Régulation de la polarité épithéliale par EFA6, facteur d'échange d'Arf6, et le système ubiquitine-protéasome

Frédéric Luton

Habilitation à Diriger des Recherches (HDR)

Université de Nice Sophia-Antipolis

Frédéric LUTON

Regulation of the development of the epithelial cell polarity by EFA6, Exchange Factor for Arf6, and the ubiquitin-proteasome system

Soutenue le 28 novembre 2007 devant le jury composé de :

Dr. André Le Bivic	Président et Rapporteur
Dr. Catherine Dargemont	Rapporteur
Dr. Claude Boyer	Rapporteur
Dr. Ellen van Obberghen	Examinateur
Pr. Keith Mostov	Examinateur

Institut de Pharmacologie Moléculaire et Cellulaire- CNRS UMR 6097
660, route des Lucioles, 06560 Valbonne Sophia-Antopolis

Table of contents

Curriculum Vitae

Luton Frédéric

Date of Birth : July 21, 1967.

Title : Chargé de Recherche 1ère classe (INSERM)

Function : Co-leader of the group : « Arf proteins, cell morphology and membrane transport » (2007) at the Institut de Pharmacologie Moléculaire et Cellulaire (IPMC), CNRS UMR 6097 – Université de Nice Sophia-Antipolis.

Address : Institut de Pharmacologie Moléculaire et Cellulaire
Université de Nice Sophia Antipolis-CNRS UMR 6097
660, route des Lucioles,
06560 Valbonne Sophia-Antipolis

Telephone : 04 93 95 77 70

e-mail : luton@ipmc.cnrs.fr

WEB site: http://www.ipmc.cnrs.fr.

Education and employment:

Chargé de Recherche 1ère classe INSERM (2004) at the IPMC.

Chargé de Recherche 2ème classe INSERM (enrolled in 2000) at the IPMC in the laboratory of Dr. Marc Chabre.

Post-doc fellow (1994-2000) at the University of California, San Francisco in the laboratory of Prof. Keith Mostov.

Graduate Student (1991-1994) at the Centre d'Immunologie de Marseille-Luminy in the laboratory of Dr. Anne-Marie Schmitt-Verhulst, under the supervision of Dr. Claude Boyer.

Undergraduate Student (1990-1991) at the Centre d'Immunologie de Marseille-Luminy in the laboratory of Dr. Anne-Marie Schmitt-Verhulst, under the supervision of Dr. Claude Boyer.

Invited talks and committees

Réunion pour le 25éme anniversaire de la Société Française d'Immunologie . Avril 1991 à Paris. Présentation orale intitulée "Implication of protein kinase activity for TCR/CD3 complex internalization".

Réunion de printemps de la Société Française d'Immunologie. Avril 1992 à Lyon. Présentation orale intitulée "Role of CD3-δ in surface expression of the T cell antigen receptor/CD3 complex and in activation for killing analyzed with a CD3-d negative cytolytic lymphocyte variant".

Réunion INSERM intitulée "Rôle des tyrosine protéines kinases dans la transduction de signal". Octobre 1993 à Paris. Présentation orale intitulée: "Kinases impliquées dans l'activation et l'endocytose du récepteur pour l'antigène des lymphocytes T".

Thirty-eigth annual meeting de l'"American Society for Cell Biology" (ASCB). Décembre 1998 à San Francisco. Présentation orale dans le Mini-Symposium "Membrane sorting and Polarity" intitulée:"Role of $p62^{yes}$ in Controlling Dimeric IgA Transcytosis into Mucosal Secretions"

ELSO meeting 2002. Juin 2002 à Nice. Présentation orale dans le Mini-Symposium "G proteins and establishment of cell polarity" intitulée: "Role of the Arf6 exchange factor EFA6 in tight junction biogenesis".

Séminaire à l'Institut Paoli-Calmettes (CNRS-U119), Marseille. Février 2004. "EFA6, facteur d'échange d'Arf6, régule le développement de la polarité des cellules épithéliales".

Examinateur de la thèse défendue par M^{elle} Fanny Jaulin, laboratoire du Dr. Jean-Paul Borg, à l'Institut Paoli-Calmettes (CNRS-U119), Marseille. Mai 2004. Etude de l'implication d'Erbin, une protéine de la famille LAP, dans l'établissement de la polarité épithéliale.

Séminaire à Mc Gill University, QC, Canada. Août 2004. "EFA6, exchange factor for Arf6, regulates the development of epithelial cell polarity".

Directeur de la thèse défendue par M^{elle} Stéphanie Klein à l'Institut de Pharmacologie Moléculaire et Cellulaire. Octobre 2005. "Rôle de la petite protéine G Arf6 et de son facteur d'échange dans la mise en place et le maintien des jonctions serrées".

Examinateur de la thèse défendue par M^{r} Arnaud Depaux, laboratoire du Dr. Nadine Varin-Blank, à l'Institut Cochin, Paris. Septembre 2006. "Régulation des complexes d'ubiquitinylation et de sumoylation par la ligase E3 hSIAH2".

Président de jury d'un concours externe pour le recrutement de trois techniciens pour le CNRS. Septembre 2006.

Supervisory positions

Technicians :	Min Zhang (1996-1997)
	Leonard Katz (1998)
	Mariagrazia Partisani TCS CNRS (2007-)
Students :	Fanny Poulain, 2002 (BTS 2ème année)
	Olympe Barre, 2006 (BTS 1ère année)
Undergraduates :	Marc Zanzouri, 2001 (DEA, stage II)
	Stéphanie Klein, 2002-2003 (DEA)
	Florian Labarrade, 2007 (Master II)
Graduate :	Stéphanie Klein (2003-2005)
Post-doc :	Delphine Théard (2006-)

Collaborations

Dr. Daniel Birnbaum
Laboratoire d'Oncologie Moléculaire,
Institut de Recherche sur le Cancer de Marseille,
UMR.599 Inserm-Institut Paoli-Calmettes, IFR 137,
27 Blvd. Leï Roure,
13009 Marseille, France

Dr. Sylvain Bourgoin
Centre de Recherche du CHUQ, pavillon CHUL
2705,Boul. Laurier,
Ste-Foy, QC
Canada, G1V 4G2.

Dr. Edward Fon
Montreal Neurological Institute
3801 University Street
Montreal, Quebec
Canada, H3A 2B4

Dr. André Le Bivic
Groupe Morphogenese et Compartimentation Membranaire
IBDM Faculte des Sciences de Luminy, UMR 6156
13288 Marseille cedex 09

Pr. Keith Mostov
Department of Anatomy; Program in Cell Biology; and Department of Biochemistry and Biophysics
University of California School of Medicine
600 16th Street
San Francisco, CA 94158-2517

Dr. Nadine Varin-Blank
Département d'Hématologie
Institut Cochin
27 rue du Fg St Jacques
75014 Paris

Dr. Stephen Wood
Child Health Research Institute
72 King William Road, North Adelaide, 5006
Australia

Fellowships and Grants

- Three year thesis fellowship MRT (sept. 1991-sept. 1993)

- Fourth year thesis fellowship Ligue Nationale Contre le Cancer (sept.1993-sept.1994)

- Thesis – post-doc transition fellowship Fondation Recherche Médicale (sept-déc 1994)

- First year post-doc fellowship Association pour la Recherche sur le Cancer (1995)

- Grant ACI-BCMS with the Dr. A. Le Bivic (2004-2006)

- International Opportunity Program- Planning and Development Grant (2004) from the Canadian Institutes of Health Research (CIHR) with the Dr. E. Fon (Montreal Neurological Institute, McGill University, Montréal).

- Post-doctoral fellowship from the CNRS for Delphine Théard (2007-2008)

- Grant Cancéropôle PACA (2007-2008)

Scientific research activities

Preamble

I started my scientific career at the Centre d'Immunologie de Marseille-Luminy in the laboratory of Dr. Schmitt-Verhulst entitled « Molecular bases for T lymphocyte function ». As an undergraduate and then graduate student, under the supervision of Dr. C. Boyer, I studied the assembly, trafficking and stimulation of the antigen T-cell receptor (TCR) in a murine cytotoxic T lymphocyte model (CTL). My initial research focused on the roles played by the CD3γ and CD3δ subunits of the CD3 signaling complex associated to the TCR. After which, I studied the relationship between the TCR endocytosis and its ligand-stimulated signaling pathways.

After my Ph. D., I wanted to further explore the field of cell biology to better understand the regulatory mechanisms that control the intracellular transport pathways. I joined the group of Prof. K. Mostov (University of California, San Francisco) studying the intracellular trafficking of the basal-to-apical transcytosis pathway taken by the polymeric immunoglobulin receptor (pIgR) in polarized epithelial cells. In contrast to the TCR for which the signaling pathways were well described but the intracellular transport poorly investigated, the pIgR trafficking cascades had been studied in large details but very little was known about its signaling properties. Nonetheless, the laboratory of Prof. K. Mostov had recently shown that ligand binding to the pIgR stimulated its transcytosis and triggered the production of second messengers (IP3, calcium). My project described a signaling pathway associated to the pIgR and led to a better understanding of how it controlled its transcytosis.

Returning to France at the Institut de Pharmacologie Moléculaire et Cellulaire (IPMC) in the laboratory of Dr. Chabre under the supervision of Dr. Chardin, I made a new change in my research interests and began to study the development of epithelial cell polarity. After showing that the exchange factor for the small G protein Arf6, EFA6, was implicated in regulating the development of epithelial cell polarity by acting on the rearrangement of the actin cytoskeleton, I started two main lines of research : 1) analysis of the

molecular mechanisms by which EFA6 affects the actin cytoskeleton organization, 2) regulation of EFA6 activity: we have found that the quantity of EFA6 is tightly regulated during epithelial cell polarization by the ubiquitin-proteasome system (UPS). We are currently studying the molecular and cellular mechanisms that regulate EFA6 ubiquitinylation and de-ubiquitinylation in a coordinate manner with the development of the cell polarity.

In the first part of the manuscript I have summarized the research accomplished during my Ph.D. and my post-doctoral fellowship, followed by a common discussion about signaling compartmentalization. In the second part of the manuscript, I have briefly presented the published work and then I go on to further develope the current projects, for which not all the data is shown. In the final discussion, I tried to put our results in perspective with the general understanding of cell polarity in order to present our working models and future research directions.

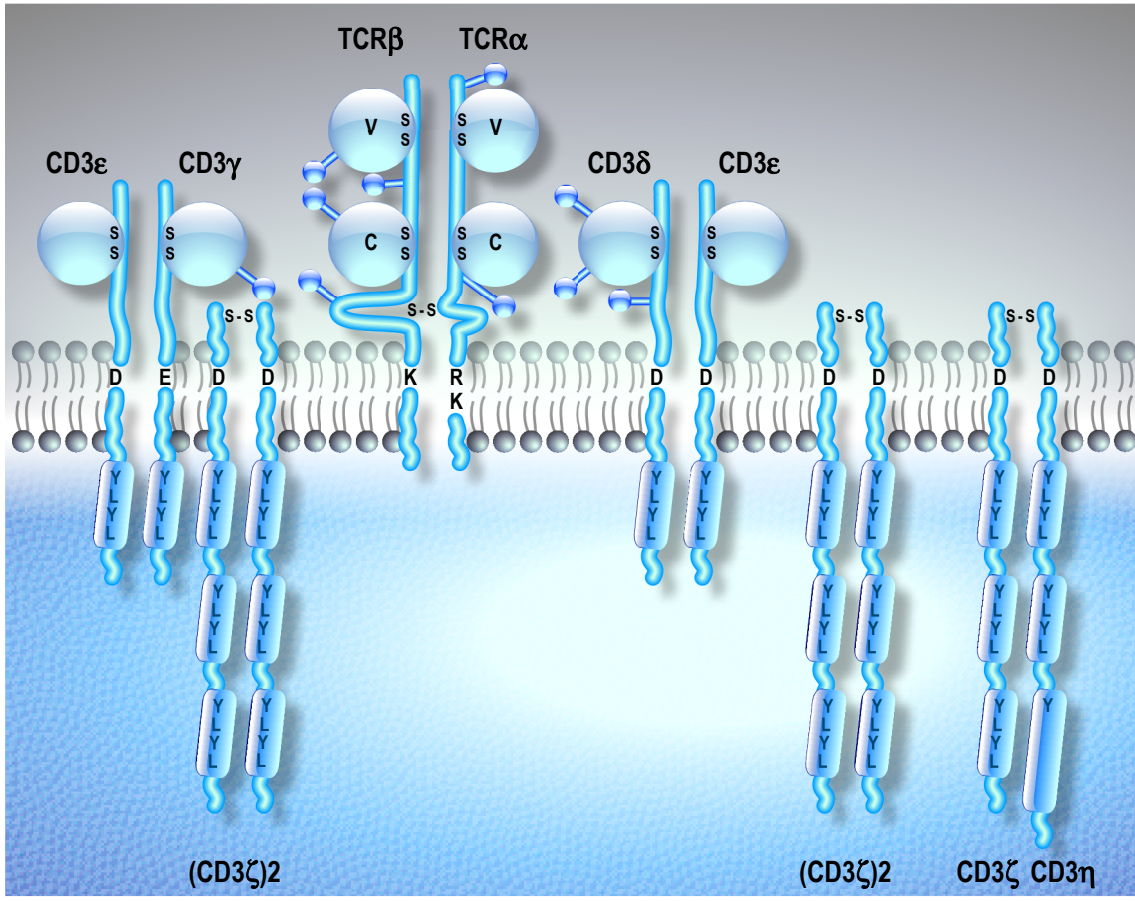

Figure 1: Structure of the TCR/CD3 complex. The subunits of the CD3 complex contain activation modules providing potential linkage to multiple pathways such as effector functions, developmental signaling pathways and down-modulation of cell surface TCR.

Study of the regulation of the cell surface expression of the TCR/CD3 complex: role of the protein kinases and functional implications

1) Introduction

I carried out my doctoral research at the Centre d'Immunologie de Marseille-Luminy in the laboratory of Dr. Schmitt-Verhulst with Dr. Boyer as my Ph.D. supervisor. When I joined the laboratory, they were studying the response to the antigen of two murine cytotoxic T lymphocytes clones and their corresponding TCR transgenic mice. The intensity of the TCR stimulation governs the response and fate of the immature and mature T lymphocytes. This includes anergy and apoptosis that contribute respectively to either the immune tolerance or activation of the effector functions, such as the production of lymphokines and the cytolytic activity. The outcome of the encounter of the T lymphocyte with its specific ligand depends, among other factors, on the number of stimulated TCRs and the duration of the stimulation (Valitutti and Lanzavecchia, 1997). Thus, the regulation of the level of expression of the TCR, before and in response to the antigen stimulation, is a determining element of the immune response.

The TCR is composed of two polypeptides, TCRα and TCRβ, accounting for the specific recognition of the antigen presented by the major histocompatibility complex. These molecules have a very short cytoplasmic domain. However, the TCR is associated to the signaling CD3 complex that comprise several transmembrane proteins with short extracellular extremities but long cytoplasmic tails (Fig.1). This complex is formed of dimers: CD3δε, CD3γε and CD3ζζ (or heterodimers between ζ and the molecules of the same family η or θ) (Malissen and Schmitt-Verhulst, 1993). A central question was the role played by each of these molecules for the cell surface expression and signal transduction properties of the TCR/CD3 complex.

2) Role of CD3δ and CD3γ for cell surface expression and function of the TCR/CD3 complex

We selected two CTL variants that had spontaneously lost the cell surface expression of their TCR. One clone had lost the expression of the CD3δ chain and the other one the expression of both CD3δ and CD3γ. When the full-length or tail-less proteins were re-expressed the CTL variants recovered the cell surface expression of their TCR together with their cytotoxic activity, production of interferon-γ and proliferation in response to the antigen. These experiments demonstrated that the cytoplasmic tails of the CD3δ and CD3γ proteins were dispensable for the antigen-stimulated TCR signal transduction. However, their transmembrane and/or extracellular domains are required for the assembly and cell surface expression of the TCR/CD3 complex (Luton et al., 1997a).

3) Role of CD3δ and CD3γ for cell surface endocytosis of the TCR/CD3 complex

The levels of cell surface expression are the results of several intracellular events: the protein synthesis of each subunit composing the TCR/CD3 complex, their appropriate assembly in the endoplasmic reticulum, the transport of the pre-assembled complex to the cell surface, and then its cell surface endocytosis followed by its recycling and/or degradation. The analysis of the clones described above allowed us to show that the cytoplasmic tails of the CD3δ and CD3γ are necessary for the internalization of the TCR/CD3 complex stimulated by an anti-CD3 antibody or induced by direct activation of a PKC with a phorbol ester (Luton et al., 1997a). This observation was in agreement with a study showing that a di-leucine motif within the cytoplasmic tail of the CD3γ chain was necessary to target the TCR/CD3 complex towards the degradative pathway (Letourneur and Klausner, 1992). Later, the laboratory showed that the CTL did not respond equally when stimulated by antibodies directed against the TCR/CD3 complex and by the specific antigen presented by the MHC (Legendre et al., 1999). At this point, more work was needed to describe the endocytotic pathway and the role played by each molecule of the TCR/CD3 complex.

4) TCR/CD3 complex and their associated protein kinase activities along the endocytotic pathway

Our initial experiments brought to light that the endocytosis of the TCR was a complex multi-step process for which we did not have the appropriate assays to analyze the discrete stages. Thus, a better knowledge of the endocytotic pathway and the identification of regulatory partners associated to the TCR/CD3 complex were needed to define the pathway at the molecular level. By analogy with the EGF and Insulin receptor tyrosine kinases, I decided to explore the relationship between the PTK-dependent signaling pathways associated to the TCR/CD3 complex and its endocytosis. First, by electron and confocal microscopy and using subcellular fractionation, we showed that internalization of the TCR/CD3 complex required as a first step its redistribution at the cell surface, followed by endocytosis through the early and late endosome, and finally degradation in the lysosomes (Boyer et al., 1991; Luton et al., 1994a; Luton et al., 1997b). Second, by measuring the kinase activities associated to the TCR/CD3 complex within the different endosomal fractions, we showed that the cell surface redistribution that precedes endocytosis is dependent on a tyrosine kinase activity but that initiation of endocytosis was kinase independent (Luton et al., 1994a). These results are similar to those obtained with the EGF and Insulin receptors. In addition, in the early and late endosomal compartments the TCR/CD3 complex is associated to kinase activities with distinct specificities for the target amino-acid (Luton et al., 1997b). These results show that the TCR/CD3 complex is still capable of signaling on its way to degradation. One proposed two non exclusive explanations are: 1) the TCR signaling pathways are compartmentalized, 2) the different kinase activities associated to the TCR/CD3 complex insure its successive targeting towards degradation rather than recycling to the cell surface.

5) TCR Endocytosis and thymic selection

Finally, in a model of TCR transgenic mice designed to study positive selection, we measured the kinetics of endocytosis of the TCR expressed by immature and mature T lymphocytes. In addition, we examined their sensitivity to different kinase inhibitors. These preliminary experiments revealed that TCR

expressed by immature thymocytes and mature CTL have very different endocytotic properties (Luton et al., 1994b). It would be interesting to study whether these properties fulfill selective purposes, that is contribute to the selection of the immature thymocytes and the functional response of the mature CTL.

6) Publications

Boyer C, Auphan N., Luton F., Malburet J.-M., Barad M., Bizozzero J.-P., Reggio H, & Schmitt-Verhulst A.-M. (1991). T cell receptor/CD3 complex internalization following activation of a cytolytic T cell clone: evidence for a protein kinase C-independent staurosporine-sensitive step. *Eur J Immunol.21 :1623*

Wegener A.-M., Letourneur F., Hoeveler A., Brocker T., Luton F., & Malissen B.(1992) The T cell receptor / CD3 complex is composed of at least two autonomous transduction modules. *Cell. 68 :83*

Buferne, M., F. Luton, F. Letourneur, A. Hoeveler, D. Couez, M. Barad, B. Malissen, A.-M. Schmitt-Verhulst & C. Boyer. (1992). Role of CD3δ in surface expression of the T cell antigen receptor/CD3 complex and in activation for killing analyzed with a CD3δ negative cytolytic T lymphocyte variant. *J. Immunol. 148: 657*

Luton, F., M. Buferne, J. Davoust, A-M. Schmitt-Verhulst & C. Boyer. (1994). Evidence for protein tyrosine kinase involvement in ligand-induced TCR/CD3 internalization and surface redistribution. *J. Immunol. 153:63*

Luton, F., V. Legendre, M. Buferne, A-M. Schmitt-Verhulst & C. Boyer. (1994). Developmental control of T cell receptor internalization. *Thymus 23: 15*

Luton, F., Legendre, V., Gorvel, J.-P., Schmitt-Verhulst, A.-M., & Boyer, C. (1997). Tyrosine and serine protein kinase activities associated with ligand-induced internalized TCR/CD3 complex. *J Immunol., 158:3140*

Luton, F., Buferne, M., Legendre, V., Chauvet, E., Boyer, C., & Schmitt-Verhulst, A.-M. (1997). Role of CD3γ and CD3δ cytoplasmic domains in cytolytic T lymphocyte functions and TCR/CD3 down modulation. *J. Immunol., 158:4162*

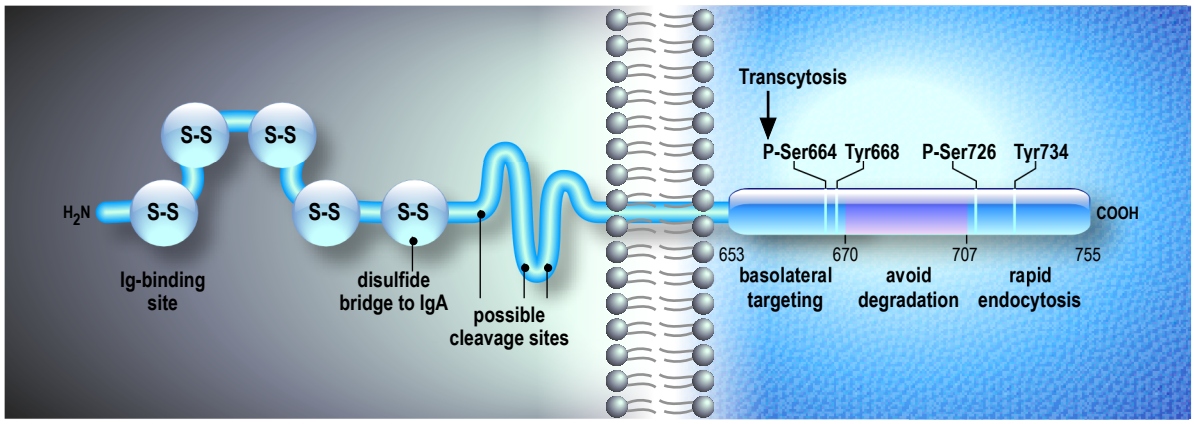

Figure 2: Structure of the the polymeric immunoglobulin receptor (pIgR). The pIgR is a single transmembrane protein that belongs to the immunoglobulin superfamily. Its cytoplasmic domain contains targeting signals that control its transport from the Golgi to the basolateral surface and its transcytosis from the basolateral to the apical surface where it is cleaved to release the extracellular domain called the secretory component or SC fragment.

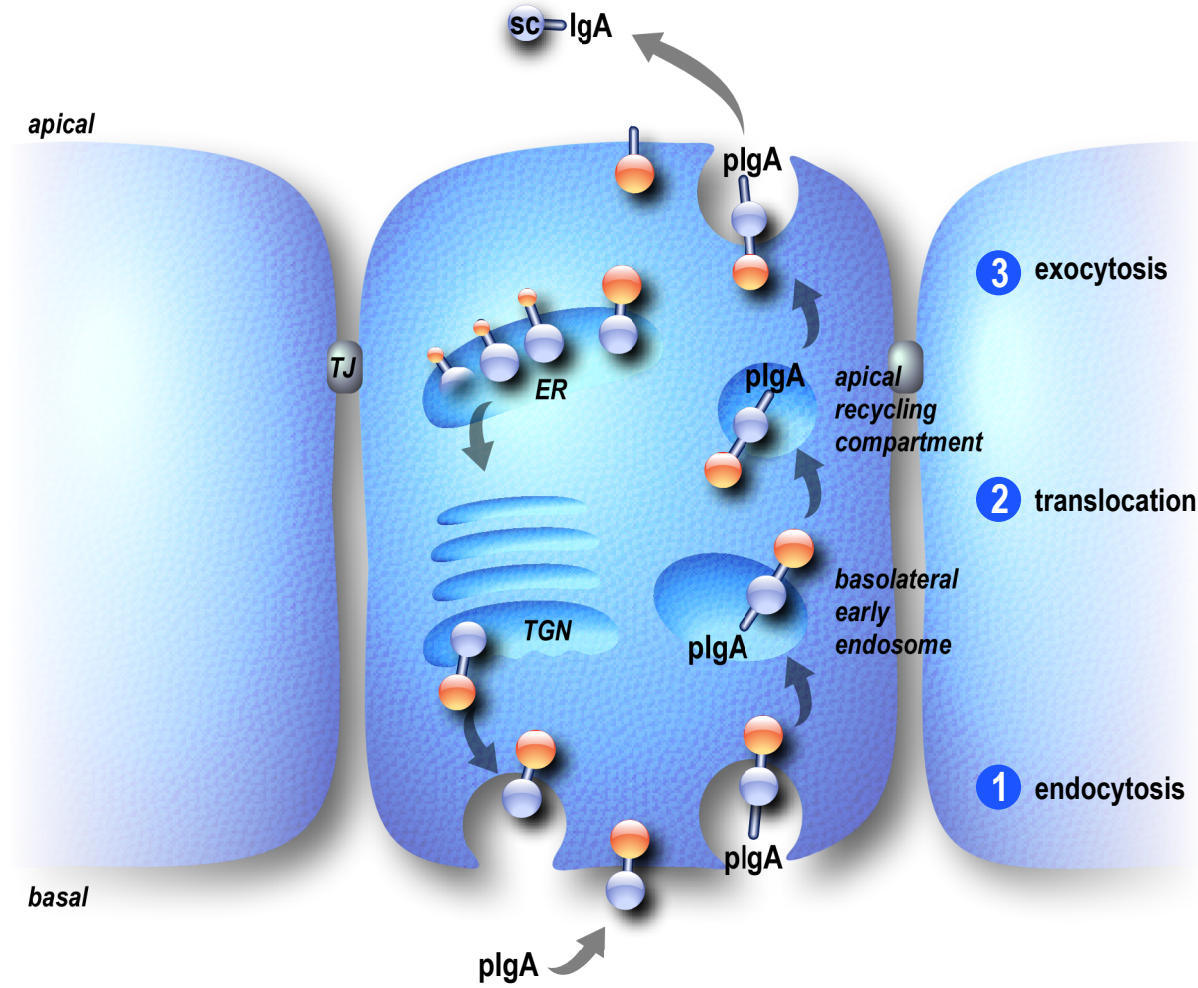

Figure 3: Trafficking pathways of the pIgR. In polarized cells, newly synthesized pIgR molecules are delivered to the basolateral membrane before engaging into the basal-to-apical transcytosis pathway. At the basal surface the pIgR can bind its ligand: polymeric-immunoglobulins pIgA or pIgM. In any case, the pIgR enters the transcytotic pathway constitutively even in the absence of ligand. Transcytosis can be broken down into three steps : 1) endocytosis into basolateral early endosome, 2) translocation into the apical recycling compartment and 3) transport to the apical membrane where the SC fragment is released by proteolytic cleavage of the extracellular domain.

Post-doctoral training (University fo California, San Francisco)

Signaling pathways regulating the ligand-stimulated transcytosis of the polymeric-immunoglobulin receptor in polarized epithelial cells

1) Introduction

After my Ph.D., I decided to undertake my post-doctoral research in a laboratory specialized in the study of the intracellular trafficking pathways. In this respect, the group of Prof. Keith Mostov was an appropriate choice, as they were a leading laboratory studying polarized trafficking using the polymeric immunoglobulin receptor (pIgR) in epithelial cells (Fig.2) (Mostov et al., 1995). In polarized cells, the pIgR goes through sequential transport pathways including direct delivery to the basolateral domain followed by transcytosis from the basal to the apical domain of the plasma membrane (Fig.3). Its function is to transport antibodies such as the polymeric IgA and IgM into the mucosal secretions. After synthesis and transport through the Golgi apparatus, the pIgR is directed to the basolateral surface where it is rapidly engaged into the transcytotic pathway that comprises three steps : 1) internalization into the basolateral endosomes, 2) translocation towards the apical recycling compartment (ARC), and 3) delivery to the apical membrane where the extracellular domain is cleaved off and released into the lumenal secretions as secretory component (SC). At the time I joined the group, the intracellular compartments traversed by the pIgR and its ligand were well described and most of the addressing signals within the cytoplasmic tail had been identified. PIgR transcytosis had long been considered as a constitutive pathway equally followed by the receptor empty or bound to its ligand. However, shortly before my arrival, the graduate student Michael Cardone had demonstrated that ligand binding could stimulate pIgR transcytosis by accelerating the third step between the ARC and the apical plasma membrane. Moreover, ligand binding was shown to lead to the membrane translocation of the PKCε, the production of inositol-3-

phosphate (IP3) and a calcium signal revealing the existence of an unsuspected signaling pathway associated to the pIgR (Cardone et al., 1996). My project consisted in identifying the signaling pathway, defining its mode of action and establishing its physiological relevance *in vivo*.

2) Characterization of a signaling pathway that accelerates the IgA-stimulated pIgR transcytosis

The experience acquired during my graduate research on tyrosine kinase associated signaling pathways enabled me to show that ligand binding to the pIgR stimulates a non-receptor tyrosine kinase from the src family, that controls the tyrosine phosphorylation and activation of the phospholipase C-γ1(PLC-γ1), itself responsible for the production of IP3. By combining pharmacological studies, enzymatic assays and genetic tools we demonstrated that the ligand-stimulated transcytosis of the pIgR was dependent on the production of IP3 and the elevation of the intracellular calcium (Luton et al., 1998).

3) Compartmentalization and specificity of the signaling pathway that stimulates pIgR transcytosis.

Numerous receptors upon ligand binding are capable of promoting the production of IP3 and a calcium signal. The question was to understand how these ordinary messengers affected selectively the pIgR. We have shown that the binding of the ligand at the basolateral surface had two consequences : 1) the activation of a signal of stimulation generated only at the basolateral membrane acting at a distance on the third step of transcytosis (ARC-to-apical plasma membrane), and 2) the sensitization of the receptor upon ligand-binding in order to respond to the signal of stimulation once the engaged receptor reaches the ARC (Luton and Mostov, 1999). This mechanism of compartmentalization explains how the cell controls the specificity within a complex environment that integrates multiple signaling pathways. For example, we have observed that the stimulation of the HGF (hepatocyte growth factor) that leads to the activation of the PLCγ1 and the production of IP3 does not stimulate pIgR transcytosis. Our model suggests that it is because the pIgR had not been sensitized by the HGF. Similarly, the activation of pIgR that generates signals to stimulate transcytosis

is not capable of stimulating the apical transport of the transferrin receptor. We propose that this is because the transferrin receptor was not sensitized by pIgR stimulation. The obligation to dispatch two separate signals acting at two different locations along the transport pathway is an efficient mechanism of regulation to maintain spatially the specificity of the signal of stimulation until the end point.

4) Identification of the protein tyrosine kinase associated to the pIgR and physiological role of the pIgR-stimulated transcytosis *in vivo*

The vast majority of pathogens enter the body through the mucosal surfaces, such as the linings of the gastrointestinal, respiratory and genito-urinary tracts. The primary immunological defense against such infections is polymeric IgA (pIgA), which is produced locally by plasma cells found in the lamina propria underlying the mucosal epithelium. PIgA is transcytosed by the pIgR to the apical membrane. At the apical surface, the ectoplasmic domain of the pIgR is cleaved off and released together with the pIgA into the mucosal secretions. Hepatocytes also transport pIgR and pIgA from the blood into the bile. During the immune response to an infection, the amount of pIgA that is transported through the epithelium to the site of infection is increased by several mechanisms. The most rapid response is due to the binding of pIgA to pIgR, which stimulates the rate of pIgR transcytosis, thereby increasing the amount of pIgA delivered locally at the site of infection. This was first demonstrated in MDCK cells transfected with pIgR (Song et al., 1994a; Song et al., 1994b). Later, it was shown that intravenous injection of pIgA into rats resulted in an increase in the amount of total SC released into the intestinal secretions (Giffroy et al., 1998).

In our quest for partners of the pIgR, we had observed that it was highly enriched in an endosomal compartment called RRC (receptor recycling compartment) of the transcytotic pathway of rat hepatocytes. In agreement with our pharmacological data obtained in MDCK cells, our enzymatic studies indicated that pIgR isolated from the RRC was associated to a PTK displaying enzymatic properties of the src family. By co-immunoprecipitation we showed that the pIgR was associated to $p62^{yes}$ but not $p60^{src}$ both of which are present in the RRC (Luton et al., 1999).

The existence of knock-out (KO) mice for $p62^{yes}$ and $p60^{src}$ provided us with the possibility to check the association of the pIgR with $p62^{yes}$ and to assess *in vivo* the physiological basis of pIgR-stimulated transcytosis. In $p62^{yes}$ KO mice, the kinase activity associated to the pIgR was undetectable, whereas in $p60^{src}$ KO mice the PTK activity was normal, thus certifying that the kinase was $p62^{yes}$ and that there was no redundancy with $p60^{src}$ or any other PTK. Then, we tested the stimulation of transcytosis *in vivo* by intravenous injection of a bolus of pIgA containing a small radioactive fraction as a tracer to measure the transcytosis into the bile. We observed in the $p62^{yes}$ KO mice a decrease of 30 to 50 times of the rate of pIgA transcytosis (Luton et al., 1999). This effect is specific because : 1) we did not detect any difference in the $p60^{src}$ KO mice compared to control mice, 2) we did not observe any difference regarding the transcytosis of the asyaloglycoprotein receptor in control, $p62^{yes}$ KO or $p60^{src}$ KO mice.

In conclusion, in this study we have identified the PTK associated to the pIgR responsible for its stimulated transcytosis, we have demonstrated the physiological importance of the pIgA-stimulated transcytosis and finally, we have assigned the first function discovered so far to $p62^{yes}$.

5) Identification of new pIgR partners

After the identification of $p62^{yes}$, I wished to look for its substrates involved in pIgR transcytosis. To this goal, I used a new strategy developed by Kevan Shokat for $p60^{src}$ and adapted it to $p62^{yes}$. The ATP binding site of the kinase is modified so that it accommodates an ATP analog but not the normal ATP. Thus, when mixing this protein and the radio active ATP analog *in vitro* with a lysate one can identify the specific substrates of the modified kinase regardless of the presence of other kinases or normal ATP (Shah et al., 1997). We incubated the modified $p62^{yes}$ and its cognate radio active ATP analog with RRC and have identified the EGFR as one of the major substrates. The subsequent work accomplished by Tao Su, a post-doc fellow in Prof. K. Mostov's laboratory, showed that in response to pIgA stimulation, $p62^{yes}$ phosphorylates and activates the EGFR leading to the activation of the MAPK. Repression of the expression of $p62^{yes}$ using shRNA and use of selective

inhibitors indicate that the pIgA-stimulated transcytosis of pIgR is dependent on the activation of the EGFR and of the MAPK pathway by $p62^{yes}$ (Su *et al.*, in revision).

Another approach had been undertaken to isolate partners of the pIgR from the RRC fraction. Analysis by mass spectrophotometry of co-immunoprecipitated proteins with the pIgR identified the sub-complex of the retromer Vps35-Vps29-Vps26. This study was continued by Marcel Vergés a post-doc fellow in Prof. K. Mostov's laboratory. The analysis of the depletion or over-expression of Vps35 combined with the use of a series of pIgR mutants showed that the retromer contributes positively to pIgR transcytosis. Nevertheless, it is not completely ruled out that the retromer could also act by rerouting some of the pIgR on its way to degradation back towards the transcytotic pathway (Verges et al., 2004).

6) Publications

Mostov K.E., Altschuler Y., Chapin S.J., Enrich C., Low S.H., Luton F., Richman-Eisenstat J., Singer K.L., Tang, K. & Weimbs T. (1994). Regulation of protein traffic in polarized epithelial cells: the polymeric immunoglobulin receptor model. *Cold Spring Harb. Symp. Quant. Biol. 60: 775*

Luton, F., Cardone, H.M., Zhang, M. & Mostov, K.E. (1997). Role of tyrosine phosphorylation in ligand-induced regulation of transcytosis of the polymeric Ig receptor. *Mol. Biol. Cell, 9:1787*

Malmborg A.-C., Shultz D.B., Luton F., Mostov K.E., Richly E., Leung P.S.C., Benson G.D., Ansari A.A., Coppel R.L., Gershwin M.E. & Van de Water J. (1998). Penetration and co-localization in MDCK cell mitochondria of IgA derived from patients with primary biliary cirrhosis. *J Autoimmun. 11 :573*

Luton, F. & Mostov, K.E. (1999) Transduction of basolateral-to-apical signals across epithelial cells: Ligand stimulated transcytosis of the polymeric Ig receptor requires two signals *Mol. Biol. Cell., 10 :1409*

Luton, F., Vergés, M., Vaerman, J.-P., Sudol, M. & Mostov, K.E. (1999) The SRC family protein tyrosine kinase $p62^{yes}$ controls polymeric IgA transcytosis *in vivo. Mol. Cell, 4 :627*

Vergés, M., Luton, F., Gruber, C., Tiemann, F., Reinders, L. G., Huang, L., Burlingame, A. L., Haft, C. R. & Mostov, K. E. (2004). The mammalian retromer regulates transcytosis of the polymeric immunoglobulin receptor. *Nat. Cell Biol, 6: 763*

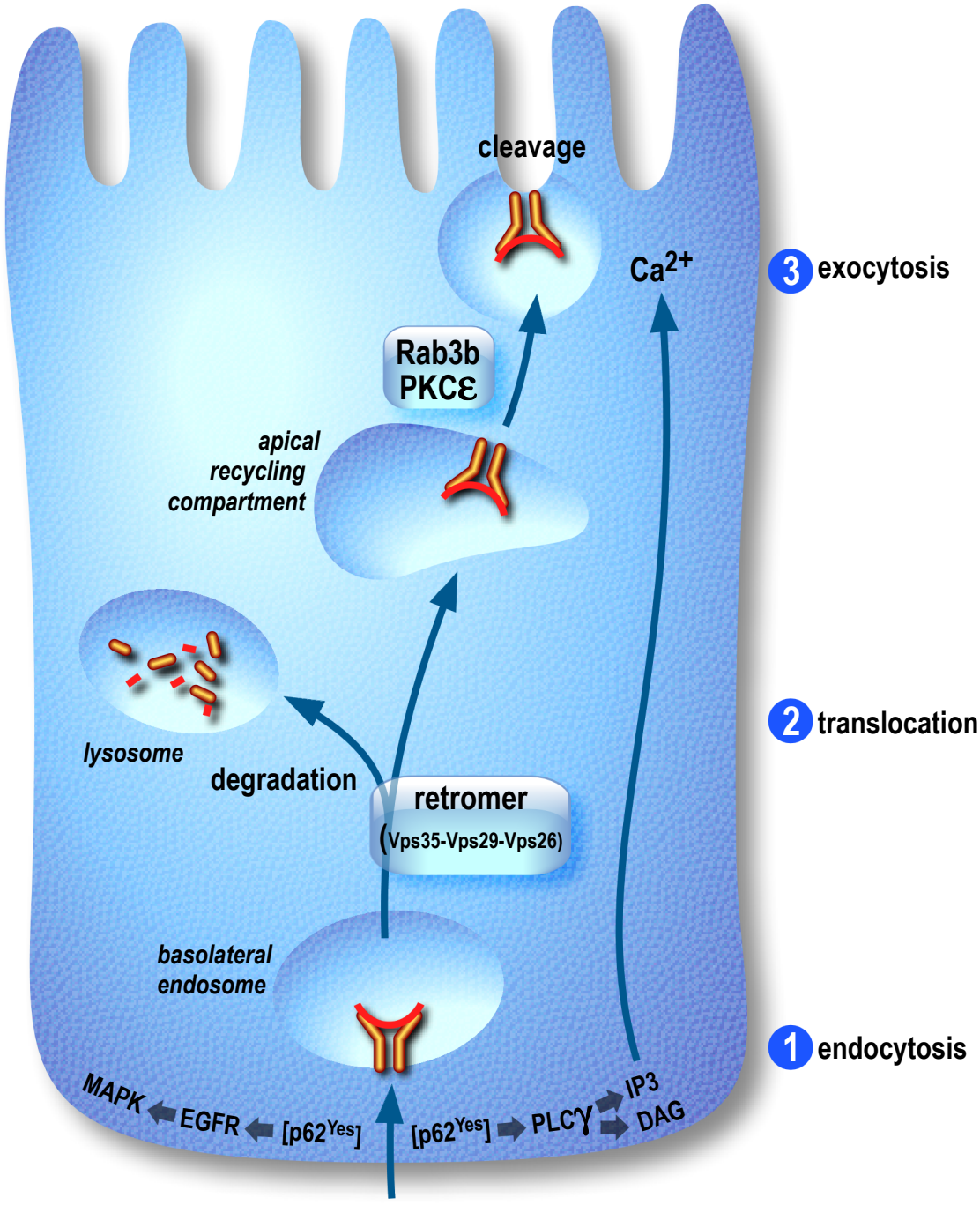

Figure 4: Ligand-stimulated signaling pathways regulating pIgR transcytosis. Upon ligand binding at the basal surface, the protein tyrosine kinase $p62^{yes}$ is recruited leading to tyrosine phosphorylation, the activation of the phospholipace Cγ and the production of the secondary messengers inositol tri-phosphate (IP3) and diacyl-glycerol (DAG). The IP3 leads to the release of calcium from intracellular stores controlled by IP3 receptors, while DAG activates the PKCε. A second pathway is initiated by $p62^{yes}$ through the tyrosine phosphorylation and activation of the EGF receptor and the downstream MAPK pathway. On its way to the apical surface the pIgR is actively directed by the retromer that may also help to prevent its degradation. Finally, the small G protein Rab3b was shown to promote the apical transport of ligand-bound pIgR from the apical recycling compartment. Although, all these molecules have been clearly implicated in ligand-stimulated pIgR transcytosis the molecular basis of their contribution and coordinate action has yet to be defined. Nevertheless, we have defined a signal of stimulation and a signal of sensitization that ensure proper stimulated transcytosis of pIgR-ligand complexes (see text for details).

7) Discussion

The two models, TCR and pIgR, display some analogies regarding the operating system used by the cell to achieve the respective functions performed by the two receptors. They both seem to resort to the compartmentalization of signal transduction: biological processes are sometimes so complex that they need to be hierarchized in successive steps separated spatially. The pIgR is a simpler model than the TCR in the way that it is associated to a signaling pathway that seems to solely control its transport. It has been possible to assign to each of pIgR transport steps a small domain within its cytoplasmic tail and for some of these domains we have identified the binding partner ($p62^{yes}$, Vps35, Rab3b) (Fig.4). Why such a segmentation ? First, they correspond to the successive steps followed by the receptor : thus the presence of the different domains and the organization of the hierarchy insures that the receptor goes first to the basolateral surface to bind the pIgA before it is directed to the apical surface. Second, it secures the physiological function that imposes to distinguish between empty and ligand-bound receptors: the signals of stimulation and sensitization allow for the cell to insure that the ligand is transported faster than an empty receptor useless for the antigen specific immune response. In the case of the TCR, our knowledge is more sketchy and this model involves several signaling pathway controlling the effector functions of the lymphocyte and the intracellular transport of the TCR (Fig.5). Similarly to the pIgR, the signaling pathways may control the transport of the TCR, but they also transduce, as the TCR goes through the endosomes, signals to stimulate in turn different effector functions. Thus, endocytosis down modulates the T lymphocyte response by decreasing the number of TCRs at the cell surface, but may also contribute positively by allowing the TCR to connect successively to different signaling pathways along its transport to the lysosome.

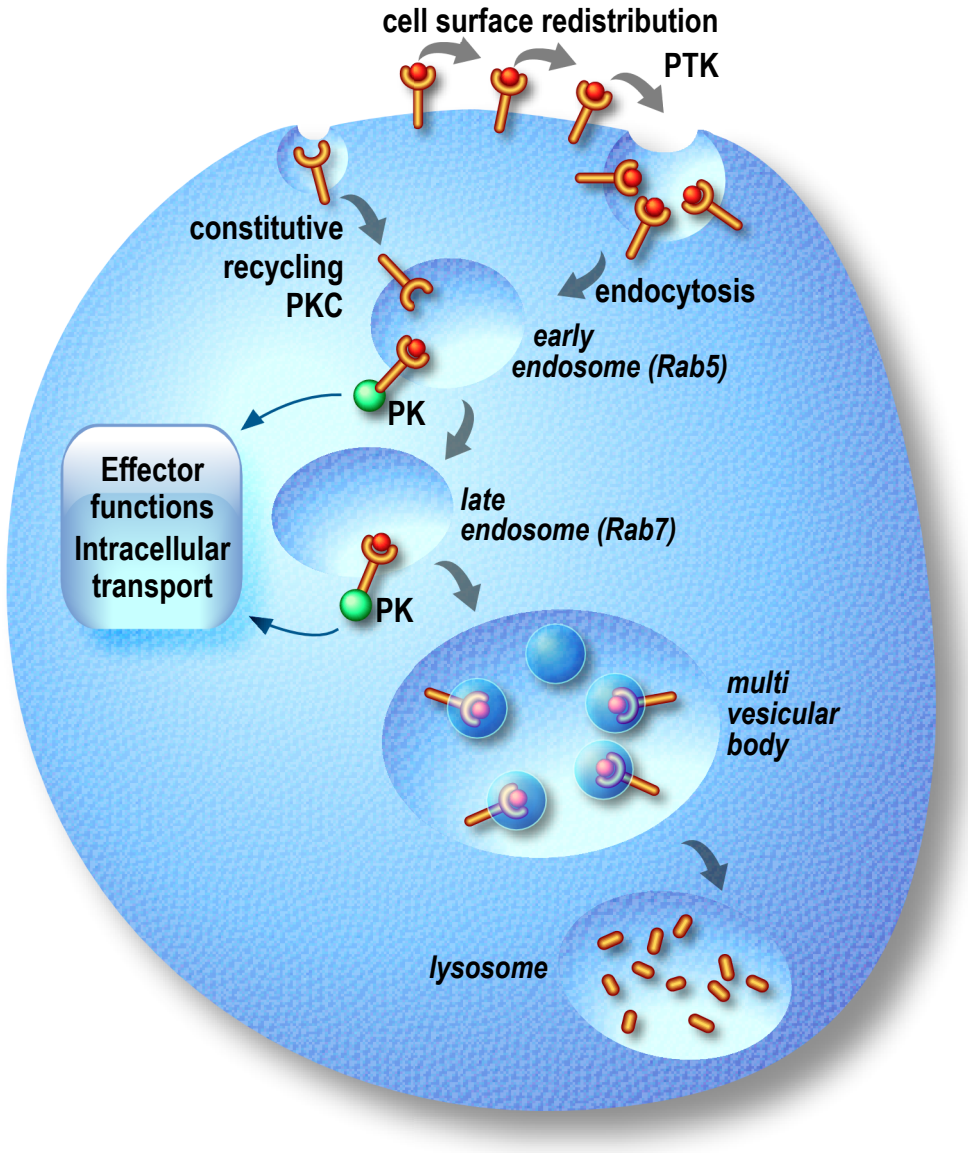

Figure 5: TCR down-modulation and associated signaling pathways. In the absence of ligand the TCR is constitutively recycled to the plasma membrane through the early endosome possibly in a PKC-dependent manner. Upon ligand binding, a protein tyrosine kinase activity allows for the cell surface redistribution of the stimulated TCR into the endocytotic pathway. Successively, in early and late endosomes the TCR associates to multiple tyrosine kinase activities proposed to control the TCR effector functions and its intracellular transport following a model of compartmentalization of the signaling pathways. Eventually, the TCR and its ligand reach the multivesicular body and the lysosomal compartment for degradation.

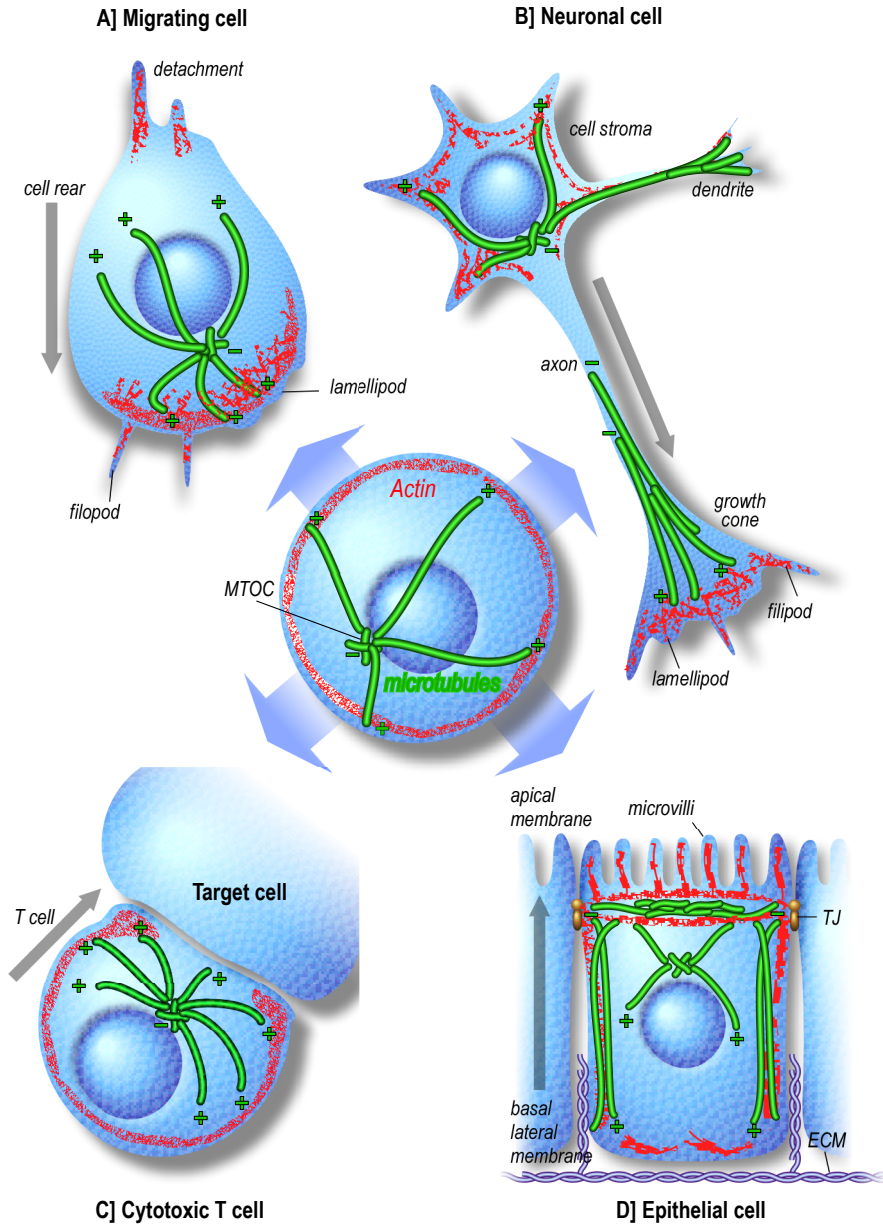

Figure 6: Schematic representation of a few examples of polarized cells. In the center is represented a non-polarized cell displaying a simple circular cortical actin cytoskeleton (red) and a non-uniformly organized microtubule network (green) associated to the microtubule organizing center (MTOC). A) In migrating cells, the microtubules are organized along the axis of migration. The actin cytoskeleton comprises a highly dynamic structure required for the membrane extension at the leading edge and a contractile structure for the retraction of the rear of the cell. B) In the axon of neuronal cells the non-centrosomal microtubules are uniformly oriented with their plus ends distal from the cell body, whereas in dendrites the centrosomal microtubules are non-uniformly oriented. The actin cytoskeleton in the terminal axon resembles the one of the leading edge of migrating cells with filopod and lamellipod structures. C) Upon cell-cell contact with its specific target, the cytotoxic T lymphocyte reorganize its cytoskeleton structures towards the contact zone. D) In polarized epithelial cells the minus ends of the microtubules are associated with the MTOC or the apical cell surface with the plus ends oriented towards the basal surface. There also exists in the apical pole a non-centrosomal meshwork of microtubules non-uniformly oriented. The actin cytoskeleton is made of several distinct elements : the stress fibers lying on the basal membrane that attach the cell to the extracellular matrix (ECM) through their terminal focal complexes, the long cortical bundles of actin filaments that run along the lateral membrane associated to the adherens junctions, the thick apical contractile ring of acto-myosin that supports the tight junction (TJ) and the specific cables of actin emerging from the apical ring of acto-myosin that structure the microvilli.

Chargé de Recherche (Institut de Pharmacologie Moléculaire et Cellulaire)

Role of EFA6, Exchange Factor for Arf6, in/for the development of epithelial cell polarity

1) Introduction

1-1) Cell polarity

Cell polarity, its establishment and regulation, are essential to the completion of numerous physiological functions and pathological events. This includes the early stages of embryo development, organogenesis, tissue repair, cancer emergence and progression, cell migration, antigen recognition, neuronal growth cone extension, pathogen invasion, etc. Cell polarity is observed in all kinds of living organisms, it can be transient or short-term (cell migration, antigen recognition, yeast budding) or permanent or long-term such as in the case of the neuronal or polarized epithelial cells that display stable distinct plasma membrane domains: cell stroma and axon or else basolateral and apical domains, respectively (Fig.6). Cell polarity consists as organized and discrete spatial domains in which the components required for the realization of one or several functions are brought together. The size of this domain varies from few microns diameter (rafts or immunological synapse) to very large surfaces such as the apical membrane of the enterocytes. The work presented in this manuscript focused on the establishment and maintenance of the apico-basal polarity of mammalian epithelial cells. Polarized epithelial cells form a monolayer that lines the internal cavity of organs and preserves the difference of composition between the serosal and lumenal compartments. They insure the adsorption, secretion and vectorial exchanges of fluids and small molecules by regulating the paracellular diffusion and the transcellular transport.

1-2) The development of the epithelial cell polarity

The development of the epithelial cell polarity is strictly dependent upon cell-to-cell adhesion, which is mediated by the successive homotypic interaction of two types of integral membrane proteins, the nectins and the E-cadherin

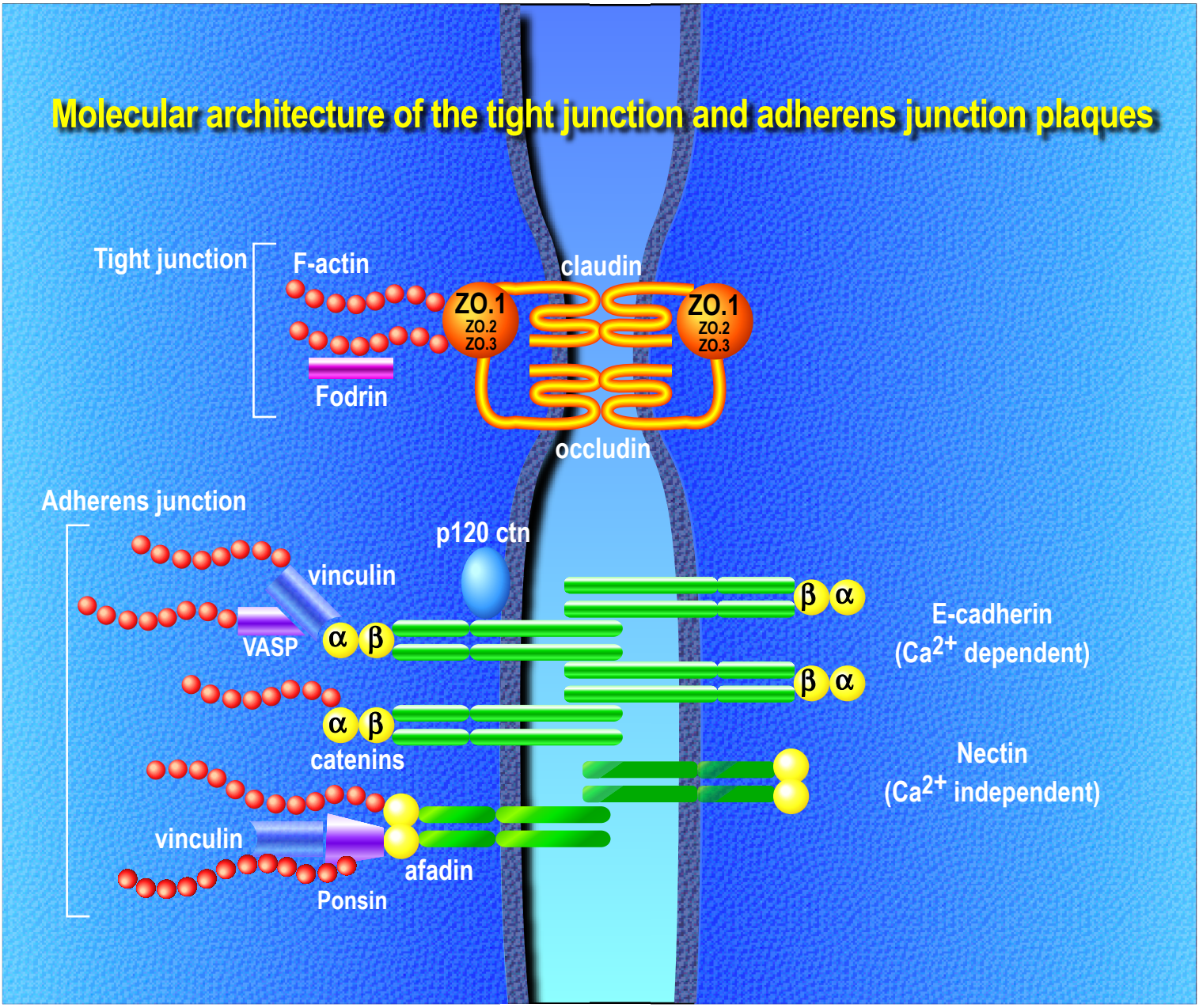

Figure 7: Molecular architecture of the tight junction and adherens junction plaques. The general organization of cell-cell junctions involves integral membrane proteins forming homotypic interactions through their extracellular domains. Their cytoplasmic tails bind adaptor proteins that serve as bridges to anchor the junctions to the actin cytoskeleton and recruit signaling molecules. The members of the claudin family represent the basic unit of the TJ and were shown to directly govern the barrier function. The function of occludin, another integral membrane protein of the TJ, is still debated. These two molecules associate to the cytosolic proteins of the ZO family. The nectin molecules represent the primary adhesion molecules involved in the initial cell-cell contact and are connected to the actin cytoskeleton through the afadin/AF6 complex. Subsequent to the contact detected by nectins, the E-cadherin molecules form the mature and stable adherens junction. The α- and β-catenin cytosolic proteins link the E-cadherin molecules to the structural and signaling networks of the adherens junction plaque.

molecules, and upon cell-to-substratum adhesion maintained by the molecules of the integrin family that act as receptors for the proteins composing the extracellular matrix (Drubin and Nelson, 1996; Zegers et al., 2003). Although intensively investigated, the signaling pathways initiated upon E-cadherin and integrins engagement are poorly defined. However, we do know that it is the adhesion cues which specify the apico-basal axis along which the cell is polarizing. This way the cell acquires its basal, lateral and apical membrane domains. This is accompanied by an extensive rearrangement of the underlying cortical actin cytoskeleton that takes place during the acquisition of the polarized phenotype. Most notably, this includes the basal stress fibers which are terminated by focal complexes that serve as anchoring points to the underlying ECM, the lateral actin cables, the apical actin ring in which the TJ is anchored and the actin filaments supporting the microvilli (Fig.6).

1-3) The tight junction and cell polarity

The plasma membrane of polarized epithelial cells is characterized by two distinct domains: the apical domain exposed to the lumen and the basolateral domain in contact with neighboring cells (lateral) and the lamina propria (basal). These two domains display different protein and lipid compositions and are physically separated by a junctional complex, the tight junction (TJ). The TJ forms a diffusion barrier that prevents mixing of the two membrane domains and provides a tight seal between cells. The TJ is assembled by the juxtaposition of tetraspan transmembrane proteins of the claudin family forming a circular belt of interdigitated strands surrounding the polarized cell at the most apical region of the lateral membrane domain (Fig.7). The members of this family are believed to represent the basic unit of the TJ and were shown to directly govern the barrier function (Tsukita et al., 2001). The role of occludin, the first integral membrane protein purified from the TJ (Furuse et al., 1993) is still under debate. It may be required at special stages of the TJ assembly and/or in the regulation of its dynamic functioning. One report described a new TJ associated protein, tricellulin, selectively localized at the intersection of three cells upon whose suppression disorganized the whole TJ and epithelial barrier (Ikenouchi et al., 2005). Numerous cytosolic molecules associate with the TJ, among which are the proteins ZO-1 and the closely related ZO-2 and ZO-3

(Schneeberger and Lynch, 2004; Stevenson et al., 1986). ZO-1 binds to many proteins including ZO-2 and ZO-3, occludin, the claudins, ZONAB (ZO-1 associated nucleic acid-binding protein) and α-catenin. In addition, the C-terminal domain of ZO-1 binds directly to actin filaments and serves as a bridge between the TJ and the underlying actin cytoskeleton (Mitic and Anderson, 1998). Recently, gene silencing of ZO-1 showed that it is involved in the assembly of the TJ (McNeil et al., 2006). In contrast, ZO-2 and cingulin (another cytosolic TJ-associated protein) do not appear to control TJ formation (Guillemot and Citi, 2006; Guillemot et al., 2004; McNeil et al., 2006).

1-4) The apical actin cytoskeleton

An important characteristic of the TJ is its association with the dense apical ring of acto-myosin. Several functions have been ascribed to this distinctive actin cytoskeleton which are important not only in fully polarized cells, but are also active during the development of cell polarity. The latter includes acting as a sorting apparatus by selective retention of actin-binding proteins at the cell surface, a platform for structural and signaling molecules where they organize as functional complexes to transduce inwards the polarity signals, and an anchoring structure for the TJ with contractile activity (Drubin and Nelson, 1996; Nusrat et al., 2000). While the small G proteins of the Rho family have been extensively studied for their role in organizing the actin cytoskeleton in non-polarized cells, little is known about their role in polarized epithelium and much less in cell polarity development. Original studies on RhoA, Rac1 and Cdc42 in polarized MDCKII cells indicated their involvement in regulating TJ functions (Jou and Nelson, 1998; Jou et al., 1998; Rojas et al., 2001). However, the expression of the mutants had multiple effects on the actin cytoskeleton and cell polarity, probably as a result of altering numerous signaling pathways. Hence, rendering it difficult to draw definitive conclusions as to where they are really involved in epithelial polarity development and maintenance.

Our studies focused on the small G protein Arf6 that has been implicated in both intracellular transport and actin rearrangement in fibroblastic cell lines (D'Souza-Schorey and Chavrier, 2006). In polarized epithelial cells, Arf6 appeared to affect the morphology and transport at the apical pole of the cells

(Altschuler et al., 1999). Likewise, we had observed that its specific exchange factor, EFA6 (Exchange Factor for Arf6), was mostly located at the apical pole of the cells and in the neighboring of the TJ. Furthermore, EFA6 had been described as a potent molecule capable of rearranging the organization of the actin cytoskeleton (Franco et al., 1999). These observations led me to explore the possibility that EFA6 might contribute to the extensive actin cytoskeleton rearrangement that takes place during the development of the epithelial cell polarity and the epithelio-mesenchymal transformation (EMT).

1-5) EFA6: an exchange factor for Arf6

EFA6A was identified based on its homology with the Sec7 catalytic domain of ARNO (Arf nucleotide-binding site opener) an exchange factor for Arf1 (Franco et al., 1999). There exists 4 isoforms of EFA6 (Fig.8) encoded by 4 different genes with various patterns of tissue distribution as determined by northern-blot and *in situ* hybridization: EFA6A is expressed in neuronal cells and the polarized epithelial cells of the intestine, EFA6B is ubiquitous, EFA6C and EFA6D are both highly expressed in neurons (Derrien et al., 2002). The general structure of the EFA6 proteins comprises a variable N-terminal domain, the catalytic Sec7 domain bearing the nucleotide exchange activity, a PH domain responsible for its membrane localization and a C-terminal region involved in actin cytoskeleton rearrangement (Franco et al., 1999). When over-expressed in fibroblastic cell lines, EFA6A and EFA6B induced the formation of structures enriched in polymerized actin such as large peripheral membrane ruffles and micro-spikes observed at the contact-free plasma membrane domain. EFA6 could be found localized in these same structures (Derrien et al., 2002; Franco et al., 1999). In addition, EFA6A was shown to perturb the membrane trafficking of transferrin (Franco et al., 1999). Together, these results suggested that EFA6 might coordinate endocytosis with cytoskeletal rearrangements (Derrien et al., 2002; Franco et al., 1999).

2) Roles of EFA6 during the development of epithelial cell polarity

Our experimental model is the MDCKII (Madin-Darby Canine Kidney) cells that form a regular polarized monolayer when cultivated on a permeable

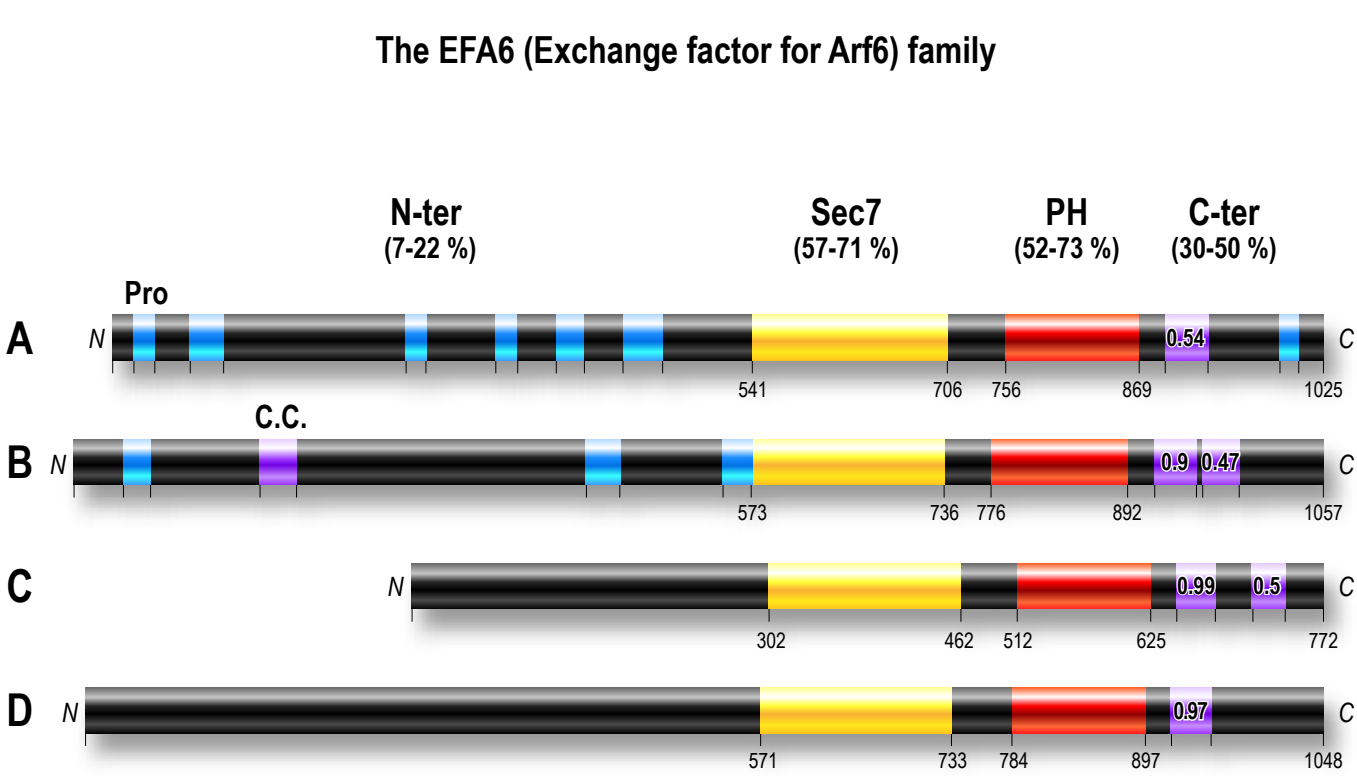

Figure 8: The EFA6 family. Four members encoded by distinct genes have been identified with protein sequence identities ranging from 26% (EFA6B versus EFA6D) to 46% (EFA6A versus EFA6C). They share a common structure with a variable N-terminal domain, the highly conserved catalytic Sec7 and Pleckstrin Homology domains, and a C-terminal domain containing at least one coiled-coil. Many proline rich regions can be found specially in EFA6A and EFA6B. Indicated are the regions with a minimum size of 15 amino-acids that contain more than 30% of proline residues.

support. They are the most studied cells in the field of epithelial polarity and can be used for inducible expression of genes under the control of the tetracycline responding element. We have expressed our proteins of interest, including EFA6, Arf6 and many of their mutants using a modified procedure of the Tet-off system developed by Dr. Altschuller in Prof. Keith Mostov's laboratory.

At the onset, we analyzed the effects of the exogenous expression of EFA6A on a polarized MDCK cell monolayer. The cells were morphologically identical to control cells, displayed correct asymmetric distribution of the apico-basal markers, as well as unchanged localization of the tight and adherens junctional complexes. In a second approach, we looked at the effects of EFA6A expression on the development of the polarity of an epithelial monolayer. We found that the cells developed the hallmarks of polarity much faster, notably the assembly of a functional TJ. However, once fully polarized the EFA6A expressing cells were undistinguishable from control cells (Luton et al., 2004).

Next, we asked by which molecular mechanism EFA6A could accelerate the epithelial cell polarization. We had shown that EFA6A expression increased the amount of endogenous active form of Arf6 (Arf6GTP) in MDCK cells. Thus, based on the observations that Arf6GTP affects the intracellular transport in various cell types, and that both Arf6 and EFA6A modify the actin cytoskeleton organization in non-polarized cells, we considered two possible non-exclusive modes of action: a) an increase in the rate of vesicular transport of the jonctional proteins controlled by Arf6, b) or else the actin cytoskeleton is affected by EFA6 in a way that it facilitates the phenotypic transformation of the cells. In a first set of experiments, we showed that the transport to the cell surface of the TJ proteins was unchanged in EFA6 expressing cells. However, they have a prolonged time of residency at the plasma membrane, while the one of the transferrin receptor is unchanged. This accumulation leads to a large increase of the total amount of the TJ proteins at the cell surface that accounts for the accelerated development of cell polarity (Luton et al., 2004).

How does EFA6 promote the retention and accumulation at the cell surface of the TJ proteins? In the beginning of the 1980s, studies on the polarized expression of the Na^+/K^+ ATPAse had revealed that the rearranging actin cytoskeleton at cell-cell contact could serve as a platform to trap and concentrate proteins that would be localized at the basolateral membrane domain

in polarized cells. The actin cytoskeleton could act as a sorting machinery by exerting a retention effect towards actin-associated proteins (Nelson and Veshnock, 1987). Likewise, TJ proteins are linked to the actin cytoskeleton and possess basolateral targeting signals within their cytoplasmic tails (Hartsock and Nelson, 2007; Matter and Balda, 1998). We had observed by immunofluorescence that the cells over-expressing EFA6A reorganized their actin cytoskeleton more rapidly. Hence, we explored the possibility that EFA6 could stabilize the apical actin ring during its formation, thus allowing for the rapid accumulation of the associated TJ proteins at the plasma membrane. We used Latrunculin B, a drug that would enable us to assess the stability of the filamentous actin structures. In cells expressing the exogenous EFA6A, the apical actin ring and the TJ structures were much more resistant to Latrunculin B. However, the adherens junction (AJ) and the other actin structures such as the stress fibers or the lateral cortical actin were equally sensitive to Latrunculin B in both cell types. Thus, EFA6 is capable of selectively stabilizing the apical actin ring and its associated TJ. Looking at the Tx-100 insoluble fraction, we observed an accumulation of the TJ proteins in EFA6A expressing cells, but not the AJ proteins nor the Tf-R. We also found that the endocytotic rates of the TJ proteins were decreased in cells over-expressing EFA6A. Thus, further indicating that EFA6A promotes the retention of the TJ to polymerized actin structures (Luton et al., 2004).

In conclusion, we proposed that EFA6 facilitates the development of epithelial cell polarity by stabilizing the apical actin ring onto which the TJs are anchored.

3) Contribution of Arf6 and its cooperation with the C-terminal domain of EFA6

3-1) Arf6 acts together with the C-terminal domain of EFA6 to facilitate the development of cell polarity

Two functional domains have been characterized in EFA6: the Sec7 domain which supports the catalytic activity of nucleotide exchange on Arf6 and the C-terminal domain whose expression induces important morphological changes, such as the formation of numerous and often long membrane

extensions enriched in polymerized actin. We have found that mutations that abolish either the nucleotide exchange activity or delete the C-terminal domain abrogate the effects of EFA6 on the epithelial cell polarization indicating that the two domains are necessary (Luton et al., 2004). We started our studies by first analyzing the contribution of Arf6 activated by EFA6.

The study of several Arf6 mutants (constitutively active, dominant negative, locked in GDP form, fast-cycling, etc) confirmed that the activation of Arf6 was necessary but insufficient to mimic the effects of EFA6A over-expression (Klein *et al.*, in preparation). We reasoned that it is not the activation *per se* of Arf6 that is required but its activation either by or in the presence of EFA6. We postulated that the signaling pathway downstream of Arf6 would cooperate with the one associated with the C-terminal domain of EFA6. To test this hypothesis we performed complementation experiments in which we studied the effects of the individual or simultaneous over-expression of a constitutively active mutant of Arf6 (Arf6T157N) with or without the C-terminal domain of EFA6A coupled to the Pleckstrin-Homology (PH) domain to warrant its correct plasma membrane localization (PHCter). While the sole expression of the PHCter domain does not have any obvious effect, the expression of Arf6T157N slowed down the development of epithelial polarity as assessed by measuring the gain of the barrier function, the assembly of the TJ, the polarized reorganization of the actin cytoskeleton or the asymmetric distribution of the apical and baso-lateral polarity markers. In contrast, the co-expression of the two proteins reverted the phenotype of Arf6157N to mimic the one of EFA6A, that is, it accelerated every aspect tested of epithelial cell polarization and delayed the loss of polarity (Fig.9A). The results of these experiments strongly suggested that there is cooperation between the two signaling pathways associated with the activated Arf6 and the C-terminal domain of EFA6A. In other words, the contribution of Arf6 needs to be envisioned in the context of its activation by EFA6 (Klein, *et al.* in preparation).

3-2) <u>Arf6 and the C-terminal domain of EFA6 cooperate to stabilize the apical actin ring</u>

As described above in section 2), we have demonstrated that the exogenous expression of EFA6A stabilizes the apical actin ring associated with

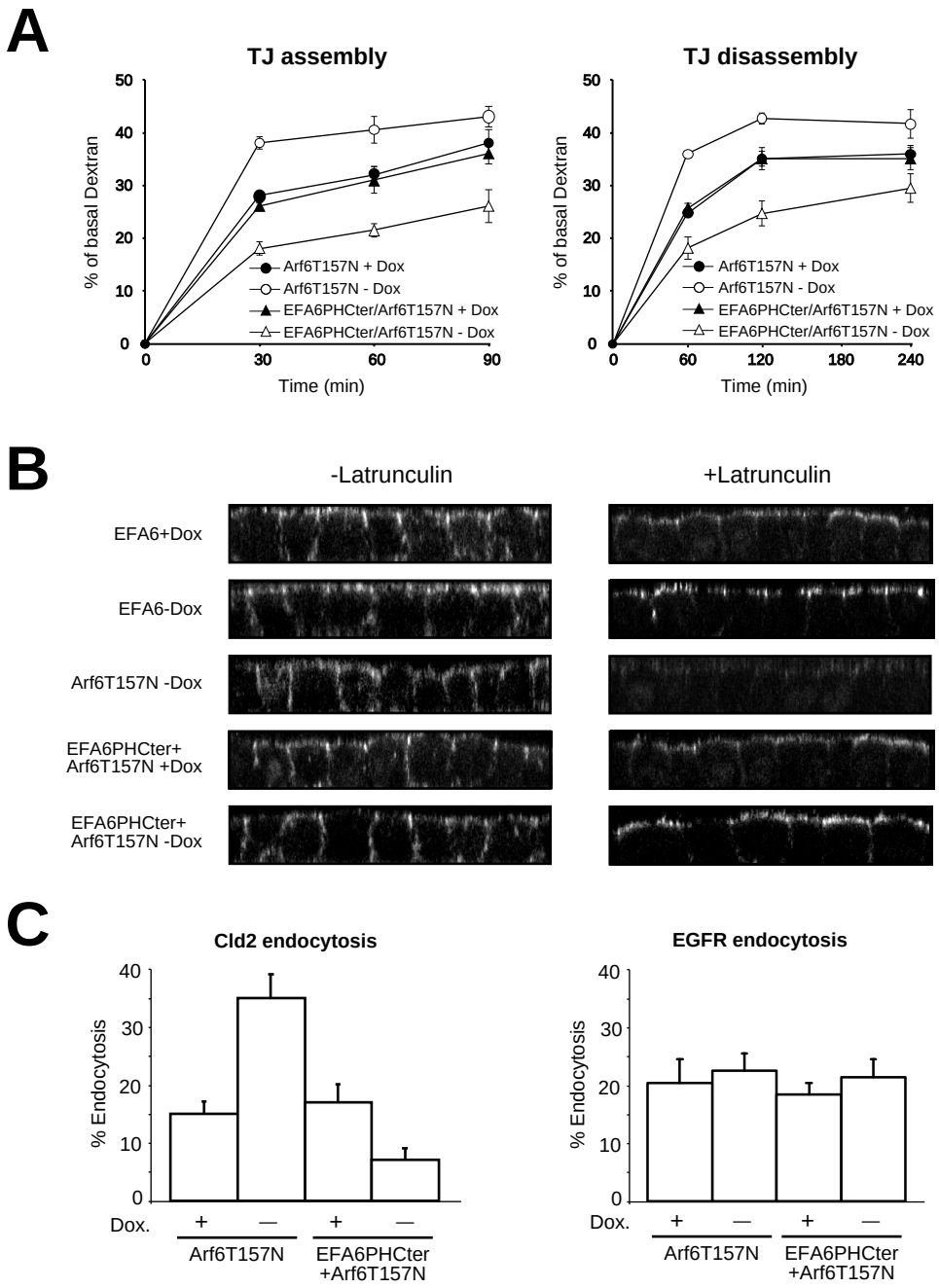

Figure 9: Co-expression of Arf6 and the C-terminal domain of EFA6 mimics the effects of EFA6 expression on the apical actin cytoskeleton stability and the TJ assembly. A) The indicated MDCK cell lines expressing or not Arf6T157N under the control of doxycyclin (Dox.) were plated either on filters at confluence in the absence of calcium or cultivated on filters for 3 days in the presence of calcium to obtain a tight polarized monolayer. At t=0, the monolayers were submitted to a calcium-switch and the gain or loss of permeability to fluorescent Dextran added apically was analyzed over time. B) The indicated polarized cell monolayers were exposed or not to 5μM Latrunculin B for 10 min and processed for confocal immunofluorescent microscopy. The images show representative XZ sections after staining of the filamentous actin with Texas-red coupled phalloidin. C) Endocytosis of Claudin 2 (Cld2) and the EGFR in the indicated polarized cell monolayers assessed after cell surface biotinylation. The graph represent the percentages of endocytosis at 30 min obtained in three independent experiments.

the TJ. Thus, to support the Arf6/PHCter cooperativity, we have assessed whether the co-expression of Arf6T157N and the PHCter domain was also capable of stabilizing the actin cytoskeleton similarly to EFA6A. In the first place, we analyzed the effects of the expression of the two proteins on the morphology of non-polarized cells, which when expressed separately has been well described. The expression of Arf6T157N was shown to induce the formation of large lamellipodia and rounding of the cells, while the PHCter domain promotes the formation of long membrane extensions giving a filamentous aspect to the cell periphery. Co-expression of the two proteins mimicked the morphological changes caused by EFA6A exogenous expression: cells with small lamellipodia at the periphery and short filopodia on the dorsal face, in both of which EFA6 is concentrated. Further, by expressing various amounts of the two proteins we could observe a dose effect which suggests that the level of activation from the two signaling pathways must be finely regulated *in vivo* to perform the biological functions. These experiments show that the two signaling pathways involving EFA6 act in a coordinate manner to regulate the actin cytoskeleton in non-polarized cells (Fig.10).

Next, we assessed whether these two pathways were also controlling the general reorganization of the actin cytoskeleton that accompanies the epithelial cell polarization. As mentioned before, the co-expression of both Arf6T15N, which expressed by itself delayed the actin polarization, and the PHCter domain, which by itself had no effect, mimicked the accelerated appearance of the polarized actin cytoskeleton. More significant were the results obtained by assessing the sensitivity of the apical actin ring to the Latrunculin B. The polarized Arf6T157N expressing cells were more sensitive to Latrunculin B than control cells (and thus than EFA6A that had a protective effect) displaying a rapid destruction of all of its actin cytoskeleton structures including the apical actin ring. On the other hand, the PHCter domain did not appear to modify the sensitivity of the cells to the drug. When the two proteins were co-expressed, the cells displayed the same phenotype as EFA6: the basolateral structures of the actin cytoskeleton were sensitive to the drug but the apical actin ring and the associated TJ were more resistant to the Latrunculin B treatment (Fig.9B). Similar results were obtained when looking at the endocytosis of the TJ protein claudin 2 compared to the control EGF receptor (Fig.9C)

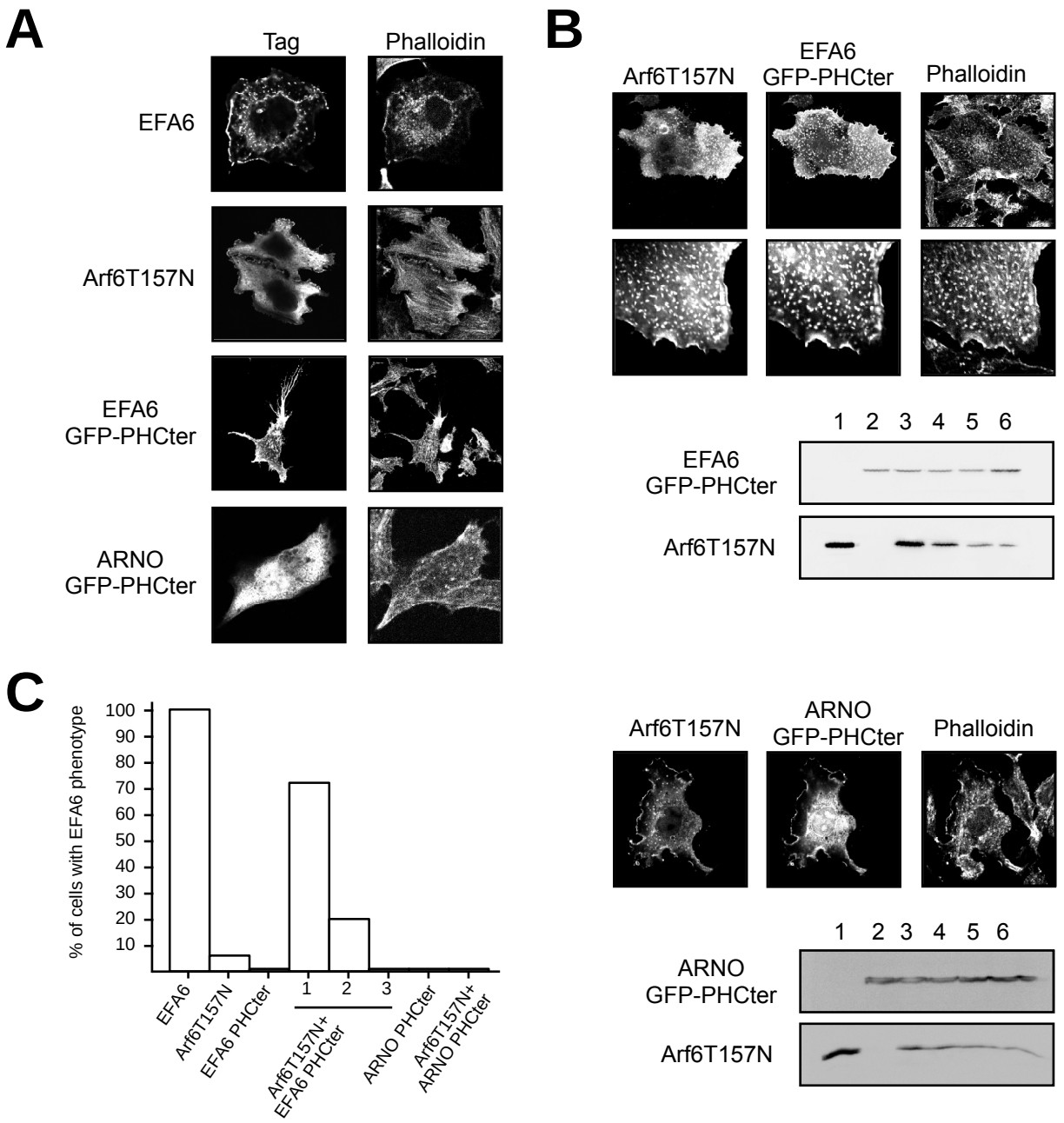

Figure 10:Co-expression of Arf6 and the C-terminal domain of EFA6 mimics the effects of EFA6 expression on cell morphology and actin cytoskeleton rearrangement. A) BHK ce lls were transfected with the indicated protein and processed for confocal immunofluorescence. The left panels display the labeling of the proteins of interest and the right panels the co-labeling of the actin cytoskeleton with phalloidin. B) The upper panels represent data of the co-expression of Arf6T157N and EFA6-PHCter analyzed by confocal microscopy and immunoblot. For immunofluorescence two examples are shown including a zoomed image to document the complete co-localization between the two proteins and the actin. Samples 1 and 2 show individual expression of Arf6T157N and EFA6-PHCter, respectively. Samples 3 to 6 show the different levels of expression of the two proteins after transfection aimed at co-expressing different ratios. The lower panels show t he same experiment performed with Arf6T157N and the PHCter of ARNO. C) For each transfected population, the phenotype of the cells was analyzed. The graph show the % of cells with an « EFA6-like » phenotype. 1, 2 and 3 represent three different groups of ce lls exhibiting relative staining intensities of Arf6T157N and EFA6-PHCter ranging from a high Arf6T157N intensity, similar staining to high EFA6-PHCter intensity. For each category about 100 cells were analyzed in two to three independent experiments by a blind test procedure.

Altogether, these results demonstrate that both EFA6 signaling pathways (one associated with Arf6 and the other with the C-terminal domain of EFA6) are necessary and sufficient, and act in a strict cooperation to contribute to the positive biological effects of EFA6 on the apical actin cytoskeleton during epithelial cell polarization (Klein, *et al*. in preparation).

3-3) Arf-GAPs: effectors of the Arf proteins

Next, we investigated the pathway downstream of Arf6 acting on the actin cytoskeleton. It had been observed that cells over-expressing Arf6 wild-type (Arf6WT) and exposed to aluminum fluoride (AlFx) formed large lamellipodia (Radhakrishna et al., 1996). Studying large G proteins, the group of Dr. Chabre had shown that AlFx mimics the third phosphate in the nucleotide binding pocket where it associates with the GDP to reproduce the presence of a GTP (Chabre, 1990). In small G proteins of the Rho family, the AlFx needs to be stabilized by a residue provided by the GAP (GTPase activating protein) to mimic the third phosphate. It produces a stable complex Rho-GDP-AlFx-GAP in which the small G protein is in the active conformation but the GAP activity is blocked (Wittinghofer, 1997). The phenotype observed in the cells expressing Arf6T157N is very similar to the one over-expressing Arf6WT and exposed to AlFx. In addition, AlFx has no effect on Arf6157N expressing cells. Thus, we postulated that an Arf6-GAP could act as an effector for Arf6GTP to rearrange the actin cytoskeleton (Klein et al., 2006). By abrogating the catalytic activity of the GAPs, thus the dissociation of the complex, the AlFx is a precious tool to test whether the GAPs can act as true effectors. In the first stage, we demonstrated *in vitro* and *in vivo* that the Arf1 and Arf6 proteins are capable of forming stable Arf-GDP-AlFx-GAP complexes. In the second stage, we tested our hypothesis in a model of focal adhesion formation that implicates Arf1 and its GAP, ASAP1. This model was interesting because the mode of action of ASAP1 was controversial. Depending on whether one analyzed a point mutation in the catalytic domain or its entire deletion, it could be reasoned that the catalytic activity was not necessary or to the contrary that it was necessary to impact the formation of focal adhesion, respectively. We have shown that in the presence of AlFx, ASAP1 could still affect the formation of the focal adhesion. Thus, indicating that the Arf1-GAP was acting as a genuine effector of Arf1GTP

and not solely as a terminator through its GAP activity (Klein et al., 2005).

3-4) Arf6 acts on the actin cytoskeleton through an Arf6-GAP

In our model, the Arf6-GAP suspected to be involved in the reorganization of the actin cytoskeleton is not known. Thus, we searched for Arf6 mutants that would not be able to bind to the Arf6-GAPs. There are only two amino-acids (Q37 and S38) different between Arf1 and Arf6 within their Switch I domains. It had been proposed that the mutation of these two residues abolished the effects of Arf6 on the cell morphology and the actin cytoskeleton without changing those on the intracellular transport (Al-Awar et al., 2000). Interestingly, we found that cells expressing the double-mutant became insensitive to the AlFx. *In vitro*, we showed that Arf6Q37ES38I can not bind nor be a substrate of two Arf-GAPS, ASAP1 and ACAP1, but was still capable of binding the effector ARHGAP10. Hence, the double mutation appeared to affect selectively the binding of the Arf-GAPs to Arf6. When the double mutation is introduced in Arf6T157N, the formation of the lamellipodia is completely abrogated indicating that the effector responsible for the remodeling of the actin cytoskeleton can not bind to Arf6 anymore. We concluded that the effector linking Arf6GTP to the signaling pathway that controls the reorganization of the actin cytoskeleton is likely an Arf6-GAP that has yet to be identified (Klein et al., 2006).

4) Ongoing project : regulation of EFA6 by the ubiquitin-proteasome system

4-1) EFA6, a protein whose intracellular levels are tightly regulated during the development of epithelial cell polarity

Quantitation of the total amount of EFA6 during cell polarization reveals rapid modulation of large amplitude. A cell monolayer synchronized to develop polarity exhibits an important increase of EFA6 quantity within the first 30 minutes, which peaks close to 1hr and decreases quickly to return to normal levels after 4 to 6 hours. The increase is accompanied by the translocation of EFA6 to the developing apical plasma membrane domain and its detection within a Triton X-100 insoluble fraction. The accumulation of EFA6 precedes

the formation of the TJs while the rapid diminution occurs before the TJs are fully assembled and the cells are fully polarized (Luton et al., 2004). Given that we have shown that the over-expression of EFA6 accelerates an early stage of cell polarization, one can speculate that the increase of EFA6 expression is required for the efficient reorganization of the actin cytoskeleton and the assembly of the TJs leading to cell polarization.

The finding that the quantity of EFA6 is being controlled so abruptly, up and down, suggests that it is a post-translational regulatory process. For this to be possible, it implies that the protein possesses a rapid turnover, that is, a fast rate of protein synthesis and a short life expectancy. We have measured these two parameters by classical metabolic labeling using radio-active cysteine and methionine. The half-life of EFA6 is of about 20 minutes, and its synthesis rate is 50 times more rapid compared to the Tf-R. Hence, according to the circumstances the cells can produce EFA6 in large amounts very rapidly or eliminate it promptly by a simple positive or negative regulation of its degradation. These quantitative data are congruous with the variations of the quantity of EFA6 observed during cell polarization.

4-2) EFA6 is a substrate of the ubiquitin-proteasome system

The most efficient and reactive degradative pathway for cytosolic proteins is the ubiquitin-proteasome system (UPS). Ubiquitinylation is a post-translational modification that consists of covalently linking ubiquitin, a small protein 76 amino-acids long, on lysine residues of target proteins. When a chain of 4 molecules of ubiquitin is formed, the modified protein becomes a substrate for the proteasome. The proteasome is a multi-protein complex that recognizes and degrades poly-ubiquitinylated cytosolic proteins (Fig.11) (Pickart and Cohen, 2004; Weissman, 2001).

To assess whether EFA6 could be a substrate of the UPS we had to determine if 1) EFA6 could be degraded by the proteasome and 2) if EFA6 could be poly-ubiquitinylated. Treatment of MDCK cells with two proteasome inhibitors, MG-132 and lactacystin, caused a rapid increase in EFA6 levels in a dose-dependent manner (Fig.12A). In addition, the kinetics of accumulation were consistent with the metabolic parameters of EFA6. Also, by co-expression of EFA6 and ubiquitin, in the presence of proteasome inhibitors, one detected

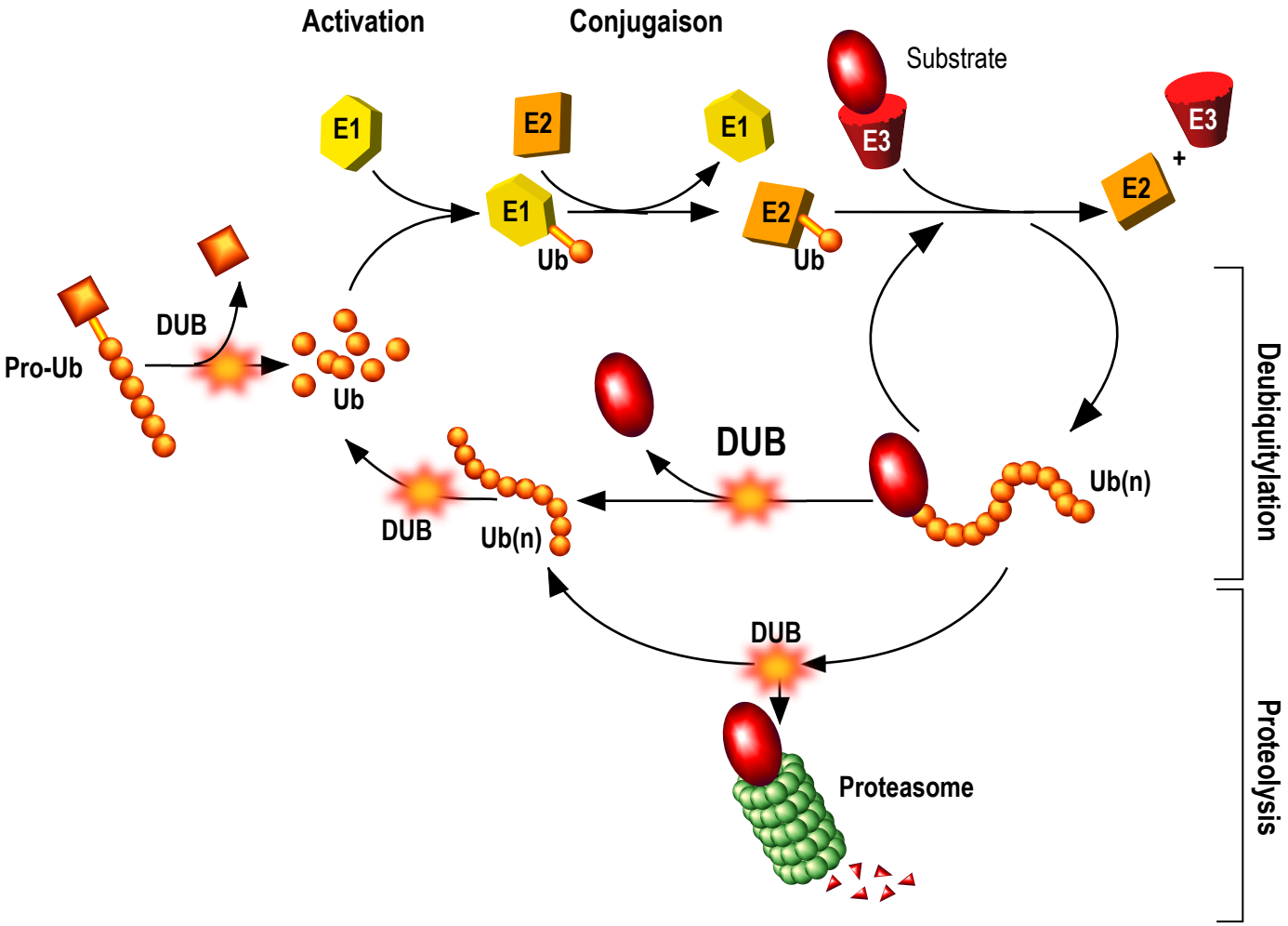

Figure 11: The ubiquitin-proteasome system. Ubiquitinylation occurs through several steps involving first the enzyme E1 that activates the ubiqtuitin molecule, which is then transferred onto a second enzyme E2. The E2-ubiquitin associates with E3 that has already bound or not its cognate substrate. Depending on the class of E3, RING or HECT, the ubiquitinylation of the substrate is realized by the E2 or by the E3 itself after it has received the ubiquitin from the E2, respectively. This sequential reaction is repeated to attach a chain of poly-ubiquitin to the substrate to be degraded by the proteasome. Several steps along the ubiquitinylation procedure can be reverted by de-ubiquitinylase proteins (DUB). One can distinguish between the ones involved in preparing or recycling the ubiquitin molecules from the one that reverts in a substrate specific manner the reaction of ubiquitinylation to protect the substrate from the proteosomal degradation.

high molecular weigh forms of poly-ubiquitinylated EFA6. Pull-down experiments using the ubiquitin binding domain of S5a (the subunit of the proteasome that recognizes the ubiquitin chains of target proteins) indicates that the poly-ubiquitinylated EFA6 can be retrieved by the proteasome. As a control, the ubiquitin binding domain of another protein that is more selective of mono-ubiquitinylated protein, did not bind to poly-ubiquitinylated EFA6 (Fig.12B). We conclude that in resting cells EFA6 is constitutively poly-ubiquitinylated and degraded by the proteasome to maintain a low level of expression.

4-3) <u>During cell polarity development EFA6 successively accumulates, becomes poly-ubiquitinylated and then is degraded.</u>

Next, we asked whether the UPS could be responsible for the variations in EFA6 quantity during cell polarization. Using a synchronized monolayer of MDCK cells, we immunoprecipitated EFA6 at different times during the development of cell polarity. Poly-ubiquitinylated forms of EFA6 were detected at the peak of accumulation of EFA6 just before its levels decrease (Fig.12C). The prevailing ubiquitinylated form corresponded to the addition of 4 molecules of ubiquitin, the minimal chain length required to be recognized and degraded by the proteasome. What it is worth noting and testifies to the amplitude of the phenomenon observed is that this observation is made on the endogenous EFA6 in the absence of exogenous ubiquitin expression or treatment with proteasome inhibitors. The poly-ubiquitinylated forms appeared just before the boost of EFA6 is degraded and brought back to its normal levels of expression which strongly supports a causal relationship between poly-ubiquitinylation and degradation.

At this point, our results suggested that in resting cells EFA6 is constitutively poly-ubiquitinylated and degraded by the proteasome to maintain a low level of expression. During cell polarization, large quantities of EFA6 are required and we observed its accumulation. This could be the result of either an arrest of ubiquitinylation, or by de-ubiquitinylation. To discriminate between these two possibilities we have looked for the ubiquitin ligase and the de-ubiquitinylase.

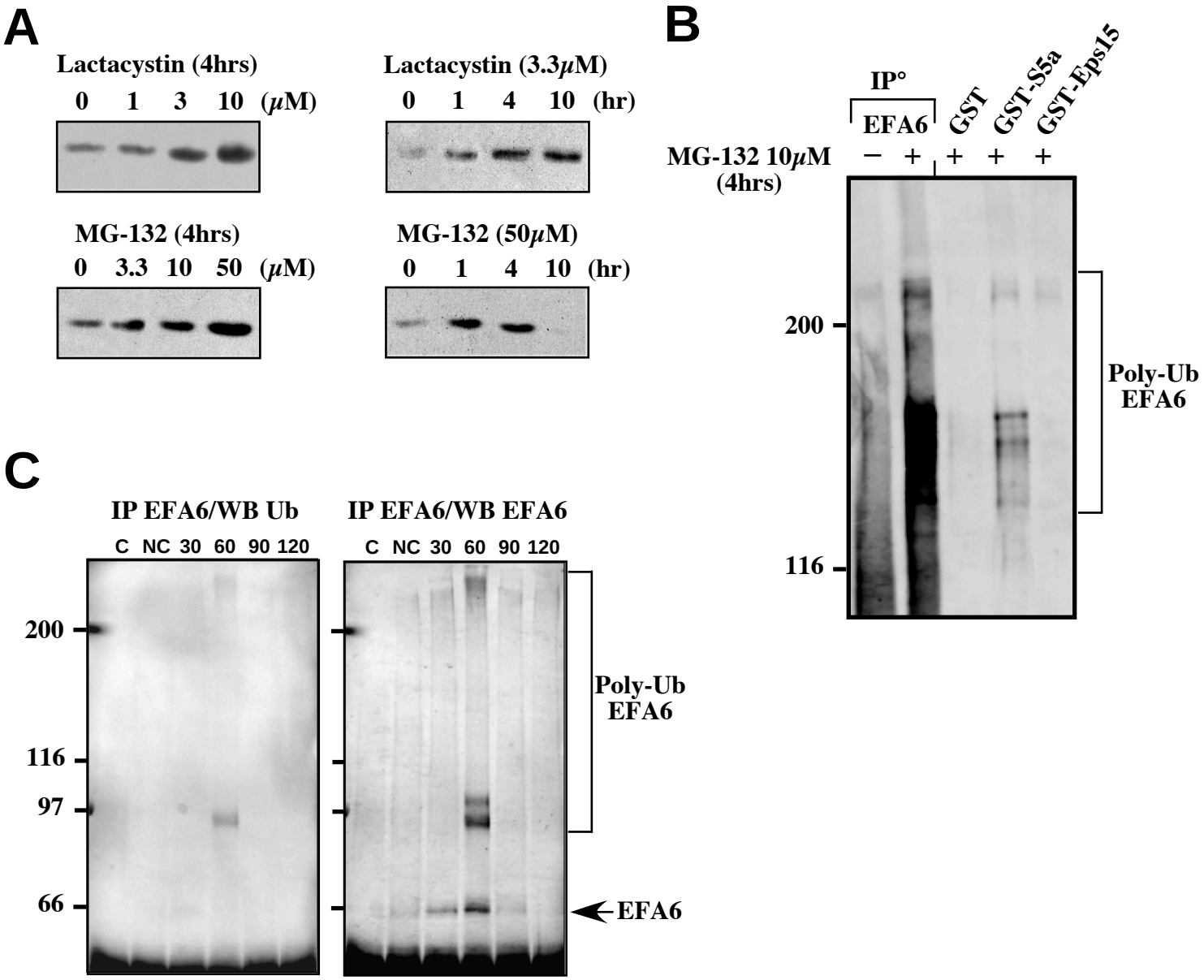

Figure 12: EFA6 is a substrate for the UPS during cell polarity development. A) MDCK cells were exposed to the proteasome inhibitors lactacystin or MG-132 at different concentrations and for different periods of time. The total amount of EFA6 from the cell lysates was determined by western-blot analysis. B) The lysates of MDCK cells treated or not to MG-132 were subjected to immunoprecipitation with an anti-EFA6 antibody or pull-down using GST, or the ubiquitin binding domains of S5a or Eps15 fused to GST. The eluates were analyzed by SDS-PAGE and western-blot with an anti-EFA6 antibody. C) MDCK cells plated on filters were subjected to a calcium switch. At different time points anti-EFA6 immunoprecipitates were analyzed by SDS-PAGE and western-blot. The membrane was first revealed with an anti-ubiquitin antibody and after stripping with an anti-EFA6 antibody.

4-4) The ubiquitin ligase of EFA6

In a two-hybrid screen using the C-terminal domain of EFA6 as a bait we have isolated the ubiquitin ligase Siah. It is a RING (Really Interesting New Gene) domain-containing E3 ligase (Fig.13A) acting as a single or as part of a multiprotein complex analogous to SCF (Skp1/Cullin/F-box) (Liu et al., 2001; Matsuzawa and Reed, 2001). A direct association of Siah and EFA6 has been confirmed *in vitro* by using a pull-down assay with the purified proteins. Furthermore, using small fragments of Siah confirms that EFA6 is binding to the C-terminal substrate binding domain (Fig.13B). In MDCK cells, we have localized Siah in the cytosol with no apparent concentration in any particular place within the cell. We are currently analyzing the capacities of Siah to ubiquitinylate EFA6.

Siah ubiquitinylates numerous substrates including the ß-catenin present in the epithelial junctional complexes (Liu et al., 2001; Matsuzawa and Reed, 2001). Therefore, it did not seem informative to assess the role of Siah during cell polarization (via its effects on EFA6) using RNA interference to repress its expression. Instead, we designed mutants of EFA6 that would no longer bind to Siah. Based on the large number of substrates of Siah, mutagenesis analyses, as well as the crystal structure of Siah with one of its substrate a binding consensus sequence for Siah was deduced (Fig.13C) (House et al., 2006; Hovey and Frank, 1995; Santelli et al., 2005). A matching sequence is present in the C-terminal domain of EFA6 that was used in the two-hybrid screen. We have mutated two amino-acids within this sequence that we thought would contribute the most to the interaction with Siah. Pull-down experiments confirmed that the mutant no longer binds to Siah (Fig.13B). We are now pursuing the functional study of this mutant in MDCK cells.

4-5) The de-ubiquitinylase of EFA6

A large number of de-ubiquitinylases have been identified in the human genome, however our knowledge of these proteins regarding their functions and mode of action is very scarce (Millard and Wood, 2006; Nijman et al., 2005). Among the most studied, USP9 has three substrates involved in junctional complexes: afadin, E-cadherin and ß-catenin (Murray et al., 2004; Taya et al., 1999; Taya et al., 1998). In epithelial cells, USP9 has been co-localized with

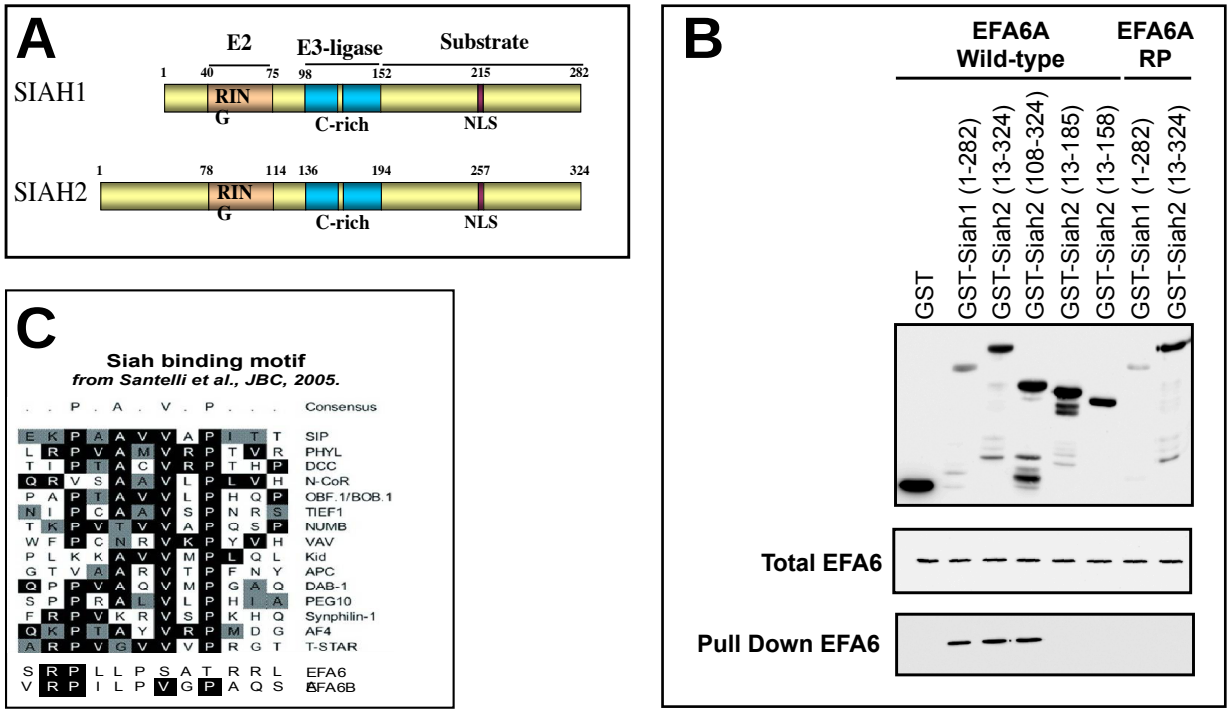

Figure 13: EFA6 binds to the E3 ligase Siah. A) Schematic representation of the Siah 1 and Siah 2 mammalian proteins. They contain a RING domain that binds the E2 protein, the cysteine-rich catalytic domain and a C-terminal substrate binding domain. B) Pull-down using different GST-Siah constructs and purified EFA6 wild-type or mutant proteins. The total amount of the various GST and EFA6 proteins were determined using an anti-GST (top panel) or anti-EFA6 (middle panel) antibody, respectively. The eluates were analyzed by SDS-PAGE followed by immunoblot probed with an anti-EFA6 antibody (bottom panel). C) The consensus Siah binding sequence is also present in EFA6A and EFA6B.

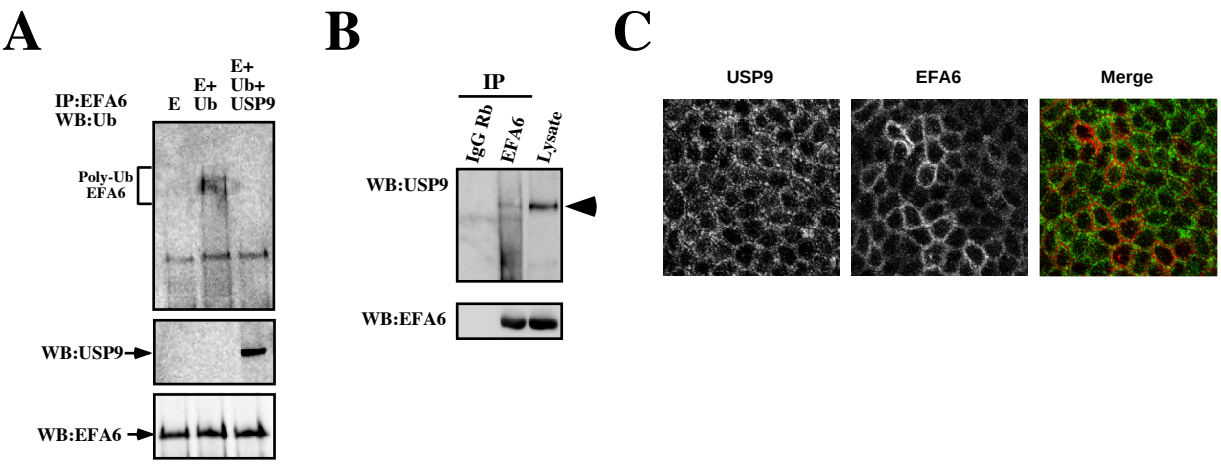

Figure 14: EFA6 is a substrate for USP9 and can be co-immunoprecipitated from synaptosomes. A) BHK cells transfected with EFA6 (E), EFA6 and ubiquitin (Ub) or EFA6, ubiquitin and USP9 were lysed and EFA6 immunoprecipitated. The eluates were analyzed by SDS-PAGE followed by immunoblot revealed with an anti-EFA6 antibody. The bottom panels show the immunoblot to control for the presence of the transfected US9 and EFA6 proteins B) Solubilized synaptosomes were submitted to immunoprecipitation with an antiserum anti-rabbit IgG or an anti-EFA6 antibody. The eluates and a fraction of the lysate were analyzed by SDS-PAGE and immunoblot probed with an anti-USP9 antibody. C) Polarized MDCK cells expressing EFA6 under the control of the tetracycline transactivator were processed for immunofluorescence to localize the endogenous USP9 (green) and the exogenous EFA6 (red).

these proteins at the AJs and TJs. It seems to be capable of protecting the protein ß-catenin from degradation by the proteasome. An ubiquitin-ligase involved in the degradation of occludin, a major component of the TJs, is also a substrate of USP9 (Mouchantaf et al., 2006; Traweger et al., 2002). Thus, this de-ubiquitinylase appears to have an active role on the epithelial junctional complexes and presents itself as a good candidate to protect EFA6 from its proteosomal degradation. To test this hypothesis, we have expressed EFA6 with or without USP9 and found that the wild-type USP9, but not its catalytic mutant, could de-ubiquitinylate EFA6 (Fig.14A). Pull-down and co-immunoprecipitation experiments confirmed the interaction of the two proteins (Fig.14B). Next, to evaluate the role of USP9 *in vivo* on EFA6 ubiquitinylation we repressed its expression using RNA interference. In non-polarized cells, we found that the extinction of USP9 was accompanied by that of one of its substrates, afadin. However, we could not observe any effect on EFA6 nor ß-catenin. This observation suggested that afadin, but not EFA6, is a constitutive substrate of USP9. We repeated the same experiment in cells synchronized to develop polarity. At all times, the levels of expression of USP9 and afadin were strongly reduced. Most interestingly, we did not observe poly-ubiquitinylated forms of EFA6 nor the variations in the expression of the unmodified EFA6. We deduced that EFA6 did not accumulate because it could not be protected anymore from the constitutive ubiquitinylation by Siah followed by proteosomal degradation. Thus, we could not detect variations in the levels of expression of EFA6 nor the appearance of poly-ubiquitinylated forms. These experiments suggest that EFA6, in contrast to afadin, is not a constitutive substrate of USP9, but becomes temporally a substrate during cell polarity development.

To further explore the temporal association of USP9 and EFA6, we looked at their spatial localization at different stages of cell polarization to determine if they co-localize at a precise time during cell polarity development. We had observed that in polarized epithelial cells EFA6 localizes nearby the TJ. In contrast USP9 is totally excluded from the plasma membrane and the TJ (Fig.14C). In isolated MDCK cells, the USP9 staining is mostly cytosolic as small punctates and absent within the peripheral membrane structures in search of cell contacts, such as lamellipodia or long protrusions (Fig.15). However, USP9 is detected as a thin line at the edge of the lamellipodia and at the very end

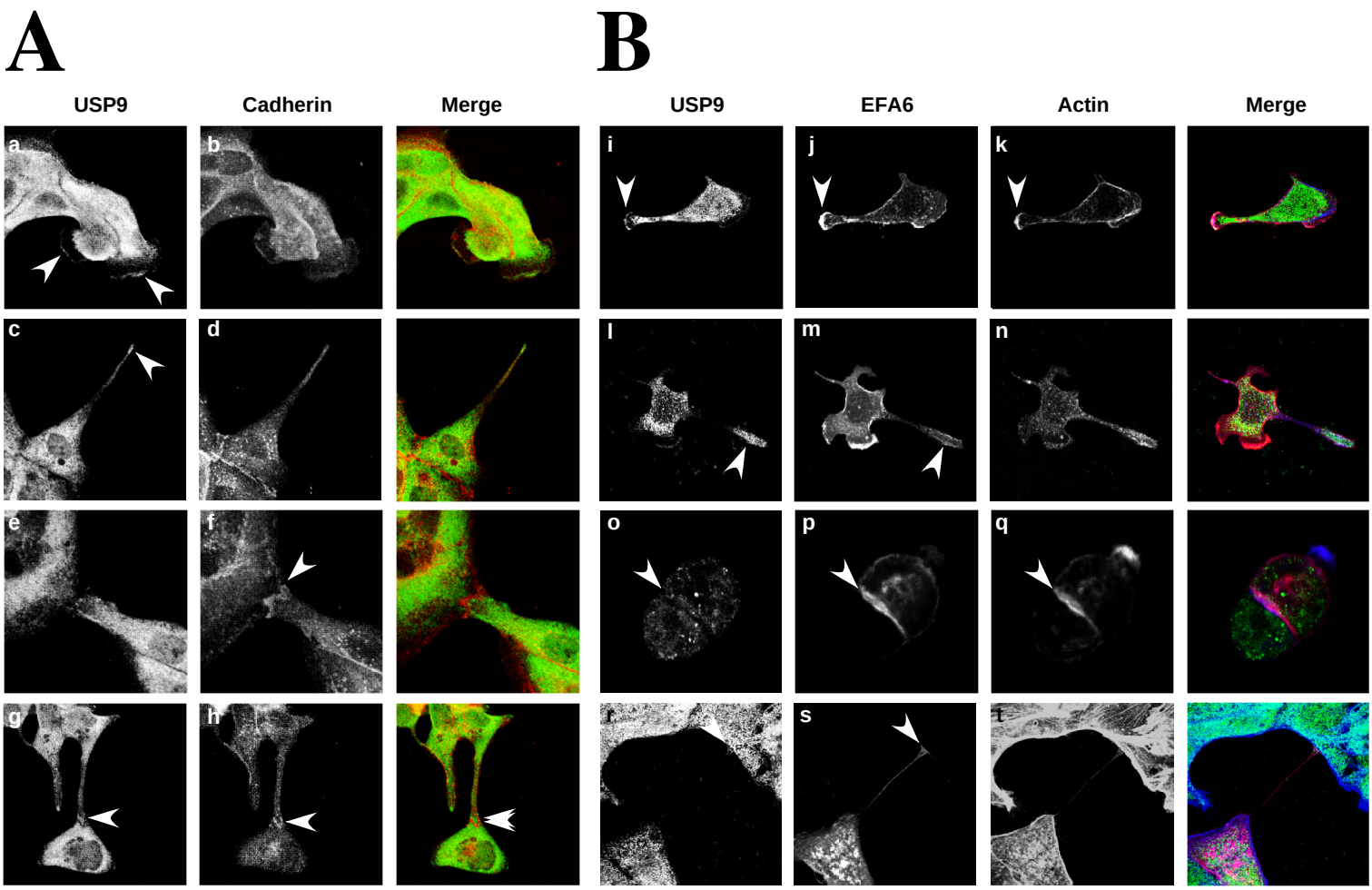

Figure 15: Comparative localization of USP9 and EFA6 in MDCK cells. A) MDCK cells grown on glass coverslips were processed for immunofluorescence to localize the endogenous USP9 (green) and cadherin (red) molecules. In a, the arrows point to the presence of USP9 at the edge of the lamellipodia. In c, the arrow points to the accumulation of USP9 at the tip of the protrusion. In f, the arrow points to the cell-cell contact marked by the presence of E-cadherin staining. In g, the arrow points to the USP9 staining that is right behind the newly formed cell-cell contact marked by the presence of E-cadherin indicated by the arrow in h. In the merge image the two arrows in g and h are placed identically to indicate the close but distinct localization of USP9 and E-cadherin. B) MDCK cells transfected with EFA6 were processed for immunofluorescence to localize USP9, EFA6 and the actin cytoskeleton. In i, j, and k, the arrows have been placed exactly at the same place to mark the exact co-localization at the edge of the lamellipodia. In l, the arrow points to the accumulation of USP9 at the tip of the protrusion. In m, the arrow placed at the same position shows the presence of EFA6. In o, p and q the three arrows placed similarly point to the cell-cell contact in which EFA6 accumulates but USP9 is excluded. In r and s, the two arrows placed at the exact same place point to a newly-formed cell-cell contact where EFA6 accumulates and USP9 is merely detectable.

of the protrusions extremities. EFA6 does not co-localize with the intracellular USP9, but is co-localized at the periphery of lamellipodia and membrane protrusions. When these structures establish a contact, USP9 rapidly withdraws from the adhesion site, while EFA6 persists in large quantities. Then USP9 disappears completely from the contact zone. In confluent cells, USP9 is totally absent from the mature adhesion structures and EFA6 present at very low levels (Fig.14C and Fig.15). In conclusion, USP9 and EFA6 are co-localized in the peripheral areas of the plasma membrane involved in establishing cell contacts and right at the time the contact is forming. After which USP9 withdraws from the zone of contact and disperse.

Altogether, our results suggest that USP9 protects EFA6 from proteosomal degradation selectively during the initial stages of contact formation when EFA6 participates in the actin cytoskeleton reorganization and TJ assembly in polarizing cells (Fig.16). Thus, the increase of EFA6 levels of expression during cell polarization appear to be due to its de-ubiquitinylation rather than an arrest of its ubiquitinylation, that is otherwise constitutive to maintain a low level of expression.

5) Discussion and perspectives

We have described an important role for EFA6 in the development of the epithelial cell polarity. EFA6 acts during the early stage in response to the engagement of the E-cadherin molecules and prior to the formation of the tight junction. In light of the most recent work, I will discuss the possible signaling cascade that links E-cadherin to EFA6 and present few experimental approaches to test our working model. Preliminary studies point to a role for EFA6 on the actin cytoskeleton, however we have very little information as how EFA6 contribute to the rearrangement of the actin cytoskeleton. Based on the discovery of new EFA6 binding partners, I will expose our strategies to decipher the molecular mechanisms by which EFA6 modulates the actin cytoskeleton. The development of the epithelial cell polarity is a complex process that requires the hierarchized and timely organized cooperative action of many players among which the three highly conserved polarity complexes (Crumbs, PAR and

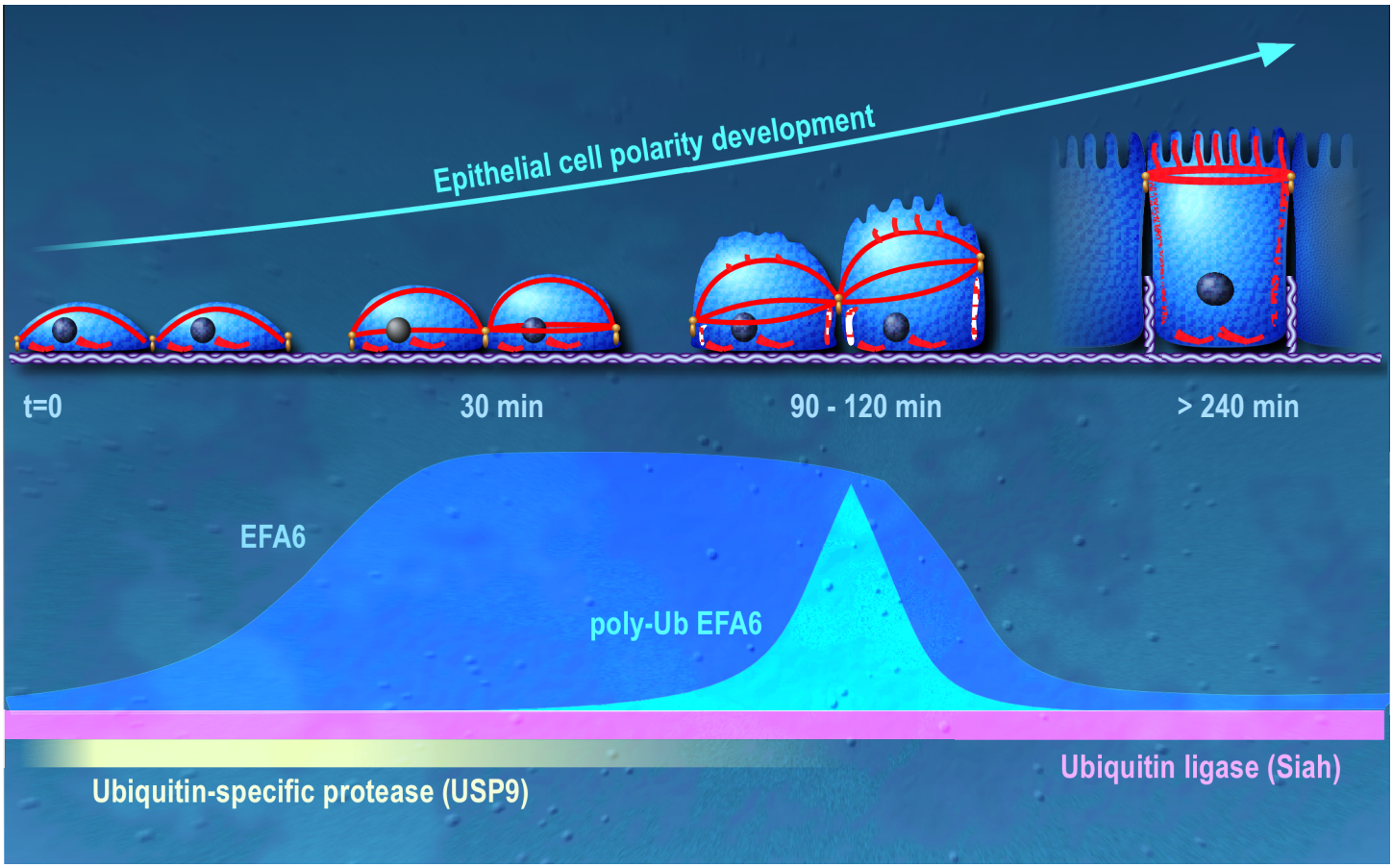

Figure 16: Model for EFA6 regulation by ubiquitinylation/de-ubiquitinylation during epithelial cell polarization. In non-polarized epithelial cells, EFA6 is submitted to a constitutive ubiquitinylation by the E3 ligase Siah followed by its degradation through the proteasome. As soon as the cells engage in the cell polarization program EFA6 is protected from degradation by the de-ubiquitinylase USP9, this leads to a rapid accumulation of EFA6, which will contribute to the reorganization of the actin cytoskeleton and the formation of the TJ. Once the latter is formed, EFA6 is no longer protected from the UPS and its protein expression under the control of Siah drops down. Since USP9 leaves very rapidly from the contact zone where EFA6 remains concentrated, we postulate that EFA6 could be protected from Siah or the proteasome, by a USP9-independent mechanism.

Scribble) (Dow and Humbert, 2007; Nelson, 2003; Wang and Margolis, 2007). From the growing knowledge of cell polarization in mammalian cells, as well as in other organisms such as flies and worms, I will attempt to position EFA6 together with the polarity complexes. Finally, I will discuss preliminary data suggesting a possible role for EFA6 in epithelial tumorogenesis which might expand our future research towards clinical studies.

5-1) Signaling pathways downstream from EFA6

Based on our findings, we propose that the engagement of the E-cadherin molecules at the initial cell-cell contact zone induces a marked increase in the levels of EFA6 through its de-ubiquitinylation followed by or coincident with its localization at the contact zone. There, EFA6 interacts with and contributes to the remodeling of the actin cytoskeleton, which leads to the formation of the apical actin ring and its associated TJ. These events are mediated by two EFA6-associated signaling pathways activated in a coordinated manner: one pathway downstream of the Sec7 domain responsible for Arf6 activation and the recruitment of an Arf6-GAP, and the other pathway downstream from the C-terminal domain.

Regarding the latter, we have recently identified by a two-hybrid screen several partners known to be integral components or regulators for polymerization and/or structuration of the filamentous actin. We are currently studying the role of EFA6 on the polymerization/structuration of the actin in the presence of these proteins. In addition, we will look at their effects on EFA6 exchange activity. Finally, *in vivo* experiments will investigate their role on the development of the epithelial cell polarity. Among these proteins, and non exclusively, some may act upstream as docking proteins to localize EFA6 to the nascent contact zone and others as downstream effectors to mediate EFA6 effects on the actin cytoskeleton organization.

Among the Arf6-GAPs, several of them have been shown to play a role at the plasma membrane: AMAP1/2, ACAP1/2 and Git1/2. Of these, AMAP1 has already been described as a true effector for Arf6. Both proteins when expressed individually, alter the morphology of the peripheral membrane, are involved in the formation of invadopodia and confer invasive properties to mammary tumors (see below paragraph 5-4). On the other hand, AMAP2 and

ACAP1/2 have been implicated in cell surface endocytosis, and the most recent studies have revealed a role for Git1/2 in controlling cell migration and adhesion (Hashimoto et al., 2006; Hashimoto et al., 2004; Randazzo and Hirsch, 2004; Sabe et al., 2006). In any case, to determine which Arf6-GAP is acting downstream of Arf6GTP to rearrange the actin cytoskeleton, the most straightforward approach would be to repress their expression individually and measure the effect on the formation of the apical actin ring. After which, generation of catalytic and binding mutants as well as the search for new partners will help to dissect the molecular mechanisms of the contribution of the suspected Arf6-GAP. It will also be important to determine if the Arf6-GAP identified is acting cooperatively with the C-terminus domain of EFA6 to regulate the formation of the apical actin ring and the TJ.

5-2) Signaling pathways upstream of EFA6

One important question to address is how EFA6 is physically recruited at the contact zone and then segregated to the membrane domain that will give rise to the apical plasma membrane. It has recently been proposed that the phospho-inositide PI(4,5)P2 could determine the apical domain membrane while the PI(3,4,5)P would determine the basolateral domain. PI(4,5)P2 apical production by PTEN was shown to recruit Annexin2, which in turn enrolls Cdc42 which binds the aPKC (Gassama-Diagne et al., 2006; Martin-Belmonte et al., 2007). We have established that the PH domain of EFA6 is specific for the PI(4,5)P2 (Macia *et al*. manuscript in preparation). Thus, we postulate that the enzymes (PTEN, for instance) that control the production of the PI(4,5)P2 are activated upon E-cadherin engagement leading to the recruitment of EFA6 to the plasma membrane. To ascertain that PIP2 is responsible for EFA6 recruitment we will conduct studies where the polarized distribution of the different species of PIs will be disturbed and assess the consequences on EFA6 membrane localization. In parallel, we will also analyze the properties of EFA6 mutated in its PH domain to modify its affinity for the PIs. By fluorescent microscopy we will compare the time course of EFA6 recruitment to the Annexin2-Cdc42-aPKC cascade. Using mutants of these proteins and the siRNA technology we will determine if there is a relationship between EFA6 and this ternary protein complex.

Another element upstream of EFA6 is its regulation by the UPS. We assume that EFA6 is constitutively ubiquitylated and degraded in resting cells (polarized or non-polarized) where it is expressed at a low level. In contrast, following the engagement of the E-cadherin molecules, the de-ubiquitinylase USP9 is set into play to protect EFA6 locally and raise its quantity at the contact zone. One needs to understand how USP9 itself is engaged. To this aim, we will try to identify within USP9 active domains responsible for its dynamic sub-cellular localization upon cell-cell contact formation. We will also attempt to identify binding partners by a two-hybrid screen and proteomic techniques. We do not believe that USP9 and EFA6, if at all, are sufficient to recruit each other at the contact zone as we found that the over-expression of USP9 or EFA6, even at very high levels, did not affect the intracellular localization of the other protein.

Our working model for the recruitment and mode of action of EFA6 is as follows (Fig.17): PI(4,5)P2 and an actin binding protein of the FERM (protein 4.1, Ezrin-Radixin-Moesin) family such as the ezrin (see below for further details) which also binds PI(4,5)P2 and that we found to be highly co-localized with EFA6, together would help to recruit EFA6 at the initial contact zone. There, EFA6 through the cooperative signaling pathways associated to Arf6 and its C-terminus domain, will initiate the reorganization of the actin cytoskeleton. This will be amplified by the action of USP9 that will prevent the removal and degradation of EFA6. The rearrangement and stabilization of the actin cytoskeleton by EFA6 will help to mature the contact zone and prepare for the formation of the apical actin ring. PI(4,5)P2 produced apically ensure that EFA6 is segregated to the apical plasma membrane. The newly formed apical actin cytoskeleton will then serve as a platform for the assembly of the TJ. Once the TJ is formed, protection of EFA6 by USP9 is terminated and its protein levels would rapidly drop to normal.

5-3) Relationship between EFA6 and the polarity complexes

Three complexes, highly conserved throughout evolution, have been described to orchestrate in a coordinate manner the program of development of epithelial cell polarity and assembly of the TJ (Dow and Humbert, 2007; Nelson, 2003; Wang and Margolis, 2007). The complex PAR composed of 4 proteins

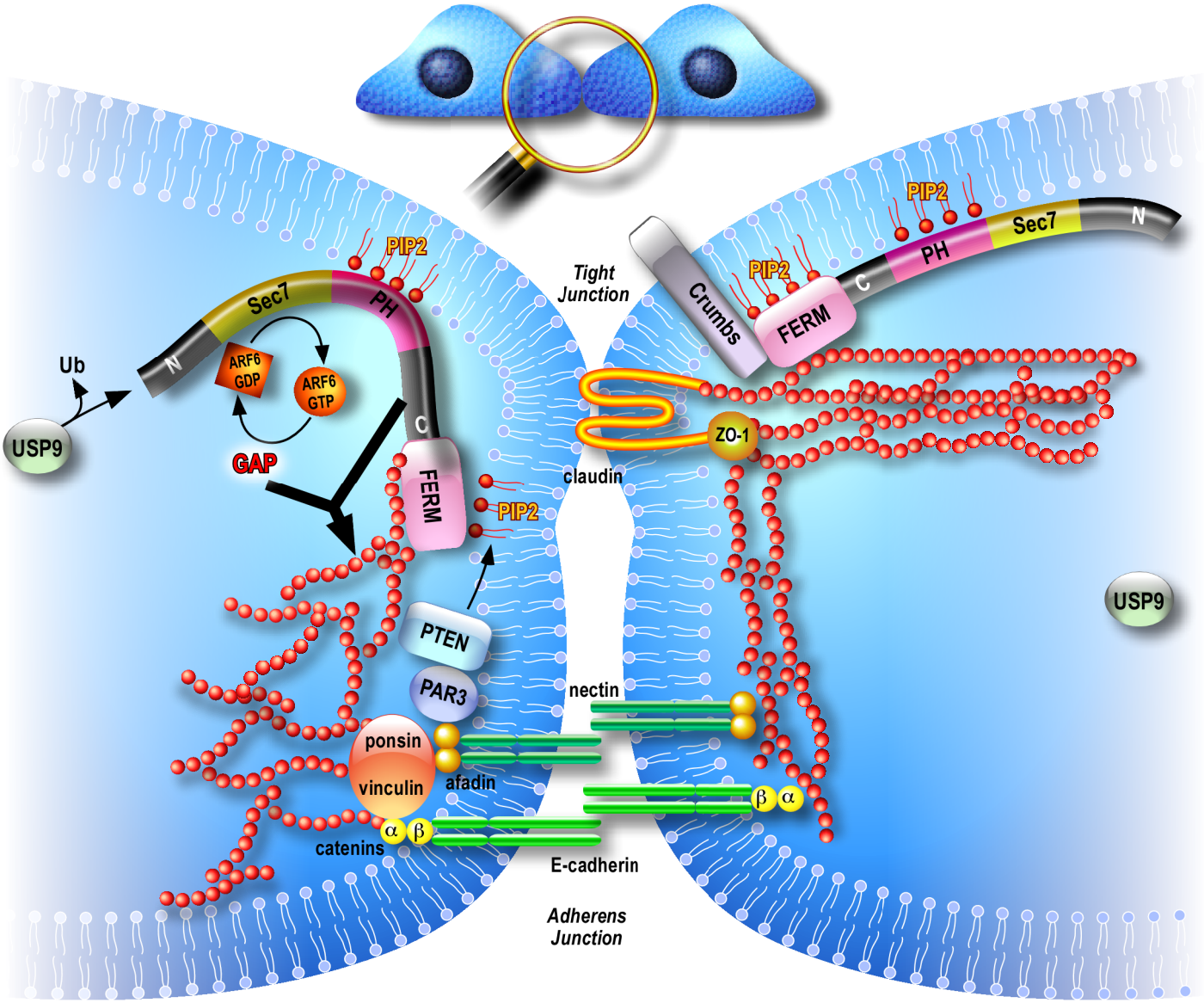

Figure 17: Model for EFA6 recruitment and mode of action at the onset of epithelial cell polarization. In the left cell are depicted the initial steps of the cell-cell contact formation and a more mature cellular junction is represented on the cell to the right. The nectin molecules recruit PAR3 and PTEN leading to the production of PIP2. A FERM protein (e.g. ezrin) and PIP2 recruit EFA6 to the contact zone. Through activation of Arf6 and its effectors, such as the Arf6GAP, and its own C-terminal domain, EFA6 reorganizes the apical actin cytoskeleton (left cell). The segregation of EFA6 to the apical surface and the nearby forming tight junction could be mediated by the enrichment of the PIP2 at the apical plasma membrane, and its association to the crumbs complex via a FERM protein (right cell). The EFA6 signal would be amplified by its accumulation due to its protection from proteosomal degradation by USP9 (left cell). At the same time, the nectin continues to participate in the formation of the AJ by stabilizing the association of the E-cadherin molecules to the actin cytoskeleton through the ponsin/vinculin or ADIP/α-actinin (not shown on the schema) actin-binding proteins (left cell).

(aPKC, PAR3, PAR6 and cdc42) is localized at the TJs, while the complexes Crumbs (Crumbs3, Pals1 and PATJ) and Scribble (hScrib, Dlg, Lgl) are distributed respectively on the apical and basolateral domains of the plasma membrane. The two latter entities are proposed to act antagonistically to delineate the two separate membrane domains, whereby the apical assembly of the TJ is in a zone marked by the PAR complex. The molecular bases of the regulation by these complexes in mammalian cells is just starting to be elucidated but remains largely unknown. It is interesting to note that, the effects of EFA6 on the assembly of the TJs are very similar to those obtained by the expression of mutants or by the repression of the expression of certain members of the PAR and Crumbs complexes. This observation suggests at least a functional link between EFA6/Arf6 and these two complexes. The PAR complex appears to act very early upon cell-cell contact with the recruitment of PAR3 by the nectins, even before the formation of E-cadherin mediated contacts. In Drosophila, PAR3 was shown to act through PTEN, the production of PI(4,5)P2 and the recruitment of Bitesize a synaptotagmin-like protein that is necessary for the proper organization of the actin which in turn stabilizes E-cadherin during embryonic development. Interestingly, this protein binds to and recruits Moesin (the only member of the Ezrin-Radixin-Moesin family in Drosophila) to the apical junctional region. Furthermore, expression of a dominant-negative of ezrin that does not bind to actin phenocopies the loss of Bitesize (Pilot et al., 2006). Another study showed that Moesin could also be stabilized at the apical membrane by the Crumbs complex (Medina et al., 2002). In the worm *C. elegans* the ERM-1 protein (the only member of the FERM family in *C. elegans*) is necessary for apical junction remodeling and tubulogenesis in the intestine (Van Furden et al., 2004). Finally, in mammalian cells ezrin was shown to be one of the downstream target of PI(4,5)P2 (Martin-Belmonte et al., 2007). Altogether, these data point to an important role for FERM proteins, in particular the ezrin. As mentioned earlier, in mammalian cells EFA6 and ezrin largely co-localize. Ezrin together with PI(4,5)P2 could be responsible for EFA6 localization at the contact zone. Thus, ezrin could be the link between the PAR and/or Crumbs complexes and EFA6 that would be responsible for the reorganization of the actin cytoskeleton to allow for the formation of the TJ (Fig.17). In turn, the remodeling of the actin cytoskeleton by

EFA6 could facilitate the recruitment of the PAR and/or Crumbs complexes to facilitate the assembly and apical positioning of the TJs. Additionally, PAR3 was shown to facilitate TJ assembly by sequestering Tiam1 to down-regulate Rac activity (Chen and Macara, 2005). This could allow for decreasing the formation of lamellipodia, which together with EFA6 activity would help to stabilize the junctional/contacting plasma membrane at the contact/adhesion zone.

5-4) Role of EFA6/Arf6 in the development of breast cancer

Among the most prevalent pathologies for public health in developed countries is cancer. In 2004, it became the first cause of mortality in France (30%) with up to 50% mortality for men between 45-64 years old. Whereas, women in the same age group die primarily of breast cancer. It is also worth noting a marked increase in the prevalence of lung cancer for women; most likely reflecting the progression of their smoking habit in the past 30 years. Overall 90% of the non-lymphoid tumors are from epithelial origin, the most frequent being trachea, lung, and colorectal cancers for men, and breast, colorectal, and lung cancers for women. The epithelial cancers are characterized by the loss of cell polarity and cell contact inhibition followed by unlimited cell proliferation. Tumor development may then lead to the acquisition of a migratory phenotype responsible for metastases. A study suggested a role for Arf6 consistent with the acquisition of aggressive properties manifested by the formation of invadopodia in human mammary cell lines (Hashimoto et al., 2004). These membrane structures, rich in polymerized actin, support the digestion of the extracellular matrix allowing the cells to reach the blood stream and disseminate. The same study mentioned a possible correlation between the quantity of Arf6 and the aggressiveness of the cell lines examined. Based on these data we have started to analyze the levels of expression of EFA6 and Arf6 in human mammary tumors by immuno-histochemistry. In a preliminary test we have observed an elevation of the expression of EFA6 in the majority of the tumor samples. A library of about 600 human mammary tumors collected and characterized by the group of Dr. Birnbaum (Institut Paoli-Calmettes, Marseille) is currently under analysis.

Note, that this research is directly related to our study on the regulation

of the levels of expression of EFA6 by the UPS during the development of the epithelial cell polarity. A dysfunction of the regulation of the quantity of EFA6 could have serious consequences on the establishment and maintenance of cell polarity. As such, the fact that both USP9 and Siah have been found to have significant altered levels of expression in epithelial cancers, including mammary tumors is highly relevant (Deng et al., 2007; Kim et al., 2004; Roh et al., 2004). Likewise, ezrin was shown to be required for metastasis of murine breast carcinoma cells (Elliott et al., 2005).

Although there is no indication that EFA6 or Arf6 present transforming power, they seem implicated in the development and the maintenance of cell polarity. Dissection of the mechanisms by which they operate might provide the tools and the knowledge necessary to comprehend the formation and/or progression of malignant tumors. The analysis of the regulation of the quantity of EFA6 by the UPS coupled to a pre-clinical research program developed with the Dr. Birnbaum could extend to the use of EFA6 or Arf6 as diagnostic, prognostic or clinical markers of human mammary tumors.

6) Publications

Luton, F., Klein, S., Chauvin, J.-P., LeBivic, A., Bourgoin, S., Franco, M. & Chardin, P. (2004). EFA6, Exchange Factor for Arf6, regulates the actin cytoskeleton and associated tight junction in response to E-cadherin engagement. *Mol. Biol. Cell, 15: 1134*

Macia, E., Luton, F. Partisani, M., Chardin, P. & Franco, M. (2004). The GDP-bound form of Arf6 is located at the plasma membrane. *J. Cell Sci., 117: 2389*

Mesmin, B., Robbe, K., Geny, B., Luton, F., Brandolin, G., Popoff, M. & Antonny, B. (2004). A phosphatidylserine-binding site in the cytosolic fragment of *Clostridium sordellii* lethal toxin facilitates glucosylation of membrane-bound Rac and is required for cytotoxicity. *J. Biol. Chem. 279:49876.*

Fournier, H-N, Dupet-Manet, S., Bouvard, D., Luton, F., Degani, S., Retta, S. F., Block, M. R. & Albiges-Rizo, C. (2005). Nuclear translocation of ICAP-1 stimulates cellular proliferation. *Mol. Biol. Cell, 16:1859.*

Paléotti, O., Macia, E., Luton, F., Klein, S., Partisani, M., Chardin, P., Kirchhausen T. & Franco, M. (2005). The small G-protein ARF6GTP recruits the AP-2 adaptor complex to membranes. *J. Biol. Chem., 280:21661.*

Pauchet, Y., Luton, F., Castella, C., Charles, J.-F., Romey, G. & Pauron, D. (2005). Effects of a mosquitocidal toxin on a mammalian epithelial cell line expressing its target receptor. *Cell. Microbiol., 7:1335.*

Klein, S., Franco, M., Chardin, P. & Luton F. (2005). AlFx affects the formation of focal complexes by stabilizing the Arf-GAP ASAP1 in a complex with Arf1. *FEBS Lett., 579: 5741.*

Luton, F. (2005). The role of EFA6, Exchange Factor for Arf6, for tight junction assembly, functions and interaction with the actin cytoskeleton. *Methods in Enzymology, 40: 332.*

Klein, S., Franco, M., Chardin, P. & Luton F. (2006). Role of the Arf6 GDP/GTP cycle and Arf6GTPase-activating proteins in actin remodeling and intracellular transport. *J. Biol. Chem., 281: 12352.*

7) References

Al-Awar, O., Radhakrishna, H., Powell, N. N., and Donaldson, J. G. (2000). Separation of membrane trafficking and actin remodeling functions of ARF6 with an effector domain mutant. Mol Cell Biol *20*, 5998-6007.

Altschuler, Y., Liu, S.-H., Katz, L., Tang, K., Hardy, S., Brodsky, F., Apodaca, G., and Mostov, K. (1999). ADP-ribosylation factor 6 and endocytosis at the apical surface of Madin-Darby canine kidney cells. J Cell Biol *147*, 7-12.

Boyer, C., Auphan, N., Luton, F., Malburet, J. M., Barad, M., Bizozzero, J. P., Reggio, H., and Schmitt-Verhulst, A. M. (1991). T cell receptor/CD3 complex internalization following activation of a cytolytic T cell clone: evidence for a protein kinase C-independent staurosporine-sensitive step. Eur J Immunol *21*, 1623-1634.

Cardone, M. H., Smith, B. L., Mennitt, P. A., Mochly-Rosen, D., Silver, R. B., and Mostov, K. E. (1996). Signal transduction by the polymeric immunoglobulin receptor suggests a role in regulation of receptor transcytosis. J Cell Biol *133*, 997-1005.

Chabre, M. (1990). Aluminofluoride and beryllofluoride complexes: a new phosphate analogs in enzymology. Trends Biochem Sci *15*, 6-10.

Chen, X., and Macara, I. G. (2005). Par-3 controls tight junction assembly through the Rac exchange factor Tiam1. Nat Cell Biol *7*, 262-269.

D'Souza-Schorey, C., and Chavrier, P. (2006). ARF proteins: roles in membrane traffic and beyond. Nat Rev Mol Cell Biol *7*, 347-358.

Deng, S., Zhou, H., Xiong, R., Lu, Y., Yan, D., Xing, T., Dong, L., Tang, E., and Yang, H. (2007). Over-expression of genes and proteins of ubiquitin specific peptidases (USPs) and proteasome subunits (PSs) in breast cancer tissue observed by the methods of RFDD-PCR and proteomics. Breast Cancer Res Treat *104*, 21-30.

Derrien, V., Couillault, C., Franco, M., Martineau, S., Montcourrier, P., Houlgatte, R., and Chavrier, P. (2002). A conserved C-terminal domain of EFA6-family ARF6-guanine nucleotide exchange factors induces lengthening of microvilli-like membrane protrusions. J Cell Sci *115*, 2867-2879.

Dow, L. E., and Humbert, P. O. (2007). Polarity regulators and the control of epithelial architecture, cell migration, and tumorigenesis. Int Rev Cytol *262*, 253-302.

Drubin, D. G., and Nelson, W. J. (1996). Origins of cell polarity. Cell *84*, 335-344.

Elliott, B. E., Meens, J. A., SenGupta, S. K., Louvard, D., and Arpin, M. (2005). The membrane cytoskeletal crosslinker ezrin is required for metastasis of breast carcinoma cells. Breast Cancer Res *7*, R365-373.

Franco, M., Peters, P. J., Boretto, J., van Donselaar, E., Neri, A., D'Souza-Schorey, C., and Chavrier, P. (1999). EFA6, a sec7 domain-containing exchange factor for ARF6,

coordinates membrane recycling and actin cytoskeleton organization. Embo J *18*, 1480-1491.

Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S., Tsukita, S., and Tsukita, S. (1993). Occludin: a novel integral membrane protein localizing at tight junctions. J Cell Biol *123*, 1777-1788.

Gassama-Diagne, A., Yu, W., ter Beest, M., Martin-Belmonte, F., Kierbel, A., Engel, J., and Mostov, K. (2006). Phosphatidylinositol-3,4,5-trisphosphate regulates the formation of the basolateral plasma membrane in epithelial cells. Nat Cell Biol *8*, 963-970.

Giffroy, D., Langendries, A., Maurice, M., Daniel, F., Lardeux, B., Courtoy, P. J., and Vaerman, J.-P. (1998). In vivo stimulation of polymeric Ig receptor-transcytosis by circulating polymeric IgA in rat liver. Int Immunol *10*, 347-354.

Guillemot, L., and Citi, S. (2006). Cingulin regulates claudin-2 expression and cell proliferation through the small GTPase RhoA. Mol Biol Cell *17*, 3569-3577.

Guillemot, L., Hammar, E., Kaister, C., Ritz, J., Caille, D., Jond, L., Bauer, C., Meda, P., and Citi, S. (2004). Disruption of the cingulin gene does not prevent tight junction formation but alters gene expression. J Cell Sci *117*, 5245-5256.

Hartsock, A., and Nelson, W. J. (2007). Adherens and tight junctions: Structure, function and connections to the actin cytoskeleton. Biochim Biophys Acta.

Hashimoto, S., Hirose, M., Hashimoto, A., Morishige, M., Yamada, A., Hosaka, H., Akagi, K., Ogawa, E., Oneyama, C., Agatsuma, T., *et al*. (2006). Targeting AMAP1 and cortactin binding bearing an atypical src homology 3/proline interface for prevention of breast cancer invasion and metastasis. Proc Natl Acad Sci U S A *103*, 7036-7041.

Hashimoto, S., Onodera, Y., Hashimoto, A., Tanaka, M., Hamaguchi, M., Yamada, A., and Sabe, H. (2004). Requirement for Arf6 in breast cancer invasive activities. Proc Natl Acad Sci U S A *101*, 6647-6652.

House, C. M., Hancock, N. C., Moller, A., Cromer, B. A., Fedorov, V., Bowtell, D. D., Parker, M. W., and Polekhina, G. (2006). Elucidation of the substrate binding site of Siah ubiquitin ligase. Structure *14*, 695-701.

Hovey, A. K., and Frank, D. W. (1995). Analyses of the DNA-binding and transcriptional activation properties of ExsA, the transcriptional activator of the *Pseudomonas aeruginosa* exoenzyme S regulon. J Bacteriol *177*, 4427-4436.

Ikenouchi, J., Furuse, M., Furuse, K., Sasaki, H., and Tsukita, S. (2005). Tricellulin constitutes a novel barrier at tricellular contacts of epithelial cells. J Cell Biol *171*, 939-945.

Jou, T.-S., and Nelson, W. J. (1998). Effects of regulated expression of mutant RhoA and Rac1 small GTPases on the development of epithelial (MDCK) cell polarity. J Cell Biol *142*, 85-100.

Jou, T.-S., Schneeberger, E. E., and Nelson, W. J. (1998). Structural and functional regulation of tight junctions by RhoA and Rac1 small GTPases. J Cell Biol *142*, 101-115.

Kim, C. J., Cho, Y. G., Park, C. H., Jeong, S. W., Nam, S. W., Kim, S. Y., Lee, S. H., Yoo, N. J., Lee, J. Y., and Park, W. S. (2004). Inactivating mutations of the Siah-1 gene in gastric cancer. Oncogene *23*, 8591-8596.

Klein, S., Franco, M., Chardin, P., and Luton, F. (2005). AlFx affects the formation of focal complexes by stabilizing the Arf-GAP ASAP1 in a complex with Arf1. FEBS Lett *579*, 5741-5745.

Klein, S., Franco, M., Chardin, P., and Luton, F. (2006). Role of the Arf6 GDP/GTP cycle and Arf6 GTPase-activating proteins in actin remodeling and intracellular transport. J Biol Chem *281*, 12352-12361.

Legendre, V., Guimezanes, A., Buferne, M., Barad, M., Schmitt-Verhulst, A. M., and Boyer, C. (1999). Antigen-induced TCR-CD3 down-modulation does not require CD3delta or CD3gamma cytoplasmic domains, necessary in response to anti-CD3 antibody. Int Immunol *11*, 1731-1738.

Letourneur, F., and Klausner, R. D. (1992). A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. Cell *69*, 1143-1157.

Liu, J., Stevens, J., Rote, C. A., Yost, H. J., Hu, Y., Neufeld, K. L., White, R. L., and Matsunami, N. (2001). Siah-1 mediates a novel beta-catenin degradation pathway linking p53 to the adenomatous polyposis coli protein. Mol Cell *7*, 927-936.

Luton, F., Buferne, M., Davoust, J., Schmitt-Verhulst, A.-M., and Boyer, C. (1994a). Evidence for protein tyrosine kinase involvement in ligand-induced TCR/CD3 internalization and surface redistribution. J Immunol *153*, 63-72.

Luton, F., Buferne, M., Legendre, V., Chauvet, E., Boyer, C., and Schmitt-Verhulst, A. M. (1997a). Role of CD3gamma and CD3delta cytoplasmic domains in cytolytic T lymphocyte functions and TCR/CD3 down-modulation. J Immunol *158*, 4162-4170.

Luton, F., Buferne, M., Schmitt-Verhulst, A. M., and Boyer, C. (1994b). Developmental control of T-cell receptor internalization. Thymus *23*, 15-25.

Luton, F., Cardone, M. H., Zhang, M., and Mostov, K. E. (1998). Role of tyrosine phosphorylation in ligand-Induced regulation of transcytosis of the polymeric Ig receptor. Mol Biol Cell *9*, 1787-1802.

Luton, F., Klein, S., Chauvin, J. P., Le Bivic, A., Bourgoin, S., Franco, M., and Chardin, P. (2004). EFA6, exchange factor for ARF6, regulates the actin cytoskeleton and associated tight junction in response to E-cadherin engagement. Mol Biol Cell *15*, 1134-1145.

Luton, F., Legendre, V., Gorvel, J. P., Schmitt-Verhulst, A. M., and Boyer, C. (1997b). Tyrosine and serine protein kinase activities associated with ligand-induced internalized TCR/CD3 complexes. J Immunol *158*, 3140-3147.

Luton, F., and Mostov, K. E. (1999). Transduction of basolateral-to-apical signals across epithelial cells: Ligand stimulated transcytosis of the polymeric immunoglobulin receptor requires two signals. Mol Biol Cell *10*, 1409-1427.

Luton, F., Verges, M., Vaerman, J. P., Sudol, M., and Mostov, K. E. (1999). The SRC family protein tyrosine kinase p62yes controls polymeric IgA transcytosis in vivo. Mol Cell *4*, 627-632.

Malissen, B., and Schmitt-Verhulst, A. M. (1993). Transmembrane signalling through the T-cell-receptor-CD3 complex. Curr Opin Immunol *5*, 324-333.

Martin-Belmonte, F., Gassama, A., Datta, A., Yu, W., Rescher, U., Gerke, V., and Mostov, K. (2007). PTEN-mediated apical segregation of phosphoinositides controls epithelial morphogenesis through Cdc42. Cell *128*, 383-397.

Matsuzawa, S. I., and Reed, J. C. (2001). Siah-1, SIP, and Ebi collaborate in a novel pathway for beta-catenin degradation linked to p53 responses. Mol Cell *7*, 915-926.

Matter, K., and Balda, M. S. (1998). Biogenesis of tight junctions: the C-terminal domain of occludin mediates basolateral targeting. J Cell Sci *111 (Pt 4)*, 511-519.

McNeil, E., Capaldo, C. T., and Macara, I. G. (2006). Zonula occludens-1 function in the assembly of tight junctions in Madin-Darby canine kidney epithelial cells. Mol Biol Cell *17*, 1922-1932.

Medina, E., Williams, J., Klipfell, E., Zarnescu, D., Thomas, G., and Le Bivic, A. (2002). Crumbs interacts with moesin and beta(Heavy)-spectrin in the apical membrane skeleton of Drosophila. J Cell Biol *158*, 941-951.

Millard, S. M., and Wood, S. A. (2006). Riding the DUBway: regulation of protein trafficking by deubiquitylating enzymes. J Cell Biol *173*, 463-468.

Mitic, L. L., and Anderson, J. M. (1998). Molecular architecture of tight junctions. Annu Rev Physiol *60*, 121-142.

Mostov, K. E., Altschuler, Y., Chapin, S. J., Enrich, C., Low, S. H., Luton, F., Richman-Eisenstat, J., Singer, K. L., Tang, K., and Weimbs, T. (1995). Regulation of protein traffic in polarized epithelial cells: the polymeric immunoglobulin receptor model. Cold Spring Harb Symp Quant Biol *60*, 775-781.

Mouchantaf, R., Azakir, B. A., McPherson, P. S., Millard, S. M., Wood, S. A., and Angers, A. (2006). The ubiquitin ligase itch is auto-ubiquitylated in vivo and in vitro but is protected from degradation by interacting with the deubiquitylating enzyme FAM/USP9X. J Biol Chem *281*, 38738-38747.

Murray, R. Z., Jolly, L. A., and Wood, S. A. (2004). The FAM deubiquitylating enzyme localizes to multiple points of protein trafficking in epithelia, where it associates with E-cadherin and beta-catenin. Mol Biol Cell *15*, 1591-1599.

Nelson, W. J. (2003). Adaptation of core mechanisms to generate cell polarity. Nature *422*, 766-774.

Nelson, W. J., and Veshnock, P. J. (1987). Ankyrin binding to (Na^+, K) ATPase and implications for the organization of membrane domains in polarized cells. Nature (Lond) *328*, 533-536.

Nijman, S. M., Luna-Vargas, M. P., Velds, A., Brummelkamp, T. R., Dirac, A. M., Sixma, T. K., and Bernards, R. (2005). A genomic and functional inventory of deubiquitinating enzymes. Cell *123*, 773-786.

Nusrat, A., Turner, J. R., and Madara, J. L. (2000). Molecular physiology and pathophysiology of tight junctions. IV. Regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells. Am J Physiol Gastrointest Liver Physiol *279*, G851-G857.

Pickart, C. M., and Cohen, R. E. (2004). Proteasomes and their kin: proteases in the machine age. Nat Rev Mol Cell Biol *5*, 177-187.

Pilot, F., Philippe, J. M., Lemmers, C., and Lecuit, T. (2006). Spatial control of actin organization at adherens junctions by a synaptotagmin-like protein Btsz. Nature *442*, 580-584.

Radhakrishna, H., Klausner, R. D., and Donaldson, J. G. (1996). Aluminum fluoride stimulates surface protrusions in cells overexpressing the ARF6 GTPase. J Cell Biol *134*, 935-947.

Randazzo, P. A., and Hirsch, D. S. (2004). Arf GAPs: multifunctional proteins that regulate membrane traffic and actin remodelling. Cell Signal *16*, 401-413.

Roh, M. S., Hong, S. H., Jeong, J. S., Kwon, H. C., Kim, M. C., Cho, S. H., Yoon, J. H., and Hwang, T. H. (2004). Gene expression profiling of breast cancers with emphasis of beta-catenin regulation. J Korean Med Sci *19*, 275-282.

Rojas, R., Ruiz, W. G., Leung, S.-M., Jou, T.-S., and Apodaca, G. (2001). Cdc42-dependent modulation of tight junctions and membrane protein traffic in polarized Madin-Darbey Canine kidney Cells. Mol Biol Cell *12*, 2257-2274.

Sabe, H., Onodera, Y., Mazaki, Y., and Hashimoto, S. (2006). ArfGAP family proteins in cell adhesion, migration and tumor invasion. Curr Opin Cell Biol *18*, 558-564.

Santelli, E., Leone, M., Li, C., Fukushima, T., Preece, N. E., Olson, A. J., Ely, K. R., Reed, J. C., Pellecchia, M., Liddington, R. C., and Matsuzawa, S. (2005). Structural analysis of Siah1-Siah-interacting protein interactions and insights into the assembly of an E3 ligase multiprotein complex. J Biol Chem *280*, 34278-34287.

Schneeberger, E. E., and Lynch, R. D. (2004). The tight junction: a multifunctional complex. Am J Physiol Cell Physiol *286*, C1213-1228.

Shah, K., Liu, Y., Deirmengian, C., and Shokat, K. M. (1997). Engineering unnatural nucleotide specificity for Rous sarcoma virus tyrosine kinase to uniquely label its direct substrates. Proc Natl Acad Sci U S A *94*, 3565-3570.

Song, W., Apodaca, G., and Mostov, K. (1994a). Transcytosis of the polymeric immunoglobulin receptor is regulated in multiple intracellular compartments. J Biol Chem *269*, 29474-29480.

Song, W., Bomsel, M., Casanova, J., Vaerman, J.-P., and Mostov, K. E. (1994b). Stimulation of transcytosis of the polymeric immunoglobulin receptor by dimeric IgA. Proc Natl Acad Sci USA *91*, 163-166.

Stevenson, B. R., Siliciano, J. D., Mooseker, M. S., and Goodenough, D. A. (1986). Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. J Cell Biol *103*, 755-766.

Taya, S., Yamamoto, T., Kanai-Azuma, M., Wood, S. A., and Kaibuchi, K. (1999). The deubiquitinating enzyme Fam interacts with and stabilizes beta-catenin. Genes Cells *4*, 757-767.

Taya, S., Yamamoto, T., Kano, K., Kawano, Y., Iwamatsu, A., Tsuchiya, T., Tanaka, K., Kanai-Azuma, M., Wood, S. A., Mattick, J. S., and Kaibuchi, K. (1998). The Ras target AF-6 is a substrate of the fam deubiquitinating enzyme. J Cell Biol *142*, 1053-1062.

Traweger, A., Fang, D., Liu, Y. C., Stelzhammer, W., Krizbai, I. A., Fresser, F., Bauer, H. C., and Bauer, H. (2002). The tight junction-specific protein occludin is a functional target of the E3 ubiquitin-protein ligase itch. J Biol Chem *277*, 10201-10208.

Tsukita, S., Furuse, M., and Itoh, M. (2001). Multifunctional strands in tight junctions. Nat Mol Cell Biol *2*, 285-293.

Valitutti, S., and Lanzavecchia, A. (1997). Serial triggering of TCRs: a basis for the sensitivity and specificity of antigen recognition. Immunol Today *18*, 299-304.

Van Furden, D., Johnson, K., Segbert, C., and Bossinger, O. (2004). The C. elegans ezrin-radixin-moesin protein ERM-1 is necessary for apical junction remodelling and tubulogenesis in the intestine. Dev Biol *272*, 262-276.

Verges, M., Luton, F., Gruber, C., Tiemann, F., Reinders, L. G., Huang, L., Burlingame, A. L., Haft, C. R., and Mostov, K. E. (2004). The mammalian retromer regulates transcytosis of the polymeric immunoglobulin receptor. Nat Cell Biol *6*, 763-769.

Wang, Q., and Margolis, B. (2007). Apical junctional complexes and cell polarity. Kidney Int. [Epub ahead of print].

Weissman, A. M. (2001). Themes and variations on ubiquitylation. Nat Rev Mol Cell Biol *2*, 169-178.

Wittinghofer, A. (1997). Signaling mechanistics: aluminum fluoride for molecule of the year. Curr Biol *7*, R682-685.

Zegers, M. M., O'Brien, L. E., Yu, W., Datta, A., and Mostov, K. E. (2003). Epithelial polarity and tubulogenesis in vitro. Trends Cell Biol *13*, 169-176.

Appendixes: selection of publications referred to in the manuscript

Luton, F., Legendre, V., Gorvel, J.-P., Schmitt-Verhulst, A.-M., & Boyer, C. (1997). Tyrosine and serine protein kinase activities associated with ligand-induced internalized TCR/CD3 complex. *J Immunol., 158:3140*

Luton, F., Vergés, M., Vaerman, J.-P., Sudol, M. & Mostov, K.E. (1999) The SRC family protein tyrosine kinase p62^{yes} controls polymeric IgA transcytosis *in vivo*. *Mol. Cell, 4 :627*

Luton, F., Klein, S., Chauvin, J.-P., LeBivic, A., Bourgoin, S., Franco, M. & Chardin, P. (2004). EFA6, Exchange Factor for Arf6, regulates the actin cytoskeleton and associated tight junction in response to E-cadherin engagement. *Mol. Biol. Cell, 15: 1134*

Klein, S., Franco, M., Chardin, P. & Luton F. (2005). AlFx affects the formation of focal complexes by stabilizing the Arf-GAP ASAP1 in a complex with Arf1. *FEBS Lett., 579: 5741.*

Klein, S., Franco, M., Chardin, P. & Luton F. (2006). Role of the Arf6 GDP/GTP cycle and Arf6GTPase-activating proteins in actin remodeling and intracellular transport. *J. Biol. Chem., 281: 12352.*