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Utilization of a Storage Protein in the Embryonic Development of *Drosophila* and *Xenopus*

Young Eui Jeong, Hae Moon Chung, and Tae In Ahn*

School of Biological Sciences, Seoul National University, Seoul 151-742, Korea

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Yolk platelets, one of the main food stores in the embryonic development, are composed of proteins. However, little is known about the identity of proteins utilized at certain stages of embryogenesis. In this study, we followed the fates of embryonic storage proteins by using an anti-polyubiquitin monoclonal antibody (mAB) as a probe. The mAb recognized the major storage proteins of *Drosophila, Xenopus* and chicken eggs. In the *Drosophila* embryo, the mAb-reactive 45-kDa protein was not used until stage 11 but was used up at stage 16 when the embryo completed segmentation. In the *Xenopus* embryo, the mAb-reactive 111 kDa protein was mostly utilized between stages 42 and 45 implying that the protein might be an energy source used just prior to feeding on food. By N-terminal sequencing the storage protein of *Xenopus* embryo was identified as a lipovitellin 1. This study confirms that storage proteins are used almost simultaneously at certain stages of embryogenesis and that vitellogenin 1 is the last storage protein in *Xenopus* embryogenesis.

One of the most obvious characteristics of egg cells of oviparous animals is their large size which results, to a major extent, from the deposition of nutritional reserves (Nardelli et al., 1987). Many animals store considerable quantities of yolk platelets, lipid droplets and glycogen granules in the eggs as a source of nourishment necessary for early embryogenesis. Among these constituents, yolk platelets are the main reserve and composed mainly of proteins derived from vitellogenin. Vitellogenin undergoes posttranslational modifications during secretion and transport, and is taken up by the growing oocytes via receptor-mediated endocytosis (Fagotto and Maxfield, 1994). Once internalized, vitellogenin is cleaved into multiple polypeptides, i.e., the two lipovitellin subunits plus phosvitin, which are then stored as microcrystals within the yolk platelets.

Yolk proteins must be degraded to their constituent amino acids at some stage of the embryonic development. In eukaryotic cells, protein degradation depends on two distinct systems: the lysosomal system and the ubiquitin-dependent proteasomal system. Lysosomal degradation is performed by lysosomal proteases and requires an acidic environment and transport of substrate proteins to the lysosome (Bohley and Seglen, 1992). The protein delivery may occur by endocytosis or by the process of autophagy (Seglen and Bohley, 1992). As for the proteasomal degradation, modification of substrate proteins covalently with ubiquitin, a small 76-residue-long protein, is a prerequisite. The polyubiquitin-conjugated cytosolic proteins are recognized and degraded by 26S proteasomes (Coux et al., 1996). The ubiquitin/proteasome-dependent proteolysis is involved in diverse cellular processes (Hershko and Ciechanover, 1998). Although ubiquitination is generally considered not being involved in lysosomal degradation system, a number of evidence have been reported which suggest the link between ubiquitination and lysosomal degradation, such as in plasma membrane receptors (Bonifacino and Weissman, 1998) and carriers (Springael and Andre, 1998).

However, little is known about mechanisms involved in yolk degradation. During embryogenesis, yolk platelets become progressively more acidic (pH < 5), and the pH is developmentally regulated and is involved in triggering yolk degradation (Fagotto and Maxifield, 1994). Recently, degradation of yolk platelets by fusion with late endosomes has been suggested through electron microscopy of the early amphibian embryo (Komazaki and Hiruma, 1999).

From a study of polyubiquitin in *Amoeba proteus*, we obtained hybridoma cells producing monoclonal antibodies (mAbs) against polyubiquitin and found the mAb to cross-react with ubiquitin-conjugated proteins and a single major protein (> 100 kDa) of *Xenopus* embryo (Lee et al., 1998, Oh and Ahn, 2000). In order to elucidate the degradation mechanism of yolk proteins, we utilized the mAb to identify the reactive yolk protein and followed the fate of the protein during the embryonic development.

* To whom correspondence should be addressed.
Tel: 82-2-880-7771, Fax: 82-2-880-7771
E-mail: ahntaein@snu.ac.kr

Materials and Methods

Eggs and embryos

Fertilized eggs of *Xenopus laevis* were obtained after artificial induction of spawning by administration of human chorionic gonadortopin (Chung et al., 1989). Eggs were collected and treated with 2.0% L-cysteine (pH 7.8) to remove egg jelly. The eggs were then cultured in 0.1× Marc's modified Ringer's solution (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4) at 20℃. The embryos were staged according to the criteria of Nieuwkoop and Faber (1967).

Drosophila melanogaster (wild type) was housed in egg laying chambers covered with 5 cm apple juice plates. In a chamber, seventy of each male and female *D. melanogaster* were cultured for 24 h at 25℃ and embryos were collected from the top of the apple juice plate using a soft brush and dechorionized in 14% hypochloride followed by several washes in distilled water. Embryos were staged according to the criteria of Campos-Ortega and Hartenstein (1997).

Production of mAbs

Cells producing mAbs against polyubiquitin were cultured and ascitic fluids were produced by routine procedures (Lee et al., 1998).

Gel electrophoresis and immunoblotting

Proteins of chicken egg yellow or of embryos of *Xenopus* and *Drosophila* were dissolved in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, and 50 mM Tris, pH 8.0) and sonicated three times for 5 sec each. The homogenate was then centrifuged for 5 min at 12,000 g and the supernatant was collected. Proteins were quantitated by phenol reagent and analyzed by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). For estimation of molecular mass, both high and low molecular mass markers (Sigma) were run in parallel. After electrophoresis, gels were stained with 0.25% Brilliant Coomassie blue (BCB) or processed for immunoblotting. For immunoblotting, proteins separated by gel electrophoresis were transferred to nitrocellulose membranes (Schleicher and Schuell) using a semi-dry transfer apparatus and immunostained using the ascites fluid diluted in PBS (1: 500) as the primary antibody and horseradish-peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) as the secondary antibody. Immunoblots were developed with enhanced chemiluminescence reagent (Amersham) (Lee et al., 1998).

Characterization of the mAb-reactive proteins

The storage proteins of *Xenopus* embryo reacting with the anti-polyubiquitin mAb was purified by preparatory gel electrophoresis (8% SDS-gel). The proteins in the gel were eluted by electrophoresis in a tube gel. Then the eluted proteins were dialyzed overnight against distilled water, precipitated with cold methanol and dissolved in 0.01 M Tris HCl, pH 7.4. An aliquot (28 µl) containing 80 µg protein was mixed with equal volume of digestion buffer (0.125 M Tris HCl, pH 6.8 and 0.2% SDS) and incubated at 37℃ after the addition of proteases, trypsin (0.352 unit) or chymotrypsin (0.288 unit). At time intervals, an aliquot of the reaction mixture was withdrawn and stopped by adding the sample buffer for SDS-gel. Proteolytic products were analyzed by SDS-PAGE and immunoblotted with the mAb. Because ubiquitin-conjugated polypeptides have multiple N-termini, we selected the polypeptide not reacting with the mAb as a candidate polypeptide for partial sequencing. For the selection of candidate polypeptides, proteolytic polypeptides of the SDS-gel transferred onto polyvinylidene difluoride membrane were visualized by BCB staining and compared with the corresponding immunoblot. Polypeptides not reacting with the mAb were excised and N-terminal amino acids were determined by custom sequencing at the Korea Basic Scientific Institute (Seoul, Korea).

Results and Discussion

In order to confirm the reactivity of the anti-polyubiquitin mAb with major embryonic proteins, various embryonic and egg proteins were analyzed by SDS-PAGE and immunoblotting (Fig. 1). In the chicken egg, the mAb was reactive with the major storage protein (116 kDa), and three minor proteins of 97, 76 and 66 kDa. In *Xenopus*, a 111 kDa protein was the only major protein and was reactive with the mAb. However, the protein profiles of *Drosophila* embryos were quite different from those of vertebrates. Yolk proteins of the *Xenopus* embryo are known to be composed of lipovitellins, phosvitin and phosvettes in a stoichiometry of 1 : 0.69 : 0.25 (Wiley and Wallace, 1981). Among these, lipovitellins are composed of lipovitellin 1 (111-121 kDa) and lipovitellin 2 (30-34 kDa). Since the mAb-reactive protein in the *Xenopus* embryo was the only major protein, this appeared to be a storage protein composed of lipovitellin 1. In the *Drosophila* embryo, among the three major proteins, a 45-kDa protein was reactive with the mAb. The mAb also recognized several minor small proteins (35-24 kDa) of *Drosophila* embryo. In the *Drosophila* three vitellogenin polypeptides (44, 45, and 46 kDa) are known (Warren et al., 1979). Thus, the mAb appeared to recognize one of the major storage proteins and a few minor proteins as well. The embryonic storage proteins of *Drosophila* were quite different from those of *Xenopus* or chick in their the molecular mass. However, they were reactive with the mAb, which implied that they were ubiquitin-conjugated proteins.

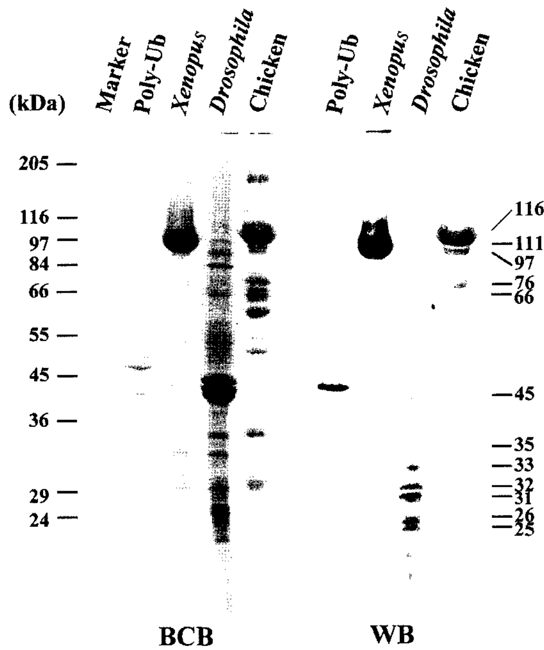

Fig. 1. Reactivity of anti-polyubiquitin mAb to embryonic proteins. SDS-gel (10%) was stained with BCB and with mAb against polyubiquitin (WB). Lanes; Marker, molecular mass markers; Poly-Ub, proteins from *E. coli* expressing polyubiquitin of *Amoeba proteus*; *Xenopus*, whole lysate of *X. laevis* embryo (stage10); *Drosophila*, whole lysate of *D. melanogaster* embryo (stage 5); Chicken, chicken egg yellow. The bars on the right side indicate the major reactive bands.

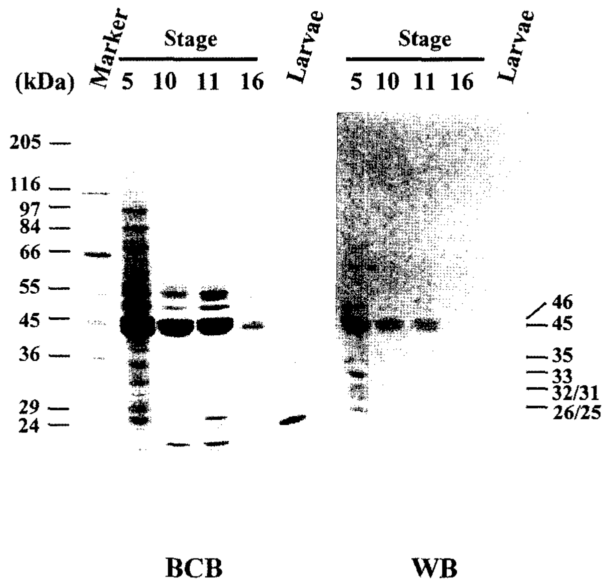

Fig. 2. Change in protein profiles of *Drosophila* embryo at various stages of development stained with BCB and with mAb against polyubiquitin (WB). Lanes; Marker, molecular mass marker; Larvae, larval proteins of *Drosophila*. The 46-kDa protein was not detectable after stage 10, whereas 44 and 45 kDa proteins were largely utilized prior to stage 16.

Since the embryonic storage proteins were simple in composition and the mAb was reactive with the major storage proteins, we followed the fate of these proteins with the embryonic development. In *Drosophila* embryo the 46 kDa protein and small storage proteins (35-25 kDa) were maintained until stage 5 and used up by stage 10 (Fig. 2). On the other hand, 44 and 45 kDa proteins were not significantly changed in their level until stage 11, mostly disappeared at stage 16 and not detectable at larval stage. Apparently, most of these two storage proteins were likely to be used up between stages 12-16 when segmentations were apparent (Campos-Ortega and Hartenstein, 1997). Stage 5 of *Drosophila* embryo corresponds to blastula. At stages 10 and 11, the embryo completes gastrulation and initiates segmentation. Stage 16, which is immediately precedent to larval stage, corresponds to the period during which embryos complete segmentation. Thus, the 46 kDa and small storage proteins are most likely used during gastrulation, while 44 and 45 kDa storage proteins are probably used during segmentation.

The level of storage proteins in *Xenopus* embryo was not changed until stage 35/36 (Fig. 3). The pattern and level of most storage proteins were consistent with those of unfertilized eggs. At stage 41, the level of the major 111 kDa protein still remained, but most of the 3 minor storage proteins (30-34 kDa) were disappeared. At stage 45, much of the 111 kDa protein was used and about a third was left. However, a 43 kDa protein increased significantly from stages 45 to 48. Thus, the major storage protein appeared to be used up mostly between stages 42 and 45. Since *Xenopus* larvae begin to feed from stage 45, the storage protein appeared to be utilized as a source of energy metabolism during the final stage of early embryonic development. These results coincide well with the temporal distribution of ubiquitin mRNA in the abdominal region of *Xenopus* embryo (Cho et al., 2001). The newly increased 43 kDa protein from stages 41 to 48 could actin for mobility of the embryo. Stage 48 has been characterized as the period when forelimb buds are first visible and yolk platelets in the alimentary canals are degraded completely (Nieuwkoop and Farber, 1967). Cysteine proteinase, which plays a key role in the digestion of yolk during the development of *Xenopus* embryo, increases gradually from stages 13 to 35. Thereafter, the activity increases sharply and doubles at stage 40 (Yoshizaki and Yonezawa, 1998). Thus, the decrease of the 111 kDa protein temporally coincides well with the protease activity.

In order to identify the mAb-reactive storage proteins in *Xenopus* embryos, we purified the protein by preparatory gel electrophoresis and made partial digestion using proteases (Fig. 4). After confirming appropriate protease and condition for partial digestion of the embryonic 111 kDa protein, the tryptic polypeptides were analyzed by SDS PAGE and immunoblotted. Many of the tryptic polypeptides were still reactive with the mAb (Fig. 5). As we questioned that those reactive bands might contain conjugated

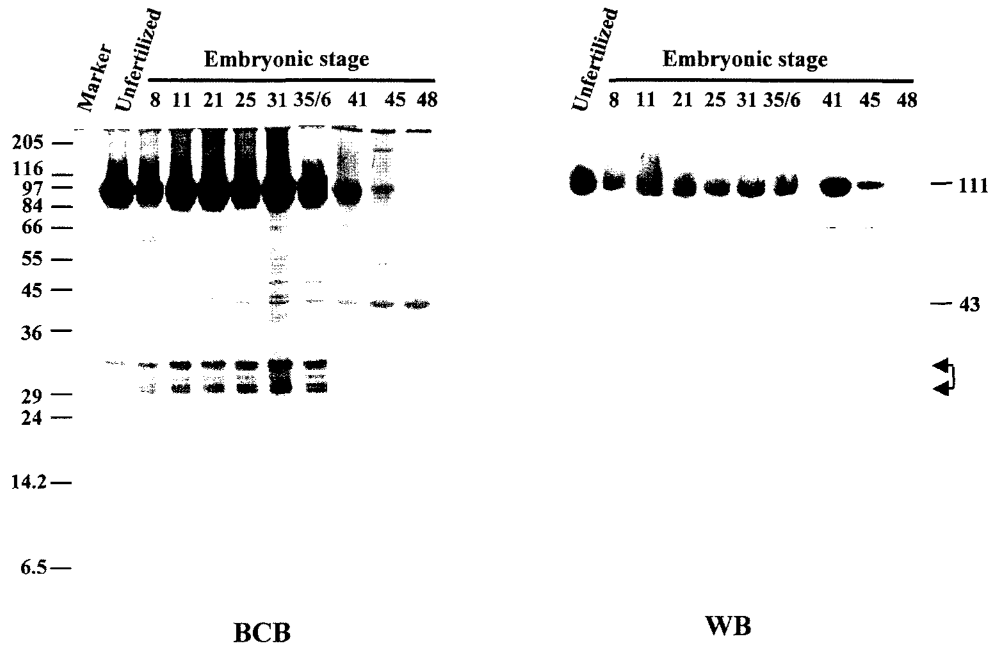

Fig. 3. Change in protein profiles of *Xenopus* embryo at various stages of development stained with BCB and with anti-polyubiquitin mAb (WB). Lanes; Marker, molecular mass marker; Unfertilized, unfertilized egg lysate of *Xenopus*. The arrows indicate the small storage proteins stained with BCB.

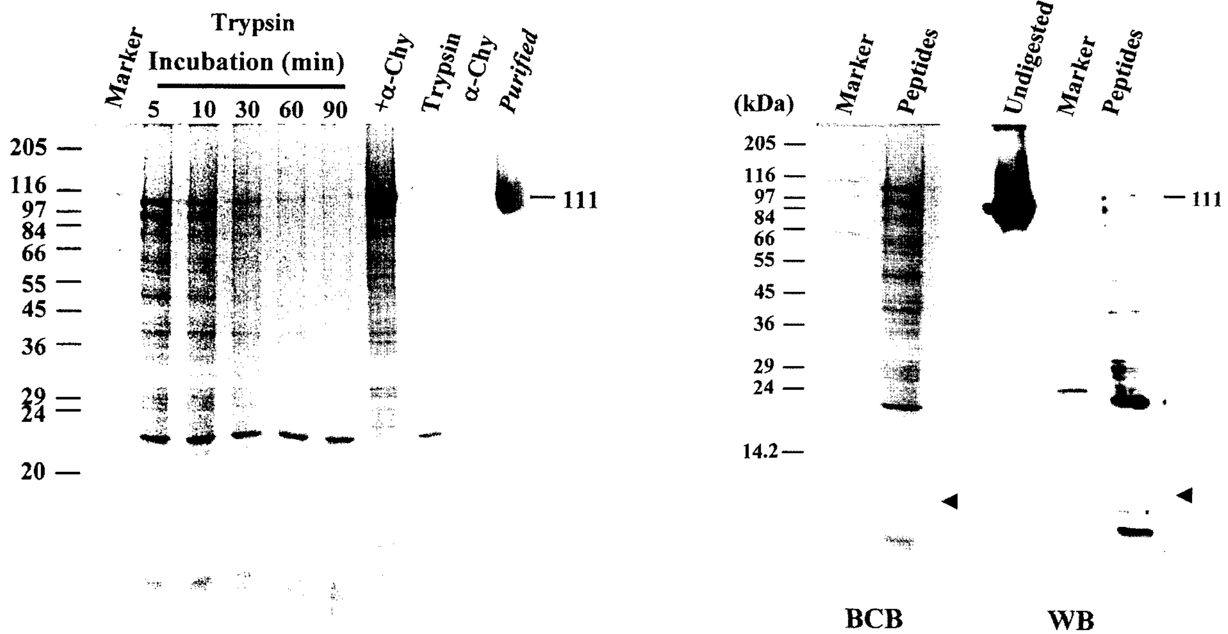

Fig. 4. SDS-PAGE patterns of proteolytic fragments of 111 kDa protein from *Xenopus* embryos. Purified 111 kDa proteins from *Xenopus* embryos were incubated for various time duration with trypsin or α-chymotrypsin (+α Chy) to determine the optimal condition for the fragmentation.

Fig. 5. Tryptic peptide map of 111 kDa protein from *Xenopus* embryo stained with BCB and with anti-polyubiquitin mAb (WB). An aliquot (80 µg) of 111 kDa protein was digested with 0.352 U trypsin for 10 min and analyzed in 10-15% SDS gel. The band (arrowhead) non-reactive to anti-polyubiquitin mAb was subjected for N-terminal amino acid sequence analysis.

ubiquitin, we selected a polypeptide band that did not react with the mAb for N-terminal sequencing. The resultant sequence, Ile-Val-Pro-Thr-Ala-Val-Gly-Leu-Pro, was searched for homologous proteins in databases and aligned (Fig. 6). The sequence was homologous to vitellogenin A2 precursor in vertebrates and its analogous proteins in invertebrates. Thus, the anti-polyubiquitin mAb-reactive storage protein of *Xenopus*

Organisms		Sequences		Sources
111 kDa protein		**IV PTAVG LP**		This study
Xenopus laevis	794	II **PTIVG LP**	802	Gerber-Huber et al., 1987
Chicken	781	VV **PSCLG LP**	790	van het Schip et al., 1987
Oncorhynchus mykiss	791	IF **PTAVG LP**	799	Goulas et al., 1996
Pimephales promelas	791	IL **PTAVG LP**	799	Korte et al., 2000
Acipenser transmontanus	785	IL **PTCIG LP**	793	Bidwell & Carlson, 1995
Fundulus heteroclitus	790	IL **PTVAG** IP	798	LaFleur, et al., 1995
Caenorhabditis elegans	862	RV **PTPMG LP**	870	Spieth et al., 1985
Anthonomus grandis	930	SF **PTEMG LP**	938	Trewitt et al., 1992

Fig. 6. Alignment of partial amino acid sequences of 111 kDa protein from *Xenopus* embryo with those of vitellogenins from other organisms.

embryo was identified as a protein originated from vitellogenin (VTG).

VTG is the sex-linked yolk precursor protein in oviparous vertebrates (van het Schip et al., 1987). Steroid-induced hepatic precursor of VTG is a phospholipoglycoprotein with molecular mass of about 460 kDa (Wallace, 1970). In the blood, VTG exists as a 200-kDa protein composed of two identical polypeptide chains. In chicken, there are two lipovitellins: one as a subunit of the four subunits of lipovitellin and the other as one of the two subunits of lipovitellin derived from parent VTG (Groche et al., 2000). In *Xenopus,* four different VTG molecules have been postulated and three distinct molecular forms (197, 188, and 182 kD) have been detected in the blood (Wiley and Wallace, 1978). VTG is the precursor form for those characterized yolk proteins. There are three main classes of yolk proteins: 111-121 kDa lipovitellin 1, 30.5-34 kDa lipovitellin 2 and 33-kDa phosvitin (Wallace 1985). Lipovitellin 1 has been further resolved into three components: α (121 kDa), β (116 kDa) and γ form (111 kDa) in 5% SDS-gel (Wiley and Wallace, 1981). Thus, the mAb-reactive 111 kDa protein of *Xenopus* and 116 kDa protein of chicken egg yellow resolved in 10% SDS gel (Fig. 1) belong to lipovitellin 1.

Reactivity of a protein with specific antibody depends on the presence of an antigenic determinant in the molecule. Since the major storage proteins of *Xenopus* and chicken eggs were all reactive with anti-polyubiquitin mAb, they may be ubiquitin-conjugated proteins or share common antigenic motifs in the molecule. In the immunoblot of tryptic polypeptides of 111 kDa protein, there were many bands reacting with the mAb (Fig. 5). This could be due to the conjugation of ubiquitins at multiple sites or multiple homologous antigenic sites in the 111 kDa protein. In order to find a common antigenic determinant between lipovitellin and polyubiquitin of *A. proteus,* we performed a homology search and found that they share 33.3% identity in amino acid sequence between 430-435 of lipovitellin and 65-70 of polyubiquitin. Thus, the 111-kDa protein appeared to be an ubiquitin-conjugated protein. Changes in the immuno-reactivity of the protein after deubiquitination will further confirm ubiquitin conjugation.

During embryogenesis, yolk platelets are considered to be degraded in the acidic compartment (Fagotto and Maxifield, 1994) by fusion with late endosomes (Komazaki and Hiruma, 1999). In this study, we confirmed a storage protein of the yolk platelet as lipovitellin 1, and that the protein was reactive to anti-polyubiquitin mAb. This implies that lipovitellin can be another example of ubiquitin-linked lysosomal degradation comparable to plasma membrane receptors (Bonifacino and Weissman, 1998) and carriers (Springael and Andre, 1998).

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