

NHK-1 phosphorylates BAF to allow karyosome formation in the *Drosophila* oocyte nucleus

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Accurate chromosome segregation in meiosis requires dynamic changes in chromatin organization. In *Drosophila melanogaster*, upon completion of recombination, meiotic chromosomes form a single, compact cluster called the karyosome in an enlarged oocyte nucleus. This clustering is also found in humans; however, the mechanisms underlying karyosome formation are not understood. In this study, we report that phosphorylation of barrier to autointegration factor (BAF) by the conserved kinase nucleosomal histone kinase-1 (NHK-1; *Drosophila* Vrk1) has a critical function in karyosome formation.

We find that the noncatalytic domain of NHK-1 is crucial for its kinase activity toward BAF, a protein that acts as a linker between chromatin and the nuclear envelope. A reduction of NHK-1 or expression of nonphosphorylatable BAF results in ectopic association of chromosomes with the nuclear envelope in oocytes. We propose that BAF phosphorylation by NHK-1 disrupts anchorage of chromosomes to the nuclear envelope, allowing karyosome formation in oocytes. These data provide the first mechanistic insight into how the karyosome forms.

Introduction

Meiosis is a specialized cell division in which recombination of homologous chromosomes is followed by two rounds of chromosome segregation. In early prophase I, Spo11 initiates recombination by introducing DNA double-strand breaks (Keeney et al., 1997). An elaborate structure, the synaptonemal complex, is also formed to stabilize pairing of homologues (Page and Hawley, 2004). Once recombination has completed and double-strand breaks have been repaired, the synaptonemal complex is disassembled. Later in prophase I, exchange sites are seen as chiasmata, which serve to link homologues, ensuring their separation to opposite poles at the first meiotic division. As the events of chromosome reorganization during prophase I are largely meiosis specific, molecular mechanisms governing this process are likely to go beyond our understanding of mitotic cell division.

Upon the completion of recombination in prophase I, all meiotic chromosomes cluster together to form a compact spherical structure called the karyosome within the enlarged oocyte nucleus in *Drosophila melanogaster* (King, 1970). This clustering of meiotic chromosomes in the oocyte nucleus is also observed in humans (Parfenov et al., 1989). Within the karyosome,

chromosomes are arranged in an organized way. Homologous chromosomes are paired at centromeric heterochromatin, but their arms are often separated. Furthermore, centromeric heterochromatin of different chromosomes tends to be clustered together (Dernburg et al., 1996).

Although very little is known about the molecular mechanism of karyosome formation, a class of mutants (called the spindle or karyosome class) has been reported to have defective karyosome organization in addition to axis patterning defects in oocytes (Morris and Lehmann, 1999). These mutants have been shown to activate the meiotic checkpoint pathway, but it remains to be understood how activation of the meiotic checkpoint leads to defective karyosome structure.

Recent studies have shown that nucleosomal histone kinase-1 (NHK-1) is essential for karyosome formation and maintenance (Cullen et al., 2005; Ivanovska et al., 2005). NHK-1 was originally identified as a kinase that phosphorylates histone 2A in vitro (Aihara et al., 2004). NHK-1 is conserved from nematodes to humans (Vrk-1 in *Caenorhabditis elegans* and Vrk1-3 in mammals), and multiple substrates have been reported for the homologous kinases in other organisms (Lopez-Borges and Lazo, 2000; Sevilla et al., 2004a,b; Nichols et al., 2006; Gorjanacz et al., 2007). Female sterile *nhk-1* mutants fail to form or maintain the karyosome in the oocyte nucleus (Cullen et al., 2005; Ivanovska et al., 2005). Later in female meiosis, *nhk-1* mutants show the formation of separate metaphase I spindles around each bivalent chromosome (Cullen et al., 2005).

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Abbreviations used in this paper: BAF, barrier to autointegration factor; MBP, maltose-binding protein; NHK-1, nucleosomal histone kinase-1; UASp, upstream activating sequence.

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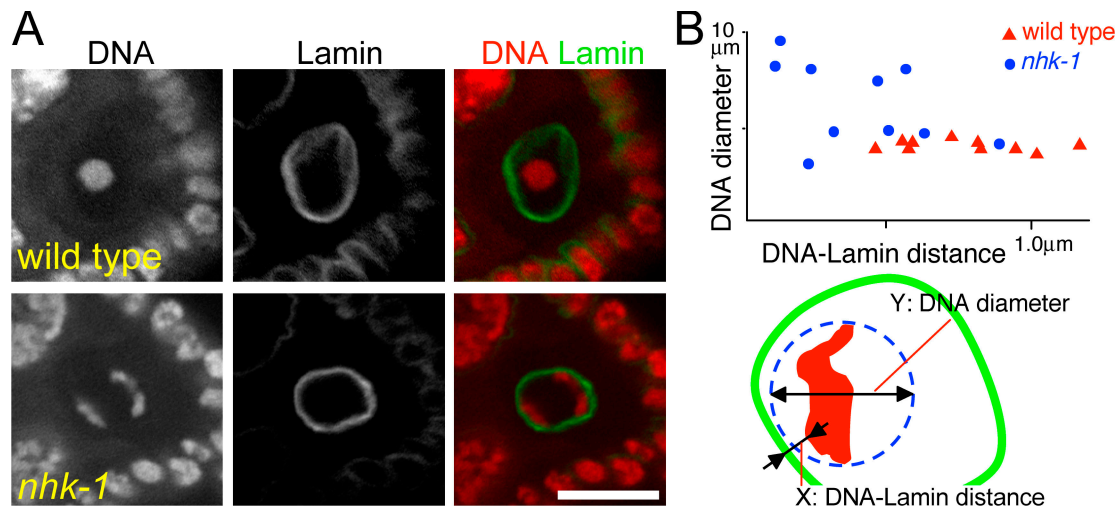


Figure 1. **Anchorage of meiotic chromosomes to the nuclear envelope in an *nhk-1* mutant.** Karyosome defects caused by an *nhk-1* mutation. (A) Ovaries from wild-type and *nhk-1*^{E24/Df} were immunostained for lamin and DNA. (B) The closest distance between chromosomes and the outline of the nuclear envelope was measured. The degree of karyosome compaction is defined here as the diameter of the smallest circle able to encompass all chromosomes. Bar, 10 μ m.

This suggests that one function of the karyosome is to facilitate the formation of a single spindle by holding meiotic chromosomes in close proximity.

In *nhk-1* mutant oocytes, the phosphorylation of H2A, loading of condensin, and synaptonemal complex disassembly are defective (Ivanovska et al., 2005). It was suggested that the phosphorylation of H2A promoted a certain pattern of histone modifications that together play an instructive role in changing chromosome architecture and promoting karyosome formation in meiosis (Ivanovska et al., 2005; Ivanovska and Orr-Weaver, 2006). Although this meiotic histone code hypothesis is attractive, H2A phosphorylation alone may not be responsible for all of the multiple functions of NHK-1.

The discovery of NHK-1 has given us a unique opportunity to begin identifying the molecular pathway of karyosome formation. In this study, we report the identification of barrier to autointegration factor (BAF), a linker between the nuclear envelope and chromatin, as a critical substrate of NHK-1 in karyosome formation. Our results indicate that BAF phosphorylation by NHK-1 breaks this link, allowing formation of the karyosome. This study provides the first truly mechanistic insight into how this meiosis-specific organization of chromatin forms in oocytes at the molecular level.

Results and discussion

A reduction of NHK-1 results in chromosomes being anchored to the nuclear envelope

Upon completion of recombination in prophase I, the oocyte nucleus dramatically changes its architecture and organizes meiotic chromosomes into a single cluster called the karyosome. The molecular mechanism of karyosome formation is not understood. Recently, female sterile *nhk-1* mutations were identified that lead to abnormal morphology of the karyosome (Cullen et al., 2005; Ivanovska et al., 2005). To understand the role of NHK-1 in karyo-

some formation, we first performed detailed examination of the karyosome abnormality induced by *nhk-1* mutations.

We (Cullen et al., 2005) and Ivanovska et al. (2005) have previously identified female sterile mutations in the conserved kinase NHK-1 that both disrupt karyosome organization. One allele (Z3-0437) is a missense mutation that alters a residue within the kinase domain (Ivanovska et al., 2005), whereas the other (*triplet*) is a nonsense mutation that truncates the noncatalytic domain of NHK-1 (Cullen et al., 2005). As it is not certain how these mutations affect the activity or regulation of NHK-1, we studied a third female sterile allele (*E24*) of *nhk-1* in which the karyosome had not previously been examined. This allele has a small insertion in the 5' untranslated region of NHK-1 (Cullen et al., 2005), and hemizygous mutants (*nhk-1*^{E24/Df}/*ro-80b*, which we refer to as *nhk-1*^{E24/Df}) contain the full-length NHK-1 protein, but the amount of the protein is reduced to 15–20% of wild-type ovaries.

At wild-type oogenesis stage 2 (pachytene stage), meiotic chromosomes occupy the entire region of the oocyte nucleus. At later stages (postpachytene stages), all meiotic chromosomes are clustered together within the enlarged nucleus to form a compact structure called the karyosome. The wild-type karyosome usually adopts a spherical shape away from the nuclear envelope (Fig. 1). In *nhk-1*^{E24/Df}, karyosome morphology became generally more extended. Most strikingly, immunostaining for lamin and DNA revealed that chromosomes tended to form extensive contact with the nuclear envelope (Fig. 1A). Quantitative analysis confirmed that the karyosome was less compact and tended to be located closer to the nuclear envelope in *nhk-1* mutants, although the exact defects varied from oocyte to oocyte (Fig. 1B).

Identification of BAF as an NHK-1 substrate

Identification of NHK-1 substrates would be key to understanding how the karyosome forms during female meiosis. To identify substrates of NHK-1, we first produced full-length NHK-1 in

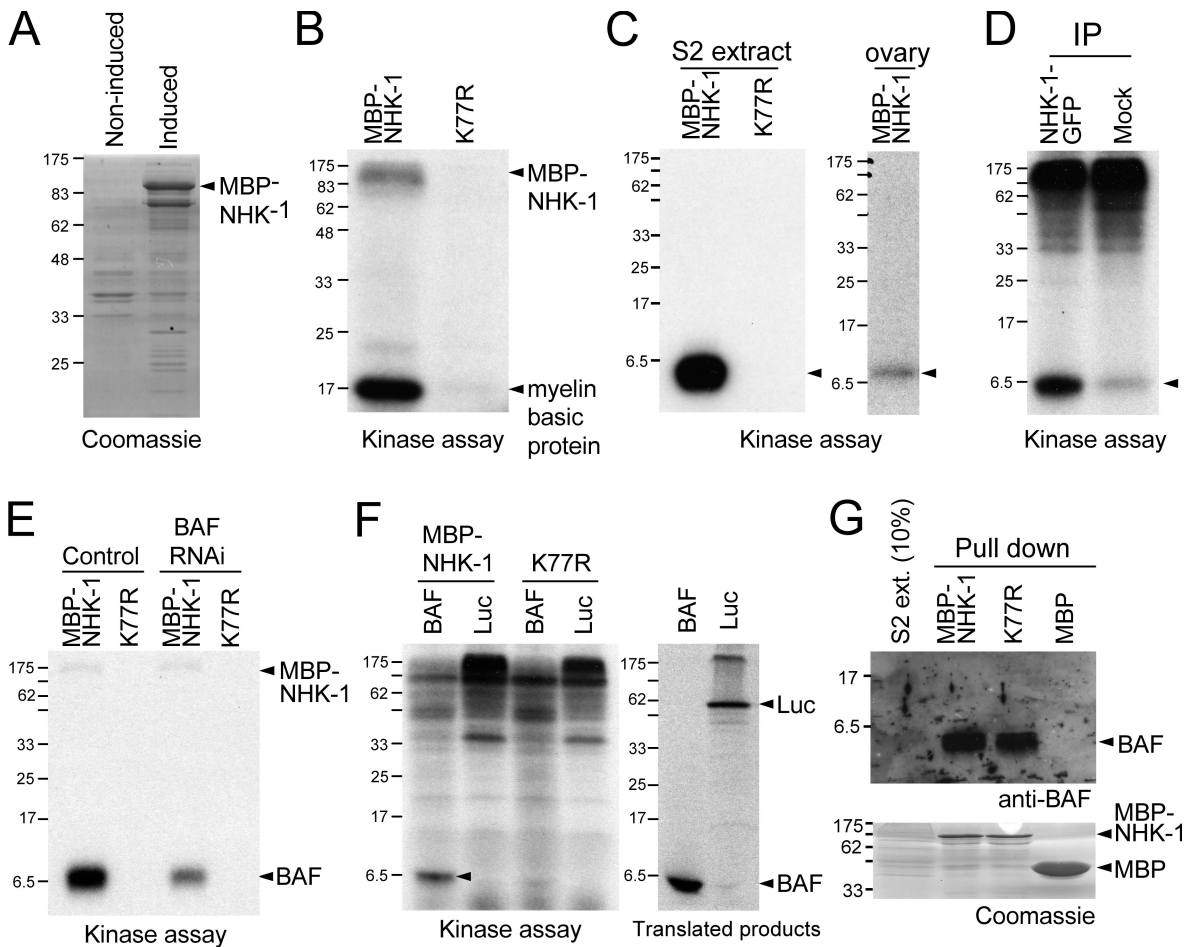


Figure 2. NHK-1 phosphorylates BAF. (A) Purified MBP-NHK-1 from bacteria. (B) Phosphorylation of myelin basic protein by MBP-NHK-1, not by inactive MBP-NHK-1K77R. (C) Phosphorylation of a 6-kD protein in a heat-inactivated S2 cell and ovary extract by MBP-NHK-1. (D) Specific phosphorylation of a 6-kD protein by NHK-1-GFP. Cell extracts were made from S2 cells that transiently expressed NHK-1-GFP or had received mock transfection procedures. Cell fractions pulled down by a GFP antibody were used in a kinase assay without exogenous substrates. (E) BAF is the substrate of NHK-1 in the S2 cell extract. Cell extracts from S2 cells treated with double-stranded RNA for BAF and β -lactamase (control) were used in an NHK-1 kinase assay. (F) NHK-1 phosphorylates BAF. BAF and luciferase (Luc) were translated in vitro and used in a kinase assay. The right panel shows equivalent translated products labeled with [35 S]methionine. Only BAF was specifically phosphorylated by MBP-NHK-1. (C, D, and F) The arrowheads point to specific phosphorylated proteins. (G) S2 cell fractions pulled down with MBP-NHK-1, MBP-NHK-1K77R, or MBP were immunoblotted with a BAF antibody. S2 cell extract (10% equivalent of pull-downs) was run in parallel.

bacteria as a fusion protein to maltose-binding protein (MBP; Fig. 2 A). In addition, inactive NHK-1 (NHK-1K77R) with a mutation in a conserved ATP-binding residue (K77R) was produced. Purified MBP-NHK-1 and MBP-NHK-1K77R were tested for their activities in an in vitro kinase assay against myelin basic protein as a substrate. Myelin basic protein has many phosphorylatable residues and is commonly used as an artificial kinase substrate. Autoradiography indicated that myelin basic protein was phosphorylated by MBP-NHK-1 but not by MBP-NHK-1K77R (Fig. 2 B). This demonstrated that bacterially produced MBP-NHK-1 kinase is active in vitro.

Next, we used this bacterially produced kinase to search for potential substrates in cells. First, extracts from *Drosophila* cultured S2 cells were heat treated to inactivate endogenous kinases (Fig. S1; available at <http://www.jcb.org/cgi/content/full/jcb.200706067/DC1>; Tavares et al., 1996). Then, this heat-inactivated extract was added to NHK-1 kinase to assay for potential substrates in vitro. One band with a mobility equivalent to 6 kD

showed strong incorporation of phosphates when wild-type NHK-1 was used but not inactive NHK-1K77R (Fig. 2 C, left). We also found this 6-kD band when we used heat-inactivated extract from ovaries (Fig. 2 C, right).

This 6-kD band was also detected in a kinase assay using NHK-1 purified from S2 cells. S2 cells were first transfected with the NHK-1-GFP gene or received transfection procedures without the plasmid (mock transfected). NHK-1-GFP was pulled down from cell extracts using a GFP antibody and was used for a kinase assay. We found that even without adding exogenous substrates, one band of 6 kD was specifically phosphorylated in a pull-down fraction from NHK-1-GFP-transfected cells but not from mock-transfected cells (Fig. 2 D). These results indicated that a protein of 6 kD in S2 cells binds to NHK-1 and is a good in vitro substrate of NHK-1.

In the course of our study, phosphorylation of BAF by Vrk-1 (the NHK-1 orthologue) was reported (Nichols et al., 2006; Gorjanacz et al., 2007). As BAF is a highly conserved

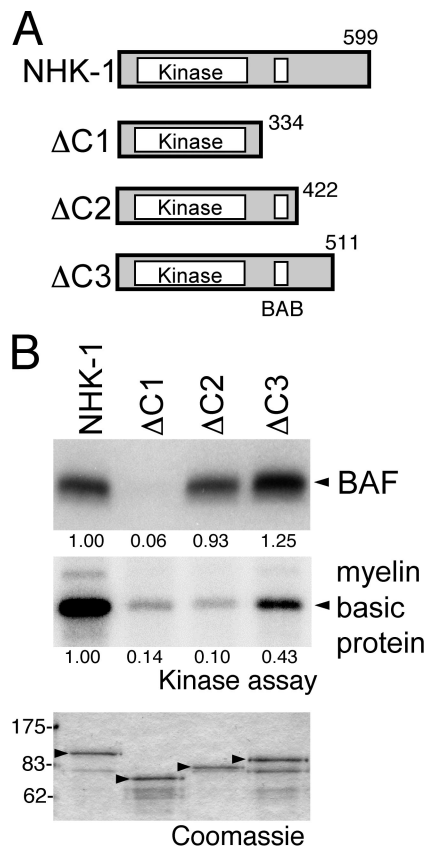


Figure 3. The noncatalytic domain targets NHK-1 activity toward BAF. (A) A summary of NHK-1 truncations (Δ 1–3). Small white boxes indicate the basic-acidic-basic motif commonly found in the C-terminal regions of the NHK-1 orthologues. (B) Kinase assays of bacterially produced MBP–NHK-1 and various truncations against heat-inactivated S2 cell extract or myelin basic protein. The numbers indicate the signal intensities relative to those of the full-length NHK-1. The arrowheads indicate full-length MBP–NHK-1 and truncations.

small protein, we thought that *Drosophila* BAF (Furukawa et al., 2003) would be a good candidate for the 6-kD protein phosphorylated by NHK-1. To test this possibility, we partially depleted BAF from S2 cells by RNAi. The cell extract was heat inactivated and used as substrate in an *in vitro* NHK-1 kinase assay. Autoradiography showed that the 6-kD band was greatly reduced in BAF-depleted cell extract (Fig. 2 E), indicating that the protein phosphorylated by NHK-1 was indeed BAF. For further confirmation, BAF protein was translated *in vitro* in reticulocyte lysate and used as a substrate in a kinase assay (without heat treatment). BAF protein was specifically phosphorylated by MBP–NHK-1 but not by MBP–NHK-1K77R (Fig. 2 F).

Furthermore, we tested for physical association between NHK-1 and BAF. S2 cell extracts were incubated with bacterially produced MBP–NHK-1, MBP–NHK-1K77R, or MBP, and MBP fusion proteins were pulled down on amylose resin. Immunoblotting indicated that BAF was pulled down by MBP–NHK-1 and MBP–NHK-1K77R but not by MBP alone (Fig. 2 G). This result indicated that BAF is able to physically interact with NHK-1 and that the interaction is independent of its kinase activity. Altogether, BAF binds to NHK-1 and is a good substrate of NHK-1 *in vitro*.

The noncatalytic domain of NHK-1 is critical for its kinase activity

NHK-1 consists of an N-terminal kinase domain and a C-terminal noncatalytic region (Fig. 3 A; Aihara et al., 2004). The *triplet* allele of *nhk-1* has a nonsense mutation that results in loss of the noncatalytic domain and showed a female sterile phenotype with karyosome defects (Cullen et al., 2005).

To establish the role of the C-terminal noncatalytic region, we made a series of truncations to NHK-1 in bacteria and tested them for their kinase activity against myelin basic protein and heat-inactivated S2 cell extract (Fig. 3). We found that truncations of the noncatalytic domain greatly influenced kinase activity. Interestingly, kinase activities toward BAF and myelin basic protein were differently affected by each truncation. In particular, Δ C1 and Δ C2 showed similar activities against myelin basic protein, but Δ C1 had much weaker activity toward BAF than Δ C2, indicating that the small region (335–422 aa) contains the residues specifically important for NHK-1 kinase activity toward BAF. This region includes the basic-acidic-basic motif (368–408 aa; Aihara et al., 2004), the only recognizable feature in the noncatalytic region commonly found among the NHK-1 orthologues.

Expression of nonphosphorylatable BAF disrupts karyosome formation

BAF is known to physically interact with DNA and inner nuclear envelope proteins, including LEM-domain proteins (Furukawa, 1999; Segura-Totten and Wilson, 2004). Furthermore, biochemical experiments have indicated that the phosphorylation of BAF by Vrk-1 (a mammalian NHK-1 homologue) reduces its interaction with both DNA and LEM-domain proteins *in vitro* (Nichols et al., 2006). Our working hypothesis is that BAF anchors meiotic chromosomes to the nuclear envelope in early female meiosis and that the phosphorylation of BAF by NHK-1 disrupts this link and allows chromosomes to form one compact cluster (the karyosome) within the enlarged oocyte nucleus (see Fig. 5). This hypothesis predicts that an ectopic expression of nonphosphorylatable BAF in wild-type oocytes would prevent the formation of proper karyosome organization even in the presence of endogenous BAF. We chose to investigate the role of the phosphorylation in this way.

The three N-terminal serine and threonine residues of mammalian BAF have been shown to be phosphorylated *in vitro* by Vrk-1 (Fig. 4 A; Nichols et al., 2006). To test our hypothesis, we replaced equivalent residues of *Drosophila* BAF with alanines (BAF-3A) to make a nonphosphorylatable version of BAF (Fig. 4 A). To examine the role of the phosphorylation in oocytes, the nonphosphorylatable BAF-3A and wild-type BAF were expressed in oocytes under an upstream activating sequence (UASp) controlled by GAL4, whose expression, in turn, was driven in oocytes by a maternal α -tubulin promoter. Immunoblots confirmed that both versions of BAF proteins were produced in oocytes.

To test that the mutated residues of BAF are the sites phosphorylated by NHK-1, extracts of ovaries expressing wild-type BAF and BAF-3A were heat inactivated and used in an *in vitro* NHK-1 kinase assay. Oocytes expressing BAF-3A showed a greatly reduced phosphorylation of BAF compared with oocytes expressing wild-type BAF (Fig. 4 B). This result indicated that

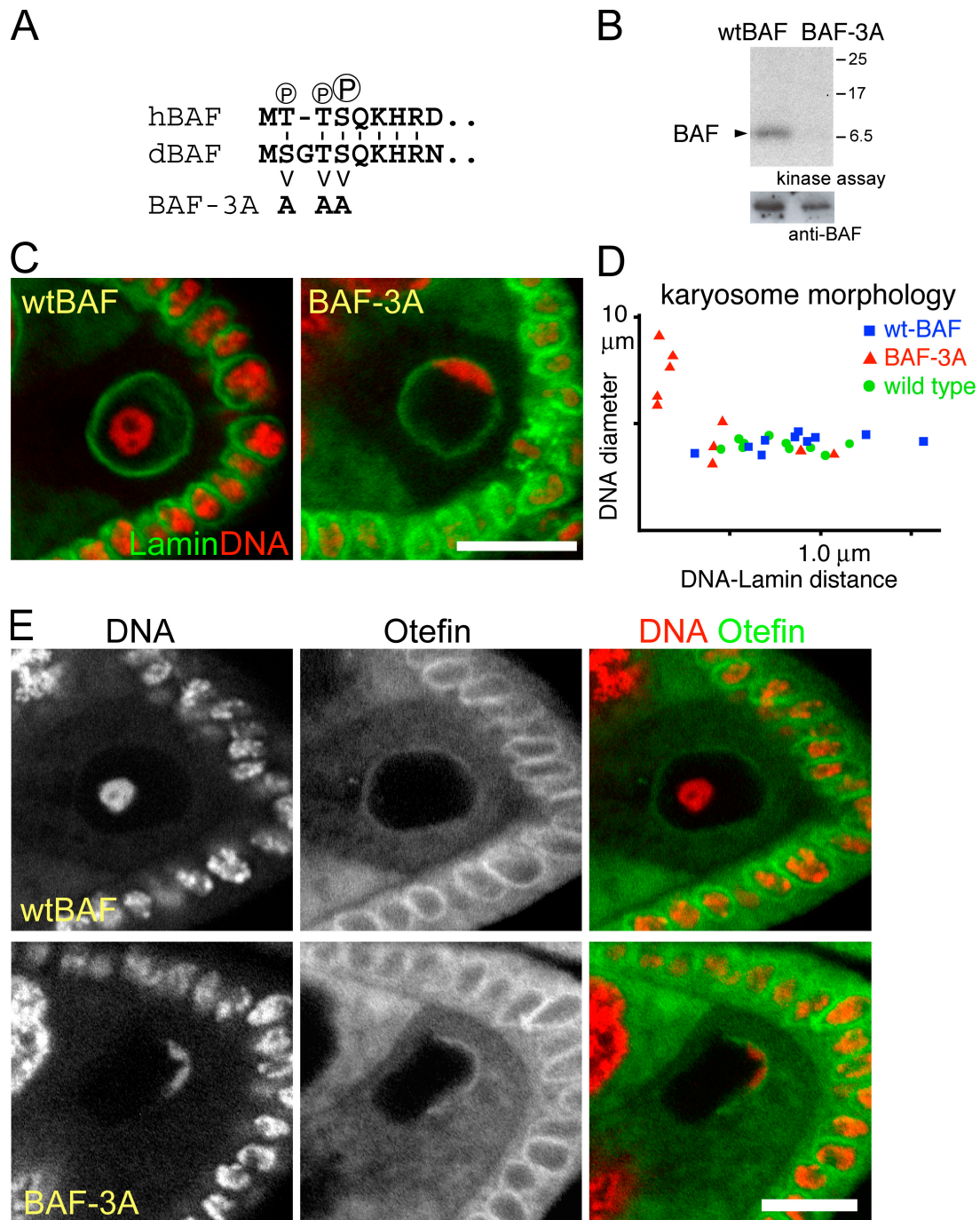


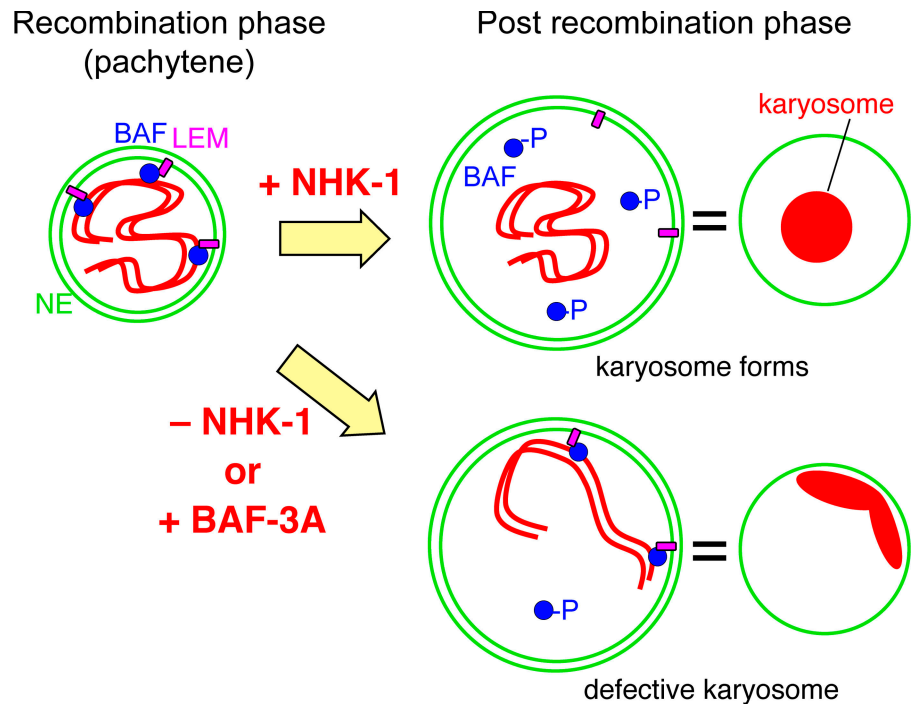
Figure 4. Expression of nonphosphorylatable BAF disrupts karyosome formation. (A) Primary sequences of the N terminus of BAF from humans and *Drosophila*. P indicates the phosphorylation sites of mammalian BAF by Vrk-1. Ser4 is the most preferred site. Three potential phosphorylation sites were mutated to create nonphosphorylatable BAF (BAF-3A). (B) BAF-3A is not phosphorylated by NHK-1. Extracts from ovaries expressing wild-type BAF (wtBAF) and BAF-3A were heat inactivated and used as a substrate in an MBP-NHK-1 kinase assay. The bottom panel (an immunoblot) shows that comparable amounts of BAF are expressed. (C) Expression of nonphosphorylatable BAF (BAF-3A) resulted in karyosome defects. Oocytes expressing wild-type BAF and BAF-3A were immunostained for lamin and DNA. (D) The karyosome is less compact and located close to the nuclear envelope in BAF-3A-expressing oocytes. This was quantified as in Fig. 1 C. (E) BAF-3A expression but not wild-type BAF induced accumulation of the LEM-domain protein Otefin to a region of the nuclear envelope attached to chromosomes. Bars, 10 μ m.

the mutated residues of BAF include the major sites phosphorylated by NHK-1.

We found that the expression of BAF-3A in oocytes led to female sterility, whereas the expression of wild-type BAF did not. We then examined whether the expression of BAF-3A

or wild-type BAF affects karyosome morphology. Immunostaining showed that overexpression of wild-type BAF did not alter karyosome morphology (Fig. 4 C). Meiotic chromosomes were clustered into a compact spherical body away from the nuclear envelope.

Figure 5. **BAF phosphorylation by NHK-1 kinase in karyosome formation.** See the last section of Results and discussion for details. P, phosphorylation.



In contrast, the expression of nonphosphorylatable BAF-3A disrupted karyosome morphology (Fig. 4, C and D). Strikingly, chromosomes often formed extensive contact with the nuclear envelope (Fig. 4, C and D), which closely resembled the phenotype seen in *nhk-1* mutants. In addition, the karyosome often lost spherical morphology and became more extended. We noted that this karyosome morphology defect is not completely identical to the *nhk-1* mutant. This may be caused by the presence of wild-type BAF or other fully phosphorylated NHK-1 substrates. Expression of BAF-3A in a null background and identification of additional substrates could provide more information on this issue. In conclusion, BAF phosphorylation is critical for the detachment of chromosomes from the nuclear envelope and for the formation of a proper karyosome.

Expression of nonphosphorylatable BAF maintains a link between a LEM-domain protein and chromatin

BAF is proposed to link chromatin DNA to LEM-domain-containing inner nuclear envelope proteins by binding to both simultaneously (Shumaker et al., 2001). BAF phosphorylation reduces the affinity of BAF for DNA and LEM-domain proteins in vitro (Nichols et al., 2006). Therefore, we expected that LEM-domain proteins would be involved in anchorage of chromosomes to the nuclear envelope induced by nonphosphorylatable BAF-3A.

To test this possibility, we determined the localization of a *Drosophila* LEM-domain protein, Otefin, in oocytes that express wild-type BAF or BAF-3A. In oocytes expressing wild-type BAF, Otefin was localized to the nuclear envelope uniformly as in wild type. In oocytes expressing nonphosphorylatable BAF-3A, Otefin was often accumulated in a region of the nuclear envelope that has close contact with meiotic chromo-

somes (Fig. 4 E). These results confirmed the involvement of the LEM-domain protein in the link between chromosomes and the nuclear envelope.

Phosphorylation of BAF by NHK-1 allows karyosome formation

This study, together with previous studies (Furukawa, 1999; Shumaker et al., 2001; Bengtsson and Wilson, 2006; Nichols et al., 2006; Gorjanacz et al., 2007), lead us to propose the following model (Fig. 5). During meiotic recombination phase, BAF in its unphosphorylated form anchors chromosomes to the nuclear envelope by binding to both DNA and LEM-domain nuclear envelope proteins in oocytes. Upon the completion of recombination, NHK-1 phosphorylates BAF to reduce its affinities for DNA and LEM-domain proteins. This allows the release of chromosomes from the nuclear envelope and the formation of the karyosome. When NHK-1 activity is reduced or nonphosphorylatable BAF is expressed, unphosphorylated BAF keeps chromosomes tethered to the nuclear envelope and prevents the formation of the karyosome.

Anchoring of chromosomes to the nuclear envelope appears to be a common feature during meiotic recombination across eukaryotes. Association of clustered telomeres with the nuclear envelope during early prophase I is found in many organisms (Scherthan, 2007). In fission yeast, this association is required for efficient recombination (Ding et al., 2004). In *C. elegans*, pairing centers are associated with nuclear envelope proteins (Him8 and Zim1-3) that are required for pairing and synapses (Phillips et al., 2005; Phillips and Dernburg, 2006). After the completion of recombination in female meiosis, meiotic chromosomes have to be detached from the nuclear envelope to form the karyosome, as it facilitates the formation of a single spindle, which takes place in the absence of centrosomes (Cullen et al., 2005).

Our identification of BAF as an NHK-1 substrate is a breakthrough in the molecular understanding of karyosome formation. BAF was originally identified as a cellular protein associated with the preintegration complex of retroviruses (Lee and Craigie, 1998) and was later shown to bind to DNA and multiple proteins (Segura-Totten and Wilson, 2004). Because of its simultaneous interactions with DNA and inner nuclear envelope proteins, BAF is proposed to act as a linker between chromatin and the nuclear envelope (Shumaker et al., 2001). Consistently, BAF has been shown to be required for reassembly of the nuclear envelope around chromosomes after mitosis (Segura-Totten et al., 2002; Gorjanacz et al., 2007). However, it has been an open question as to whether BAF has structural roles in chromatin or nuclear architecture once the nucleus is formed.

Recent studies (Nichols et al., 2006; Gorjanacz et al., 2007) showed that Vrk-1, an NHK-1 orthologue, phosphorylates BAF in mammals and nematodes. The cellular function of the phosphorylation was unclear, although it affects the localization of BAF and emerlin in interphase (Bengtsson and Wilson, 2006; Nichols et al., 2006). Vrk-1 and BAF are both essential for nuclear envelope reassembly, but the role of BAF phosphorylation in this process was not tested. This study is the first demonstration of a cellular role for BAF phosphorylation and of a structural role for BAF in organizing the nucleus, which is already formed.

Materials and methods

Drosophila genetics

Standard techniques of fly manipulation were followed (Ashburner et al., 2005). All stocks were grown at 25°C in standard cornmeal media except in some cases in which females were matured at 18°C. *w¹¹¹⁸* was used as wild type. Details of mutations, chromosome aberrations, and common vectors can be found in Lindsley and Zimm (1992) or at Flybase (Drysdale et al., 2005). Transgenic flies of pUASp-BAF and pUASp-BAF-3A were made by Genetic Services and crossed with a maternal Gal4 driver (V2H) under the α -tubulin67C promoter.

In vitro kinase assays

Culture of S2 cells, transfection, and RNAi were performed as described previously (Brittle and Ohkura, 2005). For a kinase assay, MBP-NHK-1, its mutated versions, and MBP were expressed in *Escherichia coli* (BL21) at 18°C and purified using amylose beads (GE Healthcare). As substrates, we used myelin basic protein (Sigma-Aldrich) and ovary or S2 cell extracts lysed in kinase buffer (10 mM HEPES, pH 7.6, 50 mM KCl, and 5 mM MgCl₂) and inactivated by incubation at 65°C for 20 min. In a typical kinase reaction, substrate and around 1 μ g MBP-NHK-1 were mixed with 5 μ Ci γ -[³²P]ATP (GE Healthcare) in 20 μ l of kinase buffer and incubated at room temperature (20°C) for 60 min before the addition of 20 μ l of 2 \times protein sample buffer. The samples were analyzed by SDS-PAGE, and dried gels were exposed to x-ray films with an intensifier screen or a phosphor screen (Molecular Dynamics). For a kinase assay from immunoprecipitated NHK-1-GFP fractions, transfected S2 cells were lysed in lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.5, 5 mM EGTA, 1 mM DTT, 1 mM PMSF, and protease inhibitors [Roche]), incubated with a GFP antibody for 1 h at 4°C and further with protein G beads (Invitrogen) for 1 h, and washed in lysis buffer and subsequently kinase buffer. BAF and luciferase were translated in reticulocyte lysate using a TNT Quick-Coupled Transcription/Translation system (Promega) and were directly used for kinase assays.

Molecular and immunological techniques

Standard molecular techniques were used throughout (Harlow and Lane, 1988; Sambrook et al., 1989). pUASp-BAF was made by inserting BAF cDNA into pUASp. pUASp-BAF-3A was made by PCR amplification of BAF cDNA using a 5' primer containing the desired mutations followed by insertion into pUASp. A plasmid expressing MBP-NHK-1 was made by inserting the NHK-1 coding sequence into pMalc2 (GE Healthcare). MBP-NHK-1K77R was created using the QuikChange Site-Directed Muta-

genesis kit (Promega). Truncations of MBP-NHK-1 were made by introducing a premature stop codon by site-directed mutagenesis. The P-element plasmid expressing NHK-1-GFP was made by inserting the GFP coding region in *nhk-1* cDNA driven by the *nhk-1* promoter. The absence of unwanted mutations in all constructs was confirmed by DNA sequencing using BigDye (Applied Biosystems).

For the pull-down assay, bacterially produced MBP fusion proteins were purified on amylose beads and incubated with soluble S2 cell extract in lysis buffer. After washing three times in lysis buffer, the beads were analyzed by SDS-PAGE and immunoblotting. Peroxidase-conjugated antibodies (Jackson ImmunoResearch Laboratories) were used as secondary antibodies in Western blotting and were detected using an ECL kit (GE Healthcare).

Cytological analysis

Ovaries were immunostained as described previously (Theurkauf et al., 1992). Secondary antibodies conjugated with Cy3, Cy5, or AlexaFluor488 (Jackson ImmunoResearch Laboratories or Invitrogen) were used at 1:250–1:1,000 dilutions. The primary antibodies used in this study include those against lamin (1:250; gift from P. Fisher, State University of New York, Stony Brook, NY; Stuurman et al., 1995), Otefin (1:10; gift from Y. Gruenbaum, Hebrew University, Jerusalem, Israel; Ashery-Padan et al., 1997), BAF (1:1,000; gift from P. Fisher; Furukawa et al., 2003), and GFP (3E6; 1:100; Invitrogen). DNA was counterstained with 0.4 μ g/ml DAPI (Sigma-Aldrich) and 2 μ g/ml propidium iodide (Sigma-Aldrich). Immunostained ovaries were mounted in 85% glycerol/2.5% propyl gallate. A series of 1- μ m optical sections were taken using a plan-Apochromat 63 \times 1.4 NA lens (Carl Zeiss, Inc.) attached to a microscope (Axiovert 200M; Carl Zeiss, Inc.) with a confocal scan head (LSM510meta; Carl Zeiss, Inc.). A single midsection of the oocyte nucleus has been presented. All digital images were imported to Photoshop (Adobe) and adjusted for brightness and contrast uniformly across entire fields.

Online supplemental material

Fig. S1 contains additional information on heat inactivation of endogenous kinases in S2 cell extract. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200706067/DC1>.

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