binding to unfolded substrates has been described (9,15). Thus, the evidences suggest that DNAJB4 is not only important under cellular stress, but also in control conditions, and can be involved in misfolding recognition of its putative substrates.

It has been demonstrated that DNAJB4 acts as a tumor suppressor in non-small cell lung cancer model, while inhibiting proliferation, anchorage-independent growth, motility and invasion and promoting apoptosis (16). The invasion suppressor role of DNAJB4 partially relies on the induction of Ecad expression at the transcriptional level, supposedly due to the downregulation of the transcriptional repressor Slug (17). There are no reports exploring the potential of DNAJB4 as a molecular chaperone of Ecad.

We used a Drosophila-based genetic screen to demonstrate that DnaJ-1 (DnaJ-like-1) interacts with human Ecad in vitro. We confirmed that DNAJB4 is the closest human homolog of DnaJ-1 and investigated the mechanism through which this DNAJ protein can regulate Ecad expression. To achieve this, we used gastric cancer cell lines and germline Ecad missense mutations associated to HDGC, where Ecad loss is a cancer initiating event. In our cell models, DNAJB4 regulates Ecad subcellular localization, influencing its expression at the plasma membrane (PM). Interestingly, we demonstrate that this regulation is independent of the proximal promoter of Ecad, occurs at the post-translational level, and that DNAJB4 directly interacts with Ecad, acting as its molecular chaperone. DNAJB4 distinguishes native WT from unfolded Ecad mutants, determining their divergent fate. It recognizes unfolded Ecad for degradation in the proteasome, and significantly reduces the half-life of a misfolded mutant Ecad identified in a HDGC family (2). In the other hand, it promotes the folding of WT Ecad, resulting in increased PM expression and induction of cell–cell adhesion. The biological significance of DNAJB4 in a gastric cancer cell model was analyzed, and we demonstrate that it stimulates the anti-invasive function of Ecad but depends on Ecad to act as an anti-invasive molecule in vivo. These results corroborate the observed concomitant decrease of Ecad and DNAJB4 expression in human gastric carcinoma samples. Our results demonstrate that DNAJB4 is a molecular chaperone of Ecad that is able to sense Ecad folding, leading to stabilization of a natively folded Ecad, or to degradation of its unfolded counterpart, determining the invasiveness of gastric cancer cells in vitro and in vivo.

RESULTS

Drosophila DnaJ-1/Ubp64E regulate human E-cadherin in vivo

We used a previously described Drosophila transgenic model (18) expressing human wild-type Ecad (hEcad WT) or two HDGC-associated pathogenic mutants (hEcad A634V and V832M) in the developing retina (Fig. 1A). By combining these strains with a collection of GS lines expressing different Drosophila genes in the same tissue context, we identified the lines capable of modifying the eye phenotype (19). Using this approach, we could identify new genes that interact with Ecad within a tissue context. Among the genes that were found to interact with WT and mutant Ecad, we found a GS strain that expresses DnaJ-1 and Ubp64E. The simple overexpression of DnaJ-1/Ubp64E does not interfere with the eye morphology, but significantly disrupts the phenotype of the eyes overexpressing human Ecad (Fig. 1A). We quantified the areas of the fly eyes (Fig. 1B) and found that DnaJ-1/Ubp64E significantly disrupt WT or mutant Ecad eyes, but reaches higher significance in the interaction with mutant Ecad, reflecting a higher reproducibility of the phenotype, which indicates increased specificity of this interaction in the context of a mutant Ecad.

We used Uniprot Protein Blast Similarity search to determine the putative human orthologues of DnaJ-1/Ubp64E. We found that the closest human orthologues of this pair of genes are DNAJB4 and USP47, respectively. There is high similarity between DNAJB4 and DnaJ-1 (55% identity, with 70% positives) and between UBPI47 and Ubp64E (50% identity, with 65% positives), as analyzed by protein sequence alignment (Supplementary Material, Fig. S1A and B).

Once we have established that DNAJB4 or USP47 could interact with Ecad, we decided to determine which of these genes would be the stronger candidate as Ecad regulator. To do this, we mimicked the Drosophila experiment in vitro, by over expressing separately DNAJB4 and USP47 in cells with overexpression of WT or mutant Ecad [Chinese hamster ovary (CHO) cells]. At the protein level, minor differences were observed in Ecad total expression when we over expressed USP47, while the overexpression of DNAJB4 induces changes in the pattern of Ecad expression (Supplementary Material, Fig. S2). This was unexpected, because DNAJB4 has been demonstrated to induce Ecad expression through transactivation of the proximal promoter, which is absent in these cell models (16,17). The Drosophila models and the stable transduction of Ecad in CHO cells were controlled under the activity of exogenous promoters, suggesting that the regulation of Ecad by DNAJB4 might also happen at the post-transcriptional level.
DNAJB4 directly interacts with E-cadherin and influences its expression at the plasma membrane

Ecad expression is determinant for the progression of gastric cancer and its loss is demonstrated to be an initiating event in this type of cancer (20). To investigate whether DNAJB4 regulates the expression and subcellular localization of Ecad in that context, we silenced DNAJB4 in a gastric cancer derived cell line (MKN28) that expresses endogenous WT Ecad, and analyzed the total expression of Ecad by WB and its PM localization by Flow Cytometry. We found that silencing of DNAJB4 leads to a partial decrease in Ecad expression at the PM (Fig. 2A and B) despite the absence of significant differences in the total amount of Ecad in this cell model (Fig. 2C). Conversely, overexpression of DNAJB4 in the same cell model induces an increase of PM Ecad (Fig. 2D). Interestingly, MKN28 cells are described to have insignificant levels of Slug (21), suggesting that the regulation of Ecad by DNAJB4 in this gastric cancer cell model does not significantly depend on transcriptional regulation, but rather occurs at the post-transcriptional level. To test whether DNAJB4 could regulate Ecad expression and localization at the post-transcriptional level, we manipulated its expression in CHO cell lines stably expressing Ecad under the control of the exogenous promoter CMV. We confirmed that DNAJB4 stimulates the PM expression of WT Ecad and observed that it has the opposite effect in the expression of an unfolded mutant Ecad that is prematurely recognized by ERAD, E757K (Fig. 2E) (2). Going in the same direction, DNAJB4 decreases the PM expression of Ecad in another gastric cancer cell line that endogenously expresses a mutant Ecad (Kato III, Supplementary Material, Fig. S3), suggesting that DNAJB4 is able to distinguish and differently regulate WT and mutant Ecad at the post-transcriptional level, when Ecad expression is regulated by its endogenous promoter or an exogenous one.

**DNAJB4 acts as a molecular chaperone of E-cadherin**

DNAJB4 belongs to the Hsp40-like family of molecular chaperones, thought to be the drivers of the functional specificity of Hsp70 machinery, with a major function of binding selected substrates, determining their fate (9). Therefore, we hypothesized that the post-transcriptional regulation of Ecad by DNAJB4 relies on its molecular chaperone function. To address this question, we first determined whether DNAJB4 directly interacted with the putative substrate Ecad. Using CHO cell lines stably expressing Ecad, we were able to co-immunoprecipitate both proteins together (Fig. 3A and B). Interestingly, DNAJB4 exhibits stronger interaction with the mutant form, indicating that it is retained bound to the ERAD substrate. Since molecular chaperones are frequently determinants of the half-life of their substrates, we decided to test whether DNAJB4 could determine the half-life of Ecad. We tested the impact of DNAJB4 overexpression in cells expressing WT and one unfolded mutant Ecad, and found that the half-life of the E757K mutant was reduced (Fig. 3C and D), despite the intrinsic reduced stability of the mutant (8 h in control conditions, 4 h with increased expression of DNAJB4). Although DNAJB4 directly interacts with WT Ecad, it does not influence its half-life (Fig. 3C and D). To determine whether DNAJB4 could indeed sense Ecad folding, we used the previously described HDGC-associated mutant L583R (which is unfolded), and its variant L583I which is structurally tolerated (7). DNAJB4 was able to distinguish the unfolded variant (L583R), leading to its destabilization and decreased expression, while it stabilizes the neutral variant L583I, presumably natively folded (Fig. 3E). The capacity to directly bind WT or mutant Ecad, and the ability to distinguish unfolded Ecad forms leading to their premature degradation, suggests that DNAJB4 acts as a molecular chaperone of Ecad.

**DNAJB4 mediates the recognition of E-cadherin for degradation in the proteasome**

We have previously shown that a significant number of HDGC-associated Ecad missense mutants exhibit structural defects and are thus destabilized and prematurely degraded in the proteasome (2,7). To further determine whether DNAJB4...
could be a direct mediator of the recognition of unfolded Ecad for the ERAD quality control pathway, we used the ERAD-sensitive mutant E757K. We found that the interaction between DNAJB4 chaperone and Ecad is increased in conditions where the proteolytic function of the proteasome is inhibited (Fig. 4A), suggesting that it preferentially interacts with the degradation-prone fraction of Ecad. We have also previously described that dimethylsulfoxide (DMSO) is an efficient chemical chaperone that favors the accumulation of Ecad at the PM, presumably by stabilizing its structure toward a native state and inducing its trafficking to, and stability at, the PM (2,3). As a proof of concept, and to confirm that DNAJB4 preferentially binds unfolded Ecad, we analyzed the interaction between DNAJB4 and Ecad stabilized by DMSO, and found that the interaction between the chaperone and the substrate is decreased if we induce Ecad stability with the chemical chaperone (Fig. 4A). Moreover, expression of DNAJB4 increases the fraction of unfolded Ecad degraded in the proteasome (Fig. 4B, MG132), but not the amount degraded in the lysosome (Fig. 4B, CQ), suggesting that it mediates the degradation of ERAD-prone mutant Ecad by the proteasome. Interestingly, DNAJB4 indirectly potentiates the chemical chaperone effect of DMSO, stimulating Ecad expression (Fig. 4B), although not by increased interaction with the substrate (Fig. 4A).

Figure 2. DNAJB4 regulates E-cadherin expression at the transcriptional and post-transcriptional level, determining its expression at the PM. (A–C) MKN28 cells were transfected with two different siRNA against DNAJB4 and Ecad expression at the PM was analyzed by Flow Cytometry (A and B) or by WB (C). (A) The results are the average of three independent experiments. (B) Representative histograms corresponding to Ecad PM expression in control conditions (MKN28) or upon DNAJB4 silencing. (C) 40 μg of total cell extract were analyzed by WB and probed for Ecad and DNAJB4, demonstrating the silencing of DNAJB4. α-tubulin was used as a loading control. (D) Representative histograms corresponding to Ecad PM expression in control conditions (MKN28) or upon DNAJB4 overexpression. (E) CHO cells stably expressing WT or E757K Ecad were transiently transfected with DNAJB4, the amount of PM Ecad was analyzed by Flow Cytometry, and representative histograms are shown.
Figure 3. DNAJB4 acts as a molecular chaperone of E-cadherin. CHO cell lines stably expressing WT and E757K Ecad were transfected with DNAJB4. (A and B) Ecad was immunoprecipitated, and the interaction with DNAJB4 was analyzed by WB. Membranes were probed with anti-DNAJB4 and anti-Ecad antibodies. (C and D) 48 h after transient transfection of DNAJB4-V5, cells were incubated with the protein synthesis inhibitor Cycloheximide (CHX) for 0, 2, 4 or 8 h. Ecad expression was analyzed by WB, transfection was confirmed with anti-V5, and α-tubulin served as a loading control for quantification purposes. (D) In each time point, Ecad expression was normalized to the control (0 h, CHX). The results are the average of three independent experiments. Circles represent WT cells and squares represent E757K mutant cells. Dashed lines represent cells upon overexpression of DNAJB4. (E) Ecad was either transfected alone or co-transfected with DNAJB4-V5 into the CHO parental cell line, and 48 h later the expression of Ecad, DNAJB4 and α-tubulin was analyzed by WB. Ecad expression was quantified by densitometry and is presented as the ratio to control conditions (condition without DNAJB4).
DNAJB4 stimulates the anti-invasive role of native E-cadherin and its expression is decreased in gastric carcinomas

After we have partially elucidated the mechanism whereby DNAJB4 influences Ecad stability at the post-translational level, we investigated the functional consequences of this regulation. To do this, we used CHO cell models (Ecad-null cells) (22), with stable expression of WT or mutant Ecad subjected to the CMV exogenous promoter, and induced the expression of DNAJB4 by transient transfection. We observed that increased expression of DNAJB4 is not sufficient to increase cell adhesion in the absence of Ecad or in the presence of the non-functional unfolded E757K mutant Ecad, but induce increased cell aggregation in the presence of a WT Ecad (Fig. 5A). This was expected, because it increases the amount of WT Ecad in the PM (Fig. 2E). To further analyze the role of DNAJB4 in cell migration, we used the wound healing assay. The results show that, in conditions of Ecad overexpression without transcriptional regulation (due to the lack of the Ecad proximal promoter in these cell models), DNAJB4 is not enough to further reduce cell migration (Fig. 5B), suggesting that Ecad is the dominant mobility suppressor.

After we have established the role of DNAJB4 on cell adhesion and migration in a cell culture system, we analyzed the relevance of this Ecad/DNAJB4 dominance upon invasion of a tissue context, using for this purpose the chick embryo chorioallantoic membrane (CAM) assay (23). We generated MKN28 gastric cancer cells with stable silencing of Ecad (shEcad) and a control cell line (shScramble), and transiently transfected DNAJB4 in these two distinct cellular contexts (Supplementary Material, Fig. S4A). The different cell lines were inoculated in the CAM and, after 3 days of incubation, cell invasion and vascularization of the CAM were evaluated in response to the different cell lines, as described in Materials and Methods. Labeling of the human cells inoculated in the CAM revealed that DNAJB4 reinforces the anti-invasive function of WT Ecad, but this invasion-suppressor potential is lost if Ecad is not expressed (silencing by shRNA, Fig. 6), suggesting that in the gastric cancer model Ecad is the dominant anti-invasive molecule. Analysis of the neovascularizing potential of these cells over the CAM shows that DNAJB4 is anti-angiogenic, and that this tumor suppressor feature is also Ecad-dependent (Supplementary Material, Fig. S4B).

Furthermore, using the on-line database Human Protein Atlas (24,25) we analyzed the expression patterns of Ecad and DNAJB4 in human sporadic gastric carcinomas. In normal gastric tissue, DNAJB4 is moderately expressed in more than 75% of the cells, in the glands, together with Ecad (Fig. 7, Supplementary Material, Table S2). Interestingly, 67% (8 out of 12) of the gastric carcinoma samples exhibit decreased (weak) expression of DNAJB4 (Fig. 7, Supplementary Material, Tables S1 and S2). Although Ecad expression seems to be decreased in the majority of the carcinoma samples (Supplementary Material, Table S1), only 8% (1 out of 12) are annotated to have ‘Moderate’ expression, compared with the ‘Strong’ expression obtained for the normal tissue (Supplementary Material, Tables S1 and S2), which is clearly underestimated. These results suggest that decreased DNAJB4 expression conveys with Ecad loss in human gastric carcinoma, corroborating the results obtained upon silencing of DNAJB4 in the gastric cancer cell line MKN28 (Fig. 2A and B).

Altogether, these results suggest that the post-translational regulation of native Ecad by its molecular chaperone DNAJB4 stimulates cell adhesion in vitro, but the anti-invasive potential...
of DNAJB4 seems to depend also on the transcriptional regulation of Ecad. Our results also demonstrate that Ecad is the dominant invasion-suppressor in the gastric cancer context, reinforcing the need to maintain its stability for prevention of cancer invasiveness and metastization.

DISCUSSION

The role of Ecad in cell–cell adhesion is well described, and it has been shown that Ecad loss can drive gastric cancer development in mice and humans (20). Moreover, germline Ecad mutations are the only known genetic cause of HDGC, further demonstrating the causative nature of Ecad loss in gastric cancer (26–28). Germline Ecad mutations strongly predispose carriers for development of early-onset gastric cancer, and only rarely associate with lobular breast cancer (29,30), suggesting that Ecad assumes a special role in the gastric tissue environment. Several layers of Ecad regulation have been described to be important in gastric cancer development, including mutations, deletions, loss of heterozygosity, promoter hypermethylation or regulation by transcription factors and miRNAs (6). Despite this, it is common to find cases displaying Ecad protein loss without significant impairment of transcription (stable tumor CDH1 mRNA levels) (31). Accordingly, it is likely that post-translational regulation of Ecad contributes to gastric cancer development.

Using a recently described Drosophila-based genetic screen with WT and mutant human Ecad ectopically expressed in the fly eye (19), we searched for new post-translational regulators of Ecad expression in vivo. The DnaJ-1/Ubp64E pair strongly interacted with human Ecad (WT and mutant), suggesting that their human homologs DNAJB4 or USP47 could be efficient modulators of Ecad expression. We tested the effect of the overexpression of the two human homologs separately, and concluded that USP47 does not significantly influence Ecad

Figure 5. Post-translational regulation of E-cadherin by DNAJB4 is sufficient to induce cell adhesion but does not reduce cell migration in vitro. CHO cell lines stably expressing WT or mutant (E757K) Ecad, or an empty vector were transiently transfected with DNAJB4-V5 or the V5-tag, as indicated. (A) Slow aggregation assay was performed and cells were photographed 48 h after seeding. Representative images are shown. (B) Wound healing assay was performed in CHO WT and cells were photographed every 2 h, during the 8 h period. The experiment was repeated three times and representative images are shown for time points 0 and 4 h; WC, wound closure.
expression, while DNAJB4 modulates Ecad expression in the presence, or absence, of Ecad proximal promoter.

DNAJB4/HLJ1 is a DNAJ-containing Hsp40-like molecular chaperone that was previously described to act as a tumor suppressor gene in non-small cell lung cancer, partially through the indirect induction of Ecad expression at the transcriptional level (17). Interestingly, it has also been shown that in lung cancer cell models, curcumin induces transcription of DNAJB4 and consequently increases Ecad expression at the protein level (32). Because curcurin is described to act as a chemical chaperone (33) and is an inducer of the heat shock response (34), these evidences suggest that the regulation of Ecad by DNAJB4 expression can also occur at the post-translational level.

We verified that, in gastric cancer cell models, DNAJB4 is able to induce Ecad expression at the PM. We were surprised
to observe that in the presence of unfolded mutant Ecad the effect was opposite, with the overexpression of DNAJB4 resulting in reduced expression of mutant Ecad at the PM. Owing to the chaperone nature of DNAJB4 we thought that this could result from a direct post-translational regulation of Ecad. Using cadherin-null CHO cell lines stably transduced with WT and mutant Ecad (in the absence of the proximal promoter region) we show that DNAJB4 directly interacts with Ecad, and regulates the half-life of an unfolded mutant Ecad, implying that it acts as its molecular chaperone. We used mutants with known energetic penalties (as a measure of their unfolding tendency) (7) to validate the capacity of DNAJB4 to act as a sensor of Ecad structural defects, and confirmed that it destabilizes the unfolded mutant L583R while it stabilizes the tolerated synthetic variant L583I. We also demonstrate that DNAJB4 increases the rate of unfolded Ecad degradation in the proteasome, and exhibits increased binding to Ecad when the proteasome is inhibited, suggesting that DNAJB4 mediates Ecad recognition for premature proteasome-dependent degradation, supposedly contributing to ERAD of the unfolded Ecad variants. In the opposite direction, when Ecad is stabilized by chemical chaperone treatment, Ecad/DNAJB4 interaction decreases, although the molecular chaperone potentiates the DMSO effect that results in Ecad stabilization (2). This is not surprising, because DMSO was recently shown to promote the multiple functions of DNAJB4 (35). It is also in good agreement with our previous data showing that DMSO acts by stimulating trafficking machinery, promoting the expression of Ecad at the PM (3); DNAJB4 might be one of the molecular effectors of the chemical chaperone effect of DMSO, possibly contributing to the recognition of unfolded substrates, to deliver them to the Hsp70/Hsp90 folding cycle. Indeed we observe that DNAJB4 is upregulated upon proteasome inhibition in different cell lines (data not shown), supposedly as a response to the accumulation of misfolded proteins.

The functional divergence of the different members of the DNAJ protein family has been described but is not completely elucidated (9), leaving room for the identification of specific substrates corresponding to particular DNAJ members. We demonstrated that DNAJB4 directly acts as a molecular chaperone of Ecad and efficiently regulates its expression at the post-translational level, increasing Ecad-dependent cell—cell adhesion when Ecad is retained in its natively folded state. Interestingly, when Ecad is intrinsically unfolded by mutation, or not expressed, DNAJB4 does not affect the adhesion capacity of our cell models. We used a CAM assay to evaluate the role of DNAJB4 in the regulation of gastric cancer cell invasion, and we observed that its expression increases the anti-invasive potential of Ecad-expressing cells, although this feature is lost in the absence of Ecad expression. To evaluate the expression of Ecad and DNAJB4 in human gastric cancer, we collected all the available gastric carcinoma samples from Human Protein Atlas (12 adenocarcinomas, of which 8 correspond to the same patient with stainings for both proteins). Cancer samples frequently exhibit decreased expression of both proteins, when compared with the normal tissues, suggesting that DNAJB4 might also act as a tumor suppressor in gastric cancer, possibly interfering with Ecad expression at the transcriptional and post-translational level.

In summary, we suggest that DNAJB4 chaperone is a molecular determinant of Ecad fate, acting as a sensor of Ecad structural defects (see proposed model, Fig. 8). DNAJB4 is able to distinguish between native Ecad and its unfolded counterpart (resulting from hereditary/sporadic mutation, or unfolding due to a

**Figure 8.** Proposed model establishing the role of DNAJB4 in the regulation of WT and mutant E-cadherin. DNAJB4 acts as a molecular chaperone of Ecad, binding directly after synthesis, with increased affinity for unfolded mutant Ecad. High levels of DNAJB4 increase the expression of WT Ecad in the PM (B), in comparison to low DNAJB4 levels (A). In case of an unfolded Ecad, DNAJB4 recognizes the structural defects, and increased levels of DNAJB4 stimulate Ecad proteasome-dependent degradation, leading to decreased Ecad half-life and decreased PM expression (D), when compared with conditions with low DNAJB4 levels (C).
stress insult during cancer progression), directing Ecad toward the PM (native Ecad) or to the proteasome for degradation (unfolded Ecad). Owing to the prevalence of Ecad mutations in hereditary and sporadic forms of diffuse gastric cancer, we envisage that this dual role of DNAJ4 could be explored for designing new therapeutic strategies in attempt to prevent Ecad loss during cancer progression.

MATERIALS AND METHODS

Drosophila strains and genetic manipulations

The Drosophila genetic screen is detailed elsewhere (19). Gal4/UAS system was used to induce human Ecad expression (WT, A634V and V832M) in the Drosophila eye as previously described (18) and these flies were crossed with a GS line expressing DnaJ-1 (DnaJ-like-1) and Ubp64E (Ubiquitin-specific protease 64E), kindly provided by José Felix de Celis. Eyes were examined under a Leica compound microscope, and digital images were processed and quantified using Adobe Photoshop. Statistical evaluation was done using a Student t-test.

Cell culture, transfections and treatments

CHO cells (ATCC:CCL-61, Spain) were grown in MEM Alpha, MKN28 and KatoIII (IPATIMUP Cell Line Bank) in RPMI (Invitrogen), all supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin-streptomycin (Invitrogen). CHO stable cell lines were established previously (2) and maintained in antibiotic selection with 5 µg/ml blasticidin (Invitrogen). For stable silencing of Ecad, MKN28 were transduced by lentiviral plasmid (7). Protein synthesis was inhibited with 50 µm Cycloheximide (Sigma); lysosome with 25 µm Chloroquine; and 2% DMSO was used as chemical chaperone.

Western blot and immunoprecipitation

Cells lysates were obtained and processed as described elsewhere (2) and detailed in Supplementary Material. Western blots were probed with antibodies against Ecad (Invitrogen or BD Biosciences), α-tubulin (Sigma), DNAJ4 (Santa Cruz Biotechnology) or V5 (Invitrogen), and the correspondent HRP-conjugated secondary antibodies, all diluted in 5% non-fat milk (0.5% Tween-20 in TBS), followed by ECL detection (Bio-Rad). Immunoblots were quantified with Quantity One Software (Bio-Rad). For immunoprecipitation, 400 µg of protein were coupled to anti-Ecad antibody (BD Biosciences), and precipitated with Protein G beads (GE Healthcare or Millipore), according to manufacturer instructions.

Cell migration and aggregation assays

For the wound healing assay, CHO cells were transfected as described above, and grown to confluence in 6-well plates. An artificial wound was created with a yellow Gilson pipette tip, and cells were washed twice with PBS. Phase contrast photographs were acquired with the 10 x objective of a Leica DMIRE2 microscope. Wound closure was accessed by measuring the distance between wound edges at time intervals.

For the slow aggregation assay, 96-well plates were coated with 50 µl agar solution (100 mg Bacto-Agar, 15 ml of PBS). Cells were detached with trypsin-EDTA (Invitrogen), resuspended, and 2 × 10^4 cells were seeded per well. The plate was incubated (37°C, 5%CO₂) and aggregation was evaluated in an inverted microscope (Leica DMIRE2, ×10 magnification) 48 h after seeding, and images captured using a digital camera.

Chicken embryo CAM assays

MKN28 were transduced with shRNAs as described above and inoculated over the CAM as detailed in Supplementary Materials. Briefly, 1 × 10^6 cells were placed on top of E10 growing CAM into a 3 mm silicon ring under sterile conditions. After 3 days, the embryos were euthanized and the CAM was excised from the embryos, photographed ex ovo, at 20 × magnification (Olympus, SZX16 with a DP71 camera). To evaluate angiogenesis, the number of new vessels (<15 µm diameter) growing radial toward the ring area was counted in a blind fashion manner. Excised CAMs were fixed (10% neutral-buffered formalin), paraffin-embedded and stained with hematoxylin–eosin to detected invasive cells. The samples were processed as detailed in Supplementary Materials. Slides were subsequently incubated with mouse monoclonal antibody against pancytokeratin (Sigma). Invasion was evaluated under the microscope, in a blind fashion manner, by three independent users (scored 1 if invasive, and 0 if not invasive).

Immunohistochemistry

The human tissue samples are part of a large Tissue Microarray Analysis platform, annotated in the Human Protein Atlas database (24,25). The samples were collected from surgical specimens, in accordance with approval from the local ethics committee (Stockholm, Sweden). All antibodies were previously validated. The protocols used for immunohistochemistry and
digital imaging of the tissues was described previously (25). Immunohistochemistry of Ecad and DNAJB4 from the 12 available stomach cancer samples are included as supplementary data (Supplementary Material, Table S1), and the comparison between the expression of both proteins, in normal and cancer tissues, is summarized in Supplementary Material, Table S2. Representative Ecad and DNAJB4 stainings were chosen from Normal (Patient 2101) and Stomach Cancer (Patients 2105 and 2326) samples, and the antibodies HPA004812 and HPA028385 were chosen for Ecad and DNAJB4 staining, respectively. All the information about the antibodies, validation data, immunostainings, and data usage policies, is available at http://www.proteinatlas.org.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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REFERENCES


