<u>מבחן בכתב לעריכת פטנטים: ביוטכנולוגיה</u>

נבחנת/נבחן יקר,

מצורף מכתב אליך מאת ממציא מהאוניברסיטה ובו פרטים על ממצאיו שעניינם חלבון אותו הוא חקר – ABC-1. ואשר נועדו להגשה של בקשת PCT.

אנא קראי/קרא בעיון את ההנחיות הבאות, את המכתב ואת המאמר שמצורף אליו.
אגב, כדי להקל ולפשט, לא נכללו התמונות עצמן ולא נכלל אף המקרא לתמונות (די להסתמך על שכתוב בגוף המאמר).

1. על הפירוט לכלול מבוא, תיאור כללי ותיאור מפורט וכן דוגמאות רלוונטיות. אם נדרש לדעתך חומר נוסף, למשל כדי להעמיק את התיאור או לתמוך בצורות יישום נוספות, יש לציין זאת בהערה/שאלה מודגשת בסוגריים מרובעים בתוך הטקסט (<u>לא במסמך נלווה</u>). למשל:

[I want to expand the description in the previous paragraph to other diagnostic techniques. Please provide details or references]

2. את הפירוט (לרבות התיאור והתביעות) יש להכין כך שיתאים להגשה לאומית במדינות רבות, לרבות ישראל, ארה"ב, יפן, סין והודו. אם ישנן תביעות אשר מיועדות לבקשה לאומית מסוימות – למשל תביעות שמתאימות לאירופה, ליפו וכו' (ויוצאו לפני הגשת בקשה לאומיות אחרות) יש לציין זאת בטקסט, למשל:

[claims xx-yy are intended for IL and JP only]

.

3. אם נדרש לדעתך מידע נוסף מן הממציא, יש לציין זאת <u>בתוך הטקסט בלבד</u> כהערה מודגשת למשל:

[please provide experimental data to support embodiment X]

4. על מערכת התביעות לכלול את כל מה שראוי וניתן לתבוע וכן את כל צורות היישום (embodiments) הרלוונטיות. ניתן לכלול כמות תביעות ככל שנדרש. בניסוח התביעות אין להתחשב בשיקולים של אחידות האמצאה (unity) וגם אין צורך לכלול הערות לעניין זה בתוך הטקסט (השאלות וההערות בתוך הטקסט צריכות להיות ממוקדות לעניינים מהותיים בלבד).
להזכירך: נא לציין את אותן התביעות שמתאימות רק לבקשה לאומית אחת או קבוצה מוגדרת של בקשות לאומיות.

5. אם ישנן הערות מהותיות שאת/אתה סבורה/סבור שיש לכלול, ניתן לעשות זאת בתחילת הטקסט או בסופו (אחרי התביעות).

6. ציון וקריטריונים:

(א) הציון ייקבע בהתייחס לכל חלקי הפירוט, על פי החלוקה הבאה:

- הבנה והגדרה נכונה של האמצאה: **20%**
- מערכת התביעות (לרבות הבנה של דרישות לאומיות שונות): **40%**
- התיאור והתימוכין שהוא מעניק לתביעות: **20%**
- רושם כללי (לרבות בהירות הכתיבה וההגדרות, תוכן ההערות והשאלות, אם ישנם כאלה, ועוד): **20%**

(ב) שימי/שים לב ששאלות והערות מוטעות עלולות לגרום להורדת ציון.

(ג) ציון עובר: 75

בהצלחה!!!!

25 September, 2016

Dear patent practitioner,

The technology transfer office of the university asked me to approach you for the purpose of preparing a patent application relating to my findings that are reported in the attached draft manuscript.

I was told that the application is to be filed as a PCT (whatever that is! I hope you understand.

My findings concern a novel function of a cellular protein by the name of Abrakadabra 1 (ABC-1). The ABC-1 mediates repair of DNA double-strand breaks (DSBs) caused by irradiation or chemotherapy, and therefore induces radio- or chemo-resistance of the cancerous cells.

Note that the ABC-1 was originally discovered by us although it was first published as part of a genomic and protein library in December 2015 and our initial findings relating to ABC-1 were published in February of this year (2016).

Please feel free to approach me with any questions.

Prof. Allen Krispin

ABC-1 PROTEIN ENHANCES LYMPHOMA CELL RADIORESISTANCE BY PROMOTING CYTIDINE DEAMINASE-DEPENDENT DNA REPAIR

INTRODUCTON

DNA double-strand breaks (DSBs) are highly genotoxic lesions, constituting the primary damage induced by ionizing radiation (IR) and specific anti-tumor drugs. The two major DSB repair pathways are non-homologous end-joining (NHEJ), in which broken DNA ends are directly processed and ligated without the requirement for extensive sequence homology between the DNA ends, and homologous recombination (HR), which depends on a homologous chromatid or chromosome as a template for repair. DSB repair via both mechanisms is initiated by sensory proteins complexes, which bind directly to broken DNA ends in a cell-cycle-dependent manner (Scott et al, 2014). These complexes were shown to mediate DNA end-synapsis, an initial stage of bringing two broken DNA termini at close proximity required for further processing, joining and cell cycle checkpoint signaling. Still, the mechanism whereby DNA end-synapsis occurs is not fully understood and may involve the function of yet unidentified proteins (Kirby et al, 2015).

Ionizing radiation and the majority of anticancer agents inflict deleterious DNA damage on tumor cells, predominantly DNA double-strand breaks (DSBs) and covalent DNA crosslinks. The response of various cancers to genotoxic agents generally reflects cells ability to repair or tolerate DNA damage. Unrepaired persistent DSBs in human cells pose a prominent threat to genomic integrity and cause cell death or senescence. Survival of cancer cells in the face of genotoxic treatment may accelerate tumor progression by forcing clonogenic selection of radio resistant and chemo resistant cells in advanced tumors.

Abrakadabra (ABC) proteins catalyze deamination of cytidines in single strand DNA (ssDNA), providing innate protection against retroviral replication and retrotransposition (Jones et al, 2016). The native form of ABC-1 is multimer. ABC-1 multimers consist of dimeric subunits are suggested to engage in protein-protein interactions, or protein-RNA interactions. ABC-1 contains two zinc-coordinating (Z) motifs which bind ssDNA with similar affinity, a C-terminal catalytic domain (CTD) and an N-terminal pseudo-catalytic domain (NTD).

As shown by Jones et al, 2016, there are seven Abracadabra (ABC) genes, designated ABC-1, 2, 3, 4, 5, 6 and 7, are encoded within a single chromosomal cluster. The deamination of cytidine is catalyzed by ABC proteins through a catalytic domain containing a conserved zinc-coordinating motif. ABC-1, as well as ABC-2 and ABC-5, contains two zinc-coordinating motifs which contribute unequally to the biological functions of this enzyme.

We have previously shown (Krispin et al, 2016) that ABC-1 is not expressed in most differentiated tissues but is highly expressed in proliferating tissues, including the testis, mitogen-activated PBMCs and various lymphoid malignancies. Moreover, high expression of ABC-1 in B cells of patients with diffuse large B-cell lymphoma treated with anthracycline-containing chemotherapy was associated with poor survival of the patients, suggesting that ABC-1 may be involved in tumor progression. Here we show that high expression of ABC-1 in lymphatic malignancies is associated with efficient DSB repair and enhanced cell survival after IR. ABC-1 cytidine deaminase activity was specifically required for promoting DSB repair. These findings support a role for ABC-1 in promoting lymphoma radioresistance by mutational-biased DNA repair.

RESULTS

ABC-1 expression is inversely correlated with DSB occurrence

We have recently revealed that catalytically inactive multimers of ABC-1, disassemble upon binding to single strand DNA (ssDNA) forming active monomers. We have shown that these active monomers promote terminal cytidine deamination and moreover, promote tethering of ssDNA termini (**unpublished data**). This surprising discovery that ABC-1 can tether ssDNA, prompted us to investigate the possible involvement of ABC-1 in cellular DNA damage repair. It is interesting to note that deaminase activity in bacterial cells has been correlated in certain specific conditions with DNA repair. DNA double strand breaks are considered the most lethal form of DNA damage for eukaryotic cells. DSB can either be properly repaired, restoring genomic integrity, or misrepaired resulting in drastic consequences, such as cell death, genomic instability, and cancer. It is well established that exposure to DSB-inducing agents is associated with chromosomal abnormalities and leukemogenesis. To explore the possible link between ABC-1 and the response of lymphoma cells to genotoxic treatment, we employed γ-radiation to generate DSBs in a panel of lymphocytic cell lines expressing differential ABC-1 protein levels. More

specifically, to assess the correlation, if any, between ABC-1 expression and DSB occurrence, we analyzed lysates of five different lymphoma and three leukemia cell lines for ABC-1 expression, as depicted in Figure 1A. The analysis demonstrated significant differences in ABC-1 expression between the lines, with generally higher levels of ABC-1 found in lymphoma cell lines. Phosphorylation of histone H2AX at specific sites of DNA DSBs occurs rapidly following DSB formation and is therefore indicative of the presence and location of DSBs. Thus, to determine the occurrence of DSBs, we fixed and stained lymphoma and leukemia cell lines for γ-H2AX. As illustrated in Figure 1B, lymphoma cell lines which express a relatively high level of ABC-1, such as H9 and Raji showed lower cellular DSB incidence following IR, inversely dependent on ABC-1 expression level. Ly-4, a lymphoma cell line expressing a rather low ABC-1 level displayed moderate DSB occurrence, whereas leukemic SupT1 or CEM (Fig. 1D) cells which expresses almost no ABC-1 displayed a large number of DSBs. A plot of the fraction of cells with DSBs and the relative ABC-1 expression level (fold from H9 ABC-1 expression) presented in Figure 1C clearly demonstrates an inverse relationship between ABC-1 expression and DSB frequency. Thus, the degree of DSB repair in leukemia and lymphoma cell lines correlates with ABC-1 expression level. More specifically, high expression of ABC-1 results in reduced DSBs, possibly by enhanced repair of DSBs and may thereby lead to increased resistance of the cells to IR or to any other drug that cause DNA damage (chemotherapeutic drugs).

ABC-1 is recruited to the nucleus following DNA damage and is associated with DSBs

To investigate the role of ABC-1 in the DNA damage response, we probed ABC-1 and γ-H2AX sub-cellular localization in H9 cells exposed to 4 Gy γ-radiation. As shown by Figure 2A, ABC-1 localizes predominantly to the cytoplasm of human peripheral blood mononuclear cells (PBMCs) and H9 T cells (time 0). To determine whether ABC-1 is recruited to genomic DSBs, we probed ABC-1 and γ-H2AX sub-cellular localization in H9 cells exposed to 4 Gy γ-radiation. At 30 minutes following irradiation, ABC-1 was more uniformly distributed throughout the cell, and multiple IR-induced DSBs were evident by the formation of γ-H2AX nuclear foci, as demonstrated by Figure 2B. Remarkably, one hour following irradiation, ABC-1 formed distinct nuclear foci which co-localized with γ-H2AX (Figs 2B and 2C). ABC-1 accumulation at the breakage sites intensified 4 hours following irradiation, and coincided with reduction in the number and magnitude of DSBs as reflected by reduction in γ-H2AX foci. Following 8 hours, ABC-1 was again redistributed

throughout the cell, with sporadic nuclear foci still evident at sites of minor γ-H2AX accumulation. These results suggest that ABC-1 is involved in DSB repair. Consistently, ABC-1 was also detected in H9 nuclear fractions 4 to 6 hours after IR, coinciding with a parallel reduction of ABC-1 levels in the cytoplasm (Figure 2D). These nuclear fractions were associated with cytidine deaminase activity measured on an oligunocleotide substrate (not shown). These results indicate that catalytically active ABC-1 is transiently recruited to the nucleus and that ABC-1 localizes to DSB repair foci in response to IR.

In case ABC-1 is required for repair of IR-induced DSBs, knocking-down ABC-1 expression in cells should result in defective DSB repair. ABC-1 knockdown or control H9 cells were generated by expression of specific ABC-1-directed shRNA (H9-shABC-1) or control shRNA (H9-shCtrl). As shown by Figure 2E, ABC-1 expression in H9-shABC-1 cells was reduced by approximately 70-80 percent compared to H9-shCtrl cells. To determine whether ABC-1 is required for DSB repair, we compared the dynamics of γ-H2AX dephosphorylation in IR-exposed H9-shCtrl versus H9-shABC-1 cells. Figure 2F shows that γ-H2AX foci formation in the first hour following IR was similar in both H9-shCtrl and H9-shABC-1 cells, and resembled the parental H9 cells. At 8 hours after IR, most H9-shCtrl cells that express normal levels of the ABC-1 protein, were negative for γ-H2AX staining, reflecting efficient DSB repair. However, γ-H2AX foci in H9-shABC-1 cells that do not express ABC-1, did not decrease in the following hours. Instead, these cells had increased γ-H2AX foci formation over 8 hours, encompassing the vast majority of cells and signifying extensive DNA damage.

Depletion of ABC-1 in ARH-77 multiple myeloma cells also resulted in higher DSB incidence 6 hours after IR, indicating that ABC-1 activity is not restricted to H9 cells (Figure 2G). Thus, knockdown of ABC-1 clearly reduces DSBs repair in cells exposed to IR. resulting in increased levels of DSBs and thereby increased radio-sensitivity of the cells.

ABC-1-mediated DSB repair is cytidine deaminase dependent

To assess whether ABC-1 cytidine deaminase activity is required for DSB repair, we examined whether IR-induced γ-H2AX focus formation occurred differentially in SupT11 cells stably expressing ABC-1, an E259Q catalytic mutant or empty vector (EV). As shown in Figure 3A, the SupT11 cell line is a single-cell subclone of SupT1 that is nearly devoid of all endogenous ABC proteins, including ABC-1. The average

DSB load as assessed by γ-H2AX focus formation was approximately 2-fold lower in wild-type ABC-1-expressing cells compared with the EV control cells 24 hours after IR, demonstrating that overexpression of ABC-1 in these cells enhances DSB repair and thereby results in reduction in their amount (Figure 3B). In contrast, enhanced repair was not observed in cells expressing the deaminase catalytically mutated protein ABC-1 E259Q. Therefore, the deaminase activity of ABC-1 is required for DSB repair and for the resulting resistance of the cells to genotoxic insult caused by IR and chemotherapy.

Native HIV-1-associated ABC-1 has reduced specific activity

The surprising involvement of ABC-1 in cellular DSB repair prompted us to search for compounds that modulate, and specifically inhibit, its deaminase activity or expression. Since the viral infectivity derived factor (Def) is known as promoting the degradation of ABC-1 in cells infected with the virus, we next explored the relationship between the viral protein Def and ABC-1. In the absence of Def, ABC-1 deaminates dC residues in the viral DNA and thereby prevents viral propagation. We were therefore interested to reveal whether the viral protein Def directly inhibits ABC-1 enzymatic activity. Figure 4 shows that in cells infected with *wt* virions that express the viral Def protein, the expression of ABC-1 and its deaminase activity are markedly reduced. However, in cells infected with Δ*Def* virions that do not express the Def protein, the levels and the activity of ABC-1 were not affected. Thus, the viral Def protein reduces the amount and activity of ABC-1 in cells.

Inhibition of ABC-1 deaminase activity by HIV-1 Def

To determine whether Def molecules associated with HIV-1 particles inhibit ABC-1 activity, we performed exogenous reactions using purified recombinant ABC-1. ABC-1 alone deaminated 20% of the dC in the oligonucleotide substrate. Adding viruses which contain Def, clearly decreased ABC-1 activity. In contrast, adding the same amount of Δ*Def* viruses to the reaction had no effect (Figure 5A). These results indicate that Def molecules associated with HIV-1 are able to inhibit ABC-1 activity *in vitro*.

Next, an in-depth biochemical analysis of Def-mediated inhibition of ABC-1 enzymatic activity was preformed. To this end we expressed and purified recombinant Def protein and examined ABC-1 activity in the presence of Def. Purified ABC-1 was incubated with increasing amounts of purified Def and the effect on ABC-1-mediated deamination levels of the ss-deoxyoligonucleotide substrate was measured. The

results clearly show that Def inhibits ABC-1 deaminase activity in a dose-dependent manner(Fig. 5B.)

Identifying the Def-derived peptides which inhibit ABC-1 deaminase

In order to identify the inhibitory domains in Def, a battery of 15-mer 46 Def-derived peptides (Table 1) with 11aa overlaps covering the full-length protein was screened for the inhibition of ABC-1-mediated deamination. Peptides corresponding to sequences throughout the Def protein exerted a vast inhibitory effect at a concentration of 100 μM. As shown by Figure 6A, at a lower concentration of 10 μM, a distinct inhibitory pattern was observed by peptide sequences corresponding to Def N-terminus 1- 51aa and to a central region 101-127aa. Six peptides inhibited the ABC-1 deaminase activity at a lower concentration of 1 μM, mapping the inhibitory sequences to Def9-23 (SEQ ID NO.:3), Def25-39 (SEQ ID NO.:7) and Def37-51 (SEQ ID NO.:10) at the N-terminal region, and Def101-115 (SEQ ID NO.:26), Def105-119 (SEQ ID NO.:27 and Def113-127 (SEQ ID NO.:29) at the central region, as seen in Figure 6B. These peptides were further analyzed for the inhibition of ABC-1 activity at lower concentrations ranging down to 40 nM. As illustrated by Figure 6C, the peptides Def25-39 (SEQ ID NO.:7) and Def105-119 (SEQ ID NO.:27), significantly reduced the ABC-1 activity at 1 μM and 0.2 μM with an IC_{50} of approximately 0.6 μM and 0.1 μM, respectively. Figure 7 present an experimental repeat focusing on the inhibition of ABC-1 deaminase activity by Def25-39 as compared to the control peptide Def89-103. The Def25-39 peptide specifically inhibited the deaminase activity of purified ABC-1 with an IC_{50} of approximately 1 μM, unlike Def89-103 which did not inhibit ABC-1 at a concentration of 100 μM and therefore used as a control peptide (Figure 7).

Table 1. ***Def-derived peptides***

SEQ ID NO	From position	To position	Peptide Sequence	SEQ ID NO	From position	To position	Peptide Sequence
1	1	15	MENRWQVMIVWQVDR	**24**	93	107	RYSTQVDPDLADQLI
2	5	19	WQVMIVWQVDRMRIR	**25**	97	111	QVDPDLADQLIHLYY
3	9	23	IVWQVDRMRIRTWKS	**26**	101	115	DLADQLIHLYYFDCF
4	13	27	VDRMRIRTWKSLVKH	**27**	105	119	QLIHLYYFDCFSESA
5	17	31	RIRTWKSLVKHHMYI	**28**	109	123	LYYFDCFSESAIRNA
6	21	35	WKSLVKHHMYISGKA	**29**	113	127	DCFSESAIRNAILGH
7	25	39	VKHHMYISGKAKGWF	**30**	117	131	ESAIRNAILGHIVSP
8	29	43	MYISGKAKGWFYRHH	**31**	121	135	RNAILGHIVSPRCEY

9	33	47	GKAKGWFYRHHYEST	**32**	125	139	LGHIVSPRCEYQAGH
10	37	51	GWFYRHHYESTHPRI	**33**	129	143	VSPRCEYQAGHNKVG
11	41	55	RHHYESTHPRISSEV	**34**	133	147	CEYQAGHNKVGSLQY
12	45	59	ESTHPRISSEVHIPL	**35**	137	151	AGHNKVGSLQYLALA
13	49	63	PRISSEVHIPLGDAR	**36**	141	155	KVGSLQYLALAALIT
14	53	67	SEVHIPLGDARLVIT	**37**	145	159	LQYLALAALITPKKI
15	57	71	IPLGDARLVITTYWG	**38**	149	163	ALAALITPKKIKPPL
16	61	75	DARLVITTYWGLHTG	**39**	153	167	LITPKKIKPPLPSVT
17	65	79	VITTYWGLHTGERDW	**40**	157	171	KKIKPPLPSVTKLTE
18	69	83	YWGLHTGERDWHLGQ	**41**	161	175	PPLPSVTKLTEDRWN
19	73	87	HTGERDWHLGQGVSI	**42**	165	179	SVTKLTEDRWNKPQK
20	77	91	RDWHLGQGVSIEWRK	**43**	169	183	LTEDRWNKPQKTKGH
21	81	95	LGQGVSIEWRKKRYS	**44**	173	187	RWNKPQKTKGHRGSH
22	85	99	VSIEWRKKRYSTQVD	**45**	177	191	PQKTKGHRGSHTMNG
23	89	103	WRKKRYSTQVDPDLA	**46**	181	192	KGHRGSHTMNGH

*Fifteen-mer peptides derived from HIV-1 Def (sequence accession: #AAZ14773) covering the full-length protein with 11aa overlaps.

Def25-39 inhibits DSB repair in cultured cells

Having established the mode of action of Def25-39 *in-vitro*, we proceeded to determine the effects Def25-39 performs *ex-vivo*, in cultured cells. H9 cells were incubated for 2 hours with Def25-39 (SEC ID NO. 7), Def89-103 (SEQ ID NO. 23, control) or non-peptide, irradiated (4 Gy) or mock-irradiated (No IR) and stained following 8 h with anti-ABC-1 and anti-γ-H2AX antibodies. Nuclei were counter-stained with DAPI. Cells pre-incubated with the control peptide Def89-103 exhibited efficient DSB repair (<8% of cells containing DSBs), similar to cells incubated with mock or non-irradiated cells pre-incubated with Def25-39 (Fig. 8A and 8B). In contrast, 82±4.3% of irradiated cells pre-incubated with Def25-39 contained DSBs, suggesting that cytidine deamination is required for ABC-1-mediated DSB repair.

We have therefore identified novel Def-derived peptides that specifically target ABC-1 and reduce its DSB repair activity. These peptides may be therefore used as specific ABC-1 inhibitors for sensitizing cancel cells to genotoxic insult that leads to DNA damage.

DISCUSSION

Combination chemotherapy and radiotherapy are effective treatments for non-Hodgkin lymphoma, however, more so in the case of localized disease (stage I or II) rather than in advanced disease (stage III or IV). The response of various cancers to genotoxic agents generally reflects cells ability to repair or tolerate DNA damage.

Unrepaired persistent DSBs in human cells pose a prominent threat to genomic integrity and cause cell death or senescence. Notably, the rate of DSB repair is a critical determinant of radiosensitivity in human hematopoietic cell lines.

As we now show, an underlying mechanism for radio-resistance may involve recruitment of ABC-1, leading to cytidine deamination of resected DSBs ssDNA, tethering of the ssDNA and repair of the DSBs. It may be interesting to further examine the possible use of ectopically expressed ABC-1 as a potential inhibitor of its deaminase activity.

Our results suggest that ABC-1 has a dual role in promoting survival of lymphoma cells in vivo: (1) by enhancing DSB repair after genotoxic treatment, thus preventing cell death; and (2) by promoting a mutator phenotype, driving tumor progression. Hence, strategies aimed at inhibiting ABC-1 expression or catalytic activity may improve the outcome of genotoxic lymphoma therapy.

MATERIALS AND METHODS

Cells culture

T-lymphoblastic leukemia (SupT1, SupT11, CEM-SS, MOLT-4), cutaneous T-cell lymphoma (H9, Hut78), multiple myeloma (ARH-77, NCI-H929, CAG), HL-60 acute myeloid leukemia, Ly-1 diffuse large B-cell lymphoma, and Raji Burkitt lymphoma cells were maintained at 1 X 10^5 to 1 X 10^6 mL in RPMI 1640 supplemented with 2mM L-glutamine, 10% heat-inactivated FBS, 100 U/mL penicillin and 0.1 mg/mL streptomycin (Beit-haemek) complete medium. Ly-4 diffuse large B-cell lymphoma cells were maintained in complete IMDM (Beit-haemek). SupT1 and H9 cells were provided by the National Institutes of Health AIDS Research and Reference Reagent Program (AIDSP; Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health) and Ly cells by D. Stern (Hadassah Medical School). PBMCs were donated by anonymous healthy volunteers after given consent and isolated on a Ficoll-Hypaque gradient (Sigma-Aldrich). Cells were maintained at 2 X $10^6$to 4 X 10^6 mL in complete RPMI 1640. For induction of ABC-1 expression, PBMCs were activated with phytohemagglutinin (5 μg/mL) for 36 hours, followed by supplement of IL-2 (20 U/mL) for 36 hours [**6**] Human embryonic kidney 293T adherent cell lines were grown as a subconfluent monolayer in complete DMEM (Beit-Haemek).

HeLa-ABC-1 cells stably transfected with pcDNA3.1-ABC-1 expression vector encoding G418 resistance (obtained through the National Institutes of Health AIDSP) were grown in complete DMEM and with G418 (0.4 mg/mL; Invitrogen). U2OS-DR-GFP cells (obtained from S.P. Jackson, University of Cambridge) were maintained in complete DMEM not containing phenol red, and containing charcoal-treated FBS.

Culture Media

RPMI 1640, DMEM, fetal calf serum, penicillin, streptomycin and L-glutamine were purchased from Biological Industries, Beit Haemek, Israel.

shRNA-mediated knockdown of ABC-1

For short hairpin (sh) RNA expression, the following puromycin resistance-encoding vectors were used: ABC-1-specific pLKO.1.shABC-1 (TRCN0000052188, clone NM_021822.1-398s1c1; Sigma-Aldrich), having the nucleic acid sequence 5'-CCGG-GCCAGGTGTATTCCGAACTTA-CTCGAG-TAAGTTCGGAATACACCTGGC-TTTTTG-3', and the unrelated control cyprinid herpes virus 3 (CyHV3)-specific pLKO.1.shCtrl. Lentivectors were obtained by co-transfection of 293T cells with pLKO.1.shABC-1 or pLKO.1.shCtrl, the packaging plasmid pCMVΔR8.91 and vesicular stomatitis virus G envelope protein (VSV-G) expression plasmid. Culture supernatants were collected 48 h post transfection, centrifuged for 10 min at 4,000 rpm to remove cell debris and then for 1 h at 35,000 rpm using a swing SW-41 rotor (Beckman). H9 cells were transduced with the concentrated vectors by spinoculation for 1 h at 1,000 rpm and selected with puromycin (1 μg ml^{-1}) for 3-10 days post transduction.

Immunofluorescence microscopy

Cells were irradiated by exposure to a ^{60}Co source producing 1 Gy sec^{-1} γ-radiation, or mock-irradiated. Following incubation at 37 °C, cells were fixed, attached to glass slides by cytospin, permeabilized with detergent and blocked with 10% normal goat serum. Cells were then incubated with ABC-1-specific rabbit polyclonal antibody, γ-H2AX-specific monoclonal antibody, or RPA32-specific monoclonal antibody, followed by incubation with goat anti-rabbit Cy-5-conjugated antibody, goat anti-mouse Cy-2-conjugated antibody and DAPI. Slides were mounted and examined by Zeiss LSM 710 confocal microscope. Data were collected sequentially using a x63 objective with 7-fold averaging at a resolution of 1024 x 1024 pixels. Z-sections were

obtained using an optical slice of less than 1 µm. Data were analyzed with the Zen 2009.

Cell cycle analysis

PBMC, H9 or puromycin-resistant H9-shRNA cells were irradiated (4 Gy) or mock-irradiated. Following 20 h incubation at 37 °C, cells were fixed with methanol for 1 h at -20 °C, washed with phosphate buffered saline (PBS), treated with RNase A (50 µg ml^{-1}) and stained with PI (5 µg ml^{-1}). The total cellular DNA content was determined by flow cytometry using FACScan. Cell cycle data were analyzed by the CellQuest Pro software and include 10,000 events gated on singlet cells.

Purification of recombinant ABC-1 proteins

ABC-1 **and** ABC-1 W285A containing a C-terminal His_6 tag was expressed in 293Tcells and purified as previously described [**8**]. Briefly, 293T cells were transfected with pcDNA3.1-ABC-1Myc-His_6. Cells ($3x10^8$) were harvested 48 h after transfection, washed three times in PBS and suspended in lysis buffer (50 mM Tris, pH 8.0, 1 mM PMSF, 10% (v/v) glycerol and 0.8% (v/v) NP-40), to a final concentration of 20,000 cells/µl. Following 10 min incubation in ice, cell debris and nuclei were pelleted by centrifugation at 10,000 *g* for 20 min. The soluble fraction was adjusted to 0.8 M NaCl and treated with 50 µg/ ml RNase A for 30 min at 37 °C. Treated lysates were then added to 50 µl of nickel–nitrilotriacetic acid (Ni-NTA) agarose beads, mixed on an end-over-end shaker for 1 h at 4 °C and loaded onto a chromatography column (Econo-column). Following extensive washing with wash buffer (50 mM Tris, pH 8.0, 0.3 M NaCl, 10% (v/v) glycerol) containing 30–50 mM imidazole, bound proteins were eluted seven times in wash buffer containing 120 mM imidazole. Protein samples were resolved by SDS-PAGE and stained with Imperial protein stain. ABC-1 concentration and purity was assessed by densitometry and scanning of stained gels, comparing band-intensity to that of a predetermined protein marker, and by a Bradford assay.

ssDNA production

ssDNA (typically ~1500-7000 nt) was produced by in-vitro rolling circle amplification as described [**8**].

Preparation of virus stocks

Wild type HIV-1 and HIV-1 Δ*Def* were generated by transfection of 293T cells with pSVC21 plasmid containing full length HIV-1HXB2 or Δ*Def* viral DNA. Viruses

were harvested 48 and 72 h post transfection and stored at - 80 °C until infection of cultured H9 and SupT1 cells.

Infection of cultured cells

Cultured human T lymphoblastoid H9 and SupT1 cells (5 x 10^6) were centrifuged for 5 min at 500 *g*, the supernatant was aspirated and cells were re-suspended in 30µl of medium containing 5-10 ng of p24 per ml of *wt* or HIV-1 Δ*Def*. Cells were infected by spinoculation at 1200 rpm for 100 min at room temperature. Following infection, cells were re-suspended in fresh RPMI medium supplied with 10% FCS, and incubated for additional 7-10 days at 37 °C. Culture media were harvested daily starting at 5 d.p.i. and viruses were titered by the multinuclear activation of a galactosidase indicator (MAGI) assay. The harvests containing $5x10^4$ to 10^5 IU/ml were further centrifuged to remove cells and cell debris (10,000 *g* for 10 min) and concentrated by ultracentrifuge for 1.5 h at 100,000 *g* through a 20% sucrose cushion. Pelleted virus was re-suspended in small volume of phosphate buffered saline (PBS) and stored at -80 °C until use.

HIV-1 titration

HIV-1 was titered by the MAGI assay, as described by Kimpton and Emerman [Kimpton J, Emerman M, (1992) J. Virol. 66:2232-9].

Quantification of p24

HIV-1 p24 antigen capture assay kit was used to determine the amounts of p24 in the culture medium, according to the standards and instructions supplied by the manufacturers.

Expression and purification of Def

The pD10-Def-His6 plasmid [**9**] was used to express an N-terminal His_6 tagged HIV-1HXBII Def protein in *E. coli* MC-1061. Def was purified as previously described [**9**], with the following exceptions: after induction of Def expression with 0.5 mM IPTG for 1 h at 37 °C, bacteria were pelleted at 4,000 *g* for 15 min, washed with PBS and suspended in lysis buffer containing 50 mM phosphate buffer (pH 8.0), 0.3 M NaCl, 25 µg/ml DNase, 1 mM PMSF, 5 mM imidazole and 0.8% NP-40. Following sonication, insoluble cell debris and inclusion bodies were removed by centrifugation at 10,000 *g* for 20 min and the soluble fraction was subjected to Ni^{2+} affinity chromatography. Briefly, 4 ml of the sample corresponding to 200 ml bacterial culture were incubated with 1 ml 50% Ni-NTA slurry for 1 h at 4 °C. Following extensive

washing in wash buffer [50 mM phosphate buffer (pH 8.0), 0.3 M NaCl] containing 10-40 mM imidazole, Def was eluted in the same buffer containing 110 mM imidazole. The eluted sample was dialyzed against ABC-1 reaction buffer for 6 h at 4 °C. The concentration and purity of the Def preparation were assessed as described above for ABC-1.

Western blot

Transfected or infected cells were harvested, washed once in PBS, re-suspended in SDS-gel loading buffer and boiled for 10 min. Samples of $5x10^4$ cells were analyzed by SDS - 12% polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer of the proteins to polyvinylidene fluoride (PVDF) membrane. HIV-1 proteins were detected by using rabbit anti-Def and monoclonal anti-Ca-p24 antibodies. ABC-1 protein was identified by rabbit polyclonal anti- ABC-1.

Deamination assay

Deaminase activity of purified enzyme and virion-associated ABC-1 protein was examined as previously described [**8**]. Briefly, ABC-1 deamination reactions were performed in a total volume of 10 µl in 25 mM Tris, pH 7.0, and 0.01-1 fmol single-stranded (ss) deoxyoligonucleotide substrate at 37 °C. The reaction was terminated by heating to 95 °C for 5 min. One µl of the reaction mixture was used for PCR amplification performed in 20 µl buffer S, using the following program: 1 cycle at 95 °C for 3 min, followed by 30 cycles of annealing at 61 °C for 30 s and denaturing at 94 °C for 30 s. Aliquots of the PCR products (10 µl) were incubated with Eco147I restriction enzyme for 1 h at 37 °C. Completion of the restriction reaction was verified by using positive-control substrate containing CCU instead of CCC. Restriction-reaction products were loaded onto 14% gels and separated by PAGE. Gels were stained with SYBR gold nucleic acid stain diluted 1:10,000 in 0.5 x Tris-Borate-EDTA buffer (TBE, pH 7.8), visualized by UV light (312 nm), captured by an Olympus C-5050 charge-coupled device (CCD) camera and analyzed by optical density (OD) scan using the TINA2.0 densitometry software. Assessment of ABC-1 entrapped in virions was carried out with concentrated viruses stocks of 3-5 pg of p24/µl) suspended in PBS containing Triton X-100 at final concentration of 0.1% (v/v) and 50 µg/ml RNase A (Sigma-Aldrich). Deamination reactions were incubated for 1 h at 37 °C.

Inhibition of ABC-1 catalytic activity

A panel of 46 HIV-1 Def-derived 15-mer peptides (obtained through the NIH AIDS Research and Reference Reagent Program) were screened for inhibition of purified ABC-1- **His_6** catalytic activity in a standard cytidine deamination assay. The inhibitory peptide Def25-39 corresponds to amino acids 25-39 in HIV-1 HXB_{II} Def. A fluorescein-conjugated peptide (kindly provided by Dr. Sara Frond) was used to assess the peptide uptake by H9 cells. For inhibition of endogenous ABC-1 in H9 cells, cells were incubated with 100 µM Def25-39 or a control peptide for 2 h at 37 °C before exposure to IR.