Spatial and dynamic organization of molecular structures in the cell nucleus

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CHAPTER 3

Telomere movement is constrained by interactions with an inner nuclear lamin structure

Telomere movement is constrained by interactions with an inner nuclear lamin structure

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Summary

The interphase nucleus is thought to contain a three-dimensional filamentous protein network that provides structural support to chromosomes and facilitates transcription, RNA processing, DNA replication and DNA repair. The molecular composition of this network is, however, not clear. In this work we investigate whether telomeres, which are distributed throughout the nuclear interior, are attached to a nuclear matrix structure composed of lamin proteins. We used RNA interference to knockdown components of the nuclear matrix and latrunculin to depolymerize the nuclear actin network. Fluorescence time-lapse imaging revealed that telomeres become more dynamic in lamin A/C depleted cells but not in lamin B2 depleted cells. In addition, telomeres are more dynamic in emerin depleted cells and in cells that have been treated with latrunculin. These results suggest that telomeres are associated to a complex consisting of lamin A/C, emerin and actin and that the movement of telomeres is constrained by this association.

Introduction

The cell nucleus is subdivided in distinct compartments including chromosome domains and nuclear bodies. Different types of nuclear bodies have been distinguished, each type housing a unique set of proteins that creates a specific microenvironment. The nuclear bodies are positioned in the interchromatin domain space, which separates the individual chromosome domains (Spector, 2003). The separation of chromosome domains is not absolute as some intermingling between neighbouring chromosomes has been observed (Branco & Pombo, 2006). In general, however, the spatial positioning of chromosome domains in the three-dimensional space of the interphase cell nucleus is rather fixed and non-random, though may vary among cells and change with the differentiation and proliferation state of the cell. The general concept is that gene poor chromosomes are positioned at the nuclear periphery and that gene rich chromosomes are positioned towards the nuclear interior (Croft et al., 1999). This concept is consistent with observations where the positioning of specific genes at the nuclear periphery resulted in their silencing and is explained by the predominant heterochromatic nature of chromatin at this site and by the attachment to the lamina (Finlan et al., 2008; Reddy et al., 2008). Thus, the composition of the nuclear periphery might be such that it does not favour transcriptional activity. Some genes, however, are not silenced when located at the nuclear periphery and show transcriptional activity (Kumaran & Spector, 2008). This is particularly true for genes that are positioned at nuclear pores (Casolari et al., 2004; Dieppois et al., 2006). Also, genes in

centromeric and telomeric regions, which are supposed to be heterochromatic, have been found to display transcriptional activity. Hence, additional factors are required to establish a transcriptionally competent microenvironment. These could include the spatial positioning of active genes at the periphery of compact chromatin domains facing the interchromatin domain space (Mahy et al., 2002). But also, the positioning of genes at transcription factories or at speckles (Sexton et al., 2007; Sutherland & Bickmore, 2009). These speckles are distinct nuclear bodies enriched for splicing and transcription factors. Clustering genes at transcription factories or speckles would allow for a coordinated regulation of transcription, RNA processing and RNA transport. Specific associations of genes with other nuclear bodies, like PML and Cajal bodies, have been reported as well (Trinkle-Mulcahy & Lamond, 2008). Together, these findings suggest that the spatial arrangement of genomic regions and probably also that of nuclear bodies in the cell nucleus is important to control gene activity.

One of the key questions is how the spatial organization of the cell nucleus is established and maintained during interphase, and how it reorganizes in response to external factors that may either activate or silence genes. Several lines of research suggest that the nucleus contains a rigid though dynamic nuclear skeleton or matrix structure that provides anchorage sites for chromatin and nuclear bodies. DNA loops are attached to the nuclear matrix via loop anchorage regions (LARs). These genomic regions may include matrix attachment regions (MARs), topoisomerase II binding sites and other discrete sequence motifs (Razin, 1996; Vassetzky et al., 2000). At present, a clear view about the composition and localization of the nuclear matrix does not exist. Therefore, the nuclear matrix has often been defined as a three-dimensional filamentous protein network that remains present after high-salt extractions and the removal of chromatin. Among the candidate proteins that are part of the nuclear matrix structure are the lamin proteins. The lamin proteins are encoded by three genes, the lamin A, lamin B1 and lamin B2 genes. Lamin C is a splice variant of lamin A. The lamin A and B proteins form the lamina, which is a protein filament meshwork at the nuclear periphery connected to the inner nuclear membrane (Broers et al., 2006). Lamins also exist throughout the nuclear volume and have been suggested to support transcription and DNA replication (Moir et al., 1994; Spann et al., 2002; Tang et al., 2008). Consistent with a role of lamin proteins in supporting chromatin organization are observations that the expression of mutant lamina proteins leads to nuclear reorganization (Broers et al., 2005; Taimen et al., 2009).

Previous work indicated that telomeres are firmly attached to the nuclear matrix and thereby contribute to the spatial organization of chromatin in the cell nucleus (de Lange, 1992; Luderus et al., 1996; Weipoltshammer et al., 1999). The components that mediate this interaction have, however, not been identified yet. To investigate a possible role for lamin proteins in tethering telomeres we realized a knockdown of nuclear lamin proteins by expressing specific shRNAs and measured the dynamics of telomere movement in cells by fluorescence time-lapse imaging.

Results

Reduction of lamin A/C but not lamin B2 results in an increase in telomere dynamics

To knockdown the expression of lamin A and lamin B2 we selected for each gene 5 short hairpin (sh) RNA constructs from a viral-vector-based shRNA library targeting 7100 annotated human genes. Each construct was selected to target a different region of the gene sequence and tested for its efficiency for knockdown. To this end U2OS cells were transiently transfected and cultured in the presence of puromycin for 72 hours. As a control, cells were transfected with an empty vector. Cells were lysed and subjected to Western blot analysis. Finally, we selected one shRNA construct for each target gene on the basis of most efficient knockdown of protein expression. The selected constructs were packed in lentiviral particles and used to transduce U2OS cells. At 72 hours post transduction, the knockdown of lamins was analyzed by both Western blotting and immunocytochemistry. By Western blotting we observed an almost complete reduction in expression of both lamin proteins (Fig. 1A). Next, we analyzed the efficiency of lamin protein knockdown by immunocytochemistry. As shown in Fig. 1B, the majority of cells showed an absence or strong reduction of lamin A/C staining. After counting 285 cells, we calculated an almost complete knockdown of lamin A/C in 87% of cells. For lamin B2, we calculated that 97% of cells showed a strong reduction in protein expression.

Following lentiviral transductions of U2OS cells targeting lamin A/C and lamin B2 respectively, the cells were transfected after 72 hours with TRF1-DsRed together with GFP-PCNA. As a control, cells were either not transduced or transduced with a lentivirus containing a non-silencing expression vector (SHC002). In previous studies it has been shown that TRF1-DsRed is a specific marker for telomeric DNA (Molenaar et al., 2003; Brouwer et al., 2009) and that GFP-PCNA is an efficient live cell marker to discriminate cell nuclei and cell cycle phases (Leonhardt et al., 2000). 3D time-lapse images were collected every 30 seconds for 20 minutes and the mean square displacements (MSDt) of telomeres were determined using the image analysis program STACKS. Because all MSDt curves obtained for telomere movement showed the same shape we present the MSDt values at Δt 360 seconds in order to compare telomere movements measured under the various conditions tested in this study. STACKS first corrects for cell movements and then calculates the movements of telomeres in 2D and time. A correction has been made to compensate for the fact that a cell is a 3D object. MSDt plots representing measures for the space in which an individual dot is moving inside the cell nucleus during a given time-period. Fig. 2 shows an example of a graph representing the tracks of individual telomeres in a cell nucleus. Consistent with our previous data, we observed that the movement of telomeres in control cells is constrained (Molenaar et al., 2003) and we calculated an MSDt value of ~0.35 μm^2 (at Δt 360 seconds). All MSDt values in this study represent the total of telomeres measured in 10 individual cells. When G1 cells were excluded from the analysis on basis of PCNA staining, we calculated an MSDt value of ~0.25 μm^2, consistent with observations that telomeres are more dynamic during the G1 phase (Vrolijk et al., in preparation). Reduction of lamin A/C expression by shRNA interference resulted in an increase in telomere movement as compared to cells transduced with the SHC002 control virus, MSDt ~ 0.53 μm^2 (stdev ~0.46 μm^2) versus 0.22 μm^2

(stdev ~0.05 μm^2) (Fig. 3). Surprisingly, a reduction of lamin B2 did not reveal an increase in telomere dynamics (MSDt ~ 0.19 μm^2, stdev ~0.12 μm^2) (Fig. 3).

Telomere movement in emerin depleted cells

Emerin is a nuclear membrane protein, which is missing or defective in Emery-Dreifuss muscular dystrophy (EDMD). It is a member of a family of lamina-associated proteins which includes LAP1, LAP2 and laminB receptor (LBR) (Holmer & Worman, 2001). Emerin binds directly to both A- and B-type lamins *in vitro* (Clements et al., 2000; Lee et al, 2001), colocalizes with lamins *in vivo* (Manilal, 1998), and is associated with the actin network in the nucleus (Holaska et al., 2004). Since emerin has also been identified as part of the nuclear matrix (Squarzoni et al., 1998), we decided to investigate its role in telomere positioning and kinetics. From five shRNA constructs we selected one construct that when expressed in U2OS cells resulted in a 94% reduction of emerin protein as compared to endogenous levels. This construct was used to assemble lentiviral particles and then to transduce U2OS cells. Transduction resulted in a strong reduction of cells showing emerin staining. Of 452 cells that were analyzed, 85% showed absence of emerin staining (Fig. 4). Next, cells were first transduced to knockdown emerin expression and then, after 72 hours, transfected with TRF1-DsRed together with GFP-PCNA. Analysis of 3D image stacks, which were recorded every 10 seconds during 20 minutes and collected from 10 cells revealed an increase in telomere mobility (MSDt value of ~2.1 μm^2, stdev ~1.5 μm^2) as compared to cells transduced with the control construct (~0.35 μm^2).

Preventing actin polymerization causes an increase in telomere mobility

Nuclear actin has been identified as a potential nuclear matrix component (Kiseleva et al., 2004; Albrethsen et al., 2009) and as a binding partner of lamin A (Sasseville and Langelier, 1998; Zastrow et al., 2004) and emerin (Lattanzi et al., 2003). Thus telomeres could be directly or indirectly linked to a nuclear matrix by actin. To test this possibility, we treated U2OS cells expressing both TRF-DsRed and GFP-PCNA with latrunculin A, a drug that inhibits actin polymerisation *in vitro* (Coue et al., 1987; Morton et al., 2000) and *in vivo* (Spector et al., 1983). Analysis of 3D time-lapse recordings of telomere movements in 10 cells using STACKS revealed a mean MSDt value of ~0.54 μm^2 as compared to an MSDt ~0.35 μm^2 that was calculated for telomeres in untreated control cells, suggesting that telomeres become more mobile in the absence of polymerized nuclear actin (Fig. 5). To confirm this finding we also treated mouse embryonic fibroblasts (W8 MEFs) expressing TRF1-DsRed together with GFP-PCNA with latrunculin A and collected 4D image stacks. Quantitative analysis of telomere movement by the program STACKS revealed a three-fold increase in MSDt value, MSDt ~1.01 μm^2 as compared to MSDt ~0.35 μm^2 in untreated cells (Fig. 6).

Nuclear actin has been shown to be involved in the transcription process (Zhu et al., 2004). Preventing actin polymerization by latrunculin may therefore result in global transcription inhibition, which then may result in a change in chromosome organization, leading to less constrained telomere mobility. Treatment of U2OS cells with the transcription inhibitor 5,6-dichloro-1-ß-D-ribobenzimidazole (DRB) resulted in some decrease in telomere mobility (MSDt ~0.23 μm^2), suggesting that telomere mobility is not increased by changes in global transcriptional activity but more likely

by losing their association with an actin containing complex (Fig. 5). Also in W8 MEFs, we observed a decrease in telomere mobility, although not significant, after treatment with DRB, (MSDt ~0.32 μm^2 as compared to MSDt ~0.35 μm^2 in untreated cells (Fig. 6).

Discussion

The spatial positioning and dynamic properties of telomeres in the cell nucleus have been addressed by various studies. These studies show that in yeast telomeres are positioned at the nuclear periphery and anchored to the inner nuclear membrane by Ku and Sir proteins (Hediger et al., 2002). This anchoring puts a constraint on the mobility of telomeres in yeast cells and has been functionally linked to gene regulation. Unlike in yeast, in mammalian cells telomeres are positioned throughout the nucleus (Molenaar et al., 2003; Weierich et al., 2003). Still, their mobility is not very different from that of yeast telomeres and appeared to be constrained (Molenaar et al., 2003; Jegou et al., 2009). This constrained movement can be explained by a compact organization of chromatin in the cell nucleus, but also by an association to a supporting structure like the nuclear matrix.

In this work we show that telomeres are more mobile in cells with reduced expression of lamin A/C or emerin but not in cells with reduced lamin B2 expression. These findings are consistent with the idea that lamin A/C is a component of the nuclear matrix and anchors telomeres to this structure. Patients with a 433G>A mutation in the α-helical central rod domain of the A-type lamin gene show multiple nuclear alterations including mislocalization of telomeres (Taimen et al., 2009). This mutation prevents lamin A to organize in higher order structures and may thereby lose its function to anchor telomeres. Consistent with this idea, telomeres have been found to be shifted towards the nuclear periphery in MEFs devoid of A-type lamins (Gonzalez-Suarez et al., 2009). Recently, we showed that lamin redistribution in the cell nucleus is one of the first hallmarks of a senescent state of mesenchymal stem cells and that this redistribution is accompanied by a redistribution of telomeres, suggesting that telomeres are physically associated with a lamin structure (Raz et al., 2008). Previous studies suggested that telomeres are associated with a nuclear matrix structure (Gonzalez-Suarez et al., 2009). Following various nuclear extraction procedures telomeres were shown to remain attached to structures in the cell nucleus while most other DNA sequences did not (de Lange, 1992; Ludérus et al., 1996; Weipoltshammer et al., 1999). These studies, however, did not identify lamin proteins being responsible for retaining telomeric sequences. It was, however, observed that telomere binding protein TRF colocalizes with telomeric DNA in nuclear matrix preparations and that TRF was retained in the nuclear matrix even after removal of telomeric DNA, suggesting that anchoring of telomeres to the nuclear matrix is mediated by TRF (Ludérus et al., 1996). Interestingly, fluorescence recovery after photobleaching analysis showed that the association of TRF1 and TRF2 with telomeres is highly dynamic (Mattern et al., 2004). However, a fraction of TRF2 has been identified that forms more stable complexes with telomeres (Mattern et al., 2004) and might be involved in stabilizing the interaction of telomeres with a nuclear lamina structure.
One may also argue that a dynamic binding of TRF1 and TRF2 at telomeres may allow dynamic interaction with a nuclear lamina scaffold structure which may be essential for a dynamic nuclear organization. Recently, it has been shown that deletion of TRF2 in mouse cells resulted in increased telomere mobility and was dependent on 53BP1. The supposed rationale for the increased telomere mobility is to facilitate non-homologous end joining to repair DNA damage (Dimitrova et al., 2008).

A question that has not been answered yet is which factors mediate the interaction between telomeres and the nuclear matrix. Proteins in the nuclear matrix may directly interact with telomeric DNA or indirectly by association with telomere proteins. Di-

rect interactions of lamin proteins with TRF1 or TRF 2 have thus far not been observed. Recently, it has been shown that the telomere associated protein TIN2, binding both TRF1 and TRF2, associates with the nuclear matrix and thereby may mediate the binding of telomeres to the nuclear matrix (Kaminker et al., 2009).
Our observation that emerin knockdown results in an increase in telomere dynamics is consistent with a role for emerin in structuring the cell nucleus. Emerin has been shown to interact directly with A- and B-type lamins (Clements et al., 2000; Lee et al., 2001) and in patients lacking emerin lamins A, C and B2 are more soluble suggesting a disrupted lamina architecture (Markiewicz et al., 2002). Together with our observation that nuclear actin plays a role in tethering telomeres to a nuclear structure our data support a previously proposed model in which lamin A, actin and emerin form a complex which forms or is part of a network in the nucleus (Mehta et al., 2008). Telomeres could be associated with such a complex to help structuring chromosome organization in the interphase nucleus. The fact that lamin B2 knockdown does not result in increased telomere mobility suggests that telomeres are not linked to a structure composed of B type lamins. Previously, photobleaching experiments revealed that A and B type lamins show different exchange rates in the nucleoplasm, suggesting that both types are present in distinct structures (Moir et al., 2000). At the nuclear periphery, A and B type lamins were shown to be present in a separate but interconnected network (Shimi et al., 2008). This may also be true when both proteins are present in the internuclear space. Furthermore, in vitro experiments showed that lamin B does not bind to telomeric DNA while lamin A/C does (Shoeman & Traub, 1990). Thus, intranuclear B-type lamins may contribute to a stable nuclear architecture in a different fashion as compared to A-type lamins, for example by supporting a framework for RNA synthesis (Tang et al., 2008).

Materials and Methods

Cell Culture
Human Osteosarcoma cells (U2OS) and mouse embryonic stem cells (W8 MEFs) were cultured at 37 ºC on 3.5-cm glass-bottom culture dishes (MatTek) in Dulbecco's modified Eagle's medium (DMEM) without phenol red, containing 1.0 mg/ml glucose, 4% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, pH 7.2 (all from Invitrogen). Actin depolymerisation was induced by treating cells with 2 µg/ml latrunculin A for 2h. Transcription inhibition was achieved by treating cells with 30 µg/ml DRB (5,6-dichloro-1-ß-D-ribobenzimidazole) for 2h.

Plasmids and transfection
The cloning of TRF1 in the DsRedExpress vector (Clontech) has been described (Brouwer et al., 2009). The GFP-tagged proliferating cell nuclear antigen (PCNA) protein was a gift from M.C. Cardoso (Leonhardt et al., 2000). Cells were transiently transfected with 0.5 µg vector DNA using Lipofectamine 2000 (Invitrogen) according to instructions of the manufacturer. At 16 hours post transfection 1.5 µg/ml puromycin was added to the culture medium and cells were assayed 72 hours after transfection.

Protein blot analysis
Cells were lysed in NuPAGE LDS sample preparation buffer (Invitrogen). Protein samples were then size fractionated on Novex 4–12% BisTris gradient gels using a MOPS buffer (Invitrogen) and were subsequently transferred onto Hybond-C extra membranes (Amersham Biosciences) using a submarine system (Invitrogen). Blots were stained for total protein using Ponceau S (Sigma-Aldrich). After blocking with PBS containing 0.1% Tween 20 and 5% milk powder, the membranes were incubated with monoclonal mouse antibodies against lamin A/C (sc-7292, Santa Cruz Biotechnology), lamin B2 (NLC-LAM-B2, Novocastra), emerin (NCL-emerin, Novocastra) and with a mouse polyclonal antibody against tubulin (1:2000; Sigma-Aldrich). The secondary antibody used was HRP-conjugated anti-mouse (1:5,000; Pierce). Bound antibodies were detected by chemiluminescence using ECL Plus (Amersham Biosciences).

Virus production and transduction
The shRNA lentiviral plasmid vectors targeting lamin A/C, lamin B2 and emerin were selected and obtained from the Sigma human shRNA library MISSION^{TM} TRC-Hs 1.0 (Sigma-Aldrich). The pLKO.1Puro lentiviral shRNAvectors generating an efficient knockdown of lamin A/C, lamin B2 and emerin were used to generate lentiviral particles. For this purpose 293T cells were transfected with the appropriate lentiviral shRNA vector together with the vectors pCMV-VsVG, pMDLg-RRE and pRSV-Rev using calcium phosphate precipitation. At 48 and at 72 hours after transfection the viral supernatants were harvested and filtered through a 0.45 µm pore size filter. Virus titers were determined by measuring HIV-1 p24 antigen levels by ELIZA using a RETRO-TEK HIV-1 p24 antigen ELIZA system (ZeptoMetrix, Buffalo, USA). Cells were transduced with lentiviral particles (MOI 3, MOI5 and MOI 10) in the presence of 8 µg/ml Polybrene (Sigma) and incubated overnight at 37 ºC. Then, the cells were washed 3 times in medium and cultured in fresh culture medium until analysis.

Live cell imaging

Wide-field fluorescence microscopy was performed on a multi-dimensional work-station for live cell imaging (model DMI3000B; Leica Microsystems, Mannheim, Germany) equipped with a metal halide bulb and a 63× NA 1.25 HCX plan Fluotar objective. 4D image stacks, each containing 20 sections of 0.5 μm, were collected using an automated motorized z-galvo stage. During imaging, the microscope was heated to 37 ºC in a CO_2 perfused and moisturized chamber. Image stacks were collected every 30 sec for 10 min. Image deconvolution was performed using Leica software. For each experiment and cell type at least ten movies were analyzed.

Telomere movements were quantified using an in house developed object tracking program called STACKS (Vrolijk et al., in preparation). With this program tracks of moving objects in a cell can be visualized and quantified. The movements of telomeres were quantified by measuring their mean squared displacement, or MSDt, as described in chapter 2 of this thesis.

Immunofluorescence

The following antibodies were used for immunofluorescence staining of cells: a mouse monoclonal antibody against lamin A/C (sc-7292, Santa Cruz Biotechnology), a mouse monoclonal antibody against lamin B2 (NLC-LAM-B2, Novocastra) and a mouse monoclonal antibody against emerin (NCL-emerin, Novocastra). Cells were grown on coverslips, washed three times in PBS and then fixed in 2% formaldehyde in PBS for 10 minutes at room temperature. After fixation, cells were washed three times in PBS, permeabilized in PBS containing 0.2% Triton X-100 for 15 min and washed once in TBS containing 0.1% Tween 20. Then, cells were incubated with primary antibody for 45 minutes at 37 ºC, followed by three washes in TBS containing 0.1% Tween 20. Finally, cells were incubated with an appropriate Alexa-Fluor 488, Alexa-Fluor 594 (both Invitrogen) or Cy3 secondary antibody conjugate for 45 minutes at 37 ºC, washed in TBS containing 0.1% Tween 20, and mounted in Citifluor (Agar Scientific) containing 400 μg/ml DAPI (Sigma-Aldrich).

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Figures

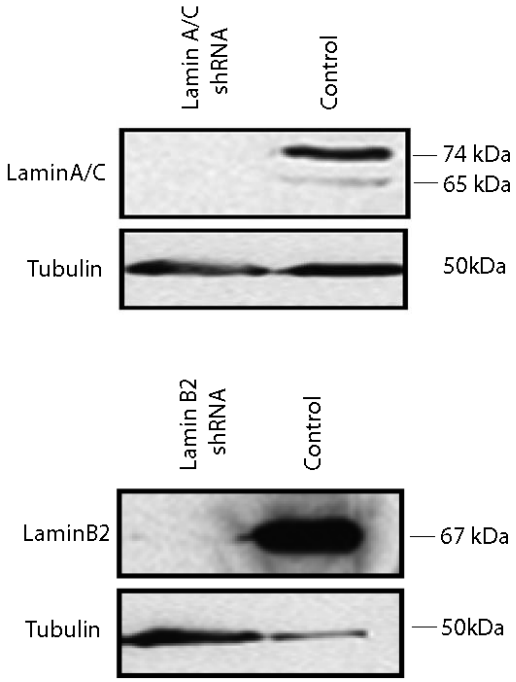

Figure 1A U2OS cells were transiently transfected with short hairpin (sh) RNA constructs from a viral based vector system directed against lamin A and lamin B. As a control, cells were transfected with an empty vector. Cells were lysed and subjected to Western blot analysis, 72 hours post transduction. An almost complete reduction in expression of both lamin A and lamin B proteins was achieved.

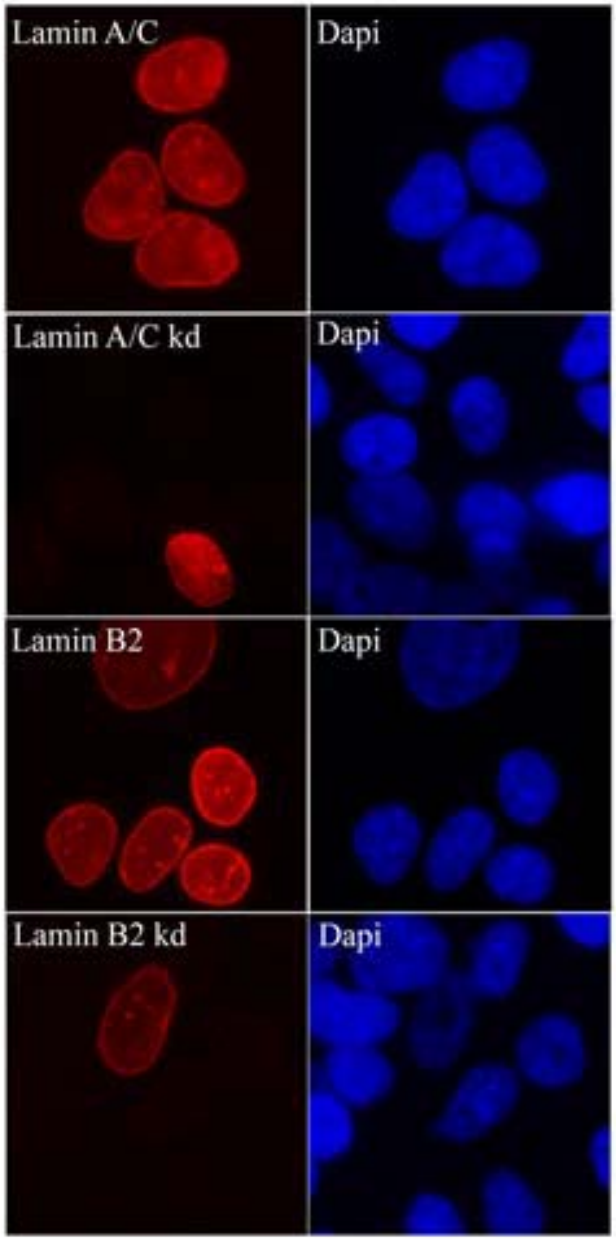

Figure 1B. Immunocytochemical images of U2OS cells transiently transfected with short hairpin (sh) RNA constructs from a viral based vector system directed against lamin A and lamin B. Cells were stained with anti-lamin A and anti-lamin B antibodies and showed an absence or strong reduction of lamin A/C and lamin B2 staining. Nuclei are stained with DAPI (blue).

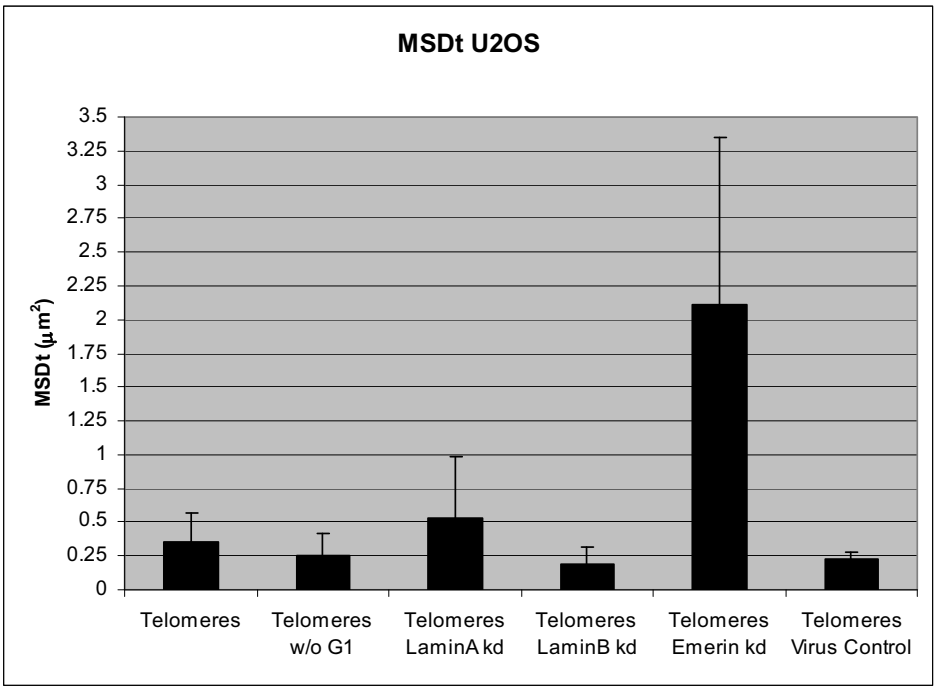

Figure 3. Reduction of lamin A/C expression by shRNA interference resulted in an increase in telomere movement as compared to cells transduced with the SHC002 control virus. Cells transduced with a lentiviral contruct to knockdown emerin expression revealed a strong increase in telomere mobility (MSDt value of ~2.1 μm^2, stdev ~1.5 μm^2) as compared to cells transduced with the control construct (~0.35 μm^2). 4D image stacks, each containing 20 sections of 0.5 μm, were collected using an automated motorized z-galvo stage multidimensional workstation for live cell imaging (model DMI3000B; Leica Microsystems). MSDt values were calculated using the STACKS tracking program.

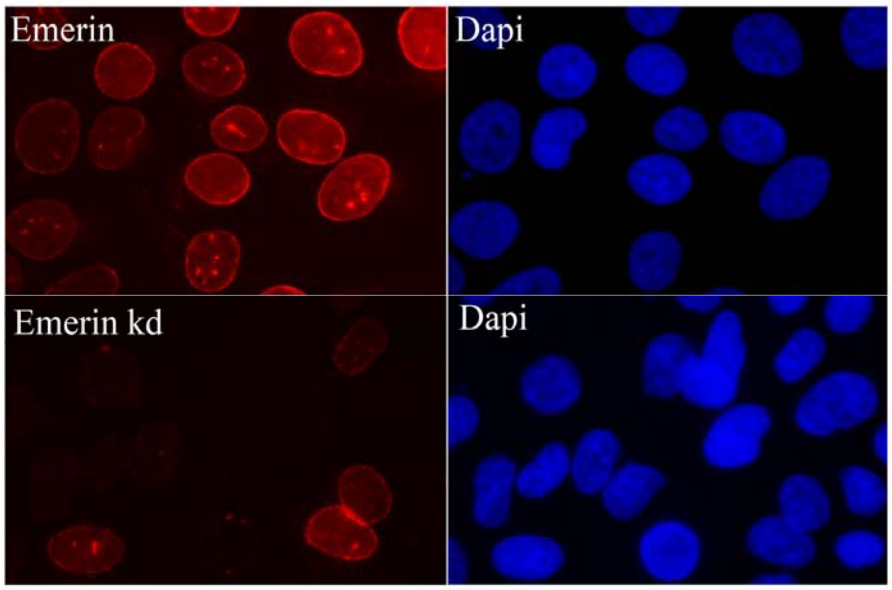

Figure 4. A reduction of emerin by shRNA interference in U2OS cells is shown in the lower panel, using an antibody directed against endogenous emerin. Untreated U2OS cells stained for emerin are shown in the upper panel. All cells are counterstained with DAPI (blue).

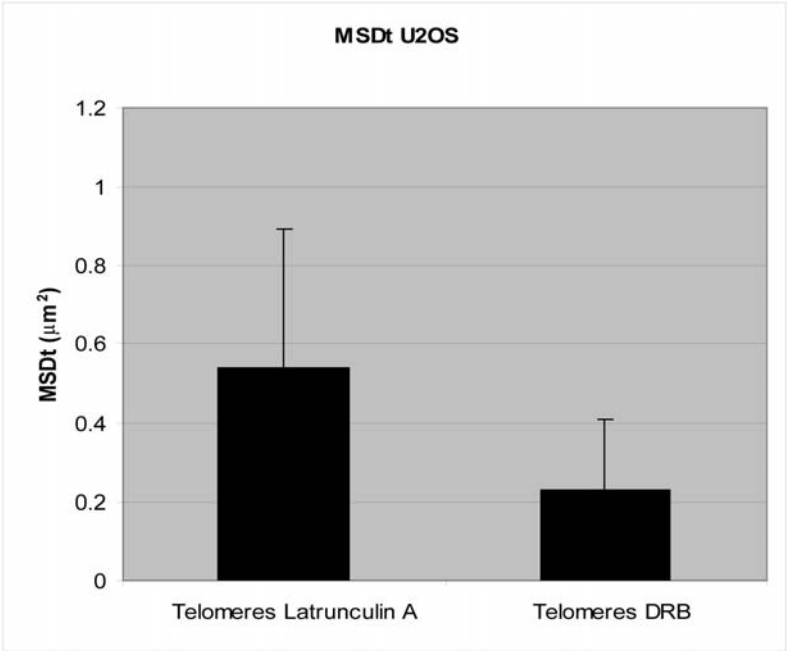

Figure 5. Measuring telomere mobility in U2OS cells treated with an actin depolymerizing agent latrunculin A revealed a mean MSDt value of ~0.54 μm^2 as compared to a MSDt ~0.35 μm^2 that was calculated for telomeres in untreated control cells. Additionally U2OS cells were treated with the transcription inhibitor 5,6-dichloro-1-ß-D-ribobenzimidazole (DRB) which resulted in a slight decrease in telomere mobility (MSDt ~0.23 μm^2). MSDt values were calculated using the STACKS tracking program.

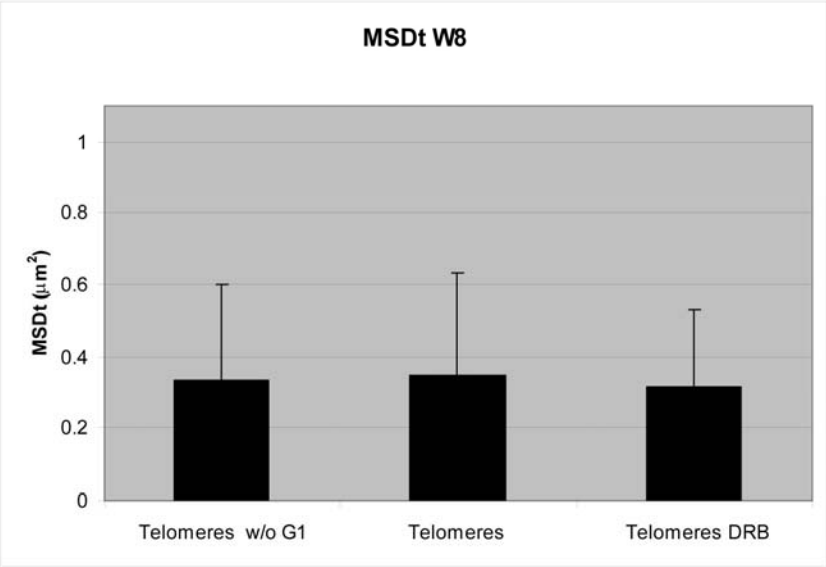

Figure 6. Measuring telomere mobility in mouse embryonic fibroblast cells (W8 MEFs) treated with the transcription inhibitor 5,6-dichloro-1-ß-D-ribobenzimidazole (DRB) resulted in a slight decrease in telomere mobility, similar to the result in U2OS cells. MSDt values were calculated using the STACKS tracking program.