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Analysis of DrkA kinase's role in STATa activation

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Abstract

Dictyostelium **STATa is a homologue of metazoan signal transducers and activators of transcription (STATs) and is important for morphogenesis. STATa is activated by phosphorylation on Tyr702 when cells are exposed to extracellular cAMP. Although two tyrosine kinase-like (TKL) proteins, Pyk2 and Pyk3, have been definitively identified as STATc kinases, no kinase is known for STATa activation. Based on homology to the previously identified tyrosine-selective TKLs, we identified DrkA, a member of the TKL**

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family and the *Dictyostelium* receptor-like kinase (DRK) subfamily, as a candidate STATa kinase. The *drkA* gene is almost exclusively expressed in prestalk A (pstA) cells, where STATa is activated. Transient overexpression of DrkA increased STATa phosphorylation, although overexpression of the protein causes a severe growth defect and cell death. Furthermore, recombinant DrkA protein is auto-phosphorylated on tyrosine and threonine residues, and an *in vitro* kinase assay shows that DrkA can phosphorylate STATa on Tyr702 in a STATa-SH2 (phosphotyrosine binding) domain-dependent manner. These observations strongly suggest that DrkA is one of the key regulators of STATa tyrosine phosphorylation and are consistent with it being the kinase that directly activates STATa.

KEY WORDS

cyclic AMP, *Dictyostelium*, phosphotyrosine signal, signal transducer and activator of transcription (STAT), tyrosine kinase-like kinase (TKL)

1 | INTRODUCTION

Phosphotyrosine (pTyr) signalling is a pivotal signal transducing mechanism widely distributed in Metazoa. pTyr signalling is called a three-component system as it is comprised of three different functional modules (three-part toolkit): protein tyrosine kinases (TKs) as "writers" to phosphorylate tyrosine residues of target proteins, protein tyrosine phosphatases (PTPs) as "erasers" to dephosphorylate targets, and Src homology 2 (SH2) domains as "readers" to bind pTyr and deliver signals downstream. More specifically, binding of the appropriate extracellular ligand to its corresponding receptor triggers activation and causes phosphorylation of specific tyrosine residues situated in the intracellular domain of the receptor. Tyrosine phosphorylation results in activation of a signal transduction pathway via protein-protein interactions in which the SH2 domain of a downstream partner specifically recognizes phosphorylated tyrosine residues on the target protein. This process starts a sequence of events, which eventually leads to alteration of gene expression patterns or other cellular responses (Lim, & Pawson, 2010; Jin, & Pawson, 2012).

Multicellular Metazoa and choanoflagellates such as *Monosiga brevicollis*, which may be the closest unicellular relatives to metazoans (King et al., 2008), harbour all parts of the toolkit for pTyr signalling. Each module of the three-part toolkit is interdependent in these organisms, and stepwise emergence of each part at different evolutionary stages is believed to have occurred (Lim & Pawson, 2010). The social amoeba *Dictyostelium discoideum* is a facultative multicellular organism and is the simplest organism known to employ primitive pTyr signalling. There are fewer than five PTPs with simple domain architectures in *Dictyostelium* and 13 proteins containing a bona fide pTyr binding SH2 domain (Eichinger et al., 2005), including four orthologues of metazoan signal transducer and activator of transcription (STAT) proteins, STATa – d (Kawata et al., 1997; Kawata, 2011). *Dictyostelium* lacks any recognizable orthodox TKs, including JAK family members, which commonly act to phosphorylate STATs, but the organism possesses a great number of tyrosine kinase-like (TKL) kinases. Therefore, to comprehend the origin and evolution of pTyr signalling, *Dictyostelium* proteins are excellent tools.

It had been a mystery as to which kinases (TKLs) specifically activate *Dictyostelium* STATs. Recently, however, two TKL kinases that phosphorylate and activate STATc were identified (Araki, Kawata, & Williams, 2012; Araki, Vu, Sasaki, Kawata, Eichinger, & Williams, 2014). STATc is activated by either hyperosmotic stress or ligand stimulation (Fukuzawa, Araki, Adrian, Williams, 2001; Araki et al., 2003; Na, Tunggal, & Eichinger, 2007), where the ligand is differentiation-inducing factor-1 (DIF-1), a chlorinated polyketide necessary to induce prestalk-specific gene expression (Williams, 2006). The TKL involved in DIF-1-induced STATc activation is Pyk2 (Araki, Kawata, & Williams, 2012) whereas Pyk2 and Pyk3 are the TKLs that activate STATc in response to hyperosmotic stress (Araki, Vu, Sasaki, Kawata, Eichinger, & Williams, 2014). Protein tyrosine phosphatase PTP3 has been demonstrated to control the level of STATc phosphorylation. In the absence of DIF-1 or hyperosmotic stress, PTP3 is active in dephosphorylating STATc. As a consequence of DIF-1 induction or hyperosmotic stress, however, PTP3 is phosphorylated at Ser448 and Ser747, which results in the reduction of PTP3 activity and, thereby, increased STATc phosphorylation on Tyr922 (Araki, Langenick, Gamper, Firtel, & Williams, 2008). Because

complex formation between STATc and Pyk2 is constitutive, Pyk2 functions as a constitutively active STATc kinase, while PTP3 serves as the principal regulator of STATc activation (Araki, Kawata, & Williams, 2012).

Among the four *Dictyostelium* STATs, STATa is the most important transcription factor, with multiple functions during *Dictyostelium* development, as evident from the defects in both early and late development observed in the STATa null strain (Mohanty et al., 1999). The null mutant exhibits delayed aggregation due to inefficient chemotaxis to pulsatile cAMP. While the null cells are capable of forming slightly short slugs, they do not display phototaxis (Kawata, Hirano, Ogasawara, Aoshima, & Yachi, 2011). In addition, the mutant shows abnormal morphogenetic cell movement (Kawata et al., unpublished), which results in the formation of aberrant terminal structures (Mohanty et al., 1999). These morphogenetic defects could be attributed to the loss or impaired function of the developmental organiser, which is located in the tip region of the migrating slug. Indeed, the *STATa* null strain underexpresses various organiser marker genes, including ones encoding several important transcription factors, such as *cudA* and *mybC* (Fukuzawa, & Williams, 2000; Shimada, Maeda, Urushihara, & Kawata, 2004; Shimada, Nishio, Maeda, Urushihara, & Kawata, 2004; Saga, Inamura, Shimada, & Kawata, 2016). STATa is activated (i.e., phosphorylated at Tyr702) when extracellular cAMP, which binds the serpentine-type receptor cAR1, is added to cells in buffered culture conditions (Araki et al., 1998). Hence, cAMP signalling is a major regulator of STATa activation. In spite of extensive analysis of the molecular mechanism of STATa function, the kinases that activate the STATa molecule have not yet been identified. In this manuscript, we characterize a TKL kinase, DrkA, and show that it is one of the direct activators of STATa.

2 | RESULTS

2.1 | DrkA harbours the high homology to kinase domain of JAKs and is expressed in the prestalk A region

To identify potential STAT kinases in *Dictyostelium*, we made a kinase domain cDNA expression library and designed a functional screen of the TKL family. Using this approach, we successfully identified two STATc kinases, Pyk2 and Pyk3 (Araki, Kawata, & Williams, 2012; Araki, Vu, Sasaki, Kawata, Eichinger, & Williams, 2014). Such a screen was not equally useful for the identification of STATa kinase(s), however, because none of overexpressed kinases in the library were associated with significant elevation of STATa phosphorylation on Tyr702 (data not shown). Therefore, in this study, we took a bioinformatic approach to identify STATa kinase(s). A BLAST search of the *Dictyostelium* genome revealed several TKLs of the *Dictyostelium* receptor-like kinase (DRK) subfamily with a high degree of homology to the kinase domain of human JAKs. Among the DRK subfamily members with the highest homology was DrkA. The E-value of the DrkA kinase domain against JAK1 is ~4e-22; against JAK2 it is ~7e-27 (the homology alignment is shown in Supporting Information Figure S1). DrkA is a protein with a predicted molecular weight of 72.3-kDa (642 amino acids [aa]) and a single TKL domain (kinase domain) situated close to the C-terminus (Figure 1A). The lysine residue in kinase subdomain II that serves as an ATP-binding site and is essential for kinase activity in protein kinase superfamily members (Hanks, & Hunter, 1995) is also located at the corresponding position (amino acid residue 401, Figure 1A) in DrkA. DrkA has a predicted N-terminal signal peptide, composed of 23 amino acids, and a centrally located (aa 323 – 343) putative transmembrane domain. There is a serine-rich region between these two domains (aa 104 – 156). β-galactosidase staining using a *drkA*/*lacZ* construct indicates that *drkA* expression is restricted to prestalk cells and is especially enriched in the prestalk A (pstA) region, in which STATa is activated after the first finger stage (Figure 1B).

2.2 | Overexpression of DrkA causes elevated phosphorylation of STATa and cell lethality

If DrkA composes part of the cAMP–STATa signalling pathway, its overexpression could cause constitutive or elevated STATa phosphorylation. To examine this hypothesis, we tried to obtain a strain overexpressing epitope-tagged DrkA but failed. On the other hand, a constitutive overexpressor of a DrkA kinase-dead form, in which the lysine at 401 (Figure 1A) was replaced by an arginine (K401R), was easily obtainable (data not shown). This observation implies that overexpression of DrkA causes cell lethality or a severe growth defect.

To test this possibility, we placed the *drkA* ORF region, with a c-Myc tag, under the control of the tetracycline (Tc)-repressive promoter element, TRE (Blaauw, Linskens, & van Haastert, 2000). The resulting vector, pMB38[TRE-P_{min}/*drkA*-myc], was transformed into MB35 cells, an Ax2 derivate that showed the most efficient tet-off induction in prior work (Morita, Amagai, & Maeda, 2004). Induction of DrkA-Myc was monitored by Western blot with the anti-Myc antibody 9E10. DrkA-Myc expression was induced rapidly after removal of tetracycline; the protein level peaked at only one day after induction and gradually decreased thereafter (Figure 2A). In the presence of doxycycline, a stable derivative of tetracycline, DrkA-Myc uniduced cells grew normally in 6-well plates, similar to cells transformed with the pMB38 control plasmid (Figure 2B). In contrast, after a few days of tetracycline removal (DrkA induced cells), the cell growth rate declined considerably compared to the pMB38 transformed cells (Figure 2B). The same result was obtained in a comparison between induced and uninduced cells in shaking culture (Figure 2C). Conversely, when pMB38[TRE-P_{min}/*drkA*(K401R)-myc], which produces kinase-dead DrkA, was transformed into cells, virtually no difference in growth rate between DrkA(K401R) induced and uninduced cells was observed (Figure 2D). After 7 days of DrkA induction in the 6-well plates, 30~70% cells were propidium iodide (PI) positive, which is indicative of dead cells, while only ~20% of DrkA uninduced cells were PI-positive (Figure 3A). These observations indicate that overexpression of the DrkA protein significantly affects cell viability.

To investigate whether DrkA overexpression affects STATa activation, we examined the phosphorylation level of STATa on Tyr702 using DrkA-Myc transformed cells. One day after DrkA induction, when the DrkA-Myc protein level peaks (Figure 2A), cells were treated with 5 mM cAMP following prior starvation in buffer for 6 h in shaken suspension. Activation of STATa by cAMP was analysed by monitoring phosphorylation on Tyr702, utilising a purified polyclonal anti-phospho-STATa antibody, pSC9 (Kawata, Hirano, Ogasawara, Aoshima, & Yachi, 2011). In DrkA induced cells treated for 10 min with cAMP, the phosphorylation level of STATa was elevated approximately 2.5-fold relative to that observed in DrkA uninduced cells following 10 min of cAMP treatment (Figure 3B). Even without cAMP treatment, the phosphorylation level of STATa in DrkA induced cells was elevated approximately 3-fold compared to that in DrkA uninduced cells. These lines of evidence strongly indicate that DrkA phosphorylates STATa on Tyr702 *in vivo*.

In a separate experiment, the effect of kinase-dead DrkA, DrkA(K401R), on the STATa phosphorylation level was investigated (Figure 3C). Cells expressing a higher level of DrkA(K401R)-Myc at one day after induction displayed less phosphorylation level of STATa on Tyr702 by ~70% compared to those expressing lower level of DrkA(K401R)-Myc at five days after induction. In addition, although direct comparison is impossible, further low level of expression of DrkA(WT)-Myc displayed even stronger STATa phosphorylation level confirms that DrkA acts as a positive effect on STATa phosphorylation, but DrkA(K401R) does not influence positively.

2.3 | DrkA autophosphorylates on tyrosine and threonine

To examine the inherent kinase activity of DrkA, GST-DrkAΔN, GST fused to DrkA that lacks the putative signal peptide, was purified from *E. coli* (we term it GST-DrkA WT hereafter). Examination with the general phosphotyrosine antibody 4G10 showed clear phosphorylation of GST-DrkA WT on tyrosine, suggestive of autophosphorylation (Figure 4A, left panel). In confirmation of this notion, GST-DrkA(K401R)ΔN, a "kinase-dead" form of DrkA (we term it GST-DrkA K401R hereafter), was not bound by the 4G10 antibody (Figure 4A, left panel).

In addition to tyrosine phosphorylation, the general phosphothreonine antibody RM102 revealed that DrkA is also capable of threonine autophosphorylation (Figure 4A, middle panel). Again, the kinase-dead form of DrkA was barely bound by the RM102 antibody (Figure 4A, middle panel). No evidence of serine phosphorylation was found when similar blots were probed with the anti-phosphoserine antibody A8G9 (data not shown). These observations indicate that DrkA harbours a kinase activity that phosphorylates both tyrosine and threonine.

2.4 | DrkA phosphorylates STATa on Tyr702 in an SH2 domain-dependent manner

If DrkA is the kinase that phosphorylates STATa, then bacterially-expressed recombinant DrkA is expected to phosphorylate STATa on Tyr702. To verify this hypothesis, GST-DrkA WT was–expressed in *E. coli* and purified using glutathione-Sepharose beads; purified His-STATa(core) (Soler-Lopez, Petosa, Fukuzawa, Ravelli, Williams, & Müller, 2004) served as a potential substrate. The reaction was performed by mixing the enzyme and substrate, followed by evaluation with the pSC9 phospho-STATa antibody. Recombinant GST-DrkA WT phosphorylated His-STATa on Tyr702 in an ATP-dependent manner. However, the GST control protein did not phosphorylate His-STATa (Figure 4B). A "kinase-dead" form, GST-DrkA K401R, was checked in parallel as an additional negative control; it was also unable to phosphorylate His-STATa in the *in vitro* kinase assay (Figure 4B).

To examine whether the SH2 domain of STATa directly interacts with a phosphotyrosine on DrkA, we made a STATa variant with a mutation in the SH2 domain. That mutant, which we designate as STATa(R577A), harbours a substitution of an alanine in place of an invariant and essential SH2 domain arginine residue (R577, shown in the diagram in Figure 4C, corresponds to R175 of v-Src). We also created another STATa mutant, STATa(Y702F), in which the site of tyrosine phosphorylation is replaced with a phenylalanine. In the *in vitro* kinase assay, His-STATa(R577A) was barely phosphorylated on Tyr702 (Figure 4C). As expected, STATa(Y702F) was not tyrosine-phosphorylated. Therefore, for STATa to serve as a substrate for DrkA, an intact SH2 domain is necessary.

2.5 | DrkA directly, but weakly, interacts with STATa

To investigate whether DrkA directly interacts with STATa, pull-down experiments were performed using recombinant GST-DrkA WT as a binding substrate for STATa. *Dictyostelium* extracts from a strain expressing the GFP-STATa(core) protein (Shimada, & Kawata, 2007) were used in binding assays with GST-DrkA WT, and the resultant Western blot was analysed using an anti-GFP antibody (Figure 5). The intensity of the band obtained using GFP-STATa(core) purified from cells not stimulated with cAMP did not differ significantly between GST-DrkA WT and negative control samples (GST alone and a kinase-dead form of DrkA). In contrast, significantly more GFP-STATa(core) protein from cells pre-treated for 15 min with cAMP was recovered in assays using GST-DrkA WT than from the corresponding negative control samples. Therefore, DrkA directly, but weakly, interacts with STATa in a cAMP treatment-dependent manner.

3 | DISCUSSION

3.1 | DrkA affects the phosphorylation level of STATa on Tyr702

Tyrosine kinase-like (TKL) kinases, found in almost all eukaryotes, except in yeast and some fungi (Kosti, Mandel-Gutfreund, Glaser, & Horwitz, 2010), are a diverse group of serine/threonine kinases with sequence similarity to protein tyrosine kinase (TKs) but without a TK-specific motif. The social amoeba *Dictyostelium discoideum* harbours a large number of TKL kinases while lacking representative TKs (Goldberg, Manning, Liu, Fey, Pilcher, Xu, & Smith, 2006; Miranda-Saavedra, & Barton, 2007). Given the existence of tyrosine phosphorylation in *Dictyostelium*, it is therefore reasonable to believe that some TKLs serve as TKs in the organism. To search for the STATa kinase, we initially undertook a functional screen of the TKL family by overexpressing a kinase domain cDNA library, but this approach was not effective in identifying the STATa kinase. This result is understandable if overexpression of the STATa TKL kinase causes cell death, and indeed this is true for DrkA (Figure 3A).

A BLAST search using human JAK1 and *Drosophila* JAK (Hopscotch) as search terms identified DrkA, which is one of the TKLs with the highest degree of homology to the kinase domain of those tyrosine kinases (Supporting Information Figure S1). DrkA harbours a putative transmembrane domain and, hence, may be a membrane bound receptor-type TKL kinase (Figure 1A). In addition, it belongs to the same family as DrkC (Vsk3), which is a receptor-type and putative tyrosine-selective kinase (Fang, Brzostowski, Ou, Isik, Nair, & Jin, 2007). Several lines of evidence presented in this study argue in favor of DrkA being one of the major kinases involved in the phosphorylation of STATa in response to cAMP. These are as follows: **1)** The β-galactosidase staining demonstrated that *drkA* gene is selectively expressed in prestalk A (pstA) cells, where STATa is activated (Figure 1B). In particular, during the slug – early culminant stages, strong expression was detected in tip-organiser cells, where the *cudA* gene, a known STATa target gene (Fukuzawa, & Williams, 2000), and several other potential STATa target genes, such as *ecmF* and *mybC* (Shimada, Maeda, Urushihara, & Kawata, 2004; Shimada, Nishio, Maeda, Urushihara, & Kawata, 2004; Saga, Inamura, Shimada, & Kawata, 2016), are expressed. **2)** Tet-off induced transient overexpression of DrkA-Myc upregulated the phosphorylation level of STATa on Tyr702 compared to the DrkA-Myc uninduced cells, independent of cAMP addition (Figure 3B). **3)** Recombinant GST-DrkA WT is autophosphorylated on its tyrosine residue(s). No tyrosine phosphorylation was detected for GST-DrkA K401R, a kinase dead form of DrkA. Therefore, DrkA can at least function as a tyrosine kinase (Figure 4A). **4)** An *in vitro* kinase experiment showed that DrkA directly phosphorylated Tyr702 of STATa (Figure 4B). Furthermore, the STATa R577A mutation resulted in the near elimination of STATa phosphorylation by DrkA. This finding demonstrates the importance of the STATa SH2 domain in the kinase-substrate interaction (Figure 4C). **5)** A weak physical interaction between STATa and DrkA was detected in a pull-down assay (Figure 5). Taken together, these data led us to conclude that DrkA is one of the key direct activators of STATa.

3.2 | DrkA roles in STATa phosphorylation and cell viability

Phenotypes of transient DrkA(WT)-Myc overexpressor clearly suggests the involvement of DrkA in STATa phosphorylation (Figure 3B). Activation of STATa phosphorylation by DrkA in the transient overexpressor cells is independent of cAMP addition, albeit STATa phosphorylation is further activated by cAMP addition (Figure 3B). This indicates that DrkA alone can phosphorylate STATa *in vivo* as observed in the *in vitro* kinase assay (Figure 4B & C), though another component(s) activated by cAMP may be necessary for the full activity of DrkA. Alternatively, such the component(s) synergistically acts with DrkA. In contrast to the result of transient DrkA(WT)-Myc overexpression, overexpression of kinase-dead form DrkA(K401R)-Myc had virtually no effect to increase STATa phosphorylation (Figure 3C). Rather, it might decrease STATa phosphorylation because of the evidence that more expression level of DrkA(K401R)-Myc caused further less STATa phosphorylation (Figure 3C). These findings suggest that lysine residue at 401 of DrkA is also vital for its *in vivo* activity to phosphorylate STATa as observed in the *in vitro* kinase assay (Figure 4B & C).

Despite the evidence of direct STATa phosphorylation by DrkA, there are a few observations concerning DrkA and STATa that we cannot fully explain. No notable difference in transcript levels of several putative STATa-target genes was observed between tet-off induced and uninduced cells (data not shown). The lack of extra expression of these genes can be explained by the presence of DrkA derived from endogenous *drkA* gene and other TKL kinases.

In addition to these observations, to support the above hypothesis, there are some experimental data from *drkA* REMI mutant (Supporting Information Figure S2) and accidentally obtained *drkA* KO strain (Supporting Information Figure S3). In both mutant cells, starved in a buffer in shaken suspension, followed by induction with extracellular cAMP, the level of STATa phosphorylated at Tyr702 was greatly reduced relative to that observed in the corresponding parental strains (Supporting Information Figures S2A & S3F). Both mutants developed almost normally morphologically, although the developmental timing is delayed by ~2 h (Supporting Information Figure S2B). The *drkA* KO mutant displayed the same

phenotype (data not shown). A slight delay in development may reflect reduced STATa activity since the *statA* null mutation delays aggregation by ~5 h (Mohanty et al., 1999). It is interesting to note that the *ptp1* (*ptpA*)-null mutant shows accelerated development (Howard, Sefton, & Firtel, 1992). Because PTP1 is a negative regulator of phosphorylated STATa (Early, Gamper, Moniakis, Kim, Hunter, Williams, & Firtel, 2001), the *ptp1*-null potentially leads to an excess of activated STATa and results in the opposite effect on the developmental rate.

However, the results from mutants are only supportive information as we cannot repeat the experiment using these mutants. Cells of REMI mutant died out possibly due to the increase in sensitivity to cryopreservation. In the case of *drkA* KO cells, they gradually lost the original phenotype during subculture or recovery from freezing, even though the *drkA* gene is kept disrupted and therefore *drkA* transcript was undetectable (data not shown). Although the reason for the above phenomena is unclear, the level of DrkA might be tightly regulated as discussed below. Therefore, the loss of DrkA might cause cells extra sensitivity during recovery from the freezing. Alternatively, it might force the mutant cells to switch to using other TKL kinase(s) acting as a STATa kinase. For instances, when DrkA is absent, TKLs with the same consensus core sequence of kinase subdomain VIb (Figure 6) as described below, would gradually replace or compensate for the function of DrkA.

In experiments using the tet-off induction system for DrkA expression, transient DrkA-Myc overexpression had a dramatic effect on cell viability (Figures 2 & 3A). Because the knockout mutant of *STATa* gene or overexpression of STATa does not affect cell viability (Mohanty et al., 1999), STATa may not be involved in this process. This observation suggests that the amount or activity of DrkA is normally tightly regulated. STATa phosphorylation levels may need to be kept as constant as possible and a mechanism strictly controlling DrkA expression levels might contribute to the process that may keep the STATa phosphorylation level.

3.3 | A Conserved motif for tyrosine-selective TKLs

Dictyostelium TKLs such as Pyk1 (SplA) (Tan, & Spudich, 1990; Nuckolls, Osherov, Loomis, & Spudich, 1996), Pyk2 (SplB) (Tan, & Spudich, 1990; Araki, Kawata, & Williams, 2012), Zak1 (Kim, Liu, & Kimmel, 1999), and Zak2 (Pyk4) (Kim, Brzostowski, Majithia, Lee, McMains, & Kimmel, 2011) have been shown to selectively phosphorylate tyrosine residues. DrkC (Vsk3) is a receptor-type kinase and is also a putative tyrosine-selective kinase (Fang, Brzostowski, Ou, Isik, Nair, & Jin, 2007). Furthermore, both Pyk2 and Pyk3 are kinases that phosphorylate STATc on Tyr922 (Araki, Kawata, & Williams, 2012; Araki, Vu, Sasaki, Kawata, Eichinger, & Williams, 2014). These TKLs share a consensus di-peptide sequence, (T/S)S, in kinase subdomain VIb (Figure 6). Consequently, the HRDL(T/S)S sequence was suggested in our previous report to serve as a characteristic motif to identify tyrosine-selective TKL kinases and to discriminate between metazoan TKs and serine/threonine kinases (Araki, Kawata, & Williams, 2012).

It is intriguing that GST-DrkA WT is autophosphorylated on threonine residue(s) in addition to tyrosine (Figure 4A). Thus, it is likely that DrkA is a dual-specificity kinase, although serine phosphorylation of DrkA was not detected using the specific phosphoserine antibody, A8G9, employed in this study. DrkA is a DrkC-like receptor-type TKL and also shares the kinase subdomain consensus with other tyrosine-selective TKL kinases, but the di-peptide sequence is slightly different as it is KS instead of (T/S)S (Figure 6). This difference may correlate with kinase specificity, because the di-peptide sequence is instead closer to that of ShkA, another dual-specificity kinase with the di-peptide sequence KT (Moniakis et al., 2001), and GskA, a serine/threonine kinase with the sequence KP (Ginger, Dalton, Ryves, Fukuzawa, Williams, & Harwood, 2000). Therefore, DrkA has an intermediate feature between authentic dual-specificity kinases and biochemically demonstrated tyrosine-selective TKLs. DrkA is likely to be the most primitive TKL that “effectively” phosphorylates tyrosine residues. In addition, a consensus di-peptide sequence within kinase subdomain VIb of a TKL kinase that possesses the activity to phosphorylate tyrosine must be (K/T/S)S. Therefore, the second serine is a more important signature for identifying these tyrosine-selective TKLs (Figure 6).

3.4 | The mechanism of DrkA in STATa activation

STATa is known to be activated in response to extracellular cAMP in a buffered suspension culture (Araki et al., 1998). Numerous components are involved in the cAMP-responsive STATa activating pathway, including the serpentine cAMP receptor cAR1 and protein kinase A (PKA) (Kawata, 2011). However, how cAMP exerts a facilitatory influence on DrkA is still unknown. STATc has been demonstrated to interact with Pyk2 constitutively, while the protein tyrosine phosphatase PTP3 predominantly deactivates STATc (Araki, Langenick, Gamper, Firtel, & Williams, 2008; Araki, Kawata, & Williams, 2012). Although PTP1 is reported to downregulate the phosphorylation level of STATa (Early, Gamper, Moniakis, Kim, Hunter, Williams, & Firtel, 2001), the relationship between DrkA and PTP1 remains to be elucidated.

Because the expression level of DrkA-Myc in the MB35 cells using the tet-off system is usually very low (see Figures 2A & 3C), we are unable to detect DrkA localisation histochemically to investigate its function. But, although the precise cellular structure where DrkA distributes remains to be elucidated, the predicted amino acid sequence of DrkA suggests that it is a potential membrane protein, as it harbours a putative transmembrane domain (Figure 1A). Fang and coauthors (2007) describe Vsk1 (DrkA) as distributed in membranes of vesicles other than those where Vsk3 (DrkC) localises.

We tested for a direct interaction between STATa and DrkA in a GST-pull down assays, using recombinant GST-DrkA WT and a lysate from *Dictyostelium* cells expressing GFP-STATa(core), and observed a slightly stronger intensity of the total STATa band when GST-DrkA WT was used for binding than that when GST was used (Figure 5). The interaction between STATa and DrkA may be weak or the two proteins may dissociate very quickly. Alternatively, binding stability may be facilitated by other, auxiliary factors. Nonetheless, an *in vitro* kinase experiment showed that DrkA is capable of directly phosphorylating Tyr702 of STATa (Figure 4B). Furthermore, the STATa R577A mutation resulted in the loss of STATa phosphorylation by DrkA (Figure 4C). This finding suggests that the SH2 domain of STATa has an important role in the kinase-substrate interaction. Regardless of the fact that the mode of

action of DrkA in STATa phosphorylation is still partially unknown, our results provide new insight into the role of DrkA, namely that it is the first identified TKL kinase activating the multifunctional transcription factor STATa. In addition, searching for the other TKL kinases with similar activity is necessary for the full understandings for the STATa activation mechanism.

4 | EXPERIMENTAL PROCEDURES

4.1 | Cell culture, development, and cAMP treatment

The Ax2 and Ax4 wild-type strains of *Dictyostelium discoideum* and their derivative mutant cells were grown axenically at 22°C in HL5 or in HL5 medium supplemented with either 10 μg/mL blasticidine S (Kaken Pharmaceutical Co. Ltd., Tokyo, Japan) or 20–40 μg/mL G418 (Nacalai tesque Inc., Kyoto, Japan). MB35 and its derivative strains were cultured as described below. For cAMP-induction assays, cells were washed twice in KK_2 buffer (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH 6.2), suspended in KK_2 buffer at a density of 2×10^7 cells/mL, and shaken for 6 h at 150 rpm, followed by addition of cAMP (Tokyo chemical industry, Tokyo, Japan) to achieve a final concentration of 5 mM (Araki et al., 1998). For multicellular development, KK_2 washed cells were plated at a density of 1.5×10^7 cells/cm^2 on an Omnipore membrane filter (JHWP04700 filter; Merck Millipore, Billerica, MA) placed on an agar plate, on a nitrocellulose filter (HABG04700 filter; Merck Millipore) placed on a buffered-soaked filter pad, or directly on an agar plate. Cells were developed until the desired stage in a humid box.

4.2 | Reverse transcription (RT)-polymerase chain reaction (PCR)

RNA isolation and semi-quantitative RT-PCR were largely performed as described (Shimada, Inouye, Sawai, & Kawata, 2010; Saga, Inamura, Shimada, & Kawata, 2016). Total RNA was purified with the RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) and treated with DNase I (RNase-Free DNase Set, QIAGEN) followed by purification with the RNeasy MinElute Cleanup Kit (QIAGEN). The primers used in this study are listed in Supplemental Table S1 and were purchased from Eurofins Genomics (Tokyo, Japan).

4.3 | Immunological analysis

The primary antibodies used in the experiments were: anti-phospho-STATa antibody pSC9 (Kawata, Hirano, Ogasawara, Aoshima, & Yachi, 2011) for STATa phosphorylated on Tyr702, anti-phosphotyrosine antibody 4G10 (Merck Millipore) for general phosphotyrosine modification, anti-phosphothreonine antibody RM102 (Merck Millipore) for phosphothreonine modification, anti-GFP monoclonal antibody (No. 11 814 460 001, Roche Diagnostics) for GFP-tagged proteins, anti-Myc antibody 9E10 (Wako Pure Chemical, Osaka, Japan) for Myc-tagged proteins, anti-polyhistidine antibody M136-3 (MBL Co. Ltd., Nagoya, Japan) for His-tagged proteins, anti-GST antibody 5A7 (Wako) for GST-tagged proteins, and anti-Actin antibodies C-11 or C4 (Santa Cruz Biotechnology, Inc., Dallas, TX) for monitoring total Actin as a loading control. Alkaline phosphatase-conjugated anti-rabbit IgG (H+L) antibody (Promega) was used as the secondary antibody for Western analysis. Proteins were detected using the ProtoBlot II AP System with Stabilized Substrate (Promega).

4.4 | Construction of vectors for the *drkA* disruption and tagged proteins

The *drkA* knockout (KO) mutant was created using the disruption construct for the *drkA*/*drkB* double KO. That construct, pTOPOΔBam[*drkA*/*drkB* double KO #1], encompasses sequences of the *drkA* promoter and a part of *drkA* TKL domain for the left arm, a part of the *drkB* TKL domain and *drkB* terminator region for the right arm, and a centrally located blasticidine S resistance gene cassette Bs^R (Figure S3A).

For the tet-off DrkA-Myc expression construct, the open reading frame (ORF) of the *drkA* gene, which harbours two introns, was amplified and inserted into pLD1ΔBX[*act15*/myc] (Kawata, Nakamura, Saga, Iwade, Ishikawa, Sakurai, & Shimada, 2015) to add a c-Myc epitope tag at its C-terminus. This Myc-tagged *drkA* ORF region was excised from the pLD1ΔBX vector and subcloned into the pMB38 vector (Blaauw, Linskens, & van Haastert, 2000) to yield pMB38[TRE-P_{min}/*drkA*-myc]. For the GST-DrkA constructs, *drkA* cDNA was amplified from total RNA of growing cells with the RNA PCR Kit (AMV) Ver.3.0 (TaKaRa Bio Inc., Kyoto, Japan) and DrkA (WT [without putative signal sequence], aa 24–642) was inserted into the *E. coli* expression vector pGEX-5X-1 (GE Healthcare Bio-Sciences, Uppsala, Sweden) to add a GST tag at its

N-terminus. For His-STATa(core), a region spanning aa 237–707 of STATa was subcloned into the pET-28a vector (Merck) to yield pET28[His-STATa(core)]. Point mutant forms of DrkA (K401R) and STATa (R577A and Y702F) were generated by PCR using primers designed to incorporate the appropriate nucleotide substitution (Supplemental Table S1). Recombinant tagged proteins expressed in *E. coli* were purified with glutathione-Sepharose 4B (GE Healthcare) or TALON metal affinity resin (TaKaRa) according to the manufacturers' instructions.

4.5 | Conditional overexpression of DrkA-Myc

The pMB38[TRE-P_{min}/*drkA*-myc] or pMB38[TRE-P_{min}/*drkA*(K401R)-myc] vector was transformed into cells of the MB35 strain. MB35 is a derivative of the Ax2 strain that contains the transactivator plasmid for the tet on/off system (pMB35) and shows the best induction efficiency (Morita, Amagai, & Maeda, 2004; obtained from Dicty Stock Center). Transformed cells were selected in HL5 medium containing 40 μg/mL G418, 10 μg/mL blasticidine S, and 20 μg/mL doxycycline-HCl (Sigma–Aldrich, St. Louis, MO). A subset of these cells was washed with KK_2 buffer, transferred into new HL5 medium including 40 μg/mL G418, 10 μg/mL blasticidine S, and 20 μg/mL tetracycline (Wako), and cultured for a further 2 days. Then, the cells were washed as above and cultured without tetracycline to induce expression of DrkA-Myc or DrkA(K401R)-Myc. Expression of DrkA-Myc or DrkA(K401R)-Myc was monitored by Western analysis using an anti-Myc antibody.

To detect elevated STATa phosphorylation on tyrosine, DrkA-Myc expressing cells were subjected to cAMP-induction assays as above. For the detection of possible dead cells, cells were harvested and suspended in PBS, and then stained with propidium iodide (PI) solution (final concentration of 10 μg/mL, -*Cellstain*- PI solution, Dojindo, Kumamoto, Japan) for 45 min at ambient temperature. Stained cells were mounted on a glass bottom dish (SF-T-D12; Fine Plus International Ltd., Kyoto, Japan) and observed with confocal microscopy a confocal laser-scanning microscope (Nicon Ti-E).

4.6 | *lacZ* fusion constructs and β-galactosidase staining

The *drkA* gene promoter region was amplified by PCR so as to add a *Xba*I site at the 5′ end and a *Bgl*II site at the 3′ end. The resulting promoter fragment was gel-purified after digestion with *Xba*I and *Bgl*II, and subcloned into *Xba*I/*Bgl*II digested pDdgal-17(H+) vector (Harwood, & Drury, 1990) to make pDd-Neo^{R}[*drkA*/*lacZ*]. To detect the activity of *drkA* promoter, cells transformed with pDd-Neo^{R}[*drkA*/*lacZ*] were developed on Omnipore membrane filters (Merck Millipore). Fixation and staining were carried out as described previously (Shimada, Maruo, Maeda, Urushihara, & Kawata, 2005).

4.7 | *In vitro* kinase assay with recombinant proteins

For the *in vitro* kinase assay, GST-DrkA (WT and the kinase dead form, K401R) and His-STATa(core) (WT, R577A and Y702F mutations) proteins expressed in *E. coli* were used. For immunological detection, His-STATa(core) (2.5 μg per reaction) served as the substrate and was incubated with GST-DrkA protein (1.0 μg per reaction) and 0.2 mM ATP in HEPES kinase buffer [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES]-KOH (pH 7.5), 30 mM NaCl, 10 mM $MgCl_2$, 1 mM glycerol 2-phosphate, 1 mM DTT and 0.02% Nonidet P-40] at 37°C for 30 min. The reaction was stopped by boiling in SDS gel sample buffer, and the extent of phosphorylation was determined were measured by Western analysis using the anti-phospho-STATa antibody pSC9.

4.8 | Pull-down assays

Bacterially expressed and purified GST-DrkA, its kinase dead form GST-DrkA(K401R) proteins, or GST (typically 30 μg) protein was combined with glutathione beads (100-μL bed volume) in GST-buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1.0 mM EGTA, 0.5 mM EDTA, 5 mM DTT, Complete Protease inhibitor mixture (Roche) and 1.0% Triton X-100] for 1 h at 4°C. After washing with GST-buffer twice and a further two washes in mNP40 buffer [50 mM Tris-HCl (pH 8.0), 150 mL NaCl, 50 mM NaF, 2 mM Na-pyrophosphate, 2 mM EDTA (pH 8.0), 2 mM benzamidine, 1 mM PMSF, 1 g/mL pepstatin, Complete EDTA-free protease inhibitor mixture (Roche) and 1.0% Nonidet P-40], the GST-protein/glutathione-Sepharose complex (100-μL bed volume per sample) was combined with cell lysate (1×10^8 cells in 1 mL) in mNP40 buffer (Araki, Langenick, Gamper, Firtel, & Williams, 2008). The cell lysate was prepared from a hypomorphic STATa strain expressing

GFP-STATa(core) (Shimada, & Kawata, 2007) that had been starved in KK_2 buffer for 4 h, followed by incubation with 5 mM cAMP to induce STATa phosphorylation. The pull-down assay mixtures were incubated at 4°C for 2 h with gentle rocking, and the beads were then washed four times with mNP40 buffer. Bound proteins were eluted by boiling in SDS gel sample buffer and detected with Western analysis.

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Figure legends

FIGURE 1. DrkA protein and spatial expression of *drkA* during development. (A) Schematic representation of DrkA primary structure. DrkA has a putative signal peptide region (yellow box), a serine-rich region (orange box), a putative transmembrane domain (grey box) and a C-terminal kinase domain (pink box). Accordingly, DrkA is presumed to be a membrane protein. The lysine residue (K) at amino acid 401, which corresponds to the position of the ATP-binding site and is important for kinase activity, is shown as a red letter. Below the diagram, alignment of the kinase subdomain II sequence is shown in the box. STK and TK denote serine/threonine kinase and tyrosine kinase, respectively. The following is the amino acid sequence information. MAPK1: *Homo sapiens* mitogen-activated protein kinase 1 (UniProtKB: P28482); DrkA: *D. discoideum* DrkA (UniProtKB: Q54H46); JAK1: *H. sapiens* JAK1 (UniProtKB: P23458); Hopscotch: *Drosophila melanogaster* JAK (UniProtKB: Q24592); Pyk2: *D. discoideum* Pyk2 (UniProtKB: P18161). (B) Spatial expression of *drkA* as detected by β-galactosidase activity. Cells of the Ax2 strain transformed with *drkA/lacZ* were developed until the desired stages; early aggregation (a), late aggregation (b), first finger (c), slug (d, e), early culminant (f), mid culminant (g), and late culminant (h). Staining was carried out at 37°C for 1 h. Scale bars indicate 250 μm.

FIGURE 2. Conditional overexpression of DrkA. (A) Time course of DrkA-Myc expression. Expression of DrkA-Myc in the MB35/[TRE-P_{min}]:*drkA*-myc strain was induced by removing the tetracycline. Cells were harvested every ~24 h (indicated as days at the top of each lane) and DrkA-Myc expression was monitored by Western analysis with an anti-Myc antibody (DrkA-Myc). Total Actin was detected as a loading control (Actin). (B) Inhibition of cell growth by inducing the overexpression of DrkA-Myc. Subcultured MB35 cells transformed with pMB38[TRE-P_{min}/*drkA*-myc] (+DrkA-Myc vector) or pMB38 empty vector (+control vector) were

used. Equal numbers of cells were transferred into medium in 6-well plates without tetracycline (induced) or medium with doxycycline (uninduced), and then photographed at 24 h intervals. The time point (day) after induction is shown at the top of each column. Strain names are shown at the left of each row. The bar denotes 200 μm. (C) Growth kinetics of DrkA-Myc induced and uninduced cells. Cells were grown in shaking culture and cell numbers were counted using an Improved Neubauer counting chamber. Blue dots (♦) and red dots (□) denote DrkA-Myc uninduced and DrkA-Myc induced cells, respectively. Because the number of input cells varied among experiments, relative cell numbers are shown on the y-axis. The relative cell number of DrkA-Myc uninduced cells at 4 days was set at 1.00 for each experiment. The relative cell numbers were calculated from two independent experiments and the standard error of the mean is shown for each point. (D) Growth kinetics of DrkA(K401R)-Myc expressing cells. Cells were grown in shaking culture and the relative cell numbers were counted as described in the legend of Figure 2B. Yellow Orange dots (♦) and pale green dots (□) denote DrkA(K401R)-Myc uninduced (control) and DrkA(K401R)-Myc induced cells, respectively. Because the number of input cells varied among experiments, relative cell numbers are shown on the y-axis. The relative cell number of DrkA(K401R)-Myc uninduced cells at 4 days was set at 1.00 for each experiment. The relative cell numbers were calculated from three independent experiments and the standard error of the mean is also shown for each point. Note that the growth rate of DrkA(K401A)-Myc transformed cells is higher than that of uninduced DrkA-Myc transformed cells. Therefore, the averaged absolute number of DrkA(K401R)-Myc uninduced cells at 4 days is higher than that observed for DrkA-Myc transformed cells and, hence, the y-axis cannot simply be directly compared between strains transformed with different plasmids.

FIGURE 3. Effect of conditional DrkA-Myc overexpression on possible cell death and STATa Tyr702 phosphorylation. (A) Cells were induced for DrkA-Myc expression by removal of tetracycline and incubated for a further 7 days (shown as DrkA-Myc induced). Cells were harvested and suspended in PBS to 1×10^6 cells/mL. Propidium iodide (PI) solution was added and the cells were incubated for 45 min at 22°C. Following staining, cells were mounted on a glass-bottom dish and observed via a confocal microscopy. Cells cultured in the presence of doxycycline were used as the DrkA-Myc uninduced negative control (shown as uninduced). The number of PI-positive cells was calculated as a fraction of the total cell number. The experiment was repeated nine times and a

beeswarm boxplot of the percentage of PI-positive cells is shown below the images. The *p* value was calculated using the Wilcoxon rank-sum test. $^{**}p < 0.01$. (B) Effect of conditional DrkA-Myc overexpression on STATa Tyr702 phosphorylation. DrkA-Myc expression was induced in suspension culture as described above. DrkA-Myc uninduced cells were used as the negative control. One day after induction, when the DrkA-Myc expression is peaking, cells were starved in KK_2 phosphate buffer (DrkA induced). DrkA-Myc uninduced cells (DrkA uninduced) were kept in the presence of doxycycline during the whole procedure. After starvation, cAMP was added at 5 mM. Protein samples were harvested at the time indicated, and STATa phosphorylation and DrkA-Myc were detected by Western analysis. The upper top panel: phosphorylated STATa (pSTATa); the upper middle panel: DrkA-Myc; the lower panel: a beeswarm boxplot of quantification of band intensities of pSTATa. STATa phosphorylation was detected by Western analysis with anti-phospho-STATa antibody, pSC9 (Kawata, Hirano, Ogasawara, Aoshima, & Yachi, 2011). The intensities of pSTATa bands of five independent experiments were quantified by normalising against that of the most intense band detected with Ponceau staining (Ponceau) using ImageJ software. Wilcoxon rank-sum test: $^{*}p < 0.05$. (C) Effects of overexpression of DrkA(K401R)-Myc on STATa Tyr702 phosphorylation. DrkA(K401R)-Myc expressing cells were harvested at 24 h (1 day) and 5 days after induction and expression of DrkA(K401R)-Myc was monitored by Western analysis. For comparison, the sample expressing DrkA-Myc after 24 h induction, used in Figure 2A, was loaded (WT). STATa phosphorylation on Tyr702 was monitored with the anti-phospho-STATa antibody pSC9 (pSTATa). The blotted filter was stained with Ponceau S and the most abundant band is shown as a loading control. The lower panel shows the quantification of band intensities of phosphorylated STATa. The band intensities of STATa normalized against the band intensity of the most abundant band with Ponceau S in the Western analysis of four independent experiments were quantified and represented as a beeswarm boxplot. Wilcoxon rank-sum test; $^{*}p < 0.05$.

FIGURE 4. Kinase activity of DrkA. (A) Tyrosine and threonine autophosphorylation of recombinant DrkA. Recombinant GST-fused DrkA wild-type protein (WT) and its kinase-dead form (K401R) were expressed in *E. coli* and affinity purified. The kinase-dead form harbours a mutation to convert lysine into arginine (K401R) at the conserved ATP-binding site within kinase subdomain II (Figure 1A). Phosphorylation on tyrosine residue(s) was detected by Western analysis with an

anti-phosphotyrosine antibody, 4G10 (pTyr) (left panel). Threonine phosphorylation was detected with an anti-phosphothreonine antibody, RM102 (pThr) (middle panel). As a loading control, total GST-fusion protein was detected with an anti-GST antibody, 5A7 (GST) (right panel). (B) Direct tyrosine phosphorylation of STATa by DrkA in an *in vitro* kinase assay. Recombinant purified His-STATa(core) was used as a substrate and mixed with GST-DrkA (WT), GST-DrkA (K401R) or GST. Each mixture was subjected to an *in vitro* kinase assay and STATa phosphorylation was detected with the anti-phospho-STATa antibody pSC9 (pSTATa). The same blots were re-probed with an anti-GST antibody (shown as GST-DrkA or GST) and an anti-polyhistidine (anti-His) antibody, M136-3 (MBL) as loading controls (shown as His). (C) SH2 domain-dependent STATa phosphorylation by DrkA. R577A is a mutant STATa in which a conserved arginine residue within the SH2 domain necessary for recognition of phosphotyrosine and reciprocal dimerisation has been converted into an alanine. In the Y702F mutant, a phenylalanine has been substituted for the tyrosine in STATa's tyrosine phosphorylation site. Protein levels and STATa phosphorylation were detected by Western analysis as described in panel (B). Note that the lanes of wild-type STATa (WT) reaction are the same image of complete mixture reaction as used in the panel (B), as all these experiments were performed at the same time. The dashed lines in the top and bottom panels indicate that those lanes were not originally adjacent. A schematic representation of STATa's primary structure is shown at the bottom. Ig-like denotes the immunoglobulin-like domain, which acts as a DNA-binding domain (Kawata, 2011).

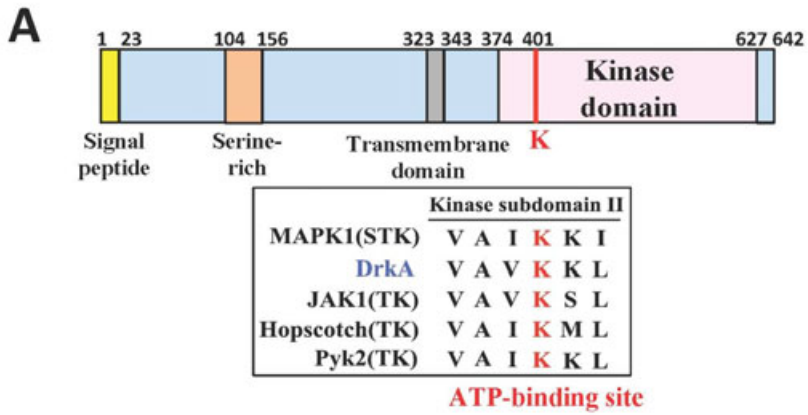
FIGURE 5. Physical interaction between STATa and DrkA. GFP-STATa(core) expressing cells (Shimada, & Kawata, 2007) were starved in buffer for 4 h in shaking culture. Cells were either kept untreated (0 min) or were treated in the presence of 5 mM cAMP for 15 min. Then, cells were lysed, and the total cell extracts were combined with either GST alone (+GST), GST-DrkA (WT), or its kinase dead form GST-DrkA (K401R) (kinase dead). These mixtures were subjected to a pull-down assay, using Glutathione beads, and GFP-STATa(core) protein and GST proteins in the resulting eluate were detected with anti-GFP (IB: anti-GFP) and anti-GST (IB: anti-GST) antibodies, respectively. Band intensities were measured using ImageJ software. The relative intensity of the STATa band, normalized to the intensity of its respective GST band, was calculated for samples from four independent experiments and the average of the untreated (0 min) GST sample was set at 100%.

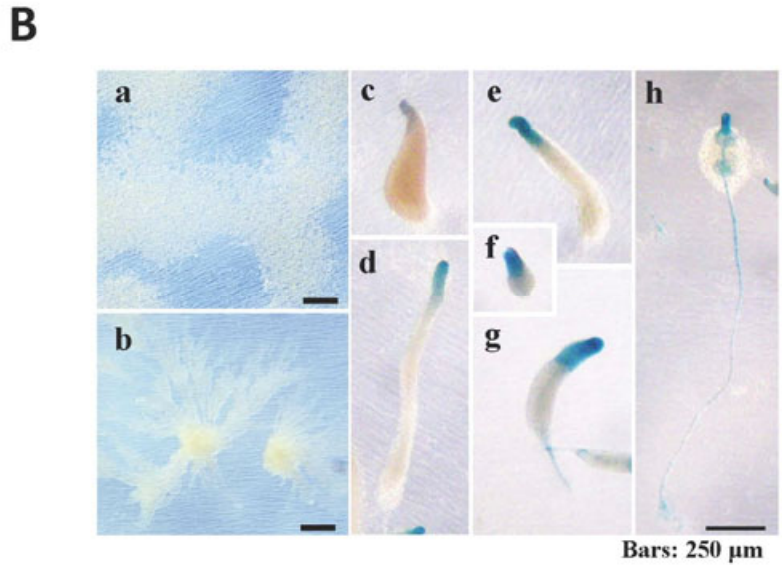
Standard error of the mean is shown for each bar. Univariate ANOVA (analysis of variance) contrast of +GST and kinase dead against WT; *$p < 0.05$.

FIGURE 6. Alignment of kinase subdomain VIb. DrkA has conserved diamino acids, K/T-S, as shown in red. These diamino acids are also found in subdomain VIb of other tyrosine-selective TKL-kinases from *Dictyostelium* such as DrkC (Vsk3) (Fang, Brzostowski, Ou, Isik, Nair, & Jin, 2007), Pyk2 (SplB) (Tan, & Spudich, 1990; Araki, Kawata, & Williams, 2012), Pyk3 (Araki, Vu, Sasaki, Kawata, Eichinger, & Williams, 2014), Zak1 (Kim, Liu, & Kimmel, 1999) and Zak2 (Pyk4) (Kim, Brzostowski, Majithia, Lee, McMains, & Kimmel, 2011). Pyk1 (SplA) (Tan, & Spudich, 1990; Nuckolls, Osherov, Loomis, & Spudich, 1996), unusually, harbours a diamino acids, SS. ShkA is a serine/threonine-selective dual-specificity TKL kinase (Moniakis et al., 2001), while GskA is a serine/threonine kinase (Ginger, Dalton, Ryves, Fukuzawa, Williams, & Harwood, 2000). A typical serine/threonine kinase sequence of human CaM kinase II gamma (CAI13965.1) is shown at the bottom. The serine residues in the diamino acids may be a difference between tyrosine-selective and serine/threonine-selective TKLs and are shaded with orange. The lysine residues in the same diamino acids may be a difference between tyrosine-selective and dual-specific TKLs and are shaded with pale green.

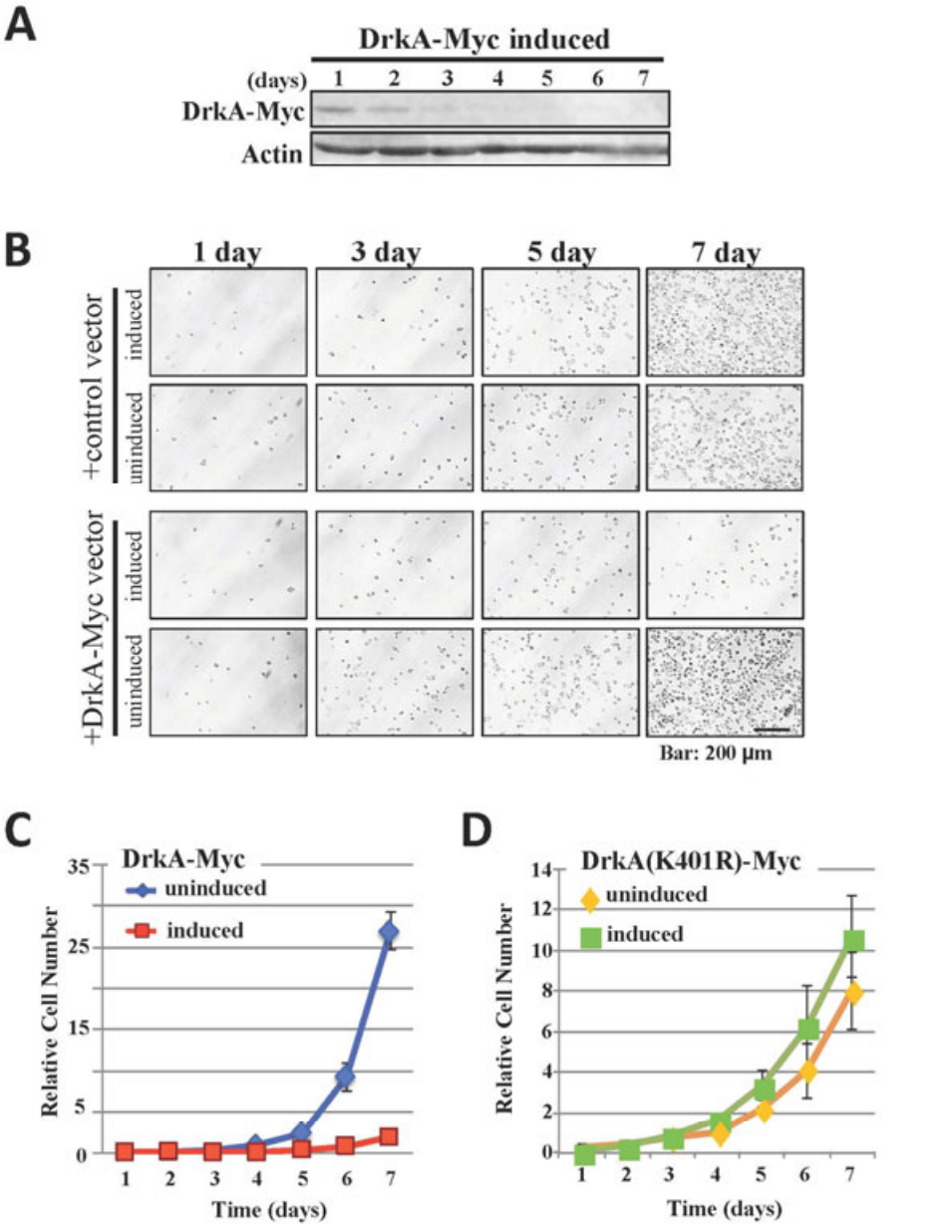
SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

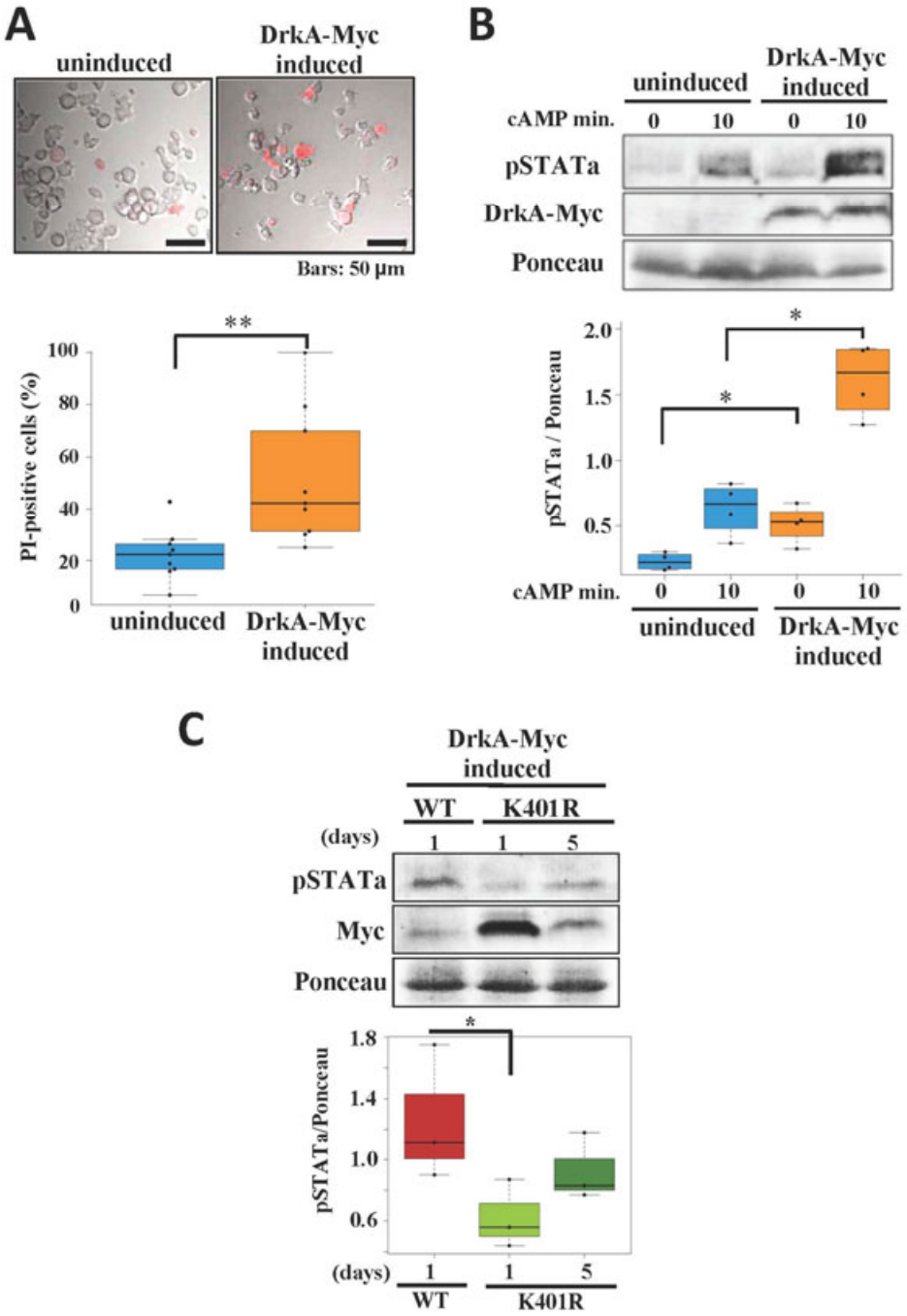




Saga *et al.* Fig. 1



Saga *et al.* Fig. 2



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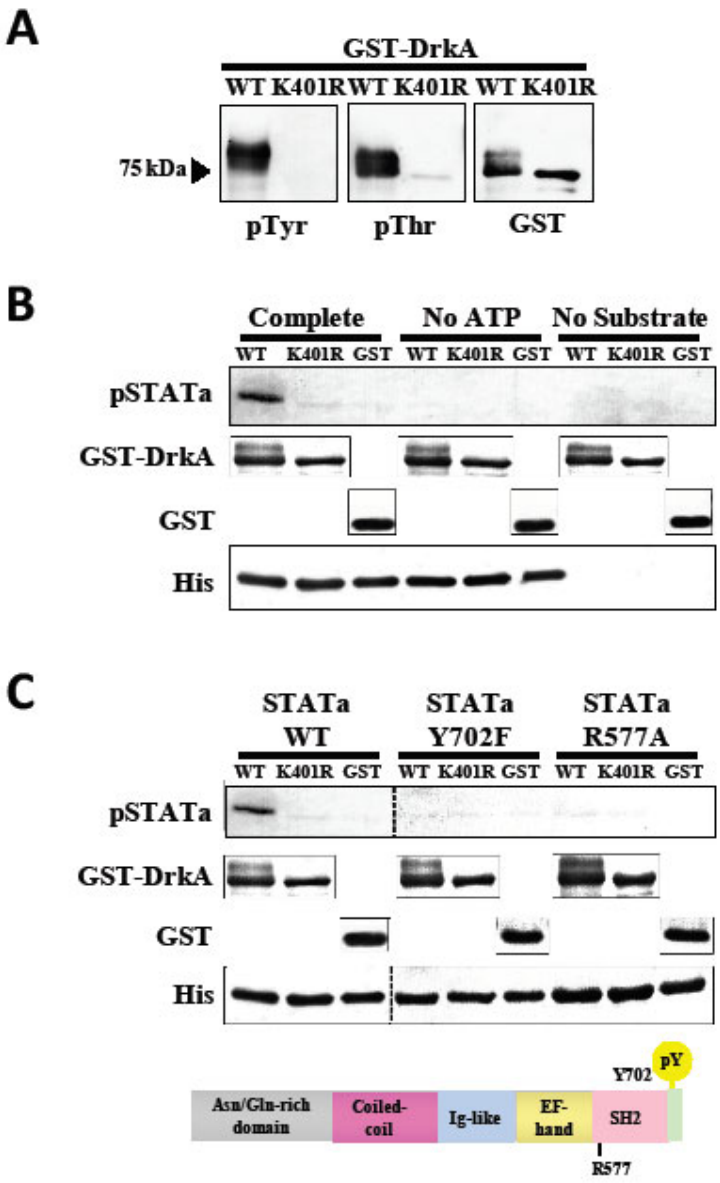
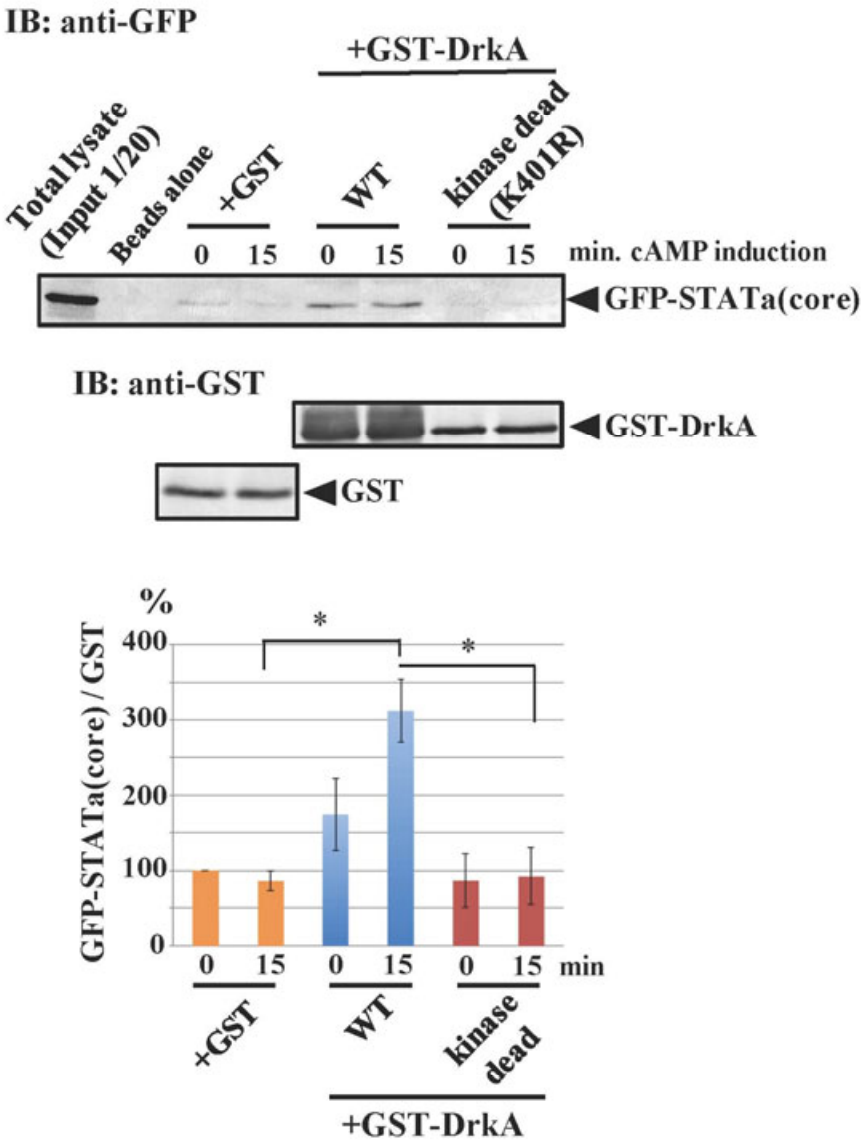
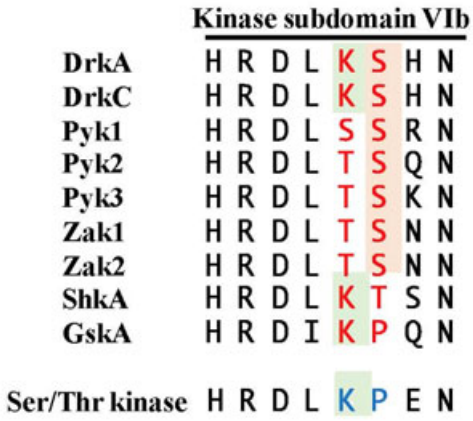


Saga *et al.* Fig. 4



Saga *et al.* Fig. 5



Saga *et al.* Fig. 6