Untangling the functional roles of a large HERC1 E3-Ubiquitin Ligase in Dictyostelium and Leukemic Cells.

Untangling the functional roles of the large HERC1 E3-Ubiquitin Ligase in *Dictyostelium* and Leukemic Cells

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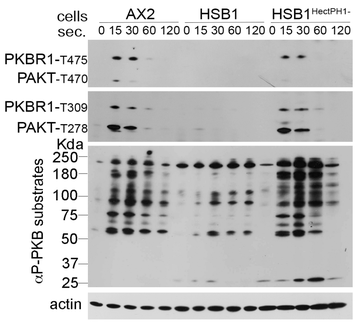
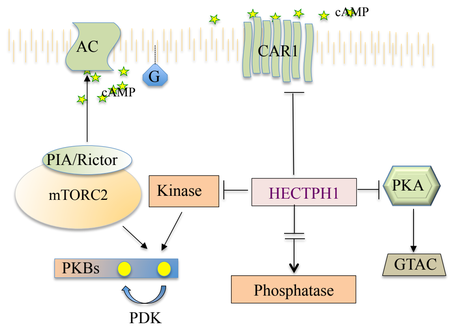
The understanding of the physiological relevance of the HERCs E3-Ubiquitin ligases has recently started to emerge, though it remains still poorly investigated. Accumulating evidence show that HERC family proteins are key components of a wide range of cellular functions including pivotal roles in cancer-related pathways. By using a simple model organism, such as the social amoeba Dictyostelium discoideum, we identified a novel E3-Ubiquitin Ligase (HectPH1) that the mTORC2-dependent activities. Due to the highest sequence homology of the HECT domain with human Herc1 counterpart, to the size and structural motifs composition the protein, HectPH1can be considered a non-conventional large HERC subfamily member. Currently, the molecular mechanisms, by which HectPH1 suppresses the TORC2 deficiency are unknown. We hypothesized that HectPH1 could act either at receptor- or to at different intracellular signalling levels. To properly address these issues it is required to identify the up- and down-stream effectors, but what is/are the regulator/s and the substrate/s of large HERC proteins is currently poorly known, both in animal and other organisms. By means of a proteomic approach, we have recently attempted to fill these gaps.

Besides the roles played by Herc1 in the nervous system of higher organisms like mammals, in the past few years it has emerged that few hematological neoplasms harbor somatic mutations affecting the HERC1 locus in different kind leukemia. However, the roles played by HERC1 in blood cells, under physiological and pathological conditions, currently remain unknown. Hence, we have recently started to assess whether HERC1 might be, or not, associated with a specific pathological condition, namely Chronic Myeloid Leukemia (CML). An in-silico survey carried out on different human neoplasia revealed that most of HECT members act as prognostic markers strengthening the hypothesis that many of them must be therapeutically targettable.

ROLE OF HectPH1 IN *DICTYOSTELIUM* CHEMOTAXIS



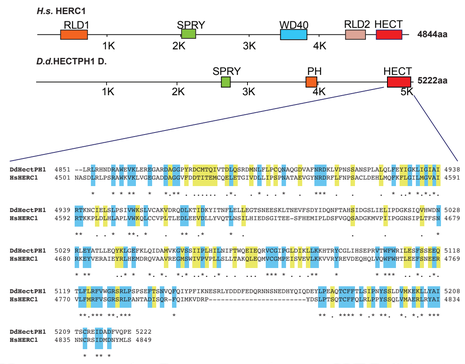


Dictyostelium discoideum (*D.d.*) development is characterized by chemotaxis-driven aggregation of starving cells and subsequent differentiation of multicellular aggregates into fruiting bodies. The schematic diagram shows how HECTPH1 could act at different levels:

1) it could directly ubiquitylate the cAMP receptor (CAR1), **2)** it could ubiquitylate components of the PKA signalling pathway, transcription factors, such as GataC, or proteins involved in mRNA maturation, regulating developmental gene expression, **3)** in addition, we propose that HectPH1 could ubiquitylate a kinase alternative to TORC2, or **4)** a factor activating a phosphatase antagonistic to TORC2, thus regulating PKBs phosphorylation.

HectPH1 IS A FUNCTIONAL HERC1 ORTHOLOGUE

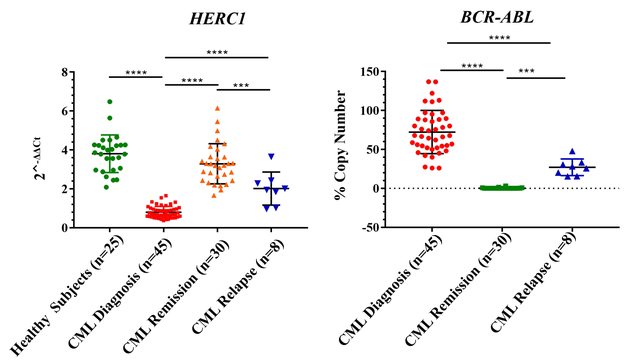
Mammalian large HERCs are defined because of their single HECT domain (a C-terminal region of approximately 350 amino acids in length with significant similarity to the C terminus of E6AP), at least one SPRY domain and a pair of the Regulator of Chromosome Condensation (RCC1)-like domains (RLD). RLD is a structurally conserved, yet functionally very versatile domain, whose roles may include interaction with other proteins or phospholipids. Interestingly, *D.d.* HectPH1 shares with HERC1 most of the structural features but it lacks the RLD, which is replaced by a PH domain that might functionally act in a similar manner to the RLD. Indeed, the isolated PH domain of HECTPH1 fused to GFP is enriched in the nuclear enveloppe and the nuclear matrix, though some labeling in the plasma membrane has been observed. Structurally, the PH domain is localized close to the HECT domain, and the latter displays the highest homology to the HECT domain of mammalian HERC1.



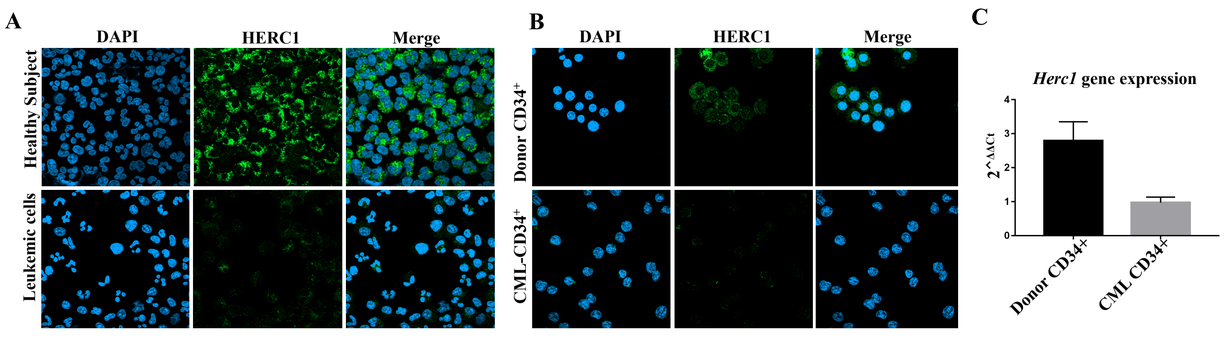
Human and *Dictyostelium* HERC1 And HectPH1Hect-domain sequences alignment: identical aminoacid residues between the two sequences are in light blue and highlighted with an asterisk whereas homologous residues are in yellow.

ANTAGONISTIC INTERPLAY BETWEEN *HERC1* AND *BCR-ABL* GENES EXPRESSION IN CHRONIC MYLOID LEUKEMIA (CML) PATIENTS

Herc1 and *BCR-ABL* genes expression were assayed in CML patients at diagnosis (n=45), remission (n=30) and relapse (n=8) by RT-qPCR and compared with normal healthy subjects (n=25). The *Herc1* mRNA quantity is expressed as $2^{-\Delta\Delta Ct}$ after normalization against *GUSB* while BCR-ABL mRNA quantity is expressed as percentage copy number after normalization against the *c-ABL* gene. *Herc1* gene expression was significantly down-regulated, both in bone marrow and in peripheral blood samples, at diagnosis when compared to control specimens while the *BCR-ABL* was up-regulated in newly diagnosed CML patients. *Herc1* gene expression at remission is comparatively similar to healthy control, while at the onset of relapse its levels decreased again. During the remission phase, in contrast to *Herc1*, *BCR-ABL* gene expression levels are dramatically reduced but increasing again at the onset of CML relapse

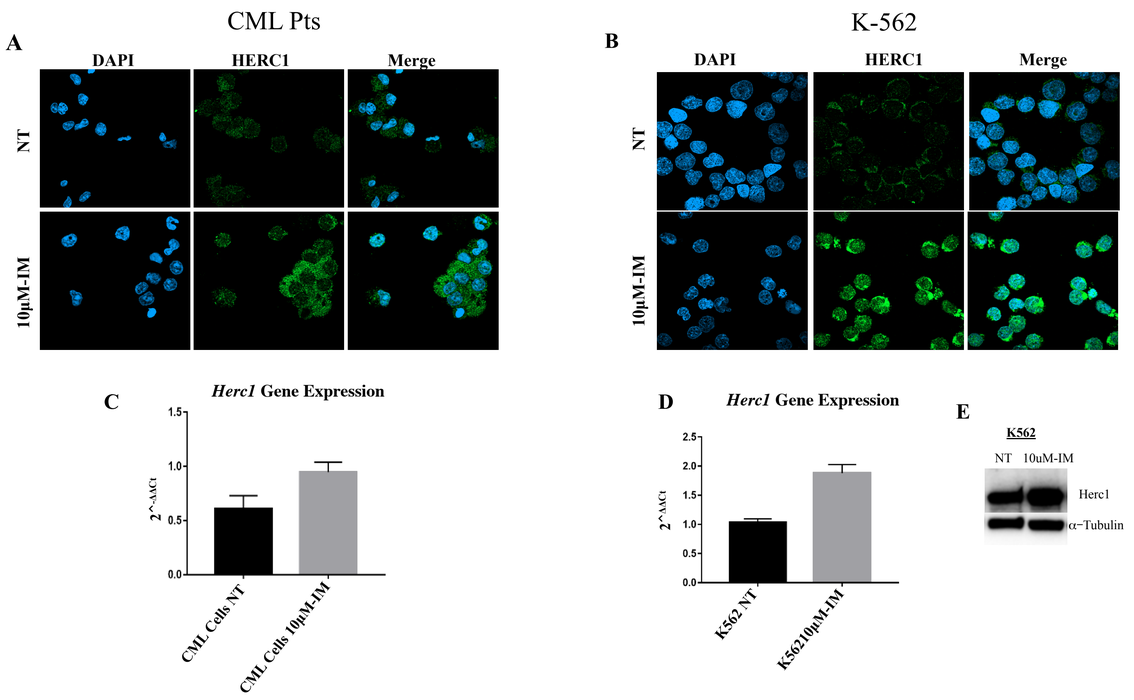


HERC1 PROTEIN EXPRESSION IS DOWNREGULATED IN CML PRIMARY AND CD34⁺ CELLS



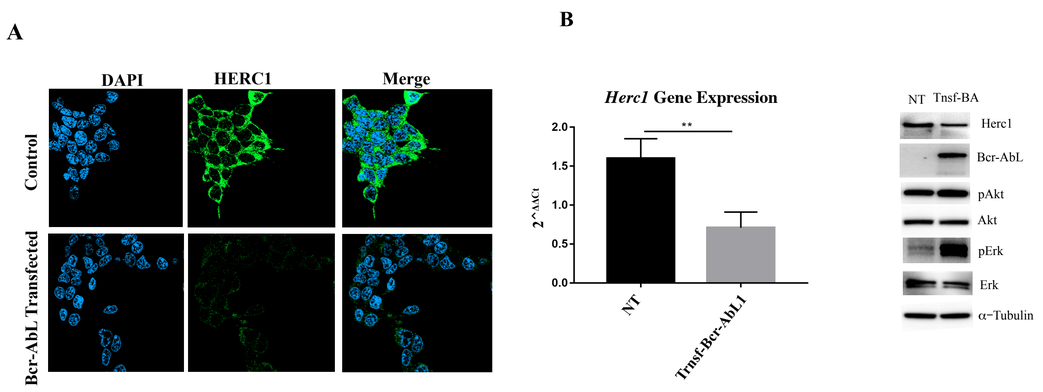
Immunofluorescence staining was performed by using rabbit polyclonal anti Herc1 antibody. DAPI staining (blue) indicates cells nuclei. The green signal, corresponding to Herc1, showed a dramatic reduction in CML patients specimens (buffy-coat and CD34+cells) when compared to healthy donors (buffy-coat and CD34+ cells) (**A**, **B**). Consistently, mRNA level of Herc1 was downregulated in leukemic CD34⁺ cells (**C**).

EFFECT OF BCR-ABL (Ph) INHIBITOR (IMATANIB) ON HERC1 PROTEIN EXPRESSION IN CML PRIMARY AND K-562 (Ph⁺) CELLS



Immunofluorescence and Western-Blot were performed by using rabbit polyclonal anti Herc1 antibody after treating, or not (NT), the K562 cell line and primary CML leukemic cells with Imatanib (IM) for 48 hours. DAPI staining (blue) indicates cells nuclei and green signal corresponds to Herc1(A, B). Similarly, cells treated with Imatinib showed an increase in Herc1 at both mRNA (C and D) and protein (E) levels protein. Tubulin was used as loading control.

EXOGENOUS BCR-ABL EXPRESSION CONTROLS THE HERC1 GENE EXPRESSION IN HUMAN EMBROYNIC KIDNEY (HEK-293T) CELLS



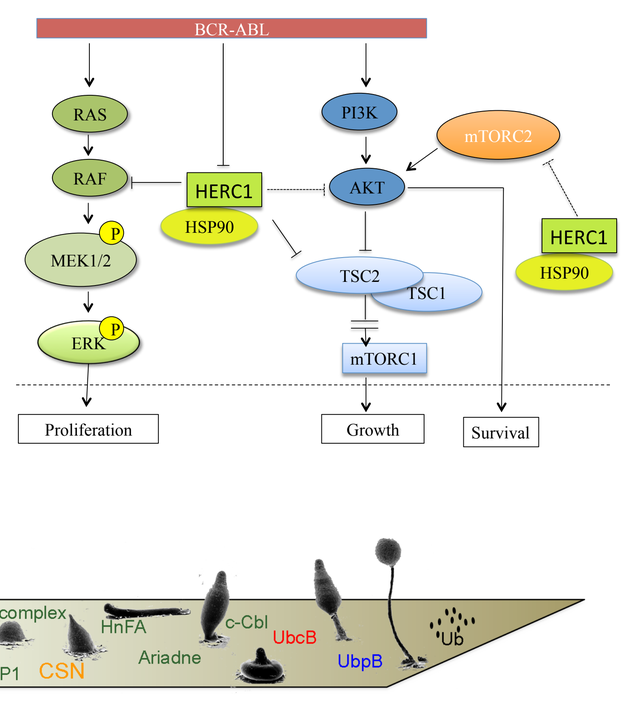
Since both CML primary cells and Ph+ cell line (K562) displayed low HERC1 gene expression levels and that its levels were sensitive to specific Bcr-Abl inhibitor, we decided to assess whether HERC1 gene expression could be affected by Bcr-Abl. HEK-293T cells were transiently tranfected, or not (NT), with *Bcr-Abl* (BA). Cells were allowed to grow on cover slip and immunofluorescence performed 48 hours post-transfection. DAPI staining (blue) indicates cells nuclei and green signal corresponds to Herc1 (A). RT-qPCR and Western-Blot analysis revealed that Bcr-Abl impaired HERC1 gene, and likely as consequence, protein expression (B).

CONCLUSIONS AND PROSPECTIVES

Our findings indicate that, in CML disorder, there is antagonistic interplay between *Herc1* and *Bcr-Abl* gene expression. Currently, the insights of this pattern is under investigation. By now, the evidence we collected, indicate that Bcr-Abl regulates Herc1 gene expression. Howerer, how this occurs and which are downstream effectors implied in this process is under investigation (depicting picture). In addition we uncovered/identified HSP90 as novel a common interactor for the Dictyostelium large HectPH1 and for the mammalian HERC1.

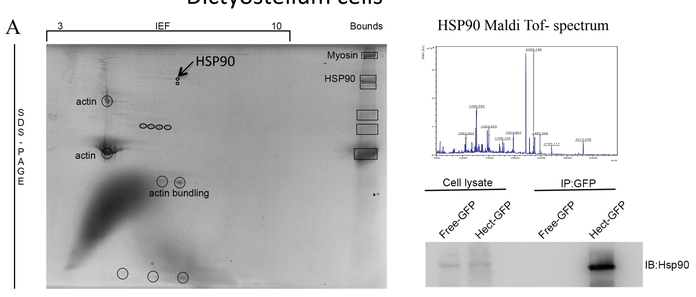
Currently, besides HERC1-mediated RAF and TSC1/2 regulation, the role/s played by this large HERC member in signal transduction is/are unknown. Based on these, and our previous, results obtained using the *Dictyostelium* as a model, we are now exploring how HectPH1 and HERC1 might regulate the mTORC2-AKT axis, crucial for cell survival and motility in a HSP90 dependent way.

So far simple model organism as *Dictyostelium* has been proved to be valuable tool to investigate basic cellular processes regulated by the ubiquitin system, which profoundly affect the whole Dictyostelium life cycle as depicted.



Representation of the characterized Ub-system components in the life cycle of *Dictyostelium*. Colour code is used to distinguish the different class of Ub-system components. Red: E2; green: E3; blue: DUBs, black: Ub and UBLs; Orange: Fanconi Anemia and CSN associated components; grey: UBDs

HSP90 AND CYTOSKELETAL PROTEINS AS NOVEL HectPH1 and HERC1 PUTATIVE BINDING PARTNERS



A) To identify HectPH1partners we performed co-ImmunoPrecipitation experiments by using a chimeric HECT domain-GFP fusion protein as bait and *Dictyostelium* total cell lysate. The co-Immunoprecipitated proteins were separated by 2D-SDS-PAGE and afterwards the spots identified by Maldi-Tof. A number of putative HectPH1 interacting partners were identified, including microfilament components. Among them, one (HSP90) has been further validated via Western-Blot to confirm the physical association. **B)** Being the Hect domain of HectPH1 highly similar to that of HERC1 we attempted to assess whether the interaction observed in *Dictyostelium* could occur also in mammalian cells. Total cell lysate from HEK-293T cells was immunoprecipitated using anti-HSP90 antibody and analyzed by immunoblotting with antibodies against the indicated proteins. As expected HSP90 specifically interacts with HERC1 but not with its closest mammals relative, namely HERC2.

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