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ATR-Dependent Phosphorylation of Emerin as a Mechanosensory Switch Governing Nuclear Envelope-Chromatin Coupling

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Abstract

As cancer cells metastasize, they navigate a highly restrictive tumor microenvironment that subjects them to severe mechanical stress. These physical forces actively deform the cell nucleus and represent a major threat to genomic integrity. Cells use specialized mechanosensors to promote dynamic structural adaptations to survive such hostile conditions. The Ataxia Telangiectasia and Rad3-related (ATR) kinase is best known for its canonical role in the DNA damage response, but recent studies indicate that it also rapidly localizes to the nuclear envelope during mechanical forces, independent of the DNA damage response, to protect nuclear integrity under mechanical load. However, the mechanisms underlying this protective response remain largely unknown. The present study addresses this issue by identifying a novel mechanobiology pathway through which ATR regulates nuclear envelope mechanics. By employing a quantitative phosphoproteomic screen of cells under mechanical compression, we identified the inner nuclear membrane protein Emerin as a key downstream effector of a compression-induced ATR-dependent signaling cascade. We demonstrated that mechanical stress induces ATR-dependent phosphorylation of Emerin at two key residues, Serine 49 and Serine 171. Using co-immunoprecipitation, we demonstrated that the physical interaction between Emerin and BAF is significantly reduced after mechanical compression, but this interaction is maintained upon ATR inhibition. To investigate the functional consequences of these modifications, we used a Tet-ON inducible system for shRNA expression to knock down endogenous Emerin and introduced phospho-mutant constructs. Mutations at these two specific sites were observed to induce structural changes, confirming that phosphorylation destabilizes Emerin's structural role. Ultimately, this biochemical modification acts as a mechanosensory switch to release BAF-tethered heterochromatin from the nuclear lamina. This transient uncoupling relieves the internal structural tension, providing the nucleus with the flexibility to safely absorb extreme physical deformation without catastrophic membrane rupture. This ATR-Emerin-BAF axis is a fundamental mechanism of nuclear mechanobiology and offers a key insight into how cells actively remodel their chromatin architecture to withstand the complex biomechanical challenges of the tumor microenvironment



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Fosforilazione dell’emerina
ATR-dipendente come interruttore
meccanosensoriale che regola
l’accoppiamento involucro
nucleare-cromatina

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Abstract (Italiano)

Durante il processo di metastatizzazione, le cellule tumorali attraversano un microambiente tumorale altamente restrittivo che le sottopone a un severo stress meccanico. Queste forze fisiche deformano attivamente il nucleo cellulare e rappresentano una grave minaccia per l'integrità genomica. Per sopravvivere a tali condizioni ostili, le cellule utilizzano meccanosensori specializzati al fine di promuovere adattamenti strutturali dinamici. La chinasi ATR (Ataxia Telangiectasia and Rad3-related) è nota principalmente per il suo ruolo canonico nella risposta al danno del DNA, ma studi recenti indicano che, in presenza di forze meccaniche, essa si localizza rapidamente anche a livello dell'involucro nucleare, indipendentemente dalla risposta al danno del DNA, per proteggere l'integrità nucleare sotto carico meccanico. Tuttavia, i meccanismi alla base di questa risposta protettiva rimangono in gran parte sconosciuti. Il presente studio affronta questa problematica identificando una nuova via di meccanobiologia attraverso la quale l'ATR regola la meccanica dell'involucro nucleare. Impiegando uno screening fosfoproteomico quantitativo su cellule sottoposte a compressione meccanica, abbiamo identificato la proteina della membrana nucleare interna Emerina come un effettore a valle chiave di una cascata dipendente dall'ATR e indotta dalla compressione. Abbiamo dimostrato che lo stress meccanico induce la fosforilazione dipendente dall'ATR dell'Emerina in due residui chiave: la Serina 49 e la Serina 171. Tramite co-immunoprecipitazione, abbiamo dimostrato che l'interazione fisica tra l'Emerina e il BAF si riduce significativamente in seguito a compressione meccanica, ma tale interazione viene mantenuta inibendo l'ATR. Per indagare le conseguenze funzionali di queste modificazioni, abbiamo utilizzato un sistema inducibile Tet-ON per l'espressione di shRNA al fine di silenziare l'Emerina endogena e abbiamo introdotto costrutti fosfo-mutanti. È stato osservato che le mutazioni in questi due siti specifici inducono alterazioni strutturali, confermando che la fosforilazione destabilizza il ruolo strutturale dell'Emerina. In definitiva, questa modificazione biochimica agisce come un interruttore meccanosensoriale per rilasciare l'eterocromatina ancorata al BAF dalla lamina nucleare. Questo disaccoppiamento transitorio allevia la tensione strutturale interna, fornendo al nucleo la flessibilità necessaria per assorbire in modo sicuro deformazioni fisiche estreme senza incorrere in una rottura catastrofica della membrana. Questo asse ATR-Emerina-BAF rappresenta un meccanismo fondamentale della meccanobiologia nucleare e offre una prospettiva chiave su come le cellule rimodellino attivamente l'architettura della loro cromatina per resistere alle complesse sfide biomeccaniche del microambiente tumorale.

Contents

Abstract	1
Abstract (Italiano)	3
Introduction	6
1 Mechanical forces in the tumor microenvironment	6
2 Main players in responding to mechanical stress	9
3 Architecture of the nucleus and chromatin at rest	11
4 Nuclear mechanotransduction: how the resting architecture responds to force .	13
5 Emerin and BAF: Anchoring Heterochromatin at the Nuclear Envelope	16
6 ATR and canonical role of ATR	18
7 Emerging roles for ATR in Mechano-transduction	21
Aim of study	26
Materials and Methods	27
Cloning shEMD-PLKO Plasmid	27
DNA Extraction (Miniprep and Maxiprep)	31
Site-Directed Mutagenesis	32
Cell culture and maintenance	32
Transfection via Lipofectamine	33
Mechanical Cell Compression	34
Protein Extraction and Immunoblotting	35
Co-Immunoprecipitation (Co-IP)	36
Phosphoproteomics	37
Sammy-seq	38
Immunofluorescence Staining	41
Results	42
1 Compression induced ATR-Dependent Phosphorylation Target Identification . .	42

2	Nuclear specific proteins that undergo ATR dependent phosphorylation following mechanical stress	45
3	Emerin phosphorylation as a regulator of chromatin tethering and compaction .	47
4	SAMMY-seq reveals ATR-dependent regulation of Lamin Associated Domain compartmentalization following compression	50
5	Construct of Mutant Emerin plasmids	52
6	Design and sequence verification of shEMD constructs	53
7	Validation of an inducible Emerin-knockdown cell line	54
8	Emerin wildtype rescue	57
9	Subcellular localization and nuclear invagination of phospho-mimetic Emerin mutants	58
10	Proposed Mechanobiology Pathway of Nuclear Adaptation	61
	Discussion	63
	Conclusion	69
	Acknowledgment	70
	References	70

Introduction

1. Mechanical forces in the tumor microenvironment

The traditional model of cancer progression is a sequence of genetic and epigenetic modifications, but there are distinct variations in the mechanical phenotype of tumor cells and their microenvironment. These factors are significantly involved in the cancer process. The balance of mechanical stresses is crucial to determine basic tumorigenic features such as cell shape, motility, proliferation, and differentiation [1]. The tumor microenvironment is a dynamic, constantly evolving ecosystem in which cancer develops. It is not just made up of cancer cells, but is a complex landscape that includes the cancer cells, the structural framework around them called the extracellular matrix (ECM), and a diverse host of supporting ‘stromal’ cells, such as fibroblasts, immune cells and blood and lymphatic vascular cells [2]. The microenvironment is important because it develops a “dynamic reciprocity” with the cancer cells. This means that the physical features of the environment - such as the stiffness, geometry and architecture of the ECM - significantly impact how cells behave, proliferate and differentiate, while the cells try to modify their surroundings [3, 4]. The physical setting of the TME varies widely across cancer progression, from the early transformation of a normal cell to invasion and metastasis. For example, the only placement of healthy epithelial cells within a very stiff, tumor-like extracellular matrix is sufficient to disturb their normal tissue architecture and induce invasive activity. Targeting the physical and stromal aspects of the TME represents an extremely promising area for innovative cancer therapeutics because the microenvironment is essentially driving the illness forward [1, 2].

Four major mechanical forces shape the tumor microenvironment (Figure 1):

Solid stress: Solid stress, or residual stress, is the compressive, tensile, and shear forces contained and transmitted by the solid and elastic components of the tumor microenvironment, including cells and the extracellular matrix (ECM) [5]. This mechanical load is the real physical forces of tissue elements pushing and pulling actively with each other. It is generated through four primary mechanisms: localized increases in tissue volume from constant cancer cell proliferation and aberrant matrix deposition, the physical displacement of surrounding normal host tissue by the expanding tumor mass, the osmotic swelling of highly absorbent ECM components like hyaluronic acid, and the continuous generation of tensile forces via actomyosin-mediated cellular contractions [5, 6]. The progressive accumulation of solid stress has profound pathophys-

iological consequences central to driving tumor progression and treatment failure. Mechanical forces can compress and collapse fragile intratumoral blood and lymphatic vessels, creating a hypoxic microenvironment that hinders drug delivery, limits immune cell infiltration, and compromises oxygen-dependent therapies like radiation [5, 7]. At the cellular level, the physical sensing of these compressive loads by cancer and stromal cells is via surface mechanosensors, leading to the nuclear translocation of transcriptional regulators such as YAP and TAZ [5, 8]. This mechanotransduction cascade profoundly modulates gene expression to override apoptotic signals and to actively drive unchecked proliferation, desmoplasia, and a highly invasive metastatic phenotype [5, 6, 9].

Fluid stresses: Fluid stress in the tumor microenvironment results from a severe breakdown of normal tissue fluid homeostasis, which is simultaneously driven by abnormal angiogenesis and impaired lymphatic drainage [5, 6]. Rapidly growing solid tumors recruit structurally unstable, hyper-permeable blood vessels that continuously leak blood plasma into the surrounding interstitial space [2, 6]. This abnormal vascular leakage, together with the collapse and compression of the internal lymphatic vessels that clear the fluid, stores fluid in the tumor, and significantly increases interstitial fluid pressure (IFP) [5–7]. Therefore, the IFP in the tumor interior becomes similar to microvascular blood pressure and effectively eliminates the typical transvascular pressure gradients required for fluid exchange [6]. This lack of a pressure difference stops the convective transport of fluids into the tumor, making the elevated IFP a strong physical barrier that makes it very hard for blood-borne chemotherapy agents to reach and enter the tumor [5, 6, 10]. The IFP is uniformly high in the core of the tumor and drops off sharply at the tumor border, resulting in a high pressure gradient that drives the trapped interstitial fluid to flow outward into the neighboring healthy tissue [5, 6, 10]. The continuous outward current causes mechanical fluid shear stress on cancer and stromal cells, altering the cellular microenvironment by activating normal fibroblasts, modulating angiogenesis, and upregulating pro-tumorigenic cell motility [5, 6, 11]. In addition, this outward fluid movement provides a mechanical channel that physically carries growth factors and detached cancer cells into peritumoral lymphatics and surrounding tissues, thereby promoting tumor cell invasion and metastatic spread [5, 6, 12].

Tissue Stiffness: Increased tissue stiffness in the tumor microenvironment is a profound mechanical abnormality primarily driven by the excessive deposition, reorganization, and enzymatic cross-linking of extracellular matrix (ECM) components, particularly collagen, by highly active cancer-associated fibroblasts [2, 5, 13]. This fibrotic, desmoplastic response is

continuously exacerbated by a process known as strain-stiffening, wherein local tumor expansion and cellular contractions pull the surrounding collagen networks tight, creating a highly rigid matrix [5, 14]. Tumor and stromal cells physically perceive this rigid environment through surface mechanosensors, most notably integrin receptors, which bind to the stiffened ECM and aggregate to form robust anchoring structures called focal adhesions [2, 8, 15]. The physical resistance encountered by these integrin clusters leads to increased actomyosin contractility in the cell, which in effect converts the extracellular mechanical rigidity into strong intracellular tension [1, 2, 8]. This increase in intracellular tension initiates a powerful mechanotransduction cascade that fundamentally changes gene expression and actively drives malignant progression [2, 8, 16]. Additionally, this stiff collagenous barrier serves as a mechanical signal for tumor cell invasion and a strong immunosuppressive microenvironment; the stiffened ECM physically inhibits the infiltration and activation of cytotoxic T lymphocytes and natural killer cells, and at the same time, drives tumor-associated macrophages toward an immunosuppressive M2 phenotype, shielding the tumor from immune destruction [8, 17].

Tissue Microarchitecture: In healthy tissues, the spatial arrangement of cells and the extracellular matrix (ECM) creates a highly specific microarchitecture optimized for organ stability and physiological function [4, 5]. The physical environment plays a crucial role in maintaining cellular homeostasis and regulating tissue morphogenesis [1, 5]. However, this highly ordered geometry is constantly disrupted in the tumor microenvironment by aberrant cell overcrowding, excessive protease activity, and dysregulated deposition of matrix proteins [2, 5]. This structural collapse is marked by the epithelial-to-mesenchymal transition (EMT), a morphogenic transition in which tumor cells give up the highly organized, polarized, two-dimensional epithelial geometry in favor of a solitary, disorganized, and highly infiltrative mesenchymal phenotype [1, 5, 18]. Simultaneously, a dramatic architectural reorganization of the extracellular space takes place; the normally flexible collagen meshwork is remodeled into thick, heavily bundled, and linearly oriented fibers driven by increased cellular tension [1, 2]. This dynamic change of tumor microarchitecture greatly enhances the malignancy and aggressiveness of the tumor. The bundled collagen fibers, aligned linearly, act as mechanical "tracks" that actively guide the directional migration of tumor cells from the primary lesion towards the vasculature, directly enabling metastatic dissemination [1, 2, 8]. Invasive cancer cells are subjected to substantial physical deformation when they are physically squeezed through the microscopic pores and tight spatial constraints of this reorganized dense matrix [1, 2, 5]. This abnormal structural

geometry provides a formidable physical and immunological barrier. The modified matrix pore sizes restrict the interstitial diffusion and convective transport of therapeutic molecules, and the aligned collagen architecture mechanically prevents infiltration and cytotoxicity of protective CD8+ T cells and Natural Killer (NK) cells [5, 8]. In addition, this mechanical compression causes severe deformation of the cell nucleus, resulting in frequent rupture of the nuclear envelope, chromatin herniation, and DNA double-strand breaks that ultimately promote malignant genomic instability [2, 5, 8]. Raising the question: what are the structural components of the nucleus that help protect against such insults?

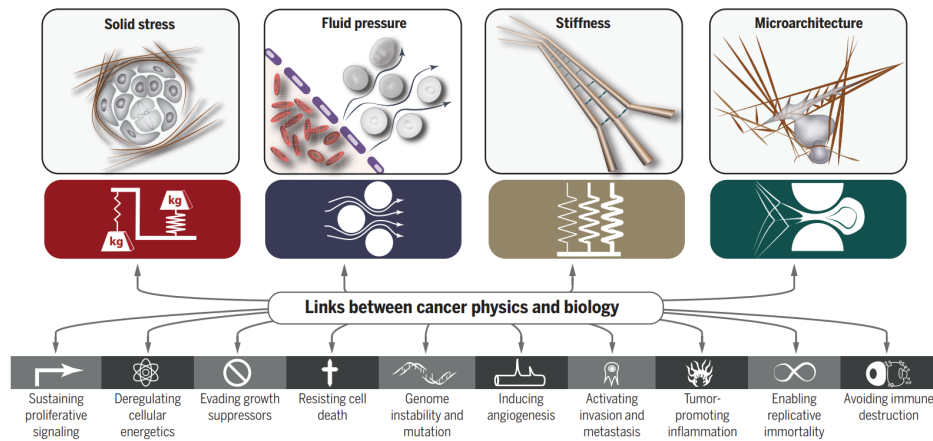


Figure 1. The intersection of cancer physics and biology. Four distinct physical traits of the tumor microenvironment—solid stress, fluid pressure, stiffness, and altered microarchitecture—act as mechanical stimuli that directly drive the downstream biological hallmarks of cancer progression. Reprinted from "Physical traits of cancer," by H. T. Nia, L. L. Munn, and R. K. Jain, 2020, *Science*, 370(6516), eaaz0868. Copyright 2020 by the American Association for the Advancement of Science.

2. Main players in responding to mechanical stress

Mechanotransduction is the crucial biological process by which external mechanical inputs are translated into internal biochemical signals, enabling cellular populations to efficiently maintain tissue homeostasis and dynamically adapt to their physical environment [19, 20]. Accurate identification of the key biological components that underlie this physical signal transduction process is important for a comprehensive understanding of how the cell responds to mechanical stress. These fundamental components of the cell are not autonomous, but are interconnected to form a highly ordered, continuous network from the outer cell membrane to the inner spaces of the nucleus (Figure 2) [21]. This cascade involves the primary biological mediators such as integrin receptors, focal adhesion kinase (FAK), specific mechanosensitive ion channels, Rho GTPases, linker of nucleoskeleton and cytoskeleton (LINC) complex, and specialized

mechanosensitive transcriptional coactivators YAP and TAZ [22, 23].

Cell-Surface Mechanosensors

Specialized surface receptors are positioned at the interface between the cell's interior and its exterior environment to serve as the primary sensors of force. Integrins serve as the primary molecular transmembrane bridges, establishing a direct physical connection between the ECM and the internal structural framework of the cytoskeleton [24]. These integrins possess the inherent capacity to be physically manipulated and to experience force-induced conformational alterations under stress, hence functioning as sensitive molecular force sensors adept at distinguishing between various mechanical environments [25]. Along with these integrin networks, certain mechanosensitive ion channels (MICs) are also employed, including Piezo1 and TRPV4. These are specialized channels situated directly at the plasma membrane. When physical strain, membrane stretching, or solid compression occurs, these channels are triggered to rapidly introduce specific calcium influxes into the intracellular space [26–28].

Intracellular Transmitters and Mediators

Once sensors on the cell surface detect the external mechanical cues, they need to be transduced into biochemical signals and transmitted further into the cell [22]. Intracellular transmitters and mediators carry out this important function. The major players in this network include the force-generating actomyosin cytoskeleton, the Rho family of small GTPases, and Focal Adhesion Kinase (FAK), which together integrate physical inputs to dictate cellular adaptation and gene expression [29]. FAK is a non-receptor protein tyrosine kinase localized to focal adhesions and serves as a major signaling hub to integrate mechanical stimuli [30, 31]. Mechanical tension induces autophosphorylation of FAK at a particular site, tyrosine 397 (Y397) [32]. This forms a recruitment site for the Src family of kinases, resulting in the formation of a robust FAK-Src signaling complex [33]. FAK directly transduces mechanical signals from surface integrins to intracellular cascades, in particular the RhoA/ROCK signaling pathways [31]. Rho family of small GTPases are the main molecular switches inside the cell that control the movement of the cytoskeleton and the transfer of mechanical signals [34]. These proteins switch dynamically from inactive (GDP-bound) to active (GTP-bound) states that are tightly controlled by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and GDP dissociation inhibitors (GDIs) [34]. FAK-Rho GTPase Crosstalk: Intracellular mediators are in a constant

bidirectional feedback loop [22]. Mechanical forces transmitted through integrins promote activation of FAK/Src, which can directly modulate Rho GTPase activity, such as by promoting RhoA activation through GEFs [35]. At the same time, Rho GTPase-dependent actomyosin contractility creates internal tension that pulls on focal adhesions, reinforcing integrin clustering and further activating FAK to sustain cellular adaptation [36]. Finally, coordinated activation of FAK and Rho GTPases gives rise to an internal “cytoskeletal prestress” (actomyosin tension) [37, 38]. This prestress is ultimately delivered to the nucleus itself, which is not a passive endpoint but an active decoding center: it displays both a passive "mechanical response" (e.g., deformation) and an active "mechanotransduction" response, converting physical stimuli into biochemical and genetic outputs via its own structural architecture [39–41]. The next two sections lay out, first, the structural components the nucleus uses to do this, and second, how those components behave mechanically once force arrives.

3. Architecture of the nucleus and chromatin at rest

Before describing how the nucleus responds to force, it is useful to establish the baseline architecture it responds with. Three structural layers are relevant: the nuclear envelope and its associated lamina, the LINC complex connecting that envelope to the cytoskeleton, and the spatial organization of chromatin itself.

The nuclear envelope and the nuclear lamina

The nuclear envelope (NE) is a rigid, double-membrane barrier that is critical for maintaining genomic integrity and mediating nucleocytoplasmic transport that physically separates the eukaryotic nucleus from the cytoplasm [42]. The NE consists of two concentric lipid bilayers, the inner nuclear membrane (INM) and outer nuclear membrane (ONM), separated by a 30- to 50-nm perinuclear space [43, 44]. The ONM is continuous with the rough endoplasmic reticulum (ER) and is studded with ribosomes while the INM has a different proteomic landscape enriched with specialized nuclear envelope transmembrane proteins (NETs) [45]. These membranes converge at nuclear pores [46] and are bridged structurally by linker complexes [47, 48]. The inner surface is supported by the nuclear lamina, a 10- to 30-nm-thick filamentous meshwork [44] located directly beneath the INM that serves as the main structural scaffold for the nuclear periphery. In addition to its scaffolding role it also serves as a central hub for chromatin organization, mechanical stability, and cellular signaling [44, 49]. Lamins are a family of type

V intermediate filament proteins that form the major structural scaffold of the nuclear lamina [44, 50]. The lamin family in mammalian somatic cells is classically subdivided into A-type lamins (lamin A and lamin C, encoded by the LMNA gene via alternative splicing) and B-type lamins (lamin B1 and lamin B2, encoded by distinct genes, LMNB1 and LMNB2) [44]. All lamin isotypes have a tripartite structure consisting of a short, unstructured amino-terminal head domain; a central coiled-coil α -helical rod domain with four helical subdomains; a nuclear localization signal; an immunoglobulin-fold domain; and a carboxy-terminal tail containing a CAAX box motif [44, 51, 52]. Assembly begins with parallel coiled-coil homodimers that polymerize head-to-tail into protofilaments, which then associate laterally in an antiparallel, stacked pattern to form stable 3.5-A tetrameric filaments [53]. Super-resolution microscopy shows that A-, B1-, and B2-type lamins form distinct, spatially segregated, overlapping, and interacting filamentous networks beneath the INM [44, 54, 55]. A number of inner-nuclear-membrane proteins, emerin among them, discussed in detail in Section 5, anchor into this lamina and couple it to underlying chromatin.

Nuclear pore complexes

Nuclear pore complexes (NPCs) are massive 120 MDa macromolecular assemblies composed of approximately thirty distinct proteins known as nucleoporins (NUPs) that serve as the selective bidirectional gates of nucleocytoplasmic transport [56]. The NPC is structurally divided into stable, structural core subcomplexes with low turnover [57–59] and a dynamic central channel lined by NUPs with intrinsically disordered domains rich in phenylalanine-glycine (FG) repeats that mediate transport by transiently binding nuclear transport receptors [60]. Although the nuclear periphery is primarily a transcriptionally repressed, heterochromatic environment, the NPC's nucleoplasmic face creates a localized euchromatin microenvironment [42]. This spatial organization is actively maintained by the nuclear basket protein TPR, which is required to form nuclear pore-associated heterochromatin exclusion zones [61]. These exclusion zones are enriched with transcriptionally active euchromatin decorated with active histone marks (such as H3K4me3), and dynamic NUPs such as NUP98 and NUP153 associate directly with active promoters, enhancers and super-enhancers to promote gene looping, epigenetic poising and transcriptional memory [42, 62, 63].

LINC complexes

LINC complexes allow physical integration of the nucleoplasm and the cytoplasm [43, 64]. Such complexes traverse the double-membrane nuclear envelope via direct physical contacts between inner nuclear membrane SUN-domain proteins (SUN1 and SUN2) and outer nuclear membrane KASH-domain proteins (nesprins) [43, 44, 64]. The SUN-KASH bridge is a stable hexameric complex (3:3) composed of three SUN protomers and three KASH peptides, in the perinuclear space [64]. On the cytoplasmic face, these nesprin adaptors connect the nucleus to all three major cytoskeletal networks: Nesprin-1 and Nesprin-2 directly bind to F-actin, Nesprin-3 recruits the crosslinker plectin to bind to intermediate filaments, and Nesprin-4 and KASH5 recruit microtubule motor proteins [64]. LINC complexes laterally associate with helper proteins in the inner membrane such as LAP1, as well as luminal chaperones such as Torsin A, to help stabilize these massive tethers. They extend biophysical connections underneath the membrane over the underlying structural intermediate filament network of the nuclear lamina [43]. Structurally, this complex is what makes the nucleus mechanically continuous with the rest of the cell rather than an isolated compartment.

Baseline chromatin organization

At rest, the genome is spatially organized in a way that itself contributes to nuclear mechanics. Densely packed heterochromatin is generally located at the nuclear periphery, conferring structural rigidity, while loosely packed euchromatin is enriched toward the nuclear center, conferring structural plasticity. Peripheral heterochromatin is organized into Lamina-Associated Domains (LADs), large blocks of chromatin physically tethered to the nuclear lamina, which link the genome directly to the lamina and, through the LINC complex, to the cytoskeleton. This baseline tethering is what allows extracellular forces to be transmitted all the way to the nuclear interior, a process described next.

4. Nuclear mechanotransduction: how the resting architecture responds to force

With this structural vocabulary in place, we can now describe how each component behaves once mechanical force is applied.

Nuclear pore complexes dilation and nuclear import:

The NE is a major obstacle for decoding mechanical signals. The NE is folded under basal conditions, during which the NPC central channel maintains a constricted diameter of 40–60 nm. However, mechanical stress stretching the nuclear membrane causes tension-sensitive nucleoporins (e.g., Nup153) to expand this channel to 55–70 nm. Such structural dilatation greatly boosts the nuclear import of mechanosensitive transcription factors like YAP, thereby directly connecting physical tension with gene regulation. [65, 66].

LINC-mediated force transmission and lamina softening:

This complex transmits mechanical impulses in the order of a microsecond, up to a million times faster than traditional biochemical diffusion, thus allowing for an almost immediate cellular reaction to a mechanical stimulus [67]. The nuclear lamina controls the mechanical stiffness and structural integrity of the nucleus [68]. Cells can actively downregulate or phosphorylate lamin A/C to achieve “nuclear softening” in response to hard physical barriers (e.g., an extremely stiff ECM). This adaptive softening improves nuclear deformability to allow tumor cells or migratory cells to traverse through tight microscopic gaps [69].

Chromatin as a Mechanosensor

Chromatin is the ultimate biophysical effector and a key mechanical support element in nuclear mechanotransduction [41]. The spatial organization of the genome determines the overall stiffness and deformability of the nucleus, with densely packed heterochromatin generally located at the periphery of the nucleus to confer structural rigidity and loosely packed euchromatin enriched in the center of the nucleus to confer structural plasticity [41, 70]. The genome is directly linked to the cytoskeleton via the LINC complex and the nuclear lamina, thereby transmitting extracellular forces to the nuclear interior within milliseconds [41]. This physical tension directly pulls on chromatin fibers, deforming nucleosome structures, and rapidly changes chromatin accessibility to bypass traditional biochemical cascades [41, 71]. This “mechanogenomic” mechanism exposes gene promoter regions within minutes, directly driving transcriptional upregulation of cancer-associated genes such as dihydrofolate reductase (DHFR) [71]. Importantly, this direct physical changing of the genomic landscape works with force-driven nuclear envelope tension [41], which leads to increased transport of master mechanosensitive co-activators like YAP and TAZ through dilated nuclear pores which then quickly bind to their newly exposed target

loci on the uncoiled DNA to facilitate the transcriptional program supporting the growth and invasion processes [23, 71]. This physical synergy is further coordinated by bimodal epigenetic adaptations, temporally regulated, orchestrating transcription factor recruitment [41]. In the short term, mechanical forces induce rapid, transient chromatin decondensation and a decrease in heterochromatin markers such as H3K9me3, which softens the nucleus for confined migration while selectively priming gene promoters for active epithelial-mesenchymal transition (EMT) transcription factors like TWIST1 [72, 73]. Conversely, with continuous mechanical loading or static 3D compression, the nucleus is driven into a protective compaction state; a static force of 1 kPa for 1 h induces nuclear accumulation of histone deacetylase 3 (HDAC3) and increases H3K9me3 and H3K27me3 levels to facilitate chromatin compaction [41, 74]. This compaction globally suppresses transcription to avoid DNA double-strand breaks during nuclear deformation, but it also selectively increases the accessibility of certain promoter regions involved in tumor invasion and DNA damage response [41, 75].

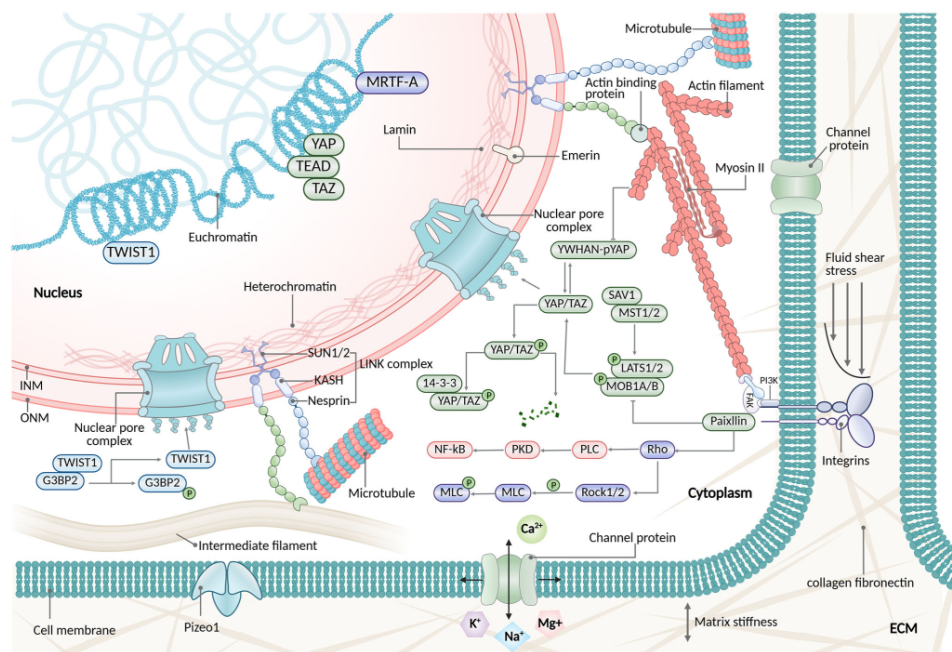


Figure 2. Mechanotransduction. Extracellular mechanical forces are detected by surface sensors like integrins and transmitted through the cytoskeleton to the LINC complex. This physical tension directly remodels the nuclear envelope and chromatin, driving the nuclear import of mechanosensitive transcription factors (such as YAP/TAZ and TWIST1) to regulate target gene expression. Reprinted from L. Song et al. 2026, Advanced Science.

Across NPC dilation, LINC transmission, and chromatin remodeling, one theme recurs: the machinery that anchors heterochromatin to the nuclear periphery under resting conditions is precisely what must be mechanically released for the nucleus to respond to force. The next section examines one such anchoring complex: emerin and BAF, in molecular detail.

5. Emerin and BAF: Anchoring Heterochromatin at the Nuclear Envelope

Emerin is a highly conserved, 254-residue type-II integral membrane protein, structurally defined by a near-C-terminal transmembrane segment and a nucleoplasmic LEM domain, that functions as a premier nucleoskeletal nexus localized to the inner nuclear membrane and ER [76]. This complex protein is far more than just a static architectural scaffold; it is a dynamic regulatory integrator that actively coordinates cyto-nuclear homeostasis by translating various biomechanical and signaling inputs through high-affinity (40 nM) stoichiometric interactions with A-type lamins [76]. This topological anchoring forms the nuclear lamina, a dynamic cytoarchitectural matrix that is crucial for the stabilization of global nuclear morphology and the maintenance of nuclear envelope elasticity against intracellular forces [76, 77]. The clinical significance of this structural node is demonstrated by X-linked Emery-Dreifuss Muscular Dystrophy, a severe myopathic syndrome marked by progressive skeletal muscle wasting and cardiomyopathy. Systemic emerin removal causes catastrophic architectural dysmorphism, such as markedly decreased nuclear shape ratios, localized chromatin protrusions, and mechanically damaged nuclear envelopes that show poor mechanotransduction and make the cell particularly vulnerable to strain-induced apoptosis [76, 77]. Beyond its basic structural topography, emerin is a fundamental mechanosensor and master transducer of bidirectional biomechanical force [76, 77]. Emerin creates a continuous mechanotransductive conduit between the cytoplasmic cytoskeleton and the nucleoskeleton by physically and functionally integrating the transmembrane LINC complex by scaffolding Nesprin and SUN-domain integral membrane proteins [78]. This molecular bridge regulates downstream signaling cascades upon dynamic mechanical strain, which requires emerin to up-regulate important anti-apoptotic and mechanosensitive genes such as IEX-1 and EGR-1 [77, 78]. Emerin-deficient cells are more vulnerable to strain-induced apoptosis due to the collapse of the mechanosensory feedback loop. This highlights the critical role of the protein in cytomechanical resilience [77]. Alongside this force-sensing role, emerin also organized the genome directly, through a distinct, chromatin-facing mechanism detailed below.

Emerin-BAF interaction

Barrier-to-Autointegration Factor (BAF) is a highly conserved, 10-kilodalton double-stranded DNA-binding protein in metazoans. BAF was first discovered to protect retroviral DNA from

suicidal autointegration. Still, its main biochemical property is its ability to bridge DNA molecules by forming a higher-order nucleoprotein complex, specifically a dodecamer that binds multiple DNA segments in a sequence-nonspecific manner [79]. In the cell, BAF acts as a structural bridge that modulates chromatin architecture, promotes mitotic progression, and organizes the nuclear envelope [79, 80]. During cell division, BAF is required for efficient chromosome segregation and capture within the reforming nuclear membrane, whereas during interphase, it acts as a rapid responder, localizing to and patching mechanical ruptures in the nuclear membrane [79, 81, 82]. It is very important to note that the way BAF interacts with DNA controls how strongly it binds to emerin [80]. Mutations that disrupt BAF's DNA-binding surface can also alter its affinity for emerin, though the relationship between the two is mutation-specific rather than a simple one-to-one rule [80]. This suggests that BAF exists in two distinct conformational states: DNA-free and DNA-bound, with altered affinity for emerin [80]. Emerin's LEM-domain is highly conserved and is vital for spatial genomic regulation and epigenetic modulation [80, 83]. The highly conserved helix-loop-helix structural fold mediates binding to the chromatin-bridging Barrier-to-Autointegration Factor (BAF), inducing spatial compartmentalization and peripheral attachment of transcriptionally inactive heterochromatin to the nuclear envelope [76, 80]. In terms of cellular function, BAF and emerin have a profound structural mutual dependency, especially during post-mitotic nuclear assembly and interphase membrane repair [81, 82]. When a cell exits mitosis, DNA-bound BAF is essential for recruiting emerin to exposed chromatin [80, 81]. Experimental depletion of BAF results in Emerin not assembling into a coherent nuclear rim but instead binding patchily, leaving large gaps in the nascent nuclear envelope that fail to safely capture and enclose the segregated chromosomes [81]. In contrast, BAF is a very sensitive marker during mechanical stress in interphase, accumulating at sites of nuclear membrane rupture within seconds, and subsequently recruiting emerin to help reseal the gap [82]. Loss of either BAF or emerin drastically delays repair of these membrane ruptures, indicating that their physical and functional partnership is the central machinery required to re-establish nuclear compartmentalization [82]. In addition, BAF is able to compete with other cellular constituents like the germ cell-less repressor for binding to emerin in vitro, so their interdependent association could also play a secondary role in regulating gene expression pathways [81]. Beyond this BAF-mediated tethering, emerin contributes to the epigenetic landscape through a second, LEM-domain-independent mechanism: it directly binds Histone Deacetylase 3 (HDAC3) and stimulates its catalytic activity [83]. Through this

direct interaction, emerin couples nuclear envelope tethering to targeted histone deacetylation, linking mechanostuctural organization to tissue-specific transcriptional silencing through a route independent of its partnership with BAF.

6. ATR and canonical role of ATR

Ataxia Telangiectasia and Rad3-related (ATR) kinase is a large serine/threonine protein kinase of the phosphatidylinositol 3-kinase-related kinase (PIKK) family [84, 85]. It is an important master regulatory protein in eukaryotic cells that is directly responsible for the maintenance of genomic stability (Figure 3) [85, 86]. ATM is another important member of the PIKK family. ATM and ATR have structural and biochemical similarities, but their cellular roles and sensitivities are quite different. ATM detects DSBs primarily, while ATR is specialized to sense and respond to a broad spectrum of DNA damage and replication stress, which is essentially characterized by ssDNA [87]. Moreover, ATR but not ATM is specifically required for the survival of mammalian cells undergoing replication, and its complete genetic ablation leads to early embryonic lethality and massive chromosomal fragmentation [85, 88]. Rather than being activated directly by the initial DNA lesion, ATR is driven by the aberrant DNA structures that naturally occur when replication forks stall or when DNA ends are biochemically resected, specifically, stretches of ssDNA coated with Replication Protein A (RPA) [89, 90]. ATR forms a stable bound heterodimer with ATRIP (ATR-interacting protein) which is essential for ATR's function. Since the RPA-coated ssDNA is directly bound by ATRIP, the kinase complex is effectively localized precisely to the site of genomic damage [87, 91]. The junction between the ssDNA and the adjacent double-stranded DNA (dsDNA) is structurally recognized by the Rad17-RFC clamp loader, and then the Rad9-Rad1-Hus1 (9-1-1) checkpoint clamp is loaded directly onto the DNA strand in an ATR-ATRIP complex-independent manner. [87, 92]. Once localized, the 9-1-1 clamp functions as a molecular marker to recruit the activator protein TOPBP1 [93]. TOPBP1 physically recruits the ATR-ATRIP complex following autophosphorylation of ATR at the Thr1989 residue [94]. TOPBP1 strongly activates the kinase activity of ATR to its full catalytic potential [95]. Once fully activated, ATR is used as the central transducer in a massive signaling cascade, actively targeting numerous downstream substrates to effectively pause the cell cycle, stabilize vulnerable replication forks, and formally initiate DNA repair mechanisms [85]. ATR plays a critical role in mediating cell cycle checkpoints that stop cell division in response to DNA damage or incomplete replication. [96]. ATR directly phosphorylates Chk1 at

specific serine residues, with the help of mediator proteins, such as Claspin [97]. Phosphorylated Chk1 then dissociates from the local chromatin to actively propagate the damage signal to the nuclear environment [85, 98]. Upon activation, CDC25 family phosphatases (including CDC25A and CDC25C) are targeted by Chk1 for either rapid degradation or cytoplasmic sequestration [99]. Indeed, the efficient inactivation of these CDC25 phosphatases strictly prevents the removal of the inhibitory phosphorylations on Cyclin-Dependent Kinases (CDKs), such as Cdc2/CDK1, and the critical cell cycle drivers are subsequently maintained in a totally inactive state [100]. By suppressing CDK activity, the ATR-Chk1 pathway successfully initiates a strong G2/M cell cycle arrest. Finally, the arrest prevents the disastrous entry of cells into mitosis with a damaged genome and the very lethal premature condensation of chromosomes [96, 101]. ATR has global effects on the cell cycle, but also local and essential roles at the site of stalled replication forks to maintain structural integrity and prevent replication failure. Physical lesions and difficult-to-replicate regions of the genome may lead to the collapse of the replicative helicase and polymerase, resulting in long stretches of single-stranded DNA and, ultimately, replication fork stalling [102]. To effectively overcome this structural weakness, multiple components of the replisome are directly phosphorylated by ATR, including the MCM2 subunit of the MCM2-7 replicative helicase [103]. Moreover, ATR-dependent phosphorylation actively targets proteins such as the helicase SMARCAL1. This critical change impairs the fork regression activity of SMARCAL1, which is essential to preventing aberrant nucleolytic processing of the stalled fork [104]. These very specific phosphorylation events serve to structurally stabilize the complex architecture of the stalled fork and thereby facilitate its eventual successful restart [85, 96]. In the absence of ATR signaling completely, stalled replication forks will inevitably lack proper molecular maintenance, rapidly leading to fork collapse and the subsequent accumulation of highly toxic double-strand breaks [105]. Furthermore, ATR protects actively replicating cells against such fork collapse through strict suppression of excessive replication origin firing and active maintenance of adequate nucleotide availability. At the same time, strict adherence to these parameters effectively prevents the global depletion of Replication Protein A (RPA), which would otherwise result in massive, lethal chromosomal fragmentation [106, 107]. Apart from cell cycle arrest and stabilization of stalled replication machinery, ATR actively regulates several specific DNA repair pathways to repair DNA damage correctly [85]. While ATM responds to double-strand breaks (DSBs), ATR repairs them via an "ATM-to-ATR switch" [108]. When a DSB is processed and resected to create long stretches of single-stranded DNA (ssDNA),

the lesion is converted into an ATR-activating structure [108]. ATR phosphorylates important recombination proteins like BRCA1 and the WRN and BLM helicases to promote error-free homologous recombination (HR) and prevent aberrant crossover events [109–111]. Also, ATR activates the kinase Chk1, which phosphorylates the recombinase Rad51 to encourage HR at stalled replication forks [84, 112]. In addition to double-strand breaks, ATR also plays a role in the repair of highly toxic lesions such as inter-strand cross-links (ICLs) and large DNA adducts. ICLs can completely block DNA replication, and ATR can regulate the Fanconi anemia pathway by phosphorylating the FANCD2 protein to fix ICLs [113]. This particular modification facilitates FANCD2 monoubiquitination and its crucial recruitment to the sites of DNA damage [85, 113]. Besides that, ATR regulates the nucleotide excision repair (NER) pathway through phosphorylation of the core NER protein XPA, which in turn affects its intracellular localization to UV-induced damage sites [114]. Finally, ATR's repair activities are closely linked to the mismatch repair (MMR) machinery and direct replisome maintenance. The MMR system can activate ATR either by generating persistent ssDNA gaps during iterative “futile cycling” or by direct physical recruitment by MMR recognition complexes [90, 115]. ATR acts directly at the replisome, phosphorylating numerous components, most notably the MCM2-7 replicative helicase, to prevent lethal replication fork collapse [85, 103]. ATR-dependent phosphorylation of MCM2 creates a specific docking site for Polo-like kinase 1 (PLK1) to override the global replication arrest and promote local resumption of DNA synthesis and fork recovery [116].

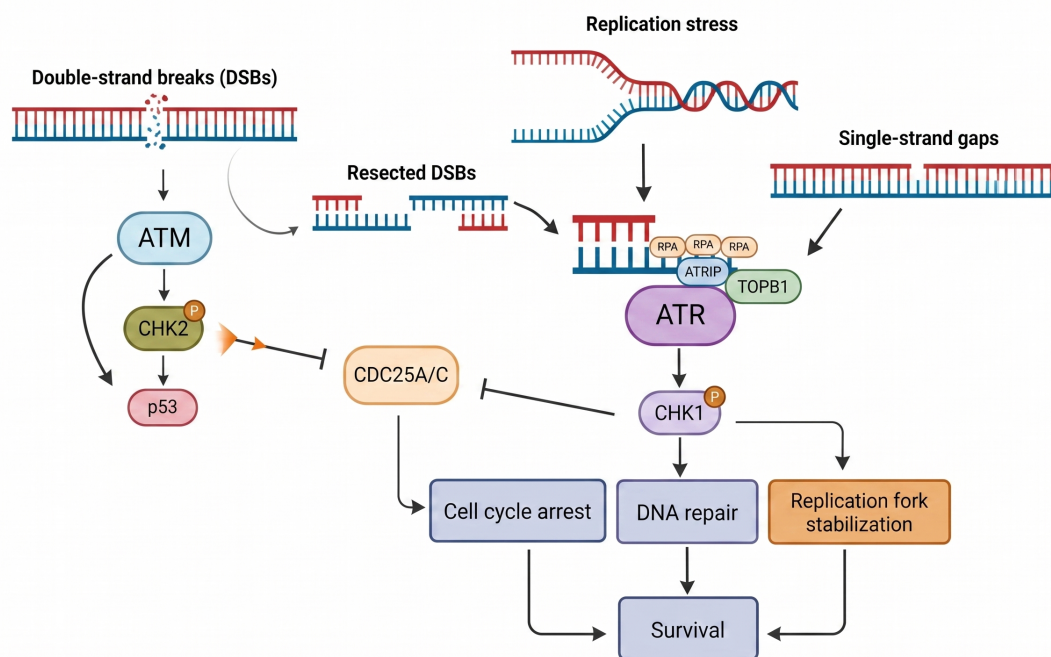


Figure 3. ATR is a major sensor of replication stress, single strand gaps and resected double strand breaks (DSBs). Activation of ATR upon binding of RPA, ATRIP and TOPBP1 to damaged DNA leads to phosphorylation of its downstream effector CHK1. Adapted from Mavroei et al., 2024

7. Emerging roles for ATR in Mechano-transduction

ATR has been classically described as a canonical DNA damage sensor [117, 118], but recent research has shed light on ATR's role as a primary cellular mechanotransducer [119–121]. Beyond its classic activation by RPA-coated single-stranded DNA following genotoxic stress, ATR also has a distinct, intrinsic capacity to sense physical strain [119, 121]. This mechanical activation occurs independently of actual DNA double-strand breaks, enabling ATR to continuously monitor physical stress rather than relying exclusively on genetic injury [119, 121]. This noncanonical mechanotransducer role is supported by strong, well-controlled experimental evidence showing it is not merely a reaction to DNA breakage; Researchers have used automated patch-clamp platforms to stretch the plasma membrane of living cells and compressive-load systems to put 15–30 nN of direct physiological weight on the (Figure 4) [119]. When physical force is applied, ATR moves and groups at the nuclear envelope within seconds to minutes. This process can be reversed once the physical force is removed. Scientists carefully checked for DNA damage during these tests of physical stretching and hyperosmotic stress. There is no accumulation of γ H2AX (the universal marker for DNA double-strand breaks), which proves that no DNA was broken [119]. Conversely, if the upstream mechanosensitive ion channel

Piezo1 or Nitric Oxide Synthase (NOS) is blocked, ATR stops responding to physical stress. Without mechanical sensors, ATR does not cluster or activate in response to osmotic swelling or nerve injury [118]. Together, these loss-of-function results demonstrate that ATR's mechanical activation runs through a genuinely separate sensing pathway rather than a byproduct of the canonical DNA damage response. ATR does not sense mechanical force directly, but is instead activated by distinct upstream mechanosensors specific to the type of physical input involved [118, 120]. When cells are subjected to severe mechanical stress, such as the aberrant nuclear deformation and increased nuclear membrane tension induced by mis-segregated chromosomes (aneuploidy), the physical strain is sensed by the mammalian target of rapamycin complex 2 (mTORC2) [120]. mTORC2 functions as a direct sensor of plasma and nuclear membrane tension, working dynamically with ATR to activate downstream survival or arrest signals [120]. A separate route senses extracellular rather than membrane-derived mechanical cues: in the nervous system, as a severed axon tries to regrow, its growth cone physically pushes against the extracellular matrix, and this microenvironment stiffness activates the mechanosensitive ion channel Piezo [118]. Opening of the Piezo channel drives local calcium influx that activates Nitric Oxide Synthase (NOS) to produce nitric oxide (NO), one of the important retrograde messengers that travels back to the nucleus to stimulate clustering and activation of ATR — coupling extracellular physical stiffness to intracellular ATR signaling [118]. Once activated by either of these upstream sensors, ATR orchestrates a set of complex, systemic physiological responses governing cell fate, tissue integrity, and neurobiology [117, 118, 120, 121]. When chromosomes fail to separate correctly during mitosis, the resulting aneuploid daughter cells inherit deformed nuclei with altered membrane tension [122]. This abnormal tension is sensed by the mTORC2-ATR mechanosensory network, which triggers a powerful cell cycle checkpoint that permanently arrests cells in G1 by stabilizing and accumulating tumor suppressors p53 and p21 immediately following mitosis [120]. In this way, mechanically activated ATR halts the proliferation of genomically unstable, cancer-prone cells based purely on their physical state, independently of any classical DNA lesions [120]. ATR's mechanosensory function also directly protects the physical integrity of the nucleus under load. During processes such as interstitial migration, for example, cancer cell metastasis, or direct mechanical compression, cells must squeeze through tight spaces, placing tremendous physical strain on the nucleus. ATR acts as a protective shield against this strain. In ATR-defective cells, the nucleus fails to properly respond to mechanical compression and undergoes irreversible deformation and

collapse. This collapse leads to severe ruptures of both the inner and outer nuclear membranes, allowing genomic DNA to leak into the cytoplasm (which is detected by the accumulation of the cytoplasmic DNA-binding protein cGAS) (Figure 5) [123]. When mechanical stress of this kind, whether from external compression or from the nuclear envelope rupture that follows severe cytoskeletal strain breaches the nuclear envelope, or even threatens to, ATR responds by driving rapid polymerization of a nuclear filamentous actin (F-actin) network that acts as a physical scaffold, increasing nuclear stiffness and effectively plugging the rupture to limit chromatin leakage [117]. ATR achieves this by two convergent pathways [117, 121]: First, upon NE rupture, ATR promotes the phosphorylation of the formin DIAPH3 at the Serine 1072 residue. This causes DIAPH3 to transiently translocate into the nucleus, where it nucleates F-actin to physically support the damaged nucleus [117]. Secondly, ATR phosphorylates the Hippo pathway scaffold protein RASSF1A at Serine 131. This phosphorylation recruits the actin-crosslinking protein Filamin-A directly to the inner nuclear membrane, driving further nuclear F-actin assembly [121]. The same ATR-RASSF1A mechanotransduction axis that protects the compressed nucleus also plays a distinct developmental role in the brain. Phosphorylation of RASSF1A by ATR promotes the differentiation of embryonic stem cells into neuronal and glial lineages. Genetic disruption of this mechanical signaling cascade, preventing ATR from phosphorylating RASSF1A, causes structural abnormalities in the brain, such as thinning of the cerebral cortex, and is strongly linked to serious mental illnesses including schizophrenia, severe neuroticism, and depression. Finally, the Piezo-NOS-ATR cascade also acts as a brake on nerve repair in the adult nervous system. After nerve damage, NO mechanically activates ATR, which phosphorylates the kinase Chk1; Chk1 in turn phosphorylates and inactivates the phosphatase Cdc25, keeping the master cell cycle regulator Cdk1 inactive and halting the axon's repair machinery. Blocking the ATR-Chk1 pathway pharmacologically or genetically removes this brake, allowing robust axon regrowth, synapse reformation, and behavioral recovery in both the peripheral and central nervous systems [118]. Taken together, ATR's noncanonical role extends far beyond monitoring broken DNA: it functions as a genuine mechanotransducer, translating physical forces, from chromosome mis-segregation, nuclear compression, and axonal growth-cone stiffness, into biochemical decisions that prevent aneuploidy-driven cancer, coordinate dynamic nuclear actin assembly for survival under mechanical load, direct healthy psychiatric brain development, and govern the regenerative limits of the mammalian nervous system [117, 118, 120, 121].

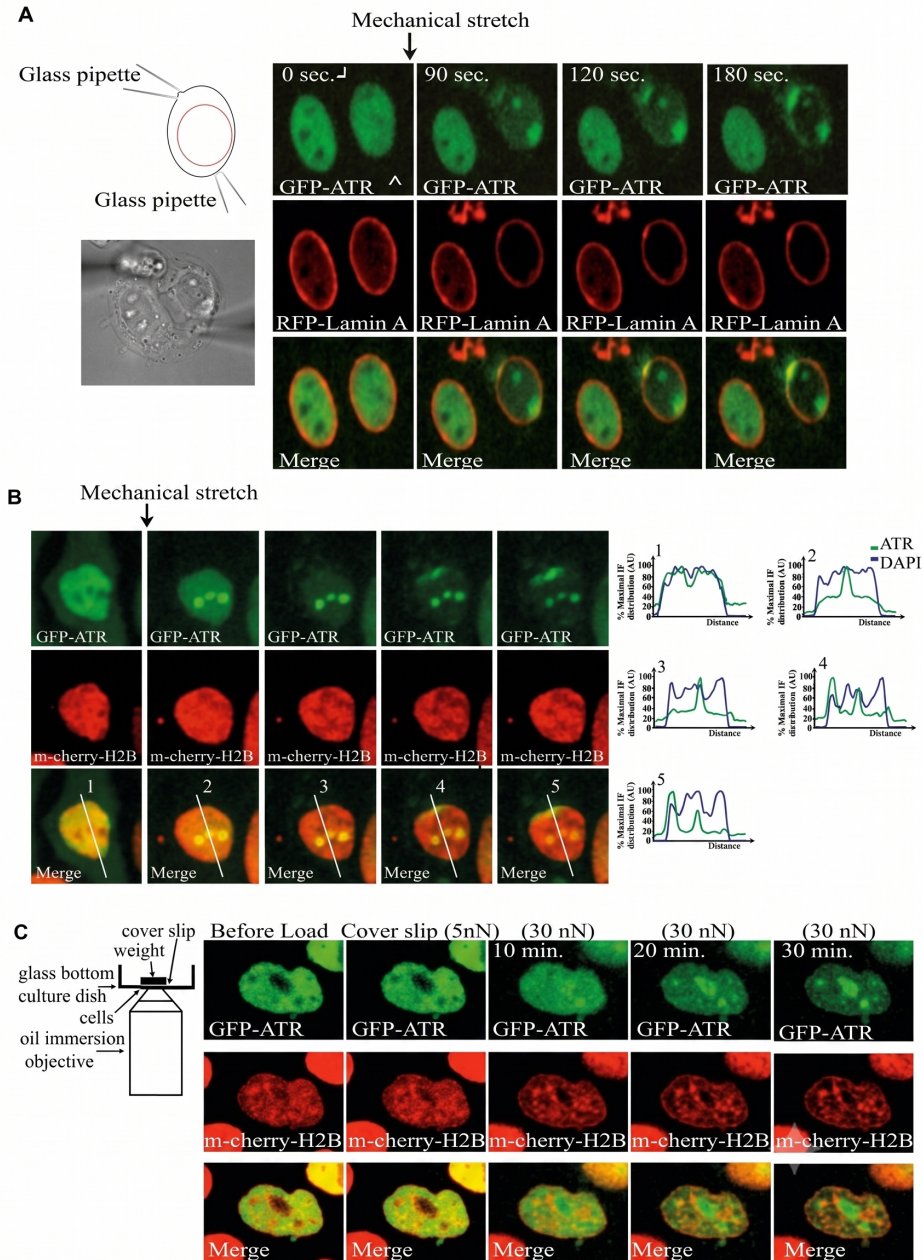


Figure 4. ATR response to membrane mechanical stress. (A) Two patch-clamp pipettes were attached to opposite sides of a HeLa cell's plasma membrane to induce mechanical stretching at specific focal points (indicated by white arrows). In this cell co-expressing GFP-ATR (green) and RFP-Lamin A (red), the rapid intracellular relocation of ATR to the nuclear envelope was captured in real time at 30-second intervals following the applied physical tension. Reprinted from A. Kumar et al., 2014, *Cell*

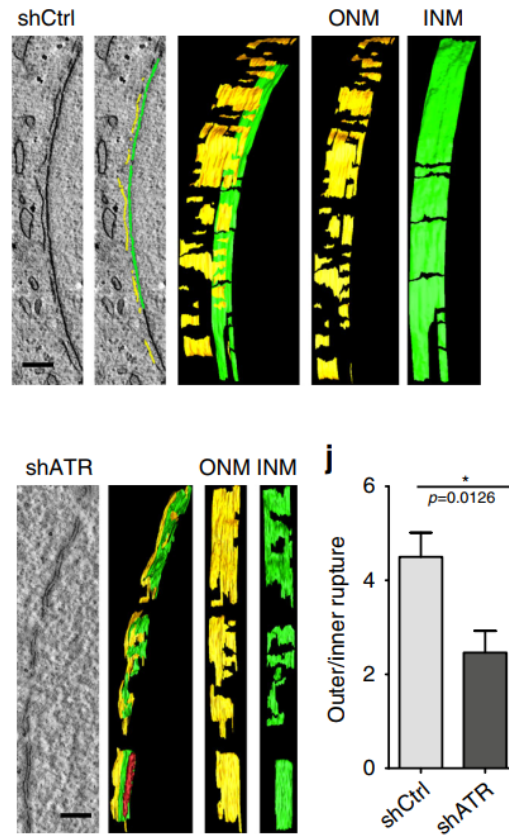


Figure 5. ATR maintains nuclear envelope integrity. 3D reconstructions of electron microscopy images show the outer (yellow) and inner (green) nuclear membranes. ATR-depleted cells (shATR) exhibit a significantly higher frequency of severe inner membrane ruptures compared to control cells (shCtrl) when forced through narrow constrictions. Adapted from G. R. Kidiyoor et al., 2020, Nature Communications.

Aim of study

During physiological growth and pathological progression, especially during expansive proliferation and active migration of cancer cells, severe mechanical stresses are continuously exerted upon the cellular architecture. To survive and adapt to these hostile physical environments, the cell uses specialized molecular mechanosensors to actively detect and biochemically respond to these forces. Among these, the Ataxia Telangiectasia and Rad3-related (ATR) kinase has recently emerged as a highly compelling and critical mechanosensor. Although the canonical functions of ATR in DNA damage response are well established, the precise molecular mechanisms and downstream structural effectors by which ATR actively participates in mechanical signal transduction remain largely unknown. This thesis aims to define the mechanobiological signaling axis by which ATR governs nuclear envelope mechanics. We propose that mechanical stress triggers ATR-dependent phosphorylation of Emerin, destabilizing its interactions with Barrier-to-Autointegration Factor (BAF) and HDAC3 and thereby releasing BAF-bound chromatin from the nuclear envelope. This transient uncoupling is proposed to protect the genome from structural damage during mechanical deformation. By characterizing this ATR-Emerin-BAF axis, this study aims to clarify how cells survive the biomechanical challenges of the tumor microenvironment.

Materials and Methods

Cloning shEMD-PLKO Plasmid

Design shRNA oligonucleotides

To effectively silence the endogenous emerin (EMD) protein while maintaining sustained expression of exogenously introduced mutant EMD constructs, a highly specific shRNA-based gene silencing strategy was designed using the Thermo Fisher website. The shRNA oligonucleotides were designed by rationally selecting sequences outside the open reading frame (ORF) of the EMD mRNA, targeting the 3' and 5' regions of the endogenous transcript. The design was such that only the endogenous emerin was degraded by the RNA interference machinery, not the exogenous mutant plasmids. Thus, these recognition sequences were not present in the exogenous mutant constructs, which were constructed to contain only the functional EMD ORF, providing a robust and accurate knockdown-rescue experimental paradigm. In parallel, a scrambled shRNA (shScramble) construct was produced to thoroughly control for any confounding variables. This non-targeting control was engineered to have the same nucleotide length and complementary cohesive restriction ends as the experimental shEMD construct, but its sequence was computationally verified to have no significant resemblance to known endogenous mammalian transcripts. Using this negative control, we could be assured that any observed biological or molecular phenotypes were specifically due to depletion of EMD and not due to off-target gene silencing, toxicity, or non-specific artifacts arising from general activation of the cellular RNA interference machinery. Thermo Fisher Scientific proprietary algorithms for optimal target identification were used to develop optimal sequences for the targeting and scrambled oligonucleotides. The shRNA oligonucleotides were cloned into the pLKO lentiviral expression vector after manufacture using a double restriction endonuclease digestion technique. The oligonucleotides were flanked by the sequences corresponding to the restriction sites of AgeI and EcoRI, respectively. The use of two enzymes allowed for directional cloning, meaning that the double-stranded shRNA insert was placed into the vector in the precise orientation needed for proper downstream transcription by the promoter. Further digestion of the vector backbone with two different restriction enzymes yielded non-compatible asymmetric cohesive overhangs at the 5' and 3' termini. This structural asymmetry was important to avoid spontaneous intramolecular self-ligation of the linearized vector during the ligation step, and thus to

maximize efficiency of insert integration and significantly decrease the background incidence of false-positive, empty-vector bacterial colonies during the subsequent transformant screening process

Annealing Oligos

The forward and reverse oligos were re-suspended in double-distilled water (ddH₂O) to a standardized final working concentration of exactly 20 μ M. An exact annealing reaction mixture was carefully formulated containing 5 μ L of the forward oligonucleotide, 5 μ L of the reverse oligonucleotide, 5 μ L of 10X NEB Buffer 2 (NEB #B7002S), and 35 μ L of ddH₂O. The mixture was then incubated for 4 min at 95°C during a first high-temperature incubation phase in a programmed automated thermal cycler or a boiling water bath. A controlled, slow cooling process was strictly required for the thermodynamically stable annealing of the complementary oligonucleotide strands. When the thermal cycler was used, the sample was incubated at an intermediate temperature of 70 °C for 10 minutes and then allowed to slowly return to ambient room temperature over a period of several hours. Alternatively, in the case of the manual water bath method, after the 95 °C incubation step the main heat source was completely removed and the volume of water surrounding the incubation mixture was allowed to passively cool to ambient room temperature over a period of several hours, providing the necessary gradual annealing process.

shEMD forward:

CCGGGGCTTCAGAGCTAGGTCTTTGCTCGAGCAAAGACCTAGCTCTGAAGCCTTTTTG

shEMD Reverse:

AATTCAAAAAGGCTTCAGAGCTAGGTCTTTGCTCGAGCAAAGACCTAGCTCTGAAGCC

■ CCGGT: AgeI restriction site

■ Stem strands

■ loop strands

AATTC: EcoRI restriction site

Digestion Vector Backbone

The recipient plasmid vector backbone was linearized to enable the integration of the newly synthesized double stranded shEMD oligonucleotides with specific cohesive overhangs. This was done by double restriction endonuclease digestion with the same enzymes used to construct

the oligonucleotide. The construction was based on the Tet-pLKO-puro lentiviral expression plasmid (Addgene #21915). We selected this particular vector system because it enables doxycycline inducible transcriptional regulation of the shEMD construct, providing temporal control of the knockdown of the target gene in downstream cell based assays. Following the protocol provided by Addgene, the restriction digest was carried out in a 50 μ L reaction volume. For dual-enzymatic activity, 2 μ g of purified, circular Tet-pLKO-puro plasmid DNA was combined with 5 μ L of AgeI, 5 μ L of EcoRI and the necessary volume of 10X NEB CutSmart Buffer. The reaction was brought to the final volume with nuclease-free water to avoid nonspecific degradation of the nucleic acids. The mixture was incubated for 2 hours at 37°C to allow complete catalytic cleavage of plasmid DNA at both recognition sites. After incubation, the small excised fragments and the undigested circular plasmid were spatially separated from the linearized vector DNA fragments by electrophoresis through a low-melting-temperature agarose gel. The band of higher molecular weight corresponding to the correctly linearized vector backbone was identified, excised from the gel matrix, and collected for further purification and ligation procedures.

Gel Extraction

DNA was extracted from agarose gel using the QIAEX II System Gel Extraction Kit. The required DNA fragment was cut from the gel by a cutter, placed in a sterile tube, and weighed. Three volumes of Buffer QC (relative to the gel weight) were added, and the sample was heated at 50°C for 10 min with mixing to fully dissolve the gel. Then a volume of isopropanol was added and mixed. The mixture was then loaded onto the center of a QIAquick column placed in a tube and centrifuged, and the flow-through discarded. The process was repeated to run all of the dissolved gel through the column. Then, 750 μ L Buffer PE was added to the column, centrifuged for 1 minute and the flow-through was discarded. The column was then centrifuged for another 2 min in the absence of buffer to remove any contamination. Finally, 30 μ L to 50 μ L of Buffer EB or nuclease-free water was added to elute the DNA, incubated for 1 min, and centrifuged for 1 min.

Ligation

After annealing, the double-stranded shEMD with cohesive overhangs was ligated into the linearized pLKO vector. T4 DNA ligase-catalyzed ligation was used to covalently join the

DNA fragments. The best insert to vector ratio was obtained with a 20 μL reaction mixture. In particular, we used 20 ng of the purified pLKO backbone and 2 μL of the annealed oligo, then 1 μL T4 DNA ligase (NEB #M0202S) was added. To provide the chemical environment and energy for the enzymatic activity, 2 μL of 10X NEB ligase buffer (NEB #B0202S) was added. Then the reaction was brought up to the final volume with 14 μL of ultra-pure water. The mixture was then incubated overnight at 16°C in a thermal cycler. The lower temperature was used to stabilize the annealing of the relatively short cohesive ends generated by the restriction enzymes. The overnight incubation also allowed sufficient time for the ligase to catalyse the formation of stable phosphodiester bonds.

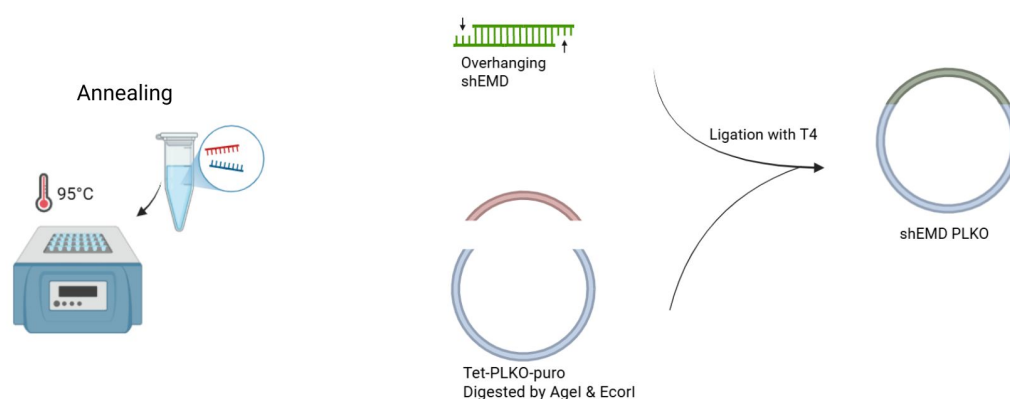


Figure 6. Cloning procedure, created by BioRender

Heat-Shock Transformation

To amplify the newly generated plasmids, a standard heat-shock transformation was performed. Half of the ligation mixture was added to 100 μL of *Escherichia coli* TOP10 competent cells and incubated on ice for 20 to 30 minutes. The cells were subjected to a heat shock at 42°C for 30 to 45 seconds and immediately transferred to ice for 2 minutes. The mixture was then supplemented with 250 to 950 μL of antibiotic-free SOC or LB medium and incubated at 37°C with shaking (200–250 rpm) for 1 hour. Finally, 50 to 100 μL of transformed cells were plated on agar plates containing ampicillin and incubated overnight at 37°C. The next day, seven colonies from both shEMD and shScrambled plates were picked and cultured in 2 mL LB broth

supplemented with ampicillin.

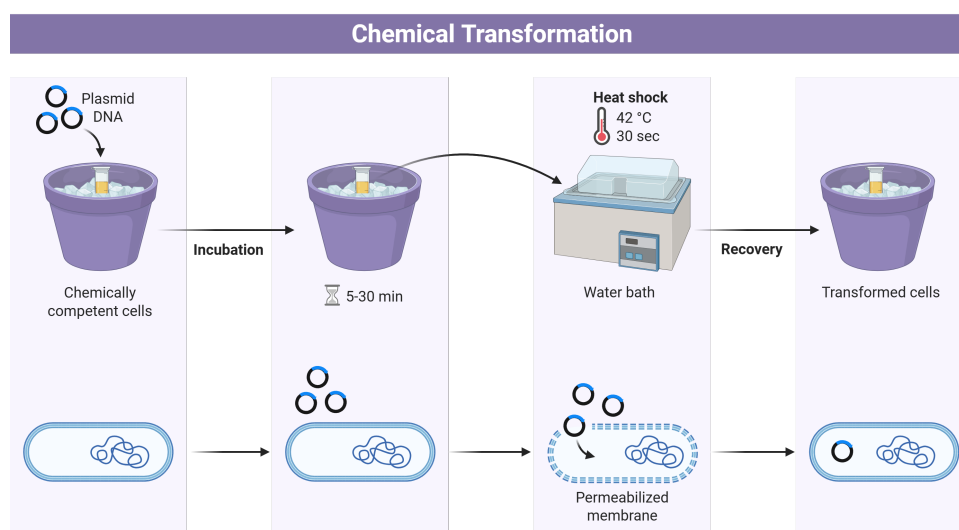


Figure 7. DNA Heat shock transformation, created by BioRender.

DNA Extraction (Miniprep and Maxiprep)

Plasmid DNA was purified using the Wizard® Plus Minipreps DNA Purification System (Promega). Bacterial cells were centrifuged to form a pellet, re suspended in 200 μ L of Cell Resuspension Solution and lysed with 200 μ L of Cell Lysis Solution. The mixture was then neutralized with 200 μ L of Neutralization Solution and centrifuged at 10,000 $\times$ g for 5 min to pellet cellular debris. The cleared lysate was loaded onto a Minicolumn assembly and passed through 1 mL Wizard® Minipreps DNA Purification Resin. The column was washed with 2 mL of Column Wash Solution and centrifuged at 10,000 $\times$ g for 2 min to ensure that the resin was completely dried. Purified plasmid DNA was eluted by adding 50 μ L of nuclease-free water directly to the column matrix and centrifuging for 20 seconds. Minipreps are used for small scale screening of plasmid DNA from low volume cultures, whereas maxipreps are used when large quantities (up to 1000 μ g) of highly purified plasmid DNA are required from larger bacterial cultures. The NucleoBond® Xtra Maxi Kit (MACHEREY-NAGEL) was used for large scale extraction. The bacterial cells harvested were re-suspended in 12 mL Buffer RES and lysed in 12 mL Buffer LYS for 5 min at room temperature. After the addition of 12 mL Neutralization Buffer NEU, the complete neutralized colorless lysate was loaded directly onto a pre-equilibrated NucleoBond® Xtra Column with column filter. The column and filter were washed with 15 mL Buffer EQU, the filter discarded and the column washed with 25 mL Buffer WASH. Plasmid DNA was eluted in 15 mL Buffer ELU and precipitated by the addition of 10.5

mL isopropanol and centrifugation at $15,000 \times g$ for 30 min at 4°C. Finally, the DNA pellet was washed with 4 ml of 70% ethanol, dried at room temperature and resuspended in TE buffer.

Site-Directed Mutagenesis

Specific mutations were introduced into the Emerin construct by site-directed mutagenesis. Custom oligonucleotide primers were designed to contain the desired mutation as a localized mismatch and used to amplify the entire circular plasmid template using a high-fidelity polymerase chain reaction (PCR) with PrimeSTAR Max DNA Polymerase Ver.2. The PCR was performed in a 50 μ L reaction volume assembled on ice. The mixture contained 25 μ L of 2X PrimeSTAR Max Ver.2 Premix, 0.2-0.3 μ M of each mutagenic primer, and 10 pg-10 ng of Emerin plasmid template, and was brought to final volume with sterile purified water. Thermal cycling was highly restricted to three cycles to reduce the likelihood of off-target amplification and to keep the overall fidelity of the construct. Cycling parameters were 98°C for 10 seconds, 55°C for [insert 5 or 15] seconds, and 68°C for [insert 5 to 10] seconds per kilobase of plasmid length. After amplification, the unmutated methylated parental DNA template was specifically degraded by direct treatment of the reaction mixture with the DpnI restriction endonuclease. This digestion step ensured that only the newly synthesized nicked mutant DNA strands were isolated. The DpnI treated products were then transformed into competent *Escherichia coli* cells, which repaired the DNA nicks *in vivo*, and replicated the mutated plasmids. Finally, plasmid DNA was extracted from the obtained transformant colonies, and DNA sequencing confirmed that the desired mutation was successfully introduced into the Emerin construct.

Cell culture and maintenance

The human colorectal cancer cell line (HCT116T) was grown at 37°C in a humidified incubator with 5% CO₂ and maintained in RPMI 1640 media (Gibco catalog #11875093) supplemented with 10% heat-inactivated FBS, fetal bovine serum (Gibco catalog #A5256801). Human embryonic kidney 293 (HEK293) was grown in Dulbecco's Modified Eagle Medium (DMEM) (Euroclone, Lot #EUM02QQ) with 10% fetal bovine serum (FBS). Cells were passaged every 2 to 3 days after reaching 70-80% confluence by dissolving the adhering layer into a single-cell suspension with a 1X Trypsin-EDTA solution. (Euroclone, Catalog #ECB3052D).

Lentiviral Transduction

The designed plasmid was transduced via lentivirus to enable stable integration into the host DNA. Lentiviral particles containing the transgene were first created by co-transfecting HEK293 packaging cells with three plasmids. Packaging plasmid psPAX2 (Addgene, Plasmid #12260) and envelope plasmid pMD2.G (Addgene, Plasmid #12259) were co-transfected with the transfer plasmid (shEMD-pLKO) by Lipofectamine®. The transfection mixture was generated by mixing 8 μ L psPAX2, 2.5 μ L pMD2.G and 10 μ g shEMD-pLKO in 500 μ L Opti-MEM medium. In a separate tube, Lipofectamine (26 μ L) was diluted in 500 μ L of Opti-MEM and incubated for 5 min at room temperature. The two solutions were then mixed and incubated for another 30 min at room temperature to facilitate the development of DNA-lipid complexes. Next, the mixture was applied drop by drop to a 10 cm plate of HEK293 cells grown in 10 mL of DMEM. The next day the culture medium was changed with 5 mL RPMI medium to concentrate the viral production. Viral supernatant was collected by syringe after an additional 24 hours and filtered through a 0.45 μ m filter to remove cell debris. Target cells were infected with 1-1.5 mL (for a 6-well plate) or 5 mL (for a 10 cm dish) of the filtered viral supernatant. Finally, polybrene was dissolved to a final concentration of 8 μ g/mL to promote viral entry and the target cells were incubated for 4 to 24 h depending on the experimental needs.

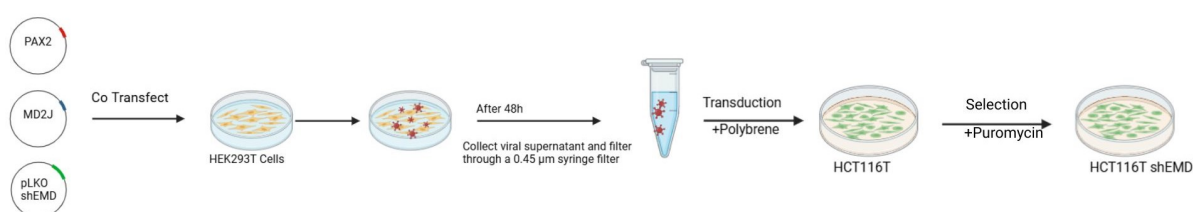


Figure 8. Lentiviral packaging and infection, created by BioRender.

Transfection via Lipofectamine

The designed plasmid was transduced by lentivirus for stable integration into the host DNA. Lentiviral particles containing the transgene were generated by co-transfection of 3 plasmids in HEK293 packaging cells. The packaging plasmid psPAX2 (Addgene, Plasmid #12260) and envelope plasmid pMD2.G (Addgene, Plasmid #12259) were co-transfected with the transfer plasmid (shEMD-pLKO) using Lipofectamine®. The transfection mix contained 8 μ L of

psPAX2, 2.5 μ L of pMD2.G, and 10 μ g of shEMD-pLKO in 500 μ L of Opti-MEM medium. In another tube, 26 μ L of Lipofectamine was diluted in 500 μ L of Opti-MEM and incubated for 5 min at RT. The two solutions were then mixed and incubated for a further 30 min at room temperature to allow DNA-lipid complexes to form. The mixture was added dropwise to a 10 cm dish of HEK293 cells cultured in 10 mL DMEM. The next day, the culture medium was changed to 5 mL of RPMI medium to enrich viral production. After another 24 h, the viral supernatant was collected by syringe and filtered through a 0.45 μ m filter to remove cellular debris. The filtered viral supernatant was applied to infect target cells (1–1.5 mL for a 6-well plate and 5 mL for a 10 cm dish). Finally, the cells were incubated with polybrene at a final concentration of 8 μ g/mL to enhance virus entry and cultured for 4–24 h based on the experimental needs.

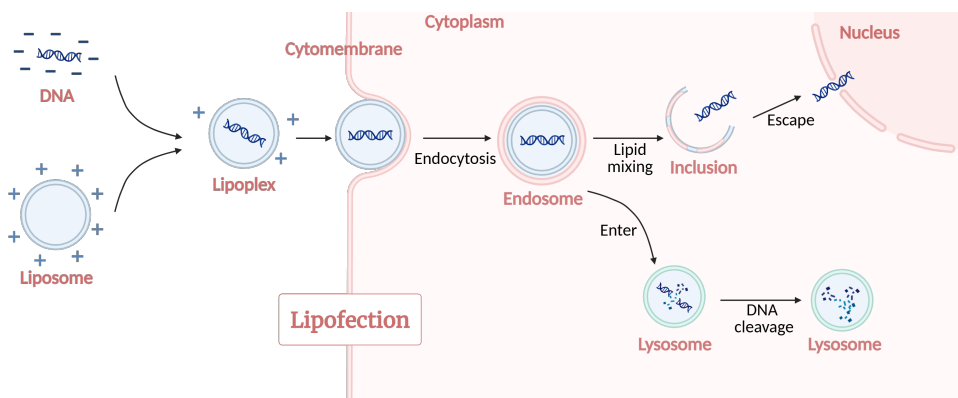


Figure 9. Transfection via Lipofectamin, created by BioRender.

Mechanical Cell Compression

Polyacrylamide gels were cast on 24 $\times$ 24 mm glass slides to match the physiological tissue stiffness and to facilitate *in vitro* cell compression. The slides were cleaned with 70% ethanol and dried. They were functionalized for 10 min with a prehydrolyzed solution of 2% APTES in 95% ethanol. The functionalized slides were washed three times in 95% ethanol and air-dried. To obtain a 10 kPa Young's modulus¹, 1 mL of prepolymer solution was prepared by diluting 40% acrylamide and 2% bis-acrylamide stock solutions (Bio-Rad) in 1X phosphate-buffered saline (PBS) to final concentrations of 7.5% and 0.95%, respectively. Polymerization was initiated by addition of 10% ammonium persulfate (APS; 1:100 v/v) and tetramethylethylenediamine

¹Young's modulus (E) quantifies a material's intrinsic elastic stiffness and resistance to deformation. In mechanobiology, it is mathematically defined as the ratio of applied mechanical stress (σ , force per unit area) to the resulting structural strain (ϵ , proportional deformation), expressed as $E = \sigma/\epsilon$. By matching this parameter to physiological levels (e.g., 10 kPa), it is ensured that the *in vitro* mechanical microenvironment accurately mimics natural tissue rigidity.

(TEMED; 1:1000 v/v). Immediately, 250 μ L of this mixture was pipetted onto a waterproof glass surface and polymerized directly against the functionalized slide. Slides coated with gel were then stored at 4°C in an appropriate culture medium until they were needed for experimentation. To mechanically compress cells, the culture medium was aspirated from the culture dish. The polyacrylamide coated slides were gently placed on top of the cell monolayer with the gel side down in even contact with the cells. Then a weight was placed on the slide to steady the confinement assembly and prevent it from moving laterally in any way. Finally, a small volume of fresh culture medium was added back to the dish to maintain the viability of the cells during the confinement period.

Protein Extraction and Immunoblotting

Proteins were extracted from cell monolayers by intracellular extraction using a Radioimmuno-precipitation Assay (RIPA) buffer. The lysis buffer was 50 mM Tris-HCl (pH 8.0) and 150 mM NaCl to maintain physiological pH and salt concentrations, and a detergent mixture of 1% NP-40, 0.5% sodium deoxycholate, and 1% SDS to lyse membranes efficiently. Immediately before use, a protease inhibitor cocktail (Calbiochem® #539134) (1:400 dilution) was added to protect the released proteins from proteolytic degradation. Total protein concentrations were determined by a standard Bradford assay (Sigma-Aldrich, #0000481629) for equalized protein loading between all samples. The matched samples were mixed with SDS loading buffer and boiled at 95°C for 10 min. The boiling step was to completely denature the proteins to allow proper size separation by SDS-PAGE. After electrophoresis, the separated proteins were electro-transferred onto Amersham™ Protran™ 0.45 μ m nitrocellulose membranes. Nonspecific binding sites were blocked by incubation of the membranes in 5% Bovine Serum Albumin (BSA) in TBS-T for 1 hour at room temperature with gentle agitation. For detection of the target, blocked membranes were incubated with primary antibodies overnight at 4 °C. Wash extensively with TBS-T multiple times the next day to remove unbound antibodies. Then the membranes were incubated with the secondary antibody conjugated to horseradish peroxidase (HRP) for 1 h at room temperature. After several final extensive washes, an enhanced chemiluminescence (ECL) substrate (Cytiva, Lot #18105283) was added to generate an HRP-mediated light signal, and the corresponding protein bands were captured using a digital imaging system.

Co-Immunoprecipitation (Co-IP)

In order to investigate specific protein-protein interactions, we performed co-immunoprecipitation using target-specific antibodies and magnetic bead separation of the proteins. Growth medium was aspirated, and cells were gently washed with ice-cold phosphate-buffered saline (PBS) in order to maintain the protein complexes in their natural non-denatured state. Depending on cell confluency, ice-cold lysis buffer (150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% NP-40) with a protease and phosphatase inhibitor cocktail was added immediately to prevent enzymatic degradation. The lysate was incubated for 10 minutes on ice. Cells were then harvested by using a cell scraper and kept on ice. Genomic DNA and nucleic acids were sheared by sonication of the cell suspension 5 times for 30 sec on and 30 sec off at 4°C. Then the suspension was centrifuged at 13000 rpm for 20 min at 4°C, and the protein-rich supernatant was collected. A pre-clearing step was introduced to reduce non-specific background binding in the immunoprecipitation procedure. The protein G magnetic bead slurry (20 μ L) was washed twice with 500 μ L lysis buffer using a magnetic separation rack. Pre-washed beads were incubated with 500 μ L clarified cell lysate (protein concentration adjusted to 250 μ g/mL or 1.0 mg/mL) with continuous rotation for 1 hour at 4°C. The beads were subsequently magnetically separated and discarded, and the pre-cleared lysate was retained. For primary immunoprecipitation, 500 μ L of the pre-cleared lysate was incubated with either target-specific primary antibodies or concentration-matched normal IgG isotype control. The mixture was rotated gently overnight at 4°C to allow formation of stable immunocomplexes. The next day, 20 to 25 μ L of pre-washed magnetic beads per sample were added to the overnight lysate mixture. The solution was then rotated for another 2 hours at 4°C to capture the antibody-protein complexes. Target-bound beads were isolated using a magnetic separation rack and washed five times with 500 μ L ice-cold wash buffer containing 150–250 mM NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.5% NP-40. The washed bead pellets were resuspended in 20 to 40 μ L of fresh 3X SDS sample buffer containing 1/10 volume of 30X DTT to elute the captured proteins. Samples were vortexed briefly, centrifuged, and then a denaturing heat treatment at 95–100°C for 5 minutes was performed. Finally, the magnetic beads were removed, and the eluted protein fraction (15–35 μ L) was loaded on an SDS-PAGE gel for further Western blot analysis.

Phosphoproteomics

For phosphoproteomic analysis cells were plated at a density of approximately 3×10^5 cells/well in standard 6-well tissue culture plates (Falcon, Cat# 353046) twenty-four hours prior to the experiment. After this adsorption time, the monolayers were mechanically compressed in a controlled way at 10 kPa by a specific elastic overlay system. At the end of the compression assay, cells were washed once with PBS and lysed in the wells with a stringent denaturing buffer containing 8 M urea in 100 mM Tris-HCl, pH 8.0. The crude lysates obtained were collected, sonicated three times in a row for shearing of genomic DNA and reduction of viscosity, and cleared from insoluble debris by centrifugation. Each cleared lysate was accurately quantified for protein content with the Pierce BCA Protein Assay (Thermo Fisher Scientific, Cat# 23225) to ensure consistent downstream processing. The enzymatic cleavage of the extracted proteins was performed on a standardized aliquot of 200 μ g of the total protein from the 8 M urea lysates by in-solution digestion. The samples were chemically reduced with TCEP to a final concentration of 10 mM to reduce the disulfide bonds, and the free cysteine residues were alkylated with 2-chloroacetamide to a final concentration of 40 mM during 30 minutes of incubation at room temperature. Samples were diluted to 5 M urea in 100 mM Tris-HCl (pH 8.0) and pre-digested with Lys-C at an enzyme to protein ratio of 1:50 for 1 hour at room temperature in order to optimize conditions for enzymatic activity. The urea concentration was diluted to 2 M and a thorough digestion was performed overnight at room temperature using trypsin at a ratio of 1:44. The peptide mixtures were acidified to a final concentration of 5% formic acid and desalted using Sep-Pak C18 cartridges (C18 Classic Cartridge, Waters). The cartridges were conditioned with 50% acetonitrile in 5% formic acid, equilibrated with 5% formic acid, loaded with the samples and washed thoroughly. Finally, the peptides were eluted using 80% acetonitrile in 5% formic acid. The purified eluates were dried down completely in a SpeedVac and resuspended in 0.2% trifluoroacetic acid. A stringent sequential enrichment strategy was applied to specifically enrich for phosphorylation peptides from the complex background mixture combining titanium dioxide (TiO₂) microparticles (Titansphere 5 μ m, GL Sciences) and Ti-IMAC magnetic beads (MagReSyn Ti-IMAC, Resyn Biosciences). Ti-IMAC beads were first equilibrated sequentially with ethanol, 1% NH₄OH and a special loading buffer (80% acetonitrile, 5% TFA, 1 M glycolic acid). Peptide samples were then incubated, washed and actively eluted. To maximize overall recovery, flow-through fractions unbound from the initial Ti-IMAC incubation were collected and subjected to a secondary enrichment step using

the TiO₂ beads. The phosphopeptide eluates from both procedures were pooled, dried down to completion in a SpeedVac, and carefully resuspended in 5% formic acid for mass spectrometric analysis. The phosphoproteomic profiling of these enriched samples was performed at high resolution using a Vanquish Neo nanoLC system coupled to a Quadrupole Orbitrap Exploris 480 mass spectrometer equipped with a Nano Flex ESI source and an FAIMS Pro interface (Thermo Fisher Scientific, Bremen, Germany). Phosphopeptides were separated on a 25-cm nano-column (75- μ m inner diameter) packed in house with ReproSil-Pur C18-AQ 1.9- μ m resin (Dr. Maisch) at a constant flow rate of 200 nL/min. Each biological sample was analysed in technical replicate with FAIMS compensation voltages set to -30/-50 V and -40/-60 V using positive Data-Independent Acquisition (DIA) mode coupled with Higher-energy Collisional Dissociation (HCD) fragmentation. Full precursor MS spectra were collected at an ultra-high resolution of 120,000 and the product fragment spectra at 30,000 resolution with a 30 m/z isolation window. Spectronaut (v18.5) was used to systematically process raw mass spectrometry data files by performing a directDIA+ algorithm search with an in-house developed phosphopeptide library extension. Trypsin was defined as the primary cleavage enzyme, allowing a maximum of two missed cleavages. Carbamidomethylation of cysteine residues was specified as a fixed modification and oxidation of methionine, N-terminal acetylation and specific phosphorylation of serine, threonine and tyrosine residues were specified as variable modifications. A high false-discovery rate (FDR) threshold of 0.01 was applied uniformly to both the peptides and protein-groups, and a post-translational modification (PTM) localization filter was applied with a probability threshold of 0.75. Quantification was based on MS1 precursor ion intensities with cross-run normalization intentionally turned off. Quantile-centering normalization was followed by subsequent statistical evaluations using Perseus (v2.0.10.0) and volcano plots were generated in R (v4.3.1). Finally, additional data visualization and basic statistical analyses throughout the larger study were performed using standard computational platforms such as Fiji/ImageJ (for microscopy), FlowJo (v10.10, BD Biosciences, for flow cytometry), GraphPad Prism 10 (v10.6.1, GraphPad Software, Inc., for general statistics), and Adobe Illustrator (v30.1, Adobe Inc., for final visual assembly).

Sammy-seq

SAMMY-seq was carried out in collaboration with the group of Francesco Ferrari and with technical assistance from Federica Lucini following the following protocol originally published

in Lucini et al., 2024 [124].

Chromatin fractionation: For 4f-SAMMY-seq, 3 million fibroblasts or 1–2 million myoblasts were detached from the culture plate by 3 min incubation in Trypsin-EDTA solution at 37°C, 5% CO₂. After two washes in cold PBS, the cells were resuspended in 600 µl of CSK-Triton buffer (10 mM PIPES pH 6.8, 100 mM NaCl, 1 mM EGTA, 300 mM Sucrose, 3 mM MgCl₂, 1 mM PMSF, 1 mM DTT, 0.5% Triton X-100, with protease inhibitors). After 10 min incubation on a wheel at 4°C, soluble proteins and the cytoskeletal structure were separated from the nuclei by centrifugation at 900g for 3 min at 4°C; the supernatant was labelled as S1 fraction. The pellet was then washed with an additional volume of CSK-Triton buffer, resuspended in 100 µl of CSK buffer (10 mM PIPES pH 6.8, 100 mM NaCl, 1 mM EGTA, 300 mM Sucrose, 3 mM MgCl₂, 1 mM PMSF, with protease inhibitors) and incubated for 60 min at 37°C with 25 U of RNase-free DNase I (Invitrogen, AM2222). To stop DNA digestion, ammonium sulphate was added in the CSK buffer to a final concentration of 250 mM. After 5 min incubation on ice, the sample was pelleted at 900g for 3 min at 4°C; the supernatant, containing digested chromatin fragments, was labelled as S2 fraction. Afterwards, the pellet was washed with 200 µl of CSK buffer and pelleted at 3000g for 3 min at 4°C, then resuspended in 100 µl of CSK-NaCl buffer (CSK buffer with NaCl final concentration increased to 2 M) and incubated for 10 min on a wheel at 4°C. At the end of the incubation, the sample was centrifuged at 2300g for 3 min at 4°C and the supernatant was labelled as S3 fraction. Finally, after two 10 min washes in 200 µl of CSK-NaCl buffer on a wheel at 4°C followed by centrifugation at 3000g for 3 min at 4°C, the pellet was solubilized in 100 µl of 8 M urea; the final suspension was labelled as S4 fraction. For 50k and 10k 4f-SAMMY-seq, 50 000 and 10 000 fibroblasts were used, respectively. They were processed following the 4f-SAMMY-seq protocol described above but using only 2 U of RNase-free DNase I (Invitrogen, AM2222). For 10kh-SAMMY-seq, 10 000 fibroblasts were processed following the 4f-SAMMY-seq protocol described above but halving the volumes in each step and using 12.5 U of RNase-free DNase I (Invitrogen, AM2222); in this way, we achieved a 150-fold increase in the ratio of enzyme units per starting number of cells, while maintaining the DNase concentration unaltered. **DNA extraction, sonication and sequencing:** Fractions S2, S3 and S4 were diluted in TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0) to a final volume of 200 µl and then incubated 90 min at 37°C with 6 µl of RNase cocktail (Ambion, AM2286), followed by 150 minutes at 55°C with Proteinase K (Invitrogen, AM2548) to a final concentration of 0.2 µg/µl. Next, DNA was isolated through

phenol:chloroform:isoamyl alcohol (Sigma, 77617) extraction followed by a back extraction of phenol/chloroform with additional volume of TE1X. DNA was precipitated in 70% ethanol, 0.3 M sodium acetate and 20 µg glycogen overnight at -20°C or 1 h in dry ice and resuspended in nuclease-free water. S2 from 4f-SAMMY-seq was additionally purified using PCR DNA Purification Kit (Qiagen, 28106) and then DNA fragments in this fraction were separated using AMPure XP paramagnetic beads (Beckman Coulter, A63880) to obtain S2S (< 300 bp) and S2L (> 300 bp) fractions. In detail, beads were added to the S2 fraction in a 0.95× (v/v) ratio, so to bind fragments larger than 300 bp while leaving smaller fragments in solution. Magnetic separation of beads from supernatant allowed the physical separation of larger fragments (on the beads) from shorter ones (in the supernatant). Larger fragments bound on the beads were then washed in 85% ethanol, resuspended in water and magnetically separated from the beads (S2L fraction). Shorter fragments in the supernatant of the first step were bound to beads by adding a further 0.85x (v/v) beads ratio to the suspension; after washing in 85% ethanol and resuspending in water, they were also detached from the beads (S2S fraction). Separation of S2S and S2L from S2 fraction of 10kh-SAMMY-seq was also tested for one sample (C004_r1); since the enrichment profile of the sequenced S2S and S2L fractions was identical, this passage was later avoided (C002_r1, C004_r2). After DNA isolation, S2 (from 10kh-SAMMY-seq), S2L (from 4f-SAMMY-seq), S3 and S4 (from both 10kh- and 4f-SAMMY-seq) fractions were transferred to screw cap microTUBEs (Covaris, 004078) and sonicated in a Covaris M220 focused-ultrasonicator to obtain a smear of DNA fragments peaking at 200bp (settings: water bath 20°C, peak power 30.0, duty factor 20.0, cycles/burst 50; duration: 125 s for S2 and S2L, 175 sec for S3 and S4). DNA in the fractions was then quantified using Qubit dsDNA HS Assay Kit (Invitrogen, Q32854) and a Qubit 4.0 fluorometer; quality control was performed by run on an Agilent 2100 Bioanalyzer System using the High Sensitivity DNA Kit (Agilent, 5067-4626). Libraries were then created from each fraction using the NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB, E7645L) and the Unique Dual Index NEBNext Multiplex Oligos for Illumina (NEB, E6440S); final qualitative and quantitative controls were performed through an Agilent 2100 Bioanalyzer System and a Qubit 4.0 fluorometer. Libraries with distinct adapter indexes were multiplexed and, after cluster generation on FlowCell, sequenced for 50 bases in paired-end mode on an Illumina NovaSeq 6000 instrument at the IEO Genomic Unit in Milan or for 100 bases in single-end mode on an Illumina NextSeq 2000 instrument at Ospedale Policlinico in Milan. A sequencing depth of at least 24.9 million raw sequencing reads was

obtained for each sample.

Immunofluorescence Staining

Glass coverslips were coated with fibronectin (20 $\mu\text{g}/\mu\text{L}$) to facilitate cell adhesion. Following a 30-minute incubation, the coverslips were rinsed twice with phosphate-buffered saline (PBS) to remove excess protein. 370,000 cells were seeded per well and incubated overnight to allow firm adhesion. The next day, cells were fixed for exactly 10 min in 4% paraformaldehyde (Elabscience, #E-IR-R113) to maintain the cellular architecture before staining. The fixative was removed and cell membranes were permeabilized for 10 minutes in 0.1% Triton X-100 in PBS to allow access of intracellular antibodies. The permeabilization buffer was aspirated and the samples were blocked with 5% Bovine Serum Albumin (BSA) in PBS-T (with 0.1% Tween) for 1 hour to reduce non-specific binding of the antibodies and reduce background signal. Cells were incubated overnight at 4°C with primary antibody diluted in 1% BSA/PBS-T to maintain protein stability (Lamin A/C Thermofischer #MA3-1000, at a dilution of 1:100 and Emerin Abcam #40688, at a dilution of 1:1000) . The next morning coverslips were thoroughly washed with PBS-T to remove any unbound primary antibodies. Fluorescent secondary antibody (1:400) diluted in 1% BSA in PBS-T was added and incubated in the dark for 30 to 60 minutes. Samples were thoroughly washed with PBS-T three times and cell nuclei were incubated with DAPI for 10min. Following immunolabeling and mounting, Images were acquired using a confocal linear laser-scanning microscope (Zeiss LSM 800) equipped with four lasers [diode laser 405 nm (5 mW), diode laser 488 nm (10 mW), diode laser 561 nm (10 mW), and diode laser 640 nm (5 mW)], two Master gain with high sensitivity, and a 63 $\times$ /1.4NA objective.

Results

1. Compression induced ATR-Dependent Phosphorylation Target Identification

Given the proposed role of ATR as a mechanoresponsive kinase acting on nuclear envelope structural components, we first undertook an unbiased phosphoproteomic screen to identify the repertoire of proteins phosphorylated by ATR specifically in response to mechanical compression (Figure 10A). The study was performed in two cell lines, colon cancer cell line HCT116 and the retinal epithelial cell line hTERT-RPE1 under control and compressed conditions. These are representative of both cancer and healthy cell lines allowing us to draw comparisons between the response to mechano-induced stresses between the two conditions. Compression was achieved through the use of overlays composed of polyacrylamide at a defined stiffness of 10kPa. We chose to work within this regime as it is the upper limits of solid stress found in solid tumors. We analyzed the phosphoproteome from six experimental conditions: uncompressed control, uncompressed control with acute treatment with ATR inhibitor (10 μ M VE821), mechanical compression (10 kPa for 10 and 60 minutes) and mechanical compression (10kPa for 10 and 60 minutes) under ATR inhibition. Following cell lysis in 8M Urea, the recovered proteins were enzymatically digested into peptides through sequential digestion with Lys-C and Trypsin. Phosphorylated peptides were selectively and sequentially enriched from a complex background mixture using titanium dioxide (TiO₂) microparticles and Ti-IMAC magnetic beads. These custom matrices have a strong chemical attraction for phosphate groups, such that non-phosphorylated peptides were washed away during the flow-through, but target phosphopeptides were retained (Figure 10A). The concentrated phosphopeptides were then extracted and analyzed by high-resolution liquid chromatography-tandem mass spectrometry (LC-MS/MS) to capture the complete phosphorylation dataset. For the purposes of this thesis we focused on the analysis performed in colon cancer cell line HCT116. Hierarchical clustering and heatmap visualization of the phosphoproteomic data for the HCT116 cell line revealed distinct shifts in the global phosphorylation landscape across all experimental conditions (Figure 10B). Crucially, the analysis identified a specific subset of peptides that exhibited compression induced changes in phosphorylation state. A fraction of these peptides displayed an ATR dependent compression induced phosphorylation. Given the aims of this work, we decided to investigate this sub-group in more detail. Focusing specifically on the ATR dependent phosphorylation

events we performed a Gene Ontology (GO) enrichment analysis to gain insight into the compartments with differential phosphorylation following compression(Figure 10C). Network analysis showed a strong enrichment in structural and biomechanical pathways with a highly prominent cluster specific for “nucleus organization”. This strongly supports the hypothesis that ATR is a mechano-responsive kinase that participates in the preservation of nuclear integrity under mechanical stress.

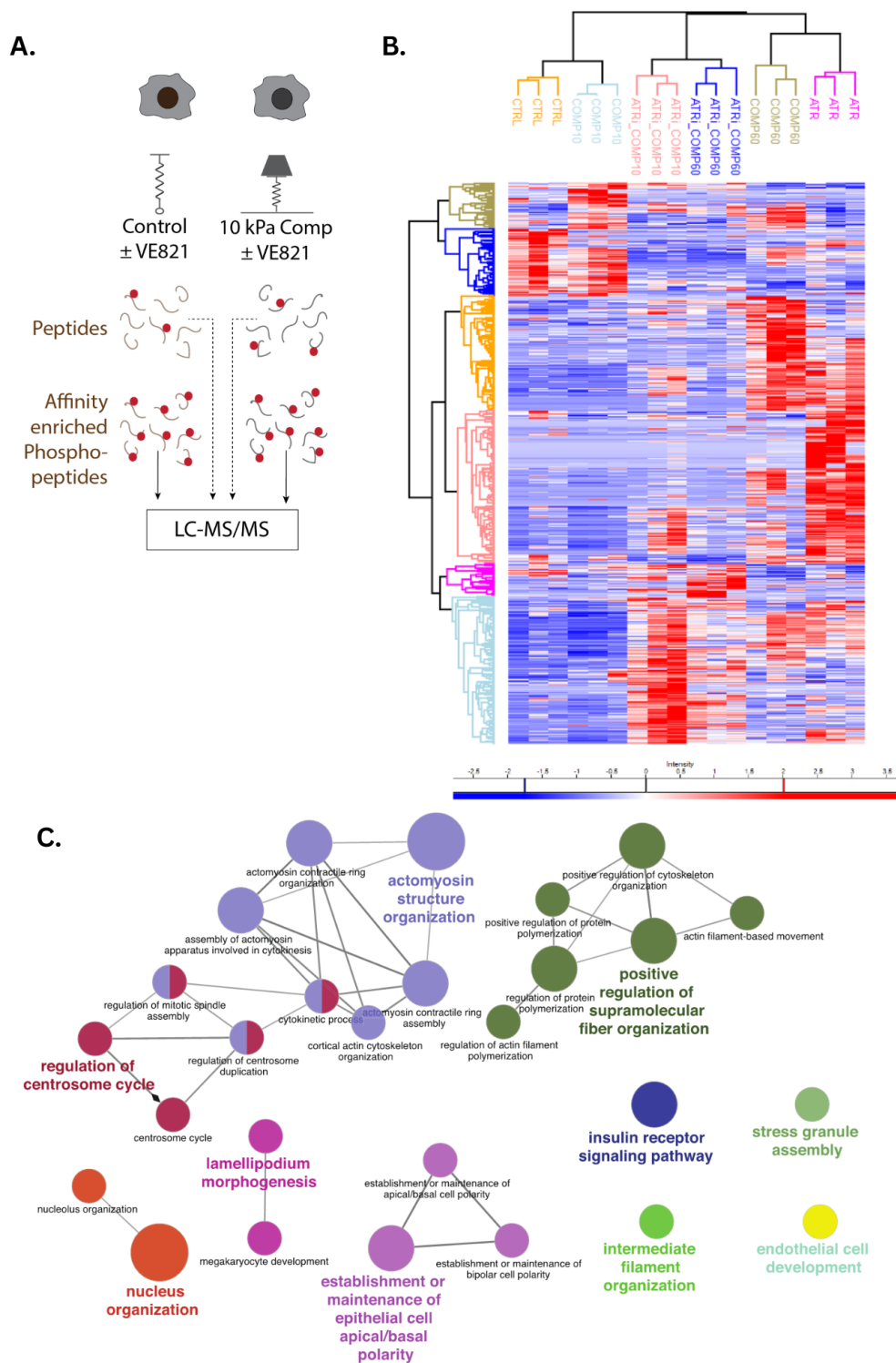


Figure 10. Compression induced ATR-Dependent Phosphorylation Target Identification. A. Schematic of phospho-proteomic screen to identify compression induced ATR targets. B. Heat-maps highlighting the comparison of differentially phosphorylated protein in control versus VE821 treated conditions following 10 minutes of compression. C. GO term enrichment analysis of differentially phosphorylated proteins.

2. Nuclear specific proteins that undergo ATR dependent phosphorylation following mechanical stress

To further understand the role of ATR in the nuclear response to compression we identified the nuclear resident ATR -dependent phosphorylation events. This detailed analysis identified a specific subset of proteins targeting the nuclear envelope, chromatin remodeling complexes, and the chromatin network itself, which are regulated by ATR-dependent phosphorylation upon mechanical compression (Table 1). ATR has previously been shown to re-localize to the nuclear envelope following mechanical stress. This previous observation from the lab drove us to focus on proteins known to localize to the inner nuclear membrane [119, 121]. Key among these proteins was Emerin (EMD), an integral protein of the inner nuclear membrane that is tightly associated with the nuclear lamina. It is also a known interactor of both BAF and HDAC3. Through these interactions it is believed to coordinate both chromatin tethering and compaction state at the inner nuclear membrane. Intriguingly ATR-dependent compression induced phosphorylation of Emerin was found in both HCT116 and hTERT-RPE1 cell lines, further strengthening it's choice as a target for us to follow-up on. Together, these findings identify Emerin as a robust and reproducible target of ATR-dependent phosphorylation under compressive stress, nominating it as a candidate mechanochemical link between ATR activity and nuclear envelope/chromatin dynamics.

Table 1. Nuclear specific proteins that undergo ATR dependent phosphorylation following mechanical stress.

Nuclear envelope	
LMNA	A-type lamin; nuclear lamina structure
EMD	Emerin; inner nuclear membrane, lamina-associated
LMNB2	B-type lamin; nuclear lamina structure
TMPO	LAP2; inner nuclear membrane/lamina
TOR1AIP1	LAP1; inner nuclear membrane
Chromatin remodeling	
ARID1A	SWI/SNF (BAF) complex subunit
SMARCA4	BRG1; SWI/SNF ATPase
BRD7	PBAF (SWI/SNF) subunit
BRD8	NuA4/Tip60 complex subunit
BRD9	ncBAF complex subunit
CHD1	Chromodomain helicase DNA-binding protein
INO80B	INO80 complex subunit
Chromatin	
H1-4	Linker histone H1
HMG1	HMG architectural chromatin protein
TASOR2	HUSH complex, heterochromatin silencing
DOT1L	H3K79 methyltransferase
PYGO2	Wnt/chromatin reader, PHD finger
SUV39H1	H3K9 methyltransferase, heterochromatin
ATAD2	Bromodomain, chromatin reader/coactivator
CHAF1A	CAF-1 subunit; replication-coupled nucleosome assembly
H4C1	Core histone H4
HMG20A	HMG-box, LSD1/CoREST complex
JADE1	HBO1 acetyltransferase complex
KDM1B	LSD2; histone demethylase
L3MBTL3	Polycomb-like repressor
MACROH2A1	Histone variant, heterochromatin/X-inactivation
TRIM28	KAP1; heterochromatin/KRAB-ZNF corepressor
TRIM33	TIF1-gamma; chromatin/TGF-beta signaling
AEBP2	PRC2 (Polycomb) accessory subunit
ARID4B	AT-rich interaction domain; Sin3/HDAC-associated
ASXL2	Polycomb-associated, PR-DUB complex
ATAD2B	Bromodomain, chromatin reader
BRD2	Bromodomain acetyl-lysine reader
CDYL	Chromodomain, transcriptional corepressor
H1-10	Linker histone H1.10
H3-3B	Histone variant H3.3
HMG1	HMG architectural chromatin protein
HMGXB4	HMG-box protein
KMT2A	H3K4 methyltransferase (MLL1)
KMT2C	H3K4 methyltransferase (MLL3)
KMT2D	H3K4 methyltransferase (MLL4)
L3MBTL2	Polycomb-like repressor
MSL1	MSL complex, H4K16 acetylation/dosage compensation

3. Emerin phosphorylation as a regulator of chromatin tethering and compaction

Following identification of Emerin as a mechanically responsive target, we mapped the modified residues to determine their functional significance. In response to compressive stress, the analysis identified two major target residues for ATR-dependent phosphorylation, which are depicted in the structural schematic of Emerin (Figure 11A): Serine 49 (S49) and Serine 171 (S171). These specific residues are located adjacent to or within functionally important domains involved in genome organization. S49 is situated immediately adjacent to the N-terminal LEM domain (amino acids 1–45) that mediates the high-affinity interaction with BAF. Serine 171 is located precisely in the intrinsically disordered region (amino acids 95–202) responsible for the interaction with HDAC3. Emerin binding to BAF and HDAC3 together is essentially required to physically tether and maintain the chromatin state (specifically heterochromatin) to the inner nuclear membrane. Therefore, we hypothesized that ATR-dependent phosphorylation of Emerin following compression could act as a regulatory switch to modulate these critical interactions between Emerin and BAF and HDAC3. We hypothesized that the incorporation of negatively charged phosphate groups at these critical interfaces would sterically hinder and weaken the molecular interactions between Emerin and its key binding partners, BAF and HDAC3. This would lead to temporary detachment of heterochromatin from the inner nuclear membrane preventing catastrophic structural rupture and preserving overall genomic integrity under mechanical stress (Figure 11B). To test the hypothesis that BAF:Emerin interactions are disrupted under mechanical stress we conducted co-immunoprecipitation (Co-IP) to test the physical interaction between Emerin and BAF under control and compressed conditions (Figure 11C). HCT116T cells were subjected to normal resting conditions or to 10 kPa of mechanical compression for 10 and 30 minutes. We then evaluated the interaction between Emerin and BAF. The Co-IP results (Figure 11C) indicated a clear change in binding dynamics between the two proteins under compression. Immunoblot analysis of BAF precipitated fractions demonstrated that in comparison the control samples there was a progressive decrease in the amount of Emerin interacting with BAF after mechanical load. The physical association between Emerin and BAF was visibly decreased at 10 minutes of compression and further decreased at 30 minutes of continuous stress. This biochemical evidence provides direct support for the working model that mechanical compression induces structural uncoupling of the Emerin-BAF complex. To test the involvement of ATR in the compression-induced detachment of Emerin from BAF,

the Co-IP was repeated in the presence of the specific ATR inhibitor VE-821 (Figure 11D). As for the phosphoproteomics, to rule out confounding effects caused by ATR's role as a checkpoint kinase we performed these assays under acute pre-treatment with 10 μ M VE821. Consistent with the previous results, the DMSO control group showed reduction in Emerin co-precipitating with BAF at 30 minutes of 10 kPa compression. However, the VE-821 treatment lead to a maintenance of BAF:Emerin interaction following mechanical stress, effectively ablating the loss observed in control cells. Taken together this data strongly suggest that ATR activity is required for modulating the Emerin:BAF interaction in response to mechanical stress.

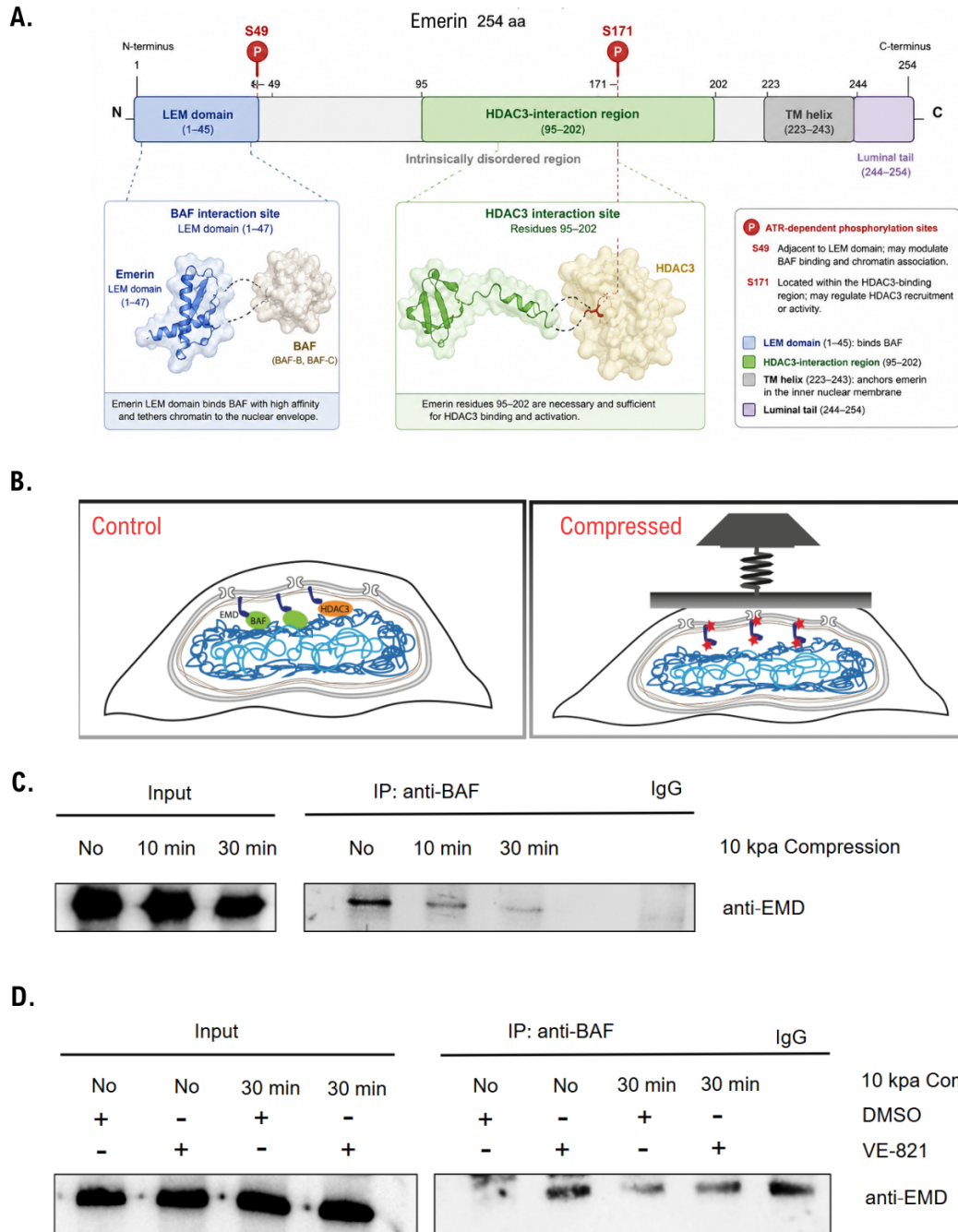
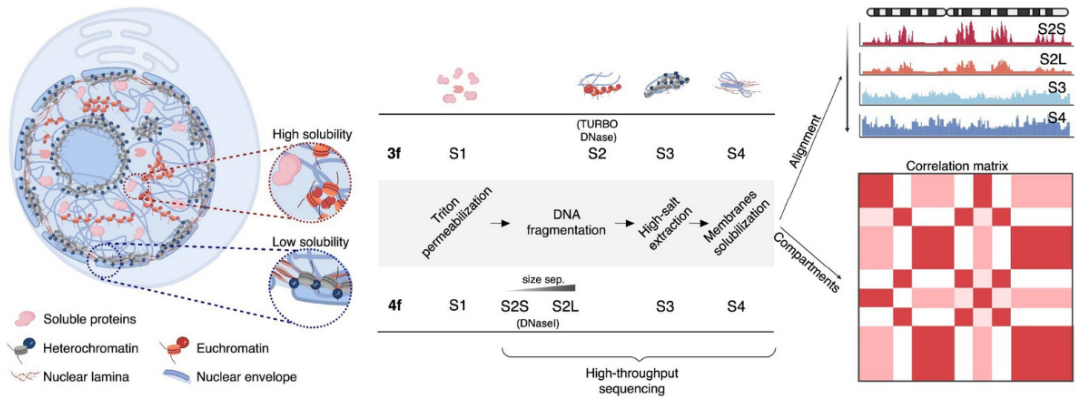


Figure 11. Emerin phosphorylation as a regulator of chromatin tethering and compaction. A. Schematic representation of Emerin detailing the location of phosphorylated serine residues with respect to the BAF and HDAC3 interaction domains. B. Proposed working model illustrating that ATR-dependent Emerin phosphorylation following mechanical compression promotes the remodeling and release of heterochromatin from the nuclear envelope. C. Co-immunoprecipitation (Co-IP) assay demonstrating a reduced interaction between native BAF and EMD following mechanical compression. D. Co-IP analysis revealing an increased interaction between EMD and BAF under both normal and compressive conditions upon ATR inhibition, contrasted with a reduced interaction following compression in the absence of ATR inhibition.

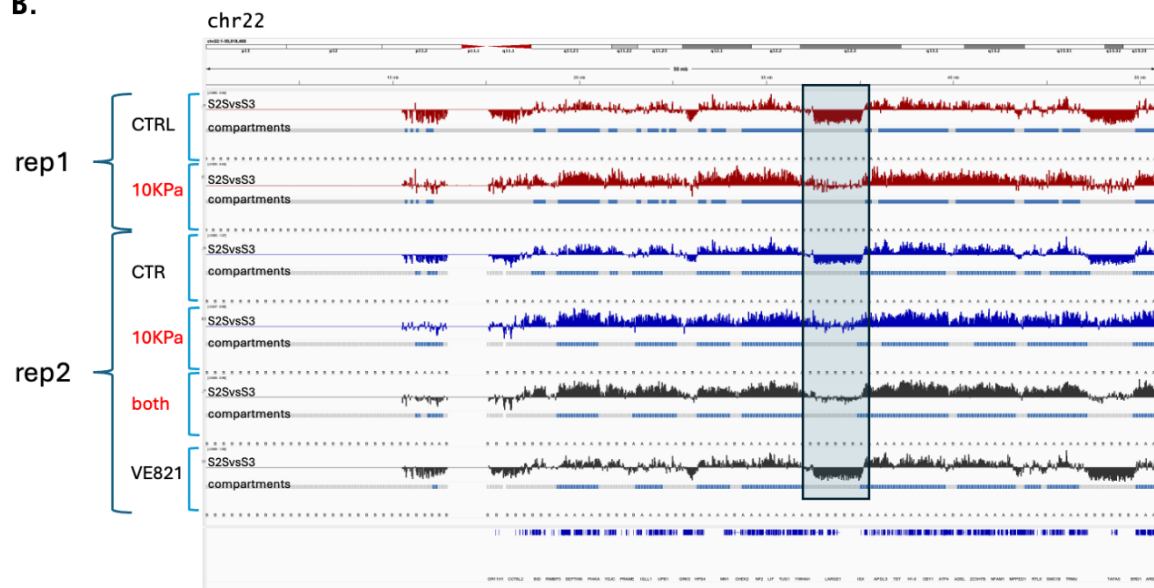
4. SAMMY-seq reveals ATR-dependent regulation of Lamin Associated Domain compartmentalization following compression

Having established that compression triggers ATR-dependent uncoupling of Emerin from BAF at the level of individual protein interactions, we next asked whether this biochemical switch translates into genome-wide consequences for chromatin organization at the nuclear lamina. To determine whether mechanical compression disrupts lamina-associated domain (LAD) organization in an ATR-dependent manner, we employed 4f-SAMMY-seq, a biochemical fractionation approach that separates chromatin into four fractions (S2S, S2L, S3, S4) of increasing solubility, enabling genome-wide identification of chromatin compartments based on differential extractability (Figure 12A). LAD association was assessed by comparing the S2S (soluble, euchromatin-enriched) and S3 (insoluble, heterochromatin/lamina-enriched) fractions across two independent experiments in cells subjected to control conditions, mechanical pressure (10 kPa, 30 min), acute ATR inhibition (VE821 10uM, 2 hrs), or their combination. Genome browser tracks across both replicate experiments showed that compression induced consistent, visible changes in the S2SvsS3 profile relative to control, (Figure 12B). To quantify this effect, we generated metaprofiles of the S2SvsS3 ratio centered on a reference LAD set (4DNFIMZGJCYC). Control cells displayed the canonical LAD signature: a sharp transition at LAD boundaries with positive S2SvsS3 values in flanking non-LAD chromatin and a pronounced negative dip across the LAD body, reflecting S3-fraction enrichment over lamina-tethered heterochromatin. Thirty minutes of 10 kPa compression substantially flattened this profile, reducing the amplitude of the boundary transitions and blunting the negative dip over LAD bodies, indicating a loss of LAD compartmentalization under short-term mechanical stress. ATR inhibition alone (ATRi) did not alter the profile relative to control, whereas combined ATR inhibition and compression (ATRi + 10 kPa) partially restored the depth of the LAD-associated dip relative to compression alone (Figure 12C, left and overlay). Overlaying all four conditions directly confirmed this pattern (Figure 12C, right): control and ATRi-alone profiles were nearly superimposable, 10 kPa compression produced the most blunted profile, and the ATRi + 10 kPa combination fell intermediate between the compressed and control/ATRi profiles. This partial rescue indicates that ATR activity significantly contributes to, the compression-induced loss of LAD heterochromatin compartmentalization.

A.



B.



C.

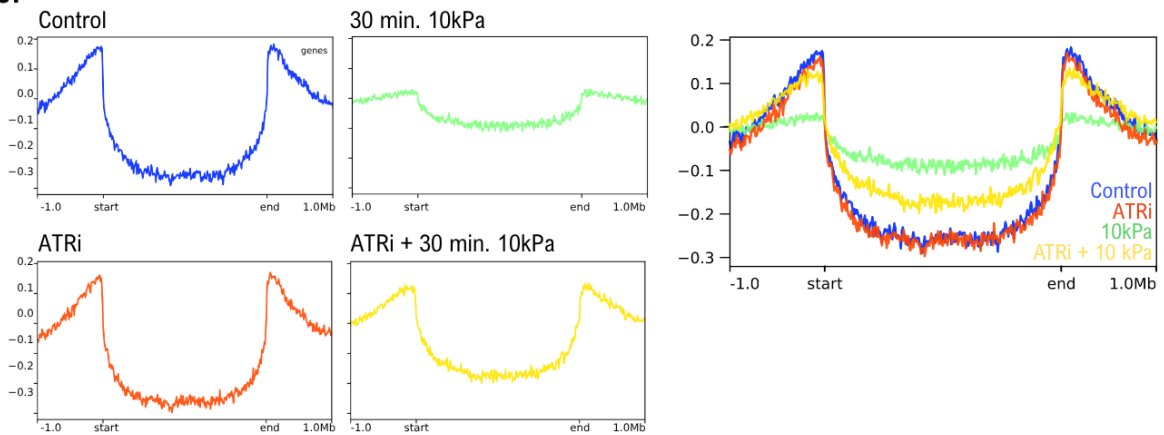


Figure 12. 4f-SAMMY-seq reveals compression induced alterations in the S2S fraction and LAD association. A. Overview of 4f-SAMMY-seq protocol that allows for fractionation based identification of chromatin compartments (taken from Nucleic Acids Res, Volume 52, Issue 12, 8 July 2024, Page e54, <https://doi.org/10.1093/nar/gkae454>) B. Genome browser tracks for two independent experiments highlighting compression induced changes in profiles C. Metaprofile S2S vs. S3 on LADs from publicly available dataset (4DNFIMZGJCYC) for control (blue), 10kPa compressed (green), VE821 treated (orange), and combination VE821 and 10kPa compression, overlay showing partial rescue by VE821 treatment (right).

5. Construct of Mutant Emerin plasmids

Having established that ATR activity is required both for compression-induced Emerin-BAF uncoupling and for genome-wide LAD disorganization, we next sought to directly test whether site-specific phosphorylation of Emerin is sufficient to drive these effects. To this end, we generated a panel of phospho-dead and phospho-mimetic Emerin mutants targeting the two ATR-dependent residues identified in our phosphoproteomic screen. Our phosphoproteomic screen identified both, Serine 49 (S49) and Serine 171 (S171) are potential targets of ATR-dependent phosphorylation in response to mechanical stress. To directly examine the functional consequences of these particular alterations and to additionally disrupt the downstream mechanobiology cascade, developed a series of Emerin mutants that either mimic (S to D mutation) or disrupt phosphorylation at these two sites. Custom-made oligonucleotide primer pairs were designed based on a wild-type Emerin expression vector (pLKO-1 GFP-EMD, a kind gift from P. Maiuri) as the genetic template to introduce targeted point mutations at these two key residues (Figure 11B). Two classes of mutants were generated to dissociate the biochemical state of Emerin with the upstream mechanosensitive signaling. First, the serine residues that were targeted were mutated to alanine (S49A and S171A) and a double mutant (S2A) where both residues were mutated simultaneously, creating "phospho-dead" variants. These mutations effectively block phosphorylation at these specific sites, as alanine lacks the hydroxyl group needed for phosphate attachment. We also mutated the serines to aspartic acid (S49D and S171D) and made a corresponding double mutant (S2D) where both sites were mutated to create "phospho-mimetic" variants. Aspartic acid's bulky, negatively charged side chain mimics the electrostatic and structural properties of a phosphorylated serine, allowing these mutants to constitutively recapitulate the phosphorylated state independent of upstream ATR activity. (Figure 13).

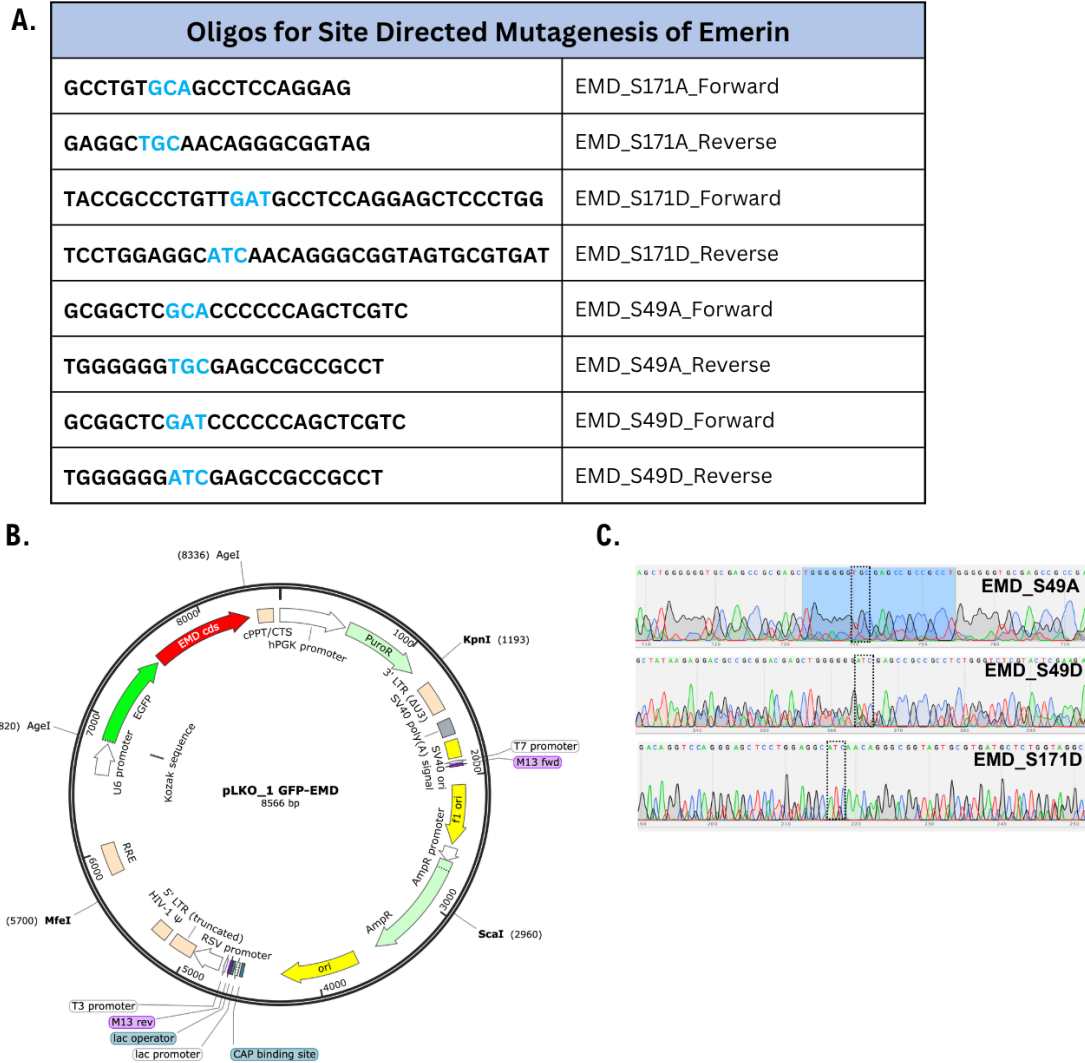


Figure 13. Construct of Mutant Emerin plasmids. A. List of oligos used for site directed mutagenesis. B. Emerin-GFP plasmid (kind gift of P. Maiuri) C. Example sequencing track and alignment from sequencing of new Emerin-GFP constructs.

6. Design and sequence verification of shEMD constructs

Efficient silencing of the endogenous Emerin background was important before introducing the phospho-mutant Emerin constructs to the cells. This step is necessary to guaranty that any observed structural or functional changes in the nucleus are only due to the mutant proteins introduced and not the endogenous wild-type pool. A specific short hairpin RNA (shRNA) silencing strategy was developed to achieve this goal. Custom oligonucleotides were designed using Thermo Fisher tools to specifically target the 3' and 5' untranslated regions of the endogenous EMD transcript. This design specifically targets the endogenous Emerin for degradation by the RNA interference machinery, while the exogenously introduced mutant constructs lacking these

specific recognition sequences would not be affected. The forward and reverse shEMD oligonucleotides were designed to have AgeI and EcoRI cohesive overhangs, respectively (Figure 14A). This allows for precise directional cloning and greatly reduces the chance of vector self-ligation during the assembly process by utilizing two different restriction sites. The annealed oligos were then ligated into a Tet-pLKO-puro lentiviral expression vector (Figure 14B). A Tet-ON inducible system was chosen on purpose so that the knockdown could be controlled in terms of time. This inducible system gives us the ability to knock down in a transient and controlled manner, allowing us to capture the immediate effects of Emerin loss. Finally, to confirm successful and accurate insertion of the shEMD sequence into the Tet-pLKO-puro vector backbone, Sanger sequencing was performed (Figure 14C), confirming that the construct was correctly assembled for subsequent lentiviral transduction.

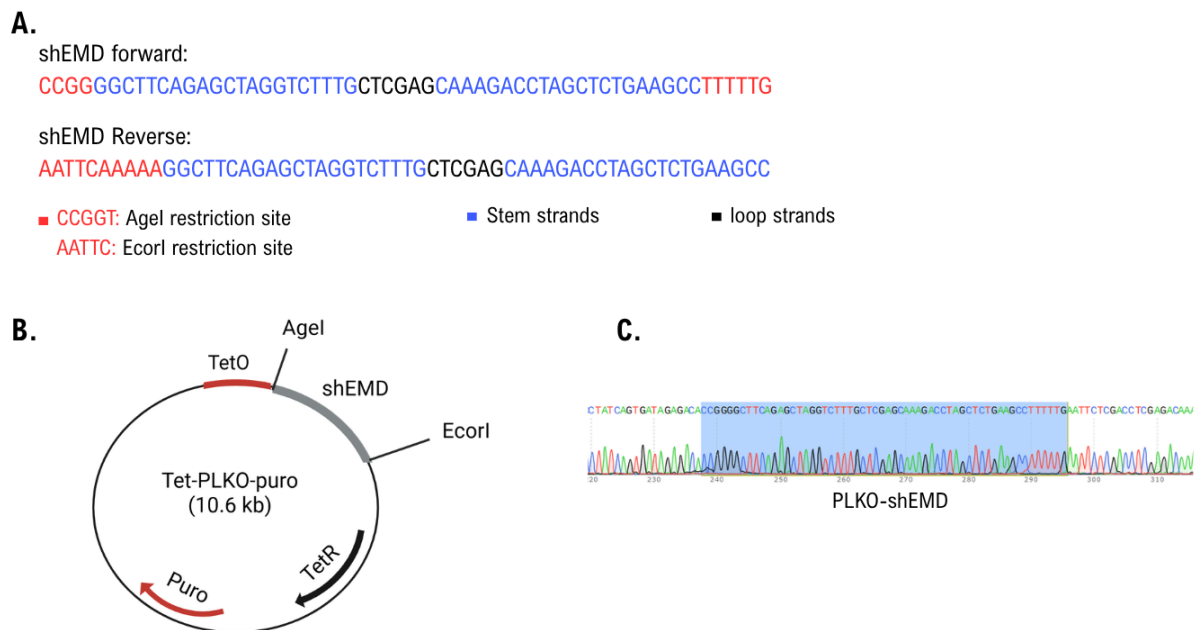


Figure 14. Design and sequence verification of shEMD constructs. A. shRNA oligonucleotides targeting the 3' and 5' regions of the endogenous EMD transcript were designed using the Thermo Fisher Scientific design tool. The sequences incorporate AgeI and EcoRI restriction site overhangs to facilitate downstream cloning into the pLKO expression vector. B. Schematic representation of shEMD oligos cloned into the pLKO vector for inducible gene expression using the Tet-On system C. A representative Sanger sequencing chromatogram confirming the successful insertion of the shEMD sequence into the pLKO vector.

7. Validation of an inducible Emerin-knockdown cell line

After the construction of the Tet-pLKO-puro shEMD plasmid, the construct was introduced into the HCT116 cell line by lentiviral transduction. To obtain a pure population of successfully transduced cells, the culture was selected with puromycin, which thanks to the presence of a

TetR cassette in the original vector selectively eliminates any cells that were not effectively transduced. This approach successfully generated a stable inducible knockdown cell line, termed HCT116-shEMD. To investigate the effectiveness and temporal control of this inducible system, HCT116-shEMD cells were treated with doxycycline at 0.5 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$. Subsequently immunoblot analysis was carried out to track the endogenous Emerin protein levels at 24 and 48 hours post-induction (Figure 15A). Immunoblot analysis shows a marked and visible reduction of total Emerin expression after 48 hours of treatment with either concentration of doxycycline. This confirms a successful and controlled depletion of the endogenous protein and thus a validated background for the subsequent introduction of the mutant constructs. Consistent with the nuclear envelope abnormalities observed in Emery-Dreifuss muscular dystrophy patient cells carrying loss-of-function EMD mutations, we predicted that shRNA-mediated depletion of Emerin would compromise nuclear lamina integrity, resulting in increased nuclear envelope blebbing, altered nuclear circularity, and heightened susceptibility to mechanically induced nuclear rupture. Immunofluorescence microscopy was used to provide visual confirmation of Emerin knockdown and to determine its immediate effect on nuclear architecture, as further confirmation of the immunoblot data. The Emerin fluorescent signal (red) at the nuclear periphery was reduced progressively and strongly in HCT116-shEMD cells upon treatment with 1 $\mu\text{g/mL}$ of doxycycline during the 48 hr induction period (Figure 15B). Imaging revealed structural consequences of Emerin depletion in line with our expectations. Co-staining with Lamin A/C (green), a major structural component of the nuclear lamina, showed prominent morphological changes. Compared with the smooth, broadly spherical nuclei in the untreated control cells, the Emerin-depleted nuclei showed severe deformation of the envelope and nuclear invaginations after 48 hours of treatment with doxycycline. The visual evidence presented here underlines the crucial structural role of Emerin in maintaining the shape and stability of the nucleus, and confirms that Emerin loss highly compromises the integrity of the nuclear envelope.

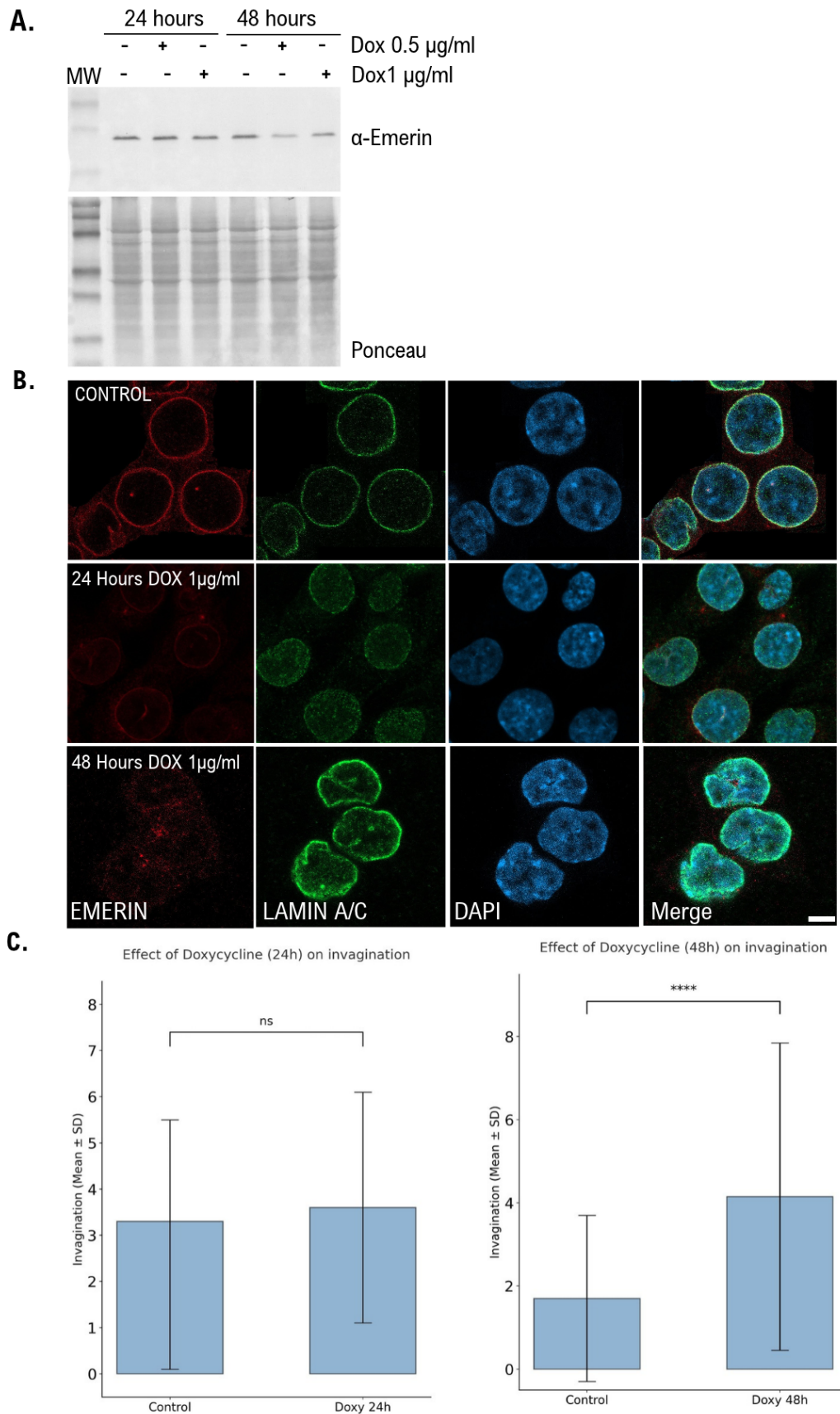


Figure 15. Validation of an inducible Emerin-knockdown cell line. A. Western blot showing endogenous Emerin knockdown in shEMD cells after 24 and 48 hours of doxycycline treatment (0.5 and 1.0 $\mu\text{g/mL}$). Ponceau indicates total protein loading. B. Immunofluorescence showing Emerin depletion (red) and subsequent nuclear envelope invaginations (Lamin A/C, green; DAPI, blue) following 1 $\mu\text{g/mL}$ doxycycline induction. C. Quantification of nuclear invaginations, demonstrating a significant increase at 48 hours, but not 24 hours (mean $\pm$ SD).

8. Emerin wildtype rescue

After successful generation of the inducible HCT116-shEMD knockdown cell line, the next step was to verify whether an exogenously introduced Emerin construct could functionally replace the depleted endogenous protein. First, immunofluorescence imaging established the baseline and knockdown phenotypes. Uninduced control cells (Figure 16A) showed normal, homogeneous localization of endogenous Emerin (green) and Lamin A/C (red) at the nuclear envelope, with a smooth, well-rounded nuclear shape (DAPI, blue). Conversely, 48 hours of doxycycline induction (Figure 16B) successfully depleted endogenous Emerin. This loss caused a severe disruption of the Lamin A/C network and the emergence of deep nuclear invaginations, confirming visually that Emerin is crucial for structural stability. To test whether this structural collapse could be reversed, we transduced the inducible kd cell line with lentivirus from the parental plasmid for wild-type Emerin-GFP and treated the cells with 1 $\mu\text{g/mL}$ amount of doxycycline for 48 hours (Figure 16C). Imaging confirmed the correct and efficient localization of exogenous WT Emerin-GFP to the nuclear envelope. Importantly, the reintroduction of wildtype Emerin-GFP successfully rescued the observed defects in nuclear morphology in the Emerin kd cells. The Lamin A/C network was re-stabilized, restoring the nuclei to their normal, rounded shape. This functional rescue demonstrates that the exogenous construct works correctly and is fully capable of supporting the structural integrity and mechanobiology requirements of the nucleus. This establishes a validated system in which mutant Emerin constructs can now be introduced to study their specific effects without interference from the endogenous protein pool.

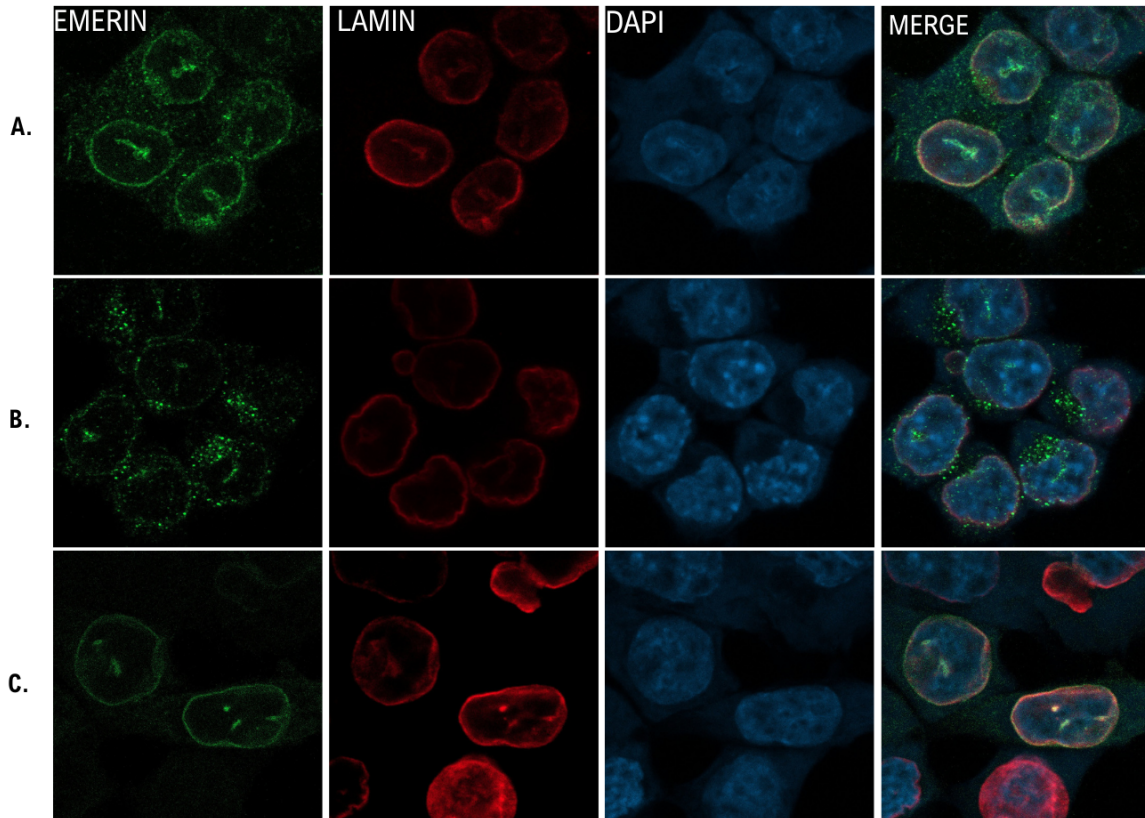


Figure 16. Emerin wildtype rescue. A. Uninduced HCT116T shEMD controls showing normal localization of endogenous Emerin (green), Lamin A/C (red), and chromatin (DAPI, blue). B. HCT116T shEMD cells after 48h Doxycycline induction, displaying Emerin depletion and Lamin A/C network disruption. C. Dox-induced cells (48h) expressing exogenous wild-type Emerin-GFP, demonstrating restored Emerin localization and rescued nuclear morphology.

9. Subcellular localization and nuclear invagination of phospho-mimetic Emerin mutants

After confirming that the inducible knockdown system worked, the next step was to look at where the phospho-mimetic Emerin mutants were found and what they would have on the structure of the nucleus. We transduced Emerin-GFP mutant S171D, S49D, and double mutant S2D constructs in the HCT116 shEMD cells. To investigate the effects of these specific variants on nuclear morphology, the cells were treated with 1 $\mu\text{g/mL}$ doxycycline for 48 hours to effectively knockdown the endogenous Emerin. Immunofluorescence imaging (Figure 17A) confirms that all three phospho-mimetic Emerin variants (green) localize properly and efficiently to the nuclear envelope, forming a discrete peripheral border. However, expression of these mutants leads to severe structural abnormalities. Cells expressing the S171D, S49D, and S2D constructs display profound nuclear invaginations, visually characterized by deep, irregular folding of the Lamin

A/C network (red) and heavily distorted chromatin organization (blue). The severity of the nuclear invaginations was quantified across populations to accurately measure this structural disruption. The mean invagination scores (Figure 17C) indicated a dramatic increase in nuclear envelope deformity in the mutant-expressing cells compared with the controls. In particular, the S171D mutation displayed the most dramatic structural collapse with the highest mean score, while S49D and S2D showed intermediate but significant levels of disruption. The invagination severity classification (Figure 17B) further describes cell populations. The control population displayed healthy nuclei with low severity (scores 0–3), but the introduction of the S171D and S49D mutations heavily shifted the distribution to moderate (4–6) and high severity (≥ 7). To investigate a potential structural basis for the effects of Emerin phosphorylation, we performed AlphaFold predictions of the Emerin–BAF–lamin A/C complex. In the unphosphorylated complex, the lamin A/C Ig-fold was predicted to interact with the BAF dimer in the context of Emerin. Consistent with the imaging data, phosphorylation of Emerin, particularly at S171, resulted in a pronounced predicted rearrangement of the lamin A/C–BAF interaction, whereas the effect was less evident following phosphorylation at S49, as in the double pS49/S171 (Figure 17D). Together, these findings support a model in which Emerin phosphorylation, particularly at S171, contributes to the regulation of nuclear envelope architecture. The pronounced nuclear abnormalities associated with the phospho-mimetic mutants, together with the predicted remodeling of the BAF–lamin A/C interaction and the compression-induced loss of Emerin–BAF association, suggest that phosphorylation-dependent remodeling of the Emerin–BAF–lamin A/C complex may contribute to the structural and chromatin abnormalities induced by mechanical stress.

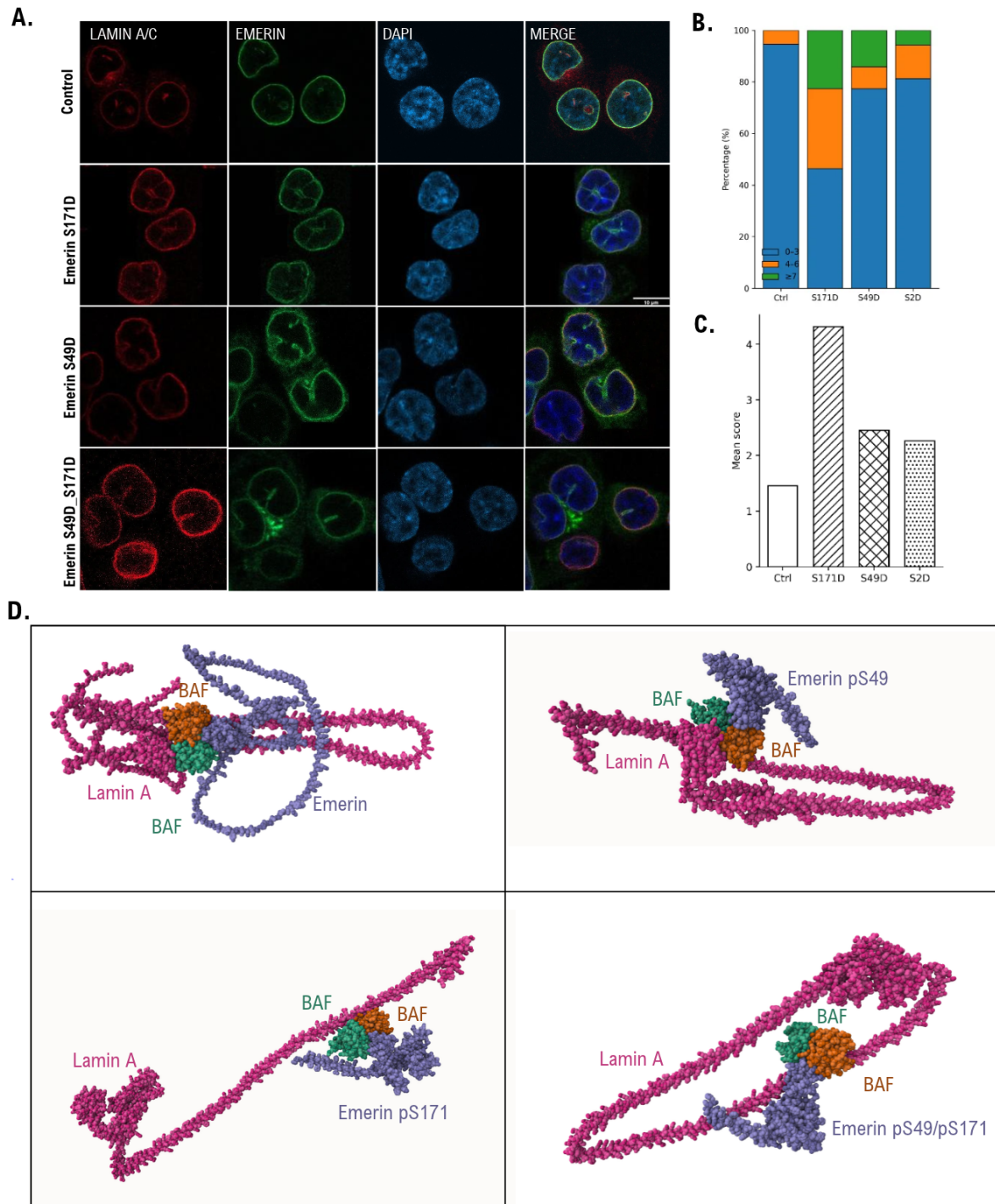


Figure 17. Subcellular localization and nuclear invagination of phospho-mimetic Emerin mutants. A. Immunofluorescence reveals S171D, S49D, and S2D Emerin mutants (green) correctly localize to the nuclear envelope but induce severe structural invaginations (Lamin A/C, red; DAPI, blue). B. Mean nuclear invagination scores for control (Ctrl) and mutant-expressing cells. C. Percentage of cells categorized by invagination severity (scores 0–3, 4–6, ≥ 7), highlighting significant structural disruption by the S171D and S49D mutations. D. AlphaFold predictions for phosphorylation induced structural alterations in the Emerin:BAF:Lamin complex. The Ig Fold of Lamin A is predicted to interact with BAF. This interaction is disrupted following phosphorylation of Emerin on S171.

10. Proposed Mechanobiology Pathway of Nuclear Adaptation

In order to connect together our structural, biochemical, and genomic results, we present a general framework that summarizes the steps of our postulated mechanobiology cascade (Figure 18). In this model, mechanical compression activates the ATR kinase that translocates to the nuclear envelope (Figure 18). As shown in our phosphoproteomic screen, this event induces ATR-dependent phosphorylation of the inner nuclear membrane protein Emerin at Serine 171 (S171) as the major site of modification and Serine 49 (S49) as a secondary site of modification. In its unphosphorylated and unstressed state, Emerin has a stable molecular architecture that allows direct docking of the Ig-fold of Lamin A/C with the BAF dimer. Computational modeling, however, predicts that this ATR-dependent phosphorylation serves as a localized structural switch. In particular, phosphorylation of S171 causes a conformational change that pushes the Lamin A/C Ig-fold away from this central anchoring node. Consistent with this predicted transition, our co-immunoprecipitation assays reveal that mechanical compression induces a dramatic decrease in the association of Emerin with BAF, an uncoupling that can be reversed by ATR inhibition. This disrupted interaction between BAF and the nuclear lamina, which acts as the tether linking heterochromatin to the nuclear lamina, is linked to a global genomic reorganization. As shown in our SAMMY-seq data, this mechanical stress is associated with the detachment of Lamina-Associated Domains (LADs) from the nuclear periphery and thus flattening the genomic architecture. Finally, constitutive expression of phospho-mimetic Emerin mutants reveals that this particular modification is sufficient to induce widespread nuclear envelope invagination, structurally validating the individual steps of this ATR-dependent mechanobiology axis.

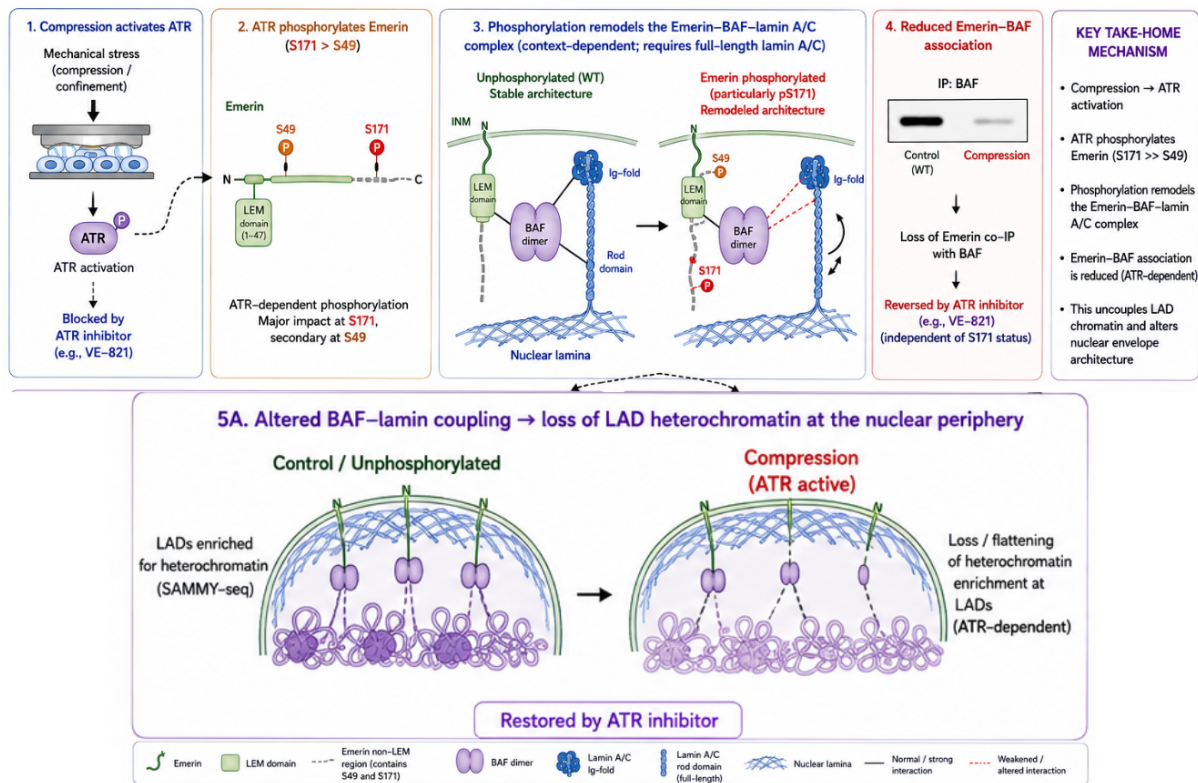


Figure 18. Proposed Mechanobiology Pathway of Nuclear Adaptation 1. Mechanical compression induces the activation of ATR kinase. 2. Activated ATR subsequently phosphorylates the inner nuclear membrane protein Emerin, primarily at Serine 171 (S171) and secondarily at Serine 49 (S49). 3. This targeted phosphorylation physically remodels the Emerin-BAF-Lamin A/C complex; specifically, the pS171 modification forces the dissociation of the Lamin A/C Ig-fold from the BAF dimer. 4. This structural rearrangement leads to a significant loss of Emerin-BAF association, an effect that is completely reversed by acute ATR inhibition. 5. Ultimately, the altered BAF-Lamin coupling triggers the detachment of Lamina-Associated Domain (LAD) heterochromatin from the nuclear periphery. This transient, ATR-driven genomic uncoupling provides the structural plasticity necessary to protect the nucleus from catastrophic damage during severe mechanical stress. This Figure is created by AI

Discussion

A dynamic adaptation of the cell nucleus to the physical forces of the microenvironment is a fundamental process in cellular mechanobiology, especially when dealing with cancer metastasis where cells must survive extreme, compressive forces [2]. Although the Ataxia Telangiectasia and Rad3-related kinase has been extensively characterized for its canonical role in coordinating replication stress and DNA damage response in the nucleoplasm [85], accumulating evidence shows that this ATR kinase also localizes to the nuclear envelope under mechanical load to protect nuclear envelope and genomic integrity [119]. However, the precise downstream structural effectors and molecular mechanisms that translate this mechanical signaling into architectural adaptations remained largely elusive. This study aims to fill this knowledge gap by directly addressing the question of whether mechanical stress activates a ATR-dependent phosphorylation cascade at the nuclear envelope. To address this we asked whether mechanical force induces ATR-dependent phosphorylation of certain structural components of the nuclear envelope. We used an unbiased quantitative phosphoproteomic screen of cells subjected to a 10 kPa compressive load ,the upper physiological limits of solid stress within solid tumors, to identify ATR dependent mechano-induced phosphorylation events. Our phosphoproteomics workflow has revealed unique global changes in the phosphorylation profile of HCT116 colon cancer cells under compressive stress. In particular, Gene Ontology enrichment analysis of the ATR dependent phosphopeptides showed a strong and highly selective enrichment of biological pathways involved in nuclear and actomyosin structure organization. The network enrichment provides strong unbiased evidence that ATR is activated by mechanical forces to modulate structural networks, rather than simply DNA repair pathways. This screen identified the inner nuclear membrane protein Emerin as an ATR-dependent compression induced phosphorylation target. Importantly, the ATR-dependent phosphorylation of Emerin in response to compression was observed in cancer (HCT116) as well as non-cancer (hTERT-RPE1) cell lines. The observation of this cascade in both healthy and cancer models suggests that this axis represents a fundamental physiological response for structural adaptation, rather than an aberrant byproduct of oncogenic transformation. To understand the molecular switch of this ATR-dependent phosphorylation, we mapped the modified residues to their particular structural and functional domains on Emerin. Our analysis revealed two major target residues for ATR-dependent phosphorylation, Serine 49 and Serine 171. These two sites map within close proximity to two important functional domains in Emerin. Serine 49 is directly adjacent to the highly conserved N-terminal LEM domain that mediates

binding of Emerin to the Barrier-to-Autointegration Factor. In contrast, Serine 171 is located within the intrinsically disordered region that interacts with and activates Histone Deacetylase 3. We hypothesized that the addition of bulky, negatively charged phosphate groups at these specific molecular interfaces would sterically block and weaken these protein-protein interactions, a mechanism supported by findings where Ser-173 modifications act as a conformational switch controlling BAF affinity [125]. This disruption is critical, since both the Emerin-BAF and Emerin-HDAC3 complexes are physically required to tether and compact heterochromatin at the inner nuclear membrane. To test this model of structural uncoupling, we performed co-immunoprecipitation assays in HCT116T cells. We were able to confirm that BAF and Emerin were physically associated under baseline, resting conditions. However, upon the application of a compressive load of 10 kPa, we observed a time-dependent and progressive loss of the physical interaction between Emerin and BAF. The loss of interaction was detectable as early as ten minutes following the onset of compression, and it was further reduced after thirty minutes of continued compressive stress. We had hypothesized that ATR's recruitment to the NE would lead to a loss of chromatin attachment. Having observed a compression induced loss of the Emerin:BAF interaction we believed that this could be a mechanism by which BAF mediated chromatin tethering was modulated by mechanical stress. Therefore, we next asked whether ATR kinase activity was required for the weakening of the interaction following compression. We performed co-immunoprecipitation in the presence of the specific ATR inhibitor VE-821 to link the uncoupling to ATR kinase activity. As expected, compression induced loss of Emerin-BAF interaction in DMSO control cells, but acute treatment with VE-821 completely prevented this dissociation, preserving the physical interaction between Emerin and BAF under compression. Biochemical evidence suggests that the catalytic activity of ATR is required to structurally uncouple the Emerin-BAF complex under mechanical compression. Importantly, although the phosphoproteomic data clearly identify Emerin as a target of this mechanobiology cascade, the biochemical nature of this modification needs critical distinction. ATR kinase classically and specifically phosphorylates Serine (S) or Threonine (T) residues immediately followed by a Glutamine (Q) residue, the canonical SQ or TQ motif. However, upon analysis of the Emerin protein sequence, it is apparent that at neither the Serine 49 nor the Serine 171 modification sites is an SQ motif present. This implies that Emerin is not a direct phosphorylation target of the ATR kinase as such. Instead, the data suggest that mechanical compression triggers a broader ATR-dependent signaling cascade, in which

activated ATR reciprocally activates an intermediate downstream kinase that then directly phosphorylates Emerin at these critical residues. Checkpoint kinase 1 is a strong candidate for this intermediate role. CHK1 is the canonical ATR effector and prior studies show that ATR dependent mechanobiology pathways are often mediated via CHK1 activation [119]. To definitively test if Emerin phosphorylation is CHK1 dependent, future work should include replication of the compressive load and co-immunoprecipitation assays in the presence of specific CHK1 pharmacological inhibitors or after targeted CHK1 siRNA knockdown. If CHK1 mediates this biochemical switch, selective inhibition of CHK1 will block Emerin-BAF dissociation upon mechanical compression, thereby directly mimicking the structural rescue seen with ATR inhibition. Using computational AlphaFold structural predictions, we modeled the tripartite Emerin-BAF-lamin A/C complex to elucidate the precise structural mechanisms by which phosphorylation of Serine 49 and Serine 171 disrupts the nuclear envelope matrix. In the unphosphorylated baseline state, the immunoglobulin-fold of Lamin A/C is expected to form a stable interface with the BAF dimer in the presence of Emerin. The modeling of phosphorylation of Emerin at Serine 171 interestingly predicted a major conformational rearrangement that promotes the physical dissociation of the Lamin A/C immunoglobulin-fold from the BAF dimer. In contrast, the phosphorylation of Serine 49 alone seemed to have a much less dramatic effect on the structure. Therefore, the phosphorylation of Serine 171 is the main driver of the structural remodeling in the larger complex. We validated this structural model by performing cellular phenotyping experiments in HCT116-shEMD cells using a doxycycline-inducible knockdown-rescue system. Emerin loss-of-function resulted in profound macroscopic structural defects, such as deep nuclear envelope invaginations, membrane folding, and a dramatic disruption of the Lamin A/C network in resting conditions, that are highly reminiscent of the nuclear envelope dysmorphism seen in muscular dystrophy patients with mutations in the EMD gene [77]. The expression of exogenous, wild-type Emerin-GFP, which localized normally to the nuclear envelope and restored the nuclei to a smooth, spherical shape, effectively rescued this phenotype. Using this validated system, we expressed phosphomimetic mutants, specifically S49D, S171D, and the double mutant S2D, which replace the serine residues with aspartic acid to mimic the negatively charged state of phosphorylation constitutively, independent of upstream ATR activity. All three mutants correctly localized at the nuclear periphery, but their expression was enough to cause severe nuclear invaginations, irregular folding of the Lamin A/C network, and distorted chromatin without any external force.

The severity of nuclear invagination quantified across cell populations was perfectly aligned with the structural hierarchy predicted by our AlphaFold models. The S171D mutant showed the greatest structural collapse, with the highest mean nuclear invagination score, and the S49D and S2D mutants showed intermediate, but still statistically significant, structural disruption compared to control cells. These results provide phenotypic evidence that these phosphorylation-mimetic mutations, particularly at Serine 171, impart an abnormal nuclear morphology that is highly similar to the structural defects caused by Emerin loss-of-function mutations. While these visual abnormalities align with the structural destabilization predicted by our AlphaFold models, definitively confirming whether these specific modifications physically uncouple the Lamin A/C-BAF-Emerin complex in cells will require future biochemical validation, such as Co-IP or proximity ligation assays (PLA) performed in these mutant backgrounds. A central question in nuclear mechanobiology is whether localized protein-protein uncouplings scale up to affect global, genome-wide organization. To address this, we employed 4f-SAMMY-seq, a biochemical fractionation and sequencing technique that assesses the compartmentalization of heterochromatin at the nuclear periphery by comparing soluble euchromatin-enriched and insoluble heterochromatin/lamina-associated chromatin fractions. Under uncompressed baseline conditions, we observed a classic, sharp Lamina-Associated Domain signature, characterized by a pronounced negative soluble-vs-insoluble ratio dip across the domain body, representing the expected physical tethering of heterochromatin to the nuclear periphery. However, subjecting the cells to thirty minutes of 10 kPa compression dramatically flattened this profile, blunting the boundary transitions and reducing the depth of the negative dip over the domain bodies. This flattening represents a widespread, physical detachment of heterochromatin from the nuclear lamina under acute compressive stress. Most importantly, our combined treatment of ATR inhibition and compression partially rescued this phenotype, restoring the depth of the domain-associated heterochromatin dip. Because the ATR inhibitor rescued the heterochromatin profile, we conclude that the global remodeling and uncoupling of heterochromatin from the nuclear peripheral envelope under mechanical load is driven in part by ATR signaling. To fully appreciate the implications of these findings, the ATR-dependent Emerin phosphorylation cascade must be situated within the broader landscape of nuclear mechanobiology. Our discovery of the ATR-Emerin-BAF pathway directly resolves key mechanistic gaps in the foundational work of Kumar and colleagues [119], who first reported that ATR activates at the nuclear envelope under mechanical stretch and osmotic stress. While they demonstrated that envelope-associated ATR

coordinates mitogenic chromatin condensation, the specific structural targets carrying out this mechanical response remained speculative. By identifying Emerin as a direct ATR substrate and mapping its Serine 49 and Serine 171 phosphorylation residues, our work provides the exact molecular link connecting physical envelope stress to the upstream checkpoint they proposed [119]. Our biochemical model also provides a detailed molecular explanation for the physical phenotypes described by Kidiyoor et al [123], who showed that ATR-deficient cells are unable to move through confined spaces and suffer severe nuclear invaginations, envelope rupture, and cGAS activation. However, they implicated several components of the LINC complex in mediating mechanical stress, but did not establish a biochemical mechanism linking force to chromatin detachment. Here, we show that the force-induced ATR activity is responsible for the dissociation of the Emerin-BAF complex, which releases the peripheral heterochromatin and results in flattened Lamina-Associated Domain profiles. This transient genomic decoupling serves as an internal shock absorber. Persistent chromatin tethering, due to loss of functional ATR signaling or phospho-dead Emerin mutants, prevents plastic nuclear deformation, accounting for the mechanical collapse and structural dysmorphism observed in ATR-depleted cells under stress. Although this study establishes a novel mechanobiology cascade from ATR activation to structural remodeling, some limitations should be noted. The experimental paradigm focused primarily on a 10 kPa polyacrylamide confinement system on 2D cultured cells to mimic solid-tissue stress. While this is consistent with the upper range of rigidity observed in solid tumors, it cannot recapitulate the complex 3D microarchitecture, fluid shear stress gradients, and dynamic ECM remodeling that are hallmarks of an *in vivo* tumor microenvironment. Furthermore, the time course of the mechanical compression and Co-IP assays was limited to acute responses from 10 to 60 min. Thus, the long-term genomic and phenotypic effects of chronic, continuous compression are yet to be fully elucidated. Future studies will need to use the S49A, S171A, and S2A phospho-dead mutants generated in this study, building on the structural consequences established by the phospho-mimetic constructs. Blocking phosphorylation at these key residues will allow future studies to determine whether preventing this mechanobiology switch forces rigid Emerin-BAF coupling under stress, which is hypothesized to increase rates of catastrophic nuclear rupture. At the same time, the precise role of HDAC3 and its downstream effects on chromatin compaction dynamics remain to be fully elucidated, as the S171 modification maps directly to the intrinsically disordered region responsible for HDAC3 binding. Future studies need to identify the mechanism by which this mechanobiology axis changes histone

deacetylation to dynamically regulate regional chromatin stiffness under mechanical load. Future experimental paradigms should shift from two-dimensional polyacrylamide compression to in vivo confinement models and three-dimensional confined migration microfluidic channels in an effort to more accurately replicate the dense matrix and tight spatial constraints of the physiological tumor microenvironment. Moreover, although this work successfully characterizes the protective structural role of ATR at the nuclear periphery, the initial mechanobiology cascade that governs the relocation of ATR to the nuclear envelope upon mechanical stress remains to be elucidated. Identifying the upstream membrane tension sensors or specific mechanosensitive ion channels that sense physical strain and respond by sending ATR will complete the biological map from the extracellular matrix to the genome and generate a full picture of nuclear adaptation during metastasis.

Conclusion

In this thesis, we find a novel, noncanonical mechanosensory pathway at the nuclear envelope where Ataxia Telangiectasia and Rad3-related (ATR) kinase functions as a regulator of nuclear mechanics and genomic stability under mechanical stress. We show that, unlike the classical role of the nucleoplasm in response to chemical replication stress, mechanical compression induces a localized signaling cascade at the nuclear interface that actively remodels nuclear envelope-chromatin coupling to prevent mechanical failure. We identify the inner nuclear membrane protein Emerin as a major mechanosensitive target for ATR-dependent phosphorylation. compressive load triggers an ATR-dependent mechanobiology cascade that drives the phosphorylation of Emerin at two important regulatory sites: Serine 49 (S49), close to the N-terminal chromatin binding domain, and Serine 171 (S171), within the regulatory region that participates in the interaction with nuclear deacetylases. This site-specific phosphorylation serves as an exact biochemical switch. The phosphorylation at S49 and S171 changes the structural conformation of Emerin, sterically hindering and disrupting the physical interaction of Emerin and the chromatin tethering protein Barrier to Autointegration Factor (BAF), as well as the structural meshwork of Lamin A/C. Cells expressing phospho-mimetic variants of these residues display severe nuclear envelope invaginations and structural abnormalities even without application of external force, suggesting that this biochemical switch is sufficient to drive nuclear membrane and lamina remodeling. Such a local molecular uncoupling translates into a global, genome-wide spatial chromatin reorganization. We find that under compressive force, the tethering of Lamina-Associated Domains (LADs) is actively released, physically detaching peripheral heterochromatin from the nuclear lamina. We show that this spatial redistribution is directly controlled by downstream ATR activity, as inhibition of ATR activation preserves the integrity of the peripheral heterochromatin-lamina architecture under mechanical load. Together, these results establish the importance of the ATR-Emerin-BAF axis in nuclear mechanoadaptation. The cell exploits the fast transient detachment of peripheral heterochromatin from the nuclear envelope under mechanical load to release internal biophysical tension. This transient decoupling provides the cell nucleus with the vital structural plasticity needed to safely deform so as to tolerate the severe physical challenges of compressed tissues and limited migration channels without suffering a catastrophic nuclear envelope rupture, genomic DNA leakage, or subsequent cell death.

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