The ATPase-dependent chaperoning activity of Hsp90a regulates thick filament formation and integration during skeletal muscle myofibrillogenesis

Thomas A. Hawkins1, Anna-Pavlina Haramis1,*, Christelle Etard2, Chrisostomos Prodromou3, Cara K. Vaughan3, Rachel Ashworth4,†, Saikat Ray1, Martine Behra1,‡, Nigel Holder1, William S. Talbot5, Laurence H. Pearl3, Uwe Strähle2 and Stephen W. Wilson1,§

The mechanisms that regulate sarcomere assembly during myofibril formation are poorly understood. In this study, we characterise the zebrafish sloth\textsuperscript{45} mutant, in which the initial steps in sarcomere assembly take place, but thick filaments are absent and filamentous I-Z-I brushes fail to align or adopt correct spacing. The mutation only affects skeletal muscle and mutant embryos show no other obvious phenotypes. Surprisingly, we find that the phenotype is due to mutation in one copy of a tandemly duplicated hsp90a gene. The mutation disrupts the chaperoning function of Hsp90a through interference with ATPase activity. Despite being located only 2 kb from hsp90a, hsp90a2 has no obvious role in sarcomere assembly. Loss of Hsp90a function leads to the downregulation of genes encoding sarcomeric proteins and upregulation of hsp90a and several other genes encoding proteins that may act with Hsp90a during sarcomere assembly. Our studies reveal a surprisingly specific developmental role for a single Hsp90 gene in a regulatory pathway controlling late steps in sarcomere assembly.

KEY WORDS: Chaperones, Myofibrillogenesis, Zebrafish

INTRODUCTION

Myofibrils are the subcellular contractile apparatus of cardiac and striated muscle cells. Within each myofibril, Actin, Myosin and many other proteins are organised into reiterative arrays of sarcomeres. We understand how the intra- and extra-sarcomeric components of muscles fit together (Clark et al., 2002; Sellers, 2004), how muscle fibres are triggered to contract and how the sarcomere transduces force (Cooke, 2004; Rossi and Dirksen, 2006). However, despite this depth of knowledge of the structure and biophysics of the sarcomere and its constituents, our grasp of myofibrillogenesis, the process by which it takes form, remains surprisingly obscure.

During myofibrillogenesis, the first true sarcomeric components to appear are Actin filaments (Ehler et al., 1999; Van der Ven et al., 1999), which assemble into I-Z-I bodies/brushes, structures consisting of Z-discs flanked on both sides by Actin filaments (Schultheiss et al., 1990). The giant sarcomeric protein Titin integrates onto the nascent sarcomere around the stage that Z-disc and I-band epitopes are first present (Fürst et al., 1989). It is thought that thick filaments are subsequently assembled onto this scaffold.

The mechanisms by which thick filaments incorporate are controversial and three models have emerged. The first proposes that thick filaments form independently from I-Z-I brushes and these two separate sarcomeric components are integrated to form striated myofibrils (Holtzer et al., 1997; Schultheiss et al., 1990). The second holds that pre-myofibrils are first constructed from non-muscle Myosin, which is later replaced by muscle Myosin to form functional sarcomeres (LoRusso et al., 1997; Rhee et al., 1994; Sanger et al., 2002). The third theory proposes that Titin is a scaffold upon which other sarcomeric components are assembled (Ehler et al., 1999; Gregorio et al., 1999; Trinick and Tskhovrebova, 1999; Van der Ven et al., 1999). The idea is that the N-terminal portion of Titin first associates with the Z-disc and I-band (forming I-Z-I brushes). These complexes are then brought into register, either by the unfolding of the Titin filament or further translation, and by the association of M-line components (Fulton and Alfline, 1997; Fulton and L’Ecuyer, 1993). Upon this scaffold, Myosin is then integrated to form the thick filaments. In support of this model, the M-line region of Titin is important for myofibrillogenesis (Gothardt et al., 2003; Musa et al., 2006). The current models of myofibrillogenesis have arisen from studies of cultured cardiomyocytes and in vivo studies have, to date, added little to help resolve between them.

The initial formation of I-Z-I brushes is common between the myofibrillogenesis models and the controversy concerns the assembly and alignment of arrays of I-Z-I brushes and the integration of thick filaments into the nascent sarcomeres. Thick filaments are elaborate structures comprising hundreds of Myosin hexamers in precise alignment with each other and other sarcomeric components. Given this inherent complexity, it is perhaps unsurprising that thick filament assembly is not well understood.

Here we describe the cloning of the sloth\textsuperscript{45} (slo\textsuperscript{45}) mutation and its phenotypic consequences. Zebrafish slo\textsuperscript{45} embryos show no morphological defects and have a normal heartbeat but lack movement of skeletal muscles. Various analyses reveal that the contractility phenotype is due to a lack of assembly of thick

\*Present address: Netherlands Cancer Institute (NKI), Department of Molecular Genetics, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

‡Present address: National Human Genome Research Center, National Institutes of Health, Building 50, Room 5537, 50 South Drive, Bethesda, MD 20892-8004, USA

Author for correspondence (e-mail: s.wilson@ucl.ac.uk)
filaments in the nascent sarcomeres of skeletal muscle fibres. Much to our initial surprise, we found that three \textit{slo} alleles all contain mutations in the \textit{hsp90a} (\textit{hsp90a.1} – ZFN) gene.

Heat-shock proteins (Hsps) are a group of proteins with transcription that is induced in response to heat or other cellular stresses. They are molecular chaperones for huge numbers of proteins and Hsp90 alone may be able to interact with more than 400 different proteins (Zhao et al., 2005). Despite extensive research on Hsps, there has been little work addressing their developmental roles during vertebrate embryogenesis. Work from \textit{C. elegans} has suggested a role for molecular chaperones during thick filament assembly and integration. The方针/\textit{Cre}p-\textit{She}4p-related (UCS) protein \textit{Unc}45 functions during assembly of thick filaments (Barral et al., 1998; Barral et al., 2002; Etard et al., 2007) and UNC-45 binds stoichiometrically with Hsp90 (Barral et al., 2002). Our findings extend these studies and show that a single, developmentally regulated \textit{hsp90} gene is necessary for thick filament assembly and for the construction of functional sarcomeres in skeletal myofibrils. Our data reveal unexpected specificity in the developmental role of Hsp90 and raise the possibility that other Hsp genes might play similarly cell-type-specific roles during vertebrate embryogenesis.

MATERIALS AND METHODS

Immunohistochemistry, phalloidin and bungarotoxin staining and in situ hybridisation

Immunohistochemistry was performed by standard methods using: ZNP-1 (DSHB, University of Iowa, IA); anti-Actinin (EA-53; \#AS044, Sigma); A4.1025 (Dan-Goor et al., 1990), which recognises all forms of sarcomeric MHC (‘pan-MHC’ antibody); F59, which is specific to slow-muscle Myosin isoforms (slow-MHC); anti-Titin (T11; Sigma), which marks an epitope in the I-band region of Titin (Fürst et al., 1988). AlexaFluor-conjugated phalloidin or bungarotoxin (Invitrogen) were applied after secondary antibodies. For in situ, DIG-labelled antisense riboprobes were prepared using standard methods from EST constructs (GenBank: \textit{hsp90a}, CCGACTTCTCAGGCATCTTGCTGTG; \textit{hsp90a2}, CTCTTCCTTATTCGTCATGAGTGCATG. cDNA was produced using Superscript II reverse transcriptase (Invitrogen) and 1 µg of quality-checked total RNA extracted using TRizol from pools of ten mutants and ten wild-type siblings. Triplicate qPCR reactions were carried out with blank controls and five standards. Copy number was determined by reference to standards normalised to a standard curve (ten triplicate 4-fold dilutions); copy number values were normalised to 1 µg of total RNA and significance determined by Student’s \textit{t}-test.

Isothermal titration calorimetry, \textit{K}_D determinations and Hsp90 ATPase assay

The heat of interaction was measured on a MSC System (Microcal), with a cell volume of 1.458 ml, in 20 mM Tris (pH 7.5), 1 mM EDTA, 5 mM NaCl, 7 mM MgCl\(_2\) at 30°C. Twenty 14.5 µl aliquots of 1 mM AMPNP were injected into 50 µM yeast Hsp90, human Hsp90\(_{\alpha}\)s or mutant protein. The heat of dilution was determined in a separate experiment by diluting protein into buffer, and the corrected data fitted using a non-linear least square curve-fitting algorithm (Microcal Origin) with three floating variables: stoichiometry, binding constant and change in enthalpy of interaction. ATPase activity of purified mutated and normal yeast and human Hsp90 protein was measured as described previously (Panaretou et al., 1998).

RESULTS

The motility defects of \textit{akinetou}^\textit{u45}/\textit{sloth}^\textit{u45} mutants are due to defective skeletal muscle fibres

\textit{akinetou}^\textit{u45} is a recessive mutation isolated at UCL in an F3 screen for ENU-induced mutations. Mutant embryos are healthy, show no obvious morphological defects and ostensibly develop as wild-type siblings for their first week. However, they do not move (see Movie 1 in the supplementary material), and die by 8 days post-fertilisation (dpf). Pairwise breeding showed that \textit{akinetou}^\textit{u45} does not complement the \textit{tu}44c and \textit{tm}201 alleles of \textit{sloth} (\textit{slo}) (Granato et al., 1996) and we therefore renamed the novel mutation \textit{slo}^\textit{u45}. \textit{slo} mutants lack spontaneous or stimulus-evoked skeletal muscle contractility but have a normal heartbeat. There is some variability in phenotype between alleles and although no movement is observed in the trunk of homozygous \textit{slo}^\textit{u45} embryos, the fns of a minority of \textit{slo}^\textit{u45} mutants show very slight twitching from 4 dpf. By contrast, around 30% of \textit{slo}^\textit{u44c} and \textit{slo}^\textit{tm201} mutants show limited trunk movements at 24 hours post-fertilisation (hpf) that disappear by 48 hpf; by 4 dpf there is substantial, though still compromised, fin movement. Transheterozygotes (\textit{slo}^\textit{u45tm44c}, \textit{slo}^\textit{u45tm201} or \textit{slo}^\textit{u44c}TM201) resemble the \textit{tu}44c and \textit{slo}^\textit{tm201} phenotype, showing some trunk movement at 24 hpf and more vigorous fin twitching later (compared with \textit{slo}^\textit{u45}). These results suggest that \textit{u45}, \textit{tu}44c and \textit{tm}201 are mutations in the same gene and that \textit{u45} is the most severe allele.

Microarray analysis

Total RNA was extracted using TRIzol (Invitrogen) from pooled \textit{slo}^\textit{u45} and wild-type embryos. RNA and microarray processing was carried out at the ICH Gene Microarray Centre (Institute of Child Health, London) according to standard methods. The \textit{MAS}5 algorithm (Affymetrix) was used to compare wild type and \textit{slo}^\textit{u45} mutants. Genes designated as showing no change in expression or where expression was designated as absent in both wild type and mutant were eliminated. The data were then split into two groups: genes with a \textit{slo} versus wild-type signal log ratio of between 0.6 and 1 or between −0.6 and −1 (1.5- to 2-fold up- or downregulated) and genes showing a signal log ratio greater than +1 or −1 (greater than 2-fold up- or downregulation). These groups were further sorted into genes absent in wild type or \textit{slo} mutants and those increased and decreased in \textit{slo} mutants (see Table S3 in the supplementary material).

Quantitative PCR (qPCR)

The following cDNA-specific primers (5’-3’) were used for qPCR: \textit{hsp90a} forward (F), CCACCTTAAAGAGGATCGTCT and reverse (R), TCTCTCCTTATCTTGGCCAT: \textit{hsp90a}7 F, GCGGCCGATCAAG-AGATC and R, CACTATGGCCTATGCGTG. cDNA was produced using Superscript II reverse transcriptase (Invitrogen) and 1 µg of quality-checked total RNA extracted using TRIzol from pools of ten mutants and ten wild-type siblings. Triplicate qPCR reactions were carried out with blank controls and five standards. Copy number was determined by reference to standards normalised to a standard curve (ten triplicate 4-fold dilutions); copy number values were normalised to 1 µg of total RNA and significance determined by Student’s \textit{t}-test.
The two most likely causes of the slo phenotype are defective innervation of muscle fibres and/or a defective contractile response of the muscle fibres to innervation. Examination of muscle innervation and calcium transients within muscle fibres revealed only minor differences between slo45 mutants and wild types (see Fig. S1 and Table S1 in the supplementary material). The absence of neuronal or excitation-coupling problems suggested a defect intrinsic to the muscle fibres. This was corroborated by polarised microscopy, mosaic labelling and cell transplantation experiments which showed that slo45 mutants had abnormally wavy muscle fibres that totally lacked birefringence (see Fig. S1 in the supplementary material). This strongly suggested a defect intrinsic to the contractile apparatus of the muscle fibres.

The filamentous organisation of myofibrils is abnormal in slo mutants
Antibodies and probes for sarcomeric components elucidated the sarcomeric defects underlying the myofibrillar phenotype of slo45 embryos (Fig. 1). We found that although filamentous Actin is present, the myofibrils of slo45 mutants lack the organised fibrillar arrangement seen in siblings (Fig. 1A,B). Sarcomeric filamentous Actin is anchored at Z-discs composed of α-Actinin, capping proteins and other molecules. Double staining for Z-discs and Actin filaments in siblings revealed evenly spaced Z-discs in register between neighbouring fibrils and even between adjacent fibres (Fig. 1C). slo45 myofibrils lacked such regimented organisation (Fig. 1D). Much of the α-Actinin staining was dispersed in the cytoplasm, although small structures resembling Z-discs were present. However, these mutant ‘Z-discs’ were neither appropriately spaced nor in register. The sarcomeric organisation of Titin evident in siblings was absent in slo45 mutants (Fig. 1E,F), and although some Titin-positive puncta were present in the slo45 mutants, they showed little evidence of organisation.

Anti-MHC immunoreactivity was considerably reduced in slo45 mutants. Unlike in siblings, the muscle of slo45 mutants lacked strong anti-MHC labelling, with no signs of striations in register between neighbouring fibres (Fig. 1G-J). Consistent with its less severe movement phenotype, MHC immunohistochemistry in the tu44c allele revealed short lengths of striation (see Fig. S2A,B in the supplementary material).
Western blots showed that MHC was almost completely absent from lysates of slo mutants at 30 hpf, when myofibrils are initially forming. By 48 hpf, when the majority of muscle fibres contain mature fibrils, Myosin levels had increased slightly in slo mutants although not near to levels in sibling lysates (F59, Fig. 1K; A4.1025, data not shown). These results suggest that either thick filaments are not formed because of a lack of Myosin, or that Myosin is degraded because thick filaments do not form correctly.

**slo myofibrils are deficient in thick filaments**

To examine more closely the defects in the contractile apparatus of slo muscle, we used transmission electron microscopy (TEM) to visualise the sarcomeric machinery at 24 hpf (Fig. 2A-D), when movement is just beginning, and at 48 hpf (Fig. 2E-H), when coordinated movement is present.

In less mature caudal myotomes of wild-type 24 hpf embryos, myofibrils were rare and those present were very immature. Z-discs were small in diameter and poorly aligned, thick and thin filaments

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**Fig. 2. Sarcomeres in slo<sup>465</sup> mutant myofibrils lack thick filaments.** (A-H) Electron micrographs of sarcomeres from wild-type (Sib) (A,C,E,G) and slo<sup>465</sup> mutant (B,D,F,H) myofibrils at the ages shown bottom left. (A,B) Caudal myotomes of 24 hpf zebrafish embryos. White arrowheads (A) show immature Z-discs with poorly positioned thick and thin filaments. In slo<sup>465</sup> mutants, bundles of thin filaments are present with putative early Z-discs (B, arrowheads). Very rarely, structures of dimensions approximating to those of thick filaments were present (box, B). (C,D) Rostral myotomes of 24 hpf embryos. The wild type has recognisable sarcomeres with almost straight Z-discs (white arrowhead, C) and more fully formed sarcoplastic reticulum with triads (black arrowhead, C). In the slo<sup>465</sup> mutant, I-Z-I brushes are formed [Z-discs (white arrowheads, D) flanked on either side by thin filaments], and a few putative thick filaments are evident (box, D). (E-H) At 48 hpf, wild-type muscle fibres are packed with mature myofibrils (E,G), possessing mature triads at each Z-disc (black arrowhead, G). Mutant muscle fibres contain no thick filaments but numerous I-Z-I brushes are evident (F,H). The Z-lines are surrounded by an electron-dense region (bracket, H; F); beyond this region the thin filaments have light striations on them (see inset, H). Triads are present, although less common (black arrowhead, H). (I) Schematic summarising the principal differences between slo<sup>465</sup> mutant and sibling sarcomere ultrastructure. At early stages of myofibrillogenesis (top), mutants have less numerous and malformed thick filaments (red), and I-Z-I brushes (green and blue) are present but less well aligned than siblings. By 48 hpf, wild-type sarcomeres are fully formed, whereas in mutants only misaligned and mis-spaced I-Z-I brushes with striated thin filaments are present (blue). T-tubules and sarcoplasmic reticulum are shown in yellow and purple, respectively. Scale bars: 500 nm in A-D,G,H; 2 μm in E,F; 100 nm in H inset.
were present but were few in number and were not yet organised into A and I bands (Fig. 2A). Structures resembling myofibrils were essentially absent from slo45 mutants (Fig. 2B). Where a presumptive nascent sarcomere could be found, it comprised small aggregations of thin filaments sometimes associated with densities (putative Z-discs).

In more mature rostral myotomes of 24 hpf wild types, myofibres often contained one or two almost complete myofibrils; such structures were absent from slo45 fibres (Fig. 2C,D). Although more fully constructed than their caudal counterparts, these structures were still immature in wild-type embryos. Z-discs were not straight and thick filaments were sporadic and not fully integrated. In slo45 mutants, the rostral myotomes contained some thin filament/Z-disc aggregates with Z-discs usually forming isolated ‘I-Z-I’ brushes. Occasionally, a few spindly thick-filament-like structures were present, appearing to face together adjacent I-Z-I brushes.

By 48 hpf, wild-type myofibres contained multiple highly organised myofibrils with Z-discs in register, whereas slo45 myofibrils contained only disorganised I-Z-I brushes (Fig. 2E,F). These were often found in groups with the Z-discs only partially in register with their neighbours and the distance between the consecutive I-Z-I brushes was also highly variable. Thick filaments were absent from slo45 myofibrils. Either side of the Z-disc was an electron-dense area, 100-200 nm wide, within which the bundle of I-band thin filaments retained the same height as the Z-disc. Beyond this region, the bundles of thin filaments were slightly striated and narrower than the diameter of the Z-disc (Fig. 2G,H). The periodicity of the striations was approximately 30 nm, suggesting that they could be Troponin complexes that would normally interact with Myosin heads (Chun and Falkenthal, 1988).

The ultrastructural appearance of slo44c muscle was broadly similar to slo45 mutants, with I-Z-I brushes lacking thick filaments. However, the I-Z-I brushes were more regularly spaced and aligned with short disorganised fibrous aggregations of electron-dense material between I-Z-I brushes (see Fig. S2 in the supplementary material). It is probable that these aggregates correspond to the striations seen with MHC immunohistochemistry.

The heart of slo45 mutants functions normally and, unsurprisingly, the ultrastructure of the heart muscle was indistinguishable between slo45 mutants and siblings (see Fig. S3 in the supplementary material).

These ultrastructural observations (summarised in Fig. 2I) reveal that the cause of the slo phenotype is a failure to assemble and integrate thick filaments into the nascent sarcomeres of myofibrils. Actin (thin) filaments and Z-lines (containing Actinin) are present, as is the excitation-coupling apparatus of T-tubules and sarcoplasmic reticulum. However, I-Z-I brushes fail to align and they lack regular spacing. The lack of aligned thick filaments underlies the birefringence deficiency and lack of organisation observed in the Myosin immunohistochemistry. Therefore, the Slo protein is not required to form a Z-disc and to assemble Actin fibres, Actinin and probably other sarcomeric proteins, but is required for assembly and integration of thick filaments.

**slo mutations are in the hsp90a gene**

Genetic mapping using SSLP markers placed the slo locus on LG20 (see Fig. S4 in the supplementary material). Bespoke SNP markers for genes in this region showed tight linkage to hsp90a, which is immediately adjacent to hsp90a2 (hsp90a2 – ZFIN). Sequencing of these genes from the three mutant slo alleles revealed mutations in hsp90a (Fig. 3A and see Fig. S4 in the supplementary material). A guanine to adenine point mutation is present in exon 3 in slo44c mutants, causing a glycine to aspartic acid change in residue 94. In exon 9 of slo44c and exon 10 of slo201, there are point mutations that respectively change tyrosine 561 and tryptophan 599 to stop codons. No mutations were found in hsp90a2 coding sequence in any slo allele.
The nonsense slo*44c and slo*m201 mutations would result in truncated molecules missing the C-domain terminal, which is important for both homo- and heterodimerisation (Ali et al., 2006); such truncations would have severe functional consequences for the molecule.

The molecular consequence of the slo*45 missense mutation was initially less obvious, but bioinformatic analysis and further experiments demonstrated that it abrogates the ATPase function of the Hsp90a protein. The N-terminal region of Hsp90 harbouring the mutation is required for ATP hydrolysis (Panaretou et al., 1998), and a ClustalW alignment of available Hsp90a sequences demonstrated the glycine mutated in slo*45 to be universally conserved (see Fig. S5 in the supplementary material). To predict the functional consequences of the Gly to Asp change, we assessed its probable consequence on the protein structure of yeast Hsp90, for which the crystallographic structure was available (Ali et al., 2006) (Fig. 3B). The Cα atom of Gly83 sits in a confined space that is 3.4 Å from the hydroxyl of Ser138, 3.8 Å from the main-chain carbonyl of Lys139, 3.9 Å from the main-chain carbonyl of Gly170, and 4.4 and 4.8 Å from the main-chain amides of Gly170 and Lys139, respectively (Fig. 3B). The large side-chain of an aspartic acid residue would not be easily accommodated and would disrupt the local folding of the protein. As the glycine is involved in critical interactions with bound ATP/ADP, it is highly likely that the side-chain change would severely affect the ability of mutant Hsp90 to bind nucleotide.

To test directly whether a Gly to Asp mutation at this site affects ATPase function, we recapitulated the mutation in yeast and human forms and tested their ATPase and ATP-binding properties in vitro. The mutations completely abrogated the ability of these molecules to hydrolyse ATP (Fig. 3C). Binding between the ATP analogue AMPPNP and mutant yeast and human Hsp90 was also negligible (see Fig. S6 in the supplementary material). Functional ATPase activity is essential for chaperoning by Hsp90 (Panaretou et al., 1998; Pearl and Prodromou, 2006). The slo*45 mutation is therefore catastrophic for the function of zebrafish Hsp90a as a chaperone.

**The slo phenotype is rescued by exogenous Hsp90a**

To confirm that the mutations identified above were indeed the cause of the muscle defects in slo mutants, we attempted to rescue the phenotype. Several BACs that covered the genomic region (see Fig. S4, and also Table S2 for clone names, in the supplementary material) were injected into batches of embryos from crosses of slo*45 or slo*44c heterozygotes. At 3 dpf, injected embryos showed normal movement, partial movement or paralysis (Table 1 and see Movie 2 in the supplementary material). Examination of muscles from partially moving embryos with polarised light revealed a mosaic pattern of birefringence in the myotomes (see Fig. S4 in the supplementary material), indicating that some cells, most likely those containing mosaically inherited BAC DNA, had functional myofibrillar assemblies. Although hsp90a and hsp90a2 are separated by only 2 kb, we found two BACs (8 and 9) with ends in this 2 kb region facing in opposite directions, thus separating the two genes. Injection of BAC 9 (hsp90a only) produced mosaically rescued embryos, as expected. Surprisingly, BAC 8 (hsp90a2 only) also rescued some embryos (Table 1), albeit less efficiently, suggesting that exogenous Hsp90a2 can partially compensate for loss of Hsp90a.

A caveat to these rescue experiments is that BACs contain more than one gene and so one cannot be certain that the rescue is due to the activity of a single gene. To address this issue, we co-injected morpholinos (Mos) against hsp90a or hsp90a2 along with the corresponding BAC. In both cases, the ability of the BACs to rescue movement of slo*45 embryos was blocked, confirming that it is the activity of the Hsp90 proteins that mediates rescue. Additionally, hsp90a Mo produces immotile embryos lacking birefringence (Table 1); conversely, neither of two hsp90a2 Mos disrupted muscle function [data not shown and see Etard et al. (Etard et al., 2007)].

The slo*45 allele is a missense mutation and, despite our study of the yeast and human mutant proteins, we could not be certain (1) that it is an amorphic (or weakly antimorphic) mutation in the zebrafish and (2) that the mutation is responsible for the lack of thick filaments. Therefore, to determine if the mutant protein has any residual activity in myofibrilligenesis, we assayed whether exogenous mutant protein could rescue movement and thick filament generation. Constructs encoding N-terminal myc-tagged wild-type (myc:WThsp90a) and u45 mutant (myc:u45Hsp90a) Hsp90a were injected into slo*45 mutants. Only the myc:WThsp90a construct rescued movement in slo*45 mutants. Mosaically distributed myc:WThsp90a-expressing muscle cells in slo*45 mutants possessed apparently normal levels of organised Myosin and resembled wild-type muscle fibres transplanted into slo*45 embryos (Fig. 4A-D and see Fig. S1K in the supplementary material), indicating a rescue of thick filament formation. Muscle cells in slo*45 mutants expressing the myc:u45Hsp90a protein had neither higher levels of Myosin immunoreactivity nor organisation in the Myosin present at low level (Fig. 4F). Sibling muscle fibres

| Table 1. Exogenous hsp90 rescues the slo phenotype |
| Construct | slo allele | Injected embryos | | | |
| BAC 2 | u45 | 73.3 | 14.4 | 12.3 | 146 | 76.2 | 0 | 23.8 | 126 |
| BAC 4 | u45 | 74.7 | 16.9 | 8.4 | 83 | 80.5 | 0 | 19.5 | 113 |
| BAC 5 | u45 | 77.2 | 21.5 | 1.3 | 79 | 77.2 | 0 | 22.8 | 127 |
| BAC 6 | u45 | 75 | 21.7 | 3.3 | 60 | 81.9 | 0 | 18.1 | 76 |
| BAC 8 (hsp90a2) | u45 | 83.1 | 9.6 | 7.2 | 83 | 73 | 0 | 27 | 74 |
| BAC 8 (hsp90a2) | tu45c | 75.7 | 6.5 | 17.8 | 185 | 78.3 | 0 | 21.7 | 230 |
| BAC 8 + hsp90a2MO | tu45c | 71.6 | 0 | 28.4 | 81 | 75 | 0 | 25 | 88 |
| BAC 9 (hsp90a) | u45 | 72.7 | 25.5 | 1.8 | 55 | 76.3 | 0 | 23.7 | 59 |
| BAC 9 (hsp90a) | tu45c | 79 | 11.2 | 9.8 | 143 | 77.6 | 0.6 | 21.8 | 174 |
| BAC 9 + hsp90aMO | tu45c | 0 | 2.9 | 97.1 | 35 | – | – | – | – |

Percentages of paralysed embryos and those showing normal and partial movement after injection with BACs, BACs plus Mos, and in non-manipulated populations.
Fig. 4. Wild-type Hsp90a rescues thick filament formation in slo\textsuperscript{445} muscle cells whereas Hsp90a\textsuperscript{445} does not. (A–D) Muscle fibres from a slo\textsuperscript{445} mutant (B,D) and wild-type (Sib) (A,C) injected with DNA encoding myc:WHsp90a double stained for myc (red) and MHC (green). MHC staining in siblings decorates the cells with organised bands (C) indicative of mature A-bands. In the slo\textsuperscript{445} mutant, striations in the MHC staining are only evident in myc+ cells (D). (E,F) Muscle fibres in sibling embryos injected with myc-tagged hsp90a\textsuperscript{445} DNA show organised MHC staining of myc+ cells (E). Myc+ cells in slo\textsuperscript{445} mutants injected with the same construct do not show organised myofibrils (F). Scale bars: 100 \mu m in A,B; 10 \mu m in C–F.

expressing myc::u45hsp90a retained the ability to express Myosin in orderly myofibrils, suggesting that the u45 mutant Hsp90a does not have detectable antimorphic activity (Fig. 4E).

The slo\textsuperscript{445} missense allele gives a slightly stronger contractility phenotype and more severe disruption to myofibrillogenesis than the truncated slo\textsuperscript{mu44c} allele (Figs 1, 2 and see Fig. S2 in the supplementary material), and we assayed whether this might be due to enhanced activity of the hsp90a2 gene in the tu44c background. However, Mo-based abrogation of Hsp90a2 activity in slo\textsuperscript{mu44c} mutants failed to block muscle twitches. This suggests that the slo\textsuperscript{mu44c} mutant Hsp90a protein retains a residual ability to mediate myofibrillogenesis. This is probably due to function of the intact ATPase region and as the dimerisation domain is missing in this allele, it suggests that the Hsp90 monomer retains some activity. Altogether, these results confirm that hsp90a is the gene affected by the slo mutations and that Hsp90a has a unique and specific developmental role in the assembly and integration of thick filaments onto nascent sarcomeres of skeletal muscle.

hsp90a and genes encoding proteins that may interact with Hsp90a to mediate sarcomere assembly are upregulated in slo mutants

Using Affymetrix arrays, we compared mRNA expression profiles of wild-type and slo\textsuperscript{445} embryos (see Tables S3, S4 in the supplementary material). Amongst the genes with lowered levels of expression were several encoding sarcomeric proteins, including Myosin light chains, Titin and Troponins. Notably, several genes encoding heat-shock proteins and factors that interact with heat-shock proteins were upregulated. hspa8l and a similar gene homologous to human HSPA1A/HSPA1B showed the largest slo\textsuperscript{445} versus wild-type differences, with 150- to 160-fold upregulation in mutants. Both hsp90a and unc45b, a gene proposed to interact with Hsp90 during myofibrillogenesis (Barral et al., 2002; Etard et al., 2007), were 4-fold upregulated in mutants. For selected genes, in situ hybridisation (ISH) analysis corroborated the expression changes found using the microarray. hspa8l was massively upregulated in the myotomes of both slo\textsuperscript{445} and slo\textsuperscript{mu44c} mutants (Fig. 5A–C), whereas both unc45b and hsp90a showed more modest increases in ISH signal in slo\textsuperscript{445} mutants (Fig. 5D–I). In surprising contrast to the upregulation of hsp90a in slo\textsuperscript{445} embryos and hsp90a morphants (see Fig. S8 in the supplementary material), hsp90a transcript levels were reduced in slo\textsuperscript{mu44c} mutants (for which no microarray analysis was carried out), despite upregulation of both unc45b and hspa8l in the morphant and both mutant alleles (Fig. 5C,F I and see Fig. S8 in the supplementary material). Complementing the changes at the mRNA level, western blot analysis revealed that Hsp90 protein levels were increased in slo\textsuperscript{445} mutants and decreased in slo\textsuperscript{mu44c} mutants when compared with siblings (Fig. 5J).

In summation, these results show that when Hsp90a function is compromised, as in slo\textsuperscript{445} mutants, levels of transcription of genes encoding certain sarcomeric proteins are reduced, whereas expression levels of genes that encode several chaperone and co-chaperone proteins (hspa8l, hsp90a, unc45b and others) are upregulated (with the allele-specific exception of hsp90a in slo\textsuperscript{mu44c} mutants).

Hsp90a2 has no role in myofibrillogenesis

Our data have revealed a requirement for hsp90a in myofibrillogenesis, but not for its neighbouring hsp90a2 gene. The reasons why hsp90a2 appears to have little or no role in muscle were not clear and so we designed experiments to assess expression of hsp90a2 and further explore its function (or lack of function). ISH analysis suggests that, like hsp90a, hsp90a2 is expressed in muscle tissue but expression levels are low (data not shown) (Etard et al., 2007). However, as expression was low and to strengthen these data, quantitative RT-PCR (qPCR) was performed to examine the differences in levels of mRNA for hsp90a2 and hsp90a2 in wild-type and slo embryos (u45 and tu44c alleles).

hsp90a2 expression levels were significantly increased in slo\textsuperscript{445} mutants compared with siblings (Fig. 5K), whereas expression levels of hsp90a2 were unaffected by either mutation (Fig. 5L). This indicates that although the genes are adjacent to each other, they are subject to differential transcriptional regulation. This conclusion is supported by analysis of the absolute levels of transcription of the two genes in wild-type embryos, with hsp90a2 expression nearly eight times that of hsp90a2 (Fig. 5K,L; see Fig. S7 in the supplementary material). This offers a likely explanation for why endogenous hsp90a2 does not compensate for mutations in hsp90a. Presumably, the low expression level of hsp90a2 that fails to be upregulated as a consequence of mutation in hsp90a means that there is very little Hsp90a2 activity in developing muscle of either wild-type or slo embryos. By contrast, the expression level of hsp90a2 following BAC injection is presumably sufficiently high to mediate partial rescue. Supporting the conclusion that hsp90a2 has no role in muscle development, hsp90a2 morphants show normal birefringence of muscle fibres and fail to upregulate hsp90a, unc45b or hspa8l, and the phenotype of hsp90a/hsp90a2 double morphants is no more severe than that of hsp90a and hsp90a2 single mutants/morphants [see Fig. S8 in the supplementary material and Etard et al. (Etard et al., 2007)].
DISCUSSION
slo mutants exhibit a developmental phenotype specifically affecting skeletal muscle myofibrillogenesis. Within slo mutant myofibres, the initial steps in construction of sarcomeres are relatively normal, leading to formation of actin-filament-decorated Z-bands. However, these I-Z-I brushes fail to align or become properly spaced and nascent sarcomeres lack properly formed thick filaments. The level of MHC protein is reduced in mutants, suggesting that Myosin and nascent sarcomeres lack properly formed thick filaments. The initial steps in construction of sarcomeres are relatively normal, leading to formation of actin-filament-decorated Z-bands. However, these I-Z-I brushes fail to align or become properly spaced and nascent sarcomeres lack properly formed thick filaments. The level of MHC protein is reduced in mutants, suggesting that Myosin and nascent sarcomeres lack properly formed thick filaments.

The developmental requirement for hsp90a is restricted to skeletal muscle cells
Given the vast amount of literature on roles for Hsp90 in many cellular events, we were initially surprised to find such a specific role for hsp90a during embryonic development. Little has been published on the requirements for Hsps during normal developmental processes in vertebrates, so the issue of tissue and cell-type specificity of function has not previously been addressed.

Two possible explanations for the specificity of the hsp90a mutant phenotype are either that skeletal muscle is the only embryonic cell type to require Hsp90 activity, or that other hsp90 genes function in other developing cells. In favour of a specific requirement in skeletal muscle cells, construction of the myofibrillar apparatus is an enormous challenge to the machinery of the cell, and muscle cells probably have the greatest load of any developing cell type in terms of protein folding and construction of multimeric protein complexes. To build a sarcomere requires balancing of the transcription, translation and folding of proteins and their incorporation into reiterative sarcomeric units. In this context, the demands upon protein chaperones will be high so Hsp90a might have been recruited for this specific developmental role. Additionally, given the large numbers of Hsp genes, it is almost certain that others will have developmental roles. With respect to hsp90a genes, in zebrafish there are at least three – hsp90a1, hsp90a2 and hsp90ab1 (formerly hsp90b) – raising the possibility that different paralogues function in different developmental events.

Hsp90a may co-operate with Unc45 in the chaperoning of Myosin during myofibril assembly
Hsp90s are well-characterised molecular chaperones with diverse and wide-ranging roles in cellular physiology (Pearl and Prodrumou, 2006). The current hypothesis for the chaperoning role of the Hsp90 homodimer is that the N-terminus ATPase domain flexes upon binding of ATP and this facilitates the maturation of client proteins. To build a sarcomere requires balancing of the transcription, translation and folding of proteins and their incorporation into reiterative sarcomeric units. In this context, the demands upon protein chaperones will be high so Hsp90a might have been recruited for this specific developmental role. Additionally, given the large numbers of Hsp genes, it is almost certain that others will have developmental roles. With respect to hsp90a genes, in zebrafish there are at least three – hsp90a1, hsp90a2 and hsp90ab1 (formerly hsp90b) – raising the possibility that different paralogues function in different developmental events.
Although this hypothesis suggests how Hsp90 chaperones, it does not explain how client specificity is managed (Pearl and Prodromou, 2006). Unlike many chaperones, Hsp90 shows much specificity for the proteins it binds, although these client proteins are very diverse and may number over 400 (Zhao et al., 2005). With this specific, yet potentially wide-ranging network of interactions, it is perhaps all the more surprising that slo mutants only show defective skeletal thick filament formation. By what mechanisms might Hsp90a specifically act to chaperone Myosin during thick filament formation?

Substrate-specific binding sites in the middle domain of the Hsp90 molecule, in addition to recruitment of various co-chaperone molecules, may contribute to the client specificity of the chaperoning activity (Pearl and Prodromou, 2006). Included among co-chaperones is the UCS-factor Unc45, identified through a C. elegans screen as being required for muscle development (Barral et al., 1998; Venolia et al., 1999). These studies led to the proposition that Unc45 acts in concert with Hsp90 to chaperone Myosin. In a recent study Etard et al. demonstrated that the steif zebrafish motility mutant is due to mutations in the unc45b gene and that Steif/Unc45b binds Hsp90 (Etard et al., 2007). These results point to a highly conserved mechanism whereby Unc45 provides specificity with Hsp90 for chaperoning Myosin during myofibrillogenesis.

The regulation of hsp90a during skeletal muscle development is likely to be independent of the normal heat-shock response pathway

During the course of our studies, we made several observations regarding the regulation of mRNAs and proteins in wild-type and slo muscles: we find that hsp90a is strongly expressed in wild-type skeletal muscle; that both hsp90a mRNA and Hsp90 protein and hspa8l and unc45 mRNA levels are increased in slo mutants whereas hsp90a2 is not; that hsp90a mRNA is not upregulated and Hsp90 protein levels are lower in slo mutants; that Myosin protein levels are severely reduced in slo mutants; and that genes encoding several sarcomeric proteins are downregulated in slo mutants. Do these observations make sense and shed any light on the transcriptional and post-translational mechanisms that operate during sarcomere assembly?

There are two obvious possibilities to explain the strong tissue-specific expression of hsp90a during normal skeletal muscle development. The first is that the stress levels that occur in developing myofibres induce the ‘heat-shock response’, a well-established trigger for upregulation of hsp90 transcription. The second is that the high-level expression of hsp90a in skeletal muscle cells is developmentally regulated, independent of the heat-shock regulation of hsp90a transcription. We favour this second possibility as our data suggest that the heat-shock response is not triggered during normal muscle development, whereas it is triggered in slo mutant muscle cells.

A well-established signature for reduced Hsp90 function is the upregulation of hsp90- and hsp70-related genes and the ubiquitination and proteasome-mediated destruction of Hsp90 client proteins (Proisy et al., 2006). In such situations, it is thought that the depletion of Hsp90 (through cellular-stress-induced client binding, mutations or for other reasons) frees the transcription factor Hsf (Heat shock factor), which triggers the heat-shock response through upregulation of various Hsp genes. Thus, the upregulation of chaperone genes and depletion of client proteins (Myosin) in slo mutants are entirely consistent with the muscle cells mounting a stress response owing to loss of Hsp90a function.

In contrast to the situation in slo mutants, there is no indication that the expression of hsp90a during normal development is a direct consequence of the cell mounting a stress response. Perhaps most notably, hspa8l expression is virtually undetectable in wild-type developing muscle in contrast to slo mutant muscle. Thus, it seems much more likely that an alternative, developmentally regulated transcriptional mechanism leads to upregulation of hsp90a during normal myogenesis. This would, in principle, not be difficult to achieve as various muscle-specific transcription factors are active during the period of myofibrillogenesis (Hinits and Hughes, 2007).

The reduction in Myosin protein levels in slo mutants could be explained by the well-established proteasome-mediated degradation of Hsp90 client proteins in the absence of Hsp90 function (Blagg and Kerr, 2006). We have no explanation for the reduced transcription of other genes involved in myofibrillogenesis, although this suggests a feedback mechanism balancing transcription levels with the translation and further processing of sarcomeric proteins.

It is curious that slo mutants show upregulation of unc45 and hspa8l, but not hsp90a itself. The fact that tu44c is a nonsense mutation might lead to nonsense-mediated mRNA decay and, if this happens, to a lowering of transcript and protein levels. However, it does not seem likely that mRNA decay would completely mask the strong constitutive expression that should be induced by the stress response. The milder phenotype of slo mutants suggests that some translation does occur and that the Slo protein retains some function, presumably in its N-terminal portion. It might still retain the ability to sequester Hsf and hence a milder stress response may be mounted in the muscle cells. However, this does not really explain why unc45 and hspa8l respond similarly in both mutant alleles. Finally, one should also consider that the truncated Hsp90 protein retains some function, presumably in its N-terminal portion. It might still retain the ability to sequester Hsf and hence a milder stress response may be mounted in the mutant muscle cells. However, this does not really explain why unc45 and hspa8l respond similarly in both mutant alleles. Finally, one should also consider that the truncated Hsp90 protein might in some unknown way suppress the hsp90a transcriptional upregulation response.

It is also curious that despite being located adjacent to hsp90a, hsp90a2 is subject to different transcriptional regulation and has no obvious role in muscle formation. Given their proximity and similarity in sequence, it seems likely that the two genes arose through a tandem duplication event. hsp90a2 still encodes a functional protein and so it must retain a function in zebrafish, although this might not be evident during embryogenesis.

Implications for myofibrillogenesis models

How do our data impact upon the three current models of myofibrillogenesis outlined in the introduction? The core of all three models is a sequential deposition of the myofibrillar components from Z-line to M-line, with 1-Z-I brushes forming first, followed by the integration of thick filaments to complete sarcomeric assembly. Our data support the notion that I-Z-I brushes form first, but that the integration of thick filaments to complete sarcomeric assembly is model dependent. The core of all three models is a sequential deposition of the myofibrillar components from Z-line to M-line, with 1-Z-I brushes forming first, followed by the integration of thick filaments to complete sarcomeric assembly.

Both Sanger’s model (Sanger et al., 2002) and the ‘Titin’ model (Trinick and Tkshovrebova, 1999) of myofibrillogenesis suggest that a well-formed scaffold of I-Z-I brushes is established prior to thick filament integration, with either non-muscle Myosin or Titin fulfilling the role of linking the I-Z-I brushes. In both cases, the lack of assembly and integration of thick filaments should not significantly impact upon the construction of the sarcomeric scaffold. In slo mutants, scaffold formation is compromised as I-Z-I brushes do not properly align, are not correctly spaced and probably do not
correctly lace up with adjacent I-Z-I brushes. This suggests either that Hsp90β has unsuspected roles in the assembly of non-muscle Myosin or Titin, or that the models might need some revision. Indeed, Titin immunohistochemistry in sl-o mutants suggests that the molecule is not even correctly integrated into the I-Z-I brush that are present, raising the possibility that sarcomeric Titin integration might lie parallel to, or even downstream of, thick filament assembly and integration. The third model of myofibrillogenesis (Ehler et al., 1999) holds that I-Z-I brushes are loaded with thick filaments at specific cellular locations and subsequently assembled into full sarcomeres. The sl-o phenotype is not inconsistent with this idea, but neither does it provide strong support.

The mutants described in this and related papers enhance our understanding of myofibrillogenesis through study of the process in vivo. We are hopeful that further genetic studies of sarcomere formation will refine existing models and help build a more complete picture of myofibrillogenesis.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/6/1147/DC1

References


