**Supplementary Table 1.** Cleavage yield quantifications in Fig. 2.

| Fig. | 2a | 2b | 2c | 2c | 2c | 2c | 2c | 2c | 2c | 2c | 2c | 2c | 2c | 2c | 2c |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lane | 2 | 2 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| Yield(%) | 17 | 80 | 67 | 75 | 70 | 55 | 60 | 35 | 67 | 20 | 70 | 67 | 20 | 60 | 60 |

| Fig. | 2d | 2e | 2f | 2f | 2f | 2f | 2f | 2f | 2f | 2f | 2f | 2f | 2f | 2f | 2f | 2g | 2g | 2g | 2g | 2g | 2g |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lane | 2 | 2 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 1 | 2 | 3 | 4 | 5 | 6 |
| Yield(%) | 20 | 90 | 88 | 94 | 91 | 87 | 85 | 50 | 80 | 30 | 95 | 97 | 30 | 50 | 70 | 90 | 92 | 92 | 90 | 92 | 30 |

**Supplementary Table 2.** Substrate phage selection result of sequence pattern –XXXGSXHXXX–.

| Sequence | output reads | control reads | Enrichment factor | Cleavage validated? | Membrane protein cleavage efficiency | Globular protein cleavage efficiency |
|---|---|---|---|---|---|---|
| GCPGSKHNCG | 23917 | 1 | 23917 | Yes | 30% | 15% |
| NFTGSQHTMS | 11320 | 0.5 | 22640 | Yes | 86% | 60% |
| NSLGSQHQAQ | 21820 | 1 | 21820 | Yes | 77% | 55% |
| KSNGSHHFMN | 10330 | 0.5 | 20660 | Yes | 85% | 70% |
| NMSGSHHNYS | 17260 | 1 | 17260 | Yes | 86% | 60% |
| FDSGSKHDIS | 7012 | 0.5 | 14024 | Yes | 85% | 45% |
| LTVGSSHDGS | 6807 | 0.5 | 13614 | Yes | 80% | 60% |
| SDKGSLHHAF | 6468 | 0.5 | 12936 | Yes | 45% | 20% |
| KPCGSTHQLP | 12195 | 1 | 12195 | no | N/A | N/A |
| RKAGSVHATS | 11610 | 1 | 11610 | Yes | 45% | 15% |
| SSRGSSHGDR | 11002 | 1 | 11002 | Yes | 91% | 60% |
| AMNGSSHFRT | 5011 | 0.5 | 10022 | Yes | 91% | 70% |
| SLNGSSHLST | 4881 | 0.5 | 9762 | no | N/A | N/A |
| SKAGSYHDTK | 4594 | 0.5 | 9188 | no | | |
| DPRGSAHRRH | 4472 | 0.5 | 8944 | no | | |
| GLRGSRHRGI | 8940 | 1 | 8940 | no | | |
| SEEGSQHGNV | 8462 | 1 | 8462 | no | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| DGNGSFHIRR | 8284 | 1 | 8284 | no | | |
| NHKGSYHNYS | 8137 | 1 | 8137 | no | | |
| LTPGSEHEGL | 7783 | 1 | 7783 | no | | |
| SSQGSRHLGK | 3839 | 0.5 | 7678 | no | | |
| YPYGSRHCTR | 3784 | 0.5 | 7568 | no | | |
| DPNGSLHISD | 7520 | 1 | 7520 | no | | |
| KILGSRHNHK | 29637 | 4 | 7409 | no | | |
| AMRGSHHPHI | 7326 | 1 | 7326 | no | | |
| MGWGSLHIIL | 14643 | 2 | 7321 | no | | |
| NKMGSPHNCT | 3466 | 0.5 | 6932 | no | | |
| LGAGSHHISQ | 3368 | 0.5 | 6736 | no | | |
| ETCGSAHLRA | 6720 | 1 | 6720 | no | | |
| LRTGSHHLLR | 3346 | 0.5 | 6692 | no | | |
| ARTGSQHVVN | 3235 | 0.5 | 6470 | no | | |
| PTYGSPHVRP | 6412 | 1 | 6412 | no | | |
| TSSGSHHLKS | 3190 | 0.5 | 6380 | no | | |
| YDGGSQHPNV | 6334 | 1 | 6334 | no | | |
| WQQGSLHGYE | 31511 | 5 | 6302 | no | | |
| LTPGSEHEGL | 6277 | 1 | 6277 | no | | |
| AMRGSHHPHI | 6248 | 1 | 6248 | no | | |
| SLTGSSHGDT | 3039 | 0.5 | 6078 | no | | |
| VKSGSAHNAF | 3000 | 0.5 | 6000 | no | | |
| YTKGSSHFRD | 2848 | 0.5 | 5696 | no | | |
| IRTGSVHYRT | 5678 | 1 | 5678 | no | | |
| HMNGSRHQVS | 21818 | 4 | 5454 | no | | |
| LVSGSVHNQS | 5400 | 1 | 5400 | no | | |
| WQRGSLHVAI | 2619 | 0.5 | 5238 | no | | |
| RHYGSDHLVH | 2603 | 0.5 | 5206 | no | | |
| AELGSSHYGR | 5190 | 1 | 5190 | no | | |
| RQVGSGHTPY | 5128 | 1 | 5128 | no | | |
| PGAGSEHLNN | 5080 | 1 | 5080 | no | | |
| WPGGSVHTSS | 5078 | 1 | 5078 | no | | |
| YQTGSAHRNK | 5005 | 1 | 5005 | no | | |

Note: Output reads is the number of the total reads of the same peptide sequence in the output library. Control reads is the number of the total reads of the same peptide sequence in the output library of the control selection when no $Ni^{2+}$ was added during cleavage step. Ranking is based on the ratio of output reads over control reads. Read number 0.5 means the specific sequence was not found in the sequencing result file.

**Supplementary Table 3.** Amino acid identity influence on cleavage efficiency at five different positions ($X_1$-$X_5$) near cleavage site in the peptide WL$X_1X_2$S$X_3$H$X_4X_5$. Cleavage conditions: 1mM $NiCl_2$, 0.1 M CHES, pH 8.2, 22 °C, 18 hours, peptide 0.2 mM.

| Sequence | Cleavage | Sequence | Cleavage | Sequence | Cleavage | Sequence | Cleavage | Sequence | Cleavage |
|---|---|---|---|---|---|---|---|---|---|
| WLGGSRHW$X_5$ |  | WLGGSRH$X_4$G |  | WLGGS$X_3$HWG |  | WLG$X_2$SRHWG |  | WL$X_1$GSRHWG |  |
| WLGGSRHWA | 45% | WLGGSRHAG | ~15% | WLGGSAHWG | ~30% | WLGASRHWG | 75% | WLAGSRHWG | ~25% |
| WLGGSRHWD | 38% | WLGGSRHDG | ~15% | WLGGSDHWG | <10% | WLGDSRHWG | 15% | WLDGSRHWG | 66% |
| WLGGSRHWE | ~40% | WLGGSRHEG | ~15% | WLGGSEHWG | ~20% | WLGESRHWG | 30% | WLEGSRHWG | 55% |
| WLGGSRHWF | ~40% | WLGGSRHFG | ~25% | WLGGSFHWG | ~50% | WLGFSRHWG | 25% | WLFGSRHWG | 55% |
| WLGGSRHWG | 45% | WLGGSRHGG | ~15% | WLGGSGHWG | ~30% | WLGHSRHWG | >93% | WLHGSRHWG | 83% |
| WLGGSRHWH | ~35% | WLGGSRHHG | ~15% | WLGGSHHWG | >95% | WLGISRHWG | <5% | WLKGSRHWG | ~25% |
| WLGGSRHWK | ~45% | WLGGSRHIG | ~27% | WLGGSIHWG | ~20% | WLGKSRHWG | 80% | WLLGSRHWG | 66% |
| WLGGSRHWL | ~45% | WLGGSRHKG | ~15% | WLGGSKHWG | ~45% | WLGLSRHWG | 40% | WLMGSRHWG | 50% |
| WLGGSRHWM | ~40% | WLGGSRHLG | ~35% | WLGGSLHWG | ~20% | WLGMSRHWG | 35% | WLNGSRHWG | 72% |
| WLGGSRHWN | ~45% | WLGGSRHMG | ~15% | WLGGSMHWG | ~20% | WLGNSRHWG | 91% | WLPGSRHWG | >95% |
| WLGGSRHWP | ~45% | WLGGSRHNG | ~15% | WLGGSNHWG | ~35% | WLGPSRHWG | <10% | WLQGSRHWG | 35% |
| WLGGSRHWQ | ~45% | WLGGSRHPG | <10% | WLGGSQHWG | ~35% | WLGQSRHWG | ~76% | WLRGSRHWG | 35% |
| WLGGSRHWR | ~45% | WLGGSRHQG | ~15% | WLGGSSHWG | ~30% | WLGRSRHWG | ~80% | WLSGSRHWG | 35% |
| WLGGSRHWS | ~45% | WLGGSRHRG | ~15% | WLGGSTHWG | ~15% | WLGSSRHWG | ~70% | WLVGSRHWG | 66% |
| WLGGSRHWV | ~45% | WLGGSRHSG | ~15% | WLGGSVHWG | ~15% | WLGTSRHWG | ~70% | WLWGSRHWG | ~20% |
| WLGGSRHWW | ~35% | WLGGSRHTG | ~20% | WLGGSWHWG | ~20% | WLGVSRHWG | <10% | WLYGSRHWG | ~11% |
| WLGGSRHWY | ~45% | WLGGSRHVG | ~15% | WLGGSYHWG | ~15% | WLGWSRHWG | <10% |  |  |
|  |  | WLGGSRHYG | <10% |  |  | WLGYSRHWG | ~20% |  |  |

**Supplementary Table 4.** Cleavage efficiency of different sequence combinations from position scanning results of Supplementary Table 3. Cleavage conditions: 1 mM $NiCl_2$, 0.1 M CHES, pH 8.2, 22 °C, 18 hours, peptide 0.2 mM. The amino acid preference at position $X_2$ of Supplementary Table 3 was not fully recapitulated in this peptide system.

| Sequence | Cleavage |
|---|---|
| `YFLGGSHHWG` | 82% |
| `YFLGASRHWG` | <20% |
| `YFLGHSRHWG` | <20% |
| `YFLGKSRHWG` | 40% |
| `YFLGNSRHWG` | 88% |
| `YFLGQSRHWG` | 80% |
| `YFLGRSRHWG` | 40% |
| `YFLGSSRHWG` | 65% |
| `YFLHGSRHWG` | 79% |
| `YFLNGSRHWG` | 65% |
| `YFLPGSRHWG` | 88% |
| `YFLPGSHHWG` | >98% |
| `YFLHGSHHWG` | 95% |
| `YFLGHSHHWG` | <10% |
| `YFLGNSHHWG` | 77% |
| `YFLGQSHHWG` | 52% |
| `YFLGRSHHWG` | 46% |
| `YFLPHSHHWG` | <5% |
| `YFLPNSHHWG` | >95% |
| `YFLHHSHHWG` | <30% |
| `YFLHQSHHWG` | 83% |
| `YFLHNSHHWG` | >98% |
| `YFLNHSHHWG` | 49% |

**Supplementary Table 5.** Different peptide sequences and gene sequences that were inserted into HB2225 and 3hbtmV2 for cleavage testing.

| | Peptide sequence | Gene sequence |
|---|---|---|
| 1 | GSHHSSPNG | ggcagccatcatagcagcccgaacggc |
| 2 | GSHHTDLPG | ggcagccatcataccgatctgccgggc |
| 3 | PGSKHNCGG | ccgggcagcaaacataactgcggcggc |
| 4 | SGSHHNYSG | agcggcagccatcataactatagcggc |
| 5 | LGSQHQAQG | ctgggcagccagcatcaggcgcagggc |
| 6 | NGSSHFRTG | aacggcagcagccattttcgcaccggc |
| 7 | NGSHHFMNG | aacggcagccatcattttatgaacggc |
| 8 | SGSKHDISG | agcggcagcaaacatgatattagcggc |
| 9 | KGSLHHAFG | aaaggcagcctgcatcatgcgtttggc |
| 10 | AGSVHATSG | gcgggcagcgtgcatgcgaccagcggc |
| 11 | RGSSHGDRG | cgcggcagcagccatggcgatcgcggc |
| 12 | TGSQHTMSG | accggcagccagcataccatgagcggc |
| 13 | VGSSHDGSG | gtgggcagcagccatgatggcagcggc |