Pak1 kinase regulates ribonucleoprotein granules through Sts5 for polarized growth and the glucose starvation response

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ABSTRACT
Fission yeast cells maintain a rod shape due to conserved signaling pathways that organize the cytoskeleton for polarized growth. We discovered a mechanism linking the conserved protein kinase Pak1 with cell shape through the RNA-binding protein Sts5. Pak1 prevents Sts5 association with P bodies by directly phosphorylating its intrinsically disordered region (IDR). Pak1 and the cell polarity kinase Orb6 both phosphorylate the Sts5 IDR but at distinct residues. Mutations preventing phosphorylation in the Sts5 IDR cause increase P body formation and defects in cell shape and polarity. Unexpectedly, when cells encounter glucose starvation, PKA signaling triggers Pak1 recruitment to P bodies with Sts5. Through retargeting experiments, we reveal that Pak1 localizes to these ribonucleoprotein (RNP) granules to promote rapid dissolution of Sts5 upon glucose addition. Our work reveals a new role for Pak1 in regulating cell shape through RNPs during normal and stressed growth conditions.

INTRODUCTION

Cell polarity signaling networks determine cell morphology by controlling growth machinery in time and space. Because active growth requires energy, these networks must also respond to changes in the environment such as glucose availability. The p21-activated kinase (PAK) family of protein kinases are key mediators of cell polarity signaling in many eukaryotes (Hofmann et al., 2004). Following activation by small GTPases like Cdc42, PAKs function in regulating the cytoskeleton and MAPK pathways, and PAK dysregulation is associated with multiple human diseases. For example, PAKs regulate the elaborate architecture of highly polarized neurons, and PAK loss-of-function mutations impair neuronal migration, neurite outgrowth, and axonal development, leading to connections with autism spectrum disorders, Alzheimer's disease, neurofibromatosis type 1, and other neurological disorders (Zhang et al., 2020). Aberrant activation of human PAK1 also drives cell migration and proliferation in multiple forms of cancer including pancreatic, breast, prostate, and thyroid (Bautista et al., 2020; Kanumuri et al., 2020; Rane and Minden, 2019). Additionally, PAK1 plays a protective role in cardiac diseases including hypertrophy (Wang et al., 2018). The mechanisms that connect PAKs with these diseases include canonical functions mediated through Cdc42 and the cytoskeleton, along with MAPK pathways. However, the diversity of these disease-related defects also suggests additional noncanonical functions and mechanisms that remain less studied. The functions of PAKs in cell morphology and signaling are conserved in a wide range of cell types and organisms including yeast and humans (Hofmann et al., 2004). This conservation is demonstrated by the finding that expression of human PAK1 suppresses defects associated with loss of the PAK Ste20 in budding yeast (Brown et al., 1996). Full understanding of PAK function in cell polarity and proliferation requires identification of noncanonical substrates and functions for PAK-family proteins.

The fission yeast *Schizosaccharomyces pombe* is a strong model system to dissect mechanisms controlling cell polarity and morphology due to its simple cylindrical shape, which is controlled by signaling pathways that are highly conserved throughout eukaryotes. This morphology can be modulated by environmental stresses and growth conditions, suggesting that cell polarity signaling pathways intersect with stress-sensing pathways. Early genetic screens identified different classes of fission yeast morphology mutants, such as round ("*orb*"), curved, bent, and branched (Snell and Nurse, 1994; Verde et al., 1995). Genes that result in similar shape defects when mutated may function together in shared and/or overlapping pathways. In this study, we connect three factors identified as *orb* mutants – the protein kinases Pak1 (also called Orb2) and Orb6, along with the RNA-binding protein Sts5 – in a shared mechanism that controls fission yeast cell shape.

Fission yeast Pak1, an ortholog of human PAK1, was initially identified as an *orb* mutant. Pak1 is essential for cell viability, and conditional *pak1* mutants become round and exhibit reduced viability during stress (Marcus et al., 1995; Ottilie et al., 1995; Verde et al., 1998). Many studies have linked Pak1

function with Cdc42 activation consistent with canonical PAK mechanisms (Marcus et al., 1995; Ottilie et al., 1995; Tu and Wigler, 1999). However, other studies identified Pak1 activation mechanisms that are independent of Cdc42 (Yang et al., 1999), consistent with similar activation mechanisms observed in other PAK kinases (Lu et al., 1997; Shin et al., 2013). Once activated, Pak1 targets regulators of Cdc42 and the cytoskeleton (Edwards et al., 1999; Moshfegh et al., 2014; Vadlamudi et al., 2005, 2002). Beyond these canonical targets, a recent phosphoproteomic screen identified potential substrates that suggest novel mechanisms for Pak1 function in cell morphology and viability (Magliozzi et al., 2020).

Similar to Pak1, the protein kinase Orb6 is a conserved regulator of cell morphology and was identified in genetic screens as an *orb* mutant (Verde et al., 1995). Orb6 is an essential NDR/LATS kinase that regulates cell polarity and morphology (Verde et al., 1998). Orb6 substrates include Cdc42 regulators and components of the exocyst, consistent with regulation of polarized growth (Das et al., 2015, 2009; Tay et al., 2019). A recent study showed that Orb6 also phosphorylates Sts5, an RNA-binding protein that associates with processing bodies (P bodies) (Nuñez et al., 2016), which are ribonucleoprotein (RNP) granules that regulate translation and degradation of associated mRNAs. Conditional mutants in *orb6* and *pak1* are synthetically lethal (Verde et al., 1998, 1995), suggesting overlapping or connected pathways between these two protein kinases. However, specific connections in their signaling mechanisms have not been identified. In this study, we demonstrate that both Pak1 and Orb6 phosphorylate Sts5 to regulate P bodies, thereby connecting these two kinases through a shared substrate.

P bodies are biomolecular condensates comprised of multiple proteins and mRNAs (Eulalio et al., 2007; Luo et al., 2018; Parker and Sheth, 2007; Rao and Parker, 2017). These cytoplasmic RNP structures are conserved from yeast through mammalian cells and regulate the translation and turnover of associated mRNAs. Fission yeast Sts5, which has similarity to human DIS3L2 and budding yeast Ssd1, associates with P bodies through an intrinsically disordered region (IDR) (Jansen et al., 2009; Kurischko et al., 2011). This association is partly inhibited by phosphorylation of the Sts5 IDR by Orb6 (Chen et al., 2019; Nuñez et al., 2016). Interestingly, Sts5 regulates mRNAs connected to cell polarity and was isolated in the same *orb* screens that identified Orb6 and Pak1, suggesting a shared cellular function (Snell and Nurse, 1994; Verde et al., 1995). Sts5 localization to RNP granules is dramatically enhanced in a reversible manner when cells encounter glucose deprivation, thereby facilitating changes in both metabolism and growth (Chen et al., 2019; Nuñez et al., 2016). The signaling mechanisms that connect Sts5 association to RNP granules with glucose availability are largely unknown but have the potential to link cellular energy state with cell morphology.

In this work, we show that Pak1 directly phosphorylates Sts5 to regulate P bodies and cell morphology. Pak1 and Orb6 phosphorylate distinct residues on Sts5 and act synergistically to control cell shape through P body formation. Unexpectedly, glucose starvation induces rapid, PKA-dependent relocalization of Pak1 from the cell periphery to RNP granules. Pak1 localization at RNP granules is required for rapid dissolution of Sts5 from these structures when glucose is restored. Our results identify a novel nutrient-modulated mechanism connecting a conserved PAK kinase with cell morphology and stress response.

RESULTS

RNA-binding protein Sts5 is a novel Pak1 substrate

We recently performed an unbiased phosphoproteomic screen that identified Sts5 as a leading candidate for phosphorylation by Pak1 in fission yeast cells (Magliozzi et al., 2020). Specifically, phosphorylation of Sts5 residues S261 and S264 was reduced 2.5-fold in *pak1-as*, a partial loss-of-function mutant, compared to wild type cells (Loo and Balasubramanian, 2008; Magliozzi et al., 2020).

This phosphorylation was further reduced 2.3-fold by 15-minute addition of 3-Brb-PP1, which completely inhibits *pak1-as* kinase activity (Magliozzi et al., 2020). S261 and S264 reside in the Sts5 intrinsically disordered region (IDR), which mediates its localization to RNP granules (Chen et al., 2019; Nuñez et al., 2016; Toda et al., 1996; Vaggi et al., 2012). To test if Pak1 directly phosphorylates Sts5, we conducted *in vitro* kinase assays using purified proteins and γ-^{32}P-ATP. Full length Sts5 was directly phosphorylated by Pak1 but not by kinase-dead Pak1(K415R) *in vitro*, confirming that Sts5 is a substrate of Pak1 (Figure 1A,B, Figure 1-figure supplement 1).

To test how phosphorylation by Pak1 might regulate Sts5 in cells, we imaged Sts5-mNeonGreen (mNG) in wild type or *pak1-as* cells. In wild type cells, Sts5 localized in a diffuse manner in the cytoplasm and formed very few granules (Figure 1C,D). In contrast, Sts5 localized to punctate granules in *pak1-as* cells (Figure 1C,D). These data show that Pak1 regulates the localization of its substrate Sts5. Consistent with a functional connection between Pak1 and Sts5, we observed a genetic interaction in the regulation of cell septation and separation. We measured an increased percentage of septated cells for *pak1-as* cells treated with 3-Brb-PP1, indicating a delay in cell separation, but this defect was suppressed by *sts5Δ* (Figure 1E). We conclude that Pak1 is required to prevent the aberrant formation of Sts5 granules, which can interfere with cell separation and potentially other cellular processes.

Dysregulation of Sts5 in *pak1-as* could reflect direct kinase-substrate regulation, or alternatively indirect consequences of altered Pak1 signaling. To distinguish between these possibilities, we generated a non-phosphorylatable Sts5(S261A S264A) mutant based on the two sites modified by Pak1 in cells. The Sts5(S261A S264A) mutant lost phosphorylation by Pak1 *in vitro* and constitutively localized to puncta in cells (Figure 1F,G,H). This localization defect was phenocopied by the single S261A mutant but not by S264A (Figure 1G,H), demonstrating that S261 is the primary site for regulation by Pak1. These combined data indicate that Pak1 phosphorylates the Sts5 IDR to prevent its localization to punctate granules in the cytoplasm.

Pak1-Orb6-Sts5 regulatory axis controls polarized growth and cell separation

Recent studies have shown that NDR/LATS kinase Orb6 similarly phosphorylates the Sts5 IDR to regulate its localization to RNP granules (Figure 2A) (Chen et al., 2019; Nuñez et al., 2016). Importantly, Orb6 phosphorylates S86 in the Sts5 IDR, while we have shown that Pak1 phosphorylates S261. These distinct sites of phosphorylation raise the possibility that Pak1 and Orb6 perform non-redundant functions on Sts5. To test this possibility, we made non-phosphorylatable mutations either at the Pak1 and Orb6 sites alone (S86A or S261A), or at both sites in combination (S86A S261A, which we refer to as Sts5-2A). Using SDS-PAGE containing Phosbind, which slows the migration of phosphorylated proteins, we found dramatic loss of phosphorylation for Sts5-2A compared to wild type or either single mutant (Figure 2B, Figure 2-figure supplement 1). Thus, mutating the primary phosphorylation sites for Pak1 and Orb6 in combination causes loss of Sts5 phosphorylation in cells.

Preventing Sts5 phosphorylation by Pak1 and Orb6 had dramatic effects on Sts5 localization and function. While either Sts5-S86A or Sts5-S261A alone increased the number of Sts5 granules per cell, combining these mutations in Sts5-2A substantially increased granule number beyond either single mutant alone (Figure 2C,D). Increased Sts5 granule localization correlated with defects in cell morphology, septation, and viability. The single mutants S86A and S261A exhibited increased cell width compared to wild type cells, but the double mutant Sts5-2A significantly increased cell width over either single mutant alone (Figure 2E,F). This increased width is consistent with a partial *orb* phenotype for the Sts5-2A mutant, which exhibited additional cell polarity defects including an increased fraction of monopolar cells (Figure 2-figure supplement 2A,B). Beyond cell polarity, the septation index of Sts5-2A was significantly increased over wild type and the single mutants, consistent with a defect in cell separation (Figure 2G). Finally, the viability of Sts5-2A mutants was dramatically decreased under a variety of stress conditions compared to wild type and the single mutants (Figure 2H, Figure 2-figure

supplement 3). In conclusion, Pak1 and Orb6 phosphorylate distinct residues in the Sts5 IDR to control whether Sts5 localizes to the cytoplasm or to granules. Defects in this combined regulatory mechanism lead to aberrant Sts5 granules, along with additive defects in cell morphology, division, and viability.

Sts5 localizes to P-bodies for translation control

Sts5 is homologous to human Dis3L2, a 3'-5' exoribonuclease, but Sts5 lacks the essential catalytic residues to degrade mRNA targets. Past studies have shown that Sts5 binds to mRNAs and targets them to P bodies, which contain machinery to reduce mRNA levels and translation in cells (Malecki et al., 2013; Nuñez et al., 2016; Uesono et al., 1997). Several transcripts related to cell morphology, including the protein kinase Ssp1, are major targets of Sts5 regulation (Nuñez et al., 2016). Sts5 localizes to P bodies through its IDR, consistent with the role of such domains in association with biomolecular condensates through a process akin to liquid-liquid phase separation (Alberti et al., 2019). In many cases, phosphorylation within an IDR prevents accumulation in condensates (Owen and Shewmaker, 2019). We hypothesized that phosphorylation by Pak1 and Orb6 could prevent Sts5 clustering and association with P bodies, thereby maintaining full expression of its targets such as Ssp1. To test this idea, we performed colocalization experiments with Sts5-mNG and Sum2-tdTomato, a marker of P bodies. Wild type Sts5-mNG rarely formed granules and therefore did not colocalize with P bodies (Figure 3A,C). In contrast, Sts5-2A-mNG formed granules that colocalized with Sum2 and increased the number of P bodies per cell (Figure 3B,C). We conclude that Pak1 and Orb6 phosphorylate the Sts5 IDR to prevent its clustering and association with P bodies. Our results also indicate that clustering of Sts5 induces formation of P bodies, which we predicted would decrease the levels of Sts5 targets including Ssp1. Consistent with this prediction, the levels of Ssp1 protein were significantly reduced in Sts5-2A cells as compared to either single mutant alone or to wild type cells (Figure 3D,E). Put together, these results demonstrate that loss of Sts5 phosphorylation by Pak1 and Orb6 leads to aberrant P body assembly and reduced levels of Ssp1 (Figure 3F). This mechanism connects with changes in cell morphology and survival under stress.

Glucose starvation promotes Pak1 localization to stress granules

Our results indicate that Pak1 and Orb6 phosphorylate Sts5 at distinct residues to regulate P body formation in an additive manner. Why would two different kinases be required for this regulatory mechanism? We considered the possibility that Pak1 and Orb6 might respond to distinct physiological states to link Sts5 regulation with a range of growth conditions. Orb6 regulation of Sts5 has been connected to both cell cycle progression and nitrogen starvation (Chen et al., 2019; Vaggi et al., 2012). We noted that glucose starvation serves as a signal for enhanced recruitment of Sts5 to large RNP granules in a manner that is reversed by reintroduction of glucose. Therefore, we tested the localization of Pak1, Orb6, and Sts5 during glucose starvation. Sts5 localized to large granules consistent with previous work (Figure 4A) (Nuñez et al., 2016). Interestingly, Pak1 was similarly concentrated into cytoplasmic granules, but Orb6 remained diffuse in the cytoplasm (Figure 4A, Figure 4-figure supplement 1B). Both Pak1 and Sts5 granules colocalized with the P body marker Sum2 (Figure 4B,C), and Pak1 and Sts5 colocalized together at RNP granules (Figure 4D). These data show that glucose starvation induces a change in Pak1 localization away from growing cell tips and into RNP granules, which contain Sts5 but not Orb6. Pak1 localization to RNP granules was independent of Sts5, as we observed this localization pattern in both *sts5Δ* and *sts5-2A* mutant cells during glucose starvation (Figure 4E, Figure 4-figure supplement 1A, Figure 4-figure supplement 2).

We next tested if Pak1 recruitment to RNP granules was specific to glucose starvation or alternatively represented a general stress response. Pak1 did not form cytoplasmic clusters in nitrogen starvation or hyperosmotic stress (Figure 4F). We observed Pak1 clusters during heat stress, but these clusters did not colocalize with Sum2 (Figure 4F). Thus, Pak1 associates with RNP granules specifically

during glucose starvation. We tested if other cell polarity kinases, in addition to Pak1, also localized to RNP granules during glucose starvation. For this experiment, we screened the localization a panel of fluorescently tagged kinases in high (2%) versus low (0%) glucose conditions. Pck2 and Kin1 localized to cytoplasmic clusters during glucose starvation (Figure 4-figure supplement 3, Figure 4-figure supplement 4). We note that Pck2 was recently shown to localize to RNP granules during high heat stress (Kanda et al., 2020). In contrast, Orb6, Nak1 and Pck1 did not form clusters during glucose starvation (Figure 4A, Figure 4-figure supplement 3). We conclude that glucose starvation induces the redistribution of Pak1 away from sites of polarized growth and into RNP granules, where it colocalizes with its substrate Sts5 and the P body marker Sum2. Comparing Pak1 and Orb6, this association with RNP granules is specific to Pak1 and identifies a clear distinction between these two regulators of Sts5.

For polarized growth in rich media conditions, Pak1 functions in a regulatory module that promotes Cdc42 activation at growing cell tips. This module includes the Pak1 substrate Scd1, which is a guanine nucleotide exchange factor (GEF) for Cdc42 (Chang et al., 1994), and the scaffold protein Scd2, which binds to both Pak1 and Scd1 (Chang et al., 1999; Endo et al., 2003). In contrast to Pak1, Scd2 and Scd1 did not concentrate at Sum2-marked RNPs during glucose starvation (Figure 5 A,B). Similarly, we did not observe RNP granule localization for Cdc42 containing a functional fluorescent tag, or for the well-characterized active Cdc42 biomarker CRIB-3xGFP (Figure 5C,D). These results indicate that Pak1 localizes to RNP granules during glucose starvation independently of its cell polarity regulatory module.

PKA signaling is required for Pak1 stress granule localization

Localization of Pak1 to RNP granules specifically during glucose starvation suggests the involvement of a glucose-modulated signaling pathway. We tested the role of proteins that signal changes in glucose availability and cellular energy status. During glucose starvation, Pak1 still localized to RNP granules with Sum2 upon deletion of Ssp2, the catalytic subunit of AMP-activated protein kinase, and upon deletion of its upstream activator Ssp1 (Deng et al., 2017; Schutt and Moseley, 2017; Valbuena and Moreno, 2012) (Figure 6A). Similarly, Pak1 localized to RNP granules with Sum2 after deletion of Sds23 (Figure 6A), which regulates glucose-dependent phosphatase activity (Hanyu et al., 2009; Ishii et al., 1996). In contrast, Pak1 was absent from RNP granules in *pka1Δ* cells (Figure 6B). Pka1 is the catalytic subunit of Protein Kinase A (PKA), which regulates cell cycle regulation and RNP granule assembly during glucose deprivation in yeast (Kelkar and Martin, 2015; Nilsson and Sunnerhagen, 2011). If PKA signaling mediates Pak1 localization, then other mutations in the PKA pathway should phenocopy *pka1Δ*. Consistent with this prediction, Pak1 did not localize to RNP granules during glucose starvation in *git3Δ*, *gpa2Δ*, or *cyr1Δ* mutants, which act in a linear activation pathway for PKA activation (Hoffman, 2005; Kelkar and Martin, 2015) (Figure 6B). Importantly, both Sts5 and Sum2 still formed granules during glucose deprivation in all of these PKA pathway mutants, which means that Pak1 localization defects are not due to overall loss of RNP granules (Figure 6C). These data identify a PKA-mediated pathway that triggers localization of Pak1 to RNP granules during glucose starvation.

Pak1 functions to promote stress granule dissolution

Finally, we sought to define a functional role for Pak1 localization to RNP granules during glucose starvation. Because Pak1 phosphorylates the Sts5 IDR to inhibit its clustering and association with P bodies, we hypothesized that Pak1 might promote Sts5 dissociation from RNP granules upon glucose addition. To test this hypothesis, we aimed to prevent Pak1 localization to RNP granules. PKA signaling has multiple effects on RNP granules (Nilsson and Sunnerhagen, 2011), so we needed a more direct method to disrupt Pak1 localization specifically. We found that addition of a lipid-modified CAAX motif forced constitutive cortical localization of Pak1 (Figure 7A, Figure 7-figure supplement 1A). In high glucose conditions, Pak1-GFP-CAAX localized to the cell cortex and concentrated at cell tips (Figure 7A,

Figure 7-figure supplement 1A). During glucose starvation, Pak1-GFP-CAAX failed to redistribute to RNP granules, and instead localized throughout the cell cortex (Figure 7A, Figure 7-figure supplement 1A).

We used this synthetic retargeting tool to test how Pak1 at RNP granules regulates Sts5 dissociation from these structures. We monitored the dissociation kinetics by measuring the concentration of Sts5-mNG per RNA granule following addition of glucose. In wild type cells, Sts5 intensity rapidly dropped and plateaued to background levels by 5-6 minutes after addition of glucose (Figure 7C). In contrast, the rate of Sts5 loss from granules was reduced in Pak1-CAAX cells. Importantly, the levels of Sts5 at granules was identical in Pak1-CAAX and control cells at the time of glucose addition (Figure 7C). However, Sts5 dissociated more slowly in Pak1-CAAX cells, and Sts5 levels did not reach background levels until 10 minutes in Pak1-CAAX (Figure 7B,C). Reduced Sts5 dissociation kinetics in Pak1-CAAX cells was observed in multiple experimental replicates (Figure 7-figure supplement 1B,C). We conclude that PKA-dependent Pak1 localization to RNP granules promotes rapid dissociation of its substrate Sts5 upon glucose addition (Figure 7D).

DISCUSSION

The main findings from our study are: (1) Pak1 directly phosphorylates the Sts5 IDR to prevent recruitment to P-bodies, (2) Pak1 and Orb6 kinases act in an additive manner on Sts5 to control cell morphology and repression of Ssp1, (3) Pak1 is recruited to RNP granules with Sts5 during glucose starvation through a PKA-dependent pathway, and (4) Pak1 localization to RNP granules promotes rapid dissolution of Sts5 from these structures upon addition of glucose. These results identify and define a new, bidirectional connection between a PAK and RNP granules. This connection ensures proper cell morphology during nutrient rich conditions, and rewires PAK signaling during glucose starvation.

The orb polarity module

We identified Sts5 as a novel Pak1 substrate through an unbiased phosphoproteomic screen followed by targeted studies *in vitro* and *in vivo*. PAKs are classically known to regulate cell morphology and signaling through Cdc42 and MAPK pathways, but our results demonstrate that Pak1 also acts through Sts5 and P bodies, thereby expanding Pak1 function in cell polarity through a glucose-dependent mechanism. This connection between a PAK and RNP granules provides a new mechanism that may operate in other organisms as well.

We found that Pak1 acts together with the NDR kinase Orb6 on Sts5. Both kinases phosphorylate the Sts5 IDR but at distinct residues. Preventing these modifications with the Sts5-2A mutant led to constitutive P body formation, increased cell width, and decreased viability during stress. Our work supports a model where phosphorylation of the Sts5 IDR by both Pak1 and Orb6 prevents Sts5 incorporation into P bodies. Sts5 binds and recruits mRNAs including *ssp1* into P bodies (Nuñez et al., 2016). Loss of regulation by Pak1 and Orb6 leads to decreased levels of Ssp1 protein, consistent with its translational repression at P bodies. In this model, phosphorylation by Pak1 and Orb6 may prevent Sts5 aggregates through multiple mechanisms. Past work has shown that phosphorylation by Orb6 enhances Sts5 binding to Rad24, a 14-3-3 protein that sequesters Sts5 away from P bodies (Chen et al., 2019; Nuñez et al., 2016). We note that phosphorylation of proteins with IDRs appears to inhibit their incorporation into biomolecular condensates through liquid-liquid phase separation both *in vitro* and in cells (Owen and Shewmaker, 2019), meaning that phosphorylation of Sts5 IDR may contribute to both Rad24-dependent and -independent forms of regulation. More generally, the connection between RNPs and cell polarity is an emerging theme that has been described in several organisms (Gerbich et al., 2020; Jansen et al., 2009; Lee et al., 2015, 2020). Our work supports a critical role for regulated assembly of P-bodies in the cell polarity program that establishes and maintains cell morphology. Given the

conservation of PAKs, NDR kinases, and P bodies, our work has implications for regulation of cell polarity by RNPs in other cell types and organisms.

In this mechanism, Pak1 and Orb6 appear to play very similar roles in regulation of Sts5, raising the question of why this system uses two distinct upstream kinases. We found that Pak1 but not Orb6 localizes to RNP granules upon glucose starvation. This localization was not observed in other stresses or during specific cell cycle stages. Meanwhile, Orb6 activity is cell cycle-regulated, and Orb6 signaling to Sts5 becomes important during nitrogen stress (Chen et al., 2019; Nuñez et al., 2016). Put together, these observations indicate that Pak1 and Orb6 respond to distinct physiological cues in their regulation of Sts5. In some cases, both Pak1 and Orb6 are active and likely to regulate Sts5 in a synergistic manner. In other cases, either Pak1 or Orb6 alone is active. By using protein kinases that respond differently to various cues, this mechanism may tune P body regulation to a range of physiological conditions.

We note that Pak1 (also called Orb2), Sts5 (also called Orb4), and Orb6 were all identified in the same genetic screen for *orb*-shaped mutant (Snell and Nurse, 1994; Verde et al., 1995). Our results unite these three factors in a shared regulatory mechanism. We observed increased cell width in the *sts5-2A* mutant, which loses phosphorylation by Pak1 and Orb6. This phenotype represents a partial *orb* shape, confirming its role in this process but also indicating that additional factors contribute to the complete *orb* shape defect. Consistent with this possibility, both Pak1 and Orb6 have additional substrates within the Cdc42 and exocytosis networks (Chang et al., 1999; Das et al., 2015; Magliozzi et al., 2020; Tay et al., 2019). Pak1 and Orb6 may control cell polarity through coordinated regulation of RNPs and Cdc42 signaling, or alternatively by acting independently on these two downstream targets. Additionally, the connection between Pak1, Orb6, and Sts5 raises the possibility that other *orb* genes also function in this mechanism. For example, Orb5 is also called Cka1, the catalytic subunit of casein kinase 2 (CK2) (Snell and Nurse, 1994). In human cells, CK2 phosphorylates and activates PAK1 (Shin et al., 2013), but this connection has not been tested in fission yeast to our knowledge. Future studies may reveal the involvement of other Orb proteins in this shared regulatory mechanism centered on phospho-regulation of RNPs.

Regulation and function of Pak1 at RNP granules

Our results show that Pak1 is recruited to RNPs during glucose starvation, raising two important questions. First, how is Pak1 recruited to RNPs? We found that Pak1 localization to RNPs is independent of Sts5 but requires PKA signaling. These combined data suggest that Pak1 binds to an unknown factor within RNPs, and this interaction depends on PKA. We note that PKA phosphorylates PAK1 in human cells (Howe and Juliano, 2000), raising the possibility of direct regulation. PKA has multiple effects on RNP granule assembly during stress (Nilsson and Sunnerhagen, 2011), so it is also possible that PKA modifies other RNP granule proteins that recruit Pak1. Future work on PKA substrates within RNP granules will provide more information on how Pak1 localizes to these sites in a regulated manner.

A second important question is the function of Pak1 at RNP granules during glucose starvation. Pak1-CAAX prevented localization to granules and led to slower dissolution of Sts5 from granules upon glucose addition. This result suggests that Pak1 at RNP granules is poised to promote rapid release of its substrate Sts5 as cells re-enter polarized growth. This model is consistent with several other protein kinases that localize to RNP granules during specific stress conditions and function to dissolve granules upon removal of stress (Rai et al., 2018; Shattuck et al., 2019; Wippich et al., 2013; Yahya et al., 2021). Alternatively, RNP granules could represent a storage depot for inactive Pak1 when cells stop growth during glucose starvation. In support of this model, PKA inhibits PAK1 kinase activity in human cells (Howe and Juliano, 2000), and several other protein kinases have been shown to be sequestered at RNPs during various stress conditions (Kanda et al., 2020; Shah et al., 2014). These two possibilities for the role of Pak1 at RNP granules are not mutually exclusive. It seems likely that these sites could both store Pak1 while keeping it close to a key substrate for rapid regulation in response to glucose. Given

that the formation and dissolution of RNP granules is highly regulated by protein kinases and by stress conditions, the regulated localization of protein kinases to these sites may generally contribute to rapid control of RNP granules.

The localization of Pak1 to RNP granules does not appear to involve its canonical ligands within the Cdc42 regulatory module. We did not observe localization of Scd1, Scd2, or Cdc42 at granules. This result is consistent with Pak1 functioning to regulate cell polarity through a new independent mechanism with Sts5, but it also raises questions about how Pak1 is activated at granules. Like other PAKs, Pak1 is thought to adopt an autoinhibited conformation that is relieved by direct binding to activated Cdc42 (Tu and Wigler, 1999). While Cdc42 may transiently activate Pak1 at granules, several lines of evidence indicate that Pak1 could be active at granules without Cdc42. First, we and other groups have shown that recombinant full-length Pak1 expressed and purified from bacteria displays strong kinase activity *in vitro* (Das et al., 2012; Loo and Balasubramanian, 2008; Magliozzi et al., 2020; Pollard et al., 2017; Yang et al., 1999), which means that it is not autoinhibited under these conditions. Second, PAKs can also be activated by various classes of lipids and proteins (Bokoch et al., 1998; Strochlic et al., 2010; Zenke et al., 1999). The proteins that activate PAKs include protein kinases such as PDK, Akt, and CK2 (King et al., 2000; Shin et al., 2013; Tang et al., 2000; Zhou et al., 2003), as well as SH3 domain-containing proteins (Parrini, 2012). In fission yeast, the SH3 domain protein Skb5 has been shown to enhance Pak1 activity in the absence of Cdc42 *in vitro* (Yang et al., 1999). We do not rule out a possible role for Cdc42 in the function of Pak1 at RNP granules, but these examples show that Pak1 could act at these sites without canonical GTPase-dependent activation.

Conclusions

Our study provides a molecular link between cell polarity signaling and the regulated assembly of RNP granules. The underlying mechanism involves two conserved protein kinases acting on a shared substrate to control its recruitment into biomolecular condensates. We anticipate that the phase separation properties of many disordered proteins in cells will follow similar regulation by multiple kinases, which facilitates combinatorial responses. Our results also unite two conserved protein kinases in a shared mechanism, raising the possibility for similar connections between PAK and NDR kinases in other organisms. Finally, recruitment of Pak1 and other kinases to RNP granules during stress points to growing roles for these enzymes in controlling the dynamics of granule assembly, disassembly, and function in additional cell types and organisms.

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FIGURE LEGENDS

Figure 1: Pak1 directly phosphorylates RNA-binding protein Sts5 to control its localization.
(A) Schematic of Sts5 domain layout. Pak1-dependent phosphorylation sites are indicated by red lines. IDR, intrinsically disordered region. CSD, cold shock domain. **(B)** In vitro kinase assay with purified proteins and γ-^{32}P-ATP. **(C)** Sts5-mNG localization in wild type versus *pak1-as* cells. **(D)** Quantification of the number of Sts5-mNG granules per cell. Values are mean $\pm$ SD (n = 50 cells per strain). ****, P <

0.0001. **(E)** Quantification of septation index of indicated strains. Values are mean ± SD from three biological replicates (*n* > 150 cells each). ***, P < 0.001; **, P < 0.01. **(F)** In vitro kinase assay with purified proteins and γ-^{32}P-ATP. GST-Sts5 protein loading was assessed by SDS-PAGE followed by silver staining. **(G)** Sts5-mNG localization in indicated strains. **(H)** Quantification of Sts5-mNG granule number per cell. Values are mean ± SD (*n* = 50 cells per strain). ****, P < 0.0001. Images in C and G are maximum intensity projections from spinning disc confocal microscopy. Scale bars, 5 μm.

Figure 1-figure supplement 1: Purified proteins for *in vitro* kinase assays.
Purified proteins were separated by SDS-PAGE and visualized by Coomassie stain.

Figure 2: Pak1 and Orb6 kinases phosphorylate distinct residues in the Sts5 IDR for cell morphology.
(A) Schematic of Sts5 domain layout. Pak1 and Orb6 phosphorylation sites are indicated by red lines. IDR, intrinsically disordered region. CSD, cold shock domain. **(B)** Phosbind western blot of indicated strains showing loss of Sts5 phosphorylation in the 2A mutant, which contains both S86A and S261A mutations. Ponceau stain included for loading. **(C)** Spinning disc confocal images of Sts5-mNG localization. Images are maximum intensity projections. **(D)** Quantification of Sts5-mNG granule number per cell. Values are mean ± SD (*n* = 50 cells per strain). ****, P < 0.0001. n.s., not significant. **(E)** Images of Sts5-mNG cells stained with Blanophor. **(F)** Quantification of cell width in Sts5-mNG strains. Values are mean ± SD (*n* ≥ 50 cells per strain). ****, P < 0.0001; ***, P < 0.001. **(G)** Quantification of septation index of indicated strains. Values are mean ± SD from three biological replicates (*n* > 150 cells each). ****, P < 0.0001. n.s., not significant. **(H)** Serial-dilution growth assays for each indicated strain. Strains were spotted onto indicated media and grown for 3-5 days at either 32°C or 37°C. Scale bars, 5 μm.

Figure 2-figure supplement 1: Phosbind western blot of all Sts5 phosphomutants.
Phosbind western blot of indicated *sts5-FLAG* strains. Ponceau stain included for loading.

Figure 2-figure supplement 2: Sts5 phosphomutants display defects in cell polarity.
(A) Representative images of Fim1-mCherry to monitor enrichment of actin patches at one cell end (monopolar) or both cell ends (bipolar) in indicated strains. Images are sum projections. Scale bar, 5 μm. **(B)** Percentage of monopolar cells in the indicated strains. Values are mean ± SD from three biological replicates (*n* ≥ 100 cells each). ****, P < 0.0001; **, P < 0.01; *, P < 0.05.

Figure 2-figure supplement 3: Mutation of Sts5 residue S264 does not impact cell growth.
Serial-dilution growth assays for each indicated strain. Strains were spotted onto indicated media and grown for 3-5 days at either 25°C or 37°C. Note that S264A does not decrease cell viability when combined with S261A or with both S86A and S261A.

Figure 3: Sts5 granules associate with P-bodies and repress levels of Ssp1.
(A) Localization of Sts5 and Sum2. **(B)** Localization of Sts5(2A) and Sum2. **(C)** Quantification of Sts5 granule number per cell (top), P-body number per cell (middle), and number of colocalized Sts5 granules and P-bodies (bottom). Values are mean ± SD (*n* = 35 cells per strain). ****, P < 0.0001. **(D)** Representative western blot showing Ssp1 protein levels in the indicated strains. Cdc2 blot shown as loading control. **(E)** Quantification of Ssp1 protein levels from western blotting. Values are mean ± SD from three biological replicates. **, P < 0.01. n.s., not significant. Images in A and B are spinning disc confocal maximum intensity projections of the top half of cells. Insets are enlarged views of boxed regions. Dotted lines show cell outlines. Scale bars, 5 μm. **(F)** Model for Sts5 phosphorylation by Pak1 and Orb6 to control both P body formation and cell shape.

Figure 4: Pak1 localizes to RNP granules with Sts5 during glucose starvation.
(A) Localization of the indicated protein kinases in the presence or absence of glucose. Images are single medial focal planes. Arrows point to granule localization of either Sts5 or Pak1. **(B)** Localization of Pak1 and Sum2 during glucose starvation. **(C)** Localization of Sts5 and Sum2 during glucose starvation. (**D**) Localization of Pak1 and Sts5 during glucose starvation. **(E)** Localization of Pak1 and Sum2 in glucose-starved *sts5Δ* cells. Images in B,C,D, and E are spinning disc confocal maximum intensity projections. **(F)** Localization of Pak1 and Sum2 under different stress conditions. Images are maximum intensity projections. Insets are enlarged views of boxed regions. Dotted lines show cell outlines. Scale bars, 5 μm.

Figure 4-figure supplement 1: Localization of Pak1 and Orb6 in the presence and absence of glucose.
(A) Localization of Pak1 and Sum2 in wild type or *sts5Δ* cells. **(B)** Localization of Orb6 and Sum2. Images are spinning disc confocal maximum intensity projections. Insets are enlarged views of boxed regions. Dotted lines show cell outlines. Scale bars, 5 μm.

Figure 4-figure supplement 2: Pak1 localizes to RNP granules during glucose starvation in Sts5(2A) phosphomutant.
Localization of Pak1 and Sum2 in Sts5-FLAG or Sts5(2A)-FLAG cells. 2A mutation denotes S86A S261A mutations in the Sts5 sequence. Images are spinning disc confocal maximum intensity projections. Insets are enlarged views of boxed regions. Dotted lines show cell outlines. Scale bars, 5 μm.

Figure 4-figure supplement 3: Localization of other polarity kinases during glucose starvation.
Localization of Nak1, Pck1, Pck2 and Kin1. Single focal planes in the cell middle are shown. Scale bar, 5 μm.

Figure 4-figure supplement 4: Pck2 and Kin1 localize to RNP granules during glucose starvation.
Images of Pck2 and Kin1 with Sum2. Images are maximum intensity projections. Red arrows indicate clustered localization of Pck2 or Kin1. Dotted lines show cell outlines. Scale bars, 5 μm.

Figure 5: Pak1 ligands in Cdc42 regulatory pathway do not localize to RNP granules.
(A) Localization of Scd2 and Sum2. **(B)** Localization of Scd1 and Sum2. **(C)** Localization of active Cdc42 biosensor and Sum2. **(D)** Localization of Cdc42 and Dcp2. Images are spinning disc confocal maximum intensity projections. Dotted lines show cell outlines. Scale bars, 5 μm.

Figure 6: PKA signaling mediates Pak1 recruitment to RNP granules during glucose starvation
(A) Localization of Pak1 and Sum2 for indicated strains. **(B)** Localization of Pak1 and Sum2 for indicated strains in 0% glucose. Note the loss of Pak1 localization to RNP granules in the mutant strains. **(C)** Localization of Sts5 and Sum2 for indicated strains in 0% glucose. Images are spinning disc confocal maximum intensity projections. Dotted lines show cell outlines. Scale bars, 5 μm.

Figure 7: Pak1 promotes rapid Sts5 dissolution from RNP granules upon glucose addition
(A) Localization of Pak1-CAAX and Sum2. Images are spinning disc confocal maximum intensity projections. Dotted lines show cell outlines. **(B)** Localization of Sts5 in indicated strains. Images are spinning disc confocal sum projections. **(C)** Quantification of Sts5-mNG fluorescence intensity per RNP granule following glucose re-feed for indicated strains. Values are means ± SD (n = 50 granules per

timepoint). ****, P < 0.0001. n.s., not significant. Scale bars, 5 μm. **(D)** Model showing PKA-dependent Pak1 localization to RNP granules to promote rapid Sts5 dissolution during glucose re-feed.

Figure 7-figure supplement 1:
(A) Images of Pak1-CAAX by spinning disc confocal microscopy. Scale bars, 5 μm. **(B)** Quantification of stress granule dissolution from multiple experiments after glucose re-feed for indicated strains. Values in this graph are means ± SD from three biological replicates (n = 50 granules per timepoint). One of these replicates is shown in Figure 7C. ***, P < 0.001; *, P < 0.05. n.s., not significant. **(C)** Quantification of Sts5 granule levels at 6-minute timepoint from panel B. Values are means ± SD of the three biological replicates (n = 50 granules per timepoint). ****, P < 0.0001.

Materials and Methods

Strain construction and media

Standard *Schizosaccharomyces pombe* media and methods were used (Moreno et al., 1991). Strains used in this study are listed in Table S1. Chromosomal tagging and deletion was performed by PCR-based homologous recombination (Bähler et al., 1998). *Psts5-sts5-mNeonGreen-Tadh1* was generated by PCR from genomic DNA of strain JM5505 and inserted into pDC99 by restriction digest and ligation. *Psts5-sts5-9gly5FLAG* was generated by PCR from genomic DNA of strain JM5522 and inserted into pDC99 by restriction digest and ligation. The non-phosphorylatable *sts5* sequences were generated by either site directed mutagenesis using QuikChange II mutagenesis (Stratagene) or mutant sequences were synthesized as a gBlocks Gene Fragment (Integrated DNA Technologies) and inserted into PCR-linearized fragment from pDC99-*Psts5-sts5-mNeonGreen-Tadh1* or pDC99-*Psts5-sts5-9gly5FLAG-Tadh1* plasmid by repliQa HiFi Assembly Mix (Quantabio). Both of these strategies to generate point mutations were done according to their respective manufacturer's protocol. All plasmids were sequenced by Sanger sequencing for verification. All pDC99 plasmids were linearized by NotI restriction digest and transformed into the *leu1* locus of JM6332 (*sts5Δ ura4-D18*). For growth assays, cells were grown in YE4S to mid-log phase and cells were spotted by 10-fold serial dilutions on YE4S media plates containing indicated compounds at 25°C, 32°C or 37°C for 3-5 days before scanning.

Protein purification and in vitro kinase assays

Full-length Pak1 constructs (wild type and catalytically inactive) and full-length Sts5 constructs (wild type and phosphomutant) were cloned into pGEX6P1 (GST tag) vector (GE Healthcare) for expression in BL21 DE3 *Escherichia coli*. Transformants were grown to log phase at 37°C and then switched to 16°C for 30 minutes. Protein expression was induced by addition of 1-thio-β-galactopyranoside to a final concentration of 200 μM and cells were incubated overnight at 16°C. Cells were then harvested by centrifugation and lysed 3 times by French press in lysis buffer (1x PBS, 300 mM NaCl, 200 mM EDTA, 1 mM DTT and complete EDTA-free protease inhibitor tablets (one tablet/50 mL buffer)(Roche)). Triton-X 100 was added to cell lysate to a 1% W/V final concentration for 10 minutes on ice. Cell lysates were then clarified by centrifugation for 20 minutes at 14,000 x g at 4°C in a Sorval SS-34 fixed angle rotor (Thermo Scientific). Lysates were then incubated with glutathione-agarose resin (Sigma-Aldrich) for 2 hours rotating at 4°C. Purified proteins were either released from resin by overnight incubation with 3C protease at 4°C or by elution with 20mM glutathione (pH 8.0).

In vitro kinase assays were performed by incubating purified proteins in kinase assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10mM $MgCl_2$ and 1 mM $MnCl_2$) supplemented with 10 μM ATP and 2 μCi γ-^{32}P-ATP (blu002z250uc; Perkin Elmer) in 15μl reactions. Reactions were incubated at 30°C for 30 minutes and reactions were quenched by adding SDS-PAGE sample buffer (65mM Tris pH 6.8, 3% SDS,

10% glycerol and 10% 2-mercaptoethanol) and boiling. Gels were dried for 1 h, and the signal of γ-^{32}P-ATP was detected using a PhosphorImager scanned by Typhoon 8600 Variable Mode Imager (GE Healthcare). Bands on the autoradiograms correspond to the molecular weight where Coomassie-stained protein migrates (Figure 1-figure supplement 1).

Widefield microscopy and analysis

Cells were imaged in either YE4S, EMM4S or EMM at 25°C, 32°C or 42°C using a DeltaVision imaging system (Applied Precision Ltd.) composed of an IX-inverted widefield microscope (Olympus) with a 100x or a 60x oil objective, a CoolSnap HQ2 camera (Photometrics), and an Insight solid-state illumination unit (Applied Precision Ltd.). Images shown as Z-stacks in Figure 2-figure supplement 2A and Figure 4F were acquired and processed by iterative deconvolution using Soft-WoRx software (Applied Precision Ltd.). For Figure 1E, all strains were grown in YE4S at 32°C and treated with 30 µM 3-Brb-PP1 (3-[(3-Bromophenyl)methyl]-1-(1,1-dimethylethyl)- 1H-pyrazolo[3,4-d]pyrimidin-4-amine (Abcam) for 1 h and fixed in ice-cold 70% ethanol. Fixed cells were stored at 4°C for no longer than 24 h and cells were stained with Blankophor before imaging to measure septation index. Septation index quantification in Figure 2G was performed for the indicated strains grown in EMM4S at 32°C and cells were stained with Blankophor before imaging. Cell polarity quantification in Figure 2-figure supplement 2B was judged by observing actin patch intensity marked by Fim1-mCherry in the indicated strains. Cells with a greater number of patches at one cell end versus the other were considered monopolar. For Figure 2E, cells were grown in EMM4S at 32°C and stained with Blankophor before imaging and non-deconvolved single focal plane images are shown with an inverted lookup table. Quantification of cell width in Figure 2F was measured by drawing a line across the division plane in septating cells stained by Blanophor. For Figure 4A and Figure 4-figure supplement 3, cells were grown in EMM4S 2% glucose to mid-log phase and cells were spun down and washed 4x in EMM4S 0% glucose, resuspended and incubated in EMM4S 0% glucose shaking at 25°C for 60 minutes before imaging. Single-channel fluorescence non-deconvolved single focal plane images are shown with an inverted lookup table. For Figure 4F, -Nitrogen cells were grown in EMM and switched to EMM lacking nitrogen source overnight at 25°C before imaging, 1M KCl stress occurred for 30 minutes in EMM4S + 1M KCl 25°C before imaging and heat shock stress proceeded with cells grown in EMM4S at 25°C and switched to 42°C for 20 minutes before imaging. All image analysis was performed on ImageJ2 (National Institutes of Health). Statistical differences were assessed by one-way ANOVA using GraphPad.

Spinning Disc microscopy and analysis

Images for Figure 3A,B were taken with a spinning-disc confocal system (Micro Video Instruments) featuring a Nikon Eclipse Ti base equipped with an Andor CSU-W1 two-camera spinning-disc module, dual Zyla sCMOS cameras (Andor), an Andor ILE laser module and a Nikon 100x Plan Apo λ 1.45 oil immersion objective. Images shown are maximum intensity projections of the top half of cells. Colocalization analysis, number of Sts5 granules and number of P-bodies in Figure 3C was performed using ImageJ2 plugin ComDet v.0.5.0. Square ROIs of with height = 22.23 microns and width = 26.52 microns were used for analysis. Channel 1 was set to 3.00 pixels and a threshold of 12. Channel 2 was set to 4.00 pixels and a threshold of 12. Both channels were set to include larger particles and segment larger particles. For calculating colocalization, maximum distance between colocalized spots was set to 3.00 pixels and ROI shapes were set for ovals.

All other spinning disc confocal microscopy was performed using a Nikon Eclipse Ti-E microscope stand with a Yokogawa, two-camera, CSU-W1 spinning disc system with a Nikon LU-N4 laser launch. This system has Photometrics Prime BSI sCMOS cameras. For Figure 1D,H and Figure 2D, Sts5-mNG granules quantification was done using ImageJ2 plugin Trackmate using a 0.4 micron size and 10 threshold setting

for each strain using maximum intensity projections of the whole cell. Spinning disc images images showing indicated protein localization in 0% glucose, cells were grown in EMM4S 2% glucose to mid-log phase and cells were spun down and washed 4x in EMM4S 0% glucose, resuspended and incubated in EMM4S 0% glucose shaking at room temperature for 40 minutes before imaging. Sts5-mNG stress granule dissolution quantification was done by washing cells in EMM4S 0% glucose media 4x and cells were resuspended in EMM4S 0% glucose and incubated shaking at 25°C for 40 minutes. After 40 minutes, timepoint 0 in stress granule dissolution sample was spun down and fixed in ice-cold 70% ethanol and stored at 4°C until imaging. For the timepoints following timepoint 0, cells in EMM4S 0% glucose were spun down and washed into EMM4S 2% glucose, incubated shaking at 25°C, and samples were removed at the indicated timepoints and fixed in ice-cold 70% ethanol and stored at 4°C until imaging. For Figure 7C and Figure 7-figure supplement 1B,C, background subtracted integrated densities of clustered Sts5-mNG signal were measured using circular ROIs drawn around the clustered signal from sum projections of the whole cell. For each timepoint, 50 clusters were measured and plotted. Statistical differences were assessed by one-way ANOVA or Welch's *t* test using GraphPad.

Western blotting and analysis

For all western blots, samples of two OD_{595} mid-log phase cells were harvested and snap-frozen in liquid nitrogen. Whole cell extracts were generated by lysing cells in 3x sample buffer (65 mM Tris, pH 6.8, 3% SDS, 10% glycerol, 10% 2-mercaptoethanol, 50 mM NaF, 50 mM β-glycerolphosphate, 1 mM sodium orthovanadate and protease inhibitor cocktail) with acid-washed glass beads (Sigma) in a Mini-BeadBeater-16 (BioSpec) for 2 minutes in a cold room. Samples were then heated to 99˚C for 5 minutes, briefly centrifuged to pellet insoluble material, and the supernatant was used as whole-cell extract. Western blots were probed with anti-HA (Covance), anti-Cdc2 (SC-53217) and anti-FLAG M2 (Sigma). Western blots were developed using an Odyssey CLx Imaging System (LI-COR). For quantification of 6His2HA-Ssp1 protein levels in Figure 3E, a rectangular ROI was drawn around each band. Mean intensity was measured, and background was subtracted for both HA and Cdc2 loading control signals using Image Studio Lite (LI-COR). The ratio of HA to Cdc2 signal was calculated for three biological replicates of each strain indicated. Statistical differences were assessed by one-way ANOVA using GraphPad. For Figure 2B and Figure 2-figure supplement 1, samples were run on a 6% SDS-PAGE gel containing 100 μM Phosbind (Apexbio) according to the manufacturer's protocol to separate phosphorylated isoforms of Sts5. Membranes were treated with Ponceau stain and scanned to display total protein loaded in each lane.

Statistical analyses

Welch's *t* test was used to assess differences for Figure 1D, Figure 3C, Figure 7C and Figure 7-figure supplement 1B,C. One-way ANOVA followed by Dunnett's multiple comparison test was used to assess differences for Figure 2F,G and Figure 3E. One-way ANOVA followed by Tukey's multiple comparison test was used to assess differences for Figure 1E,H, Figure 2D and Figure 2-figure supplement 2B. GraphPad Prism 7 was used for all statistical analyses.

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Figure 1

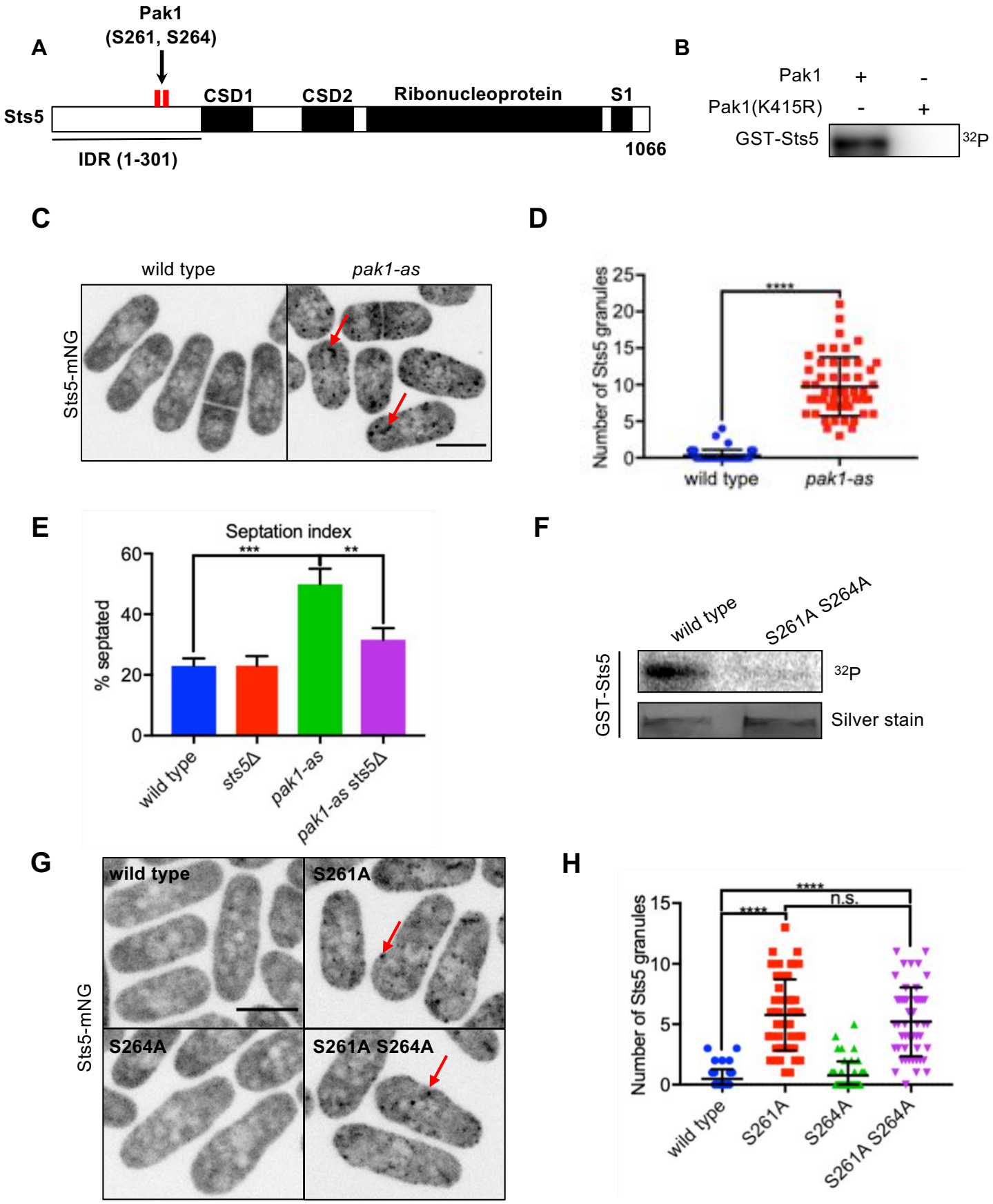

A
Pak1
(S261, S264)
Sts5
CSD1
CSD2
Ribonucleoprotein
S1
IDR (1-301)
1066
B
Pak1
Pak1(K415R)
GST-Sts5
32P
C
wild type
pak1-as
Sts5-mNG
D
Number of Sts5 granules
****
wild type
pak1-as
E
Septation index
% septated
***
**
wild type
sts5Δ
pak1-as
pak1-as sts5Δ
F
wild type
S261A S264A
GST-Sts5
32P
Silver stain
G
wild type
S261A
S264A
S261A S264A
Sts5-mNG
H
Number of Sts5 granules
****
n.s.
wild type
S261A
S264A
S261A S264A

Figure 1 Supplement 1

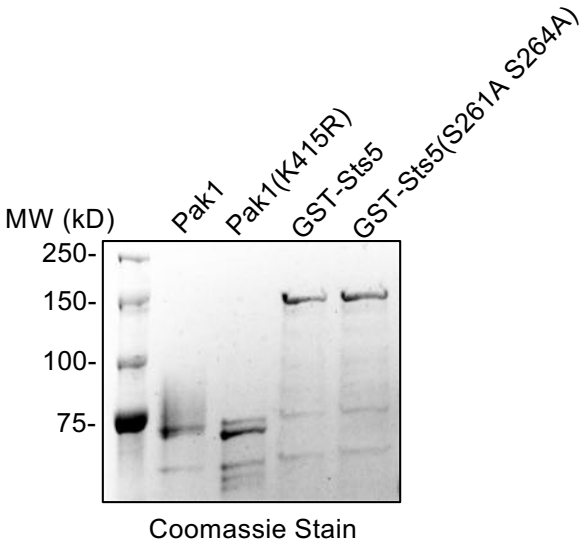


Figure 2

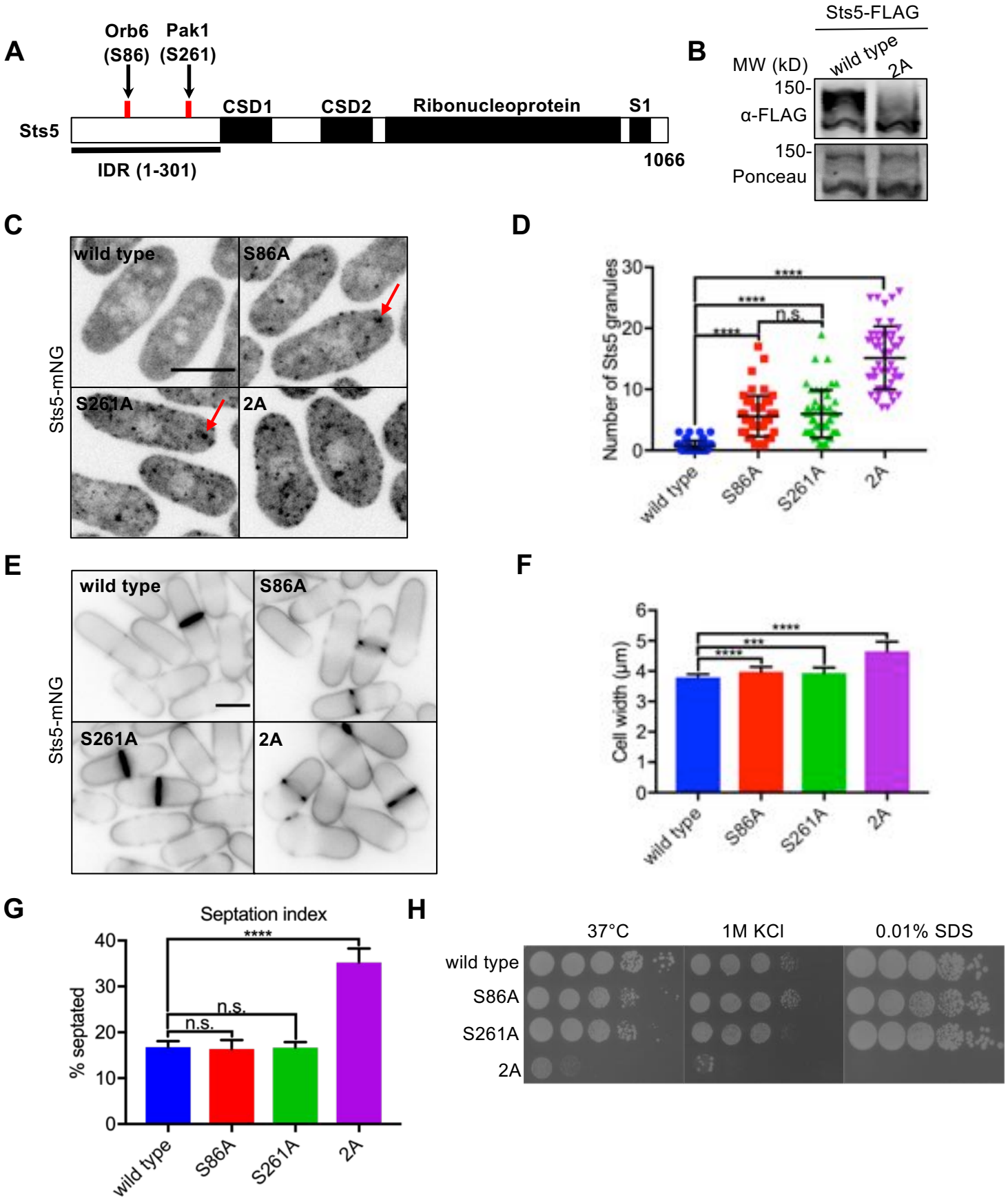
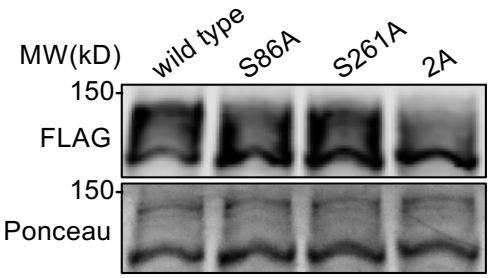

A
Orb6 (S86)
Pak1 (S261)
Sts5
CSD1
CSD2
Ribonucleoprotein
S1
IDR (1-301)
1066
B
Sts5-FLAG
MW (kD)
wild type
2A
150-
α-FLAG
150-
Ponceau
C
wild type
S86A
S261A
2A
Sts5-mNG
D
Number of Sts5 granules
****
n.s.
wild type
S86A
S261A
2A
E
wild type
S86A
S261A
2A
Sts5-mNG
F
Cell width (µm)
****
***
wild type
S86A
S261A
2A
G
Septation index
% septated
****
n.s.
n.s.
wild type
S86A
S261A
2A
H
37°C
1M KCl
0.01% SDS
wild type
S86A
S261A
2A

Figure 2 Supplement 1



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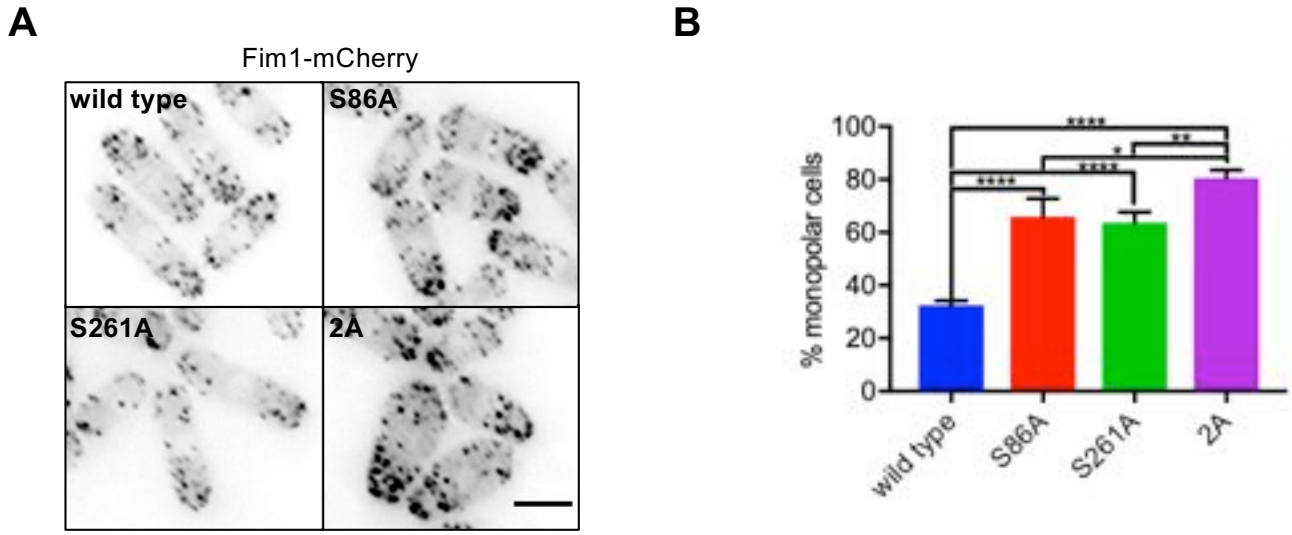
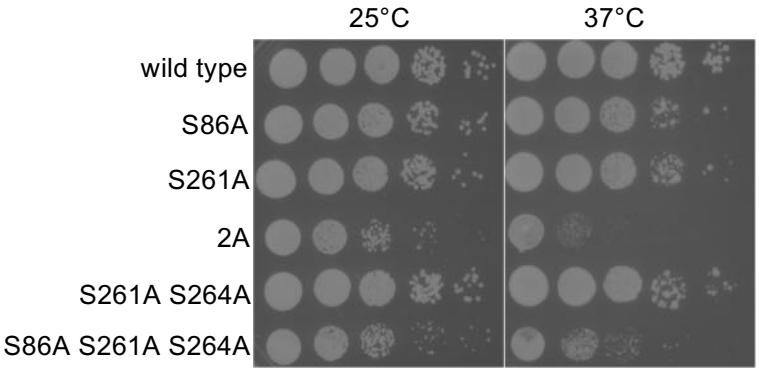


Figure 2 Supplement 3



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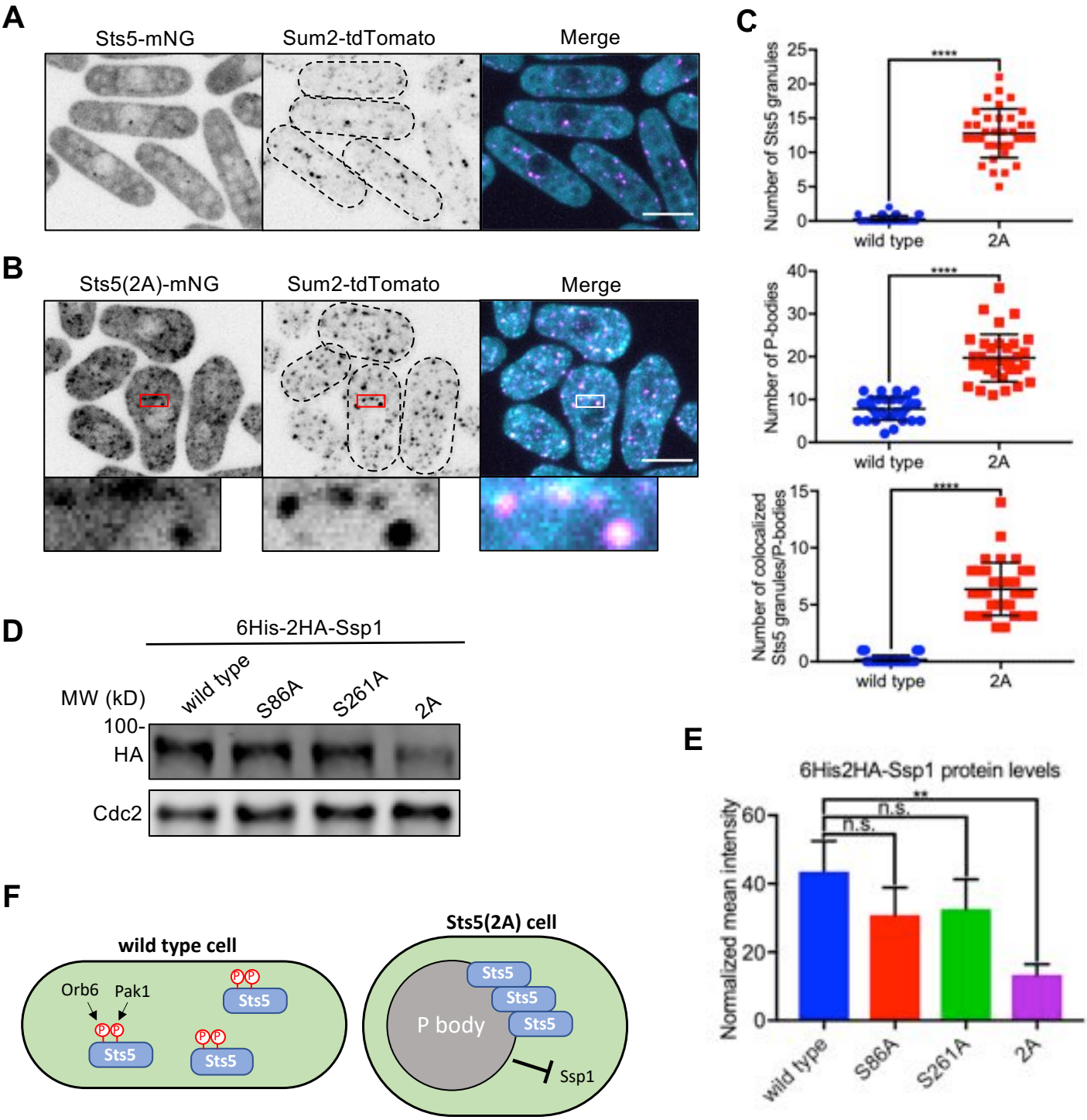

A
Sts5-mNG
Sum2-tdTomato
Merge
B
Sts5(2A)-mNG
Sum2-tdTomato
Merge
C
Number of Sts5 granules
Number of P-bodies
Number of colocalized Sts5 granules/P-bodies
wild type
2A
****
D
6His-2HA-Ssp1
MW (kD)
wild type
S86A
S261A
2A
100-
HA
Cdc2
E
6His2HA-Ssp1 protein levels
Normalized mean intensity
n.s.
**
wild type
S86A
S261A
2A
F
wild type cell
Orb6
Pak1
Sts5
Sts5(2A) cell
P body
Ssp1

Figure 4

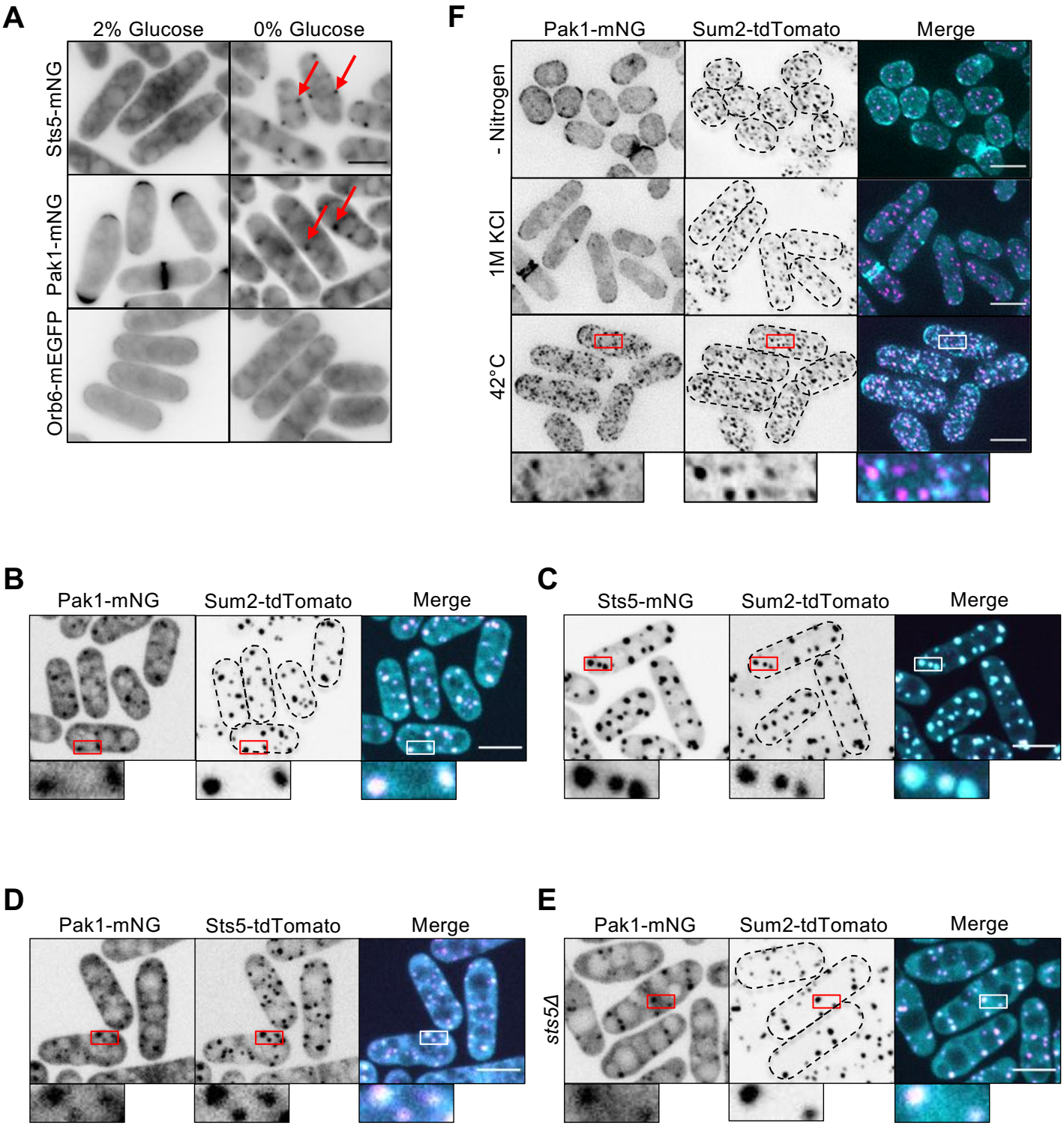

A
2% Glucose
0% Glucose
Sts5-mNG
Pak1-mNG
Orb6-mEGFP
F
Pak1-mNG
Sum2-tdTomato
Merge
- Nitrogen
1M KCl
42°C
B
Pak1-mNG
Sum2-tdTomato
Merge
C
Sts5-mNG
Sum2-tdTomato
Merge
D
Pak1-mNG
Sts5-tdTomato
Merge
E
Pak1-mNG
Sum2-tdTomato
Merge
sts5Δ

Figure 4 Supplement 1

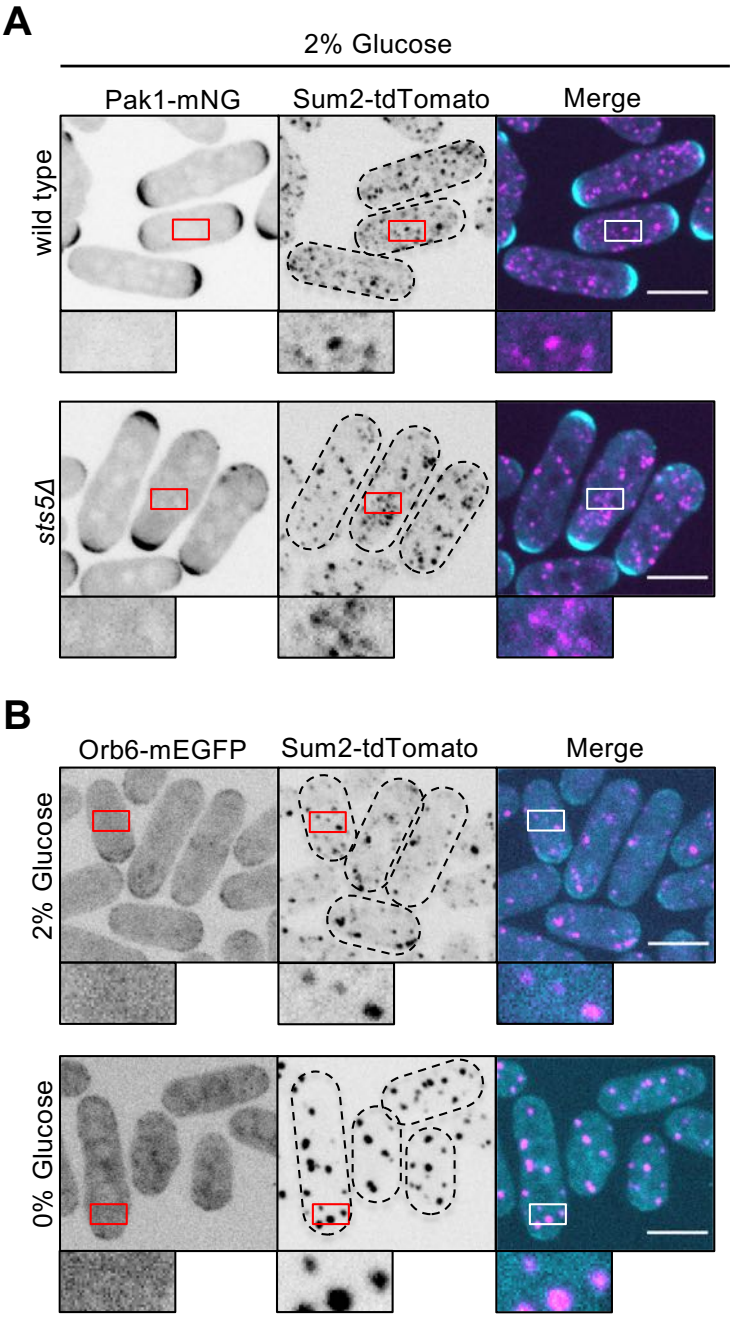


Figure 4 Supplement 2

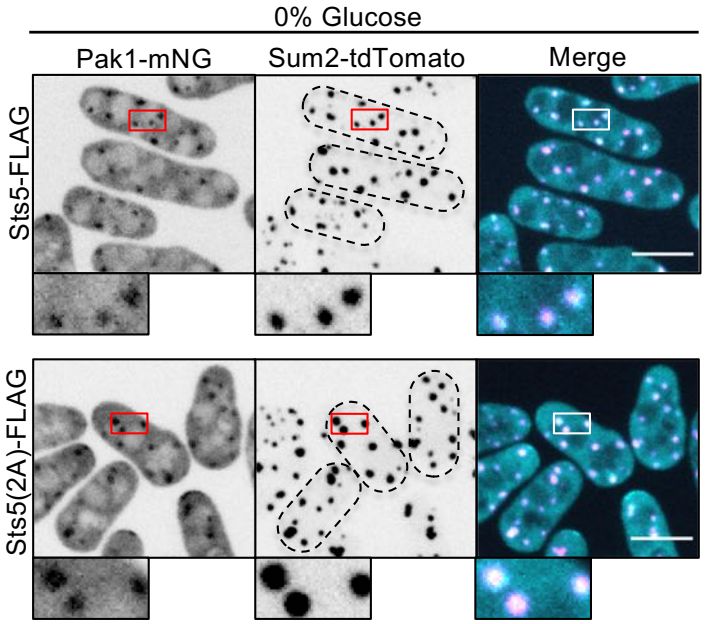


Figure 4 Supplement 3

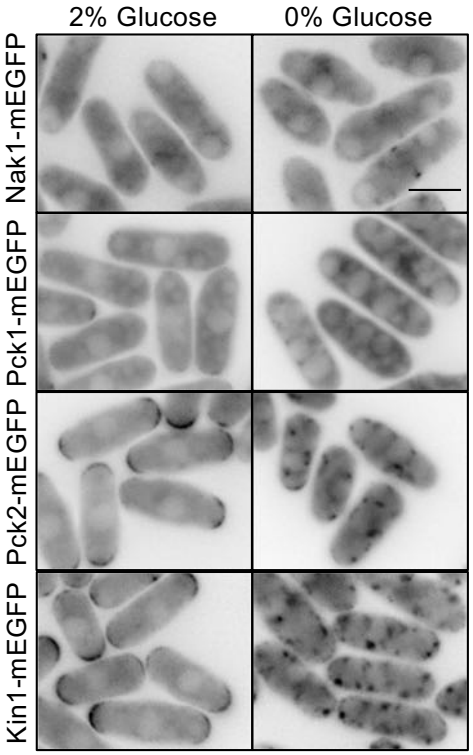
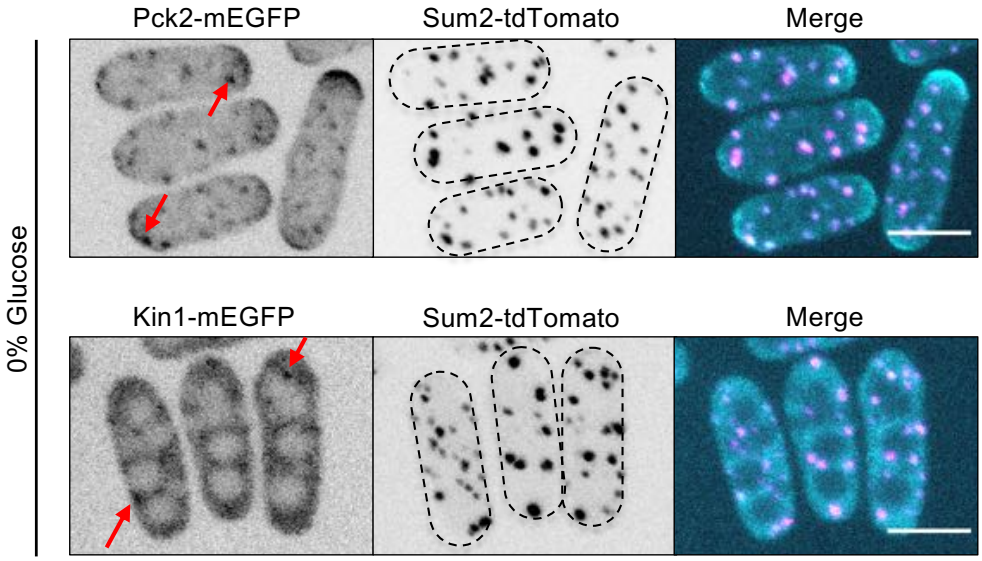


Figure 4 Supplement 4



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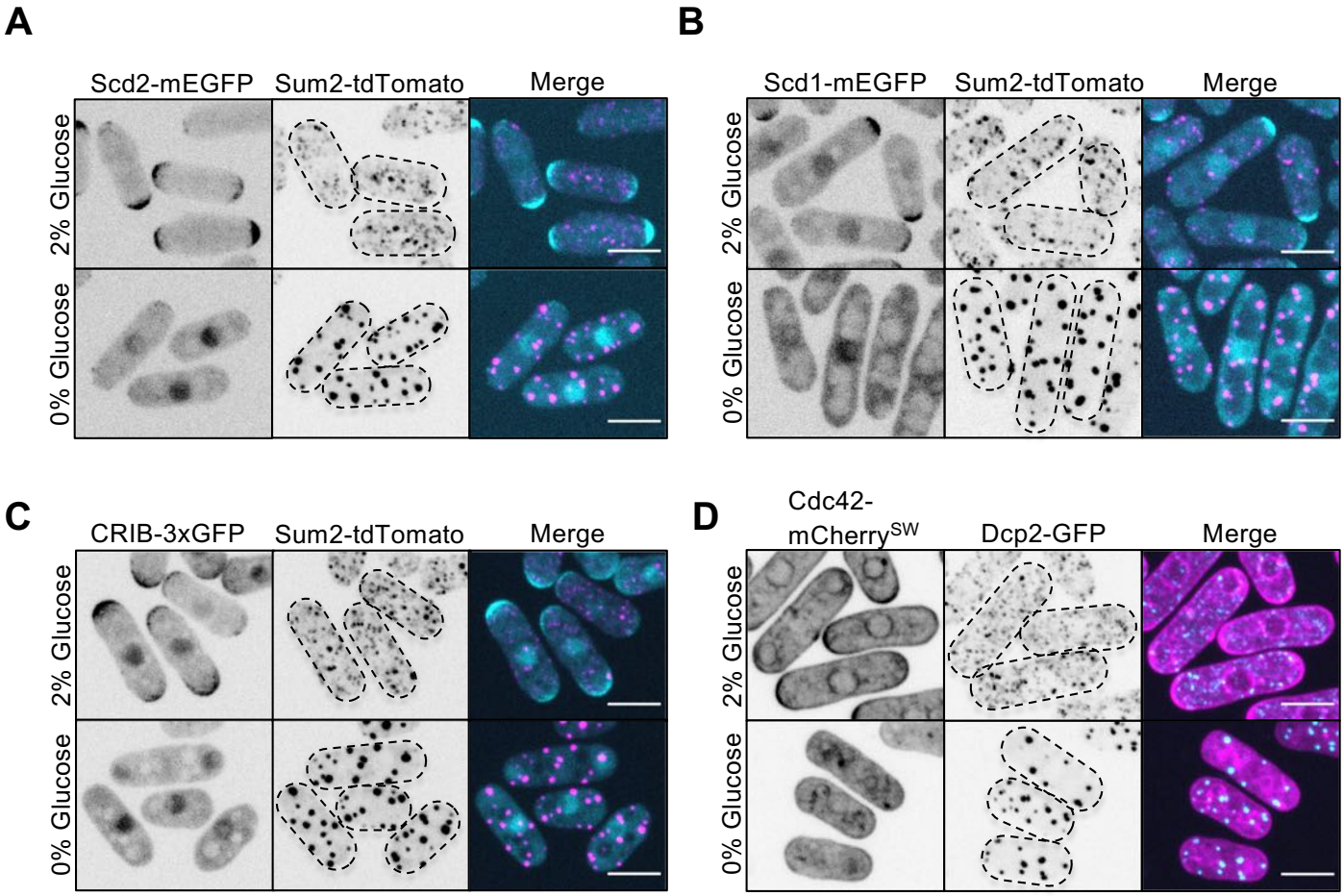

A
Scd2-mEGFP
Sum2-tdTomato
Merge
2% Glucose
0% Glucose
B
Scd1-mEGFP
Sum2-tdTomato
Merge
2% Glucose
0% Glucose
C
CRIB-3xGFP
Sum2-tdTomato
Merge
2% Glucose
0% Glucose
D
Cdc42-mCherry^{SW}
Dcp2-GFP
Merge
2% Glucose
0% Glucose

Figure 6

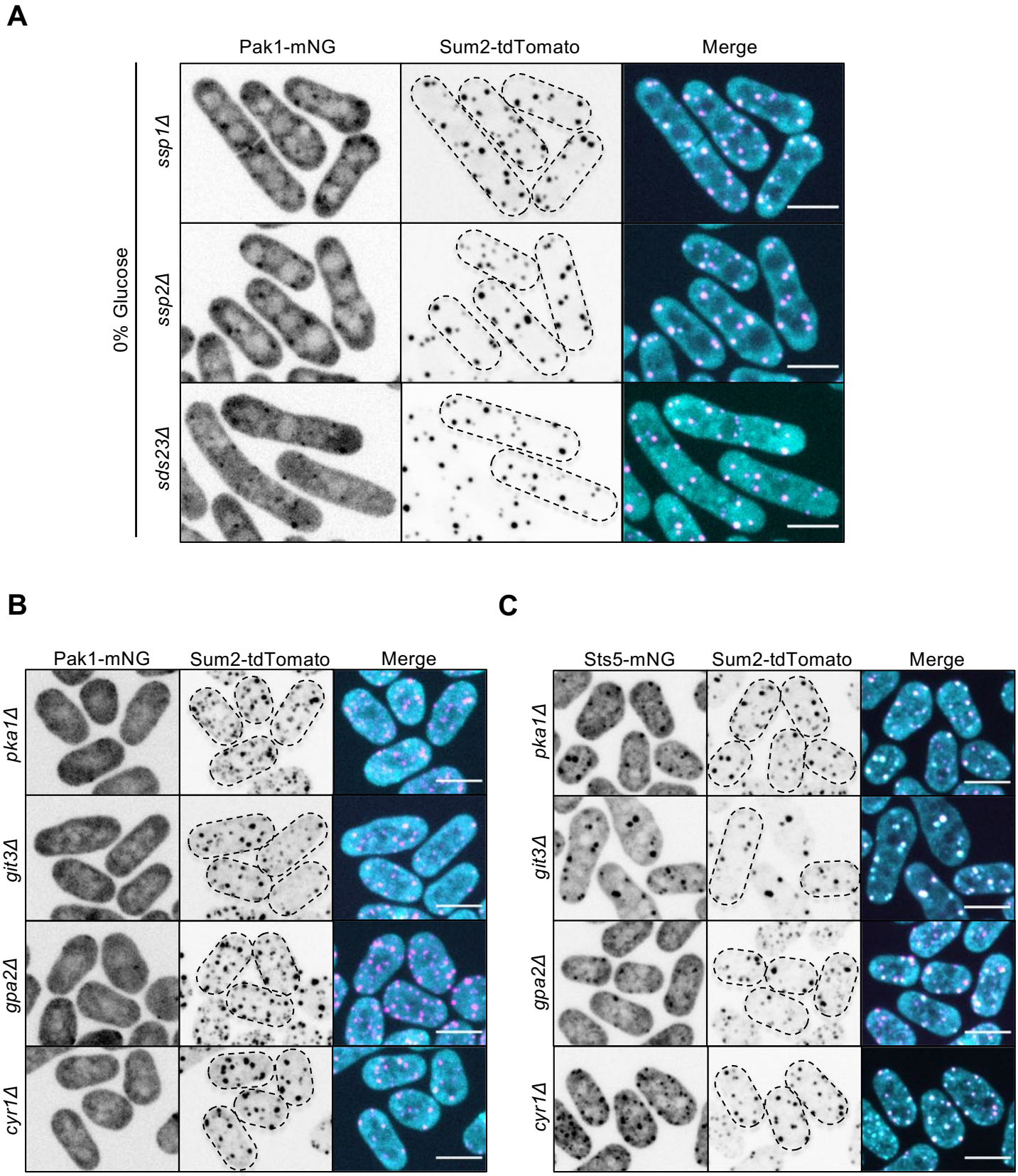
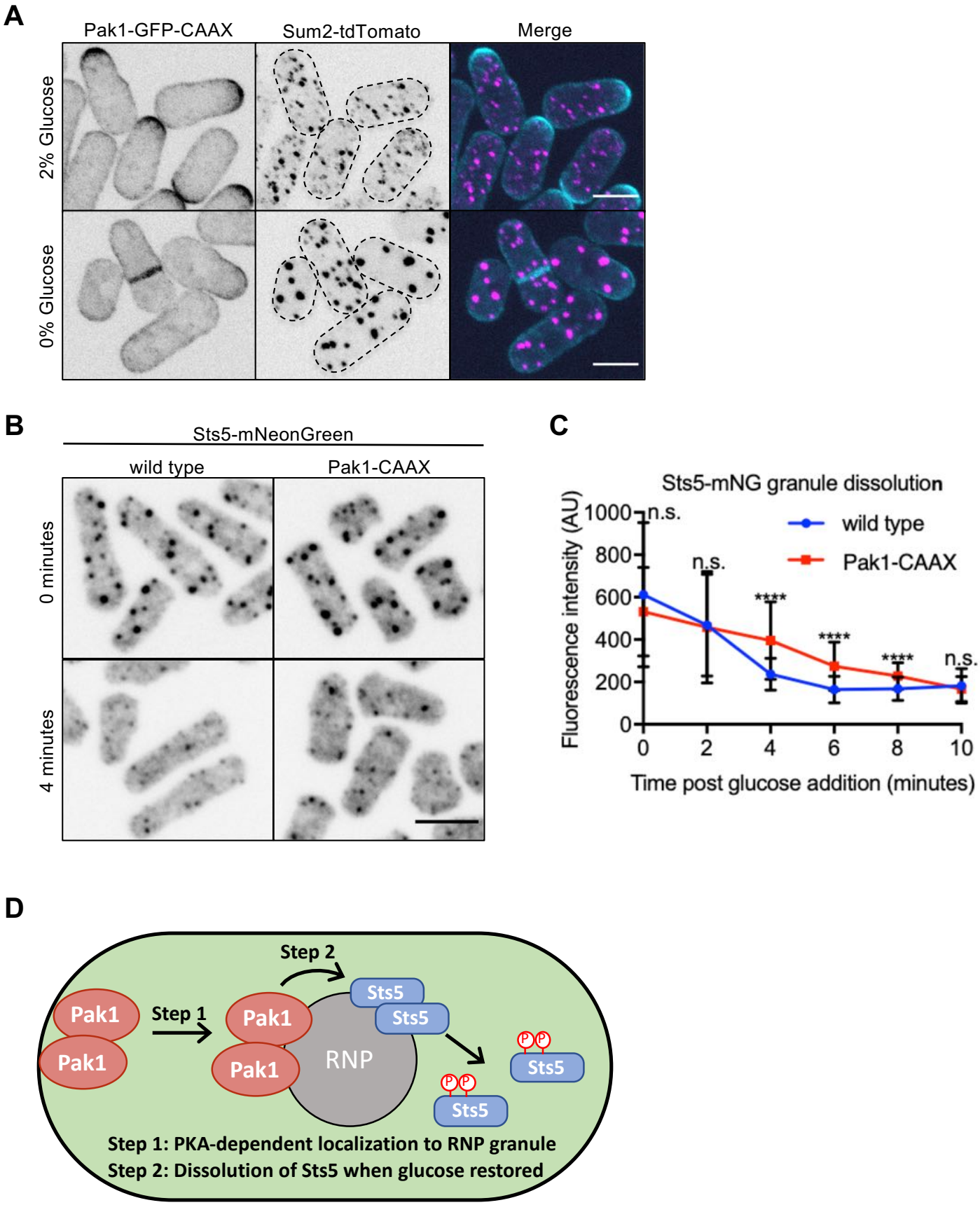

A
Pak1-mNG
Sum2-tdTomato
Merge
0% Glucose
ssp1Δ
ssp2Δ
sds23Δ
B
Pak1-mNG
Sum2-tdTomato
Merge
pka1Δ
git3Δ
gpa2Δ
cyr1Δ
C
Sts5-mNG
Sum2-tdTomato
Merge
pka1Δ
git3Δ
gpa2Δ
cyr1Δ

Figure 7


A
Pak1-GFP-CAAX
Sum2-tdTomato
Merge
2% Glucose
0% Glucose
B
Sts5-mNeonGreen
wild type
Pak1-CAAX
0 minutes
4 minutes
C
Sts5-mNG granule dissolution
Fluorescence intensity (AU)
1000
800
600
400
200
0
n.s.
n.s.
****
****
****
n.s.
wild type
Pak1-CAAX
0
2
4
6
8
10
Time post glucose addition (minutes)
D
Step 2
Step 1
Pak1
Pak1
Pak1
Pak1
RNP
Sts5
Sts5
Sts5
Sts5
P
P
Step 1: PKA-dependent localization to RNP granule
Step 2: Dissolution of Sts5 when glucose restored

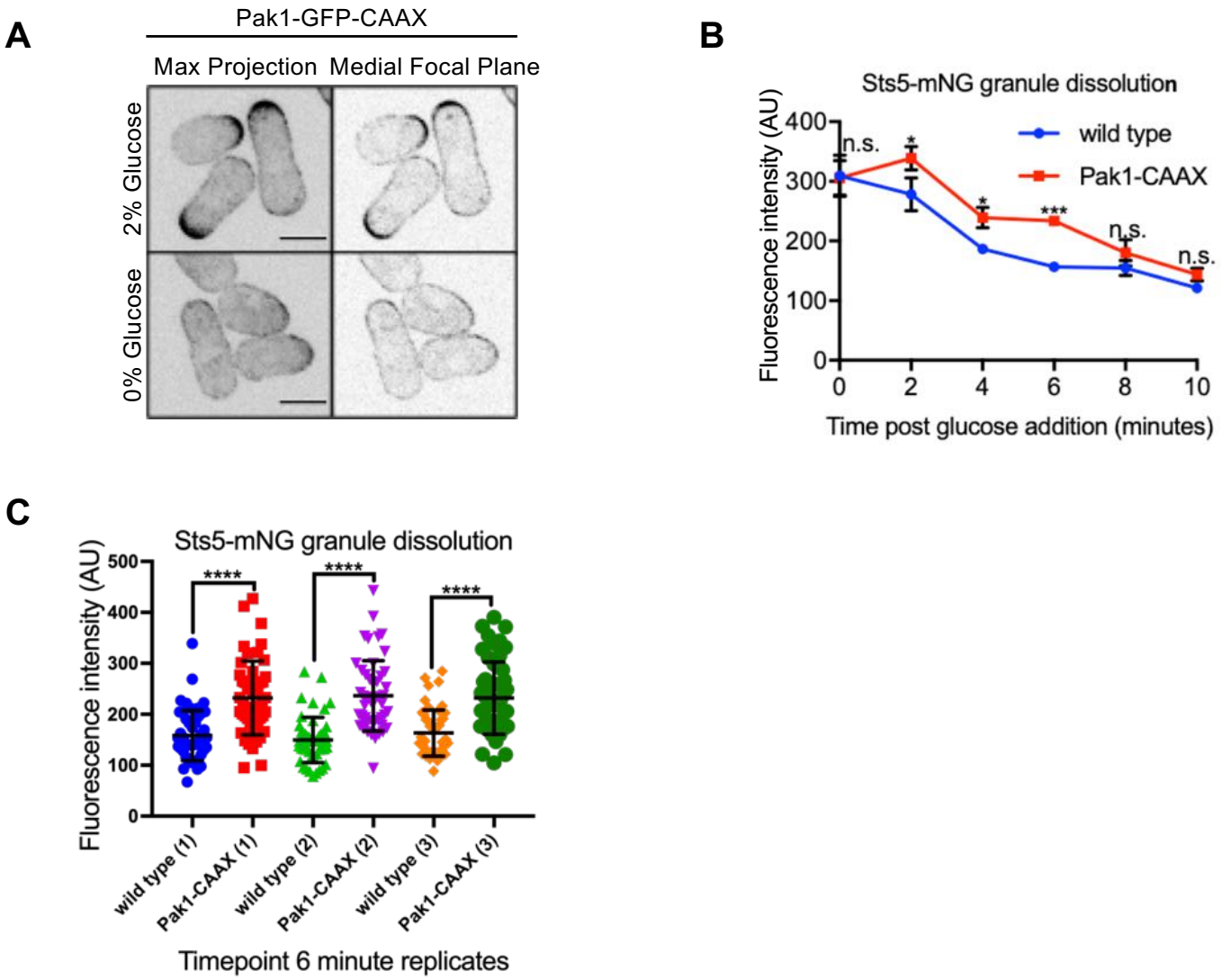



Figure 7 Supplement 1