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RETROSPECTIVE

From Rabbit Reticulocytes to Clam Oocytes: In Search of the System That Targets Mitotic Cyclins for Degradation

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By the late 1980s, the basic biochemistry of ubiquitin-mediated protein degradation had already been elucidated by studies that used reticulocyte lysates. However, the scope and biological functions of this system remained largely obscure. Therefore, I became interested at that time in the mechanisms by which mitotic cyclins are degraded in exit from mitosis. Using a cell-free system from clam oocytes that faithfully reproduced cell cycle stage–specific degradation of cyclins, we identified in 1995 a large ubiquitin ligase complex that targets mitotic cyclins for degradation. Subsequent studies in many laboratories showed that this ubiquitin ligase, now called the anaphase-promoting complex/cyclosome, has centrally important roles in many aspects of cell cycle control.

By the late 1980s, the basic biochemistry of ubiquitin-mediated protein degradation was already known, at least in broad outline (Hershko, 1988). This was mainly due to biochemical studies on the mode of action of an ATP-dependent cell-free proteolytic system from rabbit reticulocytes. In these studies we used nonphysiological, model protein substrates, such as lysozyme from hen eggs or bovine serum albumin. However, there was very little information available on the biological scope and cellular functions of this system. Thus, before 1990, the ubiquitin system had been implicated in the degradation of only a few proteins, such as phytochrome, a plant photoreceptor (Shanklin *et al*., 1987) and yeast MATα2 repressor (Hochstrasser and Varshavsky, 1990). Therefore, at that time I thought that even though important questions remained open on the basic biochemistry of the ubiquitin system, I wanted to turn to the problem of how this system degrades a biologically important cellular protein in a selective and regulated manner. It was thus that I became interested in the cell cycle stage–specific degradation of mitotic cyclins. These proteins were discovered by Tim Hunt in 1983 during laboratory exercises with students in the physiology course at the Marine Biology Laboratory (MBL) in Woods Hole (Evans *et al*., 1983). In examining proteins synthesized in fertilized eggs of marine invertebrates, they noted two proteins (cyclins A and B) that displayed very unusual behavior: they accumulated during interphase and then were rapidly destroyed in mitosis. The authors suspected that cyclins had some important roles in the control of cell division (Evans *et al*., 1983). However, their function was discovered only in 1988 when several groups of investigators reported that cyclin B is a subunit of maturation promoting factor (MPF), a protein kinase whose level oscillates in the cell cycle (reviewed in Hunt, 1989). The activity of MPF rises in early mitosis due to the synthesis of cyclin B. MPF promotes entry into mitosis and major mitotic events. Subsequently, MPF is inactivated due to the rapid degradation of cyclin B in the metaphase–anaphase transition. Thus, the programmed destruction of cyclin B is essential for exit from mitosis and for resetting the cell cycle oscillator. The mysterious activity that destroyed cyclin was called at that time "cyclin protease" (Hunt, 1989) and an important question was what is the "cyclin protease" and why is it active only at the end on mitosis? When I first learned about the cyclin story, I thought that maybe it was not a cyclin-specific protease but a cell cycle–regulated ubiquitin ligase that selectively acts on cyclin. This looked like a fascinating and challenging problem to investigate.

To examine the mechanisms of cyclin degradation by a biochemical approach, a cell-free system was required that faithfully reproduced this process in a cell cycle stage–specific manner. For this purpose, I could not use my favorite reticulocyte system, because reticulocytes are terminally differentiated, nondividing cells. Some laboratories used "cycling extracts" from *Xenopus* eggs, in which periodic DNA synthesis and fluctuations in MPF activity and in cyclin B levels could be initiated by the addition of sperm nuclei (reviewed in Hunt, 1989). This was a great cell-free system, but it required much expertise and experience in the proper handling of *Xenopus* frogs and in the preparation of concentrated and activated egg extracts. At that time there was no frog expert in Haifa, Israel, but I set up a small *Xenopus* facility and ordered frogs from the United States. The frogs landed in Israel all right, but then they were apparently not too happy in my primitive *Xenopus* facility because they laid poor quality eggs, and we could not get a decently active cell-free system.

Soon after my unlucky adventure with frogs, I started one of my numerous sabbaticals at the Fox Chase Cancer Center (FCCC) in Philadelphia (1989–90). There I had a good friend

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Leonard Cohen, who was using sea urchin embryos for developmental studies. For two months I tried to set up a cell-free system from fertilized sea urchin eggs, but although cyclins were dramatically degraded in intact eggs, as described originally (Evans *et al*., 1983), they were completely stable in extracts prepared in various ways. Cell-free systems that reproduce complex processes are tricky, and the choice of the source is crucial, since the inactivation of a single component by a protease or phosphatase released during cell lysis may inactivate the whole system. At that time, I read a recently published article by Frank Luca and Joan Ruderman in which they showed that cell cycle stage–specific degradation of mitotic cyclins could be reproduced in cell-free extracts from fertilized oocytes of the surf clam, *Spisula solidissima* (Luca and Ruderman, 1989). It was possible to get shipments of clams from Woods Hole to Philadelphia, and I got much help from Leonard Cohen at FCCC and from Bob Palazzo at the MBL in learning how to handle clams and their oocytes. We could then use the clam oocyte cell-free system to examine the question of whether mitotic cyclins are degraded by the ubiquitin system. We found that this was indeed the case, based on the finding that the degradation of both cyclin A and cyclin B was inhibited by methylated ubiquitin, an inhibitor of polyubiquitin chain formation (Hershko *et al*., 1991). The inhibition was specific, because it could be reversed by the addition of an excess of native ubiquitin. Earlier in that year (1991), the Kirschner lab published other and more extensive evidence from *Xenopus* egg extracts, reaching a similar conclusion that cyclin B is degraded by the ubiquitin system (Glotzer *et al*., 1991).

The next task was to find out which specific enzymes of the ubiquitin system are involved in cyclin degradation and how are they regulated in the cell cycle. This required fractionation of clam oocyte extracts and purification of the relevant enzymes. Enzyme purification needs large amounts of starting material. For this purpose, a weekly shipment of a dozen clams (about half of which were males and thus quite useless) from Woods Hole to Philadelphia was no longer sufficient. Luckily, Joan Ruderman invited me at that time to collaborate on this project in her summer laboratory at the MBL. The Marine Resources facility at the MBL had an abundant supply of clams. Extracts of clam oocytes could be frozen without loss of activity and could be shipped on dry ice to Israel, where much of the purification work was done. By 1994, we had fractionated extracts of clam oocytes and identified three components required for cyclin–ubiquitin ligation: the ubiquitin-activating enzyme E1, a novel E2 that we called E2-C and an E3 ubiquitin ligase whose activity was cell cycle regulated (Hershko *et al*., 1994). E2-C from clam (Aristarkhov *et al*., 1996) and from human (also called UbcH10; Townsley *et al*., 1997) origin were subsequently cloned by the Ruderman lab. In 1995, we partially purified and characterized the relevant E3 ubiquitin ligase: it was a large, ~1500-kDa complex that acted on mitotic cyclins, but not on many other proteins. Its activity was dramatically regulated in the cell cycle: it was inactive in the interphase, became active at the end of mitosis, and the interphase form could be converted to the active form by phosphorylation with MPF (Sudakin *et al*., 1995).

After the discovery of this interesting and apparently important ubiquitin ligase, Joan and I had two problems: what to call it and where to publish our findings. We came up with the name "cyclosome," to indicate its large size and important roles in cell cycle control. As for publication, I tended to submit it to *The Journal of Biological Chemistry*, a good and solid biochemical journal where I had published most of my work. Joan thought that this paper should reach a readership of cell biologists and suggested Molecular Biology of the Cell (MBoC), which was then a new and not well-known journal. I looked up the policy of MBoC and found that unlike some "flashy" journals, it insisted on description of experiments in sufficient detail to be reproduced by interested readers. I liked that approach and therefore agreed to Joan's suggestion. This is how this paper got submitted and published in MBoC in 1995 (Sudakin *et al*., 1995). I understood from the invitation of the editor of MBoC to write this Retrospective article that our 1995 paper became one of the most widely cited in the history of MBoC. I never thought that we would win a popularity contest!

I also did not guess that this specific ubiquitin ligase would become the subject of intensive research in so many laboratories and so soon. Two months after the publication of our MBoC article, three adjoining articles were published in *Cell* on this subject (Irniger *et al*., 1995; King *et al*., 1995; Tugendreich *et al*., 1995). One of these, an elegant genetic screen by the Nasmyth laboratory, identified yeast genes *CDC16* and *CDC23* as being required for the degradation of cyclin B and for sister chromatid separation (Irniger *et al*., 1995). Another article from the Kirschner laboratory described the isolation from *Xenopus* egg extracts of a ubiquitin ligase complex similar to our cyclosome and showed that the complex contained products of homologues of the yeast genes mentioned above (King *et al*., 1995). They called the same ubiquitin ligase anaphase-promoting complex or APC (King *et al*., 1995); now it is known by the combination of these names: APC/cyclosome or APC/C. Since then, a great body of work showed that APC/C is highly conserved in eukaryotic evolution and targets for degradation not only mitotic cyclins, but also some other cell cycle regulatory proteins essential for exit from mitosis and for prevention of premature entry into the S phase. The activity of APC/C is intricately controlled in the somatic cell cycles and is the target of the mitotic (or spindle assembly) checkpoint system that ensures the accuracy of chromosome segregation. For further information on these subjects, the interested reader is referred to several recent reviews (Peters, 2006; van Leuken *et al*., 2008; Musacchio and Salmon, 2007). The purpose of this article was not to review the field, but to provide a perspective of how it all started.

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