A requirement for polymerized actin in DNA double-strand break repair

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Introduction

DNA double-strand breaks (DSBs) are considered to be among the most deleterious, potentially lethal DNA lesions which can arise from both natural cellular processes and external damaging agents. Consequently DSB repair is essential to cell survival. DSB repair consists of a complicated set of events that allow for the sensing of the DSB itself, amplification of the damage signal, exposure of the damaged ends and relaxation of the local chromatin structure, recruitment of DNA repair proteins to the site of damage and finally repair of the break itself. Failure at any step can have catastrophic consequences such as genomic instability, oncogenesis or cell death. Many proteins involved in this repair process have been identified and their roles characterized. We discovered that some DNA double-strand break repair factors are capable of associating with polymeric actin in vitro and specifically, that purified KU70/80 interacts with polymerized actin under these conditions. We find that the disruption of polymeric actin inhibits DNA double strand break repair both in vitro and in vivo. Introduction of nuclear targeted mutant actin that cannot polymerize, or the depolymerization of endogenous actin filaments by the addition of cytochalasin D, alters the retention of KU80 at sites of DNA damage in live cells. Our results suggest that polymeric actin is required for proper DNA double-strand break repair and may function through the stabilization of the Ku heterodimer at the DNA damage site.

Nuclear actin is involved in several nuclear processes from chromatin remodeling to transcription. Here we examined the requirement for actin polymerization in DNA double-strand break repair. Double-strand breaks are considered the most dangerous type of DNA lesion. Double-strand break repair consists of a complex set of events that are tightly regulated. Failure at any step can have catastrophic consequences such as genomic instability, oncogenesis or cell death. Many proteins involved in this repair process have been identified and their roles characterized. We discovered that some DNA double-strand break repair factors are capable of associating with polymeric actin in vitro and specifically, that purified KU70/80 interacts with polymerized actin under these conditions. We find that the disruption of polymeric actin inhibits DNA double strand break repair both in vitro and in vivo. Introduction of nuclear targeted mutant actin that cannot polymerize, or the depolymerization of endogenous actin filaments by the addition of cytochalasin D, alters the retention of KU80 at sites of DNA damage in live cells. Our results suggest that polymeric actin is required for proper DNA double-strand break repair and may function through the stabilization of the Ku heterodimer at the DNA damage site.

HR requires a region of DNA sequence homology on the sister chromatid to act as a template for the repair of a DNA lesion and is thought to be restricted to late S and G2 phases of the cell cycle. The NHEJ pathway is active throughout the cell cycle and while it is a more error prone mechanism of DNA repair, it is preferred over allowing the persistence of the damaged DNA. Several of the players involved and the general chain of events in these two pathways are now known yet many of the complex mechanistic and regulatory details are yet to be characterized. There is also increasing evidence of crosstalk between the two pathways adding yet another level of potential regulation and complexity.

It is now accepted that actin resides within the nucleus where it has been implicated in a myriad of nuclear functions.© 2012 Landes Bioscience. Do not distribute
demonstrates that nuclear actin is important to many nuclear processes. In one example, nuclear actin had originally been found to affect serum response factor (SRF) activity through its interaction with the SRF cofactor MAL, where there were strong indications that the monomeric form of actin was responsible. Recently, it was discovered that a nuclear targeted, polymerization incompetent actin mutant has the ability to specifically decrease SRF gene activity confirming the functional importance of nuclear actin polymerization. One of the earlier indicators that polymeric nuclear actin even existed came from a study that found actin-containing filaments projecting inward from the nuclear pore in Xenopus oocytes. The large size of these nuclei, however, may make this a special case. However, the presence of a pool of polymeric nuclear actin has been established in cultured mammalian cells using fluorescence recovery after photobleaching (FRAP). In this study, a dynamic equilibrium of monomeric vs. polymeric actin was confirmed with approximately 20% of the nuclear actin pool existing in a polymeric state. Further support for both the presence and function of polymeric nuclear actin comes from studies where nuclear actin and nuclear myosin I (NMI) were both found to play a role in RNA polymerase I transcription and using both mutant actins and actin disrupting drugs, transcription was found to be inhibited in the absence of polymeric actin. Thus, there is accumulating evidence that both monomeric and polymeric actin are important in the nucleus, for review see references 21 and 33. It is also important to note that several actin binding proteins known to modulate the polymerization state of actin in the cytoplasm such as cofilin, profilin, thymosin β4, CapG and gelsofin to name a few, have all been identified in the nucleus under various conditions. Therefore, nuclear actin dynamics may be regulated in a similar manner to the cytoplasmic pool and be functionally important. For example, the neuronal Wiskott-Aldrich syndrome protein (N-WASP), a known cytoplasmic regulator of actin polymerization, has been found in the nucleus where it both affects the polymerization of nuclear actin and participates in the regulation of RNA polymerase II-based transcription. JMY, a protein cofactor that promotes actin filament nucleation, primarily resides within the nucleus where it acts as a regulator of p53 activity. JMY has shown to be a DNA damage responsive protein by its accumulation in response to DNA damaging agents and its ability to promote apoptosis although any link to its effect on nuclear actin polymerization has yet to be established. As well, the overexpression of cofilin, an actin depolymerization factor, was found to alter nuclear actin dynamics and sensitize cells to radiation induced DNA damage suggesting a possible role for polymerized actin in DNA repair. In this study, we examined the potential role of polymerized actin in DSB repair. Using an in vitro actin binding assay, we discovered that some, but not all, DNA repair proteins can bind specifically to polymerized actin. We found proteins involved in the recognition and processing of the break site (Ku80, Mre11 and Rad51) associated with polymeric actin whereas a repair factor involved in damage signaling (Chk2) does not. Non-homologous end-joining (NHEJ), as determined by the in vitro DNA end-joining assay, decreases upon the disruption of polymerized actin. Importantly, the in vivo repair of gamma irradiation-induced DNA damage is dramatically inhibited in cells treated with actin disrupting drugs as determined by both the comet assay and the FAR assay. Western blot analysis indicates that the disruption of polymeric actin does not cause a change in the overall phosphorylation of histone H2AX in gamma irradiated cells nor does it affect the phosphorylation of the DNA damage cell cycle checkpoint regulator, Chk2. Rather, we found that polymerized actin is capable of interacting with the purified Ku heterodimer, possibly through the Ku70 subunit. Using live cell imaging, we determined that the introduction of a polymerization incompetent actin into the nucleus reduces the retention of Ku80 at sites of micro-irradiated DNA damage. In total, these results strongly indicate that polymerized nuclear actin participates in the process of DNA DSB repair, possibly at the level of stabilizing Ku heterodimer recruitment to the DNA damage site. Results

Latrunculin-mediated disruption of actin polymerization alters the association of DSB repair proteins with chromatin. When cells are exposed to ionizing radiation, many double-strand break repair proteins move from the soluble to the insoluble cellular fraction when nuclei are extracted under mild conditions. This is thought to reflect an association with chromatin, which remains insoluble in this preparation. Previously, we determined that pre-treating cells with the actin disrupting drug latrunculin caused a dramatic change in the solubility of several chromatin remodeling proteins during a standard salt extraction of isolated nuclei. Thus, as a first assessment of whether there was a relationship between actin polymerization and DNA double-strand break repair, we tested whether or not treatment with latrunculin altered the cellular fractionation behavior of selected DSB repair proteins. We found that the disruption of actin structure altered the solubility of repair proteins from both the NHEJ and the HR pathway using the previously established procedure (Fig. 1). DNA repair proteins associated with polymerized actin as determined by the in vitro, high-speed F-actin pull down assay. If this change in solubility reflects a direct interaction with polymeric forms of nuclear actin, these proteins should associate with F-actin in vitro. Therefore, we tested whether or not polymeric actin is capable of associating with components involved in DNA repair using an F-actin pull-down assay. These experiments exploit the fact that polymeric actin is pelleted by centrifugation at speeds of 100,000 × g while monomeric actin and other smaller macromolecular complexes remain soluble in the supernatant. Based on the results in Figure 1, nuclear extracts isolated from latrunculin treated cells were used. This increases the abundance of repair proteins present and reduces the possibility that a failure to detect an interaction reflected the actin-dependent insolubility of a specific subset or pool of the nuclear
protein population of each factor. Briefly, nuclear extracts were pre-cleared at 100,000 g to remove any proteins or protein complexes that pellet at these speeds on their own. The pre-cleared nuclear extracts were incubated with purified polymeric actin to allow potential interactions to occur. The reaction mixture was centrifuged at 100,000 × g and the distribution of specific proteins in the resulting supernatant and pellet determined by immunoblotting. Nuclear extracts combined with buffer only (Fig. S1) or an unrelated protein (BSA) resuspended in actin buffer were used as controls to ensure specificity. Under these conditions we found that both Ku80 and Mre11 were brought down in the high speed pellet through an association with polymeric actin (Fig. 2). In the case of Mre11, we found that the majority of molecules present in the pre-cleared nuclear extract are capable of associating with polymeric actin. However, only a portion of the Ku80 population appears to associate with polymeric actin in this assay. Attempts to examine whether NBS1 also associated with polymeric actin were inconclusive due to the sedimentation of NBS1 from the nuclear extract during the pre-clearing centrifugation.

Both Ku80 and Mre11 are involved early on in DNA repair at the actual site of damage. Rad51 is a recombinase involved in strand invasion of DNA repair via HR. A substantial portion of the detectable Rad51 was found in the high-speed pellet from the pre-cleared extracts incubated with polymeric actin indicating that Rad51 also associates with polymeric actin in this spin down assay (Fig. 2). We also examined whether polymeric actin associates with chk2, which serves as an example of a protein that is required for downstream signaling of DNA damage and not directly involved in the repair process itself. Upon DNA damage, chk2 is phosphorylated and subsequently is involved in activating the cell cycle checkpoint. Under these conditions, chk2 does not associate with polymeric actin (Fig. 2). It is important to note, while these results cannot differentiate between direct interactions and indirect associations with other components of multi-protein complexes that they are found in, they do indicate that polymeric actin associates with DNA repair proteins involved at the site of the double-strand break. The fact that polymeric actin does not associate with chk2 supports the specificity of this assay and suggests that aktcin’s involvement is at the level of repair, not at the level of cell cycle checkpoint control. Inhibition of actin polymerization impairs in vitro non-homologous end joining (NHEJ). We next wanted to determine whether or not the DNA repair proteins isolated in the nuclear soluble extracts were capable of repairing DNA in vitro. Nuclear extracts, isolated in the same manner as for the immunoblotting experiments, were used in an established in vitro DNA NHEJ assay.43,44 Briefly, in this assay, plasmid DNA is cut with two different restriction enzymes and isolated to yield a linear plasmid DNA with non-homologous ends. The purified DNA is then incubated with nuclear soluble extract from control or latrunculin-treated cells together with appropriate levels of MgCl₂ and ATP. DNA repair is detected as bands of increasing size on an agarose gel. This represents increasing numbers of ligated linear DNA molecules and reflects the NHEJ activity of the extract. In the absence of either extract, no DNA repair was detected verifying that the plasmid DNA did in fact have non-compatible ends and was not simply re-ligating spontaneously (Fig. 3A). As well, to ensure that we were indeed looking at a measure of NHEJ we chose to immuno-deplete the nuclear extract used of Ku80 and found that the detectable DNA repair was lost in the absence of Ku80 (Fig. 3B).

In the presence of control nuclear extract, a low level of DNA repair does occur. However, in the presence of the nuclear extract isolated from latrunculin-treated cells, a significant increase in the amount of DNA repair is detected (Fig. 3A). This is consistent with our previous findings that latrunculin treatment increases the amount of repair proteins present in the soluble nuclear fraction (see Fig. 1) and indicates that the repair proteins isolated from these extracts still maintain their competency to repair DNA.

![Image](https://example.com/image.png)

**Figure 1.** The distribution of DNA repair proteins in subcellular fractions. (A) Nucl-irradiated cells were treated with actin-depolymerizing drug latrunculin or vehicle control. Both Ku80 and Mre11 are found in the nuclear soluble fraction (see Fig. 1c) and are present in the nuclear insoluble fraction (C). Nbs-1 and ATM are found in all three of the cellular fractions examined. Treatment with latrunculin increases the solubility of both Ku80 and Mre11. Upon irradiation, Ku80 and Mre11 accumulate in the nuclear insoluble fraction. The recruitment of Ku80 or Mre11 to the insoluble fraction upon gamma irradiation is no longer evident in cells treated with latrunculin. (C) Mre11 and (D) ATM distribution are not detectable in the cytoplasm but found in both the nuclear soluble and nuclear insoluble fractions of control cells. ATM and Nbs-1 are enriched in the nuclear insoluble fraction upon gamma irradiation. Latrunculin treatment increases the solubility of ATM and Nbs-1 allowing them to be fully extracted into the nuclear soluble fraction after gamma irradiation. (E) Chk2 distribution is the same as for Ku80 found in (A).
To determine whether or not polymerized actin played a role in this in vitro repair of plasmid DNA, the same purified plasmid DNA and latrunculin-treated nuclear extracts used above were incubated with added polymerized actin or latrunculin in the end-joining reaction mixture. We found there was no consistent enhancement of DNA repair when the extract was supplemented with polymerized actin (Fig. 3C). Since actin is already present in the extract, this indicates that actin is either not required or that polymerized actin was not limiting in the reaction. The addition of latrunculin to the assay did cause a clear reduction in NHEJ (Fig. 3C). However, in the presence of polymerized actin and latrunculin, inhibition DNA repair was even more pronounced (Fig. 3C), possibly suggesting an inhibitory effect of the presence of excess monomeric actin. The inhibition of DNA repair in the presence of actin disrupting drugs implicates the participation of polymeric actin in DNA repair under these in vitro, non-homologous end-joining conditions.

Polymerized actin is essential for DSB repair in vivo as measured by the neutral comet assay and the FAR assay. The change in solubility of the various DNA repair proteins in response to actin disruption and in vitro association of some repair proteins with polymeric actin both suggest a potential role for nuclear actin in DNA repair. The decrease of in vitro NHEJ repair in response to latrunculin treatment suggests that polymeric actin is participating in the actual repair process itself. To address whether or not polymerized actin plays a physiologically relevant role in DSB repair in vivo, we used the neutral comet assay to determine the efficiency of repair of DNA DSBs in the presence or absence of latrunculin. The comet assay involves embedding cells in agarose, spotting small amounts onto slides, lysing the embedded cells and then eluting the DNA from the nucleus by electrophoresis. Under the electrophoretic current applied, damaged DNA migrates faster (“tail”) than undamaged DNA (“head”). This allows for the determination of the level of DNA damage at any given time by measuring the amount of DNA present in the “tail” vs. the total DNA content present in the cell. In our experiments, latrunculin-treated and untreated cells were embedded in agarose and subsequently gamma-irradiated to induce DNA damage. The latrunculin concentration was maintained by keeping the embedded cells immersed in growth media containing the appropriate concentration of latrunculin throughout the experiment. The gamma-irradiated cells were lysed either immediately or after various recovery times to allow repair to occur.

Significant DNA damage is detected immediately after gamma irradiation (Fig. 4A) (p < 0.0001) in both untreated and latrunculin-treated cells. We examined the amount of repair from this initial level of damage after 1.5 h and 4 h of recovery after irradiation treatment (Fig. 3). In untreated cells, the DNA damage is rapidly repaired as seen by the decreasing amount of DNA detected in the comet tail (Fig. 4A). Approximately 63% of the DNA damage inflicted is repaired in untreated cells within the 4 h timeframe of the experiment (Fig. 4A and B). In contrast, latrunculin-treated cells manage only modest repair of the DNA damage during the 4 h experimental timeframe representing 44% of the inflicted DNA damage being repaired (Fig. 4).

We confirmed our in vivo DNA repair results using an alternative in vivo DNA repair assay known as the FAR (Fraction of Activity Released) assay and tested the effect of additional actin disrupting drugs. While the FAR assay is not as sensitive as the comet assay, the versatility of this assay lies in the ability to assess larger cell populations at one time rather than examining individual cells. This assay is extremely similar to that of the
by latrunculin treatment dramatically reduced DNA repair ability of the cells (Fig. 5). This inhibition of repair was also seen in cells treated with swinholide and to a lesser extent with cytochalasin D (Fig. 5). The difference in the effect of cytochalasin D vs.
Figure 6. Immunoblot determination of the phosphorylation of histone H2AX and the phosphorylation of Chk2 in response to gamma irradiation. (A) Phospho-H2AX and H2AX levels present in acid extract histones after gamma irradiation (8 Gy) and the indicated recovery times in untreated (-) or latrunculin treated (+) cells. (B) Phospho-Chk2 levels present in whole cell extracts after gamma irradiation (8 Gy) and the indicated recovery times in untreated (-) or latrunculin treated (+) cells.

latrunculin and swinholide may be a reflection of the mechanism of action of each of these drugs. Latrunculin binds to and sequesters actin in its monomeric state whereas swinholide binds to actin dimers. However, cytochalasin D binds to actin and blocks any further potential polymerization resulting in the accumulation of varying lengths of actin from monomers to short polymers of varying length. In light of these differences, it both interesting and informative that the drugs that cause the accumulation of monomeric or dimeric actin are more potent inhibitors of the in vivo DNA repair measured in the FAR assay than the drug that allows for the accumulation of varying short actin polymers. This data along with the comet assay data all support the concept of polymeric actin playing a role in the repair of DNA DSBs.

Disruption of polymerized actin does not affect the phosphorylated H2AX or phosphorylated Chk2 responses to DNA damage. To further understand the manner in which polymerized actin is affecting DNA DSB repair, we chose to examine the phosphorylation of H2AX in acid extracted histones from gamma-irradiated cells with and without latrunculin. H2AX and phospho-H2AX histone populations were visualized by immunoblotting. Immunoblot analysis revealed that the phosphorylation of H2AX after gamma irradiation (8 Gy) and the indicated recovery times in untreated (-) or latrunculin treated (+) cells with and without exposure to gamma irradiation. The level of Chk2 phosphorylation was then determined by immunoblotting with phospho-Chk2 specific antibodies. After gamma irradiation, phosphorylation of Chk2 is detectable in both untreated and latrunculin treated cells (Fig. 6B). Of the various recovery time points examined, the phosphorylation of Chk2 peaked 15 min after irradiation and slowly diminished over the 4 h of the experiment regardless of whether or not cells were treated with latrunculin (Fig. 6B). The disruption of polymerized actin clearly does not appear to affect the ability of the cell to sense DNA damage or amplify the damage signal nor does it affect the activation of the DNA-damage cell cycle checkpoint as seen by the appropriate phosphorylation of H2AX and Chk2, respectively. Therefore, the requirement of polymeric actin for efficient DSB repair to occur as seen in this study may be through the timely recruitment or stabilization of repair factors at the DNA damage site while leaving the DNA repair signaling pathways intact.

Polymeric actin specifically interacts with the Ku70/Ku80 heterodimer. In light of our findings that several repair factors associate with polymeric actin, the disruption of polymeric actin inhibits DSB repair both in vitro and in vivo and this effect appears to be at the level of repair itself rather than on DNA damage signaling, we decided to examine whether polymeric actin interacts with a DNA repair factor directly. We again used the high-speed F-actin pull down assay. Here we incubated purified Ku70/Ku80 heterodimer and polymerized actin followed by high speed centrifugation at which point the resulting supernatant and pellet were immunoblotted with antibodies specific for Ku70 and Ku80. We found that both Ku70 and Ku80 from the purified heterodimer associated with polymerized actin as seen by their enrichment in the high speed pellet in the presence of actin (Fig. 7A). We then decided to examine whether the heterodimer was required for the association with polymeric actin or if Ku70 could associate with polymerized actin in the absence of Ku80. For this, we generated nuclear extracts from Ku80 deficient and Ku80-GFP reconstituted cells® and performed the F-actin pull down assay. We found that Ku70 is capable of associating with polymerized actin in the absence of Ku80, which is evident in the enrichment of Ku70 in the high speed pellet in the presence of actin using the nuclear extract from the Ku80 deficient cells.
damage consistent with previously published findings. The Ku70/Ku80 heterodimer is one of the repair factors known to recruit to damaged DNA ends early on in the repair process and given our data indicating that polymeric actin can interact with the purified Ku70/Ku80 heterodimer (Fig. 7A), we next examined whether polymeric actin played a role in the recruitment of Ku to sites of DNA damage. In these experiments we utilized both drugs and a wild type actin, or Ku deficient and Ku-GFP transfected into cells alone to demonstrate the ataxia-telangiectasia mutated protein (ATM), the main kinase responsible for phosphorylating H2AX in the DSB repair process. The Ku70/Ku80 heterodimer and the Ku70 subunit appears sufficient for this interaction.

Modulation of actin polymerization alters the retention of Ku80-GFP at sites of DNA damage. The Ku70/Ku80 heterodimer is one of the repair factors known to recruit to damaged DNA ends early on in the repair process and given our data indicating that polymeric actin can interact with the purified Ku70/Ku80 heterodimer (Fig. 7A), we next examined whether polymeric actin played a role in the recruitment of Ku to sites of DNA damage. In these experiments we utilized both drugs and a wild type actin, or Ku deficient and Ku-GFP transfected into cells alone to demonstrate the ataxia-telangiectasia mutated protein (ATM), the main kinase responsible for phosphorylating H2AX in the DSB repair process. The Ku70/Ku80 heterodimer and the Ku70 subunit appears sufficient for this interaction.

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polymers. The differing response to the different actin disrupt-
ing agents may be very informative. The fact that cytochalasin D allows for the accumulation of short polymers and has less of an impact on DNA repair supports the notion that polymeric actin is required for proper DNA repair to occur. The effective inhibition of DNA repair by latrunculin or swiholide treatment also indicates a requirement for polymerized actin in the DNA repair process. However, at this point, we cannot rule out the possibil-
ity that it is the presence of excess monomeric or dimeric actin that acts as an inhibitor of DNA repair as opposed to the lack of polymeric actin.

In our attempts to gain some mechanistic insight into the nature of actin’s role in DNA repair, we were able to establish that the hallmark H2AX phosphorylation response to gamma-
irradiation induced DNA damage is unaffected by the disruption of polymeric actin structure. Likewise, the phosphorylation of Chk2 is also unaffected indicating that the DNA damage cell cycle checkpoint is intact and functioning. At this point, poly-
meric actin appears to be required for proper, timely DNA repair and its effects are not at the level of signaling or activa-
tion of the DNA damage cell cycle checkpoint.

It is reasonable to assume that many factors could affect the stability and integrity of the repair complexes that form at the break site. This study indicates that the integrity of the DNA repair process is, at least in part, reliant on the pres-
ence of polymerized actin. The ability of polymerized actin to interact directly with the Ku heterodimer in vitro may provide insight into the manner in which polymeric actin exerts its effect on DNA repair in vivo. The Ku70/ Ku80 heterodimer acts as a mediator in the NHEJ repair pathway by binding to the free ends of the damaged DNA and ultimately exposing them in a manner allowing subsequ-
ent ligation. Evidence exists that shows the ends of damaged DNA are reasonably stationary. In an elegant study by Soungzhou et al., the potential roles of several different DNA repair factors in the positional stability of damaged DNA ends were examined. It was determined that Ku80 was specifically responsible for restricting the local movement of the damaged DNA ends. Here, we found that the manipulation of the polymerization state of nuclear actin has a distinct impact on the retention of fluorescently tagged Ku80 at sites of DNA damage in live cells. Specifically, the introduction of the polymerization incompetent actin mutant (NLS-actin G13R) into the cell nucleus resulted in a decrease in the amount of Ku80-GFP retained at sites of DNA damage. In light of our findings and the requirement of Ku80 for the proper immobili-
ization of damaged DNA ends we would suggest that polymeric nuclear actin is involved in the recruitment to and/or maintenance of the Ku70/Ku80 heterodimer at DNA DSBs. In doing so, it may contribute to the immo-
obilization of the double-strand break. Notably, however, the reduced effectiveness of cytochalasin D relative to the other inhibitors of actin polymerization in the in vivo DNA repair assay indicates that only short oligomers are necessary for the efficient repair of DNA. The specific effect on Ku retention, which is required for the nonhomologous end-joining pathway, may be distinct from the requirement in repair generally, which appears less sensitive to cytochalasin D.

This study provides evidence that polymeric actin is required for proper DNA DSB repair. Based on the intricate nature of DSB repair and the understanding that there is still much to learn, at this point, we believe that actin is providing integrity and support at the break site through the Ku70/Ku80 heterodi-
meter. One could then speculate that loss of appropriate levels of nuclear polymeric actin could lead to an increase in chromosomal translocations and subsequent genomic instability. It is also pos-
sible that a dynamic equilibrium of actin polymerization in the nucleus will prove important to multiple stages of DNA repair including those yet to be determined. Regardless, this study indi-
Bilizes the foundation for further studies into the intricate mecha-
nisms and regulation of the DNA repair process.

Materials and Methods

Antibodies. Primary antibodies used in the study were as fol-
lows: anti-ATM (clone Y170, 1549-1, Epitomics), anti-phos-
pho-ATM (S1981) (clone 10H11.E12, AM-0173-200, Lake

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Plaid Biologicals), anti-53BP1 (NB100-305A, Novus Biologicals), anti-phospho-HE2AX (S139) (clone JBW301, 05-636, Millipore), anti-H2A (acidic patch) (07-146, Millipore), anti-Ku80 (clone F3, 608302, Biolegend), anti-MDC1 (ab11169, abcam), anti-CHk2 (clone 7, 05-649, Millipore) and anti-Rad51 (rabbit polyclonal against purified full-length Rad51). Secondary antibodies used were either goat anti-mouse Alexa 488 (Molecular Probes), goat anti-rabbit Cy3 (GE Healthcare Life Sciences), goat anti-mouse Alexa Fluor® 750 (Molecular Probes), goat anti-rabbit Alexa Fluor® 680 (Molecular Probes), goat anti-mouse Alexa Fluor® 750 (Molecular Probes) and anti-rabbit Cy3 (GE Healthcare Life Sciences), goat anti-rabbit Alexa Fluor® 680 (Molecular Probes), goat anti-mouse Alexa Fluor® 750 (Molecular Probes).

Nuclear extractions. For solubility experiments, HeLa S3 cells were subcultured the previous day and used at 70–80% confluency on the day of the experiment. Cells were incubated in Dulbecco's modified Eagle's medium and 10% fetal bovine serum with or without 2 μM latrunculin A (BioMol) for 60 min at 37°C, 5% CO₂, harvested using 0.53 mM EDTA in phosphate-buffered saline and washed with cold phosphate-buffered saline. High salt nuclear extracts were prepared as previously described. Cells were treated in the same manner for histone extractions or whole cell extracts as for the solubility experiments as stated above. Nuclei were released by re-suspending cells in nuclei isolation buffer (250 mM sucrose, 350 mM NaCl, 20 mM Tris pH 8, 1.5 mM MgCl₂, 0.2 mM CaCl₂, 0.1% NP-40 with added complete protease and PhosSTOP phosphatase inhibitor cocktails (Roche)) and collected by centrifugation at 3,500 × g, for 15 min at 4°C. Histones were extracted from the pelleted nuclei with 0.4 N sulfuric acid for 1 hr on ice. The extract was centrifuged at 20,000 × g for 10 min, supernatant collected and protein concentration determined using the DC Protein Assay (BioRad) or the Comassie blue Plus protein assay reagent (Pierce). Clarified histone solutions were aceton precipitated overnight at −20°C, washed twice with cold acetone, allowed to air dry and re-suspended in ddH₂O. Whole extracts were obtained by resuspending cells in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 0.1% SDS with freshly added protease and phosphatase inhibitors indicated above, incubated on ice for 1 h and clarified by centrifugation at 20,000 × g.

F-actin pull-down assay. Purified non-muscle actin was purchased and polymerized as per the manufacturer's protocol (Cytoskeleton Inc.). The pull-down assay and its buffers were derived from the manufacturer's protocol (Cytoskeleton Inc.). With minor changes. Briefly, approximately 300–500 μg of nuclear extract was incubated with 8 ug polymerized actin or an equivalent volume of actin polymerization buffer for 30 min at room temperature. The reaction was then centrifuged at 100,000 × g for 1.5 hrs. The supernatant was recovered and TCA precipitated or concentrated using a 10,000 MWCO membrane concentrator (Millipore). The pellet was washed once with 1x actin polymerization buffer and subsequently recovered directly into 3x SDS-PAGE sample loading buffer. Equal relative amounts of the supernatants and pellets were separated by 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted as indicated.

For the pull-down with the Ku heterodimer, approximately 400 ng of the Ku70/Ku80 heterodimer was diluted in 20 mM Hepes pH 7.9, 100 mM KCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 10% glycerol and incubated with polymerized actin. The purified Ku heterodimer was a kind gift from Dr Ola Hammarsen.

Gel electrophoresis and immunoblotting. Sulfuric acid extracted histones or whole cell extracts were separated by SDS-PAGE (18% or 10% gels, respectively) and transferred to nitrocellulose (0.2 μm) for subsequent immunoblotting. Immunoblotting was performed as previously described with one modification. Secondary antibodies were conjugated with infrared specific dyes (either Alexa Fluor® 680, Alexa Fluor® 750, IRDye® 800, listed above) and all immunoblots were imaged on the Odyssey Infrared Imaging system (Li-Cor Biosciences).

DNA end-joining assay. Plasmid DNA with non-homologous ends was generated by restriction digest of eGFP-C1 (Clontech) with restriction enzymes NheI and EcoRI (New England Biolabs). The linearized plasmid was separated by 1% agarose gel electrophoresis and purified using the QIAquick gel extraction kit (Qiagen). The resulting linear, purified plasmid DNA was used as the DNA substrate for all subsequent end-joining assays. Non-muscle actin (Cytoskeleton...
Ten micrograms of nuclear extract was diluted with end-joining assay buffer with freshly added ATP and DTT (final concentrations: 0.4 mg/mL nuclear extract, 75 μM Tris pH 8.0, 0.2 mM CaCl₂, 3 mM MgCl₂, 50 mM KCl, 1.2 mM ATP and 0.5 mM DTT) with or without polymerized actin (2 μg) or latrunculin A (15 μM) and allowed to stand for 30 μM overnight. The transfection was initiated by the addition of 250 ng of prepared plasmid DNA, incubated at 25°C for 15 min and stopped by the addition of 0.4% SDS, 40 mM EDTA and 0.4 mg/mL proteinase K and incubation for 1 hr at 37°C. Repaired DNA was separated by agarose gel (0.7%) electrophoresis for 4.25 h at 45 V, stained with SYBR® Green I nuclear acid stain (Molecular Probes) and visualized on a Typhoon® Image (GE Healthcare Life Sciences).

Neutral comet assay. The neutral comet assay was performed essentially as previously described with minor modifications. Briefly, normal human fibroblasts (GM38) were grown at 37°C/5% CO₂ in Dulbecco’s modified Eagle/F12 medium supplemented with 10% fetal bovine serum and subcultured the day prior resulting in 70–80% confluency the day of the experiment. Cells were pre-incubated with or without latrunculin A (2 μM) for 1 hr at 37°C/5% CO₂ prior to being trypsinized, washed and re-suspended with Ca²⁺/Mg²⁺ free phosphate buffer (PBS). Cells were mixed at a 1:10 ratio with 1% low melting point agarose. Comets were fixed in cold 70% ethanol for 5 min, removed and air-dried overnight. The neutral comet assay was performed as described in Kruhlak et al. (2006). Approximately 2–24 h after transfection, U2OS cells were incubated with 2 μM Hoechst 33342 (Invitrogen), for 5 min and in the case of cytochalasin D analysis cells were incubated with 1 μg/mL cytochalasin D (Sigma Aldrich) for 2 h. Coverslips were then washed three times with PBS. While imaging, cells were incubated in phenol red free RPMI (Invitrogen) media supplemented with 10% FBS (Sigma) and maintained at 37°C. A 405 nm UV laser was used to induce DNA DSBs in transfected cells and the recruitment of GFP-tagged repair proteins to the site of damage was followed in real-time. Images were processed and analyzed using ImageJ and analyzed using MetaMorph® imaging software. DSB damage was conducted essentially as described in Kruhlak et al. (2006). Approximately 20–24 h after transfection, U2OS cells were plated back into media containing the appropriate actin disrupting drugs and irradiated (40 Gy). At each of the designated recovery times a cellular plug was removed and placed in lysis solution at 4°C for approximately 24 h. Each plug was then cut into segments. Duplicate segments were placed into a 0.7% agarose gel and overlaid with 1% low melting point agarose. The lysed cell plugs were electrophoresed at 1 V/cm overnight, the migrating DNA stained with ethidium bromide and visualized on a Typhoon® Image (GE Healthcare Life Sciences).

Live cell imaging and determined DNA damage. Human osteosarcoma (U2OS) cells were grown at 37°C/5% CO₂ in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum. Cells were co-transfected with 100 ng Ku80-GFP and either 100 ng of NLS-actin WT or NLS-G13R actin constructs along with 900 ng of Bluescript as a carrier. Each construct was driven by a CMV promoter. Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. For live-cell imaging, cells were visualized on a custom built Zeiss Axioobserver Z1 inverted microscope (Intelligent Imaging Innovations), equipped with a 37°C incubator (LAS, USA), a CSU-M1 spinning disk confocal scanner and 4 laser lines (405, 488, 560 and 633 nm). The cells were observed using a 40x objective (1.3 N.A) and images were recorded using an Evolve cooled charge-coupled device (CCD) camera (Photometrics) and Slidebook 5.0 software (Intelligent Imaging Innovations). UV-laser induced DNA damage was conducted essentially as described in Krühlak et al. (2006). Approximately 20–24 h after transfection, U2OS cells were transfected with 2 μM Hoechst 33342 (Invitrogen), for 5 min and in the case of cytochalasin D analysis cells were incubated with 1 μg/mL cytochalasin D (Sigma Aldrich) for 2 h. Coverslips were then washed three times with PBS. While imaging, cells were incubated in phenol red free RPMI (Invitrogen) media supplemented with 10% FBS (Sigma) and maintained at 37°C. A 405 nm UV laser was used to induce DNA DSBs in transfected cells and the recruitment of GFP-tagged repair proteins to the site of damage was followed in real-time. Images were processed and analyzed using Slidebook and Adobe Photoshop CS6. Quantification of fluorescence intensity was measured within laser track regions and compared with regions outside the track for 30 cells at every time point in the 40 sec time course. Statistical analyses (two-tailed t-test) were conducted using Excel 2007 software (Microsoft). Videos frames were compiled in Photoshop CS1 (Adobe) and converted to an avi file in ImageJ 1.44i (NIH) with a frame rate of 5 frames per second.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Material
Supplemental materials may be found here: http://www.landesbioscience.com/journals/nucleus/article/21055/
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