Systematic Functional Analysis of Bicaudal-D Serine Phosphorylation and Intragenic Suppression of a Female Sterile Allele of BicD

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Abstract

Protein phosphorylation is involved in posttranslational control of essentially all biological processes. Using mass spectrometry, recent analyses of whole phosphoproteomes led to the identification of numerous new phosphorylation sites. However, the function of most of these sites remained unknown. We chose the Drosophila Bicaudal-D protein to estimate the importance of individual phosphorylation events. Being involved in different cellular processes, BicD is required for oocyte determination, for RNA transport during oogenesis and embryogenesis, and for photoreceptor nuclei migration in the developing eye. The numerous roles of BicD and the available evidence for functional importance of BicD phosphorylation led us to identify eight phosphorylation sites of BicD, and we tested a total of 14 identified and suspected phosphoserine residues for their functional importance in vivo in flies. Surprisingly, all these serines turned out to be dispensable for providing sufficient basal BicD activity for normal growth and development. However, in a genetically sensitized background where the BicD40V protein variant provides only partial activity, serine 103 substitutions are not neutral anymore, but show surprising differences. The S103D substitution completely inactivates the protein, whereas S103A behaves neutral, and the S103F substitution, isolated in a genetic screen, restores BicD40V function. Our results suggest that many BicD phosphorylation events may either be fortuitous or play a modulating function as shown for Ser103. Remarkably, amongst the Drosophila serines we found phosphorylated, Ser103 is the only one that is fully conserved in mammalian BicD.

Introduction

Reversible phosphorylation of proteins at Ser, Thr and Tyr residues is a particularly important type of a posttranslational modification because it is involved in the control of essentially all biological processes. For this reason, protein phosphorylation has drawn widespread interest, and a number of techniques have been developed and were successfully applied to investigate the phosphorylation states and sites of isolated proteins. A combination of recent advancements in genomics and mass spectrometric analysis of peptides opened the possibility to analyze phosphorylation of whole proteomes, allowing the identification of many new phosphorylation sites (see e.g. [1–3]). However, the functions of these newly discovered phosphorylation events are usually not clear.

To estimate the importance of individual phosphorylation events, we set out to systematically verify the phosphorylation sites of one polypeptide, and to test these genetically for their functional importance. To increase the chances of identifying functional roles for these sites, we chose the Drosophila Bicaudal-D (BicD) protein that is phosphorylated [4] and has many essential functions during various phases of the life cycle of the fly [5]. In addition, there is evidence that the kinases polo [6] and missiphen [7] may phosphorylate Drosophila BicD, and the human Glycogen synthase kinase-3β (GSK-3β) can phosphorylate human BicD1 in vitro [8]. Furthermore, phenotypic correlations between phosphorylation and mutant phenotypes had already been described. The BicD allele BicD3466, an alanine to valine substitution at amino acid 46, is a recessive hypomorphic mutant that is viable, but female sterile because their ovaries fail to differentiate an oocyte and egg. In these mutant ovaries, phosphorylation of the BicD40V protein is markedly reduced [4]. In addition, a suppressor of this allele, BicD3466 Su(66b), restores female fertility as well as BicD phosphorylation levels [4]. BicD functions in numerous different processes, and we will therefore briefly summarize these functions to give an impression of the various tests we set up.

During early oogenesis, BicD is required for the determination and differentiation of an oocyte from a cluster of 16 interconnected germ cells. While the remaining 15 become nurse cells, the oocyte relocates in a BicD-dependent manner to the posterior of the developing egg chamber. In this process, BicD works together with egl, Lis-1 and Dynein in a microtubule based process (reviewed in [5]), and the same machinery seems to function subsequently in delivering primary axis determination mRNAs (see e.g. [9]). Also during oogenesis, but as part of different processes with distinct requirements for additional genes, BicD localizes organelles and


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proteins to specific subcellular compartments [10–14]. During embryogenesis, the BicD-dependent RNA transport machinery is used again for the apical localization of pair-rule and segmentless transcripts [15–17]. At the third instar larval stage, formation of the ommatidia of the compound eye starts and the nuclei of the differentiating photoreceptor cells migrate to the apical surface [18]. This apical migration is dependent on BicD, Lis-1, and the microtubule motors Dynein and Kinesin [7,10,19].

The highly regular geometry of the compound eye makes this a very sensitive system to study the effect of slight alterations of the activities of genes involved in its development. To systematically test the function of suspected and identified phosphorylation sites in BicD, we made mutants in vivo that cannot be phosphorylated at these sites (Ser to Ala or Asn substitutions) and mutants that mimic permanent phosphorylation of some of these sites (Ser to Asp). We then produced transgenic lines and crossed them into the BicDfunctional mutant background [20] to test whether the mutant alleles were capable to substitute for the normal BicD in the various processes described.

Surprisingly, these phosphosite mutants turned out not to be essential for any of the described BicD functions, suggesting that they are either redundant, only modulating or even fortuitous events. While limited tests for redundant functions also failed to uncover such events, one site turned out to be important for overall BicD function of suspected and identified phosphorylation sites in BicD.

To investigate the importance of phosphorylation for BicD function, a systematic mutagenesis study was carried out. Mutations changing the identified and candidate phosphorylation target sites were introduced by site directed mutagenesis into a functional, untagged mini-BicD gene, that is expressed from its native promoter and is also used as the wild type control gene (BicDfunctional; see Materials and Methods section for details). Accordingly, we made phosphorylation-impaired mutants for the serines shown in Figure 1C by substituting the respective codons (Ser to Ala or Thr to Asn) with alanine codons. To investigate the possibility that phosphorylation of the serines in the vicinity of the PA66 mutation (serines 33, 34, 36, 39, 45, 51 and 84) plays a functional role, we also tested mutations that change these Ser into Thr. For Ser134, Ser144 and Ser149, we additionally made triple, quadruple and quintuple mutation in which these Ser were replaced with Ala or Thr. For Ser134, Ser144 and Ser149, we additionally made triple, quadruple and quintuple mutation in which these Ser were replaced with Ala or Thr. For Ser134, Ser144 and Ser149, we additionally made triple, quadruple and quintuple mutation in which these Ser were replaced with Ala or Thr. For Ser134, Ser144 and Ser149, we additionally made triple, quadruple and quintuple mutation in which these Ser were replaced with Ala or Thr. For Ser134, Ser144 and Ser149, we additionally made triple, quadruple and quintuple mutation in which these Ser were replaced with Ala or Thr. For Ser134, Ser144 and Ser149, we additionally made triple, quadruple and quintuple mutation in which these Ser were replaced with Ala or Thr.
Figure 1. Location of BicD phosphorylation sites. A, B: MS/MS spectra of the [M+2H]²⁺ ions of the peptide T⁹⁰QIEQEDALLNESAR²⁰⁶ (A) and its serine phosphorylated form (B). The intense, neutral loss fragment at m/z = 850.4 (marked with an asterisk) in B indicates the extensive loss of phosphoric acid. Upon collision induced fragmentation in the iontrap, peptide bond fragmentation allowed unambiguous characterization of the amino acid sequence and the presence of a phosphorylated Ser. Note the m/z shift of 80 mass units corresponding to the phosphorylation of Ser at y(4) and following y-ions between A and B. Furthermore, y-ions showed also extensive loss of phosphoric acid corresponding to a y-ion series with 98 mass units difference in the same MS/MS spectrum in B. C: Summary of phosphopeptides and phosphorylation sites of BicD identified by MS analysis. Phosphorylation of Ser²⁸⁵ was only observed when Ser²⁸⁸ was phosphorylated as well. Of Ser³⁰⁵ and Ser³¹⁰, both, single and double
phosphorylations, were found. The peptide 12–24 is an incomplete tryptic fragment, whereas the shown peptide 299–315 has two missed cleavage sites. Due to its small size, the peptide S310PDGTK315 could not be identified individually.

**Table 1. Phenotypes of BicD phosphorylation mutants.**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>MS identification</th>
<th>Phenotype</th>
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<tr>
<td>wild type</td>
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<td>ff</td>
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<tr>
<td>S14A</td>
<td>+ + + ff</td>
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<tr>
<td>S14D</td>
<td></td>
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<tr>
<td>S33A</td>
<td>+ + + ff</td>
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<td>S34A</td>
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<td>+ + + ff</td>
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<tr>
<td>S36A</td>
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<td>– + + + ff</td>
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<td>S39A</td>
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<td>– + + + ff</td>
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<tr>
<td>S45A</td>
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<td>– + + + ff</td>
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<tr>
<td>multiple</td>
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<tr>
<td>S51A</td>
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<td>S54A</td>
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<td>S103A</td>
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<td>S103D</td>
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<td>S103F</td>
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<td>S109A</td>
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<td>S186A</td>
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<td>S285A</td>
<td>+ + + + + + + + +</td>
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<td>S288A</td>
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<td>S285A–S288A</td>
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<tr>
<td>S30S</td>
<td></td>
<td>+ + + + +</td>
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<tr>
<td>S310D</td>
<td></td>
<td>+ + + + +</td>
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<tr>
<td>S305A–S310A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A40V</td>
<td></td>
<td>ff, 16 nurse cells</td>
</tr>
<tr>
<td>A40V–S303A</td>
<td></td>
<td>ff, 16 nurse cells</td>
</tr>
<tr>
<td>A40V–S303D</td>
<td>lethal with few adults, 16 nurse cells</td>
<td></td>
</tr>
<tr>
<td>A40V–S303F</td>
<td>ff, few egg chambers with 16 nurse cells</td>
<td></td>
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Peptides containing the indicated serines were identified (+) as serine phosphorylated form (phos.) or non-phosphorylated (non-phos.), or the peptide was never identified (–). All mutants are viable, except for BicDA40V; S103D; BicDA40V,S103D consists of the five substitutions S33A, S34N, S36N, S39A, S45A. ff females fertile. 

Wild type BicD preferentially accumulates in the oocyte, and a significant amount of the protein is hyperphosphorylated. In BicDP166 mutants however, no oocyte is formed and the levels of hyperphosphorylated BicD are reduced [4]. To further test the correlation between BicD function and the presence of the hyperphosphorylated isoform of BicD, we analyzed the BicD isoform pattern in the newly established phosphomutants. For this purpose, BicD was immunoprecipitated from wt and mutant ovary extracts and its isoforms were separated by gel electrophoresis and visualized by western blotting. In control extracts from rescued wild type and OregonR females, there were two distinct isoforms of BicD present (Figure 2A, lanes 1 and 2, respectively). The BicD-antibody complex immunoprecipitated from OreR extracts was additionally treated with Calf Intestinal Phosphatase (CIP), which led to the disappearance of the slow migrating isoform (lane 5). Treatment in the presence of phosphatase inhibitors did not change the isoform pattern (lane 4), confirming that the slow migrating band is indeed a hyperphosphorylated isoform of BicD. Consistent with its normal physiological function, the isoform pattern of the wild type rescue construct was comparable to the one of the OreR controls (lanes 1 and 2), while the reconstructed BicDA40V mutant lead to a significant reduction of the slowest migrating band (lane 6), as had been reported for endogenous BicD310[4].

Mutating the serines 14 through 288 has little effect on the global phosphorylation levels of BicD, and the patterns and the individual band intensities of BicD in these mutants appear similar to the wild type controls (Figure 2A, lanes 5, 6 and 12–23). Interestingly, in the case of Ser14 and Ser103, neither mimicking permanent phosphorylation nor preventing phosphorylation influences overall phosphorylation levels of BicD (Figure 2B and 2A, lanes 12, 13 and 5, 6, respectively). To further verify this visual impression, we quantitatively assessed the amount of phospho-BicD in these mutants. The graph in Figure 2B shows the amount of the phospho-isoforms of every mutant relative to the wild type rescue construct that was set to 100% (black columns). Because we could not see a major change of BicD protein content in any of the Ser mutants, we assumed that reduced hyperphosphorylation of BicD leads to a higher level of the hypophosphorylated isoform, rather than to degradation. Accordingly, the values of hyperphosphorylated BicD were normalized to the sum of both BicD bands in each individual sample. The quantification results confirmed the visual impression and revealed only minor differences in hyperphosphorylation of BicD between most mutants. We also repeated this phospho-BicD analysis with loading ovarian extracts directly on the gel and obtained the same results (Figure 2B, grey columns), indicating that the ratios are not distorted by differential immunoprecipitation of different isoforms.

While these findings are in agreement with a positive correlation between phospho-BicD levels and BicD function, the Ser310 mutants seems to be an exception to this. They are viable and normally fertile, but show a markedly reduced amount of hyperphosphorylated BicD, irrespective of whether phosphorylation of this residue is prevented or mimicked (Figure 2B and 2A, lanes 24 and 25). Because the Ser310 alleles are functional, while...
A40V is not, it is unlikely that the cause of the malfunction of the A40V mutation is solely reduced BicD phosphorylation. BicD has additional zygotic functions later in development, where it is required for the positioning of the photoreceptor nuclei [10]. Analyzing the eyes of BicDnull flies reveals a rough eye phenotype with irregularly shaped ommatidia (data not shown; see later). To test whether one of our serine mutants plays a role specifically in this pathway, we inspected the eyes of these mutants and found them to be normal, suggesting that BicD phosphorylation is also dispensable for this process (data not shown).

Substituting Ser103 by phenylalanine in BicDA40V suppresses the BicDPA66 phenotype and increases phospho-BicD levels

While phosphorylation of the 14 tested serines is not essential for basal BicD function, it may play a more modulating role that can be detected under less favorable conditions. A genetic screen for a suppressor of the phosphorylation mutant BicDPA66 lead to the isolation of the Su(66) mutation that significantly restores the accumulation of hyperphosphorylated BicD protein [4]. In addition, the suppressor mutation also restores female fertility and oocyte localization of BicD. As these observations point to a functional importance of BicD phosphorylation, we set out to study the molecular basis of this phenotype. The Su(66) mutation maps to the second chromosome and recombination mapping experiments placed Su(66) in the immediate vicinity of BicD (A. Swan and B. Suter, personal communication). In order to identify this suppressor mutation, we sequenced BicD and its four proximal neighboring genes Sgt, Aac11, fws and CG5110 from homozygous BicDPA66 Su(66) flies. The sequences were compared to the parental BicDPA66 strain. No polymorphism was detected in the four proximal genes and the BicDPA66 mutation was present on the BicDPA66 Su(66) chromosome as expected. In addition, we found in the BicD gene a single nucleotide transition C→T that was not present in the parental BicDPA66 strain. This mutation changes the codon 103 from TCC to TTC, causing the normally present serine to be substituted by a phenylalanine in Su(66). This substitution was of exceptional interest, because our MS analysis identified this Ser103 to be phosphorylated. In order to test whether the S103F substitution indeed acts as suppressor of the BicDPA66 allele, we reconstructed this BicD allele with both

**Figure 2. BicD phosphorylation in phosphorylation target site mutants.** BicD protein isoforms were separated by PAGE and detected by Western blotting. A. A representative blot with BicD protein immunoprecipitated from the indicated ovarian extracts. All samples were processed simultaneously. The slowest migrating isoform corresponds to hyperphosphorylated BicD (marked with an arrowhead), as demonstrated by treatment of immunoprecipitates from OregonR females without (OreR) and with Phosphatase (+CIP), or with Phosphatase in presence of inhibitors (+CIP +Inh.). In addition, the reconstruction of the BicDPA66 mutant (A40V) served as internal control, where the slow migrating isoform is reduced. Beads: mock IP from OreR extracts without antibody. The dashed vertical line indicates the border between different gels/blots. Due to the small ovary size of the female sterile mutants, less total material was loaded in lanes 8 and 9. B. For quantification, the amount of phospho-BicD was determined relative to the not mutated rescue construct (wt) that was set to 100%. All values are normalized to the sum of both bands to compensate for different total amounts loaded. Black columns: percentage of phospho-BicD determined from immunoprecipitation and the blot shown in panel A. Grey columns: percentage of phospho-BicD obtained from ovarian extracts without IP. BicD multiple consists of the five substitutions S33A, S34N, S36N, S39A, S45A. Su(66): extracts from homozygous BicDPA66 Su(66) cn females. PA66: extracts from homozygous BicDPA66 cn bw females. *: no data.

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mutations. Indeed, females with one copy of this double mutant chromosome $BicD^{A40V, S103F}$ were viable and fertile, while the ones with $BicD^{A40V}$ alone are viable but sterile.

In order to study the effects of this mutation, we analyzed the influence of residue 103 on the distribution of the protein during oogenesis. At first glance, ovaries of $BicD^{A40V}$ flies appear largely normal and contain mostly egg chambers with normal morphology (Figure 3). The mutant $BicD$ protein accumulates in the oocyte and displays a normal subcellular distribution. However, the accumulation appears less pronounced compared to the wild type situation (Figure 3A, F), suggesting that the double mutant $BicD$ protein is less active than wild type $BicD$. Moreover, such $BicD^{A40V, S103F}$ ovaries contain a few egg chambers that failed to form an oocyte, and, instead, contain 16 polyploid nurse cells (arrow in Figure 3C, H), like all egg chambers from control $BicD^{A40V}$ females do (Figure 3B, G). This is consistent with our previous observations [4], confirming that the S103F substitution is sufficient to partially suppress the effects of the $BicD^{A40V}$ mutation.

Ser$^{103}$ substitutions alter the $BicD^{A40V}$ phenotype in a mutant specific manner

Because phenylalanine is not phosphorylatable, we wondered whether preventing Ser$^{103}$ phosphorylation is sufficient to suppress the $BicD^{A40V}$ phenotype. To test this, we constructed the $BicD^{A40V, S103A}$ allele, where the Ser$^{103}$ is replaced by alanine, which cannot be phosphorylated either. Surprisingly however, such $BicD^{A40V, S103A}$ females were sterile with ovaries consisting of egg chambers with 16 nurse cells and no oocyte (Figure 3D, I), indistinguishable from the phenotype of $BicD^{A40V}$ females that have the wild type serine at position 103. Therefore, the suppression effect of the S103F substitution on $BicD^{A40V}$ cannot be caused simply by inhibition of phosphorylation of Ser$^{103}$.

We next wondered how mimicking permanent phosphorylation of Ser$^{103}$ in $BicD^{A40V}$ affects the function of the protein. Strikingly, $BicD$ with both substitutions, A40V and S103D, does not rescue $BicD^{A40V}$ alleles and thus behaves like a recessive lethal mutant. Only very few $BicD^{A40V, S103D}$ adults were obtained, they hatched 2–4 days later and were smaller than control siblings with an endogenous copy of wild type $BicD$ from the $CyO$ chromosome (Figure 4A–C), appearing weak and lethargic and displaying uncoordinated behavior. These mutants died within a few days. In addition, they had a variable rough eye phenotype because of irregular ommatidia (compare Figure 4D–F), while the viable $BicD^{A40V, S103A}$ and $BicD^{A40V, S103D}$ mutants displayed normal eyes (Figure 4G and H, respectively). Ovaries of $BicD^{A40V, S103D}$ females consist of egg chambers lacking an oocyte and containing only 16 polyploid nurse cells (Figure 3E and J), similar to ovaries from $BicD^{A40V}$ and $BicD^{PA66}$ flies (Figure 3B, and [20,24]). In addition, the $BicD^{A40V, S103D}$ protein does not accumulate into a single cell. All these described phenotypes were previously reported for $BicD^{null}$ flies [10,20]. These findings therefore strongly suggest that mimicking permanent phosphorylation at amino acid position 103 of $BicD^{A40V}$ severely inhibits even the essential zygotic functions of $BicD^{A40V}$.

Effect of amino acid 103 on $BicD$ function

In an otherwise wild type $BicD$, substituting the Ser$^{103}$ by A, D or F does not yield any obvious phenotypes. We were therefore surprised by the strikingly different phenotypes observed when these substitutions were combined with the A40V mutation found in $BicD^{A40V}$. Consequently, we also analyzed the function of residue 103 in the wild type protein in more detail. The S103A/ D/F mutants were viable and fertile (Table 1). However, the phospho-mimic $BicD^{S103D}$ mutant seems to have slightly less hyperphosphorylated $BicD$ than the phosphorylation defective mutants $BicD^{S103F}$ and $BicD^{PA66}$ (Figure 2B and 2A, lanes 5–7). Ovaries of $BicD^{S103X}$ females appear normal, and while the S103A mutant protein accumulates normally in the oocyte, the $BicD^{S103D}$

![Figure 3. A S103F substitution in $BicD^{A40V}$ suppresses the $BicD^{PA66}$ phenotype.](https://www.plosone.org/doi/10.1371/journal.pone.0004552.g003)
protein shows somewhat reduced and the S103F protein slightly increased oocyte accumulation (Supporting Figure S1). These findings suggest that mimicking permanent phosphorylation of BicD at Ser103 acts inhibitory on the protein’s oocyte localization.

Another assay to test the activity of the Ser103 mutants is to investigate whether these mutants affect the dominant BicD phenotype. Females with the BicD2 allele produce embryos with defective anterior structures [24,25] caused by a partial mislocalization of osk mRNA to the anterior of the oocyte and the embryo [26,27]. Embryos from females hemizygous for BicD2 and with one copy of the wild type rescue transgene (BicDwt) or a Ser103 mutant construct were inspected for defective anterior structures (Figure 5). With the BicD wt transgene, such mothers produce mostly normal embryos (wt). Similarly, low numbers of aberrant embryos are observed when the mothers had the S103A or S103F substitution in the BicD transgene. In contrast, a markedly increased amount of defective embryos was found when mothers carried the BicDS103D allele. These results provide further evidence that the amino acid at position 103 is important for full BicD function, and they suggest that transient phosphorylation of the native serine at this position plays a role in modulating BicD function.

Discussion

Protein phosphorylation is a posttranslational modification that is used to regulate the function of proteins involved in many different cellular processes. The widespread interest in this reversible protein modification recently led to the analysis of phosphoproteomes [see e.g. [28,29]] which revealed many new phosphorylation sites. However, the function of these phosphorylation events usually remained to be elucidated. To obtain an estimate of the function of the numerous phosphorylation sites determined with this technique, we picked a protein that is known to be phosphorylated, determined its phosphorylation sites, compared these sites with the proteomics data, analyzed the evolutionary conservation of the sites, and tested the requirement for phosphorylation of these residues. Our choice of example protein was the Drosophila BicD because it is conserved up to humans, is involved in different cellular processes that act during different phases of the life cycle, and because null mutants are available that allow us to test the functions of phosphorylation in all these phases. Using mass spectrometric analysis of immunoprecipitated BicD and BicD::GFP, we identified the serines 14, 103, 186, 285, 288, 305 and 310, and either Thr108 or Ser109 to be phosphorylated. Some of the sites found here have been determined independently by large scale screens for phospho-sites in Drosophila Kc167 cells [30] and in Drosophila embryos [31].

To find out more on the function of the BicD phosphorylation sites, we performed in silico analysis on them. Only three of the experimentally identified BicD phosphosites were also predicted with a bioinformatic analysis using the programs NetPhos [32] and Scansite [33]. The three broad phosphorylation motif classifications each fit one of the BicD phsophoserines. Ser109 corresponds to a basophilic site (K/R-X-X-S/T), while Ser288 fits an acidophilic motif (S/T-X-X-D/E), and Ser310 corresponds to a proline directed site (S/T-P). Very recently, a study on mitotic phosphorylation identified the unique phosphorylation motif S-G/A-X-K/R [29]. While the kinase for this site is yet unknown, the Ser109 matches this consensus phosphorylation motif.

In addition, we found that Ser14 is a perfect match to a Polo kinase target site D/E-X-S/T-Φ-X-D/E [22]. However, neither
BicD phosphorylation is markedly reduced in both, the S310A isoform mobility. The exception is the serine 310. Surprisingly, that the absence of a single one of them does not alter the Ser 310 substitutions, global BicD phosphorylation levels is essential for any BicD function, and that, with the exception of (Figure 1E) do not fit the known GSK-3 manner [8], but the phosphorylated serines identified in this study provide further evidence that Ser103, and possibly its phosphorylation, play a modulating role on BicD function. Strikingly, these mutants exhibited very different phenotypes: while the S103A substitution does not change the PA66 phenotype, the S103D mutation inactivates the BicD A40V protein, causing BicD A40V, S103D to behave as a null mutant. The suppression effect of the S103F mutation in Su(66) cannot be caused by the absence of phosphorylation of Ser103, because the S103A substitution is neutral and does not change the PA66 phenotype. Instead, the results suggest that the bulky side chain of phenylalanine introduces a structural change in the mutant protein that compensates for the loss of function caused by the A40V mutation. How this substitution can suppress over the relatively large distance of some 60 amino acids in the primary structure is presently unknown. However, Ser103 is located at the beginning of a predicted coiled-coil motif of BicD (Figure 1D), and it is tempting to assume that phosphorylation of Ser103 may regulate the formation of this coiled-coil region. Unfortunately, an earlier functional analysis of coiled coil regions of BicD did not test the importance of this particular coiled coil domain and only included one of the phosphoserines, S186 [35].

At the onset of our experiments we envisioned that individual phosphorylation events may control individual localization processes. The fact that all the biochemically identified phosphosites are non-essential for normal BicD function in all the different processes examined, however, suggests that most of the phosphorylation events are either fortuitous or play only minor modulating roles. The only modulating role we could find so far is one that we identified with tools created by a classical genetic approach, isolating a suppressor mutation that can restore the activity of a partially inactivated and hypophosphorylated variant of BicD. In this genetically sensitized background, the 103 position that gets phosphorylated in the wild type protein becomes decisive for the function of the entire protein. Interestingly, this Ser is also the only one that is fully conserved in mice and humans. According to our findings on BicD phosphorylation, the identification of protein phosphorylation sites needs to be treated with caution as such sites are often not crucial for the function of a protein.
Materials and Methods

Isolation of genomic DNA and sequencing of the Su(66) region

DNA from a pool of 15 flies was isolated according to [36]. The coding sequences of BicD and four proximal neighboring genes were amplified by PCR, skipping 7 kb of the first, large BicD intron. All PCR products were purified and sequenced on an ABI Prism 3100 Sequence Analyzer (Applied Biosystems).

Generation of vectors, in vitro mutagenesis and transgenesis

We constructed the pw-SNattB vector that is designed to harbor constructs driven by their own promoter. pw-SNattB contains a multiple cloning site, a white selectable marker, a lacF site, and an attB fragment that allows its integration into attP landing platforms [37] using the phiC31 integrase. Briefly, the UAS-SV40 cassette from pUASTattB [37] was replaced with a modified multiple cloning site of pLimaus20 (New England Biolabs) to yield pw-SNattB. The sequence is available from the EMBL/GenBank data libraries under accession no. EU729722. A similar strategy was used to construct pUAS-K10attB that is useful for gene expression in the female germline using the UAS-Gal4 system. The UAS-SV40 cassette was removed from pUASTattB [37] and replaced with the UASp cassette from pUASp [38] to yield pUAS-K10attB. The sequence is available from the EMBL/GenBank data libraries under accession no. EU729723.

The mini-BicD::GFP fusion construct [21] was transferred as kpnI/Nde fragment into pw-SNattB. The BicD 3’ part with the GFP fusion was then replaced with the corresponding native 2.75 kb BicD 3’ part lacking GFP that was taken from pBS4.2RV [24]. This yielded the mini-BicD::GFP vector that served as wild type control BicDWT. A BsoN1/SalI fragment of mini-BicD::GFP was subcloned into pLimaus28 (New England Biolabs), and the mutations were introduced in this construct by high fidelity PCR using suitable primers. Plasmids containing the correct mutations were further verified by sequencing. The individual mutations were then transferred into mini-BicD-pw-SNattB to replace the respective wild type sequence. The fragments containing the mutations S14A/D-S54A were cloned using BstII/EcoRI or BsrGI/BstEEI, while S84A-S310A/D were transferred using EcoRI/BstEEI or PshAI/BstEEI. Most clones were error-free, however, a small number showed 1–3 nucleotide long deletions at the PshAI site in the intron 1.

These BicD mutants were introduced into attP landing platforms using the phiC31 integrase [37]. The mutants S33A, S34A, S36A, S45A, multiple, A40V, S103A/D/F, A40V+S103A/D/F and the wild type control were integrated into the landing platform ZH-102F on the fourth chromosome. S14A/D, S51A, S109A, S168A, S285A, S288A, S305A, S310A/D and the double mutants S285A/S288A and S305A/S310A were inserted into ZH-44A on the third chromosome, and S39A in ZH-2A on the X chromosome. All transgenic constructs in the flies were verified by preparation of genomic DNA and sequencing of the relevant regions containing the mutation(s).

Fly stocks

The landing platforms and the integrases were described [37]. The BicDmutant allele BicDmutant was described earlier [20], and Df(2L)Exel7068 was obtained from Bloomington Stock Center (stock no. 7833). All BicD mutants generated for this study were kept as stocks with the BicDmutant allele on the second chromosome (genotype w; BicDmutant/SM1; +/+ with a BicD transgene on the 1st, 3rd or 4th chromosome as described above). Males were crossed to w; Df(2L)Exel7068/CyO b females as needed to generate flies carrying one copy of a BicD construct in a hemizygous BicDmutant background. These progeny were then used for the experiments.

Eye implants and embryo cuticle preparations

The eye implants were done as described in [39]. Images were recorded on a Leica DM6000 B microscope using a 40 x DIC objective. For cuticle preparations, embryos were collected for 24 h at 18°C from w; BicD+/BicDmutant/+; +/+ 102F/BicDmutant/+ mothers, with BicDmutant as indicated in Figure 5. The embryos were aged for 24 h at 25°C, and cuticles were prepared as described [40], mounted in a 1:1 mixture of lacitic acid/Hoyer’s medium and incubated at 58°C for 30 h. Cuticle phenotypes were scored on a Nikon Eclipse E600 microscope.

Immunostainings

Immunohistochemical stainings were essentially done as described earlier [4], with denoted modifications. After fixation, ovaries were incubated in PBTM (1 x PBS with 0.2% Tween-20, 0.1% Triton X-100 and 5% non-fat dry milk), and then with the appropriate antibodies in PBTM for 4 hours at room temperature. Mouse monoclonal anti-BicD antibodies 4C2 and 1B11 [4] were used each at 1:15 dilution. The secondary Cy3-conjugated anti-mouse antibodies (Jackson ImmunoResearch) was used at a dilution of 1:1,000. During the final washing steps, DNA and F-actin were stained with 2.5 µg/ml Hoechst 33258 (Molecular Probes) and 0.05 µg/ml FITC-conjugated phalloidin (Molecular Probes), respectively. The ovaries were embedded in AquaPolyexam (Polysciences), and images were recorded using a Leica DM6000 B fluorescence microscope or a Leica TCS-SP2 confocal microscope. To exclude artifacts resulting from small differences in buffers and incubation times, all samples were processed simultaneously, and images were recorded using identical settings on the microscope and the software.

Large scale immunoprecipitations and mass spectrometry

Embryos were collected during 12 hour time periods, dechorionated and stored at −80°C. Ovaries from 10 g of flies were collected as described [41]. The egg chambers were washed twice with IP buffer (25 mM HEPES pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, supplemented with complete protease inhibitors; Roche) and frozen in liquid nitrogen. In a glass homogenizer, 4 g of embryos or 3.5 ml of ovaries were homogenized in 8 ml (3.5 ml) IPpi buffer (IP buffer including sodium molybdate, 50 mM sodium fluoride). The homogenate was centrifuged for 1 h at 16,000 g at 4°C. The soluble phase was centrifuged again for 25 min at 16,000 g at 4°C. Two hundred µl GammaBind Plus Sepharose beads (GE Healthcare) were washed three times in PBS and incubated with 4 ml 1B11 anti-BicD antibody for two hours. Beads with bound antibodies were washed three times in PBS and once in IP buffer, added to the homogenate supernatant and incubated for 3 h at 4°C with constant mixing. The beads were then washed six times with IP buffer. Finally, the beads were resuspended in Nu-PAGE sample buffer (Invitrogen) containing 0.1 M DTT, boiled for 5 min, and proteins were separated by SDS PAGE. The gel was stained with Coomassie Blue (Invitrogen), bands of interest were excised, and proteins were digested in gel with sequencing grade trypsin (10 ng/ml; Promega) overnight at room temperature. Peptides were extracted from the gel with 20% formic acid (FA) by incubation for 15 min at room temperature and analyzed by
LC-MS/MS (Exquise3000+ ion trap mass spectrometer with a capillary ESI source (Bruker Daltonics) equipped with an Alliance HT2795 HPLC system from Waters). CID spectra interpretation was performed with the Phenyx software (GeneBio) using the Uniprot Drosophila protein database, release 54.0.

To immunoprecipitate BicD::GFP from embryonic extracts, anti-GFP antibodies were coupled covalently to Sepharose beads. One hundred μl GammaBind Plus Sepharose beads were washed three times in PBS and incubated with 2 μl anti-GFP antibody (mouse monoclonal 3E6, gift from A. Marcil, BR1, Montreal) for two hours. Beads with bound antibody were washed three times in PBS, and finally resuspended in 400 μl PBS. To this, 100 μl diisuccinimidyl suberate solution (13 mg/ml in DMSO; Pierce Biotechnology) was added and incubated for 1 h with constant mixing. The beads were sedimented by centrifugation for 3 min at 1,300 g, washed once with 0.2 M ethanolamine pH 8.0 for 2 min and another time for 2 hours with constant mixing. The beads were sedimented and washed twice with 0.1 M glycine pH 2.8 for 10 min, and then 3 times 10 min with PBS. Finally, the beads were resuspended in RIPA buffer (50 mM Tris-HCl pH 8.5, 300 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet-P40, 1 mM EDTA supplemented with protease and phosphatase inhibitors). Extracts of BicD::GFP embryos in RIPA buffer were obtained as described above, and incubated with the anti-GFP beads over night at 4°C with constant mixing. The beads were washed on ice six times in RIPA buffer and once for 3 min on ice in 0.1 M glycine pH 2.8. Beads were then sedimented by centrifugation, and bound proteins were eluted by incubation with 8 M urea/50 mM Tris-HCl pH 8.0 for 5 min at room temperature. The elution was repeated once, and the fractions were pooled. DTT was added to 5 mM, and the mixture was incubated at 37°C for 45 min. Sulfhydryl groups were derivatized for 30 min in the dark by addition of 0.5 M iodoacetamide to C in the dark by addition of 0.5 M iodoacetamide to 12.5 mM. The proteins were precipitated with acetone, resuspended and digested with trypsin (4 mg/ml) overnight at room temperature. The digest was acidified with 20% FA and incubated with TiO2 slurry in 1.5× loading buffer (3.7 mg/ml TiO2 in 1.5% trifluoroacetic acid, 60% acetonitrile [MeCN], 1.5 M acetic acid) for 15 min. Beads were washed once in 1× loading buffer and Twice with 5% MeCN. Bound peptides were eluted for 5 min with 50 mM phosphate, 5 mM sodium orthovanadate, 1 mM NaF at pH 10.5, acetylated with 20% FA and dried in a vacuum centrifuge. Peptides were reconstituted in 25 μl 20% FA, and 20 μl were analyzed by nano-LC-MS/MS (LTQ-orbitrap-XL equipped with a nanospray probe and two Rheos micro/nanoflow HPLC systems; ThermoFisher Scientific). MS/MS spectra were searched with Phenyx software (GeneBio) against the Uniprot-Swissprot Drosophila melanogaster database, version 54.6. All identifications on phosphorylation were manually validated for correctness.

Small scale immunoprecipitations and western blotting
To prepare ovary extracts, ovaries from 1–2 day old females were dissected in Drosophila Ringer’s solution (182 mM KCl, 46 mM NaCl, 3 mM CaCl2, 10 mM Tris-HCl, pH 7.2) and snap-frozen on dry ice. For every sample, 35 ovary pairs were used, except for BicD40V and BicD40V, where 70 pairs were used each, and OreR with 90 pairs. The ovaries were homogenized in 500 μl RIPA buffer, and the homogenate was centrifuged twice for 5 min at 16,000 g at 4°C. The supernatant was combined with 30 μl of each anti-BicD antibody 1B1 and 4C2 and incubated for 2 h at 4°C with constant mixing. GammaBind Plus Sepharose beads were resuspended in RIPA buffer, and 30 μl of this mixture was added to the ovary extracts and incubated for 1.5 h at 4°C with constant mixing. The beads were allowed to sediment by gravity and were washed 4 times with wash buffer 1 (RIPA with only 0.5% Nonidet P-40).

For phosphatase treatment, beads were washed twice with wash buffer 1, once with wash buffer 2 (wash buffer 1 lacking phosphatase inhibitors) and once in a 1:1 mixture of wash buffer 2 and NEB3 buffer (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl2, 1 mM DTT, pH 7.9; New England Biolabs). The beads were split into 3 portions, resuspended in 50 μl NEB3 buffer, and incubated at 37°C for 1 h with 10 units of calf intestinal phosphatase (CIP; New England Biolabs). Controls were incubated without CIP, and with CIP in the presence of inhibitors (10 mM Na3VO4 and 4 mM Na2MoO4). The reaction was stopped by washing the beads 3 times with wash buffer 2 containing 1 mM EDTA. Finally, the beads were resuspended in 2× sample buffer (100 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.2 M DTT and a trace amount of bromophenol blue) and boiled for 5 min before being loaded on a gel.

To analyze ovary extracts without IP, ovaries were dissected and extracts were prepared as described [42]. Phospho-isosforms of BicD were separated on standard 8.5% polyacrylamide gels (Acrylamide:Bis = 95:1) lacking SDS, which was only provided in the running buffer. Gels were run at 20 mA in the stacking gel and at 38 mA in the separating gel with cooling to 15°C, using a Protean II xi cell (Bio-Rad). After transferring the proteins to nitrocellulose membranes, BicD was detected using the mouse anti-BicD antibodies 1B1 and 4C2 at a 1:20 dilution each. Horseradish peroxidase-conjugated anti-mouse antibodies (GE Healthcare) were used at a dilution of 1:5,000. The blots were probed with ECL plus reagents (GE Healthcare), and chemiluminescence signal was detected using a LAS-1000 detection system (Fujifilm). Western blots were evaluated using AIDA software (Raytest GmbH).

Supporting Information
Figure S1 Effect of amino acid 103 on BicD localization
Found at: doi:10.1371/journal.pone.0004552.s001 (0.57 MB PDF)

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Author Contributions
Conceived and designed the experiments: RK OU MH BS. Performed the experiments: RK RL OU MH. Analyzed the data: RK RL OU MH BS. Contributed reagents/materials/analysis tools: BS. Wrote the paper: RK MH BS.

References