Nascent Adhesions: From Fluctuations to a Hierarchical Organization

Integrins assemble a complex network of molecular interactions at cell–matrix adhesion sites. Fluorescence correlation microscopy has now shed light on the spatial, temporal and numerical distributions of protein complexes during assembly and stabilization of nascent adhesions.

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A hallmark of integrins is their ability to sense the highly dynamic and complex biochemical and mechanical properties of extracellular matrices. To accomplish these tasks, integrins cluster and assemble numerous ancillary proteins in specialized adhesion structures that differ in their morphology, subcellular localization, lifespan and protein composition. Nascent adhesions are the smallest adhesive structures. They emerge at the edge of protruding membranes, are less than 1 μm in diameter and either disassemble after a lifespan of around 1 minute or mature in an actomyosin-dependent manner into large (up to 10 μm^2) and long-lived (several minutes) focal adhesions [1]. The functions that emanate from the various cell–matrix adhesion sites depend on a highly complex network of interacting proteins. Although most adhesion proteins have been identified, little is known about where and when they interact, and to what extent their numbers change during the assembly and stabilization of cell–matrix adhesion sites. In a recent issue of *Current Biology*, Bachir *et al.* [2] used fluorescence correlation microscopy to address such questions during nascent adhesion development [2]. Their results provide a hierarchy of assembly and define the chronology and stoichiometry of protein complexes associated with integrins.

The formation of integrin-containing adhesive sites proceeds in three major steps. It is assumed that all integrins require an activation step that switches the unbound form of integrins from a low-affinity (inactive) state to a high-affinity (active) state [3]. This activation triggers ligand binding and initiates cell adhesion. Integrin activation is induced upon binding of the two adaptor proteins, talin and kindlin, to β integrin cytoplasmic domains. Upon activation, integrins aggregate and recruit integrin-associated proteins into small, signalling-competent clusters that eventually become visible by conventional microscopy as nascent adhesions. Clustering is observed with all integrins and is required to stabilize the integrin–ligand complex, most likely by increasing the probability for dissociated integrin–ligand complexes to rebind before they diffuse away from the adhesion site [4]. Although it is not clear how integrin clustering is achieved at the molecular level, different reports have assigned roles to talin [5], kindlin [6] and the glycocalyx [7]. Finally, a small number of nascent adhesions mature into large and stable focal adhesions, which requires the linkage to the F-actin cytoskeleton and the activation of non-muscle myosin II. Active myosin II generates pulling forces that change the conformation of proteins associated with the integrin tail, such as talin [8] and Cas130 [9], leading to the recruitment of further adhesion-associated proteins. In addition, a force-induced conformational change in the integrin ectodomain leads to increased stability of the integrin–ligand complex, termed adhesion reinforcement [10]. Notably, the newly recruited proteins operate in a positive-feedback manner to further increase F-actin dynamics, myosin II activation and focal adhesion size.

Talin and kindlin are assumed to be the first proteins that bind to integrin cytoplasmic domains [11]. Their recruitment to adhesion sites is thought to be primarily governed by their ability to bind NPxY motifs in β tails — talin to the membrane-proximal motif and kindlin to the membrane-distal motif. It is not clear whether they bind simultaneously or sequentially to integrin tails. Also their function is only partially understood; it is even debated whether both control integrin activation. Similarly, it is also unclear how they regulate integrin clustering and adhesion reinforcement, and whether they share these functions for all integrins and in all cells. For example, although there is ample evidence supporting an essential role for talin in activating integrins in hematopoietic cells, it seems that fibroblasts lacking both talin isoforms (talin-1 and -2) can still adhere to fibronectin and initiate membrane protrusions, although these are short-lived and inappropriate for sustaining cell spreading [12]. Similarly, loss of both talin isoforms in mouse myoblasts leads to severe muscle defects but does not apparently impair activation of β1 integrins and substrate adhesion [13]. Also the function of kindlin is ambiguous. Loss of the hematopoietic isoform (kindlin-3) affects the binding of multiple blood cell types to their substrates and this was thought to be due to an impaired activation of their integrins [14]. A recent study, however, reveals a role for kindlin-3 in integrin clustering rather than activation [6].

Integrin activation and clustering are associated with the recruitment to adhesion sites of a large protein ensemble, which is collectively termed the adhesome [15]. The adhesome was first determined in a meta-survey of the adhesion literature [16] and further refined by systematic studies using high resolution quantitative mass spectrometry [17–20]. The adhesome contains at least 250 proteins, several of which are recruited in a myosin-II-dependent manner. The large inventory of proteins makes it difficult to comprehend the underlying logic of their assembly. Moreover, their recruitment dynamics, stoichiometry, networking, modifications and linkage to the cytoskeleton are poorly understood. In an attempt to address these questions, Bachir *et al.* [2] used fluorescence correlation microscopy to determine the dynamics, stoichiometry and associations of α5β1 integrin, the integrin-binding proteins talin and kindlin-2, and the actin-binding proteins talin, vinculin and α-actinin during the nucleation/assembly and stabilization of nascent adhesions. They fluorescently labelled these proteins, expressed them in CHO cells and analysed their fluorescence

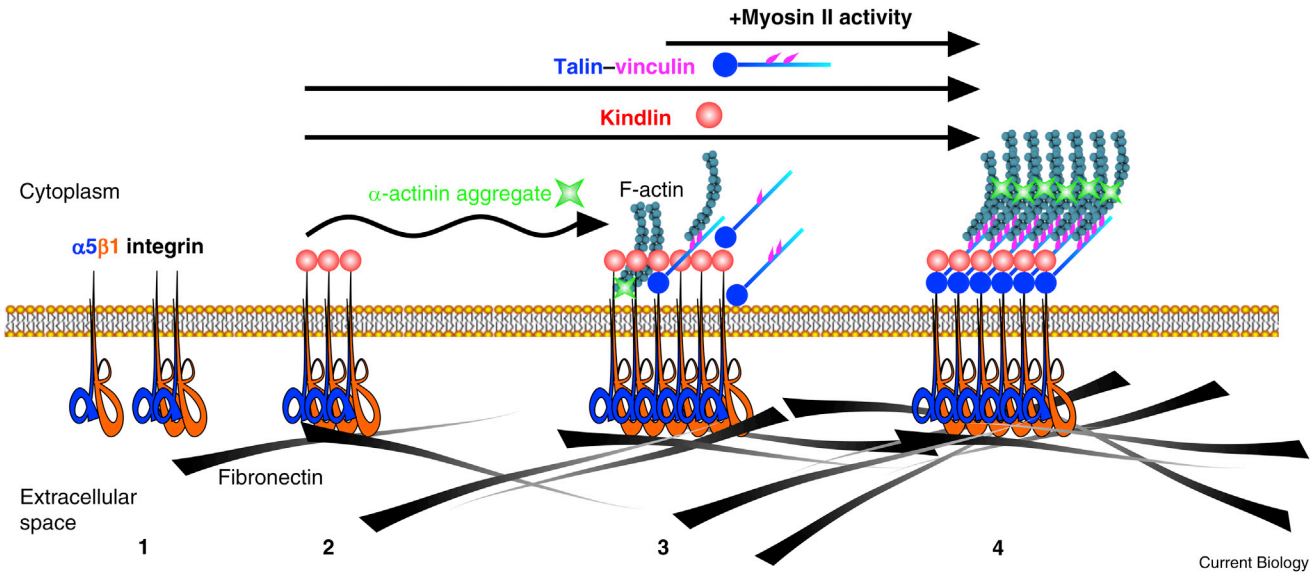

Figure 1. Model of nascent adhesion assembly.

Kindlin-2, talin, vinculin and α-actinin are all recruited to nascent adhesions during their entire lifetime but in different complexes and distinct stoichiometries. Outside of adhesive areas (1), α5β1 integrins remain singly or in clusters of two or three. Kindlin-2 binding to the β1 integrin cytoplasmic domain promotes integrin activation and initiates the nucleation of nascent adhesions (2). Developing nascent adhesions (3) contain talin, which is not associated with α5β1 but forms a complex with vinculin. Aggregates of α-actinin are periodically recruited to nascent adhesions. This is associated with periodic incorporations of further integrins and the initial connection between nascent adhesions and the actin cytoskeleton (3). In stable nascent adhesions (4), integrins reach a 2–3-fold higher degree of aggregation, α-actinin's interaction with integrins is replaced by the talin–vinculin complex, leading to a stable integrin–actin linkage. Myosin II creates pulling forces, resulting in an increased stability of the integrin–ligand interaction, further recruitment of vinculin to the strained talin, and reinforcement of the integrin–actin linkage.

intensity fluctuations in these adhesions using time-lapse total internal reflection fluorescence (TIRF) microscopy. This methodology capitalizes on the movements of the fluorescently labelled proteins, which allows for the determination of their stoichiometry by analyzing the fluctuations of the fluorescence signal with respect to their average intensity, and provides insight into their interactions by simultaneously imaging the fluctuations of two differently labelled proteins and calculating cross-correlations in fluorescence intensities. The results by Bachir *et al.* [2] reveal that the proteins chosen for their study are present throughout the lifetime of nascent adhesions — assembly and stabilization phase — although with different recruitment rates and numbers and in different molecular complexes.

The authors found that neither talin nor kindlin-2 associated with α5β1 integrin in areas adjacent to nascent adhesions, which indicates that α5β1 integrins are not in an activated state outside of adhesion sites. Within nascent adhesions, kindlin-2 and α5β1 co-exist in a 1:1 ratio throughout the lifetime of these adhesions and their positive cross-correlation variance indicates that they are constitutively associated within these adhesions. The association of α5β1 and kindlin-2 is not influenced by the inhibition of myosin II. In contrast, talin, which is also present throughout the lifetime of nascent adhesions, is stably associated with β1 tails only after nascent adhesion assembly is completed, and this interaction is myosin II dependent. Moreover, the stoichiometry of α5β1 and talin is 2:1 in assembling nascent adhesions and 1:1 in mature adhesions.

These findings have significant implications and lead to several important conclusions and hypotheses. The most surprising finding is that kindlin-2 and β1 tails are stably associated in developing nascent adhesions, while talin and β1 tails are not. This suggests that the two integrin activators bind sequentially and that kindlin-2 is probably priming the β1 tails for talin binding. It also suggests that activation of α5β1 is mediated by kindlin-2, while adhesion reinforcement of α5β1 in mature nascent adhesions is mediated by both kindlin-2 and talin, likely by linking the ternary protein complex to actomyosin. The ability of kindlin-2 to activate integrins would be in line with previously published work showing that talin is dispensable for fibroblast adhesion to fibronectin [12] and activation of integrins on mouse myoblasts [13]. However, one should keep in mind that transient interactions of talin with β1 integrin tails that escape detection may very well occur in developing nascent adhesions. Such transient interactions could be sufficient for inducing integrin activation, which is then stabilized by kindlin-2. Nonetheless, the absence of a stable talin–α5β1 integrin complex in developing nascent adhesions excludes a role for talin in integrin clustering during the nucleation of these adhesions. The authors propose that α-actinin may perform this task. Their hypothesis is based on the ability of α-actinin to homodimerize as well as their observation that α-actinin forms aggregates, which are transiently incorporated into developing nascent adhesions. An alternative candidate for integrin clustering and nucleation of nascent adhesions could be kindlin-2, as kindlins were shown to facilitate integrin binding to multivalent ligand [6]. Finally, the lack of association of talin with α5β1 in developing nascent adhesions also indicates that

other adhesome proteins (e.g. RIAM, FAK, vinculin) or lipids (e.g. phosphatidylinositol(4,5)bisphosphate) rather than the integrin tails are responsible for talin recruitment.

In summary, the work of Bachir *et al.* [2] suggests a new model of nascent adhesion assembly and maintenance (Figure 1): kindlin-2 binds to β1 integrin and induces the high-affinity state of α5β1; α-actinin promotes β1 integrin clustering and sets up a transient connection between the integrin cluster and the actin cytoskeleton; and kindlin-2 paves the way for talin recruitment, which replaces α-actinin and establishes a more stable integrin–actin linkage with the help of vinculin, leading to adhesion reinforcement. This sequence of molecular events is both intriguing and provocative, although it may not operate in all cell types and for all integrins, so we look forward to further confirmation in future studies.

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http://dx.doi.org/10.1016/j.cub.2014.07.061

Neuroscience: Waiting for Serotonin

Serotonin dysfunction is implicated in many neuropsychiatric disorders yet the precise behavioral functions of this neuromodulator are not well understood. A new study employs optogenetic methods to activate serotonin neurons during an effort-demanding waiting behavior and demonstrates that serotonin release increases patience, the capacity for self-control.

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It is downtown Manhattan on a Saturday evening. You decide to go to Ippudu Ramen for dinner. There are no reservations, so the person at the door takes down your name and says, "45 minutes". You are not in a rush but as time passes you get hungrier by the minute and less and less patient. At some point, you give up the wait and decide to look for a slice of pizza instead. We all have been in these situations when we lose the ability for self-control and make impulsive decisions. In a new study in the current issue Miyazaki *et al.* [1] shed light (literally and figuratively) on the neural mechanisms underlying patience. The authors show that patient waiting is enhanced by serotonin, an important neurochemical long hypothesized to be involved in inhibition of impulsive actions.

Serotonin is a major neuromodulator implicated in a broad assortment of behavioral and physiological functions, including aggression, appetite, aversion, behavioral inhibition and impulsivity. Serotonergic neurons are located deep in the midbrain and from there they send extensive, highly divergent projections to virtually all areas of the brain (Figure 1A). The serotonin system is one of the most important targets for the treatment of depression, anxiety, panic and mood disorders and other psychiatric conditions. It has been difficult, however, to explain the diverse effects of serotonin on adaptive behavior within a unified framework.

One of the main theories about serotonin proposes that it is important for behavioral inhibition and self-control. Indeed, a prominent behavioral effect of serotonin manipulation is observed in studies of impulsive choice, in which subjects choose between a small immediate