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SUMO as a nuclear hormone receptor effector

New insights into combinatorial transcriptional regulation

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Animal development is driven by robust, cell-specific gene expression programs. Understanding mechanistically how a single transcription factor (TF) can govern distinct programs with exquisite precision is a major challenge. We view TFs as signal integrators, taking information from co-regulator interactions, post-translational modifications, other transcription factors, chromatin state, DNA sequence and in some cases, specific noncovalent ligands, to determine the collection of genes regulated by a TF at any given time. Here, we describe a reductionist approach to combinatorial transcriptional regulation, focusing on a single *C. elegans* TF, the nuclear hormone receptor NHR-25, and a single post-translational modification, SUMO. We suggest that the ratio of sumoylated to unsumoylated NHR-25 could specify a switch-like cell-fate decision during vulval development. Direct examination of this "SUMO ratio" in vivo is challenging and we discuss possible solutions going forward. We also consider how sumoylation of multiple substrates might be coordinated during vulval development. Finally, we note that iteration of this approach could leverage our sumoylation findings to define the roles of other effectors of NHR-25 in the developing vulva and in other tissues.

Keywords: NHR-25, SMO-1, sumoylation, cell fate, vulva development, gene expression, nuclear hormone receptor, transcription, signaling, *C. elegans*

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Introduction: Combinatorial Regulation of Gene Expression

Animal development demands intricate coordination of gene regulation across many cell types, tissues and organs. Genes must be expressed at the correct time and place during development. Aberrations in this regulation can cause developmental abnormalities or animal death. Transcription factors (TFs) play a major role in coordinating metazoan gene regulation, creating gene expression networks that govern development, physiology, homeostasis and other complex biological processes. TFs must integrate information from diverse sources, such as co-regulatory proteins and other TFs, post-translational modifications, chromatin modifications, the DNA sequence to which they bind, and in some cases, noncovalent ligands. The integrated output of a given TF is the precise and reproducible activation or repression of tens to thousands of genes, with the expression of each gene specified by its unique combinatorial input code.

Given the vast permutations possible from these myriad inputs, unraveling combinatorial regulation of gene expression is a daunting challenge. In a recent *PLoS Genetics* article,[1] we described a reductionist approach to this question. We focused on a single, evolutionary conserved, broadly expressed TF, the *C. elegans* nuclear hormone receptor NHR-25, examining the consequences of a single post-translational modification, sumoylation, on development of a single organ, the vulva. Our results enhance our understanding of mechanisms by which post-translational modifications modulate TF activities at the cellular and organ levels during development in *C. elegans* and other metazoans. In turn, this approach will provide insights on the

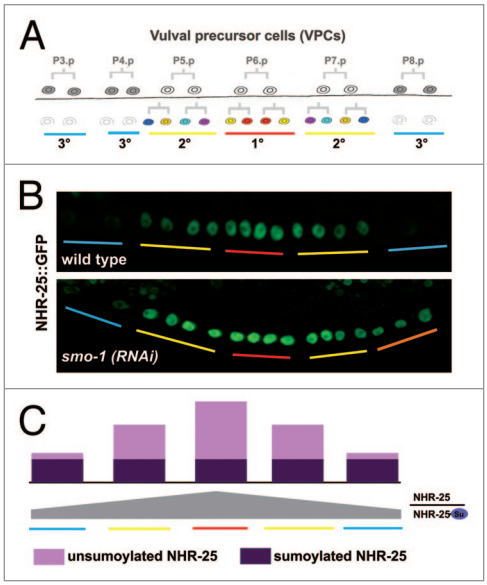

Figure 1. Sumoylation of NHR-25 controls 3° cell fate. (A) During vulval development six vulval precursor cells (VPCs) named P3.p to P8.p undergo a coordinated series of divisions, fate choices, migration and fusions to produce the final 22-cell organ. The P3.p, P4.p, and P8.p cells divide once and their daughter cells execute a 3° fate, indicated by the blue bars. The remaining VPCs divide an additional two times with P6.p executing a 1° fate (marked by the red bar) and P5.p and P7.p executing 2° fates, marked by the yellow bars.[10] (B) A *smo-1*-dependent gradient of NHR-25 forms during the course of vulval development. Using an integrated, low-copy NHR-25::GFP translational fusion we observed decreasing levels of NHR-25 in 2° and 3° cells, relative to 1° cells. This gradient is abolished following inactivation of *smo-1*, and the 3° cells execute a 1° and/or 2° fate (marked by the orange bar). (C) The "SUMO ratio" model. The NHR-25 gradient combined with constant, limiting sumoylation results in high levels of unsumoylated NHR-25 relative to sumoylated NHR-25 in 1° cells. As the NHR-25 levels drop in 2 ° and 3° cells, an increasing fraction of the total NHR-25 protein is sumoylated. At a particular set point, there is a sufficient amount of sumoylated NHR-25 relative to unsumoylated NHR-25 to allow adoption or maintenance of the 3° fate. Modified from Ward et al., 2013.

impact of NHR-25 and its sumoylation in other cells, other organs and other points in development, as well as the effects of other signaling inputs on these activities. In this commentary, we (i) compare the mechanisms of sumoylation of NHR-25 to its mammalian homologs (SF-1 and LRH-1), and (ii) consider challenges and possible solutions for testing our model that sumoylation of NHR-25 produces a switch-like behavior that regulates developmental processes.

Sumoylation of NR5 Family Nuclear Hormone Receptors

Humans and *C. elegans* share similar small ubiquitin like modifier (SUMO) proteins, which serve as post-translational modifications directly conjugated onto substrate proteins, similar to ubiquitin. Sumoylation affects a broad range of cellular processes including DNA repair, transcription, chromosome dynamics, and metabolism.[2] SUMO is conjugated onto lysines in substrate proteins using E1 and E2 enzymes, similar to ubiquitination.[2] Unlike ubiquitination, E3 enzymes are not essential for SUMO conjugation, and sumoylation is not a typical signal for proteasomal degradation.[2] Like all post-translational modifications, SUMO is a dynamic mark. Indeed, the SUMO protease that renders SUMO competent for conjugation can also release SUMO from substrate proteins.[2]

We explored the interplay between the single *C. elegans* SUMO gene, *smo-1*, and the conserved nuclear hormone receptor, *nhr-25,* during vulval development.[1] We identified that SMO-1 interacts with NHR-25 through an unbiased yeast two-hybrid screen for novel regulators of NHR-25. In vitro biochemical analyses revealed that NHR-25 is sumoylated, and we demonstrated that sumoylation of NHR-25 is important for ensuring correct cell fate determination or maintenance during vulval development. Our data show that sumoylation restricts NHR-25 activity in vivo: a reporter gene that contains NHR-25 binding elements is hyper-activated in *smo-1* deficient animals. This observation was further supported in heterologous cell culture assays: mutations in NHR-25 at SUMO interacting sites enhanced the induction of the luciferase reporter gene expression. Consistent with these observations, *smo-1* inactivation results in vulval cell fate defects (e.g., multivulva induction) which phenocopy the overexpression of an NHR-25 protein which cannot be sumoylated.

The mammalian orthologs (SF-1 and LRH-1) and *Drosophila melanogaster* orthologs (Ftz-F1) of NHR-25 are sumoylated as well, demonstrating strong evolutionary conservation of this regulatory input across the 600–1200 million years since divergence of the last common ancestor of humans and nematodes.[4-7] The SUMO acceptor sites on the human orthologs occur at corresponding positions in NHR-25, with the site near the DNA-binding domain being duplicated in NHR-25. NR5 family member sumoylation has been reported to affect both receptor localization and function. Sumoylation of LRH-1 alters the location of LRH-1 within the nucleus, driving it to promyelocytic leukemia (PML) protein nuclear bodies, away from chromatin.[4,6] Sumoylation of SF-1 affects DNA binding site preference (as discussed below), but not localization.[3,5] While the NHR-25::GFP fusion protein is constitutively nuclear, similar to SF-1 and LRH-1,[4,8] we saw no obvious changes in sub-cellular localization of NHR-25::GFP in vulval cells following *smo-1* depletion; rather, we observed ectopic expression of NHR-25::GFP, implying that SMO-1 may reduce NHR-25 protein levels in vulval cells.[1]

Sumoylated SF-1 binds in vitro to a nine base-pair consensus binding sequence, and with reduced affinity to specific target sites carrying non-consensus motifs.[5] An unsumoylatable SF-1 protein displayed a dominant gain-of-function in mice, causing endocrine abnormalities and cell fate changes, presumably through binding genomic sites deviating from the consensus.[3] NHR-25 can bind to an NR5 family consensus site upstream of the *lin-3* gene,[9] and our studies demonstrated that sumoylation impaired NHR-25 binding to and activity at canonical SF-1 response elements,[1] whereas sumoylation of SF-1 has no effect on binding to this site in mammalian cells.

How Does Sumoylation Regulate Cell Fate in Vulval Precursor Cells?

The *C. elegans* vulva is a paradigm of organogenesis. From six vulval precursor cells, the mature 22 cell organ is formed through a coordinated series of cell division, fate choice, migration, and

fusion, over an 8–10 h period beginning in the early L3 stage of larval development (**Fig. 1A**).[10] Tthe six precursor cells adopt one of three fates termed 1°, 2°, or 3°; NHR-25 sumoylation is important for ensuring correct cell fate promotion and/or maintenance in the 3° lineage.[1,10] How does NHR-25 sumoylation regulate cell fate? A model that accounts for our observations is that sumoylation is limiting in these cells. Although SUMO proteins are robustly expressed throughout eukaryotes, human Sumo1 was shown to be limiting in sumoylation of the viral protein BZLF during infection.[12] Consistent with this notion, the single *C. elegans* SUMO protein, SMO-1, is expressed evenly across the vulval precursor cells.[11]

If sumoylation is indeed limiting, a fixed amount of NHR-25 would be sumoylated across the vulval precursor cells (**Fig. 1C**).[1] Increasing NHR-25 levels would not affect the amount of sumoylated NHR-25 in the cell (**Fig. 1C**). Instead, an increasingly larger fraction of NHR-25 would be unsumoylated (**Fig. 1C**). Thus, the ratio of unsumoylated to sumoylated NHR-25, rather than the absolute amount of sumoylated NHR-25, could generate a robust, switch-like developmental decision (**Fig. 1C**). The switch point could be set either at steady-state, or through a kinetic determinant such as a molecular timer.[13-17] When the unsumoylated form is in excess a particular cell fate might be adopted (1° or 2°), and when the sumoylated form is in excess an alternate cell fate (3°) is adopted. Notably, such a scheme could sensitize the system to levels of other sumoylated proteins. Overexpression of another highly sumoylated substrate, such as RanGAP or LIN-11,[11,18,19] might squelch NHR-25 sumoylation, and phenocopy the 3° cell phenotype caused by loss of *smo-1* or overexpression of NHR-25.

The Challenge of SUMO Ratios

The biggest question raised by our work is what proportion of NHR-25 (or any other protein for that matter) is sumoylated at any given time? This question is difficult enough to address using cell culture methods in which homogenous populations of cells can be assayed. The question becomes significantly more challenging when different cell types and time points in development are considered. Moreover, as sumoylation is reversible, sumoylation ratios may be highly dynamic. Although it may be illuminating to examine E1, E2 and SUMO protease expression across the vulval precursor cells, protein levels may not correlate with activity. Development of methodologies to measure enzymatic outputs of sumoylation and desumoylation enzymes in vivo, are sorely needed. Current methods used to monitor sumoylation in vivo typically give static snapshots. Mass spectrometry-based methodologies can report on global sumoylation and on changes in response to stimuli, however determination of sumoylation ratios would require internal peptide standard spanning the modification site. Alternatively, sumoylation ratios could be deduced from quantitative immunoblots, assuming the sumoylated species is sufficiently represented. Given the emerging power of phage-display generated Fab antibodies,[20,21] it may be possible to generate an antibody highly specific to the sumoylated NHR-25 isoform(s). Clearly, however, such approaches are well-suited for homogenous populations of cells rather than whole animal extracts.

A proximity-based fluorescent assay is potentially a promising approach to probe in vivo sumoylation ratios. We unsuccessfully attempted to use a bimolecular fluorescence conjugation system (split-Venus) in which the Venus fluorophore is separated into two non-fluorescent fragments, which are then fused onto a pair of interacting proteins.[22] Failure may have been due to insufficient fluorophore intensity, non-optimal placement within NHR-25 or SUMO of the Venus fragment fusions, or to a short-lived NHR-25-SUMO interaction in vivo. A possible alternative is an ultrabright FRET system in which each partner would be tagged with a fluorophore, thus providing measures both of the sumoylation substrate protein and the ratio of sumoylated to unsumoylated protein in different cells. The interaction of SUMO and a given substrate could in principle be followed through development in a living animal. Ideally, such FRET tags would be integrated into the endogenous loci using CRISPR/Cas9 genome editing.[23] Such a tool would determine whether sumoylation was indeed limiting across the vulval precursor cells, and could assess the consequences of modifying levels of sumoylation and desumoylation enzymes and substrate levels.

Regulation of Sumoylation during Vulval Development

We could not detect NHR-25 sumoylation by western blot or immunoprecipitation-western blots from worm lysates, and NHR-25 was not identified in a proteomic screen of *C. elegans* sumoylated proteins.[24] Yet, our in vivo NHR-25 activity reporter indicates *smo-1* depletion causes activation of NHR-25 in major tissues in which NHR-25 is expressed.[1] A "memory" of NHR-25 sumoylation while the protein is actually unsumoylated is an attractive model; this idea was invoked to explain the "SUMO enigma": the paradoxical discrepancy between the functional importance of sumoylation of a given substrate and the difficulty in biochemically detecting its sumoylation.[25] As sumoylation is dynamic, SUMO can be removed from a substrate but perhaps imprint a "memory" on the protein or complex. Thus the function of the protein/complex can be regulated by sumoylation without depleting the limiting amounts of SUMO. Potential mechanisms in which a sumoylation "memory" could be imparted include altered subcellular localization, SUMO-dependent post-translational modifications, recruitment of co-regulators, or altered complex composition.[25,26] Sumoylation "memory" would provide an interesting mechanism to extend the functional output of this single modification and allow the regulatory impact of sumoylation to persist.

Many of the best-characterized *C. elegans* sumoylation substrates are involved in vulval development.[11,27-29] It is clear that sumoylation must be correctly controlled temporally and spatially, as loss of LIN-11 sumoylation or overexpression of an allele

that mimics constitutive sumoylation both caused distinct vulval defects.[11] It is unclear, however, how sumoylation of multiple substrates during organogenesis might be separately modulated from a limiting pool of SUMO and perhaps a handful of E3s. Substrate localization or scaffolding may bring specific substrates into proximity of sumoylation machinery, thus organizing sumoylation pathways similarly to those for protein kinases. Sumoylation of multiple components of a complex is another route of regulation. Three of the ten dosage compensation complex proteins are sumoylated, which is thought to enhance interactions between factors that target this complex to the X chromosome and condensin subunits.[30] Interestingly, sumoylation of these condensin subunits is specific to the dosage compensation complex; sumoylation did not occur in the context of chromosome condensin complexes involved in chromosome segregation.[30] The notion of coordinated complex sumoylation in response to developmental stimuli, in turn producing enhanced complex formation and function, is a framework concept from which to view global sumoylation during vulval development.

Perspectives

Determination that sumoylation regulates NHR-25 opens new avenues for future investigation in which sumoylation (or the memory of it) can serve as a "lever" for exploration of the roles of other post-translational modifications and co-regulators, which together, serve as determinants of NHR-25 activity in vulval precursor cells and in other tissues. Building on this reductionist strategy will inform our understanding of how these signals are integrated by NHR-25 during development and will provide new insight into combinatorial regulation of transcription.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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