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Citation for published version:
Yeyati, PL, Bancewicz, RM, Maule, J & van Heyningen, V 2007, 'Hsp90 selectively modulates phenotype in vertebrate development' PLoS Genetics, vol 3, no. 3, pp. e43. DOI: 10.1371/journal.pgen.0030043

Digital Object Identifier (DOI):
10.1371/journal.pgen.0030043

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
PLoS Genetics

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Hsp90 Selectively Modulates Phenotype in Vertebrate Development

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Compromised heat shock protein 90 (Hsp90) function reveals cryptic phenotypes in flies and plants. These observations were interpreted to suggest that this molecular stress-response chaperone has a capacity to buffer underlying genetic variation. Conversely, the protective role of Hsp90 could account for the variable penetrance or severity of some heritable developmental malformations in vertebrates. Using zebrafish as a model, we defined Hsp90 inhibitor levels that did not induce a heat shock response or perturb phenotype in wild-type strains. Under these conditions the severity of the recessive eye phenotype in sunrise, caused by a pax6β mutation, was increased, while in dreumes, caused by a sufu mutation, it was decreased. In another strain, a previously unobserved spectrum of severe structural eye malformations, reminiscent of anophthalmia, microphthalmia, and nanophthalmia complex in humans, was uncovered by this limited inhibition of Hsp90 function. Inbreeding of offspring from selected unaffected carrier parents led to significantly elevated malformation frequencies and revealed the oligogenic nature of this phenotype. Unlike in Drosophila, Hsp90 inhibition can decrease developmental stability in zebrafish, as indicated by increased asymmetric presentation of anophthalmia, microphthalmia, and nanophthalmia and sunrise phenotypes. Analysis of the sunrise pax6β mutation suggests a molecular mechanism for the buffering of mutations by Hsp90. The zebrafish studies imply that mild perturbation of Hsp90 function at critical developmental stages may underpin the variable penetrance and expressivity of many developmental anomalies where the interaction between genotype and environment plays a major role.

Introduction

Human malformations frequently show no clear Mendelian inheritance pattern, even when familial recurrence suggests a strong underlying genetic component. Such phenotypic variability is generally defined as incomplete penetrance or variable expressivity [1,2], and it may be influenced by genetic background as well as by environmental factors. Predicting phenotypic outcomes for such cases is often an impossible challenge in clinical genetics. As a corollary, it has become clear that robustness of the wild-type (WT) phenotype to extensive genetic and environmental variation may be ascribed to the complexity, and hence strong buffering capacity of gene networks and cellular surveillance mechanisms [3,4]. These homeostatic systems are of major clinical relevance as potential prophylactic and therapeutic targets. Understanding the molecular events that can alter the balance between cryptic and overt phenotypes is therefore an important endeavour.

Based on observations in Drosophila [5], we set out to assess the role of stress-response pathways, particularly of heat shock protein 90 (Hsp90) function, in phenotype modification, using zebrafish as a vertebrate model. Hsp90 proteins are environmentally responsive chaperones, encoded at multiple loci in vertebrates. Under normal conditions they assist the maturation and folding of newly synthesised proteins and escort metastable regulatory molecules such as kinases and steroid hormone receptors [6]. In response to mutation or environmental stress, additional Hsp90 capacity is required to maintain newly destabilized proteins in a folding competent state. An unexpected consequence of this dual role is that Hsp90 can become functionally limiting, so that overt phenotypes may be uncovered from previously cryptic genetic variants, in spite of Hsp90 being further induced under stress [7]. Two recent studies showed that Hsp90 perturbation affects nearly all body parts in Drosophila and numerous morphological phenotypes in Arabidopsis [5,8]. Most changes were recurrent in a strain-specific manner and enriched by subsequent inbreeding, suggesting that they arose from previously silent but preexisting genetic variants [5]. Recently, it was proposed that Hsp90 can affect the epigenetic state at specific loci, leading to phenotype modulation [9]. Interfering with Hsp90 buffering therefore has phenotypic consequences, which are dependent on underlying genetic and epigenetic components, although it is not clear whether some preexisting genetic change is required for epigenetic inheritance of the trait [7].

The Hsp90 loci identified in humans have distinct
Author Summary

Genetic variation is not always expressed as a single consistent phenotype even in familial diseases. Unilateral malformations in paired organs, such as the failure of an eye to develop on one side only, also remind us that gene function is often modified by environmental factors. Following observations by others in fruit flies, we explored the underlying mechanisms for such phenotypic fluctuation, using zebrafish as a vertebrate model. Earlier work suggested involvement of chaperone proteins like Hsp90, which assist with normal protein folding during development and also work overtime to keep proteins functional in response to environmental stress. Using specific drugs at defined times in early development for the limited reduction of Hsp90 activity, we showed that different cryptic genetic variants could be revealed consistently in genetically distinct fish strains. Once uncovered, the frequency of these variants was increased by inbreeding, confirming the role of underlying genetic factors. Similarly, we could modify the phenotypic severity of some—but not all—known gene variants, worsening some and improving others. It emerged that the most susceptible variants were those carrying amino acid alterations, in which assisted protein folding may either restore near normal function or facilitate malfunction, thus worsening phenotype. This insight may allow us to prevent recurrent malformations by minimizing or perhaps even countering the effects of exposure to environmental stress during development.

subcellular localisation: HSP90 alpha and beta, encoded by HSP90AA1 and HSP90AB1, are expressed in the cytoplasm; while GRP94, a glucose responsive form encoded by HSP90B1 resides in the endoplasmic reticulum; and there is a mitochondrial form, TRAP1 (TNF receptor-associated protein 1). Genetic studies in mice suggest that despite the close similarity between Hsp90aa1 and Hsp90ab1, these paralogues are not functionally redundant [10]. The cytosolic Hsp90 proteins are thought to work as homodimers, but in association with several cochaperones [11]. The homodimer binds and releases its “client” proteins, aided by cochaperones, in an ATP-driven cycle [12–14]. Hsp90 function from all four loci is specifically inhibited by compounds like geldanamycin or radicicol, which compete with ATP for the nucleotide-binding pocket, so disrupting Hsp90 activity [15,16]. Inhibition shifts the cells from protein folding or activation, to degradation of most, but not all, unfolded client proteins by the proteasome system [17–20]. Complete loss of Hsp90 function is lethal, as multiple essential pathways are inactivated, a feature exploited in cancer treatment, where tumour-cell growth is heavily dependent on mutated oncogenic proteins that require Hsp90 assistance to function during malignant progression (reviewed in [21] and [22]). Increased binding affinity of Hsp90 inhibitors to these mutated protein complexes preferentially sensitises them to Hsp90 inhibition, allowing differential killing of malignant versus normal cells [23]. Use of Hsp90 inhibitors has also been proposed for amelioration of protein-aggregation-associated neurodegenerative diseases [24,25]. Hsp90 protein function is required at all times in eukaryotic cells [26,27], but under stress conditions higher levels are achieved through induction of a heat shock response. Recent genome-wide approaches have expanded the set of Hsp90 client proteins and required cofactors that are thought to confer substrate specificity [28–30]. These new findings are extending the role of Hsp90 from protein folding to gene regulation (reviewed in [31]) with potential impact on cellular epigenetic states (reviewed in [32]). Hsp90 inhibitors used above a threshold level induce a heat shock response through the activation of heat shock factor 1, which is normally silenced by association with Hsp90 [33].

Classical examples of developmental anomalies with variable severity and penetrance include holoprosencephaly, neural tube defects, cleft lip and palate, and heart malformations. Recently our human disease interests have focused on severe eye malformations, such as anophthalmia, microphthalmia, and coloboma [34–36]. These provide additional good examples of non-Mendelian inheritance patterns, where the relative contribution of environmental and genetic factors to the complex phenotypes remains controversial. If vertebrate phenotypes are also modulated by Hsp90, these could provide the molecular link between periodic environmental fluctuation and the overt manifestation of genetic susceptibility. If the chaperone system plays a buffering role in the variability of certain mutant phenotypes, compromising Hsp90 function may alter the severity or penetrance of some defined traits without affecting overall development.

Here we report that previously unobserved specific developmental anomalies were uncovered following partial inhibition of Hsp90 function in zebrafish. The spectrum of phenotypes observed and the organ systems affected depend both on the strain used and on the developmental stage at which the carefully controlled Hsp90 inhibitor is administered. Breeding studies clearly indicate that the phenotypes are manifestations of underlying cryptic genetic variation. Under our conditions, WT strains do not give rise to the rare malformations we observed in some mutant strains but do reveal more common anomalies that may reflect the uncovering of generally silent common gene variants. In addition to the de novo exposure of cryptic variation, we also showed that Hsp90 inhibition can modify the severity of some, but not all, already characterized developmental anomalies. Phenotype amelioration was observed in one case and increased severity in another. Both of the modified phenotypes were characterized as missense mutations at different loci. No phenotype modulation was observed at two different loci where the mutations had been identified as nonsense changes. Molecular studies show that in the presence of Hsp90 inhibition one of the mutant proteins studied is rapidly decreased, while its WT allele is largely unaffected, providing a molecular model for Hsp90 modulation of phenotype at the organisinal level.

Results

Establishing Drug, Morpholino Doses, and Treatment Timing in WT Strains

Geldanamycin (GA) has been used previously to examine the requirement for Hsp90 during zebrafish development [37]. The drug dose used (35 μM GA) led to developmental malformations in 80% of the treated embryos regardless of the strains tested [37], and therefore independently of underlying genetic background.

To investigate treatment conditions that could affect the phenotypic outcome selectively in a strain-specific manner, we initially assessed the developmental stages and highest drug doses at which Hsp90 function is decreased without affecting viability, or inducing highly penetrant or severe
Hsp90 Modulates Phenotype in Vertebrates

Table 1. Zebrafish Strains Used in This Study

| Strains   | Origin       | Mutated Gene     | Original Phenotype |
|-----------|--------------|------------------|--------------------|
| WIK       | Wild         | WT               | –                  |
| *AB       | Wild         | WT               | –                  |
| Tuebingen | Wild         | WT               | –                  |
| Golden    | Wild         | SLC24AS*         | Reduced pigmentation |
| Albino    | Wild         | alb              | Reduced pigmentation |
| syu       | ENU          | shh (stop)       | U-shaped somites   |
| yot       | ENU          | gli2 (stop)      | U-shaped somites   |
| sri       | ENU          | pax6b (missense) | Lens, eye size     |
| dre       | ENU          | sufi (missense)  | Lens, eye size     |
| AMN       | ENU          | Unknown          | Eye, axis duplication |

WT strains were originally obtained from the Zebrafish International Resource Centre [80]. Mutant strains were isolated from the Tuebingen ethylmethanesulfonate mutagenesis screen [79].

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Developmental defects in WT strains (Table 1). Hsp90 function was reduced by treating embryos with radicicol (a macrolactone), which is functionally equivalent to but structurally distinct from GA (a benzoquinone ansamycin). Subsequently we also used the GA class inhibitor 17-allylamino-17-demethoxygeldanamycin (17AAG) [38], which has low toxicity and is now in clinical trials for cancer therapy [21]. Morpholinos, for knocking down the activity of the two Hsp90 alpha (hsp90a [Danio rerio] and HSP90AA1 [Homo sapiens]) and Hsp90 beta (hsp90ab1 [D. rerio] and HSP90AB1 [H. sapiens]), were also used as additional controls for drug-specific inhibition of Hsp90.

We tested a range of doses at 30% and 50% epiboly and recorded novel phenotypes and viability. When treatment was initiated at 30% epiboly (defined as 4.6 h postfertilization [hpf]), both drugs caused multiple phenotypes and reduced viability assessed at 24–48 hpf. For example, among those surviving to 24 hpf following treatment with 10 μM radicicol or 3.3 μM 17AAG, we repeatedly observed growth retardation, decreased pigmentation, pericardial oedema with or without abnormal heart morphology, fin-fold, and notochord defects (Figure 1B–1K). Both drugs caused mild oedema and rarely severe oedema with altered heart morphology (Figure 1I and 1J; Video S1). 17AAG also elicited inversion of left-right heart looping or heterotaxia in very high frequencies (Figure 1M; Videos S2 and S3), while after radicicol treatment only a few embryos displayed abnormal heart looping in the absence of oedema (Figure 1N, Videos S4–S6). Radicicol also produced a few cases of severe growth retardation. Most of these phenotypes were present in all WT strains tested, but with clearly different frequencies (Figure 1A).

Higher survival rates and lower frequencies of developmental malformations were achieved by initiating treatment with the same doses but later in development, 50% epiboly (5.25 hpf). Many of the elicited phenotypes were milder and transient, like growth delay, fin-fold defects, or mild heart oedemas mostly with normal morphology and circulation. These embryos were generally indistinguishable from sibling controls by 5 d postfertilization (dpf). Notochord folds were noted but heart looping was unaffected. These phenotypes were again shared by most strains tested, albeit at slightly different frequencies. The penetrance of these common phenotypes in each strain was apparently decreased compared to earlier treatment (Figure S1). In agreement, these drug doses had no adverse developmental effects when treatment was initiated at 75% epiboly (8 hpf). We assessed the molecular events elicited by initiating treatment at 50% epiboly with the indicated drug doses by monitoring heat shock protein 70 (Hsp70) levels in treated embryos. Hsp70 was not induced at any of the inhibitor doses tested (Figure 2A). Hsp90 functional reduction was confirmed by monitoring the Hsp90 client protein RAF1, whose levels decrease with Hsp90 inhibition in a dose-dependent manner (Figure 2B) [39]. Although this gives an indirect estimation of Hsp90 inhibition, the residual presence of RAF1 protein, even at the highest inhibitor dose used, demonstrates that only partial Hsp90 functional reduction was elicited.

Morpholinos are injected at two to four cell stage, which decreases Hsp90 protein levels at earlier developmental stages than the selected conditions for chemical treatment; but doses that do not give rise to severe phenotypes when injected into WT embryos still help to validate chemical inhibition studies. Injecting the double morpholino knockdown (Hsp90 a+b: 6 ng of hsp90a and 6 ng of hsp90b) partially decreased Hsp90 protein levels (Figure 2C) and had no noticeable effect on embryos derived from the WT strains Golden (n = 71). Higher doses occasionally produced heart defects, such as inverted looping (Golden: 4/100) (Figure 1O) or no looping (WIK: 5/36) (Figure S2A), oedema (Golden: 10/100 and WIK: 0/36), and notochord defects (Golden: 2/100 and WIK: 0/36), but also decreased viability (Golden: 40/100). The low frequency of heterotaxia is in agreement with our observation that trait penetrance decreases when Hsp90 function is chemically reduced before 30% epiboly (unpublished data). Doses above 10 ng of each morpholino (Hsp90 a+b) almost completely erased Hsp90 protein, greatly reducing viability, with severe oedema in most embryos (17/20 injected) (Figure S2B). While the specific chemical inhibitors reduce the functional capacity of Hsp90 without decreasing Hsp90 protein levels, in morpholino-injected embryos Hsp90 levels were reduced in a dose-dependent manner with the consequent Hsp70 induction and decreased RAF1 levels (Figure 2C).

The two chemically unrelated drugs and morpholino knockdown produced an overlapping spectrum of phenotypes, showing different frequencies and severities that may be due to differences in drug metabolism (between radicicol and 17AAG) [40–41], or treatment timing (between drugs and morpholino). Most of the phenotypes observed by initiating drug treatment at 30% epiboly were shared by all strains tested but revealed at different frequencies and therefore represent Hsp90 buffering of common gene variants or polymorphisms presumably present in different strain specific combinations. Treatment timing was critical for the penetrance of these traits.

Importantly, when drug treatment was initiated at 50% epiboly, rather than 30%, there was no significant impairment of development in embryos derived from WT strains. The selected drug concentrations reduce Hsp90 functional activity without inducing a heat shock response, which could obscure the effect of Hsp90 reduction during development. Consequently, to investigate the effect of Hsp90 phenotype...
modulation on genetic mutants, we treated embryos at 50% epiboly with 10 μM radicicol or 3.3 μM 17AAG, or lower, so minimizing the potential appearance of common developmental defects by narrowing the developmental window for the perturbation.

Hsp90 Modifies the Severity of Mendelian Eye Mutants

Variable severity, age of onset, and intrafamilial variability are often observed in classical Mendelian monogenic diseases. In order to investigate possible factors that could alter the phenotypic outcome of simple traits, we assessed the effect of
Partial Hsp90 inhibition on two Mendelian eye mutants: sunrise (sri) and dreames (dre) (Table 1).

The Pax6b mutant sri (R.M. Bancewicz, unpublished data) and the sufu mutant dre (Table 1) [42] both show abnormal lens development (Figure 3) [43], with complete penetrance but variable severity [1]. This is demonstrated by a decrease in lens size relative to the retina (Figure 3B and 3C) reflecting abnormal development of surface ectoderm-derived structures [44]. Occasional failure to close the optic fissure (coloboma) is also observed (Figure 3D). Upon Hsp90 functional reduction, lens size relative to the area of the retina was significantly decreased in homozygous sri embryos while homozygous dre remained unaltered (Figure 3I; Table 2). Lens shape was significantly altered in both homozygous mutants (Figure 3J; Table 2); but while in treated sri embryos the lens became more elliptical, in treated dre embryos it became more circular, more closely resembling lens shape in WT fish.

The significant effect of Hsp90 inhibition on the sri background prompted us to investigate whether dominance relationships could be altered in vertebrates, as previously observed in Drosophila and Arabidopsis [7]. Notably, in heterozygous sri embryos and in an additional genetically unrelated strain (AMN strain) (Table 1), these parameters were unaffected by the drug treatment (Table 3).

Factors That Contribute to Phenotype Modification by Hsp90 Inhibition

The observation that both lens size and shape can be affected in sri derived embryos but only lens shape was altered in dre embryos suggests that the two developmental processes can be uncoupled. As described above, Hsp90 chemical inhibition by the structurally distinct drugs radicicol or 17AAG significantly reduced lens size and worsened lens eccentricity of sri homozygous embryos (Table 4). Geldamycin (GMP), a structurally related but inactive form of 17AAG, was used as a negative control to discount direct drug toxicity as the mechanism for this phenotype modulation [45]. GMP had no effect on these parameters (Table 4).

We then decreased Hsp90 activity with the combined morpholinos: Hsp90 a+b. Coinjecting 6 ng of each morpholino significantly affected lens size without altering lens shape (Table 4), confirming that the two developmental processes can be uncoupled. Hsp90 can also be diverted from its developmental role by the induction of a generalised stress response [46]. Brief heat exposure of homozygous sri mutants also resulted in altered phenotypic severity not observed in control siblings (Table 4). Interestingly, lens size and shape were affected by heat shock, but in contrast to Hsp90 chemical inhibition, lens shape was ameliorated instead of worsened (compare t-values, Table 4). Several factors may be contributing to these phenotypic outcomes: functional reduction of Hsp90 by these treatments may be milder than Hsp90-specific chemical inhibition. This seems likely as RAF1 levels observed after morpholino injections are comparable to those seen in control embryos, despite the reduced Hsp90 protein levels (6 ng Hsp90 a+b, Figure 2C). Higher morpholino doses drastically reduced RAF1 levels but mostly produced severely oedematous embryos whose eyes could not be analysed. More significantly, we observed a positive correlation between Hsp70 levels and amelioration of the lens eccentricity (6 ng Hsp90 a+b, Figure 2C; Hsp70 levels, Table 4). Hsp70 is constitutively expressed during normal lens development from 28 hpf [47]. Earlier induction by heat treatment or morpholino knock down may contribute to the partial rescue of the sri lens phenotype, in contrast to drug treatment conditions that were specifically selected to avoid inducing a heat shock response. Heat shock also induces Hsp90 expression, and this may contribute additional capacity to cope with the increased requirement for chaperone activity following heat shock.

Mendelian Mutants Resistant to Mild Hsp90 Functional Reduction

To address how commonly Hsp90-mediated release of genetic variation is observed, we selected Mendelian mutants that affect somitogenesis, a developmental process previously found to require the chaperone function of Hsp90 [37,48].
We used the same experimental conditions as for the eye mutants.

We studied the semidominant you-too (yot) mutant, with U-shaped somites, carrying a premature protein truncation mutation in the gli2 gene (Table 1) [49]. Although live heterozygous yot embryos are indistinguishable from wild type, histological analysis had shown a reduction in the MyoD expression [50]. Murine MyoD has been previously identified as an Hsp90 client protein [51], which dissociates from the cochaperone cdc37 in the presence of GA potentially interfering with myoblast differentiation programs [52]. The homozygous yot phenotype is fully penetrant with variable severity. Hsp90 inhibition did not significantly worsen the homozygous phenotype of yot embryos (unpublished data), nor were somite defects uncovered when heterozygotes were treated with radicicol. Thus Hsp90 functional reduction had no effect on the gli2 pathway in spite of the already compromised low levels of MyoD protein.

The phenotypically variable, recessive somite mutant sonic-you (syu) with a stop codon mutation in the sonic hedgehog gene shh [53] was also unaffected by Hsp90 inhibition (unpublished data). Thus, mild perturbation of Hsp90 function does not affect all Mendelian phenotypes during vertebrate development emphasizing the selective interplay between Hsp90 buffering and the underlying genotype.

Hsp90 Functional Reduction Reveals Previously Cryptic Eye Trait

During our studies of the yot mutant strain, radicicol treatment revealed novel eye phenotypes not previously described in heterozygous or homozygous embryos of this

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**Figure 3. Assessing Range of Expressivity and Quantifying Phenotype Modulation in sri and dre Strains**

(A–H) Phenotype of the sri and dre eye mutants at 5 dpf. (A–D) Lateral view of live embryos; (E–H) histological sections. (A) WT; lens (white-dotted lines) and retina (red-dotted lines) measurements were used to asses phenotypic severity; (E) WT section showing retina (re), lens (le), cornea (co); (B) and (F) sri mild and (C) and (G) sri severe; (D) dre with coloboma (cb); (H) dre showing flattened anterior segment.

(I and J) Ranked plots showing distribution of quantitative eye parameters in treated (white circles) and sibling control (black circles) homozygous embryos: (I) Ratio of retina to lens area; (J) Lens shape or eccentricity.

doi:10.1371/journal.pgen.0030043.g003
strain [54,55]. These new phenotypes, ranging from bilateral anophthalmia to unilateral microphthalmia (Figure 4), were never observed in three other tested mutants, syu, dre, or sri (Table 1). Molecular analysis proved these eye defects to be independent of the gli2 mutation in the yot strain, and the yot genotype and phenotype were rapidly lost from carriers of this new variable eye trait, named AMN for anophthalmia, microphthalmia, and nanophthalmia.

In seven yot-derived mated pairs, from a total of 24, partial Hsp90 inhibition repeatedly gave rise to affected offspring; these were designated transmitter parents for the AMN eye trait (P0, Figure 5; Table 5). Their offspring revealed a significant increase in the frequency of developmental eye phenotypes compared to those from nontransmitter pairs and from genetically unrelated WT strains (Table 5). AMN-like defects were not induced repeatedly in any other mutant or in WT strains tested (n > 3,000), where even higher inhibitor doses (10 μM radicicol or 3.3 μM 17AAG and Hsp90 morpholinos) or earlier treatment (at 30% epiboly) were ineffective (Multiple Unrelated Strains, Table 5; unpublished data). Except for two embryos among several thousand scored, whenever eyes were affected in WT-treated stocks, it was in the presence of other more severe defects like oedema or incomplete development. These occasional abnormal phenotypes do not recur in subsequent generations and their frequency was unaltered by Hsp90 inhibition; they were not counted in the analysis (Multiple Unrelated Strains, Table 5). Where reduced Hsp90 function repeatedly reveals increased frequency of offspring with a particular phenotype, it is deemed to be uncovering a previously cryptic mutation in susceptible populations (Parental Transmitters, Table 5). Unlike abnormal heart looping, notochord, or fin-fold defects, the AMN phenotype was revealed uniquely in the yot population. Hsp90 inhibition therefore uncovers cryptic phenotypes selectively in susceptible populations, during vertebrate development.

### Table 2. Quantitative Effect of Hsp90 Inhibition on Embryos Derived from Eye Mutants

| Strain | Treatment | Number of Eyes | Retina Area/Lens Area | Lens Eccentricity |
|--------|-----------|----------------|-----------------------|------------------|
|        |           |                | Mean  SD  p-Value  t-Value | Mean  SD  p-Value  t-Value |
| dre (−/−) | Ethanol  | 122            | 8.45  1.40  No significance | 0.49  0.11  <0.001  −3.56 |
|         | Radicicol | 140            | 8.51  1.47  No significance | 0.44  0.11 |
| sri (−/−) | Ethanol  | 35             | 7.27  0.69  <0.001  −7.37 | 0.37  0.10  <0.001  −3.90 |
|         | Radicicol | 35             | 10.66 2.63  No significance | 0.49  0.14 |

Eye measurements (Materials and Methods) were compared by two sample t-tests assuming unequal variances. Drug doses were: ethanol (0.04 %) and radicicol (10 μM). Treatment was initiated at 50% epiboly.

### Table 3. Synergistic Effect of HSP90 Inhibition and Homozygous sri Phenotype

| Strain | Treatment | Number of Eyes | Retina Area/Lens Area | Lens Eccentricity |
|--------|-----------|----------------|-----------------------|------------------|
|        |           |                | Mean  SD  p-Value  t-Value | Mean  SD  p-Value  t-Value |
| sri (−/−) | DMSO      | 50             | 8.58  0.78  <0.001  −5.10 | 0.42  0.12  <0.001  −5.10 |
|         | 17AAG     | 49             | 9.65  1.25  No significance | 0.47  0.09 |
| sri (+/+) | DMSO      | 105            | 7.09  0.52  No significance | 0.357  0.09 |
|         | 17AAG     | 104            | 6.96  0.56  No significance | 0.355  0.11 |
| AMN*   | DMSO      | 46             | 6.66  0.47  No significance | 0.376  0.11  |
|         | 17AAG     | 47             | 6.61  0.39  No significance | 0.343  0.09 |

Drug doses: DMSO (0.03%) and 17AAG (2.5 μM) added at 50% epiboly.
*AMN-derived phenotypically WT embryos.

SD, standard deviation

doi:10.1371/journal.pgen.0030043.t002
doi:10.1371/journal.pgen.0030043.t003

[54, 55]
elicit the phenotype [57]. The complexity of the gene interactions will be further illustrated by divergent responses to Hsp90 inhibition (see below).

The inbreeding regime maintained the complex eye trait for at least five generations but never fixed it at Mendelian frequencies, most likely because only phenotypically unaffected individuals could be mated (Inbreeding, Figure 5). The low frequency of AMN defects observed in subsequent generations is another indication of the oligogenic nature of the trait. Nevertheless, the sensitivity to reduced Hsp90 levels remains. Female transmission of the AMN trait was observed after outcrossing phenotypically WT F1 carrier females to genetically unrelated WT males (F2, Female Outcross, Figure 5) with variable frequencies. The affected individuals derived from these outcrosses were viable and fertile, suggesting loss of some deleterious modifiers. However, the phenotype was not observed in the generation arising from these affected sibling crosses (F3, Female Outcross, Figure 5), but it was recovered after further inbreeding (F4, Female Outcross, Figure 5). This inheritance pattern is consistent with a requirement for a maternal recessive component with partial penetrance. In contrast, when selected males were outcrossed to genetically unrelated WT females, AMN-like embryos were not observed initially (F2, Figure 5, Male Outcross). However, AMN-like embryos were recovered in the first generation of subsequent inbreeding (F3, Male Outcross, Figure 5) suggesting that homozygosity of a recessive zygotic component is required. The recessive genetic basis of the AMN trait, suggested by these inheritance patterns, does not exclude the possibility that additional genetic or epigenetic modifiers also affect the severity and/or penetrance of the AMN phenotype.

### Table 4. Morphological and Quantitative Components Are Differentially Buffered by Hsp90

| $sri$ (−/−) Treatment | Number of Eyes | Retina Area/Lens Area | Lens Eccentricity (Shape) |
|-----------------------|---------------|-----------------------|--------------------------|
|                       |               | Mean | SD  | p-value | t-value | Mean | SD  | p-value | t-value |
| Ethanol               | 35            | 7.27 | 0.69| <0.001  | −7.37   | 0.37 | 0.10| <0.001  | −3.90   |
| Radicicol             | 35            | 10.66| 2.63| 0.49     | 0.14    | 0.49 | 0.11| 0.029   | −2.24   |
| GMP                   | 35            | 7.49 | 1.47| <0.001  | −4.30   | 0.34 | 0.11| 0.029   | −2.24   |
| 17AAG                 | 35            | 9.71 | 2.67| 0.41     | 0.16    | 0.41 | 0.16| 0.027   | −2.26   |
| STD Mo                | 67            | 8.15 | 0.84| <0.001  | −4.42   | 0.36 | 0.10| No significance |
| Hsp90 a−b             | 66            | 9.07 | 1.47| 0.34     | 0.09    | 0.34 | 0.09| 0.027   | −2.26   |
| Untreated             | 35            | 7.85 | 0.84| 0.005    | −2.90   | 0.41 | 0.09| 0.027   | −2.26   |
| Heat shock            | 35            | 8.56 | 1.17| 0.36     | 0.08    | 0.36 | 0.08| No significance |

Drug doses: ethanol (0.04%), radicicol (10 μM), GMP (2.5 μM), and 17AAG (2.5 μM) added at 50% epiboly. Injections comprised 6 ng of universal standard control morpholino or 6 ng each of the two morpholinos Hsp90 a−b (12 ng total). Heat shock response was induced by incubating $sri$ embryos for 30 min at 37°C at 50% epiboly. In all cases eye measurements were taken at 5 dpf.

SD, standard deviation

doi:10.1371/journal.pgen.0030043.t004

**Figure 4.** Eye Defects and Body-Axis Duplications Observed in AMN Population

Dorsal views shown are: (A) Unilateral anophthalmia; (B) Unilateral microphthalmia; and (C) Unilateral nanophthalmia (UN), illustrating retina (re), lens (le), cornea (co). (D) Ventral view of cyclopic embryo is shown. (E) Bilateral anophthalmia is shown (top, lateral view; bottom, dorsal view). (F) Anterior body-axis duplication (ABD), two complete heads formed; incomplete ABD were also observed (not shown). (G) Posterior body duplication (PBD), two notochords showing adjacent somite boundaries (arrows), culminating in two tails.

doi:10.1371/journal.pgen.0030043.g004

Hsp90 inhibition reveals digenic nature of the AMN trait

Although all transmitting females tested produce some affected offspring upon outcrossing to an unrelated WT male, treatment of embryos from these pairs revealed two types of AMN transmitter females: Two inbred F1 females crossed to
components of the canonical Wnt pathway may underlie the AMN complex trait

Anterior and posterior body duplications were also observed in the offspring of Group 1, but not Group 2 females. The copresentation of axis duplication with cyclopia or absent eyes is reminiscent of phenotypes caused by alterations in the canonical Wnt signalling pathway [58–60]. To test this suggestion, embryos derived from both groups of females were treated for five minutes with lithium chloride, a glycogen synthase kinase 3b inhibitor that mimics Wnt signalling [59,61]. At the selected doses and incubation times, embryos derived from WT strains, or from Group 2 females incrossed to AMN transmitting males, were barely affected (Figure 7) [59]. In contrast, lithium-treated embryos from Group 1 females outcrossed to genetically unrelated WT males show a significant increase in the number of eye defects compared to their untreated siblings ($n = 47; p = 0.001$) (Figure 7). Genes from the canonical Wnt pathway may, therefore, be implicated in the genesis of the eye trait in Group 1.

In agreement with the contrasting phenotypic outcomes following Hsp90 inhibition in embryos derived from Group 1 and Group 2 females, these results indicate that the original AMN trait comprises at least two loci sensitive to Hsp90 fluctuation. Recombining of variant alleles at these seemingly antagonistic loci may explain the sudden rise in the frequency of AMN from parental to F1 generation, observed in some of the crosses.

Modulation of Developmental Stability in Vertebrates by Hsp90

Symmetry of bilateral traits is often used as a parameter for developmental stability [62]. Measures of developmental instability include nondirectional quantitative deviation from bilateral symmetry, also called fluctuating asymmetry. Other asymmetric manifestations include unilateral absence of organ structures and perturbed organ distribution across the left-right axis or phenodeviants [63].

Table 5. Frequency of Eye and Body-Axis Defects in Parental AMN and WT Strains

| Strains | Treatment | Unilateral | Bilateral | AMN frequency | $p$-Value | Cyclopia | ABD | PBD |
|---------|-----------|------------|-----------|---------------|-----------|----------|-----|-----|
| Unaffected parental transmitters of eye traits (seven pairs) | Ethanol | 1 | 2 | 0.45% (3/664) | 0.011 | 1 | 0 | 0 |
| | Radicicol | 3 | 10 | 2.00% (13/641) | Not significant | 3 | 0 | 2 |
| | | | | | | | | |
| Unaffected parental nontransmitters (17 pairs) | Ethanol | 0 | 1 | 0.26% (1/372) | Not significant | 0 | 0 | 0 |
| | Radicicol | 0 | 0 | 0% (0/303) | Not significant | 0 | 0 | 0 |
| Multiple unrelated strains | Ethanol | 0 | 1 | 0.05% (1/1787) | Not significant | 0 | 1 | 0 |
| | Radicicol | 0 | 0 | 0% (0/2411) | Not significant | 0 | 0 | 0 |

Data represent the combined results of several spawning treated at 50% epiboly with different drug doses. For each egg lay siblings are split between solvent controls or treated. Parental carriers of AMN emerged from crosses of heterozygous yot to WT fish. Parental generation (P0) fish were assigned to transmitter or nontransmitter groups based on whether they give rise AMN embryos after Hsp90 inhibition. Golden and WK strains were used as genetically unrelated WT fish, to determine the background frequency of sporadic and drug induced defects. Drug doses: ethanol (0.0004%–0.004%), radicicol (1.6 and 10 μM), DMSO or methanol (0.016%, 0.03%, and 0.04%), GMP (2.5 μM), and 17AAG (1.4, 2.5, and 3.3 μM).
We observed a relative increase in the frequency of unilaterally affected eyes (mostly anophthalmia) in embryos from AMN females following Hsp90 functional reduction by inhibitor treatment or morpholino knock down (Table 7). Similarly, we observed a significant increase in the number of unilateral colobomas among sri-treated embryos (Table 7). The relative increase in the frequency of unilateral morphogenetic events between controls and treated embryos, suggests that Hsp90 can buffer developmental stability in vertebrates.

Inverted cardiac looping could be considered as another indication of compromised homeostasis during development. When 17AAG treatment is initiated at 30% epiboly we observed a dose-dependent increase in the frequency of heart-loop reversal or heterotaxia in WT strains (Figure 8A). Interestingly, at the same dose (2.5 μM 17AAG) the frequency of induced heterotaxias is higher in embryos from mutant strains like sri or AMN than in WT strains (incrosses, Figure 8B and 8C). When these carrier fish are outcrossed to WT strains, heterotaxia frequencies are significantly decreased (outcross, Figure 8C), suggesting that increased susceptibility to heterotaxia may be associated with homozygosity at predisposing loci. Alternatively, a previous unrelated mutation, which would not contribute to the trait under normal circumstances, could compromise development, rendering it more susceptible or dependent on fully functional buffering mechanisms like Hsp90.

Previous work has shown that in Drosophila, Hsp90 does not affect the fluctuating asymmetry of quantitative traits [64]. In contrast, Hsp90 can buffer stochastic processes in normal plant development [8]. Given the impact of Hsp90 reduction on the unilateral appearance of anophthalmia and coloboma among treated individuals over their sibling controls in some of our fish strains, we investigated whether Hsp90 could increase intra-individual quantitative differences in these mutants. Surprisingly, although Hsp90 reduction significantly affects the severity of lens defects in sri mutants (Table 3), it did not alter the variation between left and right measurements of any of these quantitative parameters (Table 8). Thus, as in Drosophila, under our experimental settings and statistical methods, Hsp90 plays no role on the developmental stability of quantitative traits. In contrast, Hsp90 clearly alters the consequences of stochastic processes on morphogenic or qualitative threshold traits like AMN, coloboma, and heart looping.

Hsp90 Can Modulate Phenotypes by Selective Chaperoning of Mutant Proteins

In order to understand phenotypic modulation of mutant phenotypes by Hsp90, we investigated the effect of compromised Hsp90 function on WT and sri mutant Pax6b proteins. Initial studies assessed the response of transfected WT and mutant expressed proteins to Hsp90 inhibitors. The sri mutation is expected to disrupt the folding of the homeo-domain that contains it (R.M. Bancewicz, unpublished data). Immunohistochemical studies show that while WT Pax6b protein remains mostly unaffected, the levels of Pax6b mutant protein are significantly decreased when Hsp90 function is impaired by 17AAG treatment (Figure 9). The

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**Table 6. Two Classes of AMN Transmitter Females Identified**

| Class | Treatment | Unilateral | Bilateral | Cyclopia | Frequency (p-Value) | ABD | PBD |
|-------|-----------|------------|-----------|----------|---------------------|-----|-----|
| Group 1: Increased penetrance upon Hsp90 reduction (two females, F1) | Controls | 5 | 5 | 1 | 12.5% (11/88) | 0.009 | 0 | 2 |
| | 17AAG | 19 | 9 | 1 | 27.6% (29/105) | 0 | 1 |
| Group 2: decreased penetrance upon Hsp90 reduction (three females, F1 to F5) | Controls | 4 | 1 | 1 | 4.18% (11/263) | 0.037 | 0 | 0 |
| | 17AAG | 1 | 1 | 0 | 0.76% (2/262) | 0 | 0 |

Data represent the combined results of several spawning treated at 50% epiboly with different drug doses. For each egg lay siblings are split between solvent controls or treated. Parental carriers of AMN emerged from crosses of heterozygous sri+ to WT fish. In subsequent generations, two groups of females were defined based on the opposing effects of Hsp90 reduction on AMN penetrance in embryos derived from outcrossing the females to males derived from WT Golden or WIK strains: Group 1 with increased frequency and Group 2 with decreased frequency of affected embryos after treatment. Phenotypes are grouped according to whether one (unilateral) or both (bilateral) eyes are affected.

Drug doses: ethanol (0.0004%–0.004%), radicicol (1.6 and 10 μM), DMSO or methanol (0.016%, 0.03%, and 0.04 %), GMP (2.5 μM), and 17AAG (1.4, 2.5, and 3.3 μM).

*Not all affected embryos were classified as unilateral or bilateral but still counted as affected, totals are therefore greater than the sum of each category.

ABD, anterior body-axis duplication; PBD, posterior body duplication.

doi:10.1371/journal.pgen.0030043.t006

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**Figure 6. Schematic Representation of AMN Phenotype Inheritance and Susceptibility to Hsp90 Reduction**

Embryos were treated with the solvent control (CN) or with radicicol (TR1), 17AAG (TR2) or Hsp90 α+β morpholino injection (TR3). Following Hsp90 inhibition, embryos derived from group 1 (G1) females showed increased penetrance, while those from Group 2 females (G2) had decreased penetrance of AMN phenotype.

doi:10.1371/journal.pgen.0030043.g006
decreased stability of the mutant protein suggests that it requires Hsp90 for maintenance in a stable conformation. The differential sensitivity of mutant and WT proteins when Hsp90 function is compromised provides a molecular explanation for the worsening of the sri phenotype upon treatment. pax6a and pax6b in zebrafish are the paradigm model for subfunctionalisation by differential distribution of long-range control elements (D. Kleinjan, personal communication). The nonoverlapping expression pattern of the two Pax6 isoforms leads to the lens-specific phenotype of the sri mutant and to the worsening of this phenotype when chaperone function is inhibited.

Table 7. Compromised Hsp90 Function Increases the Relative Frequency of Discrete Asymmetric Individuals

| Trait        | Hsp90    | Unilateral | Bilateral | Total | Asymmetry Relative Frequencya |
|--------------|----------|------------|-----------|-------|-------------------------------|
| AMN          | Control  | 15         | 21        | 502   | 41%                           |
|              | Reduced  | 38         | 28        | 549   | 57%                           |
| Coloboma     | Control  | 11         | 3         | 177   | 78%                           |
|              | Reduced  | 22         | 3         | 150   | 88%                           |

Data represents the combined results of several independent experiments, where Hsp90 was either chemically inhibited or reduced by morpholino knock down.

aRelative frequency of asymmetry calculated as the percentage of unilateral to the total of affected individuals.

bEmbryos derived from AMN strains: the affected embryos have either anophthalmia or microphthalmia. Cyclopia is considered bilateral.

cNumber of colobomas among embryos from sri homozygous parents.

doi:10.1371/journal.pgen.0030043.t007

Figure 7. Sensitivity of Group 1 Embryos to Lithium Chloride: An Inducer of Wnt Signalling

(A) Assessing duration of LiCl treatment that would not significantly affect embryos from WIK and Golden strains.

(B) LiCl treatment (0.3 M for 5 min) of embryos from Group 1 females outcrossed to WIK males gave rise to increased frequency of eye defects, while embryos derived from Group 2 females crossed to WT (not shown) or AMN transmitting males did not.

doi:10.1371/journal.pgen.0030043.g007
Within each individual was used. For WT strains, a...phology uncovered in Drosophila and Arabidopsis may be influenced by the relatively inbred nature of these organisms [5,8]. Zebrafish do not tolerate a high level of inbreeding, perhaps providing a better model for generally outbred human populations. The number of Hsp90 loci and the different spectrum of environmental triggers may also influence Hsp90 buffering capacity across phyla.

Opposite outcomes in the Mendelian microphthalmia mutants sri and dre, with the same tissues affected in both, demonstrate that Hsp90 modulation of phenotypes strongly depends upon the specific underlying genetic components. Despite the strong effect that Hsp90 inhibition had in sri homozygous embryos, it had no measurable effect in heterozygotes. This nonadditive response of the sri homozygous mutants to Hsp90 fluctuation confirms the role of Hsp90 in the maintenance of trait thresholds [67]. In contrast to plants and flies [7], these results imply that dominance relationships [1] may not be alterable in vertebrates by Hsp90—that is, previously recessive characters do not become dominant. The severity of the sri phenotype was also increased by heat shock. From the evolutionary point of view, increased temperature may not be a frequent mechanism through which Hsp90 releases variability in mammals, but this mechanism probably underlies the observations that maternal fever in humans and mild experimental hyperthermia in animals are associated with increased frequencies of developmental malformations, both mild and severe [68–70]. Similar adverse phenotypic outcomes may be elicited through induction of protein-folding stress in the developing embryo as a result of exposure to relatively low levels of environmental pollutants, or when endogenous protein aggregation levels are increased [71].

The uncovering of the complex AMN phenotypes focuses attention on the role of Hsp90 in oligogenic malformations. The involvement of multiple interacting loci, several with recessive effect, is indicated by the requirement for inbreeding, and the less than 25% peak frequency observed for AMN phenotypes. The involvement of two distinct sets of genes, but perhaps with overlapping functions, is suggested by the observation that AMN frequencies may be increased or decreased following Hsp90 inhibition in different parental groups. These opposing responses once more underline the complex mechanisms of interaction between developmental alleles and Hsp90-mediated release of genetic variation. The possibility of decreased phenotypic severity under stress conditions (Hsp90 reduction) is potentially of great evolutionary significance.

The rapid unmasking of divergent phenotypes after Hsp90 reduction helped us to break down the AMN trait into simpler components, indicating that this vertebrate model system may provide a robust approach to identifying cryptic genetic variation that can modify complex developmental traits. Similarly, heart-loop inversion was observed after earlier Hsp90 inhibition during development in all fish stocks tested, but the different frequencies among WT and mutant strains suggest underlying interacting genetic components. Further analysis of this may permit the identification of...

**Table 8. Compromised Hsp90 Function Does Not Affect Intraindividual Quantitative Differences (p-Values)**

| Strain | Hsp90 | Retina/Lens (Area) | Retina (Area) | Lens (Area) | Lens Eccentricity |
|--------|-------|------------------|--------------|------------|------------------|
| sri    | Control | 0.230 | 0.679 | 0.074 | 0.101 |
|        | Reduced | 0.519 | 0.335 | 0.661 | 0.465 |
| WT     | Control |            |            |            |                  |
|        | Reduced |            |            |            |                  |

Estimation of effects on trait symmetry of functional Hsp90 reduction by ANOVA: Logarithm of Left (L) - right (R) symmetry data were normalized to the mean trait value within each individual L - R / (L + R/2). Embryos with severe coloboma were excluded. For WT strains, a t-test of the left-right difference normalized to the mean trait value within each individual was used.

doi:10.1371/journal.pgen.0030043.t008
common susceptibility alleles at interacting loci. The observation that mutations at loci that would not normally contribute to a certain trait (e.g., \textit{pax6b} in heart looping) may potentiate the effect of compromised Hsp90 buffering, suggests that Hsp90 may act in concert with the organism's genotype. This highlights once more the different release of cryptic genetic variation in good health and disease [72].

Our results show that in vertebrates, Hsp90 can buffer the phenotypic variability between and within individuals of discrete qualitative morphological traits (mainly anophthalmia and coloboma in this study) culminating in morphologically asymmetrical individuals. The increased frequency of asymmetric unilateral eye defects upon Hsp90 inhibition implies that buffering by this chaperone system contributes to the stabilization of developmental processes that depend on the partition of signalling molecules across body axes. Asymmetric phenotypes could result from stochastic uneven left/right partitioning of signalling molecules. Altered left-right embryonic signalling can affect heart looping in a similar manner. It will be interesting to investigate how perturbation of Hsp90 activity amplifies the effect of these seemingly stochastic events, culminating in a significant increase of abnormal cases. The occurrence of unilateral and bilateral defects among AMN siblings is also strongly reminiscent of the complex human microphthalmia, anophthalmia, and coloboma spectrum [34] and indicates that environmental and stochastic factors continue to play an important role even when there are shared genetic components.

We show that in vertebrates, Hsp90 can also modulate phenotypic severity of continuous quantitative traits (e.g., relative lens size and lens shape in \textit{sri} mutants). Surprisingly, Hsp90 inhibition does not alter small quantitative left-right differences of these traits, either in \textit{sri} mutants or in WT

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**Figure 9. Differential Sensitivity of WT and \textit{sri} Mutant Pax6b Protein to Hsp90 Inhibition**

Cells were cotransfected with either WT or \textit{sri} mutant \textit{paxb} cDNA and GFP reporter. (A) Representative field of HeLa cells transfected with Pax6b-WT or Pax6b-\textit{sri} mutant immunohistochemically assessed for Pax6b protein levels (red) or control GFP frequency (green). Effects of treatment with different concentrations of 17AAG are shown compared with DMSO control. (B) Relative ratio of Pax6b positive (red cells) to GFP positive (green) cells for each treatment compared to the relevant DMSO control. The figures show one representative experiment from three independent experiments with similar results.

doi:10.1371/journal.pgen.0030043.g009
Materials and Methods

**D. rerio stocks.** Breeding fish were maintained at 28 °C in a circulating water system on a 14-h light/10-h dark cycle. Embryos were collected by natural spawning and were staged according to Kimmel [78]. The mutants *sunrise (sri)*, *d隆重 (dreno)*, *you too (yto)*, and *some you (syn)* were isolated in a large ENU mutagenesis screen [43] and were obtained from the Tuebingen Zebrafish Stockcenter [79], together with the WT strain Tuebingen. *AB, WIK, Albino, and Golden* are commonly used WT strains and were originally obtained from the Zebrafish International Resource Center at the University of Oregon [80]. Albino and Golden strains have mutations affecting only melanophore pigmentation [81–82].

**Mating strategies.** Pair mating is between two identified individuals. Incross is mating between siblings. Outcross is mating between different mutation carriers or different strains. Mutant stocks were maintained by alternate breeding to maintain between the parentalTuebingen strain and *AB, WIK, or Golden* WT strains except for subsequent inbreedings. All the Mendelian mutants used in this study are recessive. *sri mutants were found to be homozgyous viable and fertile, so that the Hsp90 inhibition study could be carried out using genetically homogeneous homozygous mating pairs. Other Mendelian mutants were studied by mating proven heterozygotes, which gave rise to 25% affected offspring. For yot, molecular analysis was used to confirm genotype and segregation of AMN eye defects among F1 embryos. Inbreeding of AMN line was carried out for at least four generations by successive increasing of identical strains that produced AMN affected embryos with or without Hsp90 inhibition.

**Treatment of D. rerio embryos with Hsp90 inhibitors and heat shock.** Embryos collected from breeding pairs were incubated in a 0.025–0.1 mg/ml solution of protease (Type I, Sigma, http://www.sigmaaldrich.com) in system water, rinsed ten times, and partially dechorionated. Treatment was initiated at 30, 50, or 75% epiboly [78] and left overnight at 28 °C, followed by drug wash-out using five rinses. Larvae were examined between 24 and 120 hpf or up to 5 dpf, with a few exceptions noted in a 0.1% solution of tricaine (Sigma), and immobilised in 4% methylcellulose (Sigma) where appropriate. To avoid variability between experiments, each spawning was split in two groups, and half was treated, while the other half was left as its respective control (treated only with the relevant solvent). Radicicol (Sigma) was dissolved in 100% ethanol, while UAMN (gift from P. Csermely) and 17AAG were dissolved in 100% DMSO or methanol (Sigma). DMSO alone increased the penetrance of the AMN phenotype in embryos from Group 1 females in a dose-dependent manner (unpublished data). Increasing doses of methanol did not alter the number of AMN affected embryos derived from these females. Neither ANN Group 2-derived embryos nor the unrelated sri-derived embryos have altered penetrance of increased severity when grown in the presence of DMSO or methanol compared to untreated siblings (Table 4). For Group 1-derived embryos 17AAG was therefore dissolved in methanol only. For all other mutants DMSO or methanol was used as solvents. Some of the controls were kept untreated (no solvent treatment and no dechorionation). Heat shock was carried out for 30 min at 37 °C in sealed petri dishes containing 30 ml system water floated on a water bath, followed by a return to 28 °C.

**Lithium treatment.** Lithium treatment was initiated at the same developmental stage as radicicol or 17AAG treatment. The embryos were incubated at 28 °C for 5–10 min with 0.3 M lithium chloride, then washed extensively, and returned to 28 °C for further scoring at 24–72 hpf.

**hsp90 morpholino microinjection.** The oligonucleotides were obtained from Gene-Tools (http://www.gene-tools.com). The sequences used were: hsp90a, 5'-CGTTGCAGAATGAGCCCCGATT-3'; hsp90b, 5'-TTGGATGGATGTTGCTAG-3'. Hela cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Gibco, http://www.invitrogen.com). We seeded 10⁵ cells onto 22 × 22-mm coverslips 18 h prior to transfection. Next 200 ng of Pax6 WT or sri mutant vectors were cotransfected with 50 ng of GFP reporter pEGFP-C1 (Invitrogen, http://www.invitrogen.com). *Hela* cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum (Gibco, http://www.invitrogen.com). We seeded 10⁵ cells onto 22 × 22-mm coverslips 18 h prior to transfection. Next 200 ng of Pax6 WT or sri mutant vectors were cotransfected with 50 ng of GFP reporter using Lipofectamine2000 (Invitrogen) as indicated by manufacturer. Lipofectamine complex was replaced 4 h posttransfection with fresh DMEM media containing 10% fetal bovine serum and 0.05% antibiotic-antimycotic. For all other treatments DMSO or methanol was used as solvents. Some of the controls were kept untreated (no solvent treatment and no dechorionation). Heat shock was carried out for 30 min at 37 °C in sealed petri dishes containing 30 ml system water floated on a water bath, followed by a return to 28 °C.
and Pax6b-sri DMSO controls were set as 100%. The effect of Hsp90 inhibition on Pax6b proteins was calculated as the relative percentage to each of these respective controls.

Western blotting. Lysates were prepared from 20–50 larvae according to Westerfield [84]. Bradford Protein Assay (Bio-Rad, http://www.bio-rad.com) was used to quantify protein levels for equal loading control by Coomassie staining of a duplicate gel. Western blot analysis was carried out using standard techniques. Specific analysis for the induction of a generalised stress response was carried out using Hsp70 antibody from Stressgen SPA-812 (http://www.stressgen.com). α-tubulin monoclonal antibody TATI [85] was used as loading control. RAF1 levels were detected with Abcam ab199297 (http://www.abcam.com), a rabbit polyclonal antibody. ECL detection with relevant secondary antibody (Amersham, http://www.amersham.com) binding was carried out according to the manufacturer’s instructions.

Scoring of D. rerio mutant phenotypes. Quantitative measurements of sri and dre were carried out using IPlab software (http://www.scanalytics.com) on digital photographs. In sri and dre embryos eye shape (eccentricity) was calculated as √[(major axis)² – (minor axis)²]/(major axis) for the region of interest, where zero is a circle and 1 an extreme ellipse. The axes and area were calculated automatically after drawing around the outer edge of the retina or lens on the digital images. The presence of coloboma was noted. Individual values and distributions are displayed in the scatter plots (Figure 5). Mean values and statistical results are represented in Table 3.

Cyclopa and other eye abnormalities were observed in both control and treated embryos. Nanophthalmia was defined as an eye structure with reduced eye size. The normal function of the eye was assessed by the ability of treated embryos to respond to light. Hsp90 inhibition induced cyclopa as a result of the AMN trait in different egg lays and pairs, the effect of Hsp90 inhibition was confirmed by Coomassie blue staining of a duplicate gel.

To assess the statistical significance of the phenotypic differences, a two-tailed Student’s t-test, under the assumption of unequal variances, was used to compare the means of the groups (Tables 2–4). Because of the variable penetrance of the AMN trait in different egg lays and pairs, the effect of Hsp90 inhibition was confirmed by Coomassie blue staining of a duplicate gel. Western blot analysis was carried out using standard techniques. Specific analysis for the induction of a generalised stress response was carried out using Hsp70 antibody from Stressgen SPA-812 (http://www.stressgen.com). α-tubulin monoclonal antibody TATI [85] was used as loading control. RAF1 levels were detected with Abcam ab199297 (http://www.abcam.com), a rabbit polyclonal antibody. ECL detection with relevant secondary antibody (Amersham, http://www.amersham.com) binding was carried out according to the manufacturer’s instructions.

**Supporting Information**

**Figure S1.** Frequencies of Common Developmental Defects Elicited in WT Strains by Decreasing Hsp90 Function at 50% or 50% Epiboly 17AAG treatment (3.3 μM) initiated at 30% epiboly (violet) or 50% epiboly (green).

Found at doi:10.1371/journal.pgen.0030043.sg001 (59 KB PPT).

**Figure S2.** Abnormal Phenotypes in Morpholino Injected Embryos (A) Abnormal looping of the heart tube in Hsp90 morpholino-injected embryos.

**Figure S3.** Abnormal Heart Looping in Hsp90 morpholino-injected embryos. (B) Severe oedema in Hsp90 morpholino-injected embryos.

Found at doi:10.1371/journal.pgen.0030043.sv001 (1.2 MB MOV).

**Video S1.** In Some Severe Cases Drug-Induced Oedema Leads to Abnormal Heart Morphology

Found at doi:10.1371/journal.pgen.0030043.sv002 (27 MB MP4).

**Video S2.** Normal Heart Looping

Found at doi:10.1371/journal.pgen.0030043.sv003 (591 KB MOV).

**Video S3.** Normal Heart Looping

Found at doi:10.1371/journal.pgen.0030043.sv004 (621 KB MOV).

**Video S5.** Abnormality of the Heart Tube in Radicicol-Treated Embryos

Found at doi:10.1371/journal.pgen.0030043.sv005 (1.2 MB MOV).

**Video S6.** Abnormality of the Heart Tube in Radicicol-Treated Embryos

Found at doi:10.1371/journal.pgen.0030043.sv006 (1.7 KB MOV).

**Accession Numbers**

The RefSeq (National Center for Biotechnology Information [NCBI]) (http://www.ncbi.nlm.nih.gov/RefSeq) accession numbers for the genes discussed in this paper are for human: Hsp90αA1 (NM_0011017963, NP_0011017962.2); Hsp90βA1 (NM_007353.2, NP_001381.2); Hsp90βB1 (NM_1198210, NP_0138553); TRAP1 (NM_016292.1, NP_0573576.1); and for zebrafish: hsp90a (NM_131328.1, NM_131403.1, NP_999891); hsp90b (NM_131310.1, NP_571358.1); pax6b (NM_131164.1, NP_571716.1); srfu (NM_689570.1, NP_094602.1); git2 (NM_130967.1, NP_571042.1); and ssh (NM_130166.1, NP_571138.1).

**Acknowledgments**

We thank Peter Csermely for gifts of GMP; Ralf Dahm and Hans-Gerold Frohnhoefer for fish stocks and advice; Peter Currie and his group for providing zebrafish expertise; Paul Perry for developing image analysis methodology; Peter League and Niall Anderson for help with statistical analysis; Nick Gilbert, Richard Meehan, Andrew Jackson, and Nicholas Hastie for advice and discussion.

**Author contributions.** PLY, RMB, and VvH conceived and designed the experiments and analysed the data. PLY, RMB, and JM performed the experiments. PLY contributed reagents/materials/analysis tools. PLY and VvH wrote the paper.

**Funding.** The authors’ work is supported by the Medical Research Council (United Kingdom).

**Competing interests.** The authors have declared that no competing interests exist.
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