Sequential Action of MalE and Maltose Allows Coupling ATP Hydrolysis to Translocation in the MalFGK₂ Transporter*

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Background: MalE and maltose together stimulate the ATPase activity of MalFGK₂.

Results: Binding of open-state MalE triggers the cleavage of ATP; maltose triggers the release of Pᵢ.

Conclusion: Open-state MalE stabilizes the transporter in the outward-facing conformation; maltose triggers return to the inward-facing state.

Significance: The complementary action of MalE and maltose allows coupling ATP consumption to transport.

ABC-four transporters are widespread and universally conserved in nature. They consist of at least two transmembrane domains (TMDs) that form the substrate passageway, as well as two nucleotide-binding domains (NBDs) that bind and hydrolyze ATP (1). In bacteria, ABC transporters are essential for pumping in nutrients and for pumping out toxic compounds. In humans, they perform equally important functions; their deregulation is often linked to diseases (2–4). Understanding the working mechanism of ABC transporters is therefore central to biomedical research. Intensive crystallographic work has led to a general model of action: ATP drives the association of the NBDs and the transition of the TMDs from an inward-facing to an outward-facing conformation. Hydrolysis of ATP leads to the dissociation of the NBDs, and thus returns the TMDs to their initial inward-facing conformation. This alternating exposure of the TMDs on either side of the membrane, termed the alternating-access mechanism, allows substrate binding, transport, and release, while preserving membrane impermeability (5–8).

An important unresolved question for ABC transporters is the mechanism by which substrate stimulates ATP hydrolysis. The degree of stimulation varies, but the characteristic is common to the family (9–14). The crystal structures of ABC exporters suggest that binding of the substrate to the inward-facing TMDs provokes pre-closure of the NBDs: a conformational state termed the primed or pre-hydrolytic state (15–17). This pre-closure of the NBDs in turn facilitates the movement of the TMDs to the outward-facing conformation, and eventually the release of the substrate on the trans-side of the membrane. Upon hydrolysis of ATP, the NBDs dissociate and the TMDs return to their initial inward-facing state. As a consequence, the binding of the substrate to the transporter augments the hydrolysis of ATP. This proposed mechanism of action is supported by structural and functional analysis on ABC exporters (15, 18, 19). In the case of ABC importers, however, the stimulatory action of the substrate needs to occur via a different mechanism for two reasons: (i) the TMDs must reach their outward-facing conformation in order for the substrate to enter the transport pathway and (ii) the transport and ATPase activities depend on a peripheral substrate-binding protein. Because the binding protein itself stimulates the ATPase cycle, the role of the substrate is furthermore obscure (9, 10, 13). The binding protein exists in equilibrium between open and closed states, but the binding of the substrate favors the closed state. It is therefore often proposed that binding of the closed-ligated protein induces the closure of the NBDs and therefore cleavage of ATP (e.g. Ref. 20).

Here, we analyze the MalFGK₂ transporter to better understand the ATP hydrolytic cycle, as well as its dependence on
MalE and maltose. This kinetic analysis is made possible by incorporating MalFGK$_2$ into nanodiscs. Previous analyses show that MalFGK$_2$ in nanodiscs behaves as it does in proteoliposomes: (i) the basal ATPase activity of MalFGK$_2$ is naturally low; (ii) unliganded MalE stimulates the ATPase activity by 4-fold (to ~40 nmol/min/mg); and (iii) the presence of MalE and maltose stimulates ATP hydrolysis by ~40-fold (~400 nmol/min/min) (9, 21). Using a transient kinetic method, we show that open-state MalE binds to the outward-facing transporter to trigger the cleavage of ATP. Maltose subsequently facilitates the release of hydrolyzed P$_i$. These findings form the basis for understanding the transport reaction.

**Experimental Procedures**

**Materials**—MalFGK$_2$, MalE, and EIIGlc$_{Glc}$ proteins were over-expressed and purified as before (22, 23). Reconstitution of MalFGK$_2$ into proteoliposomes was carried out as described (21). Nanodiscs were prepared at a molar ratio MalFGK$_2$:MSP-lipids (where MSP is membrane scaffold protein) of 1:3:600 (21). Lipid 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG) were purchased from Avanti Polar lipids. Radiolabeled nucleotides [α-$^32$P]ATP (25 Ci/mmol) and [γ-$^32$P]ATP (800 Ci/mmol) were obtained from MP Biomedicals. The EnzChek kit was from Molecular Probes (Invitrogen). Kinase-Glo reagent was from Promega. All other chemicals, including, pyruvate kinase (catalogue number P9136), lactate dehydrogenase (catalogue number L38888), TNP-ATP, and polyethyleneimine cellulose TLC plates, were supplied by Sigma.

**ATPase Assays**—Steady state ATPase assays were performed at the indicated temperature using 1 μM MalFGK$_2$ nanodiscs, 10 μM MalE, 1 mM maltose, and 1 mM [α-$^32$P]ATP in ATPase buffer (50 mM Tris-HCl, pH 7.5; 5 mM MgCl$_2$). To determine the $K_{m}$ value of MalFGK$_2$ for ATP, experiments were carried out at 25 °C with increasing concentrations of [α-$^32$P]ATP. For single turnover ATPase assays, reactions were carried out at the indicated temperature using 20 μM MalFGK$_2$ nanodiscs, 100 μM MalE, 1 mM maltose, and 1 μM [α-$^32$P]ATP in ATPase buffer. The amount of ATP hydrolyzed was determined by TLC as described previously (22), followed by autoradiography and density scanning on a Typhoon imager.

**Nucleotide Binding Assay to MalFGK$_2$**—Binding of TNP-ATP to MalFGK$_2$ was performed as described previously (22). The fluorescence data were plotted as a function of TNP-ATP concentration and fit to Equation 1,

$$F = \frac{F_{\text{max}}}{[L] + K_{d}} \quad (\text{Eq. 1})$$

where $F_{\text{max}}$ is the maximal subtracted fluorescence at saturating amount of TNP-ATP and $K_d$ is the equilibrium dissociation constant of TNP-ATP for MalFGK$_2$. To determine the affinity of MalFGK$_2$ for ATP, TNP-ATP (80 μM) was incubated with MalFGK$_2$ nanodiscs (2 μM) for 5 min at 25 °C and then titrated with an increased amount of ATP. The fluorescence data were fit to Equation 2,

$$F = \frac{F_o}{[L] + K_{\text{app}}} + F_1 \times \frac{[L]}{[L] + K_{\text{app}}} \quad (\text{Eq. 2})$$

in which $F_o$ is the fluorescence value in the absence of ATP, $F_1$ is the fluorescence value in the presence of saturating amount of ATP, [L] is the ATP concentration, and $K_{\text{app}}$ is the apparent inhibition constant of ATP at the specified amount of TNP-ATP. The $K_{d,\text{app}}$ of ATP for MalFGK$_2$ is calculated from $K_{\text{app}}$ by Equation 3,

$$K_{d,\text{app}} = \frac{F_{\text{app}}}{F_{\text{burst}}} \times \left(1 + \frac{[L]}{K_d}\right) \quad (\text{Eq. 3})$$

in which [L] is the TNP-ATP concentration, and $K_d$ is the dissociation constant of TNP-ATP for MalFGK$_2$.

**Stopped-flow Experiments**—Measurements were performed at 25 °C using an SX-18MV stopped-flow apparatus (Applied Photophysics Ltd., Leatherhead, UK). Data collection was carried out with 5 μM MalFGK$_2$, nanodiscs, 50 μM MalE, 1 mM maltose, and 1 mM ATP in ATPase buffer. Release of ADP was measured using the pyruvate kinase/lactate dehydrogenase enzyme-linked assay (24). Absorbance data were collected at 340 nm, and quantity of ADP produced was calculated using the extinction coefficient of NADH (6,220 M$^{-1}$ cm$^{-1}$). Release of phosphate was measured using the EnzChek kit. Absorbance data were collected at 360 nm, and the quantity of phosphate produced was calculated using the extinction coefficient of MESG (11,000 M$^{-1}$ cm$^{-1}$). Kinetic data of phosphate release were fit to a simple linear regression model, whereas kinetic data of ADP release were fit to Equation 4,

$$A = A_0(1 - e^{-k_{\text{burst}}}) + k_{\text{linear}} \quad (\text{Eq. 4})$$

where $A$ is the amount of calculated ADP, $A_0$ is the amount of ADP in the exponential phase, $k_{\text{burst}}$ is the rate constant for the burst phase, and $k_{\text{linear}}$ is the rate constant for the slower linear phase.

**Quenched-flow Experiments**—The kinetics of ATP cleavage were measured at 25 °C using a QFM-400 quenched-flow apparatus (Bio-Logic SAS). Experiments were carried out using 25 μM MalFGK$_2$ nanodiscs, 100 μM MalE, 1 mM maltose, and 1 mM ATP in ATPase buffer. At the indicated time points, reactions were quenched by buffer A (50 mM Tris-HCl, pH 7.5; 50 mM EDTA). The amount of ATP was quantified with Kinase-Glo reagent, and the data were fit to Equation 5,

$$A = (A_0 - B)e^{-k_{\text{burst}}} - k_{\text{linear}}t + B \quad (\text{Eq. 5})$$

where $A_0$ is the amount of ATP before the initiation of the reaction, $B$ is the amount of ATP at the end of the burst phase of the reaction, $k_{\text{burst}}$ is the rate constant for the burst phase, and $k_{\text{linear}}$ is the rate constant for the slower linear phase.

**Other Methods**—For TLC and centrifugal gel filtration assays, MalFGK$_2$ nanodiscs (20 μM), MalE (75 μM), and maltose (1 mM) were incubated with [α-$^32$P]ATP or [γ-$^32$P]ATP (100 μM) at 4 °C for 10 min. Samples were subjected to centrifugal gel filtration and analyzed by TLC.

**Results**

**Binding of ATP to MalK Is Independent of MalE and Maltose**—To understand how MalE and maltose accelerate the ATP hydrolytic cycle of MalFGK$_2$, we performed an Arrhenius analysis of its ATPase activity. The data reveal that MalE alone
The ATPase Cycle of MalFGK₂

MalE Stimulates the ATP Cleavage Reaction—We next determined the capacity of MalE and maltose to accelerate the cleavage of ATP under two different conditions: (i) when MalFGK₂ is present in 20-fold molar excess over the nucleotide, so that only a single round of ATP hydrolysis is possible (Fig. 2a), and (ii) under steady state conditions, where the nucleotide is present in 1000-fold excess over MalFGK₂, so that multiple rounds of ATP hydrolysis can occur (Fig. 2b).

MalFGK₂ was incubated with [α-32P]ATP, and ATP hydrolysis was detected by thin layer chromatography. Under single turnover conditions, ~50% of total ATP is hydrolyzed within 1 min, at 4 °C or at 37 °C. Strikingly, under the same conditions, the addition of MalE results in ~100% cleavage of ATP in a similar time period. In contrast, much less cleavage was detected when MalE and maltose are present together when compared with MalE alone (Fig. 2a). The latter result is not surprising because closed-liganded MalE has very low affinity for the transporter (26–28). Accordingly, the inhibitory effect of maltose is enhanced with the mutant MalE-DW, and this inhibitory effect is not observed with the mutant MalE-254 (Fig. 2a). We previously reported that MalE-254 does not acquire a closed conformation in the presence of maltose, whereas MalE-DW closes rapidly to capture maltose with an affinity ~60-fold higher than the

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FIGURE 1. Binding of ATP to MalFGK₂ is not stimulated by MalE and maltose. a, left panel, the $K_{cat}$ values (pmol of P/pmol of MalFGK₂/min) are determined as a function of temperature. The $y$ axis represents the natural logarithm of the $K_{cat}$ values [ln($K_{cat}$)], and the $x$ axis represents the inversed temperature values (1/T) expressed in Kelvin. Right panel, the activation energy $E_a$ was calculated from the slope of the linear plot, where the slope = $-E_a/R$ and $R$ = 8.31451 J K⁻¹ mol⁻¹. b, equilibrium titration of TNP-ATP binding to MalFGK₂. The MalFGK₂ complex was incubated with TNP-ATP, in the presence or absence of MalE and maltose. Fluorescence data were plotted against concentration of TNP-ATP. Data were fit to Equation 1 and summarized in Table 1 (mean ± S.D., $n$ = 3 independent replicates). c, MalFGK₂ ATPase activity as a function of ATP concentrations, in the presence or absence of MalE and maltose. Data were fit to the Michaelis-Menten equation, and values are reported in Table 1 (mean ± S.D., $n$ = 3 independent replicates).

**TABLE 1**

| Nucleotide     | $K_{cat}$ for ATP (µM) | $K_{cat}$ for TNP-ATP (µM) | $K_{app}$ for ATP (µM) |
|----------------|------------------------|----------------------------|------------------------|
| MalFGK₂        | 233 ± 42               | 274 ± 32                   | 283 ± 41               |
| MalFGK₂, MalE  | 9 ± 1                  | 10 ± 2                     | 9 ± 1                  |
| MalFGK₂, MalE, maltose | 211 ± 23               | 194 ± 22                   | 224 ± 33               |

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Together, these results show that open-state MalE, but not closed-liganded MalE, stimulates cleavage of ATP.

Under multiple turnover conditions, the amount of ATP hydrolyzed is increased with maltose when compared with MalE alone (Fig. 2b). Because MalE promotes the cleavage of
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ATP and because maltose inhibits cleavage under single turnover conditions, we reasoned that under these conditions, maltose must accelerate a post-hydrolysis step, such as the release of ADP or Pᵢ. As a preliminary assay of whether the release of ADP or Pᵢ is rate-limiting, we determined the amount of ADP and Pᵢ that co-purifies with MalFGK₂ (Fig. 2c and d). A control experiment using the SecA ATPase confirms that SecA, which has a stronger affinity for ADP than Pᵢ (24, 29), preferentially co-purifies with ADP (Fig. 2c). In contrast, MalFGK₂ seems to preferentially co-purify with Pᵢ (Fig. 2d).

Release of Pᵢ Limits the Kinetics of the ATPase Cycle—To determine the step that is limited during the ATP hydrolytic cycle, we analyzed nucleotide turnover in steady state and pre-steady state conditions. The rate of ATP cleavage was determined by rapid mixing, quenched-flow experiments (Fig. 3a). Two distinct kinetic phases were detected in the presence of MalE and maltose: an initial burst of ATP cleavage followed by a slower linear phase at steady state. The rate constant for the initial burst is ~7 s⁻¹, which is ~9-fold faster than the steady state rate (7.8 s⁻¹; Table 2). Thus, in the presence of MalE and maltose, the ATPase cycle is evidently limited by a step occurring downstream of ATP hydrolysis. When MalE was omitted from the reaction, the pre-steady and steady state rates were decreased by ~10-fold, in agreement with the observation that MalE stimulates the ATP cleavage reaction.

We then determined the rate of ADP release by stopped-flow kinetics (Fig. 3b). In the presence of MalE and maltose, two distinct kinetic phases were also detected: a fast burst (~66 s⁻¹) followed by a slower steady state rate (7.2 s⁻¹). Thus, in the presence of MalE and maltose, the ATPase activity of the transporter is not limited by the release of ADP. We next determined the rate of Pᵢ release using the same stopped-flow kinetics (Fig. 3c). In contrast to ATP cleavage and ADP release kinetics, there was no burst for Pᵢ production. Instead, the rate of Pᵢ release was quasi-linear, measured to be 7.5 s⁻¹. We conclude that, under transport conditions, release of Pᵢ limits the ATP hydrolytic cycle, and this step is accelerated by maltose.

MalF500 Hydrolyzes ATP Constitutively—Earlier analysis showed that ATP hydrolysis of MalF500 (MalF₃G₃Ser/NS505) is uncoupled from maltose transport: the mutant hydrolyzes a large amount of ATP in a constitutive manner (30). We therefore determined the ATP hydrolysis kinetics of MalF500 in rapid mixing quenched-flow experiments (Fig. 4). The rate of ATP cleavage in pre-steady states conditions is 90 s⁻¹, a value that is similar to that of MalFGK₂ in the presence of MalE (Table 2). The rate of Pᵢ release, which corresponds to the rate of ATP hydrolysis in steady state conditions, is also greatly enhanced (~3-fold) when compared with the wild type transporter in the presence of MalE and maltose (Table 2). Thus, MalF500 exhibits high rates of ATP cleavage and high rates of Pᵢ release. As a consequence, maltose is unable to stimulate ATP hydrolysis, which results in poor coupling efficiency (21).

The Regulator EIiA<sup>Glc</sup> Inhibits the Cleavage of ATP—The transient kinetic analysis above allowed us to examine how the regulatory protein EIiA<sup>Glc</sup> inhibits the ATPase activity of MalFGK₂, a process termed inducer exclusion (31). Recent biochemical and structural data indicate that EIiA<sup>Glc</sup> binds to the inward-facing MalFGK₂ (22, 32). We therefore tested how EIiA<sup>Glc</sup> affects the ATP hydrolytic cycle. The results presented in Fig. 5a show that EIiA<sup>Glc</sup> decreases by ~20-fold the rate of ATP cleavage in pre-steady state conditions and by ~4-fold in steady states. The rate of ATP cleavage is determined by rapid mixing, quenched-flow experiments (Fig. 3a). Two distinct kinetic phases were detected in the presence of MalE and maltose: an initial burst of ATP cleavage followed by a slower linear phase at steady state. The rate constant for the initial burst is ~7 s⁻¹, which is ~9-fold faster than the steady state rate (7.8 s⁻¹; Table 2). Thus, in the presence of MalE and maltose, the ATPase cycle is evidently limited by a step occurring downstream of ATP hydrolysis. When MalE was omitted from the reaction, the pre-steady and steady state rates were decreased by ~10-fold, in agreement with the observation that MalE stimulates the ATP cleavage reaction.

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state conditions. We conclude that EIIA\textsuperscript{Glc} inhibits the capacity of MalE-MalFGK\textsubscript{2} to cleave ATP. As a consequence, EIIA\textsuperscript{Glc} also decreases the rates of ADP release and Pi release from MalFGK\textsubscript{2} (Fig. 5, \textit{b} and \textit{c}).

Discussion

The MalFGK\textsubscript{2} transporter is a pioneering model for mechanistic studies on ABC importers; important findings have been derived from its structural and functional analysis (33, 34). In particular, earlier studies showed that MalE and maltose are necessary together to stimulate its ATP hydrolysis rate. However, how the ATPase cycle is accelerated was not determined. Here, we dissected the ATPase reaction into three steps (binding, hydrolysis, and product release) to determine the individual contribution of MalE and maltose, and therefore how substrate translocation is coupled to ATP consumption.

We show first that binding of ATP to the MalFGK\textsubscript{2} does not depend on MalE or maltose. This result was expected because the nucleotide-binding pockets are readily accessible to ATP in the resting transporter (35). Notably, ATP is sufficient to provoke the dimerization of MalK and the conversion of MalFG to the outward-facing conformation, at least in a transient manner (36–38). This ATP-dependent closure of the NBDs accounts for the basal ATPase activity of the transporter (\(-10\) nmol/min/mg). However, without MalE and maltose around, the ATP turnover cycle remains inhibited. Indeed, we show that cleavage of ATP absolutely requires MalE. Importantly, we demonstrate using two different mutants that ATP cleavage is stimulated by open-state MalE, and not closed-state MalE (Fig. 2). We reported earlier that the affinity of open-state MalE for outward-facing MalFGK\textsubscript{2} is \(-60\) nm, whereas the affinity of closed-state MalE for inward-facing MalFGK\textsubscript{2} is very low (\(>100\) \(\mu\)M) (28, 38). Because the concentration of ATP in cells is \(>1\) mm, the transporter must therefore largely exist in its outward-facing conformation, which has high affinity for open-state MalE. The stability of the complex between open-state MalE and outward-facing MalFGK\textsubscript{2} might in turn stabilize the NBDs in a conformation prone to hydrolyze ATP.

Although MalE accelerates ATP cleavage, we find that the hydrolytic cycle does not proceed forward efficiently. The rate-limiting step corresponds to the release of P\(_i\) and this step is...
accelerated ~6-fold in the presence of maltose. Without maltose, the stability of the complex between open-state MalE and outward-facing MalFGK₂ might prevent the opening of the MalK dimer, and therefore the release of the ATP hydrolysis products. Interestingly, structural analysis shows that the γ-phosphate makes multiple hydrogen bonds with Walker A and Q-loop, as well as switches histidine from one NBD and the LSGGQ motif from the second NBD (39). This high degree of coordination might decrease the dissociation rate of P_i when compared with ADP. At any rate, our current data clearly show that the \( K_{\text{cat}} \) value of ATP hydrolysis approaches the \( K_{\text{cleft}} \) value when maltose is present, indicating that the steady state complex MalE-MalFGK₂ switches from a mostly P_i-bound state to one that rapidly interconverts to the ATP-bound state. We therefore propose that maltose acts like a nucleotide exchange factor to accelerate the replacement of P_i with a new ATP molecule. Importantly, because the release of the hydrolysis products is concomitant with the opening of the MalK dimer and therefore the return of the TMDs to the inward-facing state (23, 35, 40, 41), it is logical to propose that maltose stimulates these conformational changes. Finally, we demonstrate that EIIA^{Glc} inhibits the cleavage of ATP. This last result is in agreement with structural and cross-link data that concluded that EIIA^{Glc} prevents the closure of the NBDs (22, 32).

Together, the data allow us to present a transport model (Fig. 6). We reported earlier that MalFGK₂ is converted from inward-facing to outward-facing upon binding of ATP (step i and step ii) (38). We also showed that this outward-facing transporter binds open-state MalE with a high degree of affinity (28). Here, we show that binding of open-state MalE induces cleavage of the nucleotide into ADP and P_i (primed state, step iii). Without maltose around, the transporter remains in this conformation; this assembly is stable and can readily be crystallized (42). With maltose around, the transporter rapidly returns to its initial inward-facing conformation (step iv). Maltose therefore accelerates the release of P_i and thereby the ATP turnover cycle (step v). How maltose enters the transporter, how it is released from open-state MalE, and how it triggers the return of MalFG to the inward-facing state remain to be determined; different models are possible (7, 23, 28, 43–46). In our current model (Fig. 6), maltose-bound open-state MalE forms a high-efficiency complex with MalFGK₂. This might explain why the affinity of maltose to MalE (~2–4 \( \mu \text{M} \)) is very similar to the \( K_m \) of the complex MalE-MalFGK₂ for maltose (~2 \( \mu \text{M} \)). In addition, the sequential action of MalE and maltose that we report here explains why the ATPase activity of MalFGK₂ depends on MalE and why it is coupled to maltose. The importance of this sequential action is best illustrated with the mutant MalF500:

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\begin{align*}
\text{Fig. 6. Model for the stimulation of MalFGK₂ by MalE and maltose.}
\end{align*}
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cleavage of ATP occurs in the absence of MalE, and release of P_i does not depend on maltose. It follows that the ATPase cycle is uncoupled from transport (21). The mutation G338R in MalF500 is located near the periplasmic gate. The destabilization of this region might account for the spontaneous cycle of ATP-driven conversion of the transporter.

The model in Fig. 6 contrasts with the classic model that assumes that only closed-ligated MalE binds the transporter to trigger the cleavage of ATP. This earlier model bears similarity to that proposed for ABC exporters, i.e. binding of the ligand promotes the closure of the NBDs. Interestingly, in the case of the lipid exporter MsbA, the rate-limiting step corresponds to ATP cleavage, whereas for the multidrug resistance protein 4 (ABCC4), it corresponds to ADP release (47, 48). However, the ATPase rates in MsbA and ABCC4 are only marginally stimulated by the substrate (47, 49), similar to the mutant MalF500 in which substrate transport is largely uncoupled to ATP hydrolysis. Thus, although ABC transporters share similar architecture and overall mechanism, the basal ATPase activity that characterizes each transporter might determine the substrate dependence and coupling efficiency, and even perhaps the direction of transport (50). An approach that systematically determines the ATPase kinetic parameters as developed here would be useful to address how substrate stimulates translocation in other ABC importers.

Author Contributions—H. B., K. D., and F. D. conceived the study. H. B. and K. D. designed, performed and analyzed the experiments. H. B., K. D., E. C., and F. G. wrote the paper. All authors analyzed the results and approved the final version of the manuscript.

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