A NOVEL ROLE OF THE ACTIN-NUCLEATING ARP2/3 COMPLEX IN THE REGULATION OF RNA POLYMERASE II-DEPENDENT TRANSCRIPTION
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Running title: Nuclear function of the Arp2/3 complex

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It has been well documented that actin is present in the nucleus and involved in numerous nuclear functions including regulation of transcription. The actin-nucleating Arp2/3 complex is an essential, evolutionarily conserved seven-subunit protein complex, which promotes actin cytoskeleton assembly in the cytoplasm upon stimulation by WASP family proteins. Our recent study indicates that the nuclear localized neural Wiskott-Adrich syndrome protein (N-WASP) can induce de novo actin polymerization in the nucleus and this function is important for the role of N-WASP in the regulation of RNA polymerase II-dependent transcription. Here, we present evidence to show that the Arp 2/3 complex is also localized in the nucleus and plays an essential role in mediating nuclear actin polymerization induced by N-WASP. We also demonstrate that the Arp2/3 complex physically associates with RNA polymerase II and is involved in the RNA polymerase II dependent transcriptional regulation both in vivo and in vitro. Together, these data provide strong support for the hypothesis that N-WASP and the Arp 2/3 complex regulate transcription, at least in part, through the regulation of nuclear actin polymerization in a manner similar to their function in the cytoplasm.

The rapid assembly of actin filaments in the cell is required for many basic cellular processes such as endocytosis and cell motility (1,2). The Arp2/3 complex is one of the most important mediators of actin assembly. It consists of two proteins that are closely related to actin in sequence and conformation (Arp2 and Arp3) and five other proteins including p41, p34, p21, p20 and p16 in human (3,4). Many in vitro studies using purified actin showed that the Arp 2/3 complex is capable of initiating actin polymerization and formation of new F-actin branches (4-9). This activity of the Arp2/3 complex generates space-filling networks of actin filaments which can provide the force for cell motility (10,11). The Arp 2/3 complex is activated by the members of Wiskott-Adrich syndrome protein (WASP) family including WASP, N-WASP and Scar/WAVE (12-16). These proteins have a common C-terminal Verproline-Cofilin-Acidic (VCA) region that can activate the Arp2/3 complex through interaction of the V domain with actin monomer and association of the CA domain with the Arp2/3 complex to induce filament formation (13,17,18). Despite the central function of the Arp2/3 complex in the regulation of actin filament nucleation and organization in the cytoplasm, the localization and function of the Arp 2/3 complex in the nucleus have not been studied.

Recent studies have shown that actin is localized in the nucleus and involved in a variety of nuclear functions (19,20). It has been shown that the nuclear actin is associated with small nuclear ribonucleoproteins (RNP) and heterogeneous nuclear ribonucleoproteins which are important in the regulation of mRNA processing and the nuclear export of proteins and retroviral RNA (21-26). The nuclear actin has also been found to attach to the nuclear pore complex in amphibian oocytes (27). It has also been found in association with the ATP-dependent chromatin remodeling complex and histone acetyl-transferase complex, suggesting a potential role in chromatin remodeling (19,23,28). A number of recent studies have shown that the nuclear actin plays a role in the regulation of gene transcription, which is independent on its function in chromatin remodeling (29,30). Antibodies against actin have
been shown to inhibit the transcription of naked DNA template by RNA polymerases I and II (31,32). In addition, β-actin has been shown to be necessary for the activity of partially purified RNA polymerase III. (33). The role of actin in transcription is further supported by the findings that actin is associated with all three RNA polymerases (29,31-34). Despite these progresses, one important unsolved issue is whether actin polymerization is also essential for the various nuclear functions of actin including regulation of transcription, although several recent studies suggested the presence of polymeric actins in the nucleus (35,36).

N-WASP is a member of the WASP family and functions as a key regulator of actin cytoskeleton by relaying the activation signal from small G proteins to the Arp 2/3 complex (13,37-39). Recently, we reported that the nuclear-localized N-WASP exists in a large nuclear protein complex containing PSF/NonO, nuclear actin and RNA polymerase II (40). We found that the interaction between N-WASP and NonO/PSF complex coupled N-WASP to RNA polymerase II and was important for the regulation of RNA polymerase II dependent transcription in vivo and in vitro. We also showed that N-WASP could promote nuclear actin polymerization, and this process was important for the transcriptional regulation. These results suggest the interesting possibility that N-WASP may regulate organization of nuclear actin in a similar manner as in the cytoplasm, namely through the Arp2/3 complex. In the present study, we investigated this possibility directly and demonstrate that the Arp 2/3 complex is also localized in the nucleus, where it plays a role in the promotion of de novo actin polymerization and RNA polymerase II-dependent transcription.

Experimental Procedures

Plasmid construction - DNA-vector based RNAi was generated using pBS-U6 essentially as described (41). pBS-U6-Arp2 RNAi was prepared by using oligos 5’-ggagagagagatttgaagcacca-3’ and 5’-agcttctcttggagttctc-3’. For pBS-U6-Arp3 RNAi, oligos 5’-ggtgatacgtcaagagggagagagagagtctc-3’, 5’-agcttctcttggagttctc-3’, 5’-agcttctcttggagttctc-3’ and 5’-aattcaggtgtgactcaagctcaagagggagagagagagagtctc-3’ were used. To clone the cDNA for Arp2, Arp2-5 primers 5’-cgggatccaagcttatggacagccagggcaggaaggtgg tggtg-3’ and 5’-aattcaaaaaggtgatcagctcaagagggagagagagagtctc-3’ were used for the PCR from cDNA library prepared from 293T cells using Qiagen RNaseasy kit and Superscript III RT-PCR kit (Qiagen, Invitrogen) by manufacturer’s manual. For Arp3 cDNA, Arp3-5 primers 5’-cgggatccaagcttatggacagccagggcaggaaggtgg tggtg-3’ and 5’-aattcaaaaaggtgatcagctcaagagggagagagagagtctc-3’ were used. PCR product was digested with BamH1 and EcoR1 and then ligated to a linearized pKH3 vector in the BamH1 and EcoR1 sites on the ends to generate pKH3-Arp2 and pKH3-Arp3. To make siRNA-resistant mutant of Arp2 (pKH3-Arp2-res), site-directed mutagenesis was performed using oligos 5’-aaagttgggggggagaggtttg aggcaccagaagct-3’ and 5’-agcttctcttggagttctc-3’. For siRNA-resistant mutant of Arp3 (pKH3-Arp3-res), oligos 5’-gcaaaa gttggcgcggactgagctgacagagagtgatgaaa-3’ and 5’-tttcataetctctctgagctgagctgacagagagtgatgaaa-3’ were used. To make Nuclear Exporting Sequence (NES) tagged proteins, nine amino acids of NES from Nrf2 (42) were tagged to the C-terminal of Arp2 and Arp3 by PCR. For NESm, four hydrophobic leucine of NES were replaced by glutamine. To make Myc-Arp2-NES, Arp2-5 primer and oligo 5’-ggcccgggttataggtatagtgttgatagtcgtcgttttagtcgacatgactccaaacactggattgtg-3’ were used for PCR amplification from pKH-Arp2-res. For Myc-Arp2-NESm, the same Arp2-5 primer and oligo 5’-ggcccgggttataggtatagtgttgatagtcgtcgttttagtcgacatgactccaaacactggattgtg-3’ were used for PCR from pKH-Arp2-res. PCR product was digested with BamH1 and SmaI and then ligated to a linearized pHan vector in the corresponding sites to generate pHan-Arp2-NES and Arp2-NESm. For Myc-Arp3-NES, the same strategy was used for PCR. Arp3-5 primer and oligo 5’-gggagagagatggagttctc-3’ were used. For Myc-Arp3-NESm, oligo 5’-ggtgatacgtcaagagggagagagagagtctc-3’ was used. PCR product was digested with BamH1 and EcoR1 and inserted into pHan vector at the
corresponding sites to generate pHan-Arp3 NES and pHan-Arp3-NESm. pHan vector have been described previously (43).

Reagents and cell culture - Rabbit polyclonal anti-HA (Y11), anti-PARP, anti-Arp2, anti-Arp3 and anti-GFP were obtained from Santa Cruz biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal anti-RNA polymerase II was obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-BrdU antibody was obtained from Roche Applied Science (Indianapolis, IN). Rabbit anti-Myc, mouse anti-HA, mouse monoclonal anti-vinculin, bromouridine were obtained from Sigma. RNase A was obtained from Qiagen (Valencia, CA). RNase-free DNase I and cDNA kit were obtained from Invitrogen (Carlsbad, CA). Actin polymerization biochem kit containing pyrene labeled actin was obtained from Cytoskeleton Inc. (Denver, CO). HeLa and 293 cells were cultured in DMEM supplemented with 10% FBS. Transient transfections were performed using Lipofectamine (Life Technology, Inc) according to the manufacturer’s manual.

Immunoprecipitation and western blotting - Subconfluent cells were washed with ice-cold PBS twice and lysed with 1% Nonidet P-40 lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 10% Glycerol, 1 mM NaVO₄, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin and 20 mg/ml leupeptin). Lysates were cleared by centrifugation for 20 min. at 4°C, and protein concentrations were determined by Bio-Rad protein assay. Immunoprecipitations were performed by incubating cell lysates with appropriate antibody for more than 2 hours at 4°C. For the experiment to detect endogenous association, immunoprecipitation were followed by incubation with protein-G Sepharose for another 2h. After three washings, the immune complex was resolved by SDS-PAGE. Western blotting was carried out using horseradish peroxidase-conjugated IgG as a secondary antibody and ECL system for detection.

Nuclear and cytoplasmic fractionation - Fractionation was performed as described previously (40). Briefly, cells were lifted by trypsinization, washed with PBS, then lysed in a lysis buffer (20 mM Hepes, pH 7.4, 10 mM KCl, 2 mM MgCl₂, 0.5% NP-40, 1 mM PMSF, 10 mg/ml Aprotinine, 20 mg/ml leupeptin) for 10 min. The lysates were centrifuged at 1,500 Xg for 5 min. to sediment nuclei. The supernatant was then centrifuged at 15,000 Xg for 10 min. and the supernatant formed the cytoplasmic fraction. The nuclear pellet was washed three times with lysis buffer and then resuspended in the same lysis buffer supplemented with 0.5 M NaCl to extract nuclear proteins. The extracted nuclear proteins were sedimented at 15,000 Xg for 10 min. and resulting supplement was harvested as the nuclear fraction. For, pyrene actin assay, the extract was dialysed with XB buffer at least 3 hrs.

Fluorescent microscopy - Cells were processed for immunofluorescence staining as described previously (44). GFP was observed directly by fluorescent microscope.

In vivo BrU incorporation assay - BrU incorporation for detection of nascent transcription in situ was performed essentially as described earlier (45). HeLa cells grown on coverslips were incubated for 1 hr in culture medium containing 2 mM BrU. After washing with ice-cold PBS, the samples were processed by immunofluorescent staining with mouse anti BrdU antibody (which cross react with BrU) as well as with anti-HA (to identify positively transfected cells). The fraction of BrU(+) cells among positively transfected cells were determined by blind counting. Three independent experiments were performed, and the student’s t test was used to determine the statistical significance.

Pyrene actin assay - Nuclear extracts were prepared from 293 cells as described earlier (40) and dialyzed against XB buffer (10 mM Hepes, pH 7.7, 100 mM KCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 5 mM EGTA, 1 mM DTT) for more than 3 hrs. The final concentration was generally 7 µg/µl. The reaction mix contained 180 µl extract with 10 µl energy regenerating mix (150 mM creatine phosphate, 20 mM ATP, 2 mM EGTA, 20 mM MgCl₂), 10 µl (0.4 mg/ml) pyrene actin and 10 µg of GST or GST-VCA fusion protein. Pyrene fluorescence was measured at 407 nm with excitation at 365 nm, and the kinetics of actin
filament assembly was monitored essentially as described earlier (40).

**In vitro transcription assay** - *In vitro* transcription assays were performed using the HeLaScribe nuclear extract *in vitro* transcription kit (Promega, Madison, WI) following the manufacturer’s protocol except that the nuclear extracts were pre-incubated with various affinity-purified antibodies (0.2 µg), for 15 min. before initiation of the reaction by addition of DNA templates and rNTPs. The intensity of the bands was quantified with Image J software. Three independent experiments were performed, and the student’s t test was used to determine the statistical significance.

**RESULTS**

**Nuclear localization of the Arp2/3 complex**

To examine the potential presence of the Arp2/3 complex in the nucleus, subcellular localization of Arp2 and Arp3 subunits were determined by immunofluorescence microscopy. Immunostaining of HeLa cells using antibodies against Arp2 or Arp3 showed their localization in the cytoplasm, especially in the cell periphery as observed previously (3). Both Arp2 and Arp3 are also detected in the nucleus (Fig. 1A). Confocal images also showed localization of Arp2 and Arp3 in the nucleus (Fig. 1B). The potential nuclear localization of the Arp2/3 complex was further evaluated by subcellular fractionation studies. Protein extracts from HeLa cells were subjected to fractionation as described in the Materials and Methods. The cytoplasmic and nuclear fractions were then analyzed by Western blotting using antibodies against Arp2 or Arp3. Fig. 1C shows that both Arp2 and Arp3 are detected in the nuclear as well as cytoplasmic fractions, while the nuclear (PARP) and cytoplasmic (vinculin) protein markers are only detected in their respective compartments.

Given the well characterized function of the Arp2/3 complex in the regulation of actin polymerization in the cytoplasm, our observation of Arp2 and Arp3 in the nucleus is somewhat surprising, although a previous study reported similar nuclear localization of another subunit of the Arp2/3 complex, p34 (46). Indeed, some cytoplasmic proteins could be detected in the nucleus due to non-specific recognition of other proteins by the antibodies used in these assays. To exclude such a possibility for Arp2 and Arp3, we generated specific siRNA vectors to deplete endogenous Arp2 or Arp3, and examined their effects on the detection of Arp2 or Arp3 in these assays. Fig. 2A shows that both the Arp2 and Arp3 siRNA vectors significantly reduced the endogenous levels of Arp2 and Arp3, respectively, but did not affect the expression level of control proteins PARP or vinculin. Importantly, the nuclear fraction of both Arp2 and Arp3 was reduced upon the treatments with respective siRNA constructs. Consistent with the fractionation studies, immunofluorescent staining of Arp2 and Arp3 was found to be reduced in the nucleus of the cells transfected with the respective siRNAs, but not the cells transfected with control siRNA (Fig. 2B). Taken together, these results strongly suggest that the Arp2/3 complex is specifically localized in the nucleus in addition to its cytoplasmic localization.

**The Arp2/3 complex mediates actin polymerization in the nucleus**

Our recent studies showed that the nuclear localized N-WASP could induce de novo actin polymerization in the purified nuclear extract (40). Given its well characterized role in mediating N-WASP-induced actin polymerization in the cytoplasm, the Arp2/3 complex localized in the nucleus may also be responsible for the potential actin polymerization in the nucleus. To test this hypothesis, we examined the effects of down-regulation of Arp2 and Arp3 on the actin polymerization in the nuclear extract by using pyrene actin assays as described previously (40). As observed previously (40), addition of GST-VCA domain of N-WASP, but not control GST, accelerated actin polymerization in the nuclear extracts (Fig. 3). Depletion of Arp2 or Arp3 in the nuclear extracts through expression of the corresponding siRNA constructs, but not a control GFP siRNA (see Fig. 2), significantly reduced GST-VCA stimulated actin polymerization, suggesting a role for the Arp2/3 complex in the process. Furthermore, addition of the purified Arp2/3 complex in the nuclear extracts restored the stimulation of actin polymerization by GST-VCA in a dose-dependent manner. Together, these
results suggested that the nuclear Arp2/3 complex is a major mediator of N-WASP-induced de novo actin polymerization in the nucleus.

Regulation of transcription by the Arp2/3 complex

It has been known that nuclear actin is important for the transcriptional regulation. Moreover, our previous study showed that nuclear N-WASP associates with large protein complex containing RNA polymerase II, and is involved in the regulation of RNA polymerase II-transcription through its function in promoting the polymerization of nuclear actin. Therefore, the above data showing a role of the nuclear Arp 2/3 complex in actin polymerization raised an interesting possibility for a potential function of the nuclear Arp2/3 complex in transcriptional regulation. To test this possibility, we first examined whether the Arp2/3 complex is also associated with RNA polymerase II by immunoprecipitation assay. Nuclear extracts were prepared by biochemical fractionation from 293T cells that had been transfected with plasmids encoding HA-tagged Arp2 or Arp3, or GST as control. The samples were immunoprecipitated with anti-HA and the immunoprecipitates were analyzed by western blotting to detect associated proteins. Fig. 4A shows that RNA polymerase II was detected in the samples expressing HA-Arp2 and HA-Arp3, but not control samples. To examine the association of endogenous Arp2 and Arp3 with RNA pol-II, co-immunoprecipitation was performed using nuclear extract of 293 cells with antibody against RNA pol-II. Western blotting with antibody against Arp2 and Arp3 shows co-precipitation of Arp2 and Arp3 with RNA pol-II, but not with control antibody (Fig. 4B). These results suggest that the nuclear Arp2/3 complex may also be present in the large complex containing RNA polymerase II and regulate transcription as shown for the nuclear N-WASP previously (40).

We then investigated a potential role of the Arp2/3 complex in transcription regulation by measuring the effects of depletion of Arp2 and Arp3 on the RNA polymerase II-dependent transcription in vivo using bromouridine (BrU) incorporation assays. We established the specificity of the assay for transcription by RNA polymerase II by showing that the signal of Ab-BrU staining is diminished by treatment with RNase, but not with DNase, and by actinomycin D at high concentration (that inhibits both RNA polymerase II and RNA polymerase I) but not at low concentration (that selectively inhibits both RNA polymerase I activity) (data not shown). Then, HeLa cells were co-transfected with siRNA for Arp2 or Arp3 or control gene, along with a vector encoding HA-tagged GST protein as a transfection marker. We found that knockdown of either Arp2 or Arp3 by siRNA, but not treatment of cells with the control siRNA, significantly reduced transcription activity in these cells (Figs. 5A and 5B). To verify that the inhibition of transcription by treatment of cells with siRNAs for Arp2 and Arp3 is caused by direct effect of the Arp2/3 complex on the transcription, but not by an indirect effect (e.g. potential cytoskeletal defects that may impact on transcription), we first generated Arp2 and Arp3 constructs in which the nine amino acids of Nuclear Exporting Sequence (NES) from Nrf2 (42) or its mutant form, NESm which have point mutations in four hydrophobic leucine residues of NES were fused to the C-terminus of Arp2 and Arp3. We also introduced silent mutations into these constructs so that they are resistant to siRNA-mediated depletion of Arp2 (and Arp3) (Fig. 5C). As expected, the Myc-tagged Arp2-NES and Arp3-NES were excluded from the nucleus and localized to the cytoplasm only, whereas Myc-Arp2-NESm and Arp3-NESm behaved like the endogenous Arp2 and Arp3 and localized to both the nucleus and cytoplasm (Fig. 5D). We then examined the ability of these constructs to rescue the inhibitory effects of depletion of endogenous Arp2 and Arp3 by siRNA. Fig. 5E shows that the expression of Myc-Arp2-NESm which is resistant to siRNA (lane 4), but not Myc-Arp2-NES which is resistant to siRNA but cannot enter nucleus (lane 3), in the Arp2 siRNA treated cells restored transcription. Similar results were obtained using the two Arp3 constructs (lanes 6 and 7). These results strongly suggest that the inhibitory effect of siRNA-mediated depletion of Arp2 and Arp3 is a direct effect and the nuclear Arp2 and Arp3 are critical for RNA Pol-II dependent transcription.
To further verify the direct effect of siRNA-mediated knockdown of Arp2 and Arp3 on transcription regulation, we performed in vitro transcription assays using nuclear extract of HeLa cells containing RNA polymerase II and a DNA template containing the CMV promoter for the immediately early gene. The nuclear extracts were pre-incubated with antibodies against Arp2 or Arp3 or control protein prior to initiating transcription by addition of DNA template and rNTP. Fig. 6 shows that pre-incubation with antibodies against Arp2 or Arp3, but not control antibody, significantly inhibited in vitro transcription. Furthermore, addition of the purified Arp2/3 complex to the transcription mixture for neutralization of antibodies reversed the inhibition of transcription by these antibodies. Together, these results provide strong support for a direct role of the Arp2/3 complex in the regulation of RNA polymerase II-dependent transcription in the nucleus.

DISCUSSION

The actin-nucleating Arp 2/3 complex is conserved in yeast and animals. The purified Arp 2/3 complex possesses actin nucleating activity (11,47), and its weak basal activity is synergistically stimulated by activating proteins including WASP family proteins (13). The central role of the Arp2/3 complex in the regulation of actin dynamics in the cytoplasm has been well established for a variety of cellular and developmental processes (4-9,28,48). In this report, we provide evidence that the Arp2/3 complex is also localized in the nucleus and participates in the regulation of transcription possibly also through its regulation of nuclear actin polymerization.

Actin has been observed in the nucleus for many years, although its functional significance in the nucleus has only been widely appreciated relatively recently (19,28). Several lines of evidence suggests a role of the nuclear actin in the regulation of gene transcription, including data showing nuclear actin association with chromatin remodeling complex (28,49), RNP particles and all three RNA polymerase in the eukaryotic cell nucleus (22,23,29-34). One of the key issues in the mechanisms of transcriptional regulation by nuclear actins is whether they exist in monomeric or polymeric form and whether their polymerization is essential for their nuclear functions as in the cytoplasm. Several recent studies have suggested the possibility of polymerized actins in the nucleus (27,35,50). In particular, McDonald et al quantified the properties of actins within the nucleus of living cells using fluorescently tagged actins. By using the FRAP approaches, they demonstrated that nuclear actin, like cytoplasmic actin, existed as both slowly and rapidly moving kinetic populations, and concluded that the slow moving population is comprised of polymeric (F) actin (36).

Consistent with these findings of the polymerized actin in the nucleus, we have recently demonstrated the nuclear function of N-WASP, a key upstream regulator of the Arp2/3 complex in the stimulation of actin polymerization, in the regulation of RNA polymerase II dependent transcription (40). We found that N-WASP-driven nuclear actin polymerization is sensitive to the treatment of the inhibitors of actin polymerization such as cytochalasin and latrunculin as well as N-WASP dominant negative mutant of actin polymerization Furthermore, inhibition of nuclear actin polymerization by these reagents significantly reduced mRNA transcription both in vitro and in vivo. Considering the well-established major function of the Arp 2/3 complex in nucleating actin polymerization, it is likely that the Arp 2/3 complex regulates transcription also through its function in inducing nuclear actin polymerization. Taken together, these data suggest the idea that N-WASP and the Arp 2/3 complex regulate transcription, at least in part, through their function in the regulation of nuclear actin in a manner similar to their function in the cytoplasm. It will be interesting to examine whether nuclear actin is polymerized in the transcription site in future studies.

The concentration of nuclear actin is estimated to be sufficient for spontaneous polymerization (51), suggesting that an active process to regulate polymerization/depolymerization of actin is required. Indeed,
several other critical regulators of actin polymerization have been found in the nucleus besides N-WASP and the Arp2/3 complex discussed above (52). For example, it has been reported that phosphoinositide that is known to stimulate polymerization is found in the nucleus and phosphoinositide signaling is initiated by a nucleus-specific isoform of phospholipase C (53,54). Several other actin binding proteins including cofilin, thymosin b4, gelsolin and CapG have also been observed in the nucleus (55-60). These proteins function to decrease actin polymerization and/or regulate the size of actin polymers through their severing and capping activities (41,55-60), thus antagonize the stimulatory functions of N-WASP and the Arp2/3 complex. Therefore, the regulation of nuclear actin is likely to involve both mechanisms that stimulate polymerization and depolymerization as well as those that control the size of actin filaments. Future studies will be necessary to further dissect the mechanisms by which the nuclear actins and the myriad of their regulatory components in the control of gene transcription and other nuclear functions.

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FIGURE LEGENDS

Figure 1. Nuclear localization of the Arp2/3 complex. (A) HeLa cells were plated onto fibronectin-coated coverslips, and subjected to indirect immunofluorescent staining with anti-Arp2, anti-Arp3 or control IgG, as indicated (upper panels). Nuclei were shown by Hoechst staining (bottom panels). (B) Confocal images showing nuclear localization of the Arp2/3 complex. Nuclei were shown by PI staining. (C) 293 cells were subjected to nuclear and cytoplasmic fractionation as described in Materials and Method. Equal amount of proteins were resolved by SDS-PAGE, followed by Western blotting with anti-Arp2 (left) or anti-Arp3 (right), as indicated. Anti-vinculin and anti-PARP were used as markers for the cytoplasmic and nuclear fraction, respectively.

Figure 2. Depletion of Arp2 and Arp3 proteins in the nucleus by siRNA treatments. (A) 293 cells were transfected with siRNA for Arp2 (left), Arp3 (right) or GFP as a control, as indicated. Three days later, cell lysates were prepared and subjected to fractionation as described in Fig. 1. They were then analyzed by Western blotting with various antibodies, as indicated. (B) HeLa cells plated on fibronectin-coated coverslips were transfected with siRNA for Arp2 or Arp3, along with a plasmid encoding GFP as a transfection marker. Three days after transfection, cells were subjected to indirect immunofluorescent staining with anti-Arp2 or Arp3 as indicated (upper panels). GFP were observed directly by fluorescent microscope (bottom panels).

Figure 3. Role of the Arp2/3 complex in actin polymerization in the nuclear extracts. 293 cells were transfected with siRNA for Arp2 (A), Arp3 (B), or GFP as a control (both panels). Three days after transfection, nuclear extracts were prepared by fractionation as described in Materials and method. Equal amount of samples were mixed with GST or GST-VCA with or without addition of the purified Arp2/3 complex, as indicated, and subjected to the pyrene actin
assay. The kinetics of actin filament assembly was monitored by a spectrofluorimeter. The label for the y-axis Fluorescence (a.u.) stands for Fluorescence (arbitrary units).

**Figure 4.** Association of the Arp2/3 complex with RNA polymerase II. (A) 293 cells were transfected with plasmids encoding HA-tagged Arp2, Arp3, or vector alone as control. Nuclear lysates were immunoprecipitated with anti-HA and the immunoprecipitates were analyzed by Western blotting with anti-RNA Pol II or anti-HA, as indicated (left panels). Aliquots of the lysates were also analyzed directly to verify equal amount of proteins in the samples (right panels). (B) Nuclear lysates were prepared from 293 cells and immunoprecipitated with anti-pol-II or control antibody as indicated. The immunoprecipitates and aliquots of the lysates were analyzed by western blotting with anti-Arp2 or anti-Arp3.

**Figure 5.** Role of the Arp2/3 complex in the regulation of RNA polymerase II dependent transcription in vivo. (A) HeLa cells were transfected with siRNAs for Arp2 or Arp3, along with a vector encoding HA-tagged GST as a transfection marker. Three days after transfection, cells were labeled with BrU and subjected to co-immunostaining with anti-BrU (left panels) and anti-HA (right panels) as indicated. (B) The fraction of BrU(+) cells in the transfected cells as identified by anti-HA staining were quantified and normalized to that of the cells transfected with control siRNA. The mean + s.e. from three independent experiments are shown. * P<0.05 in comparison with value from control cells. (C) HeLa cells were transfected with siRNA for Arp2 or Arp3 or control gene along with vectors encoding Myc-tagged Arp2-NES or Arp2-NESm or Arp3-NES or Arp3-NESm as indicated. Cell lysates were prepared 3 days after transfection and analyzed by western blotting with anti-Myc (upper panel) or anti-vinculin (bottom panel). (D) HeLa cells were transfected with vectors encoding Myc-tagged Arp2-NES or -NESm or Arp3-NES or -NESm as indicated and then were subject to indirect immunostaining using anti-Myc. (E) Hela cells were transfected with siRNAs for Arp2 or Arp3 or control gene along with vectors encoding Myc tagged GST as a control or Arp2-NES or –NESm or Arp3-NES or –NESm. The same assays were performed and quantified as (A) and (B). Anti-Myc antibody was used to detect transfected cells. The mean + s.e. from three independent experiments are shown. * P<0.05, ** P≥0.26 in comparison with value from control cells.

**Figure 6.** Analysis of the Arp2/3 complex in in vitro transcription assays. (A) Nuclear extracts of HeLa cells were incubated with anti-Arp2, anti-Arp3, or control antibody for 15 min in the presence or absence of the purified Arp2/3 complex before initiation of in vitro transcription, as indicated. The transcripts were isolated and resolved by 6% denaturing-PAGE, and analyzed by autoradiography. (B) The mean +s.e. of the relative levels of transcription from the three independent experiments are shown after normalization to that from control antibody. * P<0.05, ** P≥0.5 in comparison with value from control cells.
Figure 1

A)  

Arp2  
Hoechst  

Arp3  
Hoechst  

IgG  
Hoechst  

B)  

Arp2  
PI  

Arp3  
PI  

IgG  
PI  

C)  

IB : Arp2  
Nuc.  

IB : parp  
Cyt.  

IB : vinculin  

IB : Arp3  
Nuc.  

IB : parp  
Cyt.  

IB : vinculin  

Figure 2

A) Cyto. | Nuc. | Cyto. | Nuc.
--- | --- | --- | ---
GFP RNAi | Arp2 RNAi | GFP RNAi | Arp2 RNAi

IB : Arp2
IB : parp
IB : vinculin

IB : Arp3
IB : parp
IB : vinculin

B) ARP2
GFP
ARP2 RNAi
GFP RNAi

Arp3
GFP
Arp3 RNAi
GFP RNAi
Figure 3

A)

B)
Figure 4

A)  
| IP: HA | Nu. Lys. |
|-------|----------|
| HA-GST | HA-GST |
| HA-Arp2 | HA-Arp2 |
| HA-Arp3 | HA-Arp3 |

IB: RNA Pol-II
IB: HA

B)  
| Nu. lysate | Con. IP | Pol II IP |
|------------|---------|-----------|

IB: Arp2
IB: Arp3
Figure 5

A)

BrU

HA

Arp2 RNAi

Arp3 RNAi

GFP RNAi

B)

IB : Myc

IB : Vinculin

Arp2 RNAi

GFP RNAi

Myc-Arp2-NES

Myc-Arp2-NESm

IB : Myc

IB : Vinculin

C)

0

0.2

0.4

0.6

0.8

1

1.2

123

**

Relative BrU incorporation

1 : GFP RNAi

2 : Arp2 RNAi

3 : Arp3 RNAi

IB : Myc

IB : Vinculin
D) 

Myc-Arp3-NES

Myc-Arp3-NESm

Myc-Arp2-NES

Myc-Arp2-NESm

E) 

1: GFP RNAi + Myc-GST
2: Arp2 RNAi + Myc-GST
3: Arp2 RNAi + Myc-Arp2-NES
4: Arp2 RNAi + Myc-Arp2-NESm
5: Arp3 RNAi + Myc-GST
6: Arp3 RNAi + Myc-Arp3-NES
7: Arp3 RNAi + Myc-Arp3-NESm
Figure 6

A)  
1 : Mock Ab  
2 : Ab-Arp2 + PBS  
3 : Ab-Arp2 + Arp2/3 complex  
4 : Ab-Arp3 + PBS  
5 : Ab-Arp3 + Arp2/3 complex

B)  

Relative transcription level

1  2  3  4  5

**  **

0  0.2  0.4  0.6  0.8  1  1.2  1.4

*  **  **
A novel role of the actin-nucleating ARP2/3 complex in the regulation of RNA polymerase II-dependent transcription
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