the Pontifical Academy of Sciences

Wnt Signaling as a regulator of cellular endocytosis and protein stability

Edward M. De Robertis, Nydia Tejeda-Muñoz and Lauren Albrecht[*]

Introduction

Protein degradation plays a key role in cellular homeostasis. The topic of how cellular components are turned over has been one of great interest to members of the Pontifical Academy of Sciences. Academician Christian De Duve discovered that membrane proteins and external nutrients acquired through endocytosis are digested in acidic vesicular organelles called lysosomes (De Duve and Wattiaux, 1966). Cytosolic proteins are mostly degraded in proteasomes, which consist of cytosolic cysteine proteases. Proteins are targeted to proteasomes after being modified by a small 76 amino acid protein called ubiquitin (Ciechanover, 2005). When a protein is marked by a chain of ubiquitin molecules, called Lysine-48-linked polyubiquitin, it was thought to be invariably degraded in proteasomes. Ubiquitin is also involved in the targeting of membrane proteins for trafficking into lysosomes either by addition of a monoubiquitin or of Lys63-linked polyubiquitin. I had the privilege of discussing with De Duve and Ciechanover here at the Casina Pio IV the relationship between the lysosomal and proteasomal pathways, and both Nobel laureates thought there was none.

In this paper we present work from our laboratory showing that cytosolic proteins phosphorylated by Glycogen Synthase Kinase 3 (GSK3) can be degraded inside lysosomes by the process known as microautophagy, and that the transition from proteasomal to lysosomal degradation is regulated by an extracellular growth factor called Wnt. Wnt causes a great increase in non receptor-mediated endocytosis and this requires the prior activity of Protein Arginine methyltransferase (PRMT1). These studies lead to the conclusion that the proteasomal and lysosomal degradation pathways are not independent of each other as previously thought, and that the switch between them is physiologically regulated by the Wnt signaling factor.

1. Wnt signaling causes the sequestration of GSK3 inside multivesicular bodies

1.1 Wnt signaling in development and disease

The canonical Wnt/GSK3 signaling pathway was discovered by Roel Nusse and is now known to be a key regulator of tissue regeneration, stem cells, and cancer (Logan and Nusse, 2004; Nusse and Clevers, 2017). Multiple loss-of-function mutations in this pathway, in genes such as Axin, APC and #-Catenin cause stabilization of #-Catenin, increasing cell proliferation and leading to cancer.

1.2 Wnt decreases GSK3 activity

The Wnt growth factor binds to the cell surface co-receptors LRP6 (LDL-receptor related protein 6) and Frizzled. Activated receptors are phosphorylated by GSK3 and other kinases and recruit a cytosolic #-Catenin destruction complex consisting of Axin, Adenopolyposis Coli (APC), Dishevelled (Dvl), GSK3 and Casein Kinase 1 (CK1). In the absence of Wnt, the amino terminal region of #-Catenin is phosphorylated first by CK1 and then by three phosphorylations by GSK3. This generates what is called a phosphodegron. When regions of proteins are modified by multiple phosphates in Serines or Threonines in close vicinity, these phosphodegrons are recognized by ubiquitin ligases that catalyze the polyubiquitination of these proteins targeting them for degradation in proteasomes (Ciechanover, 2005). The phosphorylation of #-Catenin by GSK3 makes it an unstable protein. However, in the presence of Wnt #-Catenin is no longer phosphorylated and becomes stabilized. The accumulation of newly made #-Catenin causes its translocation into the nucleus where it binds to T-Cell Factor (TCF) on DNA, eliciting the many transcriptional effects of Wnt signaling on gene expression.

The mechanism by which Wnt signaling blocks GSK3 phosphorylations long was an enigma. We performed measurements of the enzymatic activity of GSK3 using lysates containing the detergent Triton X-100 and were surprised to find that Wnt addition did not inhibit GSK3 enzyme activity (Taelman *et al*., 2010). How could this be?

1.3 GSK3 is sequestered into multivesicular endosomes

We then remembered the classical work of Stanley Cohen, who had treated cultured cells with labelled Epidermal Growth Factor (EGF) and found that this growth factor became localized inside multivesicular bodies a few minutes after the endocytosis of its receptor (McKanna *et al*., 1979). If GSK3 were to follow its receptors into the endolysosomal pathway its sequestration inside membrane-bounded organelles could explain the reduced activity GSK3 in the cytosol while maintaining total levels of enzyme activity in detergent-treated lysates (Figure 1).

1.4 Wnt addition increases endocytosis

When cultured cells were treated with purified preparations of Wnt3a, after 10 minutes we observed the formation of prominent vesicular structures visible by phase-contrast optical microscopy (Figure 2). Thus, Wnt causes a large increase in endocytosis. When stained with antibodies against endogenous GSK3, it was clear that large amounts of GSK3 are translocated from the cytosol into these vesicles. Cryoimmuno electron microscopy, done in collaboration with David D. Sabatini of New York University, showed that GSK3 was localized inside the intraluminal vesicles (ILVs) of multivesicular bodies. Protease protection assays in cells made permeable with Digitonin showed that GSK3 and Axin were translocated into membrane-bounded organelles by Wnt signaling (Taelman *et al*., 2010; Vinyoles *et al*., 2014; Albrecht *et al.*, 2018).

MVBs form during the normal progression of intracellular membrane traffic. Early endosomal vesicles pinch off the plasma membrane. In the case of Wnt, it was known that endocytosis was required for signaling (Blitzer and Nusse, 2006) and that components of the receptor complex and the cytosolic destruction complex accumulated in the cell membrane and early endosome (Bilic *et al*., 2007). However, the decrease of GSK3 activity in the cytoplasm requires its sequestration inside the ILVs of MVBs. Once inside, the enzyme becomes separated from its cytosolic substrates by two membranes: the ILV membrane and the late endosome/lysosome limiting membrane (Taelman *et al*., 2010) (Figure 3).

The process of ILV formation is called microautophagy and results in the digestion of purely cytosolic proteins in lysosomes. The outside-inside formation of ILVs requires great effort by the cell (Piper and Katzmann, 2007). All eukaryotic cells contain an elaborate ESCRT machinery (Endosomal Sorting Complexes Required for Transport), sometimes called Vps (Vacuolar protein sorting) proteins that are necessary for membrane invagination into late endosomes.

All plasma membrane proteins must pass through the intraluminal vesicle step before they can enter the lysosome for degradation. Most growth factor receptors, such as the EGF receptor, use endolysosomes to downregulate receptor activity (Katzman *et al*., 2002). The case of Wnt is different, because the sequestration of GSK3 and Axin constitutes the signal itself. Indeed, inhibiting the activity of the ESCRT proteins HRS/Vps27 or Vps4 blocks canonical Wnt/GSK3 signaling (Taelman *et al*., 2010).

The main conclusion from these experiments is that Wnt signaling requires the sequestration of a cytosolic protein kinase, GSK3, inside endosomes.

2. Wnt regulates the stability of many proteins

2.1 How many proteins are regulated by Wnt/GSK3?

GSK3 is a very abundant protein kinase, and has the peculiarity of being constitutively active (Wu and Pan, 2010). Most other protein kinases require an activation step before they can add phosphates to proteins using ATP as substrate. GSK3 has many other substrates in addition to #-Catenin (Jope and Johnson, 2004; Kim *et al*., 2009). This raised the question of how many proteins might be stabilized by Wnt/GSK3.

GSK3 has a preference for pre-phosphorylated substrates. The priming phosphorylation can be introduced by many different kinases such as MAPK, CK1, CDK or PKA. Once phosphorylated, the substrate is recognized by the priming phosphate site in GSK3. The enzyme scans the protein and if a serine or threonine is found in the fourth position upstream of the priming phosphate, another phosphorylation is introduced (Cohen and Frame, 2001). This is a processive mechanism, so that if another Ser/Thr site is found four amino acids upstream, an additional phosphate is introduced until a Ser/Thr is no longer found.

To investigate how many proteins might conceivably be regulated by Wnt/GSK3 we analyzed the human entire proteome. We found that 20% of human proteins contain three or more consecutive GSK3 sites. This is much more than what might be expected by chance alone. The complete list of these proteins is available at: http://www.hhmi.ucla.edu/derobertis/EDR_MS/GSK3%20Proteome/Table_1-full_table.xls

Determining whether a certain protein has possible GSK3 sites is a good predictor of whether it might be stabilized by Wnt signaling. We have investigated some such proteins and found that several are indeed regulated by Wnt addition. Examples include: MITF (Microphthalmia transcription factor, a key oncogene of melanocytes) (Ploper *et al*., 2015); Tau (a microtubule-associated protein involved in Alzheimer's disease)

(Dobrowolski *et al*., 2012); HDAC4 (histone deacetylase 4) (Taelman *et al*., 2010); Smad1 (a transcription factor activated by BMP signaling) (Fuentealba *et al*., 2007); Smad4 (a transcription factor shared by the TGF# and BMP pathways) (Demagny *et al*., 2014).

We conclude that Wnt regulates the degradation of a plethora of other proteins in addition to #-Catenin.

2.2 Wnt signaling regulates total cellular protein stability

The effect of Wnt on protein stability is massive. In pulse-chase experiments with radioactive Methionine (30 minute pulse followed by chase in unlabeled medium containing a 5-fold excess of cold Methionine) the half-life of total cellular proteins in human 293 cells was extended by 25% (Taelman *et al*, 2010). This effect is so marked that it increased cell size measured by flow cytometry (Acebron *et al*., 2014). We confirmed these observations and found that HeLa cells increase 14% in size after treating with Wnt for 48 hours and demonstrated that this increase requires the ESCRT machinery (Kim *et al*., 2015).

This increase in protein stability has been designated Wnt-STabilization Of Proteins (Wnt/STOP) by Christof Niehrs (Acebron *et al*., 2014). Wnt signaling is maximal during the G2/M phase of the cell cycle (Davidson *et al*., 2009), leading to the interesting proposal that Wnt/STOP provides a means for cells to increase their volume by preventing protein degradation just prior to mitosis (Acebron *et al*., 2014).

These experiments indicate that Wnt, through the sequestration of GSK3 in multivesicular endosomes, is a potent regulator of cellular protein degradation.

3. Wnt signaling translocates Lys48-linked polyubiquitinated proteins normally degraded in proteasomes into the lysosomal pathway

3.1 Microautophagy can translocated proteins targeted to proteasomes into lysosomes in the presence of Wnt

Since GSK3 should be inhibited by Wnt, we expected that during Wnt signaling there would be less protein polyubiquitination due to lower levels of GSK3 phosphodegrons. To our surprise, we found that total polyubiquitinylated proteins accumulated after one or two hours of Wnt signaling. This accumulation could be blocked by GSK3 inhibitors. Furthermore, the increase was particularly in Lys48-linked polyubiquitin, which is the form that normally targets proteins to the proteasome. Wnt did not affect proteasomal activity. Further investigation showed that these proteins are channeled by microautophagy into the lysosomal pathway by Wnt treatment (Kim *et al*., 2015). Thus, proteins normally targeted to proteasomes are degraded in endolysosomes in the presence of Wnt (Figure 4).

The main finding from these investigations was that the proteasomal and lysosomal pathways are not independent of each other as previously thought. Remarkably, the physiological choice between proteasomal and lysosomal degradation is controlled by the Wnt extracellular signal.

4. Protein Arginine methylation is required for canonical Wnt signaling

4.1 An unexpected result

We observed that of the large number of putative GSK3 substrates, many have been shown to be also modified by a post-translational modification known as Arginine methylation. One report had shown that Arginine methylation is required to promote consecutive GSK3 phosphorylations on a cytoskeletal linker protein called Desmoplakin (Albrecht *et al*., 2015). This suggested to us that Arginine methylation might play a role in regulating GSK3 signaling.

Arginine methylation is emerging as a fundamental protein modification as it is as prevalent as phosphorylation and occurs in both the nucleus and cytoplasm (Larsen *et al*., 2016). Arginine methylation was previously thought to be irreversible. However, the discovery of the Jumonji demethylase enzymes marked a revolution in the field as it revealed that Arginine methylation can be switched on and off to dynamically regulate cell signaling (Chang *et al*., 2007). Proteomic analyses revealed that 33% of Arginine methylation substrates contained GSK3 consensus motifs (Albrecht *et al*., 2018). To test whether Arginine methylation was coordinated with Wnt signaling, cells were treated with Wnt protein and immunostained with methyl-Arginine specific antibodies. We found that Arginine methylated proteins were translocated from the cytosol into endosomal vesicles by Wnt signaling (Figure 5).

Arginine methylation is catalyzed by Protein Arginine Methyltransferase enzymes (PRMTs) (Bedford and Clarke, 2009). Of the eight PRMT enzymes, PRMT1 accounts for 85% of cellular methylation (Bedford and Clarke, 2009). In an important study, the group of R. Derynck found that PRMT1 initiates TGF-# and BMP growth factor receptor signaling (Xu *et al*., 2013). We found that in the case of Wnt signaling cytosolic PRMT1 is rapidly sequestered into multivesicular bodies (Figure 6). In addition, GSK3 is translocated into the same

vesicles as PRMT1 following Wnt protein addition (Figure 6) (Albrecht *et al*., 2018). The results suggest that PRMT1 and GSK3 function coordinately during Wnt signaling.

Conclusions

Organisms use a small number of cell signaling pathways for cell-cell communication. The study of cell signaling is very important in developmental and cancer biology. The Wnt pathway is particularly interesting because it is involved in the initiation of many tumors and also provides the first asymmetry during development of the amphibian embryo. The discovery that PRMT1 is sequestered into the same vesicles as GSK3 and is required for Wnt signaling could offer innovative therapeutic approaches to prevent the progression of cancers with an activated Wnt pathway (usually resulting from loss-of-functions mutations in APC or Axin proteins). This could be achieved by inhibiting Arginine methylation, endocytosis, or lysosomal activity. The studies discussed here are at the intersection of the cell biology of membrane trafficking, cell signaling and the regulation of cellular protein degradation. They revealed that the Wnt signaling pathway uses the normal membrane trafficking machinery of endocytosis to generate a signal that results from the sequestration of GSK3 and Axin inside multivesicular endosomes. The removal of GSK3 from cytosol stabilizes a multitude of proteins, leading to a marked increase in protein stability and cell size. GSK3 has many substrates within the Wnt receptor complex and is translocated together with it, as well as with many of its cytosolic substrates, inside the intraluminal vesicles of multivesicular endosomes. Many of these substrates will fall into a specialized cohort modified by both Arginine methylation and GSK3 phosphorylation. Wnt causes a previously unsuspected switch of protein degradation from the proteasome to the lysosome, two pathways that were thought to be independent of each other.

Bibliography

Acebron, S.P., Karaulanov, E., Berger, B.S., Huang, Y.L., and Niehrs, C. (2014). Mitotic Wnt signaling promotes protein stabilization and regulates cell size. *Mol. Cell 54*, 663-674.

Albrecht, L.V., Zhang, L., Shabanowitz, J., Purevjav, E., Towbin, J.A., Hunt, D.F., and Green, K.J. (2015). GSK3- and PRMT-1-dependent modifications of desmoplakin control desmoplakin-cytoskeleton dynamics. *J. Cell Biol. 208*, 597-612.

Albrecht, L.V., Ploper, D., Tejeda-Muñoz, N., and De Robertis, E.M. (2018). Arginine methylation is required for canonical Wnt signaling and endolysosomal trafficking. *Proc Natl Acad Sci USA 115*, E5317-E5325.

Bedford, M.T., Clarke, S.G., (2009) Protein arginine methylation in mammals: who, what, and why. *Mol Cell 33*, 1-13.

Bilic, J., Huang, Y.L., Davidson, G., Zimmermann, T., Cruciat, C.M., Bienz, M. and Niehrs, C. (2007). Wnt induces LRP6 Signalosomes and promotes Dishevelled-dependent LRP6 phosphorylation. *Science 316*, 1619-1622.

Blitzer, J.T., and Nusse, R. (2006). A critical role for endocytosis in Wnt signaling. *BMC Cell Biol. 7*, 28.

Chang, B., Chen, Y., Zhao, Y., and Bruick, R.K. (2007). JMJD6 is a histone arginine demethylase. *Science 318*, 444-447.

Ciechanover, A. (2005). Proteolysis: from the lysosome to ubiquitin and the proteasome. *Nat. Rev. Mol. Cell Biol. 6*, 79-87.

Cuervo, A.M. (2010). Chaperone-mediated autophagy: selectivity pays off. *Trends Endocr. Metabol. 21,* 142-150.

De Duve, C. and Wattiaux, R. (1966). Functions of lysosomes. *Annu. Rev. Physiol. 28*, 435-492.

Davidson, G., Shen, J., Huang, Y.L., Su, Y., Karaulanov, E., Bartscherer, K., Hassler, C., Stannek, P., Boutros, M., and Niehrs, C. (2009). Cell cycle control of Wnt receptor activation. *Dev. Cell 17*, 788-799.

Demagny, H., Araki, T., and De Robertis, E.M. (2014). The tumor suppressor Smad4/DPC4 is regulated by phosphorylations that integrate FGF, Wnt and TGF-# signaling. *Cell Rep. 9*, 688-700.

Dobrowolski, R., Vick, P., Ploper, D., Gumper, I., Snitkin, H., Sabatini, D.D., and De Robertis, E.M. (2012). Presenilin deficiency or lysosomal inhibition enhances Wnt signaling through relocalization of GSK3 to the late-endosomal compartment. *Cell Rep. 2*, 1316-1328.

Fuentealba, L.C., Eivers, E., Ikeda, A., Hurtado, C., Kuroda, H., Pera, E.M., and De Robertis, E.M. (2007). Integrating patterning signals: Wnt/GSK3 regulates the duration of the BMP/Smad1 signal. *Cell 131,*980-993.
Jope, R.S., and Johnson, G.V. (2004). The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem. Sci. 29*, 95-102.

Katzmann, D.J., Odorizzi, G., and Emr, S.D. (2002). Receptor downregulation and multivesicular-body sorting. *Nat. Rev. Mol. Cell. Biol. 12*, 893-905 (2002).

Kim, N.G., Xu, C., and Gumbiner, B.M. (2009). Identification of targets of the Wnt pathway destruction complex in addition to #-Catenin. *Proc. Natl. Acad. Sci. USA 106*, 5165-5170.

Kim, H., Vick, P., Hedtke, J., Ploper, D. and De Robertis, E.M. (2015). Wnt signaling translocates Lys48-Linked polyubiquitinated proteins to the lysosomal pathway. *Cell Rep. 11*, 1151-1159.

Logan, C.Y. and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol. 20*, 781-810.

Nusse, R. and Clevers, H. (2017). Wnt/#-Catenin signaling, disease, and emerging therapeutic modalities. *Cell 169*, 985-999.

Ohsumi, Y., (2014) Historical landmarks of autophagy research. *Cell Res. 24,* 9-23.

Piper, R.C. and Katzmann, D.J. (2007) Biogenesis and function of multivesicular bodies. *Annu. Rev. Cell Dev. Biol. 23*, 519-547.

Ploper, D., Taelman, V.F., Robert, L., Perez, B.S., Titz, B., Chen, H-W., Graeber, T.G., von Euw, E., Ribas, A., and De Robertis, E.M. (2015). MITF drives endolysosomal biogenesis and potentiates Wnt signaling in melanoma cells. *Proc. Natl. Acad. Sci. USA 112*, E420-429.

Taelman, V.F., Dobrowolski, R., Plouhinec, J.L., Fuentealba, L.C., Vorwald, P.P., Gumper, I., Sabatini, D.D., and De Robertis, E.M. (2010). Wnt signaling requires sequestration of glycogen synthase kinase 3 inside multivesicular endosomes. *Cell 143*, 1136-1148.

Vinyoles, M., Valle-Perez, B.D., Curto, J., Vinas-Castells, R., Alba-Castellon, L., de Herreros, A.G., and Dunach, M. (2014). Multivesicular GSK3 sequestration upon Wnt signaling is controlled by p120-Catenin/ Cadherin interaction with LRP5/6. *Mol. Cell 53*, 444-457.

Wu, D., and Pan, W. (2010). GSK3: a multifaceted kinase in Wnt signaling. *Trends Biochem. Sci. 35*, 161-168.

Xu, J., Wang H., Oses-Prieto, J., Makhijani, K., Katsuno, Y., Pei, M., Yan, L., Zheng, G.Y., Burlingame, A., Bruckner, K., Derynck, R. (2013). Arginine Methylation Initiates BMP-Induced Smad Signaling. *Mol. Cell 51*, 5-19.

END NOTES

[*] An early version of this work was presented in Cell Biology and Genetics, De Robertis, E.M. and Sánchez Sorondo, M., Eds. *Pontificiae Academiae Scientiarum Scripta Varia* Vol. 137, pp. 177-186 (2018). Libreria Editrice Vaticana, Rome.