Thèse de Doctorat de l'Université Louis Pasteur Strasbourg I

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Présentée et soutenue publiquement par

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Pour obtenir le grade de
Docteur de l'Université Louis Pasteur Strasbourg I

Sujet de thèse:

# Régulation de l'adhérence médiée par la DE-cadhérine au cours de la migration des cellules de bordure chez la Drosophile

Soutenue le 02 juillet 2004 devant le jury composé de:

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Thèse de Doctorat de l'Université Louis Pasteur Strasbourg I

Disciplin: Biology

Defended by

Anne PACQUELET

To obtain the degree of
Docteur de l'Université Louis Pasteur Strasbourg I

Title:

# Regulation of *Drosophila* E-cadherin mediated adhesion during border cell migration

Defended on the 2nd of July 2004 with the following jury:

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| Mr Jean-Marc Reichhart | Professeur, Strasbourg | Directeur de thèse |
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# SUMMARY

Classic cadherins are major mediators of homophilic cell-cell adhesion during animal development. In many developmental processes cadherin-dependent adhesive interactions between cells are likely to be regulated. A lot has been done to understand how adhesion is regulated in tissue culture experiments, but so far little is known about the relevance of the studied regulatory mechanisms in vivo. We used *Drosophila* as a model to study these putative regulatory mechanisms during development. Several mutant variants of *Drosophila* Epithelial cadherin (DE-cadherin) were generated. Their ability to substitute for endogenous DE-cadherin activity was analyzed in multiple cadherin-dependent processes during *Drosophila* development and oogenesis, in particular during border cell migration, a process that probably requires dynamic adhesion. Using different DE-cadherin variants, I showed that DE-cadherin/p120ctn interaction, DE-cadherin juxtamembrane domain as well as the conserved tyrosines in DE-cadherin cytoplasmic domain are surprisingly all dispensable for DE-cadherin function. DE-cadherin-Δβ/α-catenin (α-catenin fused after DE-cadherin juxtamembrane domain) partially substitutes for endogenous DE-cadherin in border cells, showing that regulation of the link between DE-cadherin and α-catenin is not strictly required for the migration. DE-cadherin-ΔCyt/α-catenin (α-catenin fused after DE-cadherin transmembrane domain) is able to mediate adhesion in the follicular epithelium but does not substitute for endogenous DE-cadherin in border cells, suggesting that some regulatory signals important for the migration may be located in DE-cadherin cytoplasmic domain. However, DE-cadherin-ΔCyt/α-catenin also have some clear subcellular localization defects and it is so far not completely clear if the observed migration defects are really due to lack of adhesion regulation. If this is the case, the precise regulatory mechanisms will still need to be identified.

# RÉSUMÉ (version courte)

Au cours du développement, les cadhérines sont des médiateurs importants de l'adhérence entre cellules. Dans de nombeux processus développementaux, ces interactions intercellulaires doivent très probablement être régulées. Bien que beaucoup d'études aient essayé de déterminer comment l'adhérence peut être régulée dans des cellules en culture, la signification physiologique des mécanismes régulateurs ainsi étudiés n'est pas connue. Nous avons utilisé la Drosophile comme modèle d'étude de ces mécanismes *in vivo*. Nous avons généré plusieurs formes mutantes de la DE-cadhérine (*Drosophila* Epithelial Cadherin) et testé leur capacité à remplacer la DE-cadhérine endogène au cours du développement et de l'ovogenèse, en particulier au cours de la migration des cellules de bordure. Différents mutants DE-cadhérine nous ont permis de montrer que ni l'interaction entre la DE-cadhérine et p120ctn, ni le domaine juxtamembranaire de la DE-cadhérine, ni les tyrosines conservées présentes dans le domaine cytoplasmique ne sont essentielles pour la fonction de la DE-cadhérine. DE-cadhérine-Δβ/α-caténine (α-caténine fusionnée après le domaine juxtamembranaire de la DE-cadhérine) se substitue partiellement à la DE-cadhérine endogène dans les cellules de bordure, montrant que la régulation du lien entre la DE-cadhérine et α-caténine n'est pas strictement nécessaire à la migration. DE-cadhérine-ΔCyt/α-caténine (α-caténine fusionnée après le domaine transmembranaire de la DE-cadhérine) se substitue à la DE-cadhérine endogène dans l'épithélium folliculaire mais pas dans les cellules de bordure, suggérant que des signaux régulateurs importants pour la migration pourraient être présents dans le domaine cytoplasmique. Des expériences supplémentaires seront nécessaires pour confirmer que les défauts de migration observés avec DE-cadhérine-ΔCyt/α-caténine sont bien dus à une absence de régulation. Si cela s'avère être le cas, il restera à identifier plus précisement le(s) mécanisme(s) régulant DE-cadhérine dans les cellules de bordure.

# RÉSUMÉ (version longue)

**INTRODUCTION - Mécanismes permettant la migration cellulaire.**

Le processus de migration cellulaire est essentiel pour la vie de nombreux organismes unicellulaires. Chez les organismes multicellulaires, il joue aussi un rôle très important dans de nombreux processus physiologiques et pathologiques. Deux grands types de stratégies sont utilisés par les cellules pour migrer. De nombreux organismes unicellulaires mais aussi certaines cellules spécialisées chez les métazoaires (par exemple les spermatozoïdes) utilisent des structures spécialisées telles que des cils ou des flagelles pour nager. La plupart des cellules des organismes multicellulaires ainsi que certains unicellulaires (tels que les amibes) migrent sans de telles structures spécialisées, « rampant » grâce à des mouvements amoeboïdes.

La migration cellulaire par mouvements amoeboïdes implique la polarisation des cellules en train de migrer et la formation dynamique de protrusions membranaires – telles que lamellipodes et filopodes - à l'avant. Au niveau de ces extensions membranaires, la polymérisation de filaments d'actine crée la force nécessaire pour faire avancer la membrane cellulaire. Les protrusions membranaires sont stabilisées grâce aux contacts adhésifs qu'elles forment avec le substrat. La contraction des filaments d'actine et de la myosine II permet de faire avancer le reste de la cellule (translocation). L'adhérence au substrat est également nécessaire pour générer une traction suffisante pour obtenir la translocation. Finalement, l'adhérence avec le substrat doit être relachée à l'arrière de la cellule pour que la translocation soit complète. Le trafic de vésicules de l'arrière vers l'avant joue probablement un rôle important dans ces différentes étapes de la migration cellulaire, en particulier en permettant le recyclage des molécules d'adhérence de l'arrière (où il y a dé-adhérence) vers l'avant (où se forment de nouveaux contacts avec le substrat) ainsi que l'extension de la membrane à l'avant de la cellule.

Ces mécanismes de base de la migration par mouvements amoeboïdes ont initialement été décrits pour des cellules en culture, migrant sur un substrat bidimensionnel. *In vivo*, les cellules migrent la plupart du temps au sein de tissus, dans un environnement tridimensionnel.

Des études plus récentes ont montré que les étapes de base sont identiques lorsque des cellules migrent dans un espace tridimensionnel. Cependant, certaines adaptations à cet environnement ont été observées, en particulier des changements de la forme des cellules leur permettant de se glisser dans de petits espaces libres. De plus, des modulations des mécanismes de base de migration semblent très fréquentes d'un type cellulaire à l'autre.

L'adhérence d'une cellule à son substrat est essentielle pour que le processus de migration cellulaire puisse se dérouler correctement. A l'avant d'une cellule en train de migrer, l'adhérence doit être suffisamment forte pour que les forces de traction exercées par le cytosquelette puissent déclencher la translocation du corps cellulaire. A l'inverse, cette translocation ne peut avoir lieu que si l'arrière de la cellule se détache du substrat. Cela signifie donc qu'il existe très probablement des mécanismes capables de réguler l'adhérence entre une cellule en train de migrer et son substrat. Les caractéristiques de cette adhérence ont principalement été étudiées dans le cas de cellules de mammifères en culture migrant sur une matrice extracellulaire. Dans ce cas, l'adhérence à la matrice extracellulaire est médiée par les intégrines. Des observations en temps réel ont permis d'analyser la dynamique de formation des contacts adhésifs avec la matrice à l'avant de cellules en train de migrer ainsi que le désassemblage des complexes adhésifs à l'arrière.

*In vivo* certaines cellules migrent en utilisant une matrice extracellulaire comme substrat de migration et les intégrines comme molécules adhésives. D'autres cellules en revanche migrent en adhérant non pas à une matrice extracellulaire mais à des cellules environnantes. Cela a en particulier été décrit dans plusieurs exemples de neurones migrant en adhérant à des cellules non neuronales voisines. Cela est également le cas des cellules de bordure chez la Drosophile qui migrent en utilisant les cellules mourricières des follicules ovariens comme substrat de migration.

## INTRODUCTION - Cadhérines et adhérence.

Les cadhérines classiques sont des protéines à un segment transmembranaire capables de générer une adhérence intercellulaire de type homophilique en interagissant avec d'autres molécules de cadhérines présentes à la surface de cellules voisines. Le domaine extracellulaire

des cadhérines contient plusieurs répétitions d'un motif appelé « répétition cadhérine » (CR). En présence de calcium, ces motifs CR permettent la formation de cis-dimères entre molécules de cadhérines présentes à la membrane d'une même cellule. La formation de cis-dimères est un prérequis pour la formation de trans-dimères antiparallèles entre molécules de cadhérines situées à la surface de deux cellules différentes. La formation de ces trans-dimères est à la base de l'adhérence homophilique entre deux cellules exprimant des cadhérines.

Les molécules de cadhérines sont reliées au cytosquelette d'actine par des protéines de la famille des caténines. Le domaine C-terminal de la région cytoplasmique des molécules de cadhérines interagit avec la β-caténine qui se lie à l'α-caténine. L'α-caténine est alors reliée aux filaments d'actine, directement ou par l'intermédiaire d'autres protéines telles que l'α-actinine et la vinculine**.**

Les cadhérines permettent l'adhérence entre cellules dans des processus très variés. Par exemple, elles sont nécessaires pour maintenir la cohésion entre cellules épithéliales, ce qui nécessite probablement une adhérence forte et stable. Elles sont aussi impliquées dans des tissus soumis à des mouvements morphogénétiques ou sont utilisées par des cellules en train de migrer pour adhérer à leur substrat. Dans ces deux exemples, l'adhérence due aux cadhérines est vraisemblablement beaucoup plus dynamique. Il est donc très probable que des mécanismes régulateurs soient requis pour que les cadhérines puissent effectuer leurs différentes fonctions au sein d'un organisme. De nombreuses expériences effectuées sur des cellules en culture suggèrent que de multiples mécanismes peuvent être impliqués pour générer une adhérence forte ou au contraire pour réduire ou rendre plus dynamique l'adhérence entre deux cellules. En particulier, des changements d'intensité d'adhérence peuvent être dus à des modifications de l'association entre cadhérine et caténines, à l'interaction avec des protéines telles que la p120 caténine (p120ctn), à la phosphorylation/ déphosphorylation des cadhérines ou caténines ou encore à l'endocytose des complexes cadhérine/caténines.

## INTRODUCTION - Un modèle d'étude : la migration des cellules de bordure.

Dans le laboratoire, nous utilisons comme modèle d'étude du processus de migration cellulaire la migration des cellules de bordure au cours de l'ovogenèse chez la Drosophile.

Les follicules ovariens de la Drosophile sont formés par 16 cellules de la lignée germinale – un ovocyte et 15 cellules nourricières – qui sont entourées par les cellules somatiques de l'épithélium folliculaire. Les cellules de bordure sont un groupe de 8 cellules initialement localisées au pôle antérieur de l'épithélium folliculaire. Au début du stade 9 de l'ovogenèse, les cellules de bordure délaminent de l'épithélium folliculaire puis migrent en direction du pôle postérieur du follicule ovarien, jusqu'à atteindre l'ovocyte au stade 10 de l'ovogenèse. Les cellules de bordure migrent entre les cellules nourricières qu'elles utilisent comme leur substrat. Il a en effet été montré que les cellules de bordure adhèrent aux cellules nourricières grâce à la DE-cadhérine (Drosophila Epithelial Cadherin). Cette adhérence homophilique médiée par la DE-cadhérine est nécessaire pour la migration. La DE-cadhérine est une cadhérine classique qui est aussi impliquée dans l'adhérence entre cellules épithéliales. Nous avons supposé que cette molécule doit pouvoir être régulée de façon à pouvoir dans certains cas créer une adhérence forte et stable - par exemple dans certains épithéliums - et dans d'autres situations médier une adhérence beaucoup plus dynamique, comme cela est probablement le cas au cours de la migration des cellules de bordure. Ce sont les mécanismes permettant cette régulation, que j'ai cherché à identifier au cours de ma thèse, en considérant principalement la fonction de la DE-cadhérine au cours de la migration des cellules de bordure.

## RÉSULTATS ET DISCUSSION - Ni l'interaction entre p120ctn et DE-cadhérine, ni le domaine juxtamembranaire de DE-cadhérine ne sont nécessaires pour la fonction ou la régulation de DE-cadhérine.

Plusieurs expériences effectuées sur des cellules en culture suggèrent que le domaine juxtamembranaire des cadhérines est important. En particulier, ce domaine interagit avec p120ctn, une autre protéine de la famille des caténines considérée comme un régulateur important des cadhérines. Afin de savoir si l'interaction de la DE-cadhérine avec p120ctn était importante pour la fonction ou la régulation de la DE-cadhérine, j'ai produit un mutant ponctuel de la DE-cadhérine (appelé DE-cadhérine-AAA) incapable de lier p120ctn. Pour tester un possible autre rôle du domaine juxtamembranaire, j'ai également produit une forme

mutante de la DE-cadhérine sans domaine juxtamembranaire (DE-cadhérine-ΔJM). Pour analyser la fonctionnalité de DE-cadhérine-AAA et DE-cadhérine-ΔJM au cours de l'ovogenèse et de l'embryogenèse, j'ai généré des lignées de mouches transgéniques exprimant ces formes mutantes de la DE-cadhérine de façon ubiquitaire. J'ai supprimé la DE-cadhérine endogène de certains tissus en utilisant des mutants nuls pour la DE-cadhérine puis testé la capacité de DE-cadhérine-AAA et DE-cadhérine-ΔJM à se substituer à la DE-cadhérine endogène, c'est-à-dire à sauver le phénotype des mutants nuls pour la DE-cadhérine.

Il s'est avéré que DE-cadhérine-AAA comme DE-cadhérine-ΔJM se substituent complètement à la DE-cadhérine endogène pour permettre la migration des cellules de bordure, à la fois dans les cellules de bordure elles-mêmes et dans les cellules nourricières. De façon tout à fait inattendue, DE-cadhérine-AAA comme DE-cadhérine-ΔJM se substituent aussi totalement à la DE-cadhérine endogène pour toutes les autres fonctions de la DE-cadhérine que j'ai testées au cours de l'ovogenèse et de l'embryogenèse. En particulier, DE-cadhérine-AAA et DE-cadhérine-ΔJM sont capables de sauver la létalité due à l'absence complète de la DE-cadhérine endogène au cours de l'embryogenèse. Ces résultats montrent, de façon très surprenante, que ni l'interaction entre la DE-cadhérine et p120ctn ni le domaine juxtamembranaire de la DE-cadhérine ne sont essentiels à la fonction ou à la régulation de la DE-cadhérine.

Un autre laboratoire a généré des mutants nuls pour p120ctn et obtenu des résultats cohérents avec ceux décrits ci-dessus: de façon tout à fait inattendue les mutants nuls pour p120ctn sont viables et n'ont pas de défauts d'adhérence apparents. De façon similaire, la déplétion de p120ctn chez *C.elegans* ne semble pas créer de défauts. Dans les deux systèmes, il a été montré que l'absence de p120ctn augmente les défauts dus à l'absence de cadhérine ou α-caténine. Ainsi p120ctn n'est pas un composant essentiel des junctions adhérentes chez la Drosophile et *C.elegans* mais semble être un modulateur positif de l'adhérence médiée par les cadhérines. Ceci contraste avec des données récentes obtenues chez les vertébrés montrant que p120ctn est essentielle au cours du développement et pour la function des cadhérines.

**RÉSULTATS ET DISCUSSION - La phosphorylation des résidus tyrosine conservés de DE-cadhérine et β-caténine n'est pas nécessaire pour la fonction ou la régulation de DE-cadhérine.**

De nombreuses tyrosine kinases et phosphatases semblent impliquées dans la régulation de l'adhérence médiée par les cadhérines. Les récepteurs de l'EGF et du PDGF étant impliqués dans la migration des cellules de bordure, en particulier pour le guidage des cellules, la (dé)phosphorylation de la DE-cadhérine ou d'autres composants des jonctions nous semblait un mécanisme plausible de régulation de l'adhérence au cours de la migration.

La DE-cadhérine contient huit tyrosines dans son domaine cytoplasmique, quatre sont conservées entre la Drosophile et les mammifères. Pour tester le rôle potentiel de la phosphorylation des tyrosines de la DE-cadhérine, nous avons choisi de muter en phénylalanine les quatre tyrosines conservées du domaine cytoplasmique de la DE-cadhérine. Nous avons ainsi obtenu DE-cadhérine-4YF. Des lignées transgéniques exprimant DE-cadhérine-4YF de façon ubiquitaire ont été générées. J'ai ensuite testé la capacité de DE-cadhérine-4YF à se substituer à la DE-cadhérine endogène dans différents tissus : DE-cadhérine-4YF peut sauver l'absence de la DE-cadhérine endogène au cours de la migration des cellules de bordure, à la fois dans les cellules de bordure elles-mêmes et dans les cellules nourricières. DE-cadhérine-4YF se substitue également à la DE-cadhérine endogène dans tous les autres processus que j'ai examinés au cours de l'ovogenèse. Au cours de l'embryogenèse, DE-cadhérine-4YF est capable de sauver la létalité due à l'absence complète de la DE-cadhérine endogène. Les 4 tyrosines conservées de la DE-cadhérine – et par conséquent leur phosphorylation - ne sont donc pas essentielles à la fonction ou à la régulation de la DE-cadhérine.

On ne peut pour l'instant pas exclure que les 4 autres tyrosines du domaine cytoplasmique de la DE-cadhérine et leur phosphorylation ne sont pas nécessaires à la fonction de la DE-cadhérine. Bien que non conservées chez les mammifères, ces 4 tyrosines semblent être conservées chez l'Anophèle. De plus, il a été montré chez les mammifères que la phosphorylation de 2 tyrosines du domaine cytoplasmique de la E-cadhérine est impliquée dans la régulation de la E-cadhérine par endocytose. Or ces 2 tyrosines ne sont pas

conservées. De facçn similaire, il n'est donc pas impossible que les 4 tyrosines de la DE-cadhérine non conservées chez les mammifères soient impliquées dans la régulation de la DE-cadhérine.

Dans des cellules en culture, la phosphorylation de la β-caténine sur une tyrosine conservée a souvent été corrélée avec une dissociation entre la β-caténine et cadhérines ainsi qu'avec une réduction de l'adhérence médiée par les cadhérines. Chez la Drosophile, cette tyrosine conservée est la tyrosine Y667. Cette tyrosine a été mutée en phénylalanine pour obtenir β-caténine-Y667F. J'ai ensuite analysé l'activité de β-caténine-Y667F au cours de l'ovogenèse en testant sa capacité à se substituer à la β-caténine endogène. Il s'avère que β-caténine-Y667F se substitue à la β-caténine endogène dans les cellules germinales et les cellules folliculaires. β-caténine-Y667F est également capable de sauver l'absence de la β-caténine endogène dans les cellules de bordure. Ainsi la tyrosine Y667 et sa phosphorylation ne sont pas essentielles à la fonction de la β-caténine au cours de l'ovogenèse et en particulier au cours de la migration des cellules de bordure.

**RÉSULTATS ET DISCUSSION - Informations apportées par l'étude de protéines de fusion DE-cadhérine/α-caténine.**

Pour déterminer si le lien entre la DE-cadhérine et l'α-caténine doit être régulé au cours de la migration des cellules de bordure, j'ai généré différentes protéines de fusion entre la DE-cadhérine et l'α-caténine : la fusion de l'α-caténine avec la DE-cadhérine élimine toute possibilité de régulation de l'association entre la DE-cadhérine et la β- ou α-caténine.

J'ai obtenu ces protéines en fusionnant l'α-caténine à la DE-cadhérine entière (DE-cadhérine-FL/α-caténine), à la DE-cadhérine sans domaine C-terminal (DE-cadhérine-Δβ/α-caténine) et à la DE-cadhérine sans aucun domaine cytoplasmique (DE-cadhérine-ΔCyt/α-caténine). J'ai exprimé ces protéines avec le système UAS-Gal4 et vérifier qu'elles étaient fonctionnelles en testant leur capacité à sauver l'absence de la DE-cadhérine endogène dans les cellules de l'épithélium folliculaire. J'ai ensuite testé leur activité dans les cellules de bordure.

Les cellules de bordure n'exprimant que DE-cadhérine-FL/α-caténine migrent normalement, suggérant qu'une régulation de l'association entre la DE-cadhérine et la β- ou α-caténine

n'est pas du tout nécessaire à la migration. Cependant, cette construction est susceptible d'interagir avec la β-caténine endogène et pourrait donc être soumise à une régulation via la β-caténine et l'α-caténine endogènes ; l'expérience devra donc être répétée en absence de toute β-caténine. Les cellules de bordure n'exprimant que DE-cadhérine-Δβ/α-caténine ont seulement de légers défauts de migration. Ceci montre qu'une régulation de l'association entre la DE-cadhérine et la β- ou α-caténine n'est pas strictement requise pour la migration des cellules de bordure. Il est cependant possible qu'une telle régulation contribue à réguler l'adhérence, sans être le mécanisme régulateur principal. Ceci pourrait expliquer les légers défauts de migration observés. Alternativement, ces défauts de migration pourraient s'expliquer par l'absence d'un signal régulateur normalement localisé dans le domaine C-terminal de la DE-cadhérine.

Les cellules de bordure n'exprimant que DE-cadhérine-ΔCyt/α-caténine sont presque totalement incapables de migrer, montrant que DE-cadhérine-ΔCyt/α-caténine ne peut se substituer à la DE-cadhérine endogène dans les cellules de bordure. En revanche, DE-cadhérine-ΔCyt/α-caténine est capable de sauver l'absence de la DE-cadhérine endogène dans les cellules folliculaires. DE-cadhérine-ΔCyt/α-caténine semble donc bien être fonctionnelle. L'absence de migration des cellules de bordure pourrait donc être due non pas à une insuffisance d'adhérence mais plutôt à une absence de régulation de la DE-cadhérine ; ceci conduirait à une adhérence entre cellules de bordure et cellules nourricières trop stable et inhibant de ce fait la migration. De facon cohérente avec cette hypothèse, la surexpression de DE-cadhérine-ΔCyt/α-caténine dans des cellules de bordure possédant également la DE-cadhérine endogène inhibe la migration. Cependant, il faut mentionner que lorsque les cellules folliculaires ou les cellules de bordure expriment seulement DE-cadhérine-ΔCyt/α-caténine, cette protéine de fusion s'accumule de façon importante à l'intérieur des cellules. On ne peut pour le moment totalement exclure que cette accumulation n'a pas d'effets negatifs sur la migration, par exemple en recrutant des éléments du cytosquelette ou des molécules de signalisation essentiels à la migration. Une protéine de fusion supplémentaire (DE-cadhérine-ΔCyt/CD2/α-caténine) a été obtenue en insérant le domaine cytoplasmique de CD2 entre DE-cadhérine-ΔCyt et l'α-caténine. Des résultats préliminaires semblent indiquer que DE-cadhérine-ΔCyt/CD2/α-caténine ne s'accumule pas de façon aussi importante que DE-

cadhérine-ΔCyt/α-caténine à l'intérieur des cellules. Si cela est vraiment le cas, l'analyse de la migration de cellules de bordure exprimant seulement DE-cadhérine-ΔCyt/CD2/α-caténine devrait alors permettre de mieux comprendre l'origine de l'absence de migration observée avec DE-cadhérine-ΔCyt/α-caténine.

Si les défauts de migration observés avec DE-cadhérine-ΔCyt/α-caténine sont bien dus à une absence de régulation de la DE-cadhérine, il reste à identifier quels sont les signaux régulateurs importants pour la fonction de la DE-cadhérine et absents dans DE-cadhérine-ΔCyt/α-caténine. Les légers défauts de migration observés avec DE-cadhérine-Δβ/α-caténine suggèrent qu'une régulation de l'association entre la DE-cadhérine et l'α-caténine ou qu'un signal régulateur localisé dans le domaine C-terminal participent à la regulation de la DE-cadhérine. La quasi-absence de migration observée avec DE-cadhérine-ΔCyt/α-caténine montrerait alors qu'un autre signal régulateur se trouve dans le domaine juxtamembranaire. Une possibilité serait que le domaine C-terminal ainsi que le domaine juxtamembranaire contiennent tous deux des signaux contrôlant la dynamique de la DE-cadhérine, par exemple en favorisant son adressage vers les vésicules d'endocytose. Jusqu'à présent, aucune interaction génétique n'a pu être détectée entre la DE-cadhérine et divers élements de la voie d'endocytose. Cependant, je n'ai testé que des interactions dans des contextes mutants hétérozygotes et il est tout à fait possible que cela ne suffise pas à révéler d'éventuels liens entre la DE-cadhérine et l'endocytose.

Il est également possible que de nombreux mécanismes contribuent à la régulation de la DE-cadhérine au cours de la migration des cellules de bordure et que la suppression d'un seul de ces mécanismes ne soit pas suffisante pour bloquer la migration. Ceci expliquerait pourquoi aucun mécanisme régulateur précis n'a pu être identifié jusqu'à présent et pourquoi seule DE-cadhérine-ΔCyt/α-caténine – qui potentiellement échappe simultanément à plusieurs niveaux de régulation - inhibe la migration.

# LIST OF ABBREVIATIONS

aa: amino-acid

ADP: Adenosine Di-Phosphate

AFG: Actin-Flipp-out-Gal4

arm: armadillo

ATP: Adenosine Tri-Phosphate

BSA: Bovin Serum Albumin

cDNA: complementary Desoxyribo Nucleic Acid

CK II: Casein Kinase II

CHO: Chinese Hamster Ovary

CR: Cadherin Repeat

DE-cadherin: *Drosophila* Epithelial Cadherin

DN-cadherin: *Drosophila* Neuronal Cadherin

dsRNA: double stranded RiboNucleic Acid

E-cadherin: Epithelial Cadherin

EGF: Epidermal Growth Factor

FAK: Focal Adhesion Kinase

FRT: Flippase Recognition Target

GFP: Green Fluorescent Protein

GTP: Guanosine Tri-Phosphate

hsFLP: heatshock-Flippase

KHC: Kinesin Heavy Chain

krGFP: kruppel-GFP

MARCM: Mosaic Analysis with a Repressible Cell Marker

MDCK: Madin-Darby Canine Kidney

MECs: Microvascular Endothelial Cells

N-cadherin: Neuronal Cadherin

NCCD: Nonchordate Classic Cadherin Domain

NGS: Normal Goat Serum

NMR: Nuclear Magnetic Resonance

NPG: N-Propyl Gallate

p120ctn: p120 catenin

PDZ: Postsynaptic density protein 95/Drosophila Disks large/Zona occludens-1

PT: PBS + 0.1% TritonX100

PDGF/VEGF: Platelet Derived Growth Factor / Vascular Endothelial Growth Factor

RNAi: RiboNucleic Acid mediated interference

shg: shotgun

shi: shibire

tubGal4: tubulin-Gal4

tubGal80: tubulin-Gal80

UAS: Upstream Activating Sequence

VE-cadherin: Vascular-Endothelial cadherin

Wg: Wingless

# 1 INTRODUCTION

## 1.1 Cell migration

### 1.1.1 Importance

Anton van Leeuwenhoek was the first to observe “animalcules” under his microscope at the end of the seventeenth century and got fascinated by their ability to move. Describing some ciliates he had observed, he wrote: *" I must have seen quite 20 of these little animals on their long tails alongside one another very gently moving, with outstretched bodies and straightened-out tails; yet in an instant, as it were, they pulled their bodies and their tails together, and no sooner had they contracted their bodies and tails, than they began to stick their tails out again very leisurely, and stayed thus some time continuing their gentle motion: which sight I found mightily diverting."*

Since then, researchers realized that cell motility is crucial for the life of many unicellular organisms that need to reach food and adapt to their environment. But cell migration also plays an essential role in multiple physiological as well as pathological processes in multicellular organisms. During development, some cells, such as the neural crest cells or the primordial germ cells, have to migrate from their site of origin towards their final destination. In the adult organism, some cells also have to migrate in order to fulfill their function. Leukocytes for instance migrate through the endothelial barrier and then through the connective tissues to reach infection sites.

Cell migration is a strictly regulated process and deregulation can result in severe pathological situations. Migration defects can result in many developmental abnormalities. Also, one of the critical changes during malignant transformation is the acquisition of motility: cells can then detach and migrate away from the primary tumour to colonize other organs and form secondary metastasis.

### 1.1.2 Swimming and crawling

Organisms have evolved two basic strategies of cell migration: swimming and crawling. Many unicellular organisms living in a liquid environment as well as some highly specialized vertebrate cells (e.g. sperm) move by swimming. This required some specialized structures, such as rotating or beating cilia or flagella (Fig1.1A-B). However, most animal cells as well

as some unicellular organisms (e.g. amoeba) move without such specialized structures, by crawling on a substratum (Fig.1.1C).

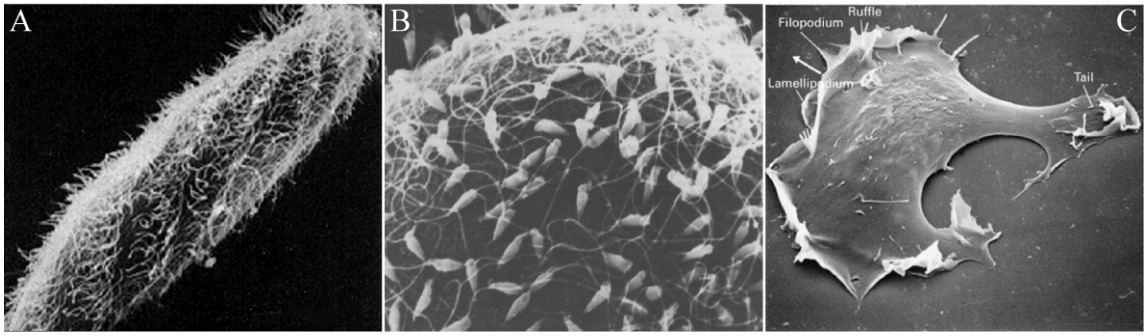



**Figure 1.1: Swimming and crawling. A.** Multiple cilia of a paramecium. **B.** Long flagella of sperms moving on the surface of an oocyte. **C.** Crawling fibroblast: the cell moves toward the left, on the front one can observe broad lamellipodia and thin filopodia. All the pictures are scanning electron micrographs.

### 1.1.3 Molecular mechanisms involved in cell crawling

Crawling involves polarization of the migrating cell and formation of very dynamic membrane protrusions at the front. Filopodia are very thin and long cylindrical extensions whereas lamellipodia are broader and shorter structures. In these extensions, the force required to push the membrane forward is provided by the polymerization of actin filaments (Mogilner and Oster, 1996). These cellular extensions are stabilized by forming adhesive contacts with the substratum. The contraction of myosin II and actin filaments is responsible for pulling the rest of the cell body along. Adhesion to the substratum is also required to obtain enough traction for this translocation. Finally, adhesive contacts have to be released at the back of the cell in order to allow the tail to retract. Thus crawling movements can be seen as a continuous cycle of (1) forming new protrusions, (2) adhering to the substratum, (3) translocation of the cell body and (4) releasing adhesive contacts and retracting the tail (Fig.1.2) (Michison and Cramer, 1996). Polarized trafficking of vesicles, from the back to the front of a migrating cell probably plays an important role for these different steps of cell crawling. In particular, exocytosis has been shown to occur at the leading edge. It is thought to participate in the formation of membrane extensions and to deliver new adhesive molecules to allow adhesion of newly formed extensions to the substratum (Bretscher, 1996). At the

back of the cell, endocytosis could participate in releasing adhesive contacts with the substratum; endocytosed adhesive receptors could then be recycled to the leading edge (see §1.1.3.3).

Basic mechanisms involved in cell crawling have initially been described in the case of cells migrating in culture, on a two-dimensional substrate. In vivo cells usually migrate inside a tissue, in a three-dimensional environment. Recent experiments have shown that the basic steps of cell migration are similar when cells migrate in a 3D-matrix (for a review see (Friedl, 2004)). However, some adaptations to the 3D-environment were observed, in particular cell shape changes allowing squeezing through narrow spaces. Such adaptations are likely to also occur in an organism. Moreover, cell type specific modulations of these basic mechanisms seem to be quite frequent (Friedl, 2004).

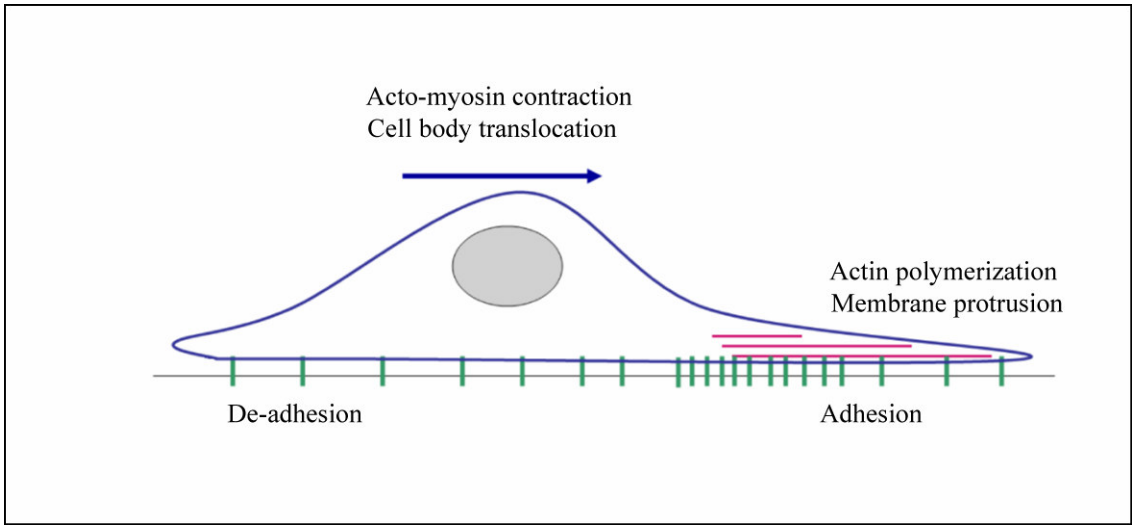



**Figure 1.2: Mechanisms of cell crawling.** Actin polymerization at the front of the cell drives the formation of membrane protrusions. Adhesion to the substratum stabilizes the protrusions. Acto-myosin contraction and adhesion provide the forces to translocate the cell body forward. De-adhesion in the back is also required for the translocation.

### 1.1.3.1 Actin polymerization

Lamellipodia contain a very dense network of actin filaments which are all oriented with their fast growing ends (also called barbed or plus ends) close to the membrane. Electromicroscopy on keratocytes lamellipodia showed that actin filaments are oriented obliquely relative to the

plasma membrane and that the back of a filament is very often linked on the side of another filament, thus forming "Y" junctions. These Y junctions are observed all over the lamellipodia but are more abundant in the front (Fig.1.3) (Svitkina et al., 1997). Actin filaments in lamellipodia are highly dynamic: actin monomers bound to ATP are added at the plus ends of filaments, close to the membrane; this is followed by a flux of actin towards the back of the lamellipodium; filaments then depolymerize at the back of the lamellipodium, providing a constant pool of actin monomers to be used for polymerization at the front (Fig.1.4). Several proteins interact with actin monomers or filaments to tightly control this actin dynamics.

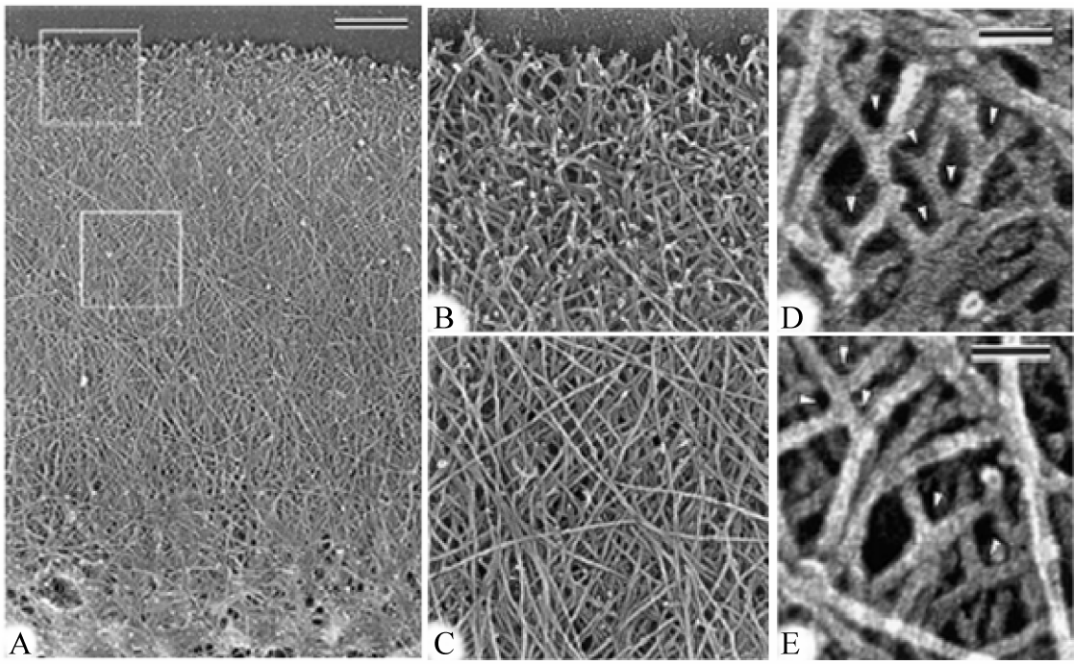



**Figure 1.3: Organization of actin filaments in keratocyte lamellipodia. A.** Actin network in a lamellipodium from the leading edge (top) to the back of the lamellipodium (bottom). **B.** Actin network at the leading edge with many short filaments and filament ends. **C.** Actin network in the middle of the lamellipodium. **D-E.** "Y" junctions between actin filaments in the front (D) and the middle (E) of the lamellipodium. Bars: A. 1µm. D-E. 50nm. From (Svitkina et al., 1997).

Initial studies of actin dynamics have lead to the following model: in the front of a lamellipodium, the Arp2/3 complex binds to the side of existing actin filaments where it nucleates a new filament with its barbed end pointing towards the membrane (Fig.1.4) (Mullins et al., 1998). Actin polymerization can then occur at this new barbed end: this

generates a new actin filament which will grow out from the existing filament towards the cortex. This mode of nucleation allows the binding of new filaments to existing ones and the assembly of the polarized actin network described above (Fig1.4). Free barbed ends may also be generated by ADF/cofilin, a protein that severs actin filaments at the back of the lamellipodium (Maciver et al., 1991). On the other hand, proteins such as the capping protein can bind to the free barbed ends, preventing further polymerization (Eddy et al., 1997): the number of free barbed ends available for polymerization can thus be tightly regulated. At the back of the lamellipodium, ADF/cofilin enhances filaments depolymerization by accelerating

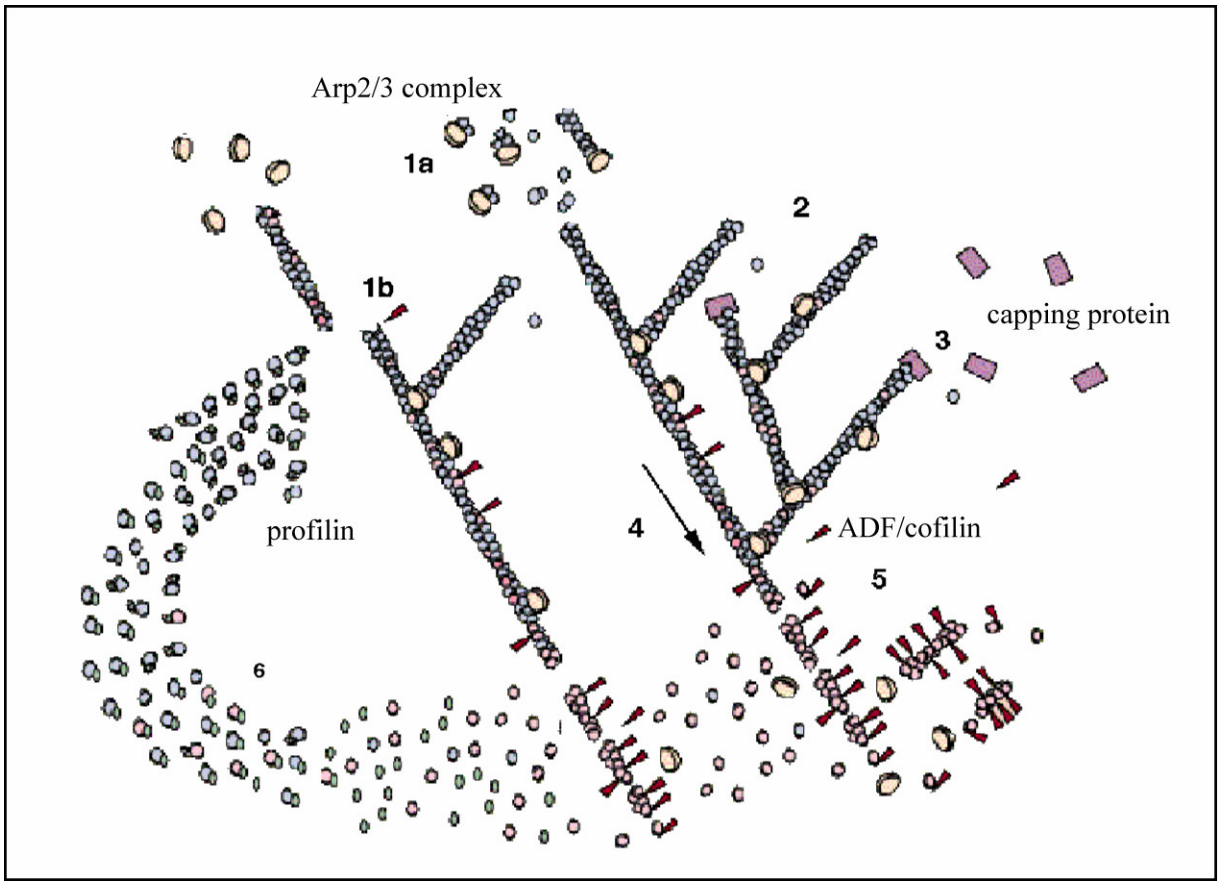



**Figure 1.4: Model of actin dynamics in lamellipodia.** Scheme is oriented with leading edge of lamellipodia on the top. **1:** formation of new free barbed ends by the Arp2/3 complex (1a) and ADF/cofilin (1b). **2:** filament elongation towards the membrane, actin monomers are bound to ATP. **3:** capping of the barbed ends. **4:** hydrolysis of actin-bound ATP. **5:** depolymerization and filament severing by ADF/cofilin. **6:** binding of actin monomers to profilin and ADP/ATP exchange. Modified from (Machesky and Way, 1998).

the dissociation of actin monomers and by severing filaments (Carlier et al., 1997; Maciver et al., 1991). After the depolymerization, actin monomers are associated with ADP. They can bind to profilin which stimulates the ADP/ATP exchange (Goldschmidt-Clermont et al., 1991). Actin monomers bound to ATP can then be presented by profilin to the barbed ends of actin filaments at the front of the cells and thus be incorporated in new filaments (Fig1.4).

More recent studies suggest that Arp2/3 is not generally required for actin polymerization. Processes involving Arp2/3 independent actin polymerization have been described in several systems (e.g. (Evangelista et al., 2002; Hudson and Cooley, 2002; Severson et al., 2002)). In yeast and *C.elegans*, proteins of the Formin family have been shown to mediate this Arp2/3 independent actin polymerization (Evangelista et al., 2002) (Severson et al., 2002).

#### 1.1.3.2 Generation of traction forces

Translocation of the cell body requires traction forces which are generated by myosin II (Doolittle et al., 1995). Myosin II is localized at the back of a migrating cell and one can observe a band at the boundary between the lamellipodium and the cell body where myosin II and actin filaments colocalize (Svitkina et al., 1997). Myosin II may cause the contraction of actin filaments in this region which would pull the cell body forward. Probably myosin II and/or actin filaments have to be somehow linked to the nucleus, to organelles and to cytoplasmic components in order to pull them forward. Also, the contraction forces cause the translocation only if actin filaments are connected to the substratum through adhesion molecules. The detailed mechanisms of how actin-myosin contractility can account for the net movement of the cell body are still not known.

#### 1.1.3.3 Adhesion

Adhesion of a migrating cell to its substratum is essential for the stabilization of newly formed membrane protrusions as well as for the traction of the cell body. New adhesive complexes are assembled at the front of the cell, however the translocation can occur only if there is concomitantly de-adhesion at the rear of the cell. Adhesion between a migrating cell and its substratum is thus a very dynamic process which is likely to be regulated. It has been shown that there is an optimum value for the adhesive strength of a cell to its substratum that

gives maximal speed of migration; changes of adhesive strength above or below this value slows the migration down: too low adhesiveness inhibits lamellipodia stabilization whereas high adhesiveness seems to prevent cell body translocation (Palecek et al., 1997).

Adhesion during cell migration has mostly been studied in cultured mammalian cells migrating on extracellular matrix. In those cases, adhesion is mediated by integrins, which are single pass transmembrane molecules that function as dimers of α and β subunits and are linked to actin filaments. Integrins have a large extracellular domain that binds to proteins of the extracellular matrix and a short cytoplasmic domain which binds to many cytosolic proteins important for cytoskeletal interactions and adhesion regulation (reviewed in (Petit and Thiery, 2000)). Time-lapse observations of migrating cells have shown that small adhesive complexes (also called focal complexes) form at the leading edge. In the back of the lamellipodium, these complexes either disassemble or mature in larger and more stable structures (also called focal adhesions) (Laukaitis et al., 2001). Analysis of the traction forces exerted by migrating fibroblast showed that the small adhesive complexes near the leading edge transmit strong propulsive forces, whereas the mature focal adhesions exert only weaker forces (Beningo et al., 2001). Hence, as focal adhesions mature, changes in structure, protein content, or phosphorylation may cause the focal adhesion to change its function, from the transmission of strong propulsive forces to a more passive anchorage function. At the back of the cell, disassembly of adhesive complexes allows de-adhesion of the cell from the substratum. A severing of the link between integrins and cytoskeletal components has been observed in the back of migrating fibroblasts (Laukaitis et al., 2001). At least part of the integrin molecules is then incorporated into vesicles and then transported forward (Laukaitis et al., 2001; Palecek et al., 1996). This integrin recycling has been shown to be essential for motility (Lawson and Maxfield, 1995). Integrins dynamics is regulated in particular by the Focal Adhesion Kinase (FAK): fibroblasts lacking FAK or FAK kinase activity have more mature focal adhesions and migrate more slowly than fibroblasts with normal FAK activity (for a review see (Parsons, 2003)).

In vivo many migrating cells also use the extracellular matrix as a substratum and integrins as adhesion molecules. However, some other cells use neighbouring cells as their adhesive substratum. In particular several cases of neurons migrating on non neuronal cells have been described: N-cadherin or other adhesive molecules mediate the interaction between migrating

neurons and the surrounding non neuronal cells and this adhesive interaction is essential for the migration (e.g. (Barami et al., 1994; Letourneau et al., 1990)). N-cadherin is also expressed in some highly invasive tumour cell lines and promotes the migration of those cells on other cells (Hazan et al., 2000) (Li et al., 2001). Finally, during *Drosophila* oogenesis DE-cadherin serves as an adhesion molecule between migrating border cells and the surrounding nurse cells which constitute the substratum for border cell migration (see §1.3.5) (Niewiadomska et al., 1999).

One should also mention that when cells migrate in vivo, they can extend and retract protrusions for long periods of time until one of these protrusions get stabilized by adhesion molecules (Knight et al., 2000). This stabilization may occur in response to extracellular signals, in particular to the presence of guidance cues thus allowing directed migration.

## 1.2 Cadherin-mediated cell-cell adhesion

Cell-cell contacts play extremely important roles during the development and the life of an organism. A great variety of transmembrane proteins involved in cell-cell adhesion exists in vertebrates and invertebrates. They can form very stable junctions that hold cells together and provide the resistance of a tissue to mechanical stresses, in particular in epithelia. They can also mediate much more transient contacts, for example between a leukocyte or a lymphocyte and an endothelial cell during the rolling preceding the extravasation of the leukocyte/lymphocyte.

One important family of proteins involved in the formation of cell-cell contacts is the classic cadherin family. In a mature epithelium, classic cadherins accumulate at discrete junctions, called adherens junctions, which are very organized structures that can be recognized by electron microscopy. But cadherins can also mediate adhesion by forming more diffuse adhesive contacts between two cells, in particular in the initial stages of cell-cell contact formation.

### 1.2.1 The Cadherin superfamily

The first identified members of the cadherin superfamily, or classic cadherins, have been characterized by their ability to mediate calcium dependent adhesion between cells (Hyafil et al., 1981; Hyafil et al., 1980), hence their name.

Since then, other transmembrane proteins containing conserved extracellular repeats also present in classic cadherins have been identified and thus considered to belong to the cadherin superfamily. This cadherin superfamily contains a large number of proteins. For instance, *C. elegans*, *Drosophila melanogaster* and human have respectively 14, 16 and around 80 different cadherin genes, among them respectively only 1, 3 and about 20 classic cadherins genes. Members of the cadherin superfamily show a high degree of variability in the structure of the extracellular and cytoplasmic domains as well as in the number of transmembrane domains (see below and Fig.1.5). The main subfamilies that can be distinguished based on these differences are described below. However some cadherins can not be assigned to one of these subfamilies. For instance, only about half of *C.elegans* and *Drosophila melanogaster* cadherins belong to identified families.

Although classic and desmosomal cadherins are well known for their ability to mediate cell-cell adhesion, it should be mentioned that the adhesive properties of other cadherins are far from being all well characterized.

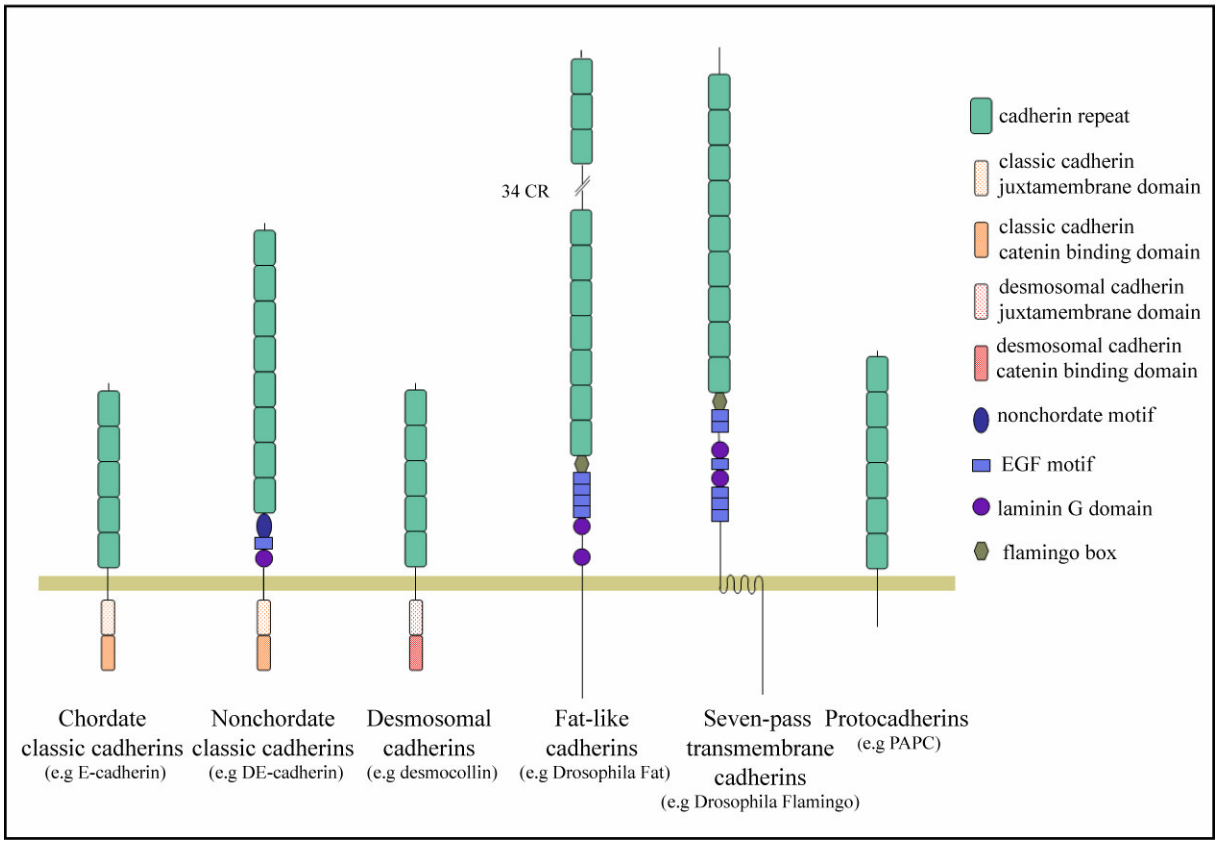



**Figure 1.5: The cadherin superfamily.** The domain composition of representatives of five cadherin subfamilies is shown. Classic cadherins, Fat-like cadherins and seven-pass transmembrane cadherins are found in both vertebrates and invertebrates. Desmosomal cadherins and protocadherins are found only in chordates.

### 1.2.1.1 A common feature: the cadherin repeat (CR)

The common feature of all the members of the cadherin superfamily is the presence in the extracellular region of conserved domains called cadherin repeats (CR). These cadherin repeats are 110 amino-acid peptides able to bind calcium. The three-dimensional structure of the aminoterminal CR of mouse epithelial cadherin has been solved by NMR (Overduin et al., 1995): this CR contains seven β strands organized in a β-barrel (Fig.1.6). A similar structure was determined by crystallography for the aminoterminal CR of mouse neuronal cadherin

(Shapiro et al., 1995). The identification of the residues involved in calcium binding (Overduin et al., 1995) together with the structure of peptides containing two consecutive CR (Nagar et al., 1996) (Pertz et al., 1999; Tamura et al., 1998) were used to determine the structure of the calcium binding site. It consists in a negatively charged pocket formed by three short amino acid motifs present in one CR and by one motif present in the following CR. This pocket can bind three calcium ions (Nagar et al., 1996) (Fig1.6). The binding of calcium between two consecutive CR could explain the conformational changes and the rigidification of the cadherin extracellular domain observed upon calcium addition (Pokutta et al., 1994).

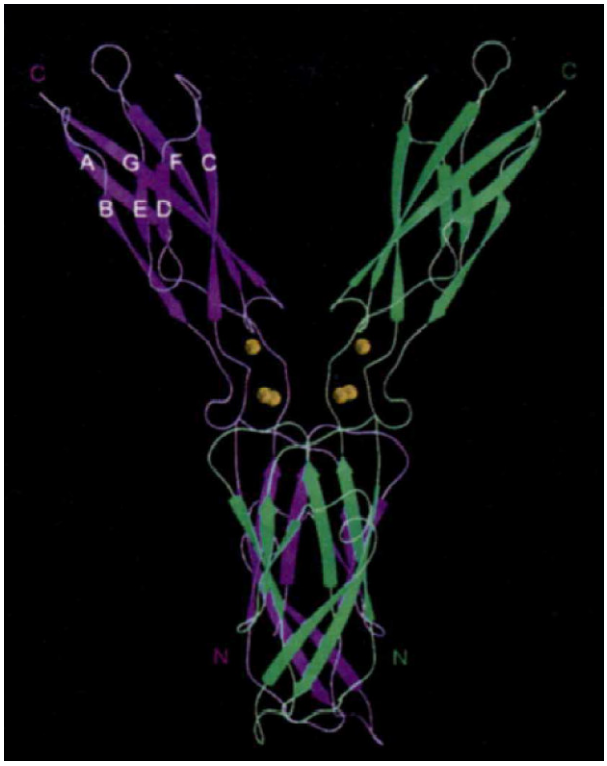



**Figure 1.6: Structure of the cadherin repeats and calcium binding pocket.** Each color corresponds to one E-cadherin molecule. The structure of the 2 aminoterminal cadherin repeats of each molecule is represented. Each cadherin repeat is a 7-stranded β-barrel (A-G). 3 calcium ions (yellow spheres) bind in the calcium binding pocket which is located at the linker region between two consecutive cadherin repeats. From (Nagar et al., 1996).

#### 1.2.1.2 Classic cadherin

Classic cadherins have only one transmembrane domain, are characterized by their highly conserved cytoplasmic domain and linked to the actin cytoskeleton. They mediate homophilic cell-cell adhesion.

The cytoplasmic domain can be subdivided in a juxtamembrane domain and a C-terminal domain (Fig.1.7). The C-terminal domain binds to β-catenin (Ozawa et al., 1989) (Ozawa et al., 1990). This binding is required to link cadherins to actin filaments (Ozawa et al., 1990) (Fig.1.7; see §1.2.4.1). The juxtamembrane domain contains a binding site for members of the p120 catenin (p120ctn) family and is possibly also involved in mediating lateral clustering of cadherins (Yap et al., 1998) (Fig1.7; see §1.2.5.3 and 1.2.5.4).

Contrary to the cytoplasmic domain, the extracellular domain of classic cadherins is different between chordates and non chordates. All vertebrate classic cadherins have five CR, whereas nonchordate classic cadherins have a varied number of CR (Fig.1.5). For example, *Drosophila* epithelial and neuronal cadherins have respectively 8 and 19 CR. Moreover, the extracellular region of nonchordate classic cadherins also contain three domains which are absent in chordate cadherins (Oda and Tsukita, 1999). These domains are located between the cadherin repeats and the transmembrane domain (Fig.1.5). One is rich in cystein residues and similar to EGF repeats. One is similar to the laminin G domain. The third domain is unique to nonchordate classic cadherins and called the nonchordate classic cadherin domain (NCCD). This NCCD motif has been shown to be required for proper translocation of *Drosophila* epithelial cadherin (DE-cadherin) to the membrane but does not seem to be required for its adhesive function (Oda and Tsukita, 1999). The NCCD motif is also cleaved along the secretory pathway, giving rise to two polypeptides that seem to be non covalently bound (Oda and Tsukita, 1999).

Vertebrates have many different classic cadherins. For instance, there are about 20 classic cadherins in human, including the well characterized epithelial cadherin (E-cadherin), and neuronal cadherin (N-cadherin). *Drosophila melanogaster* has three classic cadherins: an epithelial cadherin (DE-cadherin) encoded by the gene *shotgun* (*shg*), and two neuronal cadherins, DN- and DN2-cadherin (although the DN2-cadherin gene maybe a pseudogene (Hill et al., 2001)). *C. elegans* has only one classic cadherin gene called HMR-1. This gene encodes for two isoforms: a short isoform similar to E-cadherin and a long isoform similar to N-cadherin (Hill et al., 2001).

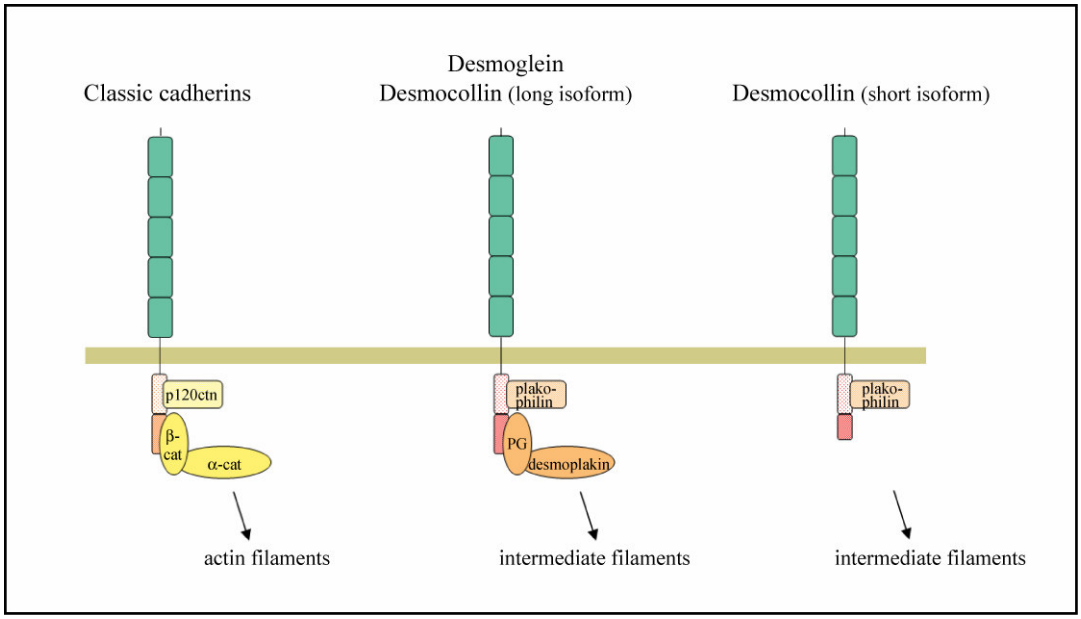



**Figure 1.7: Classic and desmosomal cadherins and associated proteins.** β-catenin, plakoglobin (PG), p120ctn and plakophilin all contain armadillo repeats. Plakoglobin is more closely related to β-catenin and plakophilin to p120ctn. β-catenin and plakoglobin respectively bind to the C-terminal domain of classic and desmosomal cadherins, whereas p120ctn and plakophilin bind to their juxtamembrane domain.

#### 1.2.1.3 Desmosomals cadherins

Desmocollins and desmogleins are cadherins found in desmosomes where they are linked to intermediate filaments (for review see (Garrod et al., 2002) and (Green and Gaudry, 2000)). Similarly to chordate classic cadherins, their extracellular domain contains five cadherin repeats (Fig.1.5). Adhesion is due to the heterophilic binding of desmocollin to desmoglein. The desmoglein cytoplasmic domain contains binding sites for two armadillo repeat containing proteins, plakophilin and plakoglobin. The desmocollin cytoplasmic domain is a little bit shorter and has two splice isoforms; both isoforms bind plakophilin but only one binds plakoglobin (see Fig.1.7). Desmosomal cadherins are linked to intermediate filaments via proteins of the plakin family, such as desmoplakin and plectin. Those proteins are bound to plakoglobin or directly to desmosomal cadherins.

Desmocollins and desmogleins are found only in chordates. The lack of desmosomal cadherins was to be expected for *Drosophila* which lacks intermediate filaments. It is more surprising for *C.elegans* which does have intermediate filaments.

#### 1.2.1.4 Fat-like cadherins and seven-transmembrane cadherins

Two subfamilies of cadherin are involved in the establishment of planar cell polarity (Adler et al., 1998) (Chae et al., 1999; Usui et al., 1999). One is composed of Fat-like cadherins, which are very large cadherins (see Fig.1.5). In *Drosophila*, there are three such large cadherins: Fat, Fat-like and Dachsous. The second subfamily regroups cadherins which have seven transmembrane domains, such as *Drosophila* Flamingo (Fig.1.5).

#### 1.2.1.5 Protocadherins

Protocadherins are found only in chordates and constitute the largest cadherin subfamily: more than 60 protocadherins are known in mouse and human. They contain a variable number of CR (often six or seven), one transmembrane domain and a short cytoplasmic domain (Fig.1.5, for a review see (Frank and Kemler, 2002)). Some protocadherins have been shown to mediate homophilic adhesion in cell culture assays, nevertheless it seems to be only weak adhesion (Frank and Kemler, 2002). Many protocadherins are highly expressed in the brain. In particular, α-protocadherins, also known as cadherin-neuronal receptors (CNR), are receptors for reelin, a secreted molecule required for proper migration of neurons in the cerebral cortex (Senzaki et al., 1999). It is also worth mentioning the paraxial protocadherin (PAPC), which is involved in convergence-extension of the mesoderm during Xenopus and zebrafish gastrulation (Kim et al., 1998; Yamamoto et al., 1998).

### 1.2.2 Adhesion mechanisms

We will now focus on classic cadherins, which mediate calcium dependent homophilic adhesion (Miyatani et al., 1989) (Nose et al., 1988).

Adhesion seems to occur in two steps. In the presence of calcium, CR repeats can form cis-dimers. This cis-dimerization seems to be a prerequisite for the formation of antiparallel trans-dimers (Pertz et al., 1999; Tomschy et al., 1996). Brieher et al. used a purified recombinant extracellular fragment of Xenopus C-cadherin to show that, in vitro, cis-dimers have substantially higher homophilic activity than monomers (Brieher et al., 1996).

The molecular details of trans-dimers formation are still controversial. Several structural studies indicate that cadherin trans-dimers are formed by the interaction of the N-terminal CR ((Shapiro et al., 1995) (Pertz et al., 1999; Tomschy et al., 1996) (Boggon et al., 2002) (He et al., 2003)). However, direct measurements of interaction forces between cadherin monolayers showed that the strongest adhesion forces are obtained when the two monolayers are separated by about 25 nm, a distance that would correspond to fully interdigitated antiparallel cadherins (Sivasankar et al., 1999).

### 1.2.3 Role of classic cadherins during development

Genetic studies in various organisms have shown the essential role of cadherins during development, in particular in the formation and maintance of tissue architecture. E-cadherin, initially called uvomorulin, was first identified as an adhesive molecule required for the compaction of early mouse embryos: antibodies directed against E-cadherin prevent embryo compaction, due to a lack of adhesion between embryonic cells (Hyafil et al., 1980). In E-cadherin K.O embryos, compaction does occur normally, thanks to the sufficient presence of maternal E-cadherin (Larue et al., 1994; Riethmacher et al., 1995). However, those embryos die before implantation due to a subsequent “decompaction”, which corresponds to a loss of adhesion (Larue et al., 1994; Riethmacher et al., 1995). Thus E-cadherin is required both for establishing and maintaining proper adhesion between embryonic cells. Similarly, vascular-endothelial (VE) cadherin is essential for both formation (Gory-Faure et al., 1999) and maintenance (Corada et al., 1999) of vascular endothelium in mouse.

In *Drosophila*, DE-cadherin zygotic null mutants show very strong defects in some epithelia, such as the ventral epidermis or the malpighian tube epithelium. Other epithelia such as the dorsal epidermis for instance do not show any defects in those mutants, probably indicating that maternal DE-cadherin is sufficient in those epithelia (Tepass et al., 1996; Uemura et al., 1996). The epithelia affected by the lack of zygotic DE-cadherin seem to be those undergoing important cell rearrangements during embryogenesis. For instance in the ventral neurectoderm, neuroblasts delaminate from the ectodermal epithelium and the remaining cells have to close the gaps to form the ventral epidermis. All these rearrangements would explain

why the formation of the ventral epidermis requires more DE-cadherin than the formation of the dorsal epidermis (Tepass et al., 1996).

Cadherins have been shown to have much more diverse roles than simply maintaining epithelial integrity. In particular, E-cadherin is sufficient to establish polarity in cultured cells (McNeill et al., 1990). Similarly, DE-cadherin is required for the orientation of asymmetric cell division (Le Borgne et al., 2002). In several cases – both in mammals and in *Drosophila* - cadherins have also been shown to mediate adhesion between somatic cells and germ line cells (González-Reyes and St. Johnston, 1998) (Godt and Tepass, 1998) (Jenkins et al., 2003) (Newton et al., 1993). This kind of interaction is essential for proper gonad morphogenesis and gametogenesis. For instance, during *Drosophila* oogenesis, DE-cadherin is required both in the oocyte and in the somatic follicular cells for the proper positioning of the oocyte at the posterior of the egg chamber (González-Reyes and St. Johnston, 1998) (Godt and Tepass, 1998).

Moreover, the link between cadherins and the actin cytoskeleton may also provide some specific functions during morphogenesis. For instance, in *C. elegans*, the loss of the cadherin/catenin complex surprisingly does not cause any epithelial breakdown (Costa et al., 1998), showing that other adhesive molecules can be functionally redundant with cadherins in adherens junction formation. However, embryos lacking the cadherin/catenin complex show defects in body elongation, a morphogenetic process that involves cell shape changes of epidermal cells (Costa et al., 1998). In those embryos, some specialized actin filament bundles that may contract to cause cell shape changes detach from adherens junctions. The cadherin/catenin complex has thus been proposed to be required to anchor these actin bundles to adherens junctions, thus allowing the force of the bundle contraction to be transmitted into cell shape changes (Costa et al., 1998). In some cases, cadherins also mediate adhesion of migrating cells to a cellular substratum allowing that the traction forces which are exerted inside the migrating cells effectively result in migration. DE-cadherin for instance is required for the adhesion of migrating border cells to the surrounding nurse cells ((Niewiadomska et al., 1999) see §1.3.5).

Cadherins, in particular N-cadherin, also play essential roles in the nervous system. In vitro, N-cadherin supports neurite outgrowth (Neugebauer et al., 1988; Tomaselli et al., 1988). Studies in the chick optic tectum (Treubert-Zimmermann et al., 2002) and in *Drosophila* N-cadherin mutants (Iwai et al., 1997) show that in vivo cadherins are required for axon fasciculation and pathfinding. Regulation of cadherin-mediated adhesion seems to be important for proper development of the nervous system. For instance, axon repulsion by the guidance cue Slit could be due to inhibition of N-cadherin mediated adhesion (Rhee et al., 2002). Another example where cadherin regulation could be involved is the migration of neurons in the cerebral cortex. The serine/threonine kinase cdk5 has been proposed to prevent stable N-cadherin mediated adhesion while cortical neurons migrate through the cerebral wall: in the absence of cdk5 activity, the first cohorte of cortical neuron migrate normally but subsequently born neurons fail to migrate over, which could be due to the inappropriate formation of stable contacts with previously generated cortical neurons (Kwon et al., 2000). N-cadherin is also involved in synaptogenesis, both for establishing synaptic contacts and generating synaptic specificity (for review see (Bruses, 2000) (Huntley, 2002)). It could also play a role in synaptic plasticity and long-term potentiation (Bozdagi et al., 2000; Tanaka et al., 2000). A recent study showed that N-cadherin intracellular domain can be cleaved by presenilin-1, which produces a peptide that promotes the proteosomal degradation of the transcription factor CBP: this suggests that N-cadherin function in the nervous sytem may not only involve its ability to mediate homophilic adhesion but also the ability to regulate transcription (Marambaud et al., 2003).

Finally, cadherins and their regulation are not essential only during development: loss of E-cadherin is observed in most human epithelial cancers and contributes to proliferation at the early stages of tumorigenesis as well as to the transition from benign tumors to malignant, invasive tumors. Invasiveness consecutive to E-cadherin loss has been thought to be due to a decrease in cell-cell adhesion. However, at least in some tumor cell lines, both proliferation and invasiveness can be suppressed by E-cadherin in an adhesion-independent manner (Gottardi et al., 2001), (Wong and Gumbiner, 2003). In both cases, E-cadherin suppression activity requires the ability of E-cadherin to bind β-catenin. Growth suppression seems to result from an inhibition of the β-catenin/TCF signaling pathway (see §1.2.4.1), following

depletion by E-cadherin of a pool of β-catenin that can bind TCF (Gottardi et al., 2001). Contrary to E-cadherin, N-cadherin is expressed in some highly invasive tumor cell lines and seems to promote motility, invasion and metastasis (Hazan et al., 2000).

### 1.2.4 Molecules associated with classic cadherins at junctions

#### 1.2.4.1 β-catenin and α-catenin: linking cadherin to actin

As mentioned above, the C-terminal domain of cadherin binds to β-catenin (Ozawa et al., 1989) (Nagafuchi and Takeichi, 1989) (Ozawa et al., 1990). In vertebrates, this domain can also bind to plakoglobin, another member of the catenin family which also binds to desmosomal cadherins (Cowin et al., 1986) (Ozawa et al., 1989). β-catenin (or plakoglobin) binds in turn to α-catenin which is linked to actin filaments (Ozawa et al., 1990) (Aberle et al., 1994; Jou et al., 1995) (Fig.1.7). α-catenin binds to actin directly or through interactions with the actin-binding proteins α-actinin or vinculin (Knudsen et al., 1995; Rimm et al., 1995; Weiss et al., 1998) (Watabe-Uchida et al., 1998). Pulse-chase experiments have shown that newly synthesized E-cadherin associates with β-catenin in the endoplasmic reticulum, whereas α-catenin joins the cadherin/β-catenin complex only at the cell surface (Hinck et al., 1994).

β-catenin contains 12 armadillo repeats which bind to the C-terminal domain of cadherin (Hulsken et al., 1994) (Pai et al., 1996)**.** Its N-terminal domain is responsible for the interaction with α-catenin (Fig.1.8) (Aberle et al., 1994; Pai et al., 1996)). The N-terminal domain of α-catenin is involved in α-catenin homodimerization and in binding to β-catenin (Jou et al., 1995; Koslov et al., 1997; Pai et al., 1996; Pokutta and Weis, 2000): α-catenin can form homodimers but, in the presence of β-catenin, forms α/β heterodimers (Koslov et al., 1997). Binding domains to actin, α-actinin and vinculin have also been identified (Rimm et al., 1995) (Nieset et al., 1997; Watabe-Uchida et al., 1998) (see Fig.1.8).

The interaction between cadherin and α- and β-catenin has been shown to be essential for cadherin function in cell culture experiments (Nagafuchi and Takeichi, 1988), (Nagafuchi and Takeichi, 1989), (Ozawa et al., 1990). This has been confirmed by the phenotypic analysis of

α- and β-catenin mutants in *C.elegans* and *Drosophila*. *C. elegans* and *Drosophila* have one gene encoding α-catenin. There is so far no mutant available for the *Drosophila* α-catenin. In *C. elegans*, α-catenin has been shown to be required for proper morphogenesis and viability; α-catenin mutants exhibit defects in the linkage of actin filaments to cadherin complexes, which are probably responsible for the observed morphogenetic defects (Costa et al., 1998) (Pettitt et al., 2003). *Drosophila* has only one gene encoding β-catenin (also known as *armadillo* (*arm*)) whereas *C.elegans* has 3 genes encoding β-catenin homologues. Lack of β-catenin during *Drosophila* oogenesis results in defects similar to those observed in DE-cadherin mutants, showing that β-catenin is an essential component of cadherin junctions (Peifer, 1993).

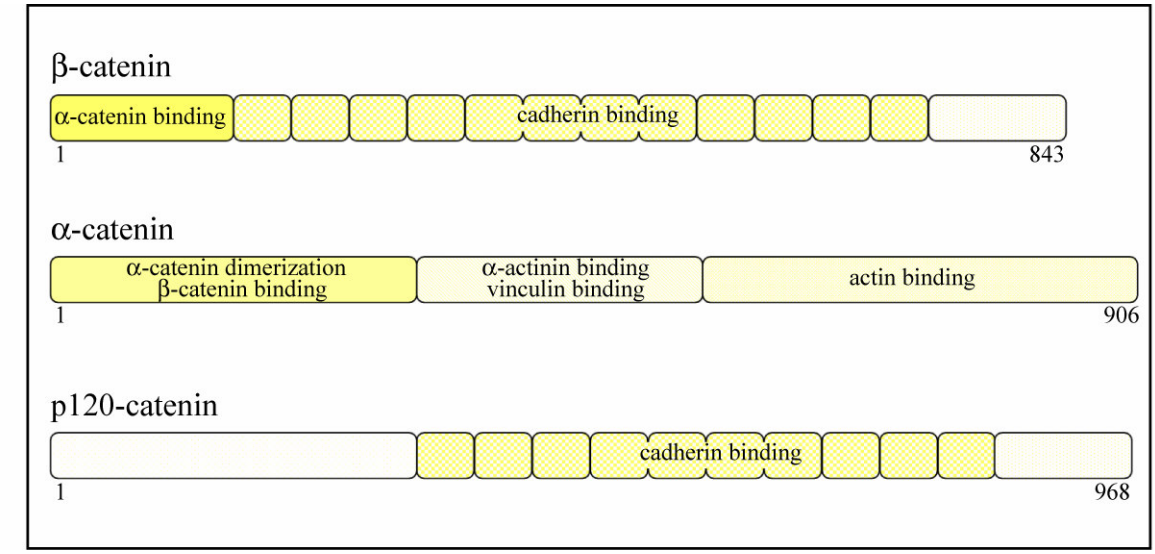



**Figure 1.8: Domain composition of β-catenin, α-catenin and p120 catenin.** Different binding sites are indicated. β-catenin and p120ctn contain respectively 12 and 10 repetitions of 42 amino-acids motifs known as armadillo repeats. These repeats are in particular involved in cadherin binding. The N-terminus of β-catenin binds to α-catenin. The N-terminus of α-catenin can either mediate α-catenin homodimerization or heterodimerization with β-catenin. The N-terminus of vertebrate p120ctn contain putative phosphorylation sites but is not conserved in invertebrates.

It should be mentioned that, in addition to adhesion defects, *armadillo* mutants also show defects similar to the defects observed in the absence of Wingless (wg) signalling (Peifer et

al., 1991). β-catenin has indeed been shown to be an essential component of the wg/wnt signalling pathway (for review see (Dierick and Bejsovec, 1999)). In the absence of wg signalling, β-catenin is phosphorylated by the GSK3 kinase and then targeted for degradation. Upon activation of wg signalling, GSK3-mediated phosphorylation of β-catenin is inhibited and β-catenin stabilized. β-catenin can then enter the nucleus, form complexes with transcription factors of the TCF/LEF family and thus regulate target gene transcription.

#### 1.2.4.2 Other cytosolic proteins associated with cadherins

Many cytosolic proteins have been described to associate with cadherin/complexes. These include many proteins (such as p120 catenin, a number of kinases and phosphatases (see §1.2.5.5), presenilin-1 (Baki et al., 2001)) which are regulating cadherin-mediated adhesion by binding directly to cadherin or to catenins.

Another class of proteins found to associate with cadherin complexes is composed of PDZ domain containing proteins, such as ZO-1 (Imamura et al., 1999), LIN-7 (Perego et al., 2000) or 1-afadin (Takahashi et al., 1999). These proteins are thought to have a structural/scaffolding role by mediating interactions between different components of cadherin junctions. For instance, 1-afadin is a PDZ domain containing protein that is required for the association between E-cadherin and the adhesion molecule nectin (see §1.2.4.3). They may also be involved in the regulation of adhesion. ZO-1 binding to α-catenin is for example required for the strong state of E-cadherin mediated adhesion in L cells (Imamura et al., 1999). ZO-1 also binds to AF-6/canoe, another PDZ domain containing protein (Takahashi et al., 1998). AF-6/canoe binds in turn to activated Rap1 (Linnemann et al., 1999), a small GTPase required for the even distribution of adherens junctions in *Drosophila* epithelial cells (Knox and Brown, 2002). In *Drosophila*, a novel protein containing two PDZ domains and called Arc has also been shown to colocalize with β-catenin. Arc is expressed in the embryo in epithelia undergoing morphogenesis (Liu and Lengyel, 2000). However, despite this highly regulated pattern of expression, complete absence of Arc does not cause any detectable defects during embryogenesis, suggesting possible functional redundancy with other proteins.

#### 1.2.4.3 Transmembrane proteins associated with cadherins

Transmembrane proteins other than cadherins are found associated with adhesion complexes. Some of them are able to regulate cadherin-mediated adhesion. This is for example the case of several growth factor receptors, which seem to regulate adhesion via their tyrosine kinase activity (e.g.(Dumstrei et al., 2002), see §1.2.5.5). Another transmembrane protein called Fear of Intimacy (Foi) and regulating cadherin-mediated adhesion has been identified in *Drosophila*: Foi is required for proper DE-cadherin function or expression in germ cells during gonad morphogenesis; Foi belongs to a novel protein family and its mechanism of action is still unknown (Jenkins et al., 2003; Van Doren et al., 2003).

E-Cadherin also associates with nectin, an adhesion molecule of the immunoglobin family (Takahashi et al., 1999). The association of E-cadherin and nectin requires interaction between their respective cytoplasmic associated proteins, α-catenin and 1-afadin (Tachibana et al., 2000). E-cadherin and nectin have been shown to cooperate to recruit to cell-cell contacts the exocyst, a complex of eigth proteins which is essential for the targeting of exocytic vesicles in polarized cells (Yeaman et al., 2003). In the inner ear sensory hair cells cadherin/catenins associate with vezatin, a novel transmembrane protein which binds to the non conventional myosin-VIIA (Kussel-Andermann et al., 2000). The role of the association of cadherin/catenins with vezatin is not known yet. During *Drosophila* oogenesis, DE-cadherin and β-catenin have been shown to associate with another non conventional myosin, myosin VI (Geisbrecht and Montell, 2002). Depletion of myosin VI results in a reduction of DE-cadherin and β-catenin levels, suggesting that it may be involved in stabilizing DE-cadherin/β-catenin complexes.

### 1.2.5 Regulation of cadherin mediated adhesion

Cadherins mediate intercellular adhesion in many different processes: for instance, they maintain very stable adhesion between epithelial cells, they are also involved in adhesion in tissues undergoing morphogenesis and they can be used by migrating cells to adhere to a cellular substratum. In the last two cases, adhesion has to be somehow dynamic for morphogenesis or migration to occur. Moreover, during development a tissue can undergo

changes such as epithelial-mesenchymal transition which requires important changes in term of cell-cell adhesion. This supposes that some mechanisms can regulate the strength of cadherin-mediated adhesion. Spatial and temporal regulation of cadherin expression is essential during development. A well known example is the regulation of cadherin expression during neural crest cell migration. As they start migrating out of the neural tube, neural crest cells downregulate N-cadherin expression and express cadherin-7. At the end of the migration, when the cells aggregate to form a ganglion, they re-express N-cadherin. Downregulation of N-cadherin has been proved to be essential for neural crest cells to escape from the neural tube (for review see (Pla et al., 2001)). However transcriptional regulation is too slow to account for the quick changes in adhesion observed in many situations. Some other mechanisms, such as association/dissociation or phosphorylation/dephosphorylation of adhesion complexes components, as well as endocytosis seem to be more appropriate for rapid regulation.

#### 1.2.5.1 Modulation of the link to actin filaments

Since linking cadherin to actin filaments through catenins is essential for adhesion, modulation of this link has been proposed as a mechanism to regulate adhesion strength. A fusion protein obtained by fusing α-catenin to the cytoplasmic domain of E-cadherin can mediate adhesion as efficiently as E-cadherin, as measured with an aggregation assay in mouse L cells (Nagafuchi et al., 1994). However, L cells expressing this E-cadherin/α-catenin fusion protein seem to be unable to regulate adhesion. In particular, they do not downregulate cell-cell adhesion during cytokinesis. They are also unable to repeatedly attach and detach from neighboring cells to move actively as L cells expressing E-cadherin do (Nagafuchi et al., 1994). It was thus suggested that the link between cadherin and α-catenin can be regulated and that this could be important for cadherin regulation in vivo. Consistently, a significant decrease in adherens junction-associated β-catenin has been observed in epithelial cells from the archenteron as they undergo convergent-extension movements during sea urchin gastrulation (Miller and McClay, 1997a).

The link between cadherin and α-catenin could be regulated either at the level of the interaction between cadherin and β -catenin or at the level of the β/α-catenin interaction.

Serine/Threonine as well as tyrosine phosphorylations seem to be involved in regulating these two levels of interaction (see §1.2.5.5). IQGAP1, an effector of Cdc42 and Rac1, also regulate β/α-catenin interaction: it binds directly to β-catenin thus inducing the dissociation of α-catenin from cadherin complexes (Kuroda et al., 1998) (Fukata et al., 1999).

#### 1.2.5.2 Endocytosis

The amount of cadherin protein expressed on the cell surface clearly influences cadherin adhesiveness (Angres et al., 1996). Removing cadherin from the cell surface thus appears as a possible mechanism to rapidly regulate cadherin-mediated adhesion. Cadherin endocytosis has indeed been reported to occur in different cell types. In MDCK cells, E-cadherin is constitutively internalized and then recycled back to the plasma membrane (Le et al., 1999). In microvascular endothelial cells (MECs), VE-cadherin is internalized and then degraded through the endosomal/lysosomal pathway (Xiao et al., 2003b). In these two cell types, cadherin endocytosis is constitutive but can also be upregulated in some circumstances. For instance, in MDCK cells, the pool of E-cadherin undergoing endocytosis and recycling is markedly increased in cells without stable cell-cell contacts (Le et al., 1999). In MECs, cadherin endocytosis increases when MECs are induced to migrate (Xiao et al., 2003b). Similarly, during sea urchin gastrulation, ingression of primary mesenchymal cells is accompanied by the rapid loss of junctional cadherin staining and the coincident accumulation of cadherin in intracellular vesicles; this suggests that deadhesion of mesenchymal cells from neighboring epithelial cells involves removal of cell surface cadherin molecules through endocytosis (Miller and McClay, 1997b).

Cadherin endocytosis has been reported to occur through both clathrin-dependent (Palacios et al., 2002) and clathrin–independent pathways (Paterson et al., 2003). This may be cell-type specific. Several signaling molecules, such as Rac1 (Akhtar and Hotchin, 2001), Protein Kinase C (Le et al., 2002), the tyrosine kinase Src (Fujita et al., 2002) have been shown to regulate cadherin endocytosis. In MDCK cells tyrosine phosphorylation of E-cadherin by Src induces an interaction between two phosphotyrosine residues and the E3-ubiquitin ligase Hakai. Hakai ubiquitinates E-cadherin, which is then endocytosed (Fujita et al., 2002). However, regulation of cadherins by Hakai may not be a general mechanism as the two

tyrosine residues necessary for the interaction with Hakai are E-cadherin specific and are absent from other cadherin cytoplasmic tails.

#### 1.2.5.3 Regulation of lateral clustering

Forcing lateral clustering of C-cadherin significantly increases adhesion strength of CHO cells in a laminar flow assay (Yap et al., 1997). This can probably be explained by the observation that cadherin cis-dimers have substantially higher homophilic activity than monomers (Brieher et al., 1996). The juxtamembrane domain of C-cadherin has been shown to be necessary and sufficient to obtain cadherin clustering in CHO cells (when not artificially forced) as well as full adhesive activity in a laminar flow assay (Yap et al., 1998). However it should be mentioned that in these experiments the C-terminal domain of C-cadherin (which binds to β-catenin) is required neither for cadherin clustering nor to get full adhesive activity, which is quite surprising considering that binding to β-catenin is essential for cadherin function.

#### 1.2.5.4 Regulation by p120 catenin

Mammalian p120 catenin (p120ctn) was initially identified as a prominent substrate for the Src tyrosine kinase. It turned out to contain armadillo repeats (Reynolds et al., 1992) and to bind to classic cadherins (Reynolds et al., 1994). Several related proteins (such as δ-catenin, ARVCF and p0071) were subsequently identified in mammals. *Drosophila* and *C.elegans* seem to have only one p120ctn.

p120ctn is composed of an NH2-terminal head domain, 10 armadillo repeats and a short COOH-terminal tail (Fig1.8). Sequence comparison of mammalian p120ctn with *Drosophila* and *C.elegans* p120ctn show that they share little similarity outside the armadillo repeat domain. p120ctn armadillo repeats interact directly with the juxtamembrane domain of cadherins (Daniel and Reynolds, 1995; Yap et al., 1998) and site directed mutagenesis of this juxtamembrane domain allowed the precise identification of the cadherin residues involved in p120ctn binding (Anastasiadis et al., 2000). At their C-terminus, δ-catenin, ARVCF, p0071 and *C. elegans* p120ctn (but not mammalian p120 nor *Drosophila* p120ctn) have a conserved

PDZ binding motif and some of them have indeed been shown to interact with PDZ domain proteins (for instance (Deguchi et al., 2000; Ide et al., 1999; Laura et al., 2002)). The N-terminal domain of mammalian p120ctn has been shown to contain tyrosine phosphorylation sites (Mariner et al., 2001).

Binding of p120ctn to the cadherin juxtamembrane domain together with the requirement of this domain for cadherin clustering and strong adhesion suggested that p120ctn itself could be required for adhesion (Yap et al., 1998). Thoreson et al, have mutated E-cadherin to specifically inhibit p120ctn binding (Anastasiadis et al., 2000). They have shown that cells expressing only p120ctn-uncoupled cadherin have a reduced adhesive activity, as measured by an aggregation assay. However, another study suggested that p120ctn was a negative regulator of E-cadherin (Aono et al., 1999). This study has been done in Colo 205 cells, a cell type in which p120ctn appears to be highly phosphorylated (on serines and threonines). This has led to the hypothesis that, depending on its phosphorylation state, p120ctn could act both as a positive and a negative regulator of cadherin (Anastasiadis and Reynolds, 2000) (see §1.2.5.5). A model in which p120ctn regulates adhesion by regulating cadherin clustering has been proposed but there is no direct evidence for this mechanism (Anastasiadis and Reynolds, 2000).

#### 1.2.5.5 Phosphorylation of cadherin and catenins

A number of growth factor receptors as well as various cytosolic kinases (both tyrosine and serine/threonine kinases) and phosphatases have been shown to induce phosphorylation/dephosphorylation of cadherin complexes components and to regulate cadherin-mediated adhesion. This has been reported in many different cell lines (e.g. (Hazan and Norton, 1998), (Behrens et al., 1993), (Takeda et al., 1995)) but also in more in vivo system. For instance, inhibition of the serine/threonine kinase cdk5 in primary cortical neurons results in an increase of N-cadherin mediated adhesion (Kwon et al., 2000). During *Drosophila* optic placod morphogenesis, loss of EGF receptor and DE-cadherin overexpresion give very similar phenotypes suggesting that EGF receptor is involved in DE-cadherin downregulation (Dumstrei et al., 2002).

Tyrosine phosphorylation of cadherin, β-catenin as well as p120ctn correlates with a decrease in cadherin-mediated adhesion. Phosphorylation of cadherin itself has been reported in a few cases. In particular, Src phosphorylates E-cadherin in MDCK cells which leads to binding of E-cadherin to the E3-ubiquitin ligase Hakai and E-cadherin endocytosis (see §1.2.5.2, (Fujita et al., 2002)). Phosphorylated tyrosine residues of cadherin have also been shown to interact with the adaptor protein Shc (Xu and Carpenter, 1999).

Tyrosine phosphorylation of β-catenin seems to be often more prominent than cadherin phosphorylation itself. In several cell types, decrease in adhesion induced by tyrosine kinases (such as Src or Fer for instance) correlates with β-catenin phosphorylation (Behrens et al., 1993) (Takeda et al., 1995) (Rosato et al., 1998). Moreover tyrosine phosphatases, such as PTP1B and LAR, can be associated with cadherin complexes and regulate both adhesion and β-catenin phosphorylation state (Balsamo et al., 1998). In many cases, a correlation between tyrosine phosphorylation of β-catenin and dissociation of β-catenin from cadherin has been observed (Rosato et al., 1998) (Rhee et al., 2002).(Balsamo et al., 1998; Balsamo et al., 1995; Muller et al., 1999). The structure of β-catenin bound to the E-cadherin cytoplasmic domain shows that this conserved tyrosine of β-catenin is tightly packed against E-cadherin and that its phosphorylation is very likely to decrease the binding of β-catenin to E-cadherin (Huber and Weis, 2001). Consistently it was observed in vitro that phosphorylation of this β-catenin conserved tyrosine causes a 5-fold reduction in the affinity of β-catenin for E-cadherin (Roura et al., 1999). Nevertheless, Src also causes a decrease of adhesion in L-cells expressing the fusion protein E-cadherin/α-catenin, showing that β-catenin phosphorylation is not responsible for the observed decrease in adhesion, at least in this cell type (Takeda et al., 1995).

p120ctn can be phosphorylated on tyrosine residues in cells transformed with Src (Reynolds and al, 1989) or stimulated by growth factors (Downing and Reynolds, 1991). The Fer tyrosine kinase has also been shown to bind to and phosphorylate p120ctn (Kim and Wong, 1995) (Rosato et al., 1998). Phosphorylation sites are located in the N-terminal domain of p120ctn (Mariner et al., 2001). In L cells, expression of a form of p120ctn lacking the N-terminal domain or of a tyrosine point mutant (Y217F) increases E-cadherin mediated adhesion (Ozawa and Ohkubo, 2001).

Ser/Thr kinases have also been involved in adhesion regulation. In particular, Casein Kinase II (CKII) and GSK-3β phosphorylate the C-terminal domain of E-cadherin, which results in an increased binding of β-catenin to E-cadherin. Preventing this phosphorylation by using Ser/Thr-mutant E-cadherin reduces E-cadherin mediated cell-cell adhesion in transfected NIH3T3 fibroblasts (Lickert et al., 2000).

CKII also phosphorylates Ser and Thr in the N-terminal domain of β-catenin. Ser/Thr-mutant β-catenin does not properly associate with cadherin/catenin complexes in 293 cells. In vitro, phosphorylation of β-catenin by CKII increases its binding to α-catenin (Bek and Kemler, 2002). In embryonic cortical neurons, the Ser/Thr kinase cdk5 coimmunoprecipitates with N-cadherin and β-catenin and inhibition of cdk5 results in an increase of N-cadherin-mediated adhesion. Moreover, in COS cells, this kinase decreases the amount of N-cadherin associated with β-catenin (Kwon et al., 2000).

p120ctn function may also be dependent on the state of phosphorylation of its Ser/Thr residues: treatment of Colo205 cells with serine-threonine kinase inhibitors correlates with decreased p120 phosphorylation and restored adhesiveness (Aono et al., 1999).

#### 1.2.5.6 Small GTPases

Three members of the Rho small GTPase subfamily (RhoA, Cdc42 and Rac1) have been reported to be required for maintaining cadherin-mediated cell-cell adhesion in various cell types (Braga et al., 1997; Takaishi et al., 1997) (Fukata et al., 1999). Activated Cdc42 and Rac1 bind to IQGAP1 (see §1.2.5.1), thus preventing binding of IQGAP1 to β-catenin and resulting in the stabilization of the cadherin-catenin complex (Fukata et al., 1999). Inhibition of RhoA results in a reduced accumulation of E-cadherin at cell-cell contacts and in a reduced association of α-catenin with cadherin/β-catenin complexes. Stabilization of adherens junction components by RhoA goes through its effector Diaphanous and requires the ability of Diaphanous to promote actin polymerization (Sahai and Marshall, 2002). Another member of the Rho family, RhoC, is able to destabilize adherens junctions. This seems to be mediated

by activation of the Rho Kinase (ROCK) and acto-myosin contraction (Sahai and Marshall, 2002).

In *Drosophila*, Rho1, the homologue of RhoA, colocalizes with DE-cadherin in the embryo and in the ovary (Magie et al., 2002). Overexpression of a dominant-negative form of Rho1 in the embryonic epidermis results in loss of epithelial integrity and abnormal cell shape which are likely to be due to defects in adhesion. Consistently, it also causes a complete loss of DE-cadherin from the cell surface. It also results in a loss of cell polarity but it is not known if this a cause or a consequence of the absence of DE-cadherin at the cell surface (Bloor and Kiehart, 2002). Zygotic Rho1 mutant embryos do not show defects in the epidermis - probably due to the presence of maternal Rho1 – but exhibit dorsal closure defects. In those mutants, DE-cadherin as well as α- and β-catenin localization in cells undergoing dorsal closure seems to be more diffuse than in wild type embryos (Magie et al., 2002).

## 1.3 Border cell migration

### 1.3.1 Oogenesis and border cells

Border cell migration takes place during oogenesis. The ovary consists in several ovarioles, which contain egg chambers of increasing maturity (Fig1.9). The germarium, located at the anterior tip of each ovariole, contains both somatic and germ line stem cells, usually two of each (Margolis and Spradling, 1995). When a germ line stem cell divides, it gives rise to one stem cell and a germ cell, which undergoes four rounds of cell division with incomplete cytokinesis, resulting in a cyst of 16 germ cells still connected via cytoplasmic bridges called ring canals (for a review see (Spradling, 1993)). One of these germ cells becomes the oocyte and enters meiosis. The other 15 cells differentiate into nurse cells, undergo several rounds of endoreplication and become polyploid. The nurse cells produce RNA and proteins required for early embryogenesis and deposit them in the oocyte. At the end of the germarium, each germ cell cluster is enveloped by a monolayered epithelium of somatic follicular cells, thereby forming a separate egg chamber (Spradling, 1993) (Fig1.9).

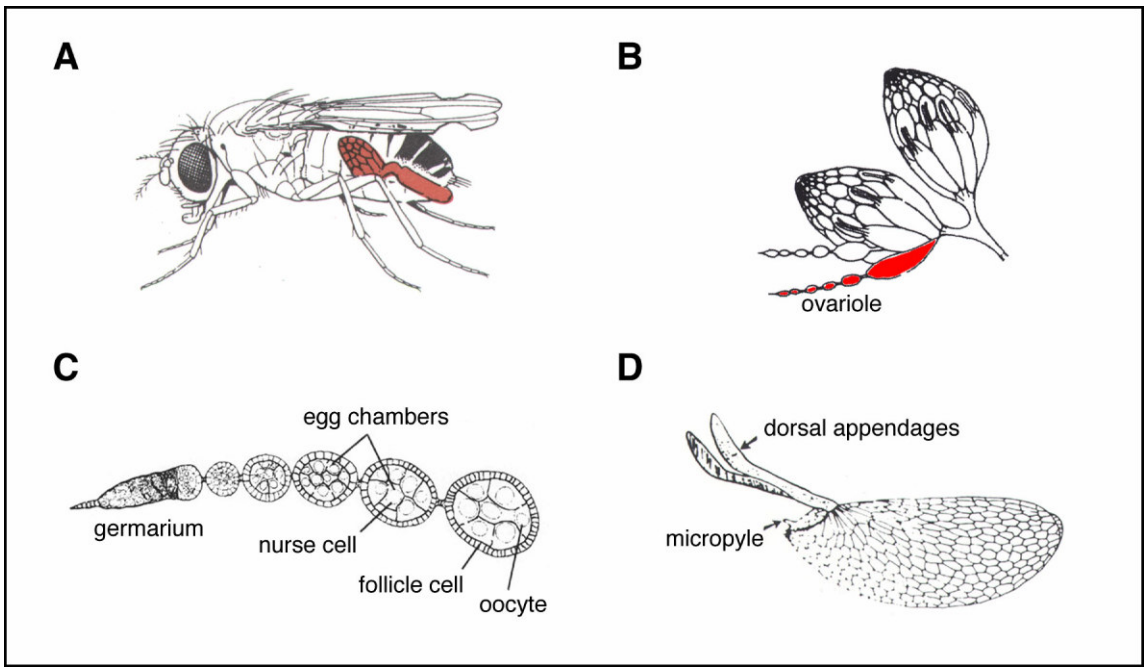



**Figure 1.9: Overview of Drosophila oogenesis. A.** The ovary (red) is located in the abdomen of females. **B.** An ovary consists of multiple ovarioles, which contain egg chambers of increasing maturity. **C.** Each egg chamber consists of 16 germline cells (15 nurse cells and the posteriorly located oocyte), surrounded by the follicular epithelium. **D.** Structure of a mature *Drosophila* egg, with two prominent dorsal appendages for respiration and an anterior micropyle, which provides an opening for sperm entry.

Follicular cells have their apical surface facing the germ cells; they produce and secrete some of the yolk components which are taken up by the oocyte. Another important function of the follicular cells is to make the eggshell, consisting of the vitelline layer and the chorion, which provides an impermeable layer to prevent the laid egg from drying out (Spradling, 1993). Follicular cells lacking β-catenin have an irregular morphology at early stages of oogenesis and degenerate at later stages showing that cadherin/catenin complexes are required for maintaining the follicular epithelium structure (Tanentzapf et al., 2000). At early to mid-oogenesis, both DE-cadherin and DN-cadherin are expressed in follicular cells and removing only DE-cadherin does not affect follicular cell shape, probably reflecting a functional overlap between DE-cadherin and DN-cadherin (Tanentzapf et al., 2000). At stage 10, DN-cadherin disappears from the follicular epithelium and follicular cells lacking DE-cadherin show cell shape defects (Tanentzapf et al., 2000).

The oocyte is always positioned at the posterior of the egg chamber. Its positioning occurs in the germarium and is dependent on the presence of DE-cadherin both in the oocyte and in the posterior follicular cells (González-Reyes and St. Johnston, 1998) (Godt and Tepass, 1998).

At the anterior and posterior pole of each egg chamber are two specialized follicular cells called polar cells. They can be distinguished from the rest of the follicular cells by their rounded morphology and molecularly by several markers that they express. Polar cell specification has been shown to require Notch signaling: Delta is produced in the germline and activates Notch in polar cell precursors to induce polar cell fate (Lopez-Schier and St Johnston, 2001). However, Notch signaling is not sufficient to specify polar cells showing that an additional signal – yet unknown - must also contribute to polar cell specification (Grammont and Irvine, 2001). Once specified, polar cells produce Unpaired, a ligand activating the JAK/STAT pathway upon binding to the Domeless receptor (Ghiglione et al., 2002; Silver and Montell, 2001). This signal emanating from polar cells has been shown to pattern the anterior follicular cell epithelium (Xi et al., 2003): graded activity of the JAK/STAT pathway induces the adoption of the different anterior cell fates so that the cells closest to the anterior polar cells will become border cells, followed by stretched cells and then centripetal cells. Moreover, the coordinated activities of the JAK/STAT and EGF

receptor (EGFR) pathways are required to specify the posterior terminal cell fate (Xi et al., 2003).

During stage 9 of oogenesis, follicular cells undergo a major morphogenetic rearrangement. While the oocyte-associated follicular cells adopt a more columnar shape, the three anterior follicular cells populations move posteriorly towards the oocyte (Fig1.10) (Spradling, 1993). Border cells migrate as a cluster in between the nurse cells while the stretched follicular cells elongate and flatten to cover the nurse cells. The centripetal cells move during stage 9 on top of the nurse cells towards the oocyte and then at stage 10 migrate in between the nurse cells and the oocyte to completely cover the anterior of the oocyte.

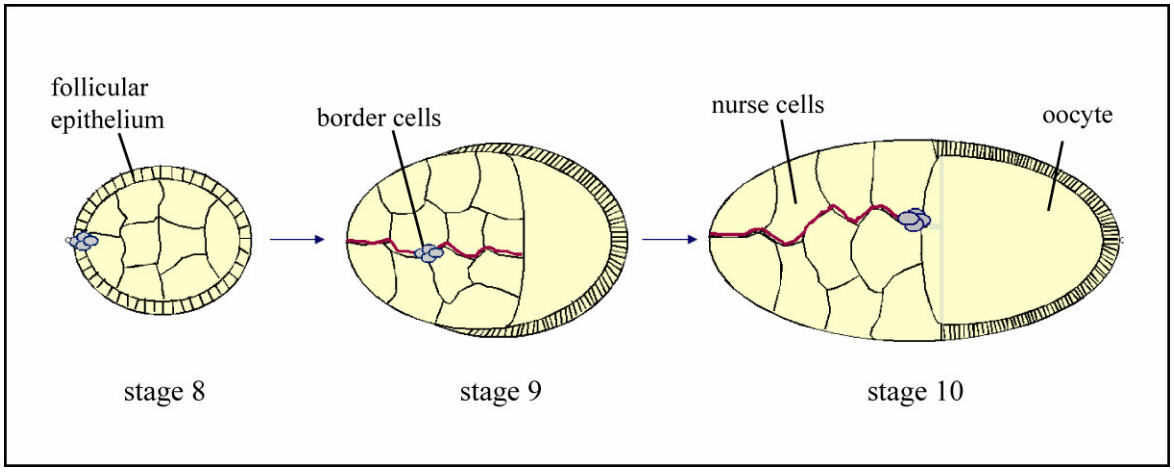



**Figure 1.10: Border cell migration during Drosophila oogenesis.** At the end of stage 8 / beginning of stage 9 of oogenesis, border cells delaminate from the anterior follicular epithelium and migrate in between the nurse cells toward the oocyte. They reach the oocyte at stage 10 of oogenesis. During stage 9, the other anterior follicular cells also move towards the posterior, on the surface of the nurse cells.

The border cell cluster is formed by the two anterior polar cells plus on average six outer border cells. It delaminates from the anterior follicular epithelium at late stage 8 / early stage 9 of oogenesis and starts to migrate in between the nurse cells (Fig1.10). Border cells first migrate posteriorly until they reach the border between the nurse cells and the oocyte, at stage 10 of oogenesis (Fig1.10). They then migrate dorsally, towards the oocyte nucleus. During the first phase, border cells migrate approximately 150 µm towards the oocyte, which takes about

5-6 hours, whereas the subsequent dorsal migration covers 10-20µm. Once they have reach their final destination, border cells participate in the formation of the hole in the micropyle, a special structure at the anterior-dorsal surface of the eggshell that provides a hole through which the sperm can enter and fertilize the egg. Border cells are thus required for female fertility: in their absence or if they do not migrate properly, the hole in the micropyle does not form and the egg can not be fertilized (Montell et al., 1992).

During the migration, the two polar cells remain rounded up and in the center of the border cell cluster. Polar cells are not motile themselves, since they do not migrate in the absence of the outer border cells (Han et al., 2000). It is therefore assumed that polar cells do not actively contribute to the migration process, but are carried along by the outer border cells. Border cells do not seem to undergo a classical epithelial to mesenchymal transition, but rather retain

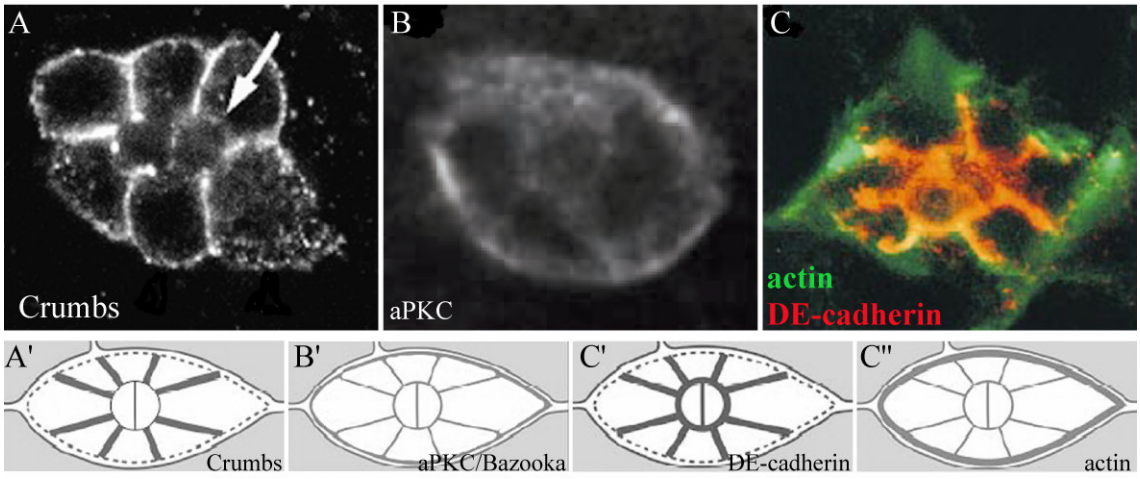



**Figure 1.11: Polarity markers in border cell clusters during the migration. A-C.** Localization of Crumbs (A), aPKC (B), DE-cadherin (C, red) and actin filaments (C, green, stained with phalloidin) in border cell clusters at stage 9. A'-C''. Schematic representation of the localization of Crumbs (A'), aPKC and Bazooka (B'), DE-cadherin (C') and actin (C''). Dashed lines correspond to punctuate stainings. Crumbs accumulates between border cells and to a lesser extent between border cells and nurse cells. It is not found at the membrane between border cells and polar cells (arrow). aPKC is present at the membrane between border cells and seems to slightly accumulate at the membrane between border cells and nurse cells. DE-cadherin (red) strongly accumulates between adjacent border cells and between border cells and polar cells. It is present at lower amount at the membrane between border cells and nurse cells. Actin filaments accumulate at the membrane between border cells and nurse cells. Pictures from (Niewiadomska et al., 1999) and (Abdelilah-Seyfried et al., 2003). Schemes modified from (Niewiadomska et al., 1999).

some polarity. For instance, the apical marker Crumbs accumulates at contact sites between adjacent border cells and to a lower extent at the interface with nurse cells whereas it is absent at the membrane in contact with polar cells (Niewiadomska et al., 1999) (Fig.1.11A). Two components of the Par complexe, Bazooka and aPKC, may be present at slightly higher levels at the membrane between border cells and nurse cells compared with the membrane between adjacent border cells (Abdelilah-Seyfried et al., 2003) (Fig.1.11B). DE-cadherin accumulates at high levels at the membrane between adjacent border cells and also at the membrane in contact with polar cells whereas actin filaments accumulate at the membrane between border cells and nurse cells (Niewiadomska et al., 1999) (Fig.1.11C).

### 1.3.2 Border cell specification

As mentioned above, it has been shown that polar cells induce border cell fate: polar cells produce Unpaired, which activates the JAK/STAT pathway in adjacent follicular cells thus inducing their specification as migratory border cells by controlling target gene expression (Beccari et al., 2002; Silver and Montell, 2001).

One of the transcriptional targets of the JAK/STAT pathway is *slbo* (Silver and Montell, 2001). *slbo* was the first gene identified for its role in border cell migration and encodes a basic region-leucine zipper (bZIP) transcription factor most similar to vertebrate CCAAT/enhancer binding protein (C/EBP) (Montell et al., 1992). *slbo* is expressed in border cells from stage 8 on, just before the onset of migration. Later on, it can also be detected in centripetal cells, but it is only required for the migration of border cells. However, a partial loss of STAT activity results in the absence of migration without interfering with *slbo* expression in border cells (Beccari et al., 2002). This suggests that STAT induces the expression of other target genes essential for border cell specification. Consistently, *slbo* expression is not sufficient to induce border cell fate (P. Rørth, unpublished data).

Another transcription factor required for border cell migration is Taiman, a steroid hormon receptor co-activator, which functions together with the ecdysone receptor in a ligand dependent manner. The functional *Drosophila* ecdysone receptor complex is a heterodimer of Ultraspiracle (Usp) and the Ecdysone receptor (EcR). *usp*, like *taiman*, is required for border cell migration (Bai et al., 2000).

### 1.3.3 Guidance

Once specified, border cells also need some signals telling them where to migrate. It has been shown that two redundant pathways are involved in guiding border cells during the first phase of the migration. Simultaneous inhibition of the *Drosophila* EGF and PDGF/VEGF receptors strongly inhibits border cell migration to the oocyte, showing that signaling through the two receptors is required for border cell migration (Duchek et al., 2001). At least one ligand for the PDGF/VEGF receptor (PVF1) and one ligand for the EGF receptor (Gurken) are produced by the oocyte during the migration and could thus in principle serve as guidance factors. Two other ligands for the EGF receptor (Spitz and Vein) are produced by follicular cells next to the oocyte and could therefore also be used as guidance factors. Uniform overexpression of these ligands in the germline as well as constitutive activation of the receptors inhibits the migration, indicating that those signaling pathways are not only permissive for the migration, but indeed serve as guidance signals for border cells (Duchek et al., 2001). Activation of the guidance receptor PVR stimulates actin polymerization, through a signaling pathway involving the small GTPase Rac and the Rac activator Mbc (Duchek et al., 2001).

Signaling through the EGF receptor is responsible for guiding border cells towards the oocyte nucleus during the second phase of the migration (Duchek and Rørth, 2001). Consistently, the three EGF receptor ligands are differentially expressed on the dorsal side of egg chambers and border cells fail to migrate dorsally in *gurken* mutants (Duchek and Rørth, 2001).

### 1.3.4 Extension formation and traction

In response to guidance cues, one border cell forms a single long and straight cellular extension at the initiation of migration. In situations where acto-myosin interactions are impaired, this extension becomes very long, is no longer straight and border cells do not move forward. Thus, acto-myosin contraction following extension formation seems to be critical for the translocation of the cell body (Fulga and Rorth, 2002).

### 1.3.5 Adhesion during border cell migration

Border cells migrate in between nurse cells and electron microscopy studies seem to indicate that there is no extracellular matrix between border cells and nurse cells but rather that border cells are in close contact with nurse cells; this suggests that border cells may adhere to nurse cells rather than to extracellular matrix during the migration (Verkhusha et al., 1999). It has been shown that it is indeed the case and that DE-cadherin is the molecule mediating adhesion between border cells and nurse cells.

DE-cadherin is expressed in nurse cells and in all follicular cells. At stage 8 of oogenesis, DE-cadherin is upregulated in border cells and keeps being expressed at high levels until the end of the migration (Niewiadomska et al., 1999) (Fig.1.12A-C). During the migration, DE-cadherin accumulates strongly at the membranes between adjacent border cells. It also localizes at the membrane between border cells and nurse cells: DE-cadherin stainings at these membranes look quite diffuse, which have led to the hypothesis that some of this staining may be due to DE-cadherin localized in subcortical vesicules (Niewiadomska et al., 1999) (Fig.1.12D).

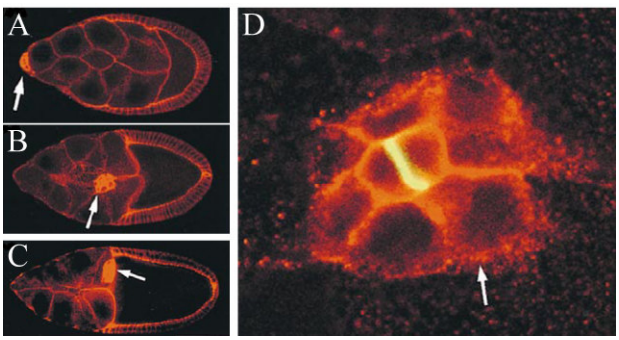



**Figure 1.12: DE-cadherin expression during border cell migration. A-C.** DE-cadherin is highly expressed in border cells (arrow, compared with follicular cells) from stage 8 on (A: stage 8; B: stage 9; C: stage 10). It is also expressed in nurse cells. **D.** Higher magnification of a border cell cluster during the migration (stage 9). DE-cadherin (red) strongly accumulates between adjacent border cells and between border cells and polar cells. DE-cadherin is present at lower amount and in a punctuate pattern (arrow) at the membrane between border cells and nurse cells. Green stains Fasciclin III which is expressed at the membrane between polar cells. Pictures from (Niewiadomska et al., 1999).

If DE-cadherin is removed from either border cells or nurse cells, border cells completely fail to migrate (Niewiadomska et al., 1999) (Oda et al., 1997), indicating that DE-cadherin mediates homophilic adhesion between border cells and nurse cells and that this adhesion is required for the migration. In the absence of DE-cadherin, border cells not only stay at the

anterior tip of the egg chamber, they are also unable to form long cellular extensions (Fulga and Rorth, 2002). It is worth noting that DE-cadherin is surprisingly not required to maintain adhesion between border cells (Niewiadomska et al., 1999).

## 1.4 Aim of the project

Regulation of cell-cell adhesion as well as cell-matrix adhesion is likely to be essential for proper development, in particular in tissues undergoing morphogenetic movements or in the case of cell migration. Although a lot has been done to understand how adhesion is regulated in tissue culture experiments, so far little is known about the relevance of the studied regulatory mechanisms in vivo.

During *Drosophila* development, DE-cadherin is very likely able to mediate stable adhesion – in particular in cases where it is required to maintain epithelial integrity – but also some much more dynamic adhesion, for instance during border cell migration. It thus seemed reasonable to assume that DE-cadherin-mediated adhesion would be regulated during *Drosophila* development. The aim of the work presented here was to identify the mechanisms regulating DE-cadherin-mediated adhesion in vivo, focusing mainly on the role of DE-cadherin during border cell migration.

# 2 RESULTS

## 2.1 p120ctn and juxtamembrane domain

### 2.1.1 Strategy

As mentioned in the introduction, p120ctn was described in the literature as an important regulator of cadherins. Moreover, during my DEA, I worked on a PDZ domain containing protein called Arc which has been shown to colocalize with adherens junction components (Liu and Lengyel, 2000). Arc overexpression inhibits border cell migration. The severity of the observed migration defects increases with the amount of DE-cadherin present in border cells. Moreover, I showed that Arc interacts with p120ctn. This suggested that Arc overexpression inhibits migration by increasing DE-cadherin mediated adhesion through its interaction with p120ctn. Although analysis of *arc* null mutants showed that Arc is not required for border cell migration, the overexpression data prompted us to test wheter p120ctn is involved in DE-cadherin function/regulation during border cell migration.

When I initiated this project there was no *Drosophila* p120ctn mutant available. I thus decided to test if the interaction between p120ctn and DE-cadherin was important by making a mutant form of DE-cadherin that can not bind p120ctn. To do so, I mutated to alanine a conserved triplet of glycine in the juxtamembrane domain of DE-cadherin. The corresponding mutation in human E-cadherin disrupts the interaction with p120ctn without affecting the interaction with β-catenin (Thoreson et al., 2000). I thus obtained DE-cadherin-AAA (Fig.2.1). To test possible other functions of the juxtamembrane domain, I also made a mutant form of DE-cadherin lacking the whole juxtamembrane domain. This mutant is called DE-cadherin ΔJM (Fig.2.1). As a negative control for our experiments, I also made a mutant form of DE-cadherin lacking the C-terminal domain; this mutant, called DE-cadherin-Δβ (Fig.2.1), is unable to bind β-catenin and is thus expected to be non functional.

To analyze the biological activity of the mutant DE-cadherin proteins during development, the following strategy was used to replace endogenous DE-cadherin with mutant forms: (1) I generated transgenic flies expressing DE-cadherin-wt, DE-cadherin-AAA, DE-cadherin ΔJM or DE-cadherin-Δβ ubiquitously, under the control of the tubulin-α1 promoter; (2) endogenous DE-cadherin was removed from specific cells by generating homozygous *shg* mutant clones or zygotic expression was removed by analyzing *shg* homozygous mutant embryos. I then determined whether the transgene encoded DE-cadherin could substitute for

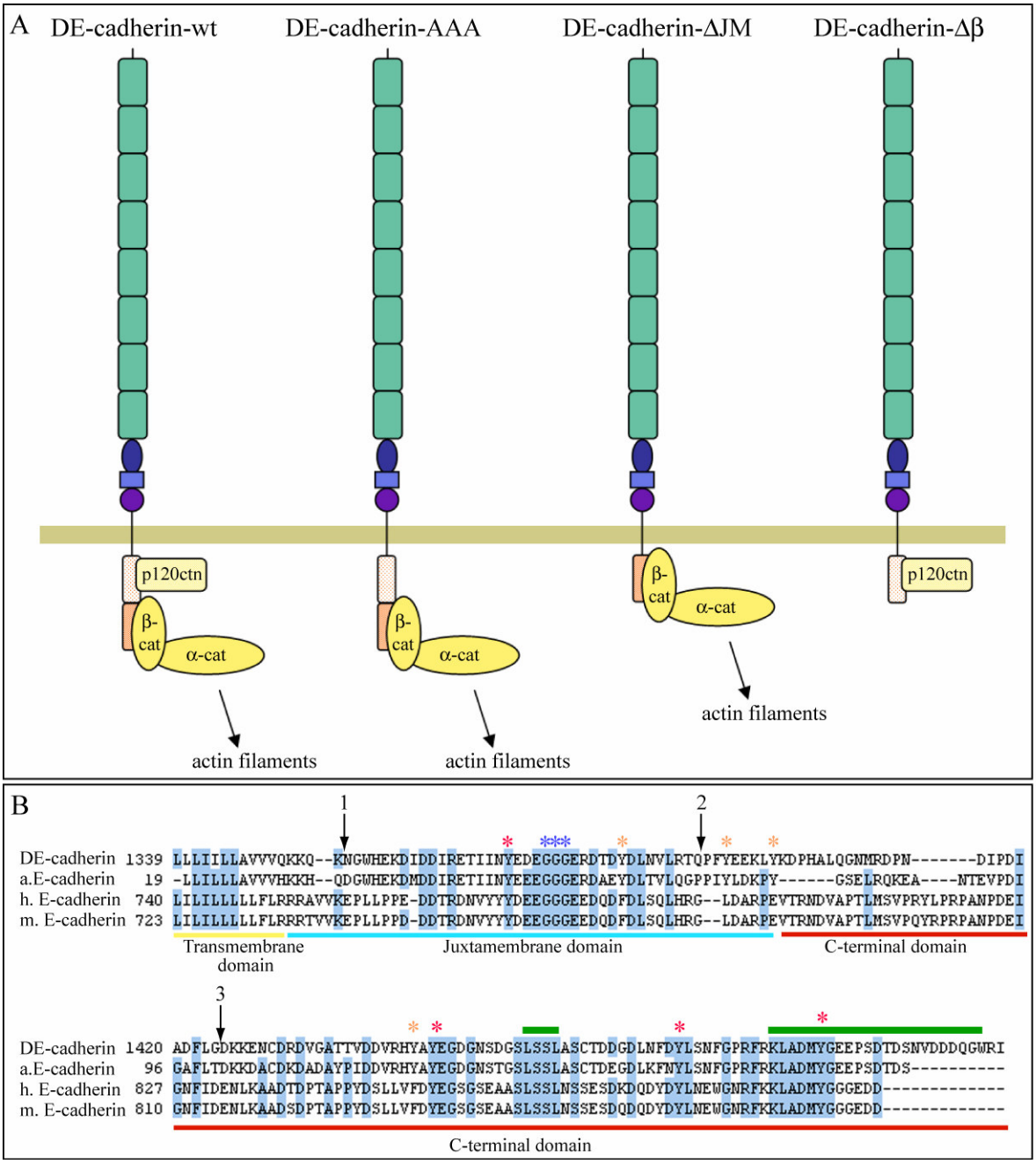



**Figure 2.1: DE-cadherin variants. A.** Schematic representation of DE-cadherin variants and catenins associated. **B.** Sequence of *Drosophila*, *Anopheles*, human and mouse E-cadherin transmembrane and cytoplasmic domains. In DE-cadherin-AAA, 3 glycines (marked with blue asterix) were mutated to alanines. In DE-cadherin-ΔJM, aa between arrows 1 and 2 were deleted. In DE-cadherin-Δβ, aa after arrow 3 were deleted. Red and orange asterix indicate respectively conserved (between vertebrates and invertebrates) and non-conserved tyrosines. Green boxes indicate LSSL and PEST sequences, which are both thought to be sequences involved in protein degradation. The limit between juxtamembrane and C-terminal domain was drawn after studies on mammal E-cadherin. In DE-cadherin, the region between arrow 2 and 3 is quite divergent, it is thus not clear if it would be involved in β-catenin binding or not.

endogenous DE-cadherin, that is, rescue the *shg* mutant phenotypes. Three *shg* alleles were used: $shg^{R69}$ is a molecular null allele, $shg^{1}$ and $shg^{2}$ are not molecularly characterized but $shg^{2}$ is a genetic null and $shg^{1}$ embryos have stronger phenotypes than $shg^{R69}$ and $shg^{2}$ embryos. An antibody directed against DE-cadherin extracellular domain does not detect any protein in any of these three mutants. In the ovary, both somatic and germline *shg* mutant clones were generated by mitotic recombination and identified by the absence of GFP (negatively marked clones, see materials and methods, §4.3.2).

### 2.1.2 Controls

Membrane localization and expression levels of the different DE-cadherin transgenes were checked by immunostaining of egg chambers expressing a DE-cadherin transgene and containing *shg* mutant clones. *shg* mutant cells express only the transgene encoded DE-cadherin, which allowed me to check the subcellular localization and the expression levels of the different DE-cadherin variants. Wild type as well as mutant cadherin proteins are enriched at the cell cortex, presumably the plasma membrane, in follicular cells (fig2.2A-D) and nurse cells (Fig2.2F-I). DE-cadherin-Δβ also accumulates in some intracellular aggregates, which may correspond to vesicles (Fig2.2D). In follicular cells, one can not see a difference in DE-cadherin levels between cells expressing only DE-cadherin-wt, DE-cadherin-AAA or DE-cadherin-ΔJM transgenes (non GFP cells) and cells expressing both the transgene encoded DE-cadherin and endogenous DE-cadherin (GFP cells) (Fig.2.2A-C). This shows that DE-cadherin-wt, DE-cadherin-AAA and DE-cadherin-ΔJM transgenes are expressed at somewhat higher levels than endogenous DE-cadherin. Comparison with endogenous expression levels in wild type egg chambers show that these DE-cadherin variants are only moderately overexpressed. DE-cadherin-Δβ was expressed at slightly lower levels (Fig.2.2D). In the germline, DE-cadherin-Δβ had expression levels similar to endogenous DE-cadherin whereas DE-cadherin-wt, DE-cadherin-AAA and DE-cadherin–ΔJM were expressed at higher levels (Fig.2.2E-I).

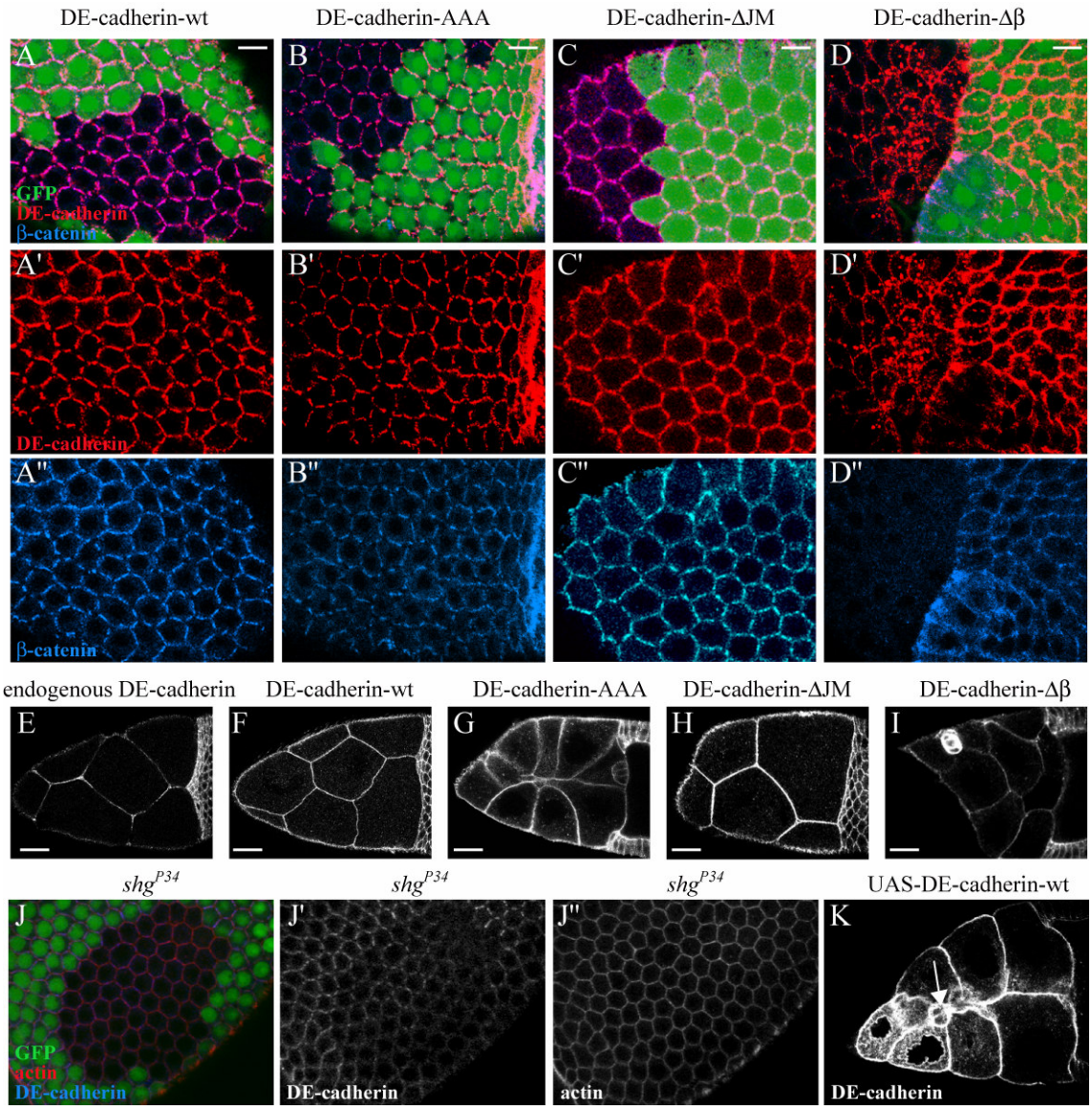



**Figure 2.2: Expression of DE-cadherin variants in the ovary. A-D.** Follicular epithelium from females carrying tubulin-DE-cadherin-wt (A), tubulin-DE-cadherin-AAA (B), tubulin-DE-cadherin-ΔJM (C) or tubulin-DE-cadherin-Δβ (D) transgenes. Absence of GFP (green) marks *shg* mutant cells. DE-Cadherin is in red and endogenous β-catenin in blue. Bars: ~10 µm. **E-I.** DE-cadherin levels in nurse cells expressing only endogenous DE-cadherin (E) or the indicated tubulin-DE-cadherin transgene (F-I, germ line cells are *shg* mutant clones.). Bars: ~20 µm. **J.** DE-cadherin levels in $shg^{P34}$ mutant follicular cells. DE-cadherin is in blue (and in J') and phalloidin stains actin filaments (in red and J''). Absence of GFP (green) marks *shg* mutant cells. **K.** DE-cadherin levels in nurse cells overexpressing a UAS-DE-cadherin-wt transgene. Overexpression is driven by nanosGal4; this driver gives not totally uniform overexpression in nurse cells. Note that border cells (arrow) are delayed.

To know in which range DE-cadherin expression levels can vary in the ovary without being deleterious, I checked levels of expression and phenotypes both in a *shg* hypomorph and in the case of DE-cadherin overexpression. $shg^{P34}$ is a hypomorphic allele of *shg* that has quite severely reduced levels of DE-cadherin (Fig.2.2J). Follicular cells look normal and border cell migration is only weakly affected in this mutant (about 10% of border cell clones have some migration delays). Overexpression of a UAS-DE-cadherin-wt transgene in border cells at quite high levels does not seem to affect the migration (see §2.3.1). By contrast overexpression of UAS-DE-cadherin-wt in nurse cells – which gives much higher expression levels than the tubulin driven DE-cadherin transgenes (Fig.2.2K) - inhibits border cell migration (more than 90% of border cell clusters do not reach the oocyte at stage 10).

To check that DE-cadherin-AAA does not interact with p120ctn, I first performed co-immunoprecipitation experiments from Schneider cells overexpressing DE-cadherin-wt or DE-cadherin-AAA and p120ctn. Since I do not have an antibody against p120ctn, I used a tagged version of p120ctn (HA-p120ctn). HA-p120ctn co-immunoprecipitates with DE-cadherin-wt but not with DE-cadherin-AAA (Fig.2.3D). This shows that the ability to interact with p120ctn is disrupted in DE-cadherin-AAA. I also tested the ability of the different forms of DE-cadherin to bind β-catenin and p120ctn by looking at their ability to recruit β-catenin and p120ctn to the cortex in follicular cells. In the presence of wildtype DE-cadherin, endogenous β-catenin localizes to the cortex in follicular cells (Fig.2.2A). In the absence of DE-cadherin, β-catenin is no longer detectable at the cortex; it also does not accumulate in the cytoplasm, presumably due to its instability. DE-cadherin–AAA and DE-cadherin–ΔJM can both recruit and/or stabilize β-catenin at the cortex (Fig2.2B-C), whereas DE-cadherin-Δβ cannot (Fig2.2D). Overexpressed HA-p120ctn in follicular cells is mostly cytoplasmic (Fig2.3A); only a small amount (arrow in Fig2.3A) is present at the cell cortex, presumably due to association with endogenous DE-cadherin. Co-overexpression of DE-cadherin-wt results in efficient recruitment of HA-p120ctn to the cortex (Fig2.3B), whereas co-overexpression of DE-cadherin-AAA does not (Fig2.3C). Hence, as expected, DE-cadherin-AAA and DE-cadherin-ΔJM but not DE-cadherin-Δβ can bind to β-catenin and DE-cadherin-AAA can not bind to p120ctn.

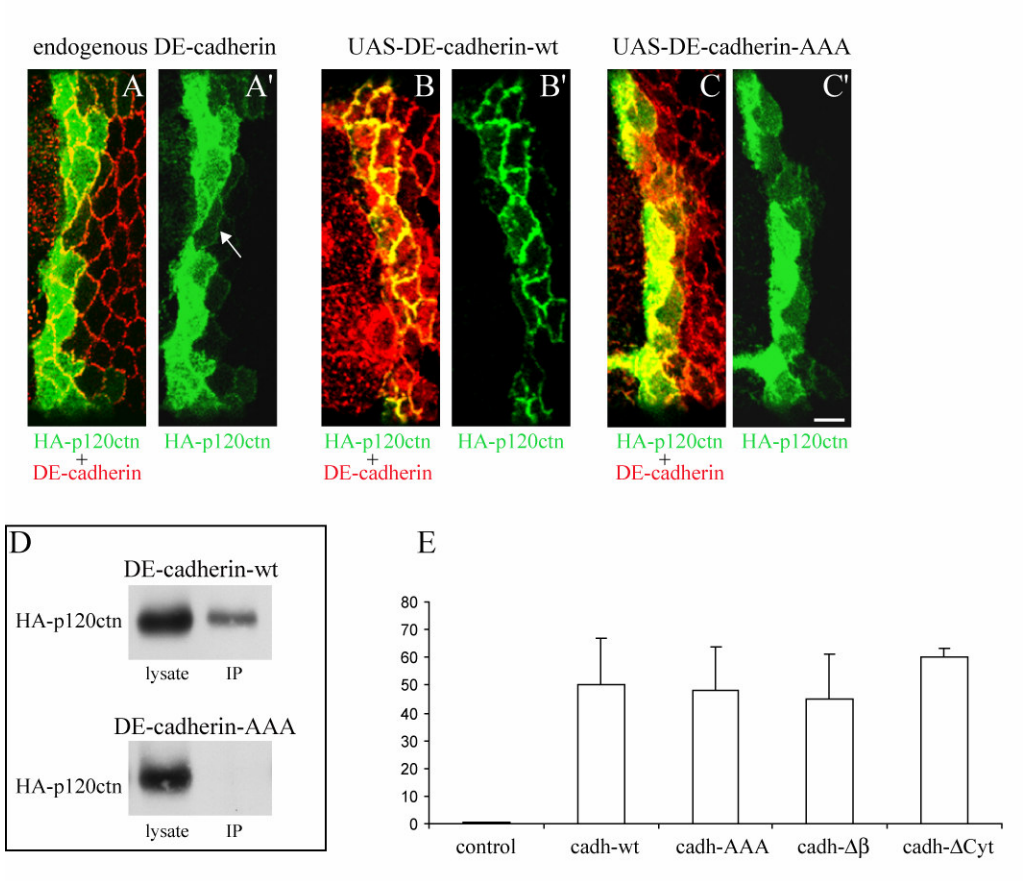



**Figure 2.3: DE-cadherin variants, binding to p120ctn and adhesive activity in S2 cells. A-C.** Localization of overexpressed HA-p120ctn (green and A'-C') in follicular cells expressing only endogenous DE-cadherin (A), or overexpressing DE-cadherin-wt (B) or DE-cadherin-AAA (C) (red). Levels of endogenous DE-cadherin are too low to recruit all overexpressed p120ctn to the cortex, except in cells where overexpression is milder (arrow in A). UAS-HA-p120ctn and UAS-DE-cadherin transgenes were overexpressed with slboGal4 (Rørth et al., 1998). Bars: ~20 µm. **D.** Immunoprecipitation from Schneider cells overexpressing HA-p120ctn and DE-cadherin-wt or DE-cadherin-AAA. 4% of lysates and all immunoprecipitates (IP) were loaded. **E.** Percentage of S2 cells forming aggregates when transfected with the indicated DE-cadherin variants. The average size of aggregates was also quantified and no significant difference observed between all four constructs.

Cell aggregation is commonly used to measure cadherin-dependent adhesion and the importance of cytoplasmic interactions in mammalian cells. As a first functional analysis of our DE-cadherin mutants, I thus compared the ability of DE-cadherin-wt, DE-cadherin-AAA and DE-cadherin-Δβ to induce aggregation of Schneider cells (see materials and methods, §4.2). An additional form of DE-cadherin lacking the full cytoplasmic tail (DE-cadherin-

ΔCyt) was also tested. All four constructs gave similar adhesion, showing that DE-cadherin mediated aggregation of Schneider cells is independent of DE-cadherin cytoplasmic tail (Fig.2.3E). Consistently, Oda and Tsukita had already observed that a mutant form of DE-cadherin lacking most of the cytoplasmic domain had the same ability as wildtype DE-cadherin to mediate aggregation of S2 cells (Oda and Tsukita, 1999). I also tried to do this assay in CHO cells, but was not able to obtain proper expression of the *Drosophila* cadherin in this heterologous system.

### 2.1.3 Neither interaction with p120ctn nor juxtamembrane domain are required for DE-cadherin function during border cell migration

I first analyzed the activity of mutant DE-cadherin proteins in border cells as well as in the substratum, the nurse cells. In both border cells and nurse cells, expression of DE-cadherin-wt as well as DE-cadherin-AAA and DE-cadherin–ΔJM completely rescued the migration (Fig.2.4E-F). Thus DE-cadherin/p120ctn interaction as well as DE-cadherin juxtamembrane domain do not appear to be required for the migration. DE-cadherin-Δβ expression in border cells did not rescue the *shg* phenotype at all (Fig.2.4E), suggesting that DE-cadherin must be anchored to the actin cytoskeleton via β-catenin to function in the migrating cells. In nurse cells, DE-cadherin-Δβ does not completely block the migration (Fig.2.4F).

It should also be briefly mentioned that Arc overexpression still inhibits migration in border cells expressing only DE-cadherin-AAA or DE-cadherin-ΔJM. This shows that Arc overexpression does not simply induce regulation of DE-cadherin via p120ctn binding as we initially hypothezised. The analysis of Arc overexpression phenotype was not pursued further.

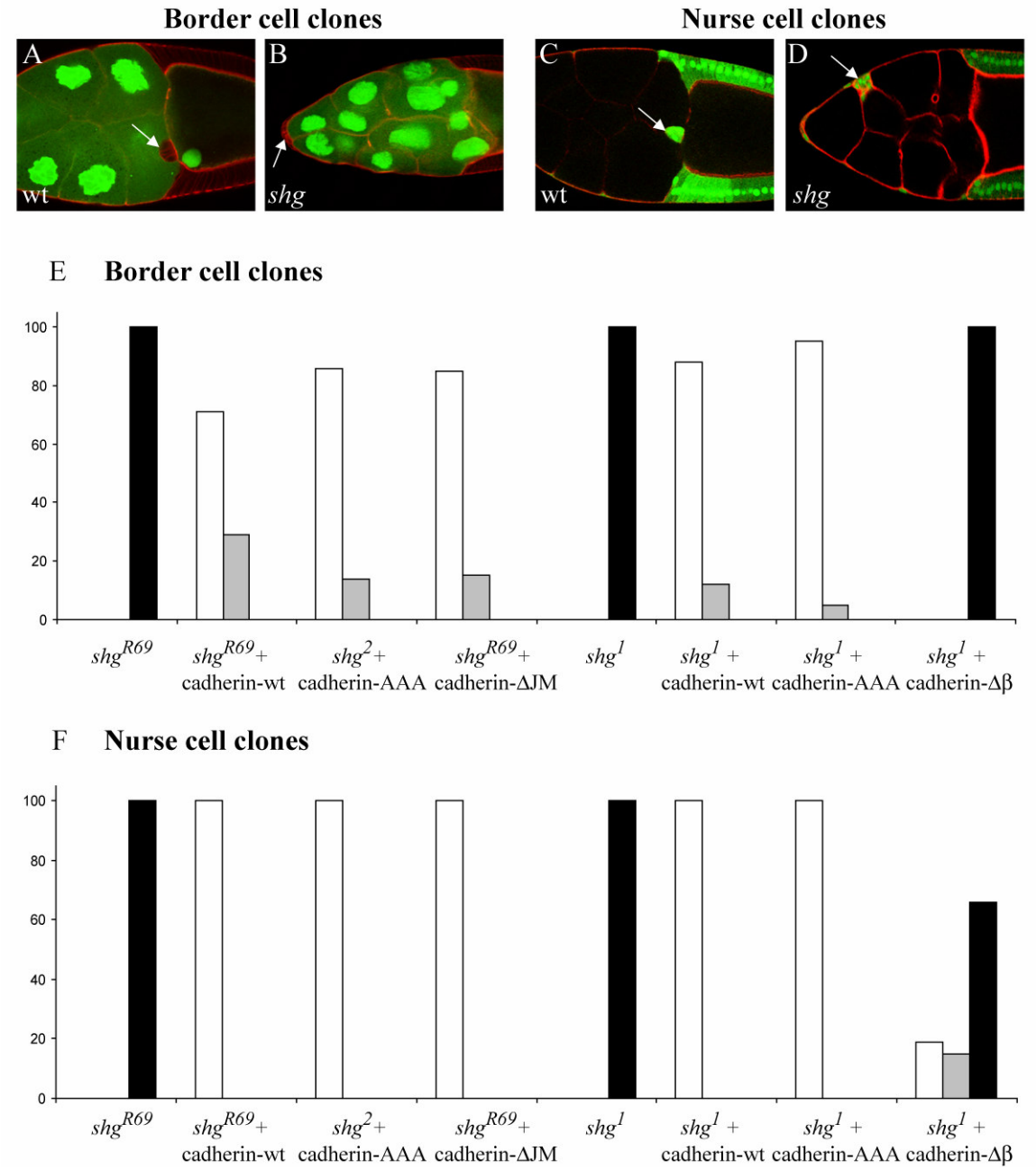



**Figure 2.4: DE-cadherin-wt, DE-cadherin-AAA and DE-cadherin-ΔJM but not DE-cadherin-Δβ transgenes can rescue *shg* phenotype during border cell migration. A-D.** In stage 10 wild type egg chambers, border cells (arrow) have reached the oocyte (A and C). Lack of DE-cadherin in border cells (B) or in nurse cells (D) prevent border cells from penetrating between the nurse cells. Phalloidin (red) stains the actin cytoskeleton of egg chambers and non-GFP (non green) cells are wild type (A,C) or *shg* mutant (B,D) clones. **E-F.** Migration defects in *shg* mutant border cell (E) and germ line (F) clones and in the presence of the indicated transgenes ($15 \leq n \leq 73$). White: full migration; grey: incomplete migration; black: no migration.

### 2.1.4 Neither interaction with p120ctn nor juxtamembrane domain are required for DE-cadherin function during oogenesis and development

I next addressed whether the DE-cadherin/p120ctn interaction was required for other types of adhesion. Early in oogenesis, DE-cadherin mediated adhesion is necessary to correctly position the oocyte at the posterior pole of the egg chamber. If either the germ line or the somatic cells are mutant for *shg*, the oocyte is often mispositioned (Fig.2.5; (Godt and Tepass, 1998; González-Reyes and St. Johnston, 1998)). DE-cadherin-wt, DE-cadherin-AAA and DE-cadherin–ΔJM all rescue the *shg* defects in both germ line and follicular cell clones (Fig.2.5E-F). Thus adhesion between the oocyte and the follicular cells requires neither DE-cadherin/p120ctn interaction nor DE-cadherin juxtamembrane domain. DE-cadherin-Δβ is completely unable to rescue the mispositioning phenotype (Fig.2.5E-F).

Within the follicular epithelium, *shg* mutant cells sort away from wild type cells generating a smooth boundary at the interface (Fig.2.6A-B; (González-Reyes and St. Johnston, 1998)). This is thought to reflect preferential adhesion between DE-cadherin expressing cells. If DE-cadherin/p120ctn interaction alters adhesion, cells expressing only DE-cadherin-AAA might sort away from cells also expressing wild type DE-cadherin. However, this was not observed (Fig.2.6E). Similarly, no sorting is observed in egg chambers expressing DE-cadherin-ΔJM (Fig.2.6E). In contrast, cells expressing only DE-cadherin-Δβ do sort from cells also expressing wild type DE-cadherin (Fig.2.6E). Thus, the lack of interaction between DE-cadherin and β-catenin results in a decrease of adhesion strong enough to obtain sorting, whereas the lack of interaction between DE-cadherin and p120ctn or the lack of juxtamembrane domain do not.

At early to mid-oogenesis, *shg* mutant follicle cells maintain correct cell shape within the epithelium. This may reflect functional overlap between DE-cadherin and DN-cadherin - which are both expressed at these stages - as *arm* mutant clones have stronger defects (Tanentzapf et al., 2000). Late in oogenesis, N-cadherin is no longer expressed and *shg* mutant follicle cells show cell shape defects in the epithelium (Fig2.6C-D) (Tanentzapf et al., 2000). This defect is also rescued by DE-cadherin-AAA and DE-cadherin-ΔJM but not DE-cadherin-Δβ.

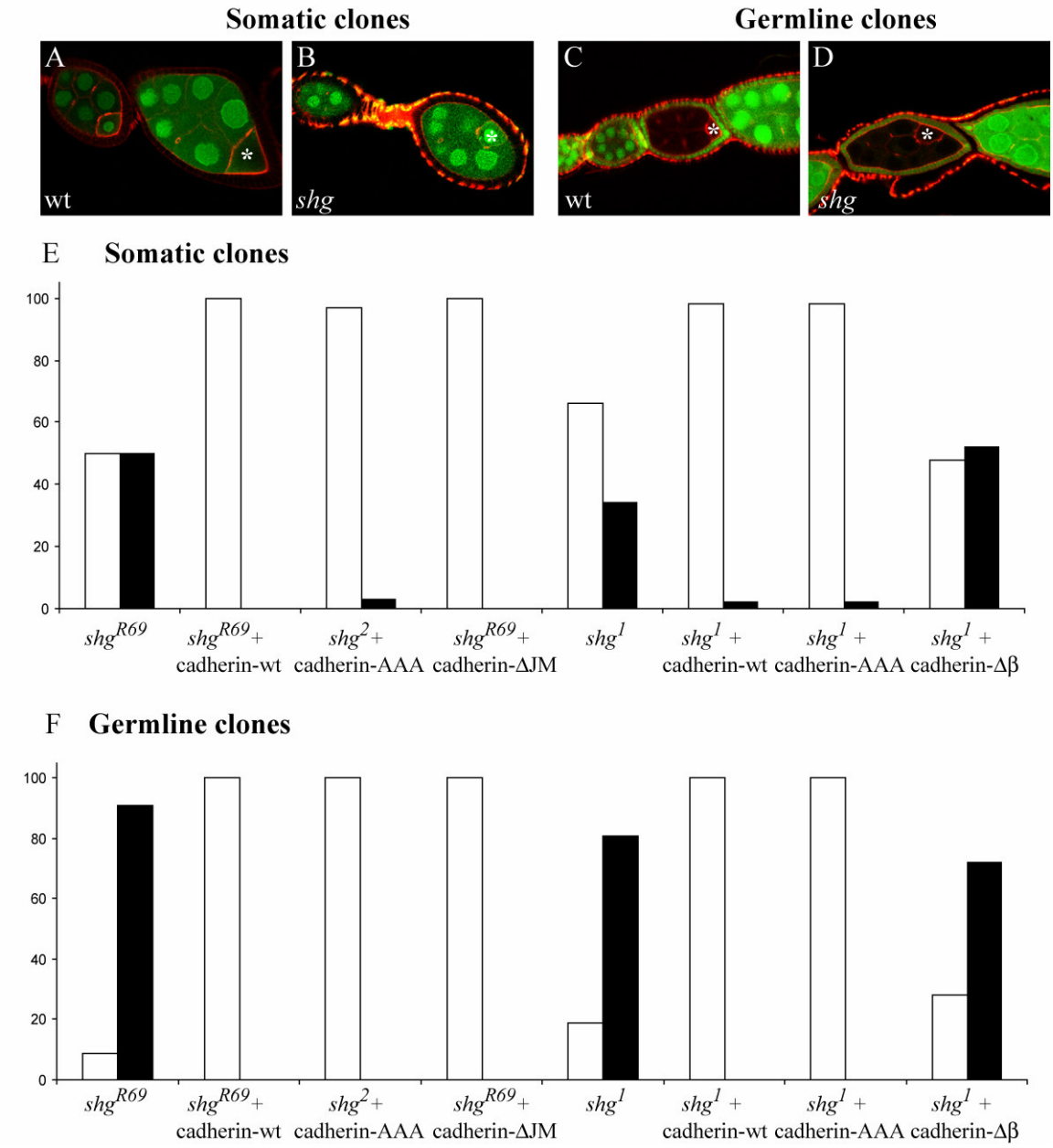



**Figure 2.5: DE-cadherin-wt, DE-cadherin-AAA and DE-cadherin-ΔJM but not DE-cadherin-Δβ transgenes can rescue *shg* phenotype during oocyte positioning. A-D.** In wild type egg chambers, the oocyte (asterix) is posteriorly located (A and C). Lack of DE-cadherin in follicular cells (B) or in germ line cells (D) often causes a mislocalization of the oocyte. Phalloidin (red) stains the actin cytoskeleton of egg chambers and non-GFP (non green) cells are wild type (A,C) or *shg* mutant (B,D) clones. All ovarioles are represented anterior (younger egg chambers) to the left. **E-F.** Oocyte mispositioning in *shg* mutant follicular cells (E) and germ line (F) clones and in the presence of the indicated transgenes ($25 \leq n \leq 63$). White: oocyte posteriorly located; black: mispositioned oocyte.

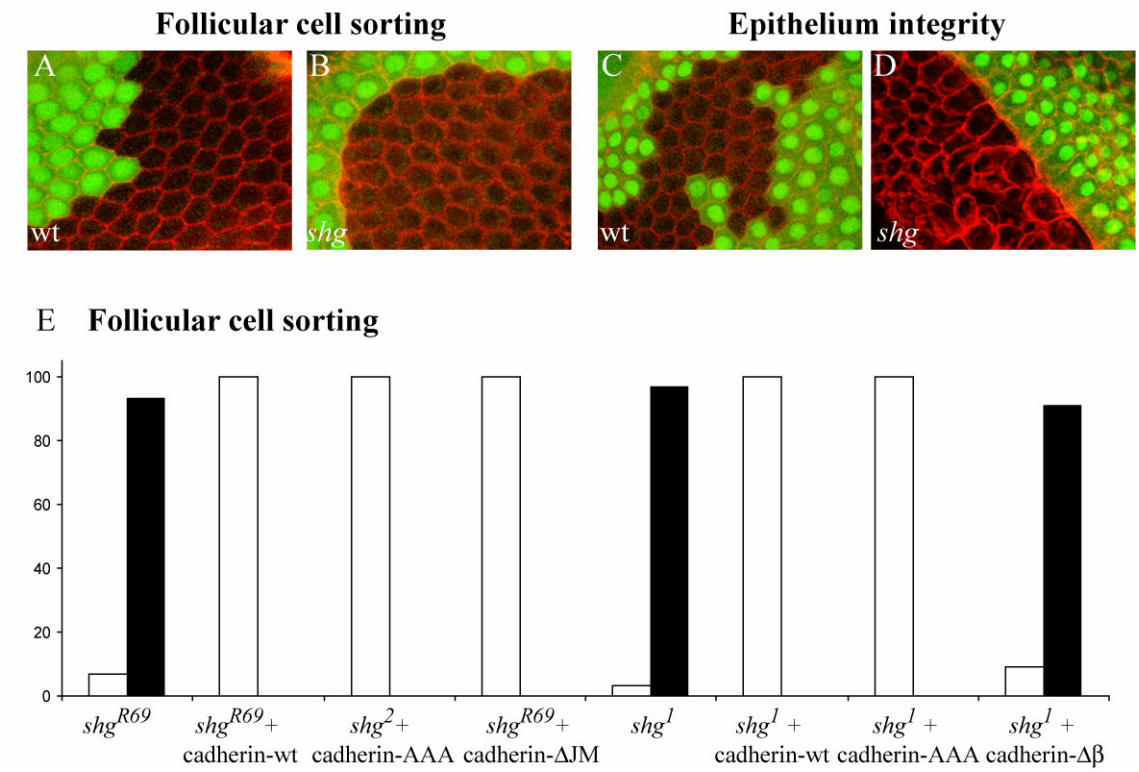



**Figure 2.6: DE-cadherin-wt, DE-cadherin-AAA and DE-cadherin-ΔJM but not DE-cadherin-Δβ transgenes can rescue *shg* phenotypes in the follicular epithelium. A-B.** Wild type follicular clones intermingle (A) whereas *shg* mutant clones sort (B). **C-D.** At stage 10-11, the follicular epithelium loses its integrity in *shg* mutant clones (D), compared to wild type clones (C). Phalloidin (red) stains the actin cytoskeleton of egg chambers and non-GFP (non green) cells are wild type (A,C) or *shg* mutant (B,D) clones. **E.** Sorting in *shg* mutant clones and in the presence of the indicated transgenes ($28 \leq n \leq 68$). White: no sorting; black: sorting.

To analyze additional types of DE-cadherin mediated adhesion in other tissues, I turned to the embryo. DE-cadherin is maternally supplied but zygotic expression is required for embryonic development, for example in the embryonic epidermis. Homozygous $shg^1$ mutant embryos lack the head and ventral epidermis which results in the lack of head and ventral cuticle (Fig.2.7A-B). Both DE-cadherin-wt and DE-cadherin-AAA transgenes fully rescue this phenotype (Fig.2.7C). I also tested rescue of the embryonic lethality due to the absence of zygotic DE-cadherin (see materials and methods, §4.3.3). Despite the use of a heterologous promoter to express DE-cadherin, 10 to 25% of $shg^1/ shg^1$ embryos carrying a DE-cadherin-wt or DE-cadherin-AAA transgene hatch (Fig.2.7D). Observation of the resulting larvae did

not show any obvious defects in terms of general morphology, cuticle, trachea, malphigian tubules and gut formation. When using the $shg^{R69}$ null allele, DE-cadherin-AAA and DE-cadherin-ΔJM can even rescue most $shg^{R69}/shg^{R69}$ mutants to adulthood (Fig.2.7E). The rescued females allowed us to test the ability of DE-cadherin-AAA and DE-cadherin-ΔJM to substitute for maternally provided DE-cadherin. Females expressing only DE-cadherin-AAA or DE-cadherin-ΔJM give rise to viable progeny also carrying only DE-cadherin-AAA or DE-cadherin-ΔJM: DE-cadherin-AAA and DE-cadherin-ΔJM can substitute for both zygotic and maternal endogenous DE-cadherin. These results indicate that DE-cadherin/p120ctn interaction and DE-cadherin juxtamembrane domain are not essential for any critical process during development.

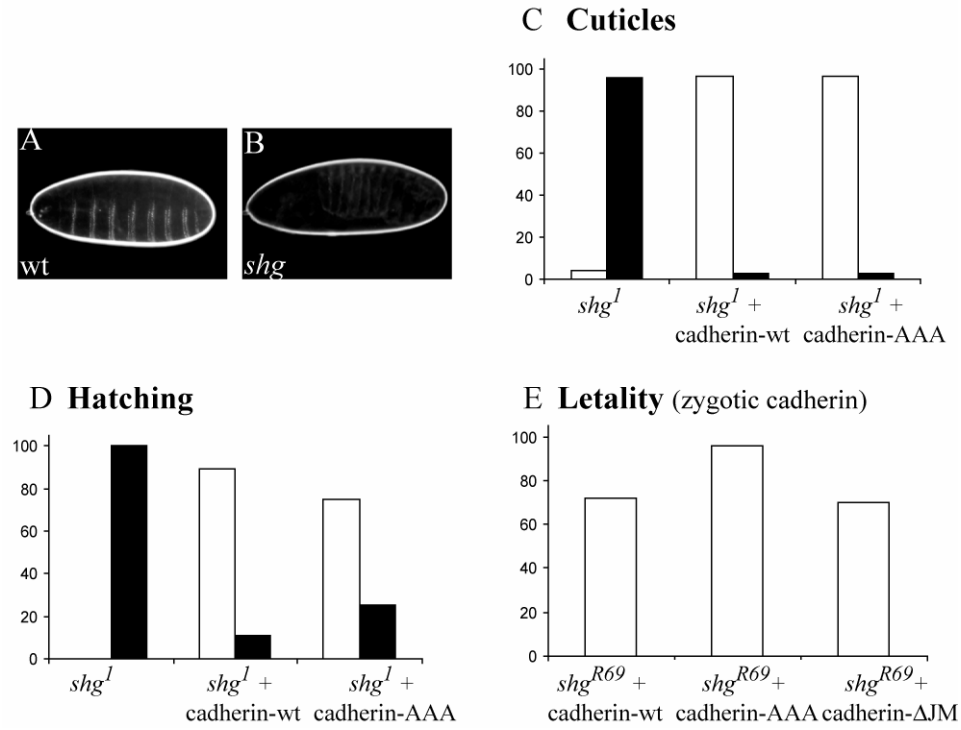
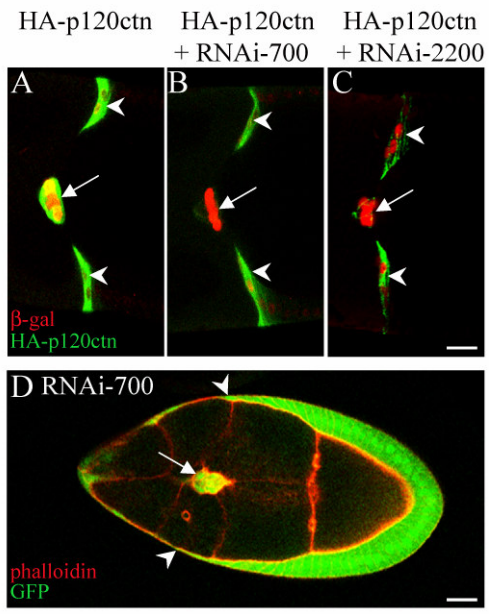



**Figure 2.7: DE-cadherin-wt, DE-cadherin-AAA and DE-cadherin-ΔJM transgenes can rescue *shg* phenotypes during embryogenesis. A-B.** Cuticle of a wild type embryo (A) and of a homozygous *shg* mutant embryo (B). Bar: ~50 µm. **C.** Cuticle defects in homozygous *shg* mutant embryos and in the presence of the indicated transgenes (32≤n≤232). White: wild type cuticle; black: cuticle with *shg* phenotype. **D.** Hatching of homozygous *shg* mutant embryos and in the presence of the indicated transgenes (n≥275). White: hatching; black: no hatching. **E.** Rescue of the lethality due to the absence of zygotic endogenous DE-cadherin by the indicated transgenes. 100% corresponds to the situation where the proportion of *shg/shg*; tub-DE-cadherin flies in the progeny would be the expected mendelian proportion if tubulin-DE-cadherin transgenes fully rescue *shg* lethality (50≤n≤630).

### 2.1.5 p120ctn itself does not seem to be required for Drosophila development

In an attempt to compromise p120ctn function directly, I used double-stranded RNA mediated interference (RNAi). Two p120ctn-RNAi transgenes (RNAi-700 and RNAi-2200, for details see materials and methods §4.1) were able to severely reduce the levels of co-overexpressed HA-p120ctn (Fig.2.8A-C), suggesting that levels of endogenous p120ctn would also be strongly reduced. Expression of p120-RNAi in somatic cells does not cause any detectable phenotype in the ovary (Fig.2.8D-E). Similarly, maternal and zygotic expression of p120ctn-RNAi transgenes (see materials and methods §4.3.3) does not affect embryonic viability (Fig.2.8E). This is consistent with p120ctn not being important for DE-cadherin function during oogenesis and development. However, the lack of phenotype may also reflect incomplete removal of p120ctn.




**E**

| | UAS-RNAi -700 | UAS-RNAi -2200 | wildtype |
|---|---|---|---|
| normal border cell migration | 100% (n>240) | 100% (n>120) | nd |
| oocyte correctly positioned | 100% (n>200) | 100% (n>130) | nd |
| follicular cell clones non sorted | 100% (n>120) | 100% (n>70) | nd |
| hatching rate (zyg) | 91% (n>2500) | 74% (n>2500) | 84% (n>1200) |
| hatching rate (mat+zyg) | 86% (n>2000) | 98% (n>2500) | 90% (n>2500) |

**Figure 2.8: Effect of p120ctn-RNAi transgenes. A-C.** Levels of HA-p120ctn in border cells (arrow) and centripetal cells (arrowheads) overexpressing HA-p120ctn (A) and one RNAi construct (B and C); stage 10b egg chambers. All constructs are expressed using slboGal4 which starts expressing in border cells at stage 9 and in centripetal cells at stage 10. HA-p120ctn levels are severely reduced in border cells, estimated to have been expressing RNAi constructs for 12-24 hours. Bar: ~10µm. **D**. Stage 9 egg chamber from a female of the genotype hsFLP/actin-Flip-out-Gal4; UAS-RNAi/+; UAS-GFP/+. Larval heat-shock was used to initiate constitutive expression of the RNAi in somatic cells (marked by GFP) one week before analysis. Border cell migration is normal (border cells (arrow) migrated as far as follicular cells (arrowheads)). Bar: ~15 µm. **E**. Quantification of phenotypes upon expression of p120ctn-RNAi transgenes in egg chambers or embryos (maternal [mat] and zygotic [zyg] expression).

## 2.2 Tyrosine phosphorylation

There is growing evidence suggesting that tyrosine phosophorylation of cadherin complexes components may be involved in regulating adhesion. In addition, two receptor tyrosine kinases – the receptors for EGF and PDG/VEGF – play an essential role during border cell migration, in particular for guidance. It thus seemed possible that tyrosine phosphorylation of DE-cadherin or catenins could be involved in regulating adhesion during border cell migration: we chose to test wheter DE-cadherin or β-catenin tyrosine phosphorylation was important for the migration.

### 2.2.1 DE-cadherin tyrosine phosphorylation

#### 2.2.1.1 Strategy

There are 8 tyrosine residues in the cytoplasmic tail of DE-cadherin, 4 of them are conserved between *Drosophila* and mammals. The four other tyrosines are not conserved between *Drosophila* and mammals but seem to be conserved between *Drosophila* and *Anopheles* (Fig.2.1B). However, when we initiated this part of the project, the *Anopheles* genome was not available yet; we therefore focused on the four tyrosines that were conserved between *Drosophila* and mammals and mutated them to phenylalanine to obtain DE-cadherin-4YF (Fig.2.9A). I then tested if DE-cadherin-4YF could substitute for endogenous DE-cadherin. As described above for DE-cadherin-AAA and DE-cadherin-ΔJM, I generated transgenic flies expressing DE-cadherin-4YF ubiquitously and tested if DE-cadherin-4YF could rescue the lack of endogenous DE-cadherin in *shg* mutant clones.

Immuno-staining of egg chambers expressing a DE-cadherin-4YF transgene and containing *shg* mutant clones showed that DE-cadherin-4YF is enriched at the cell cortex in follicular cells (Fig2.9B) and nurse cells (Fig.2.9D). Similarly to DE-cadherin-wt, DE-cadherin-AAA or DE-cadherin-ΔJM transgenes, DE-cadherin-4YF is expressed at somewhat higher levels than endogenous DE-cadherin in follicular cells (Fig.2.9B). In the germ line, DE-cadherin-4YF is expressed at higher levels than endogenous DE-cadherin (Fig.2.9C-D). I also tried to check whether wild type DE-cadherin is indeed tyrosine phosphorylated and whether DE-cadherin-4YF would be also phosphorylated or not. Preliminary attempts turned to be unsuccessfull: no tyrosine phosphorylation could be detected when DE-cadherin-wt was

immunoprecipitated from Schneider cell extracts. But it is very possible that Schneider cells are not a relevant system to study DE-cadherin regulation. To really determine if wild type DE-cadherin is phosphorylated in vivo, the experiment should be repeated for instance from ovarian extracts.

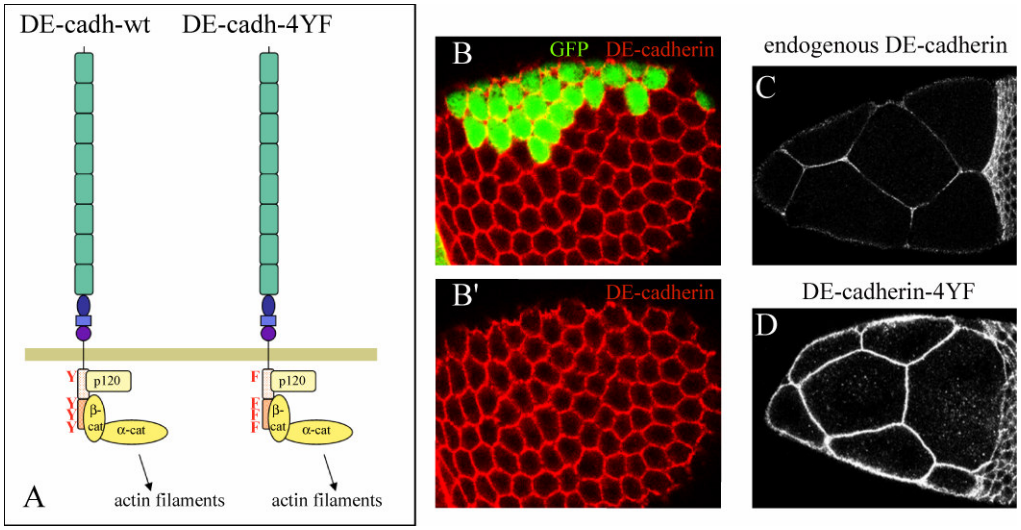



**Figure 2.9: DE-cadherin-4YF. A.** Schematic representation of DE-cadherin-4YF. **B.** Follicular epithelium from a female carrying a tubulin-DE-cadherin-4YF transgene. Absence of GFP (green) marks *shg* mutant cells. DE-cadherin is in red. **C-D.** DE-cadherin levels in nurse cells expressing only endogenous DE-cadherin (C) or tubulin-DE-cadherin-4YF transgene (D, germ line cells are *shg* mutant clones).

#### 2.2.1.2 DE-cadherin-4YF substitutes for endogenous DE-cadherin during border cell migration

First, I analyzed the ability of DE-cadherin-4YF to substitute for endogenous DE-cadherin during border cell migration. In both border cells and nurse cells, expression of DE-cadherin-4YF completely rescues the migration (Fig.2.10). Thus the four conserved tyrosine residues in DE-cadherin cytoplasmic domain, and hence their phosphorylation, are not essential for the migration.

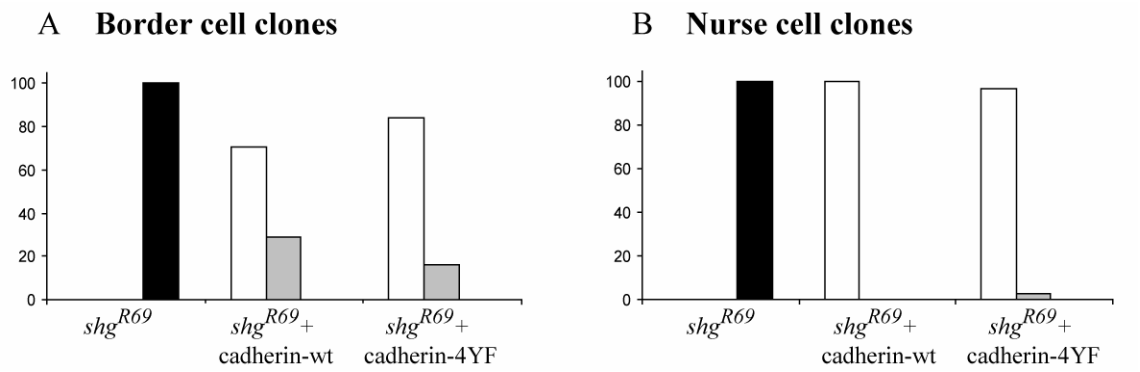



**Figure 2.10: DE-cadherin-4YF can rescue *shg* phenotype during border cell migration. A-B.** Migration defects in *shg* mutant border cell (A) and germ line (B) clones and in the presence of the indicated transgenes (15≤n≤38). White: full migration; grey: incomplete migration; black: no migration.

### 2.2.1.3 DE-cadherin-4YF substitutes for endogenous DE-cadherin during oogenesis and development

I next tested whether the four conserved tyrosines in DE-cadherin cytoplasmic domain are required for DE-cadherin function during oogenesis. It turned out that DE-cadherin-4YF can substitute for endogenous DE-cadherin for proper oocyte positioning, both in the germ line and in follicular cells (Fig.2.11). DE-cadherin-4YF also rescues the lack of endogenous DE-cadherin in the follicular epithelium, both in term of cell sorting and epithelial integrity.

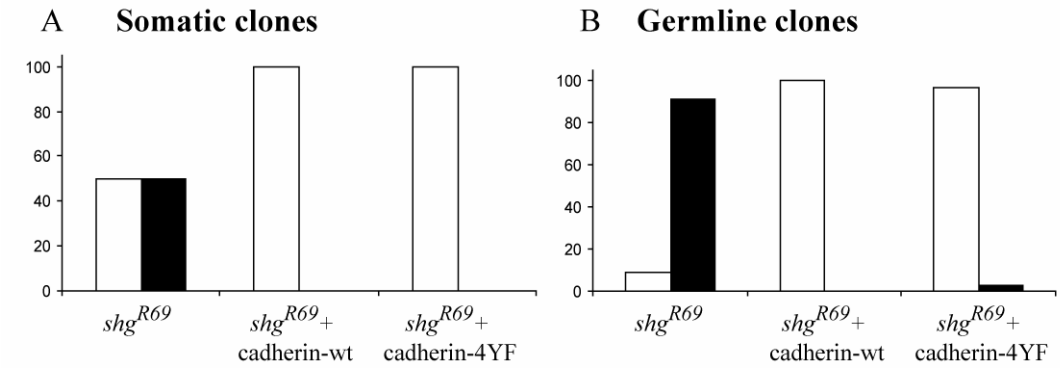



**Figure 2.11: DE-cadherin-4YF can rescue *shg* phenotype during oocyte positioning. A-B.** Oocyte mispositioning in *shg* mutant follicular cells (A) and germ line (B) clones and in the presence of the indicated transgenes (32≤n≤47). White: oocyte posteriorly located; black: mispositioned oocyte.

Finally, I analyzed the activity of DE-cadherin-4YF during embryogenesis: DE-cadherin-4YF is able to rescue the lethality due to the absence of both zygotic (Fig.2.12) and maternal endogenous DE-cadherin. Thus, the four conserved tyrosines in the cytoplasmic tail of DE-cadherin and their phosphorylation are not essential for any critical process during development.

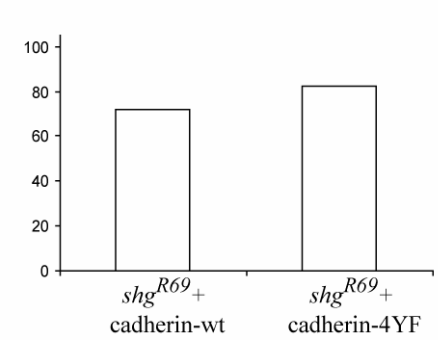



**Figure 2.12: DE-cadherin-4YF rescues the lethality due to the absence of zygotic DE-cadherin.** Rescue of the lethality due to the absence of zygotic endogenous DE-cadherin by the indicated transgenes. 100% corresponds to the situation where the proportion of *shg/shg*; tub-DE-cadherin flies in the progeny would be the expected mendelian proportion if tubulin-DE-cadherin transgenes fully rescue *shg* lethality ($50 \leq n \leq 378$).

#### 2.2.1.4 There is no redundancy between p120ctn and tyrosine phosphorylation of DE-cadherin during border cell migration

It is very possible that there is not a single mechanism involved in DE-cadherin regulation during border cell migration. I thus tested if there was some redundancy between p120ctn and tyrosine phosphorylation. To do so, I generated *shg* mutant border cell clones expressing DE-cadherin-4YF and knocked-down p120ctn by expressing a p120ctn-RNAi transgene (see materials and methods §4.3.3). All border cell clones obtained had fully migrated ($n>20$). This suggests that tyrosine phosphorylation of DE-cadherin and binding to p120ctn are not (the only two) redundant mechanisms involved in DE-cadherin regulation during border cell migration.

### 2.2.2 β-catenin tyrosine phosphrylation

#### 2.2.2.1 Strategy

As described in the introduction, phosphorylation of one highly conserved tyrosine residue in β-catenin is correlated with decrease in cadherin-mediated adhesion in many systems. In *Drosophila* β-catenin, this corresponds to tyrosine Y667. I mutated this tyrosine to phenylalanine to obtain β-catenin-Y667F (Fig.2.13). If phosphorylation of this tyrosine is essential for adhesion regulation during border cell migration, β-catenin-Y667F should not be able to substitute for endogenous β-catenin during this process.

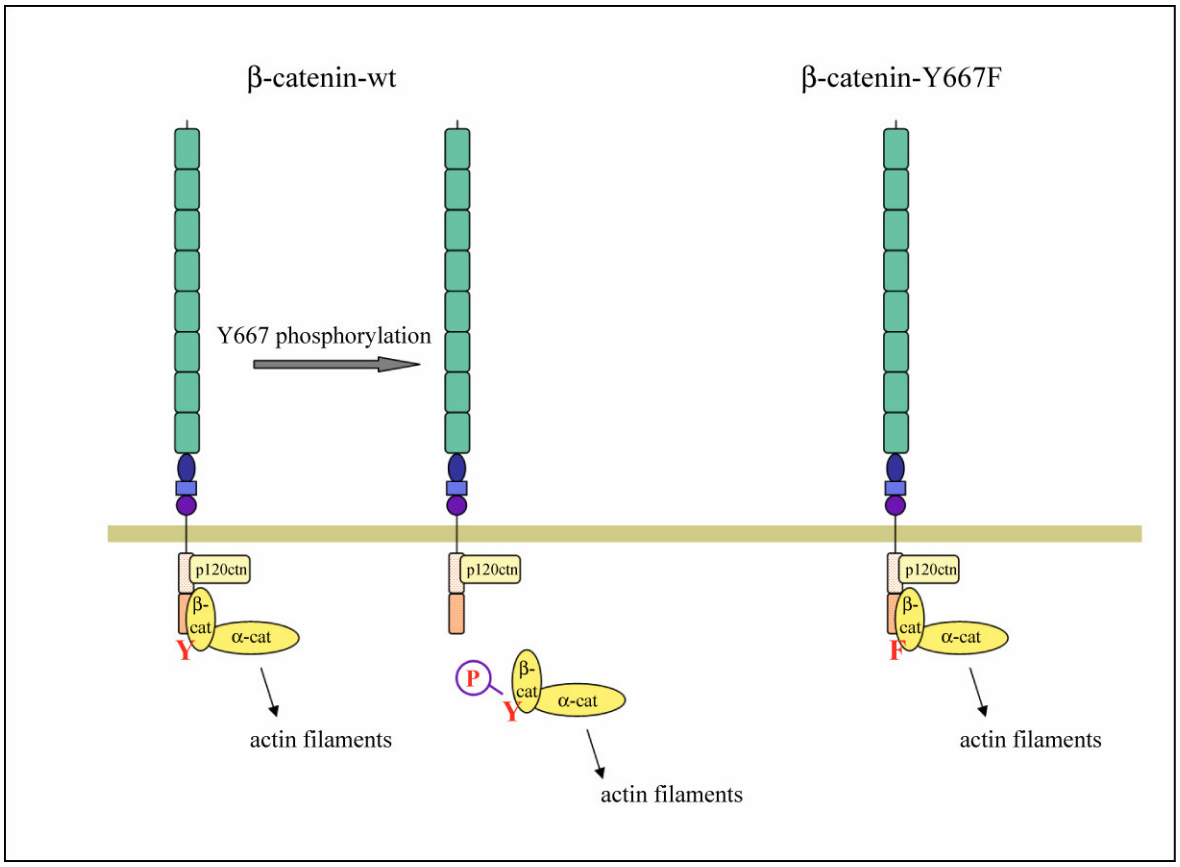



**Figure 2.13: β-catenin-Y667F.** In wild-type β-catenin, phosphorylation of a conserved tyrosine (Y667 in *Drosophila* β-catenin) is thought to induce the dissociation of β-catenin from cadherin. In β-catenin-Y667F, Y667 was mutated to phenylalanine and can thus no longer be a regulatory point.

I generated transgenic flies expressing β-catenin-wt or β-catenin-Y667F ubiquitously (under the control of an armadillo/β-catenin promoter) or with the UAS-Gal4 system. I used an

*armadillo* (*arm*) null allele (*arm[4]*) to generate *arm* mutant clones and tested the ability of β-catenin-wt or β-catenin-Y667F transgenes to rescue the lack of endogenous β-catenin. When expressed under the control of the armadillo promoter, β-catenin-wt could rescue the lack of endogenous β-catenin in germ line cells but not in follicular cells. Antibody stainings showed that β-catenin levels obtained with the armadillo-β-catenin-wt transgene in follicular cells are much lower than endogenous β-catenin levels; this probably explains the defects observed in follicular cells. To test if β-catenin-wt or β-catenin-Y667F transgenes could rescue *arm* phenotypes in follicular cells, I thus expressed them to higher levels using the UAS-Gal4 system and MARCM clones (see materials and methods §4.3.2).

#### 2.2.2.2 Phosphorylation of β-catenin tyrosine Y667 is not required for border cell migration

I first analyzed the activity β-catenin-Y667F during border cell migration, in border cells as well as in nurse cells. In border cell clones, UAS β-catenin transgenes were expressed using the MARCM system; in germ line clones, transgenes were expressed under the control of an armadillo promoter. In both border cells and nurse cells, expression of β-catenin-wt as well as β-catenin-Y667F completely rescues the migration (Fig.2.14).

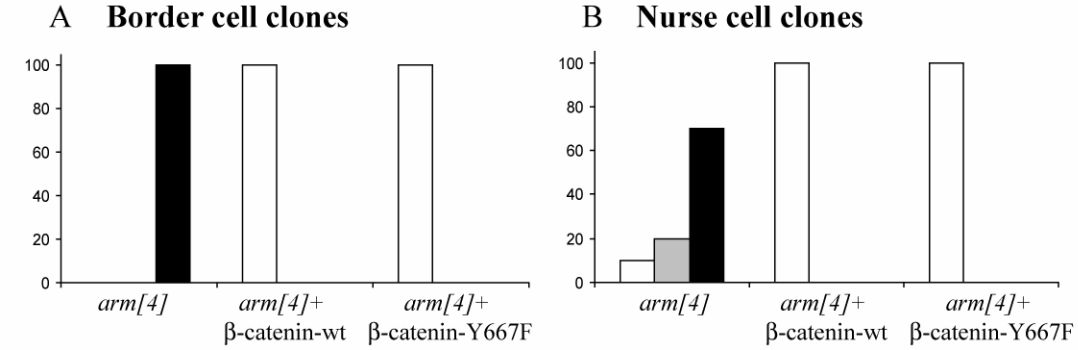



**Figure 2.14: β-catenin-Y667F can rescue *arm* phenotype during border cell migration. A-B.** Migration defects in *arm* mutant border cell (A) and germ line (B) clones and in the presence of the indicated transgenes. (A: *arm[4]*: n=2; *arm[4]*+β-cat-wt: n=5; *arm[4]*+β-cat-Y667F: n=16; B: *arm[4]*: n=66; *arm[4]*+β-cat-wt: n=16; *arm[4]*+β-cat-Y667F: n=23). White: full migration; grey: incomplete migration; black: no migration.

Thus β-catenin tyrosine Y667, and hence its phosphorylation, does not appear to be required for the migration. It is worth noting, that in complete absence of β-catenin in the nurse cells, border cell migration is not complety blocked (Fig.2.14B). This is similar to what was observed when nurse cells express only DE-cadherin-Δβ (§2.1.3, Fig.2.4F).

#### 2.2.2.3 Phosphorylation of β-catenin tyrosine Y667 is not required during oogenesis

I then tested whether β-catenin-Y667F would substitute for endogenous β-catenin for its other functions during oogenesis. Within the follicular epithelium, *arm* mutant cells sort away from wild type cells (Fig.2.15B) and lose their epithelial integrity quite early during oogenesis (Fig.2.15A-B) (Tanentzapf et al., 2000). Expression of UAS-β-catenin-wt or UAS-β-catenin-Y667F transgenes rescues these two phenotypes (Fig.2.15C-D).

Similarly to DE-cadherin, β-catenin is required in the germ line for proper positioning of the oocyte: oocyte mispositioning is frequently observed in egg chambers with *arm* mutant germ line cells (Fig.2.15E) (Peifer et al., 1993). Both β-catenin-wt and β-catenin-Y667F (expressed under the control of an armadillo promoter) are able to rescue this *arm* phenotype (Fig.2.15E).

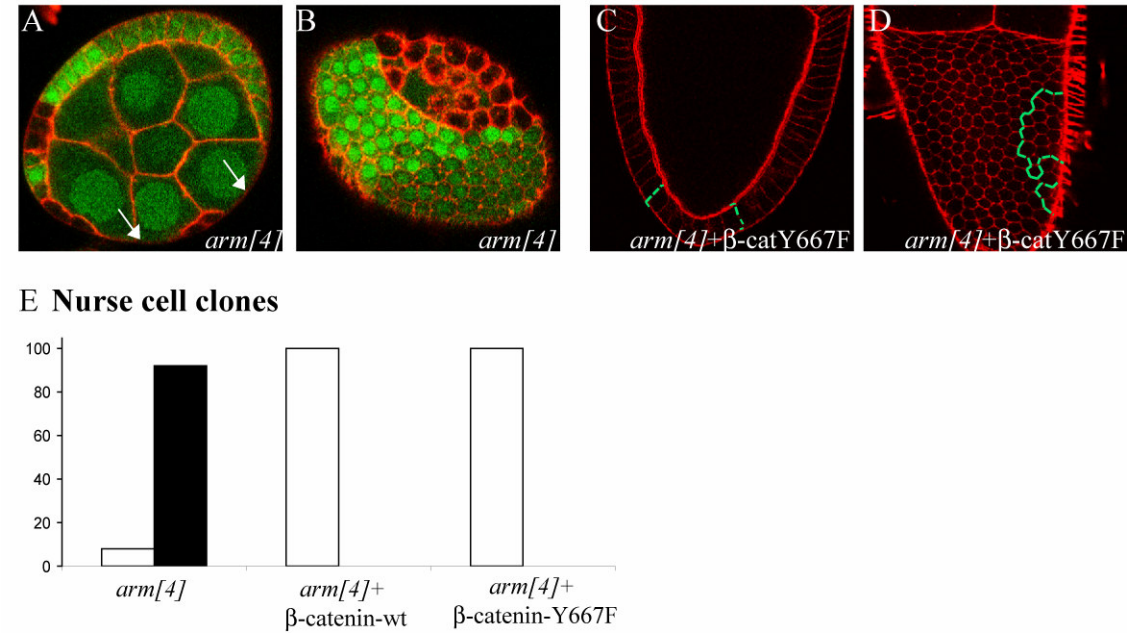



**Figure 2.15: β-catenin-Y667F can rescue *arm* phenotypes during oogenesis. A-B.** *arm* mutant follicular clones lose their epithelial integrity (A,B) and sort out from wild type cells (B). Phalloidin (red) stains the actin cytoskeleton of egg chambers and non-GFP cells are *arm* mutant clones. In A, arrows indicate discontinuities in the follicular epithelium due to the absence of β-catenin. **C-D.** Expression of a UAS-β-catenin-Y667F transgene in *arm* mutant clones rescues the loss of epithelial integrity (C-D) and the sorting phenotype (D). Phalloidin (red) stains the actin cytoskeleton. Position of *arm* mutant clones is indicated by the green line. **E.** Oocyte mispositioning in *arm* mutant germ line clones and in the presence of the indicated transgenes ($37 \leq n \leq 45$). White: oocyte posteriorly located; black: mispositioned oocyte.

## 2.3 Link with the actin cytoskeleton

### 2.3.1 DE-cadherin-ΔCyt/α-catenin fusion

#### 2.3.1.1 Strategy

Multiple mechanisms putatively involved in regulating adhesion by modulating the link between cadherin and actin have been described. To simultaneously abolish all these possible regulatory mechanisms, I generated fusion proteins between DE-cadherin and catenins. The first fusion (DE-cadherin-ΔCyt/α-catenin) was obtained by fusing full length α-catenin directly after the transmembrane domain of DE-cadherin (Fig.2.16A, arrow 1 in Fig.2.1B). This construct prevents any regulation of the link between DE-cadherin and β-catenin as well as between β-catenin and α-catenin. It also eliminates any possibility of other regulation at the level of DE-cadherin cytoplasmic domain.

If the link between DE-cadherin and α-catenin has to be regulated during border cell migration or if regulation of DE-cadherin involves its cytoplasmic domain, one would expect that DE-cadherin-ΔCyt/α-catenin is not able to substitute for endogenous DE-cadherin in border cells. To test this, I first tried to generate transgenic flies expressing DE-cadherin-ΔCyt/α-catenin ubiquitously, under the control of the tubulin-α1 promoter. However, I could not obtain transformants, which may indicate that ubiquitous expression of DE-cadherin-ΔCyt/α-catenin is lethal. I thus next decided to generate transgenic flies carrying a UAS-DE-cadherin-ΔCyt/α-catenin transgene. The ability of this transgene to rescue *shg* phenotypes was then analyzed by expressing it in *shg* mutant clones using the MARCM system (see materials and methods §4.3.2).

#### 2.3.1.2 Expression and subcellular localization

First, I checked DE-cadherin-ΔCyt/α-catenin expression and subcellular localization: in follicular cell and border cell clones, DE-cadherin-ΔCyt/α-catenin is expressed at very high levels; it shows a very strong accumulation inside the cells (Fig.2.16C,G). When expressed in a similar way, DE-cadherin-wt also accumulates to some extend inside the cells (Fig.2.16B,F), but much less than DE-cadherin-ΔCyt/α-catenin. The intracellular accumulation of DE-cadherin-ΔCyt/α-catenin made it quite difficult to determine the amount

of DE-cadherin-ΔCyt/α-catenin present at the membrane. To do so, I used a staining protocol to specifically stain DE-cadherin at the plasma membrane (see materials and methods §4.4.3): by incubating samples with the primary antibody before fixation and permeabilization, the antibody can not penetrate inside the cell and binds only to extracellular epitopes. Since the anti-DE-cadherin antibody I use is directed against DE-cadherin extracellular domain, this protocol allows specific staining of membrane DE-cadherin. With such a protocol, I could show that DE-cadherin-ΔCyt/α-catenin is indeed targeted to the membrane; in the follicular epithelium, there is actually slightly more DE-cadherin-ΔCyt/α-catenin at the membrane in *shg* mutant cells than endogenous DE-cadherin in wild type cells (Fig.2.16E). This extracellular staining protocol works well for follicular cells since they are located on the surface of the egg chambers. However, it can not be used for border cells which are located inside the egg chambers, in between the nurse cells. Hence, I could not check how much DE-cadherin-wt and DE-cadherin-ΔCyt/α-catenin are actually present at the membrane in border cells.

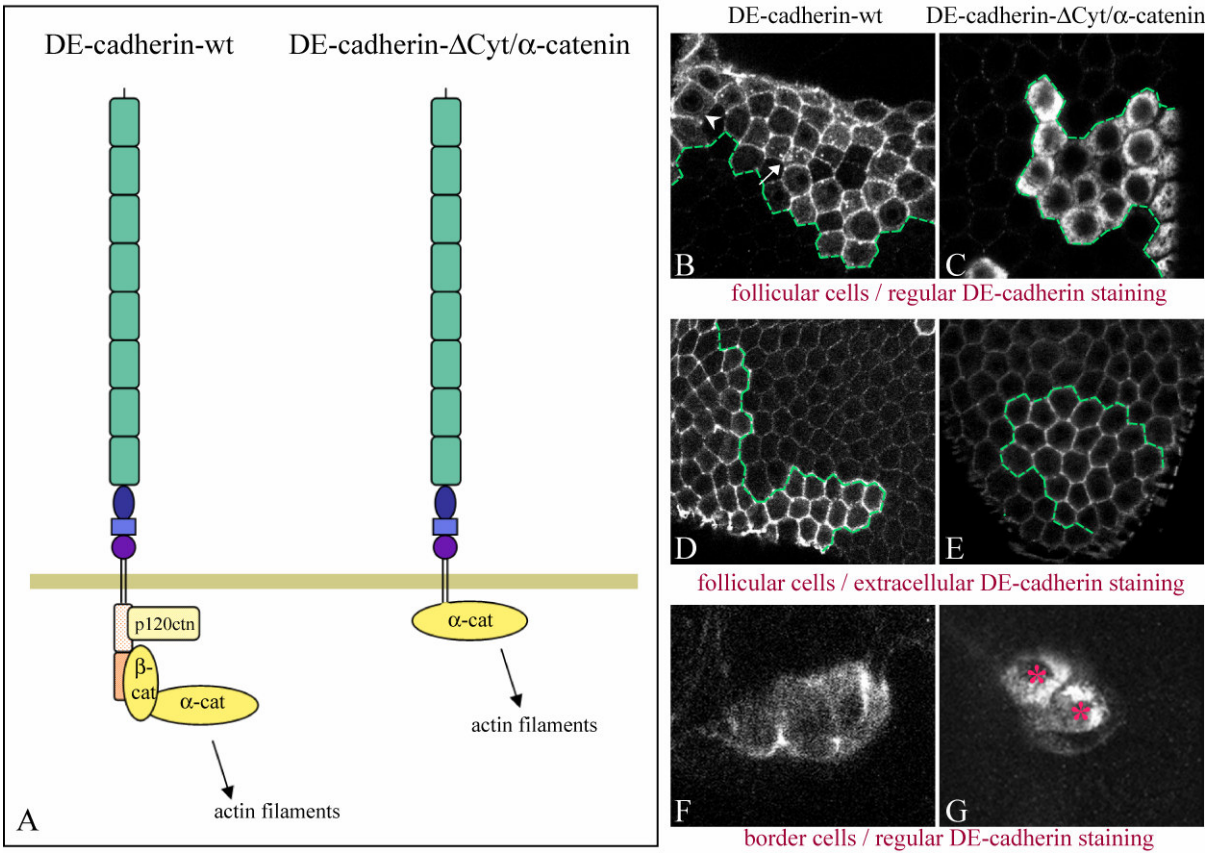



**Figure 2.16: DE-cadherin-Δcyt/α-catenin. A.** Schematic representation of DE-cadherin-ΔCyt/α-catenin. **B-G.** Expression levels and localization of wild type cadherin (B, D, F) and DE-cadherin-ΔCyt/α-catenin (C, E, G) in follicular cells (B-E) and border cells (F-G). UAS-DE-cadherin-wt and UAS-DE-cadherin-Δcyt/α-catenin transgenes are expressed in *shg* MARCM clones. In B-E, position of the clones is indicated by the green line. In B arrow indicates punctuate intracellular staining and arrowhead indicates perinuclear staining. Note that in B and C, picture settings are such that one can barely see endogenous DE-cadherin. In F all border cells are *shg* mutant and express UAS-DE-cadherin-wt. In G, 2 border cells (asterix) are *shg* mutant and express UAS-DE-cadherin-ΔCyt/α-catenin. In B, C, F, G both membrane and intracellular DE-cadherin are stained; in D and E, only membrane DE-cadherin is stained.

#### 2.3.1.3 DE-cadherin-ΔCyt/α-catenin rescues *shg* phenotypes in follicular cells

It was then important to check that DE-cadherin-ΔCyt/α-catenin is functional, that is it can rescue *shg* phenotypes in some tissues. During oogenesis, both UAS-DE-cadherin-wt and UAS-DE-cadherin-ΔCyt/α-catenin transgenes rescue the various phenotypes (follicular cell sorting, loss of epithelial integrity, oocyte mispositioning) observed in *shg* mutant follicular

cell clones (Fig2.17). Thus, DE-cadherin-ΔCyt/α-catenin can substitute for endogenous DE-cadherin in follicular cells, showing that it is able to mediate adhesion, at least in those cells.

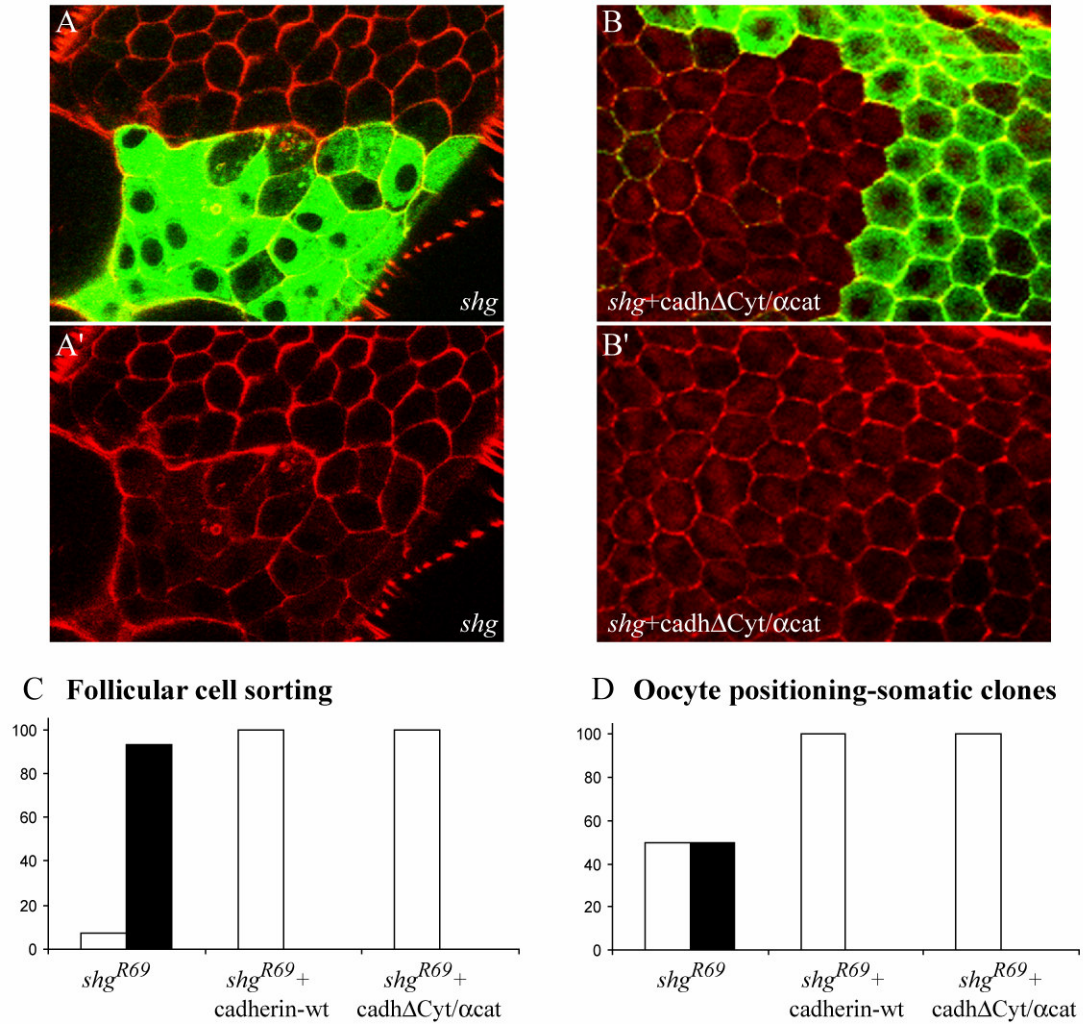



**Figure 2.17: DE-cadherin-ΔCyt/α-catenin rescues *shg* phenotypes in follicular cells. A.** *shg* mutant follicular cell clones sort out and lose their epithelial integrity. **B.** UAS-DE-cadherin-ΔCyt/α-catenin transgene expressed in *shg* mutant MARCM clones rescues *shg* phenotypes. Phalloidin (red) stains the actin cytoskeleton of egg chambers and GFP (green) cells are *shg* mutant clones. **C.** Sorting in *shg* mutant clones and in the presence of the indicated transgenes ($28 \leq n \leq 80$). White: no sorting; black: sorting. **D.** Oocyte mispositioning in *shg* mutant follicular cell clones and in the presence of the indicated transgenes ($31 \leq n \leq 42$). White: oocyte posteriorly located; black: mispositioned oocyte.

#### 2.3.1.4 DE-cadherin-ΔCyt/α-catenin does not rescue *shg* phenotype in border cells

I next tested if DE-cadherin-ΔCyt/α-catenin could substitute for endogenous DE-cadherin in border cells. Although overexpressed, a UAS-DE-cadherin-wt transgene fully rescues the migration defects of *shg* mutant border cells. On the contrary, UAS-DE-cadherin-ΔCyt/α-catenin gives almost no rescue (Fig.2.18) showing that it does not substitute for endogenous DE-cadherin in border cells. Since DE-cadherin-ΔCyt/α-catenin is able to mediate adhesion in follicular cells, it is likely to be also functional in border cells. One possibility is that the very strong migration defects observed with DE-cadherin-ΔCyt/α-catenin are due not to a lack of adhesion but rather to too stable adhesion (see discussion): it is possible that adhesion mediated by DE-cadherin-ΔCyt/α-catenin can not be downregulated and that de-adhesion of border cells from the nurse cells can not occur, resulting in strong inhibition of the migration.

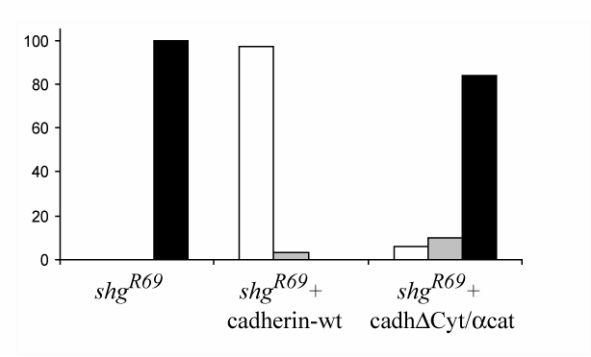



**Figure 2.18: DE-cadherin-ΔCyt/α-catenin does not rescue *shg* phenotype in border cells.** Migration defects in *shg* mutant border cell clones and in the presence of the indicated transgenes (28≤n≤32). White: full migration; grey: incomplete migration; black: no migration.

#### 2.3.1.5 Overexpression of DE-cadherin-ΔCyt/α-catenin inhibits border cell migration

If the migration defects observed when border cells express only DE-cadherin-ΔCyt/α-catenin are due to too stable adhesion, one would expect that overexpressing DE-cadherin-ΔCyt/α-catenin in wild type border cells should also affect migration.

I overexpressed UAS-DE-cadherin-ΔCyt/α-catenin in follicular cells and border cells with the "actin-flip-out-Gal4" (AFG) system (see materials and methods §4.3.4). No obvious defects were observed in follicular cells whereas there were clear migration defects (Fig.2.19A). However, these defects are substantially weaker than the defects observed in the rescue experiment in *shg* MARCM clones (Fig.2.18). Moreover, when DE-cadherin-ΔCyt/α-catenin

is overexpressed in AFG clones, one can observe big DE-cadherin aggregates inside the cells (Fig.2.19C) which look quite different from the intracellular accumulation of DE-cadherin-ΔCyt/α-catenin in *shg* MARCM clones (Fig.2.16G). DE-cadherin protein detected by the antibody in these aggregates may be the transgene encoded DE-cadherin-ΔCyt/α-catenin and/or endogenous DE-cadherin. Notably, actin filaments colocalize with these aggregates (Fig.2.19C).

One difference between the AFG overexpression experiment and the rescue experiment in *shg* MARCM clones is the driver (actinGal4 or tubulinGal4) used to express UAS-DE-cadherin-ΔCyt/α-catenin. The other main difference is the presence or absence of endogenous DE-cadherin in border cells. To determine which of these two parameters is responsible for the observed difference in migration defects and subcellular localization of DE-cadherin-ΔCyt/α-catenin, I also overexpressed UAS-DE-cadherin-ΔCyt/α-catenin in border cells with tubulinGal4 using the MARCM system; contrary to the rescue experiment, clones generated in this overexpression experiment were not *shg* mutant but wild type clones expressing endogenous DE-cadherin. In this experiment, there is accumulation of DE-cadherin (DE-cadherin-ΔCyt/α-catenin and/or endogenous DE-cadherin) in aggregates very similar to those observed in the AFG overexpression experiment (Fig.2.19E). Preliminary results seem to indicate that migration defects are not stronger than with AFG clones. Thus the difference in DE-cadherin-ΔCyt/α-catenin behaviour between simple overexpression experiments and *shg* rescue experiment is likely to be due to the presence or absence of endogenous DE-cadherin.

The inhibition of border cell migration observed with UAS-DE-cadherin-ΔCyt/α-catenin overexpression is consistent with the idea that the strong migration defects observed in the *shg* rescue experiment are due to excess of adhesion. Nevertheless, it could also indicate that DE-cadherin-ΔCyt/α-catenin behaves as a dominant-negative. It could for instance titrate away from endogenous DE-cadherin some downstream components of adhesive complexes in border cells. In particular, the fact that the migration defects are weaker in the presence of endogenous DE-cadherin would be consistent with DE-cadherin-ΔCyt/α-catenin being a dominant-negative for DE-cadherin. If this was the case, one would expect that overexpressing DE-cadherin-ΔCyt/α-catenin in a *shg* heterozygous background would give stronger migration defects than overexpression in a wild type background. But a *shg*

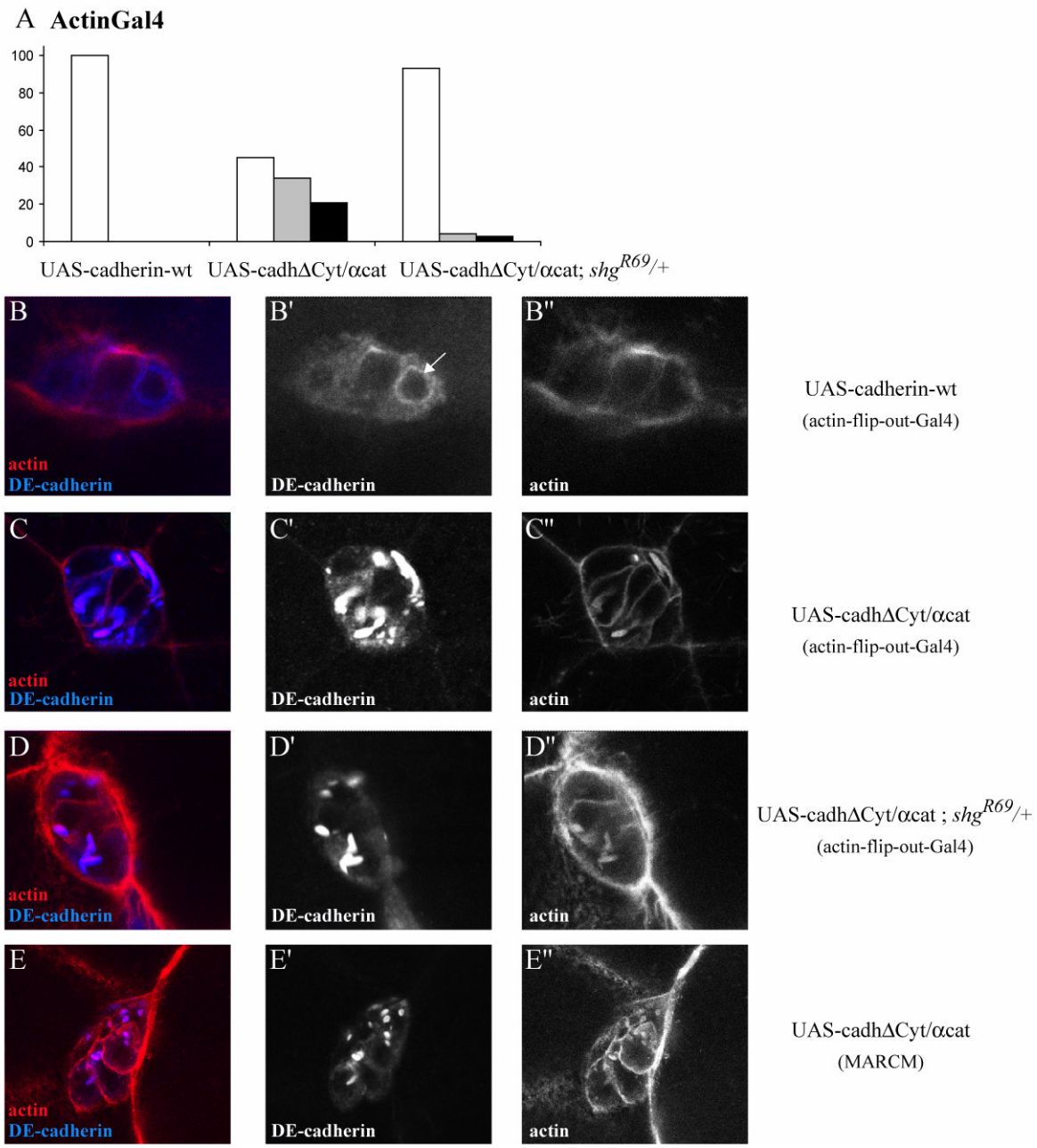



**Figure 2.19: Overexpression of DE-cadherin-ΔCyt/α-catenin in border cells. A.** Migration of border cells overexpressing UAS-DE-cadherin-wt or UAS-DE-cadherin-ΔCyt/α-catenin in AFG clones (97≤n≤195). Transgenes were expressed in otherwise wildtype egg chambers or in $shg^{R69}$/+ heterozygous egg chambers. UAS-DE-cadherin-ΔCyt/α-catenin transgenic line used is the same as in Fig.2.18. A similar result was obtained with an independent transgenic line. White: full migration; grey: incomplete migration; black: no migration. **B-E.** Subcellular localization of DE-cadherin (blue and B'-E') and actin filaments (stained with phalloidin, red and B''-E'') in border cells overexpressing DE-cadherin-wt (B) or UAS-DE-cadherin-ΔCyt/α-catenin (C-E). Overexpression was obtained in AFG (B-D) or MARCM (E) clones. In D, egg chambers are heterozygous $shg^{R69}$/+. In B, arrow indicates perinuclear staining.

heterozygous background surprisingly clearly reduces the overexpression effect of DE-cadherin-ΔCyt/α-catenin (Fig.2.19A).This does not seem to affect DE-cadherin distribution in border cells (Fig.2.19D). This result is somewhat difficult to explain (see discussion §3.7.1) but seems inconsistent with DE-cadherin-ΔCyt/α-catenin being a dominant-negative for DE-cadherin.

### 2.3.2 DE-cadherin-Δβ/α-catenin and DE-cadherin-FL/α-catenin fusions

#### 2.3.2.1 Strategy

Results obtained with DE-cadherin-ΔCyt/α-catenin suggested that DE-cadherin might need to be regulated during border cell migration. If this is the case, regulation could occur either between DE-cadherin and α-catenin or at the level of DE-cadherin cytoplasmic domain. To distinguish between these two possibilities, I generated two other fusions between DE-cadherin and α-catenin. DE-cadherin-Δβ/α-catenin was obtained by fusing full length α-catenin after the juxtamembrane domain of DE-cadherin (Fig.2.20A, arrow 3 in Fig.2.1B). In the second fusion (called DE-cadherin-FL/α-catenin), the whole cytoplasmic domain of DE-cadherin was kept and α-catenin fused at DE-cadherin C-terminus (Fig.2.20A). These two fusion proteins prevent any regulation of the link between DE-cadhering and α-catenin. In DE-cadherin-Δβ/α-catenin regulation at the level of the juxtamembrane domain can still occur. DE-cadherin-FL/α-catenin can still be regulated at the level of its juxtamembrane domain as well as its C-terminal domain.

As for DE-cadherin-ΔCyt/α-catenin, I tested if DE-cadherin-Δβ/α-catenin and DE-cadherin-FL/α-catenin are able to substitute for endogenous DE-cadherin by expressing corresponding UAS-transgenes in *shg* mutant MARCM clones and analyzing their ability to rescue *shg* phenotypes.

#### 2.3.2.2 Expression and subcellular localization

Expression and subcellular localization of DE-cadherin-Δβ/α-catenin and DE-cadherin-FL/α-catenin were checked in follicular cell and border cell clones. Both constructs are expressed at

high levels (Fig.2.20B-F) and accumulate to some extent inside the cells (Fig.2.20B-C and E-F). DE-cadherin-Δβ/α-catenin intracellular accumulation was somewhat lower than DE-cadherin-ΔCyt/α-catenin accumulation and DE-cadherin-FL/α-catenin was much lower. The amount of DE-cadherin-Δβ/α-catenin actually present at the membrane in follicular cells was checked with the extracellular staining protocol: it is slightly higher than endogenous DE-cadherin levels in wild type cells (Fig.2.20D).

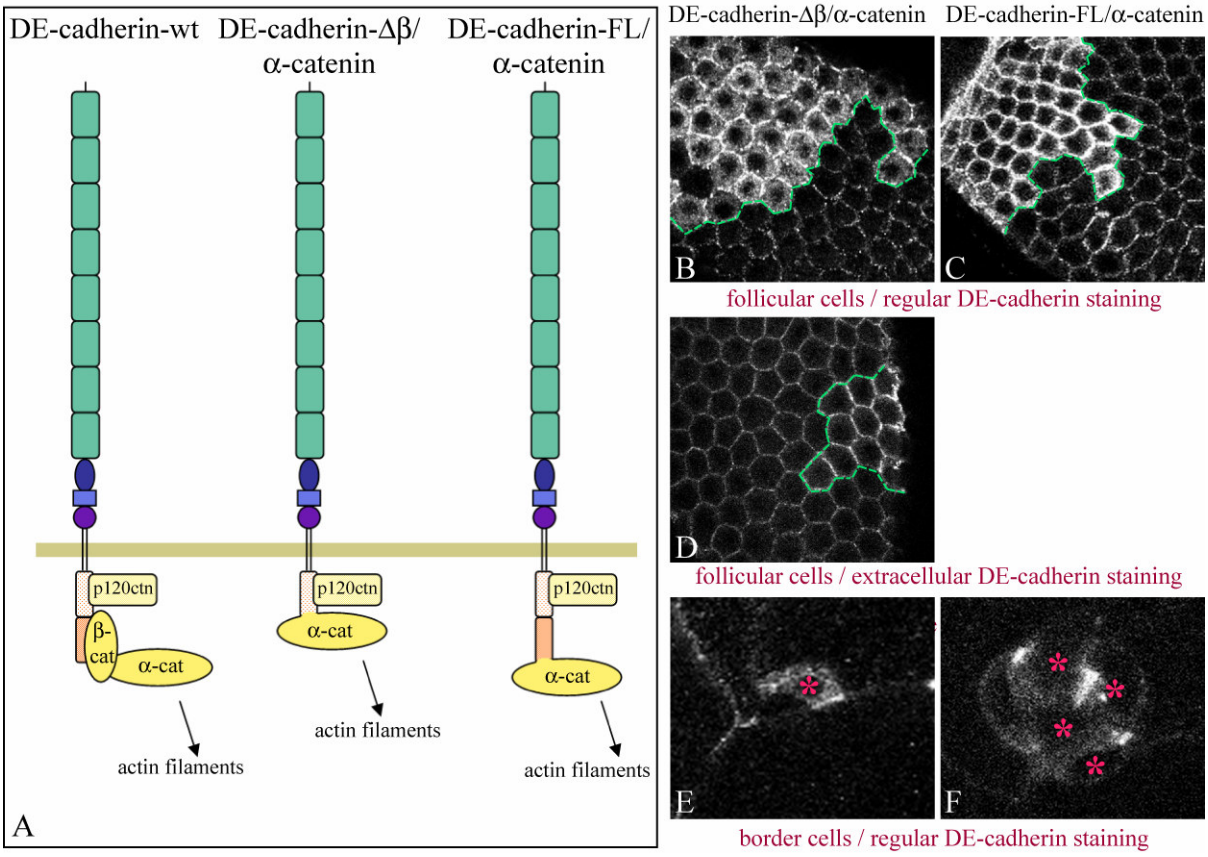



**Figure 2.20: DE-cadherin-Δβ/α-catenin and DE-cadherin-FL/α-catenin. A.** Schematic representation of DE-cadherin-Δβ/α-catenin and DE-cadherin-FL/α-catenin. **B-F.** Expression levels and localization of DE-cadherin-Δβ/α-catenin (B,D,E) and DE-cadherin-FL/α-catenin (C,F) in follicular cells (B-D) and border cells (E,F). UAS-DE-cadherin-Δβ/α-catenin and UAS-cadherin-FL/α-catenin transgenses are expressed in *shg* MARCM clones. In B-D, position of the clones is indicated by the green line. In B, note that one can see more membrane staining than in clones expressing DE-cadherin-ΔCyt/α-catenin. In E-F, border cells mutant for *shg* and expressing UAS-DE-cadherin-Δβ/α-catenin (E) or UAS-DE-cadherin-FL/α-catenin (F) are marked by an asterix. In B-C and E-F both membrane and intracellular DE-cadherin are stained; in D, only membrane DE-cadherin is stained.

### 2.3.2.3 DE-cadherin-Δβ/α-catenin and DE-cadherin-FL/α-catenin rescue *shg* phenotypes in follicular cells

When expressed in *shg* mutant follicular cell clones, both UAS-DE-cadherin-Δβ/α-catenin and UAS-DE-cadherin-FL/α-catenin transgenes rescue *shg* phenotypes such as follicular cell sorting, loss of epithelial integrity and oocyte mispositioning (Fig.2.21). Thus, both DE-cadherin-Δβ/α-catenin and DE-cadherin-FL/α-catenin can substitute for endogenous DE-cadherin in follicular cells, showing that they are able to mediate adhesion in those cells.

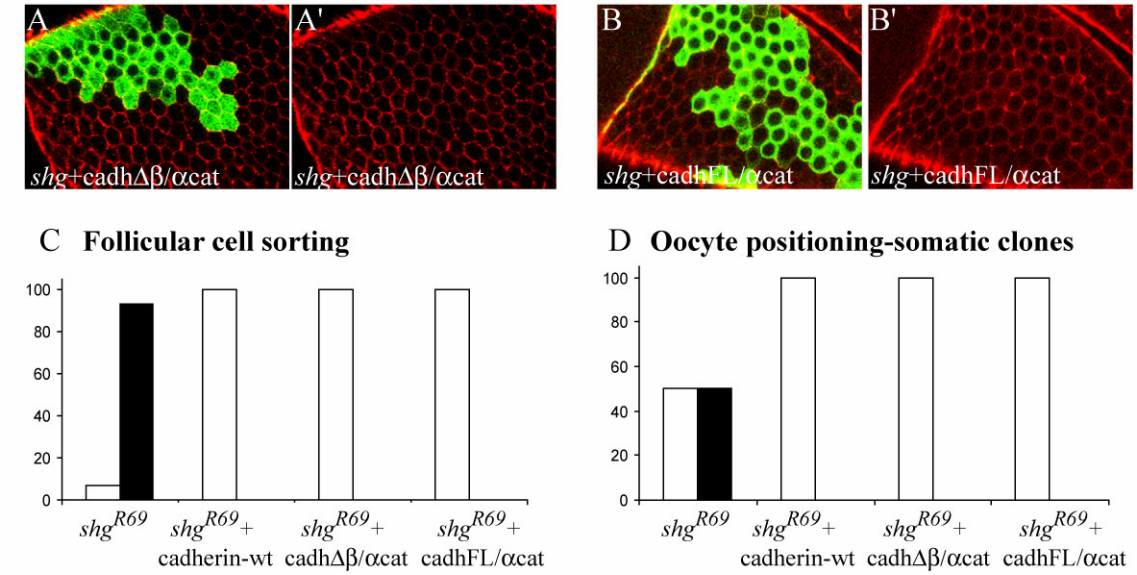



**Figure 2.21: DE-cadherin-Δβ/α-catenin and DE-cadherin-FL/α-catenin rescue *shg* phenotypes in follicular cells. A-B.** UAS-DE-cadherin-Δβ/α-catenin and UAS-DE-cadherin-FL/α-catenin transgenes expressed in *shg* mutant follicular cell clones (MARCM) rescue *shg* phenotypes (sorting and loss of epithelial integrity). Phalloidin (red) stains the actin cytoskeleton of egg chambers and GFP (green) cells are *shg* mutant clones. **C.** Sorting in *shg* mutant clones and in the presence of the indicated transgenes ($28 \leq n \leq 40$). White: no sorting; black: sorting. **D.** Oocyte mispositioning in *shg* mutant follicular cell clones and in the presence of the indicated transgenes ($32 \leq n \leq 40$). White: oocyte posteriorly located; black: mispositioned oocyte.

#### 2.3.2.4 DE-cadherin-Δβ/α-catenin and DE-cadherin-FL/α-catenin (partially) rescue *shg* phenotype in border cells

I next tested if DE-cadherin-Δβ/α-catenin and DE-cadherin-FL/α-catenin could substitute for endogenous DE-cadherin in border cells: UAS-DE-cadherin-Δβ/α-catenin partially rescues the migration defects and UAS-DE-cadherin-FL/α-catenin gives a complete rescue (Fig.2.22). For each fusion protein, similar results were obtained with two independent UAS transgenes.

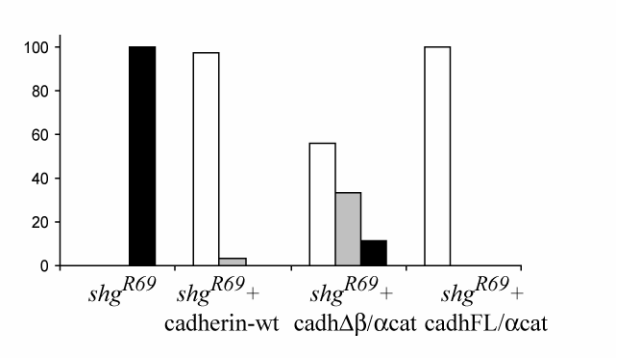



**Figure 2.22: DE-cadherin-Δβ/α-catenin and DE-cadherin-FL/α-catenin (partially) rescue *shg* phenotype in border cells.** Migration defects in *shg* mutant border cell clones and in the presence of the indicated transgenes (27≤n≤34). White: full migration; grey: incomplete migration; black: no migration.

The partial rescue observed with DE-cadherin-Δβ/α-catenin shows that regulating the link between DE-cadherin and α-catenin is not strictly required for border cell migration. The full rescue obtained with DE-cadherin-FL/α-catenin even suggests that such a regulation is not required at all. DE-cadherin-FL/α-catenin still has the β-catenin binding domain: thus, it is in principle possible that DE-cadherin-FL/α-catenin is linked to actin via endogenous β-catenin and α-catenin and some regulation of the link between DE-cadherin-FL/α-catenin and actin may occur through endogenous β-catenin and α-catenin. The lack of such a regulation in DE-cadherin-Δβ/α-catenin – which can not bind endogenous β-catenin – could account for the small migration defects observed with DE-cadherin-Δβ/α-catenin. This would mean that regulating the link between DE-cadherin and α-catenin, although not strictly required, participates in adhesion regulation. Alternatively, there may be no requirement at all for a regulation of the link between DE-cadherin and α–catenin. The difference between the partial rescue observed with DE-cadherin-Δβ/α-catenin and the full rescue obtained with DE-cadherin-FL/α-catenin would then indicate that a signal important for DE-cadherin function in border cells is located in the C-terminal domain.

It is thus important to determine whether the difference between DE-cadherin-Δβ/α-catenin and DE-cadherin-FL/α-catenin is due to the presence of the C-terminal domain in DE-cadherin-FL/α-catenin or to the binding of endogenous β-catenin to DE-cadherin-FL/α-catenin. I tried to test if DE-cadherin-FL/α-catenin would still be able to fully rescue border cell migration in the absence of endogenous β-catenin: *arm* null mutant clones were generated with the MARCM system and DE-cadherin-FL/α-catenin expressed in these clones. At early stages of oogenesis, loss of β-catenin in follicular cells affect both DN-cadherin and DE-cadherin mediated adhesion (Tanentzapf et al., 2000). Follicular cell *arm* mutant clones expressing DE-cadherin-FL/α-catenin have a normal shape, showing that DE-cadherin-FL/α-catenin can substitute for both DN-cadherin and DE-cadherin in follicular cells. However, the obtained clones are quite small, making it difficult to obtain fully mutant border cell clusters. So far, only one mutant cluster was recovered. This cluster had migrated normally. The experiment will need to be repeated on a larger scale before drawing any conclusion.

#### 2.3.2.5 Overexpression of DE-cadherin-Δβ/α-catenin weakly inhibits border cell migration

In stage 10 egg chambers, no migration defects are observed upon overexpression of UAS-DE-cadherin-Δβ/α-catenin in “actin-flip-out-Gal4” clones (Fig.2.23A). Some weak delays are observed in stage 9 egg chambers, while border cells migrate (Fig2.23A). This may indicate that border cells overexpressing DE-cadherin-Δβ/α-catenin initiate the migration a little bit later or that they migrate slightly slower than wild type border cells. In this experiment, DE-cadherin-Δβ/α-catenin accumulated in intracellular aggregates, colocalizing with actin (Fig.2.23B). This is very similar to what was observed in border cells overexpressing DE-cadherin-ΔCyt/α-catenin (Fig.2.19C).

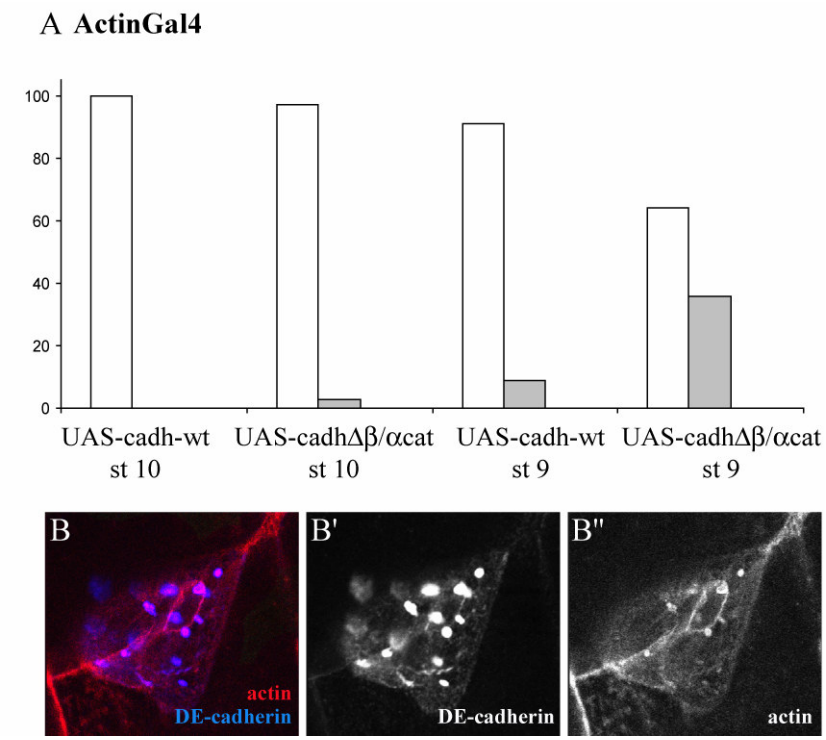



**Figure 2.23: Overexpression of DE-cadherin-Δβ/α-catenin in border cells. A.** Migration of border cells overexpressing UAS-DE-cadherin-wt or UAS-DE-cadherin-Δβ/α-catenin in AFG clones. Border cell migration was scored in stage 10 (n>100; White: full migration; grey: incomplete migration) as well as in stage 9 egg chambers. (n>100; White: border cells not delayed relative to follicular cells; grey: border cells delayed relative to follicular cells). **B.** Subcellular localization of DE-cadherin (blue and B') and actin filaments (stained with phalloidin, red and B'') in border cells overexpressing UAS-DE-cadherin-Δβ/α-catenin in AFG clones.

### 2.3.3 DE-cadherin-ΔCyt/CD2/α-catenin

In principle, at least three explanations could account for the difference between DE-cadherin-ΔCyt/α-catenin and DE-cadherin-Δβ/α-catenin in their ability to rescue the lack of endogenous DE-cadherin in border cells: (1) a regulatory signal present in DE-cadherin-Δβ/α-catenin but absent in DE-cadherin-ΔCyt/α-catenin could be required for the migration; (2) in DE-cadherin-ΔCyt/α-catenin, α–catenin could be too close to the membrane, resulting in a poor functionality of DE-cadherin-ΔCyt/α-catenin; (3) the stronger intracellular accumulation of DE-cadherin-ΔCyt/α-catenin could have negative effects on the migration, for instance by recruiting to an inappropriate location some essential components of the migrating machinery.

In an attempt to discriminate between these three possibilities, I made a supplementary construct: DE-cadherin-ΔCyt/CD2/α-catenin, which is composed of the extracellular and transmembrane domain of DE-cadherin (DE-cadherin-ΔCyt), of the cytoplasmic domain of CD2 and full-length α-catenin (Fig.2.20A). The cytoplasmic domain of CD2 was added between DE-cadherin-ΔCyt and α-catenin to move α-catenin away from the membrane; it may also help reducing intracellular accumulation of the fusion protein. Similarly to DE-cadherin-ΔCyt/α-catenin, DE-cadherin-ΔCyt/CD2/α-catenin lacks any putative regulatory signal from DE-cadherin cytoplasmic domain.

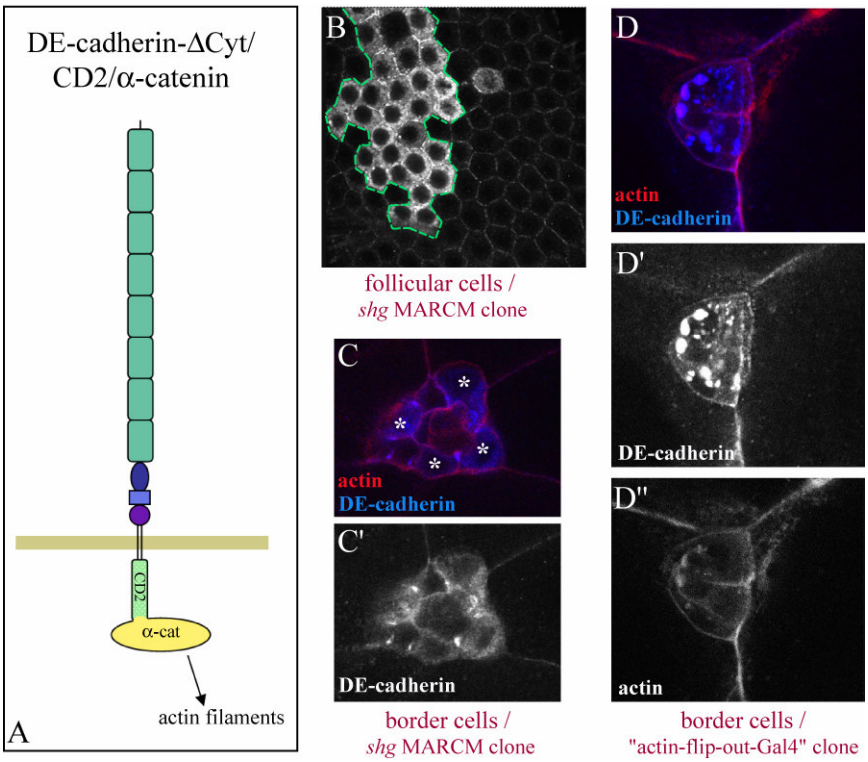



**Figure 2.24: DE-cadherin-ΔCyt/CD2/α-catenin. A.** Schematic representation of DE-cadherin-ΔCyt/CD2/α-catenin. **B-D.** Expression levels and localization of DE-cadherin-ΔCyt/CD2/α-catenin in follicular cells (B) and border cells (C-D). UAS-DE-cadherin-ΔCyt/CD2/α-catenin was expressed in *shg* MARCM (B-C) or AFG (D) clones. In B, position of the clone is indicated by the green line. Note that one can see more membrane staining than in clones expressing DE-cadherin-ΔCyt/α-catenin. In C, 4 border cells (asterix) are *shg* mutant and express UAS-DE-cadherin-ΔCyt/CD2/α-catenin. In C-D, DE-cadherin is in blue (and in C'-D') and actin (stained with phalloidin) in red (and in D'').

When expressed in *shg* MARCM clones, DE-cadherin-ΔCyt/CD2/α-catenin still accumulates intracellularly (Fig.2.20B,C). This intracellular accumulation seems to be similar to the accumulation observed with DE-cadherin-Δβ/α-catenin and somewhat weaker than the accumulation observed with DE-cadherin-ΔCyt/α-catenin. Extracellular stainings need to be done to check how much DE-cadherin-ΔCyt/CD2/α-catenin is actually at the plasma membrane. Quantification of migration defects in *shg* MARCM clones expressing DE-cadherin-ΔCyt/CD2/α-catenin has not been done yet. When DE-cadherin-ΔCyt/CD2/α-catenin is overexpressed in AFG clones, intracellular aggregates similar to those observed with DE-cadherin-ΔCyt/α-catenin and DE-cadherin-Δβ/α-catenin are observed (Fig2.20D). Preliminary results seem to indicate that overexpression of DE-cadherin-ΔCyt/CD2/α-catenin in AFG clones does cause migration defects, although it is not yet clear if they are as strong as the migration defects observed with DE-cadherin-ΔCyt/α-catenin.

### 2.3.4 Cadherin/β-catenin fusions

#### 2.3.4.1 Strategy

In parallel to the DE-cadherin/α-catenin fusions, I also generated DE-cadherin/β-catenin fusions. In case regulation of the link between DE-cadherin and α-catenin had turned out to be important, DE-cadherin/β-catenin fusions were meant to help determining at which level the regulation was occurring. Two different fusion proteins were generated: DE-cadherin-ΔCyt/β-catenin was obtained by fusing full length β-catenin directly after the transmembrane domain of DE-cadherin; in DE-cadherin-Δβ/β-catenin, full length β-catenin was fused after the juxtamembrane domain of DE-cadherin (Fig.2.25A).

As for DE-cadherin/α-catenin fusions, I tested if DE-cadherin-ΔCyt/β-catenin and DE-cadherin-Δβ/β-catenin are able to substitute for endogenous DE-cadherin by expressing corresponding UAS-transgenes in *shg* mutant MARCM clones and analyzing their ability to rescue *shg* phenotypes.

### 2.3.4.2 Expression and subcellular localization

Expression levels and subcellular localization of DE-cadherin-ΔCyt/β-catenin and DE-cadherin-Δβ/β-catenin were checked in follicular cell clones. Both constructs are expressed at high levels compared to endogenous DE-cadherin (Fig.2.25B-C). Part of DE-cadherin-ΔCyt/β-catenin and DE-cadherin-Δβ/β-catenin is targeted to the plasma membrane, but a significant amount also accumulates inside the cells (Fig.2.25B-C).

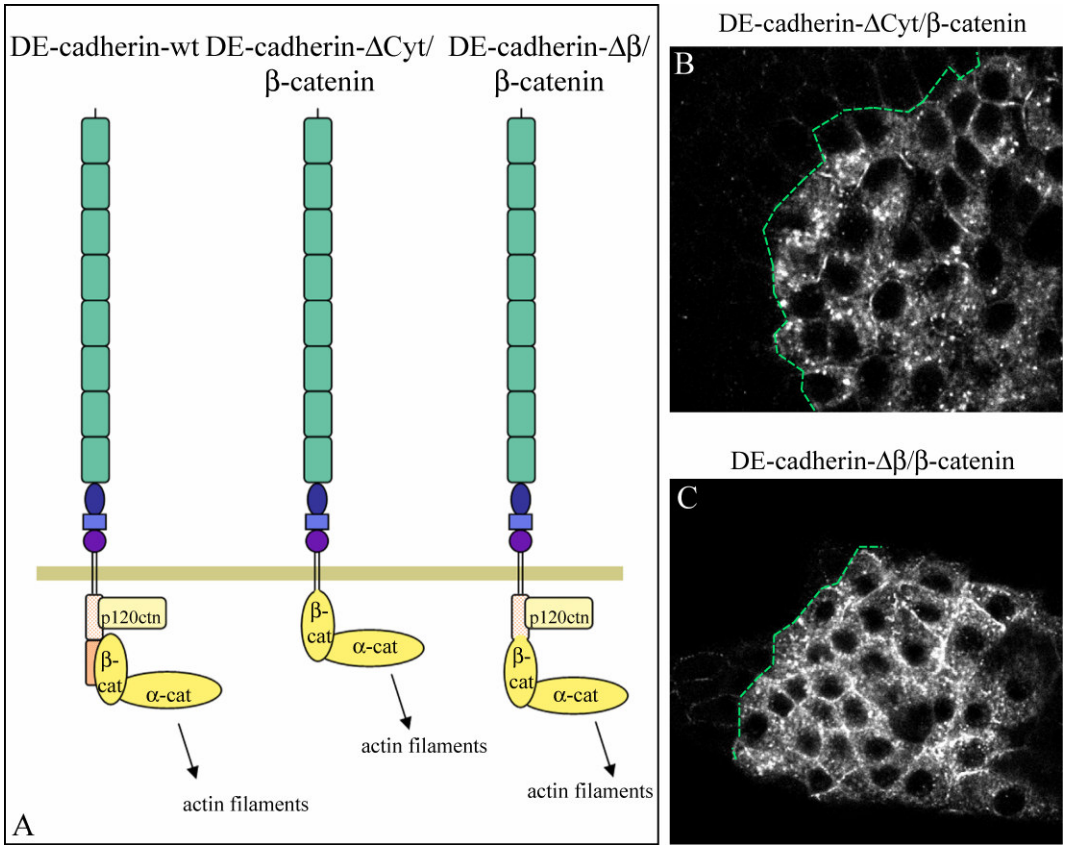



**Figure 2.25: DE-cadherin-ΔCyt/β-catenin and DE-cadherin-Δβ/β-catenin. A.** Schematic representation of DE-cadherin-ΔCyt/β-catenin and DE-cadherin-Δβ/β-catenin. **B-C.** Expression levels and localization of DE-cadherin-ΔCyt/β-catenin (B) and DE-cadherin-ΔCyt/β-catenin (C) in follicular cells. UAS-DE-cadherin-ΔCyt/β-catenin and UAS-DE-cadherin-ΔCyt/β-catenin transgenes are expressed in *shg* MARCM clones. Position of the clones is indicated by the green line. Both membrane and intracellular DE-cadherin are stained.

### 2.3.4.3 DE-cadherin/β-catenin fusions do not rescue *shg* phenotypes

To test if DE-cadherin/β-catenin fusions were functional I expressed them in *shg* mutant follicular clones and asked if they were able to rescue *shg* phenotypes in those cells. It turned out that follicular cells expressing only DE-cadherin-ΔCyt/β-catenin or DE-cadherin-Δβ/β-catenin have shape defects similar to, or even stronger than the defects observed in *shg* mutant follicular cells (Fig.2.26A,C). Moreover, mispositioning of the oocyte is also observed (Fig.2.26B,D). Thus, DE-cadherin-ΔCyt/β-catenin and DE-cadherin-Δβ/β-catenin appear to be unable to substitute for endogenous DE-cadherin. This suggests that they are non functional in mediating adhesion.

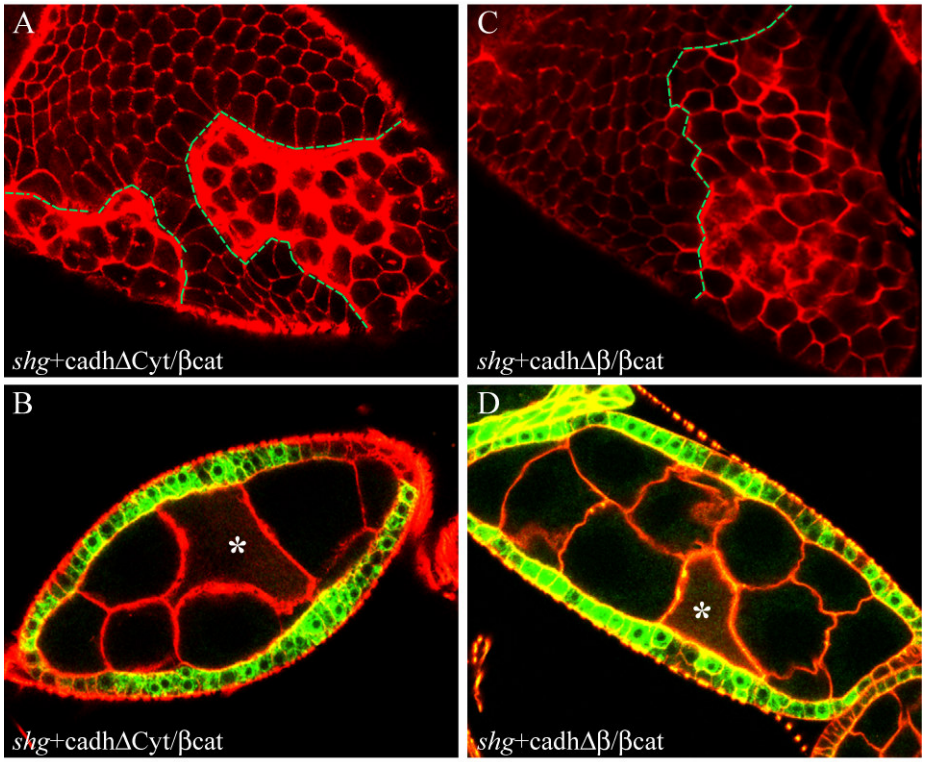



**Figure 2.26: DE-cadherin-ΔCyt/β-catenin and DE-cadherin-Δβ/β-catenin do not rescue *shg* phenotypes in follicular cells. A,C.** UAS-DE-cadherin-ΔCyt/β-catenin (A) and UAS-DE-cadherin-ΔCyt/β-catenin (C) transgenes expressed in *shg* mutant follicular cell clones do not rescue the loss of epithelial integrity. Position of the clones is indicated by the green line and phalloidin (red) stains the actin cytoskeleton. **B,D.** UAS-DE-cadherin-ΔCyt/β-catenin (B) and UAS-DE-cadherin-ΔCyt/β-catenin (D) transgenes expressed in *shg* mutant follicular cell clones do not rescue oocyte positioning defects. GFP (green) cells are *shg* mutant clones, phalloidin (red) stains the actin cytoskeleton and asterix indicate oocyte.

#### 2.3.4.4 Overexpression of DE-cadherin/β-catenin fusions inhibits adhesion in a dominant-negative manner

To ask whether DE-cadherin-ΔCyt/β-catenin and DE-cadherin-Δβ/β-catenin are simply not able to substitute for endogenous DE-cadherin or also behave as dominant-negatives, I overexpressed them in otherwise wild type egg chambers. When overexpressed in follicular cells in "actin-flip-out-Gal4' clones, both DE-cadherin-ΔCyt/β-catenin and DE-cadherin-Δβ/β-catenin give rise to defects in the follicular epithelium (Fig.2.27A-B). They also inhibit border cell migration (Fig.2.27C). Thus, overexpression of DE-cadherin-ΔCyt/β-catenin and DE-cadherin-Δβ/β-catenin seem to have negative effects on endogenous DE-cadherin.

To check that DE-cadherin-ΔCyt/β-catenin behaves as a dominant-negative I co-overexpressed DE-cadherin-ΔCyt/β-catenin and DE-cadherin-wt with slboGal4 in border cells. This clearly suppresses the migration defects observed with overexpression of DE-cadherin-ΔCyt/β-catenin alone (Fig.2.27D). Thus DE-cadherin-ΔCyt/β-catenin is a real dominant-negative for DE-cadherin. One possible explanation for this effect could be that DE-cadherin-ΔCyt/β-catenin – although not functional – is able to bind α-catenin; this could titrate out α-catenin. Indeed, when DE-cadherin-ΔCyt/β-catenin and α-catenin-GFP transgenes are coexpressed with slboGal4 in border cells and centripetal cells, some α-catenin-GFP colocalizes with intracellular pools of DE-cadherin-ΔCyt/β-catenin (Fig.2.27E). This is likely to be due to specific binding of DE-cadherin-ΔCyt/β-catenin to α-catenin-GFP, since intracellular pools of DE-cadherin-Δβ do not recruit α-catenin-GFP (Fig.2.27F). DE-cadherin-ΔCyt/β-catenin and α-catenin-GFP co-overexpression in border cells also suppresses the migration defects observed with overexpression of DE-cadherin-ΔCyt/β-catenin alone (Fig.2.27D). Thus, the dominant-negative effect of DE-cadherin-ΔCyt/β-catenin is likely to be due to titration of α-catenin away from endogenous DE-cadherin.

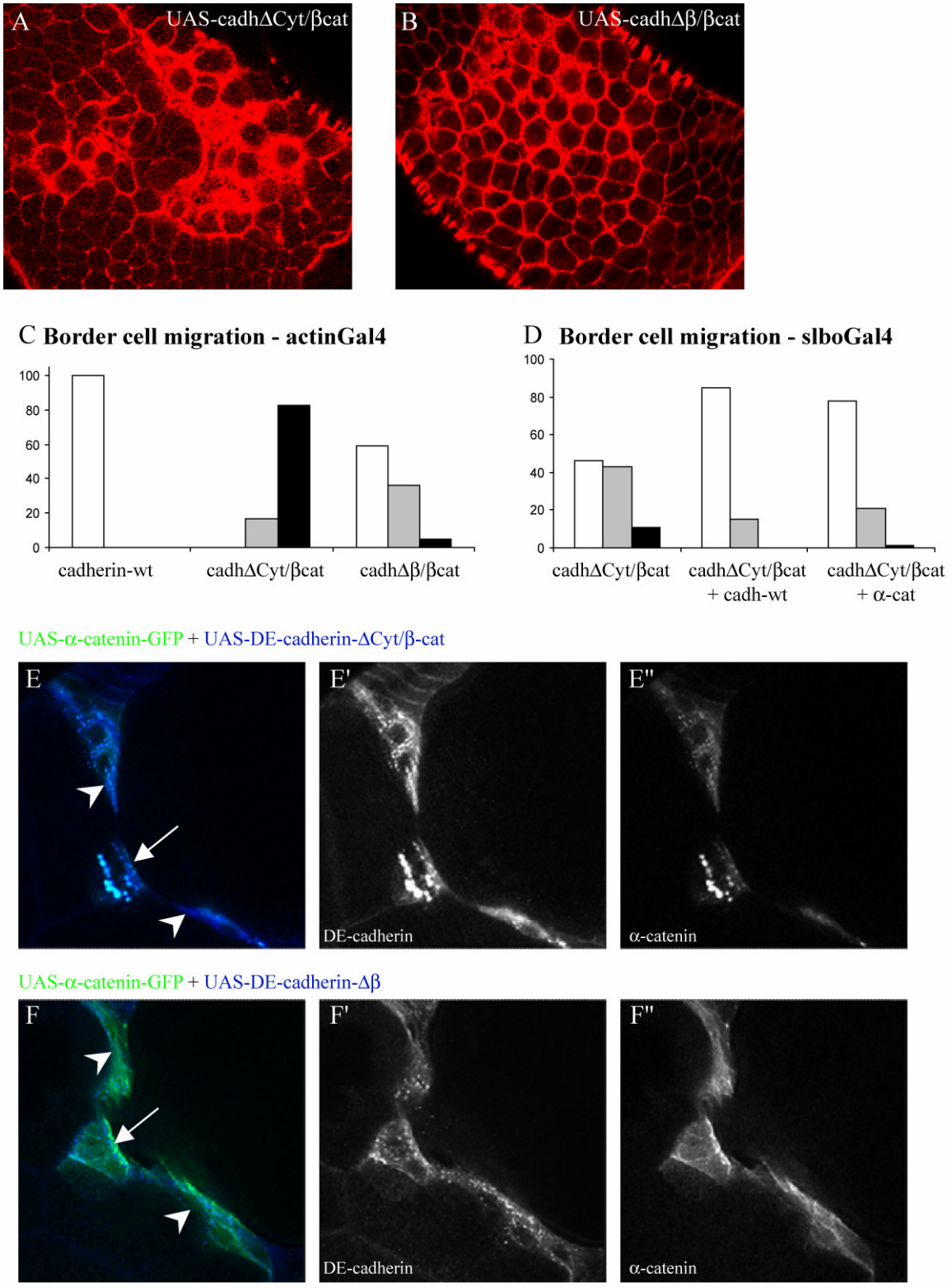



**Figure 2.27: DE-cadherin/β-catenin act as dominant-negatives. A-B.** UAS-DE-cadherin-ΔCyt/β-catenin (A) and UAS-DE-cadherin-Δβ/β-catenin (B) overexpressed in follicular cell AFG clones cause loss of epithelial integrity. **C.** Border cell migration in stage 10 egg chambers overexpressing the indicated UAS transgenes in

AFG clones (18≤n≤240). White: full migration; grey: incomplete migration; black: no migration. **D.** Border cell migration in stage 10 egg chambers overexpressing the indicated UAS transgenes with slboGal4 (142≤n≤386). The transgenic line carrying UAS-DE-cadherin-ΔCyt/β-catenin is the same as in C. The stronger migration defects in AFG clones are probably due to the earlier expression of the transgene. In D, migration defects were scored using X-Gal stainings (see §4.4.1). White: full migration; grey: incomplete migration; black: no migration. **E-F.** Localization of α-catenin-GFP (E-F: green; E''-F'') in egg chambers overexpressing DE-cadherin-ΔCyt/β-catenin (E: blue, E') or DE-cadherin-Δβ (F: blue, F'). UAS transgenes were expressed in border cells (arrows) and centripetal cells (arrowheads) with slboGal4.

## 2.4 Endocytosis

In tissue culture experiments, endocytosis of integrin complexes has been shown to be essential for migration (see §1.1.3.3) and endocytosis of cadherin has been observed in several situations where adhesion is likely to be dynamic (see §1.2.5.2). Therefore, it seemed possible that endocytosis could allow DE-cadherin turnover in border cells and regulation of adhesion during the migration. To test this hypothesis, I chose to test several possible genetic interactions between DE-cadherin and components of the endocytic pathway.

To generate a genetic background susceptible to impair endocytosis, *shibire* (*shi*) and *rab5* mutants were used (see material and methods, §4.3.5). shi encodes dynamin, a small GTPase required for vesicle pinching off. *shi* mutant follicular cell clones have severe defects early during oogenesis making it impossible to obtain border cell mutant clones (Pernille Rørth, unpublished data). Rab5 is a small GTPase involved in the fusion of early endosome vesicles. *rab5* mutant follicular cell clones also have early defects - although not as dramatic as *shi* clones - and so far no border cell cluster completely mutant for rab5 could be obtained (Hsin-Ho Sung, unpublished data). Genetic interactions between DE-cadherin and shi or rab5 were thus tested in a *shi* or *rab5* heterozygous background.

First, I tested if overexpression of DE-cadherin in a *shi* or *rab5* heterozygous background would lead to some migration defects. Overexpression of wildtype DE-cadherin in border cells does not affect the migration; but if DE-cadherin is indeed turned-over in border cells, it is possible that DE-cadherin overexpression in a context where endocytosis is reduced could lead to some migration defects. DE-cadherin was overexpressed in border cells with SlboGal4 or in “actin-flip-out-Gal4” clones. Overexpression of wildtype DE-cadherin with either driver did not give any migration defects, neither in *shi* nor in *rab5* background. I also overexpressed DE-cadherin-Δβ/α-catenin and DE-cadherin-ΔJM which may lack some regulatory signals and thus be more sensitive to perturbation of endocytosis: neither in *shi* nor in *rab5* background is any significant migration defect observed (Fig.2.28A-B).

I also tested possible genetic interactions between various DE-cadherin mutants and components of the endocytic pathway in the absence of wild type DE-cadherin. For DE-cadherin-ΔJM and DE-cadherin-4YF, I took advantage of the fact that DE-cadherin-ΔJM and DE-cadherin-4YF transgenes driven by the tubulin promoter can rescue *shg* homozygous

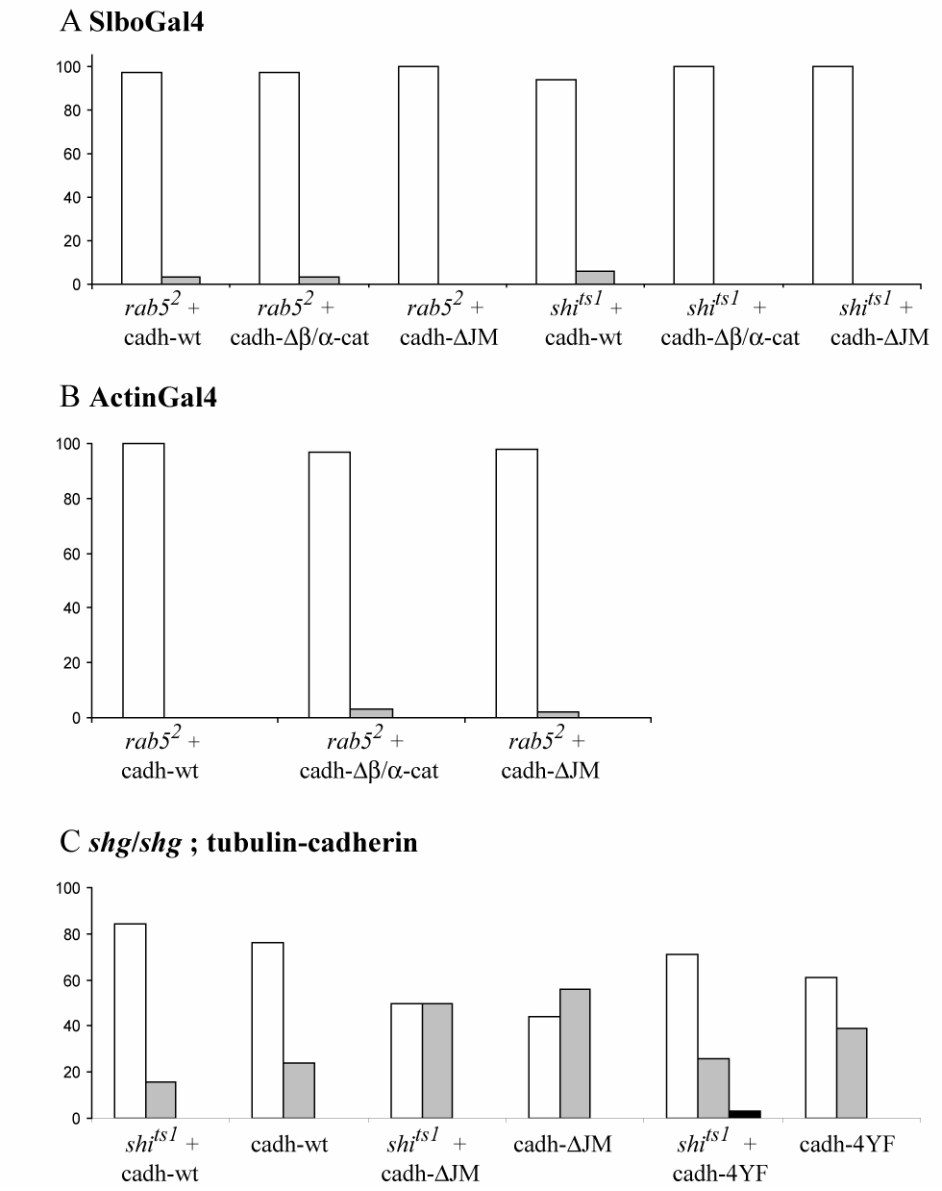



**Figure 2.28: Testing possible genetic interactions between DE-cadherin and endocytic components. A-B.** Border cell migration in flies heterozygous for *rab5* or *shi* and in which the indicated UAS-DE-cadherin variants were overexpressed in border cells with SlboGal4 (A) or in AFG clones (B) ($45 \leq n \leq 260$). **C.** Border cell migration in flies expressing only the variant form of DE-cadherin indicated (flies were *shg* homozygous mutants and express DE-cadherin transgenes under the control of tubulin promoter). When indicated, the flies were also heterozygous for *shi.* Experiment was done at 29ºC ($14 \leq n \leq 49$). White: full migration; grey: incomplete migration; black: no migration.

mutants to adulthood: border cell migration defects were analyzed in egg chambers from *shg* homozygous mutant flies expressing tubulin-DE-cadherin-ΔJM or tubulin-DE-cadherin-4YF transgenes. This was done with otherwise wild type flies as well as with *shi* heterozygous flies. No significant difference was observed between control and *shi* heterozygous flies (Fig.2.28C).

For DE-cadherin-Δβ/α-catenin and DE-cadherin-FL/α-catenin, I repeated the rescue experiment of *shg* MARCM clones with UAS-DE-cadherin-Δβ/α-catenin and UAS-DE-cadherin-FL/α-catenin in a *shi*, *rab5* as well as *sprint* heterozygous background. Sprint is a homologue of mammalian Rin1 (Szabo et al., 2001). Rin1 has been shown to activate Rab5A and to stimulate Rab5A-dependent endosome fusion as well as endocytosis (Tall et al., 2001). Overexpression of Sprint in nurse cells induces endocytosis of DE-cadherin, which is then removed from the membrane and accumulates in intracellular vesicles (Gaspar Jekely, unpublished data). Although Sprint has been shown to be not essential for the migration (Gaspar Jekely, unpublished data), it may still participates in DE-cadherin turn-over. Preliminary results do not show any obvious genetic interactions between DE-cadherin-Δβ/α-catenin or DE-cadherin-FL/α-catenin and *shi*, *rab5* or *sprint*: for DE-cadherin-Δβ/α-catenin in *shi* heterozygous, 6 full border cell clones were observed, all had migrated normally; in *rab5* heterozygous, 4 clones had migrated normally and 1 had not migrated; in *sprint* heterozygous, 6 had migrated normally, 3 were delayed and 2 had not migrated; for DE-cadherin-FL/α-catenin, in *shi*, *rab5* or *sprint* background all full border cell clones had completed migration ($1 \leq n \leq 10$).

So far, no genetic interactions were detected between various DE-cadherin mutants and components of the endocytic pathway. This absence of interactions could indicate that endocytosis is not involved in regulating DE-cadherin during border cell migration. However, I only tested interactions in heterozygous mutant backgrounds and it is possible that interactions would be detected only in a more stringent context: even if DE-cadherin is regulated by endocytosis, removing only one copy of shi or rab5 may not significantly reduce its turn-over. Therefore, we can so far not exclude that endocytosis participates in regulating adhesion during the migration.

# 3 DISCUSSION

## 3.1 Subcellular localization of DE-cadherin

The aim of the work presented here was to understand how *Drosophila* E-cadherin can be regulated in vivo, in particular during border cell migration. Several DE-cadherin mutants were generated and their ability to substitute for endogenous DE-cadherin was tested. Notably, it was observed that some DE-cadherin variants have an abnormal subcellular distribution; this may give us some information about DE-cadherin intracellular trafficking.

Similar to endogenous DE-cadherin, exogenous DE-cadherin-wt and DE-cadherin-ΔJM expressed at moderate levels (with tubulin promoter, §2.1) are cortically localized. On the contrary, DE-cadherin-wt (§2.3.1) as well as DE-cadherin-ΔJM (data not shown) overexpressed from UAS transgenes are not only cortically localized but also accumulate intracellularly, both in follicular cells and border cells: I observed diffuse DE-cadherin staining as well as punctuate and perinuclear stainings. Although I did not characterize this in details, this intracellular accumulation is likely to correspond to accumulation of overexpressed DE-cadherin along the secretory pathway (endoplasmic reticulum, Golgi, vesicles). Punctuate stainings might also correspond to recycling vesicles.

Several other DE-cadherin mutants and fusion proteins also show intracellular accumulation in follicular cells and border cells: DE-cadherin-Δβ, DE-cadherin-ΔCyt/α-catenin, DE-cadherin-Δβ/α-catenin, DE-cadherin-ΔCyt/β-catenin and DE-cadherin-Δβ/β -catenin (§2.1 and §2.3). As for DE-cadherin-wt and DE-cadherin-ΔJM, this accumulation is probably partly due to overexpression and accumulation in the secretory machinery. However, all these constructs give much stronger intracellular accumulation than DE-cadherin-wt or DE-cadherin-ΔJM. Also, DE-cadherin-Δβ gives intracellular staining when expressed at moderate levels under the control of the tubulin promoter (§2.1). Moreover, overexpressed DE-cadherin-ΔCyt/α-catenin and DE-cadherin-Δβ/α-catenin tend to be present at lower levels at the membrane than overexpressed DE-cadherin-wt (§2.3). This may be due to inefficient transport to the membrane of these DE-cadherin variants. It is possible that they lack sequences normally involved in proper membrane targeting of DE-cadherin. All these DE-cadherin variants have in common to lack the C-terminal domain of DE-cadherin, suggesting that this domain contains membrane targeting signals. This would be consistent with the work of Chen et al. who have shown that the cytoplasmic domain of E-cadherin contains sorting

signals that are necessary and sufficient for membrane targeting (Chen et al., 1999). Chen et al. generated several E-cadherin mutants lacking various parts of the cytoplasmic domain and observed that less than 10% of the mutant proteins reach the cell surface, most being retained in the endoplasmic reticulum (Chen et al., 1999). Chen et al. hypothesized that proper localization of DE-cadherin was dependent on β-catenin binding (Chen et al., 1999). However, when I express DE-cadherin-FL/α-catenin in *arm* clones, it seems to localize properly to the membrane (data not shown). Thus efficient targeting of DE-cadherin to the membrane seems to directly depend on the presence of targeting signals in its cytoplasmic domain rather than on binding to β-catenin.

Additionally to inefficient membrane targeting, it is possible that the intracellular accumulation of DE-cadherin mutants is also due to inefficient degradation. Many cadherins contain PEST sequences as well as LSSL motifs (which are both motifs targeting proteins for proteasome degradation) in their cytoplasmic domain (Huber et al., 2001). In MDCK cells, part of newly synthesized E-cadherin has indeed been shown to be degraded by the proteasome (Chen et al., 1999). DE-cadherin has one LSSL motif as well as one PEST sequence (as identified with the "PEST-find" program) in its C-terminal domain (see Fig.2.1). It is thus possible that part of wild type DE-cadherin is normally degraded by the proteasome and that DE-cadherin mutants lacking the C-terminal domain are not, due to the absence of the PEST and LSSL sequence.

In the presence of endogenous DE-cadherin, several DE-cadherin/α-catenin fusion (DE-cadherin-ΔCyt/α-catenin, DE-cadherin-ΔCyt/CD2/α-catenin and DE-cadherin-Δβ/α-catenin) give less diffuse intracellular staining and accumulate in big aggregates (§2.3). This is observed in follicular cells as well as in border cells. As cadherins are known to form lateral dimers through interactions of their extracellular domain, it is possible that these DE-cadherin/α-catenin fusions dimerize with endogenous wild type DE-cadherin, which could influence their intracellular trafficking. I did not characterize what these big intracellular aggregates are; they may correspond to some compartements of the secretory pathway (Golgi for instance) or alternatively to the endosomal/lysosomal pathway. Defining the nature of these aggregates as well as the nature of in the intracellular accumulation in the absence of endogenous DE-cadherin will be required to better understand DE-cadherin trafficking.

## 3.2 Binding to β-catenin is required for DE-cadherin function

DE-cadherin-Δβ is unable to rescue *shg* phenotypes during oogenesis (§2.1). DE-cadherin-Δβ transgene is expressed at somewhat lower levels than DE-cadherin-wt, DE-cadherin-AAA and DE-cadherin-ΔJM transgenes and maybe at slightly lower levels than endogenous DE-cadherin. Thus the observed defects could have been due to insufficient expression. However, strongly reducing endogenous DE-cadherin levels (for instance by using the hypomorph allele $shg^{P34}$), only midly affect oogenesis; in particular $shg^{P34}$/ $shg^{P34}$ mutant somatic clones do not show defects in the follicular epithelium and only mild delays during border cell migration (§2.1). Hence, the general inability of DE-cadherin-Δβ to replace endogenous DE-cadherin during oogenesis is most likely due to the non-functionality of DE-cadherin-Δβ, probably reflecting that β-catenin is an important, often essential, interaction partner for cadherin. This supports the view that linkage to the actin cytoskeleton through β-catenin and α-catenin is critical for cadherin function. I cannot rule out that other proteins bind to the deleted C-terminus of DE-cadherin, but the requirement for β-catenin interaction is supported by the similar defects observed in *arm* mutants (§2.2.2), (Peifer et al., 1993).

Absence of β-catenin or expression of only DE-cadherin-Δβ in nurse cells severely affects border cell migration but does not completely block it as absence of DE-cadherin does (§2.1 and 2.2.2). It thus appears as if linkage to the cytoskeleton via β-catenin is important but not completely essential in nurse cells. In contrast, interaction with β-catenin seems to be strictly required in border cells (§2.1 and 2.2.2). Thus, linking DE-cadherin to the actin cytoskeleton may be less critical for cadherin to function as substratum than in the actively migrating cell itself.

## 3.3 Not all functions of DE-cadherin require regulation

### 3.3.1 Tight transcriptional control of DE-cadherin is not required

As described in the introduction (§1.2.5), cadherins are transcriptionally regulated during development and, in some cases, this transcriptional control has been shown to be required for proper development. During *Drosophila* development, levels of DE-cadherin have to be somehow regulated. Indeed, insufficient levels of DE-cadherin during embryogenesis (for

instance in *shg* hypomorphs) lead to developmental defects (Tepass et al., 1996). Similarly, strong overexpression of DE-cadherin is deleterious: for instance overexpression of DE-cadherin in the embryo with a tubulin-Gal4 driver is lethal. However, ubiquitous expression of DE-cadherin at moderate levels under the control of the tubulin promoter can rescue the lack of endogenous DE-cadherin during *Drosophila* development. This indicates that tight transcriptional control of DE-cadherin expression is not essential provided that DE-cadherin is expressed at moderate levels: it does appear as if no strong tissue-specific transcriptional down- or up-regulation of DE-cadherin is essential for *Drosophila* development.

### 3.3.2 DE-cadherin regulation does not seem to be required in follicular cells

In follicular cells, DE-cadherin function requires that it is connected to the actin cytoskeleton (see §3.2). However, the fact that DE-Cadherin/α-catenin fusions can rescue *shg* phenotypes in follicular cells (§2.3.1 and 2.3.2) shows that no regulation of the link between DE-cadherin and catenins is required in those cells. Moreover, rescue by DE-cadherin-ΔCyt/α-catenin indicates that no other regulation involving the cytoplasmic domain of DE-cadherin is required. Thus, in follicular cells, DE-cadherin does not seem to require any regulation at the levels of DE-cadherin or catenins. This may correspond to a quite "simple" function of DE-cadherin in follicular cells where DE-cadherin "only" has to maintain adhesion between epithelial follicular cells or between follicular cells and the oocyte. However, it is likely that some other functions of DE-cadherin, in more complex and dynamic processes require some regulation. In particular, the border cell migration defects observed with DE-cadherin-ΔCyt/α-catenin may be due to lack of DE-cadherin regulation (§2.3.1 and §3.7).

## 3.4 p120ctn is not essential for DE-cadherin function

### 3.4.1 Interaction with p120ctn is not required for DE-cadherin function during *Drosophila* development

p120ctn protein is present in the embryo (Magie et al., 2002) and is expressed in the ovary, in a pattern identical to that of DE-cadherin (C. Magie and S. Parkhurst, personal communication). However, DE-cadherin-AAA and DE-cadherin-ΔJM were able to replace

wild type DE-cadherin in all processes that I analyzed (§2.1). Moreover, knocking-down p120ctn itself does not seem to affect oogenesis and development (§2.1.5). These results indicate that DE-cadherin/p120ctn interaction is not essential for any critical process during development. These results were quite unexpected considering the described role for p120ctn in mammalian cells. Also, DE-cadherin juxtamembrane domain is not essential for DE-cadherin function. It remains possible that DE-cadherin/p120ctn interaction or the juxtamembrane domain have subtle modulator effects on DE-cadherin function or a role in a non-essential process that I did not test directly. In particular, if the juxtamembrane domain of DE-cadherin is moderately involved in reinforcing adhesion, expression of DE-cadherin-ΔJM transgenes at levels somewhat higher than endogenous expression levels may mask this role.

Myster et al. generated null alleles of *Drosophila* p120ctn and found that mutants were surprisingly viable and fertile (Myster et al., 2003). These mutants do not show substantial changes in junction structure or function. However, p120ctn mutations enhance defects due to mutations in *shg*. This shows that *Drosophila* p120ctn is a positive modulator of DE-cadherin but not an essential core component of adherens junctions. Another study reported that embryos in which p120ctn has been knocked-down by injection of dsRNA exhibit severe morphogenetic defects (Magie et al., 2002). The discrepancy between these RNAi data and the absence of phenotype in p120ctn null mutants may be due to non specific effects of the RNAi. Alternatively, it is possible that in p120ctn null mutants, some compensatory mechanisms limit the effects of p120ctn absence whereas injecting p120ctn dsRNA in embryos could cause a very rapid loss of p120ctn that would not have time to be compensated during embryogenesis.

### 3.4.2 p120ctn in vertebrates and invertebrates

Both our results and the work of Myster et al. unexpectedly showed that p120ctn is not essential for *Drosophila* E-cadherin function. Similar results were obtained in *C.elegans*: knocking-down *C.elegans* p120ctn (called JAC-1) by RNAi does not give any obvious defects in embryonic or postembryonic development. However, it does increase the severity and penetrance of morphogenetic defects caused by a hypomorphic mutation in α-catenin (Pettitt et al., 2003). Hence, in both *C.elegans* and *Drosophila*, p120ctn appears as a positive

modulator of cadherin mediated adhesion but is not an essential component of adherens junctions as β-catenin and α-catenin are.

The non essential role of p120ctn in *Drosophila* and *C.elegans* is in contrast with results obtained in vertebrates. In Xenopus, depletion of p120ctn results in gastrulation defects and in a moderate reduction in the levels of C-cadherin. Expression of C-cadherin can partially rescue the gastrulation defects due to p120ctn depletion, suggesting that reduced C-cadherin levels contribute to the observed defects (Fang et al., 2004). Also, the murine p120ctn knock-out is embryonic lethal (Reynolds et al., unpublished data, see in (Davis et al., 2003)). Moreover, recent loss-of-function analysis in mammalian cells indicates that p120ctn is involved in regulating cadherin turnover. Knocking-down p120ctn by RNAi in various cell lines results in a dose-dependent elimination of several cadherins from the cell surface (Davis et al., 2003). In the case of E-cadherin, it was shown that this is due neither to a change in the rate of E-cadherin synthesis nor to a defect in the initial transport to the membrane. Rather, in the absence of p120ctn, membrane E-cadherin is rapidly turned-over and degraded by the proteasome and/or lysosome (Davis et al., 2003). Similarly, p120ctn downregulates the constitutive internalization and degradation of VE-cadherin in microvascular endothelial cells (Xiao et al., 2003a). p120ctn would thus positively regulate adhesion by inhibiting cadherin turnover. However, in several studies cadherin mutants unable to bind p120ctn and showing reduced adhesive activity were expressed at levels comparable to wild type cadherin (for instance see (Thoreson et al., 2000)). This suggests that inhibition of cadherin turnover is unlikely to fully explain the effects of p120ctn on adhesion. p120ctn seem to be also involved in targeting cadherins to the membrane. p120ctn-GFP associates with vesicles and moves along microtubules in REF52 cells (Chen et al., 2003). Part of this moving p120ctn-GFP is then delivered to existing cell-cell junctions and N-cadherin was shown to associate and to be transported with the cytoplasmic pool of p120ctn. Chen et al. also showed that the N-terminal region of p120ctn binds to kinesin heavy chain (KHC) and that disruption of the KHC-p120ctn-N-cadherin complex leads to a delayed accumulation of N-cadherin at cell-cell contacts during junction reassembly. Consistently, p120ctn has been shown to associate with N-cadherin very shortly after its synthesis (Wahl et al., 2003).

In *Drosophila* and *C.elegans*, how p120ctn functions as a positive modulator of cadherin-mediated adhesion is not known. It is possible that loss of p120ctn affects cadherin

intracellular trafficking and turnover. Cadherins seem to localize normally to the membrane in p120ctn *Drosophila* mutants or in *C.elegans* embryos treated with p120ctn RNAi but cadherin dynamics was not studied and it is possible that turnover or transport kinetics are slightly affected in the absence of p120ctn.

It is also not understood why p120ctn seems to be essential in vertebrates but not in *Drosophila* or *C.elegans*. It is possible that increased complexity of tissue organization and morphogenetic events in vertebrates requires more tightly regulated adhesion than invertebrate development. It is also possible that vertebrate p120ctn has additional roles compare to invertebrate p120ctn. Comparing the sequences of *Drosophila* and *C. elegans* p120ctn with mammalian p120ctn shows that they share little homology outside the armadillo repeats. In particular, several protein-protein interaction motifs and phosphorylation sites present in the N-terminal domain of mammalian p120ctn are not found in *Drosophila* and *C.elegans* p120ctn. Also, the kinesin binding domain is contained in this divergent N-terminal region of p120ctn. It is thus not clear if *Drosophila* or *C.elegans* p120ctn would also bind to kinesin.

## 3.5 Tyrosine phosphorylation may not be essential for DE-cadherin function

### 3.5.1 DE-cadherin tyrosine phosphorylation

DE-cadherin-4YF completely substitute for endogenous DE-cadherin in all the processes that I looked at during oogenesis and development (§2.2.1). This shows that the four conserved tyrosine residues in DE-cadherin cytoplasmic domain and their phosphorylation are not critically involved in regulating DE-cadherin. Although the four other tyrosines present in the cytoplasmic domain are not conserved between *Drosophila* and mammals, it still remains possible that they could be involved in DE-cadherin regulation. The two tyrosine residues required for mammalian E-cadherin regulation mediated by Hakai (§1.2.5.2) are for instance not conserved (Fujita et al., 2002). It should also be mentioned that even though the four other tyrosines in DE-cadherin are not conserved in mammals, they seem to be conserved between *Drosophila* and *Anopheles* (see Fig.2.1B). To know if phosphorylation of other tyrosines may

be involved in DE-cadherin regulation, it should first be checked if DE-cadherin can be phosphorylated in vivo or not.

### 3.5.2 β-catenin tyrosine phosphorylation

β-catenin-Y667F could fully rescue the lack of endogenous β-catenin during oogenesis, both in germ line and somatic cells (§2.2.2). These results indicate that phosphorylation of β-catenin on this conserved tyrosine is not essential for DE-cadherin regulation during oogenesis, in particular during border cell migration. I can not exclude that this tyrosine residue would have a more important role in other processes, during embryogenesis for instance.

A lot of emphasis has been put in the literature on the putative regulatory role of this tyrosine. However, only correlations between tyrosine phosphorylation of β-catenin and downregulation of adhesion have been described. It is thus not clear if β-catenin phosphorylation is a cause or a consequence of adhesion downregulation. There are actually some cell types in which this phosphorylation has been shown not to be responsible for cadherin regulation (see introduction §1.2.5.4, (Takeda et al., 1995)). Our results suggest that this is also the case during border cell migration. Consistently with the non essential role of β-catenin Y667, regulation of the link between DE-cadherin and α-catenin does not seem to be essential for border cell migration (see §3.6).

## 3.6 Regulation of the link between DE-cadherin and α-catenin is not essential in follicular and border cells

All the DE-cadherin/α-catenin fusions can substitute for endogenous DE-cadherin in follicular cells (§2.3.1 and 2.3.2), indicating that no regulation of the link between DE-cadherin and α-catenin is required in those cells.

In border cells, DE-cadherin-FL/α-catenin fully substitute for DE-cadherin (§2.3.2) unexpectedly suggesting that regulating the link between DE-cadherin and α-catenin is not required for the migration. This will still need to be confirmed by repeating the rescue experiment in the absence of endogenous β-catenin. In any case, the partial rescue observed

with DE-cadherin-Δβ/α-catenin (§2.3.2) shows that regulating the link between DE-cadherin and α-catenin is not strictly required for the migration. It can not be excluded that there is some important regulation of the link between DE-cadherin and actin in border cells, but downstream of α-catenin.

## 3.7 Why is DE-Cadherin-ΔCyt/α-catenin blocking border cell migration?

### 3.7.1 May be due to lack of regulation

DE-cadherin-ΔCyt/α-catenin gives very strong migration defects when expressed in *shg* mutant clones, showing that it is unable to substitute for endogenous DE-cadherin in border cells. Since DE-cadherin-Δβ/α-catenin can partially substitute for endogenous DE-cadherin, the migration defects observed with DE-cadherin-ΔCyt/α-catenin are not simply due to the fact that α-catenin is constitutively linked to DE-cadherin. Several explanations could account for the inability of DE-cadherin-ΔCyt/α-catenin to substitute for endogenous DE-cadherin:

- DE-cadherin-ΔCyt/α-catenin membrane levels could be too low in border cells. Strong intracellular accumulation of DE-cadherin-ΔCyt/α-catenin indeed makes it impossible to check how much is localized at the membrane in border cells. However, in follicular cells membrane level of DE-cadherin-ΔCyt/α-catenin is actually higher than endogenous DE-cadherin levels and similar to the level of DE-cadherin-Δβ/α-catenin, which gives much weaker migration defects. Also, the results obtained with DE-cadherin-ΔCyt/α-catenin contrast with what is observed in $shg^{P34}$ hypomorph mutants; in these mutants, DE-cadherin levels are quite strongly reduced but only slight migration defects are observed (§2.1). Thus insufficient membrane levels seem unlikely to be the explanation for the very strong migration defects observed with DE-cadherin-ΔCyt/α-catenin.

- DE-cadherin-ΔCyt/α-catenin may be not functional enough to mediate proper adhesion during the migration. DE-cadherin-ΔCyt/α-catenin is at least partly functional since it can substitute for endogenous DE-cadherin in follicular cells. Also, in border cells DE-cadherin-ΔCyt/α-catenin is able to mediate some adhesion. Indeed, border cells expressing only DE-cadherin-ΔCyt/α-catenin can form some long cellular extensions whereas *shg* mutant border

cells never do (§1.3.4 and 1.3.5) (Fulga and Rorth, 2002). Thus DE-cadherin-ΔCyt/α-catenin is at least functional enough to allow the formation of some cellular extensions. In theory, it is nevertheless possible that DE-cadherin has to mediate stronger adhesion for border cells to migrate than in follicular cells or than for forming some cellular extensions. It could thus be that DE-cadherin-ΔCyt/α-catenin is only partially functional, which would be sufficient in follicular cells and in border cells to obtain some cellular extensions but not for border cells to migrate. In particular, the fact that α-catenin is very close to the membrane in DE-cadherin-ΔCyt/α-catenin could affect its conformation and its interactions with the actin cytoskeleton.

The poor functionality of DE-cadherin-ΔCyt/α-catenin would not explain the migration defects observed when DE-cadherin-ΔCyt/α-catenin is overexpressed in wild type egg chambers. One possibility is that DE-cadherin-ΔCyt/α-catenin also has a dominant–negative effect on endogenous DE-cadherin. For instance, DE-cadherin-ΔCyt/α-catenin could titrate out some downstream components of the adhesive complexes away from endogenous DE-cadherin -although one would then expect that they are components specifically required in border cells as adhesion does not seem to be affected in follicular cells. However, the suppression of migration defects observed in a *shg* heterozygous background is hard to reconcile with this hypothesis.

- Alternatively, DE-cadherin-ΔCyt/α-catenin may be properly functional but its strong intracellular accumulation may have some negative effects on the migration. Intracellular DE-cadherin-ΔCyt/α-catenin may for instance titrate out some components of the cytoskeleton or some signalling molecules specifically required for the migration. It is possible that this is what accounts for the migration defects observed in the rescue experiment of *shg* mutant clones. Consistently, the severity of the migration defects observed with DE-cadherin-ΔCyt/α-catenin, DE-cadherin-Δβ/α-catenin and DE-cadherin-FL/α-catenin correlates with the level of their intracellular accumulation.

The migration defects observed when DE-cadherin-ΔCyt/α-catenin is overexpressed in “actin-flip-out-Gal4” clones in otherwise wild type egg chambers are unlikely to be due to DE-cadherin-ΔCyt/α-catenin intracellular accumulation. Indeed, overexpression of DE-cadherin-Δβ/α-catenin does not result in migration defects although intracellular DE-cadherin aggregates look very similar in both cases.

- Finally, it is possible that DE-cadherin-ΔCyt/α-catenin lacks some regulatory signals. The inability of DE-cadherin-ΔCyt/α-catenin to substitute for endogenous DE-cadherin migration would then be due its inability to be downregulated and the migration defects due to too stable adhesion. The inhibition of migration observed with simple overexpression of DE-cadherin-ΔCyt/α-catenin is consistent with this hypothesis. Why the defects are weaker than in the rescue experiment is not very clear. The difference in the type of intracellular accumulation observed in the presence or absence of endogenous DE-cadherin clearly shows that the presence of endogenous DE-cadherin affects the behaviour of DE-cadherin-ΔCyt/α-catenin, at least in terms of intracellular trafficking. It is thus possible that some regulation due to the presence of wild type DE-cadherin still occurs, either simply because there is some wild type DE-cadherin at the membrane or maybe because wild type DE-cadherin and DE-cadherin-ΔCyt/α-catenin form dimers that can be at least partially regulated. This may seem inconsistent with the very small defects observed when one copy of *shg* is taken away. One possible explanation for this could be that removal of one copy of *shg* facilitates migration by reducing DE-cadherin levels on nurse cell membranes and thus by reducing the excessive adhesion between border cells and nurse cells. Co-overexpressing DE-cadherin-ΔCyt/α-catenin and DE-cadherin-wt in border cells may help to better understand what happens in the presence of wild type DE-cadherin.

DE-cadherin-ΔCyt/CD2/α-catenin may help discriminating between these different hypotheses. DE-cadherin-ΔCyt/CD2/α-catenin should not contain any extra specific regulatory sequences compared to DE-cadherin-ΔCyt/α-catenin. Presence of the CD2 cytoplasmic domain between DE-cadherin-ΔCyt and α-catenin moves α-catenin away from the plasma membrane. It also seems to help reducing intracellular accumulation of the fusion protein. Although intracellular as well as membrane levels still need to be compared in more details, it looks like DE-cadherin-ΔCyt/CD2/α-catenin may behave more similarly to DE-cadherin-Δβ/α-catenin than to DE-cadherin-ΔCyt/α-catenin in terms of subcellular localization. Then, if the migration defects observed in the *shg* rescue experiment with DE-cadherin-ΔCyt/CD2/α-catenin are similar to those observed with DE-cadherin-Δβ/α-catenin, this would suggest that the defects observed with DE-cadherin-ΔCyt/α-catenin are not due to

the lack of a regulatory sequence but rather to its strong intracellular accumulation or to α-catenin being too close to the membrane. On the contrary, if the migration defects due to DE-cadherin-ΔCyt/CD2/α-catenin are more similar to those observed with DE-cadherin-ΔCyt/α-catenin, this would suggest that the defects observed with DE-cadherin-ΔCyt/α-catenin are neither due to its strong intracellular accumulation nor to α-catenin being too close to the membrane but rather to the lack of a regulatory sequence.

### 3.7.2 What is the nature of the regulation?

If the migration defects observed with DE-cadherin-ΔCyt/α-catenin are really due to a lack of regulation, I still need to identify which regulatory signals are missing in DE-cadherin-ΔCyt/α-catenin. The migration defects observed with DE-cadherin-Δβ/α-catenin would suggest that either regulation of DE-cadherin/catenin interactions or some regulatory signals present in the C-terminal domain contribute to adhesion regulation. The much stronger defects observed with DE-cadherin-ΔCyt/α-catenin would indicate that some other signals are localized in the juxtamembrane domain. Deletion of the first 38 aminoacids of the juxtamembrane domain (in DE-cadherin-ΔJM, between arrows 1 and 2 in Fig.2.1B) does not give any migration defects, showing that no regulatory signal essential on its own is located in this region. This could mean that a regulatory signal is localized in the downstream 32 aminoacids (between arrows 2 and 3 in Fig.2.1B), which have been deleted in DE-cadherin-ΔCyt/α-catenin but neither in DE-cadherin-ΔJM nor in DE-cadherin-Δβ/α-catenin. Alternatively, it is possible that a regulatory signal is localized in the first 38 aminoacids of the juxtamembrane domain; this signal would then be not essential on its own but redundant with a signal localized in the C-terminal domain or regulation of DE-cadherin/catenin interactions.

One particular sequence contained in the juxtamembrane domain is the p120ctn binding site. Although the interaction with p120ctn on its own is not essential for DE-cadherin function, it may be redundant with some other regulatory mechanisms involving the C-terminal domain.

To test this, the rescue experiment of *shg* mutant clones will be repeated with DE-cadherin-Δβ/α-catenin in the presence of a p120ctnRNAi transgene.

Another possibility is that the juxtamembrane as well as the C-terminal domain contain some signals involved in the regulation of DE-cadherin turnover, for instance by targeting it for endocytosis. So far, no genetic interactions were detected between various DE-cadherin mutants and components of the endocytic pathway. However, I only tested interactions in heterozygous mutant backgrounds and it is possible that interactions would be detected only in a more stringent context. Costainings of DE-cadherin and endocytic compartments may also give us some indications about a possible turnover of DE-cadherin through endocytosis in border cells. In mammals, E-cadherin endocytosis has been shown to be regulated via phosphorylation of two tyrosine residues in E-cadherin cytoplasmic domain (Fujita et al., 2002). The four conserved tyrosines in DE-cadherin cytoplasmic region are not required for the migration. However, the two tyrosine residues required for E-cadherin endocytosis are not conserved in other mammal cadherins or in DE-cadherin (Fujita et al., 2002). It is thus possible that the non conserved tyrosines participate in DE-cadherin regulation. In that regard, it is worth noting that two non conserved tyrosines are located in the 32 aminoacid region, which has been deleted neither in DE-cadherin-ΔJM nor in DE-cadherin-Δβ/α-catenin (between arrows 2 and 3 in Fig.2.1) but in DE-cadherin-ΔCyt/α-catenin. These two tyrosines being respectively present or absent in DE-cadherin-Δβ/α-catenin and DE-cadherin-ΔCyt/α-catenin might explain why DE-cadherin-Δβ/α-catenin can partly substitute for endogenous DE-cadherin during the migration whereas DE-cadherin-ΔCyt/α-catenin can not.

## 3.8 DE-Cadherin/β-catenin fusions behave as dominant negatives

Both DE-cadherin-ΔCyt/β-catenin and DE-cadherin-Δβ/β-catenin are unable to rescue *shg* phenotypes, indicating that they are probably not functional (§2.3.4). I do not know why this is the case. One possibility could be that most DE-cadherin-ΔCyt/β-catenin and DE-cadherin-Δβ/β-catenin are localized intracellularly and that they are not present in sufficient amount at the membrane. I will need to check how much is actually localized at the membrane with the extracellular staining protocol; however, even with regular stainings, one can see some DE-cadherin-ΔCyt/β-catenin and DE-cadherin-Δβ/β-catenin at the membrane making it unlikely

that insufficient amount at the membrane explains why DE-cadherin-ΔCyt/β-catenin and DE-cadherin-Δβ/β-catenin do not rescue *shg* phenotypes. Alternatively, DE-cadherin-ΔCyt/β-catenin and DE-cadherin-Δβ/β-catenin may be unable to mediate adhesion, even when localized at the plasma membrane. One possible explanation is that β-catenin can not properly orient itself relatively to DE-cadherin cytoplasmic domain and to the membrane when fused to DE-cadherin. It has indeed been shown that β-catenin cadherin-binding domain is normally oriented with its C-terminus close to the plasma membrane when it interacts with DE-cadherin (Huber and Weis, 2001). In DE-cadherin-ΔCyt/β-catenin and DE-cadherin-Δβ/β-catenin, β-catenin can not bind to DE-cadherin cytoplasmic domain; it is thus possible that it then tends to oriente with its C-terminus away from the plasma membrane, as one might expect from the cloning orientation. DE-cadherin-ΔCyt/β-catenin still seems to interact with α-catenin, but if α-catenin orientation is not correct one would expect that it can not be linked properly to the actin cytoskeleton.

DE-cadherin-ΔCyt/β-catenin and DE-cadherin-Δβ/β-catenin also give rise to *shg*-like phenotypes when overexpressed in wild type egg chambers (§2.3.4). This suggests that they are dominant-negatives for DE-cadherin. Co-overexpression of DE-cadherin-ΔCyt/β-catenin and wild type DE-cadherin suppresses the negative effect of DE-cadherin-ΔCyt/β-catenin, showing that DE-cadherin-ΔCyt/β-catenin is indeed a dominant-negative for DE-cadherin. This should be confirmed by testing what happens when DE-cadherin-ΔCyt/β-catenin is overexpressed in a *shg* heterozygous background. α-catenin is recruited by the intracellular pool of DE-cadherin-ΔCyt/β-catenin and co-overexpression of DE-cadherin-ΔCyt/β-catenin and α-catenin suppresses the negative effect of DE-cadherin-ΔCyt/β-catenin: thus, the dominant-negative effect of DE-cadherin-ΔCyt/β-catenin seems to be due to titration of α-catenin away from endogenous DE-cadherin. Although I did not characterize this in details, defects observed in the presence of DE-cadherin-Δβ/β-catenin are likely to be also due to α-catenin titration.

Defects observed in *shg* follicular cell clones expressing DE-cadherin-ΔCyt/β-catenin or DE-cadherin-Δβ/β-catenin tend to be stronger than simple *shg* phenotypes and to resemble defects observed in *arm* clones. This is likely to be due to a negative effect of DE-cadherin-ΔCyt/β-catenin or DE-cadherin-Δβ/β-catenin on DN-cadherin, which is expressed in follicular cells at

early stages of oogenesis. This is consistent with the above hypothesis that DE-cadherin-ΔCyt/β-catenin negative effect results from α-catenin titration.

## 3.9 Conclusion

When this work was initiated, we thought it was reasonable to assume that some mechanisms regulating DE-cadherin-mediated adhesion would be required for DE-cadherin-dependent border cell migration. Based on a lot of work previously done by many others in tissue culture experiments I first used a candidate approach to try to identify these regulatory mechanisms.

I first tested if DE-cadherin juxtamembrane domain in general and the p120ctn binding site in particular would be required for DE-cadherin function/regulation during border cell migration. Very surprisingly, DE-cadherin variants lacking either the whole juxtamembrane domain or the p120ctn binding site could substitute for endogenous DE-cadherin not only for border cell migration but also for all the processes requiring DE-cadherin that I studied during oogenesis and embryogenesis. This clearly shows that neither p120ctn binding site nor other regions of the juxtamembrane domain are essential on their own for DE-cadherin function/regulation. Similarly, I could also show that four tyrosine residues present in the cytoplasmic domain and conserved between DE-cadherin and mammalian E-cadherin are not essential for DE-cadherin function, in particular in border cells and more generally during oogenesis and development.

Another possible way to regulate adhesion could have been to regulate the association between DE-cadherin and β- or α-catenin. However, I showed that this is at least not strictly required for the migration, since DE-cadherinΔβ/α-catenin fusion partially substitutes for endogenous DE-cadherin during border cell migration. Also, mutating a conserved tyrosine residue of β-catenin supposedly important for the regulation of cadherin/β-catenin interaction does not cause any migration defects.

The inability of DE-cadherin-ΔCyt/α-catenin to substitute for endogenous DE-cadherin suggests that some regulatory signals may be located in the intracellular domain. However, DE-cadherin-ΔCyt/α-catenin also have some clear subcellular localization defects and it is so far not completely clear if the observed migration defects are really due to lack of adhesion

regulation or to less specific negative effects on the migration. DE-cadherin-ΔCyt/CD2/α-catenin may help discriminating between these possibilities.

The fact that no essential regulatory mechanism could be identified so far may reflect that not a single mechanism is involved in regulating adhesion during border cell migration. It is actually very possible that several mechanisms participate in regulating DE-cadherin and that suppressing only one of them is not sufficient to inhibit the migration. If the weak migration defects observed with DE-cadherin-Δβ/α-catenin and the strong defects obtained with DE-cadherin-ΔCyt/α-catenin are really due to insufficient DE-cadherin downregulation, this would clearly show that at least two levels of regulation are involved. It could be two different types of regulation (for instance interaction with p120ctn and regulation of the interaction with β- or α-catenin) or only one type of regulation (for instance via endocytosis) but controlled by several regulatory sequences present in DE-cadherin.

Alternatively, there might be no specific mechanism regulating DE-cadherin-mediated adhesion during border cell migration. As mentioned in the introduction, it has been shown in tissue culture experiments that proper migration requires that the adhesive strength between a migrating cell and its substratum is neither too high nor too low (Palecek et al., 1997). It is generally thought that some regulatory mechanisms are required to obtain proper adhesive strength and to actively down-regulate adhesion in the back of a migrating cell. However, it is in theory possible that in some migrating cells adhesion is strong enough to stabilize membrane protrusions and generate traction forces but not too strong so that simple acto-myosin contraction can pull the back of the cell forward, without the need of a regulatory mechanism to specifically disassemble adhesive complexes in the back.

# 4 MATERIALS AND METHODS

## 4.1 Cloning

DE-cadherin cDNA in pBlueScript (pBS) was provided by Bénédicte Sanson. In DE-cadherin-AAA, three glycines (aa 1376-1378) were mutated to alanines, by replacing GGTGGCGGC with GCGGCCGCC (creates a NotI site). In DE-cadherin-ΔJM, bp 4749 to 4862 were deleted. For DE-cadherin-Δβ and DE-cadherin-Δcyt , DE-cadherin cDNA was truncated at bp 4954 and 4746. For DE-cadherin-4YF, four tyrosines (aa 1372, 1452, 1478 and 1493) were mutated to phenylalanines. Tyrosine Y1372 was mutated by replacing AATTAC by GAATTC (creates an EcoRI site). For Y1452, Y1478 and Y1493, TAC was replaced by TTC.

DE-cadherin-wt, AAA, Δβ and Δcyt were subcloned from pBS to pCaSpeR-tubulin (EcoRI/KpnI fragment of tubulin-α1 promoter in pCaSpeR-4) (Asp718/XbaI). DE-cadherin-ΔJM and DE-cadherin-4YF were subcloned from pBS to pCaSpeR-tubulin with Asp718+NotI. DE-cadherin-AAA and Δβ were subcloned from pBS to pUAST (Brand and Perrimon, 1993) (Asp718/XbaI). DE-cadherin-wt, DE-cadherin-ΔJM and DE-cadherin-4YF were subcloned from pBS to pUASp (Rørth, 1998) (respectively with: Asp718/XbaI, Asp718+NotI and Asp718+BamHI). DE-cadherin-wt and AAA were cloned into pRmHa3 (Bunch et al., 1988) (Asp718/BamHI) and DE-cadherin-Δβ and Δcyt into pRmHa3b (EcoRI/NotI). DE-cadherin-wt, AAA, Δβ and ΔCyt were cloned into pCDNA-3 (Asp718/XbaI).

β-catenin cDNA (obtained from EST LD23131) was subcloned in pBS (XhoI). In β-catenin-Y667F, tyrosine 667 was mutated to phenylalanine by replacing ACATACGCCGCC with ACATTCGCGGCC (creates a NotI site). pCaSpeR-arm was obtained by subcloning the arm promoter (from pCaSpeR-armLacZ, (Vincent et al., 1994)) in pCaSpeR-4 (EcoRI-Asp718). pCaSpeR-arm-β-catenin-wt was obtained by cloning β-catenin cDNA in pCaSpeR-arm (SpeI-SnaBI/StuI). β-catenin-Y667F was subcloned in pCaSpeR-arm by replacing the SpeI-NheI fragment of β-catenin in pCaSpeR-arm-β-catenin-wt. β-catenin and β-catenin-Y667F were subcloned in pUASp2 (Asp718).

For DE-cadherin-Δcyt/α-catenin and DE-cadherin-Δβ/α-catenin fusions, α-catenin cDNA (obtained from EST LD07767) was cloned in frame with DE-cadherin-Δcyt and DE-cadherin-

Δβ . For DE-cadherin-Δcyt/β-catenin and DE-cadherin-Δβ/β-catenin fusions, β-catenin cDNA was cloned in frame with DE-cadherin-Δcyt and DE-cadherin-Δβ . For all these clonings, α- and β-catenin were fused to DE-cadherin in pCaSpeR-tubulin-DE-cadherin-ΔCyt or-Δβ. For DE-cadherin-ΔFL/α-catenin fusion, α-catenin cDNA was cloned in pBS in frame with DE-cadherin just before DE-cadherin stop codon. For this latter fusion, a linker (5 Glycine or 6 Lysine) was added between DE-cadherin and α–catenin. DE-cadherin-Δcyt/α-catenin, DE-cadherin-Δβ/α-catenin, DE-cadherin-ΔFL/α-catenin (5G and 6K linker), DE-cadherin-Δcyt/β-catenin and DE-cadherin-Δβ/β-catenin were subcloned into pUASp2 (Asp718).

DE-cadherin-ΔCyt/CD2/α-catenin was obtained by fusing DE-cadherin-ΔCyt (in pBS) in frame with the cytoplasmic domain of CD2 (aa 233 to 344) and full length α-catenin. DE-cadherin-ΔCyt/CD2/α-catenin was then subcloned in pUASp2 (Asp718 and BamHI-BglII).

p120ctn cDNA (obtained from EST LD02621) was cloned in frame with the HA tag of pRmHa-Nle-Ha (Royet et al., 1998), in place of the Nle insert (AscI/SalI). HA-p120ctn (BamHI/SalI) was then subcloned into pUAST. For p120ctn-RNAi, 1000 bp inverted repeats from the cDNA (bp 700 to1700 for RNAi-700 or bp 1200-2200 for RNAi-2200) were inserted with 500bp spacers into pUAST.

## 4.2 Cell culture, immunoprecipitation and aggregation assays

Schneider S2 and CHO cells were transfected with cellfectin (Invitrogen). pRm plasmids were induced with $CuSO_4$ (0,7 mM) for 24 hours.

For immunoprecipitation experiment, Schneider cells were transfected with pRm-HA-p120ctn and pRm-DE-cadherin-wt or pUAST-DE-cadherin-AAA+pRmGal4 (From Andreas Jenny). Immunoprecipitations were done in 1% NP-40 lysis buffer with DCAD2 antibody (Oda et al., 1994). Western Blot was done with mouse anti-HA antibody (1:10000, BabCO). Expression of DE-cadherin-wt and DE-cadherin-AAA was checked by western-blot with DCAD2 antibody.

For aggregation assays, S2 cells were cotransfected with pRm-DE-cadherin (wild type or mutants) and pRm-eGFP. CHO cells were cotransfected with pCDNA3-DE-cadherin (wild type or mutants) and pCDNA3-eGFP. S2 cells were transferred in Petri dishes and shaked for

2 hours before quantification of aggregation. Percentage of GFP cells in aggregates and average size of aggregates were quantified in at least three independent experiments. There were some variations from experiment to experiment but all four DE-cadherin constructs behave similarly in each experiment.

## 4.3 *Drosophila* genetics

### 4.3.1 Fly husbandry

Flies were grown on standard corn meal molasso agar (12 g agar, 18 g dry yeast, 10 g soy flour, 22 g turnip syrup, 80 g malt extract, 80 g corn powder, 6.25 ml propionic acid, 2.4 g methyl 4-hydroxybenzoate (Nipagin) per litre). All crosses were carried out at 25ºC, except crosses involving *shi* flies which were done at 18ºC. In order to optimise oogenesis, flies were put in vials containing dry yeast over night prior to dissection. For heat shock experiments, vials were submerged in a 37ºC waterbath for 1h. When flies were heat shocked as larvae, this was repeated three times. If adult flies were heat shocked, the females were fattened up on yeast before. After heat shock, the flies were flipped into vials with fresh yeast every day until dissection (4 or 5 days after heat-shock).

### 4.3.2 Generation of *shg* and *arm* clones – rescue experiments

To generate *shg* and *arm* mutant clones in the ovary, mutant alleles were respectively recombined onto FRT42 and FRT19A chromosomes. Mitotic recombination at FRT sites was induced by the FLP recombinase, which expression was induced upon heat-shock. Two different techniques were used to generate mutant clones in the ovary.

In the first case, mitotic recombination was induced in hsFLP ; FRT42*shg*/FRT42ubiGFP or *arm*FRT19A/ubiGFP,FRT19A ; hsFLP flies. Mutant clones were then identified by the absence of GFP (negatively marked clones). This technique is applicable to both somatic and germ line cells.

Alternatively, the MARCM (Mosaic Analysis with a Repressible Cell Marker) system was used (Lee and Luo, 1999). Flies of the following phenotypes were generated:

For *shg* clones: hsFLP,UASCD8:GFP ; FRT42D*shg*$^{R69}$/FRT42DtubGal80,hsFLP ; tubGal4/+
For *arm* clones: *arm[4]*, FRT19A/tubGal80,hsFLP,FRT19A ; UASCD8:GFP/+ ; tubGal4/+.
In the absence of mitotic recombination, Gal80 inhibits Gal4; after recombination, *arm* or *shg* mutant cells lack Gal80, Gal4 is active and drives the expression of UAS-GFP. Mutant clones are thus identified by the presence of GFP. This method can be used in somatic cells but not in the germ line.

Rescue experiments with tubulin-DE-cadherin (§2.1 and 2.2.1) and armadillo-β-catenin (§2.2.2) transgenes were done with negatively marked clones. Flies of the following genotypes were generated: hsFLP ; FRT42*shg*/FRT42ubiGFP ; tubulin-DE-cadherin/+
*arm*FRT19A/ubiGFP,FRT19A ; hsFLP ; armadillo-β-catenin/+.

Rescue experiments with UAS-DE-cadherin transgenes (§2.3) were done with the MARCM system: expression of UAS-DE-cadherin rescue transgene was driven by tubGal4 in mutant clones. Flies of the following genotypes were generated:
hsFLP,UASCD8:GFP ; FRT42D*shg*$^{R69}$/FRT42DtubGal80,hsFLP ; tubGal4/UAS-DEcadherin
*arm[4]*, FRT19A/tubGal80,hsFLP,FRT19A ; UASCD8:GFP/+ ; tubGal4/UAS-β-catenin.

### 4.3.3 Rescue of embryonic phenotypes, p120ctn RNAi

For cuticle analysis and quantification of hatching, *shg*$^{1}$/*shg*$^{1}$; tubulin-cadherin/+ embryos (18-24h) were obtained by sorting non GFP embryos from *shg*$^{1}$/CyO,krGFP; tubulin-cadherin stocks (COPAS SELECT embryo sorter, Union Biometrica).

Rescue of lethality due to the lack of zygotic endogenous DE-cadherin was tested using *shg*$^{R69}$/CyO *;* tubulin-cadherin stocks. To test zygotic and maternal rescue, *shg*$^{R69}$/*shg*$^{R69}$; tub-cadherin females were crossed to *shg*$^{R69}$/CyO ; tubulin-cadherin males. In both cases, all the flies in the progeny were carrying two copies of the tubulin-cadherin transgenes and rescue was measured by quantifying the percentage of Cyo and non Cyo flies in the progeny.

For embryo zygotic p120ctn RNAi, tubGal4/TM3Ser females were crossed to UAS-p120ctn-RNAi/UAS-p120ctn-RNAi males. For maternal and zygotic p120ctn RNAi, UAS-p120ctn-RNAi/+; nosGal4 females were crossed to tubGal4/TM3Ser.

To test if there were some redundancy between p120ctn and DE-cadherin tyrosine phosphorylation, *shg* MARCM clones were generated in flies carrying tubulin-DE-cadherin-4YF and UAS-p120ctnRNAi transgenes. Flies of the following genotype were generated: hsFLP,UASCD8:GFP/tubulin-DE-cadherin-4YF ; FRT42D$shg^{R69}$/FRT42DtubGal80,hsFLP ; tubGal4/UAS-p120ctnRNAi

### 4.3.4 Overexpression of UAS-transgenes in border cells and follicular cells

Two Gal4 drivers were used to overexpress various UAS-DE-cadherin transgenes in border cells. Slbo-Gal4 contains enhancer regulatory sequences of the *slbo* gene cloned upstream of the Gal4 open reading frame. It starts being expressed in border cells at the initiation of migration, at the beginning of stage 9 of oogenesis, and is expressed until the end of the migration. At stage 10, it also starts being expressed in centripetal follicular cells.

Slbo-Gal4 is a good driver to obtain specific overexpression in border cells but has the drawback to start driving expression of UAS-transgenes only as border cells start migration. This is too late to detect defects due to overexpression of some transgenes, probably because border cells have time to migrate quite a bit before enough overexpressed protein accumulates and starts inhibiting the migration. To circumvent this problem, I also drove expression of UAS-transgenes using the “actin-flip-out-Gal4” system. In this system, Gal4 is under the control of the actin promoter, which gives ubiquitous expression. A cassette delimited by two FRT sites and located between the actin promoter and Gal4 prevents Gal4 transcription. Heat-shock induced expression of a FLP recombinase results in “flipping-out” of this FRT cassette. The actin promoter can then drive expression of Gal4 in all clones in which the FRT cassette has been flipped out. Presence of a UAS-GFP transgene allows identification of the clones expressing Gal4 and the UAS transgene of interest. Heat-shocks were done in larvae or in adult flies, at least 4 days prior dissection. Thus the UAS-transgenes were expressed in border cells much before they start migrating. This system also allows overexpression in follicular cells.

### 4.3.5 Genetic interactions with components of the endocytic pathway

Experiments in *rab5, sprint* or *shibire (shi)* heterozygous background were done with the following alleles:

For rab5, I used $rab5^{2}$ which was generated by imprecise excision of a P-element that creates a 4kb deletion in the 5' region (UTR and first exon) of the rab5 gene. It is likely to be a null allele (only maternal protein is detected in the embryo) (Wucherpfennig et al., 2003). For the rescue experiment of *shg* MARCM clones, the $rab5^{2}$ mutation (on chromosome 2L) was recombined on a FRT42D$shg^{R69}$ chromosome.

For shi, a thermosensitive allele ($shi^{ts1}$) was used. This allele is homozygous viable at 25ºC and lethal at 29ºC. Genetic interactions in the presence of overexpressed DE-cadherin (Fig.2.28A) were tested either at 25ºC (results on graph Fig.2.28A), or at 29ºC (some small delays were observed in that case, but similar defects also occurred in wild type background). Genetic interactions in *shg/shg* ; tubulin-DE-cadherin flies (Fig.2.28C) were tested either at 25ºC (no or very little delays were observed) or at 29ºC (results on graph Fig.2.28C). The rescue experiment of *shg* MARCM clones was done at 25ºC. At 29ºC, repression of Gal4 by Gal80 is not very efficient which gives expression of UAS-GFP also in non *shg* mutant cells and makes it impossible to clearly identify the real mutant clones.

For sprint, I used a molecular null allele which was obtained by imprecise excision of a P-element (Hsin-Ho Sung, unpublished data).

## 4.4 Stainings – phenotype analysis

### 4.4.1 X-Gal stainings

For some quantifications of border cell migration, I made used of a hypomorphic allele of *slbo*: $slbo^{1310}$. This allele is an enhancer trap insertion that induces the expression of β-galactosidase under the control of *slbo* regulatory regions: β-galactosidase is expressed in border cells from stage 9 on and in centripetal cells from stage 10 on. This allows simple scoring of migration defects by X-Gal staining.

To do the stainings, ovaries were dissected in PBS and fixed with 0.5% glutaraldehyde (in PBS) for 10 min. After a brief wash with PT (PBS + 0.1% TritonX100), they were incubated for 2h at 37ºC in staining buffer (10mM $NaH_2Po_4/Na_2HPO_4$ (pH7.2), 150mM NaCl, 1mM $MgCl_2$, 3.1mM $K_4(Fe^{II}(CN)_6)$, 3.1mM $K_3(Fe^{III}(CN)_6)$, 0.3% Triton X-100) containing 0.4% X-gal. The ovaries were then washed with PT and mounted in 50% glycerol (in PBS).

### 4.4.2 Phalloidin/DAPI stainings

Ovaries were dissected in PBS and fixed with 4% paraformaldehyde (in PBS) for 20 minutes. After several washes in PT, the ovaries were pipetted several times through a yellow 200µL micropipette tip to separate egg chambers. Ovaries were incubated with Rhodamin-Phalloidin (Molecular Probes, 1:500), DAPI (1µg/mL) for 2h at room temperature. After several washes in PT, they were mounted in 80% glycerol in PBS, containing 0.4% n-propyl gallate (NPG). All images were captured using confocal microscopy (Leica).

### 4.4.3 Immunostainings

For regular immunostainings, initial steps of the protocol are as for phalloidin stainings. After washes in PT, samples are blocked with 5% normal goat serum (NGS) in PT for 30 min. Then, primary antibodies were added and incubated over night at 4ºC. Primary antibodies used were rat DCAD2 (1:100), mouse anti-HA (1:1000), mouse anti-armadillo N2-7A1 (1:10, Peifer, 1993). The ovaries were then washed 2 hours in PT+ 0.2% BSA, blocked with 5% NGS in PT for 30 min, and incubated with Rhodamin-Phalloidin (1:500), DAPI (1µg/mL) and fluorescent secondary antibodies (Jackson ImmunoResearch, 1:200) for 2h at room temperature. After several washes in PT, they were mounted in 80% glycerol (in PBS), containing 0.4% NPG. All images were captured using confocal microscopy (Leica).

To specifically stain membrane DE-cadherin, the following protocol was used (based on protocol described in (Strigini and Cohen, 2000)): ovaries were dissected in ice-cold Grace's medium. They were incubated 1h in primary antibody (DCAD2, 1:20 in PBS) at 4ºC. After several washes in PBS at 4ºC, they were fixed with 4% paraformaldehyde in PBS for 20

minutes. They were then washed in PT and the regular protocol for incubation with secondary antibodies was used.

### 4.4.4 Cuticle preparations

Cuticle preparations were done without removing the vitelline membrane to avoid loosing cuticles from *shg/shg* embryos. Embryos were dechorionated with bleach (2% in water), washed in PT and transferred onto a slide in a mix of Hoyer/acid lactic (1/1). These preparations were then backed overnight at 70ºC.

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# PUBLICATION

The work showing that the interaction between DE-cadherin and p120ctn is not essential for DE-cadherin function has also been described in the following publication:

A. Pacquelet, Lin L and Rørth P. 2003. Binding site for p120/δ-catenin is not required for *Drosophila* E-cadherin function in vivo, *The Journal of Cell Biology* 160: 313-319.

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