

Mechanisms of PP2A-Ankle2 dependent nuclear reassembly after mitosis

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This is an **important** study that reports the mechanism by which Ankle2 (LEM4 in humans) interacts with and recruits PP2A and the ER protein Vap33 to promote BAF dephosphorylation and mediate nuclear membrane reformation, using *Drosophila* as their model. Using Ankle2 mutants, they find that the ER protein Vap33 is key for the normal interphase localisation of Ankle2/LEM4 and also impacts on the function of Ankle2/LEM4 during mitosis. The conclusions on the subcellular localization of Ankle2 are drawn from overexpression of constructs. Overall, the authors use a variety of complementary techniques and provide **convincing** evidence to support the claims and advance our knowledge in the field of mitosis and nuclear envelope biology.

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Abstract

In animals, mitosis involves the breakdown of the nucleus. The reassembly of a nucleus after mitosis requires the reformation of the nuclear envelope around a single mass of chromosomes. This process requires Ankle2 (also known as LEM4 in humans) which interacts with PP2A and promotes the function of Barrier-to-Autointegration Factor (BAF). Upon dephosphorylation, BAF dimers cross-bridge chromosomes and bind lamins and transmembrane proteins of the reassembling nuclear envelope. How Ankle2 functions in mitosis is incompletely understood. Using a combination of approaches in *Drosophila*, along with structural modeling, we provide several lines of evidence that suggest that Ankle2 is a regulatory subunit of PP2A, explaining how it promotes BAF dephosphorylation. In addition, we discovered that Ankle2 interacts with the endoplasmic reticulum protein Vap33, which is required for Ankle2 localization at the reassembling nuclear envelope during telophase. We identified the interaction sites of PP2A and Vap33 on Ankle2. Through genetic rescue experiments, we show that the Ankle2/PP2A interaction is essential for the function of Ankle2 in nuclear reassembly and that the Ankle2/Vap33 interaction also promotes this process. Our study sheds light on the molecular mechanisms of post-mitotic nuclear reassembly and

suggests that the endoplasmic reticulum is not merely a source of membranes in the process, but also provides localized enzymatic activity.

Introduction

In animal cells, the disassembly of the nucleus during mitosis allows the segregation of the genetic material via the spindle apparatus, composed of cytoplasmic microtubules and centrosomes. However, this process of open mitosis complicates the transition from mitosis to interphase, as a new nucleus must reassemble around each set of segregated chromosomes. The mechanisms of nuclear reassembly after mitosis remain incompletely understood (Hampoele and Baumbach, 2023 [DOI](#); Kono and Shimi, 2024 [DOI](#); Li et al., 2024 [DOI](#); Schellhaus et al., 2016 [DOI](#); Ungricht and Kutay, 2017 [DOI](#)).

The nuclear envelope (NE) is a double membrane that is topologically continuous with the endoplasmic reticulum (ER) (Deolal et al., 2024 [DOI](#); Ungricht and Kutay, 2017 [DOI](#)). The NE is associated with multiple structural proteins that connect it to chromatin and cytoskeleton. Lamins form a semi-rigid cage-like network between chromatin and the inner nuclear membrane while the Barrier-to-Autointegration Factor (BAF) interacts with DNA, Lamins, and transmembrane LEM-Domain proteins within the inner nuclear membrane (Sears and Roux, 2020 [DOI](#)). Nuclear pore complexes composed of multiple nucleoporins ensure transport across the NE and also interact with structural cytoplasmic and nucleoplasmic proteins (Goldberg, 2017 [DOI](#); Petrovic et al., 2022 [DOI](#); Shevelyov, 2023 [DOI](#)). In addition, the LINC complex spans the entire NE to connect chromatin to the cytoskeleton and relay mechanotransduction (Hieda, 2019 [DOI](#)). During mitotic entry, the phosphorylation of many of these proteins disrupts their interactions, resulting in nuclear envelope breakdown (NEB) (Archambault et al., 2022 [DOI](#)). BAF is phosphorylated by Vaccinia-Related Kinases (VRKs, NHK-1/Ballchen in *Drosophila*) at its N-terminus, which impedes its binding to DNA (Lancaster et al., 2007 [DOI](#); Nichols et al., 2006 [DOI](#)). Similarly, CDK1, PKC and PLK1 phosphorylate Lamins A/C and B, disrupting their polymerization (Liu and Ikegami, 2020 [DOI](#); Velez-Aguilera et al., 2020 [DOI](#)). As a result of NEB, NE membranes retract into the ER during mitosis.

Correct reassembly of the nucleus after mitosis is critical for cell viability and physiology. During this process, segregated chromosomes gather together and ER membranes are recruited around chromosomes to rebuild the NE (Deolal et al., 2024 [DOI](#); Schellhaus et al., 2016 [DOI](#); Ungricht and Kutay, 2017 [DOI](#)). Failure to group chromosomes into a single nucleus results in micronuclei that are prone to rupture and DNA damage (Guo et al., 2020 [DOI](#)). The timely reformation of the nuclear envelope is essential for reestablishing nucleocytoplasmic transport that is needed for the entire gene and protein expression process, including transcription, mRNA export and ribosome biogenesis (Alberts et al., 2015 [DOI](#)). Protein Phosphatases 1 (PP1) and 2A (PP2A) dephosphorylate several proteins to promote their interactions and reassociation with the nuclear envelope (Archambault et al., 2022 [DOI](#); Huguet et al., 2019 [DOI](#)). How the activities of the enzymes are coordinated in time and space is incompletely understood.

BAF plays a central role in nuclear reassembly. It forms a dimer that binds DNA after anaphase, thereby cross-linking chromosomes into a single cohesive mass (Samwer et al., 2017 [DOI](#)). BAF also promotes the recruitment of ER membranes through its interactions with LEM-Domain proteins, and aids in the reassembly of the Lamina (Haraguchi et al., 2008 [DOI](#); Haraguchi et al., 2001 [DOI](#)). The dephosphorylation of BAF is required for its recruitment to segregated chromosomes (Archambault et al., 2022 [DOI](#); Sears and Roux, 2020 [DOI](#)). The B55 regulatory subunit of PP2A promotes this regulation in various animals, including *C. elegans*, *H. sapiens* and *D. melanogaster* (Asencio et al., 2012 [DOI](#); Mehse et al., 2018 [DOI](#)). In addition, the PP2A-interacting, ER-localized protein Ankle2 (also known as LEM4 and LEM-4L in humans and *C. elegans*, respectively) is also required for the recruitment of BAF to segregated chromosomes in the same three organisms (Asencio et al., 2012 [DOI](#); Li et al., 2024 [DOI](#); Snyers et al., 2018 [DOI](#)). In humans, Ankle2/LEM4 contains

Ankyrin repeats, a LEM domain, a transmembrane domain, and other predicted structured and unstructured regions (Fishburn et al., 2024 [↗](#)). The molecular mechanisms by which Ankle2 functions in nuclear reassembly are unclear (Fig 1A [↗](#)). *ANKLE2* function is crucial for the development of the central nervous system as mutations in this gene are associated with microcephaly (Thomas et al., 2022 [↗](#); Yamamoto et al., 2014 [↗](#)). Targeting of Ankle2 by the Zika virus also causes microcephaly (Shah et al., 2018 [↗](#)). In *Drosophila*, Ankle2 is required for asymmetric divisions in larval neuroblasts (Link et al., 2019 [↗](#)). While Ankle2 impacts the asymmetric division protein machinery, it remains unclear whether the loss of its function in nuclear reassembly also contributes to microcephaly.

To investigate Ankle2 molecular functions, we conducted studies in *Drosophila*. We found that Ankle2 forms a complex with the PP2A structural and catalytic subunits, in a mutually exclusive manner with other known regulatory subunits. Our phosphoproteomic analysis validates that BAF dephosphorylation depends on Ankle2. We also identified and characterized a novel interaction of Ankle2 with Vap33, a VAP family protein that promotes Ankle2 localization to the ER during both nuclear reassembly and interphase. We show that the interactions of Ankle2 with PP2A and Vap33 promote BAF recruitment to chromosomes after anaphase. Moreover, the Ankle2/PP2A interaction is essential for post-mitotic nuclear reassembly and development *in vivo*. We propose that Ankle2 functions as a novel PP2A regulatory subunit required for BAF dephosphorylation, and that Vap33-dependent anchoring of Ankle2 to the ER promotes this function in a localized manner during post-mitotic nuclear reassembly.

Results

Ankle2 interacts with PP2A through its Ankyrin domain

To investigate the molecular functions of Ankle2, we sought to identify its interaction partners. First, we generated D-Mel (D.mel2) stable cell lines expressing GFP-fused Ankle2 (N or C terminal). We performed GFP affinity purifications on lysate of these cells and identified co-purified proteins by mass spectrometry. Cells expressing Flag-GFP were used as control. We found that PP2A-29B (PP2A-A, structural) and Microtubule Star (Mts, PP2A-C, catalytic) were specifically co-purified with both GFP-Ankle2 and Ankle2-GFP (Fig S1A-B [↗](#) and Table S1). To test if this association occurs *in vivo*, we generated transgenic flies for the expression and purification of Ankle2-GFP in early embryos (Fig 1B [↗](#)). The maternal driver *mata4-GAL-VP16* was used to activate the expression of *UASp-Ankle2-GFP* in late oocytes and syncytial embryos. Embryos expressing GFP alone were used as control. Again, we found PP2A-29B and Mts specifically co-purified with Ankle2-GFP (Fig 1C-D [↗](#) and Table S2). Reciprocally, endogenous Ankle2 was co-purified with PP2A-29B-GFP from cells in culture and embryos, along with the regulatory subunits *Tws*, *Wdb* and *Wrd* (Fig 1E-F [↗](#), S1C-D and Tables S2, S3). Thus, the interaction of Ankle2 with PP2A is conserved between *Drosophila* and humans (Asencio et al., 2012 [↗](#)).

To map the region of Ankle2 that binds PP2A, we used a GST pulldown assay. We produced GST-fused fragments of Ankle2 covering its entire sequence in *E. coli* and tested their ability to pulldown PP2A-29B (with GFP-fused to either end) and Mts from D-Mel cells. We initially found that a fragment comprising amino acid (a.a.) residues 275-450 was sufficient to strongly associate with PP2A-29B and Mts, while fragments 1-274, 451-909 or 910-1174 showed no or very weak association with these proteins (Fig 1G-H [↗](#)). The 275-450 region comprises most of the Ankyrin domain. This domain was recently proposed to span a.a. residues 229-399 (Fishburn et al., 2024 [↗](#)), and our inspection of a structural model generated by AlphaFold3 (Abramson et al., 2024 [↗](#)) suggested a.a. residues 227-400. We found that the Ankyrin domain (a.a. 227-400 tested) was sufficient for association with PP2A (Fig S1E [↗](#) and 1H). To test if the Ankyrin domain region is required for the interaction of Ankle2 with PP2A, we used a co-purification assay in D-Mel cells. Ankle2-GFP WT or with a deletion of the Ankyrin domain were purified and the presence of co-

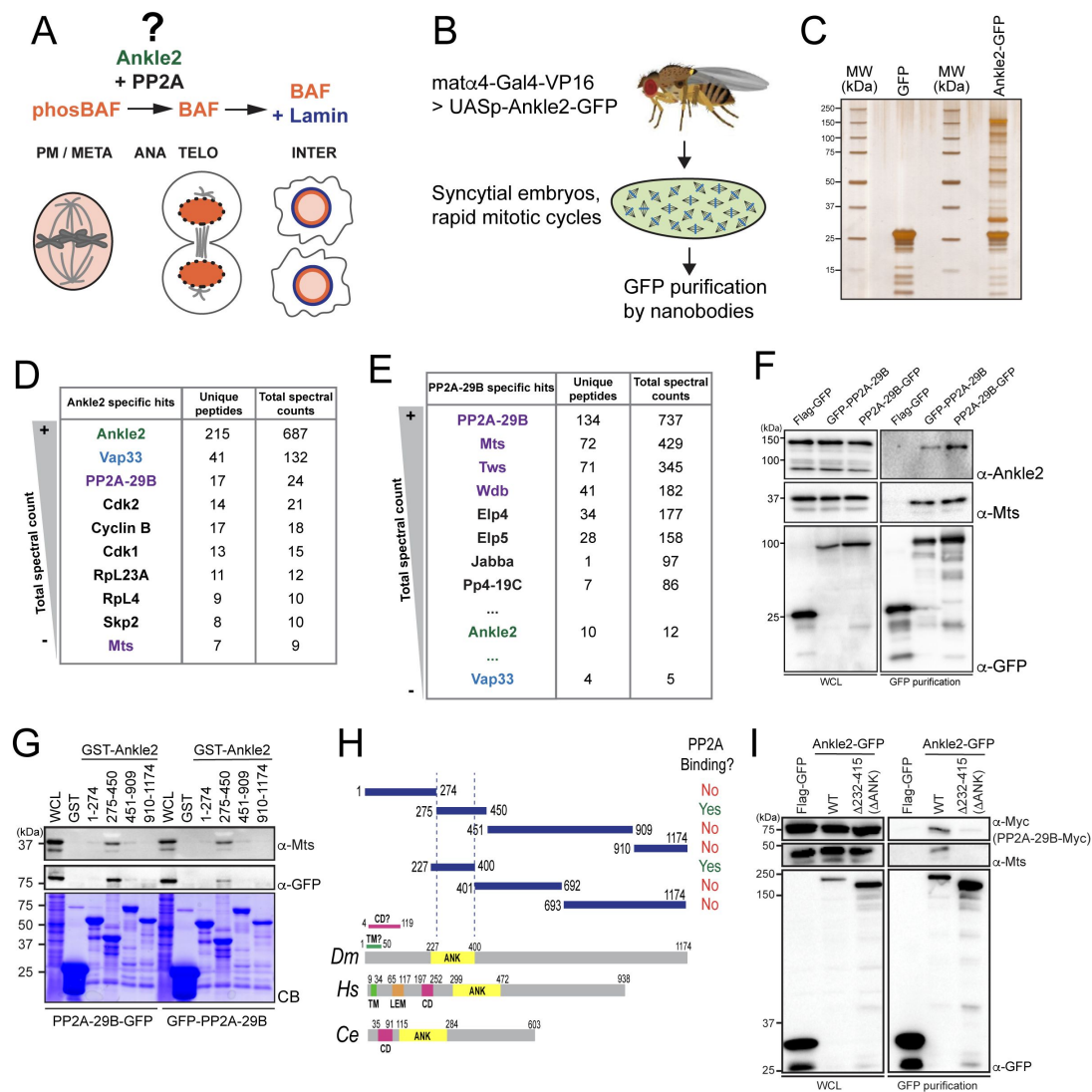


Figure 1.

Ankle2 interacts with PP2A through its Ankyrin repeats region.

A. Ankle2 functions with PP2A to allow the dephosphorylation of BAF and its recruitment to segregated chromosomes. BAF promotes the formation of a single nucleus by cross-bridging chromosomes, and the recruitment of Lamin and ER membranes containing LEM-Domain proteins (not shown). The molecular mechanisms involving Ankle2 in this process are incompletely understood. **B.** Strategy for the identification of Ankle2 interactor proteins *in vivo*. See text for details. **C-E.** Proteins obtained after purification of Ankle2-GFP, PP2A-29B-GFP or GFP from embryos. **C.** Silver-stained gel showing a fraction of the purification products. **D.** Proteins specifically identified with Ankle2-GFP. Proteins with the highest total spectral counts are shown. **E.** Proteins specifically identified with PP2A-29B-GFP after purification from embryos. Proteins with the highest total spectral counts are shown. Ankle2 and Vap33 were also identified as specific interactors further down the list. Purple names: known PP2A subunits. **F.** Ankle2 is specifically co-purified with PP2A-29B. Cells were transfected with the indicated proteins and used in GFP affinity purifications. Products were analyzed by Western blots. WCL: Whole cell extracts. **G.** A region of Ankle2 between amino-acid residues 275-450 is sufficient for interaction with PP2A. GST-fused fragments of Ankle2 produced in bacteria were used in GST-pulldowns with extracts from D-Mel cells expressing PP2A-29B-GFP or GFP-PP2A-29B. Pulled down proteins (PP2A-29B-GFP or GFP-PP2A-29B and Mts) were detected by Western blots. CB: Coomassie Blue. **H.** Top: Summary of results from GST pulldowns testing interactions of Ankle2 fragments with PP2A. Bottom: Primary structures of Ankle2 from *D. melanogaster* (Dm), *H. sapiens* (Hs) and *C. elegans* (Ce), showing known motifs or domains. ANK: Ankyrin domain region; TM: Trans-Membrane motif; LEM: LEM domain. CD: Caulimovirus Domain. **I.** The Ankyrin domain region of Ankle2 between amino-acid residues 232-415 is required for interaction with PP2A. Cells were transfected with the indicated proteins and used in GFP affinity purifications. Products were analyzed by Western blots. WCL: Whole cell extracts.

purified PP2A-29B-Myc and Mts was probed by Western blot. In this assay, we deleted a region comprising the predicted Ankyrin domain plus a few additional downstream residues showing conservation with human Ankle2 and *C. elegans* Lem-4L; (Δ ANK: residues 232-415 deleted; S1F). We found that PP2A-29B-Myc and Mts were co-purified with Ankle2^{WT}-GFP but not with Ankle2 ^{Δ ANK}-GFP (**Fig 1I**). We conclude that the region of Ankle2 comprising the Ankyrin domain is necessary and sufficient to bind the PP2A-29B/Mts complex in *Drosophila*.

Ankle2 functions as a PP2A regulatory subunit to promote BAF dephosphorylation

We hypothesized that Ankle2 functions as a regulatory protein required for PP2A to dephosphorylate some of its substrates. The knockdown of LEM-4L or Ankle2 results in the hyperphosphorylation of BAF in its N-terminus in *C. elegans* or *Drosophila* cells, respectively (Asencio et al., 2012; Li et al., 2024). To identify sites that become hyperphosphorylated in the absence of Ankle2 at a proteome-wide level, we used a phosphoproteomic approach (Emond-Fraser et al., 2023). We transfected D-Mel cells with dsRNA against Ankle2 or dsRNA Non-Target (NT). Four days later, cells were lysed, proteins were digested with trypsin and phosphopeptides were enriched, and analyzed by quantitative mass spectrometry. Among hyperphosphorylated peptides upon Ankle2 depletion, we found the expected BAF peptide phosphorylated at Thr4 and Ser5 (**Fig 2A**). Phosphorylation at the orthologous sites in human BAF was shown to negatively regulate its ability to interact with DNA, and possibly with LEM-Domain proteins (Nichols et al., 2006). Our observation of these Ankle2-dependent sites in BAF validates our approach since BAF is the only reported protein whose phosphorylation state depends on Ankle2 (Asencio et al., 2012; Li et al., 2024). Moreover, BAF could be the only obligatory substrate of Ankle2-dependent dephosphorylation for cell proliferation as lowering the dose of the BAF kinase NHK-1/Ballchen or expression of an unphosphorylatable mutant form of BAF rescues wing development defects caused by the partial depletion of Ankle2 (Li et al., 2024). Nevertheless, we identified several additional hyperphosphorylated sites in other proteins upon Ankle2 knockdown (**Fig 2A** and Table S5). Although it is likely that some of the hyper- and hypophosphorylated sites observed reflect indirect alterations of cell physiology upon Ankle2 depletion, our results point at several candidate proteins whose dephosphorylation may depend on Ankle2.

We hypothesized that Ankle2 functions as a regulatory subunit of PP2A. Interestingly, the structural (PP2A-29B) and catalytic (Mts) subunits of PP2A were detected in complex with Ankle2, while none of the known regulatory subunits of PP2A were detected (Tws, Wdb, Wrd or PR72) (**Fig 1D**, S1B and Tables S1, S2). To test if Ankle2 interacts with PP2A as a regulatory subunit, we used a competition assay (**Fig 2B**). We co-transfected cells with Ankle2-Myc and PP2A-29B-GFP or FLAG-GFP. We then proceeded to GFP affinity purifications. As expected, Ankle2-Myc was co-purified specifically with PP2A-29B-GFP. The PP2A regulatory subunit Tws was also co-purified specifically with PP2A-29B-GFP. To test if Tws and Ankle2 are mutually exclusive in the PP2A complex, we co-transfected increasing amounts of Myc-Tws plasmid along with Ankle2-Myc and PP2A-29B-GFP. As a result, increasing amounts of Myc-Tws were co-purified with PP2A-29B-GFP. Conversely, decreasing amounts of Ankle2-Myc were co-purified with PP2A-29B-GFP as the amount of Myc-Tws increased. These results suggest that Tws competes with Ankle2 for binding within the PP2A holoenzyme and reinforce the idea that Ankle2 functions as *bona fide* regulatory subunit of PP2A.

To contextualize and interpret our results, we generated structural predictions of relevant co-complexes using AlphaFold3 (Abramson et al., 2024). In models of a complex of *Drosophila* Ankle2, PP2A-29B and Mts, the Ankyrin domain consistently directly interacts with Mts (**Fig 2C** shows a representative model), consistent with our pulldown results (**Figs 1G** and S1E). Interestingly, these models also predict that additional regions of Ankle2 engage in interactions with PP2A. A region previously referred to as the Caulimovirus Domain (CD, **Fig 1H**) and proposed to mediate an interaction between human Ankle2 and PP2A (Fishburn et al., 2024) is

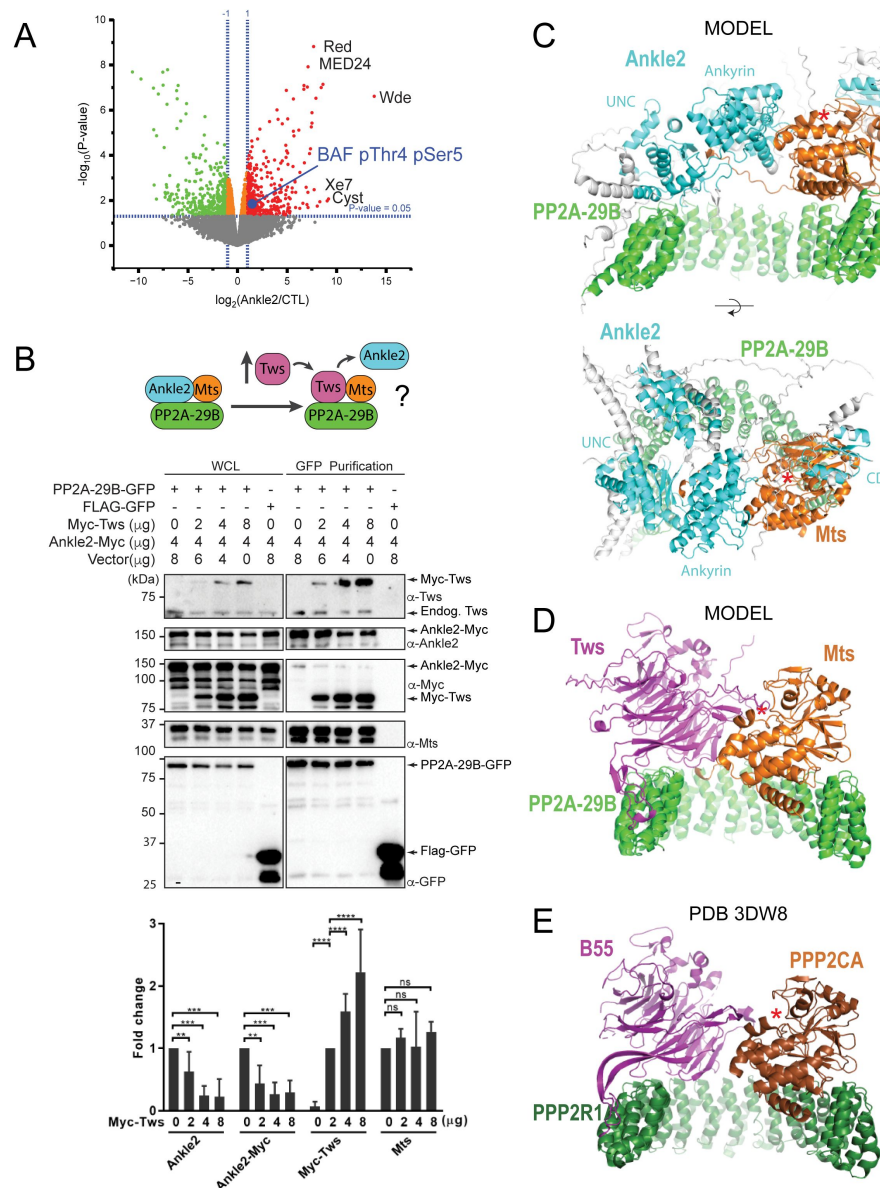


Figure 2.

PP2A-Ankle2 promotes BAF dephosphorylation.

A. Phosphoproteomic analysis identified BAF as being hyperphosphorylated at Thr4 and Ser5 upon Ankle2 depletion. D-Mel cells were transfected with dsRNA against Ankle2 or non-target dsRNA (against bacterial KAN gene). Phosphopeptides from tryptic digests were analyzed quantitatively by mass spectrometry. A few additional significantly hyperphosphorylated proteins are labeled as examples (see Table S5 for the full list). Red, green and orange dots represent peptides with significant ($p < 0.05$) hyperphosphorylation, hypophosphorylation and little change, respectively. Grey dots indicate peptides with changes below statistical significance. **B.** Competition assay. Top: schematic hypothesis. If Ankle2 occupies the position of a PP2A regulatory subunit, its presence in the PP2A holoenzyme may be outcompeted by Twis (PP2A regulatory subunit). Bottom: the interaction between PP2A-29B-GFP and Ankle2-Myc was monitored by a GFP affinity co-purification assay. Increasing amounts of Myc-Twis plasmid were co-transfected. As a result, increasing amounts of Myc-Twis, and decreasing amounts of Ankle2-Myc, are co-purified with PP2A-29B-GFP. The amounts of co-purified Mts are unchanged. Averages of 3 experiments are shown. All error bars: S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns: non-significant from paired t-tests. **C.** AlphaFold3 predicted model of a complex between *Drosophila* Ankle2, PP2A-29B and Mts. Residues of Ankle2 with confidence scores below 0.8 are coloured grey. **D.** Predicted model of a complex between *Drosophila* Twis, PP2A-29B and Mts. **E.** Crystal structure of a complex between human PPP2R1A, PPP2CA and B55 (PDB 3DW8) (Xu et al., 2008). Red asterisks denote the positions of the phosphatase catalytic site.

indeed predicted to interact with Mts in our models, on the other side from where the Ankyrin domain binds. Moreover, the modeling shows an adjacent uncharacterized domain (UNC) that is in direct contact with PP2A-29B. However, no interaction with Mts or PP2A-29B was experimentally detected for our GST-fused fragments comprising these regions alone in pulldown assays. It is possible that the UNC and CD domains interact with the PP2A subunits with low affinity. It is also possible that the fragment tested requires additional components to fold into a form that is competent for PP2A binding; in particular, AlphaFold3 predictions show that the complete UNC domain may be made of several segments distal in primary sequence (a.a. residues ~401-467 + 601-682 + 987-1050). In any case, the deletion of the Ankyrin domain of Ankle2 strongly abrogated its formation of a complex with Mts and PP2A-29B, indicating that it plays a predominant role in the formation of the complex. Importantly, these models display Ankle2 occupying an overlapping position to that of Tws in an analogous PP2A-Tws model (which itself consistent with a crystal structure of human PP2A-B55 (Xu et al., 2008) (Fig 2C-E). These models account for the mutually exclusive binding of Ankle2 or Tws to the PP2A-29B/Mts complex, and are consistent with the function of Ankle2 as a regulatory subunit of PP2A.

Ankle2 interacts with Vap33 and they colocalize at the endoplasmic reticulum

Besides PP2A, we discovered that Ankle2 interacts with Vap33, an integral protein of the endoplasmic reticulum (ER) and a member of the VAP family (Kamemura and Chihara, 2019; Murphy and Levine, 2016) (Fig 1D). Mass spectrometry data suggested that Vap33 was co-purified at the highest levels among Ankle2 interactors. Ankle2 was previously reported to localize at the ER and the NE in *Drosophila* (Link et al., 2019). We found that Ankle2-RFP co-localizes with GFP-Vap33 in D-Mel cells (Fig 3A and Video S1). This co-localization is clearest during mitosis, where both proteins are enriched around the presumed spindle in metaphase and around segregated chromosomes in late anaphase/telophase, when NE reassembly takes place. To examine the localization of Ankle2 relative to Vap33 *in vivo*, we generated transgenic flies for the inducible expression of Ankle2-GFP and RFP-Vap33. We expressed the fusion proteins in syncytial embryos and studied their localization. We found that both proteins co-localized throughout the cell cycle (Fig 3B and Video S2). In interphase, they localized to the NE and ER membranes. During mitosis, Ankle2-GFP and RFP-Vap33 became enriched on the spindle envelope until they wrapped around newly forming nuclei in telophase. In addition, both proteins were also enriched at the midbody of the central spindle, consistent with the recruitment of ER membranes at this site (Bobinnec et al., 2003). We were unable to examine the localization of endogenous Ankle2 because the antibodies that we generated failed to reliably detect Ankle2 in immunofluorescence. For the remainder of our study, we used overexpressed Ankle2-GFP, which may not perfectly reflect the localization and function of endogenous Ankle2. However, Ankle2-GFP is functional as it can rescue the phenotypes observed when endogenous Ankle2 is depleted (see below).

Ankle2 interacts with Vap33 through FFAT motifs

We confirmed the Ankle2/Vap33 interaction in a reciprocal co-purification assay. We found that endogenous Ankle2 was specifically co-purified with GFP-Vap33 or Vap33-GFP from D-Mel cells (Fig 4A). To identify the region of Ankle2 responsible for its interaction with Vap33, we used the GST pulldown described above, probing for the interaction of Vap33-Myc with GST-fused fragments of Ankle2. We found that the C-terminal region of Ankle2 (residues 910-1174) is sufficient to interact with Vap33 (Fig 4B). The VAP family of proteins is anchored to the ER via a transmembrane domain and exposes a globular MSP domain to the cytoplasm (Kamemura and Chihara, 2019; Murphy and Levine, 2016). The MSP domain interacts with proteins that contain FFAT motifs (2 phenylalanines in an acidic tract) (Loewen et al., 2003). To test if Vap33 interacts with Ankle2 in this manner, we introduced mutations in the MSP of Vap33 domain predicted to disrupt its ability to bind FFAT motifs based on results with human VAP (K89D, M91D in Vap33) (Kaiser et al., 2005). We found that this mutant form of Vap33-Myc was unable to

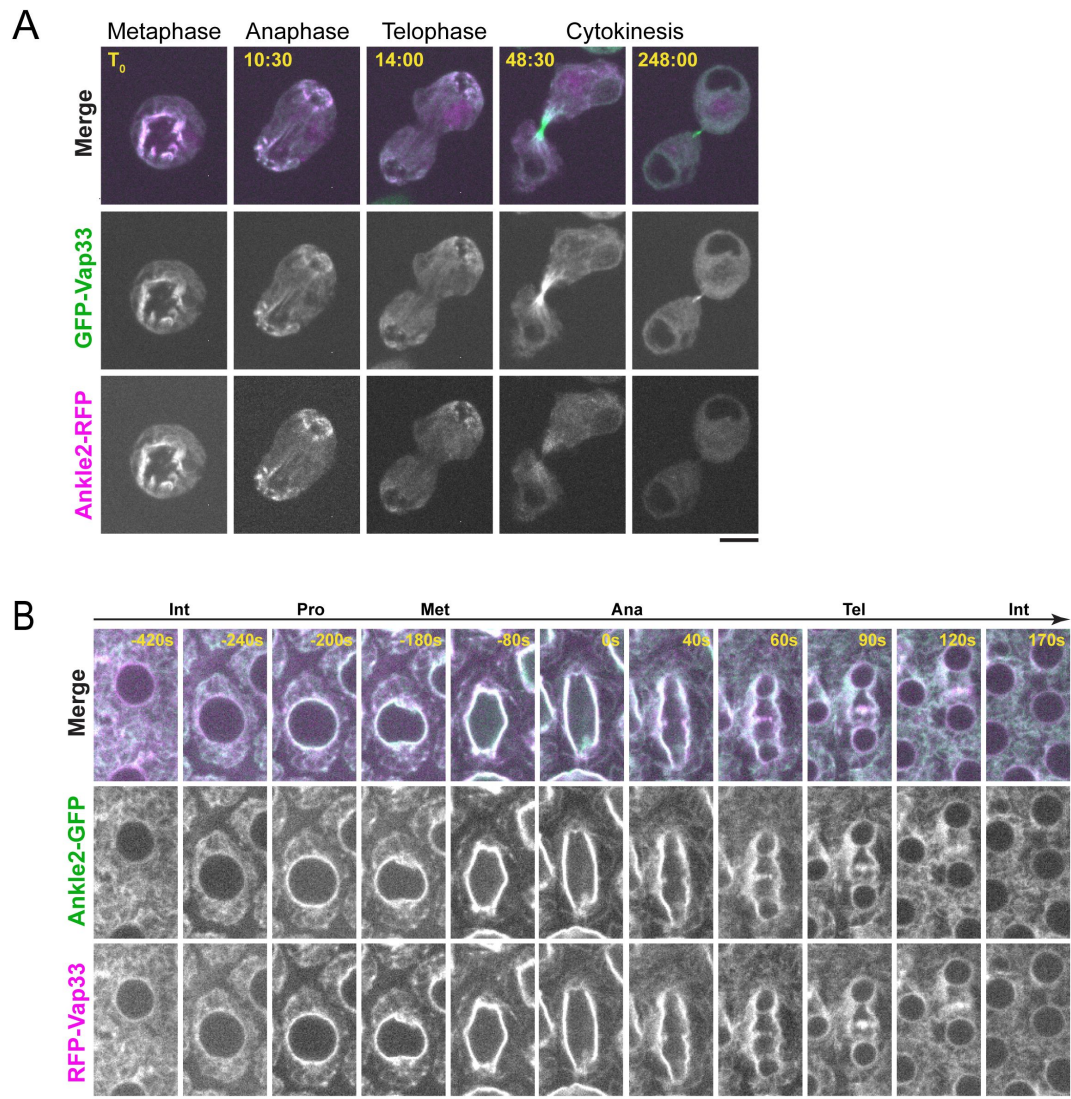


Figure 3.

Ankle2 co-localizes with Vap33.

A. Video images of a D-Mel cell co-expressing Ankle2-RFP and GFP-Vap33 through different stages of cell division. **B.** Video images of a syncytial embryo co-expressing Ankle2-GFP and RFP-Vap33 through different stages of the cell cycle. Scale bars: 5 μ m.

interact with GST-Ankle2⁹¹⁰⁻¹¹⁷⁴ (Fig 4C). We then searched for FFAT motifs in Ankle2 using published criteria (Slee and Levine, 2019) and identified three candidate motifs (Fig 4D). One of them was a particularly good fit. Mutation of this FFAT motif (Fm) abolished the interaction of the Ankle2 C-terminal fragment with Vap33 (Fig 4B-C). To test if the interaction occurs in the same way between proteins expressed in *Drosophila* cells, we used a co-purification assay. We transfected different forms of Ankle2-GFP along with Vap33-Myc and proceeded to GFP affinity purifications followed by Western blot for Myc. In this assay, mutation of the first FFAT motif in Ankle2 diminished but did not abolish its interaction with Vap33 (Fig 4E). We therefore tested the contributions of two other additional FFAT-like motifs (FL1 and FL2, Fig 4D). Mutation of FL1 (FL1m) also diminished but did not abolish the interaction of Ankle2 with Vap33, while mutation of FL2 (FL2m) did not perturb the Ankle2-Vap33 interaction. Finally, we found that the combined mutation of the FFAT and FL1 motifs in Ankle2 (Fm+FL1m) abolished its interaction with Vap33. Thus, we conclude that Vap33, via its MSP domain, can interact with Ankle2 through two redundant FFAT motifs. Interestingly, subsequent modeling using AlphaFold3 predicted that Vap33 interacts with Ankle2 preferentially through a contact between the MSP domain of Vap33 and the FL1 motif of Ankle2 (Fig 4F).

Vap33 is required for the localization of Ankle2 at the ER and reassembling NE

Drosophila Vap33 is an essential protein presumably responsible for interactions of the ER with multiple cytoplasmic proteins and structures (Pennetta et al., 2002). Nevertheless, we attempted to knock down Vap33 by RNAi in the female germline to examine the effects on Ankle2-GFP localization in embryos. Unsurprisingly, we found that those females were sterile and therefore we could not examine embryos. However, we examined ovaries. In control flies, we observed that Ankle2-GFP was weakly localized to nuclear envelopes and strongly localized to the plasma membrane and to ring canals in nurse cells (Fig S2). By contrast, in Vap33 RNAi ovaries, we found that Ankle2-GFP was largely delocalized from these structures. Whether distinct pools of Ankle2 serve specific functions is a question that remains open. Nevertheless, these results suggested that the localization of Ankle2 depends on Vap33.

To test if Ankle2 requires its interaction with Vap33 for its localization, we generated transgenic flies for the expression of Ankle2^{Fm+FL1m}-GFP. We then examined its localization in embryos co-expressing RFP-Vap33. In interphase, Ankle2^{Fm+FL1m}-GFP failed to localize to the ER and NE like Ankle2-GFP, instead appearing diffuse in the cytoplasm (Fig 4G, to be compared with Fig 3B, and Video S3). Surprisingly, as nuclei entered mitosis, Ankle2^{Fm+FL1m}-GFP became strongly localized to the nuclear/spindle envelope, similarly to Ankle2-GFP. However, while Ankle2-GFP was retained at those membranous structures in telophase, Ankle2^{Fm+FL1m}-GFP disappeared from them, returning to a diffuse, cytoplasmic localization. We conclude that the interaction of Ankle2 with Vap33 is required for the localization of Ankle2 to the ER in interphase, and for its retention at membranes during NE reassembly in telophase.

Since the ER is the source of the reassembling NE after mitosis, we hypothesized that the interaction of Ankle2 with Vap33 could mediate a localized activity of Ankle2-bound PP2A during nuclear reassembly. In this way, PP2A could dephosphorylate BAF (and potentially other substrates) locally to promote its recruitment near the reforming nuclear envelope (Fig S3A). For this model to be valid, Ankle2 should be able to interact with Vap33 and PP2A simultaneously. We used AlphaFold3 to generate structural models of a complex between Vap33, Ankle2, PP2A-29B and Mts (Fig S3B). The most probable models show that Ankle2 can simultaneously interact with Vap33, PP2A-29B and Mts without steric clash. Our attempts to detect a complex containing Vap33 and PP2A by simple co-IP and Westerns in D-Mel cells gave variable and inconclusive results. However, we detected Vap33 in complex with PP2A-29B-GFP purified from embryos (Fig 1E), despite the fact that PP2A-29B-GFP did not appear clearly enriched at the nuclear/spindle

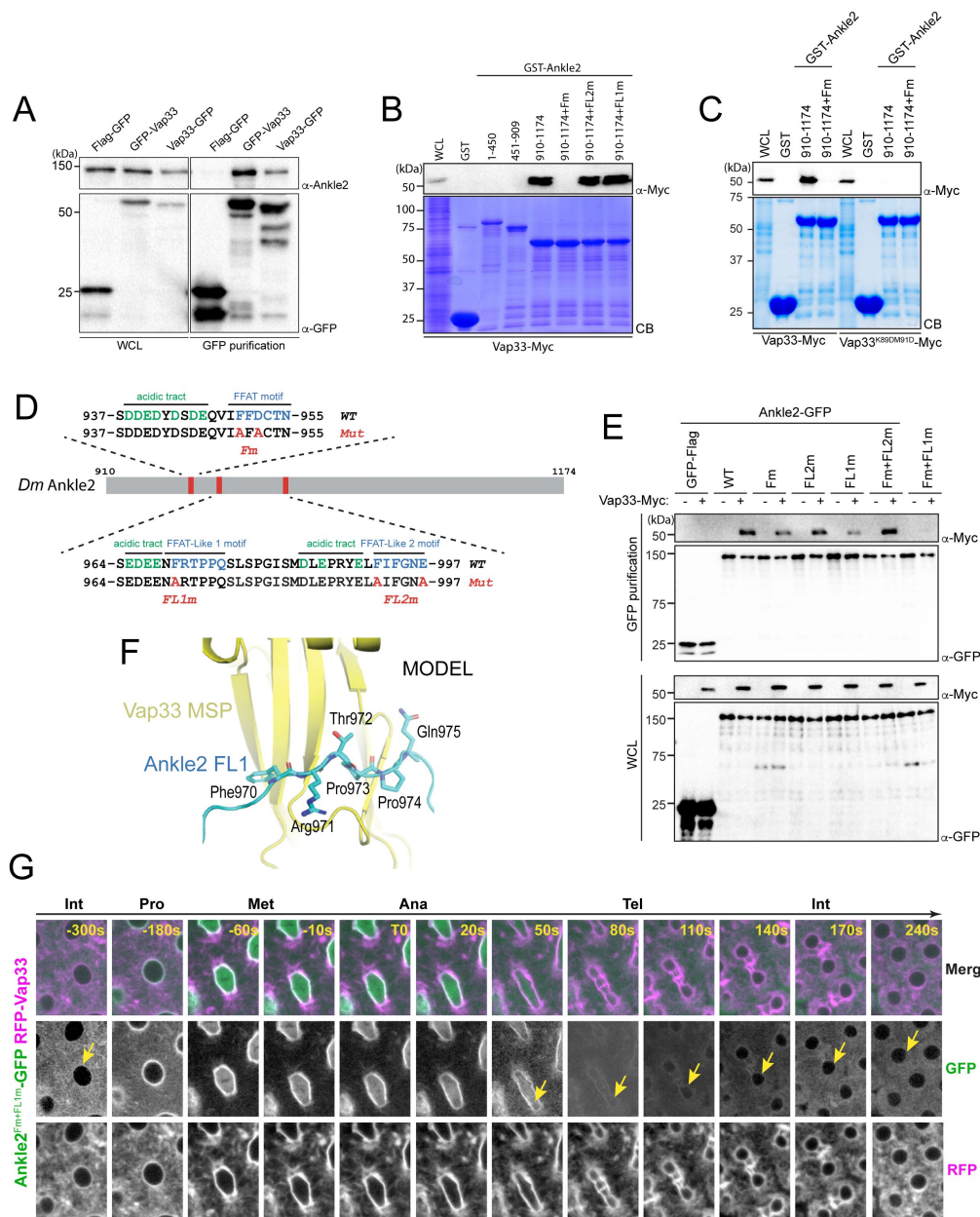


Figure 4.

Ankle2 interacts with Vap33 through FFAT motifs.

A. Cells were transfected with the indicated proteins and used in GFP affinity purifications. Products were analyzed by Western blots. WCL: Whole cell extracts. **B-C.** GST pull-down mapping Ankle2-Vap33 interaction. GST-fused fragments of Ankle2 (WT or with the indicated mutations) produced in bacteria were used in GST-pulldowns with extracts from D-Mel cells expressing Vap33-Myc or Vap33^{DD}-Myc. Pulldown products were analyzed by Western blots. CB: Coomassie Blue. WCL: Whole cell extracts. **D.** FFAT and FFAT-Like motifs identified (green) in the C-terminal end of Ankle2. Acidic residues are in blue. The mutations introduced (Fm, FL1m and FL2m) are in red. **E.** The FFAT and FFAT-Like 1 (FL1) motifs contribute to the interaction of Ankle2 with Vap33. Cells were transfected with the indicated proteins and used in GFP affinity purifications. Products were analyzed by Western blots. **F.** AlphaFold3 predicted model of a complex between *Drosophila* Ankle2, Vap33, Mts and PP2A-29B. The image shows a zoomed-in view of the contact between the MSP domain of Vap33 (yellow) and the FL1 motif of Ankle2 (blue). The full heterotetramer structure model is shown in **Fig S3B**. **G.** The FFAT and FL1 motifs are required for co-localization with Vap33 during telophase and interphase. Video images of a syncytial embryo co-expressing Ankle2^{Fm+FLm1}-GFP and RFP-Vap33 through different stages of the cell cycle. To be compared with **Figure 3B**. Note the delocalization of Ankle2^{Fm+FLm1}-GFP at the nuclear envelope indicated by the yellow arrows. Scale bars: 5 μm.

envelope in embryos (**Fig S3C**). To test if we could promote the formation of the complex, we overexpressed Ankle2-RFP in cells expressing PP2A-29B-GFP and Vap33-Myc. We found that more Vap33-Myc was co-purified with PP2A-29B-GFP under these conditions, compared with cells without Ankle2-RFP overexpression (**Fig S3D**). This co-purification reflected the specific interaction of Ankle2 with Vap33-Myc because it was abrogated with Vap33^{DD}-Myc. Consistent with this result, we found that PP2A-29B-GFP became visibly enriched at the NE upon overexpression of Ankle2-RFP in D-Mel cells (**Fig S3E**). These results suggest that a Vap33-Ankle2-PP2A complex can mediate the recruitment of a pool of PP2A at the NE.

Requirements of Ankle2 interactions and motifs for nuclear reassembly

To test the requirements of Ankle2 for its functions in mitosis, we used a rescue assay in D-Mel cells in culture. We depleted endogenous Ankle2 by RNAi and expressed different forms of RNAi-insensitive Ankle2-GFP under the copper-inducible metallothionein promoter (**Fig 5A**). Depletion of Ankle2 alone resulted in nuclear defects including dispersed or aggregated Lamin, fragmented nuclei, DNA devoid of Lamin and hypercondensed DNA (**Fig 5B-C**). We previously documented these cellular phenotypes, which we showed to occur as a result of defective BAF recruitment to chromosomes and BAF-dependent nuclear reassembly after mitosis (Li et al., 2024). In addition, Western blotting using Phos-tag revealed that BAF became hyperphosphorylated upon Ankle2 depletion (Li et al., 2024). We verified that expression of Ankle2-GFP rescued the nuclear defects and BAF hyperphosphorylation (**Fig 5B-D**). When examining cellular phenotypes, we scored only GFP-positive cells. The incomplete restoration of unphosphorylated BAF levels upon expression of Ankle2-GFP (WT and Fm+FL1m) in Ankle2-depleted cells is likely due to the inefficient expression of the GFP-tagged protein in a fraction of the cells.

To begin to map the essential regions of Ankle2, we tested the ability of Ankle2¹⁻⁵⁸⁷-GFP (N-terminal half) or Ankle2⁵⁸⁸⁻¹¹⁷⁴-GFP (C-terminal half) to rescue the nuclear phenotypes. We found that both truncated proteins were unable to rescue nuclear defects and BAF hyperphosphorylation, suggesting that each half of Ankle2 contains at least one essential region for its function (**Fig S4**). The N-terminal half of Ankle2 contains the Ankyrin domain region necessary and sufficient for Ankle2 interaction with PP2A (**Fig 1**). We found that Ankle2^{ΔANK}-GFP was unable to rescue nuclear defects and BAF hyperphosphorylation (**Fig 5B-D**). These results indicate that the Ankyrin domain is required for the essential function of Ankle2 in BAF dephosphorylation and nuclear reassembly, and suggest that the ability of Ankle2 to interact with PP2A is necessary for this function.

To map more precisely the C-terminal region(s) of Ankle2 required for its function, we made smaller truncations and deletions in Ankle2-GFP, guided in part by sequence conservation between species. We found that deletion of a.a. residues 607-680, 681-937, 938-997, 992-1040 or 1041-1174 partially or completely abrogated the ability of Ankle2-GFP to rescue nuclear defects and BAF hyperphosphorylation (Figs S4-S5). The 938-997 segment of Ankle2 contains the FFAT motifs required for the interaction with Vap33 (**Fig 4**). However, we found that the expression of Ankle2^{Fm+FL1m}-GFP rescued nuclear defects and BAF hyperphosphorylation (**Fig 5B-D**). These results suggest that the interaction of Ankle2 with Vap33, while it is required for Ankle2 localization to the reassembling NE in embryos, is dispensable for Ankle2 function in BAF dephosphorylation and nuclear reassembly in D-Mel cells in culture.

Disruption of the interaction with Vap33 prevented the localization of Ankle2 at the ER and the reassembling NE in telophase (**Fig 4G**); however, it did not abolish the localization of Ankle2 at the nuclear/spindle envelope at early stages of mitosis. We looked for another motif that could contribute to the membrane localization of Ankle2. Human Ankle2 contains a predicted trans-membrane (TM) domain in its N-terminus (**Fig 1H**) (Asencio et al., 2012). Although online

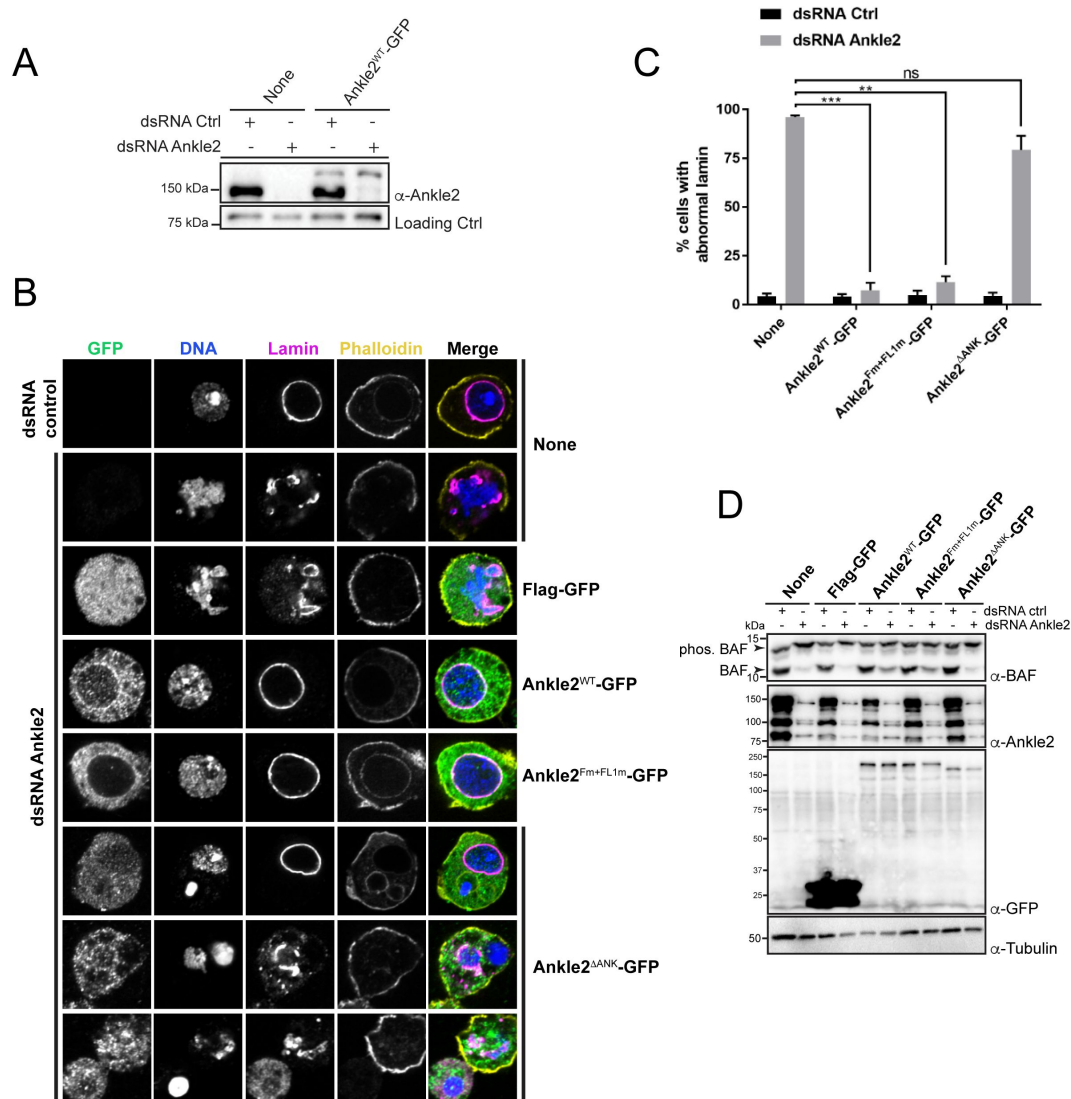


Figure 5.

Ankle2 interaction with PP2A but not with Vap33 is required for nuclear reassembly.

A. RNAi depletion of endogenous Ankle2 and simultaneous expression of RNAi-insensitive Ankle2-GFP. D-Mel cells expressing RNAi-insensitive Ankle2-GFP or not were transfected with dsRNA against Ankle2 or non-target dsRNA (NT, against bacterial KAN gene). Four days later, cells were analyzed by Western blots. Non-specific band is used as loading control. **B.** Immunofluorescence on cells expressing the indicated proteins (right labels) and transfected with the indicated dsRNAs (left labels). GFP fluorescence, DAPI staining for DNA, Lamin (Lamin B) immunostaining and Phalloidin staining for actin are shown. Red frames indicate the presence of nuclear reassembly defects. Scale bars: 5 μ m. **C.** Quantification of the abnormal Lamin phenotypes from experiments as in B. Note that Ankle2^{WT}-GFP and Ankle2^{Fm+FL1m}-GFP, but not Ankle2^{ΔANK}-GFP, rescue nuclear defects. Averages of 3 experiments are shown. All error bars: S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ **** $p < 0.0001$, ns: non-significant from paired t-tests. **D.** Western blot analysis of experiment shown in B-C. The blot at the top was obtained from a gel containing Phos-Tag to increase the resolution between phosphorylated BAF (phos. BAF) and unphosphorylated BAF (BAF). Note that BAF is hyperphosphorylated after Ankle2 depletion and that this is rescued by expression of Ankle2^{WT}-GFP and Ankle2^{Fm+FL1m}-GFP, but not by Ankle2^{ΔANK}-GFP.

prediction tools failed to predict a TM domain in *Drosophila* Ankle2, we noticed a highly hydrophobic segment in its N-terminus (a.a. 14-31). To test if it contributes to the membrane localization of Ankle2, we combined a deletion of the N-terminal 50 a.a. residues (**Fig 1H**) with the FFAT mutations. The resulting Ankle2^{51-1174&Fm+FL1m}-GFP was recruited to the NE during prophase, similarly to Ankle2-GFP and Ankle2^{Fm+FL1m}-GFP (**Fig S6**). Ankle2^{51-1174&Fm+FL1m}-GFP also largely rescued nuclear defects and BAF hyperphosphorylation, although less efficiently than Ankle2-GFP (**Fig S4**). Among various truncated and deleted forms of Ankle2-GFP, only those that lacked C-terminal a.a. residues 938-1174 (Ankle2-GFP 1-587, 1-692 and 1-937) failed to be recruited to the NE during prophase (**Fig S6**). This region comprises the 3 candidate FFAT motifs identified. These results suggest that Ankle2 relies on its C-terminus for its NE recruitment during prophase, although the FFAT motifs are not strictly required for this recruitment. We do not know how Ankle2 localizes to the nuclear/spindle envelope independently from Vap33 during early stages of mitosis, or the importance of this localization.

Overall, we conclude that in addition to its N-terminal PP2A-interacting Ankyrin domain, Ankle2 requires the integrity of its C-terminal portion for its essential function in nuclear reassembly. The C-terminus of Ankle2 mediates its interaction with Vap33, which is crucial for the localization of Ankle2 to the reassembling NE in telophase. However, our results suggest that the Ankle2/Vap33 interaction is not strictly required for Ankle2's function in D-Mel cells.

Interactions of Ankle2 with PP2A and Vap33 promote BAF recruitment to chromosomes during nuclear reassembly

To test the requirements of Ankle2 interactions *in vivo*, we expressed Ankle2-GFP transgenes in the female germline and early embryos and simultaneously knocked down endogenous Ankle2 using the maternal mata4-GAL-VP16 driver (**Fig S7**). RFP-BAF was co-expressed. We then imaged mitosis and monitored the dynamics of the fluorescent proteins (**Fig 6A** top, Video S4). As previously observed, RFP-BAF was enriched at the nuclear/spindle envelope during interphase and early mitosis, likely due to its interactions with LEM-Domain transmembrane proteins (Emond-Fraser et al., 2023). RFP-BAF also largely colocalized with Ankle2-GFP. After anaphase, RFP-BAF transferred to chromatin, becoming visible at the inner core region of the reassembling nuclei, where the chromatin periphery intersects spindle microtubules (**Fig 6B**), consistent with the role of BAF in nuclear reassembly.

To test the importance of the Ankle2-PP2A interaction, we imaged embryos expressing Ankle2^{ΔANK}-GFP. We found that RFP-BAF recruitment to the inner core of reassembling nuclei was strongly diminished and delayed compared to embryos expressing Ankle2^{WT}-GFP (**Fig 6A** middle, Video S5 and **Fig 6C**). Given that BAF recruitment promotes NE reassembly and Ankle2 is a NE-associated protein, we examined whether the recruitment of Ankle2^{ΔANK}-GFP to the inner core region of the reassembling NE was compromised. Fluorescence quantification of NE-associated proteins at the inner core region allows monitoring of NE reassembly, as this region of the reassembling NE is clearly distinct from the spindle envelope in embryos (Emond-Fraser et al., 2023) (**Figs 6B** and S8A). We found that the recruitment of Ankle2^{ΔANK}-GFP to the core region was delayed (**Fig 6D**), suggesting that the delay in BAF recruitment caused a delay in NE reassembly. Interestingly, RFP-BAF and Ankle2^{ΔANK}-GFP were both retained longer at the spindle envelope in embryos expressing Ankle2^{ΔANK}-GFP, compared with embryos expressing Ankle2^{WT}-GFP (**Fig 6E-F**). We conclude that the Ankyrin domain, required for the ability of Ankle2 to interact with PP2A, is necessary for the timely recruitment of BAF at reassembling nuclei and ensuing NE reassembly.

To test the importance of the Ankle2-Vap33 interaction, we imaged embryos expressing Ankle2^{Fm+FL1m}-GFP (also depleted of endogenous Ankle2) (**Fig 6A** bottom and Video S6). As before, we observed that Ankle2^{Fm+FL1m}-GFP failed to localize to membranes during interphase and telophase, and that it is partially recruited to the nuclear/spindle envelope from prophase to

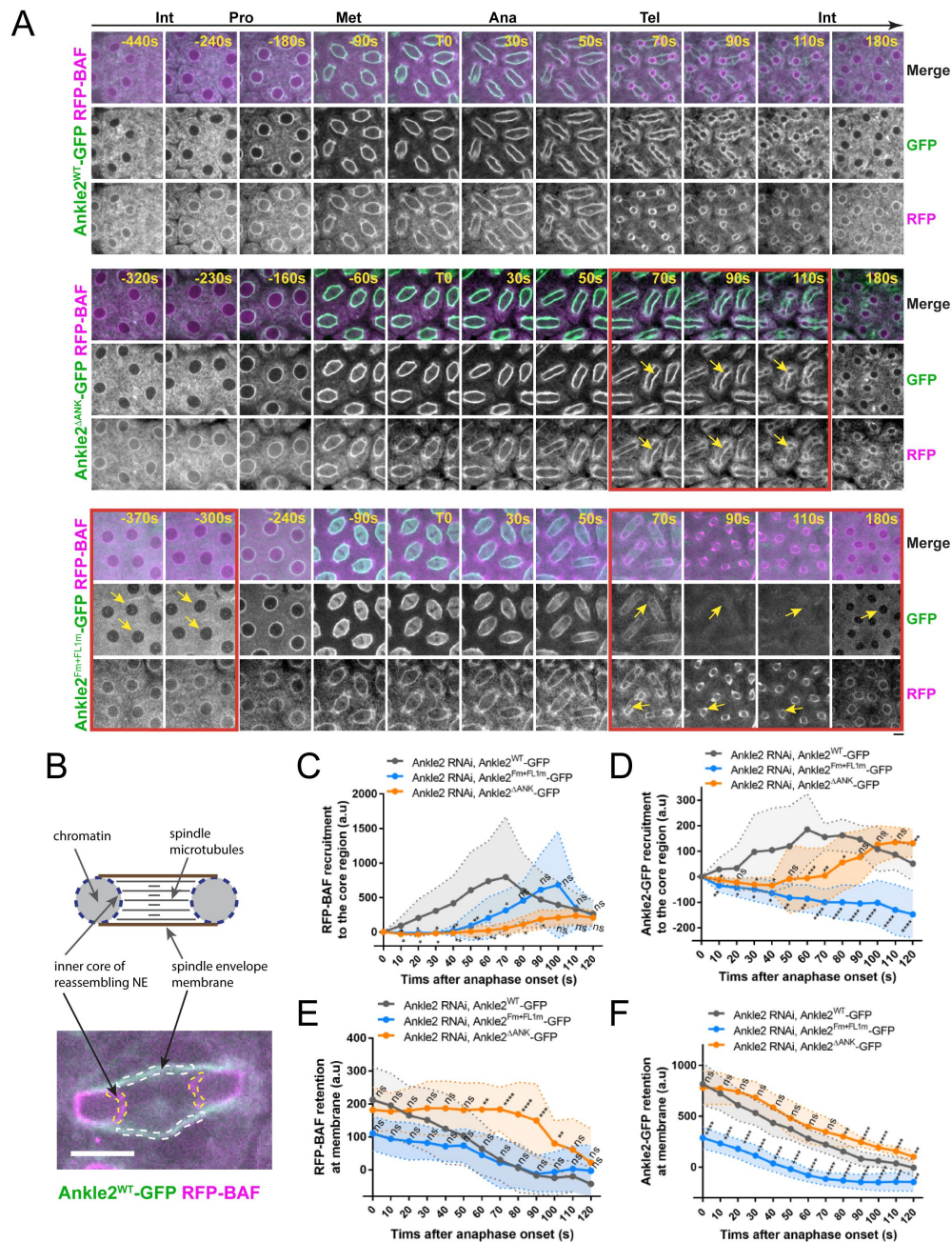


Figure 6.

Ankle2 interactions with PP2A and Vap33 promote BAF recruitment at reassembling nuclei.

A. Syncytial embryos depleted of endogenous Ankle2 and expressing RNAi-insensitive Ankle2^{WT}-GFP (top), Ankle2^{ΔANK}-GFP (middle) or Ankle2^{Fm+FL1m}-GFP (bottom) along with RFP-BAF were imaged through the cell cycle. Times frames where differences are the most pronounced are highlighted by red frames, along with yellow arrows. **B.** Illustration of fluorescence quantifications at specific structures. GFP and RFP fluorescence intensities were quantified at the inner core region of the reassembling nuclei and at the spindle envelope membranes after anaphase. **C-D.** Quantification of the recruitment of RFP-BAF (C) or Ankle2-GFP variants (D) at the inner core region of the reassembling nuclei as a function of time after anaphase onset. **E-F.** Quantification of the retention of RFP-BAF (E) or Ankle2-GFP variants (F) at the lateral spindle envelope membranes as function of time after anaphase onset. Averages of fluorescence intensity from 10 nuclear divisions taken from 5 or 6 embryos are shown per condition. All error bars: S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ **** $p < 0.0001$, ns: non-significant from unpaired t-tests. Scale bars: 5 μ m.

anaphase (**Fig S8B**). This result rules out the possibility that Ankle2^{Fm+FL1m}-GFP localizes to membranes during early mitosis because of a potential complex with endogenous Ankle2. Quantification confirmed that the interaction of Ankle2 with Vap33 is required for the maintenance of Ankle2-GFP at the spindle envelope during telophase and its recruitment to the inner core region of the reassembling NE, at the time when BAF is normally recruited (**Fig 6D**, F). Moreover, we found that the recruitment of RFP-BAF at reassembling nuclei was delayed in embryos expressing Ankle2^{Fm+FL1m}-GFP (**Fig 6C**). These results suggest that the ability of Ankle2 to interact with Vap33 contributes to the efficient recruitment of BAF at reassembling nuclei.

The interactions of Ankle2 with PP2A and Vap33 promote its function *in vivo*

We examined the importance of Ankle2 and its interactions for embryonic development. Knockdown of endogenous Ankle2 during late oogenesis using the mata4-GAL-VP16 driver did not significantly diminish the ability of females to lay eggs (**Fig 7A**). However, none of their embryos hatched, indicating a failure in embryonic development in the absence of maternally contributed Ankle2 (**Fig S9A-B**). Immunofluorescence revealed that the majority of embryos aborted development in the first mitotic cycle, with a single nucleus/spindle (**Fig S9C**). FISH for the X chromosome consistently revealed three foci in the polar body and one or two foci in the mitotic spindle, confirming that the spindle observed corresponded to mitosis and not to meiosis or sperm prior to fertilization (**Fig S9D-E**). Expression of RNAi-insensitive Ankle2-GFP in this background fully rescued embryo hatching. This rescue was strongly abrogated when Ankle2^{ΔANK}-GFP was expressed. The weak rescue observed may be in part attributed to the dilution of Gal4 between two UASp elements (UASp-Ankle2 RNAi and UASp-transgene) because a weak rescue was also observed when GFP alone was expressed (**Fig 7B**). In any case, these results suggest that the interaction of Ankle2 with PP2A, mediated by the Ankyrin domain of Ankle2, is essential during embryo development. Moreover, expression of Ankle2^{ΔANK}-GFP in the presence of endogenous Ankle2 did not cause a decrease in embryo hatching, ruling out the possibility of toxicity (**Fig 7A**). By contrast, expression of Ankle2^{Fm+FL1m}-GFP fully rescued embryo hatching, suggesting that the interaction of Ankle2 with Vap33 is dispensable for embryo development (**Fig 7B**).

To examine the requirements of Ankle2 interactions in a different context, we turned to wing development. Induction of Ankle2 RNAi in the wing disc pouch using Nubbin-Gal4 (Nub-Gal4) resulted in wingless adults. Simultaneous expression of Ankle2-GFP or Ankle2^{Fm+FL1m}-GFP rescued the development of adults with wings of the normal size. However, expression of Ankle2^{ΔANK}-GFP resulted in no rescue (**Fig 7C**). These results suggest that the interaction of Ankle2 with PP2A but not with Vap33 is essential for its function during cell proliferation in imaginal wing discs. To test if Vap33 promotes Ankle2 function in a sensitized background, we depleted Ankle2 in the wing pouch using a weaker RNAi line. As a result, smaller adult wings developed (Li et al., 2024). We found that introduction of single mutant alleles of *Vap33* in this background enhances the small wing phenotype. This result suggests that although the Ankle2-Vap33 interaction is not essential, it nevertheless promotes Ankle2 function during wing development (**Fig 7D**).

Discussion

Post-mitotic nuclear reassembly is an essential cellular process, yet its molecular underpinnings remain only partially understood. BAF has emerged as a central player required for connections between chromosomes in telophase, for the adhesion of membranes to chromatin through LEM-Domain proteins and for lamina reassembly (Haraguchi et al., 2008; Haraguchi et al., 2001; Li et al., 2024; Samwer et al., 2017). The recruitment of BAF hinges on its dephosphorylation by

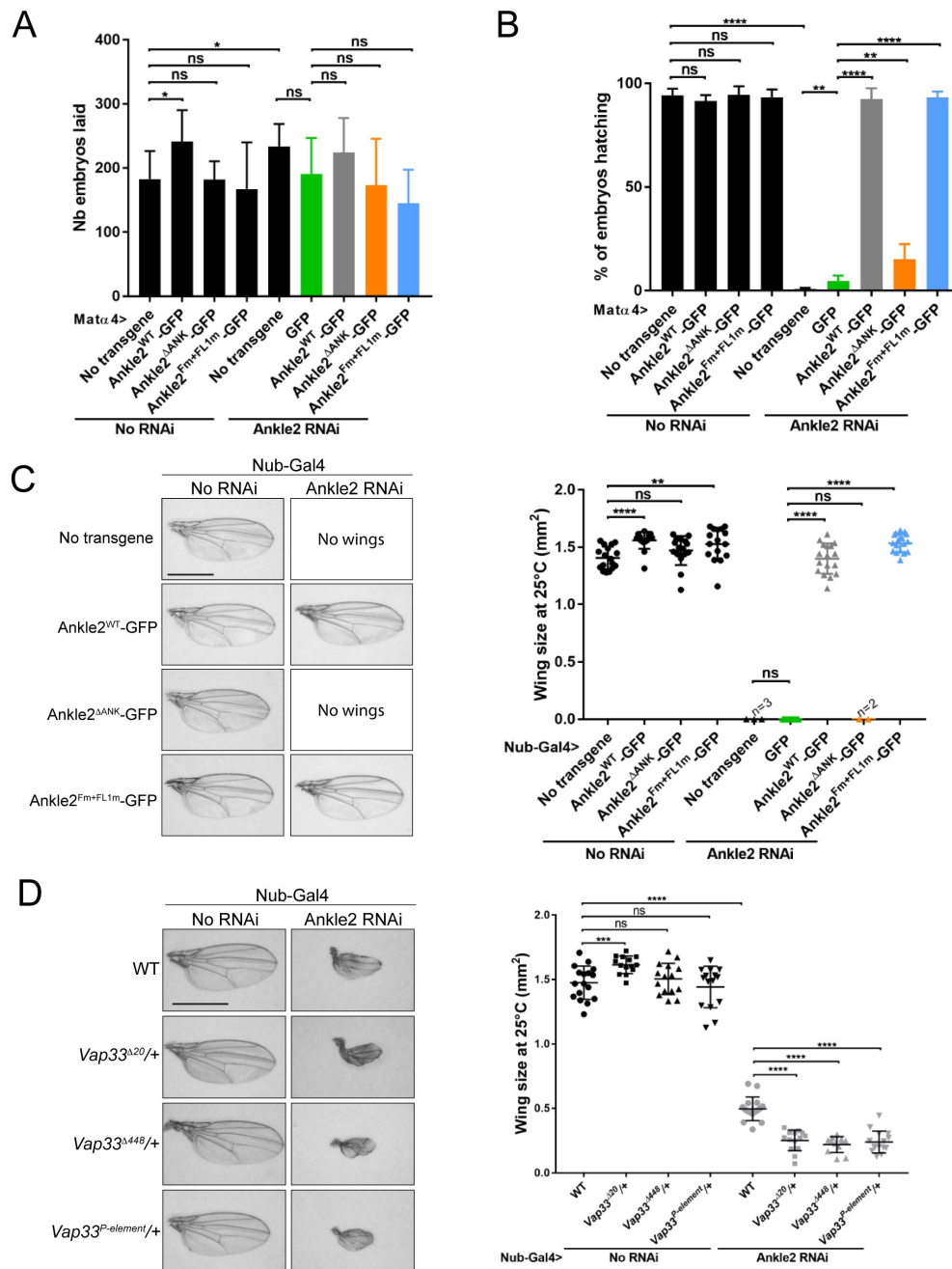


Figure 7.

Ankle2 interaction with PP2A but not with Vap3 is required for development.

A. Number of embryos laid over three days per female of the indicated genotypes. **B.** Percentage of embryos hatching per females of the indicated genotypes. In A-B, the Ankle2 RNAi insertion from line BDSC 77437 was used to deplete endogenous Ankle2 during oogenesis using the maternal *mata4*-GAL-VP16 driver and RNAi-resistant Ankle2-GFP variants were simultaneously expressed. **C.** Wing development phenotypes from flies of the indicated genotypes. The Ankle2 RNAi insertion from line BDSC 77437 was used to deplete endogenous Ankle2 in the wing pouch using Nub-Gal4 and RNAi-resistant Ankle2-GFP variants were simultaneously expressed at 25°C. Left: Examples of wings. Right: Quantification of wing size. **D.** Mutations in Vap33 enhance a small-wing phenotype caused by Ankle2 depletion. The weaker Ankle2 RNAi insertion from line VDRC100665 was used to deplete endogenous Ankle2 in the wing pouch using Nub-Gal4. Left: Examples of wings. Right: Quantification of wing size at 25°C. Averages of 12-17 wings are shown. All error bars: S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ **** $p < 0.0001$, ns: non-significant from paired t-tests with Welch's correction. Scale bars: 1 mm.

PP2A, a process dependent on Ankle2 in various animal species including humans, flies and roundworms (Asencio et al., 2012 [DOI](#); Li et al., 2024 [DOI](#); Mehse et al., 2018 [DOI](#); Snyers et al., 2018 [DOI](#)). Our study leveraged *Drosophila* as a model to elucidate how Ankle2 functions in this process.

Here we provide several lines of evidence suggesting that Ankle2 functions as a regulatory subunit of PP2A. We found that Ankle2 uses its Ankyrin domain to interact with PP2A. Ankyrin repeats are known to mediate protein interactions (Li et al., 2006 [DOI](#)). AlphaFold3 predicts an interaction of this domain with PP2A that would position Ankle2 as a regulatory subunit. Indeed, the presence of Ankle2 in the PP2A complex would be mutually exclusive with the presence of known regulatory subunits including B55 (Tws in *Drosophila*) and B56, a hypothesis further supported by our results showing that Tws competes with Ankle2 for binding to PP2A. In addition, Ankle2 co-purified PP2A-29B and Mts without any known PP2A regulatory subunits. Previous work indicated that human Ankle2 uses a similar region to interact with PP2A, comprising part of the Ankyrin domain and a segment immediately preceding it, termed the Caulimovirus Domain (CD, **Fig 1H** [DOI](#)) (Asencio et al., 2012 [DOI](#); Fishburn et al., 2024 [DOI](#)). Although the latter region is poorly conserved in *Drosophila*, our modeling suggests that it may participate in the formation of the PP2A-Ankle2 complex (Fishburn et al., 2024 [DOI](#)) (**Fig 2C** [DOI](#)). Experimental structural determination of the PP2A-Ankle2 is required to reveal the detailed structure of the complex.

Our unbiased phosphoproteomic analysis confirmed that BAF dephosphorylation depends on Ankle2 in *Drosophila* as in *C. elegans* (Asencio et al., 2012 [DOI](#); Li et al., 2024 [DOI](#)). While this dependence has not been directly tested in human cells, BAF recruitment to segregated chromosomes after anaphase, which is known to require BAF dephosphorylation, requires Ankle2 in HeLa cells (Asencio et al., 2012 [DOI](#); Sears and Roux, 2020 [DOI](#); Snyers et al., 2018 [DOI](#)). A LEM domain in human Ankle2 mediates an interaction with BAF (Snyers et al., 2018 [DOI](#)). By contrast, we did not detect an interaction between *Drosophila* Ankle2 and BAF, consistent with the absence of a LEM domain in the former, as for *C. elegans* (Asencio et al., 2012 [DOI](#); Fishburn et al., 2024 [DOI](#)). Moreover, while human Ankle2 was shown to bind and inhibit the BAF counteracting kinase VRK1 *in vitro* (Asencio et al., 2012 [DOI](#)), we detected no interaction between Ankle2 and NHK-1/Ballchen (VRK1 ortholog) in *Drosophila*. While a putative interaction between Ankle2 and NHK-1 in *Drosophila* could occur transiently, thereby escaping detection, the simplest interpretation of our results is that the loss of Ankle2 causes BAF hyperphosphorylation by preventing its PP2A-dependent dephosphorylation rather than by preventing inhibition of NHK-1. Our previous genetic analysis in the developing wing suggests that BAF is the most critical substrate of PP2A-Ankle2 during cell proliferation. Indeed, wing developmental defects upon Ankle2 depletion are rescued by lowering the dose of NHK-1 or by expressing an unphosphorylatable mutant form of BAF (Li et al., 2024 [DOI](#)). Ankle2 may enable PP2A to dephosphorylate BAF through a transient interaction independently from the LEM domain and/or by targeting PP2A to a subcellular location that is favorable to the reaction (discussed below). Interestingly, we identified several additional potential substrates of PP2A-Ankle2 (**Fig 2A** [DOI](#) and Table S5). We were so far unable to obtain sufficient amounts of the PP2A-Ankle2 complex to conduct robust *in vitro* phosphatase assays with phosphorylated BAF or other candidate substrates.

We also discovered a novel interaction between Ankle2 and the ER protein Vap33. We show that FFAT motifs in Ankle2 interact with the MSP domain of Vap33. This interaction is required for the localization of Ankle2 to the reassembling NE in telophase, and to the NE and ER in interphase. The recent identification of putative FFAT motifs in human Ankle2 (Neefjes and Cabukusta, 2021 [DOI](#)) suggests that the interaction between Ankle2 and VAP family proteins of the ER may be conserved.

These findings led us to propose a model where Vap33 targets the PP2A-Ankle2 holoenzyme to ER membranes, promoting BAF dephosphorylation and recruitment in a localized manner during nuclear reassembly. Supporting this model, we detected a complex comprising Vap33, Ankle2, PP2A-29B and Mts. Our results using cells in culture, embryos and developing wings strongly

suggest that the Ankle2-PP2A interaction is essential for BAF dephosphorylation and recruitment to nascent nuclei in telophase, as well as for nuclear reassembly and animal development. However, it remains formally possible that the deletion of Ankyrin repeats used to disrupt the Ankle2-PP2A interaction abrogated another, unknown aspect of Ankle2 function. Similarly, we found that the Ankle2-Vap33 interaction, while being less critical, also promotes BAF recruitment during nuclear reassembly in embryos. Conceptually, the novel mechanism we propose implies that the ER is more than a passive source of membranes in nuclear reassembly; it implicates the ER as a carrier of localized enzymatic activity needed for the process.

Future work should examine the functions of other protein interactions and potential substrates of PP2A-Ankle2 that we identified, which could implicate this holoenzyme in the regulation of various cellular processes. Particularly intriguing are the interactions we identified between Ankle2 and Cyclin-CDK subunits. The possibility that Ankle2, while functioning as a PP2A subunit, may also impact Cyclin-CDK functions warrants further investigation. Conversely, Cyclin-CDKs may also contribute to the regulation of PP2A-Ankle2 in the cell cycle, an area that remains to be explored.

Materials & methods

Plasmids and mutagenesis

Drosophila cells expression vectors were generated using Gateway recombination system (Invitrogen). The cDNA of interest was first cloned into the pDONR221 entry vector and then recombined into the destination vector with C-terminal or N-terminal tag containing copper-inducible or constitutively active promoters, pMT or pAc5, respectively. The RNAi resistant (RNAi res) cDNA for Ankle2 was generated by replacing codons of the original cDNA of the longest form of Ankle2 with synonymous codons. The following expression vectors were generated: pAc5-FLAG-GFP, pAc5-FLAG-Tws, pMT-Ankle2-GFP, pMT-Ankle2^{Fm}-GFP, pMT-Ankle2^{FL1m}-GFP, pMT-Ankle2^{FL2m}-GFP, pMT-Ankle2^{Fm+FL1m}-GFP, pMT-Ankle2^{Fm+FL2m}-GFP, pMT-Ankle2^{ΔANK}-GFP, pMT-Ankle2-GFP (RNAi res), pMT-Ankle2^{ΔANK}-GFP (RNAi res), pMT-Ankle2^{Fm+FL1m}-GFP (RNAi res), pMT-Ankle2^{Fm1+FL2m+ΔTM}-GFP (RNAi res), pMT-Ankle2¹⁻⁵⁸⁷-GFP (RNAi res), pMT-Ankle2⁵⁸⁸⁻¹¹⁷⁴-GFP (RNAi res), pMT-Ankle2^{Δ992-1040}-GFP (RNAi res), pAc5-Ankle2-FLAG (RNAi res), pAc5-Ankle2^{F1m+FL2m}-FLAG (RNAi res), pAc5-Ankle2^{ΔANK}-FLAG (RNAi res), pAc5-Vap33-Myc, pAc5-Vap33^{87D89D}-Myc, pAc5-Vap33-GFP, pAc5-Vap33^{87D89D}-GFP, pAc5-PP2A-29B-GFP, pAc5-GFP-PP2A-29B, pMT-RFP-BAF^{WT}, pMT-RFP-BAF^{3A}. Point mutations and deletions in the pDONR of interest were generated using QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent), as described by the manufacturer's instructions. GST-Ankle2 (WT, 1-274, 275-450, 451-909, 910-1174, 910-1174+Fm, 910-1174+FL1m, 910-1174+FL2m) expression vectors were constructed into the pGEX4T vector by classic cloning process. pUAS-Ankle2-GFP, pUAS-Ankle2-GFP (RNAi res), pUAS-Ankle2^{Fm+FL1m}-GFP (RNAi res), pUAS-Ankle2^{ΔANK}-GFP (RNAi res), pUAS-PP2A-29B-GFP, pUAS-RFP-BAF, pUAS-RFP-Vap33 and were generated by cloning PCR amplicons of interest into the pUAS-K10attB vector using restriction enzymes, NotI and BamHI.

Cell culture, transfections and cell lines

D-Mel cells were cultured in Express Five medium (Invitrogen) supplemented with glutamine, penicillin and streptomycin (Wisent) at 25°C. Transfections with plasmids were performed using X-tremeGENE HP DNA Transfection Reagent (Roche) following the manufacturer's protocol. Stable cell lines were selected by adding 20 ug/ml blasticidin into the cell culture after transient transfections of plasmids of interest. While inducible pMT-based vectors contain the gene coding for blasticidin resistance, pAc5-based vectors were co-transfected with pCoBlast to confer resistance to blasticidin to the cells. 300μM CuSO₄ were added into medium to induce expression of the plasmids containing pMT promoter, at least 16 hours before use. For RNA interference, dsRNAs were generated from PCR amplicons using a Ribomax Kit (Promega). RNAi non-target (NT)

were generated against the sequence of the procaryotic kanamycin resistance gene. 1×10^6 cells were plated in 6-well plate and treated with 1ml of medium containing 20 μg of indicated dsRNA for 4 days (rescue experiments). Cells were then harvested and analyzed by immunoblotting, immunofluorescence or live-cell imaging.

Fly genetics

Fly husbandry was done according to standard procedures. Oregon R was used as the WT strain. Transgenic flies for expression of *pUAS-Ankle2-GFP*, *pUAS-RFP-Vap33*, and *pUAS-PP2A-29B-GFP* were generated by site-directed insertions of the pUAS-K10attB-based vectors on the second chromosome in the attP40 strain (BestGene Inc, Chino Hills, CA, UAS). Transgenic lines for expression of *pUAS-Ankle2-GFP RNAi res* (WT, Fm+FL1m, ΔANK) were generated by site-directed insertions of the pUAS-K10attB-based vectors on the third chromosome in the attP154 strain (BestGene Inc, Chino Hills, CA, UAS). UAS-Ankle2 RNAi (BDRC 77437) and UAS-GFP (BDRC 4776) used in this study were purchased from Bloomington Drosophila Stock Center.

For the genetic rescue experiment, a combination of UAS-Ankle2-GFP (RNAi res, WT and mutant) and Ankle2 RNAi (BDRC 77437) were generated using a pair of second and third balancer chromosomes. UAS-GFP (BDSC 4776) was used as control for ruling out the possible effect due to Gal4 dilution. Expression of transgenes in the embryo and developing wings was driven by *mat α 4-Gal4-VP16* (BDRC 7062) and *Nubbin-Gal4* (BDRC 86108). All crosses were performed at 25°C with 60-70% humidity. Fertility tests were carried out by scoring eggs on the grape juice agar from a single female being crossed with three males for 24 hours.

Protein purifications from *Drosophila* cells and fly embryos

GFP affinity purifications were performed from *Drosophila* cells and fly embryos, as described (Emond-Fraser et al., 2023 [↗](#)). Briefly, cells stably expressing pAc5-FLAG-GFP, pMT-GFP-Ankle2 and pMT-Ankle2-GFP were harvested from four confluent 175cm² flasks and resuspended in lysis buffer (75 mM K-HEPES pH7.5, 150 mM NaCl, 2 mM EGTA, 2 mM MgCl₂, 1 mM DTT, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM PMSF, 5% glycerol, 0.5% Triton X-100). Embryos were collected every 2 hours and dechorionated in 50% bleach, washed in PBS and frozen in liquid nitrogen. Embryos were then crushed in lysis buffer as above. Cell and embryo lysates were incubated for 20 minutes at 4°C on a wheel, centrifuged at max speed for 15 minutes at 4°C, and subsequently incubated with GFP-trap nanobeads (Chromotek) for 2 hours. Beads were firstly washed 5 times with lysis buffer and then washed additionally 5 times in PBS with protease inhibitors (1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ aprotinin and 10 $\mu\text{g}/\text{ml}$ leupeptin) before sending for mass spectrometry. A small portion of the sample was also eluted in Laemmli buffer and analyzed by SDS-PAGE with silver nitrate staining.

Mass spectrometry

For proteomic analyses, samples were reconstituted in 50 mM ammonium bicarbonate urea 1M with 10 mM TCEP [Tris(2-carboxyethyl)phosphine hydrochloride; Thermo Fisher Scientific], and vortexed for 1 h at 37°C. Chloroacetamide (Sigma-Aldrich) was added for alkylation to a final concentration of 55 mM. Samples were vortexed for another hour at 37°C. One microgram of trypsin was added, and digestion was performed for 8 h at 37°C. Samples were dried down and solubilized in 5% acetonitrile (ACN)-4% formic acid (FA). The samples were loaded on a 1.5 μl pre-column (Optimize Technologies, Oregon City, OR). Peptides were separated on a home-made reversed-phase column (150- μm i.d. by 200 mm) with a 56-min gradient from 10 to 30% ACN-0.2% FA and a 600-nL/min flow rate on a Easy nLC-1200 connected to a Exploris 480 (Thermo Fisher Scientific, San Jose, CA). Each full MS spectrum acquired at a resolution of 120,000 was followed by tandem-MS (MS-MS) spectra acquisition on the most abundant multiply charged precursor ions for 3s. Tandem-MS experiments were performed using higher energy collision dissociation (HCD) at a collision energy of 34%. The data were processed using PEAKS X Pro (Bioinformatics Solutions,

Waterloo, ON) and a Uniprot database. Mass tolerances on precursor and fragment ions were 10 ppm and 0.01 Da, respectively. Fixed modification was carbamidomethyl (C). Variable selected posttranslational modifications were acetylation (N-ter), oxidation (M), deamidation (NQ), phosphorylation (STY). The data were visualized with Scaffold 5.0 (protein threshold, 99%, with at least 2 peptides identified and a false-discovery rate [FDR] of 1% for peptides).

For phosphoproteomic analyses, 500 µg of cell lysate (measured by Bradford assay) were reconstituted in 50 mM ammonium bicarbonate with 10 mM TCEP [Tris(2-carboxyethyl)phosphine hydrochloride; Thermo Fisher Scientific], and vortexed for 1 h at 37°C. Chloroacetamide (Sigma-Aldrich) was added for alkylation to a final concentration of 55 mM. Samples were vortexed for another hour at 37°C. 10 µg of trypsin was added, and digestion was performed for 8 h at 37°C. Samples were dried down in a speed-vac. For the TiO₂ enrichment procedure, sample loading, washing, and elution were performed by spinning the microcolumn at 8000 rpm at 4 °C in a regular Eppendorf microcentrifuge. The spinning time and speed were adjusted as a function of the elution rate. Phosphoproteome enrichment was performed with TiO₂ columns from GL Sciences. Digests were dissolved in 400 µL of 250 mM lactic acid (3% TFA/70% ACN) and centrifuged for 5 min at 13000 rpm, and the soluble supernatant was loaded on the TiO₂ microcolumn previously equilibrated with 100 µL of 3% TFA/70% ACN. Each microcolumn was washed with 100 µL of lactic acid solution followed by 200 µL of 3% TFA/70% ACN to remove nonspecific binding peptides. Phosphopeptides were eluted with 200 µL of 1% NH₄OH pH 10 in water and acidified with 7 µL of TFA. Eluates from TiO₂ microcolumns were desalted using Oasis HLB cartridges by spinning at 1200 rpm at 4 °C. After conditioning with 1 mL of 100% ACN/0.1% TFA and washing with 0.1% TFA in water, the sample was loaded, washed with 0.1% TFA in water, then eluted with 1 mL of 70% ACN (0.1% TFA) prior to evaporation on a SpeedVac. The extracted peptide samples were dried down and solubilized in 5% ACN-0.2% FA. The samples were loaded on an Optimize Technologies C₄ precolumn (0.3-mm i.d. by 5 mm) connected directly to the switching valve. They were separated on a home-made reversed-phase column (150-µm i.d. by 150 mm Phenomenex Jupiter C18 stationary phase) with a 120-min gradient from 10 to 30% ACN-0.2% FA and a 600-nL/min flow rate on a Easy nLC-1200 (ThermoFisher Scientific, San Jose, CA) connected to an Exploris 480 (Thermo Fisher Scientific, San Jose, CA). Each full MS spectrum was acquired at a resolution of 120,000 and followed by tandem-MS (MS-MS) spectra acquisition for 3 s on the most abundant multiply charged precursor ions. Tandem-MS experiments were performed using higher-energy collisional dissociation (HCD) at a collision energy of 27%. The data were processed using PEAKS X Pro (Bioinformatics Solutions, Waterloo, ON) and a *Drosophila melanogaster* unreviewed Uniprot database. Mass tolerances on precursor and fragment ions were 10 ppm and 0.01 Da, respectively. Variable selected posttranslational modifications were carbamidomethyl (C), oxidation (M), deamidation (NQ), acetyl (N-term) and phosphorylation (STY). The data were visualized with Scaffold 5.0 (protein threshold, 99%, with at least 2 peptides identified and a false-discovery rate [FDR] of 1% for peptides).

Western blotting and immunofluorescence

Proteins lysates were analyzed on SDS-PAGE and then transferred onto a PVDF membrane. The membrane was then blocked with 5% of milk solution for 1 hour to prevent non-specific binding. Subsequently, the membrane was probed with primary antibodies for 3 hours at 25°C or overnight at 4°C. The following primary antibodies used in this study were: anti-GFP from rabbit (1:5000, Torrey Pine Biolabs), anti-Myc 9E10 from mouse (1:2000, #sc-40, Santa Cruz Biotechnology), anti-FLAG M2 from mouse (1:2000, #F1804, Sigma), anti-tubulin DM1A from mouse (1:5000, #T6199, Sigma), anti-Ankle2 from rabbit (1:1000, custom-made by Thermo Fisher Scientific), anti-Mts from mouse (1:1000, #610555, BD Biosciences), anti-Tws from rabbit (1:2000, custom-made by Thermo Fisher Scientific). After 3 washes of 10 minutes with PBS containing 0.1% Tween (PBST, 0.1%), the membrane was probed with secondary antibody for 30 minutes at room temperature. Peroxidase-conjugated anti-rabbit or anti-mouse from goat (1:5000, Jackson ImmunoResearch) were used as secondary antibodies. The membrane being washed 3 times in PBST, 0.1% was then incubated with Clarity Western ECL Substrate (# 170-5061, Bio-Rad) and subsequently imaged using ChemiDoc™

MP Imaging system. To evaluate the phosphorylation level of the protein of interest, protein lysates were separated by SDS-PAGE in presence of the Phos-tag (Fujifilm WAKO Chemical), following the manufacturer's instructions. One wash of the acrylamide gel with the transfer buffer containing 1mM EDTA and subsequent second wash with only the transfer buffer were performed before the electrotransfer.

For Immunofluorescence, cells were first fixed with PBS containing 4% formaldehyde for 25 minutes. After three washes of 10min with PBS containing 0,2% Triton X-100(PBST), cells were permeabilized and blocked in PBST containing 1% BSA for 1 hour. Then, cells were incubated with primary antibodies for 2 hours at room temperature, washed three times with PBST, and subsequently incubated with secondary antibodies and DAPI for 1 hour avoiding light. Primary antibodies used in this study were: anti-Lamin from mouse (1:200, DSHB Hybridoma Product ADL84.12), anti-GFP from rabbit (1:1000, Torrey Pine Biolabs). Secondary antibodies from mouse and rabbit were respectively coupled to Alexa-647(1:200, Invitrogen) and Alexa-488 (1:200, Invitrogen).

Fluorescence *in situ* hybridization (FISH)

Drosophila embryos were collected, dechorionated in 50% bleach and washed three times in 0,7% NaCl, 0,05% Triton-100. Eggs were then fixed in methanol: heptane (1:1) and rehydrated successively in methanol: PBS solutions of the ratio of 9:1, 7:3 and 1:1. FISH was performed following procedures as described in, using a probe against the 359-base pair peri-centromeric repeat on the X chromosome. α -Tubulin in the embryos was stained with primary antibodies (YL1/2 from rat at 1:2000, Sigma) and subsequent secondary antibodies (anti-rat Alexa 647 at 1:1000, Invitrogen). DNA was marked with QUANT-IT Oli-green at 1:5000 (#O7582, Invitrogen). Tetrahydronaphthalene was used to mount embryos before being imaged by a confocal microscope.

GST pulldowns

D-Mel cells expressing Ac5-Vap33-Myc, Ac5-Vap33^{87D89D}-Myc, Ac5-PP2A-29B-GFP or Ac5-GFP-PP2A-29B were harvested from a confluent 175 cm² flask. Cells were then lysed in lysis buffer (75 mM K-HEPES pH 7.5, 150 mM KCl, 2 mM EGTA, 2 mM MgCl₂, 5% glycerol, 0.2% Triton X-100, 1 mM DTT) supplemented with protease inhibitors cocktail as described above at 4°C for 20 minutes. Cell lysates being centrifuged at max speed for 15 minutes were incubated with Sepharose beads bound to GST and GST-Ankle2 (truncated forms) for 1.5 hours at 4°C. Beads were washed 5 times in lysis buffer and subsequently used for SDS-PAGE analysis and Western blot.

Co-immunoprecipitation (Co-IP)

Pelleted cells from confluent 25 cm² flasks were lysed in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT, 5% glycerol, 0.5% NP40 Substitute supplemented with protease inhibitors cocktail as described above. Cell lysates being lysed for 15 minutes on a rotating wheel at 4°C were centrifuged at max speed for 10 minutes, and supernatants were incubated with 10 μ l GFP-Trap agarose beads (Chromotek) for 2 hours at 4°C. Beads were washed four times in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT, 5% glycerol, 0.1% NP40 Substitute with protease inhibitors. Samples were eluted in Lammeli buffer and analyzed using Western blot.

Microscopy

A confocal system Leica SP8 was used for imaging of fixed cells and embryos. Acquired images of cells were subsequently processed with the Lightning system. Live imaging of cells and embryos was performed by a spinning disk confocal system (Yokogawa CSU-X1 5000) mounted on a fluorescence microscope (Zeiss Axio Observer.Z1). Cells were cultured on a LabTek II chambered coverglass (#155409, Thermo Fisher Scientific) at least two hours before time-lapse imaging. For

live analysis of embryos, 0-2h embryos were dechorionated in 50% bleach and then aligned on a coverslip (#P35G-1.5-14-C, MatTek) and covered with halocarbon oil. Films of embryos were taken every 10s with six confocal sections of about 3 μ m. To monitor Ankle2-GFP (WT and mutants) and RFP-BAF levels, respectively, the mean intensity of GFP and RFP fluorescence in the region of interest were measured directly with Zen software (Zeiss) at different time points. For each point, the mean intensity of GFP and RFP was normalized by subtraction of mean intensity at anaphase onset (T0) in the core region. Images of adult flies were acquired by stereomicroscope with a camera (Canon). Developing wings of adult flies were dissected and quantified using Fiji software (National Institute of Health).

Structure Predictions

Prediction of co-complexes was performed using the AlphaFold3 (Abramson et al., 2024 <https://doi.org/10.26434/chemrxiv-2024-08-08>) AlphaFold Server (Beta) (<https://alphafoldserver.com> [↗](#)), with default seed auto-generation and with seed provided, yielding sets of 5 models. Co-complexes modelled are of Mts (NCBI accession NP_001285724.1), PP2A-29B (NP_001027225.1) and Ankle2 (NP_573221.2); Mts, PP2A-29B and Tws (NP_001287269.1); and Mts, PP2A-29B, Ankle2 and Vap33 (NP_570087.1). Each set of 5 models showed self-consistent interactions and a typical example is shown in the figures.

Statistical analysis

All the graphs and statistical analysis in this study were done by GraphPad software. In all figures, the results of quantifications are expressed as mean $\pm$ SD with indicated statistics in the legend. Overall, p values are represented as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p $\leq$ 0.0001, and n.s. (not significant) is p > 0.05.

Figure supplements

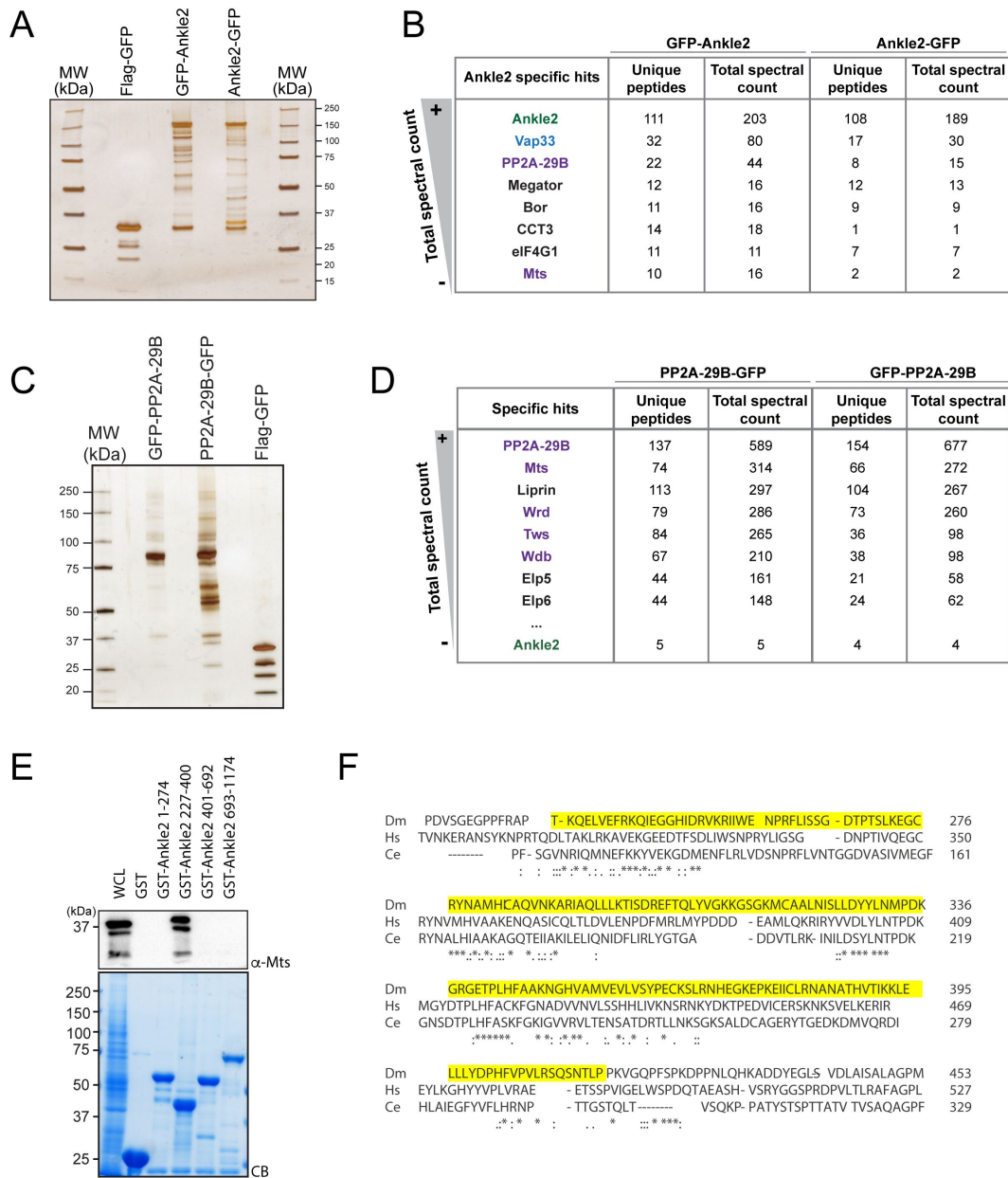


Figure S1.

Ankle2 interacts with PP2A through its Ankyrin repeats region - Complement to Figure 1.A-B

Proteins obtained after purification of Ankle2-GFP, GFP- Ankle2 or Flag-GFP from D-Mel cells. **A.** Silver-stained gel showing a fraction of the purification products. **B.** Proteins specifically identified with GFP-Ankle2 or Ankle2-GFP. Proteins with the highest total spectral counts for GFP-Ankle2 are shown. **C-D.** Proteins obtained after purification of PP2A-29B-GFP, GFP-PP2A-29B or Flag-GFP from D-Mel cells. **C.** Silver-stained gel showing a fraction of the purification products. **D.** Proteins specifically identified with PP2A-29B-GFP and GFP-PP2A-29B. Proteins with the highest total spectral counts for PP2A-29B-GFP are shown. Ankle2 was also identified as a specific interactor further down the list. Purple names: known PP2A subunits. **E.** A region of Ankle2 comprising amino-acid residues 227-400 is sufficient for interaction with PP2A. GST-fused fragments of Ankle2 produced in bacteria were used in GST-pulldowns with extracts from D-Mel cells. Mts was detected by Western blot. CB: Coomassie Blue. **F.** Alignment of the Ankyrin domain region of Ankle2 from *D. melanogaster*, *H. sapiens* and *C. elegans*. The alignment was done with full-length sequences using Clustal Omega. The Ankyrin domain region of Ankle2 (a.a. 232-415) deleted in Ankle2^{ΔANK} is shown in yellow.

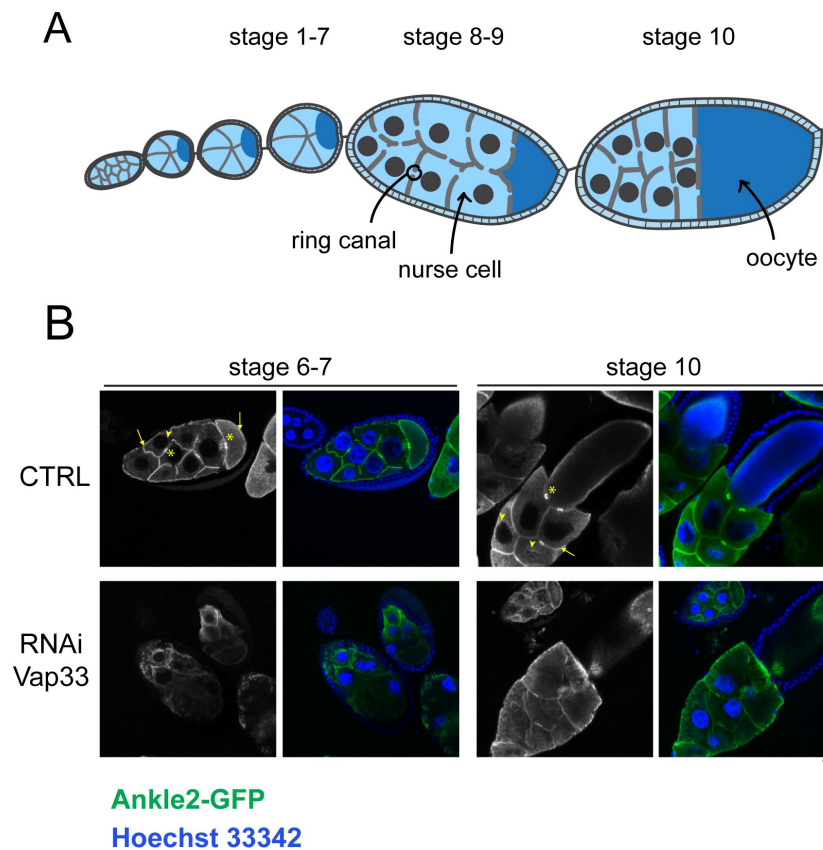


Figure S2.

The localization of Ankle2 depends on Vap33 in egg chambers.

A. Schematic representation of different stages of egg chamber maturation. **B.** Egg chambers expressing Ankle2-GFP from the *mat4-GAL-VP16* driver, with or without expression of Vap33 RNAi from the same driver, were stained for DNA with Hoechst 33342. Note that Ankle2-GFP localization around nuclei (arrowheads), the plasma membrane (arrows) and ring canals (asterisks) is abrogated upon Vap33 RNAi. Scale bar: 20 μ m.

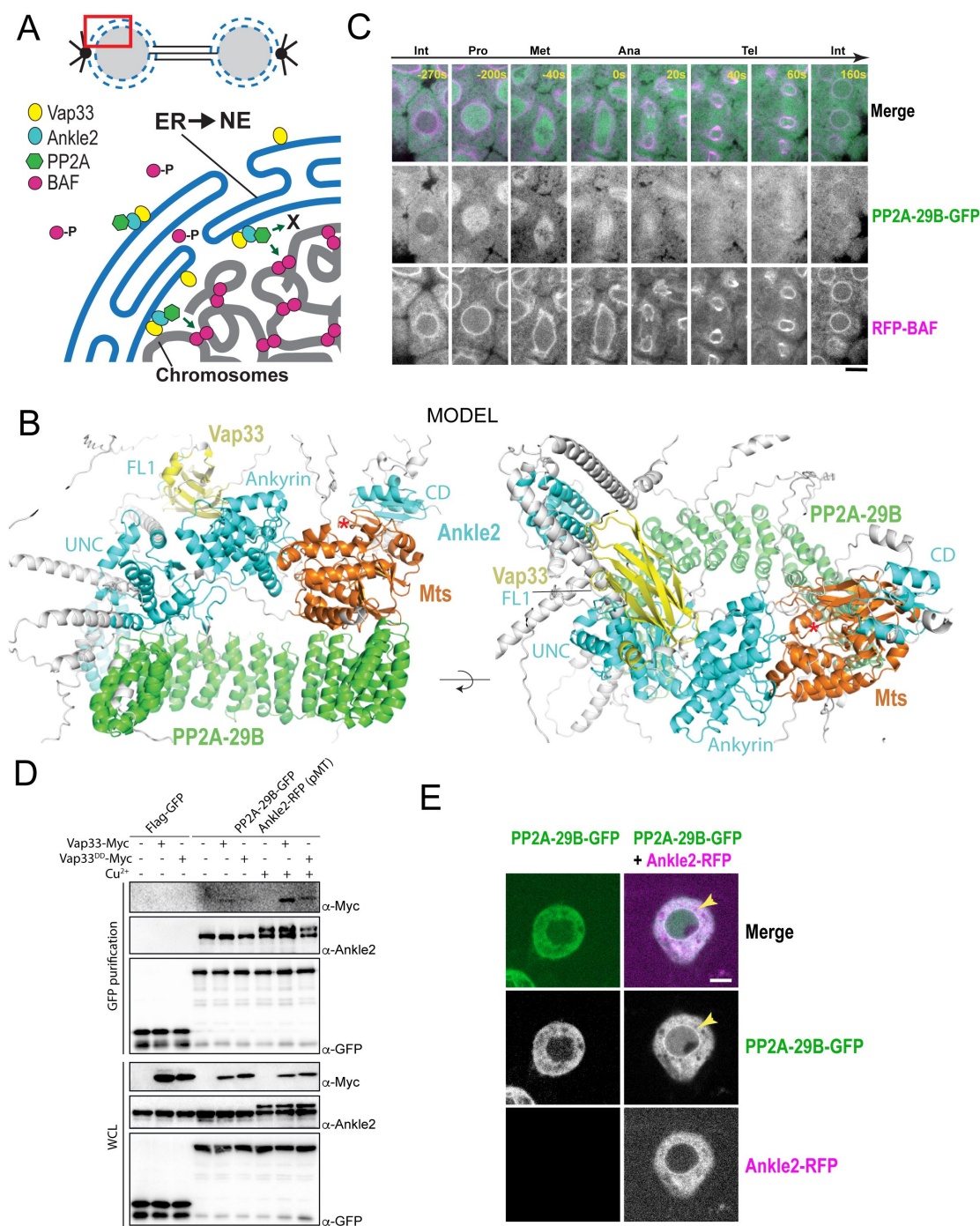


Figure S3.

A Vap33-Ankle2-PP2A complex may promote nuclear reassembly in a localized manner.

A. Hypothetical model discussed in the text. ER: Endoplasmic Reticulum; NE: Nuclear Envelope. **B.** AlphaFold3 predicted model of a complex between *Drosophila* Ankle2, PP2A-29B, Mts and Vap33. Residues with Ca confidence scores below 0.75 are coloured grey. Red asterisks denote the positions of the phosphatase catalytic site. **C.** Imaging of a mitotic cycle in an embryo expressing PP2A-29B-GFP and RFP-BAF. Scale bar: 5 μ m. **D.** Overexpression of Ankle2-RFP enhances complex formation between Vap33-Myc and PP2A-29B-GFP. Cells were transfected to express the indicated proteins and submitted to GFP affinity purifications. Cu^{2+} was added to induce the expression of Ankle2-RFP (under the pMT promoter). Products were analyzed by Western blotting. WCL: Whole cell extracts. **E.** Overexpression of Ankle2-RFP enhances the localization of PP2A-29B-GFP to the nuclear envelope (arrows). Scale bars: 5 μ m.

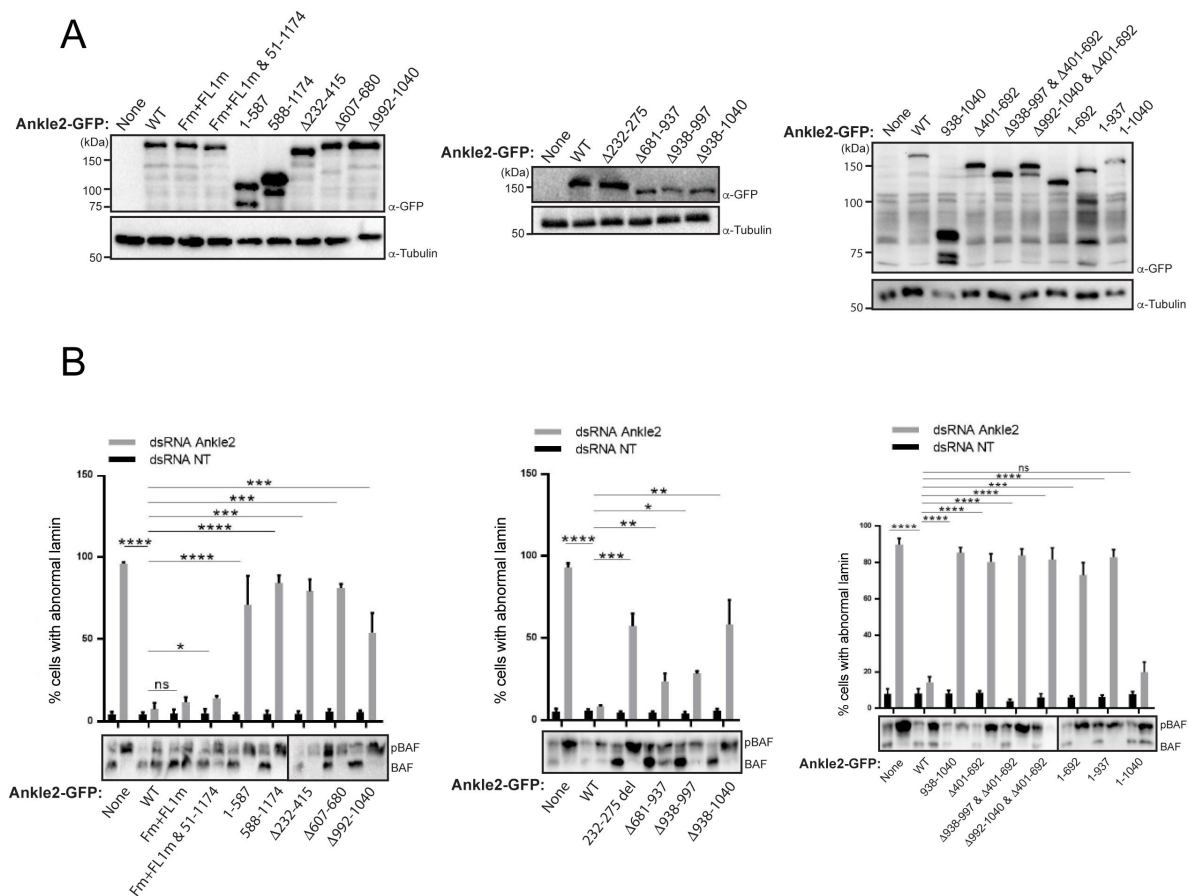


Figure S4.

Results of rescue experiments in D-Mel cells in culture.

Endogenous Ankle2 was depleted by RNAi and RNAi-insensitive Ankle2-GFP variants were simultaneously expressed as in **Figure 6**. D-Mel cells expressing RNAi-insensitive Ankle2-GFP variants or not were transfected with dsRNA against Ankle2 or non-target dsRNA (NT, against bacterial KAN gene). **A**. Western blot analyses showing expression of the different variants of Ankle2-GFP. α -Tubulin: loading control. **B**. Quantification of the abnormal Lamin phenotypes from rescue experiments (Top). Western blots (Bottom) were obtained from a gel containing Phos-Tag to increase the resolution between phosphorylated BAF (phos. BAF) and unphosphorylated BAF (BAF). Averages of 3 experiments are shown. All error bars: S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns: non-significant from paired t-tests.

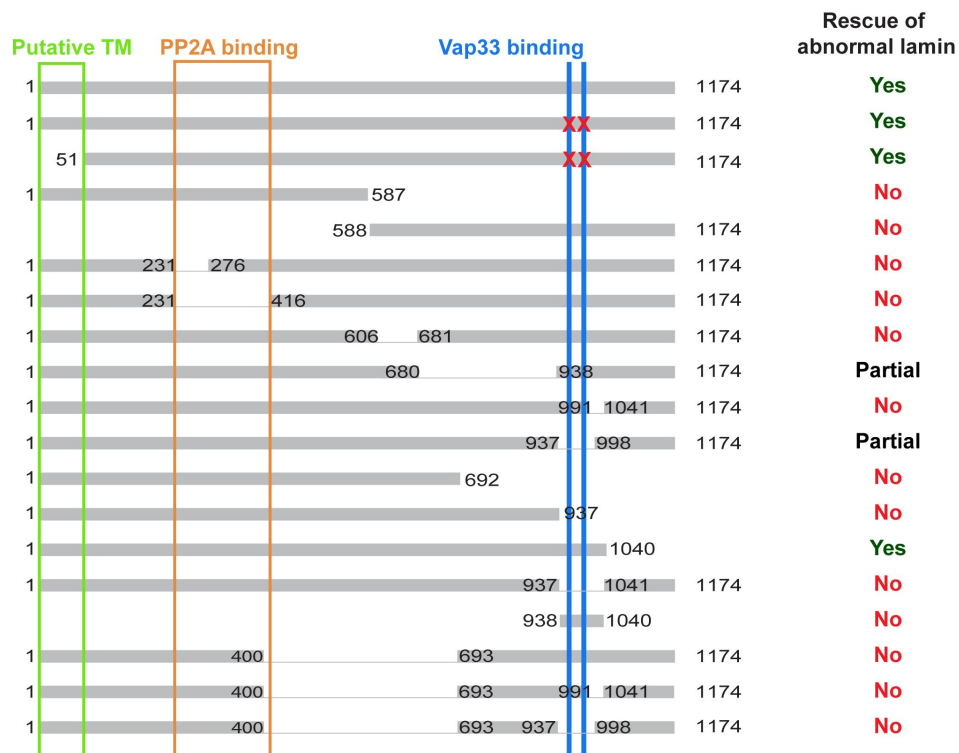


Figure S5.

Summary of results for various deletion mutants of Ankle2-GFP assessed for their ability to rescue abnormal Lamin phenotypes.

Regions of interest discussed in the text are indicated with colored boxes. Red Xs: mutations of FFAT and FFAT-Like 1 Vap33 binding motifs.

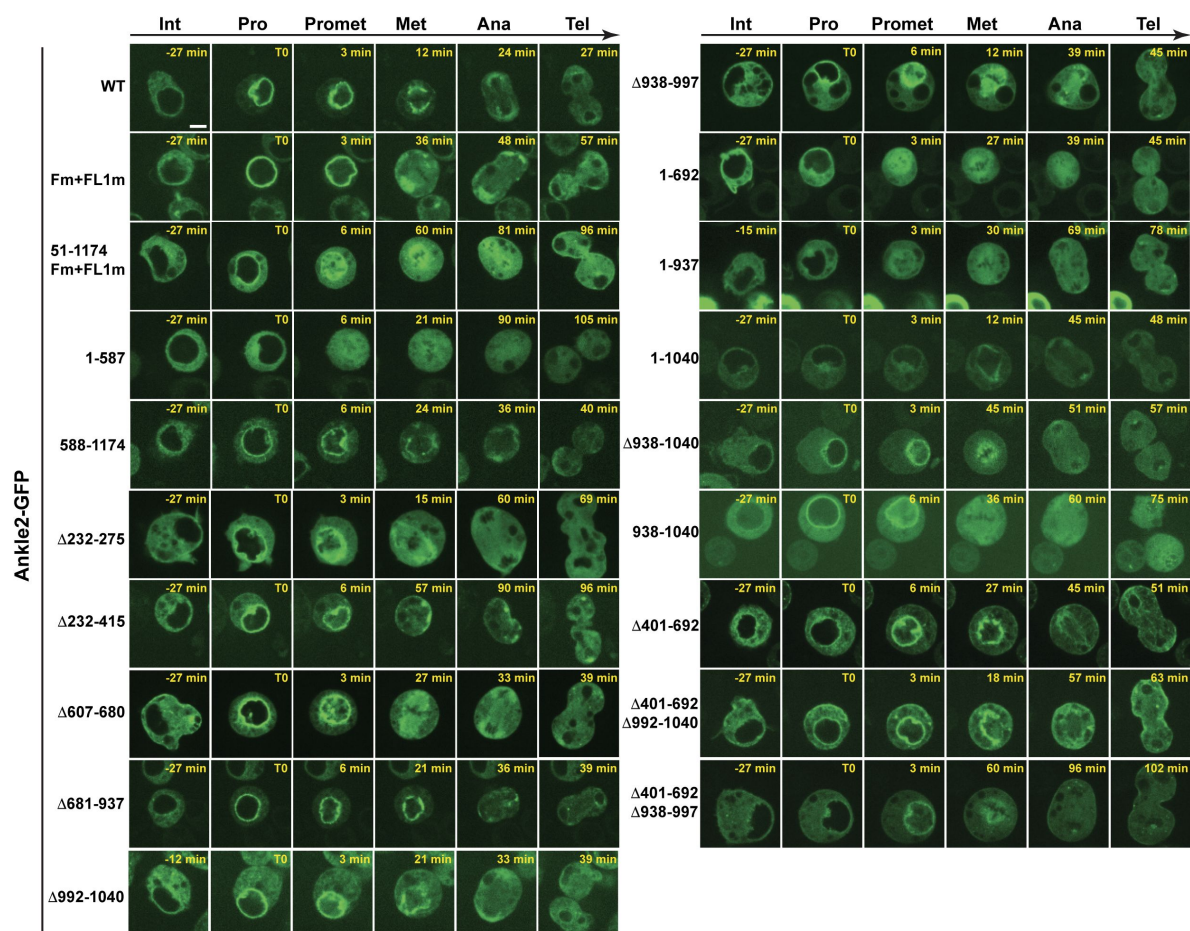


Figure S6.

Localization of Ankle2-GFP variants during the cell cycle in D-Mel cells.

Cells were imaged on a confocal microscope. Representative images of different stages of the mitotic cycle are shown. Int: Interphase; Pro: Prophase; Promet: Prometaphase; Met: Metaphase; Ana: Anaphase; Tel: Telophase. Scale bar: 5 μ m.

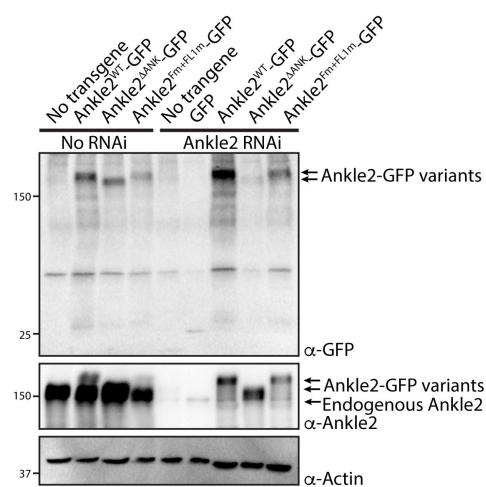


Figure S7.

Female germline expression of Ank2-GFP variants in transgenic lines.

Expression of UASp-Ank2-GFP WT, Δ ANK or Fm+FL1m (RNAi insensitive) was induced using the *mat α 4*-GAL-VP16 driver. Ank2 RNAi (line BDSC 77437) was also induced using by the same driver. Western blots from ovaries, probing for GFP, Ank2 and Actin, show relative expression levels.

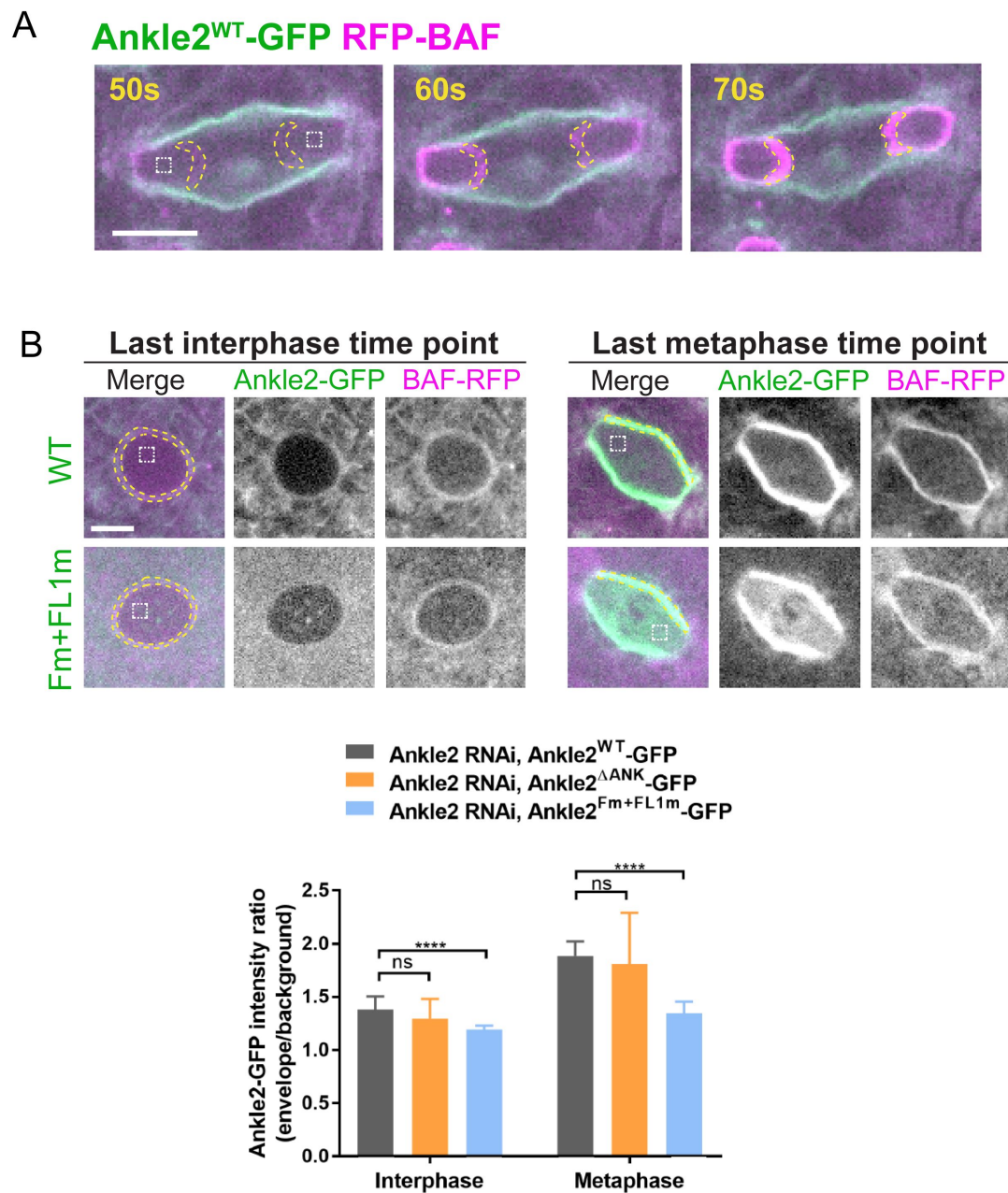


Figure S8.

Complement to Fig 6 - Quantifications in embryos.

A. Illustration of the quantification of fluorescence intensities at the inner core region of the reassembling NE in embryos expressing Ankle2-GFP and RFP-BAF. Inner core regions correspond to the area within the dotted lines where RFP-BAF is recruited in telophase. For each nuclear division quantified, the average of mean intensities from both inner core regions was calculated for each time point. **B.** Interaction of Ankle2 with Vap33 promotes Ankle2 localization during mitosis. Top: Quantification of fluorescence intensities of Ankle2-GFP (WT or Fm+FLm1) at the NE and spindle envelope at the last time points in interphase and metaphase, respectively. Mean intensities of Ankle2-GFP at the NE delineated based on RFP-BAF localization were measured (inside yellow dotted line) in interphase. Fluorescence inside the nucleus was measured as the background (white dotted boxes). For metaphase, mean intensities of Ankle2-GFP in the lateral spindle envelope (inside yellow dotted lines) and within spindles (white dotted boxes, background) were measured. Bottom: ratios of Ankle2-GFP (WT, Fm+FLm1, Δ ANK) at the membranes relative to backgrounds in interphase and metaphase. 15 nuclear divisions from 5-6 embryos were quantified for each genotype. All scale bars: 5 μ m. *** $p < 0.001$, n.s: non-significant ($p > 0.05$) from unpaired t-tests with Welch's correction.

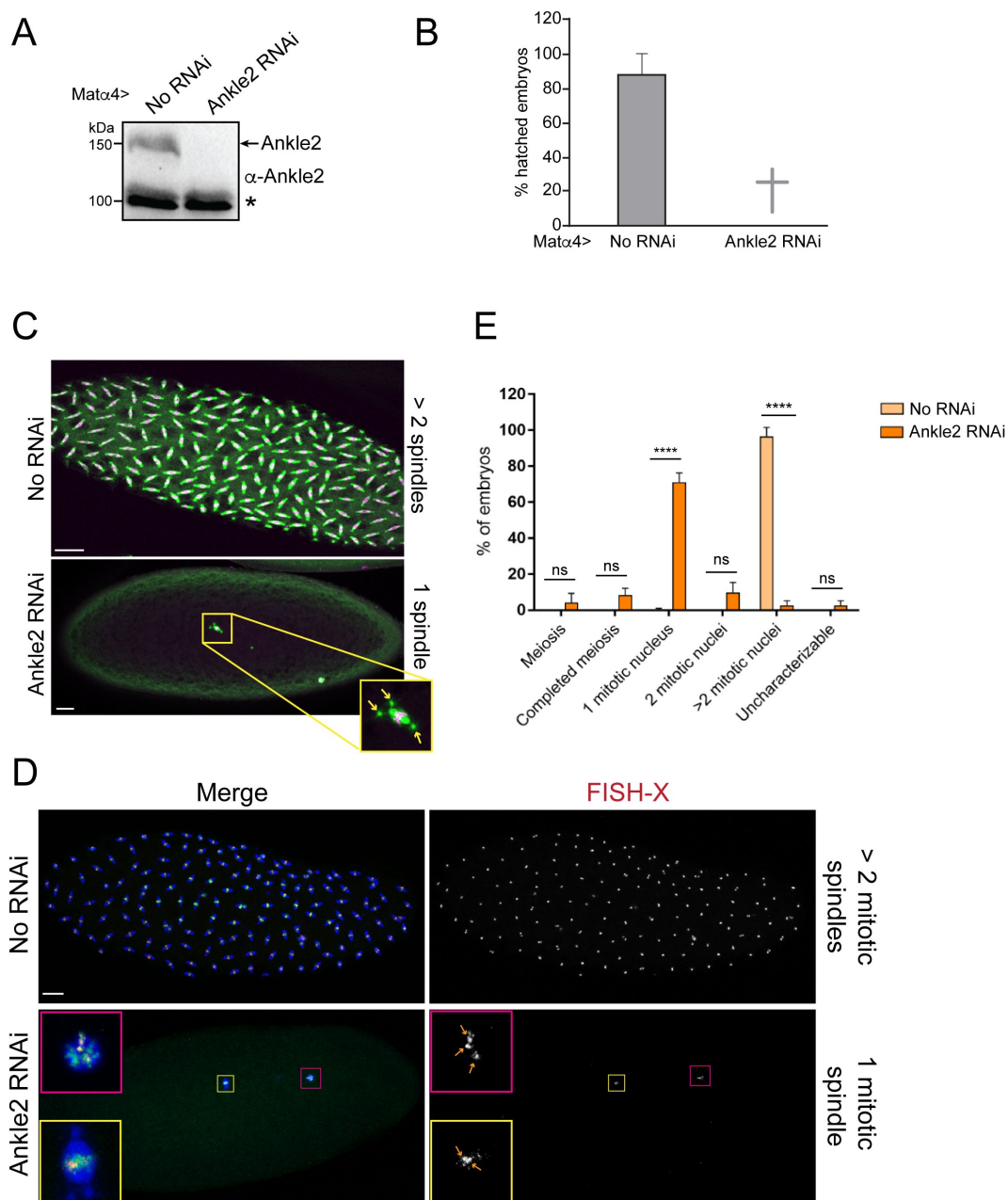


Figure S9.

Phenotypes resulting from RNAi depletion of Ankle2 during oogenesis.

Ankle2 RNAi (line BDSC77437) was using the *matα4*-GAL-VP16 driver. **A**. Western blot from ovaries showing Ankle2 depletion. The asterisk indicates a non-specific band used as loading control. **B**. Embryos fail to hatch after Ankle2 RNAi. Averages of 3 independent experiments are shown. Error bar: S.D. **C**. Representative phenotypes of embryos examined by immunofluorescence. Green: α-Tubulin; magenta: histone. Arrows indicate centrosomes, confirming that the spindle is in mitosis and not female meiosis. **D**. Embryos were examined by FISH to reveal the X-chromosome and immunofluorescence for α-Tubulin (blue) and DNA (green). Quantification of embryonic phenotypes. Yellow box: mitotic spindle; pink box: polar body. Enlarged insets are shown on the left. Arrows: X-chromosome foci. **E**. Quantification of the embryonic phenotypes using images as in D. Numbers are averages of 3 independent experiments. At least 50 embryos were scored in each replicate. Error bars: S.D. **** $p < 0.0001$. ns: non-significant from unpaired t-tests. Scale bars: 20 μm.

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Additional files

Supplementary Table 1. Proteins identified from affinity purifications of GFP-fused Ankle2 from D-Mel cells. Numbers of total spectral counts and unique peptides for GFP-Ankle2, Ankle2-GFP or Flag-GFP (control) are indicated. [↗](#)

Supplementary Table 2. Proteins identified from affinity purifications of Ankle2-GFP from embryos. Numbers of total spectral counts and unique peptides for Ankle2-GFP or Flag-GFP (control) are indicated. [↗](#)

Supplementary Table 3. Proteins identified from affinity purifications of GFP-fused PP2A-29B from D-Mel cells. Numbers of total spectral counts and unique peptides for GFP-PP2A- 29B, PP2A-29B-GFP or GFP (control) are indicated. [↗](#)

Supplementary Table 4. Proteins identified from affinity purifications of PP2A-29B-GFP from embryos. Numbers of total spectral counts and unique peptides for PP2A-29B-GFP or GFP (control) are indicated. [↗](#)

Supplementary Table 5. Phosphopeptides identification and quantification from cells after Ankle2 or control RNAi. Results are listed for 4 replicates in each condition, as well as the average fold-changes and p-values. [↗](#)

Video 1. Localization of Ankle2-RFP and GFP-Vap33 during mitosis and cytokinesis in a D-Mel cell. Orthogonal maximum intensity projection of 4 z-sections spaced by 1 μm . Images were taken every 205 seconds. [↗](#)

Video 2. Localization of Ankle2-GFP and RFP-Vap33 during the cell cycle in a syncytial embryo. A single plane is shown. Images were taken every 10 seconds. [↗](#)

Video 3. Localization of Ankle2^{Fm+FL1m}-GFP and RFP-Vap33 during the cell cycle in a syncytial embryo. A single plane is shown. Images were taken every 10 seconds. [↗](#)

Video 4. Localization of Ankle2^{WT}-GFP and RFP-BAF during the cell cycle in a syncytial embryo where endogenous is depleted by RNAi. A single plane is shown. Images were taken every 10 seconds. [↗](#)

Video 5. Localization of Ankle2 ^{ΔANK} -GFP and RFP-BAF during the cell cycle in a syncytial embryo where endogenous is depleted by RNAi. A single plane is shown. Images were taken every 10 seconds. [↗](#)

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Reviewer #1 (Public review):

Summary:

In organisms with an open mitosis, nuclear envelope breakdown at mitotic entry and re-assembly of the nuclear envelope at the end of mitosis are important, highly regulated processes. One key regulator of nuclear envelope re-assembly is the BAF (Barrier-to-Autointegration) protein, which contributes to cross-linking of chromosomes to the nuclear envelope. Crucially, BAF has to be in a dephosphorylated form to carry out this function, and PP2A has been shown to be the phosphatase which dephosphorylates BAF. The Ankle2/LEM4 protein has previously been identified as an important regulator of PP2A in the dephosphorylation of BAF but its precise function is not fully understood, and Li and

colleagues set out to investigate the function of Ankle2/LEM4 in both *Drosophila* flies and *Drosophila* cell lines.

Strengths:

The authors use a combination of biochemical and imaging techniques to understand the biology of Ankle2/LEM4. On the whole the experiments are well conducted and the results look convincing. A particular strength of this manuscript is that the authors are able to study both cellular phenotypes and organismal effects of their mutants by studying both *Drosophila* D-mel cells and whole flies.

The work presented in this manuscript significantly enhances our understanding of how Ankle2/LEM4 supports BAF dephosphorylation at the end of mitosis. Particularly interesting is finding that Ankle2/LEM4 appears to be a bona fide PP2A regulatory protein in *Drosophila*, as well as the localisation of Ankle2/LEM4 and how this is influenced by the interaction between Ankle2 and the ER protein Vap33. It would be interesting to see, though, whether these insights are conserved in mammalian cells, e.g. does mammalian Vap33 also interact with LEM4? Is LEM4 also a part of the PP2A holoenzyme complex in mammalian cells?

Weaknesses:

This work is certainly impactful but more discussion and comparison of the *Drosophila* versus mammalian cell system would be helpful. Also, to attract the largest possible readership, the Ankle2 protein should be referred to as Ankle2/LEM4 throughout the paper to make it clear that this is the same molecule.

A schematic model at the end of the final figure would be very useful to summarise the findings.

Comments on revisions:

The authors have carefully revised the manuscripts and have satisfactorily addressed the issues that were raised by the reviewers.

<https://doi.org/10.7554/eLife.104233.2.sa3>

Reviewer #2 (Public review):

The authors first identify Ankle2 as a regulatory subunit and direct interactor of PP2A, showing they interact both *in vitro* and *in vivo* to promote BAF dephosphorylation. The Ankyrin domain of Ankle2 is important for the interaction with PP2A. They then show Ankle2 also interacts with the ER protein Vap33 through FFAT motifs and they particularly co-localize during mitosis. The recruitment of Ankle2 to Vap33 is essential to ER and nuclear envelop membrane in telophase while earlier in mitosis, it relies on the C terminus but not the FFAT motifs for recruitments to the nuclear membrane and spindle envelop in early mitosis. The molecular determinants and receptors are currently not known. The authors check the function of the PP2A recruitment to Ankle2/Vap33 in the context of embryos and show this recruitment pathway is functionally important. While the Ankle2/Vap33 interaction is dispensable in adult flies -looking at wing development, the PP2A/Ankle2 interaction is essential for correct wing and fly development. Overall, this is a very complete paper that reveals the molecular mechanism of PP2A recruitment to Ankle2 and studies both the cellular and the physiological effect of this interaction in the context of fly development.

The paper is well-written and the narrative is well developed. The figures are of high quality, well-controlled, clearly labelled and easy to understand. They support the claims made by the authors.

Comments on revisions:

There are still issues with the statistics. On graphs where multiple conditions are shown, you cannot perform a T-test. You have to use other tests such as ANOVA if the data is normal, and other tests such as KS test if the data is not normally distributed.

<https://doi.org/10.7554/eLife.104233.2.sa2>

Reviewer #3 (Public review):

The authors were interested in how Ankle2 regulates nuclear envelope reformation after cell division. They show that Ankle2 can bind in a PP2A complex without other known regulatory subunits of PP2A. The authors also identify a novel interaction with ER protein Vap33 that could be important for localization. This manuscript is a useful finding linking Ankle2 function during nuclear envelope reformation to the PP2A complex. The authors present solid data showing that Ankle2 can form a complex with PP2A-29B and Mts and generate a phosphoproteomic resource that is fundamentally important to understand Ankle2 biology. The caveat should be remembered that most experiments, including subcellular localization, are based on overexpression data. Keeping this in mind, the manuscript is a valuable resource.

<https://doi.org/10.7554/eLife.104233.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public review):

Summary:

In organisms with open mitosis, nuclear envelope breakdown at mitotic entry and re-assembly of the nuclear envelope at the end of mitosis are important, highly regulated processes. One key regulator of nuclear envelope re-assembly is the BAF (Barrier-to-Autointegration) protein, which contributes to cross-linking of chromosomes to the nuclear envelope. Crucially, BAF has to be in a dephosphorylated form to carry out this function, and PP2A has been shown to be the phosphatase that dephosphorylates BAF. The Ankle2/LEM4 protein has previously been identified as an important regulator of PP2A in the dephosphorylation of BAF but its precise function is not fully understood, and Li and colleagues set out to investigate the function of Ankle2/LEM4 in both Drosophila flies and Drosophila cell lines.

Strengths:

The authors use a combination of biochemical and imaging techniques to understand the biology of Ankle2/LEM4. On the whole, the experiments are well conducted and the results look convincing. A particular strength of this manuscript is that the authors are able to study both cellular phenotypes and organismal effects of their mutants by studying both Drosophila D-mel cells and whole flies.

The work presented in this manuscript significantly enhances our understanding of how Ankle2/LEM4 supports BAF dephosphorylation at the end of mitosis. Particularly

interesting is the finding that Ankle2/LEM4 appears to be a bona fide PP2A regulatory protein in Drosophila, as well as the localisation of Ankle2/LEM4 and how this is influenced by the interaction between Ankle2 and the ER protein Vap33. It would be interesting to see, though, whether these insights are conserved in mammalian cells, e.g. does mammalian Vap33 also interact with LEM4? Is LEM4 also a part of the PP2A holoenzyme complex in mammalian cells?

We feel that conducting experiments to test the level of conservation of our findings in mammalian cells is outside the scope of our study, and we will leave it for other labs to investigate.

Weaknesses:

This work is certainly impactful but more discussion and comparison of the Drosophila versus mammalian cell system would be helpful. Also, to attract the largest possible readership, the Ankle2 protein should be referred to as Ankle2/LEM4 throughout the paper to make it clear that this is the same molecule.

We have reinforced our presentation and discussion of similarities and differences between Ankle2 from Drosophila vs humans where relevant throughout the Introduction and Discussion sections. Additionally, we have added the mention that Ankle2 is also called LEM4 in humans in the Abstract and Introduction. However, when referring to Drosophila Ankle2, we do not use LEM4 because it is not listed as an alternate name for this gene/protein in FlyBase.

A schematic model at the end of the final figure would be very useful to summarise the findings.

We have already provided a schematic model in Figure S3, where we think it is better placed.

Reviewer #2 (Public review):

The authors first identify Ankle2 as a regulatory subunit and direct interactor of PP2A, showing they interact both in vitro and in vivo to promote BAF dephosphorylation. The Ankyrin domain of Ankle2 is important for the interaction with PP2A. They then show Ankle2 also interacts with the ER protein Vap33 through FFAT motifs and they particularly co-localize during mitosis. The recruitment of Ankle2 to Vap33 is essential to ER and nuclear envelop membrane in telophase while earlier in mitosis, it relies on the C terminus but not the FFAT motifs for recruitments to the nuclear membrane and spindle envelop in early mitosis. The molecular determinants and receptors are currently not known. The authors check the function of the PP2A recruitment to Ankle2/Vap33 in the context of embryos and show this recruitment pathway is functionally important. While the Ankle2/Vap33 interaction is dispensable in adult flies -looking at wing development, the PP2A/Ankle2 interaction is essential for correct wing and fly development. Overall, this is a very complete paper that reveals the molecular mechanism of PP2A recruitment to Ankle2 and studies both the cellular and the physiological effect of this interaction in the context of fly development.

Strengths:

The paper is well written and the narrative is well-developed. The figures are of high quality, wellcontrolled, clearly labelled, and easy to understand. They support the claims made by the authors.

Weaknesses:

The study would benefit from being discussed in the context of what is already known on Ankle2 biology in C.elegans and human cells. It is important to highlight the structures shown in the paper are alphafold models, rather than validated structures.

We have enhanced our presentation of what is known about LEM-4L/Ankle2 in *C. elegans* and humans in the Introduction, and further developed comparisons of our findings regarding *Drosophila* Ankle2 with these orthologs in the Results and Discussion sections. We have also specified in all sections and figure legends that the structures shown are AlphaFold3 models.

Reviewer #3 (Public review):

Summary:

The authors were interested in how Ankle2 regulates nuclear envelope reformation after cell division. Other published manuscripts, including those from the authors, show without a doubt that Ankle2 plays a role in this critical process. However, the mechanism by which Ankle2 functions was unclear. Previous work using worms and humans (Asencio et al., 2012) established that human ANKLE2 could bind endogenous PP2A subunits. The binding was direct and was mediated through a region before and including the first ankyrin repeat in human ANKLE2. In addition to its interaction with PP2A, Asencio et al., 2012 also show that ANKLE2 regulates VRK1 kinase activity. Together PP2A and VRK1 regulate BAF phosphorylation for proper nuclear envelope reformation. Here, the authors provide more evidence for interaction with PP2A by also mapping the domain of interaction to the ankyrin repeat in Drosophila. In addition, the ankyrin repeat is essential for nuclear envelope reformation after division. They show that Ankle2 can bind in a PP2A complex without other known regulatory subunits of PP2A. The authors also identify a novel interaction with ER protein Vap33, but functional relevance for this interaction in nuclear envelope reformation is not provided in the manuscript, which the authors explicitly state. This manuscript does not comment on the activity of Ballchen/VRK1 in relation to Ankle2 loss and BAF phosphorylation or nuclear envelope reformation, even though links were previously shown by multiple studies (Asencio et al., Link et al., Apridita Sebastian et al.,). Nuclear envelope defects were rescued by the reduction of VRK1 in two of these manuscripts. It is possible that BAF phosphorylation phenotypes can be contributed by both PP2A inactivity and VRK1 overactivity due to the loss of Ankle2.

Strengths:

This manuscript is a useful finding linking Ankle2 function during nuclear envelope reformation to the PP2A complex. The authors present solid data showing that Ankle2 can form a complex with PP2A-29B and Mts and generate a phosphoproteomic resource that is fundamentally important to understanding Ankle2 biology.

Weaknesses:

However, the main findings/conclusions about subcellular localization might be incomplete since they are drawn from overexpression experiments. In addition, throughout the text, some conclusions are overstated or are not supported by data.

It is true that all experiments studying subcellular localization were done with tagged proteins overexpressed in flies and cell culture. Nevertheless, we show that Ankle2-GFP is functional since it rescues phenotypes resulting from the loss of endogenous Ankle2 in both flies and cultured cells. The antibodies we generated against Ankle2 were unable to reliably detect the endogenous protein by immunofluorescence. We have now stated this caveat in our manuscript. Regarding the validity of our conclusions in relation to our data, we address

each point raised by the reviewer under the Recommendations for the authors. In some cases, we have adjusted our conclusions and in other cases, we have provided additional clarification or justification.

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

There are a few experimental issues that should be addressed, specific comments are listed below:

(1) Figure 1F: In this experiment, the authors immunoprecipitate GFP-PP2A-29B or PP2A-B29B-GFP and Western blot for Ankle2 and Mts to demonstrate that both are co-immunoprecipitated. To demonstrate that these interactions are specific, the authors should also blot for a protein that is expected to definitely NOT co-immunoprecipitate with PP2A-B29; e.g. tubulin.

Our conclusion that GFP-PP2A-29B and PP2A-29B-GFP specifically interact with Ankle2 and Mts is also based on mass spectrometry analysis of the purification products from embryos and cells in culture, comparing with products of purification of GFP alone (Fig 1E-F, S1C-D and Tables S2, S3). The lists of identified proteins reveal that most proteins (including tubulins) are not enriched with GFP-PP2A-29B or PP2A-29B-GFP like Ankle2 and Mts are.

(2) Figure 2A: The colour coding of the dots is not explained in the figure legend.

We have now added the explanation.

(3) Figure 2B: The competition experiment is a good idea. Do the authors get the same results when they conduct the experiment the other way round, i.e. keep the concentration of Tws the same but increase the concentration of Ankle2?

We have tried this reverse experiment but saw little effect. The failure to observe displacement of Tws by Ankle2 in this context could be due to a higher affinity of Tws than Ankle2 in the PP2A complex, or to lower expression levels achieved for Ankle2 (a larger protein) relative to Tws.

(4) Figure 5D: The hyperphosphorylation of BAF is very difficult to see, and it is impossible to tell whether the hyperphosphorylation has been rescued or not by the different Ankle2 constructs. Can the phosphorylated and the hyperphosphorylated bands be separated better? This panel needs significant improvements to support the claims in the text.

In our opinion, the hyperphosphorylated (upper band) and unphosphorylated (lower band) forms of BAF are well resolved and readily distinguishable. The fainter band in the middle could correspond to a partially phosphorylated form of BAF but we do not venture to speculate on its precise identity nor do we need it to draw our conclusions. The important information from this blot is that the level of unphosphorylated BAF after Ankle2 RNAi increases when Ankle2WT-GFP and Ankle2Fm+FL1-GFP are expressed but not when Flag-GFP or Ankle2ΔANK-GFP are expressed. In these experiments, the rescue of unphosphorylated BAF is incomplete because not all cells express the GFP-tagged protein in our non-clonal stable cell lines.

Reviewer #2 (Recommendations for the authors):

(1) The alphafold models need to be labelled as such better on the figures, to distinguish them from X-ray crystallography structures. Alphafold will always propose a solution but

it is not necessarily correct.

We have added the note “MODEL” directly in Figures 2C, 2D, 4F and S3B, in addition to the information already provided in the text and figure legends specifying that these are models generated by AlphaFold3.

(2) Figure 4 F. Annotate the Ankle2 FL1 peptide.

We have indicated the amino acid residues in the figure.

(3) Problems with the statistical tests. T-tests cannot be used for comparing multiple groups, as this favors error propagation.

All of our t-tests compare only two groups at a time, as indicated. In this regard, our labeling in Fig 5C may have been misleading. We have now changed it.

(4) Close-ups of ring canal in Figure S2. In Figure S2, there seem to be lots of GFP-Ankle2 vesicles in the cytoplasm of the oocyte.

We agree that the image showing Ankle2-GFP alone in the RNAi Vap33 condition suggested a cytoplasmic granular localization of unknown nature. However, upon examination, we realized that this image did not correspond to the same z-step as the matching merged image (which also

included DNA staining). We have now replaced the image with the correct one.

Reviewer #3 (Recommendations for the authors):

Be more accurate about what conclusions can be made from reported data, particularly from overexpression and deletion studies.

(1) The domain analysis for physical interaction is quite thorough. However, localization information is taken from overexpressed constructs. While these data show what could happen, the authors are not using endogenous levels of Ankle2 in cells or tissues that are known to require Ankle2. As a result, it is difficult to determine whether localization results are biologically meaningful.

We have added the following text at the end of the third Results section:

“We were unable to examine the localization of endogenous Ankle2 because the antibodies that we generated gave inconclusive results in immunofluorescence. For the remainder of our study, we relied on the overexpression of Ankle2-GFP, which may not perfectly reflect the localization and function of endogenous Ankle2. However, Ankle2-GFP is functional as it can rescue phenotypes observed when endogenous Ankle2 is depleted (see below).”

(2) The data showing that Ankle2 is a regulator unit of the PP2A complex also relies on in vitro binding assays in an over-expression context. Data certainly show Ankle2 can bind proteins in the PP2A complex when overexpressed. However, the authors could not isolate enough of the complex from the animal to test function, so Ankle2 acting as a regulatory subunit isn't functionally shown. There are other possibilities, such as Ankle2 acts as a scaffold for complex assembly.

The competition experiments shown in Fig 2 are based on complexes assembling in cells and are not *in vitro* binding assays. We show 4 lines of evidence supporting the idea that Ankle2 functions as a regulatory subunit of PP2A: 1) Ankle2 interacts with the structural (PP2A-29B)

and catalytic (Mts) subunits of PP2A without any known regulatory subunit of PP2A. 2) Depletion of Ankle2 leads to the hyperphosphorylation of the known PP2A substrate BAF. 3) The PP2A regulatory subunit Tws/B55 competes with Ankle2 for formation of a complex with PP2A. 4) AlphaFold3 predicts that Ankle2 engages in a complex with PP2A at a position similar to that of known regulatory subunits of PP2A including Tws/B55, and consistent with their mutually exclusive presence in PP2A complexes. If Ankle2 acted as a scaffold for the formation of a PP2A complex containing other regulatory subunits, we would expect to detect Ankle2 and another regulatory subunit in the same complex.

(3) Throughout the text, some conclusions are overstated or are not supported by data. Examples are below:

a. Page 1: "we show for the first time that Ankle2 is a regulatory subunit of PP2A" The authors show binding and changes in BAF phosphorylation levels, but changes in PP2A activity with modulation of Ankle2 weren't shown.

We have replaced this phrase with this one:

"...we provide several lines of evidence that suggest that Ankle2 is a regulatory subunit of PP2A..."

b. Page 3: "The requirement for Ankle2 in the development of the central nervous system was initially discovered through its targeting by the microcephaly-causing Zika virus (Shah et al.,

This is not the first paper showing ANKLE2 plays a role in the development of the CNS. Yamamoto et al., 2014 identified mutants in Ankle2 with defects in CNS development in flies and humans, establishing it as a human microcephaly-causing gene.

We are sorry for this oversight. We have now cited this important work.

c. Page 6: "Moreover, BAF appears to be the only obligatory substrate of Ankle2-dependent dephosphorylation for cell proliferation as lowering the dose of the BAF kinase NHK-1/Ballchen rescues wing development defects caused by the partial depletion of Ankle2 (Li et al., 2024)." It is unclear why the authors conclude this since Ballchen/VRK1 can phosphorylate many things besides BAF.

Although the conclusion cannot be drawn categorically, it seems to be by far the most likely scenario. However, we agree that in principle, other mechanisms could also account for these genetic observations, such as the dephosphorylation of another, still unidentified obligatory substrate of PP2A-Ankle2 that would also be phosphorylated by NHK-1/Ballchen. However, we have also shown that expression of an unphosphorylatable mutant form of BAF rescues phenotypes observed upon loss of Ankle2 function (Li et al, 2024). We have changed our sentence as follows:

"Moreover, BAF could be the only obligatory substrate of Ankle2-dependent dephosphorylation for cell proliferation as lowering the dose of the BAF kinase NHK-1/Ballchen or expression of an unphosphorylatable mutant form of BAF rescues wing development defects caused by the partial depletion of Ankle2 (Li et al., 2024)."

d. Page 10: "These results suggest that a Vap33-Ankle2-PP2A complex can mediate the recruitment of a pool of PP2A at the NE."

There is insufficient evidence to indicate that Vap33-Ankle2-PP2A exists in a stable state in the cell and that this complex mediates recruitment of PP2A at the NE. The images do

not include Vap33, showing no evidence it is present when PP2A is at the NE and the complex could only be detected with overexpression.

We agree with this caveat and recognize the need to be cautious when proposing our model. In this regard, we feel that our wording is reasonable and appropriate, using “suggest” rather than “prove”, “show” or “indicate”.

e. Page 11: These results suggest that the interaction of Ankle2 with PP2A is essential for its function in BAF dephosphorylation and nuclear reassembly." Page 14: "these results indicate that the interaction of Ankle2 with PP2A is essential during embryo". Page 14: "These results indicate that the interaction of Ankle2 with PP2A but not with Vap33 is essential for its function during cell proliferation in imaginal wing disc development."

These experiments show that the ankyrin repeat in Ankle2 is necessary for these processes. It does not say PP2A interaction with Ankle2 is necessary because other things could bind the domain.

We have revised the segments of the text mentioned, taking the reviewer’s legitimate concerns into consideration. We have also added the following sentence to the Discussion:

“However, it remains formally possible that the deletion of Ankyrin repeats used to disrupt the Ankle2-PP2A interaction abrogated another, unknown aspect of Ankle2 function.”

f. Page 12: "Overall, we conclude that in addition to its N-terminal PP2A-interacting Ankyrin domain, Ankle2 requires the integrity of its C-terminal portion for its essential function in nuclear reassembly."

No data was shown for differences in nuclear reassembly, only the ability for ANKLE2 truncation mutants to localize to the nuclear envelope. It isn't clear whether the nuclear envelope reformation is normal in Figure S6 which the authors refer to. Lamin staining could help determine and conclude the C-terminal region is important for nuclear envelope reformation.

Our conclusion is drawn from the results shown in Figures S4 and S5 (described in the same section), where a rescue assay in cells was performed to assess the functionality of different variants of Ankle2-GFP when endogenous Ankle2 was depleted. In this assay, Lamin and DNA staining were used to examine nuclear reassembly (as in Figure 5). Figure S6 shows the localizations of the different variants of Ankle2-GFP, but endogenous Ankle2 is not depleted in these cells.

g. Page 13: "We conclude that the ability of Ankle2 to interact with PP2A is required for the timely recruitment of BAF at reassembling nuclei and ensuing NE reassembly."

It's possible the Ankyrin domain in ANKLE2 is interacting with proteins other than PP2A to recruit BAF at reassembling nuclei, especially since ANKLE2 is found to regulate VRK1 (Link 2019) which has been found to phosphorylate BAF during the cell cycle (Molitor 2014). Additionally, the images in Figure 6A appear to show fully reassembled nuclear envelopes in all mutants by 180s.

This point relates to point e, raised above by this reviewer. We have re-written the sentence as follows:

“We conclude that the Ankyrin domain, required for the ability of Ankle2 to interact with PP2A, is necessary for the timely recruitment of BAF at reassembling nuclei and ensuing NE reassembly.”

Please note that in this paragraph, we discuss a delay in RFP-BAF recruitment, rather than the complete elimination of this recruitment.

h. Page 16: "Our unbiased phosphoproteomic analysis confirmed that BAF dephosphorylation depends on Ankle2, despite the absence of a detectable interaction between Drosophila Ankle2 and BAF, which may be due to the lack of a LEM domain in the former (Fishburn et al., 2024). Moreover, while Ankle2 was shown to bind and inhibit the BAF counteracting kinase VRK1 in humans (Asencio et al., 2012), we detected no interaction between Ankle2 and NHK-1/Ballchen (VRK1 ortholog) in Drosophila. This suggests that the loss of Ankle2 causes BAF hyperphosphorylation by preventing PP2A-dependent dephosphorylation rather than by preventing inhibition of NHK-1"

There could be transient binding between Ankle2 and Ballchen/VRK1/NHK-1 or activity can be indirect, but that doesn't mean there is not a contribution of BAF phosphorylation by Ballchen/VRK1/NHK-1. Genetic evidence from three model systems, including Drosophila, indicates there is a strong genetic interaction between Ankle2 and Ballchen/VRK1/NHK-1 that includes rescue of lethality.

We agree and we have re-written in this way:

“While a putative interaction between Ankle2 and NHK-1 in *Drosophila* could occur transiently, thereby escaping detection, the simplest interpretation of our results is that the loss of Ankle2 causes BAF hyperphosphorylation by preventing PP2A-dependent dephosphorylation rather than by preventing inhibition of NHK-1.”

We do not question the fact that Ballchen/VRK1/NHK-1 phosphorylates BAF and genetically interacts with Ankle2. The antagonistic relationship between Ballchen/VRK1/NHK-1 and Ankle2 observed genetically can be explained by the fact that the kinase phosphorylates BAF while PP2A/Ankle2 dephosphorylates it, without the need to invoke an additional inhibition of the kinase by Ankle2.

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