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 BicD<sup>AA40V</sup> 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 BicD<sup>AA40V</sup> function. Our results suggest that many BicD phosphorylation events may either be fortuitous or play a modulating function as shown for Ser<sup>103</sup>. Remarkably, amongst the *Drosophila* serines we found phosphorylated, Ser<sup>103</sup> 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 post translational 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 *misshappen* [7] may phosphorylate *Drosophila* BicD, and the human Glycogen synthase kinase-3β (GSK-3β) can phosphorylate human BicD<sub>1</sub> *in vitro* [8]. Furthermore, phenotypic correlations between phosphorylation and mutant phenotypes had already been described. The BicD allele BicD<sup>AA40V</sup>, an alanine to valine substitution at amino acid 40, 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 BicD<sup>AA40V</sup> protein is markedly reduced [4]. In addition, a suppressor of this allele, BicD<sup>P166S Su(66)</sup>, 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...
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 ommatidial 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 Dynae 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 vitro 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 BicDA40V mutant background [20] to test whether the mutant alleles were capable to substitute for the normal BicD in the various processes described.

Surprisingly, these phosphosites 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 phosphorylation levels. However, this mutant did still not reveal any other BicD phenotype, further arguing against essential functions of BicD phosphorylation in normal development. With the help of a genetically identified suppressor mutation that rescues BicDA40V hypophosphorylation, we finally found evidence for a modulating role of Ser103. In the background of the only partially functional BicDA40V variant, the side chain of position 103 becomes crucial for BicD function, even though it is not in the wild type background. While a S103A substitution does not change the BicDA40V phenotype, the permanent phospho-mimic mutant S103D fully inactivates the protein, and a S103F mutant restores its activity significantly. If an extrapolation from our results is valid, most phosphorylation events may be fortuitous and play only a modulating role if any.

Results
Multiple phosphorylation sites in BicD
An initial analysis of BicD phosphorylation using in vivo 32P phosphate labeled ovaries combined with phospho-amino acid analysis revealed only significant phosphoserine signal, indicating that phosphorylation of ovarian BicD takes place preferentially at serines. CNBr mapping data further indicated that these phosphoserines are primarily present in the N-terminal region (peptide 21–138; S. Larochelle and B. Suter, personal communication). To identify BicD phosphorylation sites, we immuno-precipitated unlabeled protein from ovarian and embryonic extracts. Bands corresponding to BicD were excised from the gel and analyzed by mass spectrometry. Alternatively, BicD-GFP [21] was immuno-precipitated from embryo extracts with anti-GFP antibodies to beads and analyzed by MS without a gel purification step. Phosphopeptides were subjected to tandem MS analysis to identify phosphorylated residues, as shown exemplarily for the peptide T103R286 in Figure 1A and B (phosphorylated). The obtained data allowed unambiguous identification of phosphorylated serines at Ser2, Ser103, Ser106, Ser285 and Ser310. The latter two, Ser3 and Ser285, were also found to be simultaneously phosphorylated, as revealed by the identification of the doubly phosphorylated peptide R285/310EADLPSTELKSPDGTK with one or two missed cleavage sites. In addition, we found Ser285 to be phosphorylated, either alone, or together with Ser285, while we did not see Ser285 phosphorylation independently of pSer285. Moreover, either Thr108 or Ser109 become phosphorylated, but the lack of discriminative product ions did not allow unambiguous allocation of the phosphorylation site. Figure 1C summarizes the phosphorylation sites we identified. Phosphorylation of threonine or tyrosine was not observed in the MS analysis, which is in agreement with the earlier in vivo labeling result.

MS-identified phosphorylation sites are all located within the N-terminal half of BicD (Figure 1D). Remarkably, the Ser103 and Ser106 are conserved in human and mouse BicD, and they are situated each in regions that are highly conserved between fly, human and mouse BicD isoforms (Figure 1E), though S106 is substituted by a Thr in BicD1. All other serines, 14, 109, 285/288 and 305/310 are not conserved however, although Ser109 corresponds to a phosphorylatable tyrosine in mammalian BicD (Figure 1E). Evolutionary conservation is only one indication of functional importance and for Ser14 there is additional evidence.

The sequence around Ser14 is a perfect match to a Polo kinase target site according to the consensus sequence D/E-X-S/T-Φ-X/D/E [22], where Φ denotes any hydrophobic residue. A Polo kinase target site is of particular interest because over-expression of Polo kinase in the germ line of BicDPA66 females was reported to restore oocyte formation in most egg chambers and also normal distribution of the BicDPA66 protein [6]. Surprisingly, even though this PA66 mutation (A10V) affects overall BicD phosphorylation drastically [4] and the region around this substitution contains five serines between positions 33 and 45, none of them show any detectable Ser phosphorylation (Table 1). This was also unexpected because a similar Ser/Ala-rich region had been identified to be phosphorylated [23]. This finding indicates that these sites are either not phosphorylated or that these modifications are only short-lived.

Testing the functional importance of BicD phosphorylation
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 (BicDWT; 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 individually with alanine codons. To investigate the possibility that transient 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 Ala. At the same time we also tested for functional redundancy between sites in the serine 33–45 cluster. For this we made a quintuple mutation in which these Ser were replaced with Ala or Asn codons. For Ser14, Ser103 and Ser310, we additionally made phospho-mimic aspartic acid substitutions. Transgenic fly lines were established for all mutants and the constructs were crossed into hemizygous BicDnull flies to test for their ability to restore viability and female fertility of the BicDnull mutants. In this assay, the transgenic BicDWT construct was able to completely rescue viability and fertility of the null mutants, while a female sterile allele BicDPA66, reconstructed in the same mini gene (BicDA40V), produces viable but sterile females. Therefore, the mini-BicD rescue constructs show the same effects as the endogenous alleles and the assay system is thus validated.
Figure 1. Location of BicD phosphorylation sites. A, B: MS/MS spectra of the [M+2H]^2+ ions of the peptide T91GIEQEDALLNESAAR106 (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 Ser^285 was only observed when Ser^288 was phosphorylated as well. Of Ser^305 and Ser^310, both, single and double...
Table 1 lists all mutations that were analyzed in this study and the associated phenotypes. Surprisingly, all Ser mutant constructs were able to rescue the lethality and the sterility of the BicD*null allele, indicating that phosphorylation of these individual serines is not absolutely required to provide basal levels of essential BicD functions, and that the function of BicD in oogenesis cannot be dramatically impaired by any of these mutants. In addition, mimicking permanent phosphorylation of Ser14, Ser103 and Ser310 also appears not to have a major effect on BicD function.

Table 1. Phenotypes of BicD phosphorylation mutants.

| Mutation | MS identification | Phenotype |
|----------|-------------------|-----------|
|          | phos. | non-phos. |
| wild type|       |          |
| S14A     | +     | −        |
| S14D     | −     | +        |
| S33A     | −     | +        |
| S34A     | −     | +        |
| S36A     | −     | +        |
| S39A     | −     | +        |
| S45A     | −     | +        |
| multiple | −     |          |
| S51A     | −     | −        |
| S54A     | −     | −        |
| S103A    | +     | +        |
| S103D    | −     |          |
| S103F    | −     | +        |
| S109A    | +     | +        |
| S186A    | +     | +        |
| S285A    | +     | +        |
| S288A    | +     | +        |
| S285A+S288A|    |          |
| S305     | +     | +        |
| S310A    | +     | +        |
| S310D    |       |          |
| S305A+S310A|    |          |
| A40V     | fs 16 nurse cells |
| A40V+S103A | fs 16 nurse cells |
| A40V+S103D | lethal with few adults, 16 nurse cells |
| A40V+S103F | ff, few egg chambers with 16 nurse cells |

Wild type Bic-D preferentially accumulates in the oocyte, and a significant amount of the protein is hyperphosphorylated. In Bic-D*null mutants however, no oocyte is formed and the levels of hyperphosphorylated Bic-D are reduced [4]. To further test the correlation between Bic-D function and the presence of the hyperphosphorylated isoform of Bic-D, we analyzed the Bic-D isoform pattern in the newly established phosphomutants. For this purpose, Bic-D 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 Bic-D present (Figure 2A, lanes 1 and 2, respectively). The Bic-D 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 3). 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 Bic-D. 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 Bic-D*null mutant lead to a significant reduction of the slowest migrating band (lane 3), as had been reported for endogenous Bic-D*null [4].

Mutating the serines 14 through 288 has little effect on the global phosphorylation levels of Bic-D, and the patterns and the individual band intensities of Bic-D 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 Bic-D (Figure 2B and 2A, lanes 12, 13 and 5, 6, respectively). To further verify this visual impression, we quantitatively assayed the amount of phosphoBic-D 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 Bic-D protein content in any of the Ser mutants, we assumed that reduced hyperphosphorylation of Bic-D leads to a higher level of the hypophosphorylated isoform, rather than to degradation. Accordingly, the values of hyperphosphorylated Bic-D were normalized to the sum of both Bic-D bands in each individual sample. The quantification results confirmed the visual impression and revealed only minor differences in hyperphosphorylation of Bic-D between most mutants. We also repeated this phospho-Bic-D 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-Bic-D levels and Bic-D function, the Ser103 mutants seems to be an exception to this. They are viable and normally fertile, but show a markedly reduced amount of hyperphosphorylated Bic-D, 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 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 isofrom 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 isofrom 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. BicDmutiple 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 BicDA40V, S103D 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 BicDA40V, S103D 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 BicDA40V 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 an 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 BicDA40V 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$^{null}$ alleles and thus behaves like a recessive lethal mutant. Only very few BicD$^{A40V,S103D}$ adults were obtained, which 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 BicDP66 and BicD$^{null}$ 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

Because Ser$^{103}$ is a highly conserved residue in the BicD family and the BicDA40V, S103A double mutant is not viable, we sought to identify appropriate substitutions that allow the protein to be expressed and to test their effects. The Ser$^{103}$ residue in BicD is phosphorylated in vivo and appears to control the subcellular localization of the protein, its stability, and its function [10,20,22]. Therefore, we first constructed a phospho-mimic BicDS103D allele, where the Ser$^{103}$ is replaced by aspartate, an amino acid that cannot be phosphorylated, but mimics its effect. Strikingly, flies expressing this double mutant protein show a completely different phenotype. Most egg chambers form an oocyte, where the BicD protein accumulates to a certain extent. The egg chamber indicated by an arrow in Figure 3D, I, was selected for further analysis. The mutants A40V+ S103A and A40V+S103D (E, J) also fail to form an oocyte. Shown are maximum projections of z-stacks. Panels F–J: for clarity reasons, individual panels are composed of different optical sections.

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![Figure 3. A S103F substitution in BicD$^{A40V}$ suppresses the BicD$^{P66}$ phenotype.](image-url) Confocal images of ovaries from the indicated wt and mutant females, stained with anti-BicD antibodies (red). Blue: DNA, green: F-actin. A, F: in ovaries with wild type BicD, all egg chambers contain an oocyte, and the protein accumulates in the oocyte throughout oogenesis. The BicD mutants A40V (B, G), fail to form an oocyte, and egg chambers contain 16 nurse cells. The mutant protein does not accumulate in a single cell. C, H: Substitution of Ser$^{103}$ by phenylalanine in BicD$^{A40V}$ suppresses the BicD$^{P66}$ phenotype. Most egg chambers form an oocyte, where the BicD protein accumulates to a certain extent. The egg chamber indicated by an arrow contains 16 polyploid nurse cells and no oocyte. D, I: The mutants A40V+S103A and A40V+S103D (E, J) also fail to form an oocyte. Shown are maximum projections of z-stacks. Panels F–J: for clarity reasons, individual panels are composed of different optical sections. 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 BicDwt 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 phosphoserines. 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-Phi-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 the Ser 310 substitutions, global BicD phosphorylation levels is essential for any BicD function, and that, with the exception of mutants revealed that none of these eight phosphorylation sites multiple phosphorylation events between Ser14 and Ser288 and this could be that the hyperphosphorylated isoform contains remain unchanged in the tested mutants. One explanation for the S14A nor the S14D substitution exhibit an obvious phenotype, the phosphorylation events are either fortuitous or play only minor modulating roles. The only modulating role we could find this was a genetic screen for a suppressor of the female sterile and partially phosphorylation defective BicD440V mutant that lead to the isolation of the Su(66) mutant that revealed the only identifiable function of BicD phosphorylation. We identified this mutation as a S103F substitution in the BicD440V background and we showed that this substitution is sufficient to restore the critical functions of BicD. The analysis of additional Ser103 substitutions in the BicD440V background provided 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 BicD440V protein, causing BicD440V, 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.

Figure 5. Substitutions in amino acid 103 of BicD modulate the dominant BicD phenotype. Embryos were collected from mothers hemizygous the dominant BicD allele, and carrying one copy of transgenic BicD wt, BicD S103A, BicD S103D, or BicD PA66. The embryos were scored for anterior defects and classified according to the denoted categories. N: total number of embryos counted. The flies and the embryos were raised at 25°C and shifted to 18°C one day before egg collection.

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the S14A nor the S14D substitution exhibit an obvious phenotype, suggesting that phosphorylation of this residue is not required for proper BicD function. This finding is surprising, because a recent report suggested polo kinase to be involved in polarized transport during oogenesis, where Polo could activate BicD by phosphorylation specifically during oocyte differentiation [6]. Recently, the human Glycogen synthase kinase-3β (GSK-3β) was reported to form complexes with human BicD1 in a kinase activity-dependent manner [8], but the phosphorylated serines identified in this study (Figure 1E) do not fit the known GSK-3β consensus sequence S/T-X-X-X-S/T [34].

Our systematic in vivo analysis of BicD phosphorylation mutants revealed that none of these eight phosphorylation sites is essential for any BicD function, and that, with the exception of the Ser310 substitutions, global BicD phosphorylation levels remain unchanged in the tested mutants. One explanation for this could be that the hyperphosphorylated isoform contains multiple phosphorylation events between Ser14 and Ser100 and that the absence of a single one of them does not alter the isoform mobility. The exception is the serine 310. Surprisingly, BicD phosphorylation is markedly reduced in both, the S310A mutant that abolishes phosphorylation and the phospho-mimic S310D mutant. Even though S310 is crucial for overall BicD phosphorylation levels, this seems not to affect BicD activity much, as Ser310 mutants appear normal, further arguing against critical roles of BicD phosphorylation on its activity. In contrast, the A40V substitution that shows a similar reduction of total BicD phosphorylation, also greatly reduces the functionality of BicD. This suggests that the loss of phosphorylation in this mutant is a side effect or a consequence, rather than the cause of the loss-of-function, and that the bulky side chain of valine causes a structural change in the mutant protein and that this inactivates the protein directly. Limited redundancy tests showed that in the case of the serines 285/288, and 305/310, which we found to be doubly phosphorylated, neither site is required for BicD function (Table 1). Similarly, the five serines 33–45 in the region around the A40V mutation are also dispensable for essential BicD activity.
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 pUAS-K10attB vector that is designed to harbor constructs driven by their own promoter. pUAS-K10attB contains a multiple cloning site, a white selectable marker, a lacP 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 pLimas20 (New England Biolabs) to yield pUAS+attB. The sequence is available from the EMBL/GenBank data libraries under accession no. EU297292. 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 pUAST [38] to yield pUAS-K10attB. The sequence is available from the EMBL/GenBank data libraries under accession no. EU297293.

The mini-BicD::GFP fusion construct [21] was transferred as kpmI/VdA fragment into pUAS+attB. The BicD3 part with the GFP fusion was then replaced with the corresponding native 2.75 kb BicD3 part lacking GFP that was taken from pBS4.2RV3' [24]. This yielded the mini-BicD::pattB vector that served as wild type control BicD WT. A BsilI/Agel fragment of mini-BicD::GFP was subcloned into pLimas28 (New England Biolabs), and the mutants 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::pattB to replace the respective wild type sequence. The fragments containing the mutations S14A/D–S54A were cloned using respective wild type sequence. The fragments containing the BsiW multiple cloning site, a selectable marker, a lacP site, and an attB fragment that allows its integration into attP landing platforms [37] using the phiC31 integrase [37]. The mutants S33A, S34A, S36A, S39A in ZH-2A on the X chromosome, S285A/S288A and S305A/S310A were inserted into ZH-64A on the 3rd or 4th chromosome as described above). Males were crossed to mini-BicD::GFP/+ flies and the females were crossed to Df(2L)Exel7068/+ females as needed to generate flies carrying one copy of a BicD construct in a hemizygous BicD+ background. These progeny were then used for the experiments.

Eye imprints and embryo cuticle preparations

The eye imprints were done as described in [39]. Images were recorded on a Leica DM6000 B microscope using a 40× DIC objective. For cuticle preparations, embryos were collected for 24 h at 18°C from w; BicD+/BicD+; +; att-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 lactic acid/Hoyer’s medium and incubated at 50°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×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 1:1000. 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 phalloidine (Molecular Probes), respectively. The ovarioles were embedded in Aquamount (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) IPp buffer (IP buffer including phosphatase inhibitors 0.3 mM sodium orthovanadate, 2 mM 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 NuPAGE 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) over night 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 (Esquire3000+) 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 Phenyl 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 ml anti-GFP antibody (mouse monoclonal 3E6, gift from A. Marcil, BRI, 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 disuccinimidyl 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. 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 at 37°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) over night at room temperature. The digest was acidified with 20% FA and incubated with TiO$_2$ slurry in 1.5 × loading buffer (3.7 mg/ml TiO$_2$ in 1.5% trifluoroacetic acid, 60% acetonitrile [MeCN], 1.5 M lactic 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, acidified 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 Phenyl software (GeneBio) against the Uniprot-Swissprot *Drosophila melanogaster* database, version 54.6. All identifications on peptide spectra 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* Ringler’s solution (182 mM KCl, 46 mM NaCl, 3 mM CaCl$_2$, 10 mM Tris-HCl, pH 7.2) and snap-frozen on dry ice. For every sample, 35 ovary pairs were used, except for BicDA1 and BicDA40V, 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 1B11 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 MgCl$_2$, 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 Na$_3$VO$_4$ and 4 mM Na$_2$MoO$_4$). 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 Protein II xi cell (Bio-Rad). After transferring the proteins to nitrocellulose membranes, BicD was detected using the mouse anti-BicD antibodies 1B11 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

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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

1. Bodenmüller B, Mueller LN, Mueller M, Domon B, Aebersold R (2007) Reproducible isolation of distinct, overlapping segments of the phosphoproteome. Nat Methods 4: 231–237. 2. Collins MO, Yu L, Choudhary JS (2007) Analysis of protein phosphorylation on a proteome-scale. Proteomics 7: 2751–2768.
5. Claussen M, Suter B (2005) Bic-D-dependent localization processes: from Drosophila to human cell biology. Annu Rev Genet 39: 59–93.

6. Mironov V, Formstecher E, Couderc JL (2006) Interaction between Polo and Bic-D proteins links oocyte determination and meiosis control in Drosophila. Development 133: 4005–4103.

7. Houalla T, Hien Vuong D, Ruan W, Suter B, Rao Y (2005) The Ste20-like kinase misshapen functions together with Bicaudal-D and dynein in driving nuclear migration in the developing drosophila eye. Mech Dev 122: 97–108.

8. Funoto K, Hoogenraad CC, Kukachi A (2006) GSK-3beta-regulated interaction of BICD with dynein is involved in microtubule anchorage at centrosome. EMBO J 25: 3670–3682.

9. Clark A, Meignin C, Davis I (2007) A Dynactin-dependent shortcut rapidly delivers axis determination transcripts into the Drosophila oocyte. Development 134: 1955–1965.

10. Swan A, Nguyen T, Suter B (1999) Drosophila Lisencephaly-1 functions with Bic-D and dynein in oocyte determination and nuclear positioning. Nat Cell Biol 1: 444–449.

11. Bressel RL, Saxton WM, Dufly JR (2002) Posterior localization of dynein and dorsal-ventral axis formation depend on kinesin in Drosophila oocytes. Curr Biol 12: 1541–1545.

12. Januschké J, Gervais L, Dass S, Kaltenschmidt JA, Lopez-Schier H, et al. (2002) Polar transport in the Drosophila oocyte requires Dynactin and Kinesin I cooperatively. Curr Biol 12: 1971–1981.

13. Duncan JE, Warrior R (2002) The cytoplasmic dynein and kinesin motors have interdependent roles in patterning the Drosophila oocyte. Curr Biol 12: 1982–1991.

14. Lei Y, Warrior R (2000) The Drosophila Lisencephaly (Lis1) gene is required for nuclear migration. Dev Biol 226: 57–72.

15. Delanoue R, Davis I (2005) Bic-D-dependent localization processes: from Drosophila development to human cell biology. Ann Anat 187: 539–553.

16. Wilkie GS, Davis I (2001) Drosophila wingless and pair-rule transcripts localize apically by dynein-mediated transport of RNA particles. Cell 105: 209–219.

17. Ran B, Bopp R, Suter B (1994) Null alleles reveal novel requirements for Bic-D in Drosophila development. Curr Biol 4: 1179–1180.

18. Whited JL, Cassell A, Brouillette M, Garrity PA (2004) Dynactin is required to transport in the Drosophila blastoderm embryo. Cell 122: 97–106.

19. Claußen M, Koch R, Jin ZY, Suter B (2006) Functional characterization of BicD proteins links oocyte determination and meiosis control in Drosophila melanogaster. EMBO J 25: 375–386.

20. Mohler J, Wieschaus EF (1986)Dominant maternal-effect mutations of Drosophila melanogaster causing the production of double-abdomen embryos. Genetics 112: 803–822.

21. Ephrussi A, Dickinson LK, Lehmann R (1991) Oskar organizes the germ plasm and directs localization of the posterior determinant nanos. Cell 66: 37–50.

22. Kim-Ha J, Smith JL, Macdonald PM (1991) oskar mRNA is localized to the posterior pole of the Drosophila oocyte. Cell 66: 23–35.

23. Baibin M, Kaoru N, Hiroaki M, Gf, Oppermann FS, et al. (2008) Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. Mol Cell 31: 430–448.

24. Dephoure N, Zhou C, Villen J, Beausoleil SA, Bakalaki CE, et al. (2008) A quantitative atlas of mitotic phosphorylation. Proc Natl Acad Sci U S A 105: 10762–10767.

25. Bodenburg M, Buerler LN, Pedrioli PG, Pfieger DJ, Junger MA, et al. (2007) An integrated chemical, mass spectrometric and computational strategy for (quantitative) phosphoproteomics: application to Drosophila melanogaster Kc167 cells. Mol Biosyst 3: 275–286.

26. Zhai B, Villen J, Beausoleil SA, Minteris J, Gygi SP (2008) Phosphoproteome analysis of Drosophila melanogaster embryos. J Proteome Res 7: 1675–1682.

27. Blom N, Gammeltoft S, Brunak S (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. J Mol Biol 294: 1351–1362.

28. Okamura JC, Cantley LC, Yaffe MB (2003) Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. Nucleic Acids Res 31: 3655–3661.

29. Franke S, Cohen P (2001) GSK3 takes centre stage more than 20 years after its discovery. Biochem J 359: 1–16.

30. Oh J, Baksa K, Steward R (2000) Functional domains of the Drosophila bicaudal-D protein. Genetics 154: 713–724.

31. Bellen HJ, Levis RW, Liao G, He Y, Carlson JW, et al. (2004) The BDGP gene disruption project: single transposon insertions associated with 40% of Drosophila genes. Genetics 167: 761–781.

32. Bischof J, Maeda KK, Hefgier M, Karch F, Basler K (2007) An optimized transgenesis system for Drosophila using germ-line-specific (varphi)C31 integrase. Proc Natl Acad Sci U S A 104: 3312–3317.

33. Groth J (1998) Bali in the Drosophila female germ line. Mech Dev 78: 113–118.

34. Arya R, Lakhotia SC (2006) A simple nail polish imprint technique for examination of external morphology of Drosophila eyes. Curr Sci 90: 1179–1180.

35. Stern DL, Su cane E (2000) Preparation of Larval and Adult Cuticles for Light Microscopy. In: Sullivan W, Ashburner M, Havel ey RS, eds. Drosophila Protocols. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. pp 691–615.

36. Mancebo R, Zhou X, Shillinglaw W, Henczel W, Macdonald PM (2003) ISF bind specifically to the bcd mRNA 3’ untranslated region and contributes to stabilization of bcd mRNA. Mol Cell Biol 21: 3462–3471.

37. Cluellen M, Koch R, Jin ZY, Suter B (2006) Functional characterization of Drosophila Translin and Trax. Genetics 174: 1337–1347.

38. Deboerzi M, Speed T (2002) An HMM model for coiled-coil domains and a comparison with PSSM-based predictions. Bioinformatics 18: 617–625.

39. Notredame C, Higgins DG, Heringa J (2000) T-Coffee: A novel method for fast and accurate multiple sequence alignment. J Mol Biol 302: 205–217.