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# The blue-light receptor YtvA from *Bacillus subtilis* is permanently incorporated into the stressosome independent of the illumination state

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ABSTRACT

Higher organisms as well as bacteria rely on information on the surrounding environment. In *Bacillus subtilis*, diverse extra-cellular stimuli are transformed into an intra-cellular response via a signal integration hub, called the stressosome. The subsequent signal transduction cascade initiates the general stress response (GSR). One of these stimuli is blue light, which is sensed by the bacterial photoreceptor YtvA. We report here that YtvA is permanently incorporated into the stressosome independent of its illumination state and that RsbT stimulation occurs without direct interaction between the kinase RsbT and YtvA but in a light dependent manner. Furthermore, we show that RsbRA adopts a scaffolding function inside the stressosome explaining on a molecular level why RsbRA is required for light-mediated stress response *in vivo*.



## 1. Introduction

The common soil bacterium *Bacillus subtilis* is capable of responding to environmental stimuli via the general stress response (GSR) pathway [1] that regulates activation of sigma factor B ($\sigma^B$), indirectly affecting transcription levels of ~150 genes. This mechanism is not restricted to *B. subtilis*; similar means of extracellular signal recognition have been discovered in other bacteria, e.g. *Listeria monocytogenes* [2].

Recognition of extra-cellular stimuli and subsequent activation of the signal transduction cascade that has to follow is due to proteins of the Rsb-family (regulators of $\sigma^B$), including various kinases (RsbT, RsbW), phosphatases (RsbX) and receptor and/or mediator proteins (RsbRA, RsbRB, etc.) [3,4]. Signal integration and initiation of the environmental branch of the GSR is accomplished via the so called stressosome, a ~1.8 MDa complex consisting of at least three different proteins: RsbR, RsbS and RsbT [5]. In an unstressed cell only RsbR is partially phosphorylated while RsbS is not, thereby keeping RsbT associated with the stressosome [6]. Activation of RsbT leads to phosphorylation of RsbR and S and, ultimately, release of RsbT, which is now free to bind RsbU, its target in the signal transduction cascade [6]. The proteins of the Rsb-family except kinases and phosphatases (e.g. RsbT and RsbX) consist of either a sole STAS (sulfate-transporter/anti-sigma factor antagonist) domain (e.g. RsbS, RsbV) or a C-terminal STAS domain and a variable N-terminal domain [3]. Highly conserved phosphorylation sites are located in the STAS domain (e.g. T171 and T205 in RsbRA). RsbRA is assumed to be associated with detection of salt stress [7,8]. Although a crystal structure of the N-terminal domain or RsbRA is available the molecular details of salt detection or the stimulation of RsbT are unclear [9].

Besides high concentrations of salt or ethanol also light was identified as a trigger for the environmental branch of the GSR [10]. So far, red and blue light as two different extra-cellular signals are known to stimulate $\sigma^B$ activity [11]. While red light is able to penetrate upper layers of soil and might help the bacterium in interpreting its localization, blue light is readily absorbed at the surface, either providing indirect information on UV radiation or merely on the degree of exposition [12].

*B. subtilis* recognizes blue light via the photoreceptor protein YtvA, which is a paralog of RsbR [10,13]. Here, a FMN-binding LOV (light; oxygen; voltage) domain functions as the receptor part of the protein. Illumination with light of around 450 nm results in adduct formation of a highly conserved Cys with the chromophore [14]. The subsequent change in the hydrogen bonding network around the chromophore is supposed to trigger conformational rearrangements that lead to activation of the effector domain [15].

YtvA forms a dimer of 60 kDa irrespective of its activation state and has a dumb-bell shape with coiled-coil interactions of the linker helices separating both LOV and STAS domains [16–19]. As a direct consequence of these findings, a rotational movement of the linker helices has been proposed [20].

Beside the intra-molecular mechanism of signal transduction, the inter-molecular mechanism that underlies the conversion from an extra-cellular stimulus to an intra-cellular signal is also not yet

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fully understood. YtvA lacks the highly conserved threonine residues functioning as phosphorylation sites found in other paralogous STAS domains [3] and was shown not to bind GTP [21,22], which was originally proposed to be the function of YtvA [23]. In yeast two-hybrid and co-immunoprecipitation experiments, YtvA was shown to bind to other RsbR proteins (namely RsbRA and RsbRD) suggesting an interaction with the stressosome, but the nature of this interaction is not clear [3,13].

In this study we used well-defined illumination conditions to further investigate interactions between YtvA and the stressosomal proteins RsbR, RsbS, and RsbT *in vitro*. In addition, we analyzed whether YtvA is merely associated with the stressosome or is incorporated into the complex as the other paralogs. The light dependence of the RsbT stimulation *in vitro* is then used to discuss a mechanism for the perception of the light signal by the stressosome.

## 2. Results

### 2.1. YtvA is permanently associated with the stressosome

The Rsb proteins RsbRA and RsbRD were previously identified to be interaction partners of YtvA [3]. We chose the well characterized RsbRA (in the following termed RsbR) for our studies. From the yeast two-hybrid assays that were conducted to identify the interaction between YtvA and RsbR no information is available as towards the dependency of the interaction on the illumination state of YtvA. Therefore, we used analytical ultracentrifugation to characterize the binding in more detail.

The complex of RsbR:S:T prepared in our laboratory shows all the characteristics reported previously [4]; an approx. 1.8 MDa (from AUC) complex that elutes in the void of even a S200 gelfiltration column. Negative stain electron micrographs recorded of the complex are comparable to those used for reconstruction of the stressosome by Marles-Wright et al. [5] (Suppl. Fig. S1).

To study the light-dependency of the interaction between YtvA and various compositions of stressosomal proteins (RsbR, RsbS, RsbT) we conducted AUC experiments using sedimentation velocity experiments at relatively low speed (12,000 rpm), which is well suited to separate the high molecular weight complex from smaller species (Fig. 1A). Detection during sedimentation was accomplished at 447 nm which allowed the exclusive observation of YtvA, since no absorption of RsbR:S:T exists at this wavelength.

In a first experiment, YtvA was incubated with the three stressosomal proteins in the dark and subsequently subjected to sedimentation velocity experiments to qualitatively asses an interaction. A reference of YtvA without any other protein forms only dimers in solution and no higher oligomeric species [18]. Different concentrations (20; 40; 80 μM) of YtvA were analyzed while keeping the concentration of RsbR:S:T constant (40 μM RsbR; 20 μM RsbS; 20 μM RsbT).

One species was always present at 28 S indicating an interaction of YtvA with the stressosomal complex (Fig. 1B). A second major species is observable at 5 S corresponding to either YtvA homodimers or small YtvA:RsbR heterooligomers. Due to the relatively low speed the sedimentation of the latter, low molecular weight species is slow and at the same time governed by a strong diffusion process, which results in no precise determination of the oligomeric species.

A further increase of YtvA concentration did not change the amount of YtvA associated with the stressosomal complex while the species corresponding to a dimer or small hetero-oligomeric state increased in intensity. As a cause of the detection limit of the AUC optical system lower concentrations could not be tested. Still, the binding constant of YtvA to the complex must be below the loading concentrations ($<10^{-7}$ M).

Using a vacuum-sealed battery pack and LED mounted onto the interference laser of our analytical ultracentrifuge (cf. Jurk et al. [18]) we were also able to investigate the interaction between lit-state YtvA and the stressosomal complex. Interestingly, the interaction between light activated YtvA and RsbR:S:T persists, since no changes in the sedimentation behavior were observable.

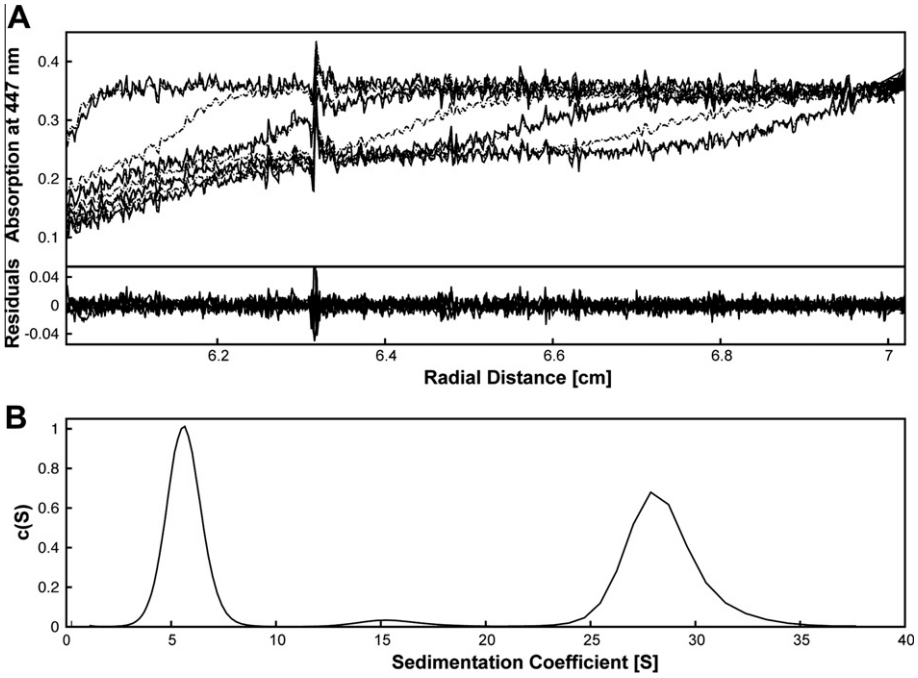


**Fig. 1.** Interaction between YtvA and the stressosome analyzed by analytical ultracentrifugation. In this exemplary result a complex of 20 μM YtvA and 40 μM RsbR:S:T was analysed. Centrifugation was performed at 12,000 rpm with scans taken every 8 min, while YtvA was selectively detected at 447 nm. (A) For reasons of clarification every eighth data curve (solid lines) but every fourth fit (broken lines) is plotted. (B) Analysis shows one major species (45%) at 5.1 S corresponding to either dimeric YtvA or a heterodimer of RsbR:YtvA. 51% are detectable as a high molecular weight species at 28 S. A small fraction (4%) is associated with an intermediate stressosomal species (15.7 S). The sedimentation process was stopped after 8 h since all of the high molecular weight fraction had sedimented.

**Table 1**
Interactions of YtvA and Rsb proteins. Results are qualitative assessments of binding using AUC sedimentation velocity experiments. Binding (+) and no detectable binding (–) are indicated.

| YtvA + | Dark state | Lit state |
|---|---|---|
| RsbR:S:T | + | + |
| RsbR | + | + |
| RsbR:S | + | n.d. |
| RsbS:T | – | n.d. |

Additionally, we tested different combinations of Rsb proteins and YtvA. A full overview of complexes that were analyzed is given in Table 1. These results show that RsbR is indeed minimally required to establish an interaction of YtvA with the stressosome and that the illumination state of YtvA does not alter the association. This finding is in line with the earlier finding that RsbR is required for light dependent stress response *in vivo* [8].

While the AUC experiments clearly show an interaction of YtvA with the stressosome independent of the illumination state, no differentiation between association and incorporation is possible. To test whether YtvA is incorporated into the stressosome and thereby replacing RsbR, we used a simple competition assay: complexes of RsbR:S:T and varying concentrations of YtvA were separated by native PAGE. RsbR used in this experiment contained an N-terminal hexa-histidine ($His_{6x}$) tag. Western blot analysis using anti-His antibodies allowed for a specific detection of RsbR.

In absence of YtvA no low molecular weight RsbR-$His_{6x}$ ("free RsbR") may be observed. Addition of YtvA on the other hand results in occurrence of a band corresponding to non-complexed RsbR-$His_{6x}$. Increasing amounts of free RsbR were detectable with increasing concentrations of YtvA (Fig. 2A). Though no reliable densitometric evaluation was possible, it is apparent that YtvA indeed displaces RsbR from the complex.

A competition assay conducted as described before only with preincubated samples of RsbR:S:T and subsequent addition of YtvA yielded no displaced RsbR (Fig. 2B). At least *in vitro*, YtvA is hence only incorporated into the stressosomal complex during association of the complex. This finding may prove crucial to further analysis of Rsb complexes.

### 2.2. YtvA regulates light-dependent stimulation of RsbT activity in vitro

Since we observed an incorporation of YtvA into the stressosome regardless of an illumination we tried to assess whether this complex is indeed functional. In order to investigate a light-induced activation of RsbT more precisely we utilized a radioactive assay using $\gamma$-$^{32}$P-ATP. Again, samples of RsbR:S:T assembled in presence or absence of YtvA in both dark and lit state were analyzed.

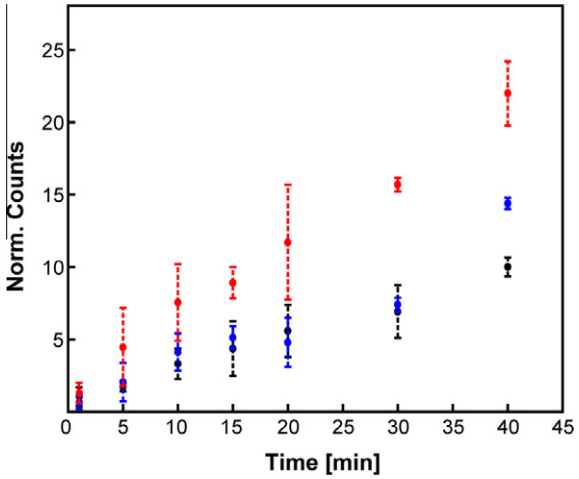


**Fig. 3.** Kinase activity of RsbT as a result of RsbS phosphorylation using $\gamma$-$^{32}$P-ATP as a substrate. Phosphorylation was measured for 15 μM RsbR:S:T (2:1:1) (reference, black), in the presence of 5 μM YtvA in the dark state (blue) or lit state (red). Error bars result from 3, 2, 5 measurements for reference, dark and lit state samples, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

As found in previous experiments by Chen et al. [4] and others, RsbT displays a basal kinase activity, which is observable in this experiment in the presence of the RsbT target proteins RsbS and RsbR. RsbS is phosphorylated primarily, while phosphorylation of RsbR is much slower (Suppl. Fig. S2), as also observed in aforementioned experiments [4].

No significant differences could be observed between samples of RsbR:S:T and RsbR:S:T:YtvA in the dark (Fig. 3). On the other hand, phosphorylation of RsbS occurs faster in the presence of lit-state YtvA. The observed increase is reproducible about two-fold. A similar increase in kinase activity was found for a dependence on the phosphorylation state of RsbR [24]. Whether RsbT kinase activity towards RsbR is likewise increased upon light-activation of YtvA could not be accurately determined due to the low signal intensity. Nevertheless, these results show that YtvA is able to stimulate phosphorylation of at least RsbS by RsbT *in vitro* despite not directly interacting with it.

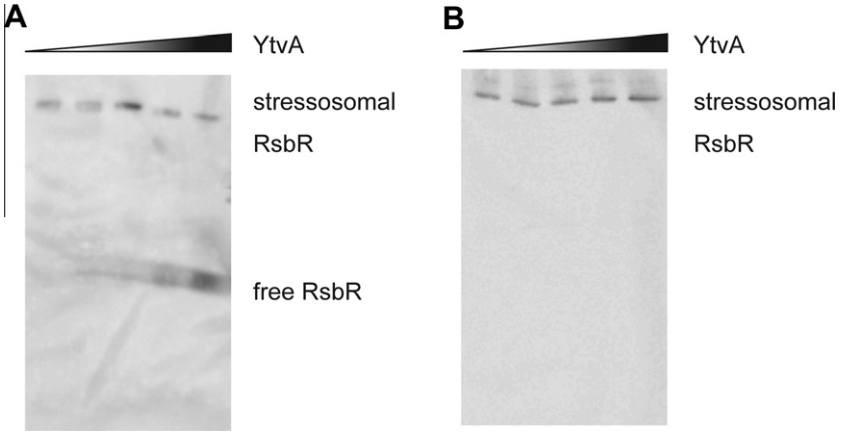


**Fig. 2.** Western blot analysis showing displacement of RsbR from stressosomal complex by YtvA. (A) RsbR:S:T (4:2:2 μM) was incubated with different concentrations of YtvA (0; 0.05; 0.2; 0.5; 1 μM). RsbR contained an N-terminal hexa-histidine tag which was detected using a penta-His antibody. Samples were subjected to native PAGE prior to Western blot analysis. (B) Same experiment as A but using preassembled stressosomal RsbR. No displacement is detectable in this case.

## 3. Discussion

While the intra-molecular mechanism of the light activation of YtvA can most likely be understood from the knowledge of its three-dimensional structure (Jurk et al., in preparation), the role and activation mechanism of YtvA inside the stressosome need further investigations. Therefore, we utilized several *in vitro* experiments to obtain information on the function of YtvA associated with an interaction with other Rsb proteins.

Our results show an incorporation of YtvA into the stressosome, independent of the illumination state. While earlier experiments were only able to show a general interaction of YtvA with the stressosome [3,13] the data presented here proves that it is always part of the stressosomal complex despite the fact that YtvA lacks the highly conserved phosphorylation sites of the other paralogous Rsb proteins [3]. In addition, YtvA is only recruited by the stressosome if RsbRA (called RsbR in this manuscript) is present inside the complex. An interaction with RsbS and RsbT is not detectable in our experiments. Resulting from the tested concentration range (maximum: 80 μM YtvA) a very weak and transient interaction cannot be completely excluded.

Our Western blot competition assay shows that RsbR is not simply required for the recruitment of YtvA but it is at the same time displaced to some extent by YtvA. As a consequence, the overall arrangement of Rsb proteins inside the stressosome is likely to remain as previously reported by Marles-Wright et al. [5]. Although a precise determination of the binding stoichiometry was not possible but given that YtvA is not able to form homooligomers other than dimers [18] a replacement of no more than every second RsbR would be possible. RsbR would in such a case be associated with a scaffolding function required for complex formation. Whether it acts also as a mediator protein remains to be elucidated. Taken together our results explain why – *in vivo* – a light mediated stress response is only generated in *B. subtilis* if both YtvA and RsbR are present [8].

Given that a definite correlation between environmental stress signals (e.g. salt) other than light and RsbR paralogs is missing, the mere knowledge of the molecular architecture of the stressosome is not sufficient to understand how the stressosome is activated. It is likely, though, that activation of RsbR proteins, which seem to adopt a YtvA-like dimeric substructure inside the stressosome [5], occurs in a same manner as for YtvA. Especially, the crystal structure of the N-terminal domain of RsbRA suggests a similar domain assembly of the full-length protein and corroborates the idea of an activation mechanism comparable to that of YtvA [9,25].

Since YtvA displaces RsbR inside the complex it is spatially close to the kinase RsbT [5]. Due to the dynamic nature of the stressosomal assembly that is assumed for the complex *in vivo* [5,26] a transient interaction between RsbT and YtvA could take place as discussed for RsbT and RsbRA [27].

It has been shown that no major structural rearrangements take place in YtvA upon illumination [18]. A slight, light induced rotational movement of the YtvA-STAS domains as suggested previously [16,20] and supported by structural studies of the full length protein (Jurk et al., in preparation) would cause a rearrangement of the STAS domains of the Rsb proteins inside the stressosome. One might imagine that either (i) the structural changes of YtvA are sufficient to trigger this rearrangment or (ii) a further conformational change in adjacent STAS domains of RsbRA or RsbS is initiated. In either way the consequence would be an exposition of the highly conserved phosphorylation sites to RsbT, thus triggering the signal transduction cascade inside the cell.

Additional information on the molecular processes is provided by the kinase activity assays conducted not only in this study but also by others [6,24], which indicate that RsbT is always in an active state. This explains the necessity for a constant equilibrium with RsbX phosphatase activity *in vivo* [24,27] and matches the assumption of structural rearrangements inside the stressosome, because an increased accessibility of phosphorylation sites for RsbT would thus be indirectly responsible for a heightened RsbT phosphorylation rate rather than a protein–protein interaction-dependent direct stimulation of RsbT. Whether a decreased accessibility for RsbX and consequently a lowered dephosphorylation is occurring at the same time remains unclear. Still, the effect of such a "always-on" system would be a faster response of the stressosome to extra-cellular stimuli.

## 4. Materials and methods

### 4.1. Cloning of RsbR, RsbS & RsbT

The DNA sequences encoding amino acids 2-274; 2-121 and 2-133 of RsbRA; RsbS and RsbT, respectively, were amplified by two-step PCR using KOD Hot Start Polymerase (Novagen; Merck KGaA, Darmstadt; Germany) and overlapping forward primers:

Forward TEV: 5′-gac gac gac aag atg gaa aac ctg tat ttc cag-3′
Forward RsbRA: 5′-aac ctg tat ttc cag gg aat gtc gaa cca gac tgt ata cca gt-3′
Reverse RsbRA: 5′-gag gag aag ccc ggt tta ttc ccc caa tga aac ga-3′
Forward RsbS: 5′-aac ctg tat ttc cag gga aga cat ccg aaa atc ccg atc ttg aaa ctg tat aat tgc tta tta gtg-3′
Reverse RsbS: 5'-gag gag aag ccc ggt tta ttc ccc caa ttc ccg ctt caa tgt ctc aag c-3′
Forward RsbT: 5′-aac ctg tat ttc cag gga aac gac caa tcc tgt gta aga atc atg aca gaa tgg gat att g-3′
Reverse RsbT: 5′-gag gag aag ccc ggt tta ccg aag cca ttt gat ggc ttg tat ctc tgt tcc ttc-3′

By means of PCR a TEV cleavage site was introduced, leaving an additional Gly at position 1 after proteolysis instead of the wild-type Met1. The N-terminally furbished expression tag consisted of a 6x His-tag and a total of 50 amino acids. As a template well separated clones of *B. subtilis* (DSM-856; German Collection of Microorganisms and Cell Cultures; Braunschweig; Germany) were subjected to colony PCR. The amplified gene was cloned into a pET30 EK/LIC vector using the ligation independent cloning technique (LIC) [28]. All materials needed for PCR and cloning were purchased from Novagen (Merck KGaA).

### 4.2. Sample preparation of RsbR, RsbS & RsbT and stressosomal assembly

Samples of full-length YtvA were prepared according to a previously published protocol [18]. RsbR, RsbS and RsbT were expressed and purified in the same way as for YtvA. Purified samples were stored in PBS [29] supplemented with 5 mM DTT. Stock solutions of RsbR, RsbS & RsbT were diluted and mixed to give a 2:1:1 ratio at least 12 h before any experiment. Stressosome assembly was allowed to happen at 8 °C.

### 4.3. Interaction analysis YtvA: stressosome

Analytical ultracentrifugation experiments were performed as sedimentation velocity experiments for YtvA and various combinations of RsbR; RsbS and RsbT using a Beckman Optima XL-I analytical ultracentrifuge. Two-sector Epon cells were used at a speed of 12,000 rpm at 20 °C. Data were acquired using the absorption optical system with scans at 447 nm or 390 nm for the dark and lit state of YtvA, respectively. Radial increments were set to 0.01 cm

without replicates. Sample illumination was achieved as described before [18]. Data evaluation was done using SEDFIT [30]. SEDNTERP [31] was used for calculation of buffer density and viscosity.

NativePAGE Novex® 3–12% Bis–Tris 10 well gradient gels (Invitrogen) were used for native PAGE analysis of samples. Sample and running buffer were prepared according to the manufacturers manual. 20 μL of 1–10 μg total amount of sample were loaded per lane. Electrophoresis was performed at 8 °C for ~2 h at 150 V and subsequently stained using recommended standard staining solution (Coomassie® G-250).

Immunodetection of RsbR-$His_6$ after native PAGE separation (without staining) was performed using a penta-His direct detection antibody (Penta-His HRP Conjugate Kit; Quiagen). Blotting onto nitrocellulose membrane (Pierce Biotechnology) was done using the semi-dry transfer protocol recommended in the antibody kit's manual. A total transfer time of 80 min at 60 mA was used (8 × 9 cm membrane size). A LAS-4000 (Fujinon Europe GmbH; Willich; Germany) was used for detection of chemiluminescence after membrane treatment (1 mL; 5 min incubation) with ECL™ Western Blotting Reagents (GE Healthcare).

### 4.4. Kinase activity

$^{32}$P-kinase assays were performed using 15 μM R:S:T in presence or absence of 5 μM YtvA in 20 mM Tris/HCl; 150 mM NaCl; 5 mM 2-ME at pH 7.4. γ-$^{32}$P-ATP was purchased from Perkin-Elmer (Rodgau; Germany) as 9.25 MBq stocks. Specific activity used in experiments was 0.12 μCi/μmol (total 50 μM ATP). Final $MgCl_2$ concentration was 11 μM. Samples of 10 μL were taken in intervals of 1; 5; 10; 15; 20; 30 and 40 min, immediately mixed with 2× SDS-sample buffer and incubated at 90 °C for 10 min. To separate proteins, free ATP and phosphate samples were loaded onto a 10 well NuPAGE® 4–12% Bis–Tris gradient gel (Invitrogen) and run for ~40 min using NuPAGE® MES SDS running buffer (Invitrogen) at 200 V. A storage phosphor screen (Molecular Dynamics; Sevenoaks; Kent; UK) was incubated with the resulting gel for 2 h. A STORM 830 phospho-imager (Molecular Dynamics) was used for read-out. Staining of gels was accomplished using SimplyBlue® staining solution (Invitrogen).

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.025.

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