Supplementary Material

PD-L1 amplification is associated with an immune cell rich phenotype in squamous cell cancer of the lung.
Goldmann T, Marwitz S, Nitschkowski D, Krupar R, Backman M, Elfving H, Thurfjell V, Lindberg A, Brunnström H, La Fleur L, Mezheyeuski A, Mattsson JSM, Botling J, Micke P, Strell C

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## Supplementary Tables

### Supplementary Table 1

Supplementary table 1: Association between PD-L1 amplification and clinico-pathologic parameters in Uppsala 06 and Uppsala 95 cohorts.

| Variable                  | No PDL1 Amp | PDL1 Amp | p-value | No PDL1 Amp | PDL1 Amp | p-value | No PDL1 Amp | PDL1 Amp | p-value | Total (n=294) | AC (n=187) | PDL1 Amp | SqCC (n=87) | Total (n=221) | p-value |
|---------------------------|-------------|----------|---------|-------------|----------|---------|-------------|----------|---------|----------------|------------|----------|--------------|----------------|---------|
| Age, years:               |             |          |         |             |          |         |             |          |         | Total (n=294) |             |          |              | Total (n=221) |         |
| ≤70                       | 175 (62.5)  | 9 (64.3) | 1.000   | 118 (65.8)  | 5 (62.5) | 1.000   | 47 (58)    | 4 (66.7) | 1.000   | 146 (69.2)    | 8 (80)    | 0.727    |              |                |         |
| >70                       | 105 (37.5)  | 5 (35.7) | -       | 61 (34.1)   | 3 (37.5) | -       | 34 (42)    | 2 (33.3) | -       | 65 (30.8)     | 2 (20)    | -        |              |                |         |
| Gender:                   |             |          |         |             |          |         |             |          |         | Total (n=294) |             |          |              | Total (n=221) |         |
| female                    | 144 (51.4)  | 8 (57.1) | 0.787   | 100 (55.9)  | 6 (75)   | 0.469   | 36 (44.4)  | 2 (33.3) | 0.692   | 94 (44.5)     | 7 (70)    | 0.192    |              |                |         |
| male                      | 136 (48.6)  | 6 (42.9) | -       | 79 (44.1)   | 2 (25)   | -       | 45 (55.6)  | 4 (66.7) | -       | 117 (55.5)    | 3 (30)    | -        |              |                |         |
| Stage (TNM):              |             |          |         |             |          |         |             |          |         | Total (n=294) |             |          |              | Total (n=221) |         |
| Ia-ib                     | 175 (62.5)  | 10 (71.5)| 0.582   | 112 (62.8)  | 6 (75)   | 0.712   | 52 (64.2)  | 4 (66.7) | 1.000   | 149 (70.6)    | 6 (60)    | 0.490    |              |                |         |
| IIa-IV                    | 105 (37.5)  | 4 (37.5) | -       | 67 (37.4)   | 2 (25)   | -       | 29 (35.8)  | 2 (33.3) | -       | 62 (29.4)     | 4 (40)    | -        |              |                |         |
| WHO performance score:    |             |          |         |             |          |         |             |          |         | Total (n=294) |             |          |              | Total (n=221) |         |
| 0                         | 175 (62.5)  | 10 (71.5)| 0.582   | 117 (65.4)  | 6 (75)   | 0.717   | 45 (55.6)  | 4 (66.7) | 0.692   | 113 (53.6)    | 3 (30)    | 0.198    |              |                |         |
| 1-3                       | 105 (37.5)  | 4 (28.6) | -       | 62 (34.6)   | 2 (25)   | -       | 36 (44.4)  | 2 (33.3) | -       | 98 (46.4)     | 7 (70)    | -        |              |                |         |
| Smoker:                   |             |          |         |             |          |         |             |          |         | Total (n=294) |             |          |              | Total (n=221) |         |
| never                     | 33 (11.8)   | 0 (0)    | 0.380   | 21 (11.7)   | 0 (0)    | 0.187   | 5 (6.2)    | 0 (0)    | 1.000   | 19 (9.1)      | 0 (0)     | 1.000    |              |                |         |
| former/current            | 247 (88.2)  | 14 (100) | -       | 158 (88.3)  | 8 (100)  | -       | 76 (93.8)  | 6 (100)  | -       | 190 (90.9)    | 10 (100)  | -        |              |                |         |
| Histology:                |             |          |         |             |          |         |             |          |         | Total (n=294) |             |          |              | Total (n=221) |         |
| AC                        | 179 (63.9)  | 8 (57.1) | 0.769   | -           | -       | -       | -          | -       | -       | 115 (54.5)    | 3 (30)    | 0.021    |              |                |         |
| SqCC                      | 81 (28.9)   | 6 (42.9) | -       | -           | -       | -       | -          | -       | -       | 77 (36.5)     | 3 (30)    | -        |              |                |         |
| AdSq                      | 5 (1.8)     | 0 (0)    | -       | -           | -       | -       | -          | -       | -       | -              | -        | -        |              |                |         |
| LCC                       | 5 (1.8)     | 0 (0)    | -       | -           | -       | -       | -          | -       | -       | -              | -        | -        |              |                |         |
| LCNEC                     | 8 (2.9)     | 0 (0)    | -       | -           | -       | -       | -          | -       | -       | -              | -        | -        |              |                |         |
| SC                        | 2 (0.7)     | 0 (0)    | -       | -           | -       | -       | -          | -       | -       | -              | -        | -        |              |                |         |

Abbreviations: Amp = amplification; WHO = world health organization; AC = adenocarcinoma; SqCC = squamous-cell carcinoma; AdSq = adenosquamous carcinoma; LCC = large cell carcinoma; LCNEC = large cell neuroendocrine carcinoma; SC = sarcomatoid carcinoma

* p-values are based on Fisher’s exact test, two-sided
### Supplementary table 2

**Cox proportional hazards regression analysis for association of PD-L1 amplification status and 5-year overall survival in Uppsala 06 and Uppsala 95 cohorts.**

| Variable                   | Uppsala 06 cohort |               |               | Uppsala 95 cohort |               |               |
|----------------------------|-------------------|---------------|---------------|-------------------|---------------|---------------|
|                            | Total (n=310)     | AC (n=196)    | SqCC (n=94)   | Total (n=224)     |               |               |
| **PD-L1 status, UVA**      |                   |               |               |                   |               |               |
| wt                         |                   |               |               |                   |               |               |
| amplified                  | 0.62 (0.25-1.51)  | 0.289         | 0.63 (0.20-1.99) | 0.430             | 0.65 (0.16-2.71) | 0.557         |
| polysomy                   | 0.68 (0.43-1.80)  | 0.730         | 0.82 (0.33-2.02) | 0.668             | 1.03 (0.32-3.35) | 0.962         |
| **PD-L1 status, MVA**      |                   |               |               |                   |               |               |
| wt                         |                   |               |               |                   |               |               |
| amplified                  | 0.65 (0.27-1.59)  | 0.344         | 0.77 (0.24-2.46) | 0.659             | 0.60 (0.14-2.51) | 0.481         |
| polysomy                   | 0.73 (0.36-1.51)  | 0.403         | 0.74 (0.30-1.83) | 0.507             | 0.69 (0.20-2.33) | 0.546         |
| **Age, years:**            |                   |               |               |                   |               |               |
| <70                        |                   |               |               |                   |               |               |
|                           | 0.91 (0.65-1.27)  | 0.569         | 0.94 (0.62-1.42) | 0.754             | 0.59 (0.30-1.16) | 0.125         |
| >70                        |                   |               |               |                   |               |               |
|                           | 1.37 (0.99-1.91)  | 0.058         | 1.21 (0.80-1.83) | 0.372             | 1.61 (0.82-3.14) | 0.166         |
| **Gender:**                |                   |               |               |                   |               |               |
| female                     |                   |               |               |                   |               |               |
|                           | 1.37 (0.99-1.91)  | 0.058         | 1.21 (0.80-1.83) | 0.372             | 1.61 (0.82-3.14) | 0.166         |
| male                       |                   |               |               |                   |               |               |
|                           | 1.37 (0.99-1.91)  | 0.058         | 1.21 (0.80-1.83) | 0.372             | 1.61 (0.82-3.14) | 0.166         |
| **Stage (TNM):**           |                   |               |               |                   |               |               |
| Ia-Ib                      | 2.08 (1.5-2.89)   | <0.001        | 2.64 (1.74-4.00) | <0.001           | 1.55 (0.82-2.93) | 0.181         |
| II-IV                      |                   |               |               |                   |               |               |
|                           | 1.38 (0.81-2.54)  | 0.222         | 1.44 (0.96-2.12) | 0.082             | 1.81 (0.94-3.49) | 0.078         |
| WHO performance score:     |                   |               |               |                   |               |               |
| 0                          |                   |               |               |                   |               |               |
|                           | 1.38 (0.81-2.54)  | 0.222         | 1.44 (0.96-2.12) | 0.082             | 1.81 (0.94-3.49) | 0.078         |
| 1-3                        |                   |               |               |                   |               |               |
|                           | 1.41 (1.01-1.96)  | 0.043         | 3.01 (1.25-7.65) | 0.015             | 0.37 (0.11-1.28) | 0.117         |
| **Smoker:**                |                   |               |               |                   |               |               |
| never                      |                   |               |               |                   |               |               |
|                           | 1.41 (1.01-1.96)  | 0.043         | 3.01 (1.25-7.65) | 0.015             | 0.37 (0.11-1.28) | 0.117         |
| Former/current             |                   |               |               |                   |               |               |
|                           | 1.41 (1.01-1.96)  | 0.043         | 3.01 (1.25-7.65) | 0.015             | 0.37 (0.11-1.28) | 0.117         |

**Abbreviations:** HR = hazard ratio; CI = confidence interval; UVA = univariable analysis; MVA = multivariable analysis; AC = adenocarcinoma; SqCC = squamous-cell carcinoma; WHO = World Health Organization

* P-values are based on Wald test
Supplementary table 3:

Differentially expressed genes (false discovery rate p-adjusted <0.05) of PD-L1 amplified cases within the adenocarcinoma subtype of the Uppsala 06 cohort.

| SYMBOL    | GENENAME                                                                 | Base Mean | log2 Fold Change | p-value | p-adj  |
|-----------|---------------------------------------------------------------------------|-----------|------------------|---------|--------|
| ETNPPL    | ethanolamine-phosphate phospho-lyase                                      | 69,23     | -23,84           | 0.0000  | 0.0000 |
| DSCAM-AS1 | DSCAM antisense RNA 1                                                     | 109,10    | -24,48           | 0.0000  | 0.0000 |
| FABP7     | fatty acid binding protein 7                                              | 229,50    | 7,57             | 0.0000  | 0.0000 |
| SNX31     | sorting nexin 31                                                         | 20,21     | 4,90             | 0.0000  | 0.0000 |
| CPS1      | carbamoyl-phosphate synthase 1                                           | 14534,93  | -5,69            | 0.0000  | 0.0020 |
| SSC4D     | scavenger receptor cysteine rich family member with 4 domains            | 84,79     | 2,52             | 0.0000  | 0.0020 |
| MUC13     | mucin 13, cell surface associated                                         | 2802,00   | -5,43            | 0.0000  | 0.0036 |
| TESC      | tescalcin                                                                | 1851,99   | -3,60            | 0.0000  | 0.0061 |
| COL25A1   | collagen type XXV alpha 1 chain                                          | 596,33    | -4,89            | 0.0000  | 0.0099 |
| PAH       | phenylalanine hydroxylase                                                | 305,05    | -5,03            | 0.0000  | 0.0101 |
| ARL14     | ADP ribosylation factor like GTPase 14                                   | 124,99    | -4,37            | 0.0000  | 0.0101 |
| STARD3    | Star related lipid transfer domain containing 3                          | 2199,24   | 0,99             | 0.0000  | 0.0107 |
| SMS       | spermine synthase                                                        | 1721,00   | 1,13             | 0.0000  | 0.0133 |
| ASCL1     | achaete-scute family bHLH transcription factor 1                          | 420,63    | -6,39            | 0.0000  | 0.0133 |
| LRAT      | lecithin retinol acyltransferase                                         | 205,16    | -2,57            | 0.0000  | 0.0140 |
| CALCA     | calcitonin related polypeptide alpha                                     | 1304,42   | -6,81            | 0.0000  | 0.0153 |
| CTNND2    | catenin delta 2                                                          | 475,09    | -4,82            | 0.0000  | 0.0153 |
| BARX1     | BARX homeobox 1                                                          | 147,18    | -6,24            | 0.0000  | 0.0161 |
| TFPI2     | tissue factor pathway inhibitor 2                                         | 6597,47   | -3,78            | 0.0000  | 0.0178 |
| FGB       | fibrinogen beta chain                                                    | 3677,79   | -6,56            | 0.0000  | 0.0178 |
| G5k       | glyceral kinase 5                                                         | 1637,82   | 1,17             | 0.0000  | 0.0178 |
| SYT13     | synaptotagmin 13                                                         | 583,13    | -4,98            | 0.0000  | 0.0197 |
| LINCO0319 | long intergenic non-protein coding RNA 319                               | 89,19     | -2,70            | 0.0000  | 0.0239 |
| CHGB      | chromogranin B                                                           | 663,52    | -4,52            | 0.0000  | 0.0329 |
| NOL4      | nucleolar protein 4                                                       | 59,24     | -4,35            | 0.0000  | 0.0335 |
| NCKAP5-AS2| NCKAP5 antisense RNA 2                                                    | 19,05     | -3,72            | 0.0000  | 0.0460 |
| ZP1       | zona pellucida glycoprotein 1                                            | 15,10     | 2,60             | 0.001    | 0.0481 |
Supplementary table 4:

Differentially expressed genes (false discovery rate p-adjusted < 0.05) of PD-L1 amplified cases within the squamous cell carcinoma subtype of the Uppsala 06.

| SYMBOL | GENENAME                                           | Base Mean | log2 Fold Change | p-value | p-adj  |
|--------|----------------------------------------------------|-----------|------------------|---------|--------|
| RCL1   | RNA terminal phosphate cyclase like 1             | 1440.42   | 2.14             | 0.0000  | 0.0000 |
| SOX1   | SRY-box transcription factor 1                    | 30.60     | -22.21           | 0.0000  | 0.0000 |
| PLPP6  | phospholipid phosphatase 6                        | 919.97    | 2.17             | 0.0000  | 0.0000 |
| DLK1   | delta like non-canonical Notch ligand 1           | 56.07     | -22.81           | 0.0000  | 0.0000 |
| AK3    | adenylate kinase 3                                | 5296.48   | 1.85             | 0.0000  | 0.0000 |
| CDC37L1| cell division cycle 37 like 1                     | 1413.59   | 1.76             | 0.0000  | 0.0000 |
| GLDC   | glycine decarboxylase                             | 613.14    | 4.91             | 0.0000  | 0.0000 |
| RIC1   | RIC1 homolog, Rab6A GEF complex partner 1         | 4138.46   | 1.47             | 0.0000  | 0.0000 |
| PRB4   | proline rich protein BstNI subfamily 4            | 24.42     | -21.90           | 0.0000  | 0.0000 |
| PLGRKT | plasminogen receptor with a C-terminal lysine     | 1174.06   | 1.76             | 0.0000  | 0.0000 |
| PUM3   | pumilio RNA binding family member 3               | 3300.58   | 1.59             | 0.0000  | 0.0000 |
| KDM4C  | lysine demethylase 4C                             | 2544.03   | 1.37             | 0.0000  | 0.0000 |
| DMAC1  | distal membrane arm assembly complex 1            | 926.28    | 1.39             | 0.0000  | 0.0000 |
| LINCO0665 | long intergenic non-protein coding RNA 665      | 1058.57   | -2.59            | 0.0000  | 0.0000 |
| UHRF2  | ubiquitin like with PHD and ring finger domains 2 | 4813.49   | 1.20             | 0.0000  | 0.0000 |
| RANBP6 | RAN binding protein 6                             | 2949.62   | 1.48             | 0.0000  | 0.0000 |
| OTOF   | otoferlin                                         | 44.25     | 2.98             | 0.0000  | 0.0008 |
| SPATA6L| spermatogenesis associated 6 like                 | 236.22    | 1.86             | 0.0000  | 0.0009 |
| SNORD11| small nucleolar RNA, C/D box 11                   | 9.11      | 2.52             | 0.0000  | 0.0012 |
| CSDE1  | cold shock domain containing E1                  | 42738.74  | -0.87            | 0.0000  | 0.0038 |
| SCIN   | scinderin                                         | 837.52    | 2.84             | 0.0000  | 0.0044 |
| ZNF829 | zinc finger protein 829                           | 282.86    | -1.82            | 0.0000  | 0.0044 |
| ZNF703 | zinc finger protein 703                           | 1101.67   | -1.91            | 0.0000  | 0.0163 |
| KIAA2026| KIAA2026                                          | 3345.84   | 1.09             | 0.0000  | 0.0183 |
| ARHGGEF5| Rho guanine nucleotide exchange factor 5       | 1100.41   | 1.30             | 0.0000  | 0.0190 |
| THEGL  | theg spermatid protein like                       | 5.98      | -5.05            | 0.0000  | 0.0191 |
| JAK2   | Janus kinase 2                                    | 3061.45   | 1.39             | 0.0000  | 0.0211 |
| ZNF212 | zinc finger protein 212                           | 929.60    | 0.85             | 0.0000  | 0.0252 |
| ZNF420 | zinc finger protein 420                           | 578.81    | -1.17            | 0.0000  | 0.0253 |
| ZFP30  | ZFP30 zinc finger protein                         | 741.18    | -1.11            | 0.0000  | 0.0269 |
| TACR1  | tachykinin receptor 1                             | 88.66     | 3.10             | 0.0000  | 0.0294 |
| CD274  | CD274 molecule                                    | 3337.90   | 2.30             | 0.0000  | 0.0326 |
| ZNF568 | zinc finger protein 568                           | 441.23    | -1.28            | 0.0001  | 0.0340 |
| SULT1E1| sulfotransferase family 1E member 1               | 528.12    | -4.35            | 0.0001  | 0.0344 |
| CXCL5  | C-X-C motif chemokine ligand 5                    | 1157.49   | -2.89            | 0.0001  | 0.0344 |
| LINCO0863 | long intergenic non-protein coding RNA 863     | 81.26     | -1.27            | 0.0001  | 0.0375 |
| IGFL3  | IGF like family member 3                          | 40.23     | -5.34            | 0.0001  | 0.0461 |
| NGF    | nerve growth factor                               | 36.70     | -2.85            | 0.0001  | 0.0484 |
| IL33   | interleukin 33                                    | 4349.46   | 2.26             | 0.0001  | 0.0491 |
| AMTN   | amelotin                                          | 2641.54   | -5.71            | 0.0001  | 0.0491 |
Supplementary Figure 1: Example images of FISH analysis for PD-L1 amplification. In normal interphase nuclei, two orange (D9Z3 at 9q12) and two green signals (CD274,PDCD1LG2 gene cluster at 9p24.1) are detected. In cells with amplification of the CD274,PDCD1LG2 gene cluster, multiple copies of the green signal or large green signal clusters are observed. Cases were defined as polysome with PD-L1 gene copy number gain when the ratios of PD-L1/centrosome 9 were 1 and there were more than two copies of PD-L1 and centrosome 9 within a single tumor cell. PD-L1 was considered as amplified, when the ratios of PD-L1 signals to centrosome 9 signals in the tumor cells were ≥ 2.
Supplementary Figure 2: (A) Percentual distribution of PD-L1 amplified cases and cases with PD-L1 polysomy among all included NSCLC patients as well as separately within the adenocarcinoma (AC) and squamous cell carcinoma (SqCC) tumor subgroups of the Uppsala 95 cohort. (B and C) Association of the PD-L1 amplification status with (B) PD-L1 mRNA expression (array-based) and (C) percentage of PD-L-positive tumor cells on protein level as determined by IHC. Box plots indicate the median with the interquartile range and the whiskers represent the 5-95 percentiles. Outliners are represented as dots. P-values are based on Kruskal-Wallis test (two-sided) with Dunn’s correction for multiple testing. *** p<0.001
Supplementary Figure 3: Correlation between the PD-L1 amplification ratio and PD-L1 mRNA expression (as FPKM for Uppsala 06 cohort and as relative expression in abbreviated units for Uppsala 95 cohort) and percentage of PD-L-positive tumor cells on protein level as determined by IHC. Spearman’s rank correlation coefficient $r$ with 95% confidence interval (CI) and two-sided $p$-value are stated as well as the number of samples included in the test ($n$).
Supplementary Figure 4: Uppsala 06 cohort; Association of the PD-L1 amplification status (black) with mutation status of 82 genes (blue) represented as binary heat map for adenocarcinoma (AC) and squamous-cell carcinoma (SqCC) tumor subgroups separately. For
the *KRAS, EGFR, PIK3CA, NRAS, BRAF, ERBB2* and *MET* genes only activating driver mutations were considered in the analysis.

**Supplementary Figure 5**: Hierarchical cluster analysis using Ward’s method and Euclidian distance of the IHC based immune marker annotation and its association with PD-L1 amplification status within the adenocarcinoma (AC) and squamous cell carcinoma (SqCC) tumor subgroups of the *Uppsala 95 cohort*. 
Supplementary Figure 6: Hierarchical cluster analysis using Ward’s method and Euclidian distance of genes related to immune exhaustion as well as HLA expression and their association with PD-L1 amplification status, immune cluster and tumor histology (AC = adenocarcinoma; SqCC = squamous cell carcinoma) within the Uppsala 06 cohort. Immune clusters are defined based on main Figure 3.