parsimonious tree conforming to each of the 15 possible phylogenetic topologies for the four ecomorphs (in the case of the two twig anoles on Hispaniola, we used *A. insolitus* because *A. sheplani* is the sister taxon of Cuban twig anoles and is nested within a clade of Cuban species) using the "backbone constraints" option in PAUP*, which constrains the relationships of a subset of the taxa but allows the remaining taxa to occur anywhere on the tree (that is, the subset of constrained taxa does not necessarily form a monophyletic group, but the relationships among these taxa must conform to the

constraint). We compared each of these trees to the most parsimonious tree (Fig. 1B) using the Wilcoxon signed-ranks test. In addition, we compared the maximum-likelihood tree with each constraint tree using the Kishino-Hasegawa test. Each of the 15 possible ecomorph topologies was rejected for at least one island. Hence, we conclude that the topology of ecomorph evolution differed among islands. In addition, when ancestral ecomorph states were reconstructed with parsimony, each island exhibited a different order of ecomorph evolution.

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Monoallelic Expression of the Interleukin-2 Locus

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The lymphokine interleukin-2 (IL-2) is responsible for autocrine cell cycle progression and regulation of immune responses. Uncontrolled secretion of IL-2 results in adverse reactions ranging from anergy, to aberrant T cell activation, to autoimmunity. With the use of fluorescent in situ hybridization and single-cell polymerase chain reaction in cells with different IL-2 alleles, IL-2 expression in mature thymocytes and T cells was found to be tightly controlled by monoallelic expression. Because IL-2 is encoded at a nonimprinted autosomal locus, this result represents an unusual regulatory mode for controlling the precise expression of a single gene.

IL-2 is a growth factor important in the regulation and differentiation of lymphocytes and natural killer cells (*1*). Produced by a subpopulation of activated T cells, IL-2 also plays a pivotal role in the generation of an adoptive immune response. Decreased secretion or the complete absence of IL-2 in humans is associated with primary and secondary immunodeficiencies (*2*). Mice homozygous for an IL-2 null mutation (IL-2$^{-/-}$) have a compromised immune system with alterations of both cellular and humoral functions (*3*). Overproduction of IL-2 results in an impaired immune response with autoimmunity, breaking of clonal anergy, and suppression of certain T cell functions (*4*). IL-2 expression, therefore, is firmly controlled by multiple signaling pathways emanating from the T cell receptor and antigen-independent coreceptors (*5*). These signals regulate the transcriptional control of ubiquitous and T cell–specific factors, which transactivate transcription of the gene encoding IL-2 in vivo through binding to the promoter and enhancer sequences using an all-or-nothing mechanism (*5*). Coreceptors also transduce signals that stabilize IL-2 mRNA (*6*).

The number of functional IL-2 alleles may also determine the amount of IL-2 produced. Therefore, we investigated whether T cells heterozygous for the IL-2 null mutation produce less IL-2 than wild-type T cells. We stimulated CD4$^+$ T cells purified from wild-type and heterozygous mice. The amount of IL-2 produced by concanavalin A (Con A)–treated IL-2$^{+/-}$ T cells was decreased by half when compared with that produced by T cells from wild-type mice (Fig. 1). As expected, Con A stimulation of IL-2$^{-/-}$ T cells did not result in detectable IL-2 secretion.

Was each heterozygous CD4$^+$ T cell pro-

ducing only half of the amount of IL-2 produced by wild-type cells, or were only half of the CD4$^+$ T cells secreting amounts of IL-2 comparable with that secreted by wild-type T cells? Concurrent transcription from both (that is, the mutant and the wild-type) alleles of the IL-2 gene would lead to the first result, whereas the latter result would be obtained if allele-specific expression occurred from only one of the two copies of the IL-2 gene. To distinguish between these two mutually exclusive models, we determined IL-2 secretion at the single-cell level. Mature CD4$^+$ thymocytes and CD4$^+$ peripheral T cells were stimulated with Con A and subsequently stained for the presence of IL-2 (*7*). About half of the CD4$^+$ T cells from 3- to 4-week-old heterozygous mice stained positively for IL-2 (Fig. 2, A and B, left). In agreement with these data, limiting dilution assays showed that the relative frequency of IL-2–secreting CD4$^+$ T cells was diminished by a third to a half in heterozygous mice in comparison with

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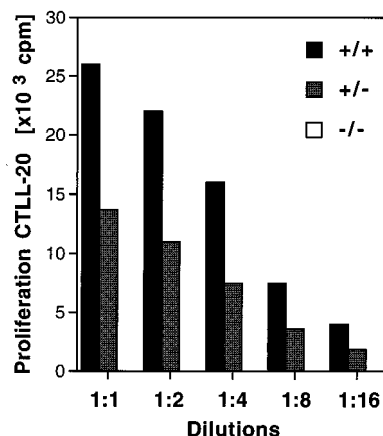
Fig. 1. The genotype of IL-2 mutant mice controls the amount of IL-2 secreted. IL-2 production in response to Con A stimulation. Purified T cells from heterozygous and homozygous IL-2 mutant mice and from wild-type mice were stimulated in vitro by Con A in RPMI 1640 medium (Gibco-BRL) supplemented with 10% fetal bovine serum (Sigma), penicillin, streptomycin, and 2-mercaptoethanol. After 24 hours in culture, serial dilutions of supernatant were assayed on 5×10^3 CTLL-20 cells in the presence of mAb to IL-4 (11B11). Proliferation was measured by [^{3}H]thymidine incorporation during the last 4 hours of a 24-hour assay. The graph is representative of three independent experiments and each experiment had less than 10% variability.

wild-type animals (Fig. 2C) (8). In contrast, older (>6 weeks) heterozygous mice displayed a relative frequency of IL-2–positive cells that had increased to about 75% of all peripheral CD4+ T cells, whereas the corresponding frequency among thymocytes remained at about 50% (Fig. 2B, right). Thus, IL-2–secreting peripheral T cells have an in vivo growth advantage over nonsecreting cells. Wild-type T cells also show an increased proliferative response to alloantigens (9) and influenza nucleoproteins (10) when compared with IL-2$^{-/-}$ T cells.

We used interactive laser cytometry to quantitate IL-2 production in single cells

(11, 12). CD4+ T cell blasts from heterozygous mice had two populations of intracytoplasmic IL-2 staining that represented a composite of the staining pattern observed for wild-type and IL-2$^{-/-}$ mice (Fig. 3A). The mean fluorescence of heterozygous cells positive for intracytoplasmic staining was comparable with that of wild-type T cells (2002 ± 392 and 1928 ± 360 relative fluorescence units, respectively; mean ± SD), whereas the mean fluorescence for the other subpopulation of IL-2$^{+/-}$ cells was equivalent to that of IL-2$^{-/-}$ T cells (1271 ± 204 and 1126 ± 210 relative fluorescence units, respectively). Single-cell fluorescence analy-

sis of a larger number of activated CD4+ T cells defined a bimodal distribution for intracytoplasmic IL-2 staining (Fig. 3B), confirming that half of all T cells in heterozygous mice do not produce IL-2, whereas the other half secrete IL-2 in amounts identical to that secreted by wild-type mice.

These results could be explained by a mechanism of allelic silencing. To test allelic expression of the IL-2 gene at the mRNA level, we analyzed activated T cells from F$_1$ crosses between *Mus musculus* (C57BL/6; female) and *M. spretus* (male). These two mouse strains exhibit allele-specific sequences that can be distinguished by digestion with restriction enzymes (13). Messenger RNAs from single, activated CD4+ T cells were reverse-transcribed, and IL-2–specific sequences were amplified by polymerase chain reaction (PCR), with the use of primers for sequences identical in both strains. Amplicons were then digested with Fnu 4HI, which cuts only C57BL/6-specific DNA of the amplified sequence (Fig. 4A) (14). Individual F$_1$ T cells contained IL-2 transcripts that derived from either the maternal or the paternal allele, but never from both.

For most genes, the initiation of DNA synthesis occurs in a temporally ordered fashion with synchronous replication of both alleles (15). In contrast, transcriptionally silenced alleles are replicated asynchronously during S phase of the cell cycle (that is, they are delayed in comparison with the transcriptionally active allele) (16). To determine whether one of two IL-2 alleles is silenced, we studied replication timing of the IL-2 locus in a single cell using fluorescent in situ hybridization (FISH). This technique allows one to establish the number of specific alleles in interphase nuclei (Fig. 4B) (17). A genomic probe for the two first exons of the IL-2 gene (located on chromosome 3) revealed one pair and an additional single hybridization spot in most activated T cells enriched for S phase (Fig. 4B, middle, and Table 1). This finding implies asynchronous replication and is compatible with transcription from only one allele (16). In contrast, three hybridization spots were detected only in the minority of
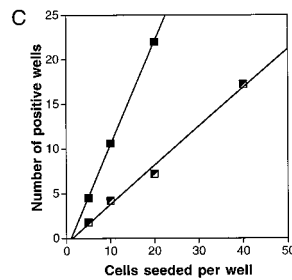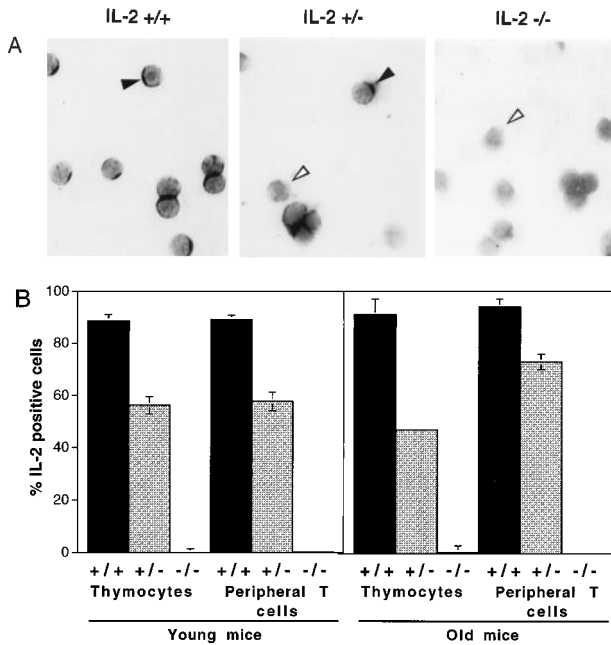

Fig. 2. Single-cell analysis of IL-2 production by CD4+ mature thymocytes and peripheral T cells from young (3 to 4 weeks) and older mice (>6 weeks). (**A**) Immunoperoxidase staining of T cells from young mice. Closed arrows, IL-2–positive CD4+ T cells; open arrows, IL-2–negative CD4+ T cells. The sensitivity and specificity of this method were verified with IL-2$^{-/-}$ T cell cultures stimulated with Con A and supplemented with recombinant IL-2 (25 IU/ml). (**B**) Immunoperoxidase staining of CD4+ T cells from young (left) and older (right) mice. (**C**) Limiting dilution analysis of peripheral CD4+ T cells from IL-2$^{+/+}$ (■, $Y = 1.163x - 1.200$) and IL-2$^{+/-}$ (▨, $Y = 0.435x - 0.565$) mice for the secretion of IL-2 (8). The frequency of false positive wells was <2 out of 386 wells in all three independent experiments (25).

Fig. 3. Digital analysis of single-cell fluorescence by interactive laser cell cytometry. (**A**) Distribution of the relative fluorescence for intracytoplasmic IL-2 in single CD4+, Con A–stimulated T cell blasts from wild-type mice and mice heterozygous and homozygous for a null mutant for IL-2 (12). The horizontal lines represent the mean (±2SD) relative fluorescence intensity measured in IL-2$^{-/-}$ and IL-2$^{+/+}$ CD4+ T cell blasts. (**B**) Bimodel distribution for intracytoplasmic IL-2 expression among IL-2$^{+/-}$ CD4+ T cell blasts (12).
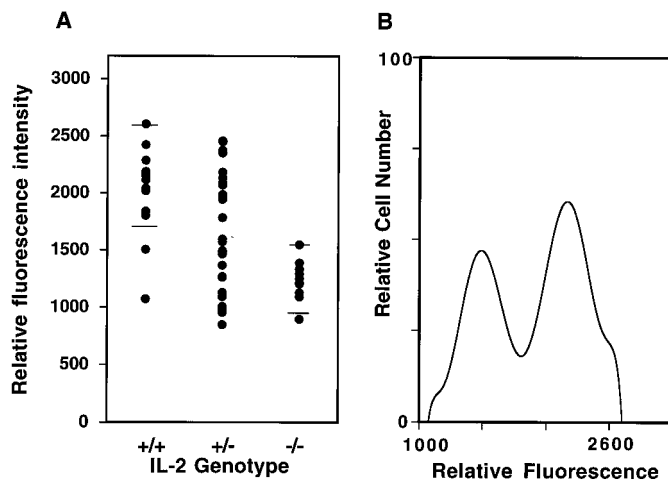

Table 1. The IL-2 gene is asynchronously replicated. Analysis representative of three independent FISH experiments counting 100 cells for each probe (22).

Number of hybridization spots	IL-2 probe (%)	c-*mpl* probe (%)
2	5	9
3	68	10
4	27	81

Fig. 4. The IL-2 gene is asynchronously replicated and its transcription is monoallelic. (**A**) (Left) Single-cell RT-PCR of stimulated CD4$^+$ T cells from (C57BL/6 × *M. spretus*) F$_1$ mice for the transcription of maternal (*M. musculus*; C57BL/6) or paternal (*M. spretus*) IL-2 mRNA (*15*). S, *M. spretus*; B, C57BL/6; F$_1$, (C57BL/6 × *M. spretus*) F$_1$. Amplicons were either digested with Fnu 4HI (+) or loaded without digestion (−). (Right) RT-PCR of a quarter of a single-cell lysate (about 2.5 to 5 pg total RNA) from C57BL/6 cells (B/4) mixed with varying amounts of total RNA from *M. spretus* activated splenocytes. (**B**) FISH of CD4$^+$ T cells stimulated with Con A (5 μg/ml) for 48 hours, stained with Hoechst 3342 (2 μg/ml) for 30 min at 37°C, and subsequently enriched for S phase (75 to 80%) by flow cytometric cell sorting. FISH was performed with a biotin-labeled genomic probe for IL-2, and cells were scored for the presence of two (left), three (middle), or four (right) hybridization dots.
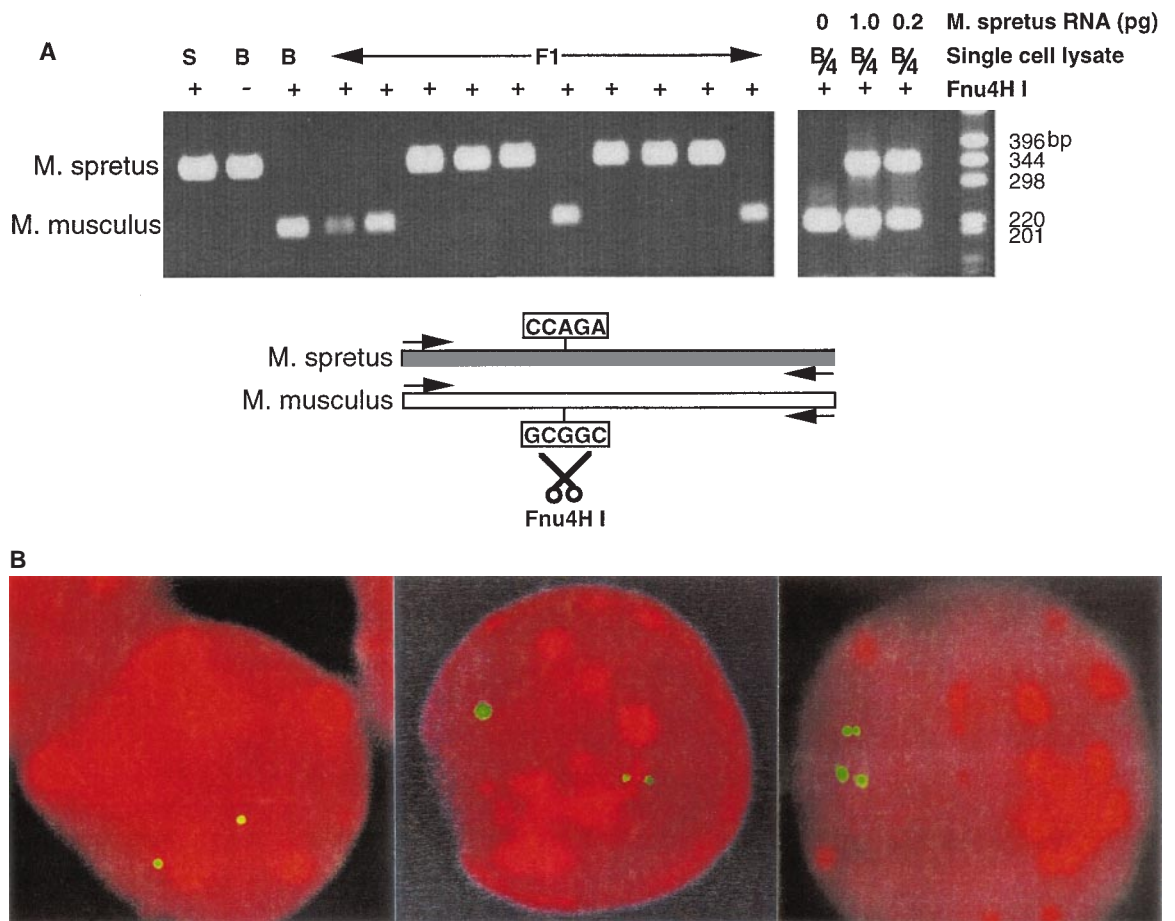
cells hybridized with c-*mpl*, the murine receptor for thrombopoietin located on chromosome 4 (Table 1), indicating synchronous replication during S phase from two active alleles. Thus, the chromosomal analysis of single wild-type T cells infers that IL-2 expression is monoallelic.

Mammals exhibit several epigenetic phenomena that prevent simultaneous gene expression from both alleles of a given locus: (i) random X chromosome inactivation in females, (ii) nonrandom parental imprinting of selected autosomal genes, and (iii) allelic exclusion of antigen receptors in lymphocytes and odorant receptor gene clusters in olfactory sensory neurons (*18–20*). The IL-2 gene does not reveal any of the features established for loci known to be allelically excluded. The gene is localized on the murine autosomal chromosome 3, which is not a known target of parental imprinting (*21*). This result is corroborated by our findings that paternal (*M. spretus*) and maternal (C57BL/6) alleles were expressed with comparable frequency. In contrast to olfactory and antigen receptors, IL-2 is encoded by a single gene (*22*). Also, the monoallelic expression at the IL-2 locus seems independent

of a feedback control through a functional gene product expressed by the other allele. Thus, the mechanism of allele-specific expression of IL-2 is different from others used by the immune system to effect allelic exclusion.

As an effective mechanism for tight transcriptional control of IL-2 at the genomic level, monoallelic expression may act as a fail-safe device to avoid harmful dysregulation of an immune response secondary to increased IL-2 production (*4*). More generally, single allele expression of growth and differentiation factors may be of critical relevance as cytokines mediate biological effects in a dose-dependent mode and chemokines form localized gradients for cell-specific homing to tissues. Moreover, growth factors also control organogenesis according to a discriminating threshold.

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genotype were obtained by cell sorting and stimulated for 8 hours with Con A (5 μg/ml) before preparation of a cytospin. Cytologic studies were performed on cytocentrifuged smears, which were air-dried, fixed in cold acetone, rehydrated in phosphate-buffered saline, and then stained with monoclonal antibody (mAb) to IL-2 (JES6-1A12) by means of an immunoperoxidase technique (23). IL-2$^{-/-}$ mutant mice demonstrate normal thymocyte subsets, confirming that IL-2 is not required for normal T cell ontogeny (24). Our observations in young and older heterozygous mice that only 50% of the mature CD4$^+$ and CD8$^-$ thymocytes stained for IL-2 further emphasizes the lack of a developmental advantage for thymocytes that produce IL-2.

8. In 96-well, round-bottom plates, 5 × 10^4 irradiated (2000 centigrays) splenic feeder cells from IL-2$^{-/-}$ mice were cultured at a final volume of 25 μl in complete RPMI 1640 medium containing Con A (5 μg/ml). Each well was subsequently seeded by flow cytometry with various numbers of CD4$^+$ peripheral T cells and grown for 30 hours at 37°C. After freezing and thawing of the plates, 50 CTLL-20 cells and mAb to IL-4 were added to each well in a final volume of 25 μl, and plates were cultured for an additional 28 hours. Live CTLL-20 cells were then rescued by exogenous IL-2 (10 U/ml) added every other day. Seven days after the addition of indicator cells, wells were scored for CTLL-20 cell growth either by examination under the microscope or by ^{3}H-labeled thymidine incorporation. Wells not seeded initially by CD4$^+$ T cells but treated identically as outlined above served as negative controls, whereas the simultaneous addition of IL-2 and CTLL-20 cells served as the positive control. This measurement of IL-2 corresponds to a modified method of (25).

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14. T cells from C57BL/6, M. spretus, and (C57BL/6 × M. spretus) F$_1$ were stimulated in bulk by phorbol 12-myristate 13-acetate and calcium ionophore A23187 for 10 hours. CD4$^+$ T cells were then sorted by flow cytometry as single cells into 10 μl of 2× reverse transcription (RT) buffer containing 0.05% NP-40 and immediately frozen on dry ice. RT was done on single-cell lysates or fractions thereof with an IL-2–specific primer (GTGTTGTAAGCAGGAGGTACATAGTTA) followed by 30 cycles of a first PCR amplification (5′: CATGCAGCTCGCATCCTGTGT; 3′: GTGTTGTAAGCAGGAGGTACATAGTTA). One microliter of a 50-μl reaction was used for a seminested second amplification of 26 cycles (5′: GAGCAGGATGGAGA ATTACAGG; 3′: GTGTTGTAAGCAGGAGGTACATAGTTA). The amplicons differ by a Fnu 4HI–sensitive sequence that allows the distinction of maternal C57BL/6 from paternal M. spretus DNA. The PCR reaction was analyzed on a 2% agarose gel after digestion of the amplicons. The expected product from the paternal M. spretus allele is 358 base pairs (bp), whereas the larger fragment of the digested C57BL/6 maternal allele is 229 bp (the smaller fragment of 129 bp is not shown in Fig. 4A). The single-cell RT-PCR used would be sufficiently sensitive to detect biallelic transcription if it were present because IL-2–specific transcripts can still be amplified from 1:4 dilution of single-cell lysates. Moreover, mixing RNA at diverse ratios followed by RT-PCR allowed for the concurrent detection of both transcripts over a broad range of different concentrations (26).

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Structure of Nitric Oxide Synthase Oxygenase Dimer with Pterin and Substrate

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Crystal structures of the murine cytokine-inducible nitric oxide synthase oxygenase dimer with active-center water molecules, the substrate L-arginine (L-Arg), or product analog thiocitrulline reveal how dimerization, cofactor tetrahydrobiopterin, and L-Arg binding complete the catalytic center for synthesis of the essential biological signal and cytotoxin nitric oxide. Pterin binding refolds the central interface region, recruits new structural elements, creates a 30 angstrom deep active-center channel, and causes a 35° helical tilt to expose a heme edge and the adjacent residue tryptophan-366 for likely reductase domain interactions and caveolin inhibition. Heme propionate interactions with pterin and L-Arg suggest that pterin has electronic influences on heme-bound oxygen. L-Arginine binds to glutamic acid–371 and stacks with heme in an otherwise hydrophobic pocket to aid activation of heme-bound oxygen by direct proton donation and thereby differentiate the two chemical steps of nitric oxide synthesis.

Nitric oxide synthases (NOSs) oxidize L-Arg to synthesize nitric oxide (NO), which is a key intercellular signal and defensive cytotoxin in the nervous, muscular, cardio-vascular, and immune systems (1, 2). Neuronal NOS (nNOS, NOS1) and endothelial NOS (eNOS, NOS3) produce low NO concentrations for neurotransmission, insulin release, penile erection, vasorelaxation, oxygen detection, and memory storage, whereas cytokine-inducible NOS (iNOS, NOS2) produces larger NO concentrations to counter pathogens and coordinate the T cell response (1). NOSs catalyze two sequential, mechanistically distinct, heme-based oxidations in the five-electron oxidation of L-Arg to L-citrulline (L-Cit) and

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