

Polyamines in spermiogenesis: Not now, darling

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“Interesting processes invariably employ interesting biochemistry,” declares Marc Kirschner (1). From this statement, one may readily conclude that antizyme (AZ) deserves our attention. AZ is the central element in a feedback loop that controls cellular polyamines. AZ is interesting for a number of reasons. First, AZ production requires the exercise of a remarkable mechanism, translational frameshifting. Second, AZ has the unique ability to cause proteasomal degradation of a protein target without using ubiquitin, thereby evoking another Kirschnerian dictum: Labile proteins are important and important proteins are labile. First identified as an inducible biochemical activity that inhibits a specific enzyme target (2), recent developments have deepened our understanding of AZ biochemistry and physiology and broadened our appreciation of the AZs as a protein family conserved in structure and means of production. In this issue of PNAS, Ivanov *et al.* (3) describe a novel AZ family member, termed AZ3. It is expressed only in the testes and is there restricted to the post-meiotic stages of spermatogenesis. Tosaka *et al.* have independently described similar findings (4), using a gene (they term *OAZ-t*) cloned from a haploid-germ-cell-specific library. The pattern of AZ3 expression suggests that it acts to sharply limit polyamine accumulation in cells that have finished DNA synthesis and meiotic reduction and are about to be remodeled into mature spermatozoa. The reported observations imply a need for adroit control of polyamines in spermatogenesis.

Role of AZ in Polyamine Metabolism. Cells make, transport, and destroy polyamines. AZ controls and limits polyamine accumulation by impeding the first and second of these processes. This regulatory circuit requires both a sensor of polyamine levels and effectors that alter pool size. The sensor: polyamines stimulate translational frameshifting required to decode the AZ mRNA (5). The effectors: AZ inhibits and sometimes destroys a key polyamine biosynthetic enzyme, ornithine decarboxylase (ODC), and inhibits cellular uptake of polyamines (6).

What are the biologically important polyamines, what do they do in cells, and

how do cells come by them? The polyamines are biologically ubiquitous small alkylamines bearing multiple amine groups that carry positive charge at physiologic pH (7). They were first observed by Leeuwenhoek in the 17th century as microscopic crystalline inclusions in human seminal fluid. The trivial names for the abundant natural polyamines, spermidine and spermine, recall this discovery. Polyamines probably play multiple biochemical roles. Both transcription and translation are stimulated by spermidine at millimolar concentrations and so are conveniently provided in the commercial kits sold to execute these reactions *in vitro*. Some specific biochemical roles of spermidine have been identified, e.g., in the posttranslational modification hypusination (8), uniquely required for function of the essential protein eIF-5A, and in gating the inward rectifier current of an ion channel (9). Genetic and pharmacological depletion experiments show that polyamines are essential for cellular life. Biosynthesis begins with the enzymatic decarboxylation of ornithine by ODC to produce putrescine (diaminobutane). Successive addition by distinct enzymes of aminopropyl groups to putrescine results in spermidine and spermine (7). The donor of the aminopropyl groups is decarboxylated *S*-adenosylmethionine, formed by the action of *S*-adenosylmethionine decarboxylase. The successive elaboration of putrescine, spermidine, and spermine produces compounds with, at physiologic pH, two, three, or four positive charges carried on amino groups. Polyamines are present at multimillimolar concentrations within all cells. Because of their high affinity for nucleic acids, spermidine and spermine are largely bound to RNA and DNA within the cell (10); hence, their free concentration is much lower than the total concentration. Polyamine biosynthesis can in effect be reversed in a catabolic series of oxidations, leading to the net conversion of spermine to spermidine and then to putrescine. The rate-limiting step for this back-conversion pathway is spermidine/spermine acetyltransferase (11). The three enzymes rate-determining in production and destruction of the polyamines, ODC, *S*-adenosylmethionine decarboxylase, and spermidine/spermine

acetyltransferase, are all rapidly turned over and strongly subject to posttranslational control (12), a characteristic of proteins that carry out critical cellular functions. It is likely that a multilevel cellular rapid-response system is in place that limits either polyamine excess or insufficiency and controls changes in availability in response to demand (13). AZ is central to this control process. Discussion of AZ will first be confined to the properties of the family member first described (AZ1) and then extended to more recently discovered AZs.

Functions of AZ. The best understood function of AZ, and the first recognized, is its action on ODC. AZ inhibits ODC and then directs its degradation by the proteasome (reviewed in ref. 6). The enzymatically active form of ODC is a homodimer; association-dissociation is rapid, with association but weakly favored. AZ has a very high affinity ($K_d \approx 10^{-11} \text{ M}^{-1}$) for the ODC monomer, so formation of the enzymatically inactive AZ:ODC heterodimer is strongly favored. The formation of AZ:ODC is reversible: e.g., by high salt concentration, with full restoration of enzyme activity. Inhibition does not produce much bang for the buck: 2 moles of AZ kill 1 mole of (ODC)₂. However, in its role as a destructive agent for ODC, AZ acts catalytically. ODC when bound to AZ is efficiently degraded by the proteasome, and AZ (usually) is recycled to act again. Almost all proteasome substrates that have been examined are marked for degradation by polyubiquitination, a covalent posttranslational modification. ODC stands as an exception; p21^{Cip1} is apparently another (14). AZ has no apparent structural resemblance to ubiquitin, but it must provide a workable substitute.

The elements of both AZ and ODC needed for association and degradation have been determined. Within the 227 amino acids of rat AZ, there are two. (i) The C-terminal half of the molecule, amino acids 121–227, binds to ODC, inactivates it, and exposes the C terminus of ODC. This AZ half molecule does not,

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however, cause proteasomal degradation. Degradation requires that (ii) a portion of the AZ N terminus, contained within amino acids 70–120, be present as well. The amino terminus of AZ is also a portable domain that, when fused to molecules containing the ODC C terminus, enhances their degradation. Within the 461 amino acids of mouse ODC, there are also two regions of importance for AZ action. (i) An \approx 40-aa C-terminal domain is needed for both AZ-independent and AZ-dependent degradation. This C-terminal domain becomes more exposed when AZ binds and can act as a portable degradation motif when fused to other proteins. (ii) An AZ binding domain is localized to the α - β barrel of ODC, with key residues contained within amino acids 117–140. Examination of the electrostatic surface potential of this region using an x-ray crystallographic model (15) suggests that an electropositive patch at the N-terminal end of the α - β barrel may help stabilize the ODC:AZ dimer.

In addition to biosynthesis, uptake from the cellular environment can provide polyamines, transport of which is also elaborately regulated (16). AZ inhibits uptake (17). The potential for regulatory complexity is further augmented by the existence in cells of a protein inhibitor of AZ (18), which has an inferred amino acid sequence very much like that of ODC, but one that lacks residues critical for enzymatic activity. Antizyme inhibitor can, like ODC, form a heterodimer with AZ (but heterodimers between ODC and antizyme inhibitor do not form). Antizyme inhibitor has higher avidity for AZ than it does for ODC and therefore should act to reduce the effective level of AZ.

Translational Frameshifting Required To Make AZ. Polyamines induce AZ by a strikingly unusual control mechanism: They induce a +1 translational frameshift required to decode AZ mRNA (5). The AZ proteins are the product of translation of a short poorly conserved ORF 1 and a well conserved ORF 2. These partially overlap. At the codon immediately 5' to the stop codon of ORF 1, translation shifts into the +1 frame to align translation with ORF 2. ORF 2 mediates all of the known functions of AZ but lacks the capacity to initiate translation. The frameshifting process is more efficient when polyamines are elevated. Within the range of the control system, cellular AZ level thus provides both a readout of the polyamine level and a means to adjust it. In vertebrate AZs, two sequences are critical for stimulating frameshifting. The first is the UGA stop codon of ORF 1 and the sequence of six codons immediately preceding it. The second is a pseudoknot that follows the UGA stop. In invertebrates, no

pseudoknot structure is apparent, but sequence information 3' to the ORF 1 stop can nonetheless contribute to frameshifting efficiency (28).

Genetic and Pharmacological Perturbations of Polyamines and Their Consequences. Examining the consequences of misregulation can help delimit the functions of a regulator. What happens when polyamine regulation is disrupted experimentally? First, it should be noted that, because of control redundancy, this is not easy to do. Synthesis, transport, and catabolic destruction are all in play. Although there is only one biosynthetic pathway in vertebrates, polyamines can move in and out of cells, and there is a catabolic pathway dedicated to their destruction, so the effect of changing any one parameter can be nullified by compensatory reactions. Second, determining whether one has succeeded in altering polyamine pools is no straightforward matter. Although measuring bulk cellular polyamines is readily accomplished, spermidine and spermine are predominantly bound to RNA and DNA and exchange slowly. Free pools, those that can take part in cellular biochemical action, could undergo radical change and yet be overlooked in the experimental noise of pool size determinations (13).

Despite these impediments, pool perturbations have been found to cause biological sequelae. Forced excess putrescine production by means of ODC overexpression can lead to transformation of cultured cells (19). The consequences of misregulation of polyamine pools are becoming more apparent through the analysis of mouse models. Mice homozygous for an AZ1 knockout are viable and fertile but have high perinatal mortality (S. Matsufuji and T. Noda, personal communication). Targeted ODC expression in keratinocytes leads to skin cancer (20). Of greatest relevance to the paper here under discussion, transgenic mice that overexpress ODC in multiple organs exhibit male infertility and disorganization of testicular architecture (21). In mice with 70-fold excess testicular ODC, microscopic analysis shows a reduced number of meiotic and particularly postmeiotic cells. This suggests that a vast excess of ODC swamps down-regulation by AZ3 in these animals, and that this has consequences, most especially for the late stages of spermatogenesis. More generally, in all of the cited cases of transgenic ODC overproduction, enzyme levels greatly in excess of normal are needed to produce a discernible biological effect, indicating, perhaps, that compensatory mechanisms must be overcome.

Turning from polyamine excess to insufficiency, genetic and pharmacological

evidence shows that polyamines are universally essential for cell growth and survival. However, in all cases that have been examined (from *Escherichia coli* to *Caenorhabditis elegans*), biosynthesis appears to be largely redundant so long as an exogenous source of polyamines is accessible, again suggesting that compensatory reactions can suffice to maintain pools at acceptable levels when synthesis is impeded. Specific pharmacological inhibitors of ODC can be used to cut off polyamine production at its source. Both cultured animal cells and unicellular organisms of multiple taxa stop growing when subjected to depletion by this means, and disruption of the ODC gene has a similar effect. The nematode worm *C. elegans* is the only multicellular organism in which an ODC gene disruption has been reported. This causes modest infertility in hermaphrodites raised on medium replete with polyamines but developmental arrest under conditions of polyamine starvation (22). Perhaps importantly, male worms are markedly infertile even in polyamine-rich medium, suggesting that male fertility requires stringent control. However, because the cause of male infertility in the *C. elegans* ODC disruption strain was not examined, it was not necessarily attributable to a defect in sperm production. Over expressing spermidine/spermine acetyltransferase in transgenic mice produces polyamine catalysis, leading, as expected, to spermidine pool reduction and putrescine increases; the magnitude of this effect was tissue-specific (23). These mice suffer permanent hair loss and ovarian hypofunction, causing female infertility. Male fertility was not impeded, perhaps because testicular polyamine pools were little affected by the transgene.

Multiple AZs. Searches of public genomic databases using sophisticated software tools have revealed that the AZs comprise a widespread family of conserved homologs [full references are available elsewhere (3, 24, 28)]. In humans, five non-allelic AZ homologs have been revealed. AZ was first cloned from mammalian cells. The full coding sequences of AZ1–2 and the testis-specific AZ3 have been cloned; in addition, two more vertebrate AZ ESTs have been detected. Two copies are present in the zebra fish *Danio rerio*. AZ has also been cloned from fruit flies (*Drosophila melanogaster* and *Drosophila virillis*). AZ is found in a number of other invertebrate species, including silkworm (*Bombyx mori*) and at least four nematode species (*C. elegans*, *Onchocerca volvulus*, *Haemonchus contortus*, and *Pristionchus pacificus*). Only one copy of AZ is present in the complete *C. elegans* genome. The fission yeast *Schizosaccharomyces pombe*

produces AZ, but no recognizable homolog was detected in the complete genome of the budding yeast *Saccharomyces cerevisiae*, despite the fact that polyamine-induced regulation of ODC shares many properties with comparable regulation in organisms that have AZs (25).

In an organism with more than one AZ, what distinct function does each AZ serve? We have as yet only partial answers. The most highly conserved region of the proteins is the C-terminal half, the ODC binding region. It seems likely that all AZs bind to and inhibit ODC; AZ3 does so both *in vitro* (3) and in cells (4). AZ1 and AZ2 resemble each other in their ability to reduce cellular polyamine uptake. However, they differ in their ability to direct ODC degradation (26). In an *in vitro* assay of proteasome-mediated degradation, AZ1 caused proteolysis of ODC, but AZ2 did not. This suggests that AZ2 may have the capacity to reversibly inhibit ODC, perhaps to provide a storage form for later use. Ivanov *et al.* (3) did not investigate the transport or degradative properties of AZ3. Its very restricted expression may imply that it will prove to have biochemical properties distinct from those of other AZs. Alternatively, its distinct function may depend simply on its special time and place of delivery.

Conjectures, Predictions, Future Developments. Several previous findings are reinforced and made more understandable by the paper discussed here. These include all of the following: First, the paper supports the finding that polyamine levels are critical in germ cell development: The previously mentioned transgenic mice with excess spermidine/spermine acetyltransferase or ODC show dramatic changes in oocyte or sperm production but are otherwise reasonably healthy. Second, the

distinct members of the AZ family are conserved in evolution, implying distinct functions for individual family members. A previously noted difference in biochemical properties (AZ1 versus AZ2) has in the paper by Ivanov *et al.* (3) been extended to a distinction in time and place of expression (AZ1 and AZ2 present almost always and almost everywhere; AZ3 present in one cell type for a limited time only). The specificity of AZ3 expression in spermatocyte development should make it easier to find out whether fine control of polyamine pools is indeed important for that process and tease out the role of polyamines. Third, the time of appearance of ODC and AZ3 transcripts seems well coordinated for managing sharp changes in polyamine synthesis. Sperm production consists of four successive phases: a series of mitotic divisions, then two meiotic divisions that reduce the ploidy from 4N to 1N, and then a series of morphologic changes (spermiogenesis) that convert the round spermatid to the mature tailed elongated spermatozoon. As discussed by Ivanov *et al.* (3), ODC transcripts are found at all of these stages but are especially abundant in late pachytene of the first meiotic division and in early-stage round spermatids (27). In contrast, AZ3 expression has a sharp onset in early spermatids, peaks in later stages, and is gone in spermatozoa. This suggests that ODC may function unopposed at or near the transition between the second meiotic division and spermiogenesis but that the door is then slammed on further polyamine accumulation. Experimental work must now turn to confirming this plausible conjecture and exploring its implications.

Spermatogenesis is a promising experimental model that may help to bridge a gap in research on polyamines: Although there has been great success in biochem-

ical and molecular investigations, there has been less success in understanding the biologic role of the processes uncovered. If a biologist knocks out his or her favorite gene and not much happens, he or she may find comfort in the mantra of redundancy: The process in question is not inconsequential but, rather, important enough that backup systems must have been deployed to safeguard it. Those working on polyamines face a similar quandary: Although mechanisms of Byzantine complexity allow for fine-tuning, disabling or misregulating these mechanisms often has no appreciable effect, suggesting that the organism is immune to polyamine discord. Examining the role of AZ3 in spermiogenesis presents an excellent opportunity to resolve this quandary because the problem of control redundancy is mitigated: AZs control not one but two of the three processes that modulate polyamines, biosynthesis and transport, and AZ3 function can be observed cleanly in this cellular context, unobscured by the presence of AZ1 or AZ2. Observations using spermiogenesis as an experimental model may offer insights concerning polyamine control and function, insights that may prove to carry general validity. Finally, it seems to be a precondition of noteworthy biology that some disease be associated with the dysfunction of our favorite gene, protein, or pathway. How significant can a process be—whatever the biochemistry involved—if nobody notices when it is missing or broken? Ivanov *et al.* (3) speculate that AZ3 mutation may underlie a specific form of heritable male infertility, a hypothesis that should be readily testable. At last, a disease on the polyamine horizon.

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