Recruitment of BAF to the nuclear envelope couples the LINC complex to endoreplication

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Abstract

DNA endoreplication has been implicated as a cell strategy to grow in size and in tissue injury. Here, we demonstrate that barrier to autointegration factor (BAF), represses endoreplication in Drosophila myofibers. We show that BAF localization at the nuclear envelope was eliminated either in mutants of the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex, in which the LEM-domain protein Otefin was similarly excluded, or after disruption of the nucleus-sarcomere connections. Furthermore, BAF localization at the nuclear envelope required the activity of the BAF kinase VRK1/Ball, and consistently non-phosphorytable BAF-GFP was excluded from the nuclear envelope. Importantly, removal of BAF from the nuclear envelope correlated with increased DNA content in the myonuclei. E2F1, a key regulator of endoreplication was found to overlap BAF localization at the myonuclear envelope, and BAF removal from the nuclear envelope resulted with increased E2F1 levels in the nucleoplasm, and subsequent elevated DNA content. We suggest that LINC-dependent, and phospho-sensitive attachment of BAF to the nuclear envelope, through its binding to Otefin, tethers E2F1 to the nuclear envelope thus inhibiting its accumulation at the nucleoplasm.
Introduction

Endoreplication emerges as an important strategy of differentiated cells to grow in size, or in rescuing tissue integrity following injury, in a wide range of non-dividing cell types (Gan et al., 2019). Recent experimental studies proposed a functional link between mechanical inputs and endoreplication events in various cell types (Cao et al., 2017; Sun et al., 2019). Moreover, mechanical signals transmitted across the nuclear membrane have been implicated in the regulation of cell cycle, epigenetic events, as well as gene transcription (Cao et al., 2017; Cho et al., 2017; Kirby and Lammerding, 2018). As part of the mechanisms linking cell cycle events with mechanical inputs, the translocation of specific essential factors into the nucleus have been proposed (Aragona et al., 2013; Driscoll et al., 2015; Dupont et al., 2011; Ho et al., 2013; Kassianidou et al., 2019). However, the molecular link between nuclear translocation of such factors, mechanical inputs on the nuclear envelope, and endoreplication is still elusive.

The linker of nucleoskeleton and cytoskeleton (LINC) complex has been suggested to mediate mechanically induced nuclear entry of essential factors (Driscoll et al., 2015; Horn, 2014; Osmanagic-Myers et al., 2015). It physically connects the cytoskeleton and the nucleoskeleton at the interface of the nuclear envelope and has been associated with various human myopathies (Horn, 2014; Mejat and Misteli, 2010; Mellad et al., 2010). The LINC complex is composed of Nesprin family protein members, which associate at their cytoplasmic N-terminal end with distinct cytoskeletal components, and on their nuclear C-terminal end with SUN domain proteins at the perinuclear space (Rajgor and Shanahan, 2013; Zhang et al., 2002; Zhang et al., 2001). SUN domain proteins bind to various nuclear lamina components, resulting with a physical link between the cytoskeleton and the nucleoskeleton (Cain and Starr, 2015; Lombardi et al., 2011; Padmakumar et al., 2005; Sosa et al., 2012). Recent results from our lab indicated that in *Drosophila* larval muscles, the LINC complex is essential for arresting endoreplication in the muscle nuclei (myonuclei), and the LINC mutants exhibit additional rounds of DNA replication, resulting with elevated polyploidy (Brayson et al., 2018; Volk, 2012; Wang et al., 2018). The molecular nature driving this process is currently elusive.
In an attempt to reveal the components downstream of the LINC-dependent arrest of DNA endoreplication, we performed a screen for genes whose transcription changes in the Nesprin/klar mutant muscles. One of the identified genes was *barrier-to-autointegration factor* (*baf*) shown to be significantly reduced at the transcription level (Wang et al., 2018). BAF is a small 89-aa protein, which binds dsDNA, as well as the nuclear envelope, and in addition forms homo-dimers (Bradley et al., 2005; Zheng et al., 2000). Furthermore, BAF had been shown to bind the inner components of the nuclear membrane, including “Lap-2, Emerin, MAN1” (LEM) domain proteins, as well as to lamin A/C, and B. Thus, BAF dimers might bridge between dsDNA and the nuclear envelope (Cai et al., 2001; Lee et al., 2001; Mansharamani and Wilson, 2005; Samwer et al., 2017). Proteomic analysis of BAF partners indicated its potential association with additional proteins, including transcription factors, damage-specific DNA binding proteins, and histones (Holaska et al., 2003; Montes de Oca et al., 2009). Furthermore, BAF binding to its potential partners may be regulated by its phosphorylation state (Birendra et al., 2017; Lancaster et al., 2007; Nichols et al., 2006). For example, phosphorylated BAF has been shown to associate with LEM domain proteins, whereas de-phosphorylated BAF favors its binding to dsDNA (Bengtsson and Wilson, 2006; Nichols et al., 2006). One kinase that has been implicated in BAF phosphorylation is the threonine-serine VRK1 kinase, whose homolog in *Drosophila* is Ballchen (Ball, also known as NHK-1) (Lancaster et al., 2007).

BAF has a crucial role in the condensation and assembly of post-mitotic DNA. Its interaction with both dsDNA and the nuclear lamina enables DNA compaction through cross-bridges between chromosomes and the nuclear envelope, a process essential for the assembly of DNA within a single nucleus following mitosis (Samwer et al., 2017). Likewise, BAF is recruited to the sites of ruptured nuclear membrane, where it is essential for resealing the ruptured nuclear membrane (Halfmann et al., 2019). Interestingly, in humans a single amino acid substitution of BAF causes Nestor–Guillermo progeria syndrome (NGPS) (Puente et al., 2011); however, the molecular basis for the disease awaits further investigation.
Previously, we demonstrated that in *Drosophila*, muscle-specific knock down of BAF increased the levels of DNA endoreplication, phenocopying the LINC mutant outcome (Wang et al., 2018). This led to the hypothesis that BAF acts downstream of the LINC complex-dependent mechanotransduction in promoting the arrest of DNA endoreplication in muscles. Here, we demonstrate that BAF localization at the nuclear envelope is critical for that process, and that it is downstream of the LINC complex, depends on nuclear-sarcomeres connections, and is phospho-sensitive. Importantly, elimination of BAF from the nuclear envelope correlated with increased DNA content in the myonuclei, and a concomitant increase in E2F1 levels in the nucleoplasm. Taken together our findings suggest a model in which a LINC-dependent localization of BAF at the nuclear envelope promotes E2F1 tethering to the nuclear envelope to inhibit its accumulation in the nucleoplasm.

**Results**

*BAF localization at the nuclear envelope depends on a functional LINC complex*

Previously we showed that the transcription of BAF is significantly reduced in the LINC mutants *klar* and *koi* in *Drosophila* larval muscles, and in addition, knockdown of BAF in these muscles led to elevated DNA content in the myonuclei (Wang et al., 2018). We used anti BAF antibodies (Furukawa et al., 2003) to reveal the cellular distribution of BAF protein in the LINC mutant larval muscles. In control myonuclei BAF accumulated at the nuclear envelope, overlapping lamin C (Fig 1 A, A”, D, D’), and in addition at the nucleolus borders as well as partially overlapping the microtubules (Fig 1 A, and Fig S1 A, B). Importantly, BAF labeling at the nuclear envelope was specifically eliminated in the LINC mutants, including either *klaroid (koi)*, lacking *Drosophila* SUN protein, or in double homozygous mutants of *klar* and *Msp300* alleles, lacking only the KASH domain (*klar;Msp300*) (Fig 1 B-C”, and E, E’, F, F’). Together, these mutants represent the entire repertoire of *Drosophila* LINC complex genes. BAF localization at the nucleolus borders did not change in the LINC mutants, (Fig 1 B-C”, E, F). Quantification of BAF-positive fluorescent voxels within the entire nuclear volume defined by lamin C borders (see Materials and Methods), normalized to nuclear volume, indicated a statistically significant reduction in the fluorescent levels of nuclear BAF, in both LINC mutants (Fig 1G). In addition, we calculated the ratio between BAF fluorescence at the nuclear envelope relative to its cytoplasmic levels, or alternatively, to its levels at the nucleoplasm (Fig 1 H and I respectively). In both
cases, a significant decrease in the relative localization of BAF at the nuclear envelope was observed. The difference between each of the mutant group relative to control was statistically significant (\(p<0.0001\)). Taken together, these results implied that the specific localization of BAF protein at the nuclear envelope requires a functional LINC complex.

Previously we showed that BAF transcription is reduced in \textit{koi}, and \textit{klar} LINC mutants (Wang et al., 2018). To exclude a possible effect of BAF reduction on its localization at the nuclear envelope, we attempted to overexpress BAF in muscles of \textit{koi} mutants. However, despite a general increase in its cytoplasmic levels, BAF overexpression in \textit{koi} mutant muscles did not rescue its localization at the nuclear envelope (Fig 2 A-B'', C, D). We noticed, however that lamin C distribution was broader in the myonuclei overexpressing BAF (Fig 2 C, D). Overexpression of BAF in control muscles did not affect BAF localization, nor did it affect lamin C distribution (Fig S2).

Taken together, these results show that BAF protein localization at the nuclear envelope depends on a functional LINC complex.

\textbf{The LINC complex is required for localization of the LEM-domain protein Otefin at the nuclear envelope}

Previous studies indicated that BAF localization at the nuclear envelope is mediated by its binding to LEM domain proteins (Cai et al., 2001; Mansharamani and Wilson, 2005). \textit{Drosophila} has three proteins with LEM domain (LEM-D), including Otefin (Ote), Bocksbeutel (Bocks), and dMAN1, shown to function throughout \textit{Drosophila} development, often redundantly (Barton et al., 2014). To address whether BAF exclusion from the nuclear envelope observed in the LINC complex mutants was downstream of a loss of LEM-D proteins, we analyzed the localization of Ote protein in the LINC mutant myonuclei. Labeling with anti Ote antibodies indicated its specific localization at the nuclear envelope of the myonuclei (Fig 3 A-A''' and D). Significantly, a substantial reduction of Ote levels at the nuclear envelope was observed in both LINC mutants myonuclei, \textit{koi} and \textit{klar};\textit{Msp300} (Fig 3, B-C'', D, and E). A significant difference between each mutant group and control was observed (\(p<0.0001\)). Assuming that BAF binds directly to Ote (as described in other organisms), its decreased distribution at the nuclear envelope is downstream of both the LINC complex and Otefin.
**BAF localization at the nuclear envelope depends on association of nuclei with muscle sarcomeres**

Our previous studies indicated that myonuclei associate with the muscle sarcomeres via a functional interaction between D-Titin (Sallimus or Sls) and Msp300 proteins (Elhanany-Tamir et al., 2012). In control larval preps, myonuclear shape is ellipsoidal at the X-Y axis, and extremely flat at the Z-axis (Fig 4A). Temporal, muscle-specific, knockdown of D-titin/sls, induced at third instar larvae (using sls RNAi combined with Gal4, and Gal80ts drivers), caused partial detachment of the myonuclei from the sarcomeres. In these larvae a significant deformation of nuclear morphology was observed, in which the nuclei become relatively more spheroid, indicative of changes in the mechanical environment of these nuclei (Fig 4 A, B, see also (Wang et al., 2015)). Importantly, BAF dissociated specifically from the nuclear envelope in the sls knocked down muscles, whereas its localization around the nucleolus remained unchanged (Fig 4 C-D'', and E,F). Measurements of BAF levels at the nuclear envelope (Fig 4 G), as well as quantification of the ratio between BAF levels at the nuclear envelope relative to its levels in the cytoplasm (Fig 4 H), or to the nucleoplasm (Fig 4 I), indicated a significant and specific decrease in BAF levels at the nuclear envelope. A statistically significant difference between the experimental group and control (p<0.0001) was observed. These results indicated that BAF localization at the nuclear envelope depends on proper connection between the myonuclei and sarcomeres.

**The serine/threonine kinase Vrk1/Ballchen regulates BAF localization at the nuclear envelope**

Previous reports indicated that BAF is phosphorylated by the nuclear serine/threonine kinase Ballchen (Ball), a homolog of vertebrates Vrk1 (Bengtsson and Wilson, 2006; Lancaster et al., 2007). We first analyzed the localization of Ball in muscle fibers, using specific antibodies (Herzig et al., 2014). Consistent with previous reports Ball labeling was specifically observed within the myonuclei as well as along the sarcomeres (Fig 5 A,A' and also Fig S3). Fig 5 B-B" shows co-labeling of Ball with Lamin C, using Expansion microscopy (Chen et al., 2015; Jiang et al., 2018). This procedure allows a roughly 4-fold increase in tissue size, while preserving its 3D constituents, allowing high resolution imaging. A fine line of Ball labeling overlapped that of Lamin C (Fig 5 B-B" arrows), indicating that in addition to its localization in the nucleoplasm, Vrk1/Ball
is localized at the nuclear envelope. To address whether Ball controls the localization of BAF at the nuclear envelope, we knocked down ball in muscles using ball RNAi, (Fig S3 indicates the efficiency of the RNAi). Notably, reduction of Ball in the muscles led to a significant decrease in BAF localization at the nuclear envelope (Fig 5 C-D”, E, F and quantified in G). The difference between the groups was statistically significant (p<0.0001). Interestingly, myonuclei of the ball knock-down muscles exhibited often an effect on nuclear position, partially phenocopying the nuclear phenotype of the LINC mutants. This implied that Ball is required for BAF localization at the nuclear envelope, presumably by promoting BAF phosphorylation. Furthermore, muscle-specific expression of either GFP-BAF, phospho-mutant BAF, in which the phosphorylated Serine-Threonine residues in its N-terminal end have been mutated to Alanine (GFP-BAF-3A), or phospho-mimic GFP-BAF (GFP-BAF-3D) (Hou et al., 2016) was performed. The GFP-BAF was localized at the nuclear membrane, and also in the cytoplasm, similarly to the endogenous BAF, (Fig 6 A-A”). However, the non-phosphorytable version of BAF, GFP-BAF-3A, was eliminated from the nuclear envelope and accumulated in the nucleoplasm (Fig 6 B-B”). Importantly, 100% of the larvae expressing the non-phosphorytable GFP-BAF-3A form were extremely sick and did not develop further into pupal stage. The phospho-mimicking BAF (GFP-BAF-3D) did show a specific localization of the GFP at the nuclear envelope and increased levels in the cytoplasm and the nucleoplasm (Fig 6 C-C”). The requirement of Ball kinase for BAF localization at the nuclear envelope, and elimination of the phospho-mutant BAF from the nuclear envelope indicated that BAF phosphorylation is critical for its localization at the nuclear envelope.

**Detachment of BAF from the nuclear envelope correlates with increased DNA levels in the myonuclei**

Previously we demonstrated that the LINC mutant muscles exhibited an increases in DNA endoreplication, leading to elevated DNA content in the myonuclei (Wang et al., 2018). We wished to address whether the localization of BAF at the nuclear envelope correlates with increased endoreplication and elevated DNA content in the myonuclei. Towards this end the DNA content in myonuclei of baf, sls, and ball knocked down muscles, was quantified. The larvae in each of these experiments were staged, fixed and labeled with lamin C and DAPI, in parallel to control larvae. Quantification of the DNA content indicated that the DNA content increased in all three experimental
conditions, relative to control (Fig 7, A, B, C p<0.0001 for each experiment). Of note, the baf RNAi line that we used led to reduction in the protein and mRNA levels of BAF (Fig S1 E, F, G, H, and I). Furthermore, the reduction of BAF was observed in all subcellular localizations, including the cytoplasm, nuclear envelope, and nucleolus (Fig S1 E-H). We followed the viability and developmental timing of 50 staged embryos expressing BAF RNAi in the muscles and compared it to control. No detectable defects in larval developmental timing nor in the percentage of flies that eclosed relative to control were observed, suggesting that the elevation in DNA content in the larval muscles did not abrogate muscle function.

For the sls RNAi larvae (Fig 7 B), driven by Mef2Gal4 combined with Gal80ts, larvae were raised at permissive temperature up to second instar stage and then transferred to restrictive temperature to allow temporal expression of the RNAi at 3rd instar larval stage. Taken together, these experiments suggested a functional correlation between dissociation of BAF from the nuclear envelope and an increase in the DNA content of the myonuclei.

To reveal a possible molecular explanation between BAF localization at the nuclear membrane and DNA endoreplication, we focused on E2F1, a key transcription factor, which regulates DNA endoreplication in flies (Zielke et al., 2011). Expansion microscopy was used to analyze the fine subcellular localization of E2F1 in the myonuclei at high resolution. Notably, we found that E2F1 accumulated at the nuclear envelope, overlapping BAF, and lamin C (Fig 7 D-G). BAF labeling at the nucleolus borders was lost in this method, possibly due to the procedure used for expansion microscopy. The specific localization of E2F1 at the nuclear envelope, and its overlapping distribution with BAF, proposed a functional coupling between both proteins.

*Increased levels of E2F1 in the nucleoplasm correlate with decreased BAF at the nuclear envelope*

Next we analyzed the levels of E2F1 in myonuclei in which BAF levels were knocked down by RNAi. Notably, E2F1 levels were significantly higher in the nucleoplasm of myonuclei in larvae expressing baf RNAi (Fig 8, compare B-B''' to control A-A''', and quantified in C). A statistically significant increase in E2F1 nuclear levels was observed in the baf RNAi expressing larvae relative to control (p<0.0001). These results suggested an inhibitory function for BAF on the nuclear accumulation of E2F1, that was consistent with the increase in DNA content in baf knockdown myonuclei.
Next, we analyzed the levels of E2F1 in the nucleoplasm in ball knocked down muscles. Significantly, E2F1 levels were specifically elevated in the myonuclei, concomitant with a decrease in the levels of E2F1 at the nuclear envelope (Fig 8 compare E-E’’’ to control in D-D’’’’, and quantified in F). The difference between the groups was statistically significant (p<0.0001). These results are consistent with an inhibitory function for Ball on E2F1 nuclear levels.

In summary, our results suggest a model in which in mature non-dividing muscle fibers the LINC complex, promotes proper localization of the LEM protein Otefin at the nuclear envelope. Ball kinase phosphorylates BAF, promoting its binding to Otefin (Fig 9 A). BAF at the nuclear envelope is essential for accumulation of E2F1 at the nuclear envelope, and for reducing its levels in the nucleoplasm (Fig 9 B). Assuming that E2F1 is a limiting factor in promoting endoreplication in the muscles, its increase in the nucleoplasm would promote DNA synthesis (endoreplication) in the myonuclei.

**Discussion**

Endoreplication is used by differentiated cells as a strategy to grow in size, and to accommodate tissue injury. Coupling between endoreplication process and the mechanical environment of the cell allows the nucleus to respond to mechanical changes following tissue injury without a need for cell division. This is especially true in myofibers, where reiterated mechanical inputs are applied on the nuclear envelope during muscle contractile waves. Here, we demonstrate the contribution of a novel mechanosensitive component, BAF for controlling the nuclear accumulation of E2F1, a critical transcription factor required for the regulation of endoreplication (Huang et al., 2005; van den Heuvel and Dyson, 2008; Zielke et al., 2011). Whereas previous reports implicated BAF in promoting the condensation and assembly of post-mitotic dsDNA into single nuclei (Samwer et al., 2017), here we demonstrate that BAF is also essential for the arrest of DNA endoreplication in fully differentiated muscle fibers. Importantly, only BAF that localizes to the nuclear envelope, appears to be relevant for this function in post-mitotic differentiated cells. The contribution of BAF to larval muscle functionality is unclear, as baf mutants do not survive up to 3rd instar larvae, and BAF knockdown in muscles using RNAi did not eliminated BAF very efficiently.
In the *Drosophila* muscle fibers, we found that BAF was detected in various subcellular sites, including the cytoplasm, the nuclear envelope, the nucleoplasm and at the nucleolus borders. Yet, only the portion of BAF localized at the nuclear envelope was found to change following elimination of a functional LINC complex. It is well accepted that LINC complex transmits cytoplasmic mechanical inputs from the cytoskeleton to the nucleoskeleton in various cell types (Chambliss et al., 2013) (Guilluy et al., 2014). Moreover, nuclear deformations (from oval into spheroid shape) observed in both larval muscles of LINC complex mutants (Elhanany-Tamir et al., 2012; Wang et al., 2015), as well as in conditions where nuclei detached from the sarcomeres (e.g. Wang et al., 2015), or following SIs knockdown) are indicative of changes in the mechanical inputs applied on the nuclear envelope. Since in both conditions BAF localization at the nuclear envelope was specifically impaired, we propose that maintenance of BAF at the nuclear envelope is mechanically sensitive.

In control myofibers, BAF exhibited a relatively broad distribution along the outlines of the nuclear envelope, often extending beyond the lamin C expression domain towards the cytoplasm, overlapping with the nuclear associated microtubules (Fig S1, C, D). This suggested that in addition to its association with the inner aspects of the nuclear membrane through binding to LEM-domain proteins and lamin A/C (Cai et al., 2001; Jamin and Wiebe, 2015; Shimi et al., 2004; Wilson et al., 2005), BAF associates with the outer aspects of the nuclear membrane. Previous experiments indicated that despite its small size BAF does not diffuse passively from the cytoplasm to the nucleus (Shimi et al., 2004). Furthermore, photobleaching experiments with GFP-BAF indicated that BAF-dependent repair of nuclear ruptures occurs when cytoplasmic BAF, but not nuclear BAF rapidly associates with the ruptured sites and further recruit LEM domain proteins to establish membrane sealing (Halfmann et al., 2019). The authors suggest that their findings are consistent with a dynamic exchange of BAF between cytoplasmic and nuclear pools, where BAF in the cytoplasm primarily responds to mechanical signals. Since our experiments indicated that BAF phosphorylation is critical for its maintenance at the nuclear membrane, it is possible that the exchange of BAF localization between the cytoplasm and the nucleus is stabilized by its phosphorylation. The contribution of the LINC complex to BAF association with the nuclear envelope could be either direct, e.g. by binding to components of the LINC complex, or indirect, e.g. through an effect of the LINC complex on the distribution of LEM proteins at the nuclear envelope. Our results
support the latter model in which the LINC complex maintains the localization of the LEM protein Otefin at the nuclear envelope, to mediate BAF association with the nuclear envelope. Hence, we suggest a model in which the contribution of the LINC complex to BAF localization at the nuclear envelope is through an effect on Otefin localization at the nuclear envelope (see Figure 9).

Endoreplication has been implicated in a wide variety of differentiated cells in a broad range of species, including human tissues (Edgar and Orr-Weaver, 2001; Fox and Duronio, 2013; Gan et al., 2019; Lee et al., 2009; Losick et al., 2013; Zielke et al., 2013). A link between mechanical tension and endoreplication has been recently suggested (Cao et al., 2017). However, the molecular mechanism coupling mechanical tension with endoreplication process is still elusive. Here, we found that a key regulator of endoreplication, E2F1 exhibits a specific distribution at the nuclear envelope in fully differentiated myofibers, where it probably resides non-active. Changes in the mechanical environment of the nuclear envelope correlated with the localization of E2F1 and promoted its accumulation within the nucleoplasm, where it is expected to promote DNA synthesis. It will be of interest to find which proteins associate directly with E2F1 at the nuclear envelope. Our attempts to co-immunoprecipitate BAF, with Msp300 or with E2F1 failed to show a specific protein interaction between these proteins. From physiological point of view, no detectable change in muscle size, or movement was observed in the baf KD muscles, and the larvae developed up to adult stage. baf homozygous mutant did not develop up to the 3rd instar larval stage, so the full physiological contribution of BAF to muscle growth awaits experiments in which a more efficient reduction of BAF levels in the muscle tissue will be induced.

In summary, our results reveal novel insight into the role of the LINC complex in coupling endoreplication with changes of the nuclear envelope composition in mature muscle fibers. In particular, the mechano-sensitive component, BAF, whose localization at the nuclear envelope is tightly regulated by the LINC complex, is shown to negatively control the nuclear accumulation of the cell cycle regulator E2F1, at the level of the nuclear envelope. The localization of Otefin in the nuclear envelope, as well as BAF phosphorylation by VRK1/Ball kinase are critical in this context. This process might be part of a mechanosensitive pathway that regulates polyplody in a wide variety of differentiated cells.
Materials and methods

Fly stocks and husbandry
All crosses were performed at 25°C and rose on cornmeal yeast agar. The following stocks were distributed by the Bloomington Drosophila Stock Center: tubP-Gal80ts/TM2 (FBst0007017), Gal4-Mef2.R (FBst0027390), baf-RNAi (FBst0036108), sls RNAi (FBgn0086906), ball RNAi (FBti0130755). Ball-GFP was obtained from Vienna BioCenter (VDRC): FlyFos016090 (pRedFlp-Hgr)(ball[41529]::2XTY1-SGFP-V5-preTEV-BLRP-3XFLAG)dFRT. UAS-GFP-BAF, UAS-GFP-BAF-3A (obtained from Lei Liu, Beijing Institute for Brain Disorders, Beijing, China). klarΔKASH(mCD4) and Msp300ΔKASH (J.A. Fischer, University of Texas, Austin, TX) were combined on Cyoy+YFP and TM6Tb balancers, koi84(FBst0025105)/Cyo-dfd-eYfp (Kracklauer et al., 2007).

Staging of the larvae was performed by 6 hours embryo collection and further growing on yeast past in vials up to early third instar stage. Temporal expression of sls RNAi or GFP-BAF, as well as GFP-BAF-3A was performed by using a combination of Mef2Gal4, tubGal80ts drivers, as follows: embryos collection was performed at 25°C for 6 hours, then embryos were transferred to permissive temperature 18°C up to first instar larval stage, and then larvae were transferred to restrictive temperature of 29°C, up to early third instar stage.

Immunofluorescence
Immunofluorescent staining was performed as previously described (Wang et al., 2015). For fixation, paraformaldehyde (4% from 16% stock of EM grade; #15710; Electron Microscopy Sciences) was used without methanol to avoid damage of native F-actin or chromosomal morphology. Specimens were fixed for approximately 30 minutes and subsequently washed several times in PBS with 0.1% Triton X-100 (PBST) on a horizontal shaker with gentle agitation. Image analyses were consistently performed on muscle 7. All specimens were mounted in Thermo Scientific Shandon ImmuMount for microscopy (Thermo Fisher Scientific). Control and experiment larvae were staged and were grown in parallel time intervals. Fixation and antibody staining of control and experiment larvae were done at the same tube marking one group by head excision.
**Antibodies and synthetic dyes**

Mouse anti-lamin C (DSHB, no. LC28.26-c) obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa. Rat anti-Tubulin alpha (1:200 dilution) (Bio-Rad MCA78G), Chicken anti-GFP (1:200 dilution) (Abcam #13970), Rabbit anti-BAF (1:300 dilution) (provided by Paul Fisher, Stony Brook, NY, and Ryszard Rzepecki, University of Wroclaw, Poland, (Furukawa et al., 2003), Rat anti-E2F1 (1:200 dilution) was used for immunofluorescence and were provided by provided by Stefan Thor (University of Queensland, Australia), (Baumgardt et al., 2014), and Jonathan Benito-Sipos, (University of Madrid). Rabbit anti Otefin (1:300 dilution) were provided by Y. Gruenbaum (HuJ, Israel). Secondary antibodies used: Alexa Fluor 488, 555 and 647 conjugated secondary antibodies against Rat, Chick, Rabbit, and mouse were purchased from The Jackson Laboratory and Thermo Fisher Scientific and were used at 1:300 dilution).

For labeling of the chromatin we used DAPI (1 µg/ml; Sigma-Aldrich). For F-actin labeling we used TRITC-Phalloidin (SIGMA-ALDRICH P1951). Labeling was performed by exposing the larvae from experimental groups and control to similar antibody mix at the same tube.

**Expansion microscopy**

The procedure was essentially as described (Jiang et al., 2018). Briefly, larvae were fixed with 4% PFA in PBS, washed with PBST, blocked with 10% BSA in PBST, incubated with first antibody overnight, and with secondary antibodies (conjugated with either Atto 647, AF555, AF488) overnight. After wash with PBST, and PBS the larvae were incubated with 1mM MA-NHSin PBS for 1hour, washed with PBS, incubated with monomer solution (2M NaCl, 8.625% Sodium Acrylate, 2.5% Acrylamide, 0.15% Bisacrylamide, 10X PBS) for 45 min at 4°C, and then transferred to gelation solution (0.2% TEMED, 0.01% TEMPO, 95% monomer solution, and 0.2% APS), for 30 min at 37°C. The gel with the larvae was incubated with chitinase (1Unit/mL) in PBS pH 6.0, 4 days at 37°C. Three wash with PBS, were followed by the addition of collagenase (1mg/mL) in 1x HBSS (w/0.01 M CaCl2, and 0.01 M MgCl2), and incubated at 37°C O.N. Washes with PBS were followed by addition of Proteinase K (8Units/mL) in digestion buffer at 37°C, for 1 Hour and additional washes with PBS. Hoechst was added for 10 min, washed and expansion was performed by the addition
of ultrapure water for 30 min. The sample was then imaged under the confocal microscope.

**Microscopy and image analysis**

Microscopic images were acquired at 23°C on confocal microscopes Zeiss LSM 800 with the following lenses: Zeiss C-Apochromat 40x/1.20 W Korr M27, 20x PlanApochromat 20/0.8. The microscopic samples were embedded with Coverslip High Precision 1.5H ± 5 μm (Marienfeld-Superior, Lauda-Königshofen, Germany). Immersion medium Immersol W 2010 (ne = 1.3339) and Immersion oil Immersol 518 F (ne = 1.518) were used, respectively. Images were analyzed with Fiji including plugins and adapted scripts. Figure panels were finally assembled using Photoshop CC 2019. Acquisition software Zen 2.3 (blue edition).

**Data Collection**

Quantification of fluorescence integrated density of proteins in myonuclei was performed using a custom-built FIJI macro which included rolling ball background subtraction. The macro has been deposited in GitHub DOI 10.5281/zenodo.3372266. In brief, lamin C was used to define the entire nuclear volume as the region of interest (ROI). In each myofibril integrated fluorescent density in different channels were measured in all the slices along the Z-direction of the ROI. This allowed us to measure the levels of specific proteins inside the myonuclei in an unbiased manner. For measuring BAF levels along the nuclear envelope, we used the manual selection tool for the lamin C channel to define the nuclear membrane as the ROI. The fluorescence integrated density of BAF along the Z-direction of the ROI was then analyzed. To assess BAF localization at the nuclear envelop in each myofibril, the nuclear envelope of myonuclei was marked using the ImageJ ‘composite Selection tool’ for lamin C channel to define the nuclear envelope as the ROI and integrated fluorescent density was measured in all the slices along the Z-direction of the ROI. The ratio of BAF along cytoplasm and nucleoplasm with respect to the nuclear envelope was measured using the line profile tool (Zeiss, Zen). BAF fluorescence at the nuclear envelope, cytoplasm, and nucleoplasm was measured along a line profile in a single, middle Z section.
**Statistical analysis**

Statistical analysis was performed using GraphPad Prism and Microsoft Excel 2016. Measurements were analyzed using a two-independent samples t-test. P-values <0.05 were considered statistically significant. BoxPlotR was used to generate box plots (Spitzer et al., 2014), in which the center lines represent the medians, box limits indicate 25th and 75th percentiles, crosses represent sample means, and outliers (data points beyond the 95% confidence intervals) are represented by dots. Furthermore, whiskers, determined using the Tukey method, extended to data points less than 1.5 interquartile ranges from the 1st and 3rd quartiles as determined by the BoxPlotR software. All experiments were repeated 3 times independently and in at least 4-5 randomly selected larvae from which muscle no. 7 was monitored in at least 4 abdominal segments, per experimental group.

**RT-qPCR**

Gene expression quantification was carried out by reverse transcription quantitative real time PCR (RT-qPCR). Briefly, total muscle RNA was isolated from larval body walls of 30 dissected Drosophila individuals using RNeasy Protect Mini Kit (Qiagen). 1 μg of total RNA were used for first strand cDNA synthesis by reverse transcription using SuperScript Reverse Transcriptase IV (Invitrogen) with oligonucleotide dT primers. qPCR was performed using gene specific primers on the ABI 7500 Real-Time PCR System (Applied Biosystems) with Fast SYBR Green Master Mix (Applied Biosystems) for detection. Each sample was run in triplicate. The primer sequences are listed in Table (see supplementary information Table 1). The difference in gene expression was calculated using the fold change (ΔΔCt method) (Schmittgen and Livak, 2008). ΔCt is the Ct value for the gene of interest normalized to the Ct value of the respective gene in both control larvae (armadillo-Gal4/YW) and BAF knockdown (armadillo-Gal4>baf RNAi). ΔΔCt values were calculated as a relative change in ΔCt of the target gene in BAF knockdown with respect to control - house-keeping gene succinate dehydrogenase (SDH). Fold changes were expressed as $2^{-\Delta\Delta Ct}$ for up-regulated genes and the negative reciprocal of the fold change for down-regulated genes (where $2^{-\Delta\Delta Ct} < 1$).
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**Figure 1: BAF dissociates from the nuclear envelope in the LINC mutant muscles**

Representative larval muscle no. 7 of control (Mef2-Gal4/YW, A-A''), or LINC mutants, including either klaroid/SUN (koi) (B-B''), or a double mutant combination of klar\textsuperscript{ΔKASH} and Msp\textsuperscript{ΔKASH} (klar;Msp300), labeled with anti-BAF and DAPI (red, blue A,B,C), lamin C and DAPI (green, blue A',B',C') and their merged images (A'',B'',C''). Arrows indicate
the nucleolus, arrowheads indicate the nuclear membrane. D) Representative line profiles of BAF (red) and lamin C (green), taken at the middle of each myonucleus of control (D) koi (E) or klarMsp300 mutants (F) and their corresponding nuclei (D’, E’, F’). Arrowheads in D, E, F indicate the lamin peaks at the nuclear envelope borders. All images represent single confocal Z stacks. G) Quantification of the fluorescence integrated density of BAF per nucleus in control, koi, klarMsp300 mutant myonuclei. Quantification in Fig 1 G, H, I, was calculated from n=66 myonuclei of control, n=136 myonuclei of koi mutants, and n=74 myonuclei of klarMsp300 mutants. One tailed t test for each of the LINC mutant groups relative to control groups indicates a significant difference between control and mutant groups p<0.0001 (****). H) The ratio between BAF fluorescence at the nuclear envelope and BAF fluorescence at the cytoplasm. I) The ratio between BAF fluorescence at the nuclear envelope and BAF fluorescence at the nucleoplasm. Each of the experiments was repeated 3 times, indicating similar trends. Bars in all images indicate 10μm.
**Figure 2: Overexpression of BAF in koi mutant muscles does not rescue BAF localization at the nuclear envelope**

Representative larval muscle no. 7 of homozygous *koi* mutant (A-A''), or *koi* mutant overexpressing BAF in muscles under the control of *Mef2Gal4* driver (B-B''), labeled with anti-BAF and DAPI (red, blue A, A'', B, B''), anti-lamin C (green, A', A'', B', B''), (blue, A, A'', B, B'') and their merged images (A'', B''). Arrowheads indicate the nuclear envelope border. The line profiles of a single nucleus of *koi* (C, arrowheads in A-A'', shown also in the right panel of C indicate the nucleus analyzed), or *koi* mutant overexpressing BAF (D, arrowheads in B-B'', shown also in the right panel of D indicate the nucleus analyzed. All images represent a single confocal Z stack. Bar indicates 10 μm.
**Figure 3: Otefin levels at the nuclear envelope decrease in the LINC complex mutants**

Representative larval muscle no. 7 of control (A-A’’, D upper panel), koi (B-B’’, D middle panel), or klar;Msp300 mutants (C-C’’, D lower panel), labeled with anti Otefin (red, A, B, C), anti Lamin C (green, A’, B’, C’), and DAPI (blue, A’’, B’’, C’’) or their merged images (A’’, B’’, C’’). The line profiles of the nuclei indicated by arrows in A, B, C are presented in D, where on the right of each profile the corresponding image of the nucleus analyzed is shown. E – Quantitative analysis of the fluorescent integrated density of Otefin for each group is presented. The analysis in (E) was based on n=69 of control nuclei, n=75 of koi nuclei, and n=72 of klar;Msp300 nuclei. A significant fluorescent reduction in Otefin distribution at the nucleus (one tailed t test p<0.0001 marked by *** ) is observed in the LINC mutants. Asterisks show a significant difference between the groups. Bar indicates 10μm.
Figure 4: Partial detachment of nuclear-sarcomeres connections promotes the removal of BAF from the nuclear envelope

Representative larval muscle no. 7 of control (Mef2Gal4, Gal80ts, A, C-C’’ and E), or larvae in which D-titin/sls was knocked down temporally at second instar larvae (Mef2Gal4, Gal80ts->D-titin/sls, B, D-D’’, and F). Labeling with Phalloidin (red) and Lamin C (green) in A, B indicates partial detachment of the nuclei from the sarcomeres and nuclear deformations (see orthogonal view in upper panels in A and B, in which the red line in each of the bottom panels indicates the position of the orthogonal optical section shown in the upper panels, and the blue line in the upper panels represents the position of the X-Y optical section shown in the bottom panels). C, D - labeling of BAF (red) and DAPI (green) of control (C-C’’) or larvae in which D-titin/sls was temporally knocked down (D-D’’). Arrowheads indicate BAF at the nuclear envelope. E, F line profile of Lamin C and BAF fluorescence of myonuclei of control (E), or larvae
in which D-titin/sls was temporally knocked down (F), and their corresponding nuclei shown in the right panels. Images (A-D’’) represent a single confocal Z stack. G- Quantification of BAF fluorescence at the nuclear envelope of control and in larvae in which D-titin/sls was temporally knocked down. Quantification was based on 72 myonuclei (control), and 66 myonuclei of larvae expressing sls-RNAi. H- The ratio between BAF fluorescence at the nuclear envelope and its levels in the cytoplasm in control or in larvae in which D-titin/sls was temporally knocked down. I- The ratio between BAF fluorescence at the nuclear envelope and its levels in the nucleoplasm in control or in larvae in which D-titin/sls was temporally knocked down. One tailed t test for each of the groups in G, H, and I indicated p<0.0001. Each of the experiments was repeated 3 times, and indicated a similar trend. Bars indicate 10µm.
Figure 5: BAF kinase Vrk1/Ball is required for BAF localization at the nuclear envelope

Larval muscle no. 7 labeled with anti Vrk1/Ball (A, green) or their merged image with lamin (blue) and Vrk/Ball (green) (A’). B-B’’ - High resolution image of control myonucleus labeled with anti Vrk1/Ball antibody (B, green) and lamin C (B’, blue), and their merged image (B’’) using expansion microscopy, B’’’: Line profile of the nucleus shown in B’’ and also in the right panel, with arrows indicating a pick of Ball labeling that overlaps with lamin C. C-D’’’ - Larval myonuclei from control (Mef2Gal4/YW, C-
C’’) or from muscles in which Vrk1/ball was knocked down by RNAi (Mef2Gal4>Vrk1/ball RNAi, D-D’’), labeled with anti-BAF (green, C, D), anti-lamin C (blue, C, D’), and DAPI (white, C’’, D’’) and their merged images (C’’’, D’’’). A significant decrease in the levels of BAF at the nuclear envelope following knockdown of Vrk1/Ball is demonstrated (C, D). E, F – line profiles of control (E) or ball knockdown (F) muscles with their corresponding nuclei images at the right panels are shown. Arrowheads show the lamin C picks (blue). G - Quantification of BAF fluorescence integrated density at the outlines of the nuclear envelope indicate a significant decrease of BAF at the nuclear envelope (one tailed t test indicates p<0.0001). Quantification was based on n=118 of control myonuclei, and n=69 myonuclei of larvae expressing ball RNAi. Each of the experiments was repeated 3 times, and indicated a similar trend. Images in all panels represent a single confocal Z stack. Bars indicate 10µm.
**Figure 6: GFP-BAF phospho-mutants localize differentially at the myonuclei**

Representative images of larval muscle no. 7 of larvae overexpressing either GFP-BAF (A-A’’’), GFP-BAF-3A (non-phosphorylatable BAF, B-B’’’), or GFP-BAF-3D (phospho-mimicking BAF, C-C’’’), labeled with anti GFP (green) and anti NPC (FG repeats, red). Line profiles of the images indicated by the corresponding empty arrows in A, B, C) are shown in A’’’, B’’’, C’’’. The image of the corresponding nucleus is shown in A’’, B’’, and C’’. The filled arrows indicate the picks of the nuclear membrane. Bar indicates 10μm.
Figure 7: Removal of BAF from the nuclear envelope correlates with increased DNA levels in the myonuclei

Quantification of the DNA content per myonucleus performed by measuring DAPI integrated density in myonuclei is demonstrated following muscle-specific knockdown of baf (A), (Quantification of DAPI fluorescence in myonuclei of baf RNAi was based on n=150 of control myonuclei, and n=106 of baf RNAi myonuclei), sls (B) (Quantification of the DAPI fluorescence was based on n=72 myonuclei of control and n=66 myonuclei of sls RNAi), and ball (C) Quantification was based on n=118 of control myonuclei, and n=69 nuclei of ball RNAi). The difference between the experimental groups and control in A, B, C was statistically significant, with one tailed \( t \) test \( p < 0.0001 \). Each of the experiments was repeated 3 times, and indicated a similar trend. D-G- High resolution images of the nuclear localization of E2F1 in myonuclei, using expansion microscopy. E2F1 (D), BAF (E), Lamin C (F), and their merged images (G) are shown. Arrows indicate co-localization of E2F1, BAF, and Lamin C, at the nuclear envelope. Bar indicates 20\( \mu \)m.
Figure 8: The levels of E2F1 in the nucleoplasm increase following knockdown of baf or vrk/ball

Myonuclei from control (A-A''), or baf knockdown muscles (B-B''), labeled with anti E2F1 (red, A, B, A'', B'') and Lamin C (green, A', B', A'', B'') and their merged images labeled also with DAPI (A'', B''). Arrows indicate the nuclear envelope. Line profiles of E2F1 and Lamin C are shown in A'', and B'', include their corresponding nuclei at...
the right panel. A specific localization of E2F1 at the nuclear envelope in control, and its enrichment in the nucleoplasm in *baf* KD muscles is noted. C- Quantification of the fluorescent integrated density of E2F1 fluorescent levels in the entire nuclear volume indicates a significant increase in E2F1 levels in the nucleoplasm of *baf* knockdown myonuclei. Quantification of E2F1 fluorescence was based on n=150 control myonuclei, and n=106 myonuclei of *baf*-RNAi expressing larvae. One tailed *t* test indicated p<0.0001. Bar indicates 10μm. D-E'': myonuclei from control (D-D''), or *ball* knockdown muscles (E-E''), labeled with anti E2F1 (red, D, E), Lamin C (green, D', E') and their merged images (D'', E'') labeled also with DAPI (blue) are shown. Empty arrows indicate E2F1 levels in the nucleoplasm (A,B, D, E). Line profiles of E2F1 and Lamin C are shown in D''', and E''', indicating the specific localization of E2F1 at the nuclear envelope in control and its accumulation in the nucleoplasm of *ball* KD muscles. Filled arrows indicate the nuclear envelope borders. F- Quantification of the fluorescent integrated density of E2F1 levels in the entire nuclear volume of *ball* knockdown muscles indicates a significant increase in E2F1 levels in *ball* KD myonuclei. Quantification in Fig 8 F was based on n=100 myonuclei of control and n=126 myonuclei of larvae expressing *ball* RNAi. One tailed *t* test indicates p<0.0001. Bar indicates 10μm.
**Figure 9: Proposed model for the coupling between LINC-dependent localization of BAF at the nuclear envelope and endoreplication**

A – The LINC complex, composed of Nesprin that associates with the microtubule cytoskeleton at its N-terminal end and binds to SUN domain proteins at the perinuclear space via its KASH domain at its C-terminal end is indicated. The LINC complex maintains the localization of the LEM domain protein Otefin at the nuclear envelope. Otefin recruits and binds to phosphorylated BAF, thus promoting BAF localization at the nuclear envelope. BAF phosphorylation is induced by the kinase Vrk1/Ball, which is found both at the nucleoplasm as well as at the nuclear envelope. B – E2F1 accumulates at the nuclear envelope through BAF at the nuclear envelope. E2F1
accumulation at the nuclear envelope lowers its free increase at the nucleoplasm. Decreased E2F1 levels in the nucleoplasm attenuate cell cycle progression and DNA endoreplication.
Figure S1: Evaluation of BAF antibody specificity

A single myonucleus labeled with anti BAF (red, A), and (B) a merged image of this nucleus labeled with BAF (red) Fibrilarin (green), and DAPI (blue). A single myonucleus labeled with BAF (red, C) and (D) a merged image of this nucleus labeled with BAF (red), α-Tubulin (green) and DAPI (blue).

Two nuclei of larval muscle (no. 7) labeled with anti BAF (red,E-H), or their merged images together with lamin C (green F, H), of control (Mef2Gal4/YW), or baf-RNAi (Mef2Gal4>baf-RNAi) larvae. Note a specific reduction in BAF labeling. 1) qPCR analysis using baf primers, as well as with primers for house-keeping gene succinate dehydrogenase (SDH) (for normalization), of control larvae (armGal4/YW), or baf RNAi larvae (armGal4>baf-RNAi). This analysis indicated a 5 fold reduction in baf mRNA levels.
Figure S2: Overexpression of BAF in muscles does not alter its localization at the nuclear envelope

Muscle nuclei from control larvae (MefGal4/YW, A, A’) or larvae overexpressing BAF (B, B’), labeled with anti BAF (red, A, B), or their merged images with lamin C (A’, B’ green). BAF subcellular localization does not change, following its overexpression in muscles.
Figure S3: Specificity of Ball antibody labeling
Myonuclei form control larvae labeled with anti Ball antibody (green, A) and Lamin C (blue, A’) indicating a specific localization of Ball in the myonuclei. Larvae expressing ball RNAi in muscles and labeled with anti Ball (green, B), and with Lamin C (blue, B’). A significant reduction of Ball labeling in the myonuclei is demonstrated. C – Quantification of the fluorescence of Ball relative to control.