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Supplemental Information

Global Analysis of Intercellular Homeodomain Protein Transfer

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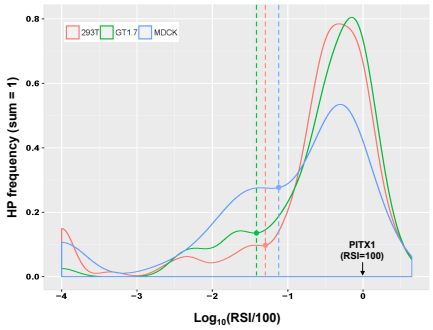
A

Classification of sHP and nsHP

Cell-lines	Cut-off (RSI)	# of sHP	# of nsHP
293T	4	136	26
GT1-7	3	141	21
MDCK	8	98	64



B

Cell type specificity

Cell-lines	Frequency (# of sHP)
293T only	5
GT1-7 only	9
MDCK only	0
293T+GT1-7	40
293T+MDCK	6
GT1-7+MDCK	7
293T+GT1-7+MDCK	85
None	10

C

Inter-cell-line correlation (log)

Cell-lines	PCC	p-values
293T vs. GT1-7	0.449	2.119E-09
293T vs. MDCK	0.399	1.478E-07
GT1-7 vs. MDCK	0.440	4.707E-09

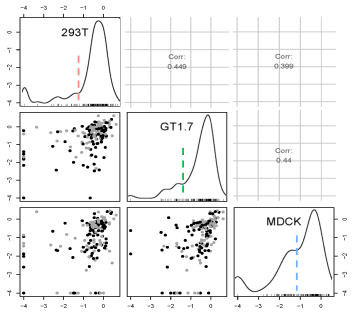


Figure S1 (related to Figure 1). Classification of secretory and non-secretory HPs. (**A**) The HPs, which were examined by DB and WB to measure their secretion efficiencies (Figure 1D; Dataset S1; Table S1), are classified to secretory (sHP) and non-secretory (nsHP) HPs based on their log-transformed RSI values ($\log_{10}$(RSI/100)). Since the distribution of the RSI is roughly bimodal shape for each cell-line, the cut-off RSI values of sHPs were defined as the baselines of major peaks (dotted lines in the graph). (**B**) Based on the classification in (A), the numbers of HPs secreted in each cell-line are listed. The results indicate that more than half of HPs were secreted commonly in all three cell-lines. (**C**) Correlation of log-transformed RSI in two cell-lines is determined by measuring the Pearson's correlation coefficient (PCC). The results show moderate (between 0.4 and 0.5) RSI correlations between cell-lines.

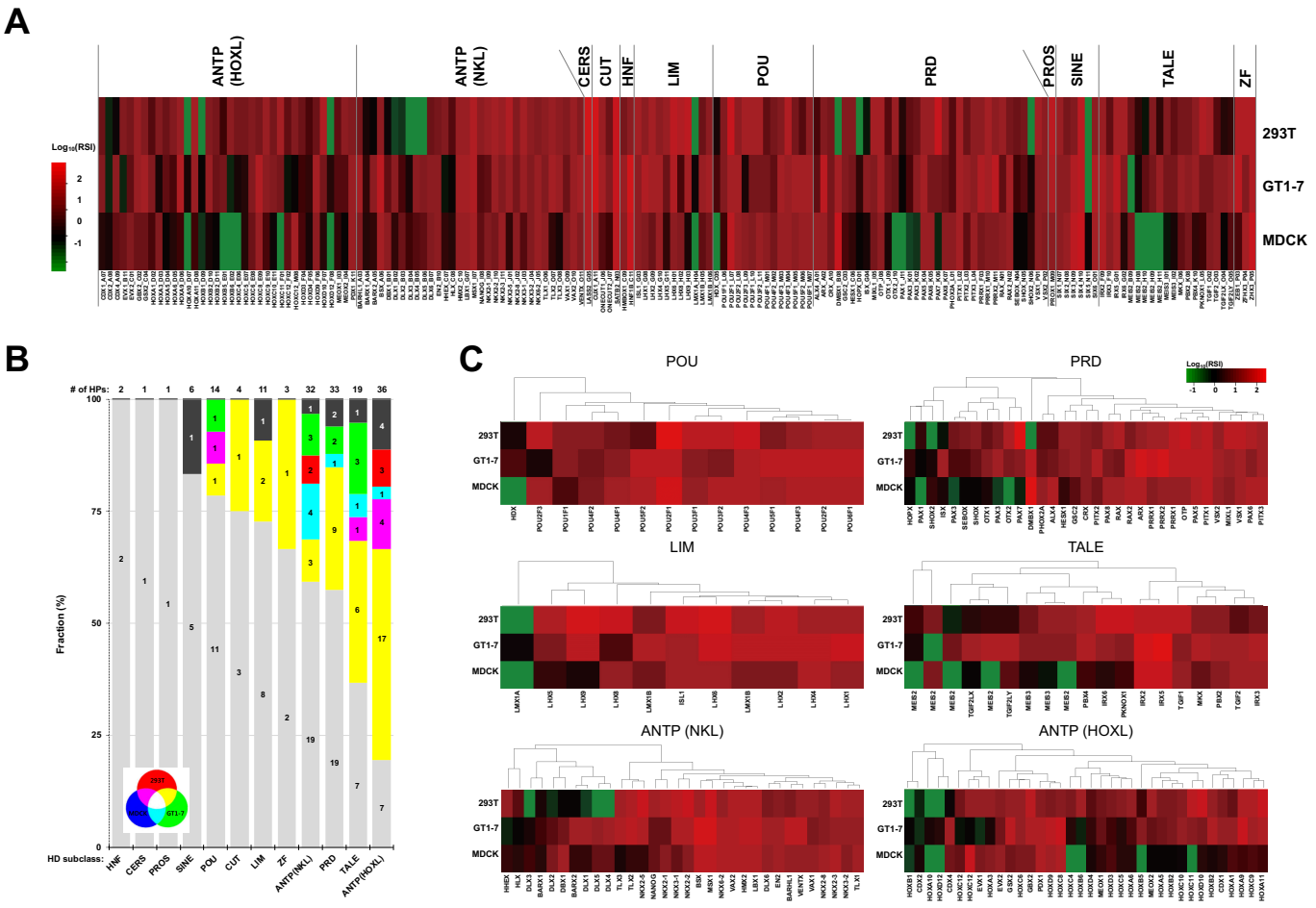


Figure S2 (related to Figure 1). Examination of HD subclass-specific secretion tendency. (**A**) Heat maps of average log-transformed RSI scores of corresponding HPs belong to each HD subclass are shown (n=3). The columns are ordered according to the hierarchical clustering of HPs by their log-transformed RSI scores across cell-lines. (**B**) To investigate a selective secretion of HD subclass in specific cell-lines, we counted the numbers of sHPs and nsHPs belonged to each HD subclass and show the results in a graph. We failed to find major HD subclasses (>10 HP members), which are secreted or not secreted exclusively in certain cell-lines. (**C**) Heat maps of average log-transformed RSI scores of corresponding HPs belong to each HD subclass are shown (n=3). The columns are ordered according to the hierarchical clustering of HPs by their log-transformed RSI scores across cell-lines.

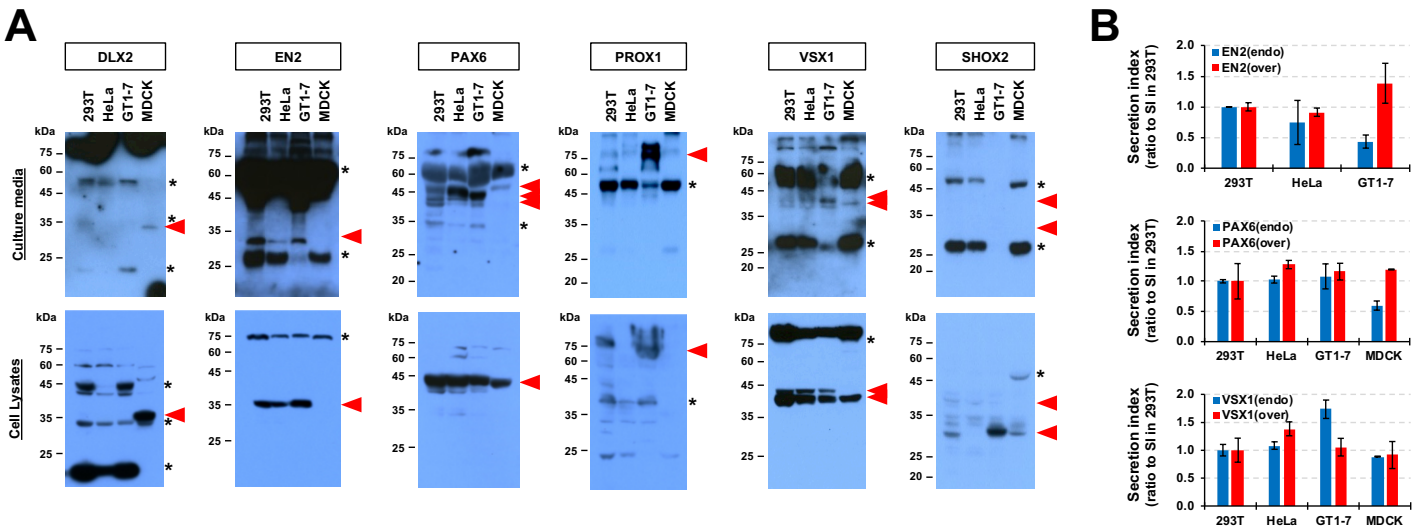


Figure S3 (related to Figure 1). Evaluation of the secretabilities of endogenous HPs. (A) (Top) Growth media from 293T, HeLa, GT1-7 and MDCK cells were collected, and the presence of the indicated HPs in the media was examined by WB with specific antibodies for corresponding HPs. (Bottom) Expression of the HPs in the cells was also determined by WB. Arrows indicate specific WB bands, and asterisks indicate non-specific bands. **(B)** Secretion indices (SI) of endogenous EN2, PAX6, and VSX1, which are expressed in multiple cell-lines, were obtained by dividing WB intensities of HPs in the growth media by cell lysates, and those are shown in the graphs as relative values to the SI of 293T cells (n=3). The SI of overexpressed V5-EN2, V5-PAX6, and V5-VSX1 are also shown to compare the secretion efficiencies of endogenous and overexpressed HPs in each cell-line. Error bars denote SDs.

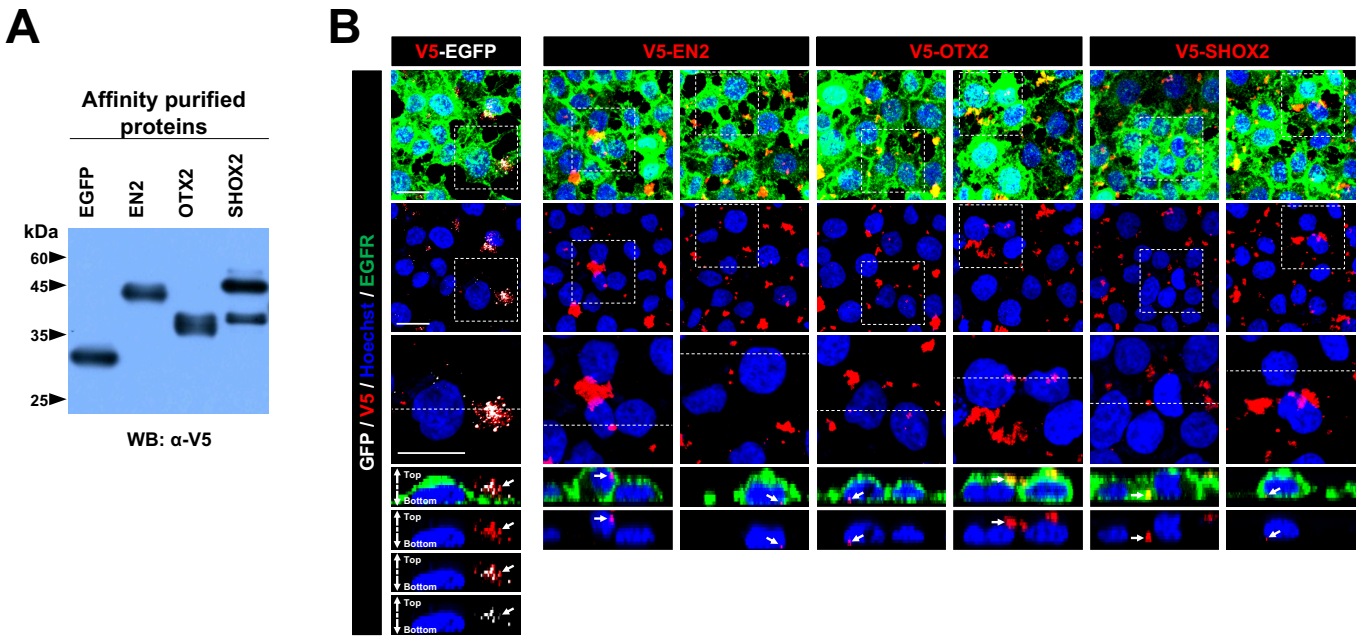


Figure S4 (related to Figure 2). Cell penetration is a unique feature of HP. (**A**) V5-tagged EGFP and HPs were overexpressed in 293T cells, and then were affinity purified with anti-V5 antibody. Relative levels of the purified proteins were determined by WB analysis using anti-V5 antibody (1 μg proteins were loaded in each lane). (**B**) To determine cell-penetrating activities of V5-EGFP and V5-HPs, the affinity purified proteins (100 ng/ml protein) were added into the growth media of HeLa cells. The V5-tagged proteins inside and outside of the cells were then visualized by immunostaining with anti-V5 antibody (red). The cell was also co-staining with anti-EGFR antibody (green), which detect EGFR in the plasma membrane and intracellular trafficking vesicles, and the nuclei were visualized by Hoechst 33342 staining (blue). Images in the third row are magnified versions of dotted-box areas in the top two rows. Images in the bottom rows are x/z-axial views of the positions indicated by dotted lines in the third row. Arrows points EGFP in the extracellular space and HPs inside the cells. Scale bars, 20 μm.

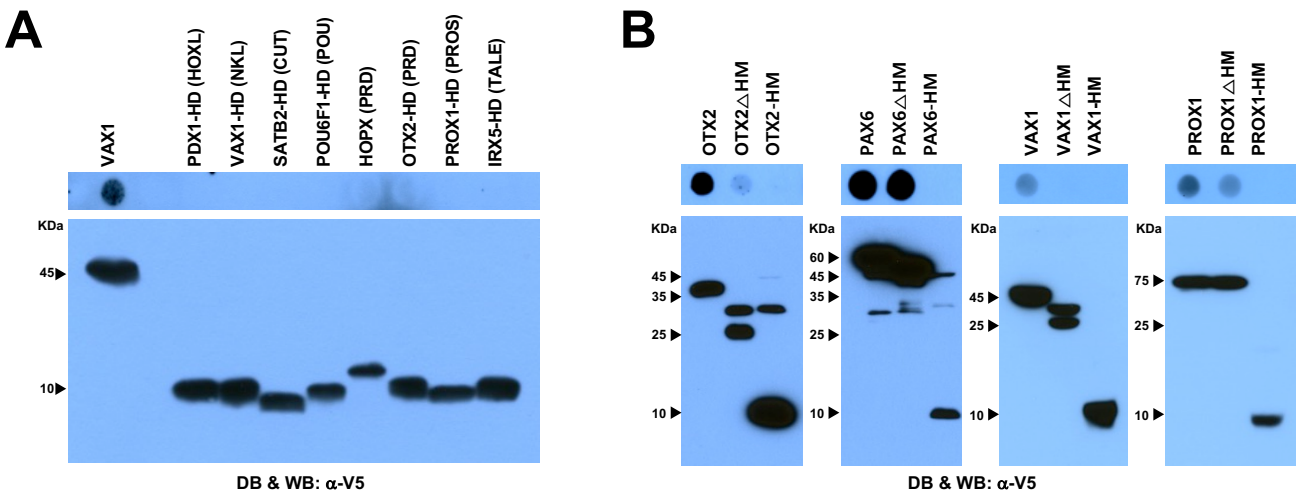


Figure S5 (related to Figure 5). The homeodomain is not sufficient for the secretion of HP. (A) The HDs of indicated HPs are isolated, fused to V5-tag, and expressed in 293T cells to examine their secretabilities by same method used in Figure 1C. Full-length V5-Vax1 was used as a positive control for the secretion. **(B)** The secretabilities of HD-deleted HP mutants were also examined.

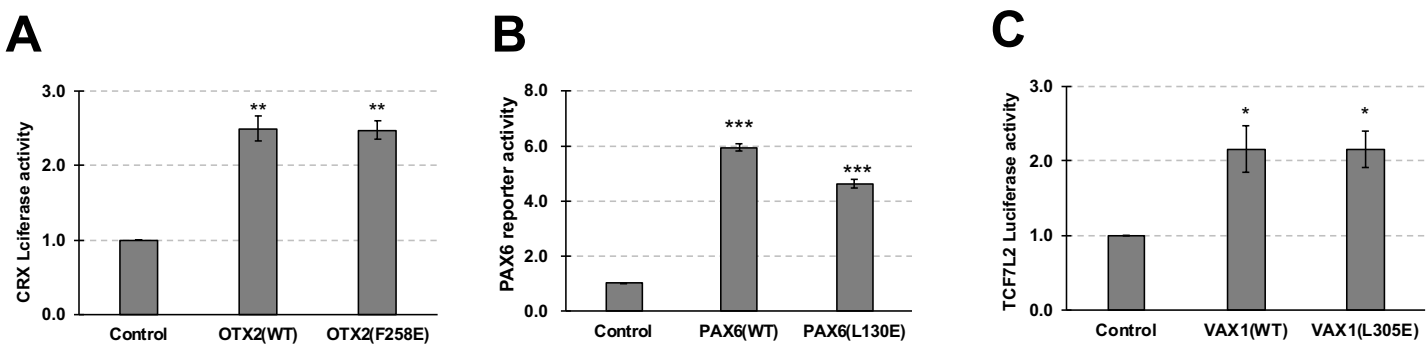


Figure S6 (related to Figure 5 and 6). Transcription factor activity of secretion-defective sHPs. **(A)** Transcription factor activities of secretion-defective OTX2(F258E) were compared with OTX2(WT) by measuring the activities of luciferase expressed at downstream of an OTX2 target *CRX* promoter in the transfected cells. Luciferase activity in each sample was normalized by co-expressed β-galactosidase activity, and relative values to reporter-only control samples are shown in a graph. **(B** and **C)** Transcription activities of PAX6 and VAX1 variants were also measured by examining the luciferase expression, which is induced by tandem (6X) PAX6 target DNA sequence or TCF7L2 promoter, respectively. Values in the y-axes of the graphs are average (n=5). Error bars denote SDs (*, $p<0.01$; **, $p<0.005$; ***, $p<0.001$; ANOVA).