of the arrest front can largely account for the effects of *CIN* on leaf morphology. Because the arrest front moves more slowly and is changed from weakly convex to strongly concave, the marginal regions of the *cin* leaf grow for a longer period relative to medial regions, both in width and length. The extra growth in width leads to a wider leaf, whereas the extra growth in length, parallel to the leaf periphery, introduces negative curvature.

To determine where *CIN* is expressed relative to the arrest front, we carried out RNA in situ hybridizations. In young leaves, expression was restricted to the adaxial side, was stronger in marginal than medial regions, and was absent from veins. *CIN* expression eventually declined from tip to base (Fig. 4D), in parallel with the decline of *H4* and *CYCLIN D3b* expression. Thus, *CIN* is expressed in the actively dividing region of the leaf lamina, proximal to, and perhaps overlapping, the arrest front. This suggests that *CIN* may act by modifying the response of cells to arrest signals, perhaps through regulation of cell-cycle gene expression. This effect seems to be local, because the regions that normally express *CIN* (interveins) show excess growth in the *cin* mutants compared with those that do not (veins).

In *cin* mutants, arrest spreads out from the leaf tip with an almost circular front (Fig. 4B). This would result in an excess of growth in the margins because they would continue to proliferate when the more medial regions have arrested. *CIN* could counteract this by making cells more sensitive to the arrest signal, particularly in marginal regions where it is more strongly expressed. This would effectively straighten and accelerate the arrest front, ensuring that zero curvature is maintained while leaf growth is progressively arrested. This suggests that an important element in the control of leaf flatness lies in precise genetic regulation of the pattern of cell-cycle arrest.

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Supporting Online Material

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Steroid Control of Longevity in *Drosophila melanogaster*

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Ecdysone, the major steroid hormone of *Drosophila melanogaster*, is known for its role in development and reproduction. Flies that are heterozygous for mutations of the ecdysone receptor exhibit increases in life-span and resistance to various stresses, with no apparent deficit in fertility or activity. A mutant involved in the biosynthesis of ecdysone displays similar effects, which are suppressed by feeding ecdysone to the flies. These observations demonstrate the importance of the ecdysone hormonal pathway, a new player in regulating longevity.

In humans, changes in steroid hormones occur during aging (*1*), but whether those changes are a cause or an effect of aging remains unclear. To investigate the role of steroids in the aging process, we used genetics to manipulate a steroid hormone in adult *Drosophila* flies.

Steroid hormones in insects are ecdysteroids, and the major form in *Drosophila* is ecdysone. Its active metabolite, 20-OH-ecdysone, is important in developmental transitions and metamorphosis in *Drosophila melanogaster* (*2*). Ecdysone is also involved in oogenesis in the adult fly, but other functions are not known (*2*, *3*). 20-OH-ecdysone circulates and binds to a heterodimeric nuclear receptor consisting of an ecdysone receptor (EcR) and Ultraspiracle (USP), a homolog of the retinoid X receptor (RXR) (*2*). In the absence of ecdysone, the EcR-USP heterodimer is thought to form a complex with one or more corepressor proteins (N-CoR and SMRT), which bind to chromosomal histone deacetylases (Sin3A/Rpd3) (*2*, *4*). When the ligand is bound to the receptor, the complex binds instead to coactivators that recruit histone acetyltransferases, thus activating the transcription of various genes, including transcription factors (*5*), chaperones (*6*), apoptosis genes (*7*), and catalase (*8*). We investigated the role of ecdysone during adulthood by studying flies with mutations in *EcR* (*9*) and in a gene involved in ecdysone biosynthesis, *DTS-3* (*10*).

The *EcR* gene encodes three isoforms. We first studied a mutant, EcR^{V559fs}, which has a 37–base pair deletion in the predicted ligand-binding domain in a region common to the three isoforms (*9*). It is homozygous lethal during development but adult viable as a heterozygote. $EcR^{V559fs}/+$ flies lived longer than the controls (Fig. 1); male and female average life-spans increased by 40 to 50%. This was true for heterozygous offspring of crosses between two independent *cinnabar brown* (*cn bw*) backgrounds: the parental line from the Bender laboratory and a *cn bw* from our own laboratory stock. The same increase in longevity was observed, regardless of whether the male or female parents were mutant for EcR^{V559fs}. Progeny of the two *cn bw* lines crossed with each other showed no differences in longevity from the two parental lines.

Developmental time and weight of the adult flies in $EcR^{V559fs}/+$ were equivalent to those of control flies (Fig. 2, A and B). However, $EcR^{V559fs}/+$ flies showed increased resistance to three stresses: oxidative challenge, heat, and dry starvation (Fig. 2C). The $EcR^{V559fs}/+$ were also more active than controls, as measured by their performance in

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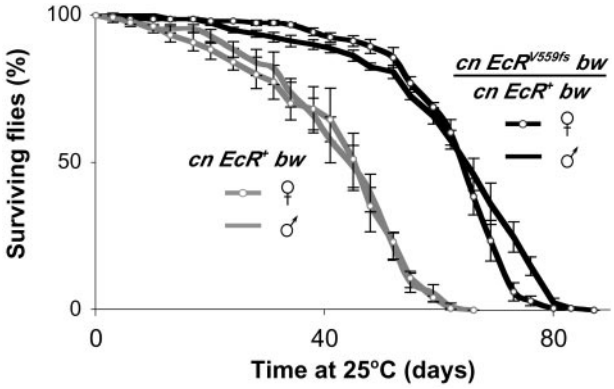

Fig. 1. Extension of life-span in an ecdysone receptor heterozygous mutant. Survival curves for *cn EcR*^{V559fs} *bw*/*cn EcR*$^{+}$ *bw* males and females, and *cn EcR*$^{+}$ *bw* controls raised at 25°C are shown. The *cn*, *EcR*, and *bw* mutations are all on the second chromosome. Male and female heterozygotes showed a 45% increase in mean life-span ($P < 0.004$; Wilcoxon rank test, one tail). Results are averages of four replicates of 40 flies per run ± SD. Flies were collected 2 to 3 days after adult eclosion to allow time for mating. All of the experiments were done using "standard" food (*22*).

fast phototaxis, a good indicator of stamina (Fig. 2D). The increase in longevity is thus not linked to a reduced activity level.

Life-span can also be extended in *Drosophila* when the costs of reproduction are eliminated (*17*). Because ecdysone is required for normal oogenesis (*2*, *3*) and spermatogenesis (*12*), reduced hormone function could increase survival through changes in reproduction. However, heterozygous *EcR* mutants showed no defect in oogenesis (*3*). Age-specific fecundity and fertility were greater in the mutant flies than in the controls (Fig. 3). Indeed, heterozygous mutant males seemed to induce greater fertility in their female mates. Thus, the increased life-span

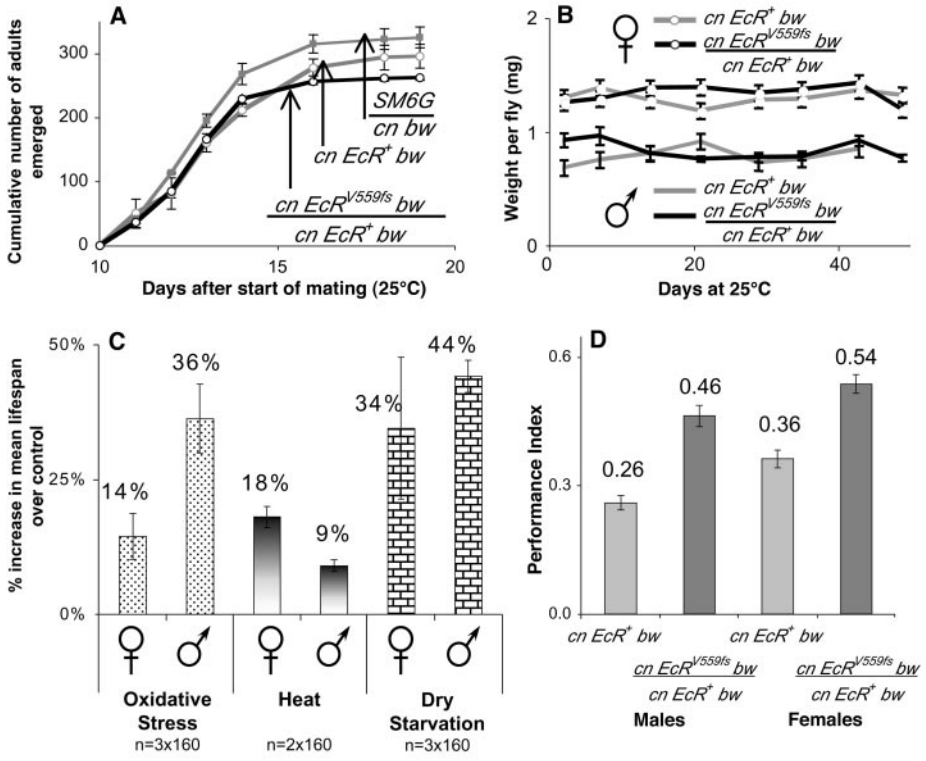

Fig. 2. Phenotypic characteristics of *cn EcR*^{V559fs} *bw*/*cn EcR*$^{+}$ *bw*. (**A**) Developmental time. The cumulative emergence of adult progeny as a function of time ± SD is shown. Two crosses were made, each in triplicate: female and male *cn EcR*$^{+}$ *bw* crossed to each other, and female *cn EcR*$^{+}$ *bw* crossed to *cn EcR*^{V559fs} *bw*/*SM6G* males. *cn EcR*$^{+}$ *bw* progeny are represented as actual numbers divided by two, because the cross with the *EcR* mutant produces two different genotypes. (**B**) Weight. Young flies were collected as in Fig. 1, with four replicates for each cross. Each point corresponds to the average weight per fly ± SD. (**C**) Resistance to various stresses. Bars represent the percentage increase in mean life-span compared to parental *cn bw* controls ± SD (n = number of runs × number of flies per run). (**D**) Phototaxis. The performance index is the total number of positive responses in five trials, divided by five multiplied by the number of flies. Each bar shows the average performance index of 14 runs ± SD. For all tests, the *cn EcR*^{V559fs} *bw*/*cn EcR*$^{+}$ *bw* flies were in a *cn bw* background; the controls were *cn bw*. Mated flies were collected as in Fig. 1.

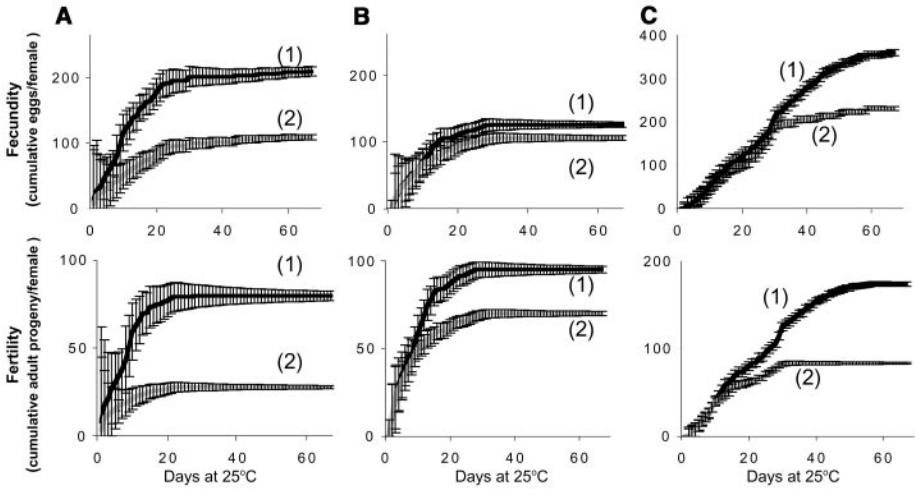

Fig. 3. Reproductive ability of *EcR*^{V559fs}/+ flies. Data are expressed as mean ± SD. (**A**) Fecundity and fertility measured under conditions similar to those of the life-span experiments in Fig. 1. In curve 1, male *cn EcR*^{V559fs} *bw*/*cn EcR*$^{+}$ *bw* were crossed with female *cn EcR*^{V559fs} *bw*/*cn EcR*$^{+}$ *bw* for 3 days, then separated. The comparison is to curve 2, which shows parental male *cn EcR*$^{+}$ *bw* crossed with female *cn EcR*$^{+}$ *bw*. (**B** and **C**) Fecundity and fertility measured in a different genetic background. (B) In curve 1, male $w^{1118}CS_{10}$ flies (w^{1118} was previously crossed 10 times with Canton-S) were crossed with female *cn EcR*^{V559fs} *bw*/*cn EcR*$^{+}$ *bw* lifelong, as compared to curve 2, in which male $w^{1118}CS_{10}$ were crossed with female *cn EcR*$^{+}$ *bw*. (C) In curve 1, male *cn EcR*^{V559fs} *bw*/*cn EcR*$^{+}$ *bw* were crossed with female $w^{1118}CS_{10}$ flies lifelong and compared to curve 2, in which male *cn EcR*$^{+}$ *bw*/*cn EcR*$^{+}$ *bw* were crossed with female $w^{1118}CS_{10}$ flies. $w^{1118}CS_{10}$, crossed to itself lifelong (*23*), gave the same result as male *cn EcR*$^{+}$ *bw* crossed with female $w^{1118}CS_{10}$ flies.

caused by reduced EcR activity did not lead to a loss in reproductive output.

Other mutations in the predicted ligand-binding domain (EcR^{A483T} and EcR^{M554fs}), in the DNA binding domain (EcR^{F288Y} and EcR^{C300Y}), in the Canton-S background, and in the *cn bw* background (*9*) also showed increased longevity (fig. S1, C to F). Note that the life-span of *cn bw* homozygotes is essentially the same as for wild-type Canton-S and for w^{1118} 10 times backcrossed to Canton-S (fig. S1, C to F). The possibility of a deleterious background in the *cn bw* controls is also counterindicated by the fact that the heterozygous mutant female progeny of the cross between thermosensitive EcR^{A483T} and *cn bw* controls do not live longer than the *cn bw* controls at 25°C. In summary, all of the mutant alleles of *EcR* that were tested as heterozygotes, in the ligand-binding domain and in the DNA binding domain, showed increased longevity.

Because ecdysone receptor mutants resulted in increased longevity, we also investigated a mutant affecting the ecdysone biosynthetic pathway. *DTS-3*, which was induced in the wild-type Samarkand strain, is a dominant lethal during development at an elevated temperature (*10*). The gene encodes a protein with Krüppel Zn-finger domains (*13*) that is specifically involved in ecdysone biosynthesis, because its developmental defect can be rescued when flies are fed 20-OH-ecdysone (*10*, *14*). After a few days at restrictive temperature (29°C), *DTS-3*/+ female adults, but not males, have a 50% lower ecdysteroid titer and reduced fertility (*10*). Female *DTS-3*/+ adults showed a temperature-dependent increase in longevity (Fig. 4A). At 29°C, females showed an increase of mean life-span of 42%. Consistent with the earlier observation that the ecdysteroid titer is reduced only in females, males did not show significantly increased life-span at any of the temperatures tested (fig. S2A). To control for genetic background, we outcrossed *DTS-3* twice with our w^{1118} line. Similar increases in longevity persisted (fig. S2B).

An increase in resistance to dry starvation was also linked to the temperature at which the flies were maintained before testing. After being exposed at 29°C, *DTS-3*/+ females showed an increase in mean survival time at 25°C of about 33%, and the result was the same when the flies were outcrossed with the w^{1118} line (fig. S2C). Again, males showed no changes in resistance (Fig. S2C). For *DTS-3*/+ females, spending only the 10 to 11 first days of adulthood at restrictive temperatures (29° or 25°C) was sufficient to induce an increase in resistance (Fig. 4, B and C). More strikingly, if the *DTS-3*/+ females spent the same period at 25°C before being shifted to 20°C, they lived longer than if they had spent their entire lives at 20°C; after 20 days at 25°C, the subsequent life-span at 20°C was as

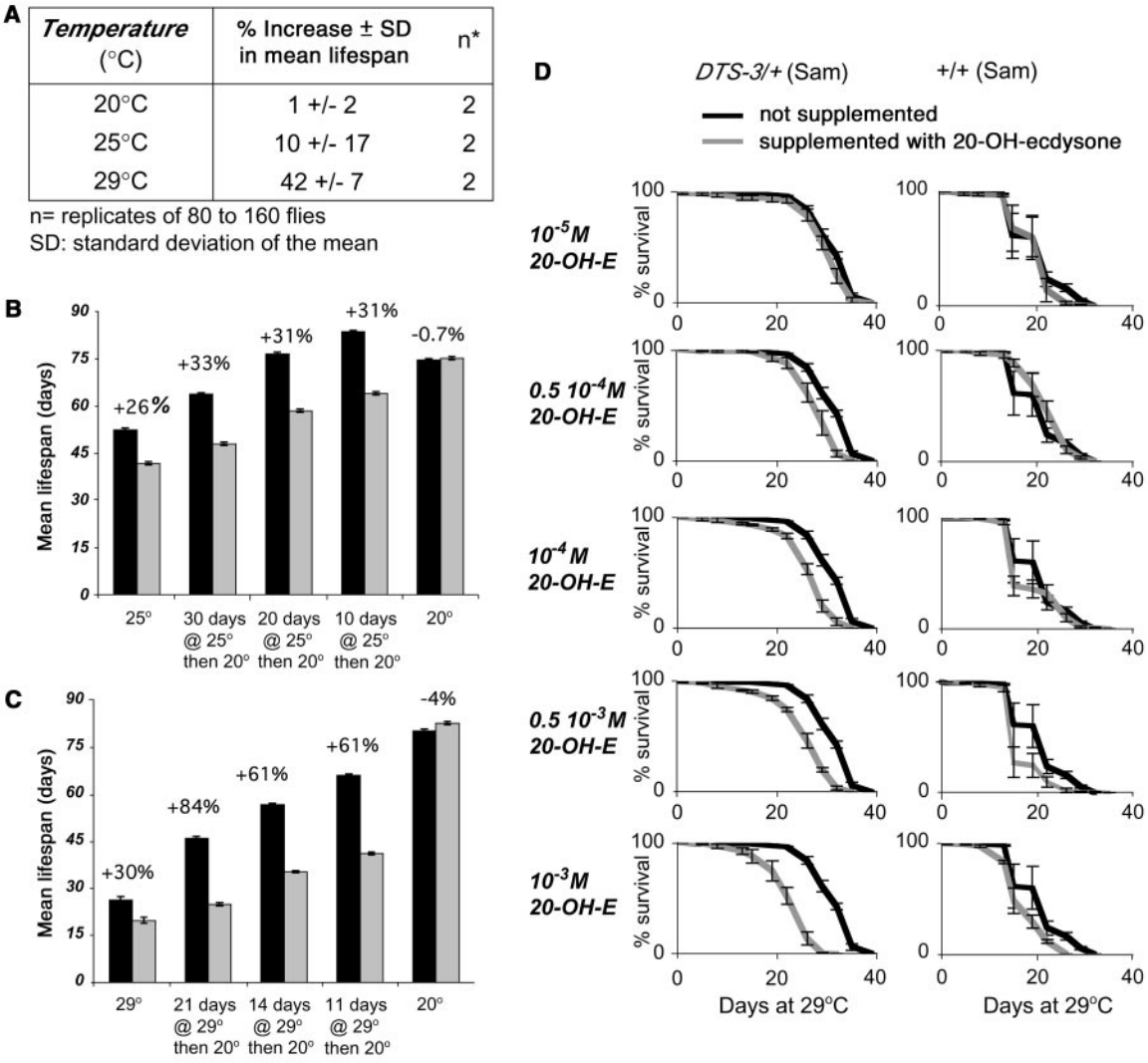

Fig. 4. *DTS-3*, a temperature-sensitive mutant affecting ecdysone synthesis in females. (**A**) Increase in life-span for *DTS-3*/+ females at higher temperature. Crosses to study longevity, as in Fig. 1, were made at 25°C (a temperature permissive for development). (**B** and **C**) Effect of shifting *DTS-3*/+ females from high to low temperature at various times during adulthood. Bars represent averages ± SD of four runs of 40 flies for the 25° to 20°C experiments (B) and four runs of 25 flies for the 29° to 20°C experiments (C). Black bars, *DTS-3*/+; gray bars, Samarkand. The percentage increase in mean life-span is shown above the bars. (**D**) Feeding 20-OH-ecdysone to *DTS-3*/+. Survival curves at restrictive temperature (29°C) with different concentrations of 20-OH-ecdysone in the food are shown. Females were placed in vials with 3 ml of food supplemented with 10^{-3} to 10^{-5} M 20-OH-ecdysone (20-OH-E) in 1% ethanol. In all cases, flies were allowed to develop at 25°C (a temperature permissive for development), until 2 to 3 days of adulthood before testing; *DTS-3*/+ was compared to the Samarkand (Sam) wild-type control, because the mutant allele was generated in a Samarkand background. Mated flies were collected as in Fig. 1.

if the flies had not aged during the time spent at the higher temperature.

When ecdysone was fed to the *DTS-3*/+ females throughout adulthood at 29°C, the increase in longevity was reduced, in a dose-sensitive manner (Fig. 4D). Feeding 20-OH-ecdysone to the heterozygous flies also reversed the increased survival under dry starvation conditions (fig. S2D). At the ecdysone concentrations used (10^{-5} to 10^{-3} M) in the standard food, there was no substantial effect on life-span in wild-type Samarkand females, which discounts a possible toxic effect of the hormone.

Our experiments show that two different components of a single pathway, the ligand and the receptor, can be manipulated to extend longevity. Hormones are known to have differential effects on males and females, and we also observed such differences with the ecdysone pathway. We have also shown, in one mutant studied in more detail, that fertility and activity levels can be raised, despite an increase in life-span.

Another hormonal pathway that is important for longevity regulation in diverse model organisms is the insulin pathway (*15*). Although the steroid and insulin pathways have very different roles, there is evidence that they do interact (*16*, *17*). In the fly, juvenile hormone (JH) is also involved in aging (*18*). Because USP has been proposed as a receptor for JH (*19*), steroids and JH might interact in regulating life-span.

The mutations in our study may be changing the balance between repression and activation of transcription of various target genes. Extension of longevity by gene silencing has been shown in *Drosophila* through mutation in *Rpd3*, another histone deacetylase interacting with the EcR-USP complex (*20*), or by feeding the flies phenylbutyrate, an inhibitor of histone deacetylase that induces one spectrum of genes while repressing others (*21*).

But which of the numerous genes that EcR regulates are crucial for the observed effects? Some of them, such as chaperones and catalase, are known to be important for longevity. Intrinsic levels of steroids may optimize certain functions but be detrimental to others, so life-span extension may require fine-tuning of the expression of appropriate combinations of genes.

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23. Materials and methods are available as supporting material on *Science* Online.

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Supporting Online Material

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Dilated Cardiomyopathy and Heart Failure Caused by a Mutation in Phospholamban

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Molecular etiologies of heart failure, an emerging cardiovascular epidemic affecting 4.7 million Americans and costing 17.8 billion health-care dollars annually, remain poorly understood. Here we report that an inherited human dilated cardiomyopathy with refractory congestive heart failure is caused by a dominant Arg → Cys missense mutation at residue 9 (R9C) in phospholamban (PLN), a transmembrane phosphoprotein that inhibits the cardiac sarcoplasmic reticular Ca^{2+}–adenosine triphosphatase (SERCA2a) pump. Transgenic PLN^{R9C} mice recapitulated human heart failure with premature death. Cellular and biochemical studies revealed that, unlike wild-type PLN, PLN^{R9C} did not directly inhibit SERCA2a. Rather, PLN^{R9C} trapped protein kinase A (PKA), which blocked PKA-mediated phosphorylation of wild-type PLN and in turn delayed decay of calcium transients in myocytes. These results indicate that myocellular calcium dysregulation can initiate human heart failure—a finding that may lead to therapeutic opportunities.

Heart failure is the leading cause of human morbidity and mortality (*1*). Reduced contractile function and pathological remodeling are recognized clinical hallmarks of heart failure, but the critical early events that impair myocyte performance are largely undefined (*2*). Intracellular Ca^{2+} handling is the central coordinator of cardiac contraction and relaxation (*3*). Contraction begins with sarcoplasmic reticulum (SR) release of Ca^{2+} into the cytosol via the ryanodine receptor; relaxation occurs with SR Ca^{2+} reuptake through the Ca^{2+} adenosine triphosphatase (ATPase) SERCA2a pump. Phospholamban (PLN), an abundant, 52–amino acid transmembrane SR phosphoprotein (*4*), regulates the Ca^{2+} ATPase SERCA2a. SERCA2a activity is decreased in human heart failure (*5, 6*), but whether this is a primary or secondary process that reflects changes in SERCA2a, PLN, and/or other molecules is unknown. Studies in mice suggest that PLN has a fundamental role: PLN protein levels correlate with cardiac contractile parameters (*7, 8*), superinhibitory PLN molecules impair heart func-