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Protein degradation machinery is present broadly during early development in the sea urchin

Vanesa Zazueta-Novoa, Gary M. Wessel *

Department of Molecular and Cellular Biology & Biochemistry, Brown University, Providence, RI 02912, USA

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ABSTRACT

Ubiquitin-dependent proteosome-mediated proteolysis is an important pathway of degradation that controls the timed destruction of cellular proteins in all tissues. All intracellular proteins and many extracellular proteins are continually being hydrolyzed to their constituent amino acids as a result of their recognition by E3 ligases for specific targeting of ubiquitination. Gustavus is a member of an ECS-type E3 ligase which interacts with Vasa, a DEAD-box RNA helicase, to regulate its localization during sea urchin embryonic development, and Gustavus mRNA accumulation is highly localized and dynamic during development. We tested if the core complex for Gustavus function was present in the embryo and if other SOCS box proteins also had restricted expression profiles that would inform future research. Expression patterns of the key members of the proteasomal function, such as the E3 core complex which interacts with Gustavus, and other E3-SOCS box proteins, are widely spread and dynamic in early development of the embryo suggesting broad core complex availability in the proteasome degradation pathway and temporal/spatial enrichments of various E3 ligase dependent targeting mechanisms.
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The ubiquitin proteasome pathway (UPP) is integral to the normal function of eukaryotic cells (Joazeiro and Weissman 2000; Tanaka et al., 2001; Pan et al., 2004; Dohmen, 2004; Pickart and Eddins, 2004; Aragon, 2005; Mani and Gelmann, 2005; Denison et al., 2005). It is ATP dependent and involves the covalent attachment of chains of ubiquitin molecules to target substrates. Proteins modified in this manner are recognized by the proteasome, a 26S multiprotein complex that catalyzes the breakdown of poly-ubiquitinated proteins. The major functions of the pathway are rapid removal of cell cycle proteins, regulation of gene transcription by degradation of transcription factors, quality control mechanism to degrade abnormally folded or damage proteins, generate amino acids for new protein synthesis, and others (Lecker et al., 2006). Several protein functions are also regulated by mono-ubiquitin attachment, and are independent of degradation, including, trafficking, chromatin re-structuring, and the modulation of protein function. Ubiquitin attachment to proteins is referred to as ubiquitination (or ubiquitylation; Sawasdikosol et al., 2000; Tyers and Jorgensen, 2000; Marmor and Yarden, 2004). Ubiquitination of target proteins proceeds in a stepwise format involving E1, E2 and E3 enzymes. E1, an ubiquitin-activating enzyme, uses ATP to catalyze the covalent transfer of ubiquitin (Ub) to the active site cysteine of an E2 Ub-conjugating enzyme. The E2 enzyme then interacts with an E3 Ub-protein ligase, which is the key enzyme in the process because it recognizes a specific protein substrate and catalyzes the transfer of activated ubiquitin to it, resulting in the ubiquitination of target proteins on specific lysine residues (Pickart, 2001; Burger and Seth, 2004; Fang and Weissman, 2004; Canning et al., 2013). Generally, the addition of one to four ubiquitin molecules to a target protein leads to a change in its localization and/or function. The addition of many ubiquitin molecules (poly-ubiquitination) leads to protein degradation by the 26S proteosome. Specificity in targeting proteins for ubiquitination lies mostly in the E3 enzyme (Amemiya et al., 2008).

Cullin-RING E3 Ub-ligases (CRLs) comprise the largest class of E3 Ub-ligases (Petroski and Deshaies, 2005). CRLs contain a substrate specificity receptor that binds the ubiquitinated target and a RING (Really Interesting New Gene) protein that is involved in recruiting an E2-conjugating enzyme. RING proteins and particular substrate specificity receptors are brought together by scaffold Cullin proteins. The two best characterized subfamilies are the SCF (Skp1/Cullin/F-box) and ECS (Elongin B/C–Cullin–SOCS box) E3 Ub-ligase families, the latter of which includes the von

Abbreviations: UPP, ubiquitin proteasome pathway; SOCS, suppressor of cytokine signaling; RING, Really Interesting New Gene; Cul-5, Cullin 5; Ub, ubiquitin; ECS, Elongin B/C–Cullin–SOCS box.

* Corresponding author. Address: 185 Meeting Street, Box G, Brown University, Providence, RI 02912, USA.

E-mail address: rhet@brown.edu (G.M. Wessel).

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Hippel–Lindau (VHL) tumour suppressor and the wider SOCS box-containing protein families (Linossi and Nicholson, 2012). SCF Ub-ligases are multiprotein complexes, and every protein in a SCF complex is homologous to a component found in ECS E3 Ub-ligases. In SCF complexes, the F-box has been shown to play a comparable role to SOCS box in ECS complexes (Kibel et al., 1995; Kamura et al., 1998; Patton et al., 1998; Zhang et al., 1999). In the SCF complex, the F-box of Skp2 binds to the adaptor protein Skp1 which in turn, binds to the cullin family member, and forms a bridge between the Cullin and the F-box (Carrano et al., 1999; Kugler et al., 2010). This is similar to the connectivity in ECS complexes, with the SOCS box or VHL box binding to elongin C, which in turn, binds to the cullin family member Cul-5 in the case of SOCS box proteins and Cul-2 in the case of VHL box proteins (Kamura et al., 1998; Zhang et al., 1999; Kile et al., 2002). For SOCS families of proteins, assembly with the E2-Ub-conjugating machinery Elongin B/C–Cullin 5 complex is predicted through the SOCS box LPXP motif, which confers Cul-5 selection (Bullock et al., 2006).

The SOCS box is a conserved domain that was initially discovered in SH2 domain-containing proteins of the suppressor of cytokine signaling (SOCS) family (Starr et al., 1997). SOCS-1 was identified simultaneously on the basis of its interaction with JAKs, its antigenic cross-reactivity to STATs and its ability to inhibit cytokine signaling. SOCS-1 has a central SH2 (Src homology 2) domain and is most similar to cytokine-inducible SH2-containing protein (CIS) and has a high degree of sequence similarity in a 40 amino acid C-terminal region that was named the SOCS box. Since then, other members of SOCS family (SOCS-2 to SOCS-7) have been identified in mouse and human. All these proteins contain SH2 domains in their N-termini and SOCS box domains in their C-termini (Hilton, 1999).

In addition to the canonical SOCS proteins, several additional SOCS protein families have been identified. Rather than containing a SH2 domain upstream of the SOCS box, these proteins contain other domains implicated in protein–protein interactions. The major families of proteins that contain a SOCS box domain are the ankyrin-repeat proteins (ASBs), the SPRY domain-containing proteins (SSBs), the WD40 repeat-containing proteins (WSBs), the Neuralized family of proteins and a previously described family of small GTPases, Rar and its relatives (Hilton, 1999).

Gustavus is an E3 Ub-ligase identified in *Drosophila* and involved in the balance of Vasa ubiquitination controlling pole plasm accumulation (Styhler et al., 2002; Kugler et al., 2010). It contains a B30.2/SPRY sequence comprising a single domain that biochemically interacts with Vasa protein *in vitro* and *in vivo* (Woo et al., 2006a,b; Styhler et al., 2002; Kugler et al., 2010) and a SOCS box that interacts with Elongin B/C–Cullin 5 complex (Woo et al., 2006a; Kugler et al., 2010). Vasa is a conserved DEAD-box RNA helicase associated with germ-line development and is expressed in multipotent cells in many animal species (Lasko and Ashburner, 1988; Raz, 2000; Gustafson and Wessel, 2010). During embryogenesis of the sea urchin, vasa transcripts are uniformly distributed through blastula formation, followed by specific expression in the small micromere lineage during gastrulation, but the Vasa protein remains uniformly distributed through only the first three cleavage divisions. In the fourth cleavage division it is enriched in the four micromeres and subsequently in the small micromeres (Juliano et al., 2006; Voronina et al., 2008). Gustafson et al. (2011) provided evidence that gustavus appears to degrade Vasa in all cells except the small micromeres thereby limiting vasa accumulation in a general background of Vasa mRNA translation (Gustafson et al., 2011).

Expression of some proteasomal components and members of the proteasome-dependent degradation system were described previously in specified adult tissues from the sea urchin (Loram and Bodnar, 2012). However, the members of the ubiquitination machinery complex in the embryo have not been documented. We were intrigued by the expression of Gus, and hypothesized that other SOCS-box proteins would have restricted, and perhaps informative expression profiles that might lead to their functional analysis. We also felt compelled to test if the other machinery for Gus function was present in the embryo – machinery that would be essential for the proposed functions of Gus in vasa regulation and of widespread function for general SOCS box protein functions.

1. Results and discussion

We obtained sequences of *Drosophila melanogaster*, human and mouse proteins of all tested genes from NCBI (http://www.ncbi.nlm.nih.gov/). Orthologous protein sequences from sea urchins were found by BLAST analysis against the published Sea Urchin Genome Database (Spbase.org). The top hits were used for reciprocal BLAST analysis to the nonredundant NCBI database to test orthology and to identify specific protein domains. *Strongylocentrotus purpuratus* Gene Expression Database (Spbase.org; Cameron et al., 2009) was used to reflect their abundance (an estimate of copy numbers per embryo as calculated by normalization to specific mRNAs at 48 h) and differential expression (Wei et al., 2006). Abundant mRNAs show signal intensities between 25,000 and 200,000 AU (Arbitrary Units) whereas those which are expressed in only a few cells during development are correspondingly lower (between 200 and 300 AU). These data provided us rough estimates of mRNA abundance that were helpful in identifying interesting candidate genes. The list of primers used for polymerase chain reaction (PCR) amplification of each gene in sea urchin and the length of RNA *in situ* probes are shown (Table 1).

1.1. Multiprotein complex ECS-type Ub-ligase components are broadly present during early development of embryos

The SOCS box motifs interact specifically with Elongin C (Kamura et al., 1998; Zhang et al., 1999) whereas Elongin B acts to stabilize the complex and has only minimal interaction with the SOCS box itself (Bullock et al., 2006). Further, one might predict that the Elongin B, Elongin C, and Cullin 5 expression profiles overlap the cells expressing Gustavus. The *S. purpuratus* Gene Expression database (Spbase.org) showed that Elongin B transcripts are present throughout development but less abundant than Gustavus transcripts (Table 1). It is clear that Elongin B transcripts accumulate throughout the early embryo. At mid-gastrula, Elongin B transcripts are enriched in the animal pole and in the blastopore (Fig. 1, red arrows). In late gastrulae, transcripts are present in the archenteron of the embryo, and within the highly prolific ciliary band (Fig. 1, red arrows). Elongin C transcripts are more abundant than Elongin B mRNAs in eggs and early cleavage divisions. During gastrulation, the areas of mRNA signal are similar to those observed for Elongin B transcripts, mainly in highly proliferating cells of the late gastrulae and in plutei (Fig. 1, red arrows). Cullin 5 mRNA is the least detectable of the mRNAs that contribute to the Elongin B/C–Cullin 5 protein complex; transcript signal is present throughout the egg and early embryos, but it decreased in mes-blastula stage. In early/mid-gastrula, a slight enrichment is seen in the middle region of the archenteron (Fig. 1, red arrow). In plutei, some transcript enrichment is seen for Cullin 5 in the gut of the larva (Fig. 1, red arrow), as also observed with Elongin B and Elongin C transcripts suggesting an important role for the expression of these components during this specific stage in the embryo. The data on expression of these genes supports the hypothesis that these gene products are widely present to target proteins for proteasome-mediated degradation.

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