

UC San Diego

UC San Diego Electronic Theses and Dissertations

Title

Functional and chemical analysis of the secretory pathway

Permalink

<https://escholarship.org/uc/item/82t7939n>

Author

Guizzunti, Gianni

Publication Date

2007

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Functional and chemical analysis of the secretory pathway

A Dissertation submitted in partial satisfaction of the requirements for the degree Doctor
of Philosophy

in

Biology

by

Gianni Guizzunti

Committee in charge:

Professor Vivek Malhotra, Chair
Professor Scott Emr
Professor Randolph Hampton
Professor Suresh Subramani
Professor Emmanuel Theodorakis
Professor Michael Yaffe

2007

The Dissertation of Gianni Guizzunti is approved, and it is acceptable in quality and form
for publication on microfilm:

Chair

University of California, San Diego

2007

This Dissertation is dedicated to my parents, Assunta and Giovanni, my brother Guido, and my wife Sandra. In these five years I spent far from home, my family has always been at my side, supporting every decision I made. A special thank goes to my wife, which left her own family to follow me in this journey. Because of you I never felt alone, I never will.

TABLE OF CONTENTS

Signature Page	iii
Dedication	iv
Table of Contents	v
List of Figures	vii
Acknowledgments	ix
Vita	x
Abstract	xii
Introduction	1
A. The secretory pathway	1
B. The Golgi Apparatus	3
C. Approaches to the secretory pathway	5
D. Chemical compounds affecting Golgi structure and function	7
Chapter 1: Functional analysis of the secretory pathway	13
1.1 Abstract	13
1.2 Introduction	14
1.3 Results	18
1.4 Discussion	42
1.5 Methods	43
Chapter 2: Chemical analysis of the secretory pathway, part I	
Norrisolide-mediated Golgi vesiculation.....	44

2.1 Abstract	44
2.2 Introduction	44
2.3 Results	50
2.4 Discussion	61
2.5 Methods	62
Chapter 3: Chemical analysis of the secretory pathway, part II	
Trifunctional probes for the study of Golgi vesiculation	64
3.1 Abstract	64
3.2 Introduction	65
3.3 Results	66
3.4 Discussion	76
3.5 Methods	77
Conclusion	82
A. Functional analysis of protein transport: TANGO genes	82
B. Tango5	84
C. Pharmacological approach: towards norrisolide target	85
References	87

LIST OF FIGURES

Introduction

Figure 1. The secretory pathway	2
Figure 2. Chemical structures of selected Golgi-disturbing agents	8
Figure 3. Compounds affecting Golgi structure.....	9

Chapter 1

Figure 4. Four classes of Tango genes	16
Figure 5. Tango genes localization	19
Figure 6. Tango11 structure, isoforms and localization	26
Figure 7. Tango11 mutants	26
Figure 8. Localization of Tango5	28
Figure 9. Human Tango5 colocalization	30
Figure 10. VSV-G transport in Tango5 expressing cells	32
Figure 11. Endogenous Tango5 in HeLa cells	34
Figure 12. Tango5 Antibody in HeLa cells	34
Figure 13. Topology, proof of concept	37
Figure 14. Tango5 topology	38
Figure 15. Tango5 cysteines	40
Figure 16. Tango5 mutants	41

Chapter 2

Figure 17. Representative structures of spongiane diterpenes	49
Figure 18. Effect of norrisolide and analogues on Golgi membranes	53

Figure 19. Structures of norrisolide-fluorescent probes	55
Figure 20. Activity and intracellular localization of norrisolide-fluorophore	57
Figure 21. Structures of norrisolide-epoxide probes	59
Figure 22. Effect of norrisolide and analogues on Golgi membranes.....	60

Chapter 3

Figure 23. Photoactivable trifunctional probe	67
Figure 24. Norrisolide-aryl azide probe on NRK cells	69
Figure 25. Western blot of norrisolide-aryl azide treated cells	70
Figure 26. Norrisolide-epoxide-biotin trifunctional probes	72
Figure 27. Norrisolide-epoxide-biotin on NRK cells	72
Figure 28. Norrisolide-bisepoxide-iodine trifunctional probe	74
Figure 29. Norrisolide-bisepoxide-iodine on NRK cells	75
Figure 30. Synthesis of compound 10 and 11	78
Figure 31. Synthesis of compound 12	79
Figure 32. Synthesis of compound 13	80
Figure 33. Synthesis of compound 14	81

ACKNOWLEDGEMENTS

I would like to thank my advisor, Vivek Malhotra, an incredible mentor, for giving me the opportunity to work in his group. Vivek could always see the big picture, and never failed to put me back on the right track the many times I got lost. Thanks for giving me the freedom to make my own mistakes, and for your encouraging words, and cursing too. This has proven invaluable for my growth as a scientist. I would also like to thank the entire Malhotra lab, past and present members, for sharing this journey with me.

Special thanks go to Dr. Emmanuel Theodorakis and his lab, for showing me the beauty of chemistry. Most of this work would not have been possible without their help. I also appreciate the positive imputes and advises given by my committee members.

Thanks also to Margaret and Iwona, my adoptive mothers, who made me feel at home in a foreign country, and to all my friends, the old ones I left in Italy and the new ones I made here. I owe you more than words can say. Thank you all.

VITA

- 1993 B.S., Biology, University of Bologna, Italy
- 2000 M.S., Molecular Biology, University of Bologna, Italy
- 2007 Ph.D., Biology, University of California, San Diego

Publications

Kinseth M.A., Anjard C., Fuller D., Guizzunti G., Loomis W.F., Malhotra V. The Golgi-Associated Protein GRASP Is Required for Unconventional Protein Secretion during Development. *Cell*. 2007 Jul 24.

Guizzunti G., Brady T.P., Malhotra V., Theodorakis E.A. Trifunctional norrisolide probes for the study of Golgi vesiculation. *Bioorg Med Chem Lett*. 2007 Jan 15;17(2):320-5.

Guizzunti G., Brady T.P., Malhotra V., Theodorakis E.A. Chemical analysis of norrisolide-induced Golgi vesiculation. *J Am Chem Soc*. 2006 Apr 5;128(13):4190-1.

Bard F., Casano L., Mallabiabarrena A., Wallace E., Saito K., Kitayama H., Guizzunti G., Hu Y., Wendler F., Dasgupta R., Perrimon N., Malhotra V. Functional genomics reveals genes involved in protein secretion and Golgi organization. *Nature*. 2006 Feb 2;439(7076):604-7.

Croci S., Strippoli P., Bonsi L., Bagnara G.P., Guizzunti G., Sartini R., Tonelli R., Messina C., Pierdomenico L., Lollini P.L. Expression of T cell receptor alpha gene (TCRA) in human rhabdomyosarcoma and other musculo-skeletal sarcomas. *Gene*. 2005 Jun 20;353(1):16-22.

Brady T.P., Wallace E.K., Kim S.H., Guizzunti G., Malhotra V., Theodorakis E.A. Fragmentation of Golgi membranes by norrisolide and designed analogues. *Bioorg Med Chem Lett*. 2004 Oct 18;14(20):5035-9.

ABSTRACT OF THE DISSERTATION

Functional and chemical analysis of the secretory pathway

by

Gianni Guizzunti

Doctor of Philosophy in Biology

University of California, San Diego, 2007

Professor Vivek Malhotra, Chair

Protein transport through the secretory pathway is an essential process for all living organisms. While studies over the last three decades have enormously increased our understanding of this event, many of the components involved in the process of protein secretion have yet to be identified, and we are still lacking mechanistic insight into many steps of the secretory pathway.

The main objective of this work is to identify new components involved in the secretory pathway. This will be achieved through the establishment of a functional genomics screen in drosophila cells and successive characterization of candidate genes as well as through a pharmacological approach in mammalian cells.

Chapter I will describe the functional genomics approach that led to the identification of new components of the secretory pathway. Genome wide RNA interference (RNAi) knockdown was performed on Drosophila cell line stably expressing a secreted form of horseradish peroxidase (HRP). In this cell line, the systematic depletion of the product (mRNA) of genes involved in secretion results in a defect in the release of HRP into the medium. The amount of HRP measured by chemiluminescence provides an indication of the level of secretion upon the loss of a certain gene product. This functional genomics screen approach has led to the identification of approximately 100 genes. The initial characterization of one of these genes is reported.

The following chapters will describe the pharmacological approach, which relies on the use of a natural product (norrisolide), a compound able to cause Golgi complex fragmentation and to block secretion in mammalian cells (Brady *et al.*, 2004; Guizzunti *et al.*, 2006; Guizzunti *et al.*, 2007).

In chapter II it is introduced norrisolide, a natural product able to induce irreversible fragmentation of the Golgi complex. The Golgi apparatus, a central organelle of the secretory pathway is a dynamic structure whose organization is maintained by a balance of membrane input and output. The fragmentation of the Golgi complex by norrisolide provides a mean to identify the mechanisms by which Golgi organization is maintained and regulated. Norrisolide activity is analyzed through the use of norrisolide-derived probes. We will show how norrisolide's core is necessary and sufficient to induce

Golgi fragmentation. A fluorescent analogue of norrisolide will be used to visualize the intracellular localization of norrisolide's target.

Chapter III shows the design of trifunctional probes based on norrisolide's structure that can be used to identify norrisolide's target. These probes are designed to contain norrisolide's core, a crosslinking agent and a tag. Different crosslinkers and tags are discussed.

INTRODUCTION

A. The secretory pathway

Secretion is the process through which proteins synthesized inside the cell are transported on to the cell surface or released in the extracellular medium. The route followed by these proteins within the cell is called Secretory Pathway.

The first breakthrough in understanding the sequential steps that secretory proteins take in order to be exported out of the cell came around 30 years ago with the work of George Palade (Jamieson and Palade, 1967; Palade, 1975). Palade and coworkers, by following the movement of secretory cargo by electron microscopy, showed that secretory proteins begin their life within the Endoplasmic Reticulum (ER), a large membrane system that comprises more than half of the total area of membranes of an average cell. Proteins that enter the ER undergo to a first round of glycosylation, as sugars are attached to asparagines residues of the protein (N-linked glycosylation). Chaperone proteins bind these oligosaccharide residues, ensuring that newly synthesized proteins are correctly folded before leaving the ER. After being packed into transport vesicles, proteins leave the ER to reach the Golgi complex, which consists of organized stacks of flattened membranes (*cisternae*) organized in a *cis-trans* (early-late) fashion. Proteins enter the *cis*-face, receive a series of complex modifications (glycosylation, sulfation, phosphorylation and methylation can all happen in the Golgi) and eventually exit from the *trans*-face. After being processed inside the Golgi, secretory cargo is sorted to its final destination. Figure 1 shows the route followed by secretory cargoes inside the cell.

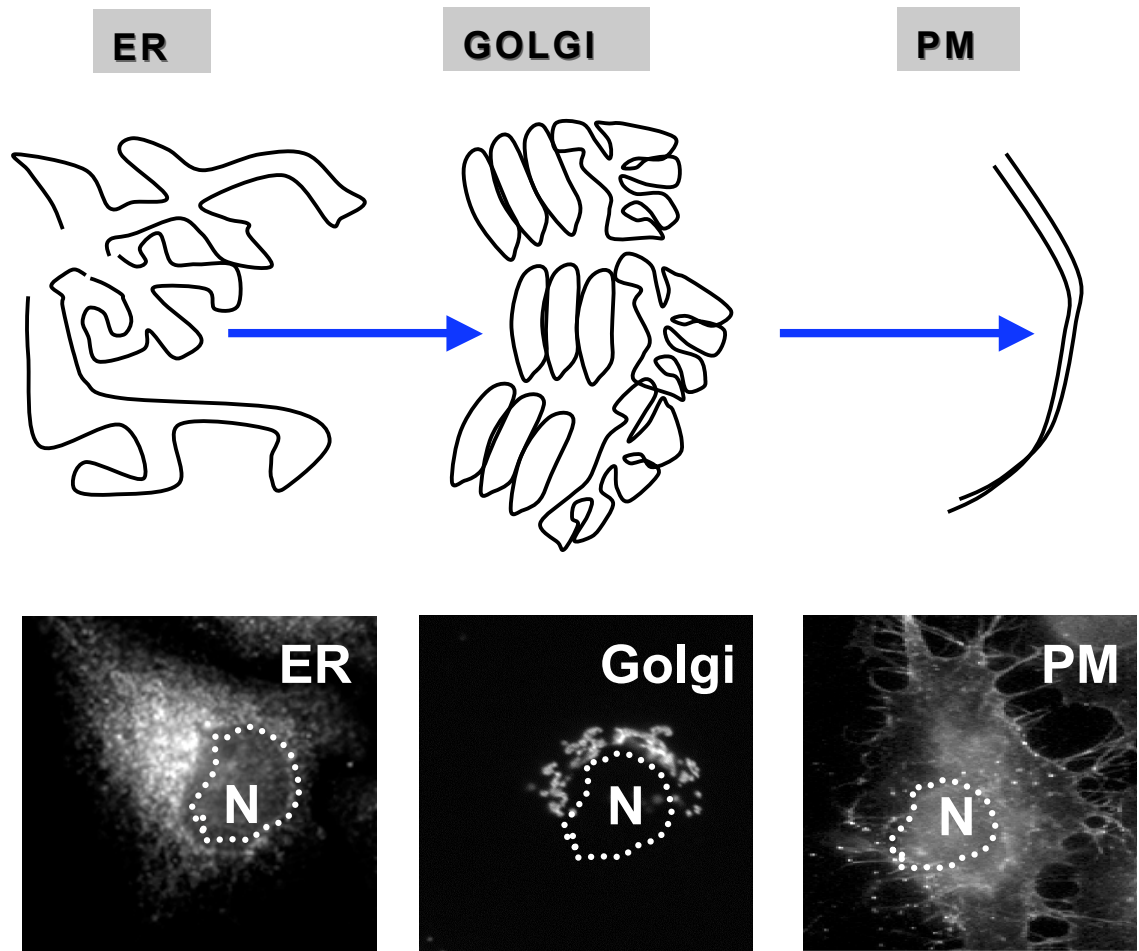


Figure 1. The secretory pathway. Newly synthesized secretory proteins enter the Endoplasmic Reticulum (ER), are packed into transport carriers, then move towards the Golgi complex. In the Golgi, proteins are sorted and delivered their final destination, like the plasma membrane (PM). Immunofluorescence pictures of each compartment are shown in the bottom panel (N indicates the position of the nucleus).

B. The Golgi apparatus

The Golgi apparatus was visualized for the first time by Camillo Golgi in the late 1800's, through a complex staining procedure. It wasn't until the early 1950's, with the advent of electron microscopy, that its existence was generally accepted (Dalton and Felix, 1954, 1956).

The Golgi complex is a central organelle of the secretory pathway whose function involves the post-translational modification and sorting of newly synthesized proteins and lipids. Ultrastructural analysis has revealed that the Golgi apparatus is composed of stacks of flattened cisternae, referred to as cis, medial and trans. On the trans face is located a complex tubulo-reticular structure known as and the Trans Golgi Network (TGN). Transport vesicles carrying secretory proteins arrive at the cis Golgi cisternae and travel across the Golgi stack in a cis-to-trans direction. Within each cisterna, the cargo undergoes specific modification such as trimming and addition of oligosaccharides, phosphorylation and sulphation. The appropriately modified proteins are then transferred from TGN to their respective destination, such as endosomes and plasma membranes, or are secreted from the cell.

How proteins move across the Golgi is still matter of debate: how does the cargo pass through the Golgi stacks leaving resident Golgi enzymes behind? In the early 1970's (Brown et al. 1970) observations of the transport of large gelatinous material in algae led to the "cisternal maturation hypothesis". This theory states that the cargo leaves the ER within a membrane bound structure that will become the cis face of the Golgi, as it receives "cis-Golgi" enzymes coming from the previous Golgi stack. Therefore the cargo will move forward without leaving the cisterna in which it was

originally packed, and Golgi resident enzymes will move backwards from late to early cisternae. This model implies that the Golgi is a transitory structure.

When Palade and colleagues used electron microscopy to visualize the movement of secretory proteins, they noticed the presence of small vesicles between and around the Golgi stacks. These vesicles were shown to contain cargo molecules, but not Golgi enzymes, that remained associated to the cisternae. They proposed the “vesicular transport” model, which views the Golgi complex as a stable organelle of the secretory pathway, which receives the cargo, modifies it and sorts it into vesicular transport carriers. In the years that followed, data supporting both theories were produced. Since the models are not mutually exclusive, it is likely that both processes are responsible to transport proteins across the Golgi apparatus, depending on the cargo transported and on the cell type involved.

The Golgi complex is unique amongst cellular organelles in the fact that it can maintain its organization despite the large amount of membranes entering and leaving the Golgi at any time during secretion. Many questions regarding the peculiar situation of the Golgi apparatus are yet to be answered. For example, how is the Golgi integrity maintained amongst the dynamic membrane traffic that moves across this organelle? How is the process of membrane fission achieved in order to produce Golgi-derived transport vesicles? And how is this process regulated, in order to avoid that the whole Golgi is converted into vesicles? As we will discuss, many approaches have been taken to understand the general process of protein transport and maintenance of organelle identity.

C. Approaches to the secretory pathway

Comprehending the mechanism of protein secretion is important not only for the basic knowledge of the biology of the cell, but it will also provide us insight into several diseases such as asthma (Fahy, 2002), Lowe syndrome (Suchy *et al.*, 1995), and cystic fibrosis (Cheng *et al.*, 1990). Moreover, toxins and pathogens have been shown to exploit the various steps of secretory pathway to gain access to the cytosol where they exert their toxic function. (Spooner *et al.*, 2006; Sandvig and Deurs 2002; Ludwig and Richards 2006).

Different approaches have been used to understand the process of protein transport and to identify the molecular machinery involved. In the late 1970's, Randy Schekman's lab performed genetic studies of mutant yeast unable to perform secretion at high temperatures. Through this genetic screen, a large collection of genes involved in secretion was identified ("SEC" genes) (Novick *et al.*, 1980). In the early 1980's, James Rothman's group was able to reconstitute the process of protein transport between two distinct *cisternae* of the Golgi stack in a cell-free system (Balch *et al.*, 1984). This in vitro biochemical approach led to the identification of the machinery (Malhotra *et al.*, 1989) responsible for trafficking across the Golgi stacks, and the proteins (SNAREs) involved in the process of membrane fusion (Rothman, 1994). Both systems, yeast genetics and cell-free biochemical approach, have some limitations: for instance, yeast secretory pathway is less complex compared to the one of higher eukaryotes. To overcome the limitations of these systems, in Chapter I we will describe a new approach, consisting in a genome wide screen in *Drosophila* cells.

All the above-mentioned approaches proved valuable to understand a large part of the process of protein transport. However, many of the components of the secretory

pathway remain unknown. Secretion is a highly dynamic process: cytoskeleton dynamics, organelle movement, the whole process of intracellular membrane trafficking is a rapid and highly regulated process. An average protein can go through (cross) the whole secretory pathway in less than 2h (Lippincott-Schwartz and Smith, 1997; (Lippincott-Schwartz et al., 2003; Lippincott-Schwartz et al., 2000). The speed and the complexity of these events make them challenging to be studied by genetic and biochemical means. The pharmacological approach, on the other hand, offer the unique advantage of being fast acting, allowing to analyze distinct steps of protein transport while they are happening. This approach will be discussed in Chapters II and III.

D. Chemical compounds affecting Golgi structure and function

Chemical reagents and natural products haven proven useful in elucidating the function and dynamics of the Golgi apparatus, like the relation between Golgi and microtubular cytoskeleton, the importance of organelle pH and the process of membrane fission that results in the production of transport carriers (Dinter and Berger, 1998). The structure (Figure 2) and the effects (Figure 3) of some of the most known “Golgi disturbing agents” are shown.

Chemical compounds affecting pH

The organelle of the secretory pathway show the existence of a pH gradient, ranging from 7.2 in the ER to 6.7 in the cis-Golgi and 6.0 in the trans-Golgi, as demonstrated by direct measurements performed from the mid-1990s by delivery of pH-sensitive fluorophores to the Golgi complex (Paroutis *et al.*, 2004; Seksek *et al.*, 1995; Kim *et al.*, 1996). The role of the pH in the secretory pathway plays a role in multiple aspects of secretion, ranging from the proper localization of enzymes, to their catalytic activity, to the modification and sorting of protein cargoes.

Compounds that can induce alterations of organelles pH, either acting as ionophores (like monensin) or inhibiting the proton pumps (H^+ -ATPases) that maintain the H^+ gradient (like bafilomycin), are useful tools to understand the role of the pH in the secretory pathway (Nachliel *et al.*, 1996; Gagliardi *et al.*, 1999)

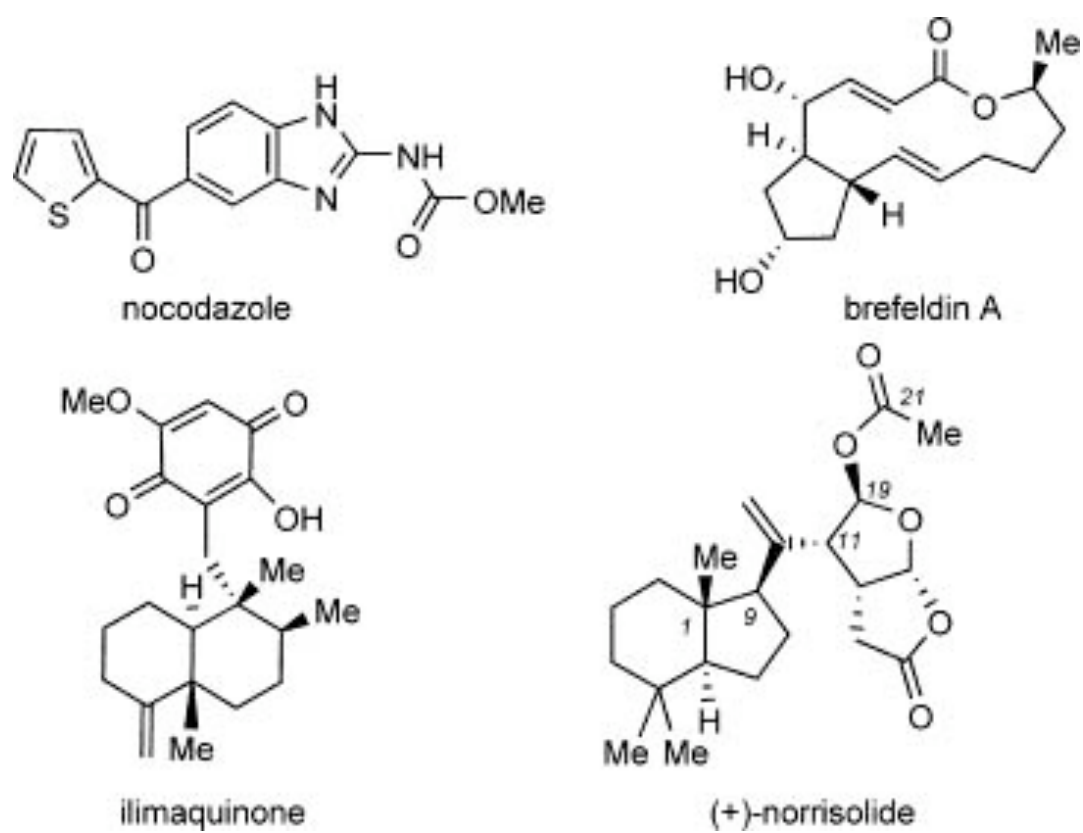


Figure 2. Chemical structures of selected Golgi-disturbing agents.

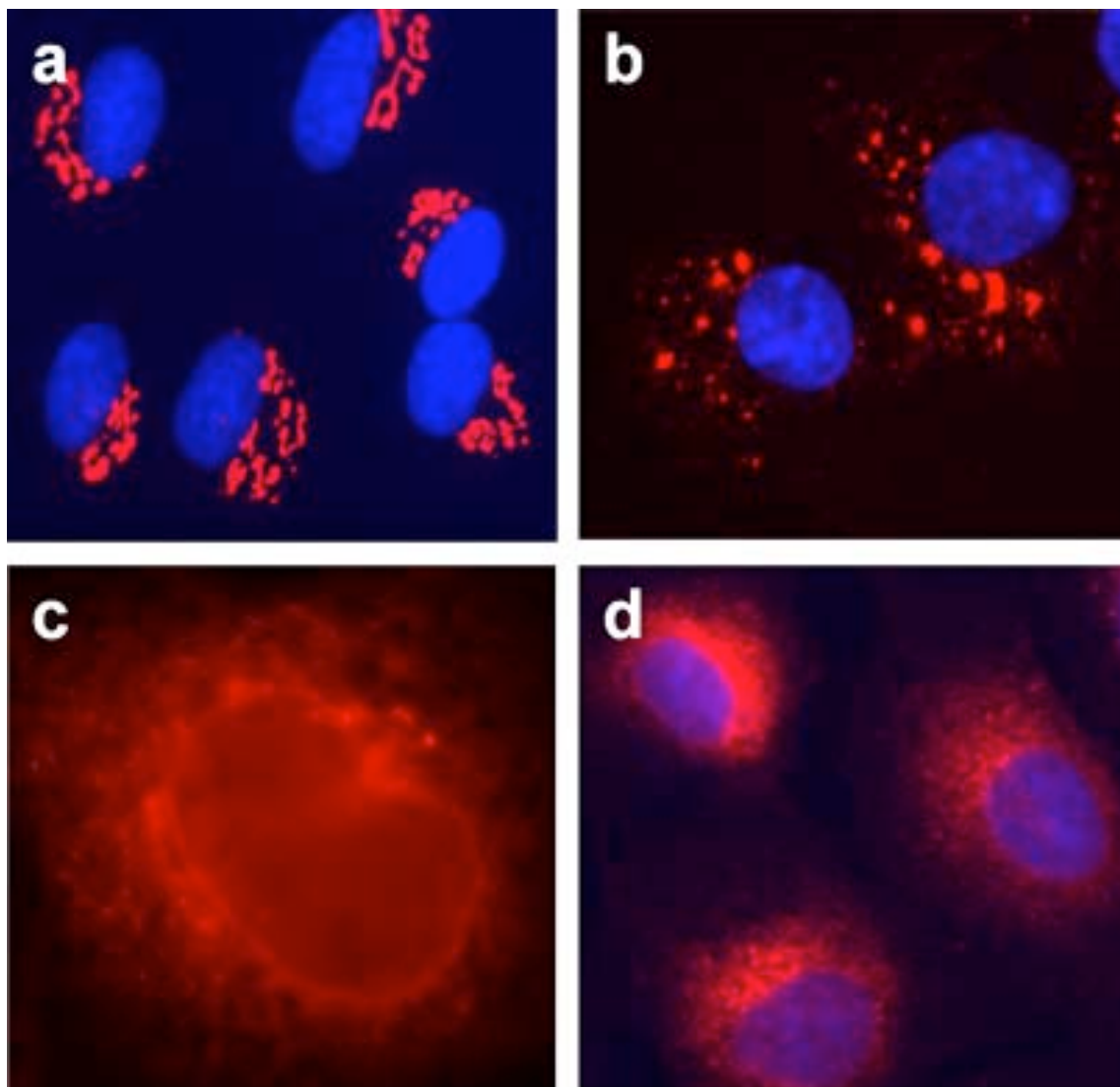


Figure 3. Compounds affecting Golgi structure. NRK cells are treated for 60 minutes with DMSO (a), nocodazole (b), BFA (c) and Ilimaquinone (d). The effect of each compound on Golgi membranes is shown through the staining of the Golgi marker Mannosidase II (red). Nuclei are stained with Hoechst (blue).

Chemical compounds affecting Microtubular cytoskeleton

Microtubules play a major role in the process of protein secretion, since transport carriers move inside the cell following the microtubular cytoskeleton (Vaughan, 2005; Allan *et al.*, 2002; Cole and Lippincott-Schwartz, 1995; Lippincott-Schwartz, 1998). Moreover, in mammalian cells, the Golgi apparatus is tightly associated with the microtubule-organizing center (MTOC), the point of origin of the microtubular cytoskeleton (Thyberg and Moskalewski, 1985).

The use of natural products disrupting microtubule organization revealed the importance of the microtubules in protein transport and in the position and organization of the Golgi complex. Studies with nocodazole (Turner and Tartakoff, 1989), for example, showed that the microtubular cytoskeleton plays a major role in the maintenance of the Golgi complex in the pericentriolar position. Application of 30 μ M nocodazole for 90 minutes leads to the depolymerization of microtubules, which results in the breakdown of the Golgi apparatus in smaller stacks that are dispersed in the cytoplasm. This process is reversible after nocodazole washout. Moreover, since nocodazole cannot block the transport of an integral membrane protein like VSV-G from the endoplasmic reticulum (ER) to the Golgi apparatus and further onto the cell surface, it was concluded that a functional tubular network is not necessary for proper transport and sorting functions of the Golgi apparatus.

Chemical compounds affecting Glycosylation

In the endoplasmic reticulum, N-linked glycosylation is connected to the process of protein folding. Therefore, compounds that interfere with this process (like the antibiotic tunicamycin) (Elbein, 1984) have very severe effects on cells, as they result in the accumulation of unfolded proteins, which cannot be further transported along the

secretory pathway. The O-linked glycosylation that takes place in the Golgi, creating complex carbohydrate structures, is essential for the function of the protein. However, in contrast to what happens in the ER, it seems that in the Golgi there is no control over the status of glycosylation. In other words, interfering with glycosylation in the Golgi does not stop the delivery of proteins containing incomplete carbohydrate chains. Nonetheless, since protein function depends on these modifications, this results in the secretion of malfunctioning proteins.

There are two general approaches to interfere with glycosyltransferases: one by using sugar analogs, like iminosugars (Goss *et al.*, 1995; Jacob, 1995); the other by using inhibitors specific for a certain glycosyltransferase, like swainsonine, an alkaloid that affect the Golgi enzyme Mannosidase II (Mohla *et al.*, 1989).

Compounds relocating Golgi into the ER

As secretory cargo moves from ER to Golgi, the molecular machinery required for the transport has to be retrieved back to the ER, to start the next round of transportation. Also, a mechanism must exist to bring back resident ER proteins that happen to escape to the Golgi (Lee *et al.*, 2004; Pelham, 1996). At the same time, endocytosed proteins may travel from the plasma membrane via the Golgi to the ER. All these processes belong to the so-called “retrograde transport”, a process that chemical natural products like brefeldin A (BFA) have helped to unravel. Brefeldin A, a fungal metabolite found to cause the fusion of the Golgi membranes with the ER (Fujiwara *et al.*, 1988), showed that anterograde and retrograde movements along the secretory pathway are in a dynamic equilibrium. (Pelham, 1991; Orci *et al.*, 1991; Klausner *et al.*, 1992).

Compounds inducing complete Golgi vesiculation

The whole process of secretion involves the accumulation of secretory proteins inside transport carriers (vesicles) followed by the detachment of the vesicle from the donor compartment (either ER or Golgi) and the transport towards its final destination. Although it is a key step in secretion, it is still not understood how the process of membrane fission is achieved. Similarly, it is not clear how are the organelles of the secretory pathway prevented from undergoing complete vesiculation during protein transport. As we will discuss, compounds inducing complete Golgi vesiculation can do so by overactivating the fission machinery, resulting in the deregulation of the formation of transport vesicles. As such, these compounds offer the possibility to unravel the molecular mechanism behind the elusive process of membrane fission and the maintenance of organelle integrity.

Ilimaquinone (IQ), a natural product isolated from a marine sponge, reversibly fragments the Golgi apparatus into small vesicles, thereby blocking cellular secretion (Takizawa *et al.*, 1993) (Veit *et al.*, 1993). IQ led to the identification of trimeric GTP-binding proteins and Protein Kinase D (PKD) as the first components required for the formation of transport carriers from the trans Golgi network (TGN) (Jamora *et al.*, 1997) (Jamora *et al.*, 1999).

CHAPTER 1

FUNCTIONAL ANALYSIS OF THE SECRETORY PATHWAY

1.1 ABSTRACT

Yeast genetics and cell-free biochemical approach have some limitations (for instance, yeast secretory pathway is less complex compared to the one of higher eukaryotes). To overcome the limitations of these systems, Vivek Malhotra's group (Bard *et al.*, 2006) recently undertook a genome wide screen in *Drosophila* cells. Fly cells are a good alternative model system to study exocytosis since their secretory compartments have similar morphological and molecular characteristics to those of mammalian cells. The screen led to the identification of more than 100 previously uncharacterized genes (named TANGO, for Transport AND Golgi Organization). Here is reported the cloning of 20 randomly selected Tango genes and the initial characterization of the most interesting amongst them: Tango5.

1.2 INTRODUCTION

The largest collection of genes involved in the process of protein transport comes from yeast genetics (Novick and Schekman, 1979). Although yeasts are suitable for this kind of analysis, their secretory pathway is relatively simple compared to the metazoans. In order to identify new components involved in the process of protein secretion, a genome wide analysis was performed in *Drosophila* cell line (Bard *et al.*, 2006). *Drosophila* is one of the best-studied genetic organisms, and has been used to identify conserved pathways from flies to humans. As metazoans, fly's secretory pathway is more complex compared to yeast, both in the variety of secreted cargo and in the organization of the organelles of the secretory pathway.

Primary Screen

A *Drosophila* cell line was generated able to synthesize and secrete horseradish peroxidase (HRP). Upon secretion, the presence of HRP in the medium is measured by chemiluminescence, providing a quantitative measure of protein secretion. The cell line was subjected to the systematic depletion by RNAi of the entire *drosophila* genome, using a library of 22,000 double-stranded RNAs (dsRNAs) (Boutros *et al.*, 2004; Armknecht *et al.*, 2005). As positive controls was used the knockdown of syntaxin 5 and bCOP, which are known to be required for protein secretion (Nichols *et al.*, 1998; Duden *et al.*, 1991; Serafini *et al.*, 1991). Knockdown of syntaxin 5 and bCOP by RNAi caused a hundred fold reduction of HRP secretion. Based on this analysis, of 23,000 *drosophila* genes tested 1,133 were identified for affecting protein secretion. These genes were analyzed in Flybase (www.flybase.org) for potential involvement in previously identified cellular processes other than secretion. Genes that could effect secretion indirectly

through their roles in apoptosis, transcription, protein translation and protein degradation were discarded from further analysis. Furthermore, genes that scored in cell survival screens previously performed (Boutros *et al.*, 2004) were removed. This selection reduced the number to 284 dsRNAs that were further tested in two additional HRP secretion assays in a 96-well plate format. The DNA-binding dye Hoechst was used to exclude dsRNAs that could affect cell number. This left us with 130 genes, 26 of which resulted to be previously characterized secretory genes, confirming the validity of the methodology used.

Morphological Effects on Golgi Membranes

The other 104 genes, which are potentially new secretory components, were named TANGO, for Transport And Golgi Organization. These genes were further screened for their involvement in the organization of the Golgi membranes. Morphological Effects on Golgi Membranes were screened on a S2 cell line stably expressing mouse Mannosidase II, a marker of the cis and medial Golgi cisternae, coupled to GFP (MannII–GFP) to test the effect of Tango genes on Golgi organization. Golgi membranes in S2 cells are organized as several unconnected stacks of cisternae (Stanley *et al.*, 1997). We incubated S2 cells expressing MannII–GFP with dsRNAs, and then imaged them by high-resolution deconvolution fluorescence microscopy. The genes were classified into four groups on the basis of the effect of their depletion on Golgi membranes. RNA-mediated interference (RNAi) of class A genes fused Golgi membranes with the ER, as shown by the relocation of MannII–GFP in a ring around the nucleus and a diffuse reticular network. RNAi of class B genes fragmented the Golgi membranes into smaller elements, RNAi of class C genes caused swelling, and RNAi of class D genes had no apparent effect on Golgi organization (Figure 4).

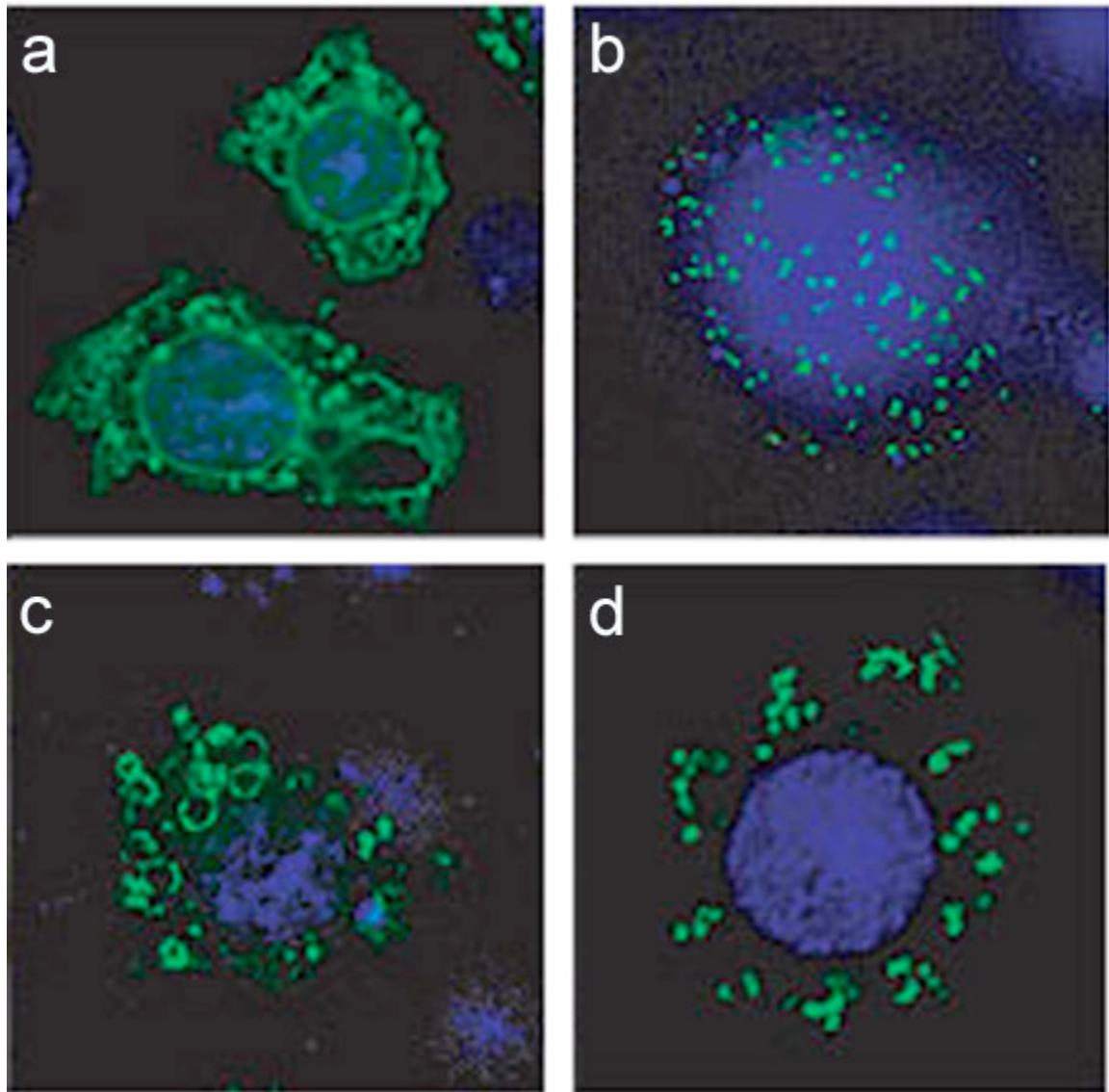


Figure 4: Four classes of Tango genes. Class A (Golgi to ER); Class B (Golgi fragmentation); Class C (Golgi bloating); Class D (no Golgi phenotype).

Characterization of TANGO genes

Among the 104 candidate genes, 20 were randomly selected for further characterization. In order to address the role of each of these genes in the process of protein transport, we need to address the following issues:

- 1) Localization of the protein coded by each gene
- 2) Process in which each gene is involved
- 3) Function of the gene product.

To address the question of protein localization, we generated a drosophila cDNA library, from which each gene will be cloned with specific primers. The strategy is to clone each gene in a tagged form through the insertion of the PCR product directly into an “entry vector”; then, a recombination reaction will transfer the gene of interest from the entry vector to a destination vector containing a choice of tags, both for visualization by immunofluorescence (IF) and for immunoprecipitation (IP). The destination vector will be transfected into drosophila S2 cells, and the protein product will be localized by IF.

The movement of a protein along the secretory pathway can be divided into discrete steps. The first step is the exit of the protein from the ER. Then, the protein has to reach the Golgi apparatus, move across it, and leave to reach the plasma membrane. Each step represents a particular process that our protein of interest can participate at. Finding where secretory cargo is blocked inside the cell after RNAi will help us answering this question.

The biological function of a protein is the more difficult question to address. We have to find what the protein interacts with, in which pathway it works, which other components are upstream and downstream. Using our knowledge of the secretory pathway we can check whether the depletion of a certain gene is affecting the function or localization of previously known secretory components. For example, if our protein

localizes in the ER and shows to block transport between ER and Golgi, we will test whether known components involved in ER to Golgi transport (like COP1 coats and SAR1) are affected by the depletion of that particular protein. An alternative is to perform a yeast two hybrid screen with that protein as bait, or IP the protein and see what else is bound to it.

1.3 RESULTS

Cloning and localization of tango genes

The 20 genes selected were cloned in an inducible expression vector with a V5 tag. Genes were cloned by RT-PCR from a library of poly(A) RNA from *Drosophila* larvae (Clontech) and subcloned into pDEST48 vector. S2 cells expressing MannII-GFP were transfected with the tagged cloned genes, and the gene products were visualized with an antibody against V5 and imaged by deconvolution microscopy. The localization of the gene products was compared with that of the Golgi marker (MannII-GFP). Of the 18 cloned genes products, 3 localized to the Golgi membranes, 2 localized to the ER, 7 were cytosolic and 6 showed nuclear localization. The result is shown in Figure 5.

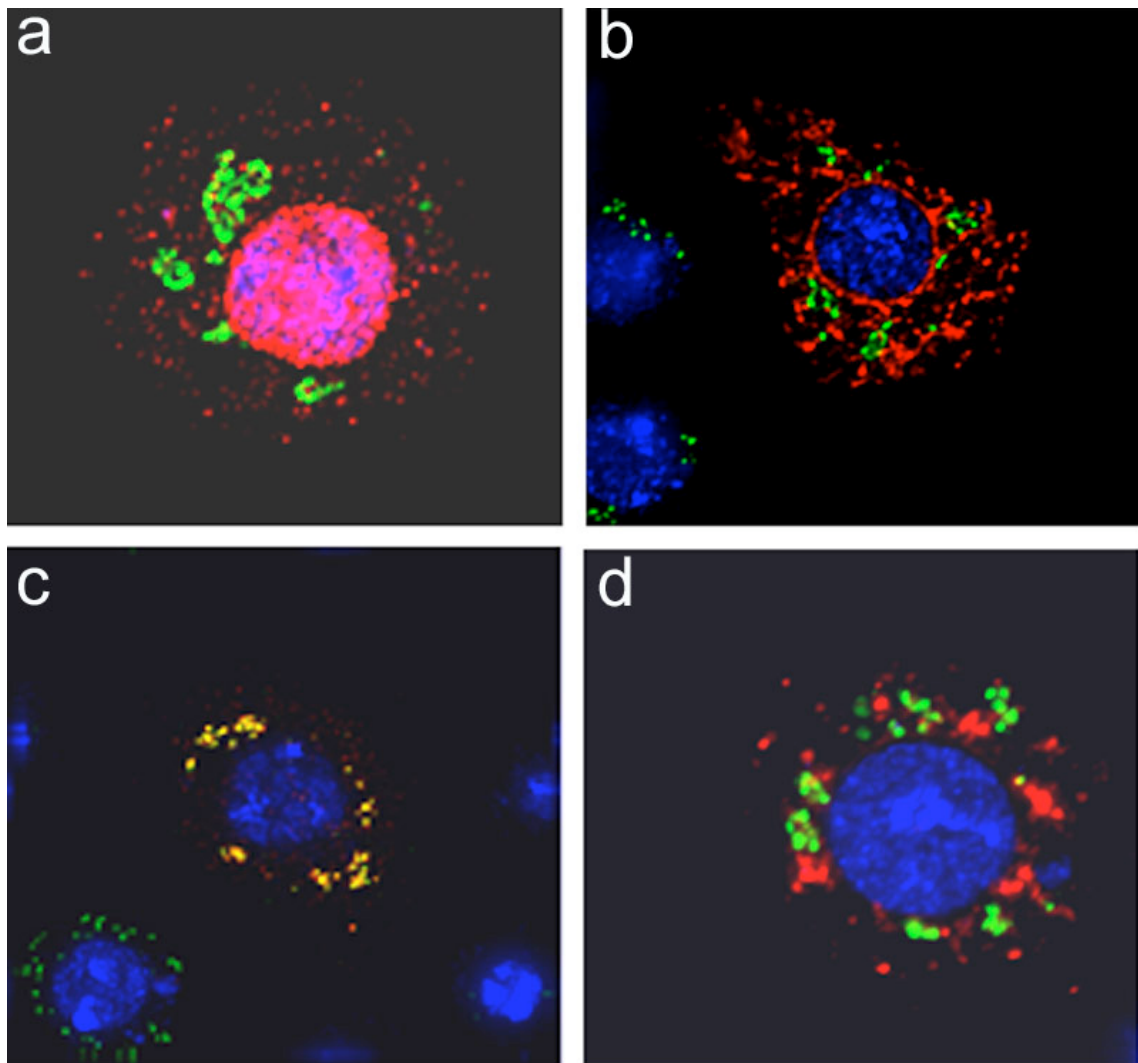


Figure 5. Tango genes localization. Candidate genes were cloned in an inducible vector with a V5 tag and transiently transfected into S2 cells expressing MannII-GFP. Cells are labeled with Hoechst (DNA staining, in blue) and an anti-V5 antibody (Tango proteins, in red). MannII-GFP is green. (a) Nuclear genes: CG2446, CG11990, CG37550, CG5674, CG8929, CG3838. (b) ER genes: CG32675, CG30404. (c) Golgi localized genes: Pgant6, CG10007, CG31052. (d) genes localized in cytosol and dot-like structures: CG1796, CG8309, CanA14F, Lsd-2, CG8588, Dalmatian, CG18561.

The list and annotations of the 20 selected tango genes follows:

Class A (Golgi to ER relocation), 4 genes:

CG2446: the gene seems to be present only in flies, not having homologues in other species. It seems not to contain recognizable domains. When its DNA was transfected into *Drosophila* S2 cells, the corresponding protein (of 550 amino acids) localized inside the nucleus.

CG32332: the gene does not have clear homologues in other species but flies. It has as many as 5 isoforms that seem to be the product of alternative splicing. Although many attempted were tried, it resulted impossible to clone the gene by PCR.

CG32675: (Tango5) is very conserved among species, being present in all organisms except yeast. Many TM domains can be identified, which vary between different organisms: there are 4 TM in flies, 6 in *Xenopus* and 7 in mammals. It encodes for a 450 amino acids protein that localizes in the ER in fly cells.

CG1796: (Tango4) very conserved gene. It contains several WD40 repeats in its C-terminal part. WD-40 repeats are short ~40 amino acid motifs, often terminating in a Trp-Asp (W-D) dipeptide. WD-containing proteins have 4 to 16 repeating units, all of which are thought to form a circularized beta-propeller structure. WD-repeat proteins are a large family found in all eukaryotes and are implicated in a variety of functions ranging from signal transduction and transcription regulation to cell cycle control and apoptosis. The underlying common function of all WD-repeat proteins is coordinating multi-protein complex assemblies, where the repeating units serve as a rigid scaffold for protein interactions. Examples of such complexes include TAFII transcription factor and E3 ubiquitin ligase.

The gene seemed to localize in the cytosol in fly cells. However, the human homologue expressed in HeLa cells showed clear nuclear localization; moreover CG1796 has also a potential homologue in yeast, the splicing factor gene Prp46. One possibility is that the tagging process in the fly homologue blocked the natural nuclear localization of the gene product. An alternative is that the gene has different localization in the two species. Moreover, if this gene is really a splicing factor, it has to be explained how the RNAi of this particular splicing factor (and not others) can induce such dramatic effect on Golgi membranes.

Class B (Golgi fragmentation), 4 genes:

Pgant6: conserved gene, present in all organisms. It shows the following domains: a signal sequence at the N-terminal; a TM domain close to the N-terminal of the protein; a “Glycosyl-Transferase-Family 2” domain and a ricin domain. The Glycosyl-Transferase-Family 2 domain is found in a diverse family of glycosyl transferases that transfer the sugar from UDP-glucose, UDP-N-acetyl-galactosamine, GDP-mannose or CDP-abequose, to a range of substrates including cellulose, dolichol phosphate and teichoic acids. The biosynthesis of disaccharides, oligosaccharides and polysaccharides involves the action of hundreds of different glycosyltransferases. These are enzymes that catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, forming glycosidic bonds. The ricin domain is a carbohydrate-binding domain found in the lectin ricin produced from the seeds of the plant *Ricinus communis*. Many carbohydrate-recognition proteins like plant and bacterial AB-toxins, glycosidases or proteases show the presence of a similar domain. This domain, known as the ricin B lectin domain, can be present in one or more copies and has been shown in some instance to bind simple sugars, such as galactose or lactose.

PGANT6 gene product was localized in the Golgi apparatus both in fly and human cells.

CG11990: conserved gene from flies to humans. It has the domain PFAM:CDC73, present in RNA polymerase II accessory factors. RNA polymerase II is responsible to produce mRNA from the DNA template (transcription); in particular, these factors appear to play an essential role in RNA elongation. The gene product localized in the nucleus of fly and human cells.

CG8309: conserved gene. It contains the HD-motif, which is found in a superfamily of enzymes with a predicted or known phosphohydrolase activity. These enzymes appear to be involved in the nucleic acid metabolism, signal transduction and possibly other functions in bacteria, archaea and eukaryotes. Towards the C-terminal of the protein there is a PINT domain, which is a domain of unclear function that occurs in the C-terminal region of several regulatory components of the 26S proteasome as well as in other proteins. Apparently, all of the characterized proteins containing PCI domains are parts of larger multi-protein complexes. The protein localized in the cytosol of fly cells. Cloning the human homologue has proven unsuccessful at this point.

CG37550: conserved gene. It contains the domain Isy1, present in protein important for the optimization of the splicing, a nuclear phenomenon part of the processing of the RNA before its translation into proteins. The gene product localizes in the nucleus in *Drosophila* cells.

Class C (Golgi bloating), 1 gene:

CG5674: the gene is present only in flies, not showing homologues in other species. It does not contain known domains. A blast search shows a very low similarity

with a putative leucine zipper transcription factor in *Dictyostelium discoideum*. The gene product localized in the nucleus.

Class D (not affecting Golgi structure), 11 genes:

CanA14F: is a very conserved gene, present from yeast to humans. It contains the PP2Ac domain. This domain is present in a large family of serine/threonine phosphatases. Protein phosphorylation, achieved through protein kinases and phosphatases, plays a central role in the regulation of cell functions causing the activation or inhibition of many enzymes involved in various biochemical pathways. The fly protein CanA14F localizes in the cytosol.

Lsd-2: the gene is present only in flies. It contains the domain perilipin. This domain is present in a family of proteins that includes lipid droplet-associated protein (perilipin) and adipose differentiation-related protein (adipophilin). Perilipin is a modulator of adipocyte lipid metabolism and adipophilin is involved in the development and maintenance of adipose tissue. Other proteins belong to this group include TIP47, a cargo selection device for mannose 6-phosphate receptor trafficking. The gene product localizes in cytosol plus spots-like structures, which could represent either protein aggregates or membrane-bound structures. Fractionations techniques or costaining with other markers can help to resolve this issue.

CG8588: it seems to be conserved only in fly and worm (*C. elegans*); no homologues could be found in higher organisms. The protein seems to contain 3 TM domains in the central part. When expressed in fly cells, the localization appears to be cytosolic plus dot-like structures, which could not be colocalized with any of our markers.

Dalmatian: the gene is present only in *Drosophila*. No domains can be found. The protein localizes in the cytosol.

CG17233: only present in fly, with no homology with any other organism. The gene is very long (over 5,200 bp), and until now has been impossible to obtain a clean PCR product in the attempt to clone the gene.

CG10007: conserved gene, with homologues present all over across species. The gene contains 7 predicted TM domains. The central part of the gene contains a domain closely related to Nucleotide-sugar transporter. This family of membrane proteins transports nucleotide sugars from the cytoplasm into the lumen of the Golgi, where sugars are used to produce complex glycoproteins. Both in flies and human cells the gene product localizes in the Golgi apparatus.

CG31052: very conserved gene, only absent in yeast. The protein is predicted to have as many as 12 TM domains. The gene product localizes in Golgi, both in *Drosophila* cells and human cells. The *C. elegans* homologue has been reported to be a sodium/hydrogen exchanger, importing H^+ into the lumen of the Golgi and exporting Na^+ . This helps to maintain the proper pH gradient across Golgi membranes. It is known that the organelles of the secretory pathway present a pH gradient, ranging from 7.2 in the ER to 6.7 in the cis-Golgi and 6.0 in the trans-Golgi. The role of the pH in the Golgi complex plays a role in multiple aspects of Golgi-related functions, ranging from the proper localization of enzymes, to their catalytic activity, to the modification and sorting of protein cargoes.

CG3838: the gene has no homologues in other species and is predicted to contain two domains: MADF domain and BESS motif. MADF is related to the Myb DNA-binding domain of c-myb, a nuclear DNA-binding protein that specifically recognize the sequence YAAC(G/T)G on the DNA. The BESS motif is 40 amino acid residues long and is composed of two predicted alpha helices. Based on the protein in which it is found and the presence of conserved positively charged residues it is predicted to be a DNA

binding domain. This domain appears to be specific to drosophila. The gene product shows nuclear localization in fly cells.

CG8929: gene present only in Drosophila, with no predicted domains. The gene product localizes in the nucleus.

CG18561: this gene is very short (270 bp), has no homologues or protein domains. Localizes in the cytosolic.

CG30404: (Tango 11) the gene is conserved between species; it exists in only one isoform in Drosophila, but at least 4 isoforms are present in humans. The 4 human isoforms derive from an alternative splicing of 3 internal small exons, producing 4 isoforms ranging from extra-long (all 3 exons are present) to short (none of the exons is present) (Figure 6 a). When tested by RT-PCD, HeLa cells show that 3 of the isoforms are expressed, with the medium form being the only one missing (Figure 6 b). All human isoforms were cloned and expressed in HeLa cells. All of them showed ER localization (Figure 6 c). The protein is predicted to have a signal sequence at the N-terminal, a coiled-coil domain at the C-terminal and a TM domain at the end the sequence. To address the role of each domain in the localization of Tango 11, mutant forms were created, lacking the coiled-coil (Figure 7 b), the TM (Figure 7 c), or both domains (Figure 7 a). From this experiment it is concluded that both coiled-coil and TM domain have to be present for the protein to properly localize in the ER.

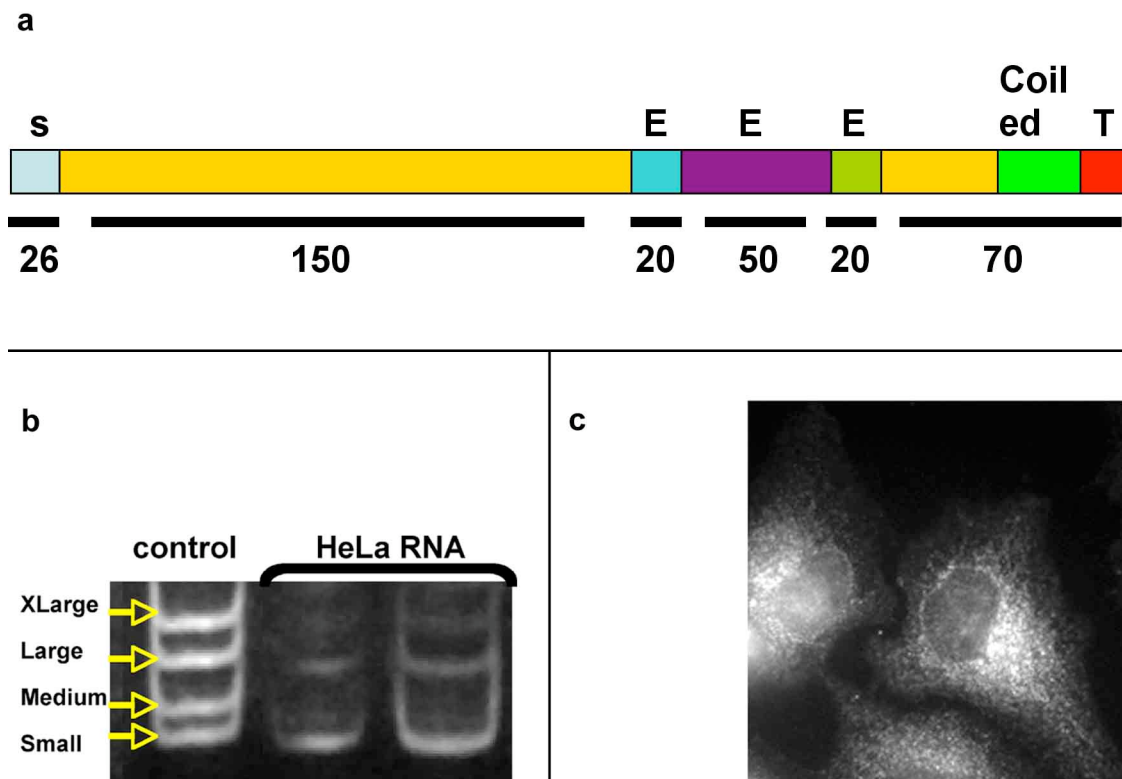


Figure 6. Tango 11 structure, isoforms and localization. (a) Tango11 (CG30404) is shown to have a signal sequence at its N-terminal (s), a coiled coil domain (in green) at the C-terminal and a TM domain at the end of the protein (TM, in red). There are 3 isoforms in humans, produced by the alternative splicing of three exons (marked as E). (b) RT-PCR in HeLa cells shows that all the isoforms, except the medium, are expressed; cDNA of the 4 cloned isoforms is loaded as control. (c) Localization of Tango11-Myc in HeLa cells.

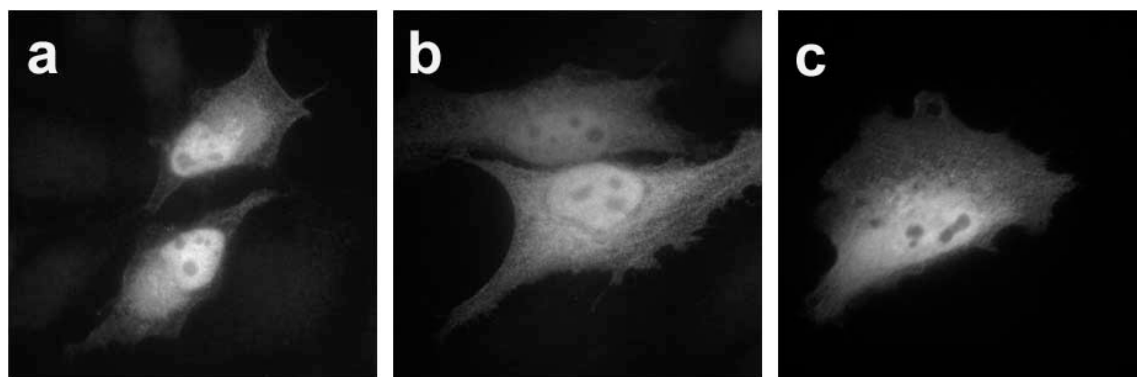


Figure 7. Tango 11 mutants. (a) Tango11 mutant lacking both Coiled-coil and TM domains. (b) only the coiled-coil domain has been deleted. (c) TM domain has been deleted.

Tango5 as putative component of the secretory pathway

The criteria to select the best gene candidate for further investigation was based on three principles:

1) Conservation amongst species. A conserved gene is most likely to be involved in a conserved process, and offers the possibility to work with different model organisms. Genes that didn't have homologues across the species were discarded: CG32332 (class A), Lsd-2, CG8588, Dalmatian, CG17233, CG18561 (all Class D).

2) Localization. Genes whose product localized in the Nucleus were discarded because of the possibility that the gene had a role in nuclear events, like transcription and DNA-RNA processing. Nuclear genes: CG1796 and CG2446 (class A); CG11990 and CG37550 (class B); CG5674, CG3838 and CG8929 (class D).

3) Phenotype after RNAi. Genes belonging to Class A, B or C were preferred over Class D genes. 4 class D genes: CG30404 (ER localization, conserved amongst species), CG10007 (Golgi localization, conserved), CanA14F (cytosolic, conserved), CG31052 (Golgi localization, conserved).

When these criteria were applied to the 20 genes mentioned above, one resulted to be the best candidate for further investigation: Tango5 (CG32675). The gene is conserved amongst species, it localizes in the ER in fly cells, and the RNAi of Tango5 induces Golgi to ER relocation. Moreover, the human homologue of Tango5 was shown to be overexpressed in cases of acute pancreatitis (Duseti *et al.*, 2002).

Human homologue of Tango5 was cloned with Myc-His tag and the DNA was transfected in HeLa cells. Whereas in *Drosophila* S2 cells the gene had ER localization (Figure 8 a), in human cells it appeared to localize in dot-like structures scattered across the cytoplasm (Figure 8 b)

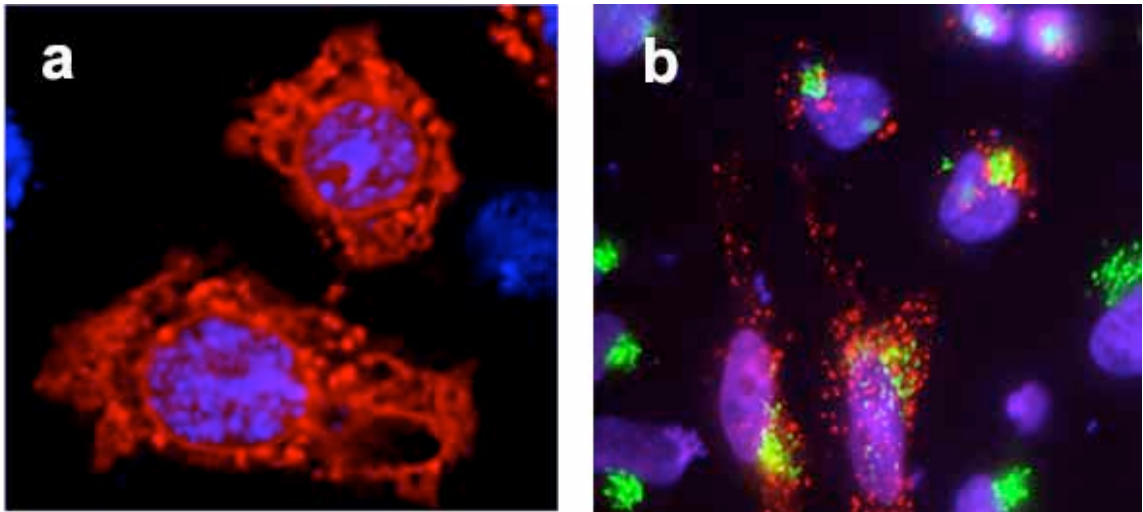


Figure 8. Localization of Tango5. (a) *Drosophila* S2 cells expressing Tango5-V5 (red). (b) HeLa cells expressing the human homologue of Tango5-Myc (red); Golgi complex in shown in green. Nuclei are in blue.

The dot-like structures containing Tango5 can potentially be any of the membrane-bound compartments that exist inside the cell. For example, the organelles of the endocytic compartment (endosomes and lysosomes) have similar looking morphology. Also, the sites of the ER from which newly synthesized protein leave (ER exit sites) show a dots-like structure when stained for some of the component of the Cop II vesicles. Moreover, in mammalian cells, between ER and Golgi, exists a tubulo-reticular compartment, known as ERGIC (ER to Golgi Intermediate Compartment) (Appenzeller-Herzog and Hauri, 2006).

To find out the exact localization of Tango5 we tested the colocalization between Tango5-Myc and some know markers of the secretory and endocytic pathway (Figure 9). No colocalization was observed with makers of ER exit sites (Sec 31 and Sec 16) and of the endocytic compartment (EEA1 and LAMP1). Markers of the ERGIC compartment didn't show costaining (ERGIC53), however a partial colocalization was observed when Tango5-Myc was coexpressed with ERGIC53-GFP (Figure 9 f).

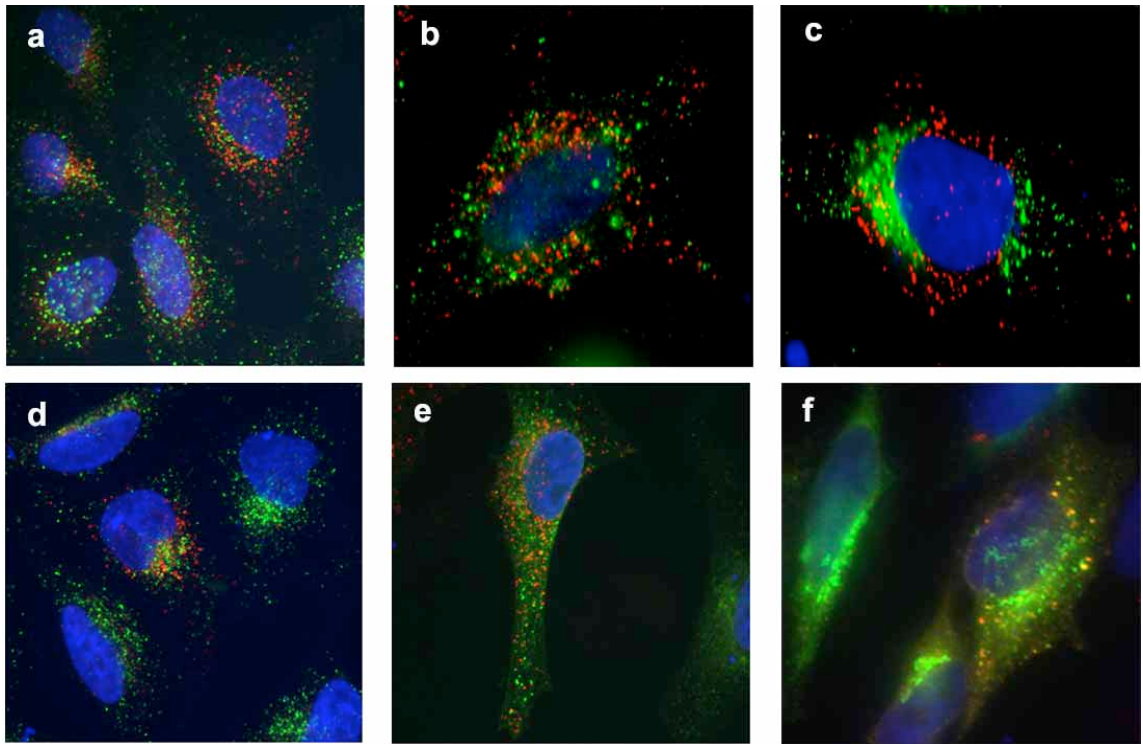


Figure 9. Human Tango5 colocalization. HeLa cells expressing hTango5-myc (in red) are stained for markers of secretory pathway and endocytic compartments (in green); nuclei are shown in blue. (a) Early Endosomes (EEA1). (b) Lysosomes (Lamp1). (c) ER-to-Golgi Intermediate Compartment (ERGIC53). (d) ER Exit Sites marker Sec31. (e) HeLa cells coexpressing hTango5 (red) and the ER Exit Site protein Sec16-GFP (green). (f) HeLa cells coexpressing hTango5 (red) and ERGIC53-GFP (green).

VSV-G transport in Tango5 expressing cells

If Tango5 is a component of the secretory pathway, it should colocalize with protein cargo at some point during their travel from the ER to the cell surface. To verify this hypothesis, cells expressing Tango5 were transfected with VSVG-GFP. This chimera protein (the viral secretory protein VSV-G is coupled to the fluorescent protein GFP) is transported from ER to Golgi to PM, and its movement can be easily followed in virtue of its fluorescence. For the experiments was used a mutant form of VSV-G (VSVG-ts045) containing a point mutation that induces the unfolding (and consequent block in the ER) of the protein when cells are kept at non-permissive temperature (40°C). After shifting the cells at permissive temperature (32°C), VSVG-ts045-GFP leaves the ER and reaches the Golgi in 30 minutes; then it is transported to the PM (which it reaches in 90 minutes). Cells were processed for imaging at time 0 (40°C block, VSVG in ER), and every 5 minutes after the shift at permissive temperature. As shown in Figure 10, VSVG-GFP enters Tango5 containing compartment after 5 to 10 minutes from leaving the ER (Figure 10 b). After 30 minutes VSVG reached the Golgi, and in 90 minutes the PM. This result indicates that the human Tango5 (hTango5) is localized along the secretory pathway in a compartment situated between the ER and the Golgi. This finding is in agreement with the data showing partial colocalization between hTango5 and ERGIC-GFP (Figure 9 f).

Other data point in the same direction: a recent paper (Marmagne *et al.*, 2006) describing organelle proteomics in *Arabidopsis* showed that plant homologue of Tango5 is an ER protein. *Drosophila* Tango5 is also localized in the ER. From these data we can conclude that in lower organisms such plants and flies Tango5 localizes in the ER. In mammals, the homologue of Tango5 appears to localize in a compartment, typical of mammalian cells, situated between ER and Golgi: the ERGIC compartment.

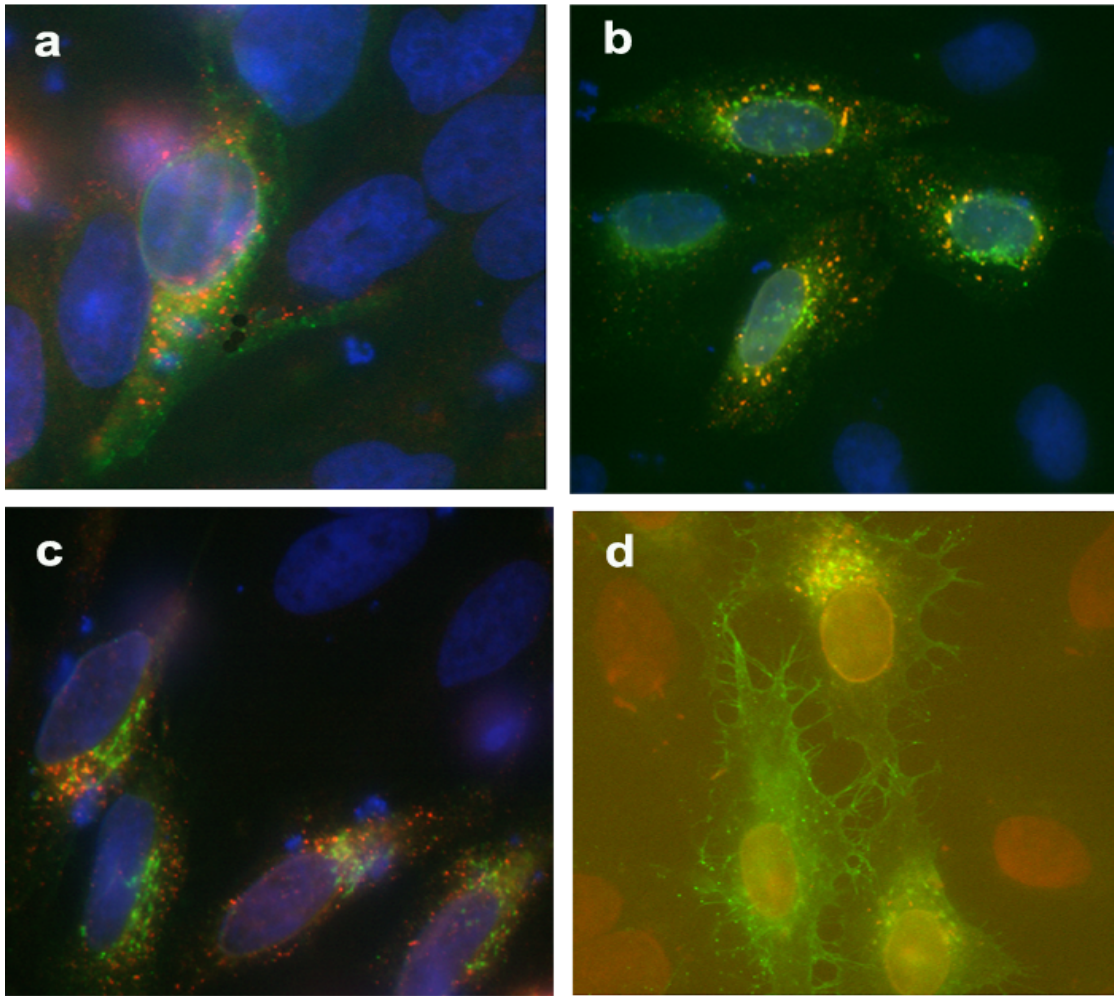


Figure 10. VSV-G transport in Tango5 expressing cells. HeLa cells are co-transfected with Tango5-Myc (shown in red) and VSVGtsO45-GFP (in green). (a) VSV-G is arrested in the ER at non-permissive temperature (40 °C). When cells are brought at permissive temperature (32 °C), VSV-G can leave the ER. (b) 10 minutes after leaving the ER, VSV-G enters Tango5-containing compartment. (c) in 30' VSV-G reaches the Golgi and (d) in 90' VSV-G arrives at the plasma membrane. Nuclei are shown in blue.

Endogenous hTango5 localization

All the findings on hTango5 localization are based on the analysis of the overexpressed protein. To check whether endogenous hTango5 also localizes in an ERGIC compartment, we decided to produce an antibody (Ab) able to recognize the endogenous protein. hTango5 has many TM domains that make the full-length protein not suitable for Ab production. Therefore we decided to use a 10 amino acid (AA)-long peptide as epitope; this sequence is located in the central portion of the protein, within a 130 AA long loop surrounded by TM domains. Before testing the ability of the Ab to recognize the endogenous Tango5, we verified that the gene was actually expressed in HeLa cells. RT-PCR performed on total HeLa cells RNA showed that this is indeed the case (Figure 11 a). Cell lysate was used to test the Ab by Western Blot (WB). HeLa cells and HeLa cells overexpressing Tango5-Myc (positive control) were used to prepare total cell lysate. The lysate was run on an acrylamide gel, and then transferred to nitrocellulose paper. As shown in Figure 11 b, amongst few unspecific bands, the Ab can specifically recognize a 45Kd band in the cell lysate containing overexpressed Tango5. No bands seem present in non-transfected HeLa cells, indicating the inability of the Ab to recognize the endogenous Tango5.

The Ab was then tested on Immunofluorescence (IF). As for the WB, the Ab was able to recognize overexpressed Tango5-Myc, as shown by the colocalization between the staining of anti-Tango5 Ab and anti-Myc Ab (Figure 12), but did not recognize the endogenous protein.

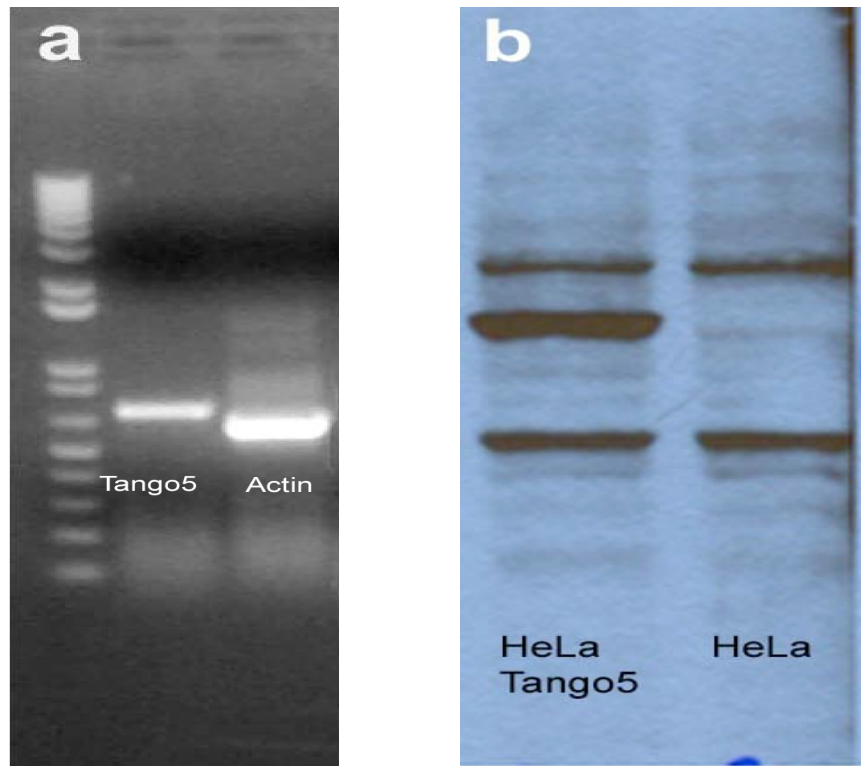


Figure 11. Endogenous Tango5 in HeLa cells, shown by RT-PCR from HeLa cells' extracted RNA (a); marker is shown in the first lane, then Tango5 PCR band, then the PCR-positive control actin. In (b) anti-Tango5 Ab is used to visualize overexpressed and endogenous Tango5 on WB.

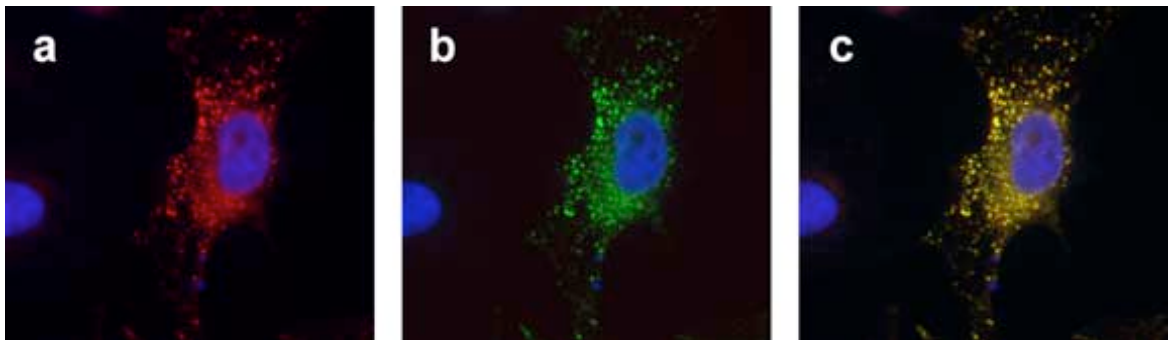


Figure 12. Tango5 Ab in HeLa cells. HeLa cells transfected with Tango5-Myc are labeled with anti-Myc Ab (a) and anti-Tango5 Ab (b). Costaining is shown in (c)

hTango5 topology

hTango5 is very conserved between species, being present in all organisms except yeast. The protein sequence (around 400 AA) can be divided in 3 regions: the first 130 AA contain a variable number of TM domains (2 in *Drosophila*, 3 in *Xenopus*, 4 in mammals); the central portion of the protein (130 AA) is a large loop; the last 130 AA also contain TM domains (2 in flies, 3 in frogs and mammals).

The two patches of 30 AA flanking the loop and 20 AA in the center of the loop itself show an amazing degree of conservation between the species (90% of AA identity). This indicates that the 3 groups of AA must be important for the function of Tango5. If Tango5 is a component of the secretory pathway, it could make contact either with the protein cargo or with the machinery responsible for its transport. If the conserved area in Tango5 loop is required for the protein's function, deciphering Tango5 topology would help to gain insights into Tango5 function. If the loop is on the cytosolic face of the lipid bilayer, Tango5 could be part of the machinery; on the other hand, if the loop is facing the lumen of the organelle, Tango5 could be interacting with the cargo.

To address Tango5 topology we decided to use an IF-based approach. The reasoning is the following: different permeabilizing agent can be used to allow an Ab to gain access to its intracellular epitope. Triton X100, for example, is able to extract lipids from membranes, therefore allowing the Ab to cross the PM and any cellular organelle. Instead, digitonin, which specifically extracts cholesterol from membranes, will preferentially permeabilize cholesterol-rich membranes (like PM), leaving intracellular membranes intact. For example, an Ab recognizing the luminal portion of the Golgi enzyme Mannosidase II will be able to bind its epitope in cells permeabilized with triton (Figure 13, a and c), but not in cells permeabilized with digitonin (Figure 13, b and d).

However, digitonin will allow the staining of a Golgi protein like GRASP65, which is situated on the cytosolic face of the organelle (Figure 13, e).

To apply this approach to Tango5 topology, we designed a construct in which a Myc tag and a Flag tag were inserted respectively on the C-terminal and N-terminal side of the last TM domain (Figure 14 a). The idea is that in digitonin permeabilized cells only the Ab whose epitope is situated on the cytosolic face will be able to recognize Tango5. As shown in Figure 14, anti-Flag, but not anti-Myc, was able to recognize Tango5 after digitonin permeabilization. This indicates that Tango5 central loop is situated on the cytosolic face of Tango5-containing organelle.

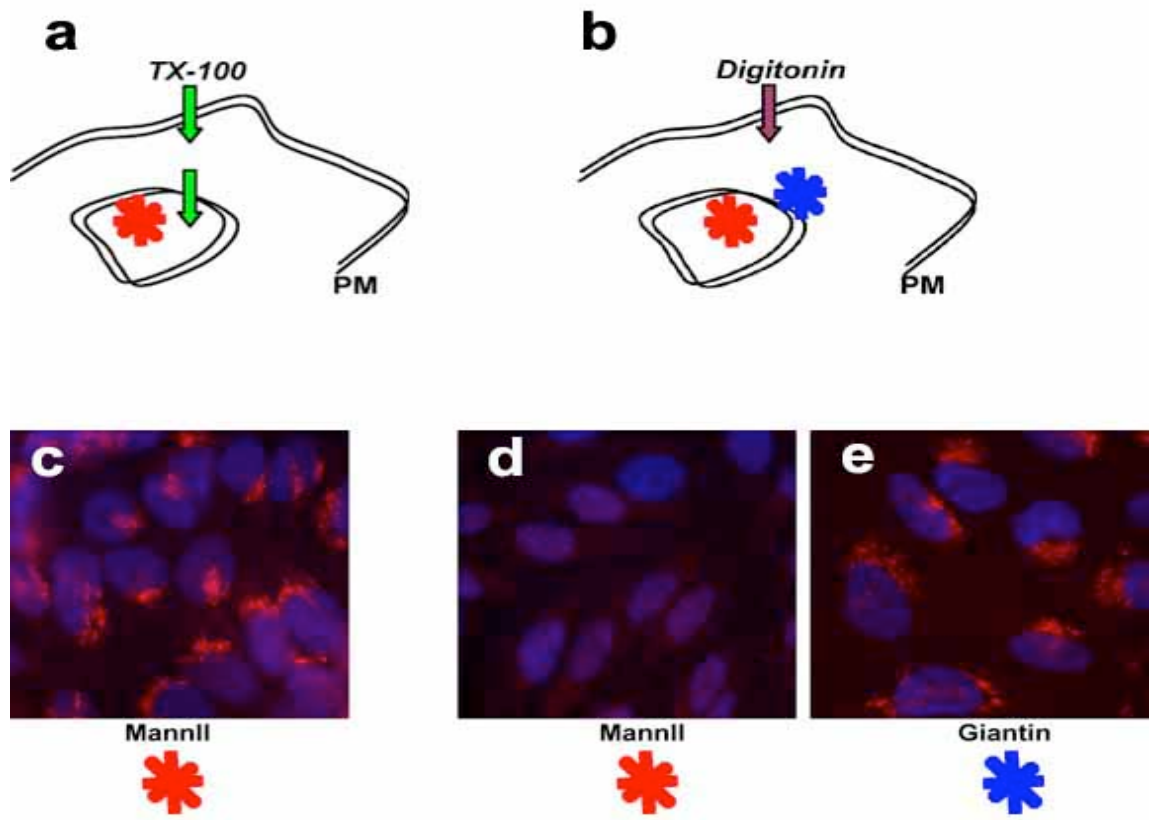


Figure 13. Topology, proof of concept. (a) shows how Triton (TX100) can permeabilize both plasma membrane (PM) and endomembranes. Cells permeabilized with TX100 are stained with anti-MannII Ab (c). Digitonin (b) preferentially permeabilize PM, leaving intracellular membranes intact. No staining is observed when using Ab anti-MannII (epitope inside the lumen of the Golgi) (d). The staining still occurs for GRASP65 (e), which is situated on the cytosolic face of the Golgi.

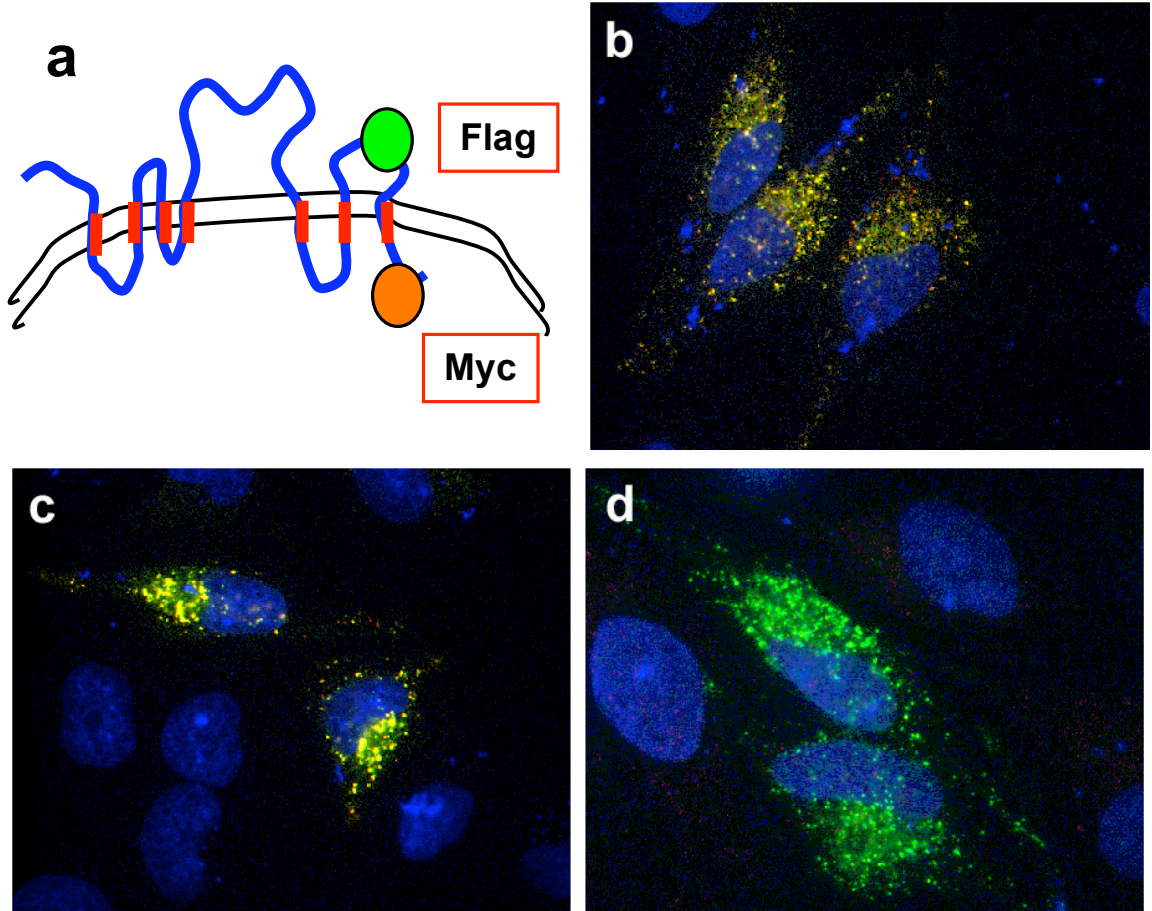


Figure 14. Tango5 topology. (a) Tango5 construct containing double tag: Myc tag is after the last TM domain and Flag tag has been placed before it. (b) cells transfected with this construct are permeabilized with TX100 in order to visualize the proper localization of the mutant protein. (c) transfected cells are permeabilized with digitonin and incubated with Ab anti-Flag (green), then they are permeabilized with TX100 and incubated with Ab anti-Myc (red); both Ab can find their targets. (d) transfected cells are permeabilized with digitonin and incubated with Ab anti-Myc (red), then they are permeabilized with TX100 and incubated with Ab anti-Flag (green); only anti-Flag Ab can recognize its target, whereas anti-Myc cannot. This indicates that the Myc epitope is situated inside the lumen, and the Flag epitope is facing the cytosol.

Tango5 mutants

The only putative domains predicted on Tango5 are the TM domains. To address which region or conserved AA are important for Tango5 localization and function, we decided to analyze how point mutations of conserved AA would affect Tango5 localization. In particular we selected the aminoacid Cysteine (Cys). hTango5 contains 7 Cys residues (Figure 15): Cys1 and 4 are conserved in vertebrates; Cys7 is only present in human Tango5; Cys2-3-5-6 are highly conserved, being present from *C. elegans* to humans. We decided to test the role of Cys in hTango5 localization by mutating Cys residues to Serine (Ser). Two mutants were prepared: one lacking the less conserved Cys (1-4-7) and one lacking the highly conserved ones (Cys 2-3-5-6). wt Tango5 and the two mutants were transfected in HeLa cells; cells were then stained for a Golgi marker (GRASP65) and for Tango5. As shown in figure 16, the mutant lacking the non-conserved Cys (Figure 16 b) showed similar localization to that of wt Tango5 (Figure 16 a). However, the mutant missing the conserved Cys showed distinct ER localization (Figure 16 c). This indicates that Cys2-3-5-6 are required for the protein to leave the ER.

We also tested the role of the C-Terminal tail in Tango5 localization. Figure 16 d shows that a mutant lacking the C-Terminal tail is also retained into the ER.

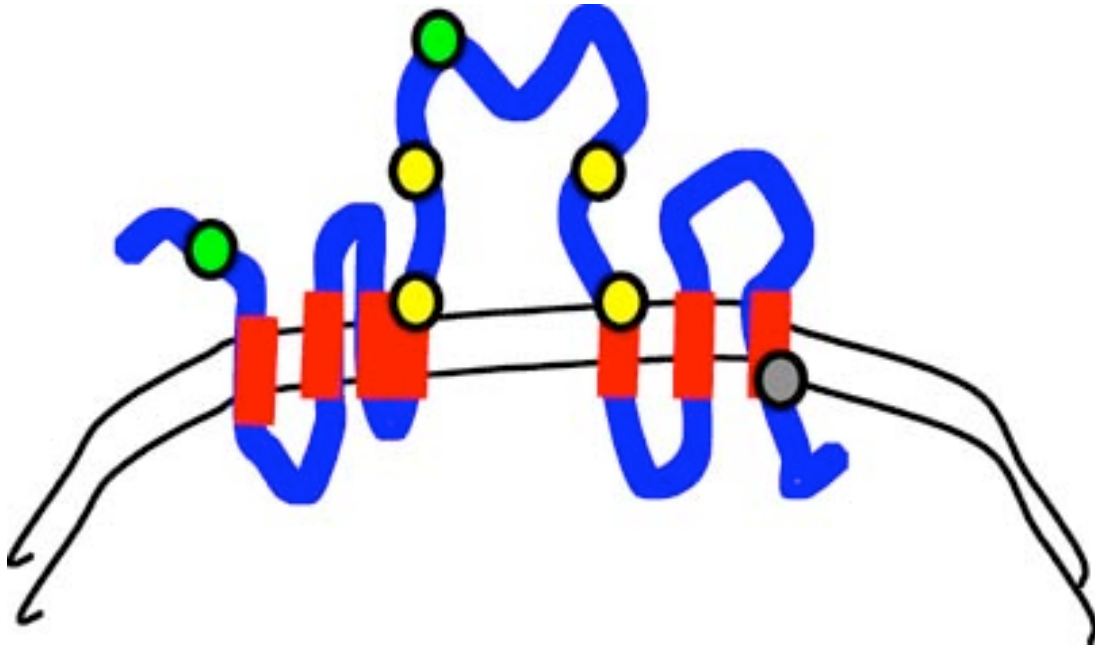


Figure 15. Tango5 cysteines. Green circles indicate Cysteines (no 1 and 4) conserved in vertebrates; in gray is a non-conserved Cys (no 7) present only in humans. In yellow are the Cys conserved from *C. elegans* to humans (no 2, 3, 5 and 6). In red are shown the TM domains.

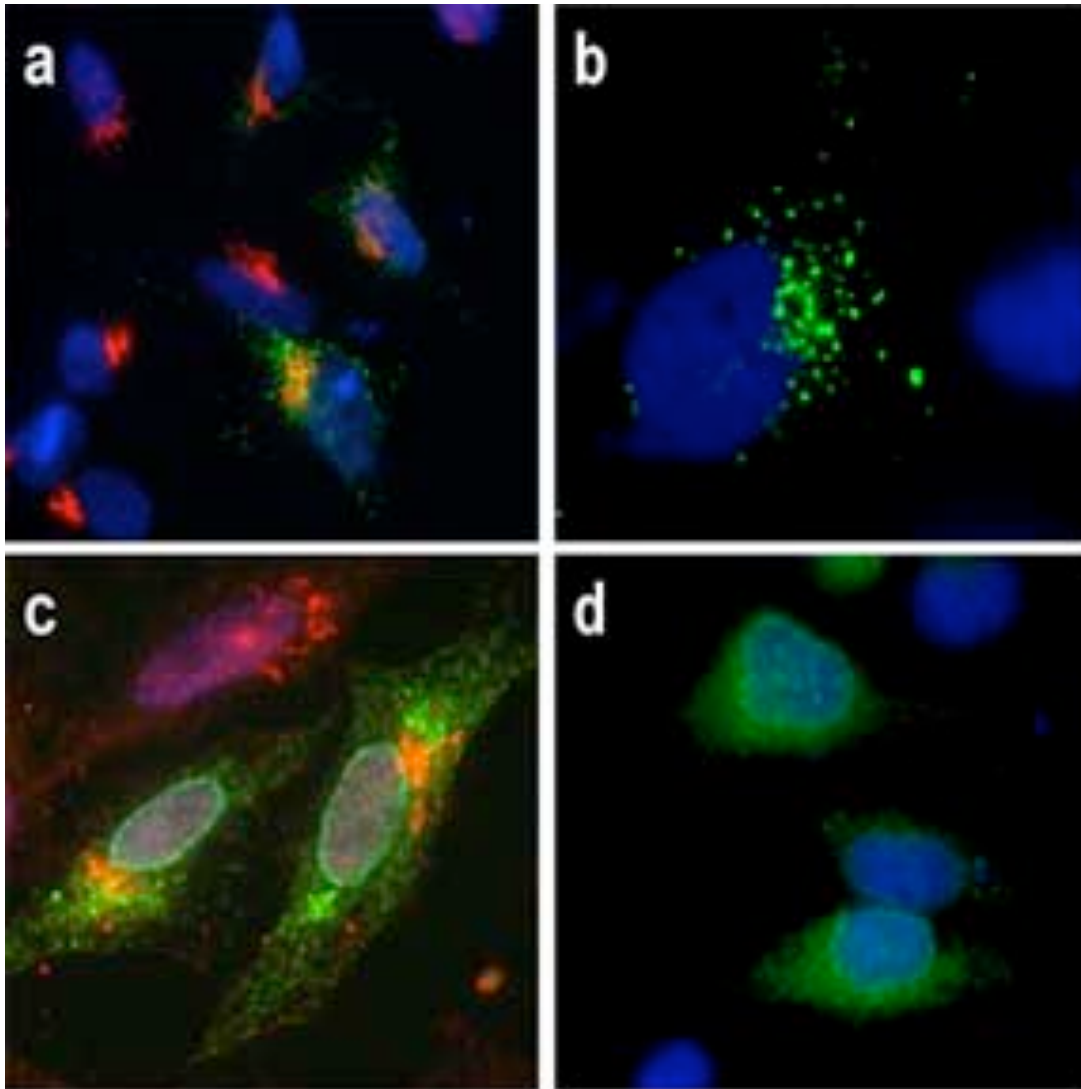


Figure 16. Tango5 mutants. Cells transfected with Tango5-myc are stained with anti-GRASP65 (in red) and anti-Myc Ab (in green). (a) Tango5 wt; (b) *tango5* Δ Cys1-4-7; (c) Tango5 Δ Cys2-3-5-6; (d) Tango5 Δ C-Terminal.

1.4 DISCUSSION

We described the cloning of 20 randomly selected Tango genes, distributed between 4 classes: 4 genes from Class A (Golgi to ER relocation), 4 genes from Class B (Golgi fragmented), 1 from class C (Golgi bloated) and 11 from class D (Golgi wt). 18 genes were successfully cloned, both fly and human homologue (when present). After localization in S2 and HeLa cells, and search for conserved domains within each gene, we decided to put aside genes showing nuclear localization (6 genes) and that were not conserved across species (6 genes). Among the remaining 6 genes, Tango5 was selected for further characterization. Tango5 appears to be the most interesting amongst the 20 Tango genes analyzed, based on its localization along the secretory pathway (ER) and Golgi to ER relocation after RNAi.

Tango5 is very conserved across species, and it was previously reported to be overexpressed in cases of acute pancreatitis. hTango5 codes for a 400 AA protein, containing two groups of TM domains separated by a 100 AA-long loop. We showed that in *Drosophila* cells Tango5 localizes in the ER. In mammalian cells hTango5 is localized in an ERGIC-like compartment, which is crossed by cargo proteins (VSVG) on their way to the Golgi apparatus.

Topology experiments indicate that the central loop of the protein is facing the cytosolic face of Tango5-containing organelle. This could indicate that Tango5 interacts with the machinery involved for transport between ER-ERGIC and Golgi.

Analysis of mutant proteins show that two pairs of conserved cysteines located at the two sides of the loop are important for Tango5 localization, as the mutant protein is unable to leave the ER. The same is true for the C-Terminal tail.

Further characterization is needed in order to establish the exact role of this protein in the secretory pathway. It will be important to perform RNAi of hTango5 and

observe the effect on secretion. Localizing where cargo proteins are blocked will tell us the step of protein transport that requires Tango5's function.

1.5 METHODS

Cloning of genes for expression in S2 cells and HeLa cells

Genes were cloned by RT-PCR from a library of poly(A) RNA using the Gateway system (Invitrogen) and subcloned into pDEST48. MannII-GFP S2 cells were transfected 2 days before gene expression was induced with Cu²⁺ for 4 h, fixed, labeled with antibody against V5 (Invitrogen), and processed for imaging as described above. The human homologues of *Drosophila*'s Tango genes (CG30404 and Tango 5) were cloned by RT-PCR from a mRNA extract from HeLa cells into pCDNA vector (Invitrogen).

Antibodies and constructs

Anti GRASP65 was produced by the Malhotra lab. Anti-Myc (SIGMA) was used to localize hTango5. For colocalization of hTango5, the following Ab were used: EEA1 (Transduction Lab), ERGIC53 (Alexis), LAMP1 (EMD Bioscience), Sec31 (Transduction Lab). Sec16-GFP and ERGIC53-GFP were gift from Ben Glick's lab.

Immunoblot

HeLa cells (wt or transfected with Tango genes) lysate was separated by SDS-PAGE then transferred by Western blot. Immunoblot was performed with anti-Tango5 Ab.

CHAPTER 2

CHEMICAL ANALYSIS OF THE SECRETORY PATHWAY, PART 1: NORRISOLIDE-MEDIATED GOLGI VESICULATION

2.1 ABSTRACT

We are investigating the effect of the natural product norrisolide and designed analogues on Golgi membranes. We found that norrisolide is the first compound known to induce an irreversible vesiculation of Golgi apparatus. To investigate the chemical origins of this effect we synthesized and evaluated a series of norrisolide analogues in collaboration with Dr. Theodorakis lab. Through structure/function studies of norrisolide, we suggest that the perhydroindane core is critical for binding to the target protein, while the C21 acetate unit is essential for the irreversible vesiculation of Golgi membranes.

2.2 INTRODUCTION

The problem of membrane fission

The Golgi complex, composed of stacks of flattened cisternae, is a central organelle of the secretory pathway, and a controlled balance of membrane input and output maintains its structure and organization. Small vesicles mediate transport into,

across, and out of the Golgi complex. To leave the Golgi apparatus, cargo molecules are packaged into transport carriers that ultimately dissociates from the TGN by membrane fission. Membrane fission involves the action of numerous components that include proteins as well as lipids. How is this process generated, and how is it coordinated with the transport carrier formation? The fission activity is most likely triggered by the arrival of cargo into the TGN and is shut off once the cargo has been transported. This process must be carefully regulated to prevent the conversion of the whole Golgi apparatus into transport vesicles (Bard and Malhotra, 2007).

Natural products able to convert the Golgi into small vesicles can be used as a tool to understand the process of membrane fission, and to identify components involved in this elusive process. The reasoning behind the use of such compounds is that they are acting through the overactivation of the fission machinery normally used by the cell to produce transport carriers. In Vivek Malhotra's lab the analysis of Golgi fragmentation induced by one of these compounds (Ilimaquinone) led to the identification of important players in the process of producing transport vesicles.

Ilimaquinone mediated Golgi vesiculation

Ilimaquinone was isolated in 1979 from the sea sponge *Hippospongia metachromia* (Luibrand *et al.*, 1979). Malhotra's group (Takizawa *et al.*, 1993) was the first to report that IQ was able to induce the complete and reversible fragmentation of the Golgi complex into small vesicular structures. The same group showed that transport of newly synthesized proteins from the ER to the cis-Golgi-derived vesicles was unaffected; however, further transport along the secretory pathway was blocked. Detailed imaging of IQ treated cells showed that while other intracellular organelles were not affected, cytoplasmic microtubules were depolymerized by IQ treatment. Since there is a

connection between the status of the MT and the organization of the Golgi, the authors used Taxol to stabilize microtubules, and showed that under these conditions IQ is still able to completely fragment the Golgi (Veit *et al.*, 1993).

In order to identify IQ target, two distinct approaches were taken: a synthetic-chemical based approach and a biochemical approach.

Snapper's group developed the total synthesis of IQ, which was used to prepare structural variants of the natural products more suitable for target identification (Radeke *et al.*, 1997). A biochemical assay was to test the effectiveness of the probes (measuring the enzymatic activity of secreted alkaline phosphatase) instead of a visual assay to test for Golgi fragmentation. A photoaffinity probe (Radeke *et al.*, 1998) led to the identification of S-adenosylhomocysteinase as the putative target of IQ (Radeke *et al.*, 1999; Casaubon and Snapper, 2001). This enzyme plays a key role in cellular methylation chemistry; however, it is not clear how disrupting cellular methylation would induce a block in protein transport and the vesiculation of the Golgi apparatus.

Malhotra's group was able to reconstitute the IQ-mediated vesiculation of the Golgi apparatus in permeabilized cells. The assay was employed to analyze the biochemical requirements for IQ activity by establishing conditions that would inhibit the effects of IQ. In other words, reagents that block the effect of IQ are likely to do so by restoring process affected by this compound, either at its target or at downstream effectors. Characterization of these components will offer insights into the molecular mechanism that leads to the vesiculation of the Golgi. After testing many of the proteins known to be involved in the process of vesicle formation, it was found that IQ-mediated Golgi vesiculation occurs through the activation of the betagamma ($\beta\gamma$) subunit of heterotrimeric G proteins (Jamora *et al.*, 1997). Interestingly, adding $\beta\gamma$ subunits in the absence of IQ is sufficient to vesiculate Golgi stacks. In the following years, the

Malhotra's lab found that Protein Kinase D (PKD) is the downstream target of $\beta\gamma$ subunit (Jamora *et al.*, 1999). The involvement of PKD in IQ-mediated Golgi vesiculation was proven without doubts through different approaches (Liljedahl *et al.*, 2001; Maeda *et al.*, 2001). For example, preincubation of cells with H89, a PKD inhibitor, blocks IQ from fragmenting the Golgi. Moreover, the expression of a mutated form of PKD lacking its kinase activity results in block in secretion and in the extensive tubulation of the Golgi complex; these tubes are a result of the inhibition of the fission reaction. All together, through the use of IQ, Malhotra's lab was able to identify some of the components involved in the fission reaction for the generation of Golgi to PM transport carriers. The working model is the following: the arriving of the cargo in the Golgi activates a trimeric G protein, through a G-protein-coupled receptor; the activated $\beta\gamma$ subunit in turns induces the production of a diacylglycerol (DAG) pool at the Golgi (Baron and Malhotra, 2002), which recruits PKD that ultimately induces the fission of the transport vesicle (Diaz Anel and Malhotra, 2005; Bard and Malhotra, 2006).

Norrisolide as Golgi vesiculation agent

Norrisolide is a natural product extracted for the first time in 1983 from *Chromodoris norrisi*, a nudibranch from Gulf of California by Faulkner and coworkers. (Bobzin and Faulkner 1991; Blunt *et al.*, 2005).

Nudibranchs (nudibranchia) are a class of shell-less, brightly colored sea slugs that comprise one of the largest groups of marine mollusks with over 3,000 species described to-date. To avoid their natural predators, these marine animals have developed a defense mechanism that enables them to incorporate in their skin the toxins produced by sea sponges they feed on.

Spectroscopic and crystallographic studies established that norrisolide belongs to a family of rearranged spongiane diterpenes, which also includes macfarlandin C and dendrillolide A (Figure 17). The biological profile of these family members includes antifungal, antimicrobial, antiviral and antitumor properties. All these compounds present a γ -lactone- γ -lactol ring attached to a hydrophobic core. In norrisolide the side chain formed by the acetylated γ -lactol- γ -lactone ring is attached to a perhydroindane core. Dr. Theodorakis lab was recently able to completed the total chemical synthesis of norrisolide (Brady et al., 2005). The synthetic compound still maintains the Golgi fragmentation activity and provides a mean to modify the molecule, to make it more suitable for biochemical studies.

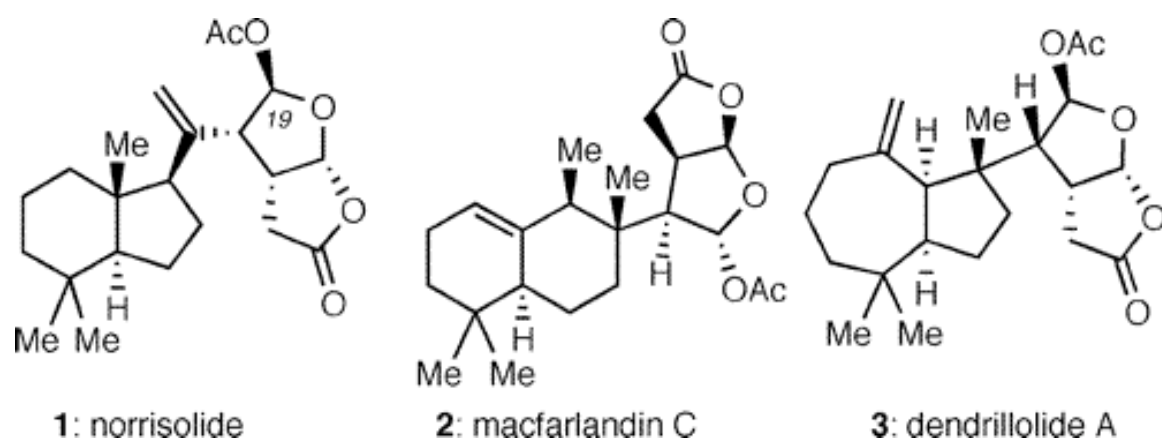


Figure 17. Representative structures of spongiane diterpenes.

2.3 RESULTS

Norrisolide induces irreversible Golgi fragmentation

The effects of norrisolide and analogues on the Golgi apparatus were investigated using normal rat kidney cells (NRK cells). Cells growing on coverslips in complete growth medium were treated with a 30 μ M stock solution of the compounds in DMSO, and then incubated for 60 min at 37 °C. The cells were then divided in two groups. One set of coverslips (Figure 18, column 1) was fixed after 60 min of incubation and processed for immunofluorescence. The other set (Figure 18, column 2) was subjected to washout with PBS, to eliminate all traces of the compounds. The cells were then allowed to recover in fresh growth medium for 90 min at 37 °C prior to the staining procedure for fluorescence microscopy. Control cells were treated only with DMSO and processed as above. All cells were visualized by immunofluorescence microscopy with antibodies specific to Golgi membranes and microtubules. The results are summarized in Figure 18. Within 60 min of incubation with norrisolide the Golgi membranes were fragmented with little change in microtubule organization (Figure 18, row 1, column 1). The fragmentation of the Golgi apparatus was irreversible since removal of norrisolide by extensive washing did not promote its reassembly (Figure 18, row 1, column 2). Under these conditions the cells died, presumably due to irreversible Golgi fragmentation.

Norrisolide is able to fragment the Golgi complex without affecting microtubules (as shown by IF imaging). Nonetheless, to exclude the possibility of undetectable changes in the microtubules structure, NRK cells were preincubated for 30 min with 10 μ g/mL of Taxol, a microtubule-stabilizing agent, and then treated with norrisolide and analogues as described above. Under these conditions, these compounds still caused Golgi membranes to vesiculate. Thus, norrisolide and perhydroindane-containing

analogues break down Golgi membranes even in the presence of taxol-stabilized microtubules.

Chemical analysis of norrisolide-induced Golgi fragmentation

As stated above, norrisolide structure is composed of a perhydroindane core coupled to an acetylated lactone-lactol side chain. We decided to investigate whether each of norrisolide constituents was able to retain the same activity of the full molecule towards the Golgi. With this in mind, two groups of compounds were synthesized.

The first group (Figure 18, row 2) had an intact perhydroindane core coupled to a modified side chain. One compound had the lactone-lactol motif truncated to a monocyclic system; another was lacking the acetoxyl functionality at the C19 center. When tested on NRK cells (Figure 18, row 2, column 1), these compounds were shown to induce complete Golgi fragmentation, therefore reproducing the activity of the natural product. However, when tested for irreversibility, both probes showed a reversible phenotype, as the Golgi quickly reassembled when the compounds were removed (Figure 18, row 2, column 2).

The second group of compounds (Figure 18, row 3) had intact lactone-lactol side chain, but the perhydroindane core was either replaced by a tert-butyl group or it was simply deleted (leaving just the lactone-lactol motif). When NRK cells were incubated with these compounds, no alteration on Golgi morphology was observed (Figure 18, row 3, column 1 and 2).

Evaluation of the above data suggests that the perhydroindane core of norrisolide and related analogues is essential for activity and appears to function as the target recognition element. This could explain why compounds lacking the perhydroindane core have no effect on the Golgi membranes. Among the latter

analogues, the fragmentation is more pronounced with compounds that contain the entire C1–C20 backbone of the natural product, and is somewhat attenuated with compounds that lack the γ -lactone subunit. Of particular significance is the finding that norrisolide induces an irreversible fragmentation of the Golgi membranes, while the partially reduced analogues lacking the C21 acetyl group induce a reversible fragmentation. This observation invites the hypothesis that a covalent modification of the target protein occurs in the case of norrisolide, presumably due to the highly electrophilic nature of the acetyl group.

A possible scenario that rationalizes these findings would involve selective binding of the perhydroindane core of norrisolide into a hydrophobic pocket of a protein that is involved in the signal transduction related to the Golgi fragmentation. This binding could bring the γ -lactol- γ -lactone side chain in close proximity to a nucleophile, which can then undergo acetylation. The identification of norrisolide dependent acetylation of a Golgi specific protein will help reveal the significance of acetylation dependent regulation of Golgi organization.

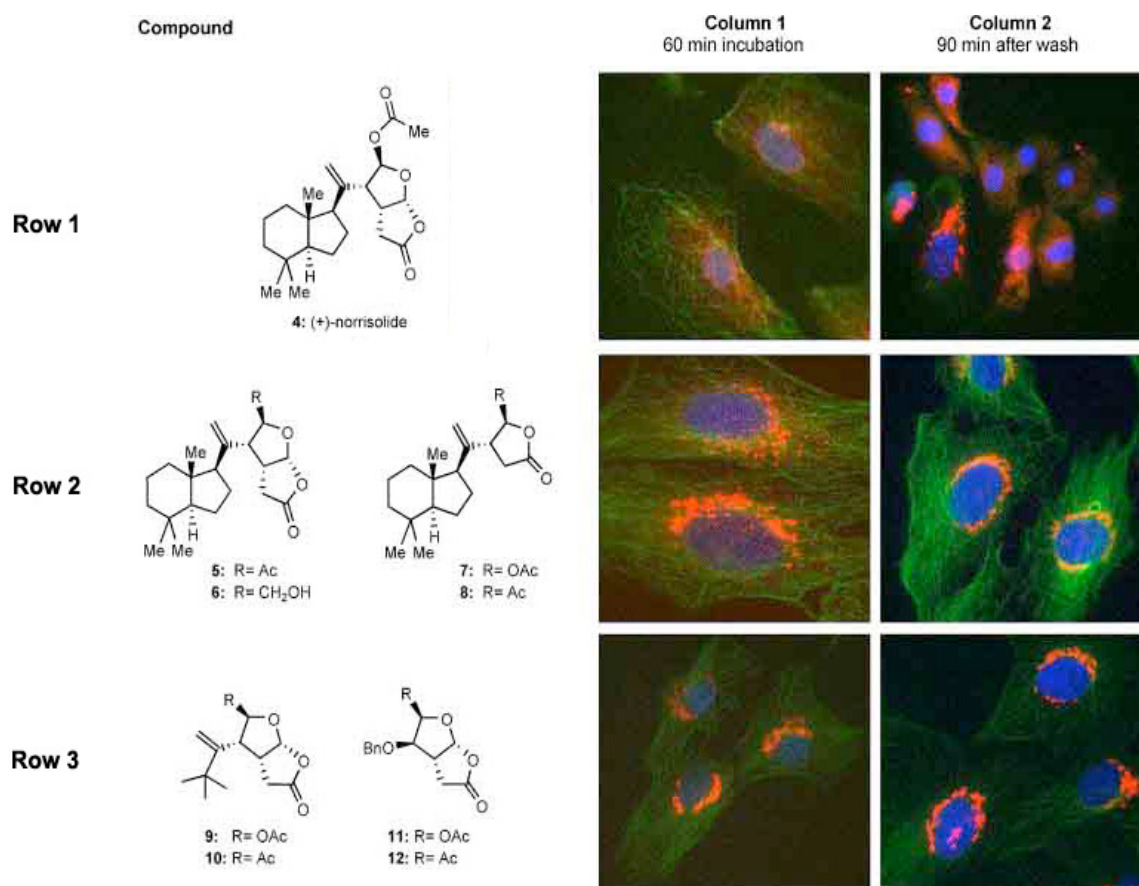


Figure 18. Effect of norrisolide and analogues on Golgi membranes. Column 1: NRK cells treated with norrisolide and analogues and incubated for 60 min; column 2: NRK cells treated as in column 2 and then washed with buffer and incubated for an additional 90 min. The Golgi apparatus is shown in red color, microtubules in green, and nucleus in blue. Row 1 shows norrisolide. Row 2 shows compounds in which the lactone-lactol motif has been perturbed. Row 3 shows compounds where the perhydroindane core has been replaced or deleted.

Norrisolide-derived fluorescent analogues

To assess the biological activity of norrisolide as a function of its structure, we studied the effect of norrisolide-derived fluorescent probes (Figure 19, probes 4, 5 and 6) on the Golgi complex. We were interested in synthesizing a fluorescent derivative of norrisolide that was able to maintain norrisolide activity. This fluorescent compound could then be used to visualize the intracellular localization of norrisolide target.

The fluorophore coumarin (Clarke *et al.*, 2005; Joullie *et al.*, 2003) was coupled to the perhydroindane core of norrisolide (compound 4), to the lactone-lactol motif (compound 5) and to norrisolide full molecule (compound 6). Normal rat kidney (NRK) cells plated on coverslips in complete growth medium were incubated with these probes (80 μ M/DMSO) for 60 min, then fixed and processed for immunofluorescence microscopy. While compound 5 had no effect on the Golgi apparatus, compound 4 was found to induce extensive Golgi fragmentation (Figure 20 b). However, in contrast to norrisolide, this fragmentation was reversed upon washing (Figure 20 c).

The intracellular localization of compound 4 was evaluated in fixed cells. After fixation, the cells were first treated with Golgi-specific antibodies (Figure 20 d) and then incubated with compound 4. Under these conditions, probe 4 was shown to localize on the Golgi complex (Figure 20 e and f). On the other hand, probe 5 did not show any specific localization.

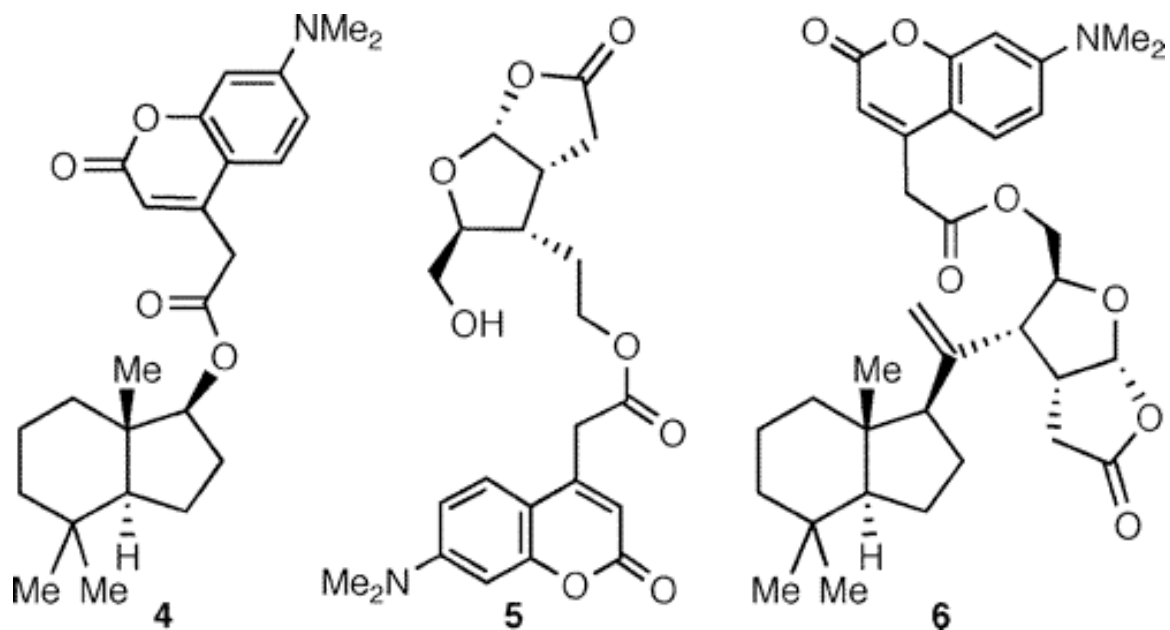


Figure 19. Structures of norrisolide-fluorescent probes: Compound 4 consists of norrisolide's core (perhydroindane motif) coupled to the fluorescent robe coumarin. Compound 5 is made of the lactone-lactol motif from norrisolide, coupled to coumarin. Compound 6 is norrisolide full molecule linked to coumarin.

Norrisolide-fluorophore binds norrisolide target

To evaluate whether compound 4 and norrisolide bind to the same receptor, we performed a competition experiment (Figure 20 g, h and i). We found that the natural product can displace compound 4 from the Golgi complex, as shown by the loss of green color, which is due to staining by probe 4, but without perturbing the overall Golgi organization, as shown by the red staining due to the anti-Golgi antibody. These studies indicate that norrisolide binds to a target on the Golgi complex. A similar localization to compound 4 was obtained using probe 6, which contains the entire framework of the natural product. Competition experiments showed again that norrisolide could displace compound 6 from its target. These results indicate that norrisolide induces phenotypic changes in cells by binding to a receptor localized at the Golgi complex and suggest that the perhydroindane core of norrisolide is essential and necessary for such a binding. In the absence of the C19 acetoxy group of norrisolide, this binding does not resist washout and induces a reversible Golgi vesiculation (Brady et al., 2004).

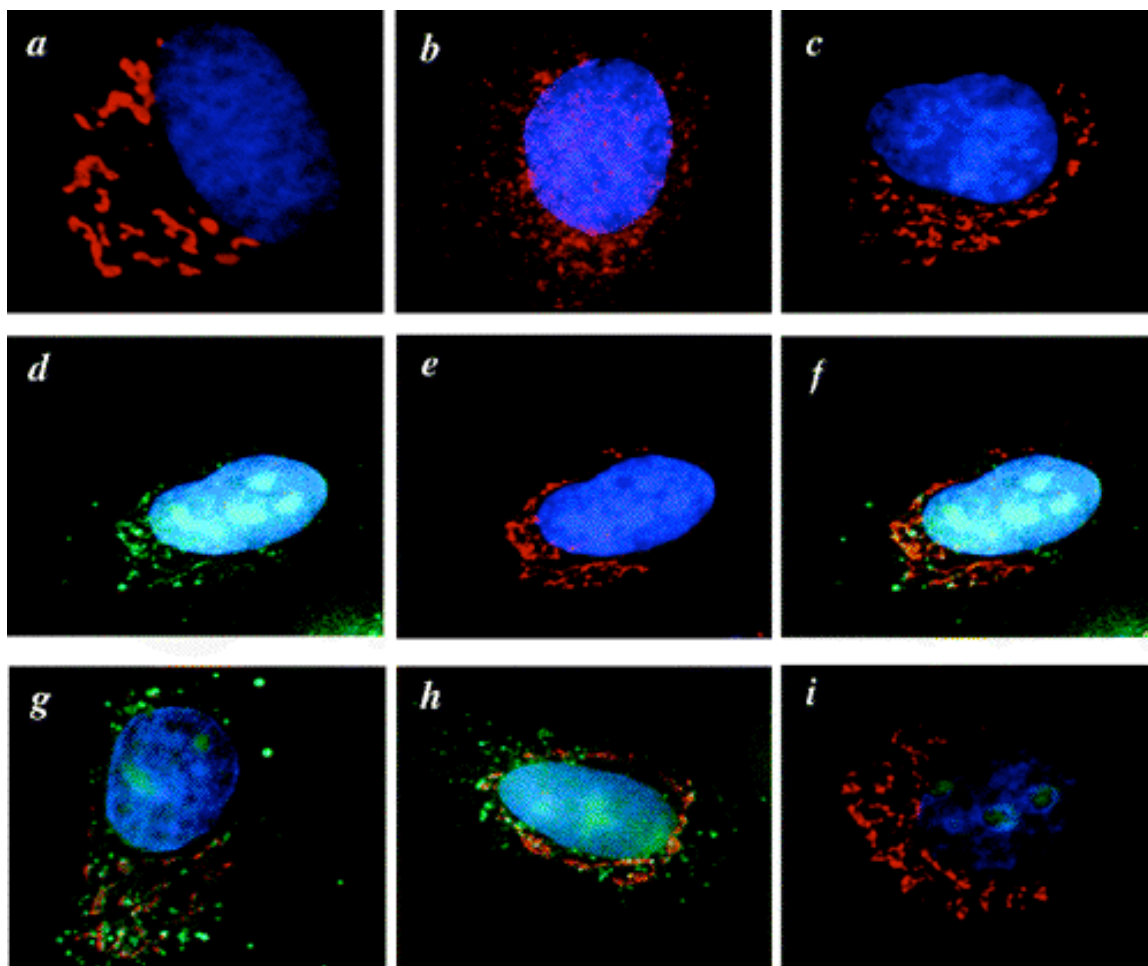


Figure 20. Activity and intracellular localization of norrisolide-fluorophore. (a-c) NRK cells treated with (a) DMSO for 60 min; (b) compound 4 for 60 min; and then (c) washed and allowed to recover for 60 min. Golgi is shown in red, nuclei in blue. (d-f) Fixed cells stained with (d) Golgi-specific antibody; and then (e) incubated with compound 4. In (f) is shown the co-localization between the Golgi antibody and compound 4 (yellow color). (g-i) Competition experiment. Fixed cells preincubated with compound 4 were treated with PBS (g), DMSO (h), and norrisolide (100 M) (i). The green coloring is due to compound 4.

Norrisolide-bisepoxide

The above findings suggest that the C19 acetoxy group of norrisolide plays an essential role in the irreversibility of the fragmentation either by stabilizing the binding or by creating a covalent bond with its target protein (Cravatt and Sorensen, 2000). Inspired by a study that identified epoxides as suitable functionalities for protein labeling (Chen *et al.*, 2003; Greenbaum *et al.*, 2000; Meng *et al.*, 1999) we replaced the entire side chain of norrisolide with a bisepoxide motif to form compound 7. Compounds 8 and 9 were also synthesized and used as controls (Figure 21).

The effect of norrisolide on the Golgi membranes is shown in Figure 22. Cells treated with norrisolide underwent extensive Golgi vesiculation (Figure 22 b) that persisted even after washing (Figure 22 c). Analogue 8, containing the core fragment of the natural product, induced a similar vesiculation (Figure 22 e) that was, however, reversible upon washing (Figure 22 f). In contrast, compound 7, in which the perhydroindane core was attached to a bisepoxide scaffold, induced an irreversible vesiculation of the Golgi membranes and reproduced the cellular phenotype of the natural product (Figure 22 g and i). On the other hand, compound 9, lacking the perhydroindane motif, had no effect on Golgi membranes, attesting to the importance of the norrisolide core in Golgi localization and structure.

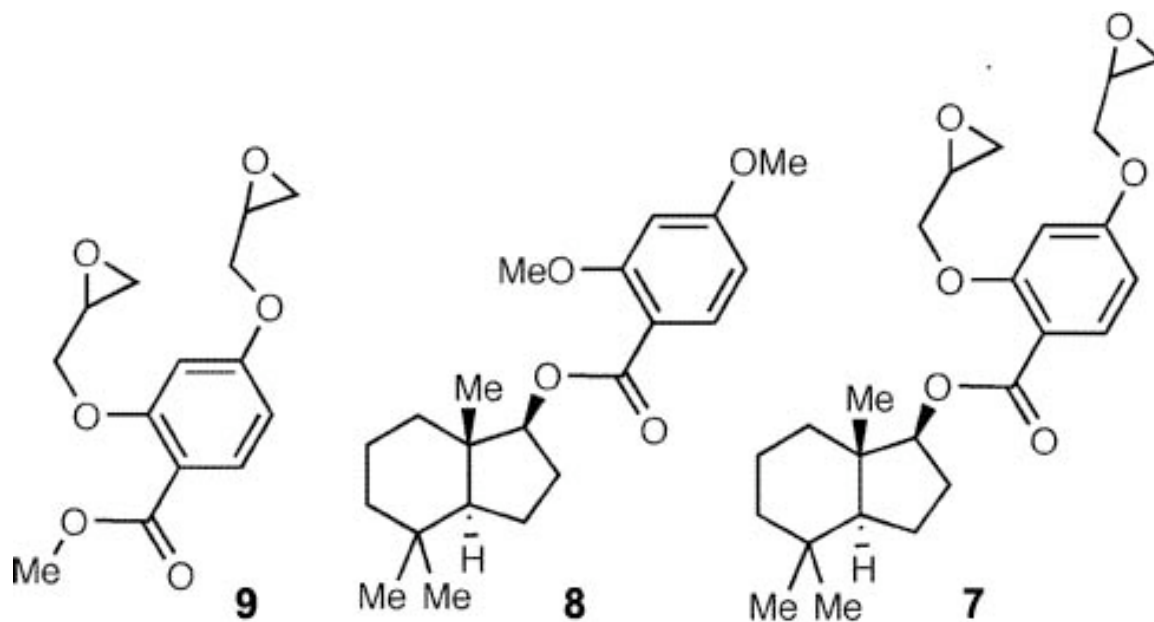


Figure 21. Structures of norrisolide-epoxide probes. Compound 7 contains the perhydroindane core and the bisepoxide motif. Compound 8 is like compound 7 but lacks the bisepoxide. Compound 9 is the negative control, containing the bisepoxide but missing norrisolide's core.

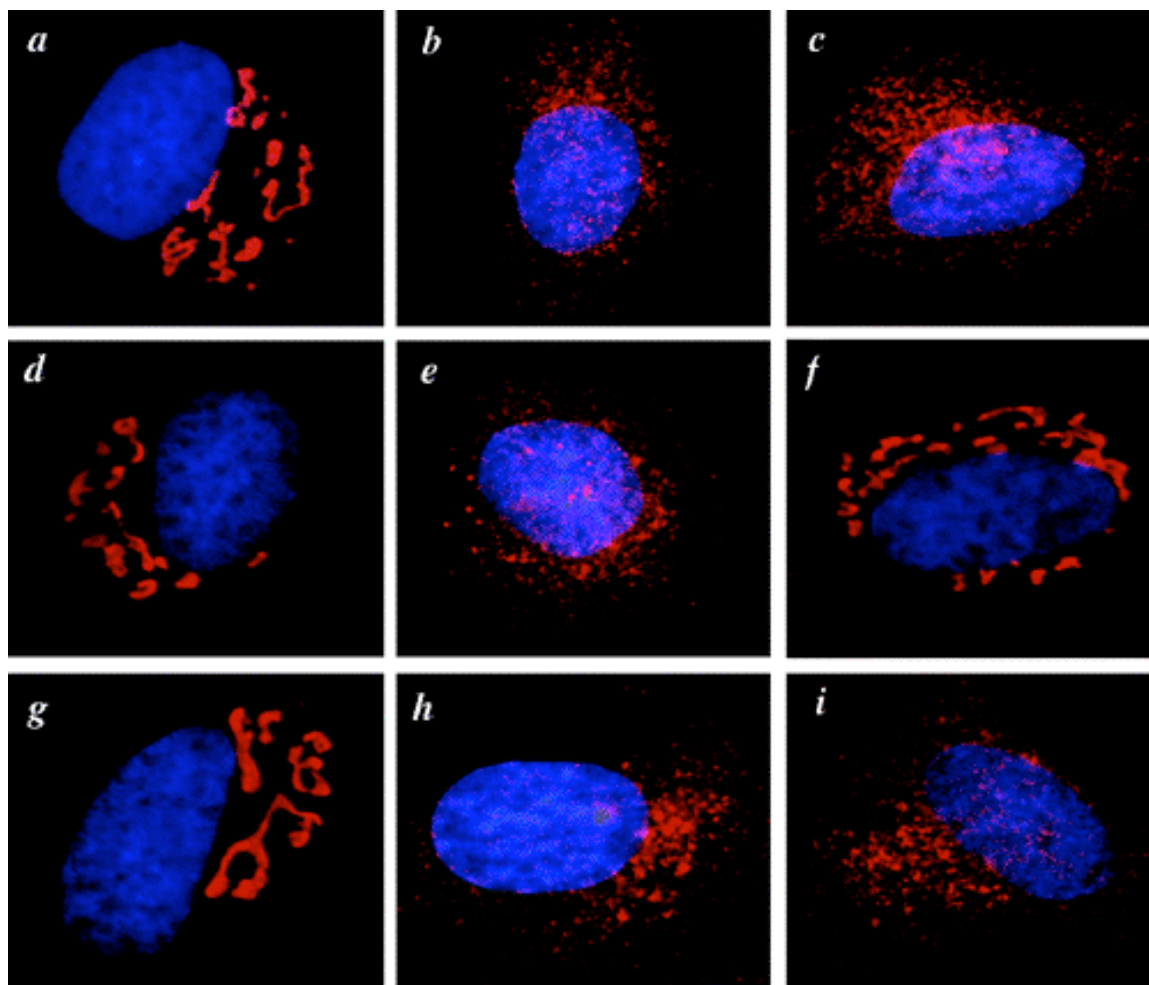


Figure 22. Effect of norrisolide and analogues on Golgi membranes. NRK cells were incubated with DMSO (*a*, *d*, and *g*) or with 40 M of norrisolide (*b*), compound 8 (*e*), and of probe 7 (*h*) for 60 min, then washed out and incubated in fresh medium for 60 min (*c*, *f*, and *i*). The cells were fixed and processed for immunofluorescence. The Golgi is shown in red (Alexa Fluor 594), and the nuclei in blue (Hoechst).

2.4 DISCUSSION

We presented here the effects of norrisolide and designed analogues on Golgi membranes. We found that the natural product induces an irreversible vesiculation of the Golgi apparatus without affecting the microtubular cytoskeleton. To the best of our knowledge, this is the first compound to have such an effect. We have also synthesized a series of norrisolide analogues in which several functionalities found in the structure of the natural product were sequentially deleted or altered. This allowed us to investigate the chemical origins of the irreversible fragmentation of the Golgi complex produced by norrisolide. Such structure/function studies suggest that the perhydroindane core is critical for binding to the target protein, while the C21 acetyl group unit is essential for the irreversible vesiculation of the Golgi apparatus.

By using fluorescent derivatives of norrisolide, we were able to show that a fluorescent probe containing norrisolide perhydroindane core is able to induce Golgi vesiculation and specifically localizes to the Golgi apparatus. This binding is attributed exclusively to the presence of the perhydroindane core that acts as the recognition element. Competition experiments done with norrisolide natural product show that the fluorescent derivative and norrisolide bind the same target.

We also concluded that is the presence of C19 acetyl group on the a γ -lactone- γ -lactol side chain is responsible of the irreversible phenotype showed by norrisolide. Replacing this group with other electrophilic functionalities, such as the bisepoxide motif of probe 7 (Figure 21) can reproduce the irreversible effect of norrisolide.

2.5 METHODS

Reagents and cells

NRK cells were plated on 12 mm glass coverslips coated with Pronectin F (Sigma) and grown in complete medium (250 μ L per coverslip), consisting of alpha MEM medium (GIBCO) with 10% fetal calf serum, 2 mM L-glutamine and 25 mM Hepes pH 7.4, at 37 °C in a 5% CO₂ cell incubator. Stock solution (5 mg/mL) of norrisolide and analogues were made in DMSO and stored at -20 °C. The working concentration of the compounds was 30 μ M for each coverslip. To half of the coverslips (70% confluent) were added norrisolide or analogues (2.5 μ L of the stock solutions). To the other half were added 2.5 μ L of DMSO as negative control. Both groups of cells were incubated at 37 °C for 60 min. Part of the treated cells and part of the control cells were then fixed with 4% formaldehyde and processed for immunofluorescence microscopy. The remaining cells were washed four times with phosphate-buffered saline (PBS) (150 mM NaCl, 1.8 mM NaH₂PO₄, 8.4 mM Na₂HPO₄). The cells were incubated in fresh complete medium at 37 °C for 90 min, then fixed with 4% formaldehyde and processed for immunofluorescence microscopy.

Immunofluorescence microscopy

For fluorescent labeling, cells were incubated in blocking buffer (PBS containing 2.5% fetal bovine serum and 0.1% Tween 20) for 30 min at room temperature. The cells were then incubated for 1 h at room temperature in primary antibody diluted in blocking buffer. Rat tubulin antibody (1:75) (Accurate Chemicals) was used to detect microtubules; rabbit Mannosidase II antibody (1:2000) (a gift from Dr. Kelly Moreman, Vanderbilt University, TN) was used to visualize Golgi apparatus. The cells were then

washed three times with PBS and incubated with secondary antibody, diluted in blocking buffer, for 1 h at room temperature. Alexa fluor 488 goat anti mouse (1:500) and Alexa Fluor 594 goat anti rabbit (1:500) from Molecular Probes were used. Cells were washed three times with PBS containing Hoechst (1:100,000) (H33342, Molecular Probes) to stain DNA. Coverslips were then mounted onto glass slides and visualized using a Nikon microphot-FXA fluorescence microscope at 60× magnification

CHAPTER III

CHEMICAL ANALYSIS OF THE SECRETORY PATHWAY, PART 2: TRIFUNCTIONAL NORRISOLIDE PROBES FOR THE STUDY OF GOLGI VESICULATION.

3.1 ABSTRACT

Inspired by the effect of norrisolide on the Golgi complex, we synthesized trifunctional probes for the identification of norrisolide target. The perhydroindane core of the parent natural product, which was shown necessary and sufficient for Golgi localization and fragmentation, was couple to a crosslinking unit (aryl azide or epoxide) for covalent binding to the target, and a tag (biotin or iodine) for subsequent target purification. We found that biotin-containing probes induced inefficient Golgi vesiculation. However, an iodinated probe induced extensive and irreversible Golgi fragmentation. This probe can be used for the isolation of the cellular target of norrisolide.

3.2 INTRODUCTION

The approach to identify the cellular receptors for natural products is generally based on two requirements. First, the bind between the natural product and its target has to be very stable. Second, the natural product has to be susceptible to the attachment of a tag, for both visualization and purification. The structure composed by the natural product and the tag is called affinity probe.

Affinity probes have proven useful to identify targets of natural products. For example, the use of a tritiated analogue of colchicine led to the isolation of tubulin as the cellular receptor for colchicine and thereby determine the molecular composition of microtubules (Borisy and Taylor, 1967). Frequently biotin is used as the tag, as it allows both affinity purification, through a streptavidin resin, and intracellular localization, through streptavidin coupled to a fluorophore. For example the Crews lab at Yale has used biotinylated analogues of epoxide-containing natural product fumagillin to identify their cellular targets (Sin *et al.*, 1997). A similar approach was chosen in the attempt to find the target of IQ by Snapper and coworkers (Radeke and Snapper 1998). In that experiment, a photo affinity probe was attached to IQ, and then bovine liver extract was incubated with this molecule in the presence of ultraviolet light, necessary to activate the crosslinking reaction. The protein S-adenosylhomocysteinase (SAHase) was co-purified with IQ. Unfortunately the authors could never prove the real involvement of such protein in IQ mediated Golgi vesiculation.

Recent studies with norrisolide have suggested that the hydrocarbon-containing perhydroindane core of this natural product binds to a receptor at the Golgi membranes and induces reversible vesiculation. Therefore, norrisolide core appears to function as the target recognition element; indeed, compounds lacking the perhydroindane core had

no effect on the Golgi membranes. The vesiculation becomes irreversible when this core is coupled to electrophilic residues. Our efforts to explore this concept led to identification a probe composed of norrisolide core linked to a bisepoxide (Figure 21, probe 7) that was shown to induce an identical phenotype to norrisolide natural product. We proposed that this effect is due to covalent reaction of the epoxide units to the cellular receptor. Based on these findings, we sought to synthesize and study trifunctional norrisolide probes that would contain the following functional groups: (a) the perhydroindane core for Golgi localization, (b) a crosslinking reagent for covalent binding to the protein target and (c) a tag for subsequent protein purification and isolation.

3.3 RESULTS

Norrisolide-aryl azide-biotin

The first probe that we evaluated was compound 10 in which the perhydroindane motif is attached to a photoactivable crosslinker (aryl azide unit) (Chen and Ebright, 1993) and biotin (Figure 23). It was expected that light-activation of the aryl azide would produce a reactive nitrene intermediate that would react covalently with its receptor and thus mimic the irreversible Golgi vesiculation observed with norrisolide natural product. The biotin tag could then allow the isolation and purification of the target via affinity chromatography on resin-bound streptavidin. As negative control we prepared a bifunctional probe (Figure 23, compound 11) containing the aryl azide functionality and the biotin but not the perhydroindane motif.

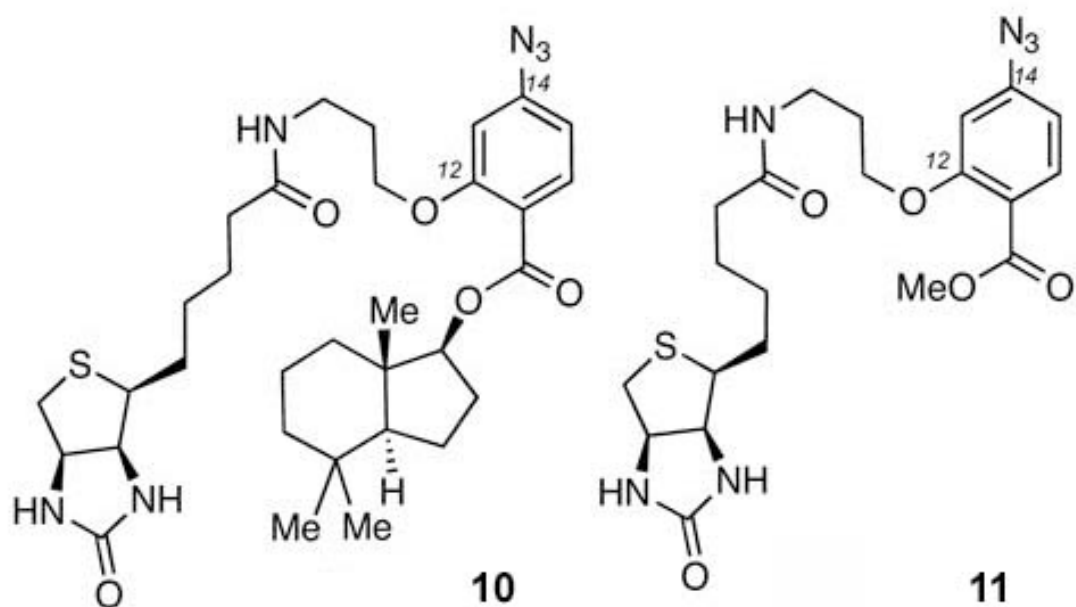


Figure 23. Photoactivable trifunctional probe. Compound 10 is a trifunctional probe consisting of norrisolide core, an aryl-azide unit (photoactivable crosslinker) and biotin as tag. Compound 11 is the negative control, having biotin coupled to the aryl azide.

The effect of probes 10 and 11 on Golgi vesiculation was evaluated using normal rat kidney (NRK) cells. Cells were treated with compounds 10 and 11 (30 μ M) for 60 min at 37 °C. The cells were then fixed and processed for immunofluorescence microscopy. Figure 24 highlights the results of this study. The trifunctional probe 10 caused Golgi fragmentation in 50% of the cell population (Figure 24, b). In contrast, the control compound 11 did not lead to any fragmentation (Figure 24 a), supporting the notion that the perhydroindane motif is essential for norrisolide activity. As expected, the fragmentation induced by probe 10 was completely reversible upon washing when the photoactivation of the aryl azide unit did not take place (Figure 24 c). UV-irradiation for 15 min caused an irreversible Golgi fragmentation in a small percent of the cells (Figure 24 d).

Despite the low extent of irreversibility, we attempted isolation of the protein captured by compound 10 after photoactivation. After UV irradiation, the cells were lysed and analyzed by Western blot to identify proteins containing biotin. As negative control we used probe 11, containing aryl azide-biotin but not the norrisolide core. The results, shown in Figure 25, indicate that both compounds 10 and 11 (lane a and b respectively) led to isolation of the same protein bands. This suggests that these protein bands are due to non-specific interactions of biotin with cellular proteins.

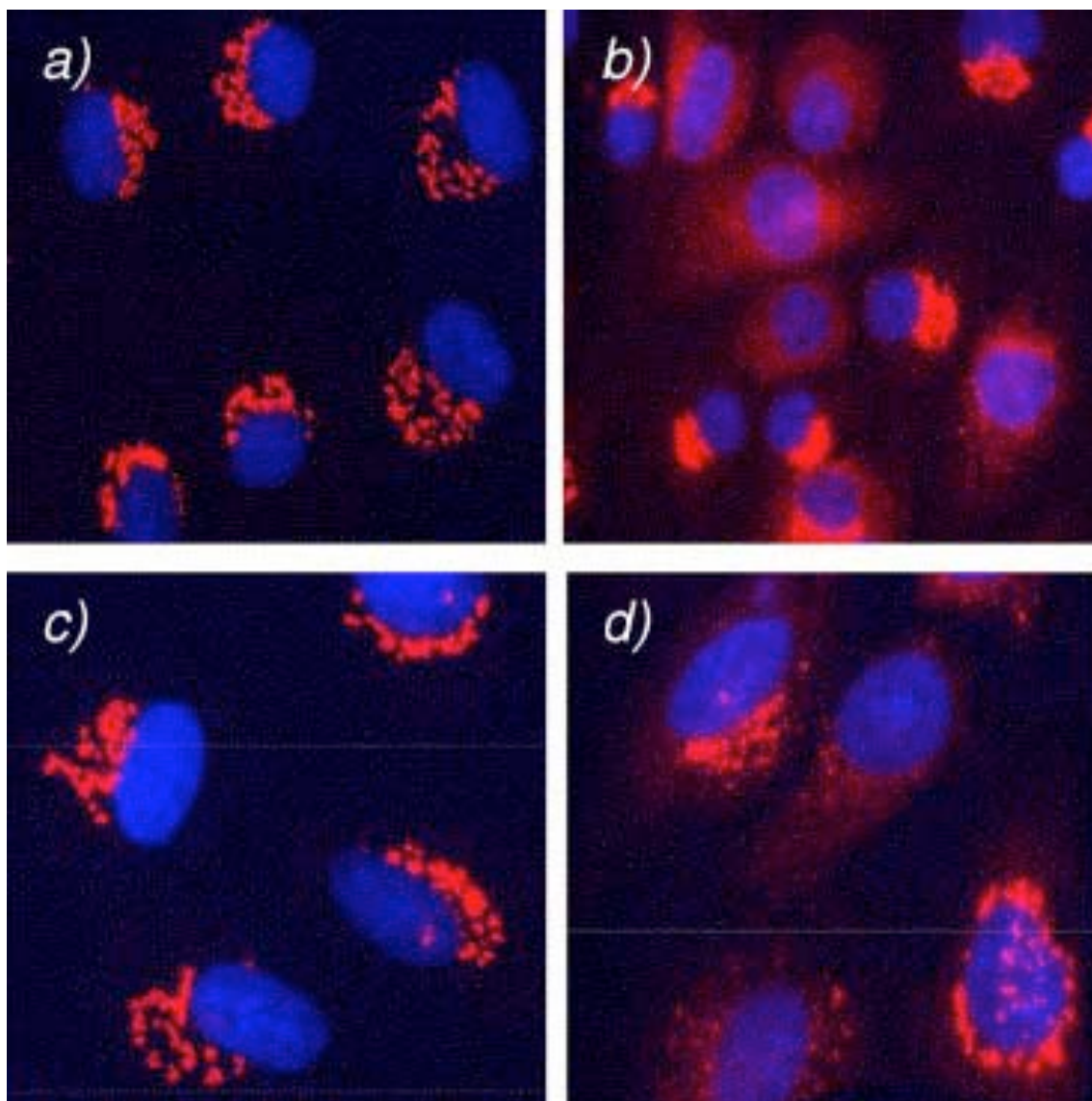


Figure 24. Norrisolide-aryl azide probe on NRK cells. Cells are incubated with control probe (a) (aryl azide-biotin) and with norrisolide-aryl azide-biotin (b) for 60 minutes, then fixed and processed for immunofluorescence. Cells treated with norrisolide-containing probe are washed and let recover for 60 min (c). Recovery after UV activation is shown in (d). Cells are then fixed and processed for imaging. Cells are stained with anti-MannII Ab to visualize the Golgi complex (red) and the nuclear dye Hoechst (blue).

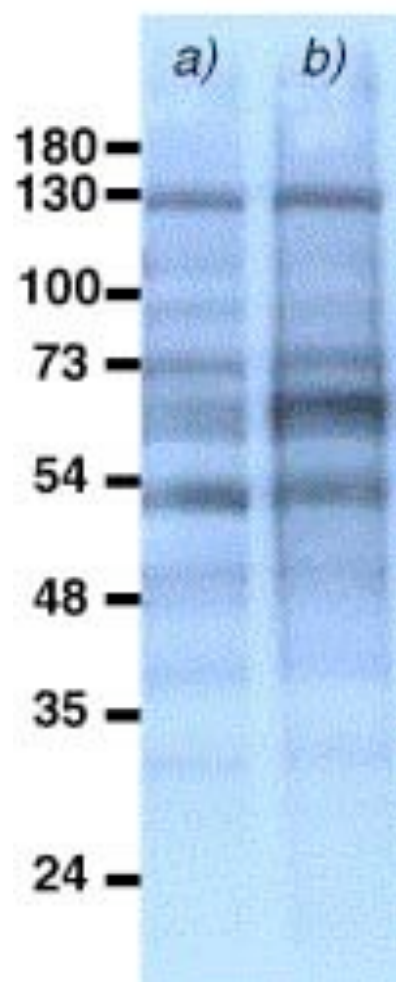


Figure 25. Western blot of norrisolide-aryl azide treated cells. Cells treated with norrisolide-containing probe 10 (a) and control probe 11 (b) and subjected to photoactivation. Total protein lysate is prepared and run on acrylamide gel. Biotin-containing proteins are visualized by Streptavidin–HRP.

Norrisolide-epoxide-biotin

In parallel with the above studies, we considered an alternative design of trifunctional probes in which the photoactivated aryl azide unit would be replaced by an epoxide functionality. This design was inspired by the observation of compound 7 in Figure 21 containing norrisolide core and a bisepoxide motif. This compound was able to induce an identical phenotype to that of norrisolide (Guizzunti et al., 2006). We hypothesized that a probe that combines the perhydroindane motif with an epoxide unit and biotin would ensure the irreversible Golgi vesiculation and allow the isolation of its target protein. This hypothesis led us to synthesize and evaluate probes 12 and 13 (Figure 26).

There are two ways to obtain a trifunctional probe containing norrisolide core, the epoxide and biotin, depending on the position in which the epoxide group is attached. Probe 12 (Figure 26) was synthesized to have the epoxide group on the C12 and biotin on C14 of the phenol group linked to norrisolide core. Probe 13 had the epoxide on C14 and biotin on C12 (Figure 26). Evaluation of probes 12 and 13 in NRK cells was performed using the conditions discussed above. Incubation of NRK cells with 12 induced less than 10% of Golgi fragmentation in the entire cell population (Figure 27, a). Moreover, this effect was fully reversible after cell washing (Figure 27, b). Similar results were obtained with compound 13. The Golgi fragmentation induced by probes 12 and 13 was clearly less effective than the one observed using norrisolide, which under identical conditions led to complete and irreversible vesiculation in more than 90% of the cell population.

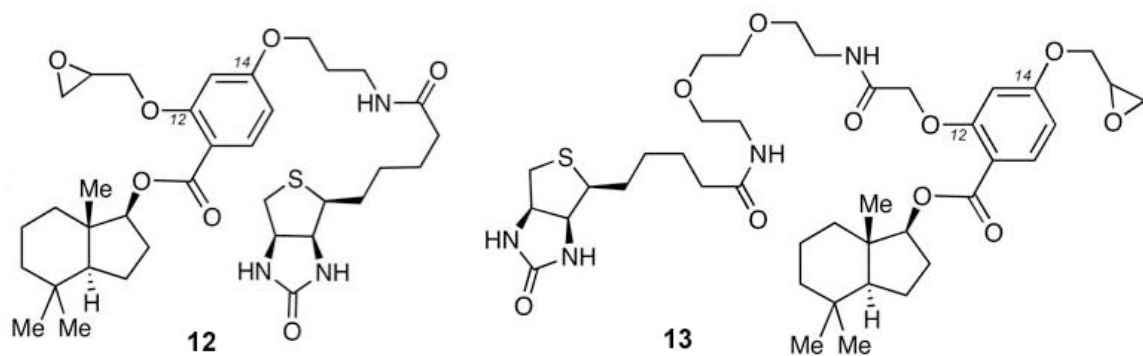


Figure 26. Norrisolide-epoxide-biotin trifunctional probes. Compound 12 carries the epoxide on C12 and biotin on C14 of the phenol group. Compound 13 has the epoxide on C14 and biotin on C12.

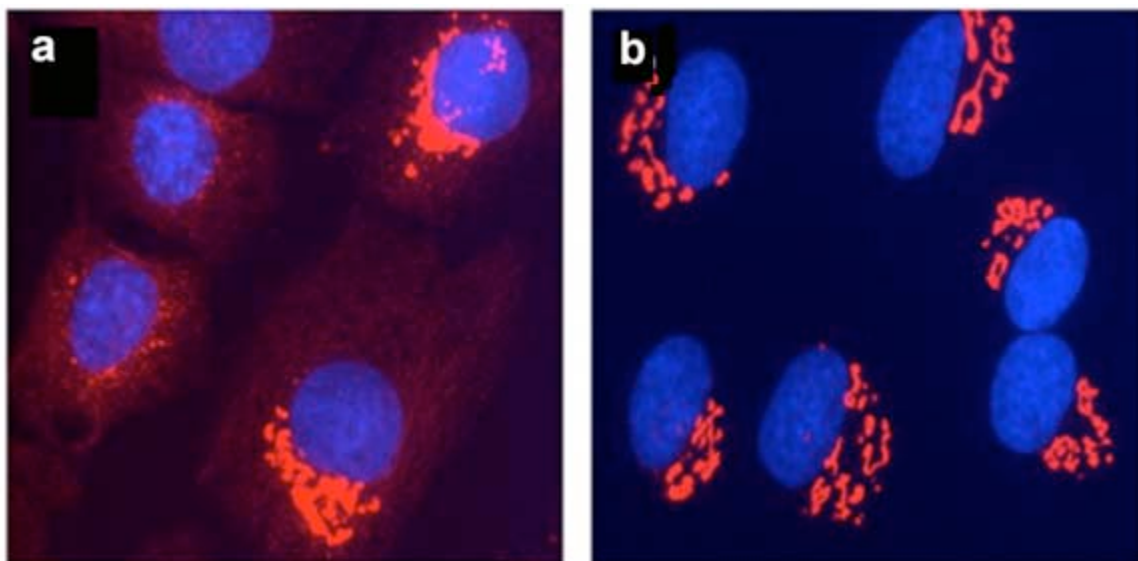


Figure 27. Norrisolide-epoxide-biotin on NRK cells. (a) NRK cells are treated with Norrisolide-epoxide-biotin (50uM) for 60 min. (b) cells are washed and let to recover for 60 min. After fixation, cells are stained with anti-MannII Ab to visualize Golgi structure (in red) and Hoechst to visualize nuclei (blue).

Norrisolide-bisepoxide-iodine

The trifunctional probes analyzed so far all contain norrisolide perhydroindane core and biotin as tag. They carry, however, different crosslinker: an aryl azide unit and an epoxide motif. These probe show little activity towards the Golgi apparatus. We hypothesized that the low extent of Golgi vesiculation induced by biotin-containing probes is due to the presence of biotin tag. It is possible that the steric hindrance caused by biotin in combination with its intrinsic affinity for biotin receptors leaves insufficient amounts of these probes on the Golgi membranes thus dramatically reducing their effect on the Golgi complex. In other words, the biotin tag could interfere with the ability of the perhydroindane motif to localize these probes on the Golgi. This analysis led us to reconsider our overall strategy and design an alternative probe in which the biotin unit would be replaced by a radioisotope. Isolation of the norrisolide target(s) could then be pursued via radiolabeling.

Compound 7 (Figure 21), containing norrisolide perhydroindane core and two epoxides mounted on a phenol group, demonstrated to be the probe with most similar phenotype to that of norrisolide natural product. The structure of compound 7 offers the unique advantage of facile incorporation of radioactive iodine at the aromatic ring at the C15 center. Compound 14 (Figure 28), containing non-radioactive iodine, was synthesized and tested on NRK cells.

Incubation of NRK cells with compound 14 induced Golgi vesiculation (Figure 29, c) at concentrations similar to those used for norrisolide and compound 7 (perhydroindane-bisepoxide) (Figure 29, b). Importantly, this fragmentation was found to occur in more than 80% of cells and was irreversible after standard washing (Figure 29, d). In fact, probe 14 induced comparable Golgi vesiculation to that obtained with analogue 6.

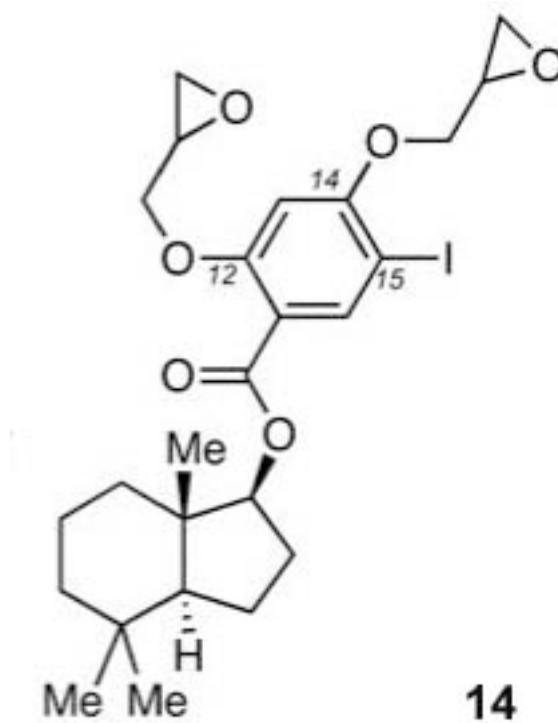


Figure 28. Norrisolide-bisepoxide-iodine trifunctional probe. This trifunctional probe was synthesized from probe 7 by adding iodine on the C15 of the phenol ring.

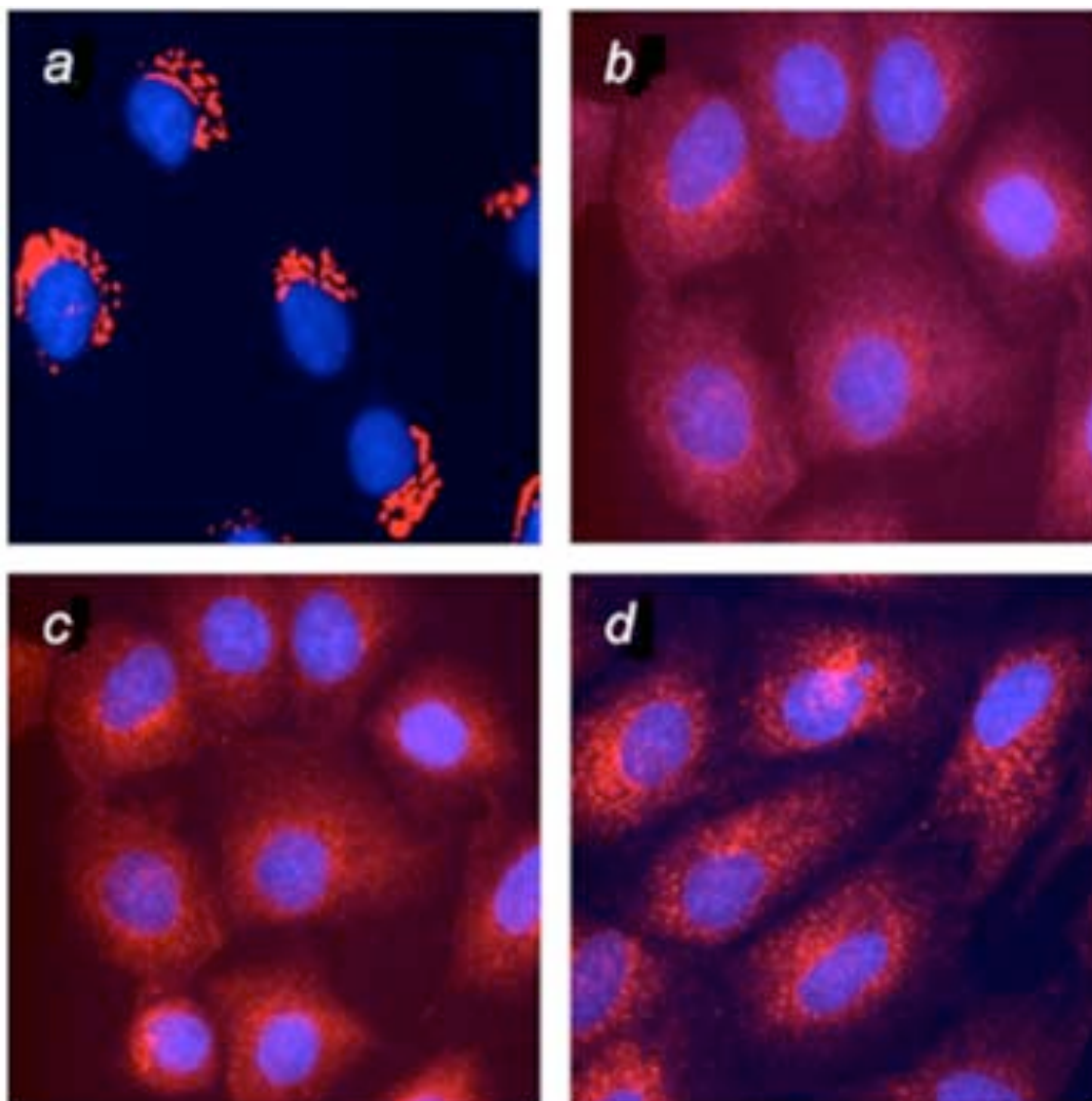


Figure 29. Norrisolide-bisepoxide-iodine on NRK cells. NRK cells are treated for 60 min with DMSO (a), norrisolide-bisepoxide (probe 7) (b) and norrisolide-bisepoxide-iodine (c). Cells incubated with the last compound are then washed and allowed to recover for 60 min. After fixation, NRK cells are labeled with anti-MannII Ab (Golgi, in red) and Hoechst (nuclei, in blue).

3.4 DISCUSSION

In conclusion, we present here the results of a study aiming to develop norrisolide-based trifunctional probes that could be used to identify the cellular target of this unusual natural product. Inspired by the finding that norrisolide and its analogue containing the perhydroindane core linked to a bisepoxide motif are able to induce an irreversible fragmentation of the Golgi apparatus, we attempted to conjugate this structure with biotin and isolate its target by affinity chromatography. None of the biotin-based probes induced significant Golgi fragmentation to justify further studies. The only proteins isolated with these compounds were non-specific biotinylated cellular proteins. However, when using iodine as tag, the trifunctional iodinated probe induced sufficient irreversible Golgi vesiculation at comparable concentrations to that of the natural product. Thus, this probe represents an appropriate reagent for further biological studies aiming to isolate the cellular target of norrisolide.

3.5 METHODS

Reagents and cells

NRK cells were grown on coverslips as described in reference 11. Stock solution (5 mg/ml) of norrisolide analogues were made in DMSO and stored at -20°C . The working concentration of the compounds was $30\text{ }\mu\text{M}$ for each coverslip and cells were incubated at 37°C for 60 min. An equal volume of DMSO was used as a negative control for each compound. For the crosslinking reaction, cells were irradiated for 15 min with UV light using a UVL-21 hand lamp ($1\text{mW}/\text{cm}^2$ at 366 nm) placed at 5 cm distance from the cells. To test the irreversibility of Golgi fragmentation, cells were washed 4 times with phosphate-buffered saline (PBS) (150 mM NaCl, 1.8 mM NaH_2PO_4 , 8.4 mM Na_2HPO_4) and then incubated in fresh complete medium at 37°C for 90 minutes. Immunofluorescence microscopy was performed as described in reference 11. Immunoblot: Cells treated with 14 and 15 for 60 min and irradiated with UV light were collected and lysated with Loading Buffer (60 mM Tris pH 6.8, 5% 2-Mercaptoethanol, 2% SDS, 0.01% Bromophenol Blue, 10% glycerol). Proteins in the lysate were separated by SDS-PAGE electrophoresis using a 10% running gel. Proteins were transferred on a nitrocellulose membrane (Western Blot) (60 min, 350 mA) that was then kept in blocking buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween20, 1% BSA) for 30 min. The membrane was incubated for 1 h at room temperature with Streptavidin-HRP (BD Pharmingen) diluted in blocking buffer. After washing 3 times with PBS, it was added the reagent for ECL (Perkin Elmer). Kodak Biomax films were used for exposure.

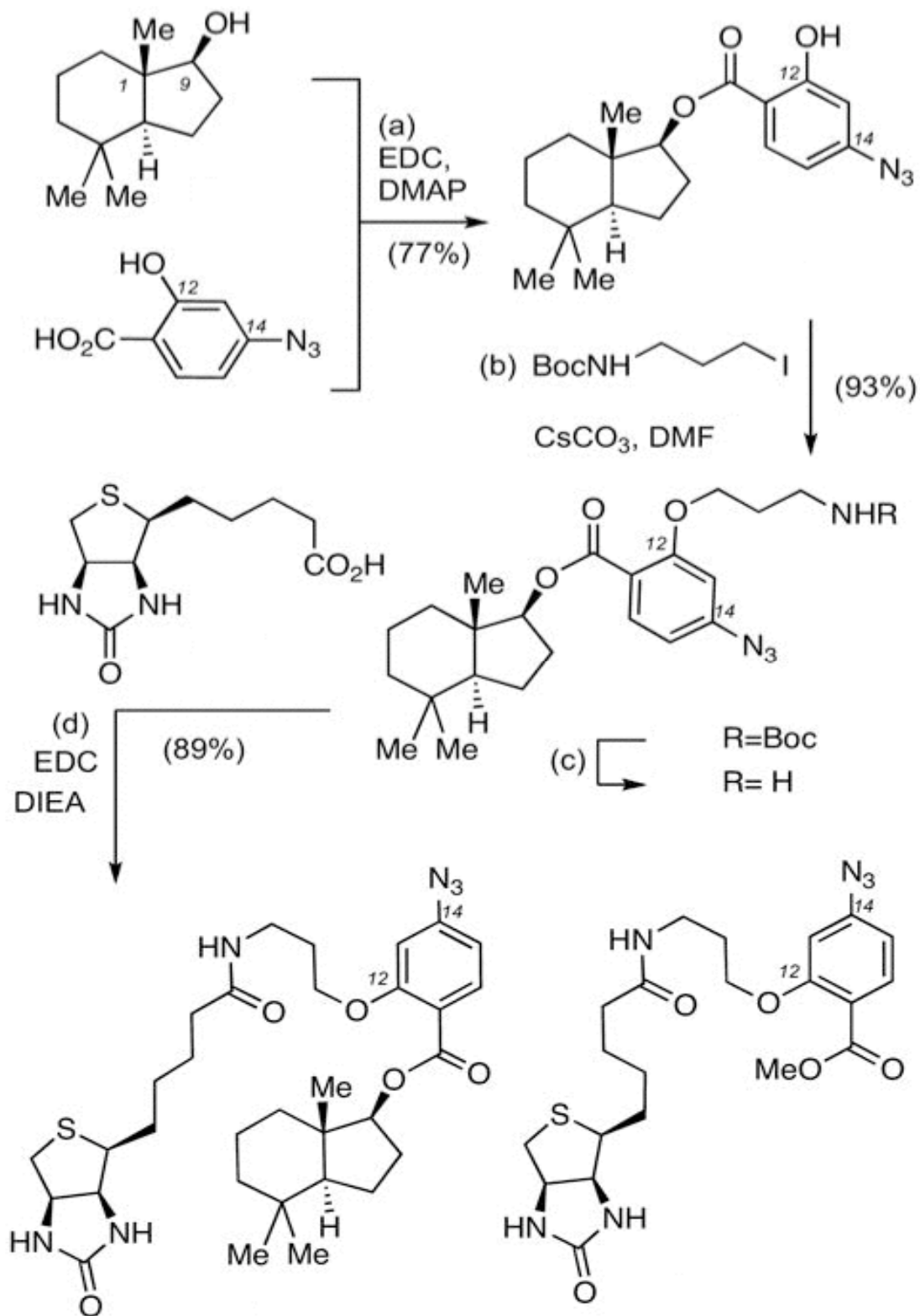


Figure 30. Synthesis of compound 10 and 11

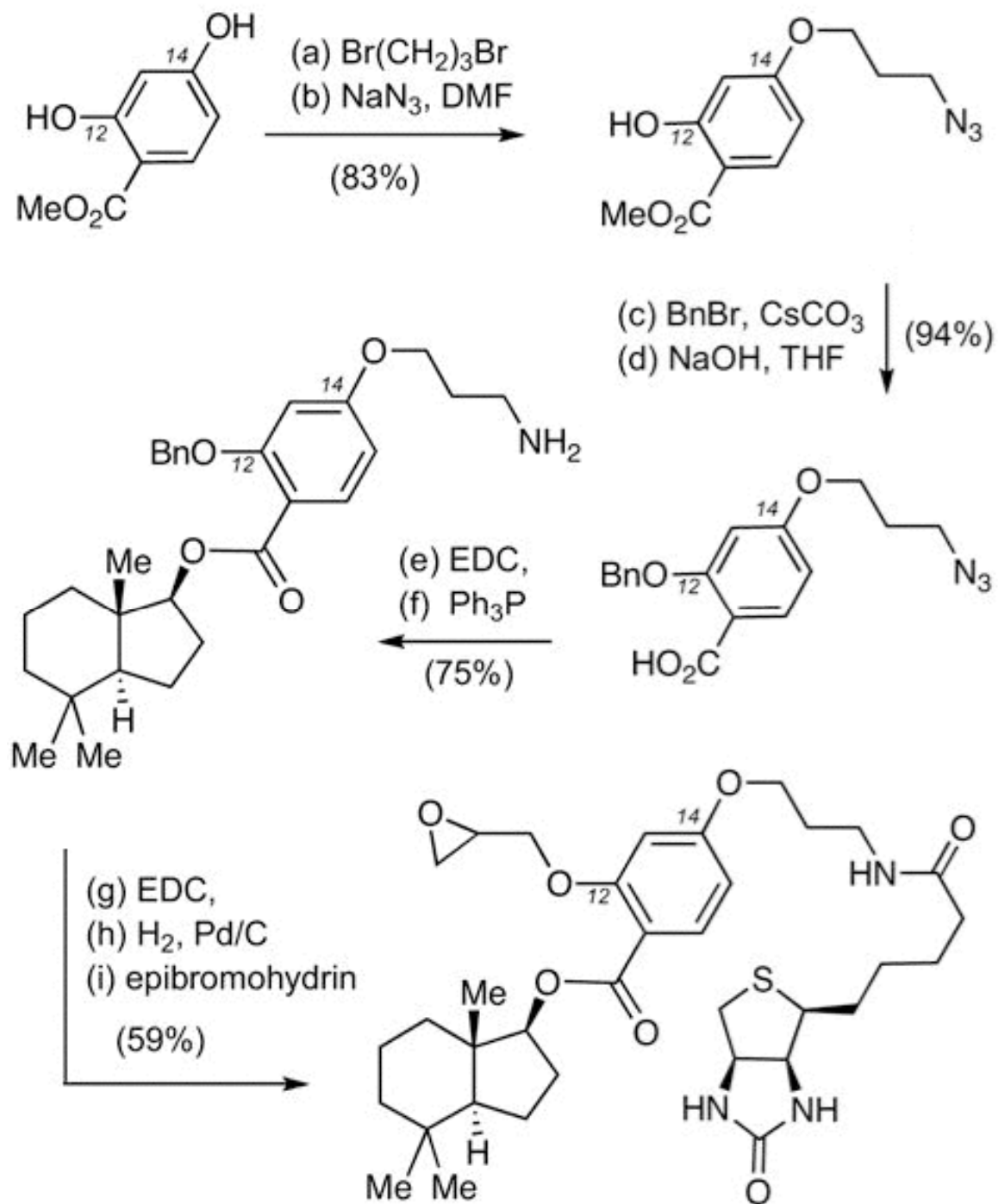


Figure 31. Synthesis of compound 12

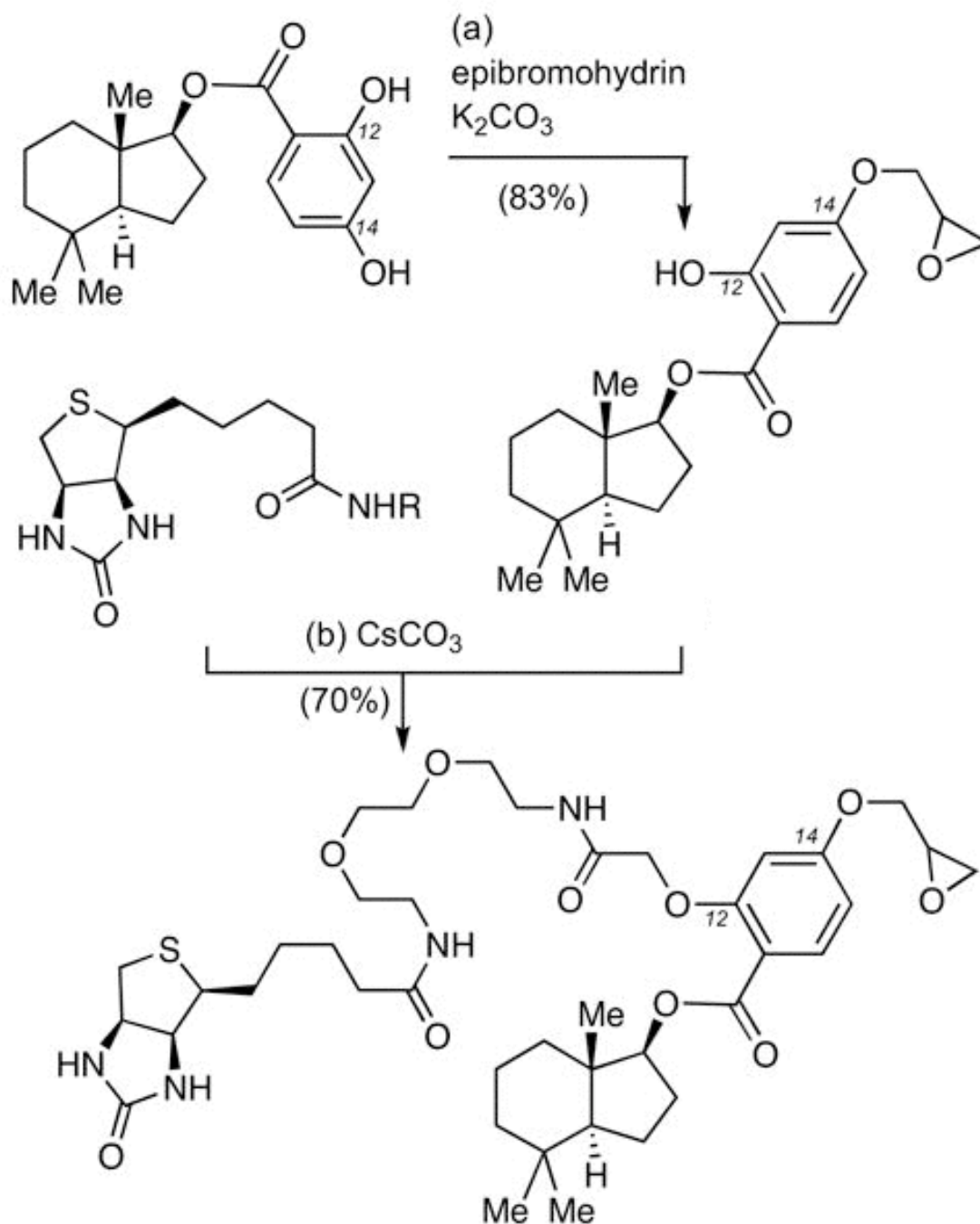


Figure 32. Synthesis of compound 13

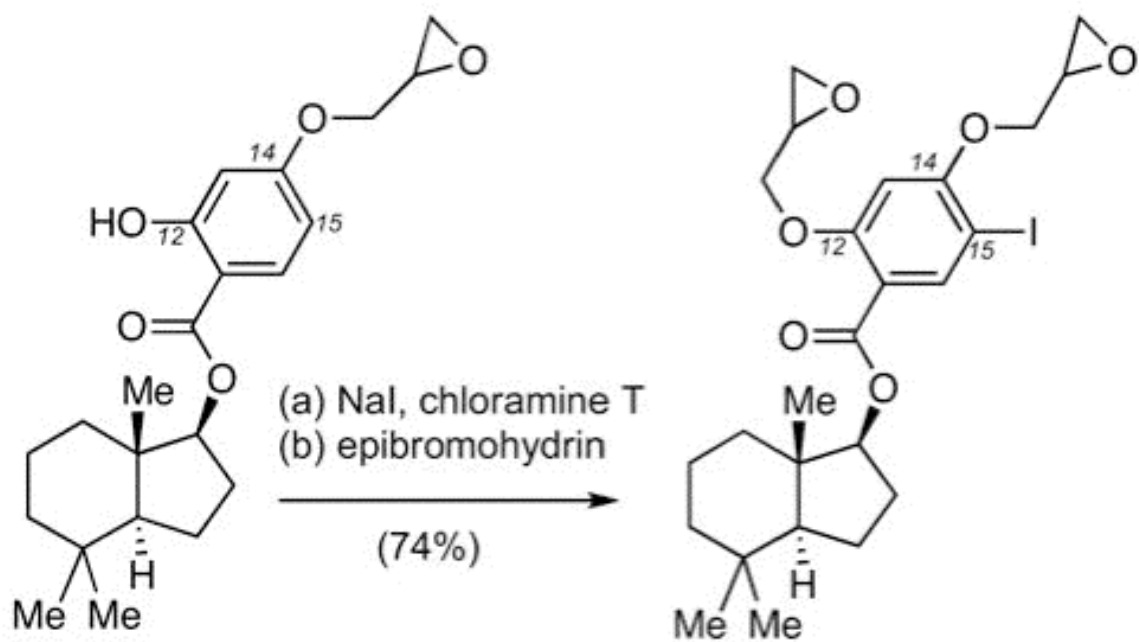


Figure 33. Synthesis of compound 14

CONCLUSION

A. Functional analysis of protein transport: TANGO genes

Yeast genetics and cell-free biochemical approach have been successful approaches used to identify many components of the secretory pathway. To overcome the limitations of these methods, Vivek Malhotra's group undertook a genome wide screen in *Drosophila* cells. Fly cells are a good alternative model system to study exocytosis since their secretory compartments have similar morphological and molecular characteristics to those of mammalian cells. The screen led to the identification of more than 100 previously uncharacterized genes (named TANGO, for Transport AND Golgi Organization).

We described how 20 randomly selected genes were cloned in a tagged version, and how the protein product was localized in *Drosophila* S2 cells. A criterion was applied to choose the best candidate gene for further investigation. This criterion left out genes otherwise potentially interesting, and many of these genes will require more attentive analysis.

Nuclear genes were discarded for their potential role in nuclear-related events, like DNA and RNA processing. However proteins involved in secretion have been reported to localize in the nucleus under particular conditions. For example, PKD2, a protein proven to be involved in TGN to cell surface transport (Bard and Malhotra, 2006), has been shown to contain a nuclear localization signal required to relocate PKD2 into the nucleus upon gastrin stimulation (Auer *et al.*, 2005). For this reason, Tango genes showing nuclear localization should be re-analyzed, taking into account that the tag used to visualize the gene product inside the cells may have affected the localization, for

example by covering nuclear export sequences. Particular attention is due to genes with effect on Golgi structure after RNAi, like Tango 4 (CG1796), a Class A gene (Golgi to ER relocation). Tango 4 has a potential homologue in yeast, the splicing factor gene Prp46. Contrary to other splicing factors, however, RNAi of Tango 4 resulted in a marked effect on Golgi membranes and its gene product localized to the cytosol and not the nucleus in fly cells, suggesting that Tango 4 regulates secretion independently from its potential role in RNA splicing.

Amongst other discarded Tango genes, 3 are of particular interest as they are conserved genes whose protein product localizes on the Golgi apparatus. Two genes seem to be involved at different levels of the glycosylation process that happens in the Golgi: Pgant6 appears to be a glycosyl transferase, and CG10007 could be a sugar transporter. In the Golgi, protein glycosylation is used to create complex carbohydrate structures, which are essential for the function of the protein. However, interfering with glycosylation in the Golgi does not stop the delivery of proteins containing incomplete carbohydrate chains (Goss *et al.*, 1995; Jacob, 1995). It will be interesting to address how the knockdown of these particular Tango genes would induce a block in protein transport. The third gene localized on the Golgi is CG31052, a putative Na^+/H^+ exchanger. It would be interesting to study what is the exact role that pH gradient in the Golgi plays in multiple aspects of Golgi functions, from the proper localization of enzymes, to their catalytic activity, to the modification and sorting of protein cargoes.

B. Tango 5

We have shown the initial characterization of Tango5, a potentially new component of the secretory pathway identified in our screen. Tango5 knockdown blocks protein transport and relocates Golgi enzymes into the ER in fly cells and unpublished evidence suggest that Tango5 role in secretion is conserved in *Dictyostelium* and *C. elegans* (Ricardo Escalante and John Audhya personal communication). The gene product appears to localize along the secretory pathway: in the ER in lower organisms (*Dictyostelium*, plants, insects) and in a pre-Golgi compartment (ERGIC) in mammalian cells.

What is the role of Tango5 in ER/ERGIC to Golgi transport? Tango5 could be a cargo receptor, required to transport proteins from ERGIC to the Golgi. Alternatively, Tango5 could be part of the machinery recruited on the cytosolic face of a budding vesicle. Another possibility is that Tango5 could have a role in maintaining the functionality of the ER/ERGIC compartment: it could be a cargo-modifying enzyme, or an ion transporter required to maintain the correct pH and osmolarity of the compartment).

To understand its role in secretion we are in the process of performing Tango5 knockdown. The analysis of cells depleted of Tango5 can provide important information on its function; for example, if Tango5 is part of the machinery required for the assembly of transport vesicles, its depletion can lead to the delocalization of these components from ER/ERGIC compartment to the cytosol. Another approach is based on the identification of Tango5 interactors by co-immunoprecipitation. Preliminary results show that when overexpressed Tango5 is extracted from HeLa cells, a few not yet characterized proteins can be co-precipitated with it. The sequence of these proteins can lead us to understanding the role of Tango5 in the secretory pathway.

C. Pharmacological approach: towards norrisolide target

Norrisolide induces complete Golgi fragmentation, which could help us to understand the process of membrane fission and how the structure of the Golgi is maintained. Amongst the few natural products inducing a similar phenotype, the sea sponge metabolite Ilimaquinone (IQ) led to the identification of PKD as a key player in Golgi to cell surface transport. The direct target of IQ has not yet been identified, in part because of the complexity of this compound. Norrisolide, on the other hand, is suitable for functional-chemistry analysis. The study of Golgi vesiculation induced by Norrisolide-derived compounds led us to the finding that the perhydroindane core of the compound is responsible to bind a receptor on Golgi membranes and to induce Golgi vesiculation. This effect can be made irreversible by coupling a crosslinking motif (epoxide) to norrisolide core. This probe can reproduce the same phenotype as the natural product, with the advantage to be suitable for chemical modifications like the attachment of a tag for target isolation. However, the synthesis of such trifunctional probe proved to be challenging. Different tags were analyzed, and eventually the fluorophore coumarin proved to be the ideal candidate. The ideal trifunctional probe, containing norrisolide core, crosslinker and fluorescent tag, is now in the process to be tested. As now, the probe demonstrated to be able to induce Golgi fragmentation (by norrisolide core) and to localize to the Golgi apparatus (visualized by the fluorophore). The fragmentation, however, was irreversible only in 10% of the cell population, indicating that the crosslinking activity (by the epoxide) was only partial. The first attempt to use this probe for target isolation has not been successful, but has shown to be promising.

The aim of this project was the identification of norrisolide target; after many years of work and extensive chemical characterization of norrisolide activity, we feel we

are now very close to the end of this venture (task, quest), and that this will only be the beginning of a new task: the molecular characterization of norrisolide-induced Golgi fragmentation.

REFERENCES

- Appenzeller-Herzog C., Hauri H.P. (2006). The ER-Golgi intermediate compartment (ERGIC): in search of its identity and function. *J Cell Sci.* Jun 1;119(Pt 11):2173-83.)
- Auer A., von Blume J., Sturany S., von Wichert G., Van Lint J., Vandenheede J., Adler G., Seufferlein T. (2005). Role of the regulatory domain of protein kinase D2 in phorbol ester binding, catalytic activity, and nucleocytoplasmic shuttling. *Mol Biol Cell.* Sep;16(9):4375-85.
- Balch W.E., Dunphy W.G., Braell W.A., Rothman J.E. (1984). Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine. *Cell.* Dec;39(2 Pt 1):405-16
- Bard F., Casano L., Mallabiabarrena A., Wallace E., Saito K., Kitayama H., Guizzunti G., Hu Y., Wendler F., Dasgupta R., Perrimon N., Malhotra V. (2006). Functional genomics reveals genes involved in protein secretion and Golgi organization. *Nature.* Feb 2;439(7076):604-7.
- Bard F., Malhotra V. (2006). The formation of TGN-to-plasma-membrane transport carriers. *Annu Rev Cell Dev Biol.*;22:439-55
- Baron C.L., Malhotra V.: (2002). Role of diacylglycerol in PKD recruitment to the TGN and protein transport to the plasma membrane. *Science.* Jan 11;295(5553):325-8
- Blunt J.W., Copp B.R., Munro M.H., Northcote P.T., Prinsep M.R. (2005). Marine natural products. *Nat Prod Rep.* 2005 Feb;22(1):15-61.
- Bobzin S.C., Faulkner D.J. (1991). Diterpenes from the Pohnpeian marine sponge *Chelonaplysilla* sp. *J Nat Prod.* Jan-Feb;54(1):225-32.
- Borisy G.G., Taylor E.W. (1967). The mechanism of action of colchicine.. Binding of colchicine-3H to cellular protein. *J Cell Biol.* Aug;34(2):525-33.
- Boutros M., Kiger A.A., Armknecht S., Kerr K., Hild M., Koch B., Haas S.A., Paro R., Perrimon N., Heidelberg Fly Array Consortium. (2004). Genome-wide RNAi analysis of growth and viability in *Drosophila* cells. *Science* 303, 832–835
- Brady T.P., Wallace E.K., Kim S.H., Guizzunti G., Malhotra V., Theodorakis E.A. (2004). Fragmentation of Golgi membranes by norrisolide and designed analogues. *Bioorg Med Chem Lett.* Oct 18;14(20):5035-9.
- Brady T.P., Kim S.H., Wen K., Kim C., Theodorakis E.A. (2005). Norrisolide: total synthesis and related studies. *Chemistry.* Dec 9;11(24):7175-90.
- Brady T.P., Kim S.H., Wen K., Theodorakis E.A. (2004). Stereoselective total synthesis of (+)-norrisolide. *Angew Chem Int Ed Engl.* Jan 30;43(6):739-42.

Brown R.M. Jr., Franke W.W., Kleinig H., Falk H., Sitte P. (1970). Scale formation in chrysophycean algae. I. Cellulosic and noncellulosic wall components made by the Golgi apparatus. *J Cell Biol.* 45(2):246-71

Casaubon R.L., Snapper M.L. (2001). S-adenosylmethionine reverses ilimaquinone's vesiculation of the Golgi apparatus: a fluorescence study on the cellular interactions of ilimaquinone. *Bioorg Med Chem Lett.* Jan 22;11(2):133-6

Chen G., Heim A., Riether D., Yee D., Milgrom Y., Gawinowicz M.A., Sames D. (2003). Reactivity of functional groups on the protein surface: development of epoxide probes for protein labeling. *J Am Chem Soc.* Jul 9;125(27):8130-3.

Chen Y., Ebright R.H. (1993). Phenyl-azide-mediated photocrosslinking analysis of Cro-DNA interaction.

Cheng S.H., Gregory R.J., Marshall J., Paul S., Souza D.W., White G.A., O'Riordan C.R., Smith A.E.: (1990). Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell.* Nov 16;63(4):827-34

Chua J.J., Ng M.M., Chow V.T. (2004) The non-structural 3 (NS3) protein of dengue virus type 2 interacts with human nuclear receptor binding protein and is associated with alterations in membrane structure. *Virus Res.* 102, 151–163

Clarke K.M., Mercer A.C., La Clair J.J., Burkart M.D. (2005). In vivo reporter labeling of proteins via metabolic delivery of coenzyme A analogues. *J Am Chem Soc.* Aug 17;127(32):11234-5.

Colanzi A., Suetterlin C., Malhotra V. (2003). Cell-cycle-specific Golgi fragmentation: how and why? *Curr Opin Cell Biol.* Aug;15(4):462-7. Review.

Connolly, C. N. , Futter, C. E. , Gibson, A. , Hopkins, C. R. & Cutler, D. F. (1994) Transport into and out of the Golgi complex studied by transfecting cells with cDNAs encoding horseradish peroxidase. *J. Cell Biol.* 127, 641–652

Cravatt B.F., Sorensen E.J. (2000). Chemical strategies for the global analysis of protein function. *Curr Opin Chem Biol.* Dec;4(6):663-8.

Dalton A.J. and Felix M.D. (1956). A comparative study of the Golgi complex. *J Biophys Biochem Cytol.* 79-84.

DasGupta, R. , Kaykas, A. , Moon, R. T. & Perrimon, N. (2005) Functional genomic analysis of the Wnt-wingless signalling pathway. *Science* 308, 826–833

De Langhe, S. , Haataja, L. , Senadheera, D. , Groffen, J. & Heisterkamp, N. (2002) Interaction of the small GTPase Rac3 with NRBP, a protein with a kinase-homology domain. *Int. J. Mol. Med.* 9, 451–459

Diaz Anel AM, Malhotra V. (2005). PKCeta is required for beta1gamma2/beta3gamma2- and PKD-mediated transport to the cell surface and the organization of the Golgi apparatus. *J Cell Biol.* 2005 Apr 11;169(1):83-91. Erratum in: *J Cell Biol.* May 9;169(3):539

Dilcher M., Veith B., Chidambaram S., Hartmann E., Schmitt H.D., Fischer von Mollard G. (2003) Use1p is a yeast SNARE protein required for retrograde traffic to the ER. *EMBO J.* 22, 3664–3674

Dinter A. and Berger E.G. (1998). Golgi disturbing agents. *Histochem. Cell Biol.* 109, pp. 371–390.

Duden R., Allan V., Kreis T. (1991) Involvement of -COP in membrane traffic through the Golgi complex. *Trends Cell Biol.* 1, 14–19

Duseti N.J., Jiang Y., Vaccaro M.I., Tomasini R., Azizi Samir A., Calvo E.L., Ropolo A., Fiedler F., Mallo G.V., Dagorn J.C., Iovanna J.L., (2002). Cloning and expression of the rat vacuole membrane protein 1 (VMP1), a new gene activated in pancreas with acute pancreatitis, which promotes vacuole formation. *Biochem Biophys Res Commun.* Jan 18;290(2):641-9.

Eggert U.S., Kiger A.A., Richter C., Perlman Z.E., Perrimon N., Mitchison T.J., Field C.M. (2004) Parallel chemical genetic and genome-wide RNAi screens identify cytokinesis inhibitors and targets. *PLoS Biol.* 2, e379

Fahy J.V. (2002). Goblet cell and mucin gene abnormalities in asthma. *Chest.* Dec;122(6 Suppl):320S-326S. Review

Gagliardi S., Rees M., Farina C. (1999). Chemistry and structure activity relationships of bafilomycin A1, a potent and selective inhibitor of the vacuolar H⁺-ATPase. *Curr Med Chem.* Dec;6(12):1197-212.

Goss P.E., Baker M.A., Carver J.P., Dennis J.W. (1995). Inhibitors of carbohydrate processing: A new class of anticancer agents. *Clin Cancer Res.* Sep;1(9):935-44.

Greenbaum D., Medzihradszky K.F., Burlingame A., Bogoy M. (2000). Epoxide electrophiles as activity-dependent cysteine protease profiling and discovery tools. *Chem Biol.* Aug;7(8):569-81.

Guizzunti G., Brady T.P., Malhotra V., Theodorakis E.A. (2006). Chemical analysis of norrisolide induced Golgi vesiculation. *J Am Chem Soc.* Apr 5;128(13):4190-1

Guizzunti G., Brady T.P., Malhotra V., Theodorakis E.A. (2007). Trifunctional norrisolide probes for the study of Golgi vesiculation. *Bioorg Med Chem Lett.* Jan 15;17(2):320-5.

Hochlowski J.E., Faulkner D.J., Matsumoto J. and Clardy J. (1983). Norrisolide, a novel diterpene from the dorid nudibranch *Chromodoris norrisi*. *J. Org. Chem.* 48, pp. 1141–1142.

J Mol Biol. Mar 20;230(2):453-60. Links

Jacob GS. (1995). Glycosylation inhibitors in biology and medicine. *Curr Opin Struct Biol.* Oct;5(5):605-11.

Jamieson JD, Palade GE. (1967). Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the peripheral elements of the Golgi complex. *J Cell Biol.* Aug;34(2):577-96.

Jamora C, Takizawa PA, Zaarour RF, Denesvre C, Faulkner DJ, Malhotra V. (1997). Regulation of Golgi structure through heterotrimeric G proteins. *Cell.* Nov 28;91(5):617-26

Jamora C, Yamanouye N, Van Lint J, Laudenslager J, Vandenheede JR, Faulkner DJ, Malhotra V. (1999). Gbetagamma-mediated regulation of Golgi organization is through the direct activation of protein kinase D. *Cell.* Jul 9;98(1):59-68

Joullie M.M., Leonard M.S., Portonovo P., Liang B., Ding X., La Clair J.J. (2003). Chemical defense in ascidians of the didemnidae family. *Bioconjug Chem.* Jan-Feb;14(1):30-7.

Kiger, A. A. et al. (2003) A functional genomic analysis of cell morphology using RNA interference. *J. Biol.* 2, 27

Liljedahl M, Maeda Y, Colanzi A, Ayala I, Van Lint J, Malhotra V. (2001). Protein kinase D regulates the fission of cell surface destined transport carriers from the trans-Golgi network. *Cell.* Feb 9;104(3):409-20

Lippincott-Schwartz J., Altan-Bonnet N., Patterson G.H. (2003). Photobleaching and photoactivation: following protein dynamics in living cells. *Nat Cell Biol.* Sep;Suppl:S7-14. Review

Lippincott-Schwartz J., Roberts T.H., Hirschberg K. (2000). Secretory protein trafficking and organelle dynamics in living cells.: *Annu Rev Cell Dev Biol.*;16:557-89.

Lippincott-Schwartz J., Smith C.L. (1997). Insights into secretory and endocytic membrane traffic using green fluorescent protein chimeras. *Curr Opin Neurobiol.* Oct;7(5):631-9. Review

Ludwig J.W., Richards M.L. (2006). Targeting the secretory pathway for anti-inflammatory drug development. *Curr Top Med Chem.*;6(2):165-78. Review

Luibrand, R.T., Erdman, T.R., Vollmer, J.J., Scheuer, P.J., Finer, J. and Clardy, J. (1979). Ilimaquinone, a sesquiterpenoid quinone from a marine sponge. *Tetrahedron* 35, pp. 609–612

Maeda Y, Beznoussenko GV, Van Lint J, Mironov AA, Malhotra V. (2001). Recruitment of protein kinase D to the trans-Golgi network via the first cysteine-rich domain. *EMBO J.* Nov 1;20(21):5982-90

Malhotra V., Serafini T., Orci L., Shepherd J.C., Rothman J.E. (1989). Purification of a novel class of coated vesicles mediating biosynthetic protein transport through the Golgi stack. *Cell*. Jul 28;58(2):329-36

Marmagne A., Salvi D., Rolland N., Ephritikhine G., Joyard J., Barbier-Brygoo H. (2006). Purification and fractionation of membranes for proteomic analyses. *Methods Mol Biol*. 323:403-20.

Meng L., Mohan R., Kwok B.H., Elofsson M., Sin N., Crews C.M. (1999). Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity. *Proc Natl Acad Sci U S A*. Aug 31;96(18):10403-8.

Mohla S., Humphries M.J., White S.L., Matsumoto K., Newton S.A., Sampson C.C., Bowen D., Olden K. (1989). Swainsonine: a new antineoplastic immunomodulator. *J Natl Med Assoc*. Oct;81(10):1049-56

Nachliel E., Finkelstein Y., Gutman M. (1996). The mechanism of monensin-mediated cation exchange based on real time measurements. *Biochim Biophys Acta*, 1285:131–145

Nichols B.J., and Pelham H.R. (1998) SNAREs and membrane fusion in the Golgi apparatus. *Biochim. Biophys. Acta* 1404, 9–31

Novick P, Field C, Schekman R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell*. Aug;21(1):205-15

Novick, P. & Schekman, R. (1979) Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA* 76, 1858–1862

Palade G. (1975). Intracellular aspects of the process of protein synthesis. *Science*. Aug 1;189(4200):347-58

Pecot M.Y., Malhotra V. (2004). Golgi membranes remain segregated from the endoplasmic reticulum during mitosis in mammalian cells. *Cell*. Jan 9;116(1):99-107.

Pelham H.R. (1991). Multiple targets for brefeldin A. *Cell* 67, pp. 449–451.

Pelham HR. (1996). The dynamic organisation of the secretory pathway. *Cell Struct Funct*. Oct;21(5):413-9.

Radeke H.S, Snapper M.L. (1998). Photoaffinity study of the cellular interactions of ilimaquinone. *Bioorg Med Chem*. Aug;6(8):1227-32.

Radeke H.S., Digits C.A., Bruner S.D., Snapper M.L. (1997). New Tools for Studying Vesicular-Mediated Protein Trafficking: Synthesis and Evaluation of Ilimaquinone Analogs in a Non-Radioisotope-Based Antisecretory Assay. *J Org Chem*. May 2;62(9):2823-2831

Radeke H.S., Digits C.A., Casaubon R.L., Snapper M.L. (1999). Interactions of (-)-ilimaquinone with methylation enzymes: implications for vesicular-mediated secretion. *Chem Biol. Sep*;6(9):639-47

Rothman, J. E. (1994) Mechanisms of intracellular protein transport. *Nature* 372, 55–63

Rudi A, Kashman Y. (1993). Three new cytotoxic metabolites from the marine sponge *Plakortis halichondrioides*. *J Nat Prod.* Oct;56(10):1827-30.

Sandvig K, van Deurs B.(2002). Membrane traffic exploited by protein toxins. *Annu Rev Cell Dev Biol.* 18:1-24.

Serafini T., Stenbeck G., Brecht A., Lottspeich F., Orci L., Rothman J.E., Wieland F.T. (1991) A coat subunit of Golgi-derived non-clathrin-coated vesicles with homology to the clathrin-coated vesicle coat protein -adaptin. *Nature* 349, 215–220

Sharp D.J. (2002) Cell division: MAST sails through mitosis. *Curr. Biol.* 12, R585–R587

Shorter J., and Warren G., (2002) Golgi architecture and inheritance. *Annu. Rev. Cell. Dev. Biol.* 18, 379–420

Sin N., Meng L., Wang M.Q., Wen J.J., Bornmann W.G., Crews C.M. (1997). The anti-angiogenic agent fumagillin covalently binds and inhibits the methionine aminopeptidase, MetAP-2. *Proc Natl Acad Sci U S A.* Jun 10;94(12):6099-103.

Snaith, H. A. , Armstrong, C. G. , Guo, Y. , Kaiser, K. & Cohen, P. T. (1996) Deficiency of protein phosphatase 2A uncouples the nuclear and centrosome cycles and prevents attachment of microtubules to the kinetochore in *Drosophila* microtubule star (mts) embryos. *J. Cell Sci.* 109, 3001–3012

Spooner R.A., Smith D.C., Easton A.J., Roberts L.M., Lord J.M.. (2006). Retrograde transport pathways utilised by viruses and protein toxins. *Virol J.* Apr 7;3:26

Stanley H., Botas J. and Malhotra V. (1997) The mechanism of Golgi segregation during mitosis is cell type-specific. *Proc. Natl Acad. Sci. USA* 94, 14467–14470

Suchy S.F., Olivos-Glander I.M., Nussbaum R.L. (1995). Lowe syndrome, a deficiency of phosphatidylinositol 4,5-bisphosphate 5-phosphatase in the Golgi apparatus. *Hum Mol Genet.* Dec;4(12):2245-50

Sutterlin C., Hsu P., Mallabiabarrena A., Malhotra V. (2002). Fragmentation and dispersal of the pericentriolar Golgi complex is required for entry into mitosis in mammalian cells. *Cell.* May 3;109(3):359-69.

Takizawa P.A., Yucel J.K., Veit B., Faulkner J.D., Deernick T., Soto G., Ellisman M. and Malhotra V. (1993). Complete vesiculation of Golgi membranes and inhibition of protein transport by a novel sea sponge metabolite, Ilimaquinone. *Cell* 73 pp. 1079–1090.

Turner J.R. and Tartakoff A.M. (1989). The response of the Golgi complex to microtubule alterations: the roles of metabolic energy and membrane traffic in Golgi complex organization. *J. Cell Biol.* 109, pp. 2081–2088.

Uhlmann, F. (2003) Separase regulation during mitosis. *Biochem. Soc. Symp.* 70, 243–251

Veit B., Yucel J.K. and Malhotra V. (1993). Microtubule independent vesiculation of Golgi membranes and the reassembly of vesicles into Golgi stacks. *J. Cell Biol.* 122, pp. 1197–1206.