Evidence for monomeric actin function in INO80 chromatin remodeling

Prabodh Kapoor1,3, Mingming Chen1,3, Duane David Winkler2, Karolin Luger2 & Xuetong Shen1

Actin has well-established functions in the cytoplasm, but its roles in the nucleus remain poorly defined. Here, by studying the nuclear actin-containing yeast INO80 chromatin remodeling complex, we provide genetic and biochemical evidence for a role of monomeric actin in INO80 chromatin remodeling. We demonstrate that, in contrast to cytoplasmic actin, nuclear actin is present as a monomer in the INO80 complex, and its barbed end is not accessible for polymerization. We identify an actin mutation in subdomain 2 affecting in vivo nuclear functions and reducing the chromatin remodeling activity of the INO80 complex in vitro. Notably, the highly conserved subdomain 2 at the pointed end of actin contributes to the interaction of INO80 with chromatin. Our results establish an evolutionarily conserved function of nuclear actin in its monomeric form and suggest that nuclear actin can utilize a fundamentally distinct mechanism from that of cytoplasmic actin.

The presence and potential functions of nuclear actin have been debated over the past several decades1-4. Early observations of biochemical copurifications of actin with nuclear proteins were dismissed as contaminations of cytoplasmic actin, which is a major protein component in the cytosol. Adding to the controversy is the fact that actin cannot be detected in the nucleus by the classical actin stain, phalloidin, which binds to filamentous actin (F-actin) and decorates the extensive actin cytoskeleton. However, a number of studies, from various organisms and using different experimental approaches, continue to suggest that actin is indeed present in the nucleus, and several lines of evidence strongly argue for its presence. First, actin can be detected in cleanly separated Xenopus oocyte nuclei5. Second, actin can be detected in the nucleus using newly developed monoclonal actin antibodies, which recognize only monomeric actin (G-actin)6. Third, nuclear export signals (NES1 and NES2) have been identified for actin that, when mutated, lead to its accumulation in the nucleus7. Finally, recent studies indicate that actin is a subunit of a number of chromatin modifying complexes, including ATP-dependent chromatin remodeling complexes and histone acetyltransferase complexes, which are all found in the nucleus8-13. These studies demonstrate that at least a fraction of actin can be found in the cell nucleus.

The presence of actin in the nucleus raises the question of its potential function. Early biochemical studies suggested that actin may be involved in transcription by RNA polymerase II (ref. 14), and more recent evidence has implicated actin in transcription by all the three classes of RNA polymerases15-18. Moreover, actin dynamics also play an important part in the regulation of transcription factors, such as serum response factor19. Consequently, it is likely that nuclear actin is utilized in mechanistically distinct ways from its cytoplasmic counterpart.

Results

Actin and Arps form a subcomplex within the INO80 complex

The mammalian BAF and Drosophila BAP chromatin remodeling complexes were initially identified as containing an actin subunit11,13. In yeast, three actin-containing chromatin modifying complexes are known: the INO80 and SWR1 chromatin remodeling complexes and the NuA4 histone acetyltransferase complex8,10,12. Using glycerol
An actin mutant defective in nuclear functions

Despite growing evidence that actin is involved in multiple nuclear functions, the function of nuclear actin in vivo remains a mystery, owing to a lack of genetic evidence. We reasoned that if actin is a functional subunit of chromatin modifying complexes such as the INO80 complex, mutations that disrupt its function in these complexes might exist.

To identify mutations that affect actin functioning in the nucleus, we screened yeast on the basis of phenotype, such as hypersensitivity to the DNA-damaging agent hydroxyurea and defects in transcription of PHO5, which encodes an acid phosphatase. We screened a collection of existing temperature-sensitive actin mutants in an S288C background for defects in nuclear function and found that only few actin mutants showed hypersensitivity to hydroxyurea or defective PHO5 transcription (unpublished observations) at permissive temperature (30 °C). Among these mutants, act1-2 (expressing an A58T substitution in actin) showed defects similar to those observed in an ino80A mutant (hereafter referred to as ino80 mutant) (Fig. 1e).

The A58T substitution occurs in subdomain 2, which is a part of the pointed end of actin (Fig. 1f and Supplementary Fig. 2b). At permissive temperature, act1-2 and ino80 mutants were hypersensitive to hydroxyurea (100 mM), unlike the wild-type strain and act1-101, a temperature-sensitive mutant expressing D363A and E364A substitutions in actin subdomain 1 (at the barbed end of the protein)25 (Fig. 1e).

We also observed markedly reduced activation of PHO5 in the act1-2 and ino80 mutants (Fig. 1g and Supplementary Fig. 2c). Furthermore, the observed defects in the act1-2 mutant were rescued by transient expression of wild-type actin (Supplementary Fig. 2d). The nuclear defects in act1-2 mutants were prominent in proliferating cells at permissive temperature26, confirming the suitability of mutant act1-2 for further analysis. Moreover, the allele specificity of ACT1 mutations in actin-containing chromatin modifying complexes such as INO80 and NuA4 suggests that specific actin mutations may have different effects on distinct complexes (Supplementary Fig. 2e–g).

Actin is required for INO80 chromatin remodeling in vitro

Given that the act1-2 mutant showed markedly affected nuclear actin function in vivo and that some defects overlapped with those
observed in the ino80 mutant (Fig. 1e), we investigated the potential contribution of actin to INO80 chromatin remodeling in vitro. To this end, we immunopurified the INO80 complex from an act1-2 mutant expressing Flag-tagged Ino80 below as the INO80 (act1-2) complex. The purified INO80 (act1-2) complex contained all the subunits found in the wild-type complex, but the wild-type actin subunit was replaced by Act1-2 (Fig. 2a), indicating that the assembly of the INO80 complex was not affected in the act1-2 mutant. Therefore, changes in the biochemical activity of the INO80 (act1-2) complex, as compared to the wild-type INO80 complex, are probably attributable to the act1-2 mutation rather than to defects in other subunits. We compared the biochemical activity of the wild-type complex to that of the INO80 (act1-2) complex using in vitro assays carried out at 30 °C (Fig. 2). Binding of the INO80 (act1-2) complex to free DNA (the 359-base-pair (bp) INO1 DNA (5 nM) as the INO80 complex (1×, 10 ng actin). Numbers (left) indicate the stoichiometry of labeled subunits, with the actin subunit normalized as 1. (the 359-base-pair (bp) INO1 DNA (5 nM) as the INO80 complex (1×, 10 ng actin). Numbers (left) indicate the stoichiometry of labeled subunits, with the actin subunit normalized as 1.


dINO80 = 7.23 ± 0.23 nM; s.e.m.) (Fig. 2d) and Supplementary Fig. 3c and Supplementary Table 1, consistent with a prominent role for actin in regulating chromatin binding.

We next compare the ATPase activities of the wild-type and mutant INO80 (act1-2) complexes. The ATPase activity of the wild-type INO80 complex is stimulated by nucleosome core particles more than by free DNA12. Compared to the wild-type complex, INO80 (act1-2) showed marked reduction in both DNA and nucleosome-stimulated ATPase activities (Supplementary Fig. 3d). Because the bulk of the ATPase activity of the INO80 complex is abrogated by the K737A mutation in Ino80 (ref. 12), the observed reductions can be attributed to effects of the A58T actin mutation on the enzymatic activity of the INO80 ATPase. We also examined the chromatin remodeling activity of the INO80 (act1-2) complex using a mononucleosome mobilization assay29,30. INO80 chromatin remodeling activity was present, as indicated by the redistribution of the nucleosome species N1, N2, and N3 (Fig. 2e). Using equimolar amounts of the wild-type and mutant protein complexes, we detected a marked reduction—but not

Figure 2 Actin contributes to INO80 chromatin remodeling. (a) SDS-PAGE and silver staining of wild-type (WT) and INO80 (act1-2) complexes. (b) Native PAGE showing 359-bp INO1 DNA (5 nM) in the presence of increasing equimolar concentrations of WT and INO80 (act1-2) complexes from 2 nM to 20 nM at 30 °C. (c) Graph showing percentage of DNA bound to WT and INO80 (act1-2) complexes as shown in b. (d) Kd values for binding of WT and INO80 (act1-2) complexes to nucleosomes containing 207 bp DNA, assessed by gel-shift assay. Data in e and d are the mean of five independent experiments ± s.d. (e) Native PAGE showing nucleosome mobilization by WT and mutant INO80 complexes (1×, 5 nM) at 30 °C using the INO1 mononucleosome substrate (5.8 nM). Chromatin remodeling is indicated by the reduction in N3 band intensity and the increase in N1 and N2 band intensity. (f) Graphical representation of e. Data in e and f are the mean of three independent experiments ± s.d. (g) SDS-PAGE and Deep Purple staining of the INO80 complex (1×, 10 ng actin). Numbers (left) indicate the stoichiometry of labeled subunits, with the actin subunit normalized as 1. Rvb1 and Rvb2 helicases appear as a single band (Rvb1&2). (h) Northern blot analysis of PHOS expression in WT and mutant strains at 30 °C after 3 h of induction and at 37 °C after 1.5 h of induction. RPL3, loading control.
Figure 3 Actin in the INO80 complex exists in a unique microenvironment. (a) Schematic representation of the structure of actin described in Figure 1f. The position of the C4 epitope (Asp24 and Asp25) is indicated. DNase I and profilin binding regions and pointed (−) and barbed (+) end regions are indicated. (b) Dot blot for actin and INO80 complex probed with anti-C4 and anti-His6 antibodies. (c) SDS-PAGE and silver staining of actin–profilin and INO80 complex purified from cells grown at 30 °C. (d) Dot blot for INO80 and actin–profilin complexes probed with anti-C4 and fluorescently labeled DNase I. 1x, amount of complex relative to actin. (e) Western blot analysis of whole cell extracts using monoclonal anti-C4. Coomasie staining (bottom) indicates equal loading of whole cell extracts. (f) Dot blot for INO80 complexes probed with anti-C4 (top) and fluorescently labeled DNase I (bottom). (g,h) Dot blot analyses for INO80 complexes purified from wild-type (WT) and act1-2 mutant cells probed with anti-C4 (g) and fluorescently labeled DNase I (h). Actin, loading control.

Actin in the INO80 complex is a monomer
A key feature of cytoplasmic actin is its ability to polymerize and form F-actin. Whether actin is able to form F-actin in the nucleus has remained controversial. In vitro studies of the BAF chromatin remodeling complex suggest that the actin-containing BAF complex can bind to F-actin in a phosphoinositide-regulated fashion. To uncover the role of nuclear actin in INO80 chromatin remodeling, we investigated whether actin polymerization could be involved. Silver and Coomasie staining of the INO80 complex indicated that the actin subunit is monomeric (Supplementary Fig. 1 and data not shown). For more accurate measurement of actin stoichiometry in the INO80 complex, we used a quantitative method based on fluorescent staining of proteins after SDS-PAGE that allows for the detection of two-fold differences in actin abundance in the complex. Fluorescence measurements of several subunits of the INO80 complex suggested that actin exists as a monomer in the INO80 complex. In contrast, the Rvb1 and Rvb2 helicases in the INO80 complex together showed a 10.6:1 stoichiometry to actin, a value close to the 12:1 stoichiometry predicted, as Rvb1 and Rvb2 are classical hexameric helicases (Fig. 2g).

Moreover, if actin polymerization is required for INO80 functional activity, actin mutants that lose the ability to polymerize should show defective INO80 functioning in vivo. The temperature-sensitive mutant act1-1 shows defects in actin polymerization even at permissive temperature; however, unlike act1-2 mutants, act1-1 mutants showed largely normal hydroxyurea sensitivity (Supplementary Fig. 3e) and PHO5 activation at 30 °C (Fig. 2h). Within minutes at nonpermissive temperature (37 °C), the act1-1 mutant loses the ability to polymerize actin; notably, the activation of PHO5 at 37 °C was relatively normal in act1-1 mutants (Fig. 2h). Given that the act1-2 mutant showed defects in PHO5 activation (Figs. 1g and 2h), the normal activation of PHO5 in the absence of actin polymerization in the act1-1 mutant suggests that actin, but not its polymerization, is required in PHO5 activation. Moreover, the INO80 complex did not bind actin filaments under conditions in which coflin interacts with actin filaments (Supplementary Fig. 3f). Taken together, our stoichiometry measurements and functional analyses suggest that actin polymerization is not required for INO80 chromatin remodeling. However, it is possible that actin polymerization is involved in other nuclear activities, such as those of the BAF complex, since the BAF and INO80 complexes are nonorthologous and distinct in composition and in the ways in which they are regulated by inositides (Supplementary Fig. 4a,b). These observations raise the possibility that actin could be used in unique ways as a monomer that is distinct from its cytoplasmic counterpart.

Unique positioning of actin in the INO80 complex
To understand how actin might function as a monomer in INO80, we investigated its microenvironment in the complex. Because actin is capable of polymerizing from either the barbed or the pointed end, we analyzed the accessibility of both ends (Fig. 3a). Using purified recombinant profilin (with a C-terminal His6 tag) as a barbed end probe in a dot-blot assay (see Online Methods), we observed that in contrast to purified actin, which readily binds to profilin, actin in the INO80 complex was unable to interact with profilin (Fig. 3b), suggesting masking of the barbed end region by other INO80 subunits. As the barbed end region of actin accepts new actin molecules during polymerization, this result suggests that the barbed end of actin in the INO80 complex is unlikely to be available for actin polymerization.

Despite the masking of the barbed end, the pointed end of actin could potentially allow actin polymerization. We probed the pointed end using DNase I, which is known to bind actin subdomain 2,
Actin subdomain 2 contributes to INO80 chromatin remodeling

To further reveal the function of the exposed, pointed end of actin in the INO80 complex, we analyzed the actin subdomains. The A58T mutation, which reduced INO80 activity, is located in actin subdomain 2. Given the reductions of DNA and nucleosome binding activities and chromatin remodeling activity in the INO80 complex containing the A58T mutation (Fig. 2), we concluded that subdomain 2 of actin may be implicated in interaction with chromatin. To test this hypothesis, we investigated the interaction between actin in the INO80 complex with its chromatin substrate. We purified the INO80 complex bound to chromatin at a lower salt concentration. Under these conditions, INO80 copurifies with its native chromatin substrate, as indicated by the presence of all core histones and DNA after gel electrophoresis of the purified complex. To probe the accessibility of actin subdomain 2 in the context of the chromatin-bound INO80 complex, we used subtilisin, a protease from Bacillus licheniformis. At a subtilisin/actin ratio of 1:1,500, subtilisin specifically cleaves actin subdomain 2, generating fragments of ~37 kDa and ~7 kDa that can be detected by actin antibodies. Notably, although subdomain 2 was accessible to subtilisin in the free INO80 complex, it was not accessible when INO80 was bound to chromatin, suggesting that subdomain 2 is involved in chromatin association. After micrococcal nuclease (MNase) treatment of the chromatin–INO80 complex, actin subdomain 2 became accessible to subtilisin, suggesting that this subdomain may be involved in interactions with MNase-sensitive features of chromatin such as linker DNA.

To test this idea, we used a reconstituted nucleosome binding system coupled with a protease mapping assay. We analyzed the accessibility of actin to subtilisin in the purified INO80 complex after adding two types of reconstituted mononucleosome: one containing linker DNA and the other containing no linker DNA. The addition of linker DNA did not substantially block subdomain 2 of actin. The addition of nucleosomes containing linker DNA at both ends nearly abolished subtilisin accessibility (Supplementary Figs. 5a,b). Actin in the INO80 complex was accessible to subtilisin in the absence of nucleosome substrates and slightly less accessible in the presence of nucleosomes without linker DNA, indicating that the binding of these nucleosomes did not substantially block subdomain 2 of actin. The addition of nucleosomes containing linker DNA at both ends nearly abolished subtilisin accessibility (Supplementary Figs. 5c,d and 3a,b). Actin in the INO80 complex was accessible to subtilisin in the absence of nucleosome substrates and slightly less accessible in the presence of nucleosomes without linker DNA, indicating that the binding of these nucleosomes did not substantially block subdomain 2 of actin. The addition of nucleosomes containing linker DNA at both ends nearly abolished subtilisin accessibility (Supplementary Figs. 5c,d and 3a,b).
Actin subdomain 2 in the INO80 (act1-2) complex was not markedly blocked by the nucleosomes containing 207 bp DNA (Supplementary Fig. 5e), consistent with the decreased ability of the INO80 (act1-2) complex to bind nucleosomes with linker DNA (Fig. 2d and Supplementary Fig. 3b). These results indicate that although the pointed end of actin in INO80 is exposed, it is involved in regulating the binding of the INO80 complex to chromatin. Taken together, our results suggest a previously unknown mechanism in which nuclear monomeric actin is contained in a unique microenvironment to regulate chromatin interaction instead of supporting actin polymerization. Although actin alone was unable to bind DNA, the INO80 complex and its actin–Arp module can bind DNA (data not shown). Thus, in the context of the chromatin remodeling complex, nuclear actin has gained the ability to either interact directly with chromatin or regulate chromatin binding indirectly through conformational changes.

To further analyze the functional relevance of actin subdomain 2 in the INO80 complex, we used DNase I to trap subdomain 2 and purified the INO80–DNase I complex (Fig. 4c). DNA- and nucleosome-dependent ATPase activity in the trapped INO80–DNase I complex was reduced by more than 50%, compared to the INO80 complex alone (Fig. 4d), confirming a crucial role for actin subdomain 2 in INO80 activity. Given that the A58T mutation, which reduces INO80 complex activity, is also located in subdomain 2, our combined genetic and biochemical data support a previously unknown mechanism in which the subdomain 2 of nuclear actin is implicated in the INO80 interaction with chromatin.

**DISCUSSION**  
A system to study nuclear actin

Despite increasing evidence suggesting that actin is in the nucleus and may have roles in many nuclear functions, research on nuclear actin has been stalled by the difficulty of unambiguously demonstrating both in vivo and in vitro, how actin functions in the nucleus. Such demonstrations require a model system in which the function of nuclear actin can be dissected genetically as well as biochemically. Here, we have established the yeast INO80 chromatin remodeling complex as a useful system for demonstrating the function of nuclear actin, owing to the defined stoichiometry and the ability to isolate and study its subcomplexes. Using techniques established to study cytoplasmic actin, we were able to probe the unique microenvironment of actin in the INO80 complex and provide insights into its mechanism. Notably, our genetic and biochemical analyses lead to the same conclusion regarding the key function of actin subdomain 2 in the INO80 complex, thus firmly establishing a previously unknown mechanism for nuclear actin.

**An ancient module of nuclear actin and Arp**

Our study refines the INO80 complex into a subcomplex comprising actin, Arp4, Arp8 and Taf14 subunits associated with the N-terminal region of the Ino80 ATPase. Notably, all subunits of this actin–Arp subcomplex, including the N-terminal region of the Ino80 ATPase, are evolutionarily conserved, suggesting that this subcomplex represents a unique and ancient module used for INO80 chromatin remodeling. Because actin and Arp4 are consistently present in several chromatin modifying complexes, including INO80, SWR1 and NuA4 (refs. 8,10,12), and the loss of Arp8 in the INO80 complex results in the loss of actin and Arp4 (ref. 30), it is possible that actin and Arp4 may form a dimer and that this dimer may represent an even more conserved and basic module involving nuclear actin. This actin–Arp4 module may also be used in combination with other Arps and proteins in chromatin modifying complexes. For example, the actin–Arp4 dimer may associate with Arp8 and the N-terminal region of the Ino80 ATPase, thus forming the actin–Arp module observed in the INO80 complex (Fig. 5). Similarly, the actin–Arp4 dimer may associate with other proteins to form functional modules in the SWR1 and NuA4 complexes. In yeast, the actin–Arp4 module may also have evolved into the less-conserved Arp7–Arp9 dimer found in the SWI/SNF and RSC chromatin remodeling complexes. On the basis of these observations, it can be postulated that actin and nuclear Arps in chromatin modifying complexes are used in a combinatorial fashion to suit specific functions.

**Mechanism of nuclear actin in chromatin remodeling**

There are two, nonexclusive models of the mechanisms of nuclear actin in chromatin modifications. In the first, nuclear actin undergoes dynamic polymerization and depolymerization and is anchored to actin-containing chromatin modifying complexes. This model is reminiscent of the actin nucleation or branching mechanism of the Arp2/3 complex, and its in vivo relevance remains to be demonstrated. We have established a second model, in which monomeric actin serves as a functional subunit in chromatin modifying complexes and participates directly in chromatin modification. This model does not invoke the polymerization of actin in the nucleus; instead, it requires a mechanism of direct interaction between monomeric actin and chromatin. However, the two models are not mutually exclusive. Our results do not exclude the possibility that some aspects of nuclear actin function still require actin polymerization, for example, or that nuclear actin may form unconventional or short filaments.

How might actin participate in chromatin modifications in the second model? It has been shown that Arps such as Arp4 and Arp8 can bind to histones. Although the precise mechanisms are still vague, it is likely that actin-containing chromatin modifying complexes such as INO80 and NuA4 interact directly with chromatin (DNA or histones) at various points and locations during ATP-dependent chromatin remodeling or histone modification; therefore, the actin–Arp complex INO80 Arp8

![Figure 5 A model for nuclear actin in the INO80 complex. Unique positioning of actin in the INO80 complex compared to its position in the Arp2/3 complex in the cytoplasm.](image)
modules may serve as interaction surfaces or chaperones for chromatin. Because actin and Arp5 form distinct modules, it can be postulated that specific combinations of actin and Arp5 in the chromatin modulating complexes are involved in the direct binding of specific features of chromatin—such as DNA, combinations of histones and histone variants or histone modifications.

Given that the Arp2/3 complex and the INO80 complex both contain Arps, it is plausible that actin–Arp subunits could also mimic the function of Arp2/3 dimers in initiating actin polymerization50. However, the orientation of actin in the nuclear INO80 complex differs from its orientation in the Arp2/3 complex in the cytoplasm (Fig. 5). In the INO80 complex, the barbed end of actin is blocked by other INO80 subunits, preventing actin polymerization. The other, pointed end of actin is exposed and could potentially be used for actin polymerization, but it does not; instead, it associates with chromatin through subdomain 2. The unique orientation and microenvironment of actin in different complexes underlies the distinct mechanisms between cytoplasmic and nuclear actin (Fig. 5).

Given that chromatin is also highly conserved evolutionarily, we propose that a previously unrecognized, yet fundamental, function of actin is to interact directly with chromatin in the nucleus. Actin subdomain 2 has evolved, perhaps together with Arps such as Arp4 or Act3p/Arp4, to cooperatively interact with chromatin. This function of nuclear actin is distinct from its cytoplasmic counterpart, but it is likely to be as ancient. We suggest that conventional actin has interdomain 2 has evolved, perhaps together with Arps such as Arp4 acted with chromatin as one of its fundamental functions since the function of Arp2/3 dimers in initiating actin polymerization40.

METHODS

Acknowledgments

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Bettinger, B.T., Gilbert, D.M. & Amberg, D.C. Actin up in the nucleus. Nat. Rev. Mol. Cell Biol. 5, 410–415 (2004).
2. Blessing, C.A., Ugrinova, G.T. & Goodson, H.V. Actin and ARPs: action in the nucleus. Trends Cell Biol. 14, 435–442 (2004).
3. Olave, I.A., Reck-Peterson, S.L. & Grabré, G.R. Nuclear actin and actin-related proteins in chromatin remodeling. Annu. Rev. Biochem. 71, 755–781 (2002).
4. Shumaker, D.K., Kuczmarski, E.R. & Goldman, R.D. The nuclear actinome: lamins and actin are major players in essential nuclear functions. Curr. Opin. Cell Biol. 15, 358–366 (2003).
5. Clark, T.G. & Merriam, R.W. Diffusible and bound actin nuclei of Xenopus laevis oocytes. Cell 12, 883–891 (1977).
6. Gonsior, S.M. et al. Conformational difference between nuclear and cytoplasmic actin as detected by a monoclonal antibody. J. Cell Sci. 112, 797–809 (1999).
7. Wang, A., Fukuda, M., Mishima, M. & Nishida, E. Nuclear export of actin as a novel mechanism regulating the subcellular localization of a major cytoskeletal protein. EMBO J. 17, 1635–1641 (1998).
8. Galanou, L. et al. Multiple links between the NuA4 histone acetyltransferase complex and epigenetic control of transcription. Mol. Cell 5, 927–937 (2000).
9. Ikuwa, T. et al. Involvement of the TIIP60 histone acetylase complex in DNA repair and apoptosis. Cell 102, 463–473 (2000).
10. Mizuguchi, G. et al. ATP-driven exchange of histone H2AZ variant catalyzed by SWI/SNF chromatin remodeling complex. Science 303, 343–348 (2004).
11. Papoulas, O. et al. The Droshaillin tritorax group proteins BRM, ASH1 and ASH2 are subunits of distinct protein complexes. Development 125, 3955–3966 (1998).
12. Shi, X., Mizuguchi, G., Hamiche, A. & Wu, C. A chromatin remodeling complex involved in transcription and DNA processing. Nature 406, 541–544 (2000).
13. Zhao, K. et al. Rapid and phosphoinositid-dependent binding of the SWI/SNF-like BAF complex to chromatin after T lymphocyte receptor signaling. Cell 95, 625–636 (1998).
14. Egy, J.M., Miyamoto, N.G., Moncollin, V. & Chambon, P. Is actin a transcriptional initiation factor for B1 RNA polymerase? EMBO J. 3, 2363–2371 (1984).
15. Hofmann, W.A. et al. Actin is part of pre-initiation complexes and is necessary for transcription by RNA polymerase II. J. Cell Biol. 313, 329–342 (2003).
16. Hu, P., Wu, S. & Hernandez, N. A role for β-actin in RNA polymerase III transcription. Genes Dev. 18, 3010–3015 (2004).
17. Percipalle, P. et al. Actin-ribonucleoprotein interaction is involved in transcription by RNA polymerase II. Proc. Natl. Acad. Sci. USA 101, 6475–6480 (2004).
18. Philimonenko, V.V. et al. Nuclear actin and myosin I are required for RNA polymerase I transcription. Nat. Cell Biol. 6, 1165–1172 (2004).
19. Miralles, F., Posern, G., Zaramytslou, A.I. & Treisman, R. Actin dynamics control SRF activity by regulation of its coactivator MAL. Cell 113, 329–342 (2003).
20. Rando, O.J., Zhao, K., Janney, P. & Crabtree, G.R. Phosphatidylinositol-dependent actin filament binding by the SWI/SNF-like BAF chromatin remodeling complex. Proc. Natl. Acad. Sci. USA 99, 2824–2829 (2003).
21. Szerlong, H. et al. The HSA domain binds nuclear actin-related proteins to regulate chromatin-remodeling ATPases. Nat. Struct. Mol. Biol. 15, 469–476 (2008).
22. Ebert, B., Birkmann, A. & Schuller, H.J. The product of the SNF2/SWI2 parologue INO80 of Saccharomyces cerevisiae required for efficient expression of various yeast structural genes is part of a high-molecular-weight protein complex. Mol. Microbiol. 32, 741–751 (1999).
23. Steger, D.J., Haswell, E.E., Miller, A.L., Wente, S.R. & O’Shea, E.K. Regulation of chromatin remodeling by inositol polyphosphates. Science 299, 114–116 (2003).
24. Shortle, D., Novick, P. & Botstein, D. Construction and genetic characterization of temperature-sensitive mutant alleles of the yeast actin gene. Proc. Natl. Acad. Sci. USA 81, 4889–4893 (1984).
25. Wertman, T.F., Drubin, D.G. & Botstein, D. Systematic mutational analysis of the yeast act1 gene. Genetics 132, 337–350 (1992).
26. Novick, P. & Botstein, D. Phenotypic analysis of temperature-sensitive yeast actin mutants. Cell 40, 405–416 (1985).
27. Udugama, M., Sabir, A. & Botstein, D. The INO80 ATP-dependent chromatin remodeling complex is a nucleosome spacing factor. Mol. Cell Biol. 31, 662–673 (2011).
28. Winkler, D.D., Luger, K. & Hib, A.R. Quantifying chromatin-associated interactions: the Enzymem. Methods Mol. Biol. 402, 233–247 (2008).
29. Hamiche, A., Sandzalopoulou, R., Gdula, D.A. & Wu, C. ATP-dependent histone octamer sliding mediated by the chromatin remodeling complex INO80. Cell 97, 833–842 (1999).
30. Shen, X., Ranallo, R., Choi, E. & Wu, C. Involvement of actin-related proteins in ATP-dependent chromatin remodeling. Mol. Cell 12, 147–155 (2003).
31. Bugyi, B. & Carlier, M.F. Control of actin filament treadmilling in cell motility. Annu. Rev. Biophys. 39, 449–470 (2010).
32. Pollard, T.D. & Cooper, J.A. Actin, a central player in cell shape and movement. Science 326, 1208–1212 (2009).
33. Kabsch, W., Mannherz, H.G., Suck, D., Pai, E.F. & Holmes, K.C. Atomic structure of crystalline profilin-actin. Science 243, 245–274 (1989).
34. Schwyter, D.H., Kron, S.J., Toyoshima, Y.Y., Spudich, J.A. & Reisler, E. Subtilisin cleavage of actin in vivo abolishes sliding movement of actin filaments over myosin. J. Cell Biol. 111, 465–470 (1990).
35. Kapoor, P. et al. Leshiniaemia actin binding and nicks kDNA as well as inhibits deacetylation activity of type II topoisomerase. Nucleic Acids Res. 38, 3308–3317 (2010).
36. Schwyter, D.H., Saha, A. & Cairns, B.R. The nuclear actin-related proteins Arp7 and Arp9: a dimeric module that cooperates with architectural proteins for chromatin remodeling. EMBO J. 22, 3175–3183 (2003).
37. Schaller, D.A. & Schroer, T.A. Actin-related proteins. Annu. Rev. Cell Dev. Biol. 515, 1–36 (2003).
38. Harata, M. et al. The nuclear actin-related protein of Saccharomyces cerevisiae, Act3p/Arp4, interacts with core histones. Mol. Biol. Cell 10, 2595–2605 (1999).
39. Goel, E.D. & Welch, M.D. The ARP2/3 complex: an actin nucleator in cell division. Nat. Rev. Mol. Cell Biol. 7, 715–726 (2006).
ONLINE METHODS

Yeast manipulations and phenotypic analysis. All S. cerevisiae strains were in the S288C background. A collection of act1 mutants was a gift from David Drubin24,25. Relevant ACT1 mutations were confirmed by PCR and sequencing. To generate strains for protein purification, the INO80 locus in act1-2 mutant was epitope-tagged with a triple-Flag sequence at the C terminus. To generate the N-terminal region expression plasmid, the region encoding amino acids 356–691 of INO80 was cloned into a modified pRS416 plasmid with a double-Flag sequence at the C terminus, together with native INO80 promoter and terminator sequences. The resulting plasmid, p-2F, was transformed into an ino80 deletion strain. pACT1 was constructed by cloning a PCR fragment spanning the ACT1 gene from –669 before the start codon to +317 after the stop codon into the pRS416 vector. To purify the actin–protein complex, the PFY1 gene encoding profilin was epitope-tagged with a double-Flag tag at the C terminus in the chromosome.

Standard yeast culture and transformation techniques were followed. Phenotypic analysis was done by plating yeast cells at five-fold serial dilutions. Plates were incubated at 30 °C for 3–5 d, then scored. For gene expression analysis, yeast strains were grown overnight, then diluted 10- to 20-fold in yeast extract peptone dextrose medium (YPD). After growth at 30 °C for 4 h, cells were collected and washed and PHO5 expression was induced in synthetic complete medium lacking phosphate at indicated temperature for 1.5–4 h. Total RNA was isolated and northern analysis was performed. The entire ORFs of PHO5, ACT1 and RPL3 were amplified by PCR and used as probes.

Purification of protein complexes. Protein complexes were purified from Flag epitope–tagged strains as described elsewhere41. All purifications were done using high salt washes (0.5 M KCl) except for INO80 bound chromatin purification, in which low salt washes were done (0.1 M KCl). For further purification, protein complexes were separated in a 5–ml 17–35% or 27–45% glycerol gradient in buffer H-0.3 (25 mM HEPES-KOH (pH 7.6), 1 mM EDTA, 0.02% NP-40 and 0.3 M KCl). SDS-PAGE followed by silver staining was done to detect proteins. Quantitative western blotting of the Flag-tagged Ino80 ATPase was used to normalize complexes used in assays. Deep Purple stain (Amersham) was used to measure the stoichiometry of the INO80 complex using a Typhoon imaging system.

DNA binding and mononucleosome mobilization assays. DNA binding and mononucleosome mobilization assays were performed as previously described42. Briefly, a 359-bp INO1 fragment spanning the INO1 promoter from positions –359 to +1 was used for the DNA binding assay. The same fragment was used to form mononucleosome substrates with recombinant yeast core histones for the mononucleosome mobilization assay. Gels from the DNA binding and mononucleosome mobilization assays were stained with SYBR Green I and documented using a Typhoon imaging system.

Quantitative mononucleosome binding assays. Nucleosomes without linker DNA (containing 147 bp DNA) or with linker DNA at both ends (containing 207 bp DNA) were prepared as described previously42. Quantitative binding assays were done by gel-shift assays using native PAGE followed by SYBR Green I staining and documented using a Typhoon imaging system for unlabeled nucleosomes and with fluorescently labeled nucleosomes using fluorescent measurements as described previously28,42.

Western blot and dot-blot assays. Monoclonal anti-C4 antibodies (Chemicon, MAB 1501), in 1:1,000 dilutions, as well as polyclonal anti–β-actin antibodies (Cell Signaling, cat. no. 4967), in 1:2,000 dilutions, were used to detect actin in western blots and dot blots. Alexa Fluor–labeled DNase I (Molecular Probes) (5 mg per milliliter of buffer) was used in dot blots to bind purified complexes (1×, equivalent to 10 ng actin) spotted on nictrocellulose membranes and DNase I binding was detected using a Typhoon imaging system. Global acetylation of histone H4 in whole cell extracts was detected by anti–H4-Penta-Ac antibodies (Upstate Biotechnology, cat. no. 06-9461:1,000 dilution) that recognize all five acetylated lysine residues in the H4 tail. As a control, under nonpermissive temperature (37 °C), a temperature-sensitive esa1 mutant (esa1-1851) was used; esa1-1851 is defective for Nua4 functions and shows a severe reduction in global histone H4 acetylation43, which was detected by the anti–H4-penta-Ac antibodies.

ATP hydrolysis assays. The ATPase assays were performed with 359-bp INO1 promoter DNA as well as with mononucleosomes prepared from same DNA using thin-layer chromatography (TLC) in 0.75 M KH2PO4 with (γ-32P)ATP, and signals were quantified on the Typhoon imaging system as described elsewhere22.

Limited proteolysis assays. Protease K and subtilisin digestions were performed using 10 μl of purified complexes (equivalent to 10 ng actin), and the amount of protease used was determined empirically. The reaction was stopped by addition of two volumes of SDS sample buffer and immediate incubation at 100 °C for 5 min. Samples were separated by SDS-PAGE (12% or 15%) followed by either silver staining or western blot analysis. Additional methods are described in the Supplementary Note.

41. Shen, X. Preparation and analysis of the INO80 complex. Methods Enzymol. 377, 401–412 (2003).
42. Winkler, D.D., Muthurajan, U.M., Hieb, A.R. & Luger, K. Histone chaperone FACT coordinates nucleosome interaction through multiple synergistic binding events. J. Biol. Chem. 286, 41883–41892 (2011).
43. Clarke, A.S., Lowell, J.E., Jacobson, S.J. & Pilus, L. Esa1p is an essential histone acetyltransferase required for cell cycle progression. Mol. Cell. Biol. 19, 2515–2526 (1999).