Mutations in the Catalytic Loop HRD Motif Alter the Activity and Function of Drosophila Src64

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Abstract

The catalytic loop HRD motif is found in most protein kinases and these amino acids are predicted to perform functions in catalysis, transition to, and stabilization of the active conformation of the kinase domain. We have identified mutations in a Drosophila src gene, src64, that alter the three HRD amino acids. We have analyzed the mutants for both biochemical activity and biological function during development. Mutation of the aspartate to asparagine eliminates biological function in cytoskeletal processes and severely reduces fertility, supporting the amino acid's critical role in enzymatic activity. The arginine to cysteine mutation has little to no effect on kinase activity or cytoskeletal reorganization, suggesting that the HRD arginine may not be critical for coordinating phosphotyrosine in the active conformation. The histidine to leucine mutant retains some kinase activity and biological function, suggesting that this amino acid may have a biochemical function in the active kinase that is independent of its side chain hydrogen bonding interactions in the active site. We also describe the phenotypic effects of other mutations in the SH2 and tyrosine kinase domains of src64, and we compare them to the phenotypic effects of the src64 null allele.

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Introduction

Src is a member of the Src Family Kinases (SFKs), a group of nonreceptor tyrosine kinases that share a common structure consisting of an N-terminal myristoylation site, an SH3 domain, an SH2 domain, a tyrosine kinase domain and a C-terminal negative regulatory domain. Myristoylation of the SFK protein tethers it to the inner face of the plasma membrane, whereas the SH3 and SH2 domains mediate interactions with proline-rich recognition sequences and phosphotyrosine-containing sequences, respectively [1,2]. Intramolecular interactions between the SH2 and SH3 domains and their recognition sites in the SFK hold the protein in a closed, inactive conformation. Release of these intramolecular interactions by dephosphorylation of the C-terminus or by SH2 binding of another phosphotyrosine protein leads to the adoption of an open, partially active conformation. Phosphorylation of the activation loop leads to the adoption of the fully active conformation. The SFK active site is located in a cleft between the N-terminal lobe and the C-terminal lobe of the kinase domain, where substrate, ATP and Mg++ cations bind. Two critical elements of the active site are the DFG motif-containing activation loop and the HRD motif-containing catalytic loop [3]. The HRD amino acids are thought to be involved in the reaction mechanism or the formation and stabilization of the active site [4–6].

SFKs have been shown to be involved in regulating the actin-based microfilament cytoskeleton [1,7]. The Drosophila genome contains two genes that encode SFKs: src42 and src64 [8]. Both act in the remodeling of the microfilament cytoskeleton during dorsal closure [9,10]. However, in ring canal growth in the egg chamber, src64 seems to function independently of src42 [9]. Ring canals are intercellular bridges linking the nurse cells to the developing oocyte, formed from the actin-rich arrested cleavage furrow that remains after incomplete cell division [11]. Src64 is required for ring canal growth [12–17].

src64 is also required for microfilament contraction during the formation of the cellular blastoderm [18]. During early Drosophila embryogenesis, synchronized nuclear division proceeds without concomitant cell division. After nuclear division stops, a single layer of cells is formed by the simultaneous and uniform invagination of plasma membrane between the peripheral nuclei [19]. The leading edge of membrane invagination, the cellularization front, consists of stable infoldings of membrane called furrow canals surrounded by microfilaments [20–25]. Contractile tension in the microfilament network maintains uniform invagination of furrow canals during early cellularization, and constriction of microfilament rings partly closes cell bases during late cellularization [18,26]. However, contraction of the microfilament network is not required for membrane invagination [18,26]. src64 mutant defects in both microfilament ring contraction and ring canal expansion are easily quantified, providing sensitive and effective means of assaying the biological function of src64 [12,18].

To understand the role of src64 in regulating microfilament ring contraction during cellularization, we identified point mutations in the src64 coding region. Of particular interest were mutations in each of the three highly conserved amino acids that constitute the HRD motif of the kinase domain catalytic loop. We analyzed the phenotypes caused by the mutation in the catalytic aspartate and
the src64 null allele and found that Src64 kinase activity is required for microfilament ring contraction. We also found that mutations in the histidine and arginine residues produce weaker cytoskeletal defects and lower reductions of kinase activity than expected. We discuss the implications of these results on the roles of the HRD amino acids in kinase domain activity and activation.

Materials and Methods

Genetics

Flies were cultured on a standard medium at 22.5°C. Embryos were collected from yeasted apple juice/agar plates at 22.5°C and counted during days three to ten. After forty hours, hatched and unhatched eggs were counted. OrecR was used as the wild-type strain. src64D372 and src64KO have been described [12,14,18]. Other alleles are described in Flybase [8]. src64 point mutations were identified by mismatch between the wild-type sequence between primers in exon five (atcgagcaccgagctgtagt) and exon eight (gcaagtgacgagaagacctaggt) and the Ziker EMS-mutagenized lines [27] by the Seattle Targeting Induced Localized Lesions in Genomes (TILLING) Project [28]. The mutations consisted of one mutation in an intron, eight silent mutations, ten unique missense mutations, a duplicate of one of the missense mutations and two additional missense mutations that were no longer available. Potential background mutations were reduced by generating additional missense mutations that were no longer available.

Immunohistochemistry and image analysis

Embryos were methanol heat-fixed [29] and stained with rabbit anti-mysin II heavy chain antibody (1:1000) [30]. Ovaries were dissected and fixed in 4% paraformaldehyde [31] and stained with either anti-HTS antibody (1:1) [11] (Developmental Studies Hybridoma Bank) or Alexafluor 488-conjugated phallolidin (Invitrogen) and Hoechst. Specimens were imaged using an Axioscimer.M1 fluorescence microscope (Zeiss) or a Fluoview 300 confocal microscope (Olympus). Images were analyzed using Axiovision 4.4 (Zeiss) and ImageJ (W. Rasband, NIH; http://rsb.info.nih.gov/ij/). Outer diameters of all ring canals were measured in stage 10A egg chambers [12]. 4–14 egg chambers were compared using Kruskal-Wallis/Dunn’s test. For amorph tests, the Wilcoxon-Mann-Whitney test was used, and 5–6 egg chambers for heterozygotes, and 11–19 egg chambers for homozygotes and trans-heterozygotes were analyzed. Cellularization microfilament rings from the embryo midsection were measured and circularity (4πA/p² where A = area, and p = perimeter) calculated [18]. 10 rings from each of 3–6 embryos were compared using one-way ANOVA/Tukey-Kramer test. For amorph tests, the Wilcoxon-Mann-Whitney test was used on 25 rings from each of 5–10 embryos.

Kinase assays

Homozygous src64 mutant embryos from homozygous src64 mutant parents were collected in a mesh basket and dechorionated in 50% bleach for two minutes [29] and washed in phosphate-buffered saline pH 6.5/0.1% Tween 20. Cellularizing embryos were selected by hand under the stereo microscopic, disrupted using a melt stained pipette tip as a pestle in hypotonic lysis buffer [32] with 50% glycerol, and centrifuged at 16,000 g for 30 min at 4°C. Supernatants were stored at −20°C. Kinase assays were conducted on 0.58 μg to 1.80 μg of total protein using the SigmaTECT protein tyrosine kinase assay system (Promega). Reactions proceeded in the presence of γ-[32P]-labeled ATP and sodium vanadate for 15 minutes at 30°C. Phosphotransfer to the manufacturer’s peptide substrate 2 was measured by liquid scintillation counting, and did not differ by cellularization stage. Src64-dependent tyrosine kinase activity was determined by subtracting measured total tyrosine phosphotransfer in src64KO mutant embryo extracts (7.57 pmol/min/mg) from measured total tyrosine phosphotransfer in the other samples. Five independent extracts were assayed from each genotype. src64KO and src64P190LN extracts were generated by pooling supernatants from multiple embryo collections because of the extremely low numbers of embryos produced by these genotypes. Kinase assays were conducted in triplicate. Kinase activity was compared to wild type using ANOVA/Dunnett test.

Results

Identification of missense mutations in src64

To identify mutations that alter the coding sequence of src64 in a phenotypically unbiased manner, mutants were screened using the Targeting Induced Localized Lesions in Genomes (TILLING) procedure [27,28]. Ten missense mutations were identified: five in the SH2 domain and five in the tyrosine kinase domain (Table 1).

Fertility and Embryonic Viability Defects

Most of the src64 mutants can be maintained as homozygous lines. Neither the null allele src64KO [14] nor the src64P190LN allele is viable as a homozygous line; however, heterozygous parents produce homozygous progeny that produce homozygous embryos that live past gastrulation. The nuclear defect in src64M17 mutant embryos [18] was not observed in other src64 mutants, suggesting that it is not caused by src64 mutation.

Src64 protein is not expressed in the male reproductive system, and src64KO mutants have no defects in spermatogenesis or male fertility (A. O’Reilly, personal communication). However, src64M17 and src64KO mutant females show fertility defects: reduced number of eggs laid and reduced proportion of eggs that hatch [12,14]. We characterized female fertility defects by crossing wild-type males to females trans-heterozygous for src64 mutations and src64KO to minimize potential fertility defects caused by background mutations, and scoring egg yield and hatch rate during the most robust period of egg production. Most, if not all, of the src64 mutations caused lower egg yields (Table 1). The src64M17, src64KO and src64P190LN mutations produced substantially lower egg yields than the wild-type allele (Table 1). Overall relative fecundity of mutant alleles is lower than indicated because egg laying drops off sooner in most mutants than in wild type. Fertility defects in src64 mutant females are not caused by defects in the germline stem cells. Activated Src64 protein is not observed in either the germline stem cells or the somatic cells of the stem cell niche [14].

Maternal viability defects were milder than egg production defects. None were observed for src64P190LN, src64D372, src64R403C, src64R403G, src64R403C, src64D372, src64P190LN and src64P190LN. The higher hatch rate of eggs laid by src64KO females was likely caused by heterozygosity for src64KO trans-heterozygous females was likely caused by heterozygosity for src64KO and src64P190LN. The lower hatch rate of eggs laid by src64KO females showed an egg laying or hatching defect as severe as that of src64KO homozygous females, suggesting that these mutations are reduction-of-function mutations.

Ring canal growth defects

src64 is required for the growth of the cytoskeletal ovarian ring canals in the Drosophila egg chamber [12–14]. We observed ring
canal size defects in the egg chambers of most of the homozygous src64 mutants; only src64<sup>src64D204V</sup>, src64<sup>src64G208E</sup> and src64<sup>src64H402L</sup> were indistinguishable from wild type (Fig. 1). Ring canal sizes varied among the other src64 mutants. Ring canal diameters of src64<sup>src64D372N</sup>, src64<sup>src64D372R</sup>, src64<sup>src64D372N</sup> and src64<sup>src64H402L</sup> mutant egg chambers were significantly smaller than those of wild-type egg chambers (Fig. 1). src64<sup>src64D404N</sup> mutants had smaller ring canals than any of the mutants described above. The ring canal diameters of these mutants were indistinguishable (Fig. 1).

src64 is also required for ring canal attachment to the nurse cell plasma membrane [12]. src64<sup>src64D372N</sup> and src64<sup>src64D372R</sup> mutants often have ring canal attachment defects and nurse cell fusion defects [12,14]. src64<sup>src64H402L</sup> mutants have severe nurse cell fusion defects similar to those of src64<sup>src64KO</sup> mutants (Fig. 2). src64<sup>src64D404N</sup> mutants have nurse cell fusion defects that occur less frequently than those in src64<sup>src64H402L</sup> mutants (Fig. 2). No nurse cell fusions were observed in src64<sup>src64P190L</sup> mutant egg chambers (Fig. 2). Detached and prematurely degenerating ring canals are sometimes observed in src64 mutants [12]. We also occasionally observed degenerating ring canals in the egg chambers of strong src64 mutants.

### Cellularization microfilament ring contraction defects

src64 is required for microfilament contraction during cellularization [18]. During early cellularization, microfilament rings are linked together, creating tension in the network through microfilament contraction [18]. We analyzed src64 mutant embryos that are both maternally and zygotically mutant: homozygous for the src64 mutation and derived from mothers homozygous for the src64 mutation. Furrow canals do not invaginate uniformly during early cellularization in src64<sup>src64D417</sup> mutant embryos; variations in the depth of the furrow canals lead to the appearance of a wavy cellularization front (Fig. 3A) [18]. src64<sup>src64D204V</sup>, src64<sup>src64G208E</sup> and src64<sup>src64H402L</sup> mutant embryos show this phenotype (Fig. 3A), as do src64<sup>src64G208E</sup> and src64<sup>src64D404N</sup> mutant embryos. The cellularization front defects of src64<sup>src64D417</sup>, src64<sup>src64D204V</sup> and src64<sup>src64G208E</sup> mutant embryos were weak and incompletely penetrant, similar to src64<sup>src64A17</sup> mutant embryos (Fig. 3A). The defect was severe in both src64<sup>src64D204V</sup> and src64<sup>src64G208E</sup> mutant embryos (Fig. 3A). None of the mutants showed temperature-sensitive defects.

To assay microfilament ring contraction defects, we determined the circularity index, a normalized ratio of area to perimeter. Deviation from circularity suggests a reduction of contractile tension in the microfilament ring [18]. Most of the src64 mutations do not cause microfilament ring defects during early cellularization. Although some src64<sup>src64G208E</sup>, src64<sup>src64D204V</sup> and src64<sup>src64H402L</sup> mutant embryos have early cellularization front defects, they do not have microfilament ring circularity defects (Fig. 3B, D). The microfilament ring circularity defects of src64<sup>src64D204V</sup> and src64<sup>src64H402L</sup> mutant embryos are indistinguishable, and significantly more severe than that of src64<sup>src64A17</sup> mutant embryos (Fig. 3B, D).

During late cellularization, microfilament rings constrict [18,20]. Microfilament rings in src64<sup>src64D204V</sup>, src64<sup>src64G208E</sup> and src64<sup>src64H402L</sup> mutant embryos show little constriction, similar to that observed in src64<sup>src64A17</sup> mutant embryos, and differ in circularity from wild-type embryos (Fig. 3C, E). The microfilament rings of src64<sup>src64G208E</sup> and src64<sup>src64H402L</sup> mutant embryos do not noticeably constrict and have more severe circularity defects than src64<sup>src64A17</sup> mutant embryos (Fig. 3C, E).

### Catalytic loop HRD mutations reduce or eliminate Src64 kinase activity

Three mutations, src64<sup>src64D204V</sup>, src64<sup>src64G208E</sup> and src64<sup>src64H402L</sup>, alter the three conserved amino acids of the catalytic loop HRD motif. To analyze the biochemical effects of these mutations, we conducted kinase assays on extracts from cellularizing embryos. Extracts from wild-type embryos and src64<sup>src64D204V</sup> mutant embryos were used as controls for full tyrosine kinase activity and Src64-independent activity, respectively. The majority of tyrosine phosphotransfer to the peptide substrate is Src64-dependent. Much of the Src64-independent phosphotransfer is likely due to the activity of Src12, an SFK present during cellularization but primarily localized to a

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**Table 1. src64 mutations and fertility of src64<sup>src64KO</sup> trans-heterozygous females.**

| Allele   | Mutation | Substitution | Domain | Eggs laid/female | Eggs hatched (%) |
|----------|----------|--------------|--------|------------------|-----------------|
| +        | -        | -            | -      | 498 (n = 4)      | 84% (n = 1992)  |
| src64<sup>src64A17</sup> | 5’ deletion [12] | -            | -      | 120 (n = 9)      | 43% (n = 1017)  |
| src64<sup>src64KO</sup> | Knockout [14] | -            | -      | 13 (n = 17)      | 15% (n = 208)   |
| src64<sup>src64D204V</sup> | CCT→CTT | P190L        | SH2    | 216 (n = 9)      | 86% (n = 1853)  |
| src64<sup>src64G208E</sup> | GAT→GTG | D204V        | SH2    | 379 (n = 5)      | 74% (n = 1812)  |
| src64<sup>src64H402L</sup> | GGA→GAA | G208E        | SH2    | 390 (n = 5)      | 84% (n = 1905)  |
| src64<sup>src64R403C</sup> | CGT→TGT | R217C        | SH2    | 411 (n = 8)      | 87% (n = 2794)  |
| src64<sup>src64D372N</sup> | TGC→TAC | C259Y        | SH2    | 322 (n = 8)      | 67% (n = 2124)  |
| src64<sup>src64D372R</sup> | GAT→AAT | D372N        | kinase | 428 (n = 5)      | 87% (n = 2138)  |
| src64<sup>src64H402L</sup> | CAT→CTT | H402L        | kinase | 323 (n = 6)      | 73% (n = 1796)  |
| src64<sup>src64P190L</sup> | GCC→TGC | R403C        | kinase | 332 (n = 5)      | 88% (n = 1659)  |
| src64<sup>src64D404N</sup> | GAT→AAT | D404N        | kinase | 93 (n = 10)      | 28% (n = 850)   |
| src64<sup>src64G208E</sup> | TCC→TTC | S440F        | kinase | 412 (n = 9)      | 96% (n = 2042)  |

Mutation is shown for each allele, + indicates the wild-type allele. For point mutations, the amino acid substitution and the protein domain containing the substitution are shown. Female fertility was assayed in females trans-heterozygous for the mutation and the src64<sup>src64KO</sup> allele. Number of eggs laid by females over days 3–10 after introduction of males is shown.

<sup>1</sup> Wild-type amino acid is indicated, followed by position and mutant amino acid.

<sup>2</sup> Altered nucleotide is underlined.

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*Plasmodium falciparum* is a human malaria parasite that causes life-threatening infections in humans. In this study, we investigated the role of Src64, an Src family kinase, in cellularization. We identified four conserved tyrosine residues in the kinase domain of Src64: Y158, Y161, Y164, and Y167. Mutations in these residues altered the activity of Src64 in vitro and in vivo. The Y164F mutation abolished tyrosine kinase activity, while the Y161F and Y167F mutations partially inhibited it. These findings suggest that Src64 is an important regulator of cellularization in *Plasmodium falciparum*.
Tyrosine kinase activity of the mutant Src64 D404N protein is consistent with the observation that the src64D404N mutants show strong defects in the egg chambers (Fig. 3). We also tested the src64D404N allele during early cellularization. src64 is maternally required for microfilament ring contraction during cellularization [33]. Maternally mutant embryos were constructed by crossing wild-type males to females of genotypes src64D404N/+, src64KO/+ and src64KO. The cellularization front defect of the src64D404N/src64KO trans-heterozygous embryos was similar to those of the homozygous embryos (Fig. 5B). The circularity defects were indistinguishable (Fig. 5C, E), suggesting that src64D404N acts as a loss-of-function allele for ovarian ring canal growth.

We also tested the src64D404N allele during early cellularization. src64 is maternally required for microfilament ring contraction during cellularization [33]. Maternally mutant embryos were constructed by crossing wild-type males to females of genotypes src64D404N/+, src64KO/+ and src64KO. The cellularization front defect of the src64D404N/src64KO trans-heterozygous embryos was similar to those of the homozygous embryos (Fig. 5B). The circularity defects were indistinguishable (Fig. 5C, E), suggesting that src64D404N acts as a loss-of-function allele for ovarian ring canal growth.

**Discussion**

**src64D404N mutation**

The catalytic loop aspartate at position 404 in Src64 (chicken c-Src D386) has a critical role in catalysis [3,5]. It has been proposed to act as a catalytic base, deprotonating the tyrosine hydroxyl to different subcellular domain [9,18]. Most Src64-dependent tyrosine phosphotransfer is likely to be directly catalyzed by Src64, but may include phosphotransfer from any other tyrosine kinases activated by Src64.

We compared the Src64-dependent tyrosine kinase activities of the HRD mutants to those of the controls. src64H402L and src64D404N mutant embryo extracts had 27%, 90% and 12% of wild-type activity, respectively (Fig. 4). Tyrosine kinase activity of the mutant Src64KO protein is indistinguishable from that of the wild-type Src64 protein (Fig. 4). This is consistent with the wild-type cellularization front and microfilament contraction phenotypes observed in src64D404N mutant embryos (Fig. 3). Tyrosine kinase activity of the mutant Src64D404N protein is indistinguishable from Src64-independent activity (Fig. 4). This is consistent with the observation that the src64D404N cellularization phenotypes are indistinguishable from those of src64KO (Fig. 3). The Src64KO mutant protein shows intermediate tyrosine kinase activity (Fig. 4), consistent with the weak to moderate phenotypes observed in src64H402L mutant embryos during cellularization (Fig. 3).

**Figure 1. Ring canal growth defects in src64 mutant egg chambers.** Ring canals are stained with antibody to HTS. (A) Egg chambers. Ring canal diameters are reduced in the src64D404N, src64D404N and src64KO mutants. Scale bar, 50 μm. (B) Typical ring canals ordered mean by diameter and oriented so diameter is presented along the vertical axis. Scale bar, 10 μm. (C) Ring canal diameters. A, does not differ from +; B, differs from + (P<0.05), but not other A alleles, except src64H402L differs from src64KO (P<0.05); C, differs from + (P<0.001) and from A alleles (P<0.05) except src64H402L, but not from group B, except src64D372N differs from src64KO (P<0.01); D, does not differ from src64D204V, but differs from all other alleles (P<0.001). Error bars are SEM. doi:10.1371/journal.pone.0028100.g001

**Figure 2. Nurse cell fusion defects in src64 mutant egg chambers.** Egg chambers are stained with phalloidin (green) and Hoechst (blue). Partial confocal projections are shown. Nurse cell fusion (arrows) occurs in src64KO, src64D404N, src64D372N and src64KO mutants. Nurse cell fusion does not occur in src64D404N mutants. Scale bar, 50 μm. doi:10.1371/journal.pone.0028100.g002

**src64D404N** is a loss-of-function mutation for ring canal growth and microfilament contraction

Since src64D404N mutants show strong defects in the egg chamber and in cellularizing embryos, we tested whether src64D404N is a loss-of-function allele for these cytoskeletal processes by conducting trans-heterozygote tests using the src64KO allele to eliminate src64 function. The diameters of ring canals from src64D404N/+ females and src64KO/+ females were indistinguishable (Fig. 5A, D) and were similar to those of wild-type homozygotes (Fig. 1), indicating that src64D404N is a recessive allele. No significant differences were observed between ring canal diameters from src64D404N/src64KO trans-heterozygous, src64D404N and src64KO egg chambers (Fig. 5A, D). These results suggest that src64D404N acts as a loss-of-function allele for ovarian ring canal growth.

We also tested the src64D404N allele during early cellularization. src64 is maternally required for microfilament ring contraction during cellularization [33]. Maternally mutant embryos were constructed by crossing wild-type males to females of genotypes src64D404N/+, src64KO, src64D404N and src64KO. The cellularization front defect of the src64D404N/src64KO trans-heterozygous embryos was similar to those of the homozygous embryos (Fig. 5B). The circularity defects were indistinguishable (Fig. 5C, E), suggesting that src64D404N acts as a loss-of-function allele for microfilament ring contraction during cellularization.
catalyze a nucleophilic attack on the γ-phosphate group of ATP as part of the phosphoryl transfer reaction. However, many studies suggest that the neutral hydroxyl group acts as the nucleophile. Aspartate hydrogen bonds, directly or indirectly, to the hydroxyl group to position it for effective nucleophilic attack and acts as a proton acceptor late in the reaction [4,5,34]. In addition, the HRD aspartate may help stabilize the inactive state through an interaction with the unphosphorylated tyrosine in the activation loop [35].

Yeast carrying an aspartate to alanine substitution in cAMP-dependent protein kinase (PKA) were nearly inviable and had only 0.4% of the kinase activity of wild type [36]. Similarly, phosphorylase kinase protein (Phk) with this mutation showed little activity. Substitution with asparagine, the neutral amide derivative of aspartate, eliminates charge without altering hydrogen-bonding interactions that do not involve the carboxyl group. This mutation strongly reduced Phk kinase activity, but not as strongly as alanine. It caused a relatively small reduction in ATPase activity, suggesting that aspartate is critical for phosphoryl transfer rather than ATP hydrolysis [37]. The asparagine mutation in the tyrosine kinase Csk also strongly reduces, but does not eliminate, kinase activity [38]. Mutation to glutamate alters structure and size but not charge; this mutation also greatly reduces Csk activity [39].

Here we describe the effect of the HRD aspartate to asparagine mutation on several well-characterized and quantifiable biological processes in a living organism. Kinase activity of the src64D404N mutant is indistinguishable from that of src64KO. src64D404N behaves like a null allele in both cytoskeletal processes examined. Therefore, the src64D404N mutation either eliminates kinase activity or reduces it below the threshold required for function. However, for fertility and embryonic viability, src64D404N acts as a reduction-
of-function allele. Low levels of Src64 kinase activity may be sufficient for some biological function. Together, these results are consistent with in vitro studies of purified PKA, PIIK and Csk proteins showing that mutation to asparagine severely reduced, but did not eliminate, kinase activity. Alternately, src64D404N may behave as a reduction-of-function mutation in fertility because Src64 has a kinase-independent activity; however, kinase-dead SFKs can titrate negative regulators and increase the activity of other SFKs, arguing against this [40,41]. Although kinase-inactive alleles of src have been reported to act as dominant-negative alleles [42–44], we did not observe dominant negative activity of src64R403C. When over-expressed in the developing eye, kinase-dead SFKs also do not act in a dominant negative manner [40].

**src64R403C mutation**

RD kinases such as SFKs have arginine in the catalytic loop HRD motif and are only fully activated by phosphorylation of the activation loop [45]. Transition to the fully active conformation is salt-dependent and the HRD arginine has different charge-based interactions in the active and inactive conformations, suggesting that activation involves an electrostatic switch [46–50]. In the inactive conformation, glutamate E327 (c-Src E310) is salt-bridged to either the HRD arginine R403 (c-Src R385) or the activation loop arginine R427 (c-Src R409) [48,49]. In the active conformation, the phosphorylated tyrosine interacts with both R403 and R427, and glutamate is salt-bridged to lysine K312 (c-Src K295), a critical linkage in the fully active conformation [48,51–53]. The arginine switch may be part of a sequential series of electrostatic switches that drive conformation change [35,50]. These arginines are likely partially redundant for phosphotyrosine coordination: the activation loop arginine plays the major role in stabilizing both active and inactive configurations in SFKs, whereas the HRD arginine plays the major role in serine/threonine kinases [35,53,54]. The HRD arginine may also stabilize the DFG aspartate in the active state [55].

**Figure 5. Cytoskeletal defects of src64D404N/src64KO trans-heterozygotes.** (A) src64D404N/+, src64D404N and src64D404N/src64KO egg chambers stained with antibody to HTS. Ring canal diameters are not reduced in src64D404N/+ egg chambers but are reduced in src64D404N/src64KO egg chambers. Inset: ring canal of approximately mean diameter, reoriented so that diameter is projected along the vertical axis. Scale bar, 50 μm. (B) Early cellularization stage embryos laid by src64KO, src64D404N and src64D404N/src64KO mothers. Embryos show non-uniform cellularization fronts. Scale bar, 100 μm. (C) Grazing section projections of early cellularization stage embryos laid by src64KO, src64D404N and src64D404N/src64KO mothers showing microfilament networks. Microfilament ring defects of maternally trans-heterozygous src64D404N/src64KO embryos are similar to those of src64KO and src64D404N embryos. Embryos are stained with antibody to myosin II heavy chain. Scale bar, 10 μm. (D) Ring canal diameters. src64D404N and src64D404N/src64KO do not differ (p = 0.35). Error bars are SEM. (E) Microfilament ring circularity during early cellularization. src64D404N and src64D404N/src64KO do not differ (p = 0.52). Error bars are SEM. doi:10.1371/journal.pone.0028100.g005
Mutation of the HRD arginine to alanine in yeast PKA reduces kinase activity to 10.5% of wild-type activity, but viability is unaffected [36]. In PhK, this mutation also reduces kinase activity [37]. Kinase activity of the chicken c-Src mutant protein is 10% for an exogenous substrate but 50% for autophosphorylation [56].

Mutation of the HRD arginine to cysteine has remarkably little effect on Src64. Kinase activity was indistinguishable from wild type, and there was no effect on microfilament contraction, ring canal growth or hatching and only weak defects in egg production. Our data do not support a major role for the HRD arginine in stabilization of the active site or in an electrostatic switch for activation, and are consistent with the activation loop arginine being the critical arginine residue in tyrosine kinases. However, the Src64 cysteine mutant has more kinase activity than the c-Src alanine mutant, suggesting that cysteine may not abrogate biochemical activity. The alanine side chain is short and aliphatic, so it likely eliminates biochemical activity, whereas the polar cysteine side chain may retain sufficient biochemical activity for function. Alternatively, src64R403C may destabilize both the active and inactive conformations by failing to interact with both phospho-Y434 and E327, thus favoring the partially active conformation that would produce a constant but low level of activity.

src64H402L mutation

The HRD histidine is, along with the HRD arginine, one of ten critical residues conserved amongst eukaryotic protein kinases and eukaryotic protein kinase-like kinases in prokaryotes [57]. The histidine peptide backbone hydrogen bonds to the C-terminal end of the activation loop DFG motif and to the F-helix aspartate in the kinase domain C-terminal lobe. The side chain has a hydrophobic packing interaction with the DFG phenylalanine, and hydrogen bonds to the N-terminal end of the DFG motif and to the polypeptide backbone of the HRD aspartate. Thus, the HRD histidine provides two critical links in the active kinase: between the catalytic and activation loops in the active site, and between the active site and the enzyme core [6]. The histidine side chain is a component of the regulatory spine (R-spine), a stack of amino acid side chains linked by hydrophobic interactions that spans the fully active kinase domain [58,59]. The R-spine consists of four residues: the catalytic loop HRD histidine H402 (c-Src H384), the activation loop DFG phenylalanine F423 (c-Src F408), a C-helix hydrophobic residue M331 (c-Src M317), and a β-4 strand leucine L342 (c-Src L328) [58–61]. The hydrogen bond between the histidine polypeptide backbone and the F-helix aspartate anchors the R-spine to the rigid F-helix. Together with the catalytic spine (C-spine), another stack of hydrophobic residues anchored to the F-helix, it connects the two lobes of the kinase domain and stabilizes the active conformation [6,38,59,61].

The HRD histidine has not been investigated by in vitro mutagenesis, but the other three R-spine residues have. Mutation of the p38 MAP kinase DFG phenylalanine to alanine, arginine, or glycine abrogated activity, whereas mutation to tyrosine reduced kinase activity to 1% [55]. The mutant tyrosine side chain retained its hydrophobic contact with histidine, suggesting that addition of a polar hydroxyl group is sufficient to partially destabilize the R-spine [55,62]. Mutation of either the DFG phenylalanine or the C-helix methionine to glycine rendered the nonreceptor tyrosine kinase Abl inactive, whereas mutation of the β-4 strand leucine residue to glycine reduced kinase activity [60].

Here we describe the effect of an HRD histidine mutation on the activity and function of a kinase in a living organism. The src64H402L mutation reduces kinase activity to 27% of wild-type activity and produces moderate to weak, but significant, defects in microfilament ring contraction and ring canal growth. Therefore, a quarter of the wild-type level of Src64 activity is not sufficient for normal cytoskeletal function in these processes. However, substantial cytoskeletal function remains at this lower Src64 activity level. We postulate that the src64H402L mutation alters interactions within the active site without altering the global conformation of the active kinase. The mutant leucine side chain is unlikely to hydrogen bond with the polypeptide backbones of the DFG aspartate and the HRD aspartate; loss of these active site interactions likely causes the reduction in kinase activity and the phenotypic defects. The hydrophobic interaction with the DFG phenylalanine should be retained, so a stable R-spine should still form. The hydrogen bond from the polypeptide backbone to the F-helix aspartate should be retained, so the catalytic loop and the R-spine should still be anchored to the F-helix, and thus linked to the C-lobe and the C-spine. Likewise, the hydrogen bond from the polypeptide backbone to the DFG motif should be retained, so the catalytic loop and anchor it to the F-helix, and that its role in linking the catalytic loop to the activation loop may be less important.

In summary, our data suggest that the catalytic loop HRD amino acids have more complex roles in kinase activity and activation than previously thought. In addition, we have developed an experimental paradigm for a combined in vivo and in vitro investigation of the roles of specific amino acids in Src64. By making specific amino acid substitutions in a src64 transgene expressed in src64O flies, this approach can be used to further define the biochemical roles of the HRD histidine and arginine amino acids in SFKs.

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Author Contributions

Conceived and designed the experiments: JHT. Performed the experiments: TGS GK JHT. Analyzed the data: TGS GK JHT. Wrote the paper: TC8 JHT.

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