Calcium Causes a Conformational Change in Lamin A Tail Domain that Promotes Farnesyl-Mediated Membrane Association

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.
Calcium Causes a Conformational Change in Lamin A Tail Domain that Promotes Farnesyl-Mediated Membrane Association

Agnieszka Kalinowski,†‡ Zhao Qin,†‡ Kelli Coffey,†§ Ravi Kodali,† Markus J. Buehler,* Mathias Lösche,†||*** and Kris Noel Dahl†‡§

†Biomedical Engineering, Carnegie Mellon University, Pittsburgh, Pennsylvania; ‡Civil and Environmental Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts; §Chemical Engineering, Carnegie Mellon University, Pittsburgh, Pennsylvania; ||Department of Structural Biology, University of Pittsburgh, Pittsburgh, Pennsylvania; ***Center for Neutron Research, NIST, Gaithersburg, Maryland

ABSTRACT Lamin proteins contribute to nuclear structure and function, primarily at the inner nuclear membrane. The post-translational processing pathway of lamin A includes farnesylation of the C-terminus, likely to increase membrane association, and subsequent proteolytic cleavage of the C-terminus. Hutchinson Gilford progeria syndrome is a premature aging disorder wherein a mutant version of lamin A, ∆50 lamin A, retains its farnesylation. We report here that membrane association of farnesylated ∆50 lamin A tail domains requires calcium. Experimental evidence and molecular dynamics simulations collectively suggest that the farnesyl group is sequestered within a hydrophobic region in the tail domain in the absence of calcium. Calcium binds to the tail domain with an affinity $K_D \approx 250 \mu M$ where it alters the structure of the Ig-fold and increases the solvent accessibility of the C-terminus. In 2 mM CaCl$_2$, the affinity of the farnesylated protein to a synthetic membrane is $K_D = 2 \mu M$, as measured with surface plasmon resonance, but showed a combination of aggregation and binding. Membrane binding in the absence of calcium could not be detected. We suggest that a conformational change induced in ∆50 lamin A with divergent cations plays a regulatory role in the posttranslational processing of lamin A, which may be important in disease pathogenesis.

INTRODUCTION

A-type lamins, including lamin A (LA), contribute to a variety of functions that include DNA transcription, heterochromatin regulation, cell senescence, DNA damage repair, and the sequestration of transcription factors (recent reviews (1,2)). These type V intermediate filament proteins are also the primary structural elements of the nucleoskeleton (3). Lamins are structurally related to other intermediate filament proteins (4), but the C-terminal tail domain (TD) of LA is much larger than that of other intermediate filaments and undergoes significant posttranslational processing. LMNA codes for the precursor prelamin A (preLA), which is transported into the nucleus where the C-terminal CaaX domain (specifically, Cys-Ser-Ile-Met) is farnesylated and carboxymethylated (5). The modified preLA is then cleaved by the enteroprotease ZMPSTE-1 at Y646 to produce mature LA. Dysfunctions of this process including loss of ZMPSTE-1 or mutations including the Y646 cleavage site are responsible for devastating human diseases. In the premature aging syndrome Hutchinson Gilford progeria syndrome (HGPS), a C1824T mutation of LMNA causes the activation of a cryptic splice site and a loss of exon 11 in the TD (6). The loss of the 50 amino acid segment, which contains the ZMPSTE-1 cleavage region, causes retention of the farnesylation, which in turn results in changes in thermodynamic stability of the mutant protein, ∆50LA or progerin (7). At a much lower frequency than in HGPS, activation of the cryptic splice site that produces ∆50LA occurs sporadically in normal cells, and this defect accumulates with aging (8).

The biochemistry of LA TD processing has been determined, but the biological benefits of the complex processing pathway and the regulation of its individual steps are unclear (9). Although farnesyl transferases have been found in both the endoplasmic reticulum and the nucleus, farnesylation of the protein likely occurs in the nucleus (5). The farnesyl modification is suggested to increase the association of preLA with the inner nuclear membrane (10,11). Similarly, the pathologies associated with the expression of ∆50LA in HGPS result, in part, from the farnesylation causing overaccumulation of structural proteins at the inner nuclear membrane (12,13). Indeed, mislocalization of LA and LA-associated proteins at the inner nuclear membrane results in altered DNA repair, transcription, and signaling (14), as well as changes in protein-protein interactions (15). On the other hand, a single farnesylation is insufficient to confer permanent membrane association of proteins in other systems (16–18). Therefore, it is unclear if the presence of the farnesyl is sufficient for the cellular pathological phenotype or if additional factors, including altered protein-protein interactions (15) or altered protein packing (7,19) are involved.

For the full-length lamin protein, the rod domain functions in dimer formation and the head and tail domains associate with the rod to form filaments; full-length filaments form aggregates and paracrystals in vitro (1–4). The TD is
responsible for association with other proteins and membrane localization. For these reasons, we limited our study to the TD to isolate the interaction between the protein and the membrane (2,10). Here, we quantify protein-membrane binding of farnesylated Δ50LA (fn-Δ50LA) by investigating purified LA TDs in solution and on model lipid bilayer membranes. We find that the binding is weak ($K_D \sim 2$ μM) and that free Ca$^{2+}$ is required for binding. The conformational response of the protein to Ca$^{2+}$ is tracked by monitoring its intrinsic fluorescence. Molecular simulations suggest that the Ca$^{2+}$-induced conformational change opens the Ig-fold, increases the flexibility of the terminal CaaX motif, and exposes the farnesyl group, which is sequestered within the TD in the absence of the ion.

**MATERIALS AND METHODS**

**Protein production, farnesylation, and characterization**

 Constructs for the expression of LA TDs were created using polymerase chain reaction from plasmids kindly provided by Mistelli at NCI/NIH (12). Plasmids were expressed in *Escherichia coli*, purified using glutathione-s-transferase fusion peptides and farnesylated in vitro using farnesyltransferase, resulting in a population of ~50% farnesylated protein (see Methods in the Supporting Material). Purified proteins were verified for size analysis using liquid chromatography electron spray ionization mass spectrometry, for purity using 14% SDS-PAGE, for aggregation by dynamic light scattering (DLS) (nanosizer). Bradford assay (Comassie Plus, Pierce) was used to determine protein concentration (see Methods).

**Solution conditions that minimize aggregation of Δ50LA and preLA TDs**

 Recombinant LA-TD variants aggregate in water, therefore accurate quantification of protein-membrane interactions required stabilization of monomer protein. We determined the minimal salt (NaCl) concentrations in the buffer necessary to suppress aggregation of Δ50LA-TD and preLA-TD by DLS. Although deconvoluted DLS spectra were not able to determine aggregation form, it clearly showed large-scale aggregation under low-salt conditions (Fig. S1 in the Supporting Material). Characteristic DLS peaks of $d \sim 10$ nm were regularly observed under high-salt conditions ([NaCl] > 50 mM), and we interpreted a 10 nm peak as a protein monomer. At low salt ([NaCl] < 50 mM), proteins aggregated ($d > 100$ nm). This analysis showed different minimal salt concentrations for the different lamin A variant TDs studied here, including with farnesylation(fn). Δ50LA-TD required 25 mM NaCl to maintain a monomeric solution of protein, whereas fn-Δ50LA-TD required 50 mM NaCl (Fig. S1 A). In contrast, preLA-TDs required a minimum of 500 mM NaCl to maintain dispersed solutions (Fig. S1 B). The addition of 2 mM CaCl$_2$ did not alter the stability of Δ50LA-TD and fn-Δ50LA-TD (stable in 50 mM NaCl and 2 mM CaCl$_2$, Fig. S1 C) or preLA-TD and fn-preLA-TD (stable in 500 mM NaCl and 2 mM CaCl$_2$, Fig. S1 D). Aggregation could also be suppressed in 10 mM sodium phosphate buffer but could not be used in these experiments because phosphate chelates calcium. Other salts, such as KCl or NaHCO$_3$, were not tried as membrane behavior under those conditions.

**Tryptophan fluorescence**

 The tryptophan amino acid residue has inherent fluorescent properties that have long been used as probes to reflect protein conformation (20). LA-TD has four tryptophan residues all of which are located on or near the Ig-fold. The peak emission intensity is at 342 nm, after excitation at 298 nm to minimize excitation of other aromatic residues. A peak emission at 342 nm indicates that the tryptophan residues are neither in a completely hydrophobic environment, nor are they fully solvent exposed (20,21).

The peak emission intensity at 342 nm (with the background intensity from the buffer subtracted) was used for analysis and normalized by the peak intensity without Ca$^{2+}$ for each sample. The binding affinity and stoichiometry of Ca$^{2+}$ association with the LA TD was determined by fitting the change in fluorescence intensity at 342 nm as a function of Ca$^{2+}$ concentration to a Hill model. For our system where the ligand is the Ca$^{2+}$ and the conformational change is assumed to be proportional to the change in fluorescence intensity at the emission peak, $\Delta I$ (342 nm), normalized by the peak emission at 342 nm without Ca$^{2+}$ in the solution, $I_c$, the Hill equation is $\Delta I/I_0 = [Ca^{2+}]^n/(K_D + [Ca^{2+}])$ gives a binding affinity of the protein to calcium $K_D$, as well as the stoichiometry of binding, n (i.e., the number of Ca$^{2+}$ that associate with one protein molecule).

**Membrane bilayer preparation and surface plasmon resonance experiments (SPR)**

 SPR was used to quantify the membrane association of the LA-TD to tethered bilayer lipid membranes (tBLMs), which provide a fluid membrane fixed to a surface for SPR to monitor membrane association without the necessity of using a protein label. The tBLM model membrane system consists of a planar lipid bilayer suspended to the surface by a minimal number of functionalized lipopolymer interspersed with β-mercaptoethanol to create a submembrane hydration layer (see Methods). The ~1.5 nm hydration layer allows the membrane to retain its fluidity and reduce the effects of the substrate on the membrane. See Methods for full details. The molecular structure, functionality, and in-plane dynamics of such tBLMs have been established by the Lösch group in extensive studies involving electrochemical impedance spectroscopy, neutron scattering, fluorescence microscopy, and fluorescence correlation spectroscopy (22–24).

**Replica exchange molecular dynamics (REMD) simulations**

 Protein structure predictions were carried out by the REMD method (25) implemented in the CHARMM c35b1 (26) software. Replica management and conformation analysis are done using the MMTSB script package (27). REMD is a simulation method with atomic detail to improve the dynamic properties of the Monte Carlo method, aiming at a global-minimum free energy state. It samples a wide conformation space without getting trapped in local-minimum free energy states because of the thermal stimulation by high-temperature replicas, and we can find stable structures at the replica with the lowest temperature. The REMD inputs include the peptide sequence and an initial guess of conformation (see Methods).

**Modeling of farnesyl diphosphate**

 The structure and interaction scheme of the farnesyl diphosphate is not defined in the standard CHARMM27 force field. We setup the initial conformation of farnesyl diphosphate according to its atomic coordinates as found in the protein data bank (with PDB ID 1KZO). The interactions (covalent bond, angle, dihedral, electrostatic, and vDW interactions) among the atoms in farnesyl diphosphate are defined in the CHARMM General force field 26b (28). We updated the CHARMM27 force field to incorporate the section of the definition of farnesyl diphosphate (see Methods for details of the modeling and validation against the atomic structures identified from experiment).
RESULTS

Membrane binding of fn-Δ50LA-TD depends on calcium

To determine the membrane affinity of fn-Δ50LA-TD, we added purified monomeric farnesylated protein (see Methods and Supporting Material information for production, characterization, and monomeric stability) to tBLMs and measured binding using SPR. The tBLMs are appropriate for precise SPR binding measurements because bilayers are virtually defect free, thus avoiding nonspecific protein adsorption (29) and tightly coupled to gold-coated substrates via hydrated oligo(ethylene oxide) spacers (30). Bilayer membranes were composed of dioleoyl-phosphatidylcholine and the charged dioleoyl-phosphatidylserine (DOPC/DOPS = 7:3) with 3 mol% cholesterol to mimic lipid composition and charge of cellular membranes.

With or without farnesylation, Δ50LA-TD associated minimally with the membrane in NaCl buffer (Fig. 1 A). However, the presence of physiological levels of free Ca$^{2+}$ in the buffer facilitated protein-membrane interaction. fn-Δ50LA-TD bound stably to tBLMs with 2 mM CaCl$_2$ in the buffer, but Δ50LA-TD binding was not detected under otherwise identical conditions (Fig. 1 A and B). Increasing concentrations of fn-Δ50LA-TD in the Ca$^{2+}$-containing buffer increased membrane binding (Fig. 1 C) and showed a binding affinity, $K_D$ ≈ 2 μM (assuming that only the farnesylated proteins associated with the membrane, i.e., approximately half the concentration of the protein added) when fit to a Langmuir model (Fig. 1 C). We note that the membrane association is a combination of binding and aggregation, which impacts the $K_D$; instantaneous high levels of fn-Δ50LA-TD do not show the same levels of binding as increased amounts of fn-Δ50LA-TD (Fig. 1 B vs. Fig. 1 C). Furthermore, protein is not easily dissociated from the interface. These and other aspects of interfacial aggregation and binding are the topic of future work. Unfortunately, the high salt required to maintain monomeric stability of fn-preLA-TD (see Methods) interfered with membrane stability, so that fn-preLA-TD binding to membranes could not be quantified.

Calcium-dependent conformational changes

To investigate the mechanism by which Ca$^{2+}$ induces membrane association, we considered Ca$^{2+}$-induced conformational changes of the TD. The TD is intrinsically disordered except for the s-type Ig-fold (31,32), therefore, direct structural examination via traditional methods (e.g., crystallography) are not possible. Conformational changes of Δ50LA-TD and preLA-TD were followed by monitoring the fluorescence of their four Trp residues located within and near the Ig-fold (W467, W498, W514, W520; see full sequence in Table S1). The chemical environment of Trp, including its solvent accessibility, affects the emission quantum yield, and exposure to the solvent has been shown to quench Trp fluorescence (20,21). Previously, we investigated thermal denaturation of LA-TD and Δ50LA-TD tertiary structure by monitoring Trp fluorescence, confirming that protein denaturation reduces Trp fluorescence (7).

The observed fluorescence emission band between 310 and 400 nm was broad, presumably because multiple Trp residues in different microenvironments contribute to the band shape. We observed that the emission decreased with CaCl$_2$ concentration (Fig. 2, A and B). Increasing $[\text{CaCl}_2]$ to the membrane. (tBLM composition: 30:70:70% w/w DOPC:DOPS:Chol)
Fluctuations of the Ig-fold of Δ50LA-TD and preLA-TD in the presence of calcium

To obtain a deeper insight into the structural responses of the LA TDs to Ca\(^{2+}\), we performed molecular dynamics (MD) simulations of the proteins in buffer. To deal with the high degree of intrinsic disorder, we simulated multiple low-energy, pseudoequilibrated structures with REMD (25,33). Starting with amino acid chains with extreme initial structures (straight or fully coiled) except for the structured Ig-fold region taken from the NMR structure in the protein database (31), we allowed the protein to relax freely in an implicit solvent environment. To enhance the sampling speed as well as to ensure that structures reached relevant local energy minima, we investigated numerous independent structures with implicit-solvent REMD to find as many low-energy states as possible (see Methods). Results were categorized according to their structural similarity, and the four most significant conformations were chosen for further studies of the structural impact of ions (7). These four conformations were further refined in REMD simulations with explicit solvent under various ion conditions (see Methods). To quantify the TD’s structural response to ion exposure, we focused on four peripheral amino acids (S429, S437, L478, and I497), which form orthogonal extremes of the two β-sheets that form the Ig-fold (Fig. 3 A).

From the positions of these four amino acids, we determined an orientation angle between the two β-sheets, θ (Fig. 3 A). The Ig-fold is slightly more open (θ is systematically larger) in the presence of Ca\(^{2+}\) (Fig. 3 B).

We computationally included a farnesyl group to the LA-TDs. For an initial conformation, we used the pseudoequilibrium structure of the unfarnesylated LA-TDs and embedded the added C-terminal farnesyl group within the hydrophobic core of the Ig-fold (see Methods for force field). We then allowed the new structure to come to a new equilibrium with explicit solvent. The Ig-fold of both Δ50LA-TD and preLA-TD were more open if farnesylated, and exposure to Ca\(^{2+}\) opened the fold further up by a larger angle than with the unfarnesylated proteins (Fig. 3 C).

FIGURE 2 Calcium caused a conformational change to Δ50LA-TD and preLA-TD. The emission spectra of tryptophan residues for purified (A) Δ50LA-TD in 50 mM NaCl and (B) preLA-TD in 500 mM NaCl excited at 295 nm were measured for increasing Ca\(^{2+}\) concentration. For Ca\(^{2+}\) concentration above 1 mM, there was no change in intensity, indicating no more substantial conformational change induced by Ca\(^{2+}\). (C) From plots of emission intensity at 342 nm versus Ca\(^{2+}\) concentration, we determined Ca\(^{2+}\) dissociation constant (\(K_D\) = 250–300 μM) and binding stoichiometry (1:1). The Δ\(I/I_0\) (342 nm) is averaged for 2–3 samples and in NaCl concentrations 50–700 mM NaCl for a given CaCl\(_2\) concentration. The Ca\(^{2+}\) dissociation constant did not change with the NaCl concentration of the solution.

FIGURE 3 Altered Ig-fold structures of preLA-TD and Δ50LA-TD exposed to calcium. (A) We determined the dihedral angle between the two β-sheets of the Ig-fold, θ, from the α-carbon of orthogonal amino acids (S429, S437, L478, and I497), which form orthogonal extremes of the two β-sheets that form the Ig-fold. (B) Statistical comparison of the dihedral angle of the Ig-fold for numerous pseudoequilibrated protein structures for preLA-TD, fn-preLA-TD, Δ50LA-TD, and fn-Δ50LA-TD showed increases in θ with Ca\(^{2+}\).
is >1.7 nm, an increase in the orientation angle \( \theta \) indicates that the space between the two \( \beta \)-sheets accommodates more water (with a van der Waals radius of 0.14 nm) in the presence of the ion than in its absence.

**Fluctuations of the C-terminus of \( \Delta 50 \)LA-TD and preLA-TD with calcium**

REMD simulations also provided insight into the mechanism by which calcium triggers membrane binding of farnesylated lamin A tail domains. We observed that Ca\(^{2+}\) bound spontaneously to the C-terminal CaaX motif, and the interaction persisted through the entire REMD simulation (cyan Ca\(^{2+}\) to red CSIM in Fig. 4 A, Fig. S3). Generally, Ca\(^{2+}\) binding increased the fluctuation and mobility of the C-terminal region for both \( \Delta 50 \)LA-TD (beyond 610\(^{\prime}\); the prime refers to the new amino acid number for the deletion) and preLA-TD (beyond 660) by 30% and 39%, respectively (Fig. 4 B).

C-terminal motility was reduced when the protein was farnesylated (Fig. 4 B), and the Ca\(^{2+}\)-mediated motility increase was enhanced for farnesylated proteins (Fig. 4 B) by 89% for fn-preLA-TD and 120% for fn-\( \Delta 50 \)LA-TD. In the absence of Ca\(^{2+}\), the farnesyl (Fig. 5, red) maintained association with the hydrophobic core (Fig. 5 A, –Ca\(^{2+}\)). In the presence of Ca\(^{2+}\), the farnesyl group escaped the hydrophobic interior of the protein and was found at the periphery of the pseudoequilibrated structures (Fig. 5 A, +Ca\(^{2+}\)). This was quantified by the increase in solvent available surface area of the farnesyl group in the presence of Ca\(^{2+}\) (Fig. 5 B) and increased end-to-end length of the TDs (Fig. 5 C). We suggest that the farnesyl group associates with the hydrophobic protein core, and the association of Ca\(^{2+}\) to the CaaX causes increased probability of farnesyl solvent exposure for membrane association.

**DISCUSSION**

**Membrane binding of farnesylated lamin A tail requires calcium**

We find that fn-\( \Delta 50 \)LA-TD binds to purified membrane bilayers with a \( K_D \sim 2 \) \( \mu \)M. This binding coefficient is markedly weaker than most biological binding coefficients; specific protein-protein binding typically occurs on the order of 10s to 100s of nM. We suggest that this weak binding affinity allows protein-membrane association while not causing irreversible association. In the case of lamin A, we suggest that the farnesyl group weakly associates the protein to the membrane, and transmembrane lamin-binding proteins then more permanently connect lamin A to the inner nuclear membrane. This weak binding is similar to qualitative observations that farnesyl groups are insufficient to stably associate proteins with membranes (16–18). During normal processing, lamin TDs, and other CaaX-domain proteins, are farnesylated, the aaX residues are cleaved by the ZMPSTE-1 protease, and the terminus is methylated in the nucleus (34). The proteolysis and methylation steps may play an additional role in stabilizing membrane association by increasing hydrophobicity. However, there have been conflicting reports regarding the additional stability conferred by methylation (35–37).

The association of the fn-\( \Delta 50 \)LA-TD is dependent on Ca\(^{2+}\). There have been previous studies examining qualitative membrane association of fn-preLA (38), but we note that the buffer used contained the divalent cation magnesium. Similarly, recoverin has a Ca\(^{2+}\)-induced...
conformational switch to expose the myristoyl group, which is responsible for membrane association (39). Unlike recov- erin, lamin A tail domains are mostly disordered, therefore the conformational switch is much less obvious. We demon- strated through experiments and simulations that D50LA- TD and pre-LA TD undergo a conformational change induced by Ca2+ that opens the Ig-fold and increases the exposure and mobility of the farnesylated C-terminus. The Ca2+-induced conformational change may also regu- late protein-membrane interactions, protein-DNA, or pro- tein-protein interactions. The large size, intrinsic disorder, and Ig-fold binding pocket of the tail domain may all allow for promiscuous binding and may be at least partially responsible for the ability of lamin A tail to bind different partners (1). Here, we have shown Ca2+-induced conformational changes, and protein binding to the lamin A of one of the many lamin A-associated proteins may also induce conformational changes in the tail domain. We find that, similar to Ca2+, Mg2+ can facilitate the membrane association of fn-D50LA-TD, and is the subject of future work.

Membrane association of farnesylated lamin A tail domain includes binding and aggregation

Simulations of the Δ50LA-TD structure show the protein diameter to be 5.2 ± 0.2 nm, and a complete monolayer of protein coverage is reached at ~1 μM of solution protein concentration. However, above 1 μM we still observe additional, stable membrane association. This additional association is likely due to a combination of aggregation coupled with binding. Because high salt levels are required to prevent aggregation of fn-preLA-TD, and proteins show no membrane association as monomers, we suggest that the protein-protein association of fn-preLA-TD overwhelms the protein-membrane association with excess protein (40). This highlights that fn-Δ50LA-TD has an apparent greater affinity for the membrane and enhanced membrane association of Δ50LA may contribute to the pathological membrane association (12,13).

Lifetime of wild-type lamin A and pathological Δ50LA

Most contemporary literature suggests that prelamin A far- nesylation is responsible for the association of protein to the inner nuclear membrane for further incorporation into the lamina (10,11). It is unclear why lamins do not embed them- selves into membrane structures outside the nucleus because farnesyltransferases have been located in the cytoplasm near the endoplasmic reticulum and inside the nucleus.

From the results presented here, we suggest changes in the tail domain of preLA from the time it is produced from LMNA mRNA until it is processed to form mature lamin A. We speculate that after transcription, the C-termi- nal CaaX domain is hidden inside the TD of preLA. The preLA (probably as a dimer) is transported into the nucleus via the TD nuclear localization sequence (NLS). In the nucleus, the higher levels of charge from DNA and counter ions induce a conformational change that exposes the CaaX for farnesylation and other modifications leading to
insertion into the inner nuclear membrane. There are high levels of divalent cations including calcium, magnesium, and zinc inside the nucleus (41), but exact levels of free divalent cations in the nucleus are difficult to determine because free ions are buffered by surrounding chromatin structure. It is also possible that small amounts of preLA may be farnesylated by cytoplasmic farnesyltransferases. We suggest that these farnesyl groups may incorporate into the Ig-fold until they are exposed to high Ca2+ levels. Ig-folds and similar β-sheet structures have been shown to act as solubilization agents for lipidated proteins (42–44). In the case of fn-preLA, the farnesylation could associate with its own Ig-fold or that of a neighbor.

**SUPPORTING MATERIAL**

Five figures and supplemental methods are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(13)00445-1.

We gratefully acknowledge the assistance by Matthew Biegler (CMU MSE) and Peter Yaron (CMU ChemE) and we are grateful to Katherine Wilson (Johns Hopkins Department of Cell Biology) for careful reading of the manuscript.

Z.Q. and M.J.B. received support from National Science Foundation (NSF) (CMMI-0642545) and Office of Naval Research- Presidential Early Career Award for Scientists and Engineers (ONR-PECASE) (N00014-10-1-0562). K.N.D. and A.K. received support from the Progeria Research Foundation, NSF (CBET-0954421 CAREER) and National Institutes of Health (NIH) National Institute on Aging (NIA) (NRSA F30-AG030905 to A.K.). M.L received support from NIH (1R01-GM101647).

**REFERENCES**


34. Kalinowski et al.


