

ORIGINAL ARTICLE

Targeting of nucleotide-binding proteins by HAMLET—a conserved tumor cell death mechanism

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HAMLET (Human Alpha-lactalbumin Made LEthal to Tumor cells) kills tumor cells broadly suggesting that conserved survival pathways are perturbed. We now identify nucleotide-binding proteins as HAMLET binding partners, accounting for about 35% of all HAMLET targets in a protein microarray comprising 8000 human proteins. Target kinases were present in all branches of the Kinome tree, including 26 tyrosine kinases, 10 tyrosine kinase-like kinases, 13 homologs of yeast sterile kinases, 4 casein kinase 1 kinases, 15 containing PKA, PKG, PKC family kinases, 15 calcium/calmodulin-dependent protein kinase kinases and 13 kinases from CDK, MAPK, GSK3, CLK families. HAMLET acted as a broad kinase inhibitor *in vitro*, as defined in a screen of 347 wild-type, 93 mutant, 19 atypical and 17 lipid kinases. Inhibition of phosphorylation was also detected in extracts from HAMLET-treated lung carcinoma cells. In addition, HAMLET recognized 24 Ras family proteins and bound to Ras, RasL11B and Rap1B on the cytoplasmic face of the plasma membrane. Direct cellular interactions between HAMLET and activated Ras family members including Braf were confirmed by co-immunoprecipitation. As a consequence, oncogenic Ras and Braf activity was inhibited and HAMLET and Braf inhibitors synergistically increased tumor cell death in response to HAMLET. Unlike most small molecule kinase inhibitors, HAMLET showed selectivity for tumor cells *in vitro* and *in vivo*. The results identify nucleotide-binding proteins as HAMLET targets and suggest that dysregulation of the ATPase/kinase/GTPase machinery contributes to cell death, following the initial, selective recognition of HAMLET by tumor cells. The findings thus provide a molecular basis for the conserved tumoricidal effect of HAMLET, through dysregulation of kinases and oncogenic GTPases, to which tumor cells are addicted.

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INTRODUCTION

Nucleotide recognition and release of inorganic phosphate ensures that energy is transferred to effector molecules necessary for cellular life.¹ Nucleotide-binding proteins include the large families of >500 kinases, G proteins, ATPases, reductases and other transferases, which share conserved nucleotide-binding motifs for ATP and GTP.^{2–4} By ATP hydrolysis, a wide range of downstream effects is achieved including phosphorylation and direct activation of ATP-dependent binding partners. Kinases are crucial for energy transfer through the binding of ATP and transfer of phosphate to specific substrates.¹ Nucleotide binding and activation triggers a specific conformational change, allowing the activation segment to bind and phosphorylate its substrates. A similar mechanism is responsible for the hydrolysis of GTP by GTPases, such as the Ras protein family, and dysregulation of kinase/GTPase homeostasis may contribute to pathology.⁵ For example, the enhanced proliferation of tumor cells is attributed to the so-called ‘pathogenic kinases’, which have been targeted by novel therapeutics.^{1,6}

HAMLET (Human Alpha-lactalbumin Made LEthal to Tumor cells) is a proteolipid complex of partially unfolded α -lactalbumin and several oleate residues.^{7,8} 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.^{9–11} In clinical studies, HAMLET has shown therapeutic efficacy against skin papillomas and dramatic

effects in bladder cancer patients.^{12,13} 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. Such mechanisms include perturbation of the plasma membrane and activation of ion fluxes and this response distinguishes tumor cells from healthy cells.¹⁴ Previously identified cellular targets for HAMLET include histones H3, H4 and H2, Hexokinase I, α -Actinin 1/4 and proteasomal subunits, but shared features explaining how HAMLET exerts its broad tumoricidal effect have remained elusive.^{15–18}

The aim of this study was to identify conserved molecular motifs targeted by HAMLET and to examine if their interaction to HAMLET might explain the ability of HAMLET to kill tumor cells of diverse origins. Using a protein microarray,¹⁹ we show that HAMLET targets protein families involved in energy metabolism and cellular homeostasis including ATPases, kinases and small GTPases. We demonstrate, in an *in vitro* kinase activity assay, that ~70% of kinases are inhibited by HAMLET and confirm kinase inhibition in HAMLET-treated cells by a phosphorylation antibody microarray. Furthermore, we show that HAMLET inhibits the Ras pathway and specifically Braf, which belongs to the ‘pathogenic kinases’. The results identify HAMLET as a new type of antagonist for nucleotide-binding proteins and suggest that the rapid death response of tumor cells to HAMLET reflects these effects on the ATPase/kinase/GTPase machinery.

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RESULTS

HAMLET interacting proteins identified by protoarray

The molecular basis of HAMLET's tumoricidal activity was approached by *in vitro* ProtoArray technology. Target proteins were detected by fluorescence after addition of AlexaFluor568-labeled HAMLET (5 and 50 ng/μl, in duplicate) to arrays spotted with more than 8000 human proteins (Figure 1a). To identify relevant HAMLET interacting proteins, we selected three variables: Fold changes (FCs) over the mean of all negative control values (M1), FCs over individual negative controls (M2) and Z-scores > 2.0 (M3, Figure 1b). The three methods produced overlapping results; 90% of the proteins identified by the Z-scores were also identified by the other methods, indicating that the HAMLET targets were

insensitive to the algorithms used. A correlation analysis of FC further excluded selection bias (Figure 1c, $R^2 = 0.95$; Supplementary Figure S1, $R^2 = 0.22$).

Nucleotide-binding domains were identified by DAVID in ~35% of all HAMLET targets, in combination with Uniprot. On the basis of the M1 analysis, 28.55% were ATP-binding and 6.45% were GTP-binding proteins (Figure 1d). HAMLET targets among ATP-binding proteins include ATPases such as the B1, noncatalytic subunit and G1 subunit of the V1-ATPase, which hydrolyzes ATP for lysosomal proton transport. HAMLET also interacts with ATP-binding cassette subfamily B member 7, which has a central role in the maturation of cytosolic iron-sulfur cluster-containing proteins. P-type ATPase (Na^+, K^+ -ATPase) and F-ATPase subunits present in the protoarray screen showed no significant binding on the basis of the three

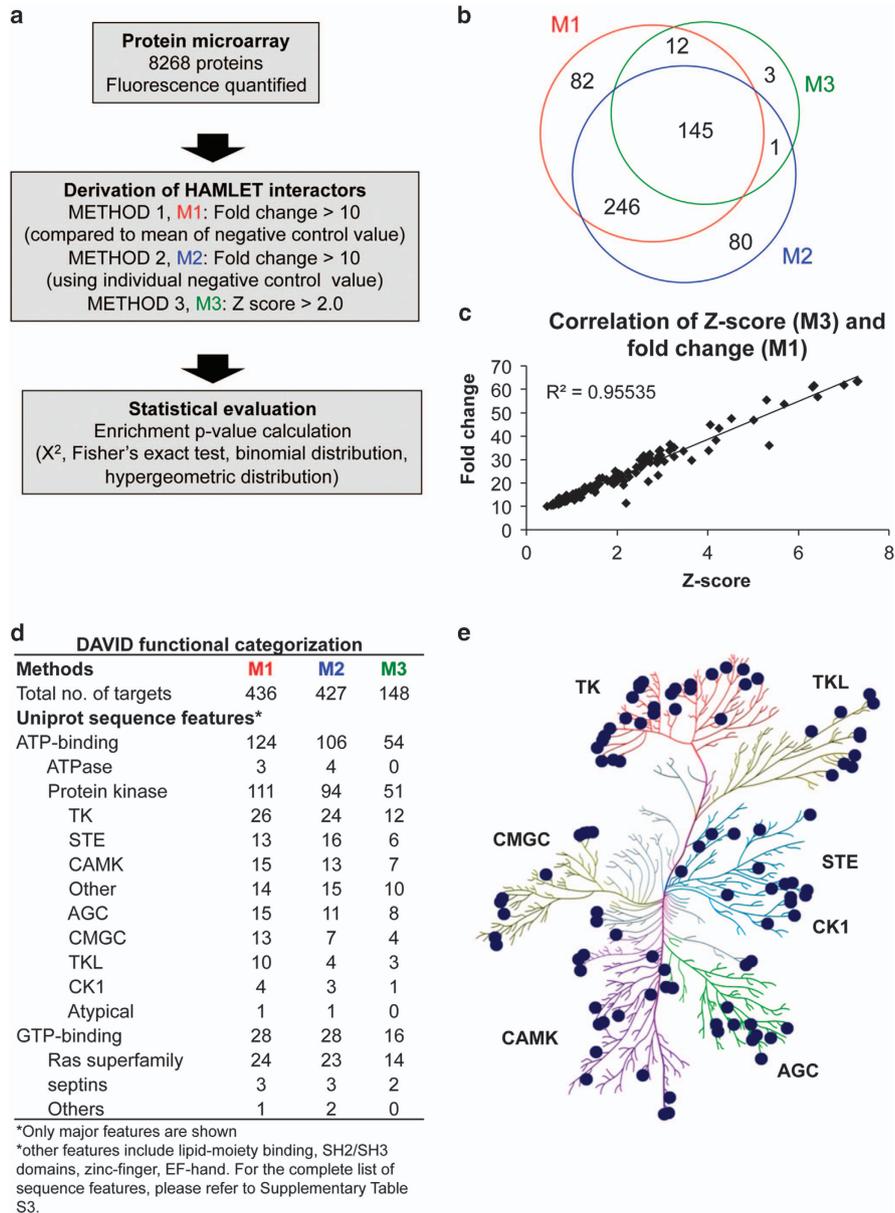


Figure 1. Identification of nucleotide-binding proteins as HAMLET targets. **(a)** Schematic workflow used to identify HAMLET targets from the ProtoArray. Binding is determined using three cut-offs (M1, M2 and M3) and the enrichment of protein cluster-based sequence features is evaluated using four statistical methods. **(b)** Venn diagram shows the large overlap of protein targets derived from the different methods. **(c)** Z-scores (M3) is highly correlated to fold change (M1). **(d)** Nucleotide-binding proteins are enriched independent of the algorithms used to derive the target protein list. The enrichment of ATPases, kinases and GTPases was highly significant ($P < 0.0001$, Chi-square and Fischer's exact test, binomial and hypergeometric distributions). Data obtained by M1 was used for the following investigations. **(e)** HAMLET binds kinases in all branches of the human Kinome tree (blue dots).

methods used in the current study. In addition, HAMLET also interacts with eight groups of typical kinases and atypical kinases.

The GTP-binding proteins were nearly all in the Ras superfamily with members from the Ras, Rheb, Arf, Rap and Rab subfamilies (Figures 2a and b, Supplementary Tables S2 and S3).

HAMLET targets were present in all branches of the Kinome tree (Figure 1e and Supplementary Table S1), including 26 tyrosine kinases, 10 tyrosine kinase-like kinases, 13 homologs of yeast sterile 7, sterile 11, sterile 20 kinases, 4 casein kinase 1 kinases, 15 containing PKA, PKG, PKC family kinases, 15 calcium/calmodulin-dependent protein kinase kinases and 13 kinases containing CDK, MAPK, GSK3, CLK families.

Broad-spectrum inhibition of individual kinases by HAMLET

To determine whether HAMLET affects kinase activity, phosphate release by individual kinases was quantified in a high-throughput HotSpot radiometric assay.²⁰ A total 347 wild-type kinases were included, as well as 93 mutant-, 19 atypical and 17 lipid kinases. A pilot study was conducted to determine a suitable screening concentration of HAMLET, using one representative member from each kinase group (Braf, Wee1, Egfr, EphA3, Aurka, Nek3, Bmpr1A). Serial dilutions of HAMLET were added to each kinase and the kinase activity was quantified as the release of $\gamma^{33}\text{P}$ from ATP. Staurosporine or kinase-specific inhibitors (LDN193189, GW5074 and Wee1) were included as positive controls.

HAMLET inhibited all kinases in the pilot study (Figures 3a and b, Supplementary Figures S2A and B). Compared with staurosporine, the IC_{50} values were two to three orders of magnitude higher for HAMLET (Aurka, Egfr, EphA3) but HAMLET was a more effective inhibitor of NEK3 (Figure 3b). The specific inhibitors of Bmpr1A, Braf and Wee1 were more efficient than HAMLET and with the exception of Wee1 and Bmpr1A, the HAMLET inhibition curves deviated from a theoretical Hill slope of 1.0, which characterized most of the controls (staurosporine, Wee1 inhibitor, Braf inhibitor). On the basis of this analysis, a concentration of 21 μM , close to the mean of the IC_{50} s in the initial screen, was selected for kinase inhibition in the high-throughput assay comprising 476 kinases.

HAMLET inhibited 69% of all kinases tested, using a cut-off of $\geq 20\%$ inhibition. Inhibition was observed in all branches of the Kinome tree, suggesting that HAMLET does not target-specific groups of kinases (Figure 3d). Some kinases were completely inhibited, including CDK4/cyclin D1 involved in cell cycle regulation, STK39/STLK3 activating the p38 MAP kinase pathway during cell stress response and MST4, which is involved in apoptosis. In addition, 31 kinases (7%) showed enhanced activity ($\geq 120\%$) relative to DMSO controls. PKN2/PRK2 and DAPK1 (death-associated protein kinase 1) showed greatly enhanced activity (385 and 1204%, respectively, Figures 3c and d). Protein Kinase N2 (PKN2) has an important role in regulating the cell cycle progression and inhibits Akt pro-survival-induced kinase activity. DAPK1 is a novel tumor-suppressor protein and a positive mediator of apoptosis.

Interestingly, kinase inhibition by HAMLET *in vitro* did not correlate with the IC_{50} values of staurosporine, which has a strong affinity for the ATP-binding site, suggesting that the broad inhibitory effect of HAMLET does not solely reflect affinity for the nucleotide-binding domain (Supplementary Figure S2C). Furthermore, Staurosporine pre-treatment of lung carcinoma cells (10 μM) did not alter the death response to HAMLET (Supplementary Figure S2D).

HAMLET perturbs kinase phosphorylation in tumor cells, affecting multiple signaling pathways

The effect of HAMLET on cellular kinase activity was quantified by microarray, using antibodies to 337 phosphorylated substrates (signal transduction protein profiling). Lysates from untreated and HAMLET-treated A549 lung carcinoma cells (21 μM HAMLET, 1 h)

were compared and kinase activity was quantified as the change in phosphorylation of relevant substrates. The effect on each phosphorylation site was defined as either 'activating' or 'inhibitory' based on a $\pm 20\%$ FC cut-off, compared with untreated cells. The results were interpreted in comparison with existing experimental data, using PhosphoNET, the curated database of Kinexus. About 17% of the substrates showed a decrease in phosphorylation ($n = 57$) and 22% showed an increase ($n = 76$). For the majority of substrates (61%), the level of phosphorylation was unchanged (Supplementary Figure S3A).

The results were further evaluated in a recently constructed activity-based phosphorylation network in human cells, representing 4417 kinase-to-phosphorylation site relationships, which connect 230 kinases to 652 substrates *in vivo*.²¹ The group of proteins with changed phosphorylation levels in response to HAMLET exhibited limited edges, indicating that these proteins were involved in distinct signaling cascades (average number of neighbors = 0.6, Supplementary Figure S3B). By mapping the group of proteins onto the complete human phosphorylation network, it was predicted that 40% of the nodes would be either up- or downregulated (average number of neighbors = 5.3, Figure 4a). Major nodes with > 10 connections included RAF1, p38alpha MAPK14, MAPK8 and MAPKAPK2, ribosomal proteins S6KA1, S6KB1 and S6KA5 and casein kinase 2A1, further confirming previous studies, which identified the MAPK and p38 pathways as targets in HAMLET-treated tumor cells.¹⁴

Implications for cancer-related networks

Changes in phosphorylation were used to predict which signaling networks were perturbed by HAMLET. By pathway analysis, HAMLET showed effects on multiple targets in the 'molecular mechanism of cancer' network. Inhibited kinases were predicted to affect the PI3K/Ras/Raf/MEK/ERK, PI3K/AKT, p38 MAPK and JNK pathways. Pathways involving CTNBN1, SMAD and Rb were activated (Supplementary Figure S4). Furthermore, HAMLET showed an overall inhibitory effect on ErbB-, ERK/MAPK-, renin-angiotensin-, UVC-induced MAPK and IL-3 signaling, consistent with a previously identified shift from ERK to p38 MAPK signaling in HAMLET-treated tumor cells.¹⁴ By modifying the canonical ERK/MAPK signaling pathway, the signaling cascade transduced from Ras was shown to be modified by HAMLET, suggesting that Ras is a critical effector of this response (Figure 4b).

The Ras GTPase protein family as HAMLET targets

Ras acts as a master regulator of multiple signaling cascades that were modified by HAMLET in tumor cells. Remarkably, 18 independent RAS-related proteins with high sequence and structural homology, bound HAMLET with high affinity (Figures 2a and b and Supplementary Table S3). The high affinity binding of HAMLET to Kras, Hras, Rab3C, Arl5a and Rasgrp3 in the protoarray (Figure 2b) was confirmed by dot blots (Figure 2c). In addition, HAMLET interacted directly with Ras and Braf as shown by co-immunoprecipitation of total cell lysates (Figure 2d).

By confocal microscopy, membrane-associated foci of Ras and HAMLET were detected at the plasma membrane after 10–15 min (Figure 2e). Colocalization of Alexa-HAMLET with four of the targets (Ras, RasL11B, Rap1B and Raf1) was prominently visible at or near the cytoplasmic leaflet, suggesting that HAMLET interacts with members of the Ras functional cluster in tumor cell membranes. The membrane accumulation of Ras was confirmed by GFP-Ras overexpression (Figure 5a). A549 lung carcinoma cells were transiently transfected with the GFP-Ras expressing plasmid RG216409, using pCMV6-AC-GFP encoding GFP as a control. In response to HAMLET treatment (35 μM , 4.5 min), GFP-Ras accumulated in membrane blebs of the transfected cells and remained there for 15–30 min.

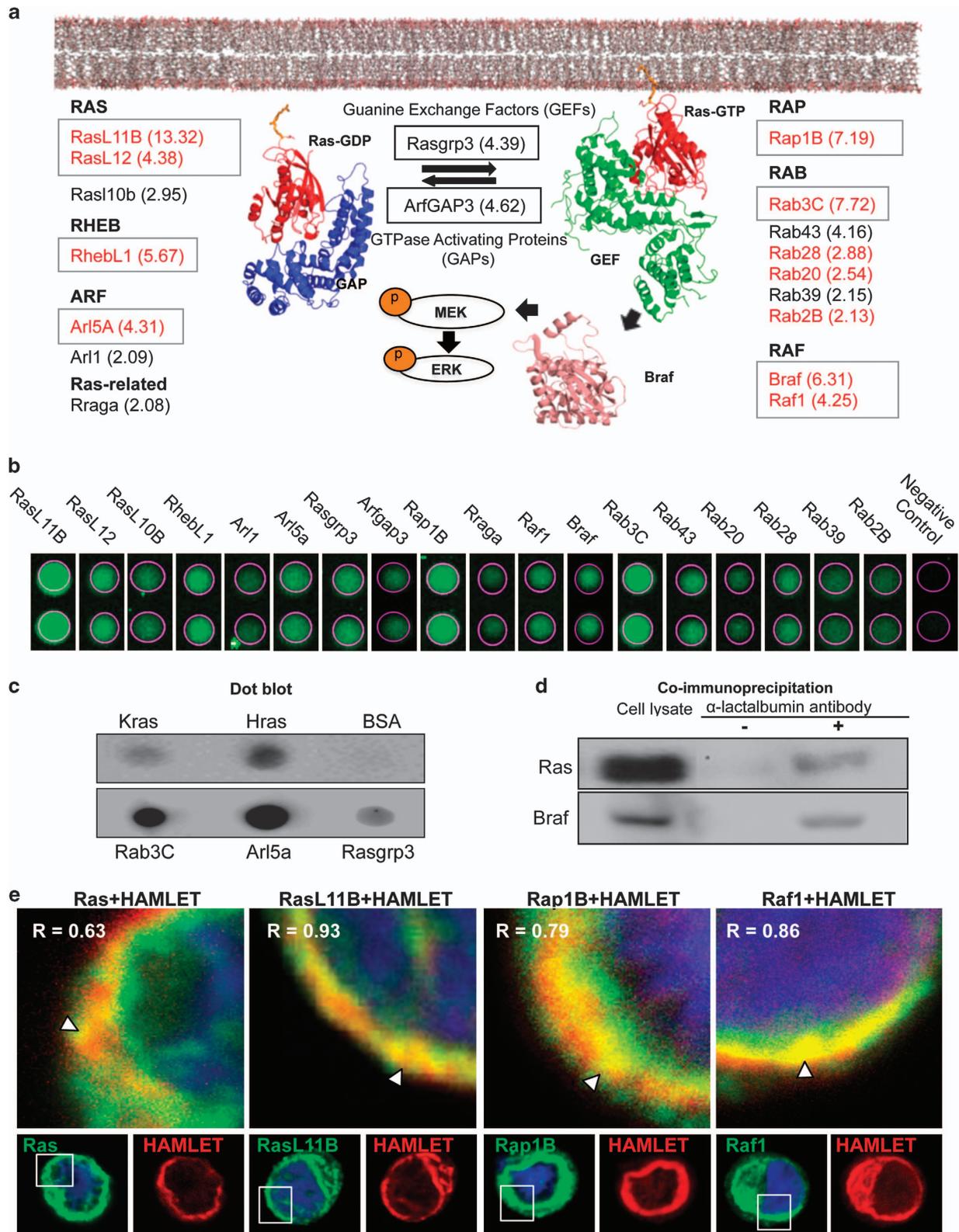
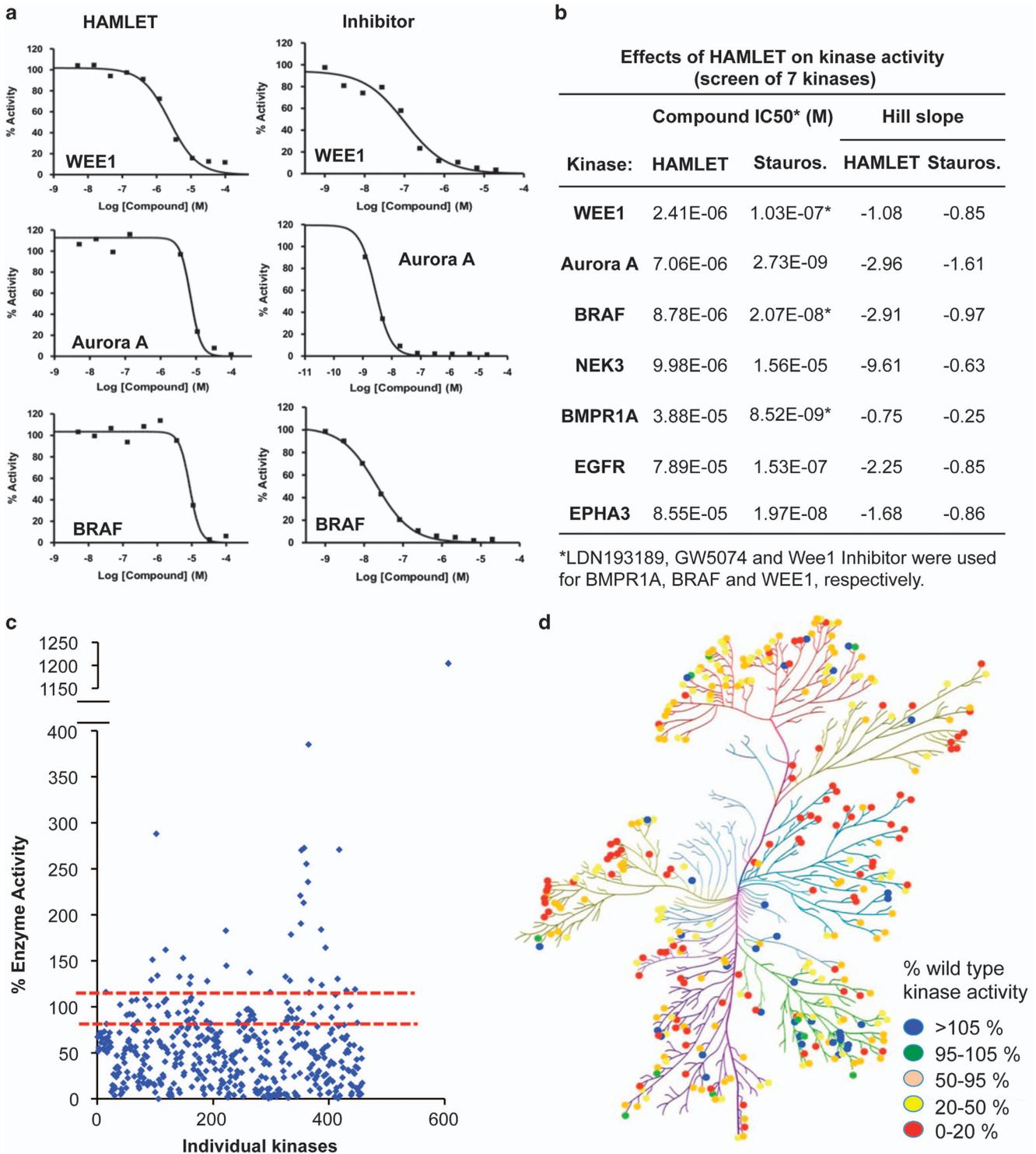


Figure 2. Interaction of HAMLET with Ras superfamily members. **(a)** ProtoArray-based identification of Ras proteins targeted by HAMLET. Targets are sorted by Ras subfamily (bold), binding intensities quantified by fluorescence are indicated by Z-scores (brackets) and targets with a propensity for lipidation are shown in red. Boxes indicate proteins selected for analysis. **(b)** Ras targets identified by protoarray. **(c)** Dot blots of HAMLET binding to Ras family proteins. **(d)** Co-immunoprecipitation of HAMLET with Ras and Braf in total cell extracts (1 h, 35 μ M HAMLET). **(e)** Membrane colocalization (yellow) of Alexa-HAMLET (red) with Ras, RasL11B, Rap1B or Raf1 (green). R, Pearson coefficient for colocalization. Scale bar, 5 μ m.



Consequences for ras activity and cell death

To determine whether the activity of Ras is modified by HAMLET, active Ras was quantified in HAMLET-treated lung carcinoma cell extracts by co-immunoprecipitation with the Ras-binding domain of Raf1. The kinetics revealed a brief activation phase (15 min) followed by inactivation at later time points (60 and 360 min,

$P < 0.05$, Figure 5b). In addition, significant effects on Braf activity were detected. HAMLET was shown to colocalize with Braf in the cytoplasm (Figure 5c) and by surface plasmon resonance, the binding of HAMLET to Braf was confirmed (Figure 5d). Furthermore, HAMLET inhibited Braf-dependent kinase activity, quantified as the phosphorylation of Mek1, which is a Braf

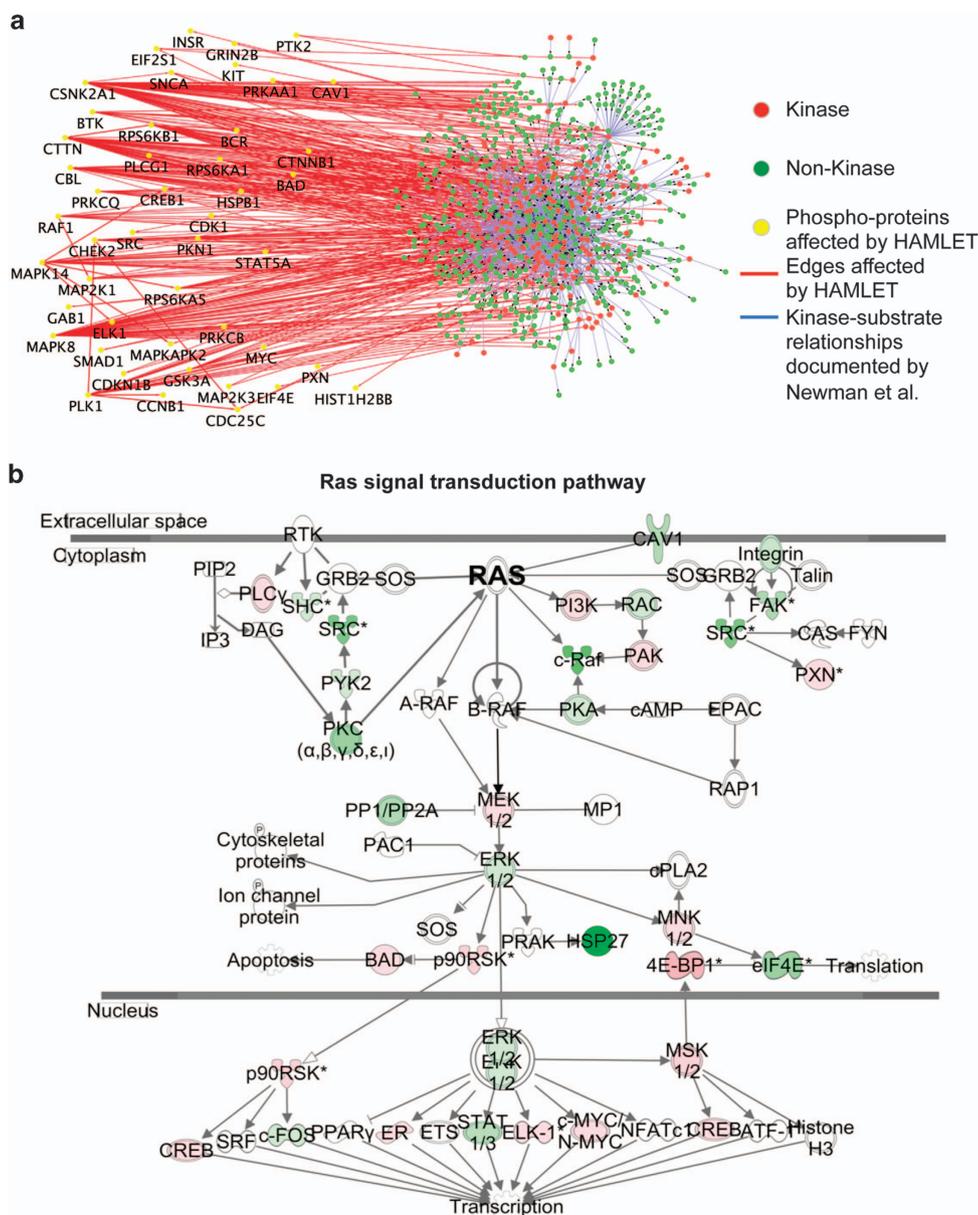


Figure 4. Effects of HAMLET on *in vivo* phosphorylation and signal transduction. **(a)** Effects of HAMLET on *in vivo* phosphorylation (4417 kinase-to-phosphorylation-site relationships connecting 230 kinases to 652 substrates) predict that HAMLET affects ~40% of the nodes, which are connected by the highlighted edges (red). Yellow nodes = proteins with changed phosphorylation in response to HAMLET, red nodes = kinase, green nodes = non-kinase substrates, blue = connections. **(b)** HAMLET modifies the phosphorylation of effector proteins in the Ras signaling cascade.

substrate (Figure 5e). These results suggest that direct interactions with HAMLET inhibit Ras and the Raf-dependent downstream effector phase of the Ras signaling pathway.

As Raf supports a network comprising at least 21 Ras pathway proteins, cell proliferation may become blocked upon HAMLET interaction (STRING database for protein-protein interaction). Furthermore, mutated, overactive Ras supports oncogenic transformation and inhibition of Ras signaling reduces the proliferation and survival of tumor cells.²² HAMLET would, therefore, be predicted to inhibit proliferation and promote cell death in synergy with Ras. Proliferation was, therefore, quantified 3 h, 24 h and 7 days after HAMLET exposure. By Ki67 staining, HAMLET was shown to inhibit proliferation of surviving tumor cells (19% of HAMLET-treated cells were Ki67 positive after 24 h, compared with 77% of untreated cells).

After 7 days, 4% of HAMLET-treated cells remained Ki67 positive, compared with 25% of untreated cells ($P < 0.001$, Figure 5f).

Finally, a Raf inhibitor specific for the ATP-binding pocket (Raf kinase inhibitor IV) was shown to synergistically enhance the tumoricidal effect of HAMLET ($P < 0.01$ or $P < 0.05$, Figure 5g). The effect, quantified as ATP levels and trypan blue exclusion, increased with increasing HAMLET concentrations.

Evidence that HAMLET interacts with ras in tumor-bearing APC^{Min/+} mice

APC^{Min/+} mice are extensively used as a model of familial and sporadic colorectal cancer as they spontaneously develop multiple intestinal polyps due to an inactivating APC mutation at nucleotide 2549.²³ Peroral administration of HAMLET protects

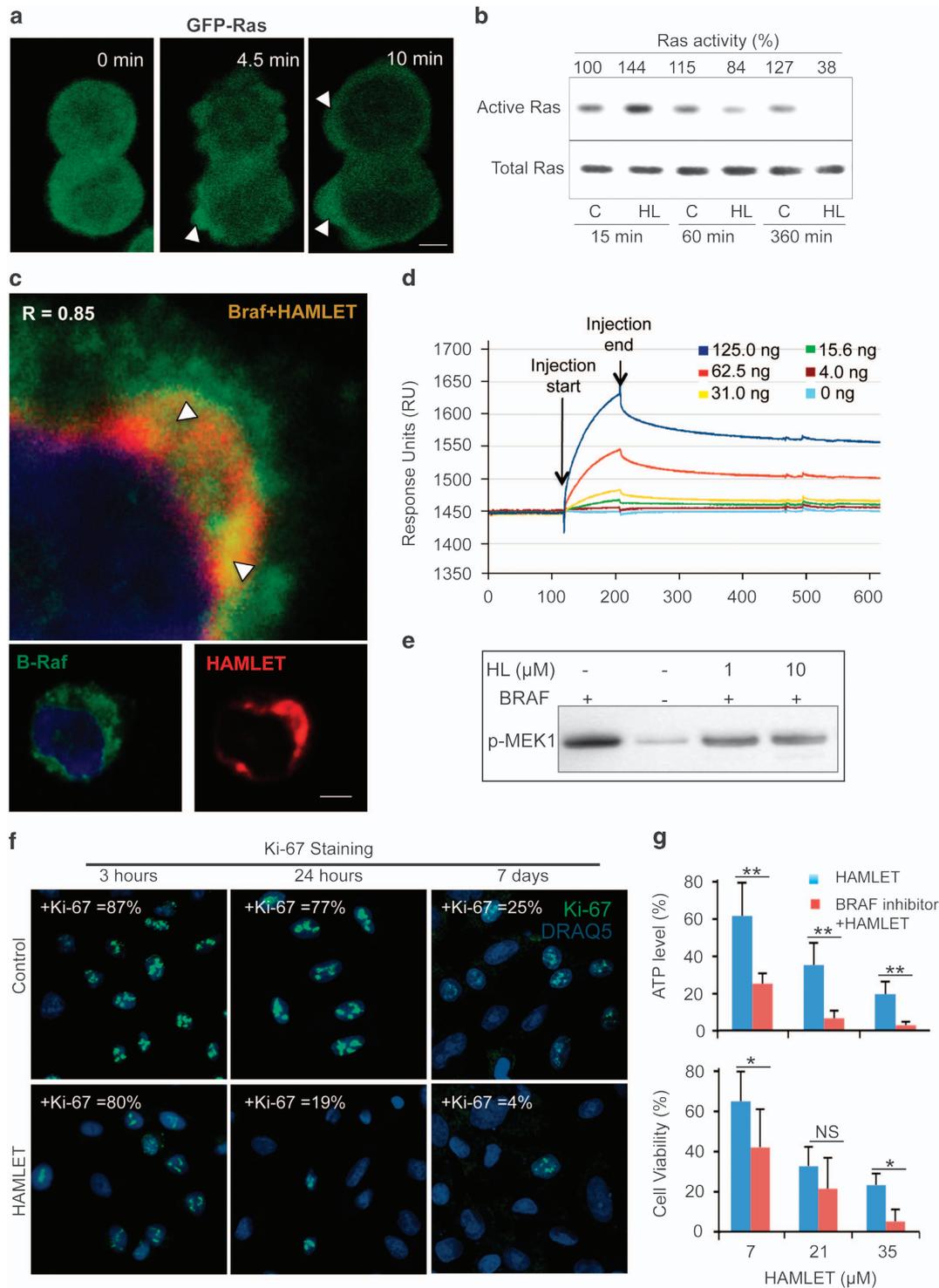


Figure 5. HAMLET inhibits the Raf-dependent Ras pathway and reduces cell proliferation. **(a)** Accumulation of GFP-Ras (green) in blebs at the periphery of Ras overexpressing, RG216409-transfected lung carcinoma cells exposed to HAMLET (35 μM). **(b)** Inhibition of Ras activity by HAMLET (21 μM) after 60 and 360 min. Pull-down assay using the Ras-binding domain of Raf1. The numbers indicated above the blots is derived from band quantification of active Ras normalized to total Ras and are expressed as percentage. **(c)** Colocalization of Braf with Alexa-HAMLET in tumor cells. Confocal microscopy, R, Pearson's coefficient for colocalization. **(d)** HAMLET binding to Braf, quantified by surface plasmon resonance. **(e)** Inhibition of Braf kinase activity (MEK1 *in vitro* phosphorylation) by HAMLET (1 and 10 μM). **(f)** Inhibition of tumor cell proliferation by HAMLET detected by Ki67 staining after 3 h, 24 h and 7 days. (100 cells/sample, $P < 0.001$, Chi-square test). **(g)** Synergistic effect on cell death of HAMLET and the Raf kinase inhibitor IV, quantified by ATP levels (** $P < 0.01$) and trypan blue exclusion. * $P < 0.05$, Student's *t*-test. All data are represented as mean ± s.d. of three independent experiments.

APC^{Min/+} mice against tumor progression, and inhibition of tumor cell proliferation has been documented in this model by Ki67 staining.¹¹ Specific uptake of HAMLET by the intestinal tumors was also observed compared with healthy tissues in the same animal.

Intestinal sections from HAMLET-treated mice were used to address whether colocalization of HAMLET with Ras proteins can be detected *in vivo* (Figure 6a). By immunofluorescence, accumulation in intestinal tumor tissue was detected 4 h after peroral HAMLET treatment with colocalization of HAMLET and Ras in individual tumor cells (Figure 6b). The majority of tumor cells that had taken up HAMLET were also stained for Ras (72%), with clear colocalization.

DISCUSSION

The HAMLET complex kills tumor cells of diverse origins and genetic backgrounds.^{7,8} This study aimed to identify molecular targets that might account for this conserved cell death response. Nucleotide-binding proteins were identified as HAMLET binding partners in a protein microarray, including ATPases, kinases and GTPases. HAMLET bound to kinases from all branches of the Kinome tree and inhibited their activity, *in vitro*. Although the interaction with individual targets did not necessarily correlate with *in vivo* activity, such as for the F-ATPase subunits,²⁴ kinase activity was broadly inhibited and kinase-dependent phosphorylation was perturbed in HAMLET-treated cells. HAMLET was shown to affect multiple kinases in cancer-associated pathways and to inhibit Ras and MAPK signaling. The Ras family of GTPases was studied in greater detail, as several members of the Ras protein family interacted with HAMLET *in vitro*. HAMLET was shown to bind to Ras, in tumor cell membranes and to inhibit Ras and Braf activity. As a consequence, the anti-proliferative and tumoricidal effects of HAMLET were enhanced in the presence of pharmacological Braf inhibitors. The results identify HAMLET as a new type of kinase inhibitor with broad and specific tumoricidal activity.

Kinase inhibitors have successfully been developed into novel therapeutic agents.⁵ Small molecule inhibitors of the 'pathogenic kinases' interact either directly with the ATP-binding pocket of the P-loop (Type I), the pocket exposed by the DFG motif in the inactive substrate-binding domain (Type II), the pocket adjacent to the ATP-binding site (Type III), a site remote from the ATP-binding pocket (Type IV) or with more than one motif, concurrently

(Type V).^{25,26} Prominent kinase inhibitors with proven clinical efficacy include Gleevec, which locks the Abl kinase in the inactive 'DFG-out' conformation with an occluded ATP-binding site.²⁷ The inhibitor PLX4032 occupies the ATP-binding site of the Braf V600E oncogenic mutant but does not target the wild-type protein, providing a basis for tumor selectivity.²⁸ As the cells used in the present study do not carry mutant Braf, we selected the RAF kinase inhibitor IV, which occupies the ATP-binding site of wild-type and mutant Braf, to demonstrate synergy of Braf inhibition with HAMLET in cell death. Like these Braf inhibitors, staurosporine binds to the P-loop and prevents ATP binding and kinase activation but with a more general effect.^{29,30} Interestingly, synergy between HAMLET and Staurosporin was not detected and we did not observe a correlation between HAMLET and staurosporine in terms of their inhibition of individual kinases *in vitro*,³¹ suggesting that HAMLET has different mechanisms of action. This is also supported by the lack of toxicity of HAMLET for healthy, differentiated cells.^{9,11}

Unlike HAMLET, kinase peptide inhibitors are often developed as structural derivatives of substrate proteins. The kinase affinity of the HAMLET complex was not predictable as HAMLET does not resemble these inhibitors structurally and is not engineered to target-specific kinases with high affinity. Furthermore, the preferential killing of tumor cells distinguishes HAMLET from most kinase inhibitors and HAMLET crosses the plasma membrane of tumor cells, reaching kinases at the inner leaflet of the plasma membrane and in the cytoplasm. Furthermore, HAMLET attacks multiple kinases in parallel, potentially giving rise to 'Kinome reprogramming' resulting from inhibition of multiple kinases, involving cMYC, Akt^{32,33} or MEK-ERK,³⁴ which are perturbed by HAMLET. The natural properties of HAMLET including its broad anti-kinase activity may also be highly favorable to avoid resistance due to degenerate and adaptable kinase networks in tumor cells.^{35,36}

Kinase overactivation drives tumor cell proliferation and other features contributing to the 'Hallmarks' of cancer. According to the 'oncogene addiction' concept, the interference with individual oncogene-driven functions can cause tumor cell death.³⁷ HAMLET showed affinity for a large number of Ras GTPases including members of the Ras subfamily, which control cell proliferation,^{22,38–40} Rab subfamily members involved in protein trafficking,⁴¹ Rap1B, which controls cell adhesion and proliferation,⁴² Arf subfamily members involved in vesicular transport⁴³ and RhebL1 in the mTOR

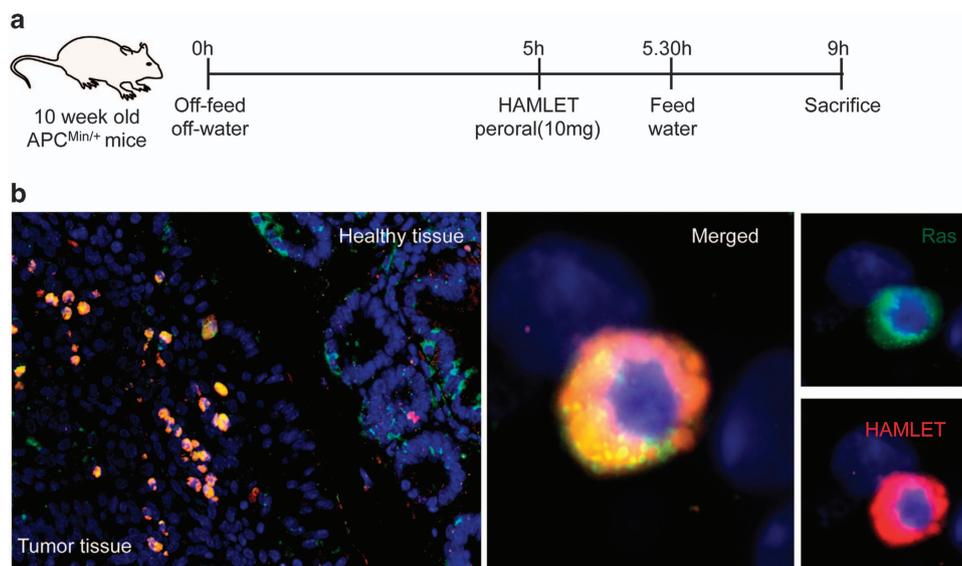


Figure 6. *In vivo* colocalization of HAMLET and Ras in intestinal tumors. (a) APC^{Min/+} mice were treated orally with 10 mg of HAMLET and killed after 4 h. (b) Colocalization of HAMLET (red) and Ras (green) in tumor cells but not in healthy intestinal tissue in the same animal.

pathway.⁴⁴ We also found that HAMLET bound to GEF Rasgrp3 and GAP ArfGAP3, which regulate GTP/GDP switching and Ras GTPase activity^{43–45} as well as Braf, Raf1⁴⁶ and RRAGA,⁴⁷ which are downstream effectors of Ras signaling. Colocalization of HAMLET with specific Ras GTPases was detected in lung carcinoma cells and inhibition by HAMLET of the Ras signaling pathway was demonstrated in a comprehensive phosphorylation antibody screen, comparing whole-cell extracts from HAMLET-treated cells to untreated cells. As the cMYC and Ras gene families were identified in a previous shRNA screen as determinants of HAMLET sensitivity, it may be speculated that Ras/Braf inhibition by HAMLET reverses the oncogene-driven phenotype, thereby killing the oncogene-addicted cancer cells.¹⁷ This concept was further supported by observations in the APC^{Min/+} model of colon cancer, where protection by HAMLET was accompanied by reduced proliferation of surviving cells. Colocalization of HAMLET with Ras was demonstrated in tumor tissue 4 h after peroral HAMLET administration.

HAMLET has shown therapeutic efficacy in several animal models of cancer and the therapeutic benefits of HAMLET have been confirmed in clinical studies. Understanding the mechanism of action is therefore essential. The present study defines HAMLET as a ligand of nucleotide-binding proteins, including ATPases, kinases and GTPases and suggests that their inhibition leads to cell death. Specifically, HAMLET inhibited Ras and Braf activity, blocking pathways involved in proliferation and survival, explaining how the sensitivity to HAMLET can be determined by oncogenes like cMYC and Ras,¹⁷ previously defined in an shRNA screen. We speculate that the affinity of HAMLET for kinases and the resulting reduction in ATP and kinase activity may be more dramatic for 'kinase addicted' tumor cells than for healthy cells and an essential mechanistic aspect of HAMLET's tumoricidal effect.

MATERIALS AND METHODS

Chemicals

DMSO (dimethyl sulfoxide), formaldehyde, Triton X-100, Tween-20, SDS, sodium deoxycholate and fluoromount were from Sigma (St Louis, MO, USA). EDTA (ethylenediaminetetraacetic acid) and Tris (hydroxymethyl) aminomethane from VWR (Volumetric solutions, BDH Prolabo, Fontenay-sous-Bois, France), DRAQ-5 from eBioscience (San Diego, CA, USA), staurosporine from Enzo Life Sciences (Solna, Sweden) and RAF kinase inhibitor IV from Merk Millipore, Calbiochem (Solna, Sweden).

HAMLET: characterization, production and quality control

α -Lactalbumin was purified from human breast milk by 25% ammonium sulfate precipitation and hydrophobic interaction chromatography, partially unfolded and converted to HAMLET on an oleic acid-conditioned DEAE ion exchanger.⁸ Human milk was obtained after signed informed consent. The samples were de-identified and steps were taken to protect the participants' identities. The procedure was approved by the human ethics committee of the Medical Faculty, Lund University, Lund, Sweden.

Protein–protein interaction profiling

A screen for possible interaction partners for HAMLET was performed on a ProtoArray Human Protein Microarray version 4.0 (Invitrogen, Madison, WI, USA) as previously described.⁴⁸ In brief, arrays were probed with Alexa Fluor 568-labeled HAMLET (5 and 50 ng/ μ l) in duplicate, and signals were compared with a control array without HAMLET. The interactions were quantified as FCs over the mean of all negative control values (M1), FCs over individual negative controls (M2) and Z-scores > 2.0 (M3).

In vitro kinase activity assay

In vitro kinase profiling was by the 'HotSpot' assay platform (Reaction Biology Corporation, www.reactionbiology.com, Malvern, PA, USA). Briefly, specific kinase/substrate pairs in reaction buffer (20 mM Hepes pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 0.02% Brij35, 0.02 mg/ml BSA, 0.1 mM Na₃VO₄, 2 mM DTT, 1% DMSO) were mixed with HAMLET, followed by the addition of ATP (Sigma) and ³³P ATP (PerkinElmer, Waltham, MA, USA) to a final

concentration of 10 μ M. Reactions at room temperature (2 h) were followed by spotting onto P81 ion exchange filter paper (Whatman Inc., Piscataway, NJ, USA). Unbound phosphate was removed by extensive washing (0.75% phosphoric acid) and kinase activity was expressed in percent of remaining kinase activity in test samples compared with vehicle (DMSO) reactions. IC₅₀ values and curve fits were obtained using Prism (GraphPad Software). Kinome tree representations were prepared using Kinome Mapper (<http://www.reactionbiology.com/>).

Mammalian cell culture

Lung carcinoma cells (A549) were from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in supplemented RPMI-1640 (Fisher Scientific, Lund, Sweden), supplemented with 1 mM sodium pyruvate, non-essential amino acids (1:100; Fisher Scientific), 50 μ g/ml gentamicin (Gibco, Paisley, UK) and 5% fetal calf serum. Cells were cultured at 37 °C, 90% humidity and 5% CO₂ in 96-well plates overnight (for trypan blue exclusion and ATP assays), in six-well plates (for western blots) and in 75-mm flasks (for immunoprecipitation).

In vivo kinase activity assay and network analysis

Cellular kinase activity was quantified by Kinex KAM-850 Antibody Microarray Services (Kinexus, Vancouver, BC, Canada). Targets with percent FC over control (% FC) > 20 were considered significant. The targets were identified in the human profiling human activity-based phosphorylation network²¹ and analyzed using Ingenuity Pathway Analysis (Qiagen, Hilden, Germany).

Structural model rendering

Molecular graphics and analyses were performed with the UCSF Chimera package,⁴⁹ developed by the Resource for Biocomputing, Visualization and Informatics at the University of California, San Francisco, CA, USA. Structural rendering of Ras protein (PDB id.: 5P21) was generated in Pymol (v1.5.0.4, Schrödinger). Subcellular localization of Ras-related proteins was derived from Uniprot (InterPro, UniProtKB, Compara, HPA and Reactome). In Figure 2a, the lipid bilayer was simulated by PACKMOL with palmitoyl and water as input molecules. The structures include Ras bound to guanine-exchange-factor region of the Son of sevenless (Sos; PDB id.: 1BKD), Ras-RasGAP complex (PDB id.: 1WD1), Braf kinase domain (PDB id.: 3C4C) and farnesyl moieties (PDB id.: 3T51). The structure of the hypervariable region of Ras was predicted using PEPFOLD. The predicted peptide structure was superimposed onto the C-terminal of the Ras structure and the farnesyl moiety was built onto the thiol group of the CAAX region of the hypervariable region in Pymol.

Confocal imaging

Lung carcinoma cells treated with HAMLET (35 μ M, 10% Alexa-HAMLET), were incubated with rabbit anti-Ras (Abcam, Cambridge, UK, ab108602), rabbit anti-Rap1A/B (Cell Signaling, Danvers, MA, USA, 4938 S), rabbit anti-Raf1 (Santa Cruz, Santa Cruz, CA, USA, sc-133), mouse anti Raf-B (Santa Cruz, sc-5284) or mouse anti RasL11b (Santa Cruz, sc-81932) primary antibodies (1:25 in 10% FCS/PBS, 2 h, room temperature), followed by secondary Alexa-488 conjugated antibodies (1:100 in 10% FCS/PBS, Molecular Probes, Eugene, OR, USA, 1 h), counterstained with DRAQ-5 (Abcam, ab108410) and examined using LSM 510 META laser scanning confocal microscope (Carl Zeiss, Jena, Germany). Colocalization analysis and fluorescence quantification were in LSM 510 image browser software and Photoshop CS5, respectively.

Dot blot

For the HAMLET far-western dot blot, 2 μ g of purified recombinant Kras, Hras (Abcam), Rab3c (Creative Biomart, Shirley, NY, USA), Arl5A (Novus Biologicals, Littleton, CO, USA) and Rasgrp3 (Abnova, Taipei City, Taiwan) were spotted onto polyvinylidene fluoride membranes, blocked with Sat-1 and Sat-2, incubated overnight at 4 °C with goat anti- α -lactalbumin antibodies (Bethyl Laboratories, Montgomery, TX, USA, A10-128P) and for 2 h, room temperature with HRP-conjugated rabbit anti-goat antibodies (Sigma Aldrich, St Louis, MO, USA).

Co-Immunoprecipitation

Total cell lysates of HAMLET-treated cells (35 μ m, 60 min) were incubated with goat anti-bovine α -lactalbumin antibody, collected with Dynabeads (magnetic) Protein G (Life Technologies, Stockholm, Sweden), analyzed by SDS-PAGE (SDS-polyacrylamide gel electrophoresis) with rabbit anti-Ras and mouse anti-Raf-B (1:1000, 5% BSA) primary antibodies followed by HRP-conjugated secondary anti-rabbit (Cell Signaling, Stockholm, Sweden, #70745) or anti-mouse (DAKO, Stockholm, Sweden, P0260) antibodies (1:4000, 5% NFDm).

GFP-ras overexpression

A549 cells (1×10^5 cells/ml), seeded in 35-mm glass bottom dishes (MatTek, Ashland, MA, USA), were transfected with RG216409 (HRas cloned in pCMV6-AC-GFP) or pCMV6-AC-GFP alone (Origene, Rockville, MD, USA) using Lipofectamine 2000 Transfection Reagent (Invitrogen).

Ras activity measurements

Ras complexes with the GST-tagged Ras-binding domain of Raf1 (Thermo Scientific, Hagersten, Sweden) were pulled down from lysates of HAMLET-treated lung carcinoma cells and detected by SDS-PAGE with mouse anti-Ras antibody (1:200 in 3% BSA, 2 h incubation at room temperature) and secondary anti-mouse (Dako, P0260) antibody (1:4000 in 5% NFDm).

Braf kinase activity assay

Braf activity was measured using Raf1 Kinase Assay Kit, Chemiluminescence Detection (US Biological, Salem, MA, USA). Recombinant Braf mixed with HAMLET or PBS, unphosphorylated MEK1 and ATP was analyzed by SDS-PAGE. Active (phosphorylated) MEK1 was probed overnight with anti-phospho-MEK1 (Ser218/222)/MEK2 (Ser222/226) antibody (1:1000 in TBST 0.05%, Merk Millipore, 05-747), followed by HRP-conjugated secondary anti-rabbit antibody (1 h). The interaction of HAMLET and Braf by surface plasmon resonance was as described.¹⁵

ATP quantification and cell viability assay

Cell viability was quantified by trypan blue exclusion, Presto blue fluorescence and ATP levels, measured by ATPlite Kit (PerkinElmer, Infinite F200).¹⁷ Briefly, cells were seeded in 96-well plates (1×10^4 cells/well), cultured overnight at 37 °C (Heracus HERAcCell 150 incubator, Thermo Scientific), washed and exposed to HAMLET in RPMI without FCS. After 60 min, 5% FCS was added to each well.

Immunohistochemistry for APC^{Min/+} mice intestinal tissue

Tumor-bearing APC^{Min/+} mice were orally gavaged with HAMLET (10 mg in 400 μ l PBS) and killed after 4 h by CO₂ inhalation.¹¹ Tissues were embedded in paraffin and 5- μ m thick sections were further processed for immunohistochemistry. Sections were blocked (10% FCS, 1 h) and incubated overnight at 4 °C with antibodies specific for α -lactalbumin (1:20 dilution, GeneTex, Irvine, CA, USA, GTX18508) and total Ras (1:50 dilution, Abcam, ab108602). Alexa Fluor 448 or Alexa Fluor 568 (1:200, 1 h, Molecular Probes) secondary antibodies were used with DAPI (Sigma) as nuclear stain. Fluorescence was quantified by microscopy (Carl Zeiss). Each experimental group contained four to five animals.

All animal protocols and procedures were reviewed and approved by the institutional animal experimental ethics committee at the Lund District Court, Lund, Sweden.

Statistical analysis

Results are presented as a means \pm s.d. Groups were compared using two-sided, Student's *t*-test (Microsoft Excel) or Chi-square (GraphPad). The enrichment of nucleotide-binding proteins was analyzed by Chi-square, Fisher's exact tests (GraphPad), binomial and hypergeometric distributions (Microsoft Excel). For all experiments, at least two replicate experiments were performed.

CONFLICT OF INTEREST

The authors declare no competing financial interests and the studies have not been funded by commercial sources. CS is a shareholder in HAMLET Pharma, a company holding patent rights to HAMLET.

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