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13. ABSTRACT (Maximum 200 Words) This proposal will test a unique hypothesis that Src-family kinases direct the ErbB receptor traffic into an intracellular compartment where proliferation signals are generated. This hypothesis is based on observations that while Cbl proto-oncoprotein facilitates the down-regulation of ErbB receptors from cell surface and functions as a negative regulator, Src tyrosine kinase enhances paradoxically enhance both the internalization of EGFR and the EGFR-mediated mitogenic signals. The proposed studies will test this hypothesis using mammary epithelial cells made to overexpress EGFR or ErbB2 together with Src. The insights gained from this model system will be directly relevant to a large proportion of breast cancers where ErbB receptors and Src-family kinases are co-overexpressed. Validation of our hypothesis will represent a shift in the current paradigm of normal and aberrant ErbB receptor signaling and may provide novel targets for therapeutic intervention relevant to ErbB-overexpressing breast cancers, which carry a significantly worse prognosis and are frequently hormone-unresponsive.				
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Introduction:

This application proposes to test a unique hypothesis that Src-family kinases direct the ErbB receptor traffic to recycling endosomal compartment, where proliferation signals are generated from the internalized pool of the activated ErbB receptor tyrosine kinases. This hypothesis is based on previous observations that the potency of mitogenic signals emanating from ErbB receptors correlates directly with the ability of these receptors to avoid the lysosomal pathway of degradation and to be sorted into the recycling pathway instead. For example, ErbB1 (EGFR) is targeted to lysosomal as well as recycling pathways, whereas the more potent ErbB2 receptor is primarily recycled. Work in our laboratory has defined the Cbl proto-oncoprotein as a novel regulator of this process. Cbl enhances the delivery of ErbB1 into the lysosomal pathway. In contrast, Cbl does not interact efficiently with ErbB2, which is primarily recycled. Paradoxically, Src tyrosine kinase enhances the EGFR internalization while at the same time enhancing the EGFR-mediated mitogenic signals. This led us to hypothesize that Src directs ErbB receptors away from lysosomes and into a recycling endosomal compartment where these receptors continue to transmit proliferation signals. The proposed studies are designed to test this hypothesis using mammary epithelial cells made to over-express EGFR or ErbB2 together with Src. This model system will provide insights directly relevant to a large proportion of breast cancers where ErbB receptors and Src-family kinases are co-over-expressed. Validation of our hypothesis will represent a shift in the current paradigm of normal and aberrant ErbB receptor signaling and may provide novel targets for therapeutic intervention relevant to ErbB-over-expressing breast cancers, which carry a significantly worse prognosis and are frequently hormone-unresponsive.

Body of Report:

Studies carried out in the course of this grant have led to the initial validation of our hypothesis that co-overexpression of Src enhances signaling through EGF receptor and have provided a series of novel cellular reagents to determine the molecular determinants of the Src-EGFR interactions within the endosomal compartment.

Despite numerous technical hurdles reported in earlier reports, we successfully generated retroviral expression vectors for co-expression of ErbB2 or EGFR with Src, using a combination of pMSCV-puro based EGFR or ErbB2 constructs together with pMSCV-hygro-Src. Using these vectors, we successfully established three distinct series of human mammary epithelial cell lines that over-express EGFR, ErbB2, Src, EGFR plus Src or ErbB2 plus Src: 16A5 (E6/E7-immortalized) and 76N-TERT (hTERT catalytic subunit immortalized) both derived from 76N cell strain with basal cell characteristics; M2-E6E7, an HPV E6/E7-immortalized derivative of a M2 milk cell strain M2, representing luminal mammary epithelial cells.

We used the soft agar growth assay to assess the impact of EGFR or ErbB2 over-expression, with and without Src, on the anchorage-independent growth, a reflection of oncogenic transformation of mammary epithelial cells (Ref #1). Indeed, the over-expression of EGFR or ErbB2 led to colony formation in soft agar; this was further increased when Src was also over-expressed (reported earlier).

A prediction of our hypothesis that overexpression of Src, which is known to increase the rate of endocytosis of EGFR (Reference # 2), will increase the intracellular pool of EGFR compared to the pool on the cell surface, but will not enhance the rate of degradation. We have tested these predictions in the following studies. When we compared the levels of EGFR on the cell surface using immunostaining with anti-EGFR antibody followed by FACS analysis, mammary epithelial cell lines co-overexpressing the EGFR and Src showed substantially lower levels of surface EGFR compared to their parental cell lines with EGFR overexpression alone (**Fig. 1**). Notably, however, the total cellular pools of EGFR as assessed by immunoblotting of cell lysates in EGFR overexpression and EGFR+Src overexpressing mammary epithelial cell lines were identical (**Fig. 2**). A clear conclusion from these results is that overexpression of Src leads to a shift of surface EGFR into an intracellular pool, located in the endosomes. Furthermore, we carried out immunoblotting analyses to demonstrate that Src overexpression, which leads to a shift of EGFR from a cell surface pool to an intracellular pool, did not affect the rate at which ligand-induced degradation of EGFR took place (**Fig. 3**). Based on these studies, our current efforts are directed at establishing that the internal pool of EGFR and Src colocalize in the recycling endosomal compartment. For this purpose, we have established the methodology to identify the recycling endosomal compartment using the accumulation of fluorescent tagged transferrin (Reference # 3) and fluorescent EHD proteins (Reference # 4) (**Fig. 4**). Notably, EHD proteins have also emerged as candidates that could regulate the traffic of ErbB receptors into and out of the recycling endosomal compartment (Reference # 4-6) and analyses of these proteins in the context of ErbB receptors has emerged as a new direction of the current research. Accordingly, we have established GFP and myc epitope tagged versions of all four human EHD proteins, have generated mutants of these proteins that are likely to disrupt their cellular function and have obtained antibodies (through an outside vendor) that are specific for each member of the family. Initial analyses (**Fig. 5**) confirm the colocalization of GFP-tagged EHD proteins with fluorescent transferrin, which is known to traverse the recycling compartment (Reference # 4-6). Overall, studies proposed under this grant have validated the basic hypothesis of the grant proposal and have given rise to two new scientific directions in the laboratory to investigate the role of endosomal signaling from ErbB receptors. One of the directions has been submitted to the NCI and approved for funding over a five-year period beginning 7/1/2004. The second direction on the role of EHD proteins in ErbB receptor signaling and traffic are expected to be submitted for NCI or other extramural support in the near future.

Key Research Accomplishments:

- Generated three distinct sets of mammary epithelial cells (16A5, 76N-TERT and M2-E6E7) with over-expression of EGFR, ErbB2, Src, EGFR+Src or ErbB2+Src.
- Established the relative over-expression of EGFR, ErbB2 and Src in transfected cells.
- Provided evidence that over-expression of EGFR plus Src or ErbB2 plus Src enhances the growth of human mammary epithelial cells.

- Provided evidence that Src overexpression leads to a shift of EGF receptor from the cell surface to an intracellular endocytic pool but does not undergo degradation.
- Established new reagents to analyze the role of the recycling endosomal compartment in ErbB receptor signaling.
- Localized the endocytic step of Cbl-mediated EGFR downregulation to sorting as opposed to internalization.

Reportable Outcomes:

Reagents:

- Generated three distinct sets of mammary epithelial cells (16A5, 76N-TERT and M2-E6E7) with over-expression of EGFR, ErbB2, Src, EGFR+Src or ErbB2+Src.
- Generated a matching pair of HPVE6 and h-TERT immortalized derivatives of an additional human mammary epithelial cell strain 81N.
- Established EGFR-expressing wildtype and Cbl-deficient MEFs.
- Established GFP and myc epitope tagged EHD1-4 proteins, their dominant-negative mutants and specific antibodies against these markers of the recycling endosomal compartment.

Funding:

Findings from research carried out under this grant formed a part of preliminary studies for the following grant application to the NIH: RO1 CA105489-01 "Endosomal ErbB Receptor and Src Signaling in Cancer" (PI, Hamid Band); approved for funding 7/1/04 – 6/30/09 (Approved budget, \$250,000 annual direct costs; awarded budget after administrative cuts, \$205,000 annual direct costs)

Conclusions:

In conclusion, we have established that co-overexpression of Src in human mammary epithelial cells, mimicking Src and ErbB receptor overexpression in breast cancer, in human mammary epithelial cells promotes oncogenic transformation. Furthermore, transfectants representing both basal and luminal cells with over-expression of EGFR, ErbB2, Src, EGFR plus Src or ErbB2 plus Src together with the appropriate vector control cell lines have been generated to test the idea that endosomal signals mediate the cooperative oncogenesis by ErbB receptors and Src. Support through this grant has led to a long term direction towards this goal that is now supported by the NCI. These studies are likely to lead to major new insights into spatio-temporal

context of oncogenic signaling in breast cancer by two eminent families of tyrosine kinases directly linked to breast cancer. In turn, these insights should be of importance in designing targeted therapies against breast cancers where ErbB and Src signaling plays an oncogenic role.

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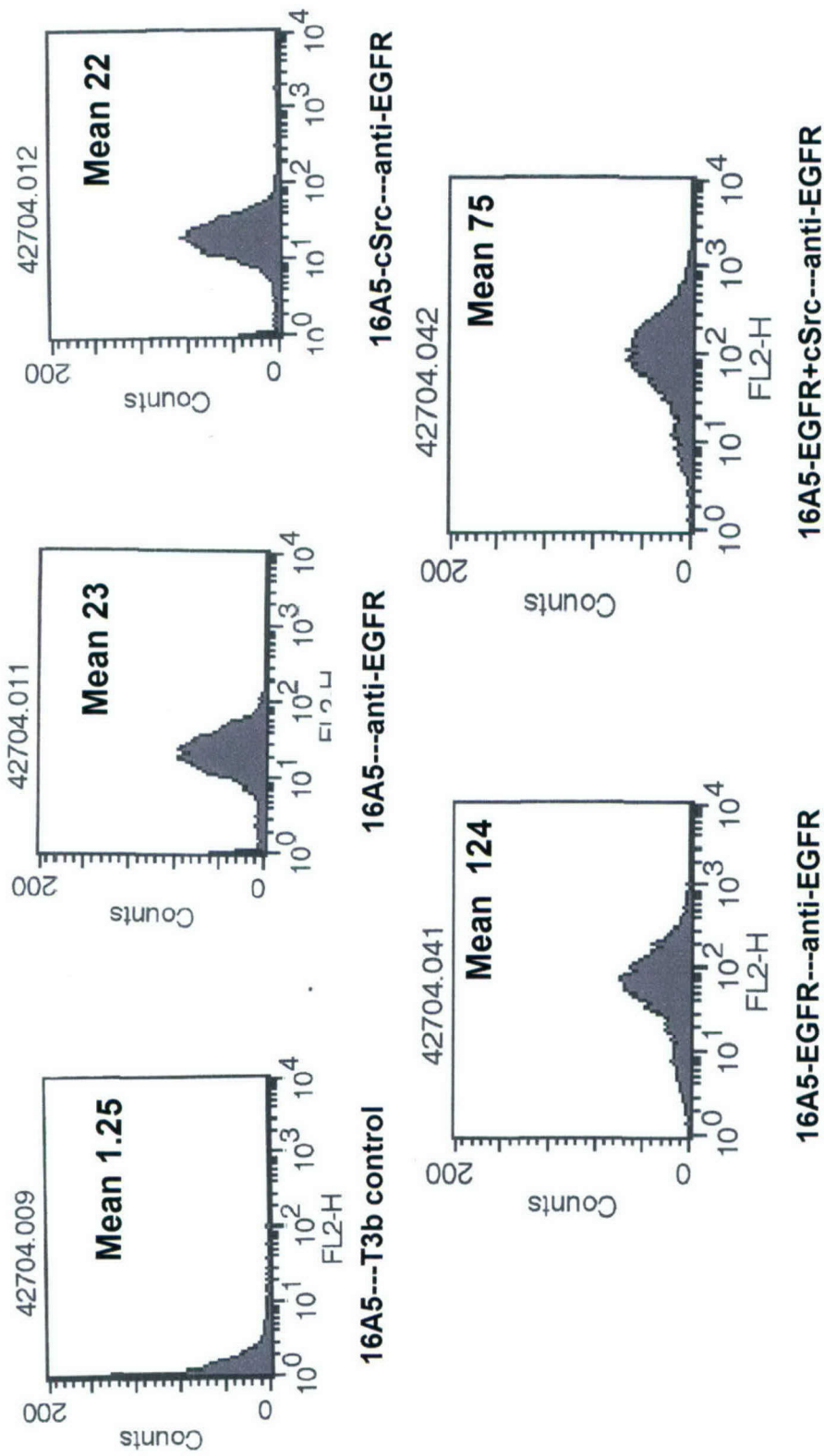


Fig. 1. Co-overexpression of Src leads to reduced cell surface levels of overexpressed EGFR in mammary epithelial cells. The 16A5 parental mammary epithelial cell line or its transfectants were deprived of EGF by growth in D3 medium for 48 hours, released with trypsin/EDTA and stained with anti-EGFR monoclonal antibody 528 or anti-CD3 antibody T3b (negative control) for 1 hr. After washing 3 times, the cells were further stained with phycoerythrin-conjugated anti-mouse secondary antibody (Jackson labs), washed 3 times and analyzed by flow cytometry in a FACSort machine. Controls were carried out with each cell line but only selected panels are shown. Note the substantially lower mean fluorescence channel intensity of anti-EGFR stained 16A5-EGFR+Src cells compared to 16A5-EGFR cells; this contrasts with the equal levels of total EGFR levels in the same cells as determined by immunoblotting (Fig. 2).

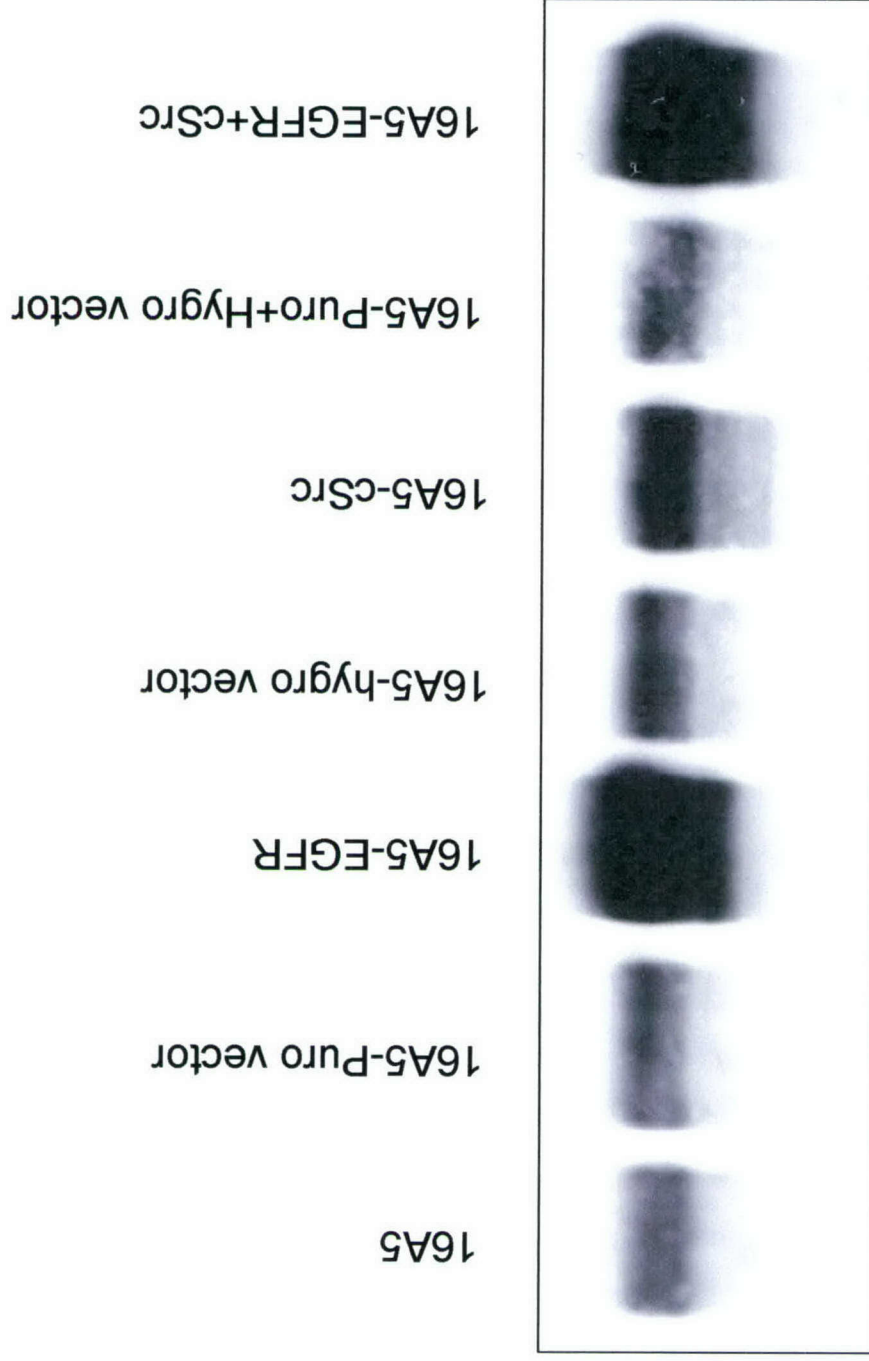


Fig. 2. Immunoblot analysis of EGFR in 16A5 transfectants. The indicated mammary epithelial cell transfectants or the parental 16A5 cell lines were EGF deprived by growth in EGF-deficient D3 medium for 48 h as in Fig. 1, and lysates were prepared in 0.5% Triton X-100 lysis buffer. 50µg aliquots of cell lysate protein were run on an 8% SDS PAGE gel and immunoblotted with a cocktail of anti-EGFR antibodies (Santa Cruz Biotech.). Note the equal levels of EGFR in 16A5-EGFR and 16A5-EGFR+Src cell lines.



EGFR
175-CTD

Fig. 3. Ligand-induced EGFR degradation is unaffected by Src overexpression. The indicated mammary epithelial cell transfectants or the parental 16A5 cell lines were EGF deprived by growth in EGF-deficient D3 medium for 48 h as in Fig. 1, and then stimulated with EGF (100ng/ml) for the indicated times (in minutes) and lysates prepared in 0.5% Triton X-100 lysis buffer. 50µg aliquots of cell lysate protein were run on an 8% SDS PAGE gel and immunoblotted with a cocktail of anti-EGFR antibodies (Santa Cruz Biotech.). Note the loss of EGFR signals over time (indicative of degradation) is comparable between 16A5-EGFR and 16A5-Src+Src cell lines.

Fig. 4. Domain structure of EHD proteins and their homology

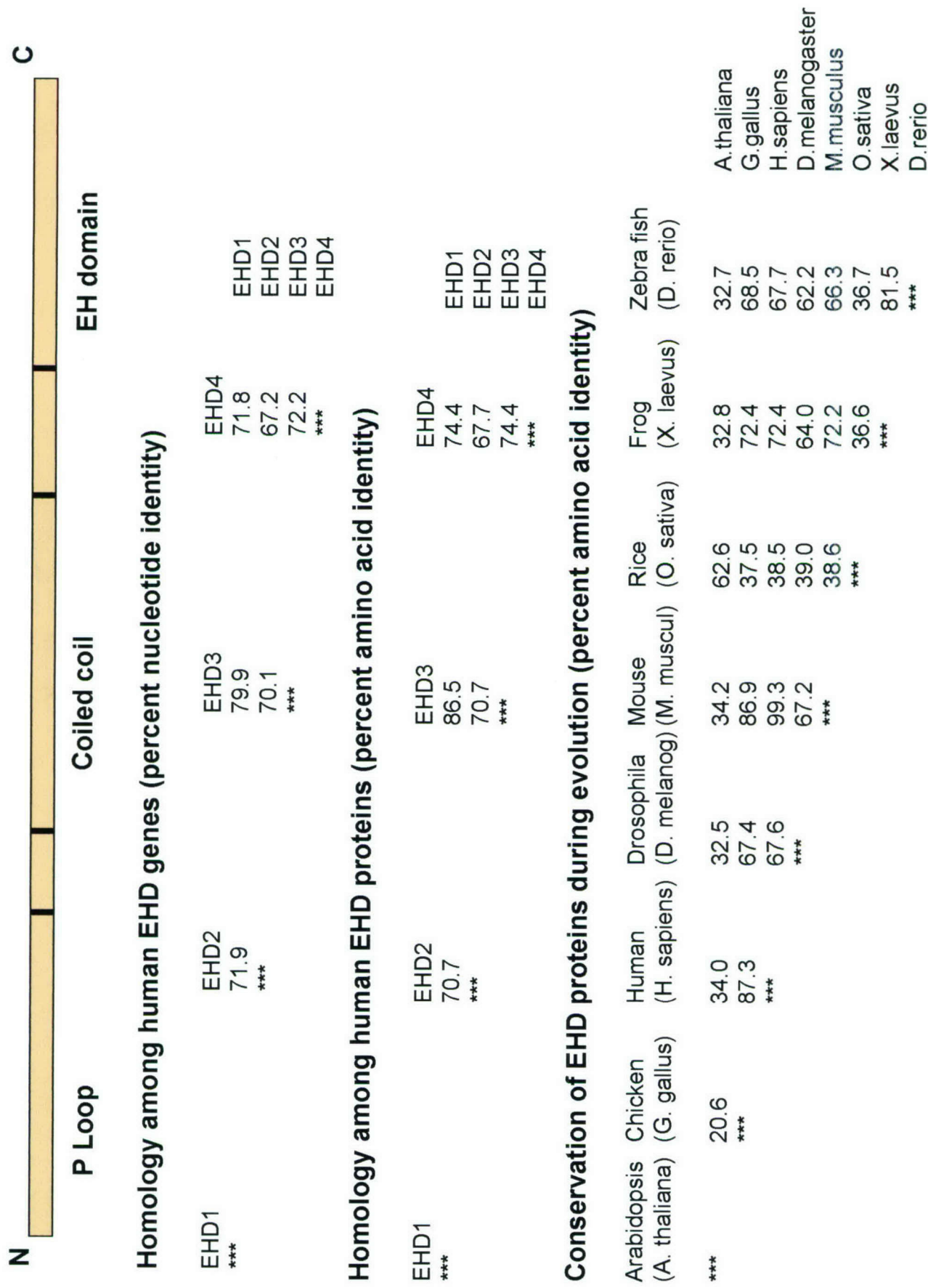




Fig. 5A. Colocalization of EHD proteins with transferrin. Human epithelial cell line Hela was transfected with an expression construct encoding the EHD1-GFP chimeric protein using the calcium phosphate transfection method. 48 hrs post-transfection, the cells were EGF deprived by growth in low serum (0.5% FCS) medium for 1 hr. Cells were then allowed to bind Alexa 594-conjugated Transferrin for 30 minutes on ice, washed and the bound transferrin allowed to internalize for 15 minutes in the presence of holo-transferrin, to demarcate the early and recycling endosomes. Confocal immunofluorescence microscopy was performed with a Nikon fluorescence microscope to visualize green (EHD-GFP) and red (transferrin) fluorescence. A merge of the two signals is shown on the right; colocalization is indicated by the similarity of the green and red signals and the yellow color in the merged picture. **Fig. 5A** shows colocalization of EHD1 and transferrin.



Fig. 5B. Colocalization of EHD2 with transferrin. The procedure was as in Fig. 5A.



Fig. 5C. Colocalization of EHD3 with transferrin. The procedure was as in Fig. 5A.

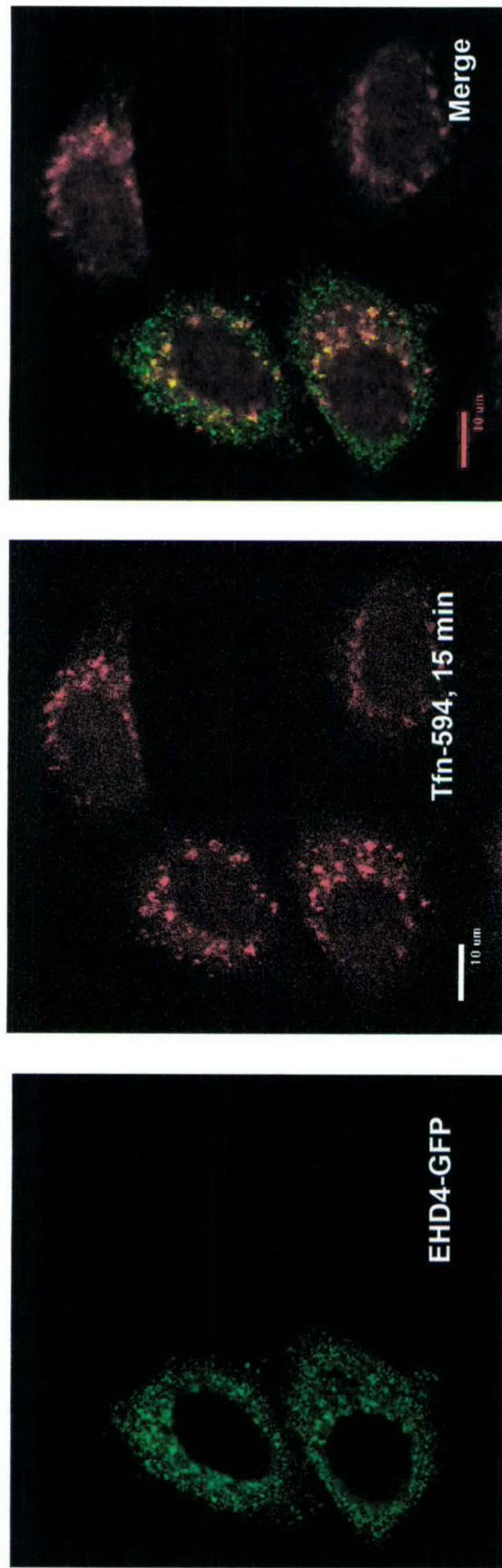


Fig. 5D. Colocalization of EHD4 with transferrin. The procedure was as in Fig. 5A.