SUPPLEMENTARY APPENDIX

# Dual cytoplasmic and nuclear localization of HTLV-1-encoded HBZ protein is a unique feature of adult T-cell leukemia

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# Supplementary Data

## *Supplementary Methods*

### Cells and Ethics Statement

Diagnosis of ATL was based on clinical and biological features including immunophenotyping, the presence of HTLV-1 specific antibodies and the detection of HTLV-1 proviral DNA. Peripheral blood mononuclear cells (PBMC) from ATL patients were purified by Ficoll-Paque TM PLUS (GE-Healthcare Bio-Science, Milan, Italy) of heparinated blood. PBMCs from healthy donors were obtained from the Blood Transfusion Center, Ospedale di Circolo, Fondazione Macchi, Varese, Italy.

### HTLV-1 Proviral Load Measurement and determination of unspliced versus spliced HBZ (mRNA)

HTLV-1 proviral load (PLV) was quantified by real-time PCR as previously described (1). Briefly, real-time PCR was carried out using primers specific for the pX region (pX forward: 5′-CAAACCGTCAAGCACAGCTT-3′, and reverse: 5′-TCTCCAAACACGTAGACTGGGT-3′) and the following probe: 5′ 6-FAM-TTCCCAGGGTTTGGACAGAGTCTTCT-TAMRA-3′. Runs were performed in 50 µL volume containing 1µg of total DNA extract, primers and probe 200nM each, 1X PCR buffer. Thermocycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 50 cycles at 95°C for 15 sec and 60°C for 1 min. Quantification was standardized with the Tarl2 cell line (HTLV-1, single proviral copy). Albumin was used for normalization. The following primers: forward 5′-GCTGTCATCTCTTGTGGGCTGT-3′ and reverse 5′-AAACTCATGGGAGCTGCTGGTT-3′, respectively, and probe: 5′-FAM-CCTGTCATGCCCACACAAATCTCTCC-TAMRA-3′, were used. Results are expressed as HTLV-1 proviral copies per 100 PBMCs.

Unspliced (us) and spliced (sp1) HBZ mRNAs were quantified as follows. Total RNA from patients (500ng) was reverse-transcribed by using SSII reverse transcriptase (Invitrogen) and random hexamers (plus strand transcripts), or the specific AS2 (5'-TCTTCCTCCAAGGATAATAGCCCGTCCA-3')

primer for HBZ transcripts following the supplier's recommendations. As an internal control, GAPDH was analyzed in parallel by using the Endogenous Control Human GAPDH kit or Ribosomal RNA Control Reagents (ThermoFischer). PCR reactions were performed with an ABI Prism 7900 HT Sequence Detection System by using 5 μl of each diluted RT sample (10 ng/μl) and 20 μl of diluted Taqman Universal PCR Master Mix (ThermoFischer) and primers and probes at the concentrations listed below. For HBZ spliced transcript: forward primer, 5'-CTCAG^GGCTGTTTCGATGCT-3' (900 nM); reverse primer, 5'-GCCCGTCCACCAATTCCT-3' (900 nM); probe HBZ, 5'(FAM)-CCTGTGTCATGCCCGGAGGACC-3'(TAMRA) (100 nM). For HBZ unspliced transcript: forward primer 5'-GTTAACTTTGTATCTGCAGGG-3' (900 nM); reverse primer, HBZ as 5'-GCCCGTCCACCAATTCCT-3' (900 nM); probe HBZ, 5'(FAM)-CCTGTGTCATGCCCGGAGGACC-3'(TAMRA) (100 nM). Each reaction was performed in duplicate. The cycling conditions comprised an initial step at 50°C for 2 min, denaturation at 95°C for 10 min, and 40 cycles at 95°C for 15 sec and 60°C for 1 min. The absolute kinetic method was applied by using standard curves constructed from 10-fold serial dilutions of a plasmid containing the GAPDH amplicon, or transcript amplicon as described (2). The absolute copy number of each transcript was determined and normalized for the copy number of the GAPDH (NCN). We scaled the NCN of HBZ Sp1 for the NCN of HBZ US.

**Cell Treatments**

Where indicated, ATL patient cells were incubated with 20nM Leptomycin B (LMB, Sigma, Milan, Italy) or the vehicle methanol for 3h at 37C, 5% $CO_2$. Cells were then washed and processed for confocal microscopy analysis as previously described (3). PBMC isolated form healthy donor were treated for three days with 1μg/ml of Phytohemagglutinin (PHA) and then processed as described above.

**Immunofluorescence, Flow cytometry and confocal microscopy**

Cell surface expression of HLA class I, HLA class II DR, CD3, CD4, CD8, CD16 and CD19 molecules was assessed by immunofluorescence and flow cytometry (BD FACSAriaTM II Cell Sorter, BD Biosciences) using commercially available specific monoclonal antibodies as previously described (4). The data were analyzed by using FlowJo 9.5.2. For confocal analysis the cells were examined by a confocal laser scanning microscope (Leica TCS SP5; HCX PL APO objective lenses, 63x original magnification, numerical aperture 1.25). Images were acquired and analyzed by LAS AF lite Image (Leica Microsystem, Milan, Italy) and/or Fiji (Image J) software. HBZ was assessed by using the 4D4-F3 monoclonal antibody whose characterization and specificity was previously described (4). 4D4-F3 mAb specifically recognizes an epitope located in the region between amino acids 97 to 133, present in both spliced and unspliced HBZ molecule. Anti-Tax-1 clone 168A51-2 mAb was used to detect Tax-1. Calreticulin, p65/RelA of the NF-kB p65 complex and JunD were assessed by using the rabbit mAbs BK12238S, BK8242S (Cell Signalling Technology) and sc-74 (Santa Cruz BioTechnology), respectively.

**References**


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**Supplementary TABLE 1. Percent sub-cellular distribution of HBZ+ and Tax-1+ PBMCs in ATL**

| Patient Code | Proviral load* | State | HBZ+ (%) | | | | Tax-1+ (%) | | | | HBZ+ Tax-1+ cells (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Total | Nuclear | Cyto-plasmic | Nuclear/ Cyto-plasmic | Total | Nuclear | Cyto-plasmic | Nuclear/ Cyto-plasmic | |
| PH131213 | 99 | Acute | 8.5 | - | 7.9 | 0.06 | - | - | - | - | - |
| PH140126 | 146 | Acute | 83.0 | - | - | 83.0 | 65.0 | 0.2 | - | 62.5 | Not Done |
| PH1612N07 | 510 | Acute | 38.0 | - | 33.0 | 5.0 | 8.4 | 0.04 | - | 8.0 | 5.8 |
| PH160822 | 296 | Acute | 65.3 | - | 62.3 | 3.0 | 43.0 | 1.0 | | 42.0 | 38.0 |
| PH150610 | 55 | Chronic | 38.0 | - | 38.0 | - | - | - | - | - | - |
| PH170706 | 100 | Chronic | 12.0 | - | 12.0 | - | 6.4 | - | 6.4 | - | 1.0 |
| PH170918 | 73 | Chronic | 97.5 | - | 68.0 | 29.0 | - | - | - | - | - |
| PH171206 | 311 | Chronic | 9.7 | - | 7.5 | 2.1 | 1.0 | - | - | 1.0 | 1.0 |

*proviral load is expressed as number of proviral copies per 100 PBMCs

## *Legends to Supplementary Figures*

**Supplementary Figure 1. Expression of cells surface markers in PBMCs of acute ATL patients**

The expression of HLA class I, HLA class II DR, CD3, CD4, CD8, CD25, CD19, and CD16 surface molecules on PBMCs from acute leukemic PH170706, PH170918, PH150610, and PH171206 patients was assessed by immunofluorescence and flow cytometry with antibodies specific for the various markers. Results are expressed as relative number of cells (ordinate) versus the mean intensity of fluorescence in arbitrary units (a.u.) (abscissa). In each histogram, negative controls, obtained by staining the cells with an appropriate isotype-matched antibody, are depicted as a dashed line.

**Supplementary Figure 2. Expression of cells surface markers in PBMCs of chronic ATL patients**

The expression of HLA class I, HLA class II DR, CD3, CD4, CD8, CD25, CD19, and CD16 surface molecules on PBMCs from chronic leukemic PH170706, PH170918, PH150610, and PH171206 patients was assessed by immunofluorescence and flow cytometry with antibodies specific for the various markers. The same markers were also analyzed on PBMC form healthy control. Results are expressed as relative number of cells (ordinate) versus the mean intensity of fluorescence in arbitrary units (abscissa). In each histogram, negative controls, obtained by staining the cells with an appropriate isotype-matched antibody, are depicted as a dashed line.

**Supplementary Figure 3. HBZ spliced (Sp1) and HBZ unspliced (US) mRNA expression in leukemic cells**

The ratio between HBZ Sp1 and HBZ US mRNA was measured as described in Methods section. Several samples were submitted to analysis including Acute ATL140126 and Chronic ATL171203 patients described in this study, the ATL-2 cell line, cells from a previously described ATL patient PH1505, cells

from two representative HAM/TSP patients (PH2163 and PH2176) and two asymptomatic carriers (AC, PH1621 and PH1307).

**Supplementary Figure 4. Comparative expression and localization of calreticulin, HBZ and Tax-1 in acute and chronic ATL cells**

Confocal microscopy analysis of **(A)** PBMC of patient PH160822 (Acute ATL) and PH170706 (chronic ATL) co-stained with the 4D4-F3 anti-HBZ mAb followed by Alexa Fluor 546-conjugated goat anti-mouse IgG1 antibody (red) and with anti-calreticulin rabbit mAb followed by Alexa Fluor 488-conjugated goat-anti-rabbit IgG antibody (green); **(B)** PBMCs of representative acute ATL patient PH160822 co-stained with the A51-2 anti-Tax-1 mAb followed by Alexa Fluor 488-conjugated goat-anti-mouse IgG2a antibody (red) and with anti-calreticulin rabbit mAb followed by Alexa Fluor 488-conjugated goat-anti-rabbit IgG antibody (green).

As control of calreticulin expression PBMC isolated form healthy donor treated for three days with 1 μg/ml of PHA were stained with the antibodies described above. Nucleus was stained with DRAQ5. (blue). DIC represents the differential interference contrast image. At least 200 cells were analyzed. All scale bars are 5μm.

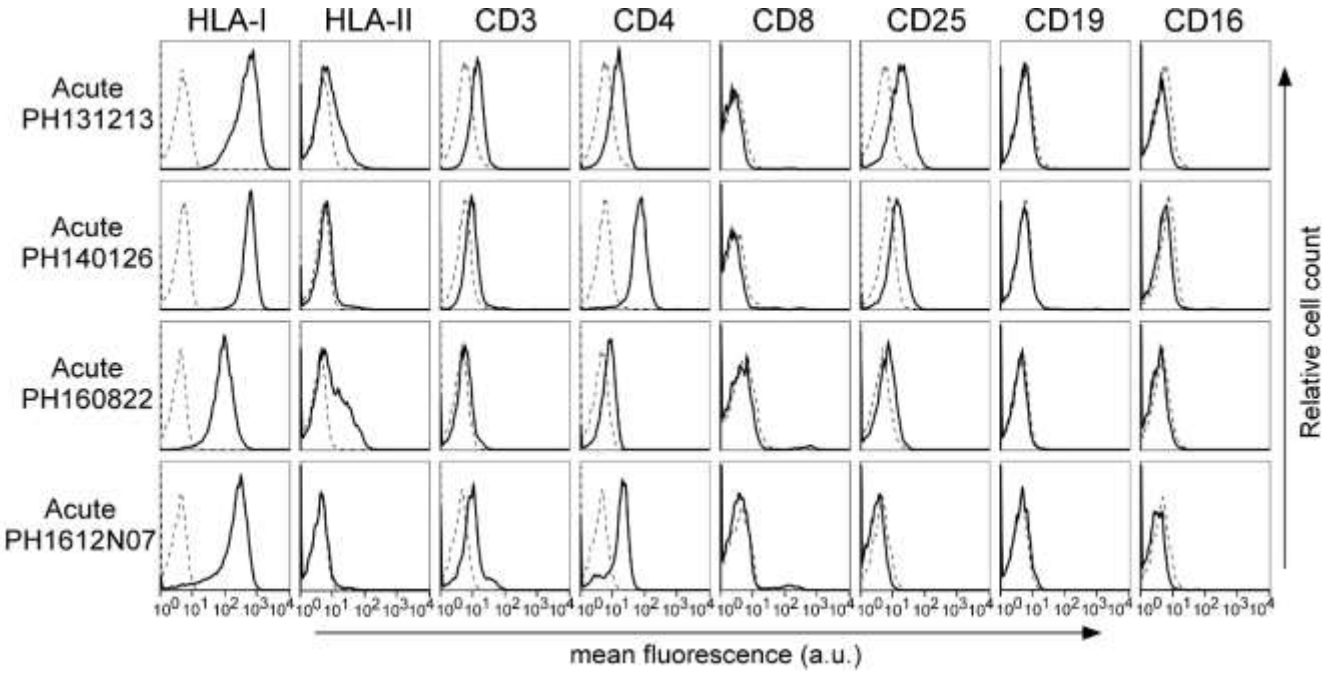
**Supplementary Figure 5. Subcellular localization of p65/RelA and JunD in ATL patient cells expressing cytoplasmic HBZ**

Representative images of patient PH160822 (Acute ATL) and PH170706 (chronic ATL) PBMC cells **(A)** co-stained with the 4D4-F3 anti-HBZ mAb followed by Alexa Fluor 546-conjugated goat anti-mouse IgG1 antibody (red), and with the anti-p65/RelA rabbit mAb followed by Alexa Fluor 488-conjugated goat-anti-rabbit IgG antibody (green); **(B)** co-stained with the 4D4-F3 anti-HBZ mAb followed by Alexa

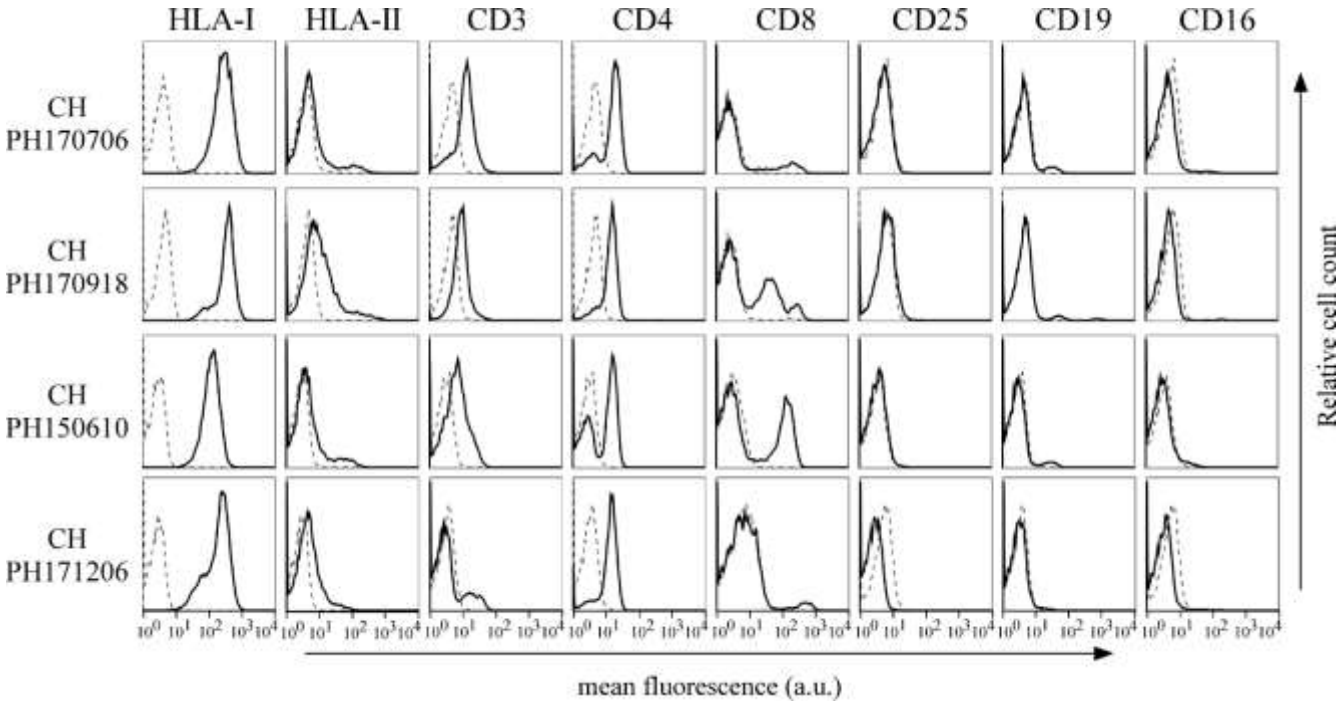
Fluor 546-conjugated goat anti-mouse IgG1 antibody (red) and with the anti-JunD Ab followed by Alexa Fluor 488-conjugated goat-anti-rabbit IgG antibody (green).

As control of both p65/RelA and JunD expression, PBMC isolated form healthy donor treated for three days with 1μg/ml of PHA were stained with the antibodies described above. DRAQ5 fluorescence probe was used to detect the nucleus (blue). DIC represents the differential interference contrast image. At least 200 cells were analyzed. All scale bars are 5μm.

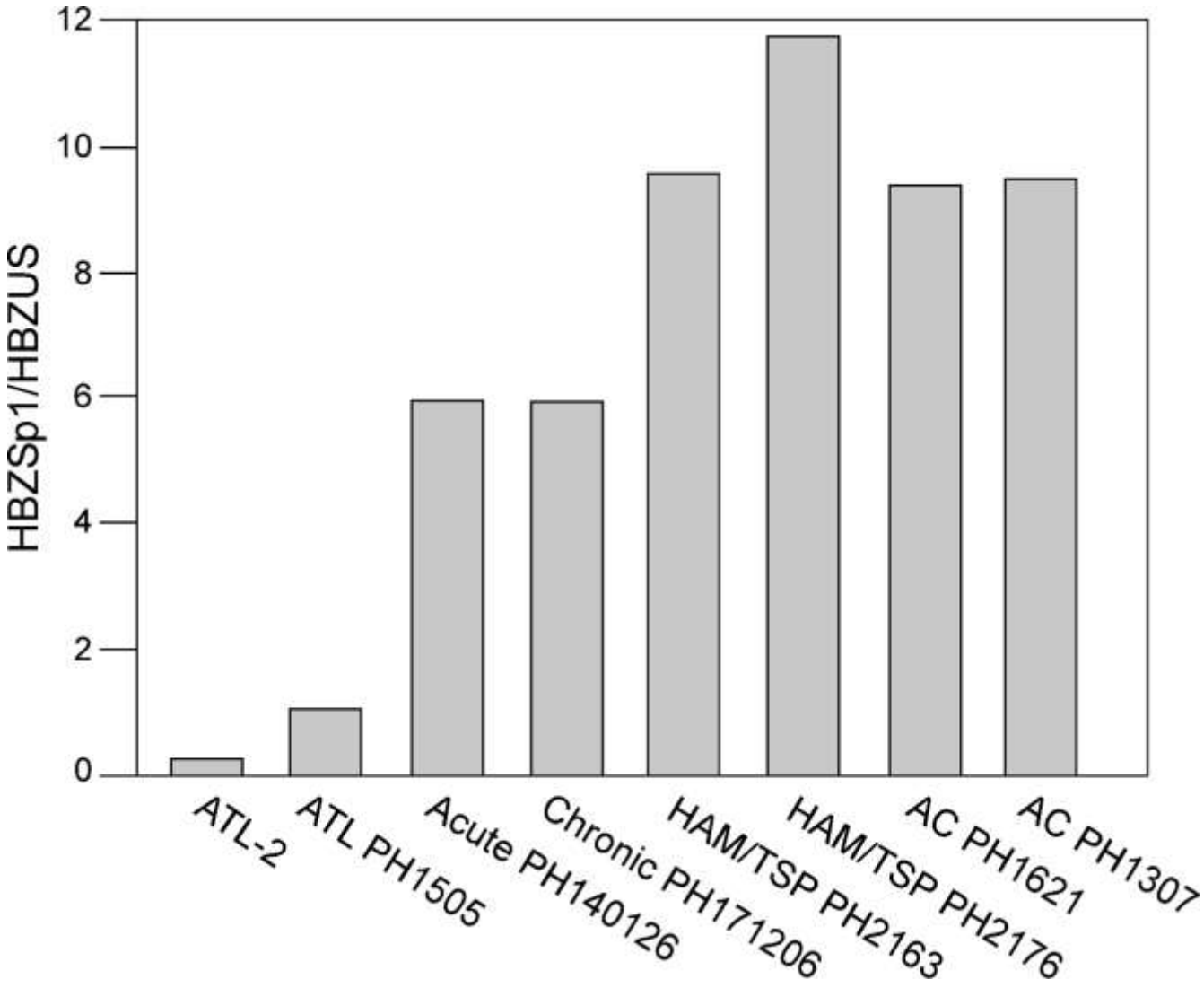
**Supplementary Figure 1**



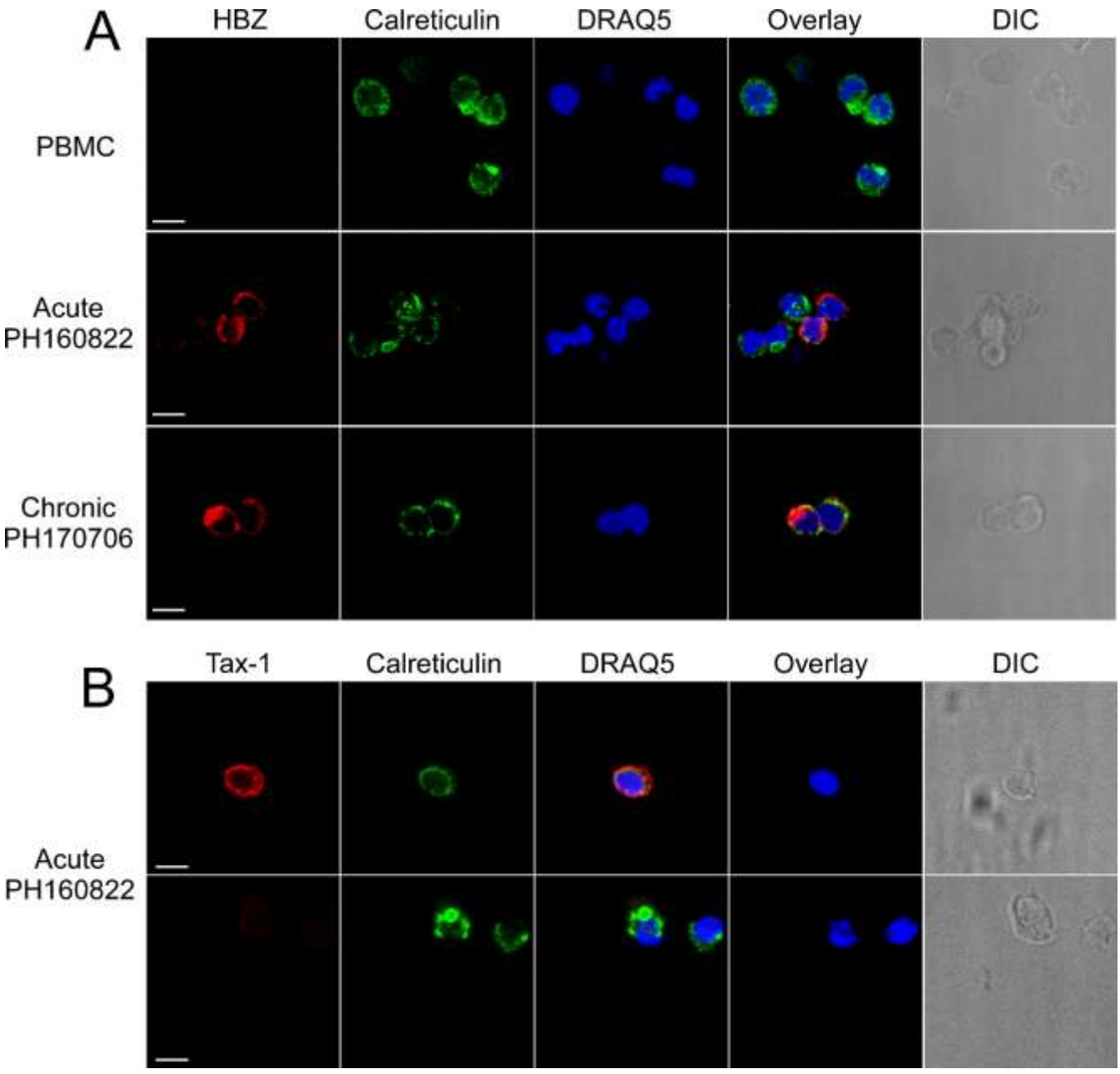
**Supplementary Figure 2**



**Supplementary Figure 3**



**Supplementary Figure 4**



**Supplementary Figure 5**