The protein kinase CK2 substrate Jabba modulates lipid metabolism during *Drosophila* oogenesis

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ABSTRACT

Lipid metabolism plays a critical role in female reproduction. During oogenesis, maturing oocytes accumulate high levels of neutral lipids that are essential for both energy production and for the synthesis of other lipid molecules. Metabolic pathways within the ovary are partially regulated by protein kinases that link metabolic status to oocyte development. While the functions of several kinases in this process are well established, the roles that many other kinases play in coordinating metabolic state with female germ cell development are unknown. Here we demonstrate that the catalytic activity of casein kinase 2 (CK2) is essential for Drosophila oogenesis. Using an unbiased biochemical screen that leveraged an unusual catalytic property of the kinase, we identified a novel CK2 substrate in the Drosophila ovary, the lipid droplet-associated protein Jabba. We show that Jabba is essential for modulating ovarian lipid metabolism and for regulating female fertility in the fly. Our findings shed light on a CK2-dependent signaling pathway governing lipid metabolism in the ovary and provide insight into the long-recognized but poorly understood association between energy metabolism and female reproduction.

INTRODUCTION

In most female animals, metabolism regulates reproductive potential, and emerging evidence indicates a clear correlation between fatty acid metabolism and oogenesis (1,2). During oogenesis, developing oocytes accumulate large amounts of sterols and triglycerides that can be oxidized for ATP production or used for the synthesis of other essential lipid species (3,4). Consequently, perturbations in lipid metabolism such as metabolic syndrome and obesity are associated with infertility and several disorders of human reproduction, most notably polycystic ovary syndrome (PCOS) (5,6). Yet despite the requirement for proper lipid homeostasis in the female reproductive system, relatively little is known regarding the molecular mechanisms that regulate lipid metabolism in the ovary.

The model organism Drosophila melanogaster has proven to be a powerful system for dissecting the fundamental role of lipid metabolism in disease states and in normal developmental processes such as gametogenesis (7-9). Oogenesis in flies occurs in 14 continuous but morphologically distinct stages. At the anterior end of the ovary, germline stem cells reside within a structure known as the germarium. These cells give rise to an oocyte that develops within an egg chamber (or follicle) composed of 15 germline-derived nurse cells enclosed by an epithelium of hundreds of somatic follicle cells (10). Neutral lipid synthesis within the nurse cells is dramatically enhanced during mid-oogenesis (11) and these lipids, primarily in the form of lipid droplets, are transferred during development from the nurse cells to the oocyte through actin-based ring canals (12).

The importance of lipid metabolism in oogenesis is highlighted by mutation of the midway (mdy) gene that encodes Drosophila DGAT (diacylglycerol acyltransferase): in mdy mutants, oogenesis fails halfway through the process with a concomitant reduction in the level of neutral lipids within the ovary (13). Likewise, mutations in the gene encoding Cct1, a phospholipid biosynthetic enzyme, result in loss of germline stem cell maintenance and inappropriate positioning of the oocyte within the Drosophila egg chamber (14). Several other studies in both flies and mammals have also demonstrated that lipids accumulate during specific stages of oocyte development and are required for oogenesis (3,11,15,16), further underscoring the importance of lipid metabolism in the ovary.

As key mediators of signaling cascades, protein and lipid kinases are crucial for regulating oogenesis and early embryonic development (17-19). During oogenesis and embryogenesis, specific kinases transduce developmental cues that establish axes of polarity, gradients of morphogens, and coordinate the activities of somatic follicle cells with germline cells for the formation of a mature oocyte (20-22). While the functions of several kinases in the metabolic control of oogenesis are well described (23-25), the identification and characterization of the complete complement of kinases whose activities couple intermediary metabolism and female reproduction remains far from complete.

Here we describe a novel role for casein kinase 2 (CK2) during Drosophila oogenesis. We report that CK2 phosphorylates the lipid droplet-
associated protein Jabba, and that Jabba is essential for mediating lipid metabolism in the ovary. These findings expand the functional repertoire of this pleiotropic kinase and highlight a novel function for CK2 in the metabolic regulation of female reproduction.

RESULTS

CK2 kinase activity is essential for oogenesis

To identify kinases with previously uncharacterized roles in oogenesis, we individually reduced expression of a subset of the Drosophila kinome by shRNA in the female germline using the Gal4-‐UAS system. Included in this RNAi screen were several kinases with known roles in Drosophila oogenesis such as polo (26) and protein kinase A (PKA) (27) that served as positive controls. While knockdown of most kinases caused no change in oogenesis, reduced expression of several kinase-‐encoding genes resulted in either an increase or decrease in the number of eggs laid (Fig. 1A). Among the genes in this latter category that most significantly affected oogenesis were those encoding the two subunits of casein kinase 2 (CK2α and CK2β). Concomitant with decreased egg production, reduction of CK2 expression resulted in a significant decrease in overall ovarian size (Fig. 1B).

CK2 is an evolutionarily conserved, ubiquitous serine/threonine kinase whose activity has been implicated in multiple physiological and pathological processes (28). The enzyme is highly promiscuous with a growing list of hundreds of putative substrates and is estimated to be responsible for phosphorylation of approximately 10-15% of the eukaryotic proteome (29). CK2 forms heterotetramers composed of two catalytic subunits (CK2α or the highly homologous CK2α’) and two regulatory subunits (CK2β) (30). CK2 is also an unusual kinase; it is considered constitutively active (31), and its precise mechanism of regulation has yet to be fully defined.

The primary sequence identity between Drosophila and human CK2α and Drosophila and human CK2β is 85% and 88%, respectively (32,33). We leveraged this high degree of homology to validate the results of our screen. Specifically, we tested whether female fertility could be restored in flies with reduced expression of endogenous CK2α or CK2β by transgenic expression of the respective epitope-‐tagged human CK2α or CK2β subunits (Fig. 1C). For these rescue experiments we used two different germline-‐specific Gal4 drivers in order to distinguish between germline stem cell autonomous vs. non-‐autonomous functions of CK2. In particular, nanos-‐Gal4 drives expression throughout all stages of oogenesis including within the germline stem cells of the germarium (34), while mato-‐Gal4 drives expression slightly later in the process beginning in stage two egg chambers outside of the germline stem cell compartment (35). Transgenic expression of both human CK2α and CK2β were able to functionally compensate for reduced expression of the respective endogenous Drosophila CK2 subunits (Figs. 1C and 1D). Moreover, the use of both Gal4 drivers produced similar results (Fig. 1C), indicating that CK2 has important functions outside of the stem cell compartment (although from these data we cannot entirely exclude a distinct germline stem cell-‐specific role for CK2). We then addressed whether the kinase activity of CK2 is essential for female fertility in the fly by expressing a catalytically dead point mutant of human CK2α (CK2α-K68M) (34) in the context of Drosophila CK2α RNAi. Western blotting of ovary lysates demonstrated equal expression of both wild-‐type and kinase-‐dead human CK2α transgenes (Fig. 1D). Results from an egg laying using these flies revealed that expression of kinase-‐dead CK2α-K68M could not rescue the reduced fertility induced by knockdown of endogenous Drosophila CK2α (Fig. 1C). Collectively these results indicate that the kinase activity of CK2 is critical for Drosophila oogenesis.

A biochemical screen identifies CK2 substrates in the Drosophila ovary

To gain additional insight into the role of CK2 kinase activity in oogenesis, we designed a novel biochemical strategy to identify CK2-‐specific substrates in the Drosophila ovary (Fig. 2A). Here we took advantage of another unusual property of CK2, namely its dual co-‐substrate specificity or its ability to use either ATP or GTP as a phosphoryl donor (36). Ovary lysate from wild-‐type flies was incubated in the presence of...
excess recombinant CK2 holoenzyme and GTPγS, allowing for thio phosphorylation of CK2-specific substrates. The alkylating agent *p*-nitrobenzyl mesylate (PNBM) was then added to the reaction generating a thiophosphate ester moiety on these CK2 substrates. This modification could be detected with an anti-thiophosphate ester antibody and could also be used to immunoprecipitate these thiophosphorylated proteins from the reaction for subsequent identification by mass spectrometry. We first confirmed the validity of this approach by incubation of ovary lysate with the CK2 kinase inhibitor CX-4945 (silmitasertib) in both the presence and absence of excess CK2 holoenzyme. Western blotting of these lysates using an anti-thiophosphate ester antibody revealed reduced total levels of thiophosphorylated proteins in the presence of CX-4945 (Fig. 2B), confirming that the observed signal was dependent upon CK2 activity. This antibody was then used to immunoprecipitate the thiophosphorylated proteins, and these proteins were resolved by SDS-PAGE and subjected to anti-thiophosphate ester Western blotting. A unique band of ~60 kDa was present upon immunoprecipitation with the anti-thiophosphate ester antibody and not with the control antibody (Fig. 2C). This band was excised from a corresponding Coomassie blue-stained gel and was submitted for protein identification by liquid chromatography/tandem mass spectrometry (LC/MS-MS).

**Jabba is a novel CK2 substrate and is essential for oogenesis**

One of the proteins identified within this band was a specific isoform (isoform F) of a recently discovered insect-specific lipid droplet-associated protein named Jabba (Fig. 2D). Long considered inert structures for the storage of neutral fats, lipid droplets have emerged as dynamic organelles that function as hubs of lipid and energy metabolism and as platforms for signaling cascades (37,38). Lipid droplets have well characterized functions in mediating lipid storage and mobilization (37) and are composed of a protein-studded phospholipid monolayer surrounding a hydrophobic core of neutral lipids (primarily triglycerides and sterol esters). The proteins associated with the lipid droplet membrane are intimately involved in the regulation of lipid metabolism and lipid droplet biogenesis, although no such function for Jabba has thus far been ascribed. Interestingly, Jabba physically interacts with histones and sequesters them on lipid droplets for use during early *Drosophila* embryogenesis (39,40). Apart from this particular function, however, little is known regarding the physiological role of Jabba in the ovary. The *Jabba* locus is predicted to encode eight isoforms of the protein (isoforms B-I) of various molecular weights that result from alternative splicing (39). The F isoform of Jabba identified here as a putative CK2 substrate is one of two larger isoforms and, like other Jabba isoforms, does not contain any known functional domains.

To validate the results obtained by mass spectrometry and confirm that Jabba is indeed a substrate of CK2, we expressed and purified GST-Jabba from bacteria. This fusion protein was used as a substrate in an in-vitro kinase assay with recombinant CK2 holoenzyme. CK2 phosphorylated GST-Jabba (Fig. 3A), demonstrating that Jabba is a bona fide substrate of this kinase in vitro.

We then addressed whether CK2 phosphorylates Jabba in a cellular context by inhibiting CK2 kinase activity in mammalian cells with CX-4945. We first determined an effective dose of the inhibitor by treating HEK293 cells with increasing concentrations of CX-4945. Cell lysates were prepared the following day and immunoblotted with an anti-phospho-CK2 substrate antibody (Fig. 3B). The results indicated significant inhibition of CK2 kinase activity at a concentration of 10 µM. HEK293 cells were then transfected or not with a construct encoding GFP-Jabba and either treated or not with 10 µM CX-4945. Cell lysates were subsequently prepared and immunoblotted with an anti-phospho-CK2 substrate antibody (Fig. 3B). The results indicated significant inhibition of CK2 kinase activity at a concentration of 10 µM. HEK293 cells were then transfected or not with a construct encoding GFP-Jabba and either treated or not with 10 µM CX-4945. Cell lysates were subsequently prepared and immunoblotted with an anti-phospho-CK2 substrate antibody (Fig. 3C). The results demonstrate a reduction in the phospho-CK2 substrate signal at the molecular weight at which Jabba migrates by SDS-PAGE suggesting that CK2 phosphorylates Jabba in cells. It should be noted here that GFP-Jabba runs at a molecular weight that is higher than expected, however the identity of this band as Jabba was confirmed by mass spectrometry, and the aberrant migration of this protein is described in more detail below (Fig. 4D). To further substantiate this finding, we determined if overexpression of CK2
would result in increased phosphorylation of Jabba. HEK293 cells were co-transfected with a plasmid encoding GFP-Jabba and a vector encoding CK2α-HA or empty vector as a control. Cells were subsequently lysed and Western blotting of whole cell lysates confirmed overexpression of CK2α and equal expression of Jabba in both samples. Jabba was subsequently immunoprecipitated with an anti-GFP antibody, and immunoprecipitates were resolved by SDS-PAGE followed by immunoblotting with anti-GFP and anti-phospho-CK2 substrate antibodies (Fig. 3D). The results demonstrate increased phosphorylation of Jabba in cells overexpressing CK2α, providing further evidence that CK2 phosphorylates Jabba not only in vitro but also in cells.

Jabba mutant embryos display reduced hatching at elevated temperatures, an altered histone H2Av/H2A ratio, and synthetic lethality upon reduced expression of histones (39,40). While these data clearly demonstrate that Jabba is important during Drosophila embryogenesis, the physiological role(s) that Jabba plays in earlier stages of development (i.e. during oogenesis) where its expression is first detected (39) is not known. We therefore tested whether isoform F of Jabba is required for oogenesis by reducing its expression in the female germline. We first determined by RT-PCR analysis that the shRNA targeting Jabba is effective at reducing Jabba mRNA in the Drosophila ovary. Ovaries from Jabba RNAi flies displayed reduced Jabba (isoform F) transcript levels compared to ovaries from wild-type flies (Fig. 4A). Moreover, knockdown of this specific Jabba isoform by shRNA resulted in reduced egg production as assessed by an egg laying assay (Fig. 4C). We confirmed that this phenotype was indeed due to reduced expression of Jabba by transgenic expression of an epitope-tagged shRNA-resistant form of the protein in the Jabba RNAi genetic background. Importantly, Western blotting of ovary lysates from these flies demonstrated levels of Jabba that were similar to expression of the transgene in the absence of the targeting shRNA (Fig. 4B), and an egg laying assay confirmed that oogenesis was, in fact, restored in these flies (Fig. 4C). Collectively, these data demonstrate that isoform F of Jabba is essential for Drosophila oogenesis, phenocopying the effect of reduced CK2 expression (Fig. 1C).

An intriguing observation made during the course of this experiment was that Jabba migrated at approximately twice its predicted molecular weight on SDS-PAGE (Fig. 4B), suggesting that this protein may form covalent dimers. To determine if this was indeed the case, Drosophila ovary lysate expressing myc-tagged Jabba was treated with the alkylation agent iodoacetamide or DMSO as a control (Fig. 4D). Treatment with iodoacetamide resulted in the appearance of a lower molecular weight band of the predicted size of Jabba, indicative of the formation of dimers in vivo and consistent with data from a recent study (41). However, this explanation cannot completely account for the aberrant migration of this protein by SDS-PAGE since other tagged forms of Jabba run at molecular weights that are inconsistent with dimer formation (Fig. 3). This is perhaps suggestive of additional, as yet unidentified, post-translational modifications of Jabba such as lipidation.

**CK2 phosphorylates multiple regions of Jabba**

CK2 is an acidophilic kinase and preferentially phosphorylates serine or threonine residues in the context of surrounding glutamates or aspartates (29). Given this defining property, we assessed which regions of Jabba may be phosphorylated by CK2. Analysis of the primary sequence of Jabba revealed two major regions of the protein containing serine or threonine residues flanked by highly acidic stretches of amino acids that appeared to be within ideal consensus sites for CK2-dependent phosphorylation. One such region was an acidic serine-rich stretch consisting of residues 222-273 while the other encompassed the C-terminal portion of the protein comprised of amino acids 274-537 (Figs. 2D and 5A). To test if either or both of these regions of Jabba are phosphorylated by CK2, we expressed and purified GST-tagged truncation fragments of these regions from bacteria for use in in-vitro kinase assays with CK2. The results revealed that both regions of Jabba were in fact phosphorylated by CK2 in vitro (Figs. 5B and 5C), suggesting that CK2-mediated Jabba phosphorylation likely occurs at multiple regions and multiple sites in vivo.
**Jabba localizes to lipid droplets in a CK2-independent manner**

The molecular mechanisms by which proteins are targeted to lipid droplets are diverse and incompletely understood. Documented modes of localization include the embedding of amphipathic helices or hydrophobic hairpins of lipid droplet-associated proteins within the phospholipid monolayer or neutral lipid core of the organelle (42). Alternative targeting mechanisms include post-translational modifications such as lipidation (43-45) or binding to other lipid droplet-associated proteins that are often phosphorylated (46,47). We therefore sought to determine if CK2 kinase activity was required for localization of Jabba to lipid droplets. To address this question in a tractable system, we tested if Jabba localized to lipid droplets in mammalian cells. 3T3-L1 murine pre-adipocytes were co-transfected with plasmids encoding GFP or GFP-tagged Jabba and myc-tagged perilipin-3, a member of the perilipin family of proteins that localizes to cytoplasmic lipid droplet membranes (48). These cells were subsequently treated with oleic acid to induce lipid droplet formation and were stained with an anti-myc antibody to visualize lipid droplets. While GFP displayed cytoplasmic and nuclear localization as expected (Fig. 6A), Drosophila Jabba strikingly localized to mammalian lipid droplet membranes as determined by its co-localization with perilipin-3 (Fig. 6B, top panels). To investigate if this localization was dependent on CK2 kinase activity, cells expressing GFP or GFP-Jabba were treated with CX-4945 and imaged by fluorescence microscopy. These results indicate that Jabba still localizes to lipid droplets in the presence of the inhibitor (Fig. 6B, bottom panels), suggesting that the mechanism by which Jabba is targeted to this organelle is not dependent on CK2-mediated phosphorylation. These results are also consistent with a recent report indicating that a hydrophobic segment at the N-terminus of Jabba is both necessary and sufficient for lipid droplet localization (41).

**Jabba and CK2 regulate ovarian lipid metabolism**

In comparison to wild-type embryos, lipid droplets in Jabba mutant embryos are misshapen and unevenly distributed, however total embryonic triglyceride levels remain unchanged (39), suggesting that Jabba is not critical for lipid metabolism, at least in embryos. Nonetheless, to gain insight into the molecular basis for the reduced fertility noted upon Jabba knockdown (Fig. 4C), we qualitatively assessed levels of neutral lipids in ovaries from flies with reduced expression of Jabba by staining with Oil Red O (Fig. 7A). As a control for this experiment, we used flies in which expression of midway was decreased by RNAi, a perturbation that results in a marked reduction in neutral lipids within the germline (13). While knockdown of midway resulted in dramatically reduced levels of ovarian triglycerides and lipid droplet density, knockdown of Jabba caused a similar decrease as determined by Oil Red O staining (Fig. 7A). Importantly, this apparent reduction in lipid droplets could be rescued by expression of shRNA-resistant Jabba in the Jabba RNAi genetic background (Fig. 7A). To corroborate these results, we used a colorimetric assay to quantify total ovarian triglyceride levels from flies in which expression of midway or Jabba was reduced by RNAi (Fig. 7B). Individual knockdown of both genes in the female germline resulted in reduced levels of ovarian triglycerides compared to ovaries from wild-type (w^{1118}) flies, and this reduction was again rescued by expression of shRNA-resistant Jabba in the Jabba RNAi genetic background (Fig. 7B). Taken together, these data indicate that the CK2 substrate Jabba is essential for maintaining neutral lipid levels and for regulating lipid homeostasis within the Drosophila ovary.

To investigate a role for CK2 in lipid metabolism during oogenesis, we used confocal immunofluorescence microscopy to analyze Jabba-labeled lipid droplets in the Drosophila ovary upon reduced expression of the kinase. In wild-type egg chambers, dense clusters of Jabba-associated lipid droplets were observed that localized primarily to the subcortical regions of nurse cells (Fig. 7C). In contrast, upon reduction of CK2 expression by RNAi, Jabba-labeled lipid droplets were markedly reduced (Fig. 7C), indicating an overall decrease in the number of ovarian lipid droplets in flies of this genotype. These results suggest that CK2 plays an integral role in regulating lipid metabolism in the Drosophila ovary, although the precise functions of this kinase and its substrate Jabba have yet to be determined.
DISCUSSION

In the present study, we have identified the lipid droplet-associated protein Jabba as a novel CK2 substrate and have defined a new role for CK2 in regulating lipid metabolism during Drosophila oogenesis. In the context of development, CK2 has been shown to be essential for early mammalian embryogenesis: mice lacking the CK2α subunit die in mid-gestation due to neural tube and cardiovascular defects (49). Interestingly, mice that are homozygous null for the highly homologous CK2α’ subunit are viable, but males are sterile and display abnormal sperm morphology and degeneration of germ cells at all stages of spermatogenesis (50,51). While these findings implicate CK2 in male germ cell development, our results now demonstrate a role for this kinase in regulating female germ cell development, expanding the functions of this already multifaceted kinase.

Kinase-mediated phosphorylation is an established mechanism for regulating the activity of metabolic enzymes via covalent modification. Among protein kinases that control lipid metabolism and lipid droplet biology, PKA is undoubtedly the best characterized, regulating a key step in the mobilization of triglycerides stored in adipose tissue. PKA phosphorylates hormone sensitive lipase (HSL) and perilipin, promoting their interaction and initiating the degradation of triacylglycerols and the subsequent release of fatty acids from lipid droplets. Phosphorylation of the lipid droplet-associated protein CGI-58/ABHD5 by PKA results in its dissociation from the lipid droplet membrane and enhances its capacity to co-activate adipose triglyceride lipase (ATGL)(52). In addition, phosphorylation of perilipin A by PKA facilitates lipolysis by causing lipid droplet fragmentation and cytoplasmic dispersion (53).

Strikingly, deletion of the two genes in yeast that encode the beta subunit of CK2 (CKB1 and CKB2) also affects lipid droplet morphology. However, deletion of CK2β results in the opposite phenotype and the accumulation of “supersized” lipid droplets (54). Notably, we observe a similar phenotype (i.e. the presence of larger lipid droplets) when CK2 kinase activity is inhibited in mammalian cells, although intriguingly, this phenotype seems to be more prominent for lipid droplets that are associated with Jabba (Figs. 6A and 6B). Collectively, these results suggest that CK2 regulates lipid droplet morphology in various ways in multiple species and, as a consequence, may modulate lipid metabolism by regulating access of cytosolic lipases to lipid droplets.

Previous work has demonstrated that Jabba is essential for Drosophila development only under certain conditions such as histone depletion (40), and that mutation of Jabba does not affect overall triglyceride levels in embryos (39). Our findings indicate that Jabba may play a more prominent role earlier in the process by regulating lipid metabolism prior to embryogenesis during female germ cell development. Indeed, expression of Jabba is first detected within the nurse cells and oocyte of the Drosophila egg chamber (39) where its function has thus far been unknown. While we have not identified the specific amino acids that are phosphorylated by CK2, we speculate that Jabba is heavily phosphorylated by this kinase on multiple serine/threonine residues. Moreover, we predict that other isoforms of Jabba are likely phosphorylated by this kinase as they all contain the highly acidic serine-rich middle region that is phosphorylated by CK2 (Fig. 5B). Taken together, these findings further suggest that there may be isoform-specific functions of Jabba perhaps mediated by post-translational modification of their divergent C-termini.

Accumulating evidence from several model organisms indicates that CK2 plays key roles in the regulation of lipid metabolism. In budding yeast, CK2 phosphorylates both Opi1p, the transcription factor that regulates phospholipid synthesis (55), and Pah1, the phosphatase that catalyzes the penultimate step in the synthesis of triacylglycerol (56). In mammalian cells, CK2 is essential for adipocyte differentiation (57,58), and in Drosophila embryos, data suggest that endogenous CK2 partially localizes to lipid droplets (59), all lending support to the notion that CK2 may function as a master regulator of lipid metabolism. Our results bolster these findings by demonstrating a functional role for CK2 in modulating lipid metabolism in the Drosophila ovary. Whether CK2 regulates lipid metabolism in the human ovary has yet to be determined, but the possibility is tantalizing and warrants further investigation.
EXPERIMENTAL PROCEDURES

Antibodies and Reagents
Anti-CK2α (C-18), anti-CK2β (FL-215), anti-GFP (E6), anti-c-Myc (9E10), anti-GST (Z-5), anti-β-tubulin (H-235), anti-GAPDH (6C5), and anti-Vasa (d-260) antibodies were from Santa Cruz Biotechnology. Anti-HA and anti-phospho-CK2 substrate ([pS/pT]DXE) antibodies were from Cell Signaling Technology. Anti-thiophosphate ester antibody (51-8) and PNBM (p-nitrobenzyl mesylate) were from Abcam. ATP, ATPγS, GTPγS, DAPI, oleic acid, Oil Red O, and iodoacetamide were purchased from Sigma Aldrich. Rhodamine phalloidin was purchased from Invitrogen. The CK2 inhibitor CX-4945 was purchased from Selleckchem.

Cell Culture
HEK293 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. 3T3-L1 cells were purchased from the American Type Culture Collection (ATCC) and grown in DMEM supplemented with 10% BCS (bovine calf serum) and 1% penicillin/streptomycin. Cells were grown in a humidified atmosphere with 5% CO2 at 37°C. Lipofectamine 2000 (Invitrogen) was used for transfection according to the manufacturer’s instructions. To induce lipid droplet formation, 3T3-L1 cells were stimulated overnight with 0.4 mM oleic acid.

Plasmid Construction
Drosophila Jabba (isoform F) was synthesized as a gene block fragment (Integrated DNA Technologies [IDT]) flanked with BgII and SalI restriction sites and was ligated into the corresponding sites of pEGFP-C1 (Clontech) and pGEX-6P-1 (GE Healthcare). shRNA-resistant Jabba was also synthesized as a gene block fragment (IDT) and contains nine silent mutations in the region targeted by the shRNA. Constructs encoding human CK2α–HA (pZW6) and myc-CK2β (pZW12) for mammalian cell expression were obtained from Addgene (plasmids deposited by David Litchfield). Murine perilipin-3 was PCR-amplified from a cDNA clone (OriGene) as an EcoRI-BgIII fragment and cloned into the corresponding sites of pCMV-myc (Clontech). For bacterial expression of GST-tagged Jabba fusion proteins, residues 1-537, 222-273, and 274-537 were PCR-amplified as BglII-EcoRI fragments and cloned into the BamHI-EcoRI sites of pGEX-6P-1 (GE Healthcare). Oligonucleotides were purchased from IDT. Primer sequences are available upon request. All constructs were fully sequenced.

Mass Spectrometry
Mass spectrometry was performed by MS Bioworks, LLC (Ann Arbor, MI). Briefly, in-gel digestion was performed on the submitted gel bands (an endogenous band of ~60 kDa from Drosophila ovarian lysate and GFP-tagged Jabba immunoprecipitated from mammalian cell lysate) using a robot (ProGest, DigiLab). The sample was washed with 25mM ammonium bicarbonate followed by acetonitrile and then reduced at 60°C followed by alkylation with 50mM iodoacetamide at room temperature. The sample was then digested with trypsin (Promega) at 37°C for 4 hours and quenched with formic acid. The supernatant was analyzed directly without further processing. Half of the digested sample was analyzed by nano LC-MS/MS with a ThermoFisher nLC-1000 HPLC system interfaced to a ThermoFisher Q Exactive mass spectrometer. Peptides were loaded onto a trapping column and eluted over a 75µm analytical column at 350nL; both columns were packed with Acclaim PepMap (Thermo). The mass spectrometer was operated in data-dependent mode, with MS and MS/MS performed in the Orbitrap at 70,000 and 17,500 FWHM resolution, respectively. The fifteen most abundant ions were selected for MS/MS. MS/MS samples were analyzed using Mascot (version 2.5.1, Matrix Science, London, UK). Mascot was used to search the UniProt D. melanogaster database assuming strict digestion with trypsin. Scaffold software (version 4.6.2, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at a greater than 50.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 90.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet...
algorithm (60). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Fly Stocks and Transgenic Fly Lines

Stocks were maintained and all crosses performed at 25°C on molasses-based food. All RNAi fly stocks were generated by the TRiP (Transgenic RNAi Project; Harvard University Medical School) and were obtained through the Bloomington Drosophila Stock Center (BDSC; Indiana University). Human CK2α, CK2α-K68M, and CK2β were PCR-amplified from vectors pZW6, pGV15, and pZW12 (Addgene; deposited by David Litchfield), respectively, using attB-modified primers and were cloned into pDONR-Zeo (Invitrogen). The cloned inserts were transferred into the relevant Drosophila destination vectors using Gateway cloning technology to create N- or C-terminal epitope-tagged constructs, as indicated, for Drosophila embryo microinjection. Transgenic flies were generated by BestGene, Inc. All transgenes map to the 2nd chromosome.

RNAi Screen and Rescue Experiments

Virgin nos-Gal4:VP16/TM3 Sb females were crossed with UAS-RNAi males. Four to six young (<7 days old) nos-Gal4:VP16/UAS-RNAi females were then crossed with w1118 males and eggs were collected overnight (16-18 hours) on grape juice agar plates. The eggs were then counted using a dissecting microscope, and the number of eggs laid per female per hour was calculated. After twenty egg collections, the egg laying rate of rescue females was compared to that of nos-Gal4:VP16/UAS-CKIIα RNAi or nos-Gal4:VP16/UAS-CKIIβ RNAi females using the Mann-Whitney U test. All experiments were performed at 25°C.

Oil Red O Staining

This protocol was performed essentially as described (61). Briefly, ovaries were dissected and fixed in 4% formaldehyde for 30 minutes, washed twice in PBS, and twice in 100% propylene glycol. Fixed ovaries were then stained in a solution of 0.5% Oil Red O (Sigma Aldrich) dissolved in propylene glycol previously filtered through Whatman #1 filter paper and preheated to 60°C. Samples were incubated for one hour at 60°C and then washed twice with 85% propylene glycol and twice with PBS at room temperature. Stained specimens were mounted on microscope slides in glycerol and imaged using an EVOS FL microscope (Life Technologies) with a 40X objective.

Colorimetric Triglyceride Assay

This protocol was performed essentially as described (61). Virgin female flies were raised on media with yeast and males for 3 days at 25°C. Ovaries were dissected in cold PBS, washed, transferred to cold PBS + 0.05% Tween 20, homogenized on ice using a motorized pestle, and heated at 70°C for 10 min. Triglycerides were measured from each sample in triplicate by incubating with Triglyceride Reagent (Sigma, T2449) at 37°C for 45 minutes. Reactions were pelleted and the supernatant was incubated with Free Glycerol Reagent (Sigma, F6428) at 37°C for 5 minutes. Absorbance was measured at 540 nm using a Spark® Multimode Microplate Reader (Tecan Life Sciences). Concentrations were determined by comparing readings to a glycerol standard curve of Glycerol Standard Solution (Sigma, G7793) subjected to the same reactions. Protein concentration was determined using a Bradford assay (Bio-Rad Protein Assay Dye Reagent Concentrate) and by measuring absorbance at 595 nm. Triglyceride levels were then compared to total protein content.
Recombinant Protein Expression and Purification

BL21(DE3) cells (Novagen) were transformed with plasmids encoding GST, GST-Jabba, GST-Jabba-222-273, or GST-Jabba-274-537. Protein expression was induced at 16°C for 24 hours with 0.5mM IPTG. Following centrifugation, the pellet was resuspended in 15 ml lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, with protease inhibitors (Roche)). The lysate was sonicated on ice and centrifuged at 20,000xg for 20 minutes at 4°C. The supernatant was added to 0.5 ml (packed volume) glutathione agarose beads (Gold Biotechnology) and incubated at 4°C for 2 hours with end-over-end tumbling. The slurry was transferred to a column and washed extensively with wash buffer. GST-fusion proteins were eluted with excess glutathione in lysis buffer (pH 7.4) and appropriate fractions were pooled. The protein was dialyzed overnight into dialysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM DTT). Glycerol was added to a final concentration of 10%, and the proteins were snap-frozen and stored at -80°C.

In-vitro Kinase Assays

Recombinant CK2 holoenzyme (New England Biolabs) was incubated with 25 ng of recombinant purified protein in kinase buffer (50 mM HEPES [pH 7.5], 0.65 mM MgCl2, 0.65 mM MnCl2, 12.5 mM NaCl) with 500 µM ATP or ATPγS, as indicated. Kinase reactions were incubated at 30°C for 30 minutes. When appropriate, PNBM (dissolved in DMSO) was added to a final concentration of 2.5 mM, and reactions were incubated at room temperature for one hour followed by the addition of 6X boiling sample buffer to stop the reaction. Reactions were then resolved by SDS-PAGE followed by immunoblotting with the indicated antibodies.

SDS-PAGE and Western Blotting

To prepare lysates for immunoblotting or immunoprecipitation, dissected Drosophila ovaries or mammalian cells were lysed in ice-cold lysis buffer (20 mM Tris, pH 8.0, 200 mM NaCl, 1% Triton X-100 with protease inhibitors [Roche] and phosphatase inhibitors [1 mM sodium orthovanadate, 10 mM sodium fluoride, and 1 mM β-glycerophosphate]). Proteins were resolved on 10% or 12.5% polyacrylamide gels and transferred to nitrocellulose membranes (GE Amersham) using the Pierce G2 Fast Blotter (Thermo Scientific). Membranes were blocked in 5% non-fat milk in TBS followed by overnight incubation with the indicated primary antibody in the same buffer. Membranes were washed three times the following day in TBS + 0.5% Tween (TBS-T), incubated for one hour with the appropriate HRP-conjugated secondary antibody (Cell Signaling Technology), washed three times again in TBS-T, and processed for signal detection using enhanced chemiluminescence (Santa Cruz Biotechnology). For detection of proteins from the same reaction/lysate that migrate at similar molecular weights (i.e. phosphorylated and non-phosphorylated forms of the same protein), samples were split and run on separate gels.

Immunofluorescence Microscopy

3T3-L1 cells were seeded on glass coverslips and transfected the following day with the indicated constructs for 6 hours. The medium was then replaced with fresh medium containing 400 µM oleic acid for overnight incubation. Cells were fixed the following day in 4% formaldehyde and processed for immunofluorescence microscopy. Coverslips were mounted on glass slides in Vectashield mounting medium (Vector Labs), and cells were imaged using an EVOS FL Auto microscope (Life Technologies) with a 40X objective. For confocal microscopy, dissected ovaries were fixed and immunostained as described (25) and imaged using an Olympus FluoView® FV3000 confocal laser scanning microscope equipped with a 30X silicone immersion oil objective (1.05 NA). Images were acquired using FluoView® software (Olympus) and processed using Photoshop CS6 (Adobe).

RT-PCR

Ovaries were dissected from flies of the indicated genotypes and total RNA was extracted using the RNasey Mini Kit (Qiagen). Complementary DNA (cDNA) was synthesized using SuperScript IV First-Strand Synthesis System (Invitrogen) and used as a template in PCR with gene-specific primers. The following primer pairs were used: Jabba-For: 5’-GCC ACC TCC TCC GAA GAC ATA TTC-3’; Jabba-Rev: 5’-CCT GGG TTC CTA GGC TAG TAT TGA A-3’; Actin42A-For: 5’-AAG AGG TTG CAG CTT TAG TGG-3’;
Actin42A-Rev: 5’-TCC CAT TCC TAC CAT TAC GCC-3’. PCR products were run on a 2% agarose gel and stained with ethidium bromide.

Statistical Analysis
Unless otherwise described, data are presented as the mean ± standard deviation. Statistical significance (p<0.05) was determined by an unpaired Student’s t-test or a Mann-Whitney U test, as indicated.
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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

T.I.S. conceived and coordinated the study and wrote the paper. E.A.M. designed and conducted experiments and analyzed the experimental data in Figures 1-4 and Figure 7. S.M.L. performed experiments and analyzed the data in Figure 2. M.D.S. and S.B. performed experiments and analyzed the data in Figure 3. B.L. and T.I.S. performed experiments and analyzed the data in Figure 6. N.K.S. performed experiments and analyzed the data in Figure 5. All authors reviewed the results and approved the final version of the manuscript.
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FIGURE LEGENDS

Figure 1: CK2 kinase activity is essential for Drosophila oogenesis.

(A) Identification of novel kinase regulators of Drosophila oogenesis. The indicated 78 kinases (on the x-axis) were knocked down in the female germline using nanos-Gal4:VP16-driven shRNA. Shown are box plots depicting egg laying rates of these flies (n=20). Bars indicate the first to third quartiles, horizontal black lines denote the median, and circles denote outliers. Red bars indicate kinases whose reduced expression resulted in decreased numbers of eggs laid, while green bars indicate kinases whose reduced expression caused an increase in the number of eggs laid. Blue bar denotes egg-laying rate of flies expressing a control shRNA. Whiskers represent the upper and lower limits of the range. Statistical significance was determined using a Mann-Whitney U test.
(B) Reduced ovarian size upon reduction of CK2 expression. Ovaries from flies of the indicated genotypes were dissected and imaged by light microscopy. Scale bar=1 mm.
(C) Egg laying rates from flies of the indicated genotypes. Expression of shRNA and/or transgenes was driven using either nanos-Gal4 (green) or matα-Gal4 (blue). Data are presented as in (A).
(D) Transgenic expression of epiotpe-tagged human CK2α and CK2β in the Drosophila female germline. Ovary lysates were prepared, resolved by SDS-PAGE, and immunoblotted with the indicated antibodies. Note that CK2α-HA migrates as a doublet.

Figure 2: A biochemical screen identifies substrates of CK2 in the Drosophila ovary.

(A) Schematic of the strategy used to identify novel ovarian substrates of CK2.
(B) Experimental validation of the approach outlined in (A). Ovary lysate was incubated for 30 minutes at 30°C with or without recombinant human CK2 holoenzyme and in the presence or absence of CX-4945 (100 µM), as indicated. GTPγS (2.5 mM) was then added and the reactions were incubated for one additional minute at 30°C followed by treatment with PNB for 60 minutes at room temperature. Kinase reactions were subsequently resolved by SDS-PAGE and immunoblotted with the indicated antibodies.
(C) Immunoprecipitation of putative CK2 substrates from Drosophila ovary lysate. An anti-thiophosphate ester antibody was used to immunoprecipitate thiophosphorylated proteins. Immunoprecipitated proteins were resolved by SDS-PAGE followed by immunoblotting with the indicated antibodies. Yellow asterisk indicates the position of the band that was excised from a corresponding Coomassie blue-stained gel for protein identification by LC-MS/MS.
(D) Primary amino acid sequence of Jabba (isoform F). Highlighted in red are unique peptides identified by mass spectrometry representing 7% coverage of the protein.

Figure 3: Jabba is a novel CK2 substrate.

(A) In-vitro kinase assay using GST-Jabba and CK2. GST-Jabba (or no substrate) was incubated in the presence or absence of recombinant CK2 holoenzyme and ATPγS followed by treatment with PNB. Kinase reactions were resolved by SDS-PAGE followed by immunoblotting with the indicated antibodies. Western blots shown are representative of three independent experiments.
(B) Inhibition of CK2 kinase activity using the small molecule inhibitor CX-4945. HEK293 cells were treated (or not) with the indicated concentrations of CX-4945 for 24 hours. Cell lysates (40 µg total protein) were resolved by SDS-PAGE and immunoblotted with the indicated antibodies.
(C) HEK293 cells were transfected (or not) with a plasmid encoding GFP-Jabba. Cell lysates (5 µg total protein) were resolved by SDS-PAGE and immunoblotted with the indicated antibodies.
(D) HEK293 cells were co-transfected with plasmids encoding GFP-Jabba and pCMV-HA (empty vector) or pCMV-CK2α-HA. Cells were lysed followed by immunoprecipitation of GFP-Jabba and
immunoblotting of the precipitated protein with the indicated antibodies. Western blots shown are representative of three independent experiments. IP=immunoprecipitate; WCL=whole cell lysate.

**Figure 4:** Jabba is essential for *Drosophila* oogenesis and forms dimers in vivo.

(A) Reduced Jabba mRNA levels in Jabba-RNAi ovaries by RT-PCR analysis. Total RNA was isolated from dissected ovaries from flies of the indicated genotypes. Resultant cDNA (or no template) was used in a polymerase chain reaction with primers to amplify specific regions of the genes encoding Jabba (expected product size=93 bp) or actin (expected product size=137 bp), as a control. PCR products were run on a 2% agarose gel, stained with ethidium bromide, and imaged.

(B) Expression of myc-Jabba and shRNA-resistant myc-Jabba in the *Drosophila* female germline. Ovary lysates were prepared, resolved by SDS-PAGE, and immunoblotted with the indicated antibodies.

(C) Egg laying rates from flies of the indicated genotypes. Data are presented as in Fig. 1A. n=20; * denotes p-value <0.05 as determined by a Student’s t-test.

(D) Jabba (isoform F) forms dimers in vivo. Ovarian lysates from flies expressing myc-Jabba in the female germline were treated (or not treated) with iodoacetamide (83.3 mM) at room temperature for 30 minutes. Lysates were resolved by SDS-PAGE followed by immunoblotting with the indicated antibodies.

**Figure 5:** CK2 phosphorylates multiple regions of Jabba.

(A) Schematic diagram of Jabba (isoform F) depicting various truncation fragments generated.

(B) In-vitro kinase assay using GST-Jabba-222-273 and CK2. GST or GST-Jabba-222-273 was incubated with recombinant CK2 holoenzyme and ATPγS in the presence or absence of CX-4945 (10µM) followed by treatment with PNBM. Kinase reactions were resolved by SDS-PAGE followed by immunoblotting with the indicated antibodies. Western blots shown are representative of three independent experiments.

(C) In-vitro kinase assay using GST-Jabba-274-537 and CK2. GST or GST-Jabba-274-537 was incubated with recombinant CK2 holoenzyme and ATP in the presence or absence of CX-4945 (10µM). Kinase reactions were resolved by SDS-PAGE followed by immunoblotting with the indicated antibodies. Western blots shown are representative of three independent experiments.

**Figure 6:** Jabba localizes to lipid droplets in a CK2-independent manner.

(A) 3T3-L1 pre-adipocytes were co-transfected with plasmids encoding myc-perilipin-3 (Plin-3) and GFP. Cells were treated with oleic acid. Cells were then treated DMSO (top panels) or CX-4945 (10µM) (bottom panels), fixed, and processed for immunofluorescence microscopy with anti-myc antibodies. Cells were counterstained with DAPI to label nuclei (blue). Scale bar=10µm.

(B) 3T3-L1 pre-adipocytes were co-transfected with plasmids encoding myc-perilipin-3 (Plin-3) and GFP-Jabba and incubated with oleic acid. Cells were then treated DMSO (top panels) or CX-4945 (10µM) (bottom panels), fixed, and processed for immunofluorescence microscopy with anti-myc antibodies. Cells were counterstained with DAPI to label nuclei (blue). Scale bar=10µm.

**Figure 7:** Jabba and CK2 regulate lipid metabolism during *Drosophila* oogenesis.

(A) Ovaries from flies of the indicated genotypes were dissected and stained with Oil Red O for visualization of neutral lipids. Shown are representative stage 10 egg chambers. Scale bar=100µm.

(B) Quantitation of total triglyceride levels from ovaries of flies of the indicated genotypes. Data are presented as the mean (n=4) with error bars denoting standard deviation. * indicates p-value<0.05; ns=not significant.
(C) Confocal immunofluorescence microscopy of egg chambers from flies of the indicated genotypes. Dissected egg chambers were fixed and stained with anti-myc antibody to detect Jabba-labeled lipid droplets (green) and counterstained with rhodamine-phalloidin to label F-actin (red). Shown are single optical confocal sections. Scale bar=50µm.
Figure 1

A

B

C

D

nos/+ nos>hCK2_−HA; CK2_−RNAi
nos/+ nos>hCK2_−K68M-HA; CK2_−RNAi
nos/+ nos>myc-hCK2_; CK2_−RNAi
nos/+ nos>myc-hCK2_; CK2_−RNAi

eggs/female/hour

mat/+ mat>hCK2_−HA; CK2_−RNAi
mat/+ mat>hCK2_−HA; CK2_−RNAi
mat/+ mat>CK2_−RNAi; CK2_−RNAi
mat/+ mat>hCK2_−RNAi; CK2_−RNAi

mat/+ mat>hCK2_−RNAi; CK2_−RNAi
mat/+ mat>hCK2_−RNAi; CK2_−RNAi
mat/+ mat>hCK2_−RNAi; CK2_−RNAi
mat/+ mat>hCK2_−RNAi; CK2_−RNAi

50- 37- 37-
37-
75-
75-

anti-HA
anti-myC
anti-Vasa
anti-Vasa
**Figure 2**

**A**

phosphorylation  alkylation  immunoprecipitation  protein identification

ovary lysate +CK2+GTPγS  →  +PNBM  →  anti-thiophosphate ester IP  →  SDS-PAGE and LC-MS/MS

**B**

|          | CK2: |          |          |
|----------|------|----------|----------|
| -        | -    | -        | +        |
| +        | -    | +        | -        |

**C**

anti-thiophosphate ester IP

**D**

**Jabba, isoform F, Accession: NP 001188970.1**

MAQETKTVATAKDQNRQDHIDEEEVIELHESRSFYDRVREQAERFASTRVGQFVIERADKALAMIEDTAKW
SLPQDKSSAPLQLPWAFLMLIVLRLTRIWLSVGAMLNCNGPGISPSTMVYFIQTRRRLRAIRVHGKL
VMRRQEQVESYGSQGTMQKLQWFSRAMCRPGVQDSSSRVVFVHSEQQLSNDFVVKRPREECNADADL
TIDQMLAKYANSENEDDSDFVPNEEEEESSSSSESESSEGSGSEGEVDEVVSQKSAGAVENGVH
KAEEKENDKGLNNTTSNGNNDEQAADVAAAPQDEEKSSPGHSAAVMNTRLNNATAAVTTDPDEPED
TQPEPESPTSQPSQSTEETDDADEDCSSSSNGTGVQYQADETOPTDAQEQILEIAATLTDHLSN
YPYTVDLTPATSSEDYFSPGISPTCFNTSLGTQALLKASIQLSIHVLAHSTPTTEMRDQPIEEANPQPT
ETEKTQQTPKTVNQNOQQQNOQRYRQHPRQYRQHGNRR
Figure 3

A

| GST-Jabba | no substrate | GST-Jabba |
|-----------|--------------|-----------|
| CK2:      | -            | +         |

- anti-thiophosphate ester
- anti-CK2α
- anti-GST

B

| CX-4945: | - | 0.5 | 1.0 | 2.0 | 5.0 | 10 (μM) |
|----------|---|-----|-----|-----|-----|---------|
| anti-phospho-CK2 substrate |
| anti-β-tubulin |

C

| untransfected | pEGFP-Jabba | pEGFP-Jabba |
|---------------|-------------|-------------|
| CX-4945:      | -           | -           | +           |

- anti-phospho-CK2 substrate
- anti-GFP
- anti-β-tubulin

D

| pCMV/HA | pCMV/CK2α-HA |
|---------|-------------|
| anti-phospho-CK2 substrate |
| anti-GFP |
| anti-HA |

IP

WCL
Figure 4

A

B

C

D

anti-myc
anti-Vasa
75-
50-
100-
150-
nos/+ nos>myc-Jabbanos>
myc-Jabba
;
      Jabba RNAi... template no template
  Jabba   actin
nos>Jabba RNAi nos>Jabba RNAiw
1118
w
1118

iodoacetamide:      -          +
  anti-myc
  anti-Vasa

eggs/female/hour
Figure 5

A

N → 1-221 → 222-273 → 274-537 → C

acridic serine-rich region

B

CX-4945: - - +

anti-thiophosphate ester

anti-GST

anti-CK2α

C

CX-4945: - - +

anti-phospho-CK2 substrate

anti-GST

anti-CK2α

by guest on July 24, 2018http://www.jbc.org/Downloaded from
Figure 7

A

cont. RNAi  mdy RNAi
Jabba RNAi; Jabba RNAi; nos>myc-Jabba

B

mg triglyceride / mg protein

---

C

nos>myc-Jabba
CK2α RNAi; nos>myc-Jabba
