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Progress in microscopy has ever yielded many discoveries in biology. Electron microscopic techniques such as freeze-fracture have provided much evidence that cell membranes are organized into functional domains. However, because these techniques are only applicable to fixed or frozen cells, they cannot visualize membrane structures in live cells. Optical methods such as fluorescence imaging have been used to follow the dynamics of specific molecules in live cell membrane. But, they require labeling and hence cannot capture the protein structure and non-tagged molecules. Atomic force microscopy (AFM) does not require complicated sample preparation and has been applied for imaging live cells, but so far molecular resolution on the live cell surface is not achieved. High speed (HS-) AFM developed by our group can visualize the dynamic molecular processes of isolated proteins at sub-molecular resolution.

In this study, we applied HS-AFM to imaging magnetic bacterial cell surfaces. This bacterium takes water, nutrient and iron into the cell interior through the outer membrane (OM). The transport function is accomplished by membrane proteins forming selective channels. For imaging, this bacterium was anchored, in solution, on substrate modified by polylysine and glutaraldehyde. HS-AFM captured the dynamic surface ultrastructure. The AFM movies revealed that OM proteins form a honeycomb structure and the constituent molecules are slowly diffusing. This is the first direct observation of dynamic molecular architectures on a live cell surface.

3K1334 AFM を用いたシングルセル RNAi スクリーンにより M 期細胞メカニクスを制御する疾病関連遺伝子が単離された

A single-cell RNAi screen for regulators of mitotic cell mechanics identifies diseases-associated genes

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The cell coordinates its mechanical properties (e.g. shape, volume, stiffness) as well as biochemical pathways for survival, function, and proliferation. While both aspects are equally important, the cell mechanics is only poorly understood. Using HeLa cells as a model, we recently reported that actomyosin contraction and osmotic pressure drive mitotic cell rounding. In order to understand mitotic cell mechanics in terms of genes and molecules, a single-cell RNAi screen was performed using atomic force microscopy (AFM). Among 400 actomyosin-related and cortex genes screened, we identified 58 genes required for cellular pressure of mitotic HeLa cells. In the hits, there were five disease-associated genes. These hits were further characterized with a combination of genetic methods and AFM. Their RNAi phenotype of altered cellular pressure was rescued in HeLa cells stably expressing the mouse ortholog transgenes, showing that they were genuine hits of the RNAi screen. To identify molecular regulations, treatments suppressing or enhancing their RNAi phenotype were searched using the existing knowledge. Along with the results of secondary screens to further characterize the 58 hits, the mechanisms in which the isolated hits function in mitotic cell mechanics will be discussed.

3K1346 FRET イメージングと統計的信号処理に基づく自発的な細胞運動を制御する細胞内シグナル伝達経路の同定

Identification of feedback loops regulating stochastic cell migration by FRET imaging and statistical signal processing

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Rho-family GTPases Rac1 and phosphatidylinositol (3,4,5) trisphosphates (PIP3) are key regulators of cell migration. Irrespective of extensive research by conventional biochemical methods, the spatial dynamics remains elusive. Here, we attempt to address this issue by fluorescence resonance energy transfer (FRET) imaging and statistical signal analysis. First, we acquired the time-lapse images of stochastic migration of HT1080 fibrosarcoma cells expressing FRET biosensors of Rac1 and PIP3. Second, we developed an image processing algorithm to extract FRET values and velocities at leading edge of migrating cells. By employing cross-correlation analysis between the edge velocity and molecular activities, we found that the edge protrusion preceded Rac1 activities. This result suggested the presence of feedback regulations between Rac1 activity and cytoskeletal dynamics. To validate the feedback regulations, we perturbed the signaling pathway with pharmaceutical inhibitors. Inhibition of

actin polymerization by Latrunculin B decreased Rac1 activity, clearly indicating the presence of a positive feedback from F-actin to Rac1 activity. Furthermore, we also noticed that PI3-kinase inhibitor, LY294002, reduced Rac1 activity only transiently. We searched for the molecular network that reproduced these experimental data, and eventually identified a negative feedback pathway from Rac1 activity to F-actin through MLCK accumulation. These two feedback pathways can be considered as the key factor of the self-organization of leading edge dynamics.

3K1358 Role of traction force and myosin II localization for cell polarity generation in freely migrating *Dictyostelium* amoeba

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Dictyostelium cells decide their migrating direction by generating anterior-posterior polarity in response to reaction forces against their own tractions. Myosin II is indispensable for this polarity generation.

To clarify the relationship between the traction forces and myosin II in polarity generation, traction forces and myosin II dynamics were recorded simultaneously under TIRF illumination during random migration. Large traction forces induced myosin II localization and prevent further expansion of pseudopod there.

To determine what function of myosin II is essential for the polarity generation by traction forces, three types of myosin II heavy-chain-null (*mhcA*⁻) cells were dispersed on a fine microstretching device, which can mimic the reaction forces against the tractions from substratum. The *mhcA*⁻ cells expressing GFP-3xALA myosin II, that constitutively assembles into filaments, and the cells expressing GFP-E476K myosin II, that cannot hydrolyze ATP, migrated perpendicular to the stretching direction. On the other hand, the cells expressing GFP-3xASP myosin II, that cannot form thick filaments, migrated randomly. GFP-3xALA myosin II and -E476K myosin II were localized bilaterally in the horizontal direction.

These results indicate that reaction force, which acts against the traction and induces myosin II localization, has a direct role in the generation of cell polarity. It requires not contraction of actomyosin but only localization of myosin II filaments.

3K1422 プロセッシブにアクチン重合している間の mDia1 の螺旋回転

P08 The helical rotation of mDia1 during processive actin polymerization

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Formins homology proteins (formins) bind around the actin barbed end and processively elongate the actin filament (F-actin). The property implies that formins transmit the actin polymerization force to cellular structures. On the other hand, formins are expected to rotate along the helical structure of F-actin during processive actin elongation (referred to here as helical rotation). Helical rotation of formins might conflict with the property to transmit the actin polymerization force. Here we show the helical rotation of a mammalian formin, mDia1 by single-molecule fluorescence polarization. Polarized fluorescence from TMR-labeled F-actin elongating from the immobilized mDia1 alternated periodically between two angles. The distance per an alternation of polarized fluorescence was 36.1 ± 0.33 nm, which corresponds with a half-pitch length of F-actin. A previous study concluded that a budding yeast formin, Bni1p, slips on the barbed end like a bearing as the authors observed buckled F-actin elongating from Bni1p without super-coiling. However, we revealed that the frequency of buckled F-actin elongating from the mDia1 decreases when mDia1 was tightly anchored to the glass surface with antibodies. These results indicate that mDia1 executes faithfully the helical rotation. Previously, it has been reported that cofilin twists the strand of F-actin and torsional stress induces destabilization of F-actin. Therefore, helical rotation of formins might modulate the actin turnover by changing axial torsion in F-actin.

3K1434 サケ鼻軟骨由来プロテオグリカンの創傷治癒促進効果の in vitro 解析

In vitro analysis of the facilitating effect of Salmon nasal cartridge proteoglycan on the wound healing in a cell monolayer

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Proteoglycan (PG), one of the major components of extracellular matrix, is a macromolecule composed of glycosaminoglycans (GAGs) chains covalently linked to a core protein. PG influences various cellular functions including cell growth, adhesion and differentiation. These features of PG indicate a potential clinical use of PG such as an ointment. In this study we investigated the effect of salmon nasal cartridge PG (SNC-PG) on the healing of a scar made on a cultured cell monolayer using the wound healing assay. Exogenously applied SNC-PG at a concentration of 10 µg/ml accelerated significantly the healing in the monolayer of murine fibroblast NIH/3T3 cells through facilitating both cell proliferation and migration. Essentially similar effects of SNC-PG were observed in the monolayer of human primary cultured fibroblasts or keratinocytes. Surprisingly, SNC-PG at a higher concentration (1000 µg/ml) had no effect. Since the EGF (epidermal growth factor)-like domain of the core protein of SNC-PG is thought to function as an EGF ligand, we tested if the EGFR kinase inhibitor CI-1033 could suppress the facilitating effect of SNC-PG on healing. Although CI-1033 inhibited completely EGF-induced MAPK phosphorylation of fibroblasts, it did not alter the effect of SNC-PG on the wound healing. This result suggests that the other portion(s) of SNC-PG is responsible for the SNC-PG effect. Actually chondroitin sulfate, one of the components of GAGs of SNC-PG, facilitated the healing in the monolayer of fibroblast cells.

3K1446 血管平滑筋細胞内ストレスファイバのレーザー切断に伴う細胞核および核内 DNA の動的挙動観察

Dynamic behavior of nucleus and intranuclear DNA induced with laser nano-dissection of stress fibers in vascular smooth muscle cells

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Actin stress fibers (SFs) play important roles in cellular mechanotransduction and regulation of various cellular functions. SFs generate internal tension and contribute to physical interactions between cells and extracellular matrices. It has recently been suggested that cytoskeletons have the potential to interact with nuclei via nuclear membrane proteins. However, it remains unclear at this stage whether SFs are involved in a mechanical interaction with the cell nucleus and their internal forces are transmitted directly to the nucleus. Here we investigated mechanical interaction between SFs and nuclei in cultured vascular smooth muscle cells using laser nano-dissection technique. Cells with fluorescently labeled SFs and nucleus were set on the stage of a microscope combined with a laboratory-built UV laser nanoscissor. We cut a SF running across the top surface of nucleus in each cell at the point adjacent to the nucleus by the laser to release its pretension, and observed their dynamic behavior. SFs shortened across the top surface of nucleus immediately after dissection. The nuclei also moved in the direction of SF retraction, and showed marked local deformation. Intranuclear DNA just below the SFs aligned in the direction of SFs, and their distribution dramatically changed following SF dissection. These results may indicate that SFs over the nucleus firmly connected to nuclear surface. The internal force of SFs may be transmitted mechanically to nucleus inducing conformation changes of peripheral chromatin near nuclear lamina.

3K1458 単一細胞内における情報伝達タンパク質 RAF の構造と RAS-RAF 情報伝達能の相関解析

Correlation between RAS-RAF signaling and RAF conformation observed in single living cells

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RAS-RAF signaling regulates various cell responses including proliferation, differentiation, and carcinogenesis. Recently we reported that the RAS-induced conformational change in RAF is essential for the accurate signaling from RAS to RAF, and that the RAF mutants, which biased to the open conformation, cannot distinguish between inactive (RASGDP) and active (RASGTP) form of RAS. Based on these results, we have proposed a possibility that the conformation of RAF in resting cells determines the sensitivity and accuracy of the RAS-RAF signaling in response to the extracellular signals, such as EGF. To confirm this hypothesis, we examined the ensemble conformation of RAF molecules in individual resting HeLa cells and the intracellular redistribution of

RAF in the same cells after stimulation with EGF. The redistribution of RAF is a result of binding between RASGTP and RAF. The localization and conformation of RAF were examined by using newly constructed FRET probe of RAF.

The FRET efficiency in individual resting cells was different from each individual cell, suggesting that the ensemble conformations of RAF were varied among resting cells. In parallel, the responsiveness of cells to EGF observed in the kinetics and intensities of RAF redistribution was varied with individual cells. Together with analyses of RAF mutants modulated its conformational equilibrium, we will discuss the relationship between the conformation of RAF and the responsiveness of RAS-RAF signaling in living cells.

3K1522 二光子蛍光寿命イメージング法による Rho GTPase 活性化の単一 P09 シナプスレベル可視化解析

1YE1100 Imaging Rho GTPases activation in single dendritic spines by 2-photon fluorescence lifetime imaging microscopy

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Rho family GTPases regulate the polymerization of actin that controls the shape and size of dendritic spines in hippocampal neurons. However, spatiotemporal dynamics of Rho GTPases activity during structural plasticity remain elusive. Here, we monitored the activity of RhoA and Cdc42, in single dendritic spines undergoing structural plasticity associated with long-term potentiation using 2-photon fluorescence lifetime imaging microscopy. To measure Rho GTPase activity, we transfected CA1 pyramidal neurons in hippocampal slices with GFP-based FRET sensors of Rho GTPases activation using ballistic gene transfer, and imaged the activity of Rho GTPases after induction of structural plasticity using 2-photon glutamate-uncaging. We found that RhoA and Cdc42 were rapidly activated in the stimulated spine, and were then followed by a phase of persistent activation lasting more than 30 min. These activities were CaMKII-dependent. Although active RhoA and Cdc42 were similarly mobile, their activity patterns were different. RhoA activation diffused out of the stimulated spine and spread over ~5 µm along the dendrite, whereas Cdc42 activation was restricted to the stimulated spine. Inhibition of the Rho pathway preferentially inhibited the initial spine growth, whereas the inhibition of the Cdc42 pathway blocked the maintenance of sustained structural plasticity. Thus, RhoA and Cdc42 relay transient CaMKII activation to synapse-specific, long-term signalling required for spine structural plasticity.

3K1534 新規ガングリオシドプローブの 1 分子追跡により明らかになった GPI アンカー型タンパク質クラスターとの短期間相互作用

Single-molecule tracking of new fluorescent ganglioside probes revealed their transient association with GPI-anchored protein clusters

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Gangliosides are considered to be strongly associated with raft domains in the cellular plasma membranes. However, how they interact with and are concentrated in raft domains have been unknown. This is largely due to the lack of their fluorescent analogues that behave like the parent molecules in assays like cold-detergent solubility and partitioning into liquid-ordered-phase domains in the blebbed plasma membrane (commercially available Bodipy-GM1 behaves like a non-raft phospholipid in these assays). Here, we succeeded in conjugating various fluorophores to specific hydroxyl group of the neuraminic acid or galactose in GM1 and GM3 exactly at a single site. Surprisingly, conjugation at the neuraminic acid better preserved the properties of the parent molecules than that at the galactose. Single-molecule observation revealed that the fluorescent GM1 and GM3 probes were transiently recruited to the clusters of five CD59 molecules (a GPI-anchored protein) for ~100 ms (exponential lifetime), whereas the association time of a non-raft reference phospholipid DOPE was ~40 ms. This result indicates that the ganglioside concentration in the CD59-cluster raft occurs dynamically. For micron-scale CD59 clusters induced by its antibody and secondary antibodies, ganglioside probes also moved back and forth between the cluster and the bulk region, whereas DOPE stayed outside the cluster. These results indicate that the assembly of gangliosides in raft domains occurs far more dynamically than assumed previously.