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Preface

Endocytosis, signaling and cancer, much more than meets the eye

Any encyclopedia definition of “Endocytosis” will include statements such as “eukaryotic cells use endocytosis to internalize plasma membrane, surface receptors and their bound ligands, nutrients, bacterial toxins, immunoglobulins, viruses, and various extracellular soluble molecules” (Schwab, 2001). In other words, the canonical view of endocytosis has, for a long time, been that of a process designed to bring nutrients and/or other types of molecules inside the cell and, at the same time, to regulate the composition of the plasma membrane. Of course, endocytosis does all of this, and more... much more. The approaches that are being used to define molecular and biological details of all these “mores” constitute the *leitmotif* that unites the contributions included in this thematic issue of Molecular Oncology. Not surprisingly, as we uncover the complexity of the physiological processes that are inextricably linked to endocytosis and membrane traffic, we also discover more ways in which things can go wrong. Endocytosis-linked ill-health spans from infectious diseases to genetic diseases, from immune system diseases to neurodegenerative diseases. What we are mostly concerned with here is cancer. Hence, the title we have selected for this thematic issue “Endocytosis, signaling and cancer, much more than meets the eye”.

1. An unauthorized biography of endocytosis (from a signaling perspective)

Perhaps a bit of history will help to guide the non-expert reader to the point from which this issue of Molecular Oncology starts. We need to travel way back in history, all the way to pre-history. Many essential small molecules and nutrients, such as sugars, amino acids, and ions traverse the plasma membrane through the action of pumps or channels. For a while, early life forms coped pretty well using only these relatively simple devices. But, as prokaryotes evolved to eukaryotes, more complex “entry portals” started to appear. Why this happened is, of course, matter of speculation. As Christian De Duve suggested (De Duve, 1991), a critical transition from environments in which nutrients were present in a concentrated form to diluted environments (from the primordial

soup to the oceans) might have supplied the selective pressure necessary to confer a proliferative advantage to life forms capable of actively searching for, and concentrating nutrients. Regardless of how these transport systems came to be, eukaryotic cells now depend on many mechanisms that internalize macromolecules within membranous vesicles derived from plasma membrane invagination and pinching-off. The spectacular evolution of these systems has led to a vast complexity of entry routes that we are just beginning to comprehend: from phagocytosis, responsible for the intake of large particles, to the various forms of pinocytosis, responsible for the uptake of fluids or solutes. Readers who are interested in understanding the molecular complexity of these routes (and the present level of knowledge and/or uncertainty about their nature) will find numerous excellent recent reviews (Conner and Schmid, 2003; Jung and Haucke, 2007; Kaksonen et al., 2006; Kirkham and Parton, 2005; Mayor and Pagano, 2007; Mercer and Helenius, 2009; Parton and Simons, 2007; Pelkmans, 2005; Pryor and Luzio, 2009; Raiborg and Stenmark, 2009; Roth, 2006; Ungewickell and Hinrichsen, 2007; Zwang and Yarden, 2009).

Space constraints require us now to skip a couple of billion years of evolutionary history, and jump to the first international symposium of the Ciba Foundation in London in 1963 A.D., when the word “endocytosis” was first used. The concept, of course, had been around for a while, and the term endocytosis was coined to encapsulate the “encyclopedia” definition of this process, i.e. an efficient mechanism to internalize plasma membrane and nutrients.

From the signaling viewpoint, our outlook on endocytosis first started to change with the discovery of receptor-mediated endocytosis, a concept that was elaborated through the pioneering work of Brown and Goldstein in the 1970s (Anderson et al., 1977). The internalization of macromolecules through their binding to surface receptors is an obvious and rather efficient way of concentrating these molecules and of reducing the consumption of energy needed for their internalization. Many forms of endocytosis rely on receptor-mediated transport and it rapidly became clear that such transport mechanisms can be either constitutive or ligand-induced.

In constitutive endocytosis, membrane receptors are continuously internalized and then recycled back to the cell surface, after sorting in the endosomal compartment (Brodsky et al., 2001; Schmid, 1997). The ligand is simultaneously internalized and can be destined to different metabolic fates. Two paradigmatic examples are provided by endocytosis of the low-density lipoprotein (LDL) receptor and of the transferrin (Tf) receptor (Brodsky et al., 2001; Goldstein and Brown, 2009; Schmid, 1997). In the former case, LDL complexed to cholesterol is internalized with its receptor. In the endosomes, the LDL receptor dissociates from the LDL-cholesterol complex and is re-directed to the cell surface for more cycles of internalization. The LDL-cholesterol complex is routed to the lysosomes, where LDL is degraded and free cholesterol made available to the cell. The Tf receptor cycle is more complex. Tf, bound to iron, is also internalized together with its receptor. In the endosomal compartment, the acidic pH causes the dissociation of iron. Iron-free transferrin (apotransferrin) remains bound to the receptor, however, and is recycled to the plasma membrane. Such constitutive internalization is mostly used by cells for the uptake of nutrients.

In the ligand-regulated process, internalization is triggered by the interaction of a ligand with its surface receptor. Both ligands and receptors are normally routed to the lysosomal compartment with ensuing degradation. However, a fraction of the internalized receptor can be re-delivered to the plasma membrane, in a recycling process not dissimilar to that of constitutive endocytosis. Ligand-mediated endocytosis is typical of signaling receptors, such as receptor tyrosine kinases (RTKs) (Sorkin and Goh, 2009). In this case, the major role of endocytosis is to remove active, signaling receptors from the plasma membrane and to destine them for degradation. Thus, in this case, endocytosis serves to extinguish signals. It is now textbook knowledge that endocytic-mediated degradation is the major mechanism of long-term attenuation of signaling receptors.

Although these latter findings linked endocytosis to signaling, another question emerged. While in transit through the endosomal compartments, RTKs remain bound to their ligands for some time, and they are therefore active. Thus, the possibility existed that signaling might persist throughout the endosomal route. More interestingly, it could be postulated that signaling receptors in the endosomal compartment could potentially be exposed to substrates that were inaccessible from their plasma membrane location. It was reasoned, therefore, that endocytosis might represent a mechanism to sustain signaling and to achieve signal diversification and specificity. Initial results did not support this contention. Rather, through the use of endocytosis-defective receptors, which nevertheless retained full biological competence, the original idea that signaling occurred only from the cell surface received corroboration (Chen et al., 1989). Then, things changed.

2. Endocytosis and signaling: the coming of age

In 1996, Sandra Schmid and coworkers performed a critically important experiment (Vieira et al., 1996). They exploited a dominant negative mutant of Dynamin, an essential component of most (not all) endocytic pathways that is required for

the fission of vesicles from the plasma membrane. This mutant blocks internalization; therefore, in its presence, signaling receptors are ‘frozen’ on the plasma membrane and signal transduction can be studied in the absence of the endocytic component. Under these conditions, they found that the mitogenic effects of EGF were potentiated, a result that was in line with the predominant notion that signaling originated from the plasma membrane. However, Schmid and colleagues also observed that the endocytic blockade had distinct effects on individual signal transduction pathways. While some were augmented, others were substantially decreased (Vieira et al., 1996). This study established the concept that endocytosis is required for at least some forms of signaling.

In the decade that followed this seminal discovery, the field witnessed a tumultuous expansion in knowledge that has firmly established the fact that signaling and endocytosis programs are deeply written into each other. This bi-univocal correspondence is witnessed by i) the regulation of signaling pathways by endocytosis; ii) the reciprocal regulation of endocytosis by signaling; iii) a shareware situation in which several molecules have a dual role in endocytosis and signaling. An in-depth review of the field is impossible here, but the issue has been extensively studied, and the sequential reading of various reviews produced over the years provides an interesting account of how rapidly our thinking on the relationships between endocytosis and signaling have evolved over a little more than a decade (Benmerah, 2004; Ceresa and Schmid, 2000; Coumleau and Gonzalez-Gaitan, 2008; Di Fiore and De Camilli, 2001; Di Fiore and Gill, 1999; Fischer et al., 2006; Kholodenko, 2003; Kramer, 2000; Le Borgne et al., 2005; Le Borgne and Schweisguth, 2003; Le Roy and Wrana, 2005a,b; Liberali et al., 2008; Miaczynska et al., 2004; Mills, 2007; Mukhopadhyay and Riezman, 2007; Polo and Di Fiore, 2006; Sorkin and Von Zastrow, 2002; von Zastrow, 2001; von Zastrow and Sorkin, 2007; Zwang and Yarden, 2009).

A brief review of the emerging concepts may nevertheless be useful:

1. Endocytosis regulates the accessibility of receptors and ligands. Endocytosis regulates signaling, both in a negative and in a positive way, depending on the receptor system, by modulating the presence of receptors, of their ligands or the accessibility of downstream effectors to the receptor at the plasma membrane (Polo and Di Fiore, 2006; von Zastrow and Sorkin, 2007).
2. The integration of different endocytic routes determines the net signaling output. At least in the case of certain receptors, such as the EGFR and the TGF β -R, there is evidence that clathrin- and non-clathrin-mediated internalization pathways preferentially destine receptors to different fates: recycling to the plasma membrane or degradation, respectively (Di Guglielmo et al., 2003; Sigismund et al., 2008). In turn, and depending on the proportion of receptors internalized through either pathway, this contributes to determining the signaling output (Di Guglielmo et al., 2003; Sigismund et al., 2008). In other systems, the opposite seems true, as signaling is sustained by non-clathrin routes, while degradation occurs through clathrin-mediated endocytosis (Mayor and Pagano, 2007; Parton and Simons, 2007). While these findings highlight

the complexity of the strategies enacted by cells, they all concur to re-enforce the general principle that the integration of signals originating from different entry routes holds the key to the final net biological output, especially in terms of potentiation vs. extinction of signaling.

3. Endocytosis regulates the assembly of signaling complexes. Both at the plasma membrane and on endomembranes, endocytosis orchestrates the construction of signaling platforms. At the plasma membrane, the best example is represented by caveolae, which mediate a form of non-clathrin-dependent endocytosis, but which also represent the site of assembly of macromolecular complexes (Mayor and Pagano, 2007; Parton and Simons, 2007). Intracellularly, the signaling competence of endocytic organelles is best exemplified by the concept of the “signaling endosome” (see below). Several other endomembranes also serve as scaffolding signaling platforms, as analyzed in detail in the contribution by Fehrenbacher et al. (2009).
4. Endocytosis confers spatial and temporal dimensions to signaling. The most relevant aspect of this issue can be summarized as follows “free diffusion will not get you very far”. In the cell, signals originating from the plasma membrane must travel considerable distances, for instance to reach the nucleus. In some specialized cells, such as neurons (in the case of retrograde signaling from nerve terminals) the distance scale can be of the order of several centimeters. Diffusion simply won’t cut it! Conversely, signaling molecules traveling on fast communication routes, such as endosomes, which move along microtubules, will get the job done. And the situation might be even more complex than this, as illustrated by Birtwistle and Kholodenko. But what happens in your average cell, where distance may be less of an issue? In this case too, as the same authors explain (Birtwistle and Kholodenko, 2009), free diffusion of signaling effectors in a membrane-unconstrained environment would lead to the creation of precipitous, biologically ineffective gradients. Both theoretical (Kholodenko, 2002, 2003, 2006) and experimental work [reviewed in (Miaczynska et al., 2004; Mills, 2007; Polo and Di Fiore, 2006; von Zastrow and Sorkin, 2007)] converge on the idea that trafficking of signaling molecules in association with endocytic vesicles contributes to signal propagation from the cell surface to distant targets. Theoretical modeling generates testable hypotheses and also helps to explain how endocytosis could control cell polarity, spatial signal propagation, signal magnitude, kinetics and dynamic synchronization with stimulus reception (Birtwistle and Kholodenko, 2009).

There is an interesting twist to this concept, i.e. that diffusion (lateral diffusion in this case) might not get effector molecules far enough and fast enough to a desired location, even when they move on the plasma membrane. Frequently, the activation of signaling receptors at the cell surface leads to the execution of polarized functions, such as cell migration. This is due to the transient polarization of the molecular machinery that has to accumulate in certain areas of the plasma membrane, where a spatially-restricted function (for instance the protrusion of a lamellipodium) must take place. Cells have had to evolve

mechanisms to move components swiftly and precisely to certain locations and they have resorted, to a considerable extent, to employing endocytosis for this purpose. As Disanza et al. portray in this issue (Disanza et al., 2009), perhaps the fastest (and most specific) way of achieving this is by internalizing the necessary components, and then recycling them, through specialized endosomes, to specific areas of the cell surface.

5. Endosomes are critical signaling stations. As already illustrated above, endosomes play a pivotal role in many aspects of the endocytic control of signaling. The pleiotropic role of endosomes in signaling is embodied in the concept of the “signaling endosome”: these vesicles are primary signaling stations with a dual role: i) they can sustain and prolong signaling that originated at the plasma membrane [with numerous kinetic implications, see (Birtwistle and Kholodenko, 2009)], ii) they serve as the origin of unique signals that cannot be generated at the plasma membrane, thus contributing to signal diversification and specificity.

How can something so small achieve so much? “Small” is part of the answer. The small volume of endosomes favors receptor:ligand association and sustains receptor activity. Endosomes also have limited surfaces, and endosome-bound molecules simply cannot diffuse away laterally, because they have nowhere to go. This creates ideal conditions for the operation of “coincidence detectors”, i.e. molecular functions needing two or more simultaneous, relatively weak interactions to exert their function (Carlton and Cullen, 2005; Krauss and Haucke, 2007a,b; Pawson, 2007). In addition, endosomes are enriched for particular lipids (such as phosphatidylinositol-3-phosphate) that function as interaction surfaces for proteins harboring lipid-binding domains (Birkeland and Stenmark, 2004; Carlton and Cullen, 2005; Krauss and Haucke, 2007a, b), as well as for specific resident scaffold proteins (such as the MP1/p14 complex) (Teis et al., 2002). All these factors allow endosomes to function as unique assembly platforms for signaling complexes. Additionally, endosomes can travel rapidly, through microtubular-mediated transport, as already discussed.

3. Do “canonical” and “non-canonical” functions of endocytosis really exist?

So, much has happened between 1996 and today that has served to reveal a complex pattern of connections between endocytosis and signaling. Today, we view these two programs as two faces of the same coin, inseparably connected to each other. And yet, what we have discussed so far – unexpected though it might have been only a decade ago – still somehow falls within a canonical view of endocytosis. In other words, endocytic routes contribute to signaling by doing what they are supposed to do, i.e. by internalizing molecules and destining them to a variety of fates.

Needless to say, endocytosis holds many more surprises in store, as demonstrated by a series of connections that are emerging between endocytic proteins and complex signaling

programs, such as transcription, cell cycle regulation, mitosis, apoptosis, and cell fate determination. I have reviewed these connections recently, and therefore refer the reader to that review (Lanzetti and Di Fiore, 2008). I also do not want to spoil the enjoyment of reading through this issue, since many of the reviews address several of these aspects in scholarly detail. Pyrzynska et al. (2009) cover the role of endocytic proteins in the regulation of nuclear signaling and transcription; Vaccari and Bilder (2009) explore the connections between endocytosis and cell polarity, proliferation and apoptosis; Fürthauer and González-Gaitán (2009) describe the role of endocytosis in asymmetric cell division and cell fate determination.

Many of these connections (albeit not all) cannot intuitively be rationalized within a canonical view of endocytosis. They appear, therefore, to identify “non-canonical” functions of the process. Regardless of nomenclature, the real question to resolve is the relationship between the molecular machinery of endocytosis and that of apparently very distant cellular processes, such as transcription, cell cycle control, apoptosis, or regulation of progression through mitosis. One possibility is that endocytic proteins participate in these events as “freelancers”, so to speak: their functions in these processes would be unrelated to the roles they play in endocytosis. There is, however, an alternative, and much more appealing hypothesis, i.e. that endocytosis is integrated with, and necessary for, the execution of a number of cellular programs. Under this scenario, the elucidation of the molecular connections involved would constitute a major advance in our understanding of the blueprint of cell regulation.

One example serves to illustrate this concept: the role of endocytic proteins in mitosis. There is increasing evidence that connects endocytic proteins – such as clathrin, dynamin, ARH, and Rab6A – both physically and functionally to the centrosome and to the spindle at mitosis (Lehtonen et al., 2008; Miserey-Lenkei et al., 2006; Royle et al., 2005; Thompson et al., 2004, 2002). The question is whether the function of endocytic/trafficking proteins at mitosis is distinct from their role in membrane trafficking during interphase (the “freelancer” hypothesis). The localization of endocytic proteins at mitotically-relevant structures involves binding partners that are distinct from those involved in trafficking pathways, a fact that favors this view (Royle et al., 2005; Thompson et al., 2004). But above all, endocytosis, and in particular clathrin-mediated endocytosis, has long been believed to cease at mitosis. In addition, some endocytic proteins are phosphorylated at mitosis, and this modification has been reported to disrupt critical endocytic interactions [reviewed in (Mills, 2007)]. Under this scenario, endocytic proteins – relieved of their endocytic obligations – might be free to serve alternative roles.

However, a recent study could change our perception of this issue. Boucrot and Kirchhausen (2007) showed that clathrin-mediated endocytosis is active throughout mitosis, while the recycling pathway slows down from prophase to anaphase. This causes a net decrease of the cell surface area with ensuing cell detachment and round-up. Later, at telophase, the recycling pathway recovers and allows spreading of newly-formed daughter cells. Changes in cell shape and size at mitosis are thought to be critical components of the mitotic program, as they might ensure the correct formation

of spatial gradients for signaling proteins, or for the local dynamics of microtubules required for mitotic spindle morphogenesis (Bastiaens et al., 2006; Meyers et al., 2006). In addition cell round-up could be important for the appropriate distribution of cell constituents to the daughter cells. If endocytosis and recycling are critical for the proper execution of mitosis, then the described mitotic functions of endocytic proteins might be less of a freelance job than first appears. In a simple scenario, a number of critical effectors may have evolved to serve a dual role in endocytosis and mitosis, in order to ensure proper coordination between these processes. From this point of view, the endocytic machinery cannot truly be thought of as providing a single service, rather it is a multifunctional machine that is seamlessly integrated with other cell functions

4. Endocytosis and cancer, at last

With this background in mind, one question of great interest, which is the topic around which all the reviews in this issue revolve, is whether endocytosis plays a role in cancer. The idea is self-evident: if the endocytic program is so deeply written into various signaling programs, then its subversion should have an impact on pathological phenotypes in which aberrant signaling is central. Cancer epitomizes such a pathological phenotype. Once again, the field has been reviewed recently, and the reader is referred to those reviews for a detailed account (Bache et al., 2004; Coumailleau and González-Gaitán, 2008; Giebel and Wodarz, 2006; Grandal and Madshus, 2008; Haglund et al., 2007; Lanzetti and Di Fiore, 2008; Mosesson et al., 2008; Polo et al., 2004).

Several connections have been made between endocytosis and cancer, only a few of which are outlined below:

1. Endocytosis is an attenuator of signaling, and therefore a potential candidate as a tumor suppressor pathway/system (Polo and Di Fiore, 2006; von Zastrow and Sorkin, 2007).
2. Endocytosis sustains signaling in various manners (signaling endosomes, recycling as a tool to prevent degradation), thus it is a potential oncogenic pathway [(Miaczynska et al., 2004; Mills, 2007; Polo and Di Fiore, 2006; von Zastrow and Sorkin, 2007); and in this issue (Birtwistle and Kholodenko, 2009; Disanza et al., 2009; Fehrenbacher et al., 2009)].
3. Endocytosis is involved in pathways leading to the activation of certain receptors whose relevance to cancer is well-established, for example, Notch [(Nichols et al., 2007); and in this issue (Fürthauer and González-Gaitán, 2009; Vaccari and Bilder, 2009)]. This again indicates a role in a potential oncogenic pathway.
4. Endocytosis is a major regulator of cell fate determination, and is therefore predicted to play a role in the maintenance of the stem cell compartment: another issue of significant relevance to cancer [(Coumailleau and González-Gaitán, 2008); and in this issue (Fürthauer and González-Gaitán, 2009)].
5. Endocytosis or, at least, endocytic proteins are involved in the regulation of the cell cycle, mitosis, and possibly apoptosis: again alterations in any of these processes are

known to play a major role in cancer (Boucrot and Kirchhausen, 2007; Colaluca et al., 2008; Lehtonen et al., 2008; Miserey-Lenkei et al., 2006; Royle et al., 2005; Thompson et al., 2004, 2002).

6. Endocytosis is involved in the spatial restriction of signals needed for directed cell movement, and for the switch between motility strategies (amoeboid vs. mesenchymal) adopted by metastatic cells. This allows us to predict a role for endocytosis in tumor progression [(Balasubramanian et al., 2007; Caswell and Norman, 2008, 2006; Jekely et al., 2005; Jones et al., 2006; McDonald et al., 2006, 2003; Montell, 2003; Palamidessi et al., 2008; Radhakrishna et al., 1999; Riley et al., 2003; Schlunck et al., 2004; Wang et al., 2006); and in this issue (Disanza et al., 2009)].
7. Autophagy, a degradative pathway that involves the delivery of cytoplasmic cargo to the lysosome, is linked to tumor suppressor and tumor promotion [(Eisenberg-Lerner and Kimchi, 2009; Levine and Kroemer, 2008; Mizushima et al., 2008); and in this issue (Brecht et al., 2009)].
8. There is ample experimental evidence to support the notion that alterations of processes involving endocytic proteins can actually cause transformation in several model systems [reviewed in (Brumby and Richardson, 2005; Coumailleau and González-Gaitán, 2008; Crosetto et al., 2005; Giebel and Wodarz, 2006; Vidal and Cagan, 2006)]. In particular, genetic studies in flies have recently provided new research impetus in this area, with the identification of *Drosophila* tumor suppressor genes [in this issue (Vaccari and Bilder, 2009)].
9. Genetic and/or regulatory alterations of several endocytic/trafficking proteins have been reported in naturally occurring human tumors [reviewed in (Lanzetti and Di Fiore, 2008); and in this issue (Pyrzynka et al., 2009)]. In addition, scenarios have been depicted under which the alteration of endocytic proteins in cancer might have an even more sizable impact in cancer than would appear from the simple catalogue of reported alterations (Lanzetti and Di Fiore, 2008).

In conclusion, it seems that endocytosis is a basic constituent of many processes of the cellular master plan, and the ramifications of this process go far beyond what the most visionary thinker might have dreamt of at the London Ciba Foundation Meeting of 1963. Understanding the functions of endocytosis will probably be an indispensable step in any attempt to reverse-engineer the cellular blueprint. This knowledge will not only considerably advance our understanding of the pathogenetic mechanisms cancer, but will also help to identify novel targets for molecular therapies and markers for patient stratification, and to optimize current medical therapies.

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Pier Paolo Di Fiore

IFOM, Fondazione Istituto FIRC di Oncologia Molecolare at IFOM-IEO Campus, Via Adamello 16, 20139, Milan, Italy

Dipartimento di Medicina, Chirurgia ed Odontoiatria, Università degli Studi di Milano, Via di Rudini 8, 20122 Milan, Italy
Tel.: +39 02 574303257; fax: +39 02 574303231.
E-mail address: pierpaolo.difiore@ifom-ieo-campus.it

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