COMMON AND DIVERGING INTEGRIN SIGNALS DOWNSTREAM OF ADHESION AND MECHANICAL STIMULI AND THEIR INTERPLAY WITH REACTIVE OXYGEN SPECIES

Running head: Integrin signaling downstream of adhesion and mechanical stimulation

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The integrin family of adhesion receptors regulates basic functions of cells, and the signals they induce are altered in tumor cells. In this review we discuss how different integrin-dependent signals are generated during cell adhesion and by physical forces acting on cells. We also describe how reactive oxygen species are integral parts of integrin signaling and highlight a few important questions in the field. Answers to those may improve our understanding of integrins and their role in the development of cancer.

Keywords: Integrin; adhesion; reactive oxygen species; signaling; mechano-stimuli.

Introduction

The integrin family of adhesion receptors has crucial roles for numerous processes such as organ development, angiogenesis, blood coagulation, and immune responses. At the cellular level integrins contribute to these processes through their structural and signaling functions. They are the central components of hemidesmosomes, focal contacts and other cell contacts, and they regulate extracellular matrix formation. The signaling reactions induced by integrins are essential for several basic cellular functions, including survival, cell cycle regulation, and migration (1,2). Integrin signals are generated by different types of cellular stimuli, i.e. ligand binding and various types of physical forces (3,4). Importantly, the "integrin signals" triggered by these stimuli are not the same (5).

Tumor development is known to depend on integrin-mediated adhesion and physical stimuli in several ways, which concern both the cancer cells themselves and the non-transformed host cells. Cell proliferation is regulated by cooperative signals from growth factors and integrins, e.g. in the activation of the ERK pathway and the passage through the G1/S checkpoint of the cell cycle (6). Integrin adhesion also controls the cytokinesis process at the end of the cell cycle by poorly understood signals (7). Integrins have been reported to cooperate with growth factor receptors by different mechanisms, including interactions in common receptor complexes, generation of intermediates

necessary for the growth factor pathway (converging pathways), and amplification of growth factor signals (8). Integrins may therefore contribute to the deregulated proliferation of cancer cells caused by defects in reactions downstream of integrins or several other receptors. Invasive growth and metastasis require cell migration, which depends on membrane protrusions at the cell front driven by actin polymerization and detachment at the rear driven by actin-myosin contraction. Both reactions are potently induced by integrins (2,9). The invasive phenotype in carcinomas is linked to TGFβ-induced epithelial-mesenchymal transition, and the activation of the latent TGFβ complex occurs mainly via binding to integrin αvβ6 in carcinomas (10). The high TGFβ activity will in addition have other effects promoting tumor growth, including the formation of a collagen-rich stiff extracellular matrix (ECM) by resident fibroblast-like cells. Host cells contribute to tumor progression also by forming new blood vessels, whose angiogenesis requires integrins both for the migration of endothelial cells and pericytes, and for the remodeling of the ECM.

In order to better understand how integrin-dependent reactions are used by cancer cells to promote invasive growth, the ability to metastasize and to resist apoptosis-inducing conditions, the effects of defined integrin stimuli on cell signals need to be identified. These efforts include characterization of (i) responses induced by the different integrin stimuli, and (ii) integrin type-specific signals.

Integrin Signaling Mechanisms

Integrin signaling has mainly been studied in re-adhesion assays where suspended cells are seeded on immobilized integrin ligands. During the attachment and cell spreading phases in such assays many signaling reactions occur as extensively documented, e.g. the activation of focal adhesion kinase (FAK), phosphatidylinositol 3-kinase (PI3K), Rho GTPases, and the pathway components downstream of these proteins. However, the initial triggering events for the reactions are still poorly understood. Ligand binding to integrins results in large conformational changes in the extracellular domain (11), but it is not known whether they are propagated across the plasma membrane. While this possibility should not be excluded, it has been shown that mere integrin clustering by polyvalent antibodies can induce recruitment and activation of cytoplasmic enzymes (3,12). The understanding of the signaling mechanism(s) is also complicated by the later emerged role of integrins as mechano-receptors, whereby signals are generated through conformational changes in force-sensitive proteins associated with integrins (4).

Ligand-induced integrin signaling

Cell attachment and spreading are driven by dynamic and incompletely understood reactions involving ligand-integrin interactions, force from actin polymerization pushing against the plasma membrane, and myosin-dependent pulling force on adhesion sites. Thus it is often unclear by which mechanism the signaling proteins were activated in published studies of re-adhering cells. Using inhibitors of myosin II (Blebbistatin) and RhoA kinase (Y27632) we found that several phosphorylation reactions during the initial stages of fibroblast attachment (<30 minutes) to fibronectin occur independently of intracellular contractile forces (5). Thus, mere ligand binding to integrin α5β1 was

sufficient to trigger phosphorylation of FAK-Y397, ERK-T202/Y204, AKT-S473, p130CAS-Y410, myosin phosphatase targeting subunit 1 (MYPT)-T853, myosin light chain (MLC)-S19 and cofilin-S3. This was the case also for the reactions driving integrin-dependent actin polymerization. The polymerization was monitored as lamellipodia protrusion from the initial contact points of attaching cells by live cell total internal reflection fluorescence (TIRF) microscopy, and this assay could detect the response to integrin ligand binding as early as after a few seconds (5,9). Possibly, the contribution of myosin-dependent contractile force becomes important at later time points or affects other signaling reactions.

Tension-induced integrin signaling

Integrins act as mechano-sensors by linking extracellular ligands to actin filaments via adaptor proteins in adhesion sites (4). Some integrin adhesion site-associated proteins can change conformation upon mechanical stimulation and thereby expose cryptic binding or phosphorylation sites. So far only few intracellular proteins have been clearly shown to be force-regulated, such as talin (13), p130CAS (14), and filamin (15), but this may change if the methodological difficulties to study such conformational changes are overcome. Mechanical force has also been reported to regulate the structure and function of integrins themselves (16), an interesting finding that is important to be investigated further. The forces acting on cells in our body include gravity, stretching by muscle work (breathing, pressure pulses from heart beats, pulling on tendons, etc.), shear stress from liquid flow, and contractile force generated by myosin II inside the cell. These physical stimuli are necessary for the development and maintenance of our body (17,18). The signaling outcome of intracellular contraction is dependent on the stiffness of the surrounding ECM, and ECM stiffness may be a dominant factor for stem cell differentiation (19). Tumor development has been reported to be affected by the tissue stiffness mainly in two ways: (i) soft ECMs foster selection of tumor initiating cells ("tumor stem cells") by induction of pluripotency genes, (ii) a stiff ECM (typical for solid tumors) promotes tumor growth and migration (20).

A variety of approaches have been used to study the role of mechanical force on cell signaling reactions, and the reported results vary considerably. The cell type studied and how the force is applied (static, cyclic, frequency, amplitude, duration) are obvious factors that will influence the outcome of the experiments. For example, cyclic stretching for hours has been reported to affect the cytoskeletal organization (21), oxidative stress levels (22), and mRNA synthesis (23), responses that will have many secondary effects in the cell. Some responses are seen within five to ten minutes of mechanical stimulation (5,23), and they are likely to be relatively direct results of the conformational changes in the force-sensitive proteins. Other factors that can affect the results are cell density and ion channel expression, information usually not provided in the published reports. Besides integrins, cadherins and several ion channels are believed to be the main mechano-receptors on cells (24,25). The contributions of mechano-signals from cell-cell contacts can be analyzed and controlled by performing the experiments at low and high cell densities. However, the involvement of mechano-stimulated ion channels is presently more difficult to study. This is due to a lack of specific inhibitors and to the large number

of different channels that makes knock-out and knock-down approaches complicated. It should also be noted that some ion channels have been reported to actually interact with and to be regulated by integrins (26).

We have recently shown that short-term cyclic stretching (ten to 30 minutes) of sparsely seeded fibroblasts triggers activation of only a small number of integrin-associated signaling proteins compared to the signals generated during cell attachment (integrin ligation). The phosphorylation of ERK1/2 appears to be a particular stretch-responsive signal (5). FAK has been suggested to become activated by unfolding in response to force and to be required for tension-induced activation of ERK (27,28). However, consistent with the absence of force-induced FAK activation in our studies, phosphorylation of ERK1/2 was induced to the same degree by cyclic stretching (1 Hz) of FAK-null and FAK-expressing fibroblasts (29). The question how this MAPK is activated by tensional force is intriguing and presently not understood as illustrated by the following examples: different reports have suggested that ERK activation by integrin-mediated tension correlates with or is dependent on activation of Ras (30), inactivation of Ras (31), influx of Ca^{2+} (24), or release of reactive oxygen species (ROS) from mitochondria (22).

Integrin Type-Specific Signals

In spite of the vast literature on integrin-mediated signaling the differences in responses between different integrins are still poorly characterized. Most cells express several integrin α and β subunits, and some of the resulting integrin heterodimers have overlapping ligand specificities. Therefore, in order to receive significant data regarding signaling specificity of the different integrins, cell systems and ligands need to be well characterized and controlled. The signals generated by β1, β2, or β3 integrin subunits have been extensively studied while less is known about β4 to β8. The information regarding contributions from the α subunits to integrin signals is scarce. β1 and β3 integrins are expressed by most cultured adherent cell lines and can trigger similar signaling reactions. Yet, important functional outcomes are known to differ between the two fibronectin receptors α5β1 and αvβ3. Integrin αvβ3 promotes the formation of large adhesion sites (32). In contrast, α5β1 induces the formation of smaller and more dynamic adhesion sites at the cell periphery (32,33) and stronger traction force (32,34). It is also much more efficient than αvβ3 in inducing fibronectin polymerization on the cell surface (35). The underlying mechanisms for these actomyosin-related differences are not clear. However, myosin II-dependent contraction requires activating phosphorylation on myosin light chain (MLC)-S19, and α5β1 was recently shown to induce this phosphorylation more efficiently than αvβ3 (5,32).

The regulation of MLC phosphorylation involves at least two signaling pathways, i.e. RhoA/ROCK-dependent inactivation of MLC phosphatase and phospholipase C (PLC)/Ca^{2+}/calmodulin-dependent activation of MLC kinase (36). Interestingly, the RhoA activity can be suppressed by β3-associated Src catalyzing an activating phosphorylation of RhoA GAP (37), while the β1 cytoplasmic domain does not bind Src (38). This is consistent with lower RhoA activity after adhesion mediated by αvβ3 compared to α5β1 (39). However, αvβ3 has also been reported to induce a much higher

RhoA activity than α5β1 in adhesion assays (32); since the traction force in this study was lower from αvβ3 than from α5β1 and therefore did not correlate with the RhoA activities, it was concluded that only α5β1 was able to promote coupling of RhoA to ROCK activation in these cells. However, we found that α5β1 and αvβ3 induced the inactivating phosphorylation of MLC phosphatase (a direct measure of ROCK activity) equally efficiently in two different cell lines (5). The varying results regarding the correlation of RhoA with MLC activity suggest that the activation via PLC/Ca^{2+}/calmodulin/MLC kinase has a dominating role in the regulation of myosin II and cell contraction. The regulation of MLC kinase by integrins (Figure 1) therefore deserves more detailed studies.

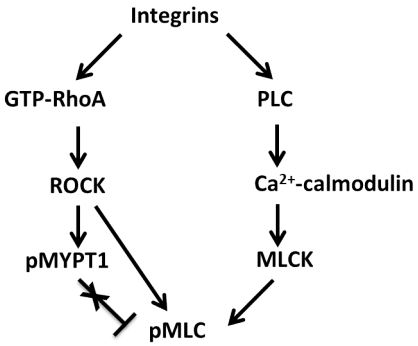

Fig.1 Phosphorylation of MLC by MLC kinase (MLCK) or ROCK activates myosin II to pull on actin filaments. ROCK also phosphorylates the MLC phosphatase subunit MYPT1 and thereby inhibits the inactivation of myosin II. ROCK can be activated by integrins via GTP-loaded RhoA (30). MLCK activation requires Ca^{2+}-loaded calmodulin and is promoted by (locally) elevated cytoplasmic Ca^{2+} levels, which can be induced by integrin-activated phospholipase C (PLC) (40). The activating steps upstream of RhoA GEFs (including p190RhoGEF, p115, GEF-H1, and LARG) (30)) and PLC triggered by integrins are incompletely understood. Adhesion via integrin α5β1 is reported to induce phosphorylation of MLC more efficiently than adhesion via αvβ3 (5,32).

Integrins and Reactive Oxygen Species

It has become increasingly clear that integrin functions are intimately associated with ROS action. Integrin stimulation generates significant amounts of ROS, and ROS affect integrin signals as well as a variety of other functions in cells. While it is important to realize that ROS is a summary name for very different molecules, each with characteristic properties and reactivity such as hydrogen peroxide, hydroxyl and superoxide radicals, cell signaling reactions are thought to be affected mainly by hydrogen peroxide due to its relatively long half-life. ROS are produced by several cellular oxidases, e.g. complexes in the mitochondrial electron transport chain, NADPH oxidases (NOXes) and 5-lipoxygenase (5-LOX) in the contexts of cell metabolism, pathogen defense and cell signaling (41). ROS production is tightly controlled and defense systems limit ROS to act mainly in a localized fashion. Thereby potentially harmful effects of these promiscuously reactive molecules are prevented or reduced. Elevated ROS production from mitochondria and NOXes are commonly found in tumor cells and may be linked to increased migration and apoptosis resistance (42,43).

Hydrogen peroxide preferentially targets redox-sensitive residues in proteins, cysteines being the prototypic example (44). Quite early on it has been realized that cysteines in proteins often play roles for their enzymatic function, and more recently it has been acknowledged that the reversible modification of these residues by oxidation or conjugation is part of signal transduction mechanisms (44,45). An important example is the reaction of hydrogen peroxide with phosphatases, e.g. the inhibition of PTEN (45) which conserves the 3´-phosphorylated phosphatidylinositols and leads to sustained activities downstream of PI3Ks. Other targets include transcription factors (41,46), kinases (e.g. Src, (47)), small GTPases (48-52), matrix metalloproteinases (41,53), actin (54,55) and actin-associated molecules (56-58). ROS acting on these targets will give rise to numerous feedback and feedforward reactions and is likely responsible for providing signal amplification and mediating cross-talk between different signal cascades (59).

In order to control ROS effects, cells contain multiple protection systems such as ROS-converting enzymes (superoxide dismutases, catalase, glutathione peroxidase, peroxiredoxins) and other scavengers (e.g. GSH-GSSG, ascorbate), but there may be differences in how effectively these mechanisms work in different cell types and even in individual cells among one population. Additionally, different cellular compartments show marked differences in their redox potential (44). These variations, together with the complicated chemistry and fast reactivity of ROS, underlie the complexity of this research field and the experimental difficulties it is facing. More detailed information can be found in several comprehensive reviews on ROS chemistry and biology (41,60-65).

ROS from several sources have been linked to integrin engagement and signaling, both during attachment and mechanical stimulation of cells. Mitochondria-derived ROS (52,66) as well as ROS from NOXes and 5-lipoxygenase (5-LOX) produced in response to integrin ligand binding (67,68) were reported to affect cell attachment, spreading, and associated cytoskeletal changes. There are also indications that FAK, important for survival and migration, is regulated by ROS in response to integrin-mediated adhesion e.g. through the reversible oxidation (i.e. inhibition) of the phosphatases LMW-PTP (67,69) and SHP-2 (66). The inhibition of these phosphatases may allow a sustained phosphorylation and activation of FAK and thus the propagation of integrin signals. Less is known about ROS production downstream mechanical cell stretching, but NOXes (70,71) and mitochondria (22) as well as cross-activation between these sources (72) have been implicated. For example, Ali *et al.* (22) reported an increased FAK phosphorylation at Y397 in endothelial cells in response to cyclic strain, which could be abolished by antioxidants and mitochondrial inhibitors. However, several of the results on the role of ROS during different integrin stimuli were obtained with reagents that have poor specificity (e.g. diphenylene iodonium (DPI), apocynin, and N-acetylcysteine) or may cause artifacts (e.g. dyes such as 2,7-dihydrodichlorofluorescein diacetate (H_2DCFDA)) (73-75). Considering the variations in experimental settings in different studies, such as stimuli parameters, measurement methods and endogenous differences in cell lines and types, both with regard to ROS production and antioxidant capacities, general conclusions are difficult to draw at present.

In our own studies, we have obtained evidence that mitochondrial ROS affect AKT and ERK1/2 signaling pathways in two different fibroblast cell lines in re-adhesion

assays (30 minutes after seeding) (5). Rotenone, an inhibitor of complex I in the respiratory chain with no known other targets, reduced AKT phosphorylation levels, what would be consistent with a higher activity of PTEN (and other phosphatases) when ROS release from mitochondria is inhibited. In this context it is interesting to note that AKT2 has been reported to co-localize with mitochondria (76) and that β1 integrins in MCF-7 cells preferentially activate the AKT2 isoform during attachment and spreading (77). Also Taddei *et al.* (66) reported data supporting the importance of mitochondrial ROS in an early phase of cell attachment. The mechanism for how integrins transfer signals to mitochondria remains unclear, although both physical coupling via actin filaments and diffusible factors have been suggested (22,52,78).

We also found that extracellular addition of catalase (a highly specific enzyme catalyzing the reaction from hydrogen peroxide to water and oxygen) enhances the stretch-induced ERK1/2 phosphorylation. A stable vitamin C derivate that is converted to active vitamin C (superoxide scavenger) by cellular enzymes inside and outside the cells has strikingly similar effects. These results, summarized in Table 1, point to a role of NOXes in stretch-induced signaling ((5) and Figure 2).

Table 1. Diverging effects of ROS derived from different cellular sources on signals during cell attachment and stretching.

	AKT-pS473		ERK-T202/Y204		p130CAS-Y410	
	BJ hTERT	GD25β1	BJ hTERT	GD25β1	BJ hTERT	GD25β1
Attachment + rotenone	−	−	−	+	=	=
Stretching + rotenone	=	=	=	=	=	=
Attachment + catalase	=	=	=	=	=	=
Stretching + catalase	=	=	+	+	=	=

= no change
− reduced phosphorylation
+ increased phosphorylation

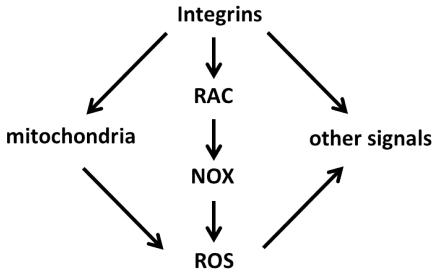

Fig. 2 Integrin stimulation generates ROS from different sources. Elevated release of hydrogen peroxide from mitochondria during cell attachment is induced by unknown mechanisms. Our data suggests that mechano-stimulation generates superoxide by NOXes located at the plasma membrane (5) and, possibly, in the case of NOX4, also at intracellular sites (79-81). Superoxide can rapidly dismutate to hydrogen peroxide, which can pass through membranes. Integrin-mediated activation of NOX1 and NOX2 involves the activation of RAC, but the mechanisms for RAC activation as well as other steps in NOX activation are incompletely characterized. The generated ROS will significantly modulate the signaling reactions downstream of integrins as well as signals induced by other receptors.

In order to obtain more informative data, better methods allowing for both temporal and spatial resolution are needed. Several new methods to monitor certain ROS types or redox states have been developed employing for example boronate-based H_2O_2-selective probes (e.g. PeroxyGreen (82,83)), the genetically encoded H_2O_2 biosensor HyPer (84) and redox-sensitive GFP (for example roGFP (85,86)); all have been used for live cell measurements. However, it is important to choose the probes carefully depending on the research question and to ensure suitable conditions with appropriate controls in order to be able to draw valid conclusions (87). For example, a measurement of the redox state does not provide relevant information about ROS concentrations. It will be interesting to follow if these promising probes work as hoped for, and to see if they can verify previous observations and provide opportunities to better understand the interplay between integrins and ROS.

Outlook

In spite of the vast amount of research that has been performed in the integrin field during the past decades, there are several key questions remaining to clarify.

(i) Integrins: What are the signals deriving from distinct members of the integrin family during ligand binding? And what are the roles of the integrin α units in signaling? We still have not yet clearly revealed if integrins themselves are mechano-sensitive, i.e. if their conformation or clustering is affected by force, and if it makes a difference if the force comes from inside or outside of the cells.

(ii) Force-induced signals: It is necessary to find ways to experimentally distinguish integrin mechano-signals from signals originating from cell-cell contacts and ion-channels. Also, the proposed interactions between certain ion-channels with integrins need to be characterized in more detail.

(iii) ROS: It would be important to clarify the mechanisms for how integrins regulate different NOXes and how they affect ROS release from mitochondria.

Although these are demanding tasks, every step towards a more detailed understanding of integrin signaling mechanisms and their interplay with other crucial molecules such as ROS, would bring us closer to understanding very important basic cellular functions and their roles in pathologies.

References

1. Legate, K. R., Wickstrom, S. A., and Fassler, R. (2009) Genetic and cell biological analysis of integrin outside-in signaling. *Genes Dev* **23**, 397-418
2. Huttenlocher, A., and Horwitz, A. R. (2011) Integrins in cell migration. *Cold Spring Harbor perspectives in biology* **3**, a005074
3. Miyamoto, S., Teramoto, H., Coso, O. A., Gutkind, J. S., Burbelo, P. D., Akiyama, S. K., and Yamada, K. M. (1995) Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. *J Cell Biol* **131**, 791-805
4. Moore, S. W., Roca-Cusachs, P., and Sheetz, M. P. (2010) Stretchy proteins on stretchy substrates: the important elements of integrin-mediated rigidity sensing. *Dev Cell* **19**, 194-206

5. Zeller, K. S., Riaz, A., Sarve, H., Li, J., Tengholm, A., and Johansson, S. (2013) The role of mechanical force and ROS in integrin-dependent signals. *PLoS One* **8**, e64897
6. Walker, J. L., and Assoian, R. K. (2005) Integrin-dependent signal transduction regulating cyclin D1 expression and G1 phase cell cycle progression. *Cancer Metastasis Rev* **24**, 383-393
7. Gupta, R. K., and Johansson, S. (2013) Fibronectin assembly in the crypts of cytokinesis-blocked multilobular cells promotes anchorage-independent growth. *PLoS One* **8**, e72933
8. Ivaska, J., and Heino, J. (2011) Cooperation between integrins and growth factor receptors in signaling and endocytosis. *Annu Rev Cell Dev Biol* **27**, 291-320
9. Zeller, K. S., Idevall-Hagren, O., Stefansson, A., Velling, T., Jackson, S. P., Downward, J., Tengholm, A., and Johansson, S. (2010) PI3-kinase p110alpha mediates beta1 integrin-induced Akt activation and membrane protrusion during cell attachment and initial spreading. *Cell Signal* **22**, 1838-1848
10. Margadant, C., and Sonnenberg, A. (2010) Integrin-TGF-beta crosstalk in fibrosis, cancer and wound healing. *EMBO Rep* **11**, 97-105
11. Luo, B. H., Carman, C. V., and Springer, T. A. (2007) Structural basis of integrin regulation and signaling. *Annu Rev Immunol* **25**, 619-647
12. Akiyama, S. K., Yamada, S. S., Yamada, K. M., and LaFlamme, S. E. (1994) Transmembrane signal transduction by integrin cytoplasmic domains expressed in single-subunit chimeras. *J Biol Chem* **269**, 15961-15964
13. Hytonen, V. P., and Vogel, V. (2008) How force might activate talin's vinculin binding sites: SMD reveals a structural mechanism. *PLoS Comput Biol* **4**, e24
14. Sawada, Y., Tamada, M., Dubin-Thaler, B. J., Cherniavskaya, O., Sakai, R., Tanaka, S., and Sheetz, M. P. (2006) Force sensing by mechanical extension of the Src family kinase substrate p130Cas. *Cell* **127**, 1015-1026
15. Ehrlicher, A. J., Nakamura, F., Hartwig, J. H., Weitz, D. A., and Stossel, T. P. (2011) Mechanical strain in actin networks regulates FilGAP and integrin binding to filamin A. *Nature* **478**, 260-263
16. Friedland, J. C., Lee, M. H., and Boettiger, D. (2009) Mechanically activated integrin switch controls alpha5beta1 function. *Science* **323**, 642-644
17. Jaalouk, D. E., and Lammerding, J. (2009) Mechanotransduction gone awry. *Nat Rev Mol Cell Biol* **10**, 63-73
18. Wozniak, M. A., and Chen, C. S. (2009) Mechanotransduction in development: a growing role for contractility. *Nat Rev Mol Cell Biol* **10**, 34-43
19. Engler, A. J., Sen, S., Sweeney, H. L., and Discher, D. E. (2006) Matrix elasticity directs stem cell lineage specification. *Cell* **126**, 677-689
20. Shin, J. W., and Discher, D. E. (2012) Cell culture: Soft gels select tumorigenic cells. *Nat Mater* **11**, 662-663
21. Kaunas, R., Nguyen, P., Usami, S., and Chien, S. (2005) Cooperative effects of Rho and mechanical stretch on stress fiber organization. *Proc Natl Acad Sci U S A* **102**, 15895-15900
22. Ali, M. H., Mungai, P. T., and Schumacker, P. T. (2006) Stretch-induced phosphorylation of focal adhesion kinase in endothelial cells: role of mitochondrial oxidants. *Am J Physiol Lung Cell Mol Physiol* **291**, L38-45
23. Lutz, R., Sakai, T., and Chiquet, M. (2010) Pericellular fibronectin is required for RhoA-dependent responses to cyclic strain in fibroblasts. *J Cell Sci* **123**, 1511-1521

24. Sukharev, S., and Sachs, F. (2012) Molecular force transduction by ion channels: diversity and unifying principles. *J Cell Sci* **125**, 3075-3083
25. Sun, Y., Chen, C. S., and Fu, J. (2012) Forcing stem cells to behave: a biophysical perspective of the cellular microenvironment. *Annu Rev Biophys* **41**, 519-542
26. Arcangeli, A., and Becchetti, A. (2006) Complex functional interaction between integrin receptors and ion channels. *Trends Cell Biol* **16**, 631-639
27. Mofrad, M. R., Golji, J., Abdul Rahim, N. A., and Kamm, R. D. (2004) Force-induced unfolding of the focal adhesion targeting domain and the influence of paxillin binding. *Mech Chem Biosyst* **1**, 253-265
28. Wang, J. G., Miyazu, M., Matsushita, E., Sokabe, M., and Naruse, K. (2001) Uniaxial cyclic stretch induces focal adhesion kinase (FAK) tyrosine phosphorylation followed by mitogen-activated protein kinase (MAPK) activation. *Biochem Biophys Res Commun* **288**, 356-361
29. Hsu, H. J., Lee, C. F., Locke, A., Vanderzyl, S. Q., and Kaunas, R. (2010) Stretch-induced stress fiber remodeling and the activations of JNK and ERK depend on mechanical strain rate, but not FAK. *PLoS One* **5**, e12470
30. Guilluy, C., Swaminathan, V., Garcia-Mata, R., O'Brien, E. T., Superfine, R., and Burridge, K. (2011) The Rho GEFs LARG and GEF-H1 regulate the mechanical response to force on integrins. *Nat Cell Biol* **13**, 722-727
31. Sawada, Y., Nakamura, K., Doi, K., Takeda, K., Tobiume, K., Saitoh, M., Morita, K., Komuro, I., De Vos, K., Sheetz, M., and Ichijo, H. (2001) Rap1 is involved in cell stretching modulation of p38 but not ERK or JNK MAP kinase. *J Cell Sci* **114**, 1221-1227
32. Schiller, H. B., Hermann, M. R., Polleux, J., Vignaud, T., Zanivan, S., Friedel, C. C., Sun, Z., Raducanu, A., Gottschalk, K. E., Thery, M., Mann, M., and Fassler, R. (2013) beta1- and alphav-class integrins cooperate to regulate myosin II during rigidity sensing of fibronectin-based microenvironments. *Nat Cell Biol* **15**, 625-636
33. Rossier, O., Octeau, V., Sibarita, J. B., Leduc, C., Tessier, B., Nair, D., Gatterdam, V., Destaing, O., Albiges-Rizo, C., Tampe, R., Cognet, L., Choquet, D., Lounis, B., and Giannone, G. (2012) Integrins beta1 and beta3 exhibit distinct dynamic nanoscale organizations inside focal adhesions. *Nat Cell Biol* **14**, 1057-1067
34. Gupta, R. K., and Johansson, S. (2012) beta1 integrins restrict the growth of foci and spheroids. *Histochem Cell Biol* **138**, 881-894
35. Wennerberg, K., Lohikangas, L., Gullberg, D., Pfaff, M., Johansson, S., and Fassler, R. (1996) Beta 1 integrin-dependent and -independent polymerization of fibronectin. *J Cell Biol* **132**, 227-238
36. Satoh, K., Fukumoto, Y., and Shimokawa, H. (2011) Rho-kinase: important new therapeutic target in cardiovascular diseases. *Am J Physiol Heart Circ Physiol* **301**, H287-296
37. Flevaris, P., Stojanovic, A., Gong, H., Chishti, A., Welch, E., and Du, X. (2007) A molecular switch that controls cell spreading and retraction. *J Cell Biol* **179**, 553-565
38. Arias-Salgado, E. G., Lizano, S., Sarkar, S., Brugge, J. S., Ginsberg, M. H., and Shattil, S. J. (2003) Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. *Proc Natl Acad Sci U S A* **100**, 13298-13302
39. Danen, E. H., Sonneveld, P., Brakebusch, C., Fassler, R., and Sonnenberg, A. (2002) The fibronectin-binding integrins alpha5beta1 and alphavbeta3

differentially modulate RhoA-GTP loading, organization of cell matrix adhesions, and fibronectin fibrillogenesis. *J Cell Biol* **159**, 1071-1086
40. Jones, N. P., Peak, J., Brader, S., Eccles, S. A., and Katan, M. (2005) PLCgamma1 is essential for early events in integrin signalling required for cell motility. *J Cell Sci* **118**, 2695-2706
41. Droge, W. (2002) Free radicals in the physiological control of cell function. *Physiol Rev* **82**, 47-95
42. Gogvadze, V., Orrenius, S., and Zhivotovsky, B. (2008) Mitochondria in cancer cells: what is so special about them? *Trends Cell Biol* **18**, 165-173
43. Ushio-Fukai, M., and Nakamura, Y. (2008) Reactive oxygen species and angiogenesis: NADPH oxidase as target for cancer therapy. *Cancer Lett* **266**, 37-52
44. Jones, D. P. (2010) Redox sensing: orthogonal control in cell cycle and apoptosis signalling. *J Intern Med* **268**, 432-448
45. Tonks, N. K. (2005) Redox redux: revisiting PTPs and the control of cell signaling. *Cell* **121**, 667-670
46. Morgan, M. J., and Liu, Z. G. (2011) Crosstalk of reactive oxygen species and NF-kappaB signaling. *Cell Res* **21**, 103-115
47. Giannoni, E., and Chiarugi, P. (2013) Redox Circuitries Driving Src Regulation. *Antioxid Redox Signal*
48. Aghajanian, A., Wittchen, E. S., Campbell, S. L., and Burridge, K. (2009) Direct activation of RhoA by reactive oxygen species requires a redox-sensitive motif. *PLoS One* **4**, e8045
49. Goitre, L., Pergolizzi, B., Ferro, E., Trabalzini, L., and Retta, S. F. (2012) Molecular Crosstalk between Integrins and Cadherins: Do Reactive Oxygen Species Set the Talk? *J Signal Transduct* **2012**, 807682
50. Hordijk, P. L. (2006) Regulation of NADPH oxidases: the role of Rac proteins. *Circ Res* **98**, 453-462
51. Nimnual, A. S., Taylor, L. J., and Bar-Sagi, D. (2003) Redox-dependent downregulation of Rho by Rac. *Nat Cell Biol* **5**, 236-241
52. Werner, E., and Werb, Z. (2002) Integrins engage mitochondrial function for signal transduction by a mechanism dependent on Rho GTPases. *J Cell Biol* **158**, 357-368
53. Svineng, G., Ravuri, C., Rikardsen, O., Huseby, N. E., and Winberg, J. O. (2008) The role of reactive oxygen species in integrin and matrix metalloproteinase expression and function. *Connect Tissue Res* **49**, 197-202
54. Fiaschi, T., Cozzi, G., Raugei, G., Formigli, L., Ramponi, G., and Chiarugi, P. (2006) Redox regulation of beta-actin during integrin-mediated cell adhesion. *J Biol Chem* **281**, 22983-22991
55. Lassing, I., Schmitzberger, F., Bjornstedt, M., Holmgren, A., Nordlund, P., Schutt, C. E., and Lindberg, U. (2007) Molecular and structural basis for redox regulation of beta-actin. *J Mol Biol* **370**, 331-348
56. Fiaschi, T., Cozzi, G., and Chiarugi, P. (2012) Redox Regulation of Nonmuscle Myosin Heavy Chain during Integrin Engagement. *J Signal Transduct* **2012**, 754964
57. Kim, J. S., Huang, T. Y., and Bokoch, G. M. (2009) Reactive oxygen species regulate a slingshot-cofilin activation pathway. *Mol Biol Cell* **20**, 2650-2660
58. Klemke, M., Wabnitz, G. H., Funke, F., Funk, B., Kirchgessner, H., and Samstag, Y. (2008) Oxidation of cofilin mediates T cell hyporesponsiveness under oxidative stress conditions. *Immunity* **29**, 404-413

59. Wu, W. S., Wu, J. R., and Hu, C. T. (2008) Signal cross talks for sustained MAPK activation and cell migration: the potential role of reactive oxygen species. *Cancer Metastasis Rev* **27**, 303-314
60. Monteiro, H. P., Arai, R. J., and Travassos, L. R. (2008) Protein tyrosine phosphorylation and protein tyrosine nitration in redox signaling. *Antioxid Redox Signal* **10**, 843-889
61. Murphy, M. P. (2009) How mitochondria produce reactive oxygen species. *Biochem J* **417**, 1-13
62. Ushio-Fukai, M. (2006) Localizing NADPH oxidase-derived ROS. *Sci STKE* **2006**, re8
63. Jaquet, V., Scapozza, L., Clark, R. A., Krause, K. H., and Lambeth, J. D. (2009) Small-molecule NOX inhibitors: ROS-generating NADPH oxidases as therapeutic targets. *Antioxid Redox Signal* **11**, 2535-2552
64. Lambeth, J. D., Kawahara, T., and Diebold, B. (2007) Regulation of Nox and Duox enzymatic activity and expression. *Free Radic Biol Med* **43**, 319-331
65. Dickinson, B. C., and Chang, C. J. (2011) Chemistry and biology of reactive oxygen species in signaling or stress responses. *Nat Chem Biol* **7**, 504-511
66. Taddei, M. L., Parri, M., Mello, T., Catalano, A., Levine, A. D., Raugei, G., Ramponi, G., and Chiarugi, P. (2007) Integrin-mediated cell adhesion and spreading engage different sources of reactive oxygen species. *Antioxid Redox Signal* **9**, 469-481
67. Chiarugi, P., Pani, G., Giannoni, E., Taddei, L., Colavitti, R., Raugei, G., Symons, M., Borrello, S., Galeotti, T., and Ramponi, G. (2003) Reactive oxygen species as essential mediators of cell adhesion: the oxidative inhibition of a FAK tyrosine phosphatase is required for cell adhesion. *J Cell Biol* **161**, 933-944
68. Dib, K., Melander, F., Axelsson, L., Dagher, M. C., Aspenstrom, P., and Andersson, T. (2003) Down-regulation of Rac activity during beta 2 integrin-mediated adhesion of human neutrophils. *J Biol Chem* **278**, 24181-24188
69. Buricchi, F., Giannoni, E., Grimaldi, G., Parri, M., Raugei, G., Ramponi, G., and Chiarugi, P. (2007) Redox regulation of ephrin/integrin cross-talk. *Cell Adh Migr* **1**, 33-42
70. De Keulenaer, G. W., Chappell, D. C., Ishizaka, N., Nerem, R. M., Alexander, R. W., and Griendling, K. K. (1998) Oscillatory and steady laminar shear stress differentially affect human endothelial redox state: role of a superoxide-producing NADH oxidase. *Circ Res* **82**, 1094-1101
71. Zhang, Y., Peng, F., Gao, B., Ingram, A. J., and Krepinsky, J. C. (2010) Mechanical strain-induced RhoA activation requires NADPH oxidase-mediated ROS generation in caveolae. *Antioxid Redox Signal* **13**, 959-973
72. Lee, S. B., Bae, I. H., Bae, Y. S., and Um, H. D. (2006) Link between mitochondria and NADPH oxidase 1 isozyme for the sustained production of reactive oxygen species and cell death. *J Biol Chem* **281**, 36228-36235
73. Karlsson, M., Kurz, T., Brunk, U. T., Nilsson, S. E., and Frennesson, C. I. (2010) What does the commonly used DCF test for oxidative stress really show? *Biochem J* **428**, 183-190
74. Meyer, A. J., and Dick, T. P. (2010) Fluorescent protein-based redox probes. *Antioxid Redox Signal* **13**, 621-650
75. Murphy, M. P., Holmgren, A., Larsson, N. G., Halliwell, B., Chang, C. J., Kalyanaraman, B., Rhee, S. G., Thornalley, P. J., Partridge, L., Gems, D., Nystrom, T., Belousov, V., Schumacker, P. T., and Winterbourn, C. C. (2011)

Unraveling the biological roles of reactive oxygen species. *Cell Metab* **13**, 361-366

76. Santi, S. A., and Lee, H. (2010) The Akt isoforms are present at distinct subcellular locations. *Am J Physiol Cell Physiol* **298**, C580-591
77. Riaz, A., Zeller, K. S., and Johansson, S. (2012) Receptor-Specific Mechanisms Regulate Phosphorylation of AKT at Ser473: Role of RICTOR in beta1 Integrin-Mediated Cell Survival. *PLoS One* **7**, e32081
78. Wang, N., Tytell, J. D., and Ingber, D. E. (2009) Mechanotransduction at a distance: mechanically coupling the extracellular matrix with the nucleus. *Nat Rev Mol Cell Biol* **10**, 75-82
79. Hilenski, L. L., Clempus, R. E., Quinn, M. T., Lambeth, J. D., and Griendling, K. K. (2004) Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **24**, 677-683
80. Chen, K., Kirber, M. T., Xiao, H., Yang, Y., and Keaney, J. F., Jr. (2008) Regulation of ROS signal transduction by NADPH oxidase 4 localization. *J Cell Biol* **181**, 1129-1139
81. Martyn, K. D., Frederick, L. M., von Loehneysen, K., Dinauer, M. C., and Knaus, U. G. (2006) Functional analysis of Nox4 reveals unique characteristics compared to other NADPH oxidases. *Cell Signal* **18**, 69-82
82. Dickinson, B. C., Huynh, C., and Chang, C. J. (2010) A palette of fluorescent probes with varying emission colors for imaging hydrogen peroxide signaling in living cells. *J Am Chem Soc* **132**, 5906-5915
83. Dickinson, B. C., Peltier, J., Stone, D., Schaffer, D. V., and Chang, C. J. (2011) Nox2 redox signaling maintains essential cell populations in the brain. *Nat Chem Biol* **7**, 106-112
84. Malinouski, M., Zhou, Y., Belousov, V. V., Hatfield, D. L., and Gladyshev, V. N. (2011) Hydrogen peroxide probes directed to different cellular compartments. *PLoS One* **6**, e14564
85. Cannon, M. B., and Remington, S. J. (2006) Re-engineering redox-sensitive green fluorescent protein for improved response rate. *Protein Sci* **15**, 45-57
86. Hanson, G. T., Aggeler, R., Oglesbee, D., Cannon, M., Capaldi, R. A., Tsien, R. Y., and Remington, S. J. (2004) Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein indicators. *J Biol Chem* **279**, 13044-13053
87. Lukyanov, K. A., and Belousov, V. V. (2014) Genetically encoded fluorescent redox sensors. *Biochim Biophys Acta* **1840(2)**,745-756