

## REVIEW

# Signaling mechanisms of glucose-induced F-actin remodeling in pancreatic islet $\beta$ cells

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The maintenance of whole-body glucose homeostasis is critical for survival, and is controlled by the coordination of multiple organs and endocrine systems. Pancreatic islet  $\beta$  cells secrete insulin in response to nutrient stimuli, and insulin then travels through the circulation promoting glucose uptake into insulin-responsive tissues such as liver, skeletal muscle and adipose. Many of the genes identified in human genome-wide association studies of diabetic individuals are directly associated with  $\beta$  cell survival and function, giving credence to the idea that  $\beta$ -cell dysfunction is central to the development of type 2 diabetes. As such, investigations into the mechanisms by which  $\beta$  cells sense glucose and secrete insulin in a regulated manner are a major focus of current diabetes research. In particular, recent discoveries of the detailed role and requirements for reorganization/remodeling of filamentous actin (F-actin) in the regulation of insulin release from the  $\beta$  cell have appeared at the forefront of islet function research, having lapsed in prior years due to technical limitations. Recent advances in live-cell imaging and specialized reagents have revealed localized F-actin remodeling to be a requisite for the normal biphasic pattern of nutrient-stimulated insulin secretion. This review will provide an historical look at the emergent focus on the role of the actin cytoskeleton and its regulation of insulin secretion, leading up to the cutting-edge research in progress in the field today. *Experimental & Molecular Medicine* (2013) 45, e37; doi:10.1038/emm.2013.73; published online 23 August 2013

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## INTRODUCTION

### Biphasic insulin secretion from pancreatic islet $\beta$ cells

In response to increased blood glucose levels, pancreatic islet  $\beta$  cells secrete insulin in a biphasic manner (Figure 1). Glucose enters the  $\beta$  cell and is rapidly metabolized to yield a net increase in the ATP/ADP ratio. ATP-sensitive potassium ( $K_{ATP}$ ) channels close and cause membrane depolarization, which opens voltage-dependent calcium channels.<sup>1</sup> Subsequent calcium influx leads to insulin granule docking and fusion with the plasma membrane in a process referred to as 'insulin granule exocytosis'.<sup>2–4</sup> Insulin granule docking and fusion events are mediated by a group of proteins termed soluble N-ethylmaleimide sensitive factor attachment receptor (SNARE) proteins. This stimulus-secretion coupling pathway results in a rapid robust spike of insulin secretion called first phase, derived from insulin granules that are both pre-docked within 100–200 nm of the plasma membrane as well as so-called 'restless newcomer' granules; this pool of release-competent

granules is referred to as the 'readily releasable pool'.<sup>5,6</sup> After the first-phase peak, the insulin release rate drops to two- to fivefold above basal secretion and is sustained at this lower rate of insulin release, called 'second phase,' which persists until euglycemia is restored.<sup>7,8</sup>

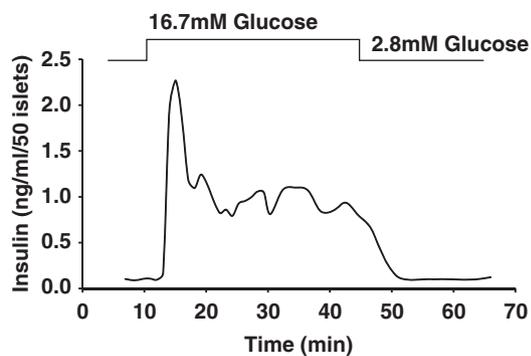
Second-phase insulin secretion requires the recruitment of granules from intracellular storage pools to the plasma membrane and involves reorganization of the filamentous actin (F-actin) cytoskeletal network (Figure 2a).<sup>9–11</sup> This reorganization takes place in the cortical F-actin web (Figure 2b). Stress fiber-localized actin and focal adhesion actin-remodeling events, which occur at cell surface and cell-cell junctions, also contribute to insulin secretion.<sup>12–14</sup> This review will focus largely on cortical F-actin remodeling, as it is cortical F-actin that oversees insulin granule access to the plasma membrane, but the importance of focal adhesions will be discussed where relevant. Pre-clinical and frank type 2 diabetes are associated with losses in both first and second

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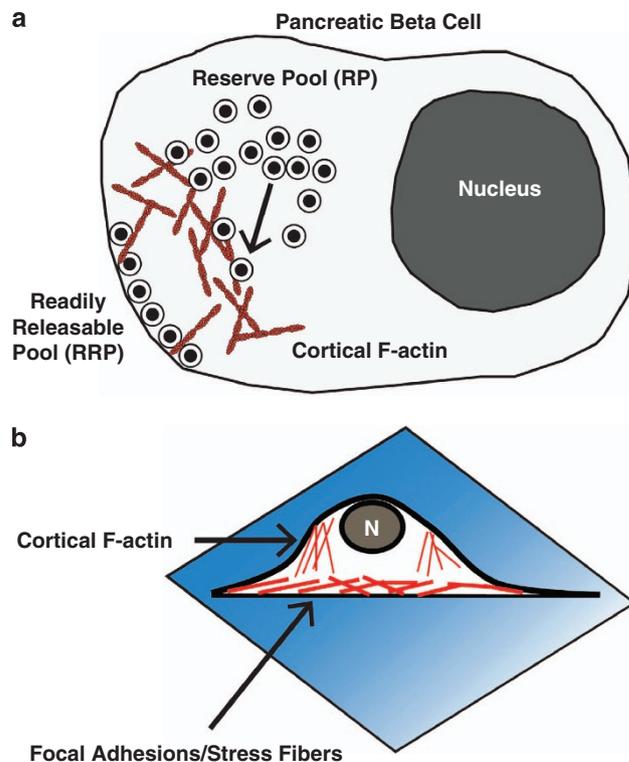


**Figure 1** Biphasic glucose-stimulated insulin secretion from perfused mouse islets. In response to a square-wave increase in glucose concentration, islet  $\beta$  cells dock/fuse  $\sim 50$ – $100$  granules in the first phase of secretion. First phase is temporally defined, complete within  $\sim 10$  min of glucose stimulation, thought to be accounted for in large part by the readily releasable pool of granules. First phase is immediately followed by a second phase, with second phase being lower in amplitude but persistent in the presence of glucose stimulation over hours of time, and thus not temporally limited. Second phase is presumed to require storage/reserve pools of granules that can be recruited to the plasma membrane for secretion.

phases of insulin secretion.<sup>15,16</sup> Of the two phases, there is far less known about the mechanistic regulation of second-phase insulin secretion. Key proteins shown by perfusion to be required for both phases of insulin secretion include, but are not necessarily limited to, the SNARE protein Syntaxin 4, the SNARE-related proteins tomosyn-2 and Doc2b, Rab27a, Rab3a, granuphilin and RalA.<sup>17–22</sup> The SNARE protein Syntaxin 1 is only required for first phase.<sup>23</sup> Currently identified regulators specific to second-phase insulin secretion include Cdc42, p21-activated kinase (PAK1), RhoGDI, oleate/GPR40/PKD1 and Munc18c.<sup>24–28</sup> Notably, many proteins required for biphasic secretion have connections to actin cytoskeleton remodeling.

### FILAMENTOUS ACTIN AS A REGULATOR OF INSULIN GRANULE EXOCYTOSIS

F-actin was first recognized as a regulator of exocytosis  $\sim 40$  years ago.<sup>29–33</sup> Over this time, use of F-actin-disrupting drugs like cytochalasins, latrunculins, clostridium C2 toxins and F-actin-polymerizing drugs like phallotoxins and jasplakinolide, have proven useful in implicating roles for F-actin in secretory/exocytotic processes across multiple cell types.<sup>29,33,34</sup> These cell types include pancreatic  $\beta$  cells and acinar cells,<sup>35</sup> enteroendocrine L cells,<sup>36</sup> chromaffin and PC12 cells,<sup>37,38</sup> melanotrophs and lactotrophs,<sup>39,40</sup> neutrophils,<sup>41–43</sup> platelets,<sup>44</sup> endothelial cells,<sup>45</sup> neurons,<sup>46</sup> adipocytes<sup>47</sup> and myocytes.<sup>48</sup> In chromaffin cells, F-actin was shown to form a cortical ring beneath the plasma membrane, function as a barrier between vesicles and the cell surface, and to colocalize with cytoskeletal proteins such as myosin, caldesmon,<sup>30,49,50</sup> gelsolin or scinderin<sup>51</sup> and fodrin.<sup>52</sup> Landmark work in 1972 by Orci *et al.*<sup>29</sup> showed electron micrographs of islet  $\beta$  cells



**Figure 2** Cortical filamentous actin (F-actin) remodeling in  $\beta$  cells regulates insulin granule exocytosis. **(a)** Schematic of a pancreatic  $\beta$  cell depicts how readily releasable pool (RRP) granules are docked at the membrane and reserve pool granules are in a more intracellular storage pool. Cortical F-actin can regulate access of reserve pool granules to the readily releasable pool. **(b)** Microscopic analysis of  $\beta$  cell-actin remodeling is often performed on cells attached to coverslips (a very different environment from the three-dimensional islet architecture) where it is important to note that focal adhesions/stress fibers form at the cell attachment interface. Conversely, cortical F-actin is visualized at the cell perimeter when focused at the mid plane of the cell. This distinction is particularly important in differentiating cortical F-actin remodeling from other types of actin remodeling.

depicting F-actin in the form of a cell web just beneath the plasma membrane, further demonstrating that depolymerization of F-actin with *Clostridium botulinum* C2 toxin, Cytochalasin B or E potentiated glucose-stimulated insulin secretion from pancreatic islets,<sup>33,53–55</sup> consistent with the ‘barrier’ model formulated in chromaffin cells. However, the role of F-actin as a simple barrier in the  $\beta$  cell was quite controversial, given additional data showing that actin depolymerization inhibited glucose-stimulated insulin secretion from the HIT (hamster)  $\beta$  cell line,<sup>33</sup> as well as an increased fraction of F-actin in pancreatic  $\beta$ -cell homogenates prepared from rodent islets stimulated with glucose.<sup>56,57</sup> Technological advances made in the last decade enabling the visualization of F-actin remodeling and insulin exocytosis in  $\beta$  cells have re-invigorated this field of research, as has the outcomes of genome-wide association studies pointing to  $\beta$  cell functional failure as a primary feature of type 2 diabetes.

### Positive and negative roles of F-actin in glucose-stimulated insulin exocytosis

To sustain insulin release, mature insulin granules located in the intracellular storage pools must be mobilized toward the plasma membrane. This process coincides with what is now referred to as glucose-induced remodeling of the actin cytoskeleton,<sup>56,58</sup> as it encompasses the simultaneous localized depolymerization and polymerization of F-actin across the cell in a concerted manner. The concept of remodeling has evolved from early studies wherein F-actin was first thought to function solely as a negative barrier to restrict insulin granule accumulation at the plasma membrane.<sup>29,30,33,54</sup> This was based upon a body of literature that relied upon pharmacological depolymerizing agents, usage of which results in more morphologically docked granules and may confer enhanced release competence to granules.<sup>10,59</sup> However, early evidence of positive effects of the cytoskeleton in stimulus-induced insulin secretion existed as well, raising controversy as to the true role and requirement for F-actin in insulin granule exocytosis.<sup>56,57,60–62</sup> Upon the advent of live-cell and time-lapse imaging of subcellular-localized  $\beta$  cell F-actin changes using confocal microscopy, and in conjunction with genetic manipulations of proteins involved in F-actin changes rather than reliance upon pharmacological agents, these positive and negative roles of F-actin in insulin granule exocytosis were understood to be the cyclic nature of F-actin remodeling.

### F-actin and the regulation of basal insulin secretion

Control of basal insulin secretion, the amount of insulin secreted under non-stimulatory or sub-threshold conditions, is a critical part of maintaining whole-body glucose homeostasis.<sup>63</sup> Aberrantly increased insulin secretion under fasting conditions can cause hypoglycemia acutely, and chronically may contribute to the development of insulin resistance in peripheral tissues.<sup>63</sup> The barrier role of F-actin is considered an integral part of maintaining low levels of insulin release under basal conditions, via restricting insulin granule access to the cell surface/plasma membrane docking and fusion machinery.

The actin cytoskeleton has been linked to roles in basal exocytosis through several factors:  $\beta$ -Pix,<sup>64</sup> Caveolin-1,<sup>65</sup> focal adhesion kinase (FAK),<sup>14</sup> EphA–Ephrin–A signaling<sup>62</sup> and Gelsolin.<sup>66</sup> Depletion from  $\beta$  cells of either  $\beta$ -Pix (also called Cool-1) or Caveolin-1, which coordinately regulate Cdc42 activation, leads to dysregulated basal insulin secretion.<sup>64,65</sup> Cdc42 is a small Rho family guanine nucleotide triphosphates (GTPase) that is kept mostly inactive (>90%) in the  $\beta$  cell by the binding of Caveolin-1, the guanine nucleotide dissociation inhibitor (GDI) for Cdc42 localized to the insulin secretory granule. Cdc42 becomes activated in response to glucose stimulation by the dissociation of Caveolin-1 and the activation of the guanine nucleotide effector protein  $\beta$ -Pix (discussed in detail in the next subsection). A body of work suggests that maintenance of Cdc42 in its inactive state is important to maintain low levels of insulin secretion under basal (~5 mM glucose) conditions. Pharmacological inhibition

of FAK, localized to focal adhesions, caused an increase in basal insulin secretion, which was related to disrupted  $\beta$  cell F-actin structure due to discontinuous cell–cell contact.<sup>12,14</sup> EphA5–Ephrin–A5 signaling mediates a paracrine signaling pathway that regulates insulin secretion. Disruption of EphA5–Ephrin–A5 signaling in MIN6  $\beta$  cells prevents normal glucose-induced Rac1 activation and F-actin remodeling.<sup>62</sup> The binding of Gelsolin, a cortical F-actin severing and capping protein, to the docking and fusion target-SNARE protein, Syntaxin 4, required for biphasic insulin exocytosis, has been shown to be essential in the maintenance of minimal insulin release under basal conditions.<sup>66</sup> While it is clear that signaling related to F-actin regulation has impacts on basal insulin secretion, further investigation of the dynamics of F-actin remodeling and how basal insulin secretion is controlled will be useful in understanding this linkage.

### SIGNALING PATHWAYS LEADING TO F-ACTIN REMODELING IN THE $\beta$ CELL

#### Small Rho family GTPases regulate F-actin remodeling and insulin secretion

Small GTPases, such as Cdc42 as mentioned above, have many roles in cell biology including, but not limited to cytoskeletal reorganization, membrane trafficking and cell growth.<sup>67</sup> These GTPases are known as the Ras superfamily due to their sequence homology; well-studied families include Ras, Rho, Rab, Arf, Rap and Ran.<sup>68</sup> Of particular importance to second-phase insulin release and F-actin remodeling is the Rho family, which contains protein members Cdc42, Rac and Rho. Small GTPases become activated when bound to GTP and remain active until the GTP is hydrolyzed to yield GDP and inorganic phosphate.<sup>67</sup> The GDI-bound GTPase is restricted from the cell surface, and upon dissociation of the GDI from the GTPase enters the cycle of GTP binding and hydrolysis, whereby the guanine nucleotide effector proteins then promote the dissociation of GDP and binding of GTP.<sup>69</sup> Active small GTPases bind to effector molecules to propagate their signal. As described in greater detail below (in the subsequent four sections), the activation of Cdc42 in  $\beta$  cells leads to subsequent activation of Rac1, through the activation/phosphorylation of the PAK1.<sup>25</sup> Arf6,<sup>70</sup> Rab27A,<sup>71–74</sup> Rab3a<sup>22</sup> and Rap1<sup>75</sup> have emerged as additional potential positive effectors of insulin release. On the other hand, initial findings suggest against positive roles for RhoA, Ras or Ran in insulin secretion.<sup>76,77</sup>

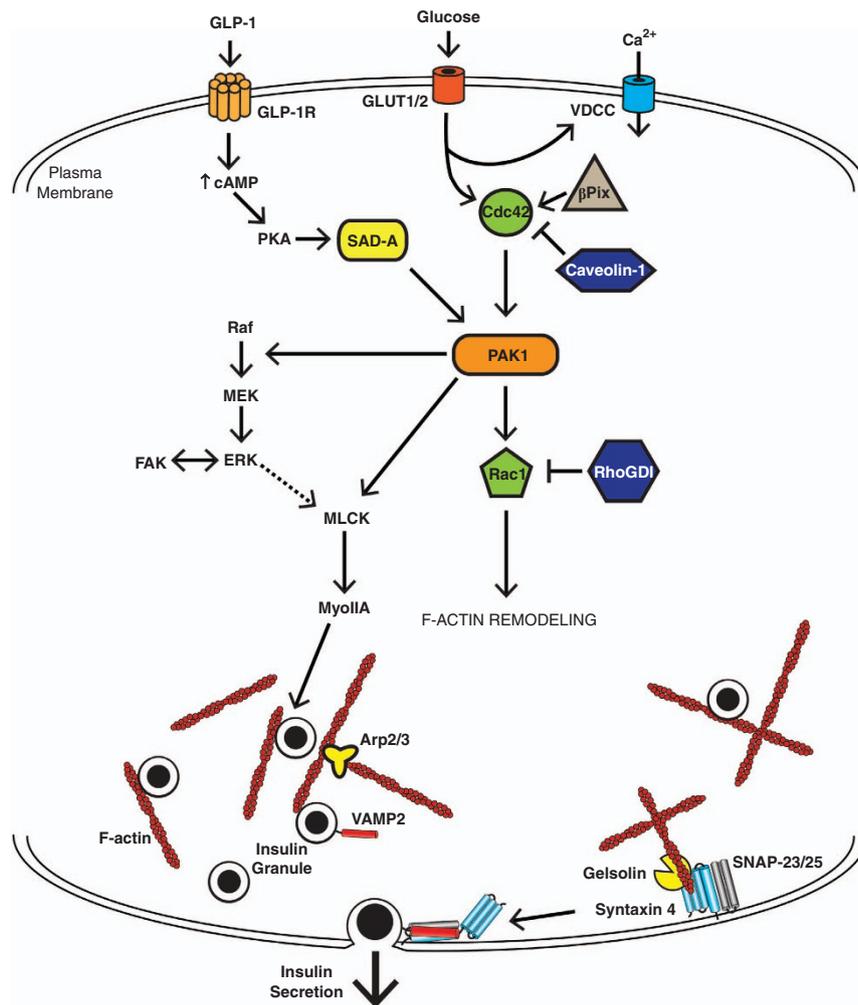
#### The Cdc42–PAK1–Rac1 signaling pathway

Early studies implicating Cdc42 and Rac1 cycling in F-actin remodeling coupled to glucose-stimulated insulin secretion involved expression of GTPase cycling mutants.<sup>34,78</sup> Subsequent studies using RNAi-mediated knockdown of each GTPase confirmed their roles to be in the second phase of insulin secretion, and further revealed their signaling itineraries in human and mouse  $\beta$  cells.<sup>25,27</sup> Most recently,  $\beta$  cell-specific Rac1 knockout mice have been characterized and the role of Rac1 in F-actin remodeling and second-phase

insulin release confirmed.<sup>79</sup> However,  $\beta$  cell-specific Cdc42 knockout mice have yet to be generated, and as Cdc42 was found to be required for formation of the pancreatic architecture,<sup>80</sup> islets of classic Cdc42 knockout mice are unavailable for study.

In mouse insulinoma MIN6 and rat insulinoma INS 832/13 clonal  $\beta$  cell lines, Cdc42 is activated within 3 min of glucose stimulation and activation declines to basal levels within the following 2 min;<sup>25,34,64,70</sup> in human islets, this has been detected indirectly by the phosphorylation and activation of Cdc42's downstream effector, PAK1<sup>27,81</sup> (Figure 3). The activation–deactivation cycling of Cdc42 coincides with

post-translational modifications such as carboxymethylation, prenylation and O-glycosylation.<sup>34,82</sup> The direct signaling factor responsible for activating Cdc42 remains at-large, although progress has been made in this regard. It has been deduced that Cdc42 activation requires glucose metabolism, as non-metabolizable glucose analogs or KCl-depolarization induced  $\text{Ca}^{2+}$  influx fail to induce Cdc42 activation.<sup>25</sup> New data from our laboratory suggest this factor signals through a Src family kinase upstream of Cdc42 activation (unpublished results, SM Yoder and DC Thurmond), wherein activation follows with the glucose-stimulated association of Cdc42 with  $\beta$ -Pix within 2 min, coordinate with the timing of Cdc42–GDP



**Figure 3** Pathways impinging specifically on glucose-mediated F-actin remodeling in the  $\beta$  cell. The  $\beta$  cell responds to certain insulin secretagogues by remodeling its cortical filamentous actin (F-actin). Glucose metabolism can signal to Cdc42, at least partially through  $\beta$ -Pix, to activate p21-activated kinase (PAK1) and evoke Rac1 activation. Under basal conditions, Cdc42 and Rac1 are held inactive by guanine nucleotide dissociation inhibitor (GDI) proteins Caveolin-1 and RhoGDI. In addition, GLP-1 activation of GLP-1R can lead to increased cAMP and PKA activation which can activate SAD-A kinase and feed into the PAK1 pathway. Active PAK1 signals to multiple effectors, such as Rac1, to facilitate F-actin remodeling. PAK1 is important for Raf/MEK activation, leading to ERK activation. PAK1 (and possibly ERK as well) may then signal to myosin light-chain kinase (MLCK)-myosin IIA (MyoIIA) to further mediate F-actin remodeling. Focal adhesion kinase (FAK) participates in cross-talk with ERK, which may indirectly modulate cortical F-actin remodeling. The F-actin binding and severing protein Gelsolin complexes directly with the t-SNARE (soluble N-ethylmaleimide sensitive factor attachment receptor) protein Syntaxin 4, ultimately impacting SNARE-mediated insulin exocytosis. Syntaxin 4 in particular is essential for second-phase insulin release, complexing with the other t-SNARE protein synaptosomal-associated protein of 25 kDa (SNAP-25) (or SNAP-23) and the incoming granule vesicle-SNARE, VAMP2.

dissociation from its GDI, Caveolin-1.<sup>64</sup> Upon activation, Cdc42 triggers phosphorylation and activation of PAK1, ~5 min from the start of glucose stimulation, followed by Rac1 activation after ~10 min more (15 min from the start of glucose stimulation).<sup>25</sup> Thus, the activation of Rac1 occurs during the second phase of insulin release, and both Cdc42 and Rac1 are required for this phase of secretion through activation of PAK1 in a sequential pathway.

Under basal unstimulated conditions, activated Cdc42 is maintained at a low level, ~7% of total Cdc42, via sequestration by GDIs;<sup>26,34,65</sup> Rac1 is also kept in the inactive state.<sup>78</sup> So far, two GDI proteins for Cdc42 in  $\beta$  cells have been identified: Caveolin-1 and RhoGDI.<sup>26,65,83</sup> In the  $\beta$  cell, Cdc42 is in the cytosol, at the plasma membrane and on insulin secretory granules;<sup>64,65</sup> Rac1 is principally cytosolic.<sup>83</sup> Caveolin-1, a membrane-bound protein important for caveolae formation as well as in signal transduction,<sup>84–86</sup> was found to function in an unconventional manner as a Cdc42 GDI protein in islet  $\beta$  cells in 2006,<sup>65</sup> binding to that quotient of Cdc42 localized to insulin granules; this new role for Caveolin-1 has since been confirmed in other cell types.<sup>87,88</sup> Conversely, RhoGDI binds to the cytosolic pool of Cdc42; RhoGDI also binds to Rac1 in the cytosol, and differential RhoGDI tyrosine and serine phosphorylation events account for the distinct spatial and temporal dissociation patterns of RhoGDI-Cdc42 versus RhoGDI-Rac1 in the  $\beta$  cell.<sup>26,83</sup> Depletion of RhoGDI from mouse islets selectively and robustly potentiates the second phase of insulin secretion, correlating with increased glucose-stimulated Cdc42 activation.<sup>26,83</sup> Whether it is the cytosolic pool of Cdc42 that is released from RhoGDI or the insulin granule pool of Cdc42 that is released from Caveolin-1 that ultimately activates PAK1 to then activate Rac1 remains to be discerned. Some evidence suggests that the cytosolic Cdc42 released from RhoGDI upon glucose stimulation transits to the insulin granule storage pool rather than to the plasma membrane (Z Wang and DC Thurmond, unpublished results). Cdc42 may be needed on the insulin granule to assist with their targeting to fusion sites at the plasma membrane for secretion, by virtue of its propensity to interact directly with the vesicle-SNARE protein VAMP2, forming heterotrimeric complexes with Syntaxins.<sup>89</sup> While it initially strikes odd that Cdc42 activation occurs early during the first phase of insulin release but is only required selectively for second-phase glucose-stimulated insulin secretion, we speculate that the early activation of Cdc42 functions to initiate signaling cascades necessary to evoke F-actin remodeling and granule mobilization to the plasma membrane to support the second phase of insulin release. This is consistent with the multiple reports of F-actin remodeling visualization. Indeed, recent live-cell imaging demonstrates that PAK1 activation is essential for F-actin remodeling to occur in  $\beta$  cells.<sup>81</sup> In this manner, the early activation of Cdc42 could facilitate insulin granule trafficking toward the plasma membrane, and/or enhance the release competence of granules that are already juxtaposed to the plasma membrane.

### The role of PAK1 signaling in the $\beta$ cell

PAK1 has recently evolved as a protein of interest in  $\beta$  cell function, as it is ~80% reduced in the islets of type 2 diabetic individuals.<sup>27</sup> Whether this deficit marks PAK1 as a point of genetic susceptibility to type 2 diabetes, or rather that PAK1 is affected as a consequence of metabolic aberrations associated with type 2 diabetes, remains to be determined. PAK1 is one of six members of the PAK family, with isoforms grouped into two main categories based upon sequence homology. Group I PAKs include PAK1, PAK2 and PAK3; group II includes PAK4, PAK5 and PAK6. The PAK (p21-activated kinase) family is named for the 21 kDa proteins that activate them, Rac1 and Cdc42. Both Cdc42 and Rac1 interact with the CRIB domain of PAK1, which in turn induces a conformational change in PAK1 to dissociate the kinase and regulatory domains to induce its activation.<sup>90</sup> PAK1 can serve as the effector or upstream activator of Cdc42 and Rac1, with its role being seemingly cell-type dependent. For example, in  $\beta$  cells, PAK1 is activated downstream of Cdc42 but is the upstream activator of Rac1<sup>25</sup> (Figure 3). In non- $\beta$  cells, Rac1 can be upstream of Cdc42,<sup>91</sup> or Rac1 and Cdc42 can exert antagonistic actions.<sup>92</sup> Recently PAK1 has been shown to be activated by SAD-A kinase (also known as BRSK2) in response to cAMP signaling in the  $\beta$  cell.<sup>93,94</sup> However, whether SAD-A kinase action on PAK1 is dependent on Cdc42 or not remains an open question, as does the true role of SAD-A kinase, as knockdown of this gene has been shown to both increase and decrease insulin secretion.<sup>93,95</sup>

The recent availability of PAK1 knockout mice and of the PAK1 activation inhibitor IPA3 have helped to elucidate the role and requirement for PAK1 in second-phase secretion and F-actin remodeling in the islet  $\beta$  cell. Classic whole-body PAK1 knockout mice exhibit *in vivo* glucose intolerance,<sup>27,93</sup> and their islets show loss of second-phase insulin secretion *ex vivo*.<sup>27</sup> Both human and mouse islets also exhibit glucose-stimulated ERK1/2 activation<sup>27,81,96,97</sup> and studies of islets from PAK1 knockout mice as well as PAK1-depleted clonal  $\beta$  cells revealed ERK1/2 activation to be dependent upon PAK1 activation and signaling (as will be discussed further in the next section). Alternatively, or in addition to signaling through ERK, PAK1 in other cell types has been demonstrated to utilize several other substrates for phosphorylation, including LIM kinase,<sup>98</sup> Filamin A,<sup>99</sup> myosin light-chain kinase (MLCK),<sup>100</sup> RhoGDI<sup>101</sup> and the p41-Arc subunit of the Arp2/3 complex.<sup>102</sup> As such, it remains possible that the modification of one or more of these targets by PAK1 could contribute to F-actin remodeling and second-phase insulin secretion. As the MLCK substrate myosin IIA has recently been implicated in  $\beta$  cell-actin remodeling and insulin secretion,<sup>103</sup> it is possible this pathway is linked through Cdc42-PAK1 signaling.

### The role of Rac1 signaling in the $\beta$ cell

Rac1 was first implicated in glucose-specific insulin secretion in 1997 in clonal  $\beta$  cells,<sup>76,104</sup> later confirmed to being refractory to KCl-stimulation in 2004.<sup>78</sup> In 2007, Rac1 was placed downstream of Cdc42 activation, and in 2010, Kowluru

*et al.*<sup>105</sup> demonstrated that Raf-1 and ERK were required for glucose-induced Rac1 activation in INS 832/13 cells. Most recently, in 2012, characterizations of  $\beta$  cell-specific Rac1 knockout mice confirmed these results: the mice exhibited impairments in glucose tolerance *in vivo*, their islets showed impaired second-phase insulin release *ex vivo* with defective glucose-stimulated F-actin remodeling, and the islets showed no impairment to either first-phase glucose-stimulated or KCl-stimulated insulin release,<sup>79</sup> confirming all prior clonal  $\beta$  cell studies. However, the question still remains about how and why Rac1 activation is detected after the initial visualization of F-actin remodeling and the onset of second-phase insulin release. One possible explanation for this discrepancy is that Rac1 has a role in perpetuating, rather than initiating, second-phase insulin release. A potential mechanism for this putative perpetuating effect may involve Rac1 signaling to Gelsolin.<sup>66,106,107</sup> Rac1-dependent PIP<sub>2</sub> production was suggested to be a driving force to inactivate Gelsolin, which in turn would alter the rate and/or localization of cortical F-actin remodeling.<sup>43</sup> The development of specific acute inhibitors of Rac1 activation, as has been done recently for Cdc42,<sup>81,108</sup> should assist future studies to parse out the role of Rac1 signaling in actin remodeling and its relationship to second-phase insulin release in the  $\beta$  cell.

#### Other small GTPases with potential roles in $\beta$ cell F-actin remodeling

Other small GTPases have been investigated for their roles in biphasic insulin secretion. For example, Arf6 was recently reported to become activated within the first minute of glucose stimulation and was proposed to be an upstream activator of the Cdc42 pathway.<sup>70</sup> However, that Arf6 can be activated by KCl<sup>70</sup> is inconsistent with the glucose-selective activation of Cdc42 and of second-phase insulin secretion,<sup>25</sup> calling into question the role of Arf6 in the Cdc42–PAK1 signaling pathway. Another GTPase, Rap1, has also been shown to function as a positive regulator of insulin secretion. Rap1 is activated downstream of GLP-1 signaling in  $\beta$  cells due to cAMP-dependent activation of the Rap1 guanine nucleotide effector Epac2.<sup>75,109</sup> Rap1 is required for the full potentiation of glucose-induced insulin secretion by cAMP,<sup>75</sup> but whether this is linked to F-actin remodeling is unknown. GTPases Rab27a<sup>21</sup> and Rab3A<sup>22</sup> are two additional positive effectors for both phases of insulin secretion.<sup>73</sup> Although it is unclear if Rab3A is related to actin remodeling, Rab27a is implicated, as it signals downstream to myosin Va to promote insulin granule transport to the plasma membrane.<sup>110,111</sup>

#### ROLE OF ERK IN INSULIN SECRETION AND ACTIN REMODELING

More than 15 years ago, ERK was shown to be activated in response to glucose in  $\beta$  cells.<sup>112,113</sup> Although early studies concluded that active ERK translocated to the nucleus to exert effects upon transcription of insulin and other targets, a significant pool of cytoplasmic active ERK remained,<sup>96,112,113</sup> and was later revealed to be necessary for glucose-stimulated

insulin secretion in rat islets.<sup>96</sup> In 2010, it was confirmed via RNAi-mediated depletion studies that ERK was indeed essential for glucose-stimulated insulin secretion, and solidified as an upstream activator of Rac1.<sup>105</sup> Substantiating this signaling role for ERK, very recent work has suggested ERK may be important for amplification of insulin secretion in response to glucose.<sup>81,114</sup>

#### The Ras–Raf–MEK–ERK pathway in the $\beta$ cell

In the  $\beta$  cell, ERK has been shown to be activated in multiple ways. For example, glucose triggers calcium influx, calcium activates calcineurin to dephosphorylate B-Raf,<sup>115</sup> and Raf then phosphorylates the MAP/ERK kinase (MEK) to subsequently phosphorylate ERK.<sup>116</sup> Alternatively, Raf-1 was recently shown to be dependent upon Cdc42 activation and PAK1 signaling, which ultimately led to ERK activation.<sup>81</sup> Early studies using pharmacological inhibition of ERK yielded conflicting results about its role in the  $\beta$  cell. Specifically, while inhibition of ERK activity had negligible impact upon stress-fiber actin remodeling,<sup>106</sup> it did impact focal adhesion remodeling.<sup>14</sup> More recent studies using a newer and more specific inhibitor support a role for ERK in glucose-stimulated cortical actin remodeling, with ERK positioned downstream of PAK1 signaling.<sup>81,114</sup> Thus, whether glucose-stimulated ERK activation in the  $\beta$  cell occurs in one sequential pathway downstream of Raf-1  $\rightarrow$  PAK1, or instead is triggered concurrently via Ca<sup>2+</sup>  $\rightarrow$  calcineurin  $\rightarrow$  B-Raf, will require further investigation.

#### ERK targets actin regulatory proteins

ERK activation is clearly coupled to the actin cytoskeleton. ERK has been shown to phosphorylate synapsin I, cortactin, myosin light-chain kinase (MLCK), FAK and paxillin in other cell types. Although synapsin I does not have an essential role in glucose-stimulated insulin secretion,<sup>117</sup> FAK and MLCK have been established as necessary factors,<sup>13,118</sup> cortactin is yet to be investigated in  $\beta$  cells. As FAK is required in  $\beta$  cells for basal and stimulated insulin secretion and focal adhesion remodeling,<sup>12–14</sup> there exists the potential for input from the ERK pathway through FAK signaling to impact F-actin remodeling (Figure 3). ERK can also phosphorylate FAK to disrupt FAK-paxillin binding,<sup>119–121</sup> and paxillin was suggested to be important for glucose-stimulated insulin secretion from primary rat  $\beta$  cells.<sup>14</sup> MLCK has also been implicated in cortical F-actin remodeling and glucose-stimulated insulin secretion,<sup>103,122</sup> although its placement downstream of ERK in  $\beta$  cells remains to be demonstrated. Details of MLCK and FAK signaling in the  $\beta$  cell will be discussed in the following sections.

#### ACTIN-BINDING PROTEINS IN INSULIN GRANULE EXOCYTOSIS

As cortical F-actin remodeling is known to couple granule mobilization to the SNARE exocytosis machinery in a dynamic and transient manner,<sup>9,12,59,66,89</sup> recent efforts have focused upon analyses of proteins that control the temporal and spatial

polymerization and depolymerization events. The idea that F-actin severing and stabilizing proteins may underlie glucose-stimulated F-actin remodeling fits with the current model and also leaves room for the potential modifiers of F-actin dynamics in the  $\beta$  cell.

### F-actin severing proteins in the $\beta$ cell

$\beta$  cells are known to express the F-actin severing proteins Gelsolin, Scinderin and Cofilin. Gelsolin is the founder of a family of calcium-activated actin-severing and -capping proteins, first cloned in 1979.<sup>123,124</sup> This protein family also includes Scinderin, CapG and many others,<sup>124</sup> although only Gelsolin and Scinderin are known to be expressed in  $\beta$  cells. Gelsolin consists of six globular domains (S1–6), each of which binds calcium; S2 also binds to phosphoinositides.<sup>124</sup> There are two isoforms of Gelsolin encoded as different mRNAs from the same gene: an intracellular cytosolic isoform and a secreted plasma isoform, which contains a 50-residue N-terminal extension.<sup>125,126</sup> The intracellular cytosolic form of Gelsolin participates in F-actin remodeling. Gelsolin was first implicated in glucose-stimulated insulin release in 2006.<sup>106</sup> In 2012, a role of Gelsolin was determined to be in clamping unsolicited basal secretion, through its interaction with the target-SNARE protein, Syntaxin 4, at the plasma membrane<sup>66</sup> (Figure 3). Scinderin shares nearly 60% sequence identity to Gelsolin,<sup>127</sup> yet unlike Gelsolin, Scinderin expression is largely restricted to neuroendocrine cells (including islet  $\beta$  cells),<sup>128,129</sup> and is differentially regulated by calcium. Scinderin becomes activated at lower levels of calcium (0.5  $\mu$ M) than Gelsolin ( $\sim$ 1–3  $\mu$ M).<sup>130</sup> In patch-clamped primary  $\beta$  cells, peptides derived from Scinderin's PIP<sub>2</sub>-binding domain were shown to inhibit both calcium- and GTP $\gamma$ S-induced insulin secretion, potentially implicating Scinderin in glucose-stimulated insulin secretion.<sup>131</sup> However, the role of Scinderin in glucose-induced F-actin remodeling and biphasic insulin release remains untested in the islet  $\beta$  cell. The least well-studied severing protein present in  $\beta$  cells is Cofilin. Cofilin is usually thought to be regulated upstream by PAK1 activation of LIM kinase. Active LIM kinase then phosphorylates Cofilin to inhibit its severing activity.<sup>132</sup> Unexpectedly however, PAK1 deficient mouse islets and MIN6  $\beta$  cells failed to show alterations in Cofilin activation, arguing against a significant role for Cofilin in glucose-induced F-actin remodeling and insulin release.<sup>27</sup>

### F-actin stabilizing proteins with potential roles in the $\beta$ cell

The two major F-actin stabilizing proteins, which may have potential roles in  $\beta$  cell F-actin remodeling, are  $\alpha$ -Fodrin and Filamin A.  $\alpha$ -Fodrin, also known as  $\alpha$ -spectrin, cross-links F-actin under low calcium and releases actin upon calcium influx in chromaffin cells.<sup>52</sup>  $\alpha$ -Fodrin can be cleaved by Calpain and increased cleaved  $\alpha$ -Fodrin is found in islet  $\beta$  cells from type 2 diabetic donors, suggesting compromised cellular cytoskeletal structure.<sup>133</sup>  $\alpha$ -Fodrin also binds to Syntaxin 4, the target-SNARE isoform operational in second-phase insulin release, *in vitro*.<sup>134,135</sup> Filamin A is the most

potent of all known F-actin cross-linking proteins and interacts with Cdc42, PAK1 and Rac1, all of which are vital for second-phase insulin secretion.<sup>25,136–138</sup> PAK1 is known to phosphorylate Filamin A, regulating its F-actin cross-linking activity,<sup>99</sup> although this is yet to be shown in  $\beta$  cells.

### F-actin-associated proteins in the $\beta$ cell

In addition to F-actin severing and stabilizing proteins, additional F-actin-associated proteins such as MLCK, myosin IIA and FAK, have been implicated in insulin release. In 1997, MLCK signaling to MLC2 was first implicated in glucose-stimulated insulin secretion,<sup>118</sup> and glucose found to phosphorylate the myosin light-chain 2 (MLC2) subunit of myosin IIA at serine 19 in 1999.<sup>139</sup> Myosin IIA is the most studied non-muscle myosin and is involved in F-actin remodeling and exocytosis.<sup>140,141</sup> In 2013, myosin IIA specifically, and not myosin IIB, was shown in primary  $\beta$  cells to be required for glucose-stimulated insulin secretion and actin remodeling.<sup>103</sup> Whether MLCK  $\rightarrow$  MLC2 requires upstream ERK activation in  $\beta$  cell cortical F-actin remodeling remains in question. This is because in  $\beta$  cells, ERK can also lie downstream of MLCK in the process of focal adhesion remodeling.<sup>103</sup> In addition to myosin IIA, myosin Va has been shown to be a positive regulator of insulin granule localization to the plasma membrane and of glucose-stimulated insulin secretion.<sup>58</sup> As such, it will be important to discern the ordered signaling events in cortical versus focal adhesion actin remodeling processes to reconcile these discordant data.

FAK regulation of focal adhesion remodeling has implications for indirect regulation of F-actin remodeling and insulin secretion. In MIN6  $\beta$  cells, FAK inhibition was shown to prevent glucose-stimulated F-actin remodeling,<sup>12</sup> decreasing glucose-stimulated insulin secretion.<sup>14</sup> Recently, islets of  $\beta$  cell-specific FAK knockout mice were found to exhibit impaired glucose-induced F-actin reorganization correlated with decreased ERK activation, coinciding with a reduced number of docked insulin granules; serum insulin levels during glucose tolerance tests suggest that both phases of insulin secretion are decreased.<sup>13</sup> Thus, FAK likely acts in coordination with F-actin remodeling in order to regulate glucose-stimulated insulin secretion, but its linkage to Cdc42 and PAK1 remains to be elucidated. Lastly, N-WASP and Arp2/3 proteins are known to cooperatively promote actin filament polymerization and are implicated in secretion from neuroendocrine cells,<sup>37</sup> yet neither has been established in glucose-stimulated insulin secretion.

### SNARE-MEDIATED INSULIN EXOCYTOSIS

The concept of SNARE protein-mediated vesicle/granule exocytosis was first elucidated in 1993 and rapidly confirmed by multiple groups and in many diverse cell types, including  $\beta$  cells.<sup>142–147</sup> In  $\beta$  cells, the target-SNARE proteins, Syntaxin and synaptosomal-associated protein of 25 kDa (SNAP-25, as well as its homolog SNAP-23), are localized to the plasma membrane, whereas the vesicle-SNARE VAMP2 (also known

as synaptobrevin 2) is localized to the insulin granule. Syntaxins have three N-terminal  $\alpha$ -helices denoted Ha, Hb and Hc (Habc) followed by a linker domain and then the H3 SNARE helix domain that associates with the other SNARE proteins.<sup>145</sup> VAMP2 and Syntaxin each donate their one  $\alpha$ -helix and SNAP-23/25 contributes two  $\alpha$ -helices to form a coiled four  $\alpha$ -helical heterotrimeric complex that brings the plasma membrane and granule membranes in close enough proximity for fusion to occur;<sup>148</sup> once fused, the insulin cargo is released from the  $\beta$  cell (Figure 3). SNARE complex formation is ATP dependent and the final initiation of regulated exocytosis requires calcium influx,<sup>149</sup> although the exact mechanisms by which this calcium influx causes membrane fusion are not fully understood. In addition, other accessory factors are important for SNARE-mediated membrane fusion such as the Munc13 proteins and Doc2 $\beta$ , which also have calcium-binding activity that could aid in sensing increased cytosolic calcium.<sup>150</sup> F-actin interacts with multiple SNARE proteins and can directly regulate exocytosis in this manner, therefore the linkage of F-actin and SNARE-mediated exocytosis will be discussed following a brief review of the role of SNAREs in biphasic insulin secretion.

#### SNARE requirement in biphasic insulin secretion

In  $\beta$  cells, the necessary SNARE isoforms for glucose-stimulated insulin secretion are Syntaxin 1,<sup>23</sup> Syntaxin 3,<sup>151,152</sup> Syntaxin 4,<sup>17</sup> SNAP-25,<sup>153</sup> SNAP-23<sup>154</sup> and VAMP2.<sup>155</sup> Indeed, islets from type 2 diabetic donors have low abundances of Syntaxins 1 and 4, SNAP-25 and VAMP2, highlighting the functional importance of these proteins in the disease state (unpublished data, E. Oh and D.C. Thurmond).<sup>156,157</sup> Syntaxin 1 is required for first-phase insulin secretion only, while Syntaxin 4 has been shown as necessary for both phases of insulin secretion.<sup>17,23</sup> Most recently, a third isoform, Syntaxin 3, has been implicated in granule recruitment to support both phases of glucose-stimulated insulin secretion.<sup>151</sup> Syntaxin protein function is tightly regulated by binding to their cognate nSec1/Munc18 (SM) proteins. Syntaxin 1 and 3 are regulated by Munc18-1,<sup>158</sup> Syntaxin 3 may also be regulated by Munc18-2 (also known as Munc18b) in  $\beta$  cells.<sup>152</sup> Distinct from these isoforms is the pairing of Syntaxin 4 with Munc18c (also known as Munc18-3) with Munc18c being the only isoform exclusively required for the second phase of insulin secretion.<sup>28</sup> It is hypothesized that upon stimulation, post-translational modification of SM proteins causes dissociation (in the case of Munc18c) or a binding mode shift (in the case of Munc18-1) to assist in the opening and binding of Syntaxin to its cognate vesicle-SNARE VAMP2.<sup>150</sup> Post-translational modification of the Syntaxin proteins also contributes to their activity, as glucose-induced production of nitric oxide causes S-nitrosylation of Syntaxin 4 in  $\beta$  cells.<sup>159</sup> Most recently, Munc13-4, the newest member of the Munc13 family of priming factors, was shown to interact with the H3 SNARE domains of Syntaxins 1 and 4 in a calcium-dependent manner.<sup>160</sup> Munc13-4 also interacts with the small GTPase Rab27a,<sup>161</sup> and therefore could be a missing link coupling

glucose-regulated GTPases to SNARE-mediated biphasic insulin exocytosis.

#### Linkage of F-actin remodeling to SNARE-mediated secretion

F-actin function is mechanically coupled to multiple SNARE and SNARE-associated proteins and many F-actin-binding proteins interact with the SNARE machinery. Immunoprecipitation of Syntaxin 1, Syntaxin 4 and SNAP-25 from  $\beta$  cells show co-precipitation of F-actin.<sup>9,12,59,66,162</sup> Of interest is the dynamic nature of the F-actin-SNARE interaction after glucose stimulation. After 5–10 min of glucose stimulation, F-actin dissociates from the Syntaxins and SNAP-25, but re-associates within 30 min after stimulation.<sup>9,12,59</sup> In the case of Syntaxin 4, this binding has been shown to be direct.<sup>9,34,59,162</sup> Direct binding of F-actin to Syntaxin 4 was determined to occur at the N-terminus of Syntaxin 4, likely through the Hb helix.<sup>59</sup> The Syntaxin 4–F-actin interaction seems to be selective for Syntaxin 4, as all other SNARE proteins tested so far (Syntaxins 1, 2 and 3, SNAP-23, VAMP2 and VAMP8) have not bound F-actin directly *in vitro*.<sup>44,59,162</sup> In  $\beta$  cells, evidence for Syntaxin 4 binding to F-actin and not G-actin comes from treatment with the actin depolymerizing drug latrunculin, which abrogates the co-precipitation of actin with Syntaxin 4, showing that Syntaxin 4 will not bind to monomeric G-actin.<sup>59,66</sup> Artificial disruption of Syntaxin 4–F-actin complexes in  $\beta$  cells using a GFP-tagged truncation of Syntaxin 4 corresponding to the F-actin-binding site led to increased insulin secretion and VAMP2-bound insulin granule accumulation at the cell surface, suggesting that F-actin binding to Syntaxin 4 has a role in restricting granule access to the plasma membrane in the basal or unstimulated state.<sup>59</sup> Syntaxin 1 and 4 can also form heterotrimeric complexes with Cdc42 and VAMP2 *in vitro*, presenting a potential avenue for localized coupling of SNARE proteins with Cdc42-mediated F-actin remodeling.<sup>89,163</sup>

#### CONCLUDING REMARKS

What mediates the glucose-specific signal for  $\beta$  cell F-actin remodeling? Glucose metabolism results in calcium influx required to trigger insulin secretion, and it is now recognized that a metabolic amplifying signal/second messenger is also produced, although the identity of this amplifying factor has remained elusive.<sup>164,165</sup> Two recent studies used the rat  $\beta$  cell line INS-1 832/13 to investigate glucose-induced changes in different metabolites.<sup>166,167</sup> These studies implicated the pentose phosphate pathway as well as AMPK kinase activation (from ZMP) as metabolites with the potential to be the glucose sensing readout that leads to insulin secretion. Future work with such metabolites may lead to the discovery of the signal that leads to activation of the Cdc42–PAK1–Rac1 pathway that is critical for F-actin remodeling and second-phase insulin secretion.

F-actin remodeling has been investigated for a role in the amplification of insulin secretion. Recently, the diazoxide paradigm was used in islets in conjunction with pharmacological disruption of F-actin using latrunculin or stabilization

of F-actin using jasplakinolide.<sup>10</sup> Polymerization or depolymerization of F-actin elicited potentiation of biphasic insulin secretion, consistent with the published literature.<sup>9,10</sup> These studies highlight how F-actin remodeling may fine-tune and regulate the amount of insulin that is secreted in response to the triggering and amplifying mechanisms. A caveat to the use of pharmacological de/polymerizing agents complicates determination of the role of F-actin, however, as pretreatment with these drugs alters the F/G-actin ratio and granule distribution at the plasma membrane prior to stimulation, and they cause global changes to F-actin, as opposed to the localized alterations that are seen to occur in response to glucose.<sup>9,34</sup> As such, it is important to study F-actin remodeling in  $\beta$  cells using reagents that can act more selectively, and in a localized manner, upon key regulators of F-actin dynamics in order to elucidate more nuanced regulatory roles of localized cytoskeletal reorganization. Future studies incorporating novel tools in the  $\beta$  cell, such as light-activated small Rho GTPases, will be instrumental in addressing the role of localized F-actin remodeling on insulin exocytosis.<sup>168–170</sup>

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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