Supplementary Material

A transcriptome-based classifier to identify developmental toxicants by stem cell testing: design, validation, and optimization for histone deacetylase inhibitors

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| Compound          | Molecular mechanism                                                                 | Human evidence for DNT | Reference relevant to DT/DNT |
|-------------------|-------------------------------------------------------------------------------------|------------------------|-------------------------------|
| MeHg              | Oxidative stress, ROS, lipoperoxidation, GSH inhibition, Disruption of Ca homeostasis, Inhibition of protein synthesis, Apoptosis, Microtubule disruption, Mitochondria toxicity, Mitotic arrest, Reaction with DNA and RNA | +                       | [1-15]                        |
| Thimerosal        | Mitochondria toxicity, Reduced oxidative reduction activity, Oxidative stress, Lipid peroxidation, Protein alkylation, Microtubuli disassembly, inhibition of tubuli assembly, Cellular degeneration, Oxidative stress | -                       | [16-22]                       |
| HgBr₂ / HgCl₂     | Cytoskeletal disassembly, tubulin degradation, Disruption of Ca homeostasis, Apoptosis, cell shrinking, chromosome condensation, Mitochondrial dysfunction, Necrosis, Oxidative stress | -                       | [23-30]                       |
| PMA               | ATPase inhibition, Peptidases / proteases inhibition                                  | -                       | [31-33]                       |
| PCMBBA            | Matrix metalloprotease, cleavage and activation                                       | -                       | [34]                          |

**Rempel et al., 2015, Supplementary Fig. S1**

**Suppl. Fig. S1: Literature data on toxicological effects of mercurials relevant for developmental toxicity (DT) or developmental neurotoxicity (DNT).** The six mercurials (methylmercury, thimerosal, mercury(II)chloride, mercury(II)bromide, 4-chloromercuribenzoic acid and phenylmercuric acid), which were used in the present study have one mercury atom in common. A literature research was performed for mechanism relevant for DT and DNT. The heterogeneous mode of action and the potential to cause DNT of the respective mercurials is listed.

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Rempel et al., 2015, Supplementary Fig. S2

Suppl. Fig. S2: Determination of the benchmark concentration (BMC) used for toxicant testing on microarrays. Compounds (here exemplified for the HDACi SAHA and the mercurial thimerosal) were tested at multiple dilutions in the standard UKN1 setup for 6 days. On DoD6, the viability was measured by a resazurin reduction assay, and data were normalized to untreated control samples. Experiments were performed in 3-4 cell preparations (each indicated by a different colour of data point and curves). An average curve (black) was calculated to determine the concentration at which viability was reduced to 90% (=BMC). Then the lower 95% confidence interval of this concentration was calculated (=BMCL). This value was used for all compounds to obtain microarray data (and is referred to as “BMC” in the text).
### A

**Genes up-regulated by x HDACi**

| x = 4 | 5 | 6 |
|-------|---|---|
| 73    | 32 | 16 |
| 58    | 41 | 13 |
| 42    | 29 | 12 |
| 22    | 28 | 16 |
| 3     | 12 | 6  |
| 0     | 1  | 1  |
| 0     | 0  | 0  |

**Sum:** 198 143 64

### B

**Genes down-regulated by x HDACi**

| x = 4 | 5 | 6 |
|-------|---|---|
| 22    | 6 | 2 |
| 53    | 14 | 6 |
| 13    | 13 | 3 |
| 33    | 14 | 4 |
| 3     | 2  | 2 |
| 0     | 0  | 0 |
| 0     | 0  | 0 |

**Sum:** 124 49 17

### C

**Genes regulated by mercurials**

| x = 3 | 4 | 5 | 6 |
|-------|---|---|---|
| UP    | 184 | 51 | 2 | 0 |
| DOWN  | 177 | 12 | 0 | 0 |

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**Rempel et al., 2015, Supplementary Fig. S3**

**Suppl. Fig. S3: Identification of HDACi consensus genes.** The UKN1 test system was exposed to 6 HDACi and 6 mercurials (as in Fig. 1) and DEG were identified (see Fig 2B). (A) The genes that were up-regulated by 4, 5, or 6 HDACi were counted (see “sum” line). The gene lists were further differentiated as to whether genes did not affect any mercurial (top line) or they were affected by the indicated numbers of mercurials. (B) The same procedure as in A was applied to down-regulated genes. (C) For a complete overview, the genes up- or down-regulated by at least 3 mercurials were compiled. For the gene list see suppl. Table S2.
Suppl. Fig. S4: Transcription factor binding sites (TFBS) overrepresented amongst up- and down-regulated HDACi consensus genes. The UKN1 test system was exposed to 6 HDACi (as in Fig. 1) and amongst the DEG, up- and down-regulated HDACi consensus genes were identified (see Fig. S3). The overrepresented TFBS in these sets of genes were determined by using oPOSSUM web tool and JASPAR database.
Supplementary Fig. S5: Visualization of a transcription factor (TF) network. (A) The CellNet database (2839 microarray sets from all major tissues) was used to construct a generic human TF network, based on statistical co-expression information and graph-theoretical design principles. Each node (n=1000) represents a TF gene, and each edge suggests co-regulation. The edge lengths is driven by the number of edges on neighbouring nodes, not by the strength of co-regulation. Nodes are placed according to the Fruchterman-Reingold algorithm. Clusters (coded by same colours) were defined by an optimization algorithm that tries to maximize the modularity of the division of the graph into clusters. Then GO term overrepresentation analysis was performed for each cluster to identify its biological role, and naming of the 16 clusters is based on these findings. The figure is similar to Fig. 4A, but it allow better reading of all node names.

Rempel et al., 2015, Supplementary Fig. S5
Suppl. Fig. S6: Identification of transcription factors (TF) with HDACi consensus genes enriched among targets. An enrichment analysis was performed using the interaction scores generated by the CLR algorithm. For each TF, the scores with the HDACi consensus genes were compared to the distribution of the scores of all genes using Wilcoxon's rank-sum test. TFs with an enrichment p-value less than $10^{-20}$ were marked red in the network.
Rempel et al., 2015, Supplementary Fig. S7

Suppl. Fig. S7: Transcriptome-based 10 PS classifier. To calculate classifier value the fold change (FC) of each PS is multiplied with the weighting coefficient $\gamma$ and summed up over all classifier components (i = 1-8 for 10 PS classifier as shown, i = 1-100 for 100 PS classifier). The offset 0.896 is added.

$$x = \sum_{i=1}^{8} (\gamma_i \cdot FC_i) + 0.896$$

Afterwards, a logistic transformation was applied. $logit(x) = \frac{1}{1+exp(Ax+B)}$

with A = -2.57 and B = 0.03 (For 100 PS classifier: A = -2.41; B = -0.12). The result is the probability of a compound to represent an HDACi. For the genes constituting the minimal HDACi classifier (10 PS, corresponding to 8 genes), the regulation (of all 6 HDACi) and the weighting factor are listed.

| Minimal HDACi classifier gene | Belinostat | Entinostat | Panobinost | SAHA  | TSA  | VPA  | weight factor $\gamma$ | Reference relevant to role and function (see also Fig. 7C) |
|-------------------------------|------------|------------|------------|-------|------|------|------------------------|--------------------------------------------------|
| FZRL2                         | 0.69       | 0.17       | 0.27       | 0.48  | 0.25 | 0.10 | 0.262                  | 1                                                 |
| TFAP2B                        | 3.79       | 1.26       | 17.08      | 8.90  | 51.84 | 5.17 | -0.102                 | 2                                                 |
| EDNRA                         | 2.44       | 0.95       | 8.69       | 7.67  | 60.13 | 9.90 | -0.014                 | 3                                                 |
| FOXD3                         | 6.44       | 3.46       | 17.99      | 7.23  | 33.41 | 4.02 | -0.26                  | 4                                                 |
| SIX3                          | 0.46       | 0.25       | 0.11       | 0.33  | 0.05  | 0.06 | 0.086                  | 5                                                 |
| MT1E                          | 0.83       | 1.26       | 1.26       | 0.92  | 1.04  | 3.12 | 0.136                  | 6                                                 |
| ETS1                          | 0.13       | 1.29       | 11.24      | 5.37  | 43.90 | 5.40 | -0.04                  | 7                                                 |
| LHX2                          | 0.45       | 0.15       | 0.14       | 0.27  | 0.04  | 0.14 | 0.094                  | 8                                                 |

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| ID     | Symbol | Weights γ | ID     | Symbol | Weights γ |
|--------|--------|-----------|--------|--------|-----------|
| 230147 | F2RL2  | 2.12      | 206588 | DAZL   | 5.17      |
| 214451 | TFAP2B | -2.17     | 236359 | SCN4B  | -1.72     |
| 204463 | EDNRA  | -1.06     | 204745 | MT1G   | 0.69      |
| 204464 | EDNRA  | -1.61     | 230895 | HAPLN1 | -1.25     |
| 155399 | TFAP2B | -2.66     | 230204 | HAPLN1 | -1.36     |
| 241612 | FOXD3  | -4.18     | 205113 | NEFM   | 0.76      |
| 242054 | SIX3   | 1.94      | 228875 | FAM162B| -5.85     |
| 212859 | MT1E   | 1.54      | 214265 | ITGA8  | 0.07      |
| 224833 | ETS1   | -2.64     | 235666 | ITGA8  | -0.51     |
| 206140 | LHX2   | 1.33      | 219179 | DACT1  | -2.37     |
| 208581 | MT1X   | 0.998     | 201468 | NQO1   | 2.82      |
| 206461 | MT1H   | 1.07      | 237056 | INSC   | -1.57     |
| 21139  | SNAI2  | -1.39     | 1555800| ZNF385B| 0.63      |
| 229125 | KANK4  | -1.47     | 206018 | FOXG1  | -1.52     |
| 238878 | ARX    | 0.1       | 209199 | MEF2C  | -0.71     |
| 221086 | FEZF2  | 1.22      | 205430 | BMP5   | 2.44      |
| 204326 | MT1X   | 2.82      | 215729 | VGLL1  | 1.13      |
| 203789 | SEMA3C | 0.0657    | 228658 | MIAT   | 2.89      |
| 209160 | AKR1C3 | 3.03      | 1555414| C6orf141| 0.592    |
| 220184 | NANOG  | -10.3     | 209200 | MEF2C  | -0.75     |
| 217165 | MT1F   | 1.37      | 208096 | COL21A1| -0.083    |
| 212185 | MT2A   | 1.69      | 211456 | MT1H1  | 1.53      |
| 216336 | NA     | 0.631     | 219054 | NPR3   | -0.845    |
| 227238 | MUC15  | -1.23     | 1556378| LOC440896| 0.755   |
| 233972 | FEZF2  | 0.695     | 210524 | NA     | 2.06      |
| 210302 | MAB21L2| 1.18      | 201012 | ANXA1  | 1.48      |
| 226213 | ERBB3  | -0.835    | 237435 | NA     | -1.5      |
| 204653 | TFAP2A | -4.28     | 243611 | MICALCL| -7.71     |
| 209875 | SPP1   | 1.28      | 206029 | ANKRD1 | -0.257    |
| 239205 | NA     | -7        | 230493 | SHISA2 | 0.722     |
| 204273 | EDNRB  | -2.71     | 230008 | THSD7A | 2.17      |
| 203665 | HMOX1  | -1.19     | 237322 | MIAT   | 4.29      |
| 206634 | SIX3   | 3.03      | 210729 | NPY2R  | 0.902     |
| 1555801| ZNF385B| 1.06      | 204932 | TNFRSF11B| -2.57    |
| 205523 | HAPLN1 | -0.91     | 229004 | ADAMTS15| -3.8     |
| 243555 | NA     | -1.54     | 210519 | NQO1   | 3.77      |
| 223044 | SLC40A1| -1.08     | 205923 | RELN   | -1.04     |
| 206801 | NPPB   | 3.02      | 228367 | ALPK2  | 2.93      |
| 240055 | NA     | -0.423    | 219197 | SCUBE2 | -0.141    |
| 214920 | THSD7A | 0.92      | 219058 | ME1    | 4.33      |
| 228347 | SIX1   | -0.744    | 227241 | MUC15  | 1.47      |
| 221950 | EMX2   | -1.53     | 242193 | MIR124-2HG| 0.228   |
| 202454 | ERBB3  | -2.41     | 204112 | HNMT   | 1.75      |
| 213629 | MT1F   | 1.3       | 209735 | ABCG2  | 1.27      |
| 209723 | SERPINB9| -4.99    | 216235 | EDNRA  | -3.25     |
| 213894 | THSD7A | -0.888    | 210303 | MAB21L2| 4.66      |
| 1552521| TMEM74 | 0.6       | 1561101| JAKMIP2-AS1| -3.27  |
| 1554012| RSP02  | 1.52      | 237449 | SP8    | 0.75      |
| 221245 | FZD5   | 1.55      | 203324 | CAVE2  | 2.74      |
| 213943 | TWIST1 | -0.0731   | 205286 | TFAP2C | -5.4      |

**Rempel et al., 2015, Supplementary Fig. S8**

**Suppl. Fig. S8: Transcriptome-based 100 PS classifier.** To calculate the classifier value, the foldchange of each PS is multiplied with the respective weighting coefficient $\gamma (=\text{weight/100})$, summed up and further processed as described in suppl. Fig. S7.
Suppl. Fig. S9: Regulation of tissue specific transcription factors (TF). (A) Tissue-specific TF were determined as follows. First, the CellNet tissue data was used to calculate the average expression of each TF in each tissue. Subsequently, the average expression was binned into 10 quantiles from 0 (lowest) to 9 (highest). To determine TF with preferential expression in one tissue, we computed the tissue specificity index \( t \) proposed in (Yanai et al. 2005).

\[
t_j = \frac{\sum_{i=1}^{N} 1 - x_{ij}/\text{max}_i(x_{ij})}{N-1}
\]

In the above formula, \( i \) runs over all tissues, \( N \) is the number of tissues, \( x_{ij} \) is the expression quantile of TF \( j \) in tissue \( i \). The top 50% TF with the highest specificity index \( t \) were then assigned to the tissue in which they were expressed most highly. This resulted in 671 TF with ‘tissue specific expression’. The absolute number for five tissues are listed.

(B) The percentage of tissue specific TF, which were up-regulated at DoD6 vs DoD0 in untreated cells was determined. (C) The percentage of tissue-specific TF found in up- and down-regulated HDACi consensus genes was counted.

| Tissue      | Number (N) | Percentage of tissue-specific TF up-regulated at DoD6 vs DoD0 in untreated cells | Percentage of tissue-specific TF found in HDACi consensus genes |
|-------------|------------|--------------------------------------------------------------------------------|-----------------------------------------------------------------|
| Neuron      | 48         | 17% (1)                                                                        | 3% (4)                                                          |
| ESC         | 96         | 13% (2)                                                                        | 11% (1)                                                         |
| Colon       | 31         | 7% (4)                                                                         | 4% (2)                                                          |
| Ovary       | 85         | 12% (3)                                                                        | 4% (2)                                                          |
| MuscleSkel  | 104        | 4% (5)                                                                         | 3% (4)                                                          |

Numbers in brackets indicate rank position in list

Rempel et al., 2015, Supplementary Fig. S9