

Flexible substrata for the detection of cellular traction forces

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By modulating adhesion signaling and cytoskeletal organization, mechanical forces play an important role in various cellular functions, from propelling cell migration to mediating communication between cells. Recent developments have resulted in several new approaches for the detection, analysis and visualization of mechanical forces generated by cultured cells. Combining these methods with other approaches, such as green-fluorescent protein (GFP) imaging and gene manipulation, proves to be particularly powerful for analyzing the interplay between extracellular physical forces and intracellular chemical events.

Cell migration is driven by the physical interactions, termed 'traction forces', that take place between cells and the surrounding environment [1–3]. Evidence strongly indicates that these traction forces are generated by the actin–myosin cytoskeleton and are coordinated with other events – for example, adhesion/de-adhesion – to drive directional movements during such important events as embryonic development, tissue formation and wound healing [1–3]. Numerous studies have also indicated that cells can respond to mechanical signals. Forces generated by fluid shear [4], sound vibration [5], physical impact [6,7] or muscle/non-muscle contractions are transmitted constantly to target cells. These mechanical signals allow cells to detect physical changes in a constant chemical environment and probably have a synergistic role with chemical signals mediated by chemoattractants or growth factors.

Over the past two decades, extensive advances have been made in our understanding of chemical communications, and of cell–cell and cell–substrate adhesions. But progress in characterizing the mechanical interactions has been held back by technical limitations, in particular the difficulty in measuring the miniscule physical forces exerted by single cells over an area no bigger than 50×50 microns.

In the past few years, several methods have been developed to address this problem. Here, we review the basic principles, strengths and limitations of these methods and discuss the biological insight provided by the analysis of traction forces exerted at the cell–substratum interface that are generated by migrating cells.

How are cell–substrate mechanical interactions detected?

Detecting the traction forces generated by single cells generally involves the use of various forms of

transparent, non-toxic, flexible substrata. Mechanical forces induce deformation (strain) of flexible substrata, which is detected with a light microscope. Several studies have used collagen gels for the detection of traction forces (e.g. Ref. [8]); however, the poor mechanical characteristics of these gels have limited their applications.

Silicone substrata

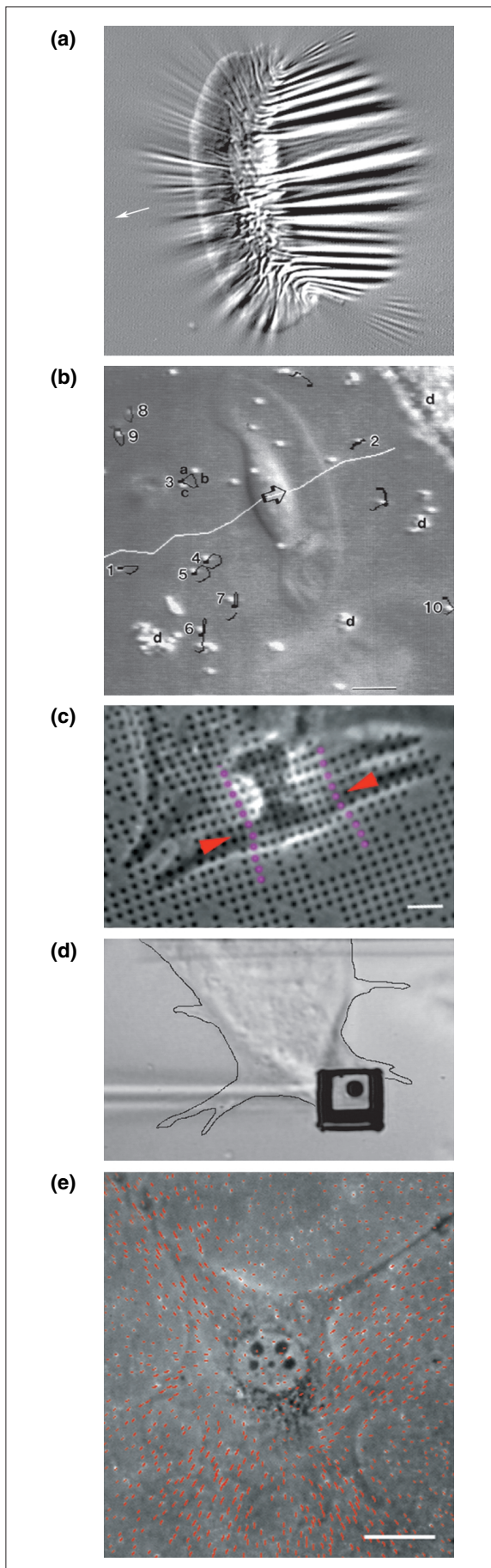
Extensive uses of artificial flexible substrata started about 20 years ago, when Harris *et al.* [9] cultured different cell types on thin films, polymerized with a flame, on the surface of silicone fluid. Compressive forces exerted by adherent cells cause the surface to wrinkle, which is easily visible under the light microscope. Subsequently, the approach was optimized by replacing the flame with ultraviolet light to allow finer control of the flexibility [10]; this generates softer films with a higher density of wrinkles for improved sensitivity and resolution (Fig. 1a).

Although this improved wrinkling method is thought to be sensitive to deformations over as little as $1 \mu\text{m}^2$ and to nano-Newton forces [11], there is neither a simple way to convert the pattern of wrinkles into a map of traction forces, nor is the method appropriate for detecting patterns of forces more complex than isolated regions of compression. Furthermore, although the magnitude of compressive forces has been calculated by multiplying the substratum stiffness with the extent of wrinkling [11], the number reflects no more than a crude estimate. Despite these limitations, wrinkling silicone remains a simple, effective means for studying compressive forces at a qualitative level.

A significant improvement to this method was introduced by adhering the silicone film along its perimeter to the inner wall of a chamber, which prevents the surface from wrinkling but still allows local deformation. In this method, deformation of the surface is detected on the basis of the movement of embedded particles that act as markers ([12]; Fig. 1b). Although the silicone film does not behave exactly like an ideal spring, this method does allow the application of physical equations for estimating the direction and magnitude of traction forces [12]. Such calculations have provided the first reliable estimate of the traction forces under migrating cells [13,14]. So far, however, the studies have been applied

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Fig. 1. Various flexible substrata used to detect traction forces. (a) Motile fish keratocyte on a wrinkling silicone substratum. Arrow indicates direction of migration. Image kindly provided by K. Burton. (b) Motile fish keratocyte on a non-wrinkling silicone substratum. Black tracings indicate the trajectories of embedded microbeads; bar, 10 μm . Reproduced, with permission, from Ref. [14]. (c) Stationary rat cardiac fibroblast causing distortions on a micropatterned silicone substratum with regularly spaced dots. Arrowheads and magenta dots underline the pinching action of the contraction on the elastomer; bar, 6 μm . Reproduced, with permission, from Ref. [15]. (d) Tail region of a chick embryonic fibroblast moving across a detection pad of a cantilever substratum. Reproduced, with permission, from Ref. [20]. (e) Motile NIH 3T3 cell on a polyacrylamide substratum; bar, 10 μm . Red arrows indicate local displacements of beads.



primarily to fish keratocytes, on a non-physiological silicone surface tagged with a limited density of marker particles. Although it should be possible to optimize the method, the complexity of the preparation procedure has limited its development and applications.

A recent development in silicone substrata involves the preparation of sheets of solid elastomers using a curing agent ([15]; Fig. 1c). This generates non-wrinkling substrata with improved mechanical characteristics. In addition, deformation of the surface is determined on the basis of micropatterns of dots or lines, generated by lithography on silicon (Si) or gallium arsenic (GaAs) molds and imprinted onto the surface of the substratum. The regular micropattern has a density of up to 1 dot per 4 μm^2 , and allows the direct visualization of strains. But this approach is currently limited by the availability of micropatterned molds. Moreover, the micropattern creates a physically or chemically textured surface, which might affect cell adhesion and migration through the contact guidance mechanism [16]. Like the other types of silicone substrata, a method has yet to be developed for coating the surface with extracellular matrix (ECM) proteins to create a more physiological environment.

Polyacrylamide substrata

As an alternative to silicone, the flexible substratum can be made from polyacrylamide sheets, which are easy to prepare and have superior mechanical and optical properties [17]. The flexibility of the material is easily controlled by the concentration of acrylamide and/or bis-acrylamide. Furthermore, the porous nature of the material provides a more physiological environment than do solid substrata. Because most cells show no detectable affinity for polyacrylamide, several chemical approaches have been developed to coat the surface with ECM proteins [18], and one can assume that mechanical interactions with such substrata are mediated by the coated ECM or associated proteins.

Deformation is detected by using embedded fluorescent microbeads as markers [18] (Fig. 1e). Because the beads are randomly distributed throughout the substratum and their movements are dependent on the depth from the surface, the image must be carefully focused near the surface of the substratum. In addition, although bead displacements can be observed directly as the cell migrates, for stationary or slow-migrating cells the full extent of deformation must be determined by comparing images of the stressed substratum with a null-force image, which must be recorded after removing the cell by physical or chemical means. The problem with focusing can be alleviated by the recently developed technique of stacking a thin layer of polyacrylamide containing beads on top of a bead-free substratum; this then confines the beads to the top surface of the substratum [19].

Micromachined cantilevers

Instead of using uniformly flexible substrata, an innovative approach has been to use micromachined cantilevers as force transducers on silicon wafers [20] (Fig. 1d). Cells adhere and exert forces on micrometer-sized pads at one end of the flexible cantilever, causing displacements that are detected with high precision on a light microscope.

Unlike flexible sheets in which strain propagates across the surface and requires sophisticated computational analysis for the calculation of traction forces (see below), strains are confined to individual cantilevers and forces can be easily calculated by multiplying the spring constant of the cantilever with the distance of movement. Furthermore, this method can be applied to cells distributed at a high density, whereas uniformly flexible substrata must be used with isolated cells. However, the device is difficult to construct and the surface topology can exert some effects on cell migration, such as those discussed above. Moreover, the spatial resolution is limited by the density of cantilevers and the detection of forces is limited to one dimension – perpendicular to the axis of the cantilever.

How are magnitude and direction of traction forces calculated?

With isolated one-dimensional springs, forces are easily calculated by the product of displacement and the spring constant. This simple approach is applicable to the cantilever method but not to uniformly flexible substrata, where strains propagate across the substratum and fall off as a function of distance from the source of stress. The distribution of deformation must be mathematically deconvolved – in a process similar to the deconvolution of optical images – to obtain the distribution of forces. Generally, the analysis involves two steps: the determination of substrate deformation, and the computation of traction forces.

Originally, substrate deformation was determined by visually identifying corresponding markers in the images with and without mechanical stress [14,21]. The coordinates of these markers were then used to construct a vectorial map. This painstaking process has since been replaced with automatic computer programs based on various forms of the optical flow algorithm [15,22], which searches for the best regional matches between a pair of images and generates vectors at a specified density. Under a correlation-based algorithm, normalized cross-correlation coefficients are used for identifying the most likely fit between image subregions [23]. Using interpolation algorithms, deformation at a given location can then be determined with a precision of 10–100 nm [23].

Two different approaches have been used to convert displacement maps acquired from flexible substrata into maps of traction forces or traction stress (force per unit area). Both approaches are

based on the elasticity theory for the semi-infinite space and can be applied to either micropatterned or bead-labeled substrata comprising different elastic materials.

The method developed by Dembo and colleagues [24,25] makes no *a priori* assumption of the distribution of forces other than that forces must be confined within the boundary of the cell and that net forces and torques equal zero (given the small mass and acceleration, the net forces and torques involved in cell migration are negligible). Because the number of deformation vectors, superimposed with noise, is generally insufficient to provide an unambiguous answer, a probability-based algorithm that favors minimal complexity (i.e. smooth transitions in forces) is used to generate a ‘most likely’ map of traction stress. This approach, although used widely in signal deconvolution, can limit the precision in regions where sharp transitions do exist. Mathematical simulations, using pre-assigned patterns of point forces [26], have placed the current resolution at roughly 2 μm (W.A. Marganski and M. Dembo, unpublished; [27]).

By contrast, the method described by Balaban *et al.* [15] for the conversion of marker displacement to force requires the assumption that forces are exerted only at focal adhesions, which significantly reduces the number of possible answers and possibly allows a more definitive determination of forces at these sites. It is unclear, however, whether forces are indeed exerted only at focal adhesions because many adherent cells show no detectable focal adhesions [26]. Furthermore, the detection of focal adhesions requires additional steps of immunofluorescence or imaging with green-fluorescent protein (GFP) and is subject to uncertainties – particularly for small focal adhesions, which might exert stronger forces than those of large focal adhesions (see below). Such uncertainties might lead to systematic errors in the calculated traction forces.

Calculated force or stress distribution can be visualized as a map of vectorial arrows (Fig. 2a) or rendered as color images after converting the stress magnitude into different colors ([27]; Fig. 2b). In essence, the latter approach functions as a new form of microscopy and has been referred to as ‘traction force microscopy’. It can be used to generate a series of force images during cell migration that can be played back as motion pictures depicting the dynamics of cell–substrate interactions.

What has been learned about cellular mechanical interactions?

The above methods have been applied to study forces exerted during processes such as cell migration [9,11,13,14,20,27,28], growth cone extension [19] and cytokinesis [10]. For migrating fibroblasts, strong traction forces pointing towards the center of the cell have been localized at the anterior and posterior regions [2,24]. Such compressive action is consistent

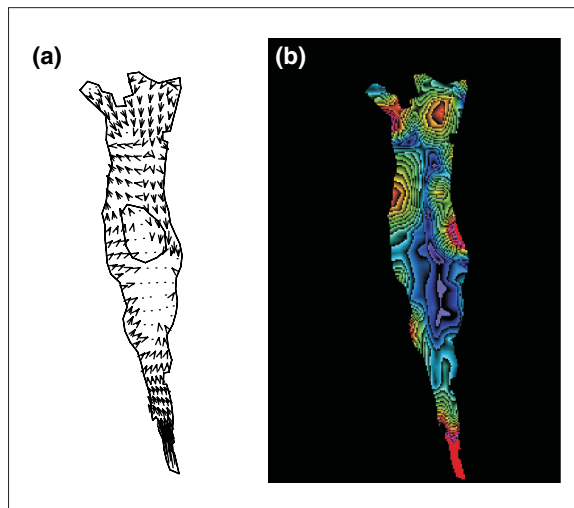


Fig. 2. (a) Vector plot of traction stress generated by a fish fin fibroblast on a polyacrylamide substratum. Arrowheads indicate direction of forces. (b) Color rendering of the magnitude of traction forces, 'hot' colors highlight areas of strongest force and 'cool' colors indicate regions of weaker force.

with the original observations using wrinkling substrata [9]. Recent studies with myosin inhibitors and regional detachment of cells with integrin-binding RGD peptides have indicated further that forces at the front can be generated by active actin–myosin contractions [21,28], whereas those in the rear serve as passive anchors [28]. The distribution of forces supports a frontal towing model of cell migration, in which the frontal regions serve as the 'engine' that tows an adhesive cargo consisting of the cell body and the tail [22].

Although early studies suggested that frontal traction forces are generally localized near focal adhesions [9,21], subsequent scrutiny indicates that not all adhesions produce detectable traction forces. Recent systematic analyses using a combination of traction force microscopy and GFP imaging has led to the surprising finding that, in the frontal region of migrating fibroblasts, it is the small, nascent focal adhesions (sometimes referred to as focal complexes) that generate the strongest traction stress [27]. The magnitude of forces decreases as focal adhesions mature and grow in size. Once the focal adhesions mature, they seem to maintain a constant stress that is independent of their size [15]. These observations corroborate the heterogeneity of size, morphology, protein composition and tyrosine phosphorylation of focal adhesions [29]. In addition, the transient propulsion at nascent focal adhesions provides an elegant, responsive strategy for the cell to coordinate contractility with migration and adhesion. Together, these results suggest that different focal contacts have different mechanical functions depending on their age and the state of cellular motility (Fig. 3).

Studies with fish keratocytes, which have a distinct half-moon morphology in which the long axis lies perpendicular to the direction of cell migration, have produced results with both similarities and

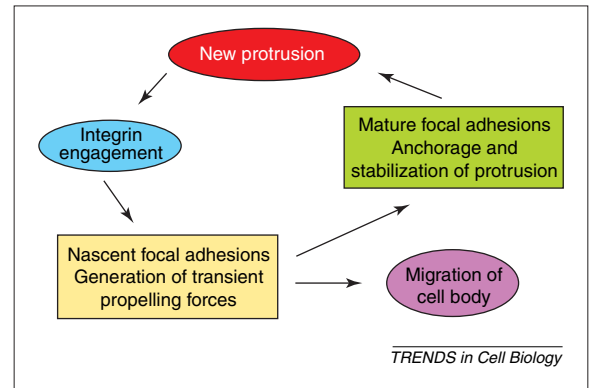


Fig. 3. Relationship between focal adhesions and mechanical forces during fibroblast migration. The formation of focal adhesions in the lamellipodium, accompanied by the generation of a pulse of propulsive forces, drives the forward movement. Cell migration is sustained by repeated formation of nascent focal adhesions, and thus repeated pulses of propulsive forces. Mature focal adhesions, such as those located in the tail, play only a passive role in anchoring cells to the substrate. Adapted, with permission, from Ref. [27].

differences compared with those obtained from fibroblasts. Similar to fibroblasts, strong traction forces lie along the long axis of the cell [30], in a region where new adhesion sites are forming [31]. In keratocytes, however, this region is located near the lateral extremities of the cell, and forces are directed primarily perpendicular to the direction of cell migration. Although a small propulsive component has been identified in a recent analysis [32,33], the results suggest that traction forces are involved not only in cell migration but possibly in other functions such as cell–cell communication and/or mechanosensing (see below).

Flexible substrates have also been used as a means to apply mechanical stimulations to adherent cells, to test the ability of cells to sense mechanical changes in the environment [6,7]. Mechanical forces are exerted by pushing or pulling on the substrate near the cell using a blunt microneedle [34,35]. Cells have been found to reorient towards pulling forces [34], accompanied by an increase in the number and/or size of focal adhesions [35,36]. Conversely, pushing forces cause approaching cells to turn around and move away.

Cells have been also challenged with substrata containing a gradient of stiffness and have been found to move preferentially towards the rigid side [34]. These observations show that cells not only respond to forces exerted through their adhesion sites, but also actively probe mechanical properties of the substratum – a phenomenon termed 'mechanosensing'.

What is likely to be learned from future investigations?

Clearly, cell–cell and cell–substrate adhesions represent both a mechanism for passive anchorage and a mechanism for active physical communications with the environment. These interactions are likely to involve transient, localized activities of the

actin–myosin cytoskeleton and signal-transduction enzymes and cannot be investigated without subcellular characterization of the traction forces, protein interactions and structural organization. Traction force microscopy represents a powerful tool that can be easily combined with other light-microscopy techniques, such as GFP imaging, ion imaging, photobleaching, photoactivation, local drug delivery and micromanipulation, to allow experimentation at a high spatial and temporal resolution. The approach has already been combined with genetic engineering to address the functions of specific proteins [36]. The simplicity of recently developed substrata makes qualitative studies of traction forces feasible for most laboratories. Although quantitative analyses were initially performed with supercomputers, a combination of hardware/software improvements and availability has enabled personal computers to handle the task.

Many important issues need to be resolved. For example, given the differences between nascent focal complexes and focal adhesions in mechanical output, it is important to identify the mechanisms that regulate the production and transduction of contractile forces during the maturation of focal adhesions. The process is likely to involve profound changes in protein–protein interactions. An intriguing observation is that stationary fibroblasts appear to maintain an overall magnitude of traction force similar to that of migrating cells [15], even though they presumably contain only mature focal adhesions. During the transition from migrating to stationary state, therefore, a separate process might cause the traction forces to stay on focal adhesions or to transfer the mechanical load from nascent focal contacts to existing focal adhesions.

Equally important is the mechanism of mechanosensing. Although focal adhesion kinase, microtubules and myosins have been implicated in this process, an integrated mechanism remains to be constructed that probably involves intricate cross-talk between Ca^{2+} , GTP, proteolysis and phosphorylation.

Besides focal adhesions, attention has to be diverted to mechanical interactions at other structures, including close contacts and cell–cell junctions. Although the current methods are designed for analyzing traction forces from isolated single cells, with some modifications they should be able to deal with forces generated by a small colony of cells. An interesting challenge would be to extend the current studies to a three-dimensional setting. In a multicellular organism, mechanical interactions for most cells occur around the whole surface. The current two-dimensional system for studying traction forces creates a marked asymmetry between the dorsal and ventral surfaces, and might yield results that deviate substantially from those in a true physiological setting.

Finally, there is strong evidence that mechanical interactions play an important role in a wide spectrum of specific processes, including embryonic morphogenesis [37], neuronal guidance [19], osteoblast maturation [38] and phagocytosis [39]. Although they probably share some common aspects, the specific functions of mechanical forces in these processes are just beginning to be unraveled. Understanding the interplay between extracellular physical interactions and intracellular chemical events is likely to exert a strong impact on many practical applications, including tissue engineering, stem cell differentiation and treatments of autoimmune diseases and cancer.

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Apoptotic DNA fragmentation and tissue homeostasis

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DNA fragmentation is a hallmark of apoptosis. The tightly controlled activation of the apoptosis-specific endonucleases provides an effective means to ensure the removal of unwanted DNA and the timely completion of apoptosis. Over the past several years, crucial progress has been made in identifying the long-awaited apoptotic endonucleases, and their importance in tissue homeostasis is beginning to unfold. Here, we focus on the most recent discoveries about the functions and mechanisms of these endonucleases in the context of apoptosis. We also discuss consequences that defective DNA fragmentation might have for tissue homeostasis and disease development.

Multicellular organisms have developed an intricate control system to balance cell proliferation and cell death to ensure proper development and tissue homeostasis. Any abnormalities in the cell death process can potentially lead to severe human diseases, including neural degeneration, autoimmunity and cancer [1–4]. Therefore, identifying the molecules involved in cell death and understanding the regulation of the death process are crucial for prevention and management of these human diseases.

In normal development and tissue homeostasis, most of the cells die through physiological or programmed cell death to remove excessive or damaged cells [4]. The term ‘apoptosis’ was first used to describe such cell death with preprogrammed morphological changes including cell and nuclear shrinkage, chromatin condensation and apoptotic body formation in vertebrates [1]. Similar morphological changes were also observed in programmed cell death in invertebrates [4]. The apoptotic morphological changes exhibited by the dying cells are followed by phagocytosis by

scavenger cells. A biochemical hallmark of apoptosis is the cleavage of chromosomal DNA into oligonucleosome-sized fragments, a process called DNA fragmentation [2]. Apoptosis eliminates excessive, mutated, infected and damaged cells and is actively and inherently controlled [1–4].

Because of the fundamental role of apoptosis in development and tissue homeostasis, a cell-suicide program utilizing evolutionarily conserved molecules is dedicated to the process [5,6]. Elegant genetic and biochemical work has identified several families of proteins, such as the Bcl-2 family, that regulate apoptosis, and caspases that mediate apoptosis by cleaving downstream molecules. The importance of these regulators and executors of apoptosis is underscored by the various developmental deficiencies and tumorigenic phenotypes of mice that either are deficient in or overexpress the genes encoding these molecules [7].

Although most research efforts have focused on the more upstream death program molecules such as the Bcl-2 family proteins and caspases, the key molecules involved in DNA fragmentation and the role of cleavage of chromosomal DNA in apoptosis and tissue homeostasis remained elusive. Over the past few years, biochemical and genetic work has identified several endonucleases that play crucial roles in apoptosis. This article focuses on the latest developments in the functions and mechanisms of these endonucleases in the context of apoptosis and discusses the consequences that compromised functions of the endonucleases might have for tissue homeostasis and disease development.

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