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OBITUARY

In memoriam – Alan Hall

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ABSTRACT

On May 3 of this year, cell biology lost a giant with the untimely passing of Alan Hall (Fig. 1). Alan didn't discover the Rho family of GTPases but, more than anyone else, he and his laboratory brought these key regulatory proteins to the prominent position that they now occupy. I first met Alan in the early 1990s shortly after his landmark papers with Anne Ridley were published (Ridley and Hall, 1992; Ridley et al., 1992). Over the years our interests frequently overlapped, we met often at conferences and became friends. Ultimately, we became collaborators, each of us directing projects within a Program Project Grant that is headed by Klaus Hahn, and that also includes Gaudenz Danuser and John Sondek. Shortly before his death we had been in conversation about this grant and were discussing when we would next get together as a group. I was looking forward to seeing him again, not only because I enjoyed his company but because I always learned something new from every interaction. Other obituaries have covered Alan Hall's career, research accomplishments and service to the research community, such as being Chair of Cell Biology at the Memorial Sloan Kettering Cancer Center and Editor-in-Chief of the *Journal of Cell Biology*. Here, I wish to share my perspective on his enormous contribution to the Rho GTPase field, particularly focusing on the decade of the 1990s when he and his laboratory thrust Rho GTPases to the forefront of cell biology.

Ras to Rho

Before his work on Rho GTPases, Alan Hall was prominent in the Ras field. Starting his own independent laboratory in the early 1980s at the Chester Beatty Laboratories at the Institute of Cancer Research in London, he had joined the search for oncogenes in human cancer. Working in close collaboration with Chris Marshall at the same institute, he discovered N-Ras, a new member of the Ras family of GTPases that was responsible for the transformation of several human cancer cell lines (Hall et al., 1983). After a number of highly significant papers on Ras, Alan began, in the late 1980s, to expand his investigations to include some of the Rho family members. Very little was known about them at that time, other than that they were cousins of Ras. Alan, in collaboration with Klaus Aktories, showed that RhoA was a substrate for ADP-ribosylation by the *Clostridium botulinum* exoenzyme C3 (Aktories et al., 1989). Based on the homology with Ras, Alan and his laboratory generated constitutively active mutant forms of RhoA (G14VRhoA) and used microinjection of the recombinant protein to explore its effects. Constitutively active RhoA induced a contracted phenotype when introduced into isolated Swiss 3T3 cells and a reorganized actin cytoskeleton in confluent cells, which, with hindsight, one can see represents the assembly of stress fibers (Paterson et al., 1990).

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Fig. 1. A recent photograph of Alan Hall in his laboratory at Sloan Kettering. Image courtesy of Memorial Sloan Kettering.

Ridley and Hall papers

From my own perspective of working on focal adhesions, Alan Hall's two papers with Anne Ridley in *Cell* in 1992 were transformative (Ridley and Hall, 1992; Ridley et al., 1992). Frankly, at that time, focal adhesions were a comparative backwater, the sort of topic relegated to the last morning of cytoskeletal or adhesion conferences when many of the conferees had already departed for the airport. The field was still preoccupied with identifying new components and looking for potential interactions between them, mainly in the context of trying to understand how F-actin links up to integrins at these sites. Indeed, in a review that I had written a few years earlier, there were just six named structural proteins that had been identified in focal adhesions together with a handful that were referred to simply by their molecular mass or monoclonal antibody designations. In terms of signaling, even less was known, although 1992 was significant not only for the Ridley and Hall papers, but also for the discovery of focal adhesion kinase (FAK) (Hanks et al., 1992; Schaller et al., 1992). Very little was known about the assembly of focal adhesions or the signaling pathways that controlled them.

The first Ridley and Hall paper in *Cell* changed all that. Although Rho proteins were already known, particularly by those working in the Ras field, most of us studying focal adhesions were unaware of them or had paid them very little attention. In that first paper in *Cell*, Anne Ridley and Alan Hall showed that the introduction of constitutively active RhoA into quiescent fibroblasts rapidly

induced assembly of both stress fibers and focal adhesions. They also identified a pathway that signaled from a component in serum, activating RhoA to drive the formation of stress fibers and focal adhesions. A crucial factor in this work was that they used Swiss 3T3 cells, which became quiescent and rapidly lost their stress fibers upon serum deprivation. I recall seeing Anne Ridley's presentation of their results a few months before publication at the 1992 Cold Spring Harbor Symposium and being puzzled over the loss of stress fibers and focal adhesions in their starved cells. I knew that the cells I worked with did not lose these structures even after a day without serum. But that was a key aspect of their work – they had found a cell type that, when cultured appropriately, responded rapidly to the removal and subsequent re-addition of serum. It allowed them to assay for and identify the component in serum that stimulated focal adhesion and stress fiber assembly. Somewhat unexpectedly, this factor was not a protein, but the lipid lysophosphatidic acid. The identification of a soluble factor in serum that signals to RhoA to regulate stress fibers and focal adhesions changed the landscape of focal adhesion research. Suddenly, many of us became interested not only in how RhoA regulates these structures through downstream effectors, but also in how upstream signaling pathways controlled RhoA activity through its regulators, the guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), as well as in the pathways that signal to them.

In the second of their landmark papers, Ridley et al. established for the first time the link between Rac and the cytoskeleton, demonstrating that Rac stimulates actin polymerization to drive membrane protrusion (Ridley et al., 1992). For several years it had been known that various growth factors induce membrane protrusions, in the form of lamellipodia and membrane ruffles, but how they exerted these effects was unclear. In general, the mechanism was thought to be through the induction of phosphatidylinositol 4,5-bisphosphate (PIP_2), followed by PIP_2-mediated uncapping of actin filaments. To test whether growth factors acted through Rac1, a technique was needed to inhibit this GTPase [the development of small interfering (si)RNA knockdown strategies was still many years away]. Here, Alan's prior experience in the Ras field led to the suggestion of the key reagent. With Ras, it was known that the mutant S17N acted as a dominant-negative construct. Ridley et al. generated the equivalent S17N mutant of Rac1 and showed that this had a tenfold lower affinity for GTP than GDP, thus favoring the inactive GDP-bound form and making it a dominant-negative construct. Expression of this S17N Rac1 mutant effectively blocked growth-factor-stimulated membrane ruffling, indicating that the requirement of Rac1 is crucial for the protrusion of these membranes and for the reorganization of the actin cytoskeleton that ruffling involves. This use of dominant-negative Rho constructs was a breakthrough. It provided the field with a powerful strategy to block specific Rho-family members and is an approach that continues to be used extensively.

Ridley et al. not only established an important role for Rac in driving actin polymerization downstream from growth factors, but they also established for the first time the concept of crosstalk between Rho-family GTPases, as well as crosstalk with other members of the Ras super family. Now we take for granted the existence of multiple levels of crosstalk and pathway intersection, but in the early 1990s, most pathways were linear with few, if any, cross-connections. In their Rac paper, however, Ridley et al. observed that agents that activated Rac also led to the Rho-dependent formation of stress fibers downstream from Rac. Subsequent studies have often observed an antagonistic relationship between Rac and Rho, with Rac generally inhibiting

Rho activity at multiple levels (Guilluy et al., 2011). However, in quiescent Swiss 3T3 cells, Rac-mediated activation of Rho was consistently observed (Ridley et al., 1992).
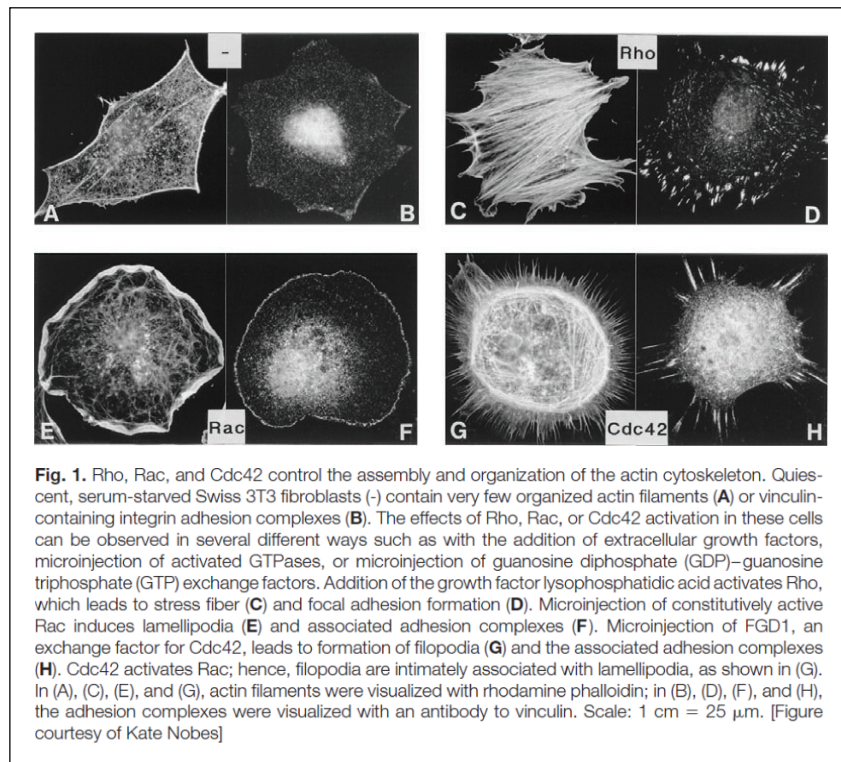
It had been known for several years that active Ras could also induce membrane ruffles and macropinocytosis (Bar-Sagi and Feramisco, 1986). Ridley and co-workers demonstrated that the activity of Ras was blocked by simultaneous expression of dominant-negative Rac, placing Rac downstream of Ras (Ridley et al., 1992). In terms of mechanism, the authors speculated that Ras could be inhibiting a Rac GAP or activating a Rac GEF. Subsequent work has indeed demonstrated that the Rac GEF Tiam1 is activated by Ras (Lambert et al., 2002).

Taken together, the two papers from Ridley and Hall in *Cell* in 1992 revealed, for the first time, the central role of Rho GTPases in regulating cytoskeletal organization. Up to that time, most attention had been focused on signaling pathways that involved cyclic AMP, Ca^{2+} and PIP_2, and how these affected myosin activity or actin-binding proteins. Suddenly, a new cast of characters had been introduced. To this day, we are still exploring the many ways that Rho proteins affect cytoskeletal organization and influence other aspects of cell behavior.

Nobes and Hall papers

Continuing on the theme of crosstalk between Rho family members, shortly after the Rho and Rac papers were published in *Cell*, Alan turned to Cdc42, the other ubiquitously expressed member of the Rho family. With his postdoctoral researcher Kate Nobes, he showed that Cdc42 acted upstream of both Rac and Rho in Swiss 3T3 cells (Nobes and Hall, 1995). At the time, Cdc42 was known to be crucial in determining the polarity of bud site formation in yeast (Johnson and Pringle, 1990), and the mammalian homolog was being studied extensively by Richard Cerione's laboratory (Shinjo et al., 1990). However, little was known about Cdc42 and its effect on the cytoskeleton in mammalian systems. The Nobes and Hall paper, as well as a parallel paper from Louis Lim's laboratory (Kozma et al., 1995), demonstrated that active Cdc42 induces the formation of filopodia. Because of downstream activation of Rac, filopodia are often obscured by Rac-induced lamellipodia that leave the filopodial bundles of actin filaments as rib-like structures within the lamellipodia. The induction of filopodia was most easily seen if the expression of constitutively active Cdc42 was combined with simultaneous inhibition of Rac activity. The Nobes and Hall paper was also significant because it showed that both Rac and Cdc42 can trigger the formation of integrin-based adhesions. These were typically smaller than the focal adhesions induced by active Rho, and Nobes and Hall coined the term 'focal complexes' for these adhesions. Many focal complexes turn over rapidly, but some mature into focal adhesions in a RhoA-dependent manner. Examples of the cytoskeletal and adhesive structures induced by active RhoA, Rac1 and Cdc42 in quiescent Swiss 3T3 cells are shown in Fig. 2, which is taken from one of the many influential reviews that Alan wrote (Hall, 1998).

With the discovery that Rac and Cdc42 stimulate actin polymerization, it was obvious that these two GTPases must play key roles in cell migration. However, how each of the 'big three' (RhoA, Rac1 and Cdc42) cooperate to drive migration deserved analysis. Using a scratch-wound model, Nobes and Hall went on to investigate the roles of each GTPase in migration, as well as the role of Ras (Nobes and Hall, 1999). Not surprisingly, inhibiting Rac prevented both the formation of lamellipodia and the movement of the cells into the wound. Inhibiting Cdc42, however, was only partially effective in reducing migration. Cells in which active

Fig. 1. Rho, Rac, and Cdc42 control the assembly and organization of the actin cytoskeleton. Quiescent, serum-starved Swiss 3T3 fibroblasts (-) contain very few organized actin filaments (**A**) or vinculin-containing integrin adhesion complexes (**B**). The effects of Rho, Rac, or Cdc42 activation in these cells can be observed in several different ways such as with the addition of extracellular growth factors, microinjection of activated GTPases, or microinjection of guanosine diphosphate (GDP)–guanosine triphosphate (GTP) exchange factors. Addition of the growth factor lysophosphatidic acid activates Rho, which leads to stress fiber (**C**) and focal adhesion formation (**D**). Microinjection of constitutively active Rac induces lamellipodia (**E**) and associated adhesion complexes (**F**). Microinjection of FGD1, an exchange factor for Cdc42, leads to formation of filopodia (**G**) and the associated adhesion complexes (**H**). Cdc42 activates Rac; hence, filopodia are intimately associated with lamellipodia, as shown in (**H**). In (A), (C), (E), and (G), actin filaments were visualized with rhodamine phalloidin; in (B), (D), (F), and (H), the adhesion complexes were visualized with an antibody to vinculin. Scale: 1 cm = 25 µm. [Figure courtesy of Kate Nobes]

Fig. 2. From Hall 1998. Reprinted with permission from AAAS.

Cdc42 had been sequestered or inhibited revealed lamellipodia that extended in multiple directions, consistent with a loss of polarized migration. Examination of the orientation of the Golgi complex in front of the nucleus and toward the leading edge confirmed that these cells had lost their oriented migration into the wound. With respect to the role of RhoA in cell migration, it had been known for a long time that stress fibers and focal adhesions are not required for cell migration and that there is often an inverse correlation between the size of these structures and the migration rate of a cell (Herman et al., 1981). Consistent with these earlier observations, Nobes and Hall found that high RhoA activity was inhibitory to migration (Nobes and Hall, 1999). However, inhibiting Rho activity was complex, because blocking RhoA activity completely with the exoenzyme C3 resulted in cell rounding and detachment due to loss of adhesion to the substratum. When they inhibited the RhoA effector Rho kinase (ROCK), the focal adhesions and stress fibers disassembled but the cells still adhered to the substratum. Under these conditions they found that wound closure was accelerated, again supporting the idea that focal adhesions and stress fibers can be inhibitory to rapid cell migration. Interestingly, blocking Ras activity also decreased cell migration, and this inhibition was rescued by inhibiting ROCK or decreasing RhoA activity. These results suggested that Ras decreased RhoA activity in these cells and that inhibition of Ras relieved this effect, retarding migration as a result of RhoA-mediated strengthening of adhesions. Subsequent work has shown that there are multiple ways by which Ras can influence RhoA activity and that, depending on the cell type and situation, it can either activate or inhibit RhoA.

Etienne-Manneville and Hall papers
One of the prominent and conserved functions of Cdc42 is in determining cell polarity. When Sandrine Etienne-Manneville joined Alan's laboratory, she set out to explore this in the context

of mammalian cell migration. Using primary rat astrocytes and a scratch-wound model, which polarizes the cells at the wound edge, they found that integrin-mediated adhesion activated Cdc42 at the cell front. In turn, this recruited the polarity protein Par6 together with PKCζ, an atypical protein kinase C (PKC) (Etienne-Manneville and Hall, 2001). Etienne-Manneville and Hall showed that this complex was responsible for the polarization of the astrocytes, as judged by determining the orientation of the centrosome and Golgi complex in front of the nucleus in the direction of migration into the wound. Additionally, they showed that this was dependent on the microtubule motor dynein. Pursuing the downstream signaling pathways from the Par6–PKCζ complex, they found that glycogen synthase kinase-3β (GSK-3β) was phosphorylated and inhibited through PKCζ in a Cdc42-dependent manner at the leading edge of the polarized astrocytes (Etienne-Manneville and Hall, 2003). In turn, the inhibition of GSK-3β promoted the association of one of its substrates, the adenomatous polyposis coli (APC) protein, with the plus ends of microtubules at the leading edge. Blocking this spatially restricted interaction of APC with the ends of microtubules prevented the establishment of cell polarity.

Later years
Just as it was a natural progression to investigate the roles of the various Rho proteins in cell migration, exploring their effect on phagocytosis was an obvious goal. When Emmanuelle Caron joined Alan's group, this was her objective. It was already known that distinct mechanisms seemed to exist for macrophage phagocytosis through the immunoglobulin receptor or the complement receptor, although both involved reorganization of the actin cytoskeleton. Unexpectedly, Caron and Hall found that these pathways were also distinguished by the Rho GTPases involved (Caron and Hall, 1998). Phagocytosis mediated through the immunoglobulin receptor was

found to be mediated by Rac and Cdc42, whereas Rho was crucial for phagocytosis mediated through the complement receptor.

In addition to revealing the importance of Rho GTPases in the assembly of adhesions between the cell and extracellular matrix, Alan was involved in the first exploration of their role in cadherin-mediated cell–cell interactions. Shortly after establishing her laboratory at University College London, Vania Braga began a collaboration with Alan and his laboratory to investigate the role of RhoA and Rac1 in epithelial cell junctions (Braga et al., 1997). The introduction of C3 into confluent keratinocytes quickly resulted in the loss of E-cadherin, although other junctional proteins remained. Similarly, dominant-negative Rac1 rapidly resulted in the loss of E-cadherin from junctions. This paper laid the groundwork for studies into the complex role of Rho GTPases in cell–cell junctions that continue to this day. Interestingly, at the time of his death, Alan and his laboratory were pursuing the multiple GEFs and GAPs that regulate Rho proteins at epithelial junctions. They have been particularly interested in the roles of GEFs, GAPs and their downstream GTPases in collective cell migration, which is essential in embryonic development but is also involved in the invasive behavior of many tumors.

Alan had received many accolades and prizes for his work on Rho GTPases. Surely there were more awards and bigger ones still to come. Despite this, he was always humble and modest. More important to him than the prizes and the recognition, I believe, was the knowledge that the field that he had done so much to initiate and shape was continuing vigorously and influencing so many other areas. His research leaves a lasting legacy, but his legacy also includes the impact he had on so many of us working in this field. In particular, I look at the list of the many outstanding cell biologists (only a few of whom I have mentioned here) who were his students, postdocs or collaborators. He launched their careers, and they will be the torchbearers for the rigorous, probing and creative cell biology that Alan Hall exemplified.

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