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# Gettin' down with ubiquitin: turning off cell-surface receptors, transporters and channels

Linda Hicke

*G-protein-coupled receptors and transporters in Saccharomyces cerevisiae are modified with ubiquitin in response to ligand binding. In most cases, the proteasome does not recognize these ubiquitinated proteins. Instead, ubiquitination serves to trigger internalization and degradation of plasma membrane proteins in the lysosome-like vacuole. A number of mammalian receptors and at least one ion channel undergo ubiquitination at the plasma membrane, and this modification is required for their downregulation. Some of these cell-surface proteins appear to be degraded by both the proteasome and lysosomal proteases. Recent evidence indicates that other proteins required for receptor internalization might also be regulated by ubiquitination, suggesting that ubiquitin plays diverse roles in regulating plasma membrane protein activity.*

Signal-transducing receptors, small-molecule transporters and ion channels reside at the plasma membrane, where their function is tightly regulated. The activity of cell-surface proteins is often regulated by controlling the level of protein localized at the plasma membrane. To reduce activity, proteins can be removed quickly from their site of action at the cell surface by endocytosis into the cell. In the case of signalling receptors, this is part of the mechanism that allows a eukaryotic cell to return to an unstimulated, basal state after receiving and responding appropriately to a signal. Internalized receptors can either be transported to the lysosome, where they are permanently inactivated by degradation, or be recycled to the plasma membrane to function again. Other plasma membrane proteins such as channels, transporters and permeases are regulated in a similar manner in response to a changing extracellular milieu. The downregulation of plasma membrane proteins is a crucial regulatory mechanism because a number of diseases result when downregulation mechanisms go awry at the cellular level. For instance, mutations that block the downregulation of epithelial $Na^+$ channels in human kidney cells result in an inherited form of hypertension[1], and cells that are unable to internalize activated receptors for epidermal growth factor (EGF) adopt the phenotypes of transformed cells[2,3].

It has become apparent that the polypeptide ubiquitin is a key participant in the downregulation of many plasma membrane proteins. Ubiquitin is a highly conserved 76-amino-acid polypeptide with a role in a fantastic variety of cellular functions (reviewed in Ref. 4). Historically, ubiquitin has played a leading role in protein degradation because it serves as a tag for the recognition of proteins by the multisubunit proteolytic particle known as the proteasome. Cytosolic proteins that are old or damaged, or proteins that undergo regulated destruction such as the cyclins and some transcription factors, are modified with a polyubiquitin chain, which is then recognized by the regulatory cap of the 26S proteasome. Subsequently, the targeted protein is thought to be unfolded, threaded into the interior of the proteasome and reduced to small peptides by the enclosed active proteolytic subunits.

Ubiquitin becomes covalently linked to substrate proteins via an isopeptide bond formed through its C-terminal glycine to the ε-amino group of lysine residues. Ubiquitination of proteins generally requires the action of either two or three enzymes. First, ubiquitin becomes activated by the formation of a high-energy thioester bond with a ubiquitin-activating enzyme (E1). Ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2), followed by


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**TABLE 1 – UBIQUITINATED PLASMA MEMBRANE PROTEINS**

| Protein | Refs |
|---|---|
| ***Saccharomyces cerevisiae*** | |
| ABC peptide transporter (Ste6p) | 8 |
| α-Factor receptor (Ste2p) | 9 |
| **a**-Factor receptor (Ste3p) | 10 |
| Uracil permease (Fur4p) | 15 |
| Multidrug transporter (Sts1p) | 42 |
| Galactose permease (Gal2p) | 43 |
| Maltose permease (Mal61p) | 44 |
| General amino acid permease (Gap1p) | 45 |
| **Animal cells** | |
| T-cell receptor ζ subunit | 20 |
| PDGF receptor | 21 |
| FcεRI receptor | 46 |
| c-Kit receptor | 47, 48 |
| EGF receptor | 49 |
| Fibroblast growth factor receptor | 50 |
| Colony-stimulating factor 1 receptor | 50 |
| Growth hormone receptor | 22 |
| Met tyrosine kinase receptor | 32 |
| Epithelial $Na^+$ channel | 35 |
| Complement receptor type 2 | 51 |

conjugation to a substrate protein, which often requires a third enzyme, a ubiquitin protein ligase (E3). Polyubiquitin chains are formed on proteins by the conjugation of additional ubiquitin moieties to one of several lysine residues in the ubiquitin molecules previously attached to the protein. Not only cytosolic proteins are modified with ubiquitin – integral membrane proteins are also substrates for ubiquitination. Membrane proteins located in the endoplasmic reticulum are ubiquitinated and degraded in a proteasome-dependent manner (reviewed in Ref. 5).

The first evidence that plasma membrane proteins are modified with ubiquitin was obtained when the N-terminal amino acid sequences of purified preparations of the growth hormone (GH) receptor and the platelet-derived growth factor (PDGF) receptor were determined. Two sequences were obtained from each receptor and, in each case, one of the sequences corresponded to the N-terminus of ubiquitin, suggesting that the protein was modified with ubiquitin. Later, it was shown that subunits of the T-cell receptor, and the GH and PDGF receptors, undergo ligand-stimulated ubiquitination on their cytosolic tails. It is now clear that a number of other plasma membrane proteins, both in *Saccharomyces cerevisiae* and animal cells, are modified with ubiquitin (reviewed in Refs 6 and 7; Table 1).

The function of ubiquitination of plasma membrane proteins is still a mystery in many cases. However, for several yeast proteins, the role of ubiquitination has been defined – and it has nothing to do with the proteasome. In yeast, ubiquitination serves to trigger the internalization of plasma membrane proteins into the endocytic pathway. This leads to their degradation in the vacuole. In animal cells, the situation is not as clear because a number of plasma membrane proteins that are ubiquitinated appear to be degraded through both the proteasome and lysosomal pathways. In at least one case, that of the GH receptor, ubiquitination machinery is required for internalization of the ubiquitinated receptor; however, it is unlikely that ubiquitination of the receptor itself triggers internalization[52]. This review describes the current understanding of the role of ubiquitin in the downregulation of plasma membrane proteins in eukaryotic cells and highlights some of the questions that we still need to answer.

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## Ubiquitin as an internalization signal in *Saccharomyces cerevisiae*

The first membrane protein reported to be ubiquitinated in yeast was the peptide transporter encoded by the *STE6* gene (Ste6p). Kölling and Hollenberg observed that Ste6p accumulated as a ladder of high-molecular-weight forms in a yeast mutant that cannot internalize plasma membrane proteins. They proved that these high-molecular-weight forms were ubiquitinated species and provided the first evidence suggesting a connection between ubiquitination and endocytosis[8]. Subsequently, a number of yeast cell-surface proteins have been shown to become modified with ubiquitin (Table 1).

Two of these proteins are G-protein-coupled signal-transducing receptors, the α-factor receptor and the **a**-factor receptor, which are expressed on yeast cells of opposite mating types. These receptors bind to mating pheromones, either α-factor or **a**-factor, and the receptor–pheromone interaction initiates a signal-transduction pathway that leads to a number of changes in the yeast cell that are required for mating. Both receptors are ubiquitinated constitutively. Upon ligand binding, they are further ubiquitinated and downregulated rapidly[9,10]. An important component of this inactivation is endocytosis of the receptor, and pheromone binding stimulates receptor endocytosis. Endocytosis of the receptors results in their transport to the vacuole, where they are permanently inactivated by degradation[11,12].

Although the α-factor receptor is modified with ubiquitin, the proteasome appears to play no role in its degradation. Instead, the receptor is degraded in the vacuole (Fig. 1). To determine why the receptor is ubiquitinated and whether ubiquitination plays any role in its vacuolar degradation, the internalization and degradation of the pheromone–receptor complex was investigated in yeast mutants in which components of the ubiquitination machinery are defective. Although yeast synthesize a large number of different ubiquitin-conjugating enzymes, mutants defective in the Ubc4p and Ubc5p conjugating enzymes are specifically unable to internalize radioactive α-factor[9]. Ubc4p and Ubc5p are also required for ubiquitination of the receptor, suggesting that ubiquitin addition to the receptor itself is required for its internalization. Proof of this idea came from experiments demonstrating that lysine residues are essential components of the internalization signals within the receptor tail[9]. Ubc4p and Ubc5p, as well as receptor lysine residues, are required for

ubiquitination and rapid vacuole-dependent degradation of the other pheromone receptor, the **a**-factor receptor, indicating that its internalization can also be triggered by ubiquitination[10,13]. Ubiquitin-dependent internalization has been demonstrated or is strongly suggested for a number of transporters and permeases expressed on the yeast cell surface (see Table 1). Experiments with the general amino acid permease and the uracil permease have established a role for another component of the ubiquitination machinery, the Rsp5p ubiquitin protein ligase, in plasma membrane protein ubiquitination and downregulation[14,15].

Why are the cytosolic tails of the pheromone receptors not recognized and degraded by the proteasome? At least part of the answer is the type of ubiquitin modification that the receptors receive. Efficient recognition and binding of proteins by the proteasome requires a polyubiquitin chain at least four subunits long[16]. The most common form of this chain appears to be linked through Lys48 of ubiquitin[17]. By contrast, the α-factor receptor is primarily mono- and di-ubiquitinated. Mono-ubiquitination of the receptor on a single lysine residue is sufficient for rapid rates of endocytosis[18] (Fig. 1). The **a**-factor receptor is also mono- and di-ubiquitinated[10]. The uracil permease, which undergoes regulated ubiquitin-dependent endocytosis, appears to be modified by short di-ubiquitin chains that are linked through Lys63. Although mono-ubiquitination is sufficient to support internalization of this permease, the formation of the Lys63 linkage is necessary for maximal rates of endocytosis[19]. Short polyubiquitin chains might be required on some internalized proteins to avoid steric hindrance of putative protein–protein interactions. The lack of a long polyubiquitin chain might preclude the recognition of cell-surface proteins by the proteasome and could protect the cytoplasmic domains, which contain internalization signals, from being inappropriately degraded by the proteasome. In animal cells, the ζ subunit of the T-cell receptor also appears to be mono-ubiquitinated and/or modified with short chains[20]. However, other receptors such as those for GH and PDGF, appear to be modified with multiple ubiquitin molecules[21,22]. Because these receptors carry multiple lysine residues in their cytoplasmic tails, it is not possible to determine whether ubiquitinated species carry long polyubiquitin chains or are ubiquitinated on multiple lysines with one or a few ubiquitin moieties.

Just as with cytosolic proteins that undergo regulated ubiquitination and degradation, plasma membrane protein ubiquitination appears to be positively regulated by phosphorylation. In addition to being ubiquitinated, the α-factor receptor cytoplasmic tail is phosphorylated on serine and threonine residues in response to pheromone binding[23], and both of these modifications are required for receptor downregulation[9,24]. In a truncated version of the α-factor receptor, three serines that are required for phosphorylation of the receptor tail are also required for receptor ubiquitination and internalization. Furthermore, mutants deficient in yeast homologues of casein kinase I are severely reduced in their ability to phosphorylate the receptor and are also unable to ubiquitinate and internalize it. Thus, phosphorylation of the receptor cytoplasmic tail precedes and positively regulates receptor ubiquitination. The phosphorylated serines could provide a site that is recognized by the ubiquitination machinery, or phosphorylation could induce a conformational change that allows access of the machinery to the tail lysines. Phosphorylation followed by

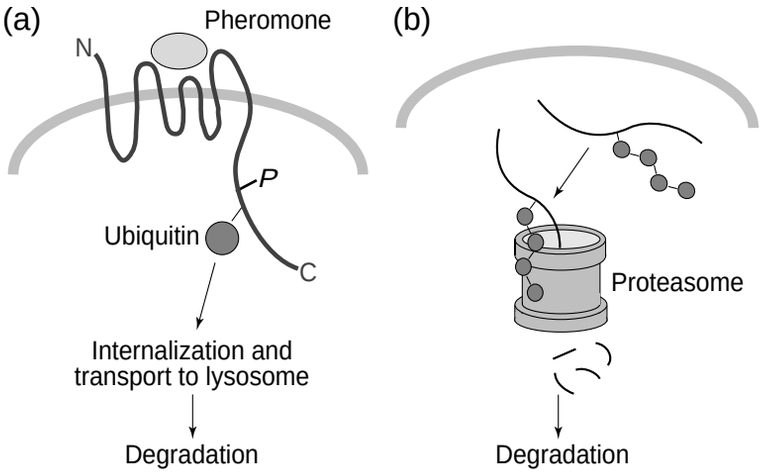


**FIGURE 1**

(a) Ubiquitin-dependent internalization of G-protein-coupled receptors in *Saccharomyces cerevisiae*. α-Factor receptor activated by pheromone binding is phosphorylated (*P*) and subsequently ubiquitinated. Modification of the receptor with a mono-ubiquitin moiety on a single lysine residue is sufficient to promote rapid internalization, followed by degradation in the vacuole. This type of ubiquitin modification is different from the polyubiquitin chain that is required for recognition of cytosolic proteins targeted for degradation by the proteasome (b).

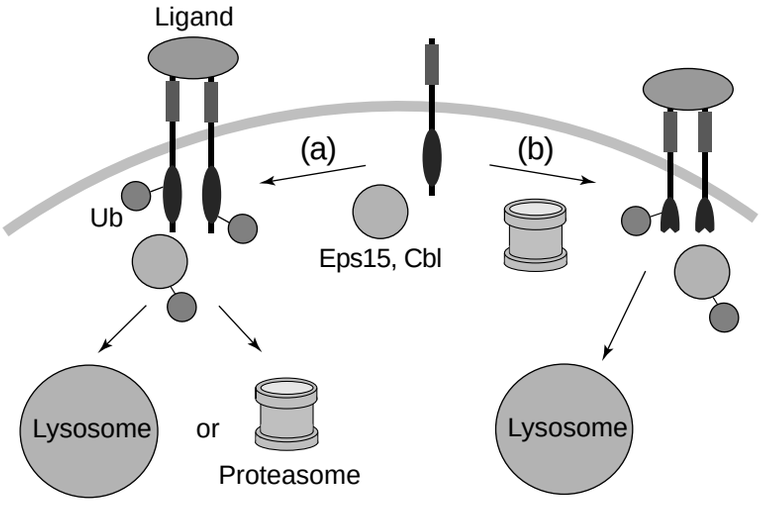


**FIGURE 2**

Ubiquitin-dependent downregulation of mammalian signalling receptors. This is a speculative model indicating that ligand-stimulated ubiquitination of mammalian receptors might play a role in both proteasome-mediated and lysosomal degradation. Individual receptors might be inactivated exclusively by one pathway or the other (a), or both proteolytic pathways could be required together to fully degrade a receptor (b). In addition, the ubiquitination of proteins such as Eps15 and/or Cbl might be required for their ability to promote the internalization and transport to the lysosome of activated receptors.

ubiquitination appears to be required for the endocytosis of a number of proteins in yeast. The uracil permease carries a PEST-like sequence that is required for permease phosphorylation, ubiquitination and internalization[25]. The **a**-factor receptor also contains a PEST-like sequence in its tail that is required for ubiquitination and internalization[10,13], although it has not been demonstrated that phosphorylation is required for either of these events.

### Mechanism of ubiquitin-induced internalization

The mechanism by which ubiquitination triggers plasma membrane protein internalization has not been defined. It is clear that mono-ubiquitin or short ubiquitin chains are sufficient to direct internalization. In addition, ubiquitin that is fused in-frame to a receptor lacking all posttranslational ubiquitination sites can serve as an efficient internalization signal[18]. Thus, it appears that the ubiquitin moiety itself, rather than the ubiquitin–receptor isopeptide linkage, is important for endocytosis. Perhaps the simplest explanation for ubiquitin-dependent internalization is that a ubiquitinated plasma membrane protein is recognized by a protein required for internalization, possibly a protein that serves an adaptor function to link ubiquitinated receptors to the endocytic machinery. The tyrosine-based and di-leucine internalization signals that have been identified in many mammalian receptors bind to adaptin complexes that link the internalized proteins to clathrin. Although at this time there is no evidence for a role of the classic adaptin homologues in endocytosis in yeast, ubiquitin might be recognized by a specialized type of adaptor. There are several examples of such specialized adaptors. One example is arrestin, the protein that recognizes activated, phosphorylated β-adrenergic receptor and couples it to clathrin to promote its internalization[26]. Another example is Nef, a protein of the human immunodeficiency virus (HIV) that induces by 5–10 fold the internalization of CD4 (a subunit of the T-cell receptor). Nef interacts with the CD4 tail and with adaptin subunits, and it has been suggested that it acts as a specific viral adaptor to facilitate the interaction of CD4 with the constitutive endocytic machinery[27]. A putative adaptor protein that recognizes and binds to mono-ubiquitin to promote the internalization of plasma membrane proteins would have to be protected from the relatively high concentration of free ubiquitin that is present in the cytosol. The ability of this adaptor to bind to ubiquitin might be regulated, or ubiquitin itself might not be sufficient for recognition. In the latter case, ubiquitin might be required together with other sequences in the receptor tail to promote internalization.

Another way in which ubiquitination could facilitate internalization would be to affect the location of a protein within the plane of the plasma membrane. There is growing evidence for the existence of plasma membrane subdomains, such as glycolipid–cholesterol rafts, and for the regulated movement of proteins into and out of these subdomains. Several receptors that undergo ligand-stimulated ubiquitination move into or out of glycolipid rafts upon ligand binding[28,29]. Ubiquitin could trigger endocytosis by inducing movement of a protein into a subdomain of the plasma membrane that is primed to invaginate to form a primary endocytic vesicle. Ubiquitination might facilitate this movement by inducing multimerization of modified receptors or by providing a necessary localization signal.

### Ubiquitin-dependent downregulation of growth factor receptors and epithelial $Na^+$ channels

Although G-protein-coupled signalling receptors in yeast clearly undergo ubiquitin-dependent internalization, there is no evidence yet for ubiquitination of mammalian G-protein-coupled receptors. However, as mentioned above, a variety of mammalian tyrosine kinase or kinase-linked receptors undergo ligand-stimulated ubiquitination at the plasma membrane (Table 1). These receptors include those for EGF, PDGF and GH, which, like the α-factor receptor, undergo rapid internalization and degradation in response to ligand stimulation.

The role of ubiquitin in mammalian receptor downregulation has been studied most thoroughly for the GH receptor. Using a temperature-sensitive Chinese hamster ovary cell mutant cell line, ts20, that is deficient in a ubiquitin-activating enzyme at the nonpermissive temperature (42°C), Strous and colleagues have demonstrated that the cellular ubiquitination machinery is required to internalize the GH receptor in response to ligand binding. By contrast, internalization of the transferrin receptor, which is internalized constitutively and recycled to the plasma membrane, is not impaired in ts20 cells at 42°C[22]. The GH receptor also undergoes ligand-stimulated ubiquitination; however, the ubiquitin-dependent internalization of a truncated GH receptor does not require lysine residues in the receptor cytoplasmic tail. Instead, a 10-amino-acid motif that lacks lysine residues is required for receptor internalization. This motif is also required for receptor ubiquitination, prompting the suggestion that GH receptor internalization requires interaction of the receptor with the ubiquitination machinery but not receptor ubiquitination itself. Receptor ubiquitination might merely be a byproduct of this interaction[52].

Another observation is that the degradation of several mammalian receptors that are known to undergo ligand-stimulated ubiquitination appears to be impeded by inhibitors of the proteasome as well as by agents that block lysosomal degradation. This has been reported for the PDGF receptor and the Met tyrosine kinase receptor[31,32]. It is possible that a fraction of these receptors is degraded by the proteasome and another fraction is degraded in the lysosome. Alternatively, both the proteasome and lysosomal proteases might function to destroy different parts of the receptor. Yet another possibility is that proteasome-dependent degradation of a protein other than the receptor might be required for efficient targeting and transport to the lysosome (Fig. 2).

The epithelial $Na^+$ channel (ENaC) is another type of plasma membrane protein that is ubiquitinated

and requires the cellular ubiquitination machinery for its degradation. In this case, interaction of the channel with ubiquitinating enzymes has been linked directly to a human disease[33]. Liddle's syndrome is a form of inherited hypertension that is due to increased activity of the ENaC. Much of the increased activity is due to an increased number of channels at the cell surface. All the mutations associated with Liddle's syndrome identified to date are point mutations or deletions in the proline-rich regions of the cytoplasmic tails of the channel β and γ subunits. In wild-type channels, these sequences are necessary for interaction with Nedd4, the mammalian homologue of the Rsp5 ubiquitin protein ligase[33]. The channel is downregulated by endocytosis, and it has been shown that mutations in the proline-rich regions of the channel tails similar to those required for Nedd4 interaction also impair the ability of the channel to be internalized[34].

The α and γ subunits of the ENaC are multiubiquitinated, and mutation of lysine residues in the subunit tail reduces the turnover rate of the channel, indicating that ubiquitination of the channel itself is required for its downregulation. Like some of the growth-factor receptors, inhibitors of both the proteasome and the lysosomal proteolytic system retard degradation of the channel subunits. To explain this observation, Rotin and colleagues have suggested that unassembled channel subunits are degraded by the proteasome, and that fully assembled ENaC complexes that reside at the plasma membrane are degraded primarily in the lysosome[35].

## Role of ubiquitin in activating the endocytic machinery

Ubiquitination of plasma membrane proteins clearly functions to trigger their internalization in *S. cerevisiae*. However, several observations suggest that the ubiquitination of proteins other than the internalized protein itself plays a role in receptor endocytosis in yeast and mammalian cells. First, although the activity of a ubiquitin-activating enzyme is clearly required for internalization of the GH receptor, only a small fraction of stimulated receptor is ubiquitinated, and the ubiquitination of the receptor itself is not required for internalization. Furthermore, blocking internalization of the receptor appears to prevent its ubiquitination, which would not be expected if ubiquitination of the receptor itself precedes and is required for endocytosis[30]. Second, in yeast, the Rsp5p ubiquitin protein ligase is required for the endocytosis of a fluid-phase marker[36] and for the internalization of proteins that carry non-ubiquitin-dependent signals (R. Dunn and L. Hicke, unpublished). These observations suggest that some other protein must be ubiquitinated for internalization to occur or that the ubiquitin protein ligase plays some role in addition to catalysing ubiquitination. Finally, Eps15, a protein that is required for the internalization of the transferrin receptor in animal cells and that is a component of clathrin-coated pits and vesicles, becomes mono-ubiquitinated in response to stimulation of cells with EGF[37], an event that also stimulates internalization of the EGF receptor. Two yeast proteins that are similar to Eps15, End3p and Pan1p, are required for endocytosis[38].

Another protein that might play a role in ubiquitin-dependent internalization is Cbl, a protooncogene product. Cbl is a 120-kDa protein that has been well characterized as a negative regulator of the activities of numerous tyrosine kinase and kinase-linked receptors. Cells that overexpress the Cbl protein show a threefold stimulation of ligand-induced ubiquitination of the PDGF receptor. Degradation of cell-surface PDGF receptors is also enhanced in the Cbl-overexpressing cells[39]. As with the PDGF receptor, the colony-stimulating factor 1 (CSF-1) receptor undergoes ligand-stimulated ubiquitination and, interestingly, Cbl itself is ubiquitinated in response to the stimulation of cells with CSF-1. Furthermore, CSF-1 induces translocation of ubiquitinated Cbl from the cytosol to membranes[40]. The *Caenorhabditis elegans* homologue of Cbl, SLI-1, interacts genetically with UNC-101, a homologue of the medium (μ) chain of the adaptin complexes that are required for clathrin-dependent internalization[41]. Although there are many pieces missing in this puzzle, these observations together suggest that ubiquitinated Cbl plays a role in enhancing the ubiquitin-dependent downregulation of stimulated signalling receptors (Fig. 2).

## Concluding remarks

At least eight plasma membrane proteins in yeast and eleven proteins in animal cells are ubiquitinated at the cell surface. In yeast, all endogenous proteins that are known to be internalized use ubiquitin as an internalization signal. In animal cells, signalling receptors and subunits of ion channels are ubiquitinated, and, in several cases, the ubiquitination machinery is required for downregulation of the protein. The function of ubiquitination as an internalization signal might be conserved in the downregulation of some mammalian cell-surface proteins; however, the modification of these proteins with ubiquitin might also play diverse roles in both targeting to the lysosome and in proteasome-mediated downregulation. The mechanism by which ubiquitin acts to trigger receptor internalization is an important area of future work, as is defining other proteins that are regulated by ubiquitination and are important in downregulating cell-surface proteins.

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