Regulation of cytokinesis and its consequences for human health

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Ithaca (1911)

As you set out for Ithaca
hope your road is a long one,
full of adventure, full of discovery.
Laistrygonians, Cyclops,
angry Poseidon - don't be afraid of them:
you' ll never find things like that on your way
as long as you keep your thoughts raised high,
as long as a rare excitement
stirs your spirit and your body.
Laistrygonians, Cyclops,
wild Poseidon - you won't encounter them
unless you bring them along inside your soul,
unless your soul sets them up in front of you.

Hope your road is a long one.
May there be many summer mornings when,
with what pleasure, what joy,
you enter harbours you're seeing for the first time;
may you stop at Phoenician trading stations
to buy fine things,
mother of pearl and coral, amber and ebony,
sensual perfume of every kind -
as many sensual perfumes as you can;
and may you visit many Egyptian cities
to learn and go on learning from their scholars.

Keep Ithaca always in your mind.
Arriving there is what you're destined for.
But don't hurry the journey at all.
Better if it lasts for years,
so you're old by the time you reach the island,
wealthy with all you've gained on the way,
not expecting Ithaca to make you rich.

Ithaca gave you the marvelous journey.
Without her you wouldn't have set out.
She has nothing left to give you now.
And if you find her poor, Ithaca won't have fooled you.
Wise as you will have become, so full of experience,
you'll have understood by then what these Ithakas mean.

Konstantinos P. Kavafis , Greek poet, 1863-1933

Table of contents

Acknowledgements **4**

List of publications **6**

Abbreviations **8**

Introduction **12**
PI3Ks Class III and PtdIns3*P* 12
The PI3K family *12*
The PI3K Class III *13*
Beclin 1 direct binding proteins *15*
Additional Beclin 1 binding proteins *18*
PtdIns3P *19*
PtdIns3P in endosomal trafficking *21*
PtdIns3P in autophagy *22*
Mechanisms of cytokinesis 24
Cytokinesis and lipids 31
Cytokinesis and cancer 34
Cytokinesis failure, aneuploidy and cancer *34*
Kinases and septins in cytokinesis and cancer *36*
Other proteins associated with cytokinesis and cancer *38*

Aims of the study **39**

Summary of included publications **40**
Paper I 40
Paper II 40
Paper III 41

Discussion **42**
Molecular mechanisms of cytokinesis 42
Impaired cytokinesis and disease 46

Experimental considerations **50**
Experimental models: cell lines and *Drosophila* 50
Confocal microscopy and quantification 51
Overexpression of proteins 51
Gene silencing using siRNA 52

Conclusions **54**

References **55**

Original publications **68**

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List of publications

I. PtdIns(3)*P* controls cytokinesis through KIF13A-mediated recruitment of FYVE-CENT to the midbody.

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Comment in:

1) Nature Cell Biology News and Views: Montagnac, G and Chavrier, P. (2010).Abscission accomplished by PtdIns(3)P. Nat Cell Biol 12:308-310.
2) Leah, E. (2010). Cytokinesis: Where PIP splits, fatty acid stops. Nature Lipidomics Gateway. doi:10.1038/lipidmaps.2010.13.

II. A tumor-associated mutation of FYVE-CENT prevents its interaction with Beclin 1 and interferes with cytokinesis.

Sagona AP, Nezis IP, Bache KG, Haglund K, Bakken AC, Skotheim RI, Stenmark H. PLoS One. 2011 Mar 24;6(3):e17086.

III. Association of CHMP4B with chromosome bridges and micronuclei: implications for cataract formation.

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Related publications not included in this thesis:

Cytokinesis and cancer.
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Divide and Pros*P*er: the emerging role of PtdIns3*P* in cytokinesis.
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*Equal contribution.

Other publications:

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Nezis,I.P., Shravage,B.V., **Sagona,A.P.**, Lamark,T., Bjorkoy,G., Johansen,T., Rusten,T.E., Brech,A., Baehrecke,E.H., and Stenmark,H. (2010). Autophagic degradation of dBruce controls DNA fragmentation in nurse cells during late Drosophila melanogaster oogenesis. J. Cell Biol. *190*, 523-531.

Nezis,I.P., Shravage,B.V., **Sagona,A.P.**, Johansen,T., Baehrecke,E.H., and Stenmark,H. (2010). Autophagy as a trigger for cell death: autophagic degradation of inhibitor of apoptosis dBruce controls DNA fragmentation during late oogenesis in Drosophila. Autophagy. *6*, 1214-1215.

Abbreviations

ADF	Actin depolymerization factor
ALFY	Autophagy-linked FYVE protein
ALIX	Apoptosis-linked gene-2 interacting protein X
Ambra 1	Activating molecule in Beclin 1 regulated autophagy protein 1
AML	Acute myeloid leukemia
Atg6	Autophagy related protein 6
Atg14 (Apg14p)	Autophagy related protein 14
Atg18	Autophagy related protein 18
Bcl-2	Apoptosis regulator Bcl-2
Bif-1	BAX-interacting factor 1
CCD	Coiled-coil domain
Cdc42	Cell division control protein 42
CDK1	Cyclin-dependent kinase 1
CENP-E	Centromere protein E
CEP55	Centrosomal protein 55 kDa
CHMPs	Charged Multivesicular body proteins/Chromatin-modifying proteins
CHO	Chinese-hamster ovary
DFCP-1	Double FYVE domain containing protein-1
DLAD	DNase II-like acid DNase
Don1	Donuts protein 1
Dpp	Decapentaplegic
EAP45	ELL-associated protein of 45 kDa
ECD	Evolutionary conserved domain
ECT2	Epithelial cell-transforming sequence 2 oncogene
EEA1	Early endosome antigen 1
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
ERM	Ezrin/radixin/moesin
ESCRT	Endosomal sorting complex required for transport
F-actin	Filamentous actin

FcγRs	Fc Receptors for Immunoglobulin G
FGD1	FYVE, RhoGEF and PH domain-containing protein 1
FGD3	FGD1 family member 3
FIP3	Rab 11 family-interacting protein 3
FYVE	Fab1, YOTB, Vac1 and EEA1
FYVE-CENT	FYVE domain containing centrosomal protein
GAP	GTPase-activating protein
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GLUE	GRAM-Like Ubiquitin-binding in EAP45
GPCRs	G-protein-coupled-receptors
H2B	Histone 2B
HMGB1	High mobility group protein B1
HRS	Hepatocyte growth factor-regulated tyrosine kinase substrate
ICP34.5	Neurovirulence factor ICP34.5
ILVs	Intraluminal vesicles
INCENP	Inner centromere protein
IP(3)R	Inositol 1,4,5-triphosphate receptor
IST1	Increased sodium tolerance
KIF4	Kinesin family member 4
KIF13A	Kinesin family member 13A
KIF14	Kinesin family member 14
LPA	Lipoprotein A
M2	Matrix protein 2
MCAK	Mitotic centromere-associated kinesin
MgcRacGAP	Male germ cell Rac GTPase-activating protein 1
MIM domain	MIT domain interacting motif
MIT domain	Microtubule-interacting and trafficking molecules domain
MKLP1	Mitotic kinesin-like protein 1
MLC	Myosin light chain
MVBs	Multivesicular bodies
MYPT1 (MBS)	Protein phosphatase 1 regulatory subunit 12A (PPP1R12A)
Nef	Negative Regulatory Factor
nPist	Neuronal isoform of protein-interaction with TC10

NuSAP	Nucleolar-spindle associated protein
OCRL	Oculocerebrorenal syndrome of Lowe
PE	Phosphatidylethanolamine
PI K3C3	Class III PI(3)Kinase
PI3Ks	Phosphoinositide 3-kinases
PINK1	PTEN-induced putative kinase 1
PLK1	Polo like kinase 1
PM	Plasma membrane
PRC1	Protein regulator of cytokinesis 1
PtdIns	Phosphatidylinositol
PtdIns3*P*	Phosphatidylinositol 3-phosphate
PtdIns(3,4,5)P_3	Phosphatidylinositol 3,4,5-triphosphate
PtdIns(4,5)P_2	Phosphatidylinositol 4,5-biphosphate
PtdIns5*P*	Phosphatidylinositol 3-phosphate
PX domain	Phox homology domain
Rab5	Ras-related protein Rab5
Raf-1	Raf proto-oncogene serine/threonine-protein kinase
RhoA	Ras homolog gene family member A
ROCK	Rho-associated protein kinase
RTKs	Receptor tyrosine kinases
RUN domain	Domain involved in Ras-like GTPase signaling
SAC	Spindle assembly checkpoint
SARA	SMAD Anchor for Receptor Activation
SCCHN	Squamous cell cancer of the head and neck
SEPT2	Septin 2
SEPT9	Septin 9
SEPT12	Septin 12
SLAM	Signaling lymphocytic activation molecule
SNARE	SNAP (Soluble NSF Attachment protein) Receptor
SLAN	Suppressed in Lung Cancer
SOPs	Sensory organ precursors
SPT	Serine palmitoyl transferase
TSG101	Tumor susceptibility gene 101
TTC19	Tetratricopeptide Repeat Protein 19

UVRAG	UV radiation resistance-associated gene
VLCFAs	Very-long-chain-fatty-acids
VMP1	Vacuole membrane protein 1
Vps15	Vacuolar protein sorting 15
Vps30	Vacuolar protein sorting 30
Vps34	Vacuolar protein sorting 34
Vps38	Vacuolar protein sorting 38
VPS4	Vacuolar protein-sorting-associated protein 4
WIPI-1	WD-repeat domain, phosphoinositide interacting protein 1

Introduction

During the last decades, many sciences have benefitted enormously from the immense technological development. Especially when it comes to biology, the progress is so great that one could say that the 21st century belongs to the biological sciences. With the contribution of computational technology and all kinds of microscopy, important knowledge has been gained concerning the plethora of biological processes that take place within the cell. This is a very important aspect, since almost all human diseases have a cellular basis, therefore, the more is known about the cellular environment the easier each kind of disease can be approached. The scientific questions to be answered are many, but a very intriguing one is the understanding of what cellular programmes orchestrate cell division and how the defects in these processes are linked to cancer. This thesis contributes to address one relevant issue. How is the final step of cell division – cytokinesis – regulated?

PI3Ks Class III and PtdIns3*P*

The PI3K family

The phosphoinositide 3-kinase (PI3K) family, is a family of enzymes that catalyzes the transfer of the γ-phosphate group of ATP to the 3' hydroxyl position of the phosphatidylinositol ring.They can be divided in three classes, according to their selective substrate specificity (Wymann and Pirola, 1998; Backer, 2008).

PI3Ks class I use phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P_2) as preferred substrate, leading to the generation of phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3). They consist of a 110-kDa catalytic subunit (p110α, β, γ, δ) in complex with a regulatory subunit. The subclass IA catalytic subunits (p110α, β and δ) are bound to a p85 regulatory subunit, of which there are five species (p85α, p85β, p55α, p55γ and p50a). They are activated by receptor tyrosine kinases (RTKs) or receptors for immunoglobulin G (FcγRs). The subclass IB catalytic subunit p110γ binds to p101 and p84 (non-p85 regulatory subunits) and is activated by GPCRs.

This class of the PI3K family plays important role in growth control, cell cycle progression and migration.

PI3Ks class II use phosphatidylinositol (PtdIns) as substrate, yielding the product phosphatidylinositol 3-phosphate (PtdIns3*P*). These kinases consist of the catalytic subunits PI3K-C2α, β, γ and are activated by external stimuli such as lipoprotein A (LPA) and insulin receptors. The biological role of this class is not clear yet (Lindmo and Stenmark, 2006; Kok et al., 2009; Backer, 2008).

Class III PI3K, which has been studied in this thesis, is described below.

The PI3K Class III

The class III PI3Ks (PIK3C3) are the only ones conserved from lower eukaryotes to plants and mammals and they represent the most ancient form of PI3Ks (Lindmo and Stenmark, 2006). The yeast homologue of class III PI3K, Vps34 (vacuolar protein sorting 34), was first described as a component of the vacuolar protein sorting machinery in *Saccharomyces cerevisiae* and is the only PI3K in yeast. The substrate of this enzyme is exclusively PtdIns, so its product in cells is PtdIns3*P*. The main reason for this high substrate specificity is that Vps34 lacks the positively charged KRER sequence, which is present in the other PI3Ks. This peptide is located in the putative substrate binding loop where it could interact with the two additional phosphate groups in the inositol ring of PtdIns(4,5)P_2. By contrast to other PI3Ks, , this region of Vps34 is relatively uncharged, thus limiting Vps34 substrates only to PtdIns (Volinia et al., 1995; Backer, 2008; Miller et al., 2010).

The class III PI3Ks consist of several subunits: the catalytic subunit (Vps34/PIK3C3), the regulatory subunit (Vps15/p150) and the accessory subunits [Vps30(Atg6)/Beclin 1], and [Atg14(Apg14p) or Vps38] (Lindmo and Stenmark, 2006). More specifically, in yeast Vps34 forms at least two multi-subunit complexes with different function: one that contains Vps15, Vps30 and Atg14 and regulates autophagy and the other that contains Vps15, Vps30 and Vps38 and sorts vacuolar proteins such as Carboxypeptidase Y at the *trans*-Golgi network and delivers them to the vacuole (Kihara et al., 2001; Funderburk et al., 2010). Both complexes contain Vps34 and Vps15, a Vps34 regulatory protein, as common factors. The sequence of Vps15 suggests that it functions as a protein kinase, even though it lacks important motifs found in other protein kinases,

such as the canonical GXGXXG motif, which is involved in ATP binding (Backer, 2008). Vps15 anchors the complex to the membrane where Vps34 is recruited and then activated by Vps15.

The complex that regulates autophagy contains Atg14, which localizes to vacuolar membranes and the pre-autophagosomal structure and is important for autophagosome formation (Suzuki and Ohsumi, 2007).The complex that is important for sorting of vacuolar proteins, contains Vps38, which localizes to the vacuolar membranes and endosomes (Funderburk et al., 2010) (Figure 1). Atg14 and Vps38 interact with Vps30/Atg6 in a competitive manner in order to commit the PI3K complex for autophagy or vacuolar sorting.

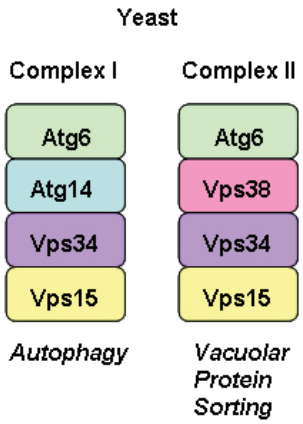

Figure 1. Vps34 complexes in yeast. In yeast there are two Atg6-Vps34-Vps15 complexes, I and II, which regulate autophagy and vacuolar protein sorting respectively.

In mammals, in a similar manner to yeast, Vps34/PIK3C3 (VPS34) makes a complex with Vps15/p150/PIK3R4. Vps34/PIK3C3 binds to Vps30/Beclin 1 via its evolutionary conserved domain (ECD) (244-337 αα), a domain that is suggested to be essential for autophagy and its tumor suppressor function (Furuya et al., 2005). Beclin 1 further on serves as a platform for the recruitment of other proteins (Figure 2).

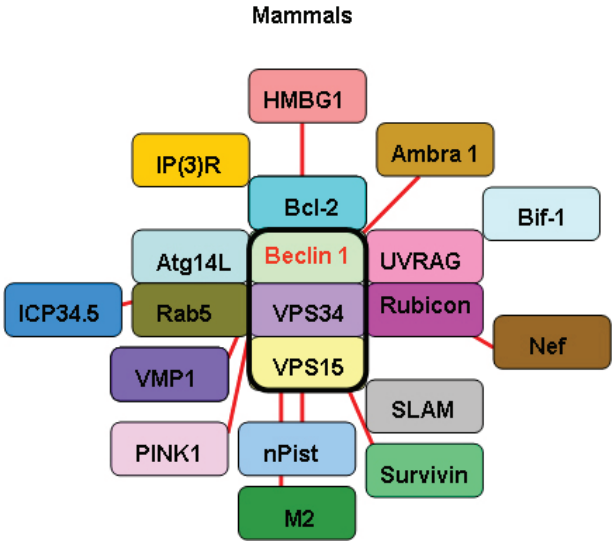

Figure 2. VPS34 complexes in mammals. In mammals, multiple Beclin 1-VPS34 complexes exist: There is the core complex Beclin 1-VPS34-VPS15, the stable binding partners UVRAG, Atg14L and Rubicon and the more peripherally associated binding partners including Bcl-2 family members, IP(3)R, Rab5, Nef, Bif-1, SLAM, Survivin, nPist, VMP1, PINK1, M2, HMBG1, ICP34.5 and Ambra 1. Among those, IP(3)R interacts with Bcl-2 and Bif-1 interacts with UVRAG.

Beclin 1 direct binding proteins

Beclin 1 is a tumor suppressor and consists of a BH3-only domain, a central coiled-coil domain (CCD) and an evolutionary conserved domain (ECD). A function for Beclin 1 in tumor suppression is further supported by the identification of additional Beclin 1 interacting proteins

(Cao and Klionsky, 2007). One of these is UVRAG, a protein with tumor suppressor activity like Beclin 1. UVRAG (UV radiation resistance-associated gene) has partial sequence similarity to Vps34, and it has been suggested that UVRAG could be a functional counterpart of Vps38 (Itakura et al., 2008). It interacts in a direct way with Beclin 1 through its CCD domain (Liang et al., 2006). The function of UVRAG and its effective role in the VPS34-VPS15-Beclin 1 complex are controversial. Even though it is clear that UVRAG plays a role in the endocytic pathway, the extent to which UVRAG, particularly as a subunit of the PI3KC3 complex functions in autophagy regulation is not clear (Funderburk et al., 2010).

Another protein that interacts directly with Beclin 1 is Atg14L/Barkor. This protein was discovered by sequence-homology searching and has been identified as a putative mammalian homolog for yeast Atg14 (Itakura et al., 2008). It contains two coiled-coil domains that are necessary for binding to the CCD regions of Beclin 1 and VPS34. Atg14L/Barkor is required for autophagosome formation and it promotes the ability of the PI3K class III complex to positively regulate autophagy (Funderburk et al., 2010).

Recently, one more protein was found to interact and form a stable complex with Beclin 1, named Rubicon, based on the conserved RUN domain (domain involved in Ras-like GTPase signalling) that it contains near the N- terminus. Rubicon also contains a cysteine-rich domain near the carboxy terminus and a central CCD region which is crucial for the binding of the protein to both VPS34 and Beclin 1. Rubicon is found to be in the same complex with UVRAG when binding to Beclin 1 and it also seems that it can bind to the core complex only in the presence of UVRAG, suggesting that Rubicon interacts with Beclin 1 via UVRAG. Concerning its function, Rubicon downregulates autophagy and in contrast to Atg14L it seems to prevent autophagosome maturation. It has also been found to decrease VPS34 activity and this effect does not require Beclin 1. Finally, Rubicon also participates in the endocytic pathway, negatively regulating the function of the complex in endosomal trafficking, even though it is not clear if this role is dependent or not of Beclin 1 (Zhong et al., 2009; Funderburk et al., 2010) (Figure 3).

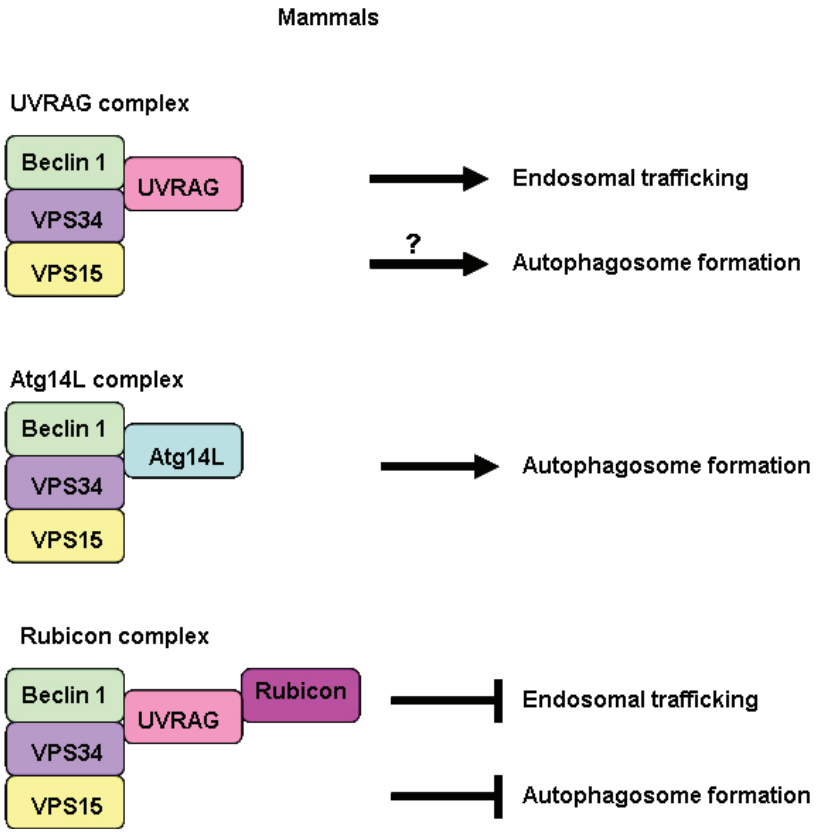

Figure 3. Function of Beclin1-VPS34 complexes in mammals. In mammals, three Beclin 1-VPS34 complexes function in autophagy and endocytic trafficking. UVRAG complex has a possible positive role in both processes, Atg14L complex functions in the formation of autophagosomes and Rubicon complex functions negatively in both processes.

Additional Beclin 1 binding proteins

All the proteins described above were detected to be interactors of Beclin 1 under the same experimental conditions. More specifically, Beclin 1-EGFP protein complexes were isolated by affinity purification from various mouse tissues and the above interacting proteins were identified using mass spectrometry, suggesting that they form stable complex with Beclin 1 (Zhong et al., 2009). There are though other proteins as well that even interact indirectly with Beclin 1 or have a more loose association with the complex but still have an effect in its regulation (Funderburk et al., 2010; Kang et al., 2011). These proteins are the following:

Bif-1 (Endophilin B1) interacts with Beclin 1 via UVRAG (Takahashi et al., 2007). It functions as a positive regulator of VPS34 activity and promotes the induction of autophagy and the formation of autophagosomes in mammalian cells (Kang et al., 2011). Ambra1(activating molecule in Beclin 1 regulated autophagy protein 1), another interacting protein of Beclin1, is required for Beclin 1 activity, favours the Beclin1-VPS34 interaction and is regarded as a key factor in autophagy regulation (Fimia et al., 2007). nPIST, neuronal isoform of protein-interaction with TC10, has also been found in a yeast two-hybrid study to interact with Beclin 1 and can act synergistically with Beclin 1 to induce autophagy (Yue et al., 2002). IP(3)R (inositol 1,4,5-triphosphate receptor) is a membrane glycoprotein complex, activated by IP3 that acts as a Ca^{2+} channel. It interacts with Beclin 1 and represses autophagy through Bcl-2-mediated sequestration of Beclin 1 (Vicencio et al., 2009). VMP1 (vacuole membrane protein 1), the pancreatitis-associated protein, interacts with Beclin 1 via its hydrophilic C-terminal region (Atg domain). It is important for autophagy induction and autophagosome formation. HMGB1(high mobility group protein B1), a chromatin-associated nuclear protein, also binds to Beclin 1 and has an important role in cross-regulating apoptosis and autophagy. PINK1 (PTEN-induced putative kinase 1) is a serine/threonine protein kinase that localizes to mitochondria. This protein as a full length interacts with Beclin 1 and promotes autophagy (Kang et al., 2011). SLAM (signalling lymphocytic activation molecule) is microbial sensor found to interact with VPS34-VPS15-Beclin1 complex mainly through interaction with Beclin 1. It functions in autophagy and in immune cell killing of Gram-negative bacteria through the phagosome (Berger et al., 2010). Survivin is a member of the inhibitor of apoptosis protein family and is a novel interactor of Beclin 1 and via this interaction provides possible mechanism regulating the cross-talk between apoptosis and autophagy (Niu et al., 2010).The endosomal protein Rab 5 also interacts with Beclin 1, but only in the presence of Vps34, suggesting that Rab5 is part of the complex that contains Vps34 and Beclin 1. Rab5 is an

activator of Vps34 and as it seems that it plays an important role both in the autophagy and endocytosis function of PIK(3)C3 complex (Ravikumar et al., 2008; Christoforidis et al., 1999).

Furthermore, Beclin 1 interacts with Bcl-2 family members. Beclin 1 was first identified as a Bcl-2 interacting protein in a yeast two-hybrid screen (Liang et al., 1998). The Bcl-2 family of proteins are important regulators of apoptosis and contain both anti- and pro-apoptotic members. The anti-apoptotic members, Bcl-2, Bcl-$_{XL}$, Bcl-w, Mcl-1 and Bfl-1 protect cells from apoptosis and contain BH domains, designated BH1, BH2, BH3 and BH4. The pro-apoptotic members of the family can be divided into those which contain two or three BH domains and those who contain only BH3 domain. Beclin 1 binds to several anti-apoptotic Bcl-2 proteins such as Bcl-2, Bcl-$_{XL}$, Bcl-w and weakly to Mcl-1 and via this interactions the autophagic function of Beclin 1 is inhibited (Erlich et al., 2007).

Finally, Beclin 1 has recently been found to interact with several pathogen-derived proteins, such as vBcl-2 of γ-herpesviruses, ICP34.5 of herpes simplex viruses, M2 (matrix protein 2) of influenza and Nef (negative regulatory factor) of HIV. In all the cases, the function of this interaction is the promotion and maturation of autophagosome formation in different pathogens, thus regulating host response in immunologic defence (Kang et al., 2011).

PtdIns3*P*

The PI3K class III complex regulates several essential cellular processes through the downstream effects of its catalytic product phosphatidylinositol 3-phosphate (PtdIns3*P*). One important step in the understanding of how PI3K class III and its catalytic product controls cellular functions was accomplished with the identification of the domains that bind to PtdIns3*P*. These domains include the FYVE domain, named by the first four proteins known to contain the domain (conserved in Fab1, YOTB, Vac1 and EEA1) and the PX domain, named by the Phox homology domain of the p47 ^{phox} and p40 ^{phox} subunits of the phagocyte NADPH oxidase. The FYVE finger domain was initially identified as a cysteine-rich motif at the C-terminus of EEA1 (early endosome antigen 1), able to bind two Zn^{2+} ions and important for the localization of EEA1 to early endosomes (Stenmark et al., 1996). It binds exclusively to PtdIns3*P*, whereas the PX domain even though it binds preferentially to PtdIns3*P*, binds also to other PIs, such as PtdIns(3,4)P_2 (Backer,

2008; Stenmark, 2010). Around 30 FYVE-domain containing proteins and 45 PX domain containing proteins have been identified, and are regarded to mediate most of the downstream functions of PtdIns3*P*. There are also additional proteins, such as Proppin/WIPI proteins that even though they do not contain FYVE or PX (Phox homology) domains, are able to bind to PtdIns3*P*, via a WD40-repeat-containing β-propeller structure and certain variant pleckstrin homology domains such as the GLUE (GRAM-Like Ubiquitin-binding to EAP45) domain (Stenmark, 2010).

The identification of PtdIns3*P*-binding domains was important for the design of probes that reveal the intracellular distribution of this lipid. One such probe was constructed, consisting of two PtdIns3 -binding FYVE domains (2XFYVE). The FYVE finger domain for this purpose derived from HRS protein and was placed in tandem so as to have greater avidity for PtdIns3*P*. The ability of 2XFYVE to be easily transfected into cells as a fusion with EGFP or another tag or expressed in bacteria and purified as a recombinant probe that can be used directly on fixed specimens, makes it very useful in studying the localization of PtdIns3*P* (Gillooly et al., 2000; Stenmark, 2010). Other probes have been constructed as well, using the FYVE domain of various FYVE domain-containing proteins such as SARA (SMAD Anchor for Receptor Activation), EEA1 and FGD1(FYVE, RhoGEF and PH domain-containing protein 1) (Hayakawa et al., 2004) or even the PX domain of certain proteins such as NADPH (Scott et al., 2002). All the above probes give comparable results, even though the 2XFYVE has been tested more thoroughly. Based on studies using 2XFYVE, both by fluorescence and electron microscopy, PtdIns3*P* localizes at early endosomes and intralumenal vesicles of multivesicular endosomes (Gillooly et al., 2000). It also localizes on the autophagosomes (weakly on the outer surface of autophagosome membranes and strongly in the inner membranes of autophagosomes), upon starvation in yeast cells (Obara et al., 2008). In mammalian cells upon amino acid starvation PtdIns3*P* localizes in membranes dynamically connected to the ER, thought to be involved in autophagosome biogenesis (Axe et al., 2008). PtdInd3*P* has an important function in various cellular processes, such as endosomal trafficking and autophagy, which will be analyzed below.

PtdIns3*P* in endosomal trafficking

The importance of PtdIns3*P* in endocytic trafficking was first revealed during the study of Golgi to vacuole/lysosome trafficking in yeast. It was obvious from this study that Vps34 appears to regulate intracellular protein trafficking decisions (Schu et al., 1993; Wurmser et al., 1999). Later on it was proven that PtdIns3*P* plays also an important role in membrane trafficking in mammals. The fungal metabolite wortmannin as well as the compound LY294002, both PI3K inhibitors, have been shown to inhibit homotypic endosome fusion in vitro (Jones and Clague, 1995). In this process, activated Rab5 is required, since it recruits complex of proteins including PI3K class III, that may play a role in activating SNARE (SNAP Receptor) protein complexes for membrane fusion (Roth, 2004). Among these, EEA1, Vac1/Rabenosyn-5 and Rabankyrin-5 play important role in this process. Even though all of these are required for efficient homotypic endosome fusion in vitro, EEA1 seems to be the most important for the heterotypic fusion of endosomes with early endosomes (Lindmo and Stenmark, 2006). Taking all these data together, it is suggested that PtdIns3*P* identifies the destination membrane for fusion of incoming vesicles through the assembly of a protein complex on the endosomes that would tether the incoming vesicle and participate in the fusion (Roth, 2004).

Following this idea, the roles of PtdIns3*P* and EEA1 were also investigated in phagosome maturation and as it was proven they are both essential molecules for phagosomal maturation (Fratti et al., 2001). This suggests a role for PtdIns3*P* in trafficking of internalized pathogens as well.

PtdIns3*P* is also important for another step in endocytic trafficking, the proper sorting of certain membrane proteins from endosomes to lysosomes (Stenmark, 2010). Very important for the understanding of this role of PtdIns3*P*, was the discovery of the ESCRT (endosomal sorting complex required for transport) machinery. The ESCRT machinery consists of four complexes, ESCRT-0, -I, -II and –III. This machinery initially recognizes ubiquitilated cargoes (e.g activated growth factor receptors) in the endosome membrane and prevents their recycling and retrograde trafficking. Next, it deforms the endosomal membrane allowing cargo to be sorted into endosomal invaginations and forms ILVs (intraluminal vesicles) that contain the sorted cargo, catalysing in that way the final abscission of the endosomal invaginations (Raiborg and Stenmark, 2009). More specifically, ESCRT-0 which contains ubiquitin-binding domains has a role in the clustering of

ubiquitylated cargo, whereas ESCRT-I and –II, which also contain ubiquitin-binding domains, are important for inducing inward budding of the endosome membrane. Once a bud is created, its separation from the limiting membrane requires scission. This is what ESCRT-III does: ESCRT-III is recruited from ESCRT-I and –II and catalyses the scission of membrane necks (Wollert and Hurley, 2010; Hurley and Hanson, 2010). PtdIns3*P* is required for the membrane recruitment of various subunits of the ESCRT machinery. One such example is Vps27/HRS (hepatocyte growth factor-regulated tyrosine kinase substrate), which is a subunit in the ESCRT-0 complex and has the ability to bind PtdIns3*P* via its FYVE domain (Gaullier et al., 1998; Burd and Emr, 1998). In this way, PtdIns3*P* binding recruits HRS and as a result ESCRT-0 complex to endosomal membranes (Raiborg et al., 2001). Vps27/HRS in turn recruits ESCRT-I via its interaction with the ESCRT-I Vps23/TSG101 (tumor susceptibility gene 101) subunit (Bache et al., 2003; Katzmann et al., 2003; Lu et al., 2003). Furthermore, Vps36/EAP45 (ELL-associated protein of 45 kDa) ESCRT-II subunit contains a GLUE domain which has the ability to bind PtdIns3*P* and is also important for the membrane recruitment of ESCRT-II (Slagsvold et al., 2005; Teo et al., 2006). Taking these data together, the contribution of PtdIns3*P* in ESCRT machinery recruitment and thereby in sorting is very important.

PtdIns3*P* in autophagy

The VPS34 complex and is catalytic product PtdIns3P are also involved in autophagy. But how does PtdIns3*P* regulate this process? Upon starvation PtdIns3*P* localizes on the autophagosomes in yeast cells (Obara et al., 2008) and in membranes dynamically connected to the ER in mammalian cells which are thought to be involved in autophagosome biogenesis (Axe et al., 2008). These observations are linked nicely with the identification of DFCP-1 (double FYVE domain containing protein-1). This protein has a FYVE domain that binds to PtdIns3*P*, it translocates from the Golgi to the ER during starvation and it forms DFCP-1 specific structures, called omegasomes, which colocalize with autophagic markers (e.g LC3) and ER markers upon starvation. Based on these data, it has been suggested that DFCP-1 positive ER membranes are important for the formation of the phagophore and thus autophagosome formation. Furthermore, PtdIns3*P* generation seems to be very critical for this process and it is suggested as a regulator of the autophagosome biogenesis pathway, by being the determining factor for the localization of autophagosome induction (Axe et al., 2008; Tooze et al., 2010).

Another PtdIns3*P*-binding protein associated with autophagy is the mammalian homologue of yeast Atg18, WIPI-1 (WD-repeat domain, phosphoinositide interacting protein 1). This protein, localizes to endosomal and Golgi membranes, but is also recruited to autophagic membranes in a PtdIns3*P*-dependant manner. Moreover, depletion of Jumpy, a PtdIns3*P* phosphatase, results in accumulation of WIPI-1 on autophagic membranes. It has also been found that yeast Atg18 together with its homologues regulate autophagy via PtdIns3*P*, so it is suggested that Atg18 and its mammalian homologues might work as PtdIns3*P* sensors, by regulating PtdIns3*P* levels and thereby autophagy (Simonsen and Tooze, 2009).

Finally, the mammalian PtdIns3*P*-binding protein ALFY (autophagy-linked FYVE protein) has also been found to play a role in autophagy and specifically in the selective degradation of protein aggregates (Simonsen et al., 2004; Filimonenko et al., 2010; Clausen et al., 2010). ALFY is a huge protein, which contains 3527 amino acids residues and has very important functional domains in its C terminus region: a BEACH domain followed by a series of WD40 repeats and a PtdIns3*P*-binding FYVE domain (Simonsen et al., 2004). ALFY, even though it contains a FYVE domain, is not found on endosomes but instead localizes to the nuclear envelope. Additionally, upon starvation or proteasomal inhibition, ALFY relocalizes to cytoplasmic structures located close to autophagic membranes and ubiquitin-containing protein aggregates, and based on electron microscopy studies similar structures can be found within autophagosomes (Simonsen et al., 2004). An interesting aspect is that ALFY interacts physically with PtdIns3*P*, Atg5 and p62 and via this interaction participates dynamically in the selective degradation of aggregated proteins such as poly-glutamine-containing mutant huntingtin (Filimonenko et al., 2010). In conclusion, ALFY can be regarded as a scaffold receptor for recruitment of misfolded, ubiquitinated proteins to the autophagosomal membrane that become degraded by autophagy (Filimonenko et al., 2010; Clausen et al., 2010). Taken together the above data highlight the important role of PtdIns3*P* in regulation of autophagy.

Mechanisms of cytokinesis

The cell cycle is divided in interphase and mitosis. Interphase consists of the phases G1, S - during which the DNA synthesis takes place- and G2. Mitosis is divided into prophase, metaphase, anaphase, telophase and cytokinesis. Cytokinesis [derived from the Greek words *cyto*-(cell) *and kinesis* (motion)] is the final step of the cell cycle, during which the two daughter cells separate completely (Sagona and Stenmark, 2010). In animal cells, cytokinesis can be divided into the following four stages: specification of the cleavage plane, ingression of the cleavage furrow, formation of the midbody and abscission (Normand and King, 2010) (Figure 4).

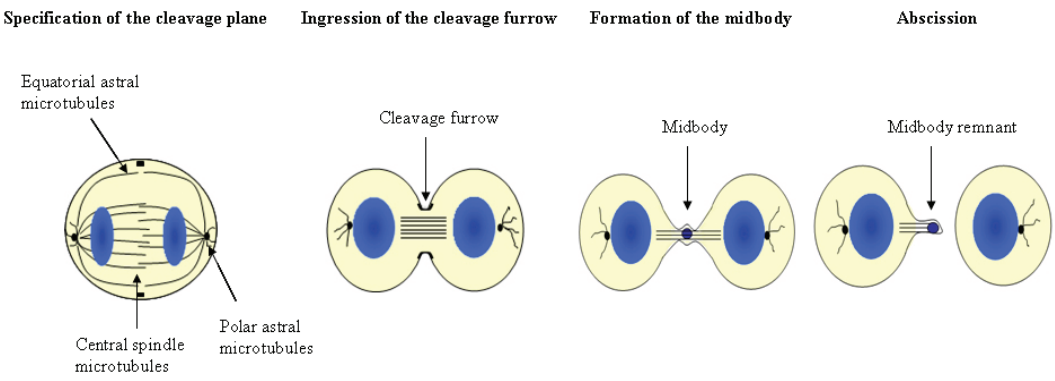

Figure 4. Schematic diagram of the different stages of cytokinesis. Cytokinesis can be divided in 4 different stages: specification of the cleavage plane, ingression of the cleavage furrow, formation of the midbody and abscission.

The first stage of cytokinesis (specification of the cleavage plane) is regulated by various subpopulations of microtubules (equatorial astral microtubules, polar astral microtubules and central spindle microtubules), who deliver positive signals that initiate furrowing at the correct place in the cell. An important event that triggers this process is the activation of the small GTPase RhoA at the site of the cleavage furrow (Normand and King, 2010). There are various activators of RhoA which are discussed below.

First, ECT2 (epithelial cell-transforming sequence 2 oncogene), a guanine nucleotide exchange factor, is an important activator of RhoA. ECT2 localizes to the central spindle by binding to centralspindlin complex (consisting of MKLP-1 and CYK-4/MgcRacGAP, which contains a GAP domain for Rho GTPases). ECT2 interacts with CYK-4 in a cell cycle regulated

manner and CYK-4 can act as an activator of ECT2, but both of them are necessary for RhoA localization (Yuce et al., 2005). In addition FIP3 (Rab 11 family-interacting protein 3), a class II Rab11 family interactive protein binds to CYK-4 in a region that overlaps with the ECT2 binding region and both the proteins form exclusive complexes with CYK-4. Importantly, removal of ECT2 from the centralspindlin complex at late telophase results to the recruitment of FIP3-containing endosomes to the cleavage furrow (Simon et al., 2008). Thus, this complex of proteins regulates cleavage furrow ingression and further on abscission, that will be discussed later.

Other proteins that regulate RhoA activity during cytokinesis include RhoGEFs (such as GEF-H1 and Myo GEF)(Birkenfeld et al., 2007; Wu et al., 2006), but also the armadillo protein p0071(Wolf et al., 2006) and the Rho effector mDia1(Kitzing et al., 2007) are important for RhoA activation. Also, the kinases Aurora B and Polo are involved in the positive signal delivered in microtubules (Eggert et al., 2006). Aurora B together with the inner centromere protein INCENP, Survivin and Borealin are part of the chromosomal passenger complex. This complex associates with chromatin in early mitosis, concentrates at the centromere in prometaphase and metaphase and then transfers to the central spindle in anaphase and is important for several steps during cytokinesis (Barr and Gruneberg, 2007).

Finally, certain proteins are essential for the inactivation of RhoA, a necessary process during the late stages of cytokinesis for the cytokinesis completion. These include CYK-4/MgcRacGAP and p190RhoGAP (Su et al., 2003). The first one as it seems has a double role, by both activating RhoA via the recruitment and activation of ECT2, but after phosphorylation by Aurora kinases, it acts as a RhoGAP, thus participating in RhoA inactivation (Minoshima et al., 2003). Furthermore, it serves as an inhibitor of GTPase Rac, necessary for the completion of cytokinesis (Yoshizaki et al., 2004).

The second stage of cytokinesis is the ingression of the cleavage furrow, which separates the two daughter cells at the end of cell division. This is driven by the assembly and contraction of actomyosin filaments that form a contractile ring. In order for successful cytokinesis to be achieved, the actomyosin filaments need to be well organized and this is accomplished by a network of cytoskeletal proteins built at the cleavage site which act as a scaffold for actomyosin filaments and connect them to plasma membrane (D'Avino, 2009). The proteins that initially participate in this process are myosin II and actin which form the contractile ring and together with formins generate the force needed for furrow ingression (Schiel and Prekeris, 2010). Myosin II (myosin) is the principal actin-dependent motor protein required for cytokinesis. Its activity and localization are regulated by phosphorylation of its regulatory light chain (MLC). More

specifically, phosphorylation of serine 19 of MLC stimulates actin-activated ATPase activity of myosin, whereas phosphorylation at threonine 18 promotes myosin assembly (Matsumura et al., 1998; Normand and King, 2010). The phosphorylation at positions 18 and 19 of MLC is accomplished via three kinases: ROCK kinase (Kosako et al., 2000), Citron kinase (Yamashiro et al., 2003) and MLC kinase (MLCK) (Chew et al., 2002). They all localize to the cleavage furrow and the two first are activated by RhoA, whereas the latter by calcium/ calmodulin.

MLC phosphorylation is affected also by the activity level of myosin phosphatase. This enzyme consists of a subunit that binds to myosin (MYPT1 or MBS), a catalytic subunit (the delta isoform of PP1c) and an additional small subunit (Kawano et al., 1999). Myosin phosphatase is inhibited during cytokinesis in order to favour MLC phosphorylation by various ways: it can be inactivated by both ROCK and Aurora B via the phosphorylation of MYPT1(Yokoyama et al., 2005), but also by other kinases, like Raf-1(Broustas et al., 2002).

Further on, actin is a key protein for this process. Actin, as mentioned above, is part of the contractile ring, where it participates in a polymerized form and its polymerization is promoted by RhoA. Once the actomyosin ring is fully constricted, the cell must undergo a process of disassembling the actomyosin ring so as the furrow ingression to start. A very important step for furrow ingression to be triggered is the actin depolymerization. The precise mechanism of this process is not clear, but it seems that very important role play proteins from the ADF-cofilin family (twinstar in *Drosophila*), which depolymerize actin (Schiel and Prekeris, 2010). The recruitment of actin to the furrow occurs by transport of filaments from elsewhere or by nucleation in the furrow and this is accomplished by formins. Most specifically, Diaphanous, a conserved forming essential for cytokinesis, functions in the nucleation of actin filaments, in a process activated by RhoA. It is not clear whether it acts in furrows or nucleates elsewhere, followed by transport of filaments to the furrow, but the role of formins in cytokinesis is established and need further investigation (Eggert et al., 2006).

In order to achieve successful cytokinesis, actomyosin filaments are assembled upon a network of cytoskeletal proteins at the cleavage site which acts as a scaffold by connecting the filaments to the plasma membrane (D'Avino, 2009). A key protein that plays that role is anillin, a highly conserved multidomain protein that interacts with cytoskeletal components as well as their regulators. Anillin interacts with F-actin, where it is supposed to act as a furrow ingression crosslinker for F-actin in furrows. It also interacts indirectly with myosin II and directly with non muscle myosin II as identified in *X. laevis* and this interaction stabilizes myosin localization at equatorial plane. It also interacts with RhoA, in an interaction via which Anillin regulates RhoA localization on one hand and on the other hand activation of RhoA is required for the localization

of Anillin to the furrow. This is further confirmed with the interaction of Anillin with ECT2, since via this interaction RhoA activation and localization is further stabilized. Moreover, Anillin interacts with septins, a family of proteins that will be analysed below as well as with many other proteins that will not be mentioned here (Piekny and Maddox, 2010). Septins, a family of GTP-binding proteins, also are scaffolding proteins that interact with Anillin and contribute to the organization of the various components of the cleavage furrow (Tasto et al., 2003). They can form filaments, they localize to the contractile ring and several members including SEPT2, SEPT9 and SEPT12, are implicated in regulation of cytokinesis. This is accomplished via their interaction with Anillin, but also via their ability to regulate actin and microtubule dynamics (Normand and King, 2010). In addition, SEPT2 containing filaments are suggested to provide a molecular platform for myosin and its kinases, so as to ensure the full activation of myosin that is essential for cytokinesis (Joo et al., 2007). Finally, septins may form a barrier that restricts the diffusion of membrane proteins in the furrow and in that way activated RhoA is retained within the narrow zone that is required for successful initiation of cytokinesis (Schmidt and Nichols, 2004). Taken together the above data show that anillin is important for the organization and recruitment of the structural components of the contractile ring, but also has the ability to link these components to signalling proteins that regulate cytokinesis.

The third step of cytokinesis is the formation of the midbody. According to Steigemann and Gerlich, midbody (also termed stembody or Flemming body) is the central region of the intercellular bridge, where overlapping antiparallel bundles of microtubules are covered by an electron-dense matrix. The intercellular bridge is the cytoplasmic connection between postmitotic sister cells at post-furrow ingression stages, with the midbody at its center (Steigemann and Gerlich, 2009). It is important to note that different nomenclature systems exist (Margolis and Andreassen, 1993; Eggert et al., 2006; Steigemann and Gerlich, 2009; Normand and King, 2010) and in paper I we have used the term “midbody ring” for the midbody and “midbody” for the intercellular bridge. The midbody is formed after the actomyosin ring has contracted and disassembled and the cleavage furrow has ingressed fully, creating an intercellular bridge with approximately 1-1.5 μm diameter (Eggert et al., 2006). An important protein for this process is PRC1 (protein regulator of cytokinesis 1), a microtubule binding and bundling protein required for the spindle midzone maintenance (Mollinari et al., 2002). This is a mitotic spindle associated CDK substrate, which is phosphorylated by CDK1 (cyclin-dependent kinase 1) in early mitosis and turns into an inactive and monomeric state. It is further dephosphorylated during the metaphase-anaphase transition and further interacts with the kinesin protein KIF4 (kinesin family member 4)

which transports PRC1 to the ends of the microtubules. PRC1 in turns recruits the centralspindlin complex and the chromosome passenger complex-both necessary for the midbody formation (Zhu et al., 2006)- but also additional mitotic kinesins such as CENP-E (centromere protein E) (Kurasawa et al., 2004), MCAK (mitotic centromere-associated kinesin) (Shimo et al., 2007) and KIF14 (kinesin family member 14) (Gruneberg et al., 2006). Finally, it serves as a docking site for PLK1(polo like kinase 1) in the central spindle (Neef et al., 2007).

The final step of cytokinesis is the abscission. This is the process that leads to the severing of the intercellular bridge between postmitotic sister cells (Steigemann and Gerlich, 2009). By the time of abscission, the intercellular bridge has a diameter approximately 0.2 microns (Normand and King, 2010). Abscission requires coordination of events at multiple cellular structures. Initially, microtubule bundles and all other cellular material need to be removed from the site of abscission. Further on, the plasma membrane has to split at the intercellular bridge and during these processes the cells need to maintain the cell cortex of the ingressed furrow tightly anchored to the intercellular bridge so as to prevent furrow ingression (Guizetti and Gerlich, 2010). The exact mechanisms that govern abscission are not clear yet, but several models have been proposed and will be analyzed further.

The first model for abscission is the mechanical force model, according to which postmitotic sister cells undergo abscission by the use of traction forces between them. The membrane tear at the site of abscission is supposed to close by a wound healing mechanism (Figure 4). Even though this model is supported by the fact that wound healing and cytokinesis share molecular similarities, abscission also proceeds efficiently in non-motile cells and also under conditions that do not create high forces between cells, therefore further investigation is needed to establish this model (Steigemann and Gerlich, 2009; Guizetti and Gerlich, 2010; Schiel and Prekeris, 2010).

The second model of abscission is the membrane fusion model. According to this, Golgi- and endocytosis-derived vesicles are targeted to the site of abscission and they fuse with each other and with the plasma membrane to complete cytokinesis (Figure 4). Support of this model comes from the fact that indeed secretory and endocytic vesicles have been found at regions close to the midbody. Also, SNARE proteins -critical components required for membrane fusion- have been implicated in cytokinesis completion. An important protein for midbody targeting of SNAREs is centriolin, which also brings the exocyst complex to the midbody. Septin proteins may also assist in the membrane fusion by restricting the diffusion of membranous components such as the exocyst complex to the area of abscission. Septins also are suggested to promote abscission via the direct recruitment of SNARE proteins. A problem with this model is that it has never been

demonstrated whether organelles accumulate in the furrow in sufficient numbers to actually mediate an abscission based on fusion. Also it is not clear at which stage vesicle trafficking and fusion would affect abscission. For these reasons, this model requires further testing (Steigemann and Gerlich, 2009; Normand and King, 2010; Schiel and Prekeris, 2010).

The third model is the ESCRT-mediated abscission model (Figure 4). As mentioned above, the ESCRT machinery mediates sorting of ubiquitinated proteins into ILVs in forming MVBs (multivesicular bodies). Among its four complexes, ESCRT-III is the one which provides scission activity. Recently, the ESCRT machinery and more specific the ESRCT-III complex is suggested to be involved in an event topologically equivalent to MVB formation, that is the abscission of the midbody during cytokinesis (Caballe and Martin-Serrano, 2011; Guizetti et al., 2011). ESCRT-III complex is composed of the CHMP1-7 (Charged Multivesicular body proteins/chromatin-modifying proteins 1-7) and its disassembly is regulated by VPS4 (vacuolar protein-sorting-associated protein 4), an AAA-ATPase necessary for the recycling of CHMPs. Most of the ESCRT-III components localize close to the midbody. CHMP4 (A-C) is targeted to the midbody via ALIX (apoptosis-linked gene-2 interacting protein X), which binds to the midbody-localized protein CEP55 (centrosomal protein 55 kDa) (Guizetti and Gerlich, 2010). CEP55 is a centrosomal protein which localizes to the mitotic spindle during prometaphase and metaphase and to the spindle midzone and midbody during anaphase and cytokinesis and is very important for the last step of abscission. It interacts with MKLP1 and is controlled by centraspindlin, since depletion of centraspindlin abolishes CEP55 localization from the midbody (Sagona and Stenmark, 2010). It also interacts with TSG101, an ESCRT-I subunit, and recruits both TSG101 and ALIX as a homodimer to the midbody. These proteins play an important role in the final step of abscission by recruiting subunits of ESCRT-III, which promote membrane severing via the formation of constricting helical oligomers. It is known that, *in vitro,* ESCRT-III components can polymerize into filaments or tubules, for example recombinant CHMP3 can form filamentous structures and a combination with truncated CHMP2 and 3 can be polymerized to helical polymer tubes (Guizetti and Gerlich, 2010). Recent studies reveal that overexpressed CHMP2B polymerizes into long, rigid tubes that protrude out of the cell and are thought to participate in the plasma membrane deformation(Bodon et al., 2011). Additionally, overexpressed CHMP4A forms spiral arrays at the cell cortex (Guizetti and Gerlich, 2010). These properties of ESCRT-III support the idea that they function during abscission (Guizetti and Gerlich, 2010; Sagona and Stenmark, 2010).

Two recent studies have shed light to the mechanisms that support the above model (Elia et al., 2011; Guizetti and Gerlich, 2010; Guizetti et al., 2011). In the first study (Elia et al., 2011) is suggested that TSG101 and CHMP4B (ESCRT-III subunit) are sequentially recruited into the

centre of the intercellular bridge where they form cortical rings. As cytokinesis evolves, CHMP4B moves to the narrow constriction zones and followed by VPS4, the abscission is completed. In the second study (Guizetti et al., 2011) with the use of high-resolution imaging it was revealed that at the site of partial microtubule disassembly the cortex of the intercellular bridge had ingressed to a narrow stalk, which contained a tightly compressed bundle of microtubules and that was deformed by regularly spaced electron-dense ripples. Further it was observed that ESCRT-III subunits (including CHMP4B) extend towards the sites of cortical constriction, suggesting that polymerization of ESCRT-III mediates the formation of the constriction zones necessary for abscission. This is further supported by the fact that CHMP2A depletion resulted in cells with intercellular bridges that didn't have these 'rippled constriction zones' and also showed that addition of a microtubule-depolymerizing drug after furrow ingression in CHMP2A depleted cells did not restore abscission, indicating that the microtubule disassembly alone cannot drive membrane scission. Additionaly, CHMP2A depleted cells lack of cortical filaments that are present under normal conditions in the constriction sites of HeLa cells, supporting the idea that these filaments are composed of ESCRT-III components (Guizetti et al., 2011) (Neto and Gould, 2011). Both of the studies contributed also in the understanding of how the cleavage of microtubule is accomplished, a step necessary for the final abscission. Consistent with that, recent studies have revealed that CHMP1B and human IST1 (increased sodium tolerance) components of the ESCRT-III complex, bind to the microtubule-severing protein spastin (Yang et al., 2008; Agromayor et al., 2009; Renvoise et al., 2010). Spastin localizes to the midbody and is recruited there via the interaction of its MIT domain with the ESCRT-III component CHMP1B (Yang et al., 2008; Connell et al., 2009). Spastin futher interacts with MIM1 (MIT interacting motif 1) of the ESCRT-III protein hIST1, which is found to be important for cytokinesis (Agromayor et al., 2009; Renvoise et al., 2010). IST1 in turn interacts with spartin and this interaction is suggested to be necessary for the recruitment of spartin to the midbody and for the participation of spartin in cytokinesis(Renvoise et al., 2010). Taken together all the above data support a model where the ESCRT-III machinery functions at specific sites within the intercellular bridge that are probably prepared for abscission by membrane trafficking and cuts the intercellular bridge by combining membrane scission with microtubule severing. When it comes to the correlation of spastin distribution with the membrane ripples or the constriction zones, two theories have arisen (Guizetti et al., 2011; Neto and Gould, 2011; Schiel et al., 2011). The first (Guizetti et al., 2011) suggests that spastin depletion can delay abscission but spastin depleted cells still have constriction zones with electron dense ripples and also once the intercellular bridge has formed, the microtubules are not required for abscission. The second (Schiel et al., 2011) suggests that the reorganization of

central spindle microtubules is driven by highly restricted zones of microtubule buckling and breaking and spastin has increased efficiency in these areas. In any case, further investigation is necessary so as to conclude in a firm model.

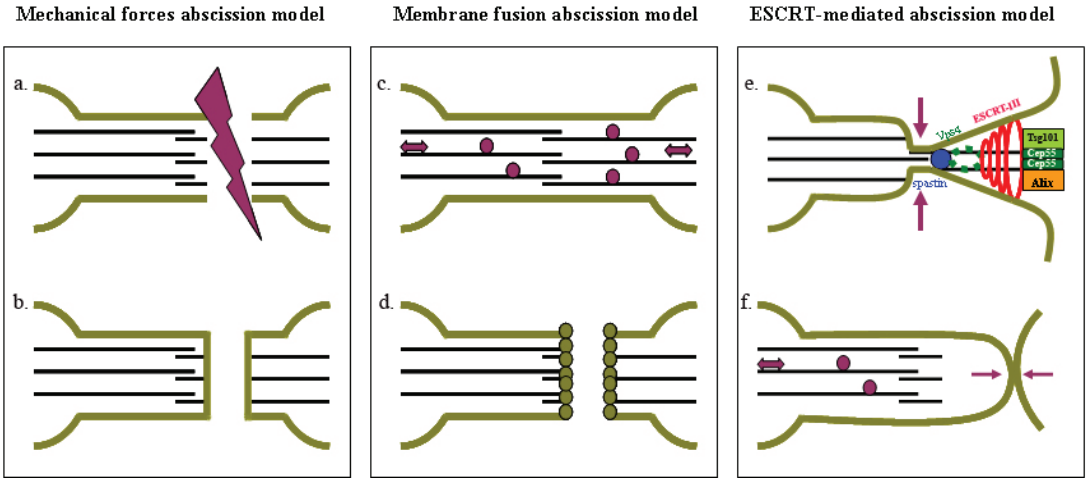

Figure 4. Schematic diagram of the various models of abscission. (a,b) Mechanical forces abscission model. (a) Mechanical forces separate the daughter cells, by rupturing the intercellular bridge. (b) The plasma membrane (PM) (in light green) repairs via wound-healing mechanisms, giving two daughter cells. (c,d) Membrane fusion abscission model. (c) Golgi- and endocytosis-derived vesicles (in dark purple) are delivered and accumulate in the intercellular bridge. (d) A simultaneous fusion event leads to abscission. (e,f) ESCRT-mediated abscission model. (e) CEP55 homodimer recruits TSG101 and ALIX to the midbody and they sequentially recruit ESCRT-III to the midbody. ESCRT-III subunits polymerize into filaments and are accompanied by Vps4, which promotes disassembly and recycling of ESCRT subunits. In the final stage of abscission, spastin is targeted to the midbody and mediates disassembly of the underlying microtubules required for abscission. (f) ESCRT-mediated abscission.

Cytokinesis and lipids

In addition to proteins, several lipids have emerged to play an important role in cytokinesis. One first example is the phospholipid phosphatidylethanolamine (PE), which normally localizes at the inner leaflet of the plasma membrane (PM), but during cytokinesis accumulates in the outer leaflet of the PM in the cleavage furrow (Nezis et al., 2010). It has been shown that in CHO cell lines defective in PE biosynthesis; cytokinesis is not completed properly, suggesting that PE is involved in cytokinesis (Emoto et al., 1999). Further studies have revealed that it is possible PE to

form unique PM domains within the furrow that may be important for the interaction between RhoA and the contractile ring, thus affecting cytokinesis (Neto et al., 2011).

Cholesterol also is implicated in cytokinesis in many organisms, including yeast and animal cells as well as zebrafish cells. It localizes at the cleavage site during cytokinesis in fission yeast and sea urchin eggs (Nezis et al., 2010). Depletion of cholesterol in zebrafish blastomeres results in an increased number of multinuclear cells as a consequence of cytokinesis impairment (Feng et al., 2002) and inhibition of cholesterol synthesis or cholesterol starvation results in cytokinesis failure in mammalian cells, suggesting that cholesterol is important for cytokinesis completion (Fernandez et al., 2004).

Sphingolipids belong to a class of complex lipids that are abundant in cell membranes and play a variety of roles in cellular environments, among those, regulating cytokinesis. More specifically, it has been found that myriocin, an inhibitor of SPT, an enzyme that calalyzes the first reaction during sphingolipid biosynthesis, causes defects in cytokinesis in various cell types, which can be rescued when sphingosine is added (Atilla-Gokcumen et al., 2010).Additionally, inhibition of glycosphingolipid biosynthesis, via the inactivation of GCS, results in failure of cleavage furrow ingression and thus cytokinesis failure (Atilla-Gokcumen et al., 2011). Further on, psychosine, a metabolite of the sphingolipid pathway has been found to induce the formation of multinucleate cells in many cell types. The same is also observed with other sphingolipid analogues as well, such as glycopsychosine, sphingosylphosphorylcholine and lysosulfatide. Finally, G_{M1}, is another sphingolipid the level of which is found to increase 7-fold in furrowing cells compared to metaphase cells in sea urchin eggs and additionally localizes to the equatorial band during contractile ring formation, suggesting that it is also involved in cytokinesis regulation (Atilla-Gokcumen et al., 2010).

Very-long-chain fatty acids (VLCFAs) are fatty acids with aliphatic tails longer than 22 carbons and participate in the stabilization of highly curved membrane domains. They are mostly found in sphingolipids and they are necessary for the formation and function of those. Elovl enzymes mediate elongation during the biosynthesis of VLCFAs. It has been addressed that a mutation in the gene bond, which encodes an Elovl protein in *Drosophila*, causes cytokinesis impairment in spermatocytes. Moreover, VLCFAs are metabolized in peroxisomes and mutants in peroxin proteins, which are necessary for the biogenesis of peroxisomes in *Drosophila*, result in cytokinesis failure in spermatocytes. Taken these data together, it is revealed that VLCFAs also play a role in cytokinesis (Atilla-Gokcumen et al., 2010; Nezis et al., 2010).

Finally, phosphoinositides are involved in cytokinesis with best studied candidate for both animal and fungal cells the phosphoinositide PtdIns(4,5)P_2. In *S. pombe*, it has been found that

both PtdIns4*P* 5-kinase and its product PtdIns(4,5)P_2 localize in the medial ring during cytokinesis and they are necessary for the completion of this process (Neto et al., 2011). Recently, it was further revealed that the PtdIns(4,5)P_2 5-phosphatase OCRL, which is mutated in Lowe syndrome patients, is an effector of the Rab35 GTPase in cytokinesis abscission. GTP-bound Rab35 interacts directly with OCRL and controls its localization in the intercellular bridge. Depletion of any of these interactors results in cytokinesis failure and causes abnormal accumulation of F-actin and PtdIns(4,5)P_2 in the intercellular bridge (Dambournet et al., 2011). A similar study was performed by another group in Drosophila, where it was revealed that depletion of the Drosophila orthologue of human OCRL 1, results in cytokinesis failure. In absence of dOCRL, various components of the cleavage furrow were found to localize abnormally on giant cytoplasmic vacuoles rich in PtdIns(4,5)P_2 and in endocytic markers (Ben El et al., 2011). It has also been addressed, both in mammalian cells and in *Drosophila* spermatocytes that there is an accumulation of PtdIns(4,5)P_2 in the cleavage furrow and in both cases it seems to be important for the completion of cytokinesis. Based on these studies, it is suggested that interference with PtdIns(4,5)P_2 production in the furrow interferes with the adhesion of PM to the contractile ring – since it results in cytokinesis failure- and also that a specific level of PtdIns(4,5)P_2 production is essential in the furrow so as the ingression can be sustained and a link with the underlying actin cytoskeleton can be maintained. This is further enhanced by the observation that PtdIns(4,5)P_2 interacts *in vitro* with septins and ERM-family proteins that are known to link the actin cortex to the PM (Neto et al., 2011).

Another phosphoinositide that is a regulator of cytokinesis is PtdIns4*P*. This phosphoinositide localizes to the cell plate during plant cytokinesis and has been suggested to promote the recruitment of both Rab11 and actin-regulatory proteins during AP-1- dependent protein sorting at the Golgi. It is also thought that PtdIns4*P*- containing organelles are essential for gathering or recruiting factors that maintain F-actin in the contractile ring. This hypothesis is supported by the fact that mutations in the Drosophila gene *fwd* -which encodes PtdIns 4-kinase- , *rab11* and *nuf* (a Rab11 effector that promotes the polymerization of actin in the furrow) results in failure to maintain actin organization during cytokinesis. Furthermore, mutations in the gene *fwd* results in defective cytokinesis during male meiosis (Neto et al., 2011).

PtdIns(3,4,5)P_3 is implicated to be involved in cytokinesis. In *Dictyostelium discoideum*, PtdIns(3,4,5)P_3 accumulates in ruffles at polar regions of the cells during cytokinesis. The spatial distribution of PtdIns(3,4,5)P_3, is regulated by the lipid phosphatase PTEN and by PI3-kinase, therefore mutant *Dictyostelium discoideum* cells that are devoid of PI3K-Class I and PI3K-Class II or PTEN, present defects in cytokinesis and also in the presence of the PI3K inhibitors wortmannin and LY294002, cytokinesis in defective (Nezis et al., 2010).

Finally, PtdIns3*P* has been mentioned to play a role in fungi and plant cells. In the fungus *Ustilago maydis*, important for secondary septum formation and thus abscission is the Rho-GEF Don1, which contains a FYVE domain that binds to PtdIns3*P*. Deletion of the FYVE domain of Don1 or mutation in a PtdIns3*P* binding region of the FYVE domain results in cytokinesis defects, suggesting that PtdIns3*P* is important for cytokinesis. Additionaly in plants, PtdIns3*P*-positive vesicles accumulate as a ring around the rim of the expanding cell plate. *Arabidopsis* plants that express an anti-sense construct to the catalytic subunit of PI3K-III, present a severe growth phenotype and in the presence of wortmannin cell plate growth is inhibited (Nezis et al., 2010). Finally, deletion of TbVps34, the *Trypanosoma* orthologue of Vps34, causes severe growth defect with a post-mitotic block in cytokinesis (Hall et al., 2006). These data suggest a role of PtdIns3*P* in cytokinesis.

Cytokinesis and Cancer

The idea that there is a link between abnormal mitosis and cancer was introduced first time by Theodor Boveri in 1888. Ever since, many studies have taken place and based on those it has been proposed that failure to complete cytokinesis promotes tumorigenesis by leading to tetraploidy and resulting chromosomal instability. Recent observations suggest that APC mutations found in human colorectal cancer inhibit cytokinesis by preventing mitotic spindle to anchor at the anaphase cortex and in that way preventing the initiation of cytokinesis (Caldwell et al., 2007; Sagona and Stenmark, 2010). Additionally, it was found that cancer cells accumulate midbodies by suppressing autophagy and as a result of this accumulation, in vitro tumorigenicity is increased (Kuo et al., 2011). There are many examples supporting this idea and more and more studies shed light in this hypothesis.

Cytokinesis failure, aneuploidy and cancer

It is now obvious from the previous descriptions, that proper cytokinesis is important for the correct inheritance of the genetic material and cytoplasm by the two daughter cells. Various events though, can lead to cytokinesis failure, with fatal consequences for the cell (Lacroix and Maddox, 2011).

First of all, inaccurate positioning of the contractile ring could result in partitioning of both daughter nuclei into one daughter cell. In the case of slow ring closure, a cytoplasmic connection between the daughter cells can occur, leading to furrow regression. Furrow regression could also occur if the mechanical forces in the contractile ring are not able to fully deform the cell. Even if the fully deformation of the cell is achieved and a narrow intercellular bridge is formed, failure of precise abscission can result to fusion of daughter cells (Lacroix and Maddox, 2011). The formation of DNA bridges across the anaphase spindle could also lead to cytokinesis failure. In normal cells, this is resolved via the DNA repair machinery or DNA damage checkpoint-mediated arrest and consequent apoptosis. In cells with cancer mutations, DNA bridging is often and cytokinesis progresses before complete chromosome segregation, resulting in cell fusion and tetraploidy (Lacroix and Maddox, 2011).

Apart from the genetic mechanisms of cytokinesis failure, recently a non-genetic mechanism of cytokinesis failure has been described, which occurs as a result of cell-in-cell formation by entosis. According to this mechanism, live cells are internalized by entosis, a process whereby viable cells are internalized into neighbouring cells, forming cell-in-cell structures. These cells have the ability to persist through the cell cycle of host cells and to block the formation of contractile ring during host cell division, resulting in cytokinesis failure and consequently aneuploidy and cancer (Krajcovic et al., 2011).

Aneuploidy is the state in which cells contain alterations in the total chromosome number but also a variety of other chromosomal rearrangements, such as amplifications, deletions and translocations (Storchova and Pellman, 2004). This state can result from an unstable tetraploid intermediate. Supporting this idea, it is demonstrated by recent studies that tetraploidy can promote chromosomal aberrations and tumorigenesis *in vivo*. Tetraploid cells can arise by various different mechanisms, such as mitotic slippage, cytokinesis failure and viral-induced cell fusion (Ganem et al., 2007). Tetraploidy triggers the activation of the tumor suppressor p53. Even though most normal mammalian cells have a tetraploidy checkpoint that leads to arrest in G1 phase, after cytokinesis failure, cells expressing p53 are able to continue mitotic progression after cytokinesis failure. This continuous mitosis leads to aneuploidy (Lacroix and Maddox, 2011). Aneuploidy is a direct consequence of chromosome segregation errors in mitosis, whereas structural aberrations are caused by improperly repaired DNA breaks. Recent studies reveal that chromosome segregation errors can also lead to structural chromosome aberrations and DNA breaks can result in unbalanced translocations in the daughter cell (Janssen et al., 2011). Aneuploidy is very firmly linked with tumorigenesis and cancer. Aneuploid cells are present in approximately 90% of solid human tumors and 75% of hematopoietic cancers (Weaver and Cleveland, 2006). The reason for

this phenomenon is thought to be the fact that aneuploidy enhances the ability of cancer cells to evolve and adapt in difficult environments (Fang and Zhang, 2011). Based on studies that use SAC (spindle assembly checkpoint)-deficient mice in combination with ATM or p53 deletions, it is proven that the aneuploidy checkpoint is necessary for preventing aneuploidy induced oncogenic transformation, indicating that possible control of this checkpoint could be used for therapeutical reasons (Li et al., 2010).

Kinases and septins in cytokinesis and cancer

A large family of proteins involved in both cytokinesis and cancer are the kinases, including Aurora kinases, polo-like kinases and others. The Aurora kinases are a family of highly conserved serine-threonine kinases, consisting of three members, Aurora A, Aurora B and Aurora C. Of those Aurora A and Aurora B play an important role during mitosis with link to cancer and they will be analyzed further. Aurora A presents multiple functions, such as centrosome maturation and separation, bipolar spindle assembly, chromosome alignment and transition from prophase to metaphase as well as cytokinesis (Sagona and Stenmark, 2010). Aurora A is frequently overexpressed in human cancers, such as hepatocellular carcinoma, and based on studies in transgenic mice which overexpress human Aurora A in the liver, it was shown that during liver regeneration a p53 dependent premitotic arrest occurs, suggesting that Aurora A is involved in tumorigenesis (Li et al., 2009). Aurora A is also significantly overexpressed in squamous cell cancer of head and neck (SCCHN). Treatment of SCCHN cell lines with Aurora kinase inhibitor results in defective cytokinesis, polyploidy and apoptosis, again confirming its oncogenic role (Hoellein et al., 2011). Additionally, it has been found recently that SLAN, a novel protein with multiple subcellular localization including spindle matrix and midbody is downregulated in lung cancer and inhibits cell proliferation and Aurora A (Yu et al., 2011). Selective inhibition of Aurora A kinase causes abnormal mitotic spindles and chromosome segregation defects and the cells as a result become aneuploid (Lens et al., 2010). Additionally, the Aurora kinase inhibitor CCT137690, which inhibits Aurora A and B kinases, decreases MYCN neuroblastoma protein expression and inhibits tumor growth in transgenic mouse model of neuroblastoma (Faisal et al., 2011).

Aurora B kinase is a chromosomal passenger protein that localizes along the chromosome arms and at centromeres in prophase, in the inner centromere region from prometaphase to metaphase, moves to the central spindle and cortex in anaphase and eventually accumulates in the midbody during telophase (Lens et al., 2010). This kinase is very important for cell cycle and

cancer progression. It was recently shown that the bromodomain protein Brd4 controls the transcription of Aurora B and as a consequence, depletion of Brd4 causes cytokinesis impairment in cancer cells and the same is the case when Aurora B is depleted (You et al., 2009). Moreover, it has been found that inactivation of Aurora B can mediate abscission which promotes the completion of chromosome segregation and in that way protects against tetraploidization and cancer (Steigemann et al., 2009). Inhibition of Aurora B kinase in cell culture leads to impaired cytokinesis, to failure to bi-orientate chromosomes, to resistance to taxol-induced mitotic arrest, and thus induction of polyploidy (Lens et al., 2010). Additionally it was recently revealed that the Aurora B inhibitor AZD1152-HQPA has antineoplastic effects in breast cancer and thus could be important for breast cancer treatment (Gully et al., 2010). Taken together the above findings reveal the important role of Aurora B in the regulation of cytokinesis and its links with cancer (Sagona and Stenmark, 2010)

Polo-like kinases belong to a family of serine-threonine kinases that consist of five members and among these PLK1 is the most prominent. PLK1 is essential for centrosome maturation, bipolar spindle formation and cytokinesis. Its inhibition results in defective mitosis and eventually cell death. In human leukemia cell lines as well as in cell samples from individuals with acute myelogenous leukemia and acute lymphoblastic leukemia, PLK1 is highly overxpressed, suggesting its role in cancer progression (Ikezoe et al., 2009; Lens et al., 2010). PLK1 is mainly expressed in proliferating tissues and is overexpressed in cancers, therefore PLK1 inhibitors are being evaluated as cancer treatment drugs (Christoph and Schuler, 2011). Finally, another serine-threonine kinase, citron, also seems to play a role in cytokinesis and cancer progression. Citron localizes to the central spindle and its localization depends on the kinesin-3 motor KIF14 and vice versa (Gruneberg et al., 2006). KIF14 is found overexpressed in retinoblastoma suggesting a possible role of these kinesins in carcinogenesis (Madhavan et al., 2009).

Another family of proteins important for cytokinesis and carcinogenesis are the septins. Septins as mentioned above regulate cytokinesis, via their interaction with anillin and via their ability to regulate actin and microtubule dynamics and to localize to the contractile ring (Normand and King, 2010). Many members, such as SEPT2, SEPT8, SEPT9 and SEPT11 are found upregulated in various tumors, whereas other members, such as SEPT4 and SEPT10 are downregulated in most cancer types, indicating a role of septins in carcinogenesis. Additionally, SEPT5, SEPT6, SEPT9 and SEPT 11 are found mutated in infant acute leukemia patients (Liu et al., 2010). Anillin is also implicated in carcinogenesis, since expression levels of anillin correlate with metastatic cancer of various types and inhibition of anillin expression is shown to suppress the growth of lung cancer cells in culture (Zhang and Maddox, 2010).

Other proteins associated with cytokinesis and cancer

Finally, many proteins that are involved in cytokinesis are also implicated in cancer. For example centrobin, is a centrosomal protein that when depleted, centriole duplication is inhibited and cytokinesis is not completed properly. Depletion of centrobin inhibits the proliferation of a lung cancer cell line and prevents the G1 to S transition of the cells, via the upregulation of p53, which is associated with activation of cellular stress induced by the p38 pathway. Importantly, inhibition of p38 activity can overcome the cell cycle arrest in which centrobin depletion leads. Additionally, according to a very recent study, it was shown that cancer cells often go through defective cytokinesis because of decreased phosphorylation of the MLC, which as mentioned also above is important for the activation of myosin II and thus cortical contraction during cell division. Overexpression of myosin phosphatase or inhibition of the MLCK in normal cells could mimic some of the mitotic defects of cancer cells, such as multinucleation and multipolar spindles, indicating that these changes are sufficient to reproduce cytokinesis failures seen in cancer cells (Wu et al., 2010). NuSAP also is an important mitotic regulator that localizes in early mitosis to the chromosome arms, in anaphase to the spindle midzone and in telophase to the midbody. Depletion of NuSAP causes G2-M arrest, abnormalities in interphase nuclei, abnormal chromosome segregation and overall defective cytokinesis. The *NUSAP1* gene that corresponds to this protein is found overexpressed in patient samples of glioblastomas and it is also found upregulated in hepatocellular carcinomas. Moreover, *NUSAP1* mRNA is downregulated in response to methionine stress, which based on experiments in pancreatic adenocarcinoma cell lines,is shown to sensitize pancreatic adenocarcinoma cell lines to chemotherapy. Moreover, NuSAP is also related to breast cancer and acute myeloid leukemia (AML) as well as with colon cancer, indicating that is an important factor for carcinogenesis (Iyer et al., 2011). Finally, *SPG20* is found hypermethylated in colorectal cancer and encodes a protein named spartin which localizes to the spindle poles and intercellular bridge.Depletion of spartin results in defective cytokinesis, reinforcing the idea of interaction between defective cytokinesis and cancer (Lind et al., 2011). Overall, all the above examples indicate the importance of correct cytokinesis in the prevention of carcinogenesis.

Aims of the study

The general aim of this study was to elucidate the role of PI3K Class III and PtdIns3*P* -binding or associated proteins in cytokinesis. Additionally, we investigated the mechanisms that govern this process and tried to clarify its role in diseases. We have approached the aim by analyzing candidates that either bind to PtdIns3*P* or interact with PtdIns3*P*-binding proteins and are all involved in diseases and have a role in cytokinesis. The specific aims that correspond to each study are mentioned below:

Paper I: PtdIns(3)*P* controls cytokinesis through KIF13A-mediated recruitment of FYVE-CENT to the midbody.

In the beginning of this study, it was known that PtdIns3*P* and PI3K Class III are involved in endocytic trafficking and autophagy, but nothing was known about the involvement of these factors in cytokinesis. The aim of this study was to examine the role of PtdIns3*P* and of PI3K Class III in cytokinesis. Additionally, another aim was to characterize a novel FYVE-domain containg protein, which we named FYVE-CENT (FYVE domain containing centrosomal protein) and to reveal its role as well as its interactors' roles in cytokinesis.

Paper II: A tumor-associated mutation of FYVE-CENT prevents its interaction with Beclin 1 and interferes with cytokinesis.

When this study started, Beclin 1 was known to be a tumor suppressor and also to have a very important function in autophagy. The aim of this study was to address the role of Beclin 1 in cytokinesis via its interaction with FYVE-CENT and further on to understand the function of this interaction and its link with cancer.

Paper III: Association of CHMP4B with chromosome bridges and micronuclei: implications for cataract formation.

CHMP4B is an ESCRT-III component, important for the last step of abscission and is found mutated in autosomal dominant cataract. The aim of this study was to investigate the localization and function of CHMP4B and to try to address its function with respect to cataract disease.

Summary of included papers

Paper I: PtdIns(3)*P* controls cytokinesis through KIF13A-mediated recruitment of FYVE-CENT to the midbody.

The role of PtdIns3*P* and PI(3)K-III complex has been well characterized in processes such as endocytosis and autophagy. In this study we have revealed a novel function for PI(3)K-III complex and its catalytic product PtdIns3*P* in cytokinesis. We have found that PtdIns3*P* positive vesicles localize along the intercellular bridge and move towards the midbody during abscission. We also found that PtdIns3*P* recruits a novel centrosomal protein that also contains a FYVE domain, which we named FYVE-CENT (ZFYVE26). FYVE-CENT interacts with TTC19; a TPR repeat containg protein, which in turn interacts with CHMP4B, an ESCRT-III subunit very important for the last step of cytokinesis. We have identified that all the above interacting proteins localize to the intercellular bridge and FYVE-CENT with TTC19 also localize to the centrosome. FYVE-CENT also interacts with KIF13A; a microtubule motor, which also localizes to the centrosome and intercellular bridge. Based on depletion studies, we have identified that translocation of FYVE-CENT and TTC19 from the centrosome to the intercellular bridge requires KIF13A. Depletion of the VPS34 or Beclin 1 subunits of PI(3)K-III results in cytokinesis arrest and in an increased number of binucleate and multinucleate cells, and the same is the case when FYVE-CENT, KIF13A or TTC19 are depleted. In conclusion, with this study we have provided a mechanism for the translocation and docking of cytokinesis regulatory machinery at the intercellular bridge during cytokinesis.

Paper II: A tumor-associated mutation of FYVE-CENT prevents its interaction with Beclin 1 and interferes with cytokinesis.

The tumor suppressor activity of Beclin 1, a subunit of class III phosphatidylinositol 3-kinase complex, has been attributed so far to its regulation of apoptosis and autophagy. In this study we have revealed a novel role for Beclin 1 in cytokinesis via FYVE-CENT. More specifically, we have identified a novel interaction between FYVE-CENT and Beclin 1. We have found that Beclin 1 as well as FYVE-CENT localize to the intercellular bridge. Since Beclin 1 has a tumor suppressor activity, we further tested the interaction between Beclin 1 and a construct of FYVE-CENT which contained a mutation (R1945Q) found in breast cancer. We revealed that in

the presence of this mutation, the interaction was abolished and the localization of both these proteins in the intercellular bridge was reduced. We further tested this idea using a breast cancer cell line containing the mutation R1945Q. We found that this cell line displayed a significant increase in arrested cytokinetic profiles and bi-multinuclear phenotype, thus confirming our observations. Based on gene expression data, both Beclin 1 and FYVE-CENT were found to be downregulated in advanced breast cancers. In conclusion, our findings suggest a positive feedback loop for recruitment of FYVE-CENT and Beclin 1 to the intercellular bridge during cytokinesis and present a novel potential tumor suppressor mechanism for Beclin 1.

Paper III: Association of CHMP4B with chromosome bridges and micronuclei: implications for cataract formation.

The ESCRT-III subunits have been characterized for their role in catalyzing the scission of membrane necks and for being involved in the last step of abscission during cytokinesis. Completion of cytokinesis strongly associates with the clearance of chromatin from the intercellular bridge and can be significantly delayed by bridged chromosomes. In the last study, based on the observation that we had from our first study concerning the localization of CHMP4B (chromatin-modifying protein/charged multivesicular body protein 4B), an ESCRT-III component in the intercellular bridge during cytokinesis, we have examined further the localization of this protein. We have found that it localized in various types of intercellular bridges in interconnected cells, including thin bridges, thick bridges and this was tested in many different cell lines. We have also found that CHMP4B associate strongly with DNA, both by immunoprecipitation of CHMP4B with various nuclear proteins such as H2B and Lamin A but also by confocal microscopy where we found that it localized in the chromosome bridges trapped inside intercellular bridges as well as in micronuclei. In the latter case we showed that the lysosomal and autophagic markers Lamp 1 and LC3 localized adjacent to micronuclei. Mutations in the *CHMP4B* gene were reported to cause autosomal dominant posterior polar cataract. Therefore, we further tested the localization of the CHMP4B mutant construct D192V, found in patients with cataract and interestingly the association in the mutant construct with DNA was much weaker compared to the wild type. Based on the idea that cataract forms when DNA is not properly degraded during lens cell differentiation, we have provided a possible explanation between CHMP4B and its function when it comes to cataract. We suggest that CHMP4B may participate to the lysosomal degradation of micronuclei and in this way protects lens cells from forming cataract.

Discussion

The studies included in this thesis aim at elucidating the role of PI3K Class III and its catalytic product PtdIns3*P* as well as its associated proteins in cytokinesis (Paper I and II). They also provide more details about the ESCRT III components in the same process and try to link their function with disease (Paper I, II and III). In the current study it is for the first time shown that PI3K Class III and its catalytic product PtdIns3*P* regulate cytokinesis via various interactors (Paper I) and a novel protein named FYVE-CENT is characterized (Paper I). Additionally, for the first time it is shown that the tumour suppressor Beclin 1 has a role in cytokinesis (Paper I and II). Finally, we reveal the localization of an ESCRT-III component CHMP4B to intercellular bridges (Paper I and III), in chromosomal bridges and micronuclei (Paper III) and try to link this functionally with the manifestation of disease.

Molecular mechanisms of cytokinesis

In Paper I, we revealed mechanistic details concerning the cytokinetic machinery that is important for the final cell abscission. Using GFP-2XFYVE, we demonstrated that PtdIns3*P*, the catalytic product of PI3K-Class III, localizes in vesicles within the intercellular bridge, presumably in transit to the midbody. These vesicles were found to partially colocalize with the recycling endosome marker transferrin. Endogenous VPS34 (PI3KC3) was found to localize at the intercellular bridge, and depletion of VPS34 and an accessory subunit of the PI3K-III complex, Beclin 1, was found to cause defects in cytokinesis as well as increased numbers of bi- and multinuclear cells. This was further confirmed *in vivo*, since it was revealed that homozygous vps34 mutant Drosophila ovarian follicle cells display a five-fold increase in the binucleate phenotype compared to wild-type cells. Recent studies have revealed that three additional accessory subunits of PI3K-III, VPS15, UVRAG and BIF-1, are also required for proper cytokinesis (Thoresen et al., 2010). In order to understand better the involvement of PtdIns3*P* in cytokinesis, in Paper I we performed a siRNA screen for PtdIns3*P* effectors regulating cytokinesis in HeLa cells. This screen led to the identification of FYVE-CENT (FYVE domain containing centrosomal protein), a 285-kDa protein that contains a PtdIns3P-binding FYVE domain which is responsible for localization of the protein to the intercellular bridge during cytokinesis. Based on a

yeast two-hybrid screen, FYVE-CENT was found to interact with the kinesin-like protein KIF13A and the tetratricopeptide repeat protein 19 (TTC19), which were both revealed to localize at the centrosome and intercellular bridge. KIF13A is a plus end-directed microtubule-dependent motor protein and is a member of the kinesin-3 family of proteins (Nakagawa et al., 2000). TTC19 contains four tetratricopeptide (TPR) repeats, which were first described as a protein-protein interaction domain in cell division cycle proteins (Blatch and Lassle, 1999). In Paper I, we showed that depletion of KIF13A and TTC19 in Hela cells results in cytokinesis arrest and in increased numbers of binuclear and multinuclear cells. We also presented immunoprecipitation and GST-pull-down experiments that indicated that TTC19 interacts with CHMP4B. In order to reveal further mechanistic details of this model we performed a series of siRNA knock-downs of the proteins involved. We found that depletion of KIF13A in Hela cells abolished FYVE-CENT and TTC19 localization to the intercellular bridge and depletion of FYVE-CENT also prevented the localization of TTC19 to the intercellular bridge. According to these data, we suggested that KIF13A transports FYVE-CENT and TTC19 to the intercellular bridge where FYVE-CENT can dock to PtdIns3*P* and TTC19 can dock to CHMP4B.

Even though the functions of PtdIns*3P* in endosomal and vacuolar sorting have been studied extensively in mammalian and fungal cells (Lindmo and Stenmark, 2006; Falasca and Maffucci, 2009; Strahl and Thorner, 2007), very little is known about the involvement of PtdIns3*P* in cytokinesis and Paper I has an important contribution in this field. One of the few cases where PtdIns3*P* is reported to have an important function in cytokinesis is in the dimorphic fungus *Ustilago maydis*. The cells in *U. maydis* grow by budding and cell separation in this fungus is accomplished by the consecutive formation of two distinct septa that are formed between the mother and the daughter cell. The physical separation of mother and daughter cell takes place in the so-called fragmentation zone that these two septa delimit as follows: the primary septum physically separates the mother and daughter cells, whereas the secondary septum is necessary for proper abscission (Weinzierl et al., 2002; Mahlert et al., 2006). For the formation of each of these two septa the establishment of a contractile actomyosin ring is required (Bohmer et al., 2008). A very important regulator of secondary septum formation in U. maydis is the Rho-GEF Don1(Weinzierl et al., 2002). Don1 activates the small GTPase Cdc42, and both Cdc42 and Don1 are necessary for the accomplishment of cytokinesis in these cells (Mahlert et al., 2006; Hlubek et al., 2008).Deletion mutants for both Cdc42 and Don1 are viable, but present serious cell separation defects (Weinzierl et al., 2002) (Mahlert et al., 2006).

Don1 shows homology to the FGD1-family of Rho-GEFs in higher eukaryotes (Pasteris et al., 1994). Interestingly, in the siRNA screen performed in Paper I, FGD3 (FGD1 family member

3), was the second highest candidate after FYVE-CENT arrested in arly cytokinesis. Proteins in this family contain a characteristic DH-PH tandem domain, but also a C-terminal FYVE domain (Pasteris et al., 1994). The Don1-FYVE domain binds specifically to PtdIns3*P* and has been shown to be crucial for the function of Don1 during cell separation (Schink and Bolker, 2009).Deletion of the FYVE domain of Don1 results in cytokinesis defects and the same is the case when the FYVE domain is mutated to a PtdIns3*P*-binding defective variant. Additionally, the FYVE domain of Don1 is important for the intracellular localization of the protein: Don1 with an intact FYVE domain localizes to endosomes and vacuoles, whereas Don1 lacking the FYVE domain, or containing a defective domain, is mislocalized to the cytoplasm (Schink and Bolker, 2009). When it comes to FYVE-CENT (Paper I), the observations are comparable: both deletion of the FYVE domain of FYVE-CENT or mutation R1835A in the FYVE domain of FYVE-CENT abolish FYVE-CENT localization from the midbody, indicating the importance of FYVE domain for targeting FYVE-CENT on the midbody.

PtdIns3*P* has also an important role in plants, in which PtdIns3*P*-positive vesicles accumulate as a ring around the rim of the expanding cell plate and deliver membrane material by fusion with the cell plate in dividing cells (Vermeer et al., 2006). Additionally, Arabidopsis plants expressing an antisense construct to the catalytic subunit of PI3K-III, AtVps34, present a severe inhibition in growth and development (Welters et al., 1994) and it has been shown that wortmannin inhibits cell plate growth (Dhonukshe et al., 2006). Moreover, Patellin1, a Sec14-like protein, localizes to the maturing cell plate in Arabidopsis roots and in tobacco BY-2 cells, and binds to PtdIns5*P*, PtdIns(4,5)*P*2 and PtdIns3*P* *in vitro*. This localization suggests a possible role in membrane recycling during cell plate maturation in plant cytokinesis. In *Trypanosoma brucei*, knockdown by RNA interference of TbVps34, the Trypanosome orthologue of Vps34, induces a severe growth defect, with a post-mitotic block in cytokinesis (Hall et al., 2006). Based on the above information it can be suggested that PtdIns3*P* serves as a docking platform for regulators of cytokinesis in fungi, plants and mammals.

Finally, even though it is not clear yet if PtdIns3*P* has a specific function during cytokinesis in Drosophila, it is detected in endosomes positive for the FYVE-domain-containing signaling protein SARA, which are targeted to the central spindle during mitosis. These are involved in the symmetric partitioning of Dpp (Decapentaplegic) morphogens among daughter cells during wing development (Bokel et al., 2006). Additionally, PtdIns3*P*-containing endosomes are enriched significantly at the midzone plane of cell division during asymmetric division of the fly sensory organ precursors (SOPs) (Coumailleau et al., 2009).

From the above information it appears that PtdIns3*P* localization during cytokinesis is similar in fungi as well as in animal and plant cells. Even though this localization implicates a possible role of PtdIns3*P* in cytokinesis, the mechanistic details of this process need further investigation. For example, it is still not clear if or how TTC19 regulates CHMP4B, but one possibility is that interaction with TTC19 controls proper oligomerization of CHMP4B at the midbody. Another intriguing question is why FYVE-CENT is not associated with PtdIns3*P*-containing endosomes in interphase cells. A possible explanation would be that transport to the intercellular bridge is required for exposure of the FYVE domain of FYVE-CENT, since FYVE-CENT is a very big protein and possibly has complicated folding.

It is well established that PtdIns3*P* regulates autophagy and is required for autophagosome formation (Simonsen and Tooze, 2009). Interestingly, it was shown r that midbody derivatives are removed by autophagy after abscission. Additionally, the autophagy-related proteins Atg8a and p62 have been found to localize at the midbody (Pohl and Jentsch, 2009). Furthermore it was recently reported that in stem cells the autophagic degradation of the midbodies is mediated by the binding of the autophagic receptor protein NBR1 to the midbody protein CEP55 (Kuo et al., 2011). Based on these data, it could be suggested that PtdIns3*P* may serve as a platform for recruiting FYVE domain containing proteins that regulate autophagy, such as WIPI-1 (Proikas-Cezanne et al., 2004), DFCP-1 (Axe et al., 2008) and ALFY (Simonsen et al., 2004), at the midbody. This has yet to be proven and could be important for the regulation of abscission and for the absorption of the midbody remnant.

The importance of ESCRT-III subunits in cytokinesis was revealed gradually, with the contribution of an increasing number of studies. Initially, it was revealed that the ESCRT-I component TSG101 and ALIX, an ESCRT-associated protein, were recruited to the midbody during cytokinesis via their interaction with CEP55, a centrosomal and midbody protein known to be required for abscission (Carlton and Martin-Serrano, 2007). Additionally, the ESCRT-III components CHMP2A, CHMP4A and CHMP5 as well as VPS4 were shown to localize to the midbody (Morita et al., 2007). Depletion of ALIX and TSG101 was demonstrated to inhibit the completion of cytokinesis and the same was the case when VPS4 was overexpressed. It was also shown that ALIX point mutants were not able to bind to CEP55 or ESCRT-III interactors, resulting in cytokinesis inhibition thus suggesting a role for ESCRT-III in cytokinesis (Carlton and Martin-Serrano, 2007; Morita et al., 2007). More detailed analysis of this model came with two other sequential studies, showing that ALIX binds to CEP55 via an evolutionarily conserved peptide (PRR)(Carlton et al., 2008) and that peptides from ALIX and TSG101 compete for binding to the TSG101 and ALIX-binding region (EABR) of CEP55. It was further revealed that EABR

forms an aberrant dimeric parallel coiled coil and that CEP55 dimerizes in order to function properly and each of the dimmers binds to ALIX and TSG101 respectively (Lee et al., 2008). Even more detail in this model came with the two very recent studies mentioned earlier (Elia et al., 2011; Guizetti et al., 2011). In total, the suggested model is as follows: the centralspindlin component MKLP1 recruits CEP55 to the midbody. CEP55 homodimerizes and interacts with TSG101 and ALIX, which then recruit ESCRT-III components. TSG101 and ESCRT-III subunit CHMP4B are recruited in the centre of the intercellular bridge where they form cortical rings. In a further step in cytokinesis, CHMP4B concentrates at abscission zones followed by VPS4 and forms two narrow cortical rings adjacent to the midbody prior to disassembly of the microtubule. CHMP4B and other ESCRT-III subunits form filaments which extend towards the site of ingression and then recruit the microtubule-severing enzyme spastin which catalyzes the final abscission (Caballe and Martin-Serrano, 2011; Guizetti et al., 2011; Elia et al., 2011; Neto and Gould, 2011). How do PtdIns3*P* and its associated proteins fit into this model? A possible model would be as follows: PtdIns3*P* positive vesicles accumulate at the intercellular bridge and move towards the midbody during abscission. PtdIns3*P* recruits FYVE-CENT which in turn recruits TTC19. TTC19 controls CHMP4B, which forms filaments and is important for the last step of abscission.

Impaired cytokinesis and disease

An interesting aspect of Paper I is the fact that *ZFYVE26*, the gene encoding FYVE-CENT, is mutated in patients with hereditary spastic paraplegia (Hanein et al., 2008). Another protein found mutated in patients with hereditary spastic paraplegia is spastin, which apart from severing microtubules and catalyzing final abscission has also been shown to localize to the midbody and to control cytokinesis (Yang et al., 2008; Connell et al., 2009). Additionally spartin, also found mutated in spastic paraplegia, localizes as well to the midbody and participates in cytokinesis (Renvoise et al., 2010; Lind et al., 2011). According to these data, it can be proposed that defective cytokinesis and hereditary spastic paraplegia could be related. Additionally, *ZFYVE26* has also be found mutated in breast cancer samples with a frequency of more than 10% and this has been confirmed from different studies (Sjoblom et al., 2006; Wood et al., 2007; Kohler et al., 2011). It has already been shown that three subunits of the PI3K-III complex, specifically Beclin 1, Bif-1 and UVRAG are known tumor suppressors (Liang et al., 1999; Qu et al., 2003; Maiuri et al., 2007) and their tumor suppressor activity so far was explained via their involvement in autophagy. Our

results in Papers I and II suggest that PI(3)K-III complex components might have tumor suppressor function via their involvement in cytokinesis, which is suggested to promote tumorigenesis. Depletion of VPS34 and Beclin 1 results in cytokinesis arrest at the midbody stage as well as in binuclear and multinuclear cells. Based on Paper I, in Paper II we present more data confirming this hypothesis. According to our data, Beclin 1 interacts with FYVE-CENT and via this interaction participates actively in cytokinesis. This interaction was found via a yeast two-hybrid screen where FYVE-CENT C-terminal part (residues 2120-2539) was used as bait and was further confirmed through immunoprecipitation with endogenous proteins and GST-pulldown with myc-tagged Beclin 1 transfected proteins. Mutations in FYVE-CENT (R1945Q) associated with breast cancer abolish the interaction with Beclin 1, suggesting an additional role of Beclin 1 in tumorigenesis via impaired cytokinesis. More specifically, when the interaction is abolished, cytokinesis failure and multinucleate cells are observed. This is confirmed both in FYVE-CENT mutant cancer cells with R1945Q mutation but also in HeLa cells transfected with a C-terminal 1807-2539 FYVE-CENT tagged construct containing R1945Q mutation. Interestingly, the R1945Q mutation does not affect the interaction between FYVE-CENT and KIF13A or TTC19, indicating that this is specific for Beclin 1, even though it corresponds to the same interacting region of FYVE-CENT. We could still observe that *ZFYVE26*, *BECN 1*, *KIF13A* and *TTC19* are found downregulated in advanced breast cancer, indicating that these genes encode possible tumor suppressors. Additionally, the R1945Q mutation is located outside the minimal interacting part of FYVE-CENT with Beclin 1, but is still very important for their full interaction, indicating that there are additional interacting surfaces outside this region. Another possible explanation could be that this mutation changes the folding of the C-terminal part of FYVE-CENT or even results in the recruitment of chaperone proteins that might prevent sterically the Beclin 1 binding. In Paper I we showed that PtdIns3*P* recruits FYVE-CENT at the midbody during cytokinesis, and that subunits of the PI3K-III complex, including Beclin 1, are required for correct cytokinesis. In Paper II, we propose a positive-feedback loop model wherein FYVE-CENT can recruit Beclin 1 at the midbody/ intercellular bridge. Further on, Beclin 1 interacts with VPS34; thereby producing more PtdIns3*P*, which in turn can recruit more FYVE-CENT. This model can give an explanation to the significant increase in cells arrested in cytokinesis and bi- and multinuclear cells in FYVE-CENT mutant cells. Additionally, it gives an explanation of how the mutation R1945Q found in FYVE-CENT breast cancer samples can promote cancer. Moreover, apart from the well established role of Beclin 1 in autophagy also a new role of Beclin 1 in cytokinesis is acknowledged. The new role of Beclin 1 in cytokinesis is associated with its tumor suppressor activity and further links Beclin 1 to carcinogenesis.

In Paper III we focus on the ESCRT-III subunit CHMP4B. This protein plays a crucial role in the final abscission step during cytokinesis by participating in the formation of helical filaments that support the constriction of the intercellular bridge and the final abscission (Elia et al., 2011; Guizetti et al., 2011). In the current study, we demonstrate that CHMP4B localizes to various types of intercellular bridges in interconnected cells, and this is the case in various cell lines. This localization pattern has a low frequency (approximately 5% of the total cell population), suggesting that these bridges are formed due to cytokinesis failure.

Additionally, we found that CHMP4B localizes to chromosome bridges and micronuclei. Micronuclei were shown to arise from chromosome bridges in cancer cell lines (Hoffelder et al., 2004). CHMP4B appears to be the first protein to localize in both structures and thus connects failure of cytokinesis with micronuclei, even though its exact role in this process is not clear yet. The gene that encodes for CHMP4B protein (*CHMP4B*) is found mutated in autosomal dominant cataract (Shiels et al., 2007). Therefore, we tested the localization of CHMP4B in the HLEB-3 human epithelial lens cell line and we observed that also in this cell line CHMP4B localizes in micronuclei. This disease is associated with improper degradation of cellular organelles and chromosomal DNA during lens cell differentiation from epithelial to fiber cells. (Nishimoto et al., 2003; Nagata and Kawane, 2011). Based on mouse model studies, DNase II-like acid DNase (DLAD) is shown to be responsible for the degradation of chromosomal DNA in the lens (Nakahara et al., 2007). DLAD is found to colocalize with the lysosomal marker Lamp 1 (Nakahara et al., 2007), possibly indicating that the degradation of DNA could occur via lysosomal degradation. In Paper III we present that lysosomal and autophagic markers Lamp 1 and LC3 localize adjacent and /or attached to CHMP4B positive micronuclei in HeLa and HLEB-3 cells, suggesting that micronuclei may be digested via lysosomal degradation. We further tested this idea by observing the localization of CHMP4B construct containing the mutation D192V which is found in cataract patients and we found that this mutation abolishes CHMP4B localization to micronuclei compared to the wild type construct. This suggests that CHMP4B may have a role in facilitating the degradation of micronuclei. We speculate that CHMP4B facilitates the fusion of lysosomes with the nuclear membrane of the micronuclei. Degradation of nuclei during lens cell differentiation has been associated with the lysosomal machinery (Vrensen et al., 1991) (Nishimoto et al., 2003; Nagata and Kawane, 2011; Nakahara et al., 2007), therefore we believe that CHMP4B participates in the lysosomal degradation of chromosomal DNA during lens cell differentiation and in this way protects the lens cells from the formation of cataract.

Degradation of organelles during lens differentiation occurs independently of the canonical autophagy machinery, since it has been found to occur normally in *Atg5* deficient mice (Matsui et al., 2006). In our experiments we have observed that autophagomes localize adjacent to micronuclei. We suggest that autophagy may facilitate the degradation of small parts of the micronuclei and may act synergistically with lysosomes. This observation is in agreement with the results reported by Nakahara et al. who showed that autophagy related *atg3* and *atg4b* were significantly upregulated during fiber lens cells differentiation (Nakahara et al., 2007). Additionally, Atg5-independent autophagy has been reported to function in the autophagic elimination of organelles during erythrocyte differentiation (Nishida et al., 2009) raising the possibility that alternative non-canonical autophagy may participate during lens differentiation.

In conclusion, in Paper III we present that CHMP4B is a novel structural component of chromosome bridges and micronuclei. We propose that CHMP4B is important for the lysosomal degradation of micronuclei, and this may have implications in DNA degradation during lens cell differentiation and cataract formation.

Experimental considerations

In this section, I will discuss possible pitfalls and limitations concerning the major methods that were used in the papers included in the thesis.

Experimental models: cell lines and *Drosophila*

In most of the experiments performed in the papers included in this thesis, immortarized cancer cell lines were used. The most frequently used cell line was HeLa cells, a cervical cancer cell line cultured in the laboratory. Other cell lines used were Hep2 cells- a human laryngeal carcinoma cell line- MCF-7, HCC1395 and HCC1954- breast cancer cell lines and U2Os, an osteosarcoma cell line. Finally, a transformed human lens epithelial cell line HLE-B3 was used in Paper III. It is obvious from the previous descriptions that cancer cell lines were mostly used in this thesis. These cell lines contain multiple mutations, which are normally not found in the human body. Additionally, they are all cultivated cell lines, grown in the laboratory for many decades and there is a high possibility that extra mutations have occurred during the years. For these reasons, the only way to confirm the generated data based on these studies is to use several cell lines and test if the experimental results obtained from the various different cell lines are similar. Ideally, as an extra confirmation, a model organism should be used. In this thesis, in Paper I, the fruit fly *Drosophila melanogaster* was used and thus our results were confirmed both *in vitro* (based on various cell lines) and *in vivo*. *Drosophila melanogaster* is a powerful genetic model organism for higher animals and has some special characteristics that make it very useful and easy to handle. It has a short life cycle, meaning that short time scale experiments are possible; it does not require expensive equipment for its cultivation, therefore it is an economical tool and since it is a very well studied organism, many genetic tools are available and many mutant RNAi lines have been generated. Additionally, behavioural studies are possible when using *Drosophila melanogaster* and this is not the case when using cells. The effect that a mutation has in humans can often be deduced from genetic studies in *Drosophila*, since the *Drosophila* homologs of human disease genes are very well characterized. In conclusion, various approaches and various different platforms need to be used when handling a scientific question, in order to obtain more accurate results.

Confocal microscopy and quantification

Confocal microscopy is a key method used in all the studies that are included in this thesis. This method has many advantages compared to conventional microscopy, such as much greater optical resolution via the elimination of out-of-focus light in specimens and via the control of the depth of the field. This specificity enables the application of quantitative measurements and the security of proper observation of fluorophores (such as colocalization studies). Despite the multiple advantages of confocal microscopy, there are some precautions and certain pitfalls that need to be taken in consideration when handling this method. First of all, the microscope settings must be exactly the same between the various fluorophores used, the laser power and detector gain must have the same intensity for all the samples and for each different condition and filter settings must be optimized in order to avoid any bleed-through effects. Additionally, when studying a process, in order to obtain unbiased results, it is important that the conclusion is excluded by a big amount of cells, by at least three independent experiments and by observations derived from various areas across the coverslip. In order to avoid the possibility of observing artefacts caused by inappropriate fixation or caused by areas in the coverslip that are not well fixed, it is necessary to try different fixation methods and always observe many cells from the whole surface of the coverslip and then compare the findings. In this thesis, in order to obtain conclusive results, we tried different fixation methods and our results were drawn from many different experiments and from scanning the whole coverslip. This was the case especially when characterizing new proteins like FYVE-CENT or when finding new roles and new localization patterns for already known proteins, such as Beclin 1 and CHMP4B. In knock-down experiments of various proteins, we kept the scanning intensity stable for all samples in order to avoid possible mistaken conclusions e.g false localization etc. In total confocal microscopy is a very important tool for biological sciences when some precautions are taken.

Overexpression of proteins

The overexpression of proteins is accomplished via transfection of foreign DNA into the cells, which encodes the protein of interest, usually fused with a tag (e.g Myc-, HA- or GFP-). This method is very useful because it allows us to visualize proteins, even if there are not any available or properly working antibodies that recognize the proteins of interest. It also enables to visualize

proteins live, using live microscopy without fixing the cell, when tagged with GFP or other fluorescent tags. Overexpression of a protein could provide information about the function of the protein and can also be used when studying protein-protein interactions. Even though there are many advantages when using this method, there are again some pitfalls that need to be taken in consideration. Overexpressed proteins usually have higher expression compared to endogenous proteins and as a result, frequently mislocalize inside the cell, leading to false results. For this reason it is important to observe cells with low level overexpression and this usually can be accomplished when generating a stably transfected cell line. Additionally, overexpressed proteins often form aggregates and in some cases the folding of the overexpressed protein is improper thus making the protein dysfunctional. In this thesis, we have often faced challenges like the above mentioned with overexpressed proteins and in each case we tried to adjust the conditions and thereby minimize artifacts. When encountering such a problem, we always tested the localization of a specific protein also endogenously, in order to confirm its proper localization. Moreover, we tried to pick cells with low level of protein expression, so as to avoid overexpression artifacts.

Gene silencing using siRNA

The small interfering RNA (siRNA) method is based on the fact that short double-stranded RNA molecules specific for a target gene, prevent protein expression by inducing degradation of the corresponding mRNA. This method can reveal very important information concerning the function of the protein of interest that corresponds to the gene silenced. However, when using this method, the analysis of the results needs to be done very carefully. The greatest problem of this method is the occurrence of off-target effects, which means phenotypes that are unrelated to the specific mRNA depletion. In order to avoid this, it is important to include in every experiment a control siRNA-a non-specific siRNA for the target gene- and also repeat the experiment at least three times and with at least two independent siRNA sequences that correspond to the target gene. Another problem of this method is that the knock-down of a protein is not equally efficient in all the cells applied and in some cases there is still some amount of endogenous protein in the cells. In order to prevent possible misinterpretations because of this, it is necessary to test the knock-down both by western blotting and by immunofluorescence analysis, so as to have an accurate idea of the efficiency of the depletion. In immunofluorescence analysis, when staining with antibody against endogenous protein in siRNA depleted cells, we can observe the reduction of the level of the protein in which cells the protein is depleted and we can then observe specifically these cells in

order to make conclusions of its function. In our studies, we always used more than two different siRNA molecules corresponding in the target gene -in some cases even four different molecules, first a pool of siRNAs and then all the four individual oligonucleotides consisting the pool-, we repeated every experiment at least three times and very carefully followed the results. Finally, we performed rescue experiments where we observed that the depletion phenotype was reversed by reintroducing the target protein in a tagged version into the cells.

Conclusions

The present work has shed light on the role of PI3K Class III as well as the role of PtdIns3*P* and PtdIns3*P*- binding or associated proteins in cytokinesis and their association with disease.

The main conclusion in Paper I is that PtdIns3*P* and PI3K Class-III are involved in cytokinesis. Additionally, the PtdIns3*P*-binding protein FYVE-CENT has an important role in cytokinesis, via its interaction with KIF13A and TTC19, which sequentially interacts with the ESCRT-III component CHMP4B.

The main conclusion in Paper II is that FYVE-CENT interacts with Beclin 1 and via this interaction is involved in cytokinesis and carcinogenesis.

Finally, in Paper III our main conclusion is that CHMP4B localizes on intercellular bridges, chromosome bridges and micronuclei. Moreover, we conclude that CHMP4B may contribute to the lysosomal degradation of micronuclei, with implications in DNA degradation during lens cell differentiation and cataract formation.

Even though more investigations on precise molecular mechanisms are needed, this work has contributed to understanding the regulation of the cytokinesis machinery, the proteins involved in this machinery and the association of those proteins with disease. An interesting topic that arises from this thesis and could be further investigated is the elucidation of the mechanism via which ESCRTs are targeted to the plasma membrane. Additionally, although some first insights have been obtained, it would be intriguing to understand the mechanism by which phosphoinositides and more specifically PtdIns3*P* ensure that the membrane during cleavage has the correct composition and biophysical properties. Moreover, it would be interesting to study the role of specific lipids which participate in the composition of the cleavage furrow and intercellular bridge, in order to understand better the mechanism of cytokinesis.

Regarding the functions of PtdIns3*P*, it will be important to investigate possible functional links between the FYVE-CENT axis and ESCRTs and also to understand how PtdIns3*P* formation controls cell-cell abscission. In order to achieve this, it would be necessary to determine the structure of the TTC19-CHMP4B complex and perform mutagenesis experiments so as to test the function of this interaction. Finally, it will be very useful to implement advanced methods of live-cell microscopy to visualize molecules and organelles during cleavage furrow formation and abscission. A combination of such methods with electron microscopy will provide more knowledge concerning the cellular events that occur during cytokinesis.

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Original publications

Paper I

PtdIns(3)*P* controls cytokinesis through KIF13A-mediated recruitment of FYVE-CENT to the midbody.

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Paper II

A tumor-associated mutation of FYVE-CENT prevents its interaction with Beclin 1 and interferes with cytokinesis.

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A Tumor-Associated Mutation of FYVE-CENT Prevents Its Interaction with Beclin 1 and Interferes with Cytokinesis

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Abstract

The tumor suppressor activity of Beclin 1 (BECN1), a subunit of class III phosphatidylinositol 3-kinase complex, has been attributed to its regulation of apoptosis and autophagy. Here, we identify FYVE-CENT (ZFYVE26), a phosphatidylinositol 3-phosphate binding protein important for cytokinesis, as a novel interacting protein of Beclin 1. A mutation in FYVE-CENT (R1945Q) associated with breast cancer abolished the interaction between FYVE-CENT and Beclin 1, and reduced the localization of these proteins at the intercellular bridge during cytokinesis. Breast cancer cells containing the FYVE-CENT R1945Q mutation displayed a significant increase in cytokinetic profiles and bi - multinuclear phenotype. Both Beclin 1 and FYVE-CENT were found to be downregulated in advanced breast cancers. These findings suggest a positive feedback loop for recruitment of FYVE-CENT and Beclin 1 to the intercellular bridge during cytokinesis, and reveal a novel potential tumor suppressor mechanism for Beclin 1.

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Introduction

Beclin 1 is a known tumor suppressor protein that regulates apoptosis and autophagy [1–3]. Importantly, Beclin 1 is a subunit of the phosphatidylinositol 3-kinase class III (PI3K-III) complex and interacts directly with VPS34, the catalytic subunit of PI3K class III complex [4–7]. It also serves as a platform for the recruitment of other proteins such as UVRAG (UV radiation resistance-associated gene) [5], BIF-1/Endophilin B1 [8], and ATG14L/Barkor [9,10] with known functions in autophagy and tumor suppression. In addition to its known roles in endocytic and autophagic membrane traffic, it was recently established that the PI3K Class III complex plays a crucial role in cytokinesis [11–13]. More specifically, the phospholipid PtdIns3*P*, which is produced by VPS34, was found to localize at the intercellular bridge, and depletion of human VPS34 and Beclin 1 resulted in an increased arrest of cells in cytokinesis as well as in an increased amount of binuclear and multinuclear cells [11]. Unsuccessful cytokinesis has been implicated in tumorigenesis but the underlying mechanisms are largely unknown [14].

Here, we uncover a novel potential tumor suppressor mechanism for Beclin 1. We find that Beclin 1 interacts with FYVE-CENT, a PtdIns3*P* binding protein involved in cytokinesis [11]. Further, we show that a tumor-associated mutation of FYVE-CENT abolishes its interaction with Beclin 1, prevents recruitment of Beclin 1 to the intercellular bridge, and is accompanied by cytokinesis arrest and multinuclear phenotype. These results suggest a novel tumor suppressor mechanism for Beclin 1, which is supported by our finding that both Beclin 1 and FYVE-CENT are downregulated in advanced breast cancer.

Results

FYVE-CENT is a novel Beclin 1 interacting protein

We have recently shown that FYVE-CENT is a critical PtdIns3*P* effector protein that regulates cytokinesis [11]. In order to identify interacting partners of FYVE-CENT, we performed a yeast two-hybrid screen in a human T-lymphocyte library, using the C-terminal part of FYVE-CENT as bait (residues 2120–2539). Using this approach, Beclin 1 was identified as a positive hit (Dataset S1). The interaction of Beclin 1 with FYVE-CENT maps to a region containing the coiled coil domain and the evolutionarily conserved domain of Beclin 1 (Figure 1). To verify this interaction biochemically, we performed a pull-down assay, incubating the C-terminus of FYVE-CENT fused to GST with myc-Beclin 1 expressed in HeLa cell lysates. The pull-down assay showed a positive biochemical interaction (Figure 2A). To further verify this interaction, endogenous FYVE-CENT and Beclin 1 were co-immuno-precipitated with an antibody against FYVE-CENT (Figure 2B), indicating that the two endogenous proteins can form a complex in vivo.

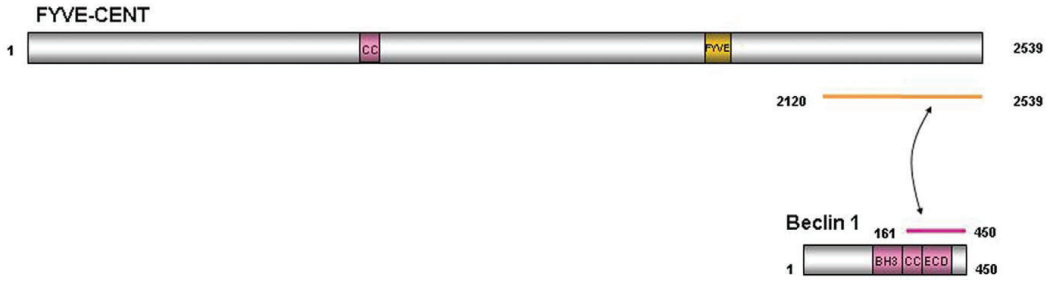

Figure 1. Two-hybrid interactions of Beclin 1 with FYVE-CENT. The figure shows schematically the domain of Beclin 1 that interacts with FYVE-CENT.
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A mutation associated with breast cancer abolishes the interaction between FYVE-CENT and Beclin 1

The *ZFYVE26* gene encoding FYVE-CENT was found mutated in breast cancer samples with a frequency of more than 10% [15]. Since Beclin 1 is a well-known tumor suppressor [2,16], we therefore wanted to test the cell biological consequence of such mutations in the context of FYVE-CENT interaction. To this end we performed a GST pull-down between the C-terminal part of FYVE-CENT (residues 1807–2539) that contains the R1945Q mutation found in breast cancer cell lines [15] and myc-Beclin 1 in HeLa cell lysates. Interestingly we observed that the FYVE-CENT R1945Q mutation abolished the interaction between FYVE-CENT and Beclin 1 (Figure 2C). This was also confirmed in co-immuno-precipitation experiments where endogenous FYVE-CENT R1945Q mutant protein extracted from HCC-1954 breast cancer cells and Beclin 1 did not co-immuno-precipitate with an antibody against endogenous FYVE-CENT, whereas wild-type FYVE-CENT from a control cancer cell line was able to co-immuno-precipitate with Beclin 1 (Figure 2D). We have previously shown that FYVE-CENT interacts with the microtubule-based motor KIF13A and the tetratricopeptide repeat protein TTC19 [11]. KIF13A was found to regulate translocation of FYVE-CENT to the midbody, and the importance of these proteins in cytokinesis is illustrated by the finding that depletion of either FYVE-CENT, KIF13A or TTC19 is sufficient to cause an increased number of cytokinetic profiles and bi- and multinucleate cells [11]. We therefore asked whether the FYVE-CENT R1945Q mutation also interferes with its interaction with KIF13A and TTC19. Interestingly, pull-down assays showed that the R1945Q mutation does not inhibit the interaction of the C-terminus of FYVE-CENT with neither TTC19 nor KIF13A in vitro (Figure 2E). These data indicate that the FYVE-CENT R1945Q mutation associated with breast cancer specifically abolishes the interaction of FYVE-CENT with Beclin 1.

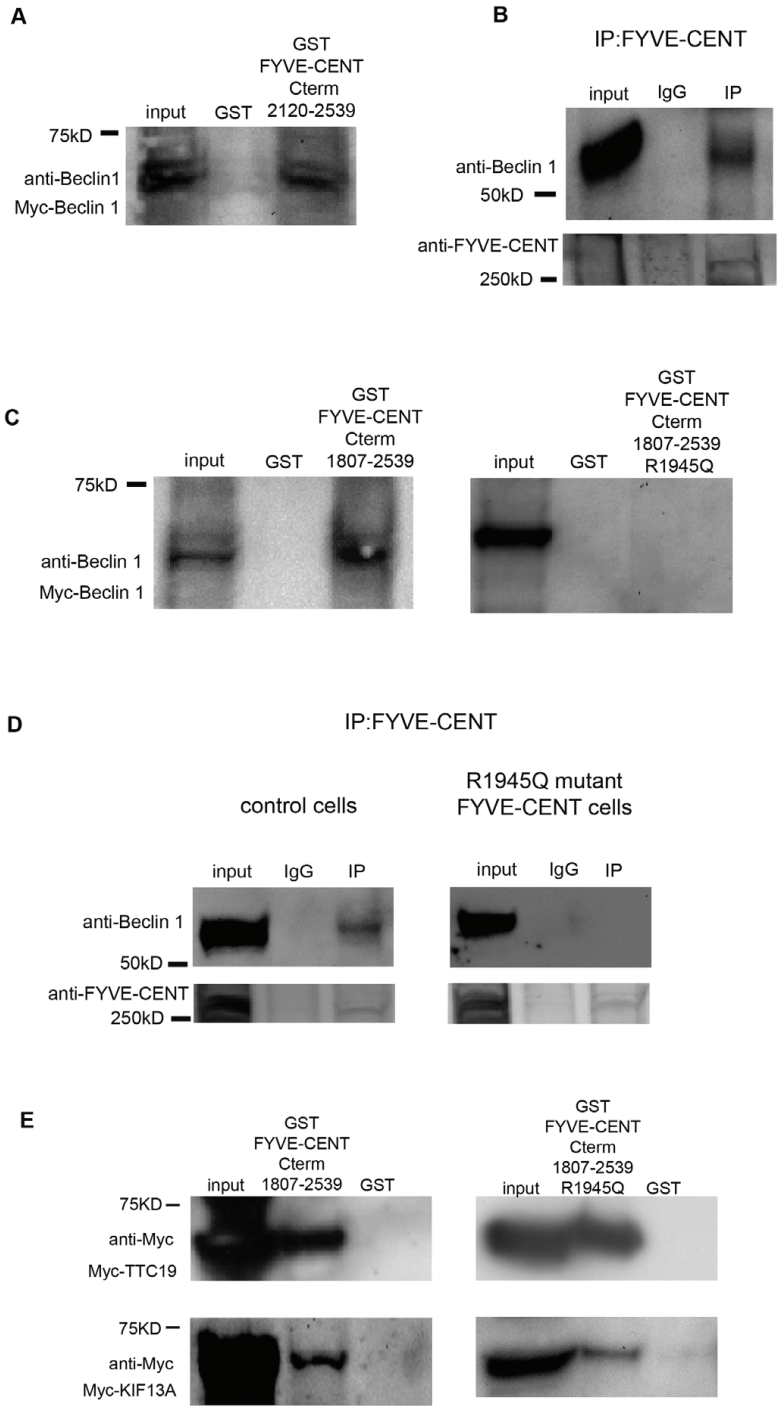
Breast cancer cells containing the FYVE-CENT R1945Q mutation display a significant increase in cytokinetic profiles and hyperploidy

In order to examine the biochemical consequences of the cancer-associated R1945Q mutation of FYVE-CENT, we investigated the HCC-1954 breast cancer cell line which contains this mutation [15]. By cDNA sequencing, we confirmed the mutation status of the cell line and also that the mutant gene is indeed expressed (Figure 3A). Interestingly, cDNA sequencing detected almost exclusively the mutant allele and only a weak signal for the wild-type, indicating a preferential expression of the mutant allele in a heterozygous cell line, or alternatively, that only the mutant allele is present in the majority of the cells, and the existence of a sub-population of cells which is heterozygous for the mutation. The protein levels of Beclin 1 were comparable in the cell line used as control (HCC-1395) and the mutant FYVE-CENT (HCC-1954) cells (Figure S1A). Consistent with this, upon siRNA depletion of FYVE-CENT, Beclin 1 protein levels remained the same (Figure S1B). Likewise, FYVE-CENT levels ramained unaffected by depletion of Beclin 1. In contrast, upon depletion of the Beclin 1 interacting protein VPS34, Beclin 1 became downregulated whereas FYVE-CENT protein levels remained the same (Figure S1B). These results show that the FYVE-CENT R1945Q mutation does not affect the protein levels of Beclin 1.

In order to identify any biological consequence of the FYVE-CENT R1945Q mutation, we examined the phenotype of mutant cells by performing immunofluorescence microscopy using the HCC-1954 and HCC-1395 breast cancer cells. We observed that FYVE-CENT R1945Q mutant cells showed an increased population arrested in cytokinesis (16%) compared to the control cells (6%) and also an increased percentage of binuclear-multinuclear profiles (31.5% versus 19%) (Figure 3B–C and Figure S2A–C). In order to examine whether this phenotype is a direct consequence of the FYVE-CENT R1945Q mutation, we tested whether R1945Q mutation can rescue the arrest in cytokinesis observed upon FYVE-CENT depletion. To examine this we back-transfected HeLa cells which were RNAi-depleted for FYVE-CENT with wild type FYVE-CENT C terminus (1807–2539) or FYVE-CENT C terminus R1945Q mutant. We observed that wild type FYVE-CENT C-terminus could rescue the arrest in cytokinesis and bi-multinuclear phenotype observed upon FYVE-CENT RNAi depletion suggesting that this part of FYVE-CENT entails the minimal functional domains. In contrast, the FYVE-CENT C terminus R1945Q mutant could not rescue the siRNA-induced phenotypes (Figure S3A–C). These data suggest that the FYVE-CENT R1945Q mutation may promote carcinogenesis by interferring with normal cytokinesis.

Beclin 1 localizes at the intercellular bridge during cytokinesis, and this localization is abolished in FYVE-CENT R1945Q mutant breast cancer cells

We next asked how the interplay between FYVE-CENT and Beclin 1 may regulate cytokinesis. We have recently shown that VPS34, the catalytic subunit of PI3K-III complex, and FYVE-CENT are localized at the intercellular bridge during cytokinesis [11]. Given the interaction of Beclin 1 with FYVE-CENT, we examined the localization of Beclin-1 during cytokinesis, and we


A
GST
FYVE-CENT
Cterm
2120-2539
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GST
75kD
anti-Beclin1
Myc-Beclin 1
B
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IgG
IP
anti-Beclin 1
50kD
anti-FYVE-CENT
250kD
C
GST
FYVE-CENT
Cterm
1807-2539
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GST
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anti-Beclin 1
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FYVE-CENT
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Myc-TTC19
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FYVE-CENT
Cterm
1807-2539
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R1945Q
GST
75KD
anti-Myc
Myc-KIF13A

Figure 2. Beclin 1 interacts with FYVE-CENT. (A) GST pull-down from HeLa cell lysates transiently over-expressing myc-Beclin 1 using recombinant GST-FYVE-CENT C-terminal fusion (2120–2539) protein or GST protein immobilized on glutathione-Sepharose beads. Proteins eluted from the beads were analyzed by SDS-PAGE and immuno-blotting using an anti-myc antibody. Equal amounts of GST-FYVE-CENT C-terminal fusion protein and GST protein were loaded. (B) HeLa cell lysates were subjected to immunoprecipitation (IP) with an antibody against FYVE-CENT. Immunoprecipitated proteins were detected by Western blotting, using anti-Beclin 1 and anti-FYVE-CENT antibodies. (C) HeLa cells transiently over-expressing myc-Beclin 1 were pulled down with recombinant GST-FYVE-CENT C-terminal fusion (1807–2539 or 1807–2539 R1945Q) protein or GST protein immobilized on glutathione-Sepharose beads. (D) HCC-1395 control cells and HCC-1954 FYVE-CENT R1945Q mutant cells were lysed and subjected to immunoprecipitation (IP) with an antibody against FYVE-CENT. Immunoprecipitated proteins were detected by Western blotting, using anti-Beclin 1 and anti-FYVE-CENT antibodies. (E) HeLa cells transiently over-expressing myc-TTC19 or myc-KIF13A were pulled down with recombinant GST-FYVE-CENT C-terminal fusion protein and GST-FYVE-CENT C-terminal R1945Q fusion protein or GST protein immobilized on glutathione-Sepharose beads.
doi:10.1371/journal.pone.0017086.g002

found that this protein also localizes at the intercellular bridge (Figure 4A, upper panels). Interestingly, Beclin 1 was also found to localize at the intercellular bridge in the control cell line HCC-1395, whereas in the FYVE-CENT R1945Q mutant breast cancer cell line this localization was significantly reduced (Figure 4A and 4C). Additionally, the localization of FYVE-CENT at the intercellular bridge was partially abolished in the FYVE-CENT R1945Q mutant breast cancer cell line (Figure. 4B and 4C). Taken together, these data suggest that the FYVE-CENT R1945Q mutation prevents localization of Beclin 1 at the intercellular bridge and interferes with proper cytokinesis.

Downregulation of FYVE-CENT and Beclin 1 in advanced breast cancer

To further explore the association of FYVE-CENT with breast cancer, we examined its expression pattern in previously published gene expression data [17,18]. We found that the average expression of *FYVE-CENT* was significantly lower in high vs. low grade breast cancers (Figure 5A). Furthermore, we found that there was a similar significant association between decreased *BECN1* mRNA levels and tumor grade (Figure 5B). More specifically, breast cancers of grade 3 had a significantly lower expression mean than grade 1 and 2 tumors. Interestingly, we also observed that the average expression of *KIF13A* and *TTC19* was significantly lower in high vs. low grade breast cancers (Figure 5C and 5D). Altogether, the associations to clinical parameters strengthen the links between FYVE-CENT, Beclin 1 and breast cancer biology.

Discussion

The tumor suppressor activity of Beclin 1 has been attributed to its interactions with proteins that regulate cell death and autophagy [2,3,7]. Our present data suggest an additional mechanism for the tumor suppressor functions of Beclin 1, namely its ability to bind FYVE-CENT and participate in the regulation of cytokinesis. Failure to complete cytokinesis has been implicated in carcinogenesis [14,19,20], and our data demonstrate that the Beclin 1 - FYVE-CENT complex may play important roles in controlling this process. Importantly, mutations in FYVE-CENT associated with breast cancer interfere with its interaction with Beclin 1. It is interesting that loss of this interaction is accompanied by cytokinesis failure, since this suggests a mechanism that may contribute to the cancer phenotype of FYVE-CENT mutant cancer cells.

The fact that the R1945Q mutation is located outside the minimal interacting part of FYVE-CENT with Beclin 1 may suggest that there are additional interacting surfaces that extend outside the 2120–2539 C-terminal part that was used as bait in the yeast two- hybrid screen. Alternatively, the R1945Q mutation might promote a conformational change in C-terminal folding that could alter its association with Beclin 1, or result in recruitment of chaperone proteins that would sterically prevent Beclin 1 binding. The R1945Q mutation does not affect the interaction of FYVE-CENT with KIF13A and TTC19, suggesting that it specifically abolishes binding to Beclin-1. The downregulation of *FYVE-CENT*, *BECN 1*, *KIF13A* and *TTC19* in advanced breast cancer is consistent with the possibility that these proteins may participate in tumor suppression.

We have recently shown that PtdIns3*P* recruits FYVE-CENT at the midbody during cytokinesis, and that subunits of the PI3K-III complex, including Beclin 1, are required for correct cytokinesis [11]. Our present data suggest a positive-feedback loop model wherein FYVE-CENT can recruit Beclin 1 at the intercellular bridge. Subsequently, Beclin 1 can interact with VPS34, thereby producing more PtdIns3*P*, which in turn can recruit more FYVE-CENT. This model (Figure 5E) would explain the significant increase in cells arrested in cytokinesis and bi- and multinuclear cells in FYVE-CENT mutant cells and highlight a role for Beclin 1 in cytokinesis. Collectively, our findings reveal a novel regulatory role of the tumor suppressor Beclin 1 and its binding partner FYVE-CENT that has potential implications for carcinogenesis.

Materials and Methods

Cell culture and transfections

HeLa cells were grown and transfected as described previously [11]. HCC-1395 (CRL-2324) and HCC-1954 (CRL-2338) cells were purchased from ATCC and grown in RPMI-1640 medium (GIBCO, Invitrogen) supplemented with 10% fetal bovine serum in a 5% CO_2 atmosphere at 37°C.

Confocal fluorescence microscopy

Immunofluorescence microscopy was performed using HeLa, HCC-1395 and HCC-1954 as previously described [11]. The following primary antibodies were used for immunofluorescence studies: rabbit anti-human FYVE-CENT antibody, used in 1:300 dilution, as described before [11], mouse anti-α-tubulin, used in 1:1000 dilution and purchased from SIGMA, rabbit anti-human Beclin 1 and mouse anti-human Aurora B antibody, both used in 1:200 dilution and purchased from Abcam. The secondary antibodies used were goat-anti-mouse Alexa Fluor® 488, in 1:500 dilution from Invitrogen and Cy3-labelled goat anti-rabbit antibody, in 1:500 dilution and Cy2-labelled goat anti-mouse antibody, in 1:200 dilution purchased from Jackson Immunoresearch. Alexa Fluor® 594 phalloidin, used in 1:750 dilution, and Hoechst 33342, used at 1 μg/μl, were purchased from Invitrogen.

Immunoblotting

To determine the cell-specific distribution of FYVE-CENT, Beclin 1, VPS34, beta-actin and the overexpressed TTC19 and KIF13A-myc tagged constructs, the various cell lines were lysed in lysis buffer (25 mM HEPES pH 7.2, 125 mM potassium acetate, 2.5 mM magnesium acetate, 5 mM EGTA, 1 mM DTT, 0.5%

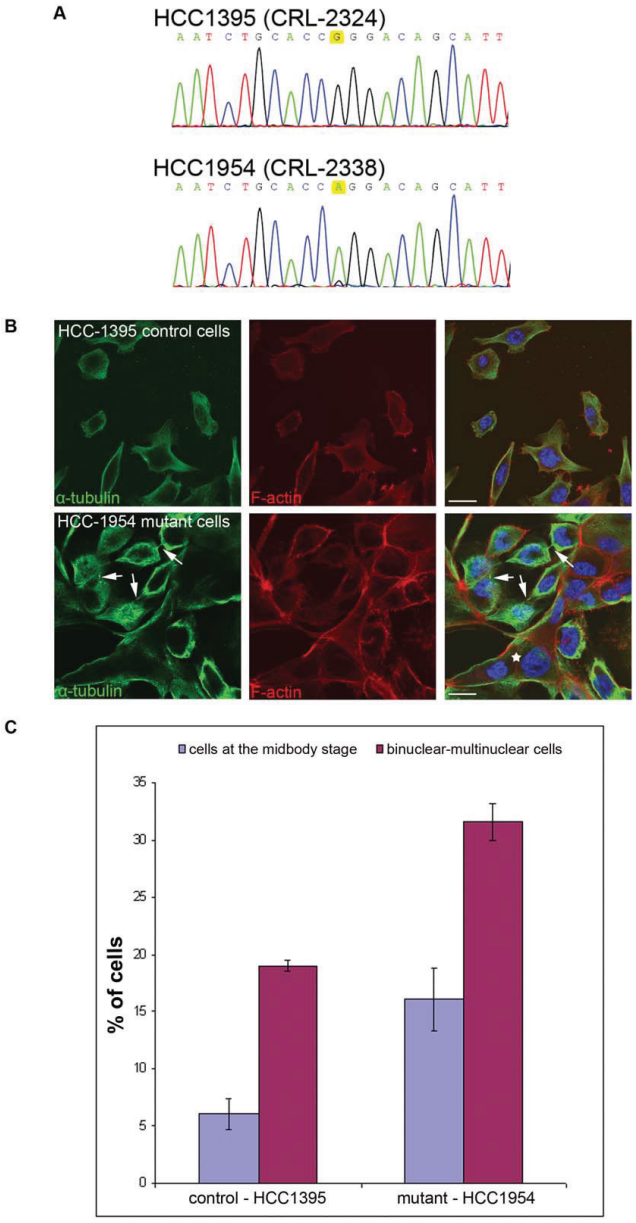
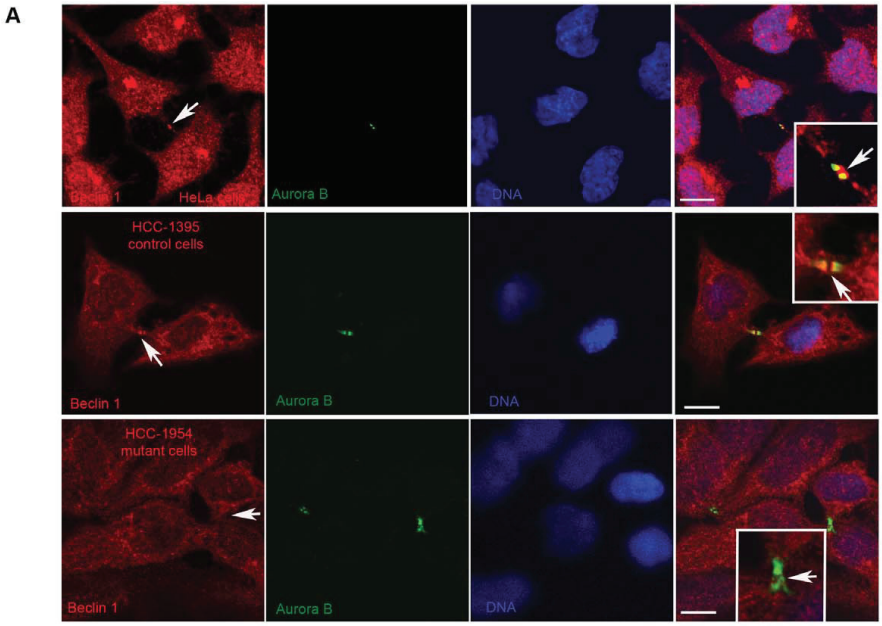
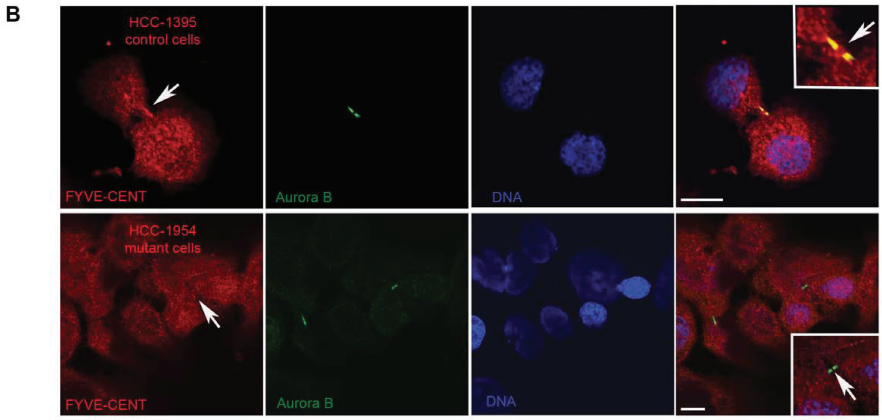


Figure 3. A FYVE-CENT R1945Q mutant breast cancer cell line exhibits an increased number of cells arrested in cytokinesis as well as bi- and multinuclear cells. (A) Sequencing of cDNA for exons 31 to 33 of *FYVE-CENT* from the HCC-1395 and HCC1954 breast cancer cell lines revealed a G to A substitution at base position 5834 in the HCC1954 cell line. (B). Confocal micrographs of HCC-1395 and HCC-1954 breast cancer cell lines cells stained with α-tubulin, Alexa Fluor® 594 phalloidin and Hoechst. In FYVE-CENT mutant cells (HCC-1954) there is a significant increase in cells arrested in cytokinesis (arrows) compared to the control as well as increase in binuclear-multinuclear cells (asterisk). Scale bars: 20 μm. (**C**) Graphic presentation of quantification of cells arrested at the midbody stage and bi-multinuclear cells in control cells (HCC-1395) and FYVE-CENT R1945Q mutant cell line (HCC-1954). Error bars show mean ± s.d. Control: 3 independent experiments, n = 1142 cells. Mutant cells: 3 independent experiments, n = 1225 cells. p value for cells arrested at the midbody stage <0.01. p value for binuclear-multinuclear cells <0.01.
doi:10.1371/journal.pone.0017086.g003


A
Beclin 1
HeLa cells
Aurora B
DNA
HCC-1395
control cells
Beclin 1
Aurora B
DNA
HCC-1954
mutant cells
Beclin 1
Aurora B
DNA


B
HCC-1395
control cells
FYVE-CENT
Aurora B
DNA
HCC-1954
mutant cells
FYVE-CENT
Aurora B
DNA

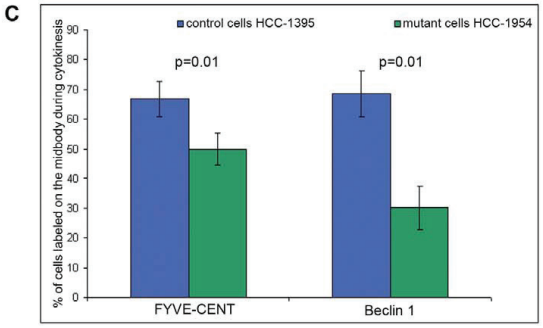

C
control cells HCC-1395
mutant cells HCC-1954
p=0.01
p=0.01
% of cells labeled on the midbody during cytokinesis
0
10
20
30
40
50
60
70
80
90
FYVE-CENT
Beclin 1

Figure 4. The localization of Beclin 1 to the intercellular bridge during cytokinesis is abolished in FYVE-CENT R1945Q mutant breast cancer cells. (A) and (B) Confocal micrographs of HeLa, HCC-1395 and HCC-1954 cells stained with antibodies against Aurora B and Beclin 1 (A) or FYVE-CENT (B), and with Hoechst. Magnifications of the intercellular bridges are shown in the insets. Scale bars: 10 μm. (C) Graphic presentation of quantification of control cells (HCC-1395) and mutant cells (HCC-1954) labeled on the midbody with anti-FYVE-CENT or anti-Beclin 1 antibodies. Error bars show mean ± s.d. Control cells stained with anti-FYVE-CENT: 4 independent experiments, n = 1769 cells. Mutant cells stained with anti-FYVE-CENT: 4 independent experiments, n = 1781 cells. Control cells stained with anti-Beclin 1: 4 independent experiments, n = 1340. Mutant cells stained with anti-Beclin 1: 4 independent experiments, n = 1521. p value for cells labeled with anti-FYVE-CENT on the midbody: 0.01. p value for cells labeled with anti-Beclin 1 on the midbody: 0.01.
doi:10.1371/journal.pone.0017086.g004

Nonidet P40, 1:100 proteinase inhibitor mix (Roche Applied Science). After centrifugation for 5 min at 5,000 *g* the samples were sonicated for 10 s at 70 volts and incubated for 10 min on ice in lysis buffer. Another centrifugation at 10,000 g separated the supernatant from the pellet and 30 μg of protein of the supernatant was subjected to SDS–PAGE (4–20% gradient) and transferred to Immobilon-P membrane (Millipore) for immunoblotting. The blot was developed with the Supersignal West Pico Chemiluminescent substrate kit or Supersignal West Femto Maximum Sensitivity Substrate kit (Pierce). The antibodies used for immunoblotting were the following: Rabbit anti-human Beclin 1 antibody used for western blotting and immunoprecipitation, was purchased from Cell Signaling Technology. Rabbit c-Myc polyclonal antibody was purchased from Abcam and the rest antibodies used (anti-FYVE-CENT, anti-VPS34, anti-beta-actin, anti-GST and HRP labeled) were described previously [11]. For quantitative Western blotting, equal amounts of cell lysates (as measured by protein content) from control and mutant cells were loaded in triplicates on a gel for PAGE. The proteins were transferred to a PVDF membrane and stained with antibodies for FYVE-CENT, Beclin1 and β-actin. The bands were detected using LiCore infrared dye secondary antibodies and the Odyssey imaging system. The bands were quantified using the Odyssey quantifying software.

GST pull-down assay

The GST–FYVE-CENT C-terminus (amino acid residues 2120–2539), the GST–FYVE-CENT C-terminus (1807–2539) and the GST–FYVE-CENT C-terminus (1807–2539) mutant R1945Q constructs were expressed in BL21 *Escherichia coli*, purified and GST-pull down assays were performed as described previously [11].

Co-immunoprecipitation analysis

Rabbit antibody against FYVE-CENT or rabbit IgG (control) were rotated at RT (room temperature) with Protein A agarose beads for 1 h. Then the beads were washed two times with PBS and two times with 0.2 M triethanolamine, pH 8.2. Crosslinking was performed by rotating the beads in 0.2 M triethanolamine containing 3 mg/ml dimethyl pimelimidate at 4°C overnight. In order to quench the unreacted beads, they were rotated with 10 mM ethanolamine, pH 8.2, at 4°C for 30 min. The beads were washed three times with PBS and were used for immunoprecipitation.

HeLa, HCC-1395 and HCC-1954 cells were grown confluent in 10-cm culture dishes and lysed in ice-cold lysis buffer (20 mM HEPES pH 7.2, 2 mM MgCl2, 100 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100) containing inhibitors (*N*-ethylmaleimide, mammalian protease inhibitor mixture, phosphatase inhibitor cocktail I and II (Sigma-Aldrich).The lysates were placed on ice and centrifuged at 10,000 g, 4°C and the supernatant was added to the Protein A-coupled magnetic beads (Dynal, Invitrogen) which had been precoupled with rabbit antibody against FYVE-CENT or rabbit IgG as a control, in PBS Tween 20. Antibody coupled magnetic beads and cell lysates were gently mixed for 1 h at 4°C. The beads were then washed with lysis buffer, eluted in 4× sample buffer plus 1 mM DTT at 95°C for 5 min. The eluted proteins were subsequently subjected to SDS–PAGE and immunoblotting as described previously.

Plasmid constructs

All the FYVE-CENT constructs used were generated by PCR with the *FYVE-CENT* cDNA (ORF) (NM_015346.2), which was cloned in a pCMV6-XL4 vector by OriGene Technologies, Inc., as template. Synthetic oligonucleotides were from MWG Biotech. The FYVE-CENT R1945Q mutant was prepared by PCR site-directed mutagenesis. PCR errors were excluded by sequencing. For expression as GST fusion proteins in *Escherichia coli* BL21 (DE3) cells, the C-terminal part (2120–2539) as well as (1807–2539) and with mutation (R1945Q) of FYVE-CENT were cloned into pGEX-6P-3 (Pharmacia Amersham). The expression plasmid encoding myc-epitope-tagged mouse KIF13A and the Myc-DDK-tagged ORF clone of Homo sapiens TTC19 (NM_017775.2) were obtained as described previously [11]. Expression in mammalian cells and purification were performed as described previously [11].

Assay of rescuing cytokinesis phenotype in RNAi FYVE-CENT depleted cells

HeLa cells were transfected with siRNA (70 nM) against human FYVE-CENT for 72 h. The siRNA-treated cells were then seeded onto coverslips in a 5 cm culture dish and were transfected with myc-tagged C- terminal 1807–2539 and myc-tagged C-terminal 1807–2539 R1945Q FYVE-CENT constructs respectively in three different series of experiments for 36 h. The cells were washed in PBS, stained with anti-myc and anti-α tubulin antibodies and processed in confocal microscopy analysis as described above. The experiment was repeated three times and in total, and 270 back transfected cells were quantified. In parallel, simple depletion experiments using control and FYVE-CENT siRNA were performed in triplicates and quantified using the same stainings and conditions.

RNA interference studies

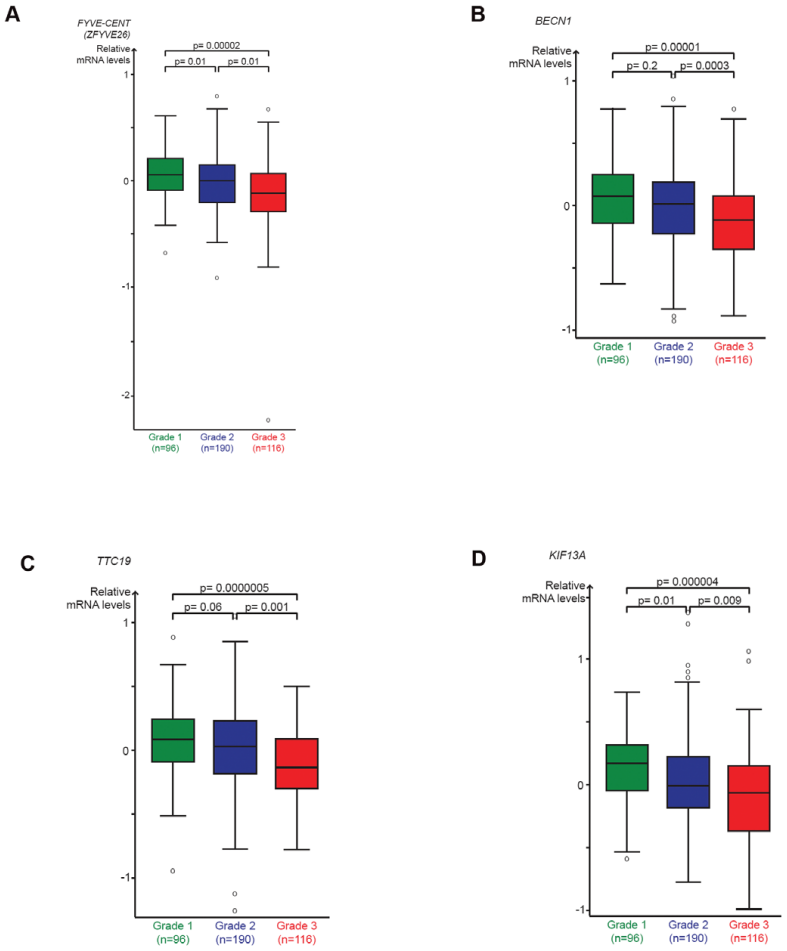
Single deconvoluted siRNAs against FYVE-CENT (cat.no. D-031136-04), VPS34 (PIK3C3)(cat. no. D-005250-04) and Beclin 1(siRNA 1: cat. no. J-010552-05) were purchased from Dharmacon Research. The siRNA experiments were performed on HeLa cells as described before [11].

Yeast two-hybrid screening

The yeast two-hybrid screening was based on the C terminus (residues 2120–2539) of FYVE-CENT as bait and performed by Hybrigenics S.A Services using a human T cells RP1 (CEMC7) library.

RNA isolation/cDNA sequencing

Total RNA was isolated from HCC-1395 (control cells) and HCC-1954 (FYVE-CENT R1945Q mutants cells) (1 well of a 6


A
FYVE-CENT (ZFYVE26)
Relative mRNA levels
p= 0.00002
p= 0.01
p= 0.01
Grade 1 (n=96)
Grade 2 (n=190)
Grade 3 (n=116)
B
BECN1
Relative mRNA levels
p= 0.00001
p= 0.2
p= 0.0003
Grade 1 (n=96)
Grade 2 (n=190)
Grade 3 (n=116)
C
TTC19
Relative mRNA levels
p= 0.0000005
p= 0.06
p= 0.001
Grade 1 (n=96)
Grade 2 (n=190)
Grade 3 (n=116)
D
KIF13A
Relative mRNA levels
p= 0.000004
p= 0.01
p= 0.009
Grade 1 (n=96)
Grade 2 (n=190)
Grade 3 (n=116)

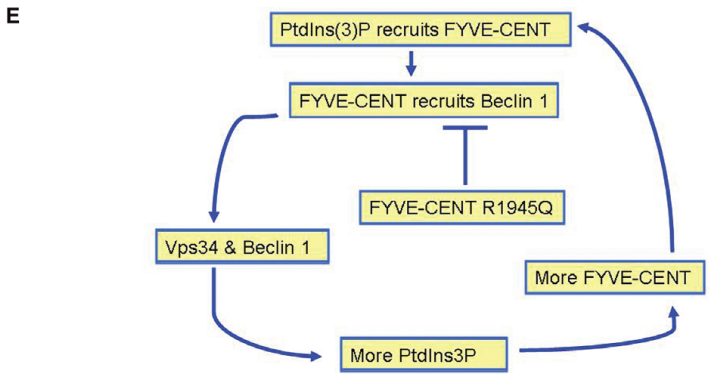

E
PtdIns(3)P recruits FYVE-CENT
FYVE-CENT recruits Beclin 1
FYVE-CENT R1945Q
Vps34 & Beclin 1
More FYVE-CENT
More PtdIns3P

Figure 5. The average expression of *FYVE-CENT* and associated genes is significantly lower in high vs. low grade breast cancers. (A)–(D) The gene expression data were derived from the Gene Expression Omnibus GSE1456 and GSE4922 datasets. The expression values were median centred within each of the series separately. The p-values were derived from comparing means by independent samples t-test (SPSS, v.16.0). (E) Proposed model: FYVE-CENT- Beclin 1 interplay in a positive feedback loop manner during cytokinesis.
doi:10.1371/journal.pone.0017086.g005

well plate for each) using the Total RNA Mini Kit (BioRad) according to the manufacturer's descriptions. One microgram RNA was converted to cDNA using the iScript cDNA Synthesis kit (BioRad). Forward and reverse primers, AGGAGGAAAATGAGCTGGTG and CAGCACATCTACCTTGCTGA, were designed with the Primer3 software using default settings, and PCR products were sequenced in forward and reverse using an ABI 3730 DNA Analyzer (Life Technologies).

Statistical analysis

Values are given as means and s.d in all figures. The p values are calculated based on t-test.

Supporting Information

Figure S1 FYVE-CENT and Beclin 1 expression in HCC-1395 and HCC-1954 breast cancer cells. (A) Whole cell lysates from HCC-1395 and HCC-1954 (FYVE-CENT R1945Q mutant) cell lines were analyzed by immunoblotting with the indicated antibodies. Equal amounts of cell lysates (as measured by protein content) from control and mutant cells were loaded in triplicates. The bands were detected using LiCore infrared dye secondary antibodies and the Odyssey imaging system. The bands were quantified using the Odyssey quantifying software, and the numbers resulting from the average of three loadings are shown. (B) Whole cell lysates from HeLa cells transfected with scrambled (scr) or the indicated siRNAs were analyzed by immunoblotting with the indicated antibodies. Experiments were repeated three times and a representative blot is shown.
(TIF)

Figure S2 A FYVE-CENT R1945Q mutant breast cancer cell line exhibits an increased number of cells arrested in cytokinesis as well as bi- and multinuclear cells. (A–B) Confocal micrographs of HCC-1395 and HCC-1954 breast cancer cells stained with the Aurora B and Hoechst (A), and HCC-1954 breast cancer cells stained with the α-tubulin, Alexa Fluor® 594 phalloidin and Hoechst (B). In FYVE-CENT mutant cells (HCC-1954) there is a significant increase in cells arrested in cytokinesis (arrows) compared to the control (A) as well as increase in binuclear-multinuclear cells (B). Scale bars: 10 μm. (C) Graphic presentation of quantification of cells arrested at the midbody stage and bi-multinuclear cells in FYVE-CENT control (HCC-1395) and R1945Q mutant cell lines (HCC-1954). Error bars show mean ± s.d. Control: 6 independent experiments, n = 1982 cells. Mutant cells: 6 independent experiments, n = 2001 cells. p value for cells arrested at the midbody stage: 0.001. p value for binuclear-multinuclear cells: 7×10^{-7}.
(TIF)

Figure S3 Back-transfection of R1945Q FYVE-CENT mutant C terminus (1807–2539) transgene does not rescue cytokinesis arrest caused by siRNA compared to FYVE-CENT C terminus (1807–2539). Hela cells were tranfected with myc- FYVE-CENT C terminus (1807–2539) and siRNA against FYVE-CENT (A), or myc- FYVE-CENT C terminus (1807–2539) R1945Q mutant and siRNA against FYVE-CENT (B) transgenes simultaneously. The cells expressing myc- FYVE-CENT C terminus (1807–2539) transgene can rescue arrest in cytokinesis compared to the adjacent cells (A) but myc-FYVE-CENT C terminus (1807–2539) R1945Q cannot (B) (arrows). (C), Quantification of the results shown in (A) and (B). Scale bars 10 μm.
(TIF)

Dataset S1 Positive hits from yeast two-hybrid screening with the C-terminus of FYVE-CENT. A list of the interacting proteins with the C-terminus (residues 2120–2539) of ZFYVE26 (FYVE-CENT) were identified in a two-hybrid screen of a human T cells RP1 (CEMC7) cell library. The data are from Hybrigenics S.A, Paris, France.
(XLS)

Author Contributions

Conceived and designed the experiments: APS IPN KGB KH RIS HS. Performed the experiments: APS IPN KGB KH ACB. Analyzed the data: APS IPN RIS. Contributed reagents/materials/analysis tools: HS RIS. Wrote the paper: APS IPN HS. Edited the manuscript: KGB KH ACB RIS.

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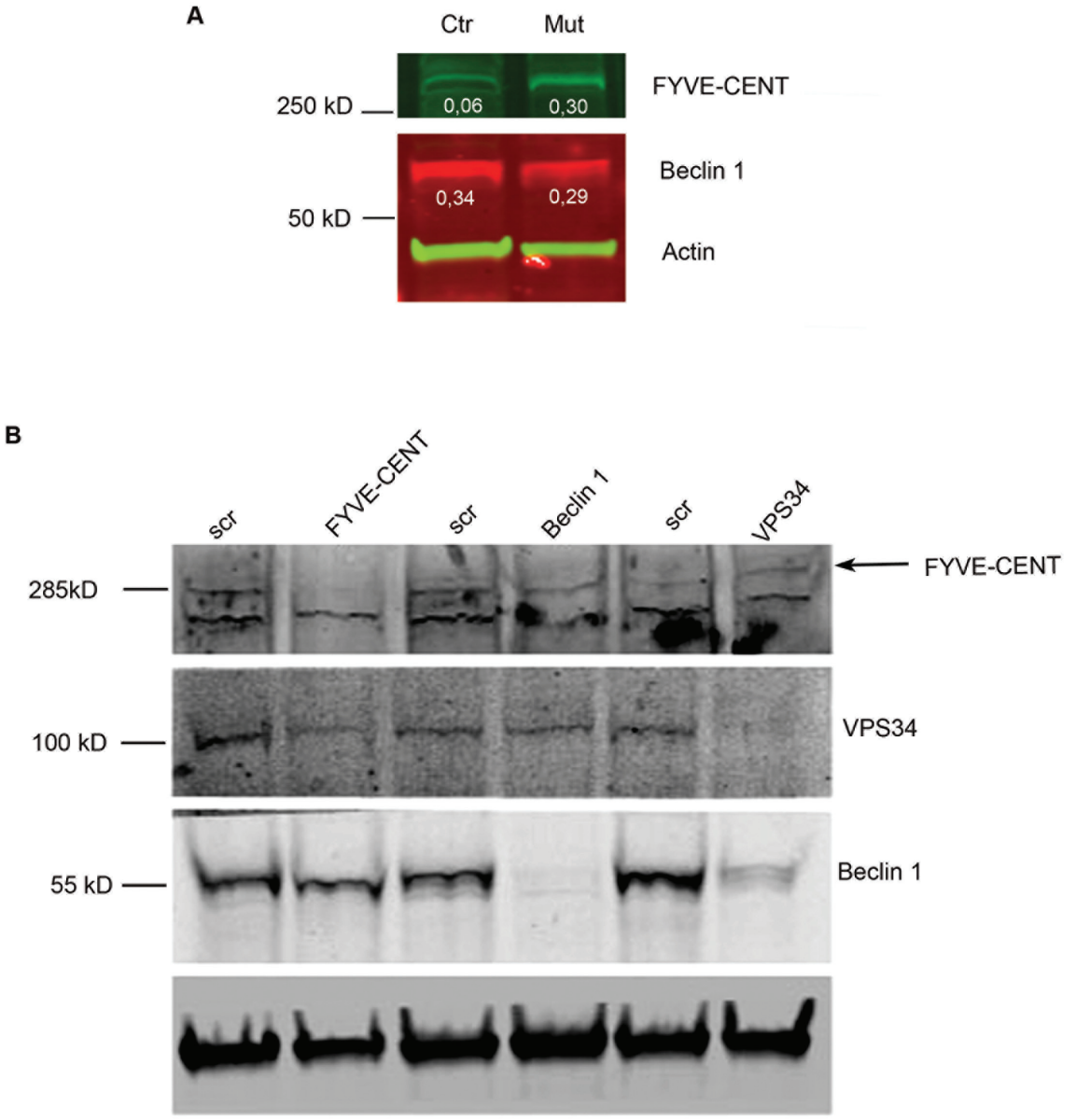
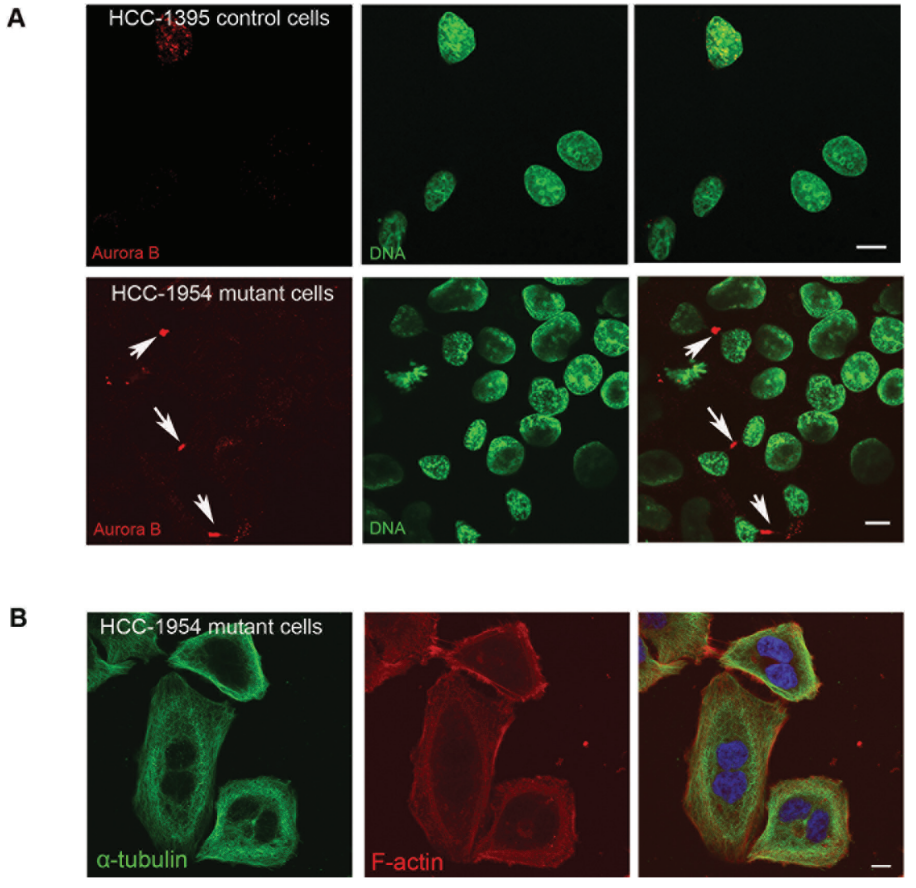
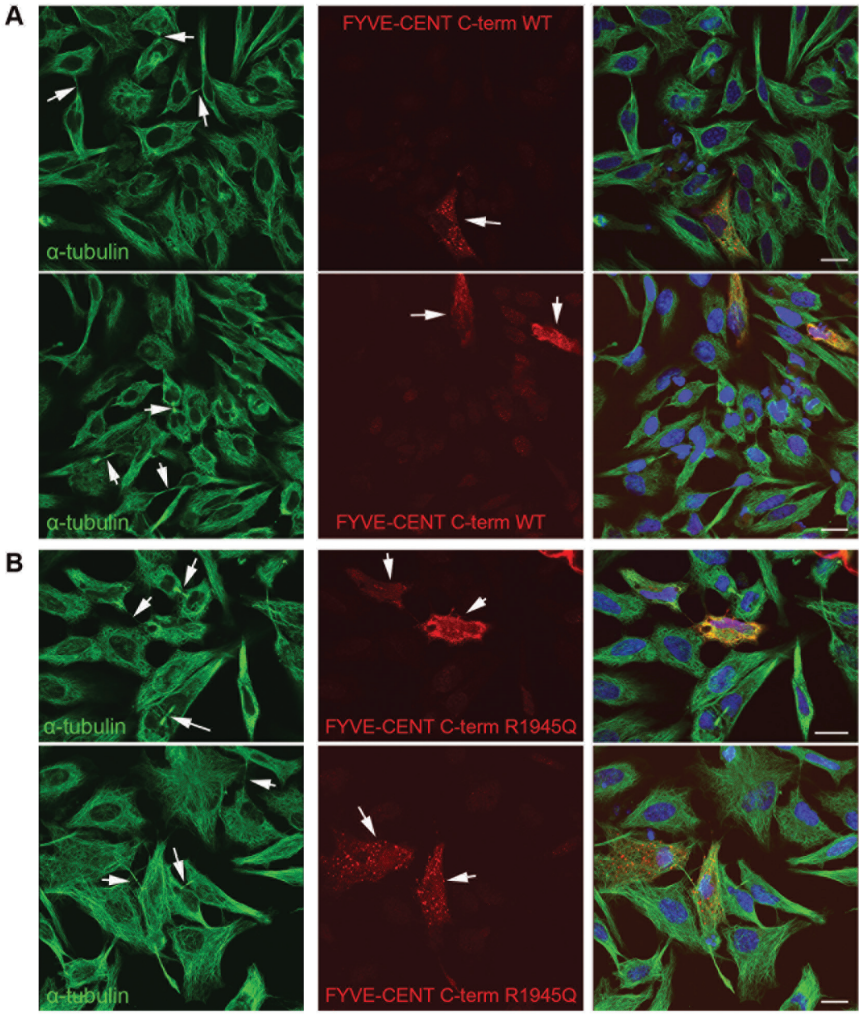
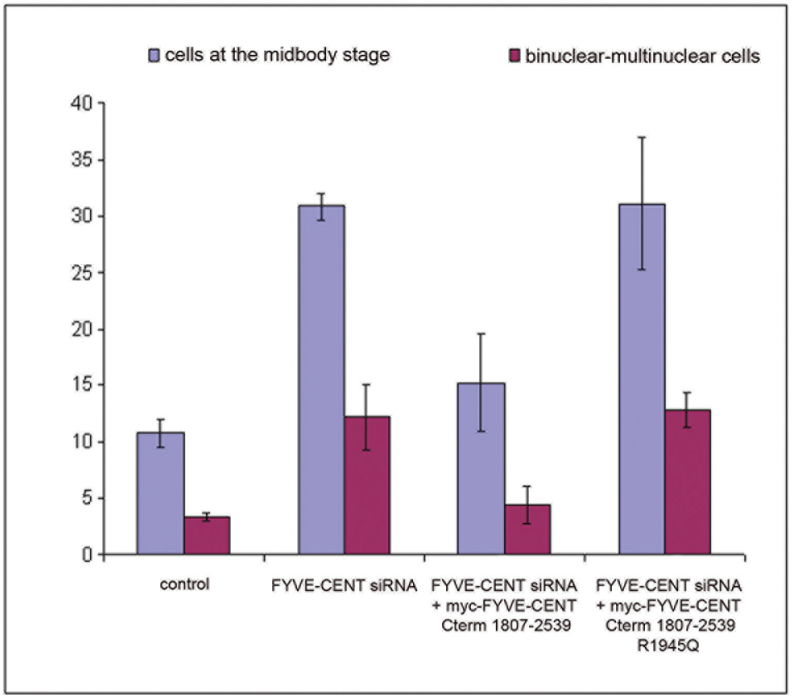


Suppl. Figure 1 Sagona et al. 2011



Suppl. Figure 2 Sagona et al. 2011





Suppl. Figure 3 Sagona et al., 2011

Paper III

Association of CHMP4B with chromosome bridges and micronuclei: implications for cataract formation.

Sagona AP, Nezis IP and Stenmark H. Manuscript.