# The Molecular Motor F-ATP Synthase Is Targeted by the Tumoricidal Protein HAMLET

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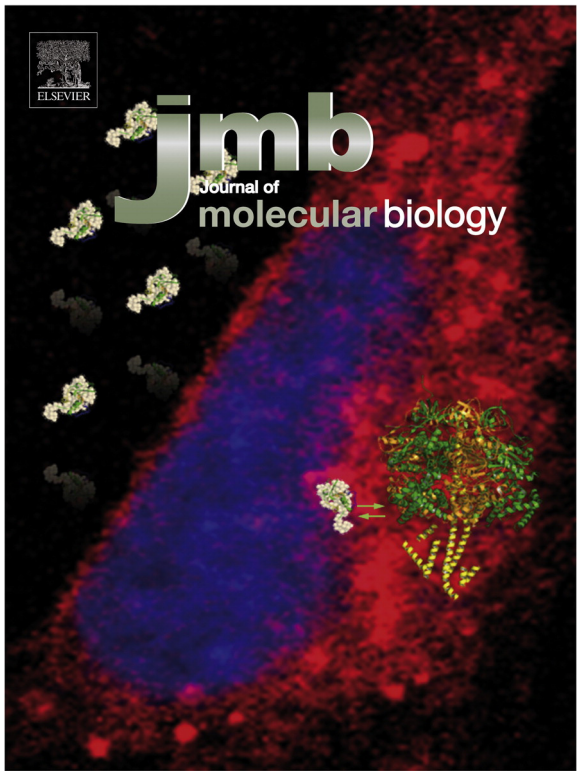
## Abstract

HAMLET (*h*uman *a*lpha-lactalbumin *m*ade *le*thal to *t*umor cells) interacts with multiple tumor cell compartments, affecting cell morphology, metabolism, proteasome function, chromatin structure and viability. This study investigated if these diverse effects of HAMLET might be caused, in part, by a direct effect on the ATP synthase and a resulting reduction in cellular ATP levels. A dose-dependent reduction in cellular ATP levels was detected in A549 lung carcinoma cells, and by confocal microscopy, co-localization of HAMLET with the nucleotide-binding subunits α (non-catalytic) and β (catalytic) of the energy converting $F_1F_0$ ATP synthase was detected. As shown by fluorescence correlation spectroscopy, HAMLET binds to the $F_1$ domain of the $F_1F_0$ ATP synthase with a dissociation constant ($K_D$) of 20.5 μM. Increasing concentrations of the tumoricidal protein HAMLET added to the enzymatically active $\alpha_3\beta_3\gamma$ complex of the F-ATP synthase lowered its ATPase activity, demonstrating that HAMLET binding to the F-ATP synthase effects the catalysis of this molecular motor. Single-molecule analysis was applied to study HAMLET–$\alpha_3\beta_3\gamma$ complex interaction. Whereas the $\alpha_3\beta_3\gamma$ complex of the F-ATP synthase rotated in a counterclockwise direction with a mean rotational rate of 3.8 ± 0.7 $s^{-1}$, no rotation could be observed in the presence of bound HAMLET. Our findings suggest that direct effects of HAMLET on the F-ATP synthase may inhibit ATP-dependent cellular processes.


***Legend:** HAMLET (beige), approaching the tumor cell and identifying the F-ATP synthase with which it associates to inhibit ATP synthesis. The structure of the $\alpha_3\beta_3\gamma$ complex of the F-ATP synthase is shown in green, orange and yellow, respectively.*

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## Introduction

HAMLET (*h*uman *a*lpha-lactalbumin *m*ade *le*thal to *t*umor cells) is a proteolipid complex of partially unfolded α-lactalbumin and several oleate residues [1]. HAMLET in solution shows a two-domain conformation with a large globular domain and an extended C-terminal part [2]. Its efficacy as a selective killer of tumor cells has been documented *in vitro* and *in vivo* in several animal models, including human brain tumor xenografts in nude rats, murine bladder cancer and colon cancer in the $APC^{Min+/-}$ mice, resembling human disease [3]. In clinical studies, HAMLET has shown therapeutic efficacy against skin papillomas and dramatic effects in bladder cancer patients [3,4]. HAMLET initiates cell death by perturbation of the plasma membrane and activation of ion fluxes and this response distinguishes tumor cells from healthy cells. The interactions of HAMLET with different cellular targets including histones H2, H3 and H4; hexokinase I; α-actinin 1/4; and proteasomal subunits have been studied [5–8]. The sensitivity of tumor cells of different origins suggests that HAMLET may act on molecular targets that are shared among tumor cells, thereby succeeding to kill those cells, rather than healthy differentiated cells with a more inert cell membrane. HAMLET is also internalized by tumor cells, changing morphology, gene expression and phosphorylation. In parallel, a reduction in the content of ATP accompanies cell death [9,10].

The enzyme catalyzing ATP synthesis is the $F_1F_0$ ATP synthase (F-ATP synthase), a membrane-bound multi-subunit complex consisting of two rotary motors in the $F_0$ and $F_1$ sector, respectively. The membrane-bound proton translocating ATP synthase catalyzes ATP synthesis and ATP hydrolysis in the $F_1$ part, which is coupled to proton translocation across the $F_0$ sector. The $F_1$ domain consists of subunits $\alpha_3\beta_3\gamma\delta\varepsilon$ [11] and the membrane-integrated $F_0$ of most bacteria is made up of subunits *a* and *b* (a:*b2*), and the *c*-ring rotor subunits, with a stoichiometry of 9–15 *c* subunits [12]. The subcomplex $\alpha_3\beta_3$ forms a hexamer with a central cavity that allows for the penetration of subunit γ. Subunits γ and ε form the soluble part of the rotor shaft, called the central stalk [13]. Rotation of the central stalk subunit γ within the $\alpha_3\beta_3$ cavity causes conformational changes in the three catalytic sites located at the α–β interfaces leading to ATP hydrolysis [14]. The two parts of the ATP synthase are connected by two stalks, that is, one central rotating shaft formed by the subunits γ and ε and a thin stalk at the periphery, composed of the subunits *b* and δ holding together the $F_1$ and $F_0$ portions [15].

Here, we demonstrate that the interaction of HAMLET with the $F_1$ sector of the F-ATP synthase results in a reduction of catalytic activity, providing one mechanism for the reduction in cellular ATP. Monoclonal antibodies against the nucleotide-binding subunits α and β of the F-ATP synthase identify the co-localization of HAMLET with the molecular

**Fig. 1.** (A) The effect of HAMLET on intracellular ATP levels of tumor cells. A549 lung carcinoma cells treated with 7, 21 or 35 μM HAMLET, which was produced as described by Hakansson *et al.* [1], showed a rapid time- and dose-dependent reduction in intracellular ATP levels. Error bars represent ± SEM (*s*tandard *e*rror of the *m*ean). The lung carcinoma cells (A549) were procured from American Type Culture Collection and were maintained in RPMI 1640 medium supplemented with 1 mM sodium pyruvate (Fisher Scientific), non-essential amino acids (1:100) (Fisher Scientific), 50 μg/ml gentamicin (Gibco, Paisley, UK) and 5% fetal calf serum (FCS). Cells were cultured at 37 °C, 90% humidity and 5% $CO_2$. Cells were grown in 96-well plates overnight (for PrestoBlue™ and ATP assays), in 6-well plates (for Western blots) and in 75-mm flasks (for immunoprecipitation). Afterwards, cells were detached from culture flasks with 10–15 ml versene (200 mg ethylenediaminetetraacetic acid dissolved in 800 ml phosphate-buffered solution (PBS) and 200 ml $H_2O$) and resuspended in RPMI 1640 complete medium with 5% FCS. Cells were seeded in a 96-well plate at a concentration of $1 \times 10^4$ cells per well, overnight at 37 °C in a humidified incubator (Hera cell 150; Heraeus). Adherent cells were washed with PBS twice and treated with different concentrations of HAMLET for different time durations. Cell culture medium was removed and cellular ATP was quantified using the ATPlite Kit (Infinite F200; PerkinElmer) according to the manufacturer's instructions. (B and C) The effects of HAMLET on the cellular localization of F-ATP synthase subunits α and β in lung carcinoma cells. (B) Suspension cells: HAMLET triggered the translocation of F-ATP synthase subunits α (top) and β (bottom) from the periphery to the cytoplasm and the nuclei. A punctate staining pattern in untreated cells was replaced by the formation of larger subunit α aggregates after HAMLET exposure (1 h). The β subunit aggregate formation was less pronounced. HAMLET showed stronger co-localization with subunit α than with subunit β. (C) Adherent cells: HAMLET caused an increase in the staining for both α and β subunits in the entire cell (see Fig. 2) and stronger co-localization (yellow) with the catalytic β subunit was observed at 21 μM HAMLET. At 35 μM, co-localization with the α and β subunits was similar. For suspension cell experiments, A549 lung carcinoma cells were allowed to partially adhere to glass slip and treated with three doses of HAMLET (7, 21 and 35 μM; 10% Alexa-HAMLET) for 1 h. For adherent cell experiments, A549 cells were grown on 8-well glass chamber slides (Lab-Tek, Chamber Slide, Thermo Fisher Scientific) at a concentration $2.5 \times 10^4$ cells per well overnight at 37 °C. Cells were washed twice with PBS and treated with HAMLET (7, 21 and 35 μM; 10% Alexa-HAMLET) for 1 h. Cells were fixed with 2% paraformaldehyde, non-permeabilized, blocked (10% FCS, 1 h), incubated with primary F-ATP synthase subunit α or β monoclonal antibodies (1:40 in 10% FCS/PBS; Life Technologies) for 2 h at room temperature, incubated with secondary Alexa-488 conjugated antibodies (1:100 in 10% FCS/PBS; Molecular Probes), counterstained with DRAQ-5 (Abcam) and examined using a LSM 510 META laser scanning confocal microscope (Carl Zeiss). Co-localization analysis and fluorescence quantification were performed using LSM510 image browser software and Photoshop CS5, respectively.

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motor F-ATP synthase *in vivo*. With the use of the enzymatic active $\alpha_3\beta_3\gamma$ complex of the thermophilic *Bacillus* PS3 F-ATP synthase, the quantitative and qualitative interaction of both proteins was investigated, and a mechanistic model of HAMLET–F-ATP synthase assembly is proposed.

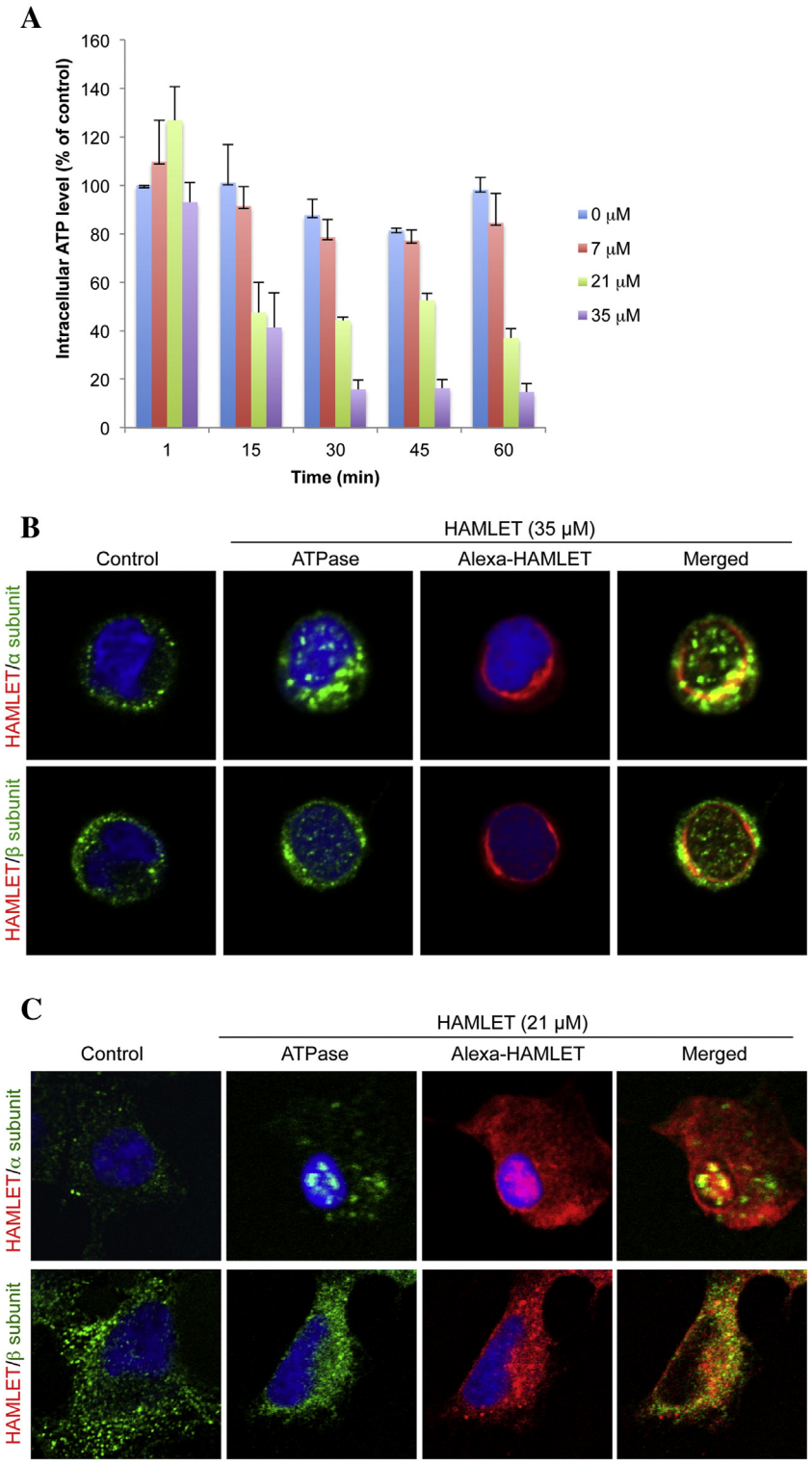


**Fig. 1** (*legend on previous page*).

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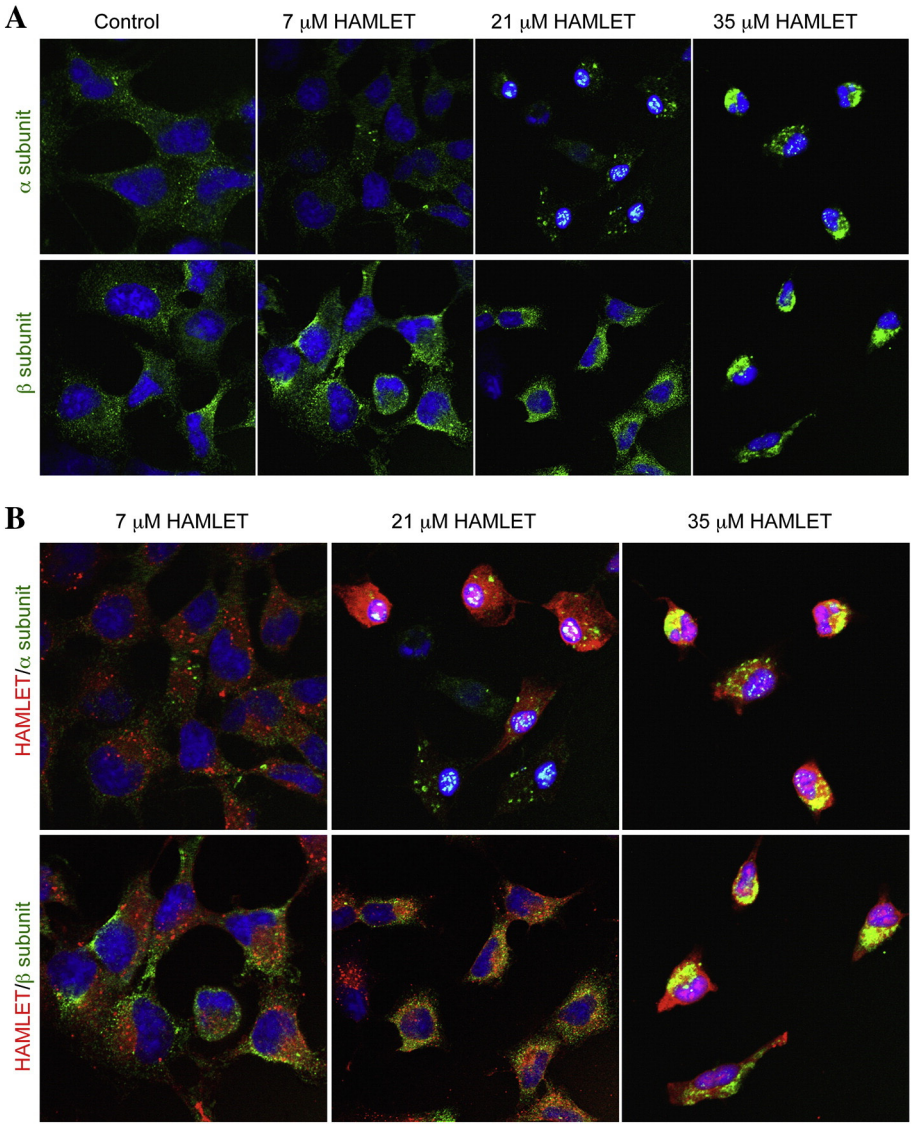


**Fig. 2.** The effects of HAMLET on the cellular localization of F-ATP synthase subunits α and β in adherent lung carcinoma cells. (A) HAMLET triggered a concentration-dependent change in F-ATP synthase subunit α and β staining from a punctate cytoplasmic staining pattern in untreated cells to the formation of larger aggregates for α subunit and occasional smaller aggregates for β subunit in cell nucleus after HAMLET exposure (7, 21 or 35 μM, 1 h). (B) Stronger co-localization (yellow) with the catalytic β subunit was observed at 21 μM HAMLET. At 35 μM, co-localization with the α and β subunits was similar.

## Results and discussion

### Reduction in intracellular ATP levels

The effect of HAMLET on intracellular ATP levels was quantified in A549 lung carcinoma cells. A dose-dependent reduction was observed after 15 min. At 35 μM HAMLET, the ATP level was reduced to about 40% of untreated cells after 15 min, and ATP levels remained low from 30 min onwards (15% of control; Fig. 1A). At 21 μM HAMLET, the ATP level was reduced to about 45% after 15 min and was further reduced to 35% after 60 min, as compared to control. Lower concentrations of HAMLET (7 μM) had a modest effect (about 20% control).

### HAMLET co-localizes with the F-ATP synthase

The enzyme responsible for ATP synthesis in eukaryotic cells is the $F_1F_0$ ATP synthase, with the alternating nucleotide-binding subunits α and β forming a hexameric headpiece of the $F_1$ part. The interface of each α–β pair forms the nucleotide-binding sites. Besides subunit *c* of the $F_0$ part, which

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is membrane embedded, both subunits α and β show the highest levels of homology among F-ATP synthases. To examine if HAMLET affects the cellular distribution of $F_1F_0$ ATP synthase, we stained Alexa-HAMLET-treated A549 cells with monoclonal antibodies directed against subunits α and β from human mitochondrial F-ATP synthase. A rapid increase in staining was observed by confocal microscopy of cells in suspension (Fig. 1B). HAMLET triggered a change in F-ATP synthase subunit α staining (Fig. 1B, top) from a punctate, peripheral α subunit staining pattern in untreated cells to the formation of larger aggregates after HAMLET exposure (15 min). At a HAMLET concentration of 35 μM, nuclear staining was observed. To further address if HAMLET is localized in the same cellular compartments as the F-ATP synthase subunits, co-localization of the α and β subunits with Alexa-HAMLET was investigated by confocal microscopy and weak co-localization was observed (Fig. 1B, right panel). The β subunit showed a similar translocation from the cell periphery but aggregate formation was less pronounced. Cytoplasmic and nuclear aggregates were formed at 35 μM HAMLET. Co-localizations of Alexa-HAMLET with the two subunits were observed at the perinuclear and the cytoplasmic region where HAMLET accumulation was the strongest.

The experiment was extended to include adherent cells (Figs. 1C and 2). HAMLET triggered a concentration-dependent change in F-ATP synthase subunit α and β staining from a punctate cytoplasmic staining pattern in untreated cells to the formation of larger aggregates for subunit α and

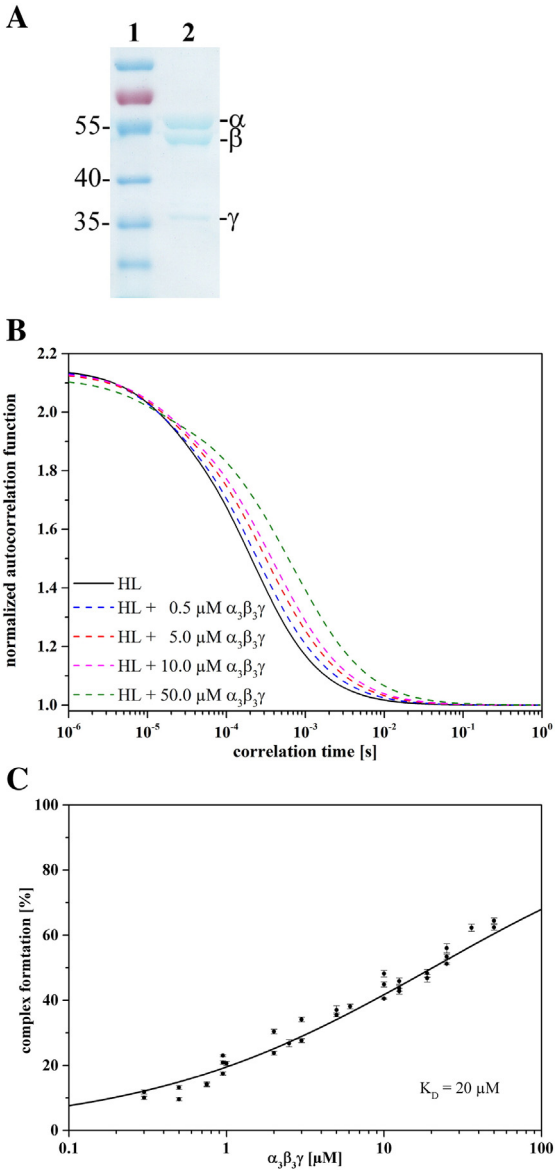


**Fig. 3.** $TF_1$–HAMLET binding studied by fluorescence correlation spectroscopy. (A) SDS-PAGE of purified $\alpha_3\beta_3\gamma$ complex of the F-ATP synthase from thermophilic *Bacillus* PS3 (lane 2) and a molecular weight marker (lane 1). (B) Normalized autocorrelation functions of the $\alpha_3\beta_3\gamma$ complex and HAMLET-Atto647N (HL) obtained by increasing the quantity of the $\alpha_3\beta_3\gamma$ complex (from left to right: 0 μM, 0.5 μM, 5 μM, 10 μM and, 50 μM). (C) Concentration-dependent binding of $\alpha_3\beta_3\gamma$ to HAMLET. The percentage of complex formation for each concentration was calculated using a two-component fitting model. The binding constant, $K_D$, was derived by fitting the data with the Hill equation. For the fluorescence correlation spectroscopy experiments, the cysteine in subunit γ of $\alpha_3\beta_3\gamma$ was labeled with Atto647N. The free dye was removed by washing the sample in buffer A [20 mM Mops (pH 7.0), 50 mM KCl and 5 mM $MgCl_2$], followed by centrifugation in a centrifugal filter column (exclusion size of 100 kDa; Centricon, Millipore) for at least three times. In case of HAMLET, lyophilized HAMLET was resuspended in 250 μl of buffer B [50 mM Tris (pH 7.5) and 250 mM NaCl] to a final concentration of 50–100 μM. Atto647N-maleimide (ATTO-TEC) was added in a protein:dye ratio of 1:0.9 and incubated for 5 min on ice in the dark, before inactivation of the maleimide moiety by adding 10 mM DTT. The reaction time and labeling ratio were kept low to avoid double labeling. The labeled protein was separated from free dye by gel filtration via a S75 column (GE Healthcare) with buffer C. Measurements were performed on a LSM510 Meta/ConfoCor 3 microscope (Carl Zeiss, Germany) with a water immersion objective (40×/1.2W Corr UV-VIS-IR; Zeiss) and the 633 nm line of a 5 mW HeNe633 laser. Samples (in buffer A) of 15 μl were placed in Nunc 8-well chambers treated with 3% gelatin to prevent unspecific binding of proteins [20]. Cy5 in water was used as references for the calibration of the confocal microscope. The fluorescence intensities of fluorescent particles in the confocal volume (HAMLET-Atto647N and $\alpha_3\beta_3\gamma$-Atto647N) were measured at 25 °C for up to 10 min with 10-s repetitions. From the fit of the autocorrelation function, the number of particles in the confocal volume, the diffusion times of fluorescent particles, the intrinsic triplet state of the dye and the percentage of complex formation were derived.

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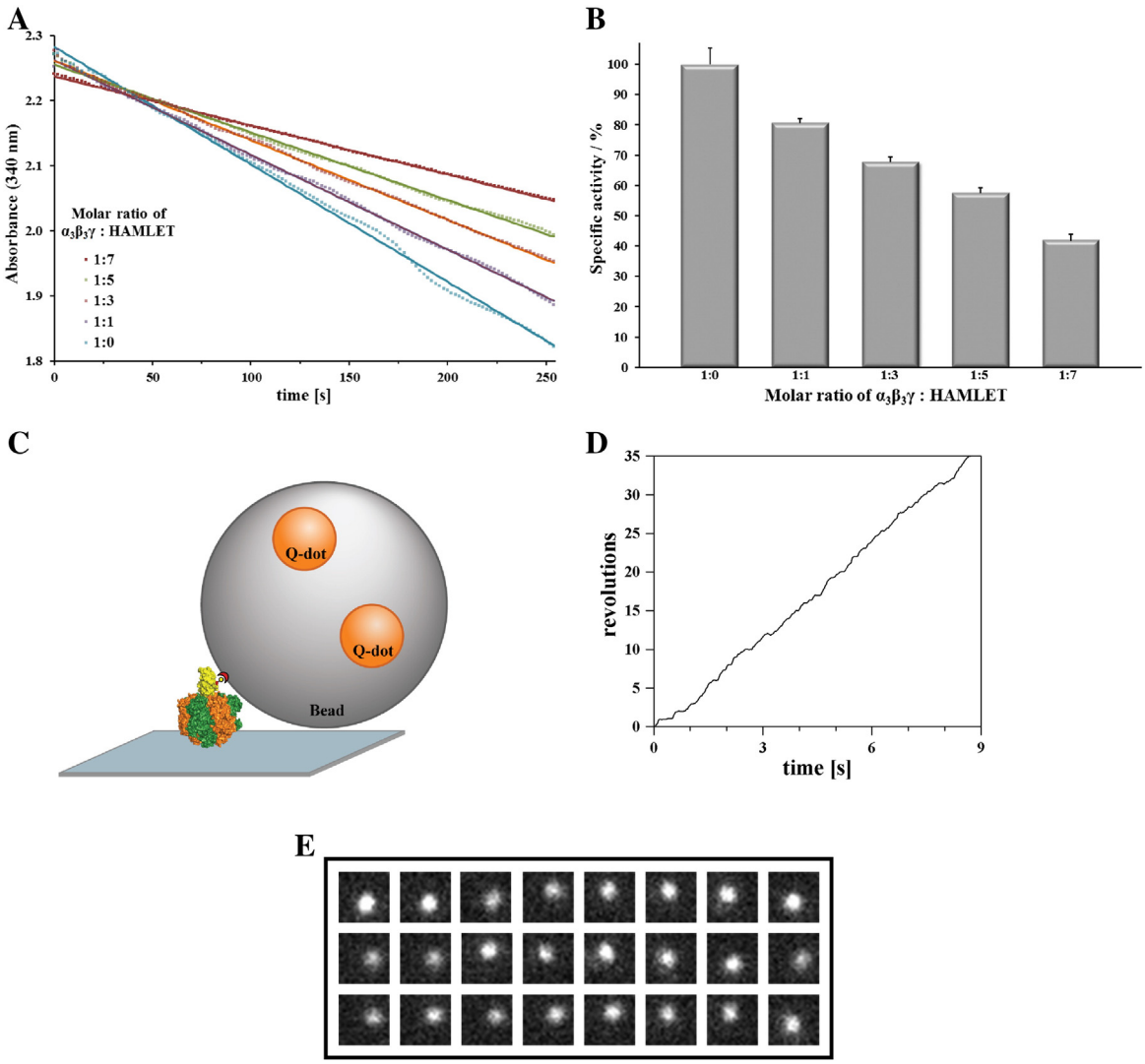


**Fig. 4.** HAMLET effects ATPase hydrolytic activity and the rotary motion. (A and B) Dose-dependent decrease in the specific ATPase activity of $\alpha_3\beta_3\gamma$ after incubation with HAMLET. A continuous ATP hydrolysis assay was applied to measure the specific activity of $\alpha_3\beta_3\gamma$. In this assay, ATP was constantly regenerated by an enzymatic reaction, while the consumption of NADH was detected at a wavelength of 340 nm. The change in absorbance was measured for 250 s in 2 s intervals at 37 °C after adding 10 μg of $\alpha_3\beta_3\gamma$ to 1 ml reaction solution [25 mM Hepes (pH 7.5), 25 mM KCl, 5 mM $MgCl_2$, 5 mM KCN, 2 mM phosphoenolpyruvate, 2 mM ATP, 0.5 mM NADH, 30 U L-lactic acid dehydrogenase and 30 U pyruvate kinase], and its activity was derived by fitting the linear part of the slope. (C) Experimental setup for the single-molecule rotation assay of recombinant $\alpha_3\beta_3\gamma$ complex. The enzyme was fixed to a Ni-NTA-coated cover slide with $His_{10}$ tag at the N-termini of the β subunits. The engineered cysteine at γ107 was labeled with biotin-maleimide to bind a streptavidin-coated bead (Ø = 0.3 μm) doped with two biotinylated quantum dots 605. An inverted fluorescence microscope (Cell^TIRF; Olympus, Japan), with an oil immersion objective (PlanApo 100×/1.49 oil), was equipped with an Orca Flash-4.0 CMOS camera (Hamamatsu, Japan) to record moving protein–bead complexes. Quantum dots were excited using a 491-nm diode laser in total internal reflection fluorescence mode. Videos of rotating single molecules were recorded on a connected computer system with a frame rate of 100 frames per second at a resulting magnification of 65 nm/pixel and analyzed using customized software to obtain the angular orientation of the bead in each frame. (D) Trajectory of a rotating $\alpha_3\beta_3\gamma$–bead complex with a rotational rate of 4 rotations per second. (E) Sequence of single video frames (30 ms per frame) showing the counter clockwise rotation of a single $\alpha_3\beta_3\gamma$–bead complex. Each frame has a resolution of 20 pixel × 20 pixel with 65 nm/pixel.

occasional smaller aggregates for subunit β in the cell nucleus after HAMLET exposure (1 h; Fig. 2). This effect was dose dependent and aggregates were located in the cytoplasm and perinuclear area. Strong co-localization was observed in the nucleus for the α subunit, while the catalytic β subunit showed moderate co-localization in the perinuclear region at 21 μM HAMLET. Strong cytoplasmic co-localization with the

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catalytic β subunit was observed at 21 μM HAMLET. Our observations are consistent with earlier studies in which F-ATP synthase subunits were also localized at cellular compartments distinct from the inner membrane of mitochondria [16,17]. Furthermore, ectopic cell surface F-ATP synthase has been shown to be a receptor for angiostatin [18]. In addition to the existing knowledge, our present findings on the drastic change in localization of F-ATP synthase subunits, from a punctate cell surface staining pattern to a cytoplasmic pattern with nuclear aggregates, might suggest a scenario whereby the motor is uncoupled from the proton gradient, leading to an inhibition of the ATP synthesis process. The outcome of this is evident as a rapid reduction in intracellular ATP level was observed after HAMLET treatment.

### Quantitative and qualitative binding of HAMLET to the $F_1$ domain of F-ATP synthase

To examine the hypothesis that HAMLET inhibition causes a malfunctioning F-ATP synthase, we applied fluorescence correlation spectroscopy to confirm and quantify the interaction of HAMLET with the F-ATP synthase. We used the mechanistically best understood F-ATP synthase from thermophilic *Bacillus* PS3 as a prototype. The enzymatically active $\alpha_3\beta_3\gamma$ complex of the $F_1F_0$ ATP synthase ($TF_1$) was purified as previously described (Fig. 3A and see Ref. [19]). Both HAMLET (14.1 kDa) and the $\alpha_3\beta_3\gamma$ complex (352 kDa) were labeled with Atto647N-maleimide and their individual diffusion times were determined from a single-component fit of the resulting autocorrelation function to be 209 μs and 440 μs, respectively, which correlate well with their molecular size. Next, we measured the diffusion time of HAMLET-Atto647N after addition of increasing concentrations of unlabeled $\alpha_3\beta_3\gamma$ complex. In these experiments, the autocorrelation function was fitted with a two-component fit, where the diffusion time of the smaller component was fixed to 209 μs. We observed that, with increasing concentrations of $\alpha_3\beta_3\gamma$, an increasing fraction of HAMLET-Atto647N showed a diffusion time of about 700 μs, which was attributable to the binding of HAMLET to the $\alpha_3\beta_3\gamma$ domain (Fig. 3B). These data proved that HAMLET-Atto647N indeed binds to the $F_1$ domain of the F-ATP synthase. Figure 3C shows the ratio of the formed complex depending on the $\alpha_3\beta_3\gamma$ complex concentration in the range from 0.3 to 50 μM $\alpha_3\beta_3\gamma$. From the fit with the Hill equation, a dissociation constant ($K_D$) of 20.5 μM was determined.

### HAMLET-F-ATP synthase interaction decreases ATPase activity

The data presented here raise the question whether the HAMLET-F-ATP synthase interaction causes enzymatic alterations in the F-ATP synthase. In order to address this question, we have studied the effect of HAMLET to F-ATP synthase binding by performing an NADH-coupled ATP hydrolysis assay with the $\alpha_3\beta_3\gamma$ complex in the presence and absence of HAMLET (Fig. 4A). The $\alpha_3\beta_3\gamma$ complex was incubated with different molar ratios of HAMLET at 37 °C. As a positive control, we used the $\alpha_3\beta_3\gamma$ domain, while HAMLET alone served as a negative control. Depending on the incubation time, the $\alpha_3\beta_3\gamma$ complex alone showed a specific activity of around 4.0 U/mg, which we set to 100%, and we calculated the specific activity of HAMLET that inhibited $\alpha_3\beta_3\gamma$ accordingly. As shown in Fig. 4A, HAMLET alone did not show any hydrolytic activity. In comparison, a dose-dependent inhibition of the hydrolytic activity of $\alpha_3\beta_3\gamma$ reaching more than 55% inhibition at 7-fold molar excess of HAMLET was observed (Fig. 4B).

### Mechanistic implications of HAMLET–F-ATP synthase interaction

The F-ATP synthase is made up of two motors: the membrane-embedded $F_0$ motor, responsible for ion translocation, and the $F_1$ motor, whose movements are coupled by the central stalk subunit γ [15]. To further confirm the enzymatic effect of HAMLET on the F-ATP synthase and to gain insight into a possible mechanistic event, we tested the effects of HAMLET in a single-molecule rotation assay. Single molecules of the enzymatically active $\alpha_3\beta_3\gamma$ complex were attached via the N-terminal His tags in the three β subunits to a Ni-NTA-covered cover slip as described previously (Fig. 4C and see Ref. [21]). On the opposite end, an engineered cysteine in the γ subunit was biotinylated in order to attach a streptavidin coated bead to the protein complex. The bead was further doped with biotinylated quantum dots to visualize its movement in an inverted fluorescence microscope. Upon addition of a saturating ATP concentration (4 mM), some beads started to rotate counterclockwise (when viewed from the membrane side), indicating that $\alpha_3\beta_3\gamma$ is hydrolyzing ATP. We actively scanned the cover slide for rotating enzyme–bead complexes and found on average one rotating bead in 7 min with a mean rotational rate of 3.8 ± 0.7 rotations per second. In some cases, when a 0.6-μm bead duplex was attached to the protein, the mean rotational rate dropped to 1.8 ± 0.4 rotations per second by 50% due to a higher hydrodynamic friction of the bead duplex. These results are inline with the rotational rate Sakaki *et al.* [21] found previously for a rotating $\alpha_3\beta_3\gamma$ complex at saturating ATP concentration (about 3 rotations per second for a 0.49 μm bead duplex). Occasionally, the complexes stopped rotating for a few seconds. Instead, they were fluctuating around a certain position with a mean angular distribution of 41° ± 10°, as if they were inhibited by Mg-ADP [15,22]. The trajectory of a rotating enzyme–bead complex

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is given in Fig. 4D, while Fig. 4E shows a sequence of 24 frames of the rotating complex (the whole video sequence is provided as Supplementary Movie S1).

In another set of experiments, $\alpha_3\beta_3\gamma$ was incubated with HAMLET in a ratio of 1:10 before it was used in the rotation assay. Under this experimental condition, the time course did not show any clear unidirectional rotation in a counterclockwise direction as observed for the $\alpha_3\beta_3\gamma$ complex alone (see above). Despite intensively scanning for rotating beads, no rotating complex was found within 45 min of total searching time. These results reveal that the rotation in the $\alpha_3\beta_3\gamma$ part is blocked due to HAMLET binding and that HAMLET is influencing the catalytic process of ATP hydrolysis of the F-ATP synthase motor protein.

## Conclusions

The present study reports qualitative and quantitative studies demonstrating the direct binding between HAMLET and the $F_1$ domain of the F-ATP synthase and functional consequences of this interaction. The HAMLET–F-ATP synthase association reduces enzymatic activity and rotary motion of the motor protein F-ATP synthase. Being the key enzyme in the process of oxidative phosphorylation, a reduction in the catalytic activity of the F-ATP synthase inhibits ATP formation and reduces cellular ATP levels. As glycolysis, which tumor cells are heavily dependent on, is driven by ATP in the first rate-limiting step, a reduced F-ATP synthase function caused by HAMLET is likely to impair glycolysis and thereby drives the energy-deprived tumor cell to their death.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2015.01.024.

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