Online supplement for Bierer et al., Intergenerational Effects of Maternal Holocaust Exposure on *FKBP5* Methylation. Am J Psychiatry (doi: 10.1176/appi.ajp.2019.19060618)

**Supplemental Methods**

***FKBP5* methylation**: *FKBP5* methylation at intron 7, site 6 was determined by pyrosequencing, using sodium bisulfite mapping in three separate batches. ***Batch 1 (Sample 1a/Study 1)***: Methylation determination was based on genomic DNA extracted from whole blood and isolated using the Gentra Puregene Kit (Qiagen, Valencia, California) (1). DNA was bisulfite-treated using the EZ-Gold Kit (Zymo Research, Irvine, California), and methylation analysis was performed at Varionostic GmbH (Ulm, Germany) using a Q24 System with PyroMark Q24 analysis software (Qiagen) (2). The following primers were used for amplification: 5'-GTTGTTTTTGGAATTTAAGGTAATTG-3' and reversed 5'-biotin-TCTCTTACCTCCAACACTA CTACTAAAA-3' and for pyrosequencing at site 6: 5'-GTTGATATATAGGAATAAAATAAGA-3'. Specific annotation for site 6 is provided in (1). ***Batch 2 (Sample 1b/Study 1)***: Samples were assayed using the same primers as in Batch 1 in the laboratory of EBB at the Max Planck Institute of Psychiatry (MPI) on a PyroMark Q96 ID system using PyroMark Q96 ID software 2.5 (Qiagen). Cycling conditions were adjusted as follows: Polymerase: Kapa Ready Mix Uracil + (Kapa Biosystems), amplification protocol: 95°C – 5 min, 2x (98°C – 40s, 62°C – 30s, 72°C – 60s), 5x (98°C – 40s, 60°C – 30s, 72°C – 60s), 8x (98°C – 40s, 58°C – 30s, 72°C – 60s), 25x (98°C – 40s, 56°C – 30s, 72°C – 60s), 72°C - 1 min). For ***Batch 3 (Study 2)*** genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) and sent to EpigenDx (Buffalo, NY) for pyrosequencing and CpG methylation quantification using a similar protocol.

For analyses of methylation data derived from whole blood (Study 1), a covariate was used to account for differences related to site of assay and specific instrumentation, although no significant difference was observed in methylation by site (Varionostic: 53.92% ± 0.77%; MPI: 54.89% ± 0.52%; $F_{(1,90)}$ = 1.08, *ns*, controlling for age, gender, BMI, and control-offspring group). For analyses based on combined samples from Studies 1 and 2, an additional covariate was introduced to account for lower methylation

values derived from whole blood vs. PBMCs (whole blood: 54.45 ± 0.63, PBMCs: 77.01 ± 0.65; $F_{(1,181)}$ = 539.49, p<.0005, controlling for age, gender, BMI, and group). In addition to site 6, methylation was also assayed for CpG sites 3, 4 and 5 (described as 'bin 2' in our prior report (1) and in (2)).

***FKBP5* rs1360780 genotype**: For ***Batches 1 and 2***, rs1360780 genotype was determined at the MPI using a differential hybridization protocol on a LightCycler 480 (Roche, Basel, Switzerland), as previously described (1, 3, 4). For ***Batch 3***, genotype was assessed at Icahn School of Medicine at Mount Sinai (ISMMS) using Applied Biosystems TaqMan SNP Genotyping Assays and amplified on the GeneAmp PCR System 9700. Plates were scanned using the Applied Biosystems™ 7900HT Real Time PCR System and sample genotypes called with SDS software from Applied Biosystems. Genotypes of rs1360780 from both laboratories were in Hardy-Weinberg equilibrium (p<.05), and were grouped according to the presence of the PTSD-risk allele (TT/CT) and protective genotype (CC).

**RNA extraction:** RNA was extracted from Trizol-dissolved PBMCs (for ***Batch 3/Study 2 only***) using RNeasy Mini Kits (Qiagen) to isolate total RNA. RNA concentration was determined using a ND-1000 spectrophotometer (NanoDrop Technologies). RNA purity and quality were evaluated using the RNA 6000 Nano LabChip kit and 2100 Bioanalyzer (Agilent Technologies). Samples had high RNA quality with a mean RNA integrity number (RIN) of 9.37 ± 0.56 (SD).

***FKBP5* expression***: FKBP5* expression was determined (for ***Batch 3/Study 2 only***) by quantitative polymerase chain reaction as previously described (5). The primers and probes used for determination of RNA expression were designed based on the National Center for Biotechnology Information (NCBI) sequence (NM_004117.2) using the ProbeFinder v2.50 software (Roche, Madison, Wisconsin, http://www.roche-applied-science.com). For the *FKBP5* gene, the following mRNA input sequence was used: gttacacttaagagcttcgaaaaggccaaagaatcctgggagatggataccaaagaaaaattggagcaggctgc. The following sequences were also used, respectively, for GAPDH: agccacatcgctcagacaccatggggaaggtgaaggtcggagtcaacggatttggtcgtattgggc; for *ACTB:*

ccaaccgcgagaagatgacccagatcatgtttgagaccttcaacaccccagccatgtacgttgctatccaggctgtgctatccctgtacgcctctgg; for *BR2M*: ttctggcctggaggctatccagcgtactccaaagattcaggtttactcacgtcatccagcagagaatggaaagtcaaatttcctga, and for *RPLPO*: gatgcccagggaagacagggcgacctggaagtccaactacttccttaagatcatccaactattggatgattatccgaaatgtttcattgtgggagcag a. The resulting Universal Probe Library (UPL) probes were #14, #60, #64, #42, and #85. Data analysis was performed using qBase v2.5 (Biogazelle NV, Belgium) with *FKBP5* as the target gene and *GAPDH, ACTB, BR2M and RPLPO* as the house-keeping genes.

**Immune cell type differentiation and proportions:**

**a. Cibersort data**: White blood cell (WBC) composition was assessed in order to investigate whether cell type distribution was a potential confound for *FKBP5* methylation or gene expression measures. Using the same RNA samples from Study 2, cDNA microarray data (HumanHT-12 v4.0 Gene Expression BeadChip, Illumina) were generated and processed as previously described (5). The frequencies of PBMC cell types were estimated based on genome wide gene expression using CIBERSORT cell type de-convolution method at the default setting (6). Normalized expression data and the LM22 signature matrix were used as input, providing the frequencies of 22 immune cell-types. Three cell types were estimated at zero frequency; thus nineteen cell types were identified, and clustered into 10 groups for which means were calculated: B cells, plasma cells, T cells (CD4), T cells (other), natural killer cells, macrophages, dendritic cells, mast cells, eosinophils and neutrophils. Of these, three cell types (plasma cells, eosinophils, and neutrophils) showed only 1 non-zero count of 88 samples assayed and thus were not amenable to correlative analysis. Of the remaining 7 cell types, only B cells differed between Holocaust offspring and controls (controls: .008 ± .001; Holocaust offspring: .010 ± .000; $F_{(1, 84)} = 4.30$, p=.041). WBC differential data from Study 1 were not available.

Comparison of *FKBP5* gene expression between Holocasut offspring and controls was not substantively altered by additionally controlling for B-cells (without B-cell covariation: $F_{(1,85)} = 7.32$,p=.008;

with B-cell covariate: $F_{(1,82)} = 5.36$, p=.023). Likewise, site 6 and mean intron 7 methylation differences between offspring and controls were significant when correcting for B-cells, in addition to age, sex, and BMI (site 6: $F_{(1,82)} = 4.19$, p=.044; mean methylation: $F_{(1,82)} = 10.64$, p=.002). Thus, the difference in B-cell proportions do not account for group differences in expression or methylation.

**b. CLIA WBC differential:** In addition to Cibersort data, clinical white blood cell differential distributions were available, again only for subjects in Study 2. There were no significant differences in CLIA based WBC types between controls and Holocaust offspring, and there were no significant or nearly significant correlations between WBC cell-type and *FKBP5* gene expression.

**Neuroendocrine methods**: Blood samples were obtained at 08:00h on two consecutive mornings for the determination of basal plasma cortisol, and for cortisol and dexamethasone (DEX) levels the morning following ingestion of 0.50 mg DEX the previous night at 23:00h. A portion of the blood was processed for isolation of PBMC with Ficoll Hypaque as previsouly described (7). Lysozyme activity was assessed in PBMCs following the *in vitro* administration of increasing concentrations of DEX to wells containing 3.5-4.0 × $10^5$ cells (PBMCs) as previously described (8). Day 1 basal plasma cortisol, and Day 2 plasma cortisol and DEX were determined by radioimmunoassay. The intra- and inter-assay coefficients of variation were 2.3% and 6.1% for cortisol, 8.0% and 9.0% for DEX, respectively.

**Supplemental Results**

**Sample characteristics:** Descriptive characteristics were compared between Holocaust offspring (n=125) and controls (n=31) for subjects comprising the 'replication sample' (Studies 1b and 2 combined). Results were similar to those shown for the entire sample, however in this sample, the gender distribution differed significantly between controls (females: n=14 (45.2%)) and Holocaust offspring (females: n=86 (68.8%); $\chi^2 = 5.82$, df=1, p=.016). Additionally, in contrast to results for the entire sample, group differences were not significant for the following measures: lifetime psychiatric care (controls: 4

(12.9%), offspring: 39 (31.5%); $\chi^2$=2.56, df=1, *ns*), current anxiety disorder (controls: 7 (25.0%), offspring: (52 (43.2%); $\chi^2$=3.00, df=1, *ns*), lifetime PTSD (controls: 0 (0.0%), offspring: 7(5.6%); $\chi^2$=3.21, df=1, *ns*), maternal care and overprotection as measured on the Parental Bonding Instrument (PBI)(9) (controls: 3.99 ± 1.61, offspring: 2.26 ± 0.78 [mean ± SE]; F(1,136) = 0.91, *ns*; paternal care and overprotection (PBI) (controls: 4.99 ± 1.54, offspring: 2.15 ± 0.49; F(1,136) = 2.69, *ns*; depressive symptom severity as determined by the Beck Depression Inventory (BDI)(10) (controls: 6.88 ± 1.53, offspring: 10.25± 0.73; F(1,145) = 3.90, *ns*). In all other respects group comparisons were similar across the replication and total samples. The replication sample, excluding subjects on whom our original report was based (Study 1a) (1), was used only to test the difference in *FKBP5* site 6 methylation between offspring and controls, and thereby validate our previously reported finding (1).

***FKBP5* Intron 7 methylation for offspring and controls:** In addition to site 6, sites 3, 4, and 5 were examined. Methylation at sites 3 through 6, and the mean of these four intronic sites are compared between Holocaust offspring and controls, as presented in ***Supplemental Table 1*** (below)***,*** using the expanded model as described in the text. These data are provided to demonstrate that methylation at site 6 was singularly reduced in the Holocaust offspring, and that methylation at this site drives the mean methylation group difference. Mean methylation for sites 3 – 6 represents methylation of the larger functional intronic unit as previously described (2).

**Genotype dependent associations with childhood adversity and PTSD:** It should be noted that in this larger sample, the previous finding (1) of a *FKBP5* rs1360780 genotype-dependent association of site 3 methylation with childhood physical abuse was apparent at a trend level of significance, such that there is a significant correlation in carriers of the risk allele (r= - .261, df=72, p=.025), whereas in carriers of the protective genotype this is not the case (r=.019, df=86, *ns*; z=1.84, p=.066). Also consistent with prior results (2), there was a signficant allele-dependent association of methylation at site 3 with lifetime

PTSD (risk allele: r=-.395, df=76, p<.0005; protective allele: r=.048, df=89, *ns*; z=2.78, p=.005) whereas for site 6 these associations are not apparent (r=.094 and r= -.035, respectively).

**Table S1. Comparison of methylation at *FKBP5* intron 7 sites 3 through 6, between Holocaust offspring and controls.**

| | Controls (n=40) | Offspring (n=146) | ANCOVA (F, df, p) [pη²] [a] |
|---|---|---|---|
| ***Limited model*** [b] | | | |
| Site 3 | 86.81 ± 0.55 | 85.88 ± 0.29 | F (1,179) = 2.21, *ns* |
| Site 4 | 87.16 ± 0.50 | 86.84 ± 0.26 | F (1,179) = 0.32, *ns* |
| Site 5 | 78.68 ± 0.49 | 78.30 ± 0.25 | F (1,179) = 0.48, *ns* |
| Site 6 | 67.45 ± 0.93 | 64.86 ± 0.48 | **F (1,179) = 6.08, p=.015 [pη² = .033]** |
| Sites 3-6 (mean) | 80.03 ± 0.38 | 78.97 ± 0.20 | **F (1,179) = 6.12, p=.014 [pη² = .033]** |
| ***Expanded model*** [c] | | | |
| Site 3 | 86.83 ± 0.69 | 85.72 ± 0.31 | F (1,155) =1.95, *ns* |
| Site 4 | 86.58 ± 0.61 | 86.81 ± 0.28 | F (1,155) = 0.11, *ns* |
| Site 5 | 78.94 ± 0.59 | 78.50 ± 0.27 | F (1,155) = 0.43, *ns* |
| Site 6 | 69.02 ± 1.15 | 65.33 ± 0.52 | **F (1,155) = 7.81, p=.006 [pη² = .048]** |
| Sites 3-6 (mean) | 80.34 ± 0.46 | 79.09 ± 0.21 | **F (1,155) = 5.65, p=.019 [pη² = .035]** |

[a] $p\eta^2$ = partial eta squared, a measure of effect size, where small (~.01), medium (~.06), and large (≥ .14) effects are estimated; [b] covariates: age, sex, BMI, batch, study; [c] covariates: age, sex, BMI, batch, study, maternal PTSD, paternal PTSD, CTQ total score, lifetime MDD, lifetime anxiety disorder, lifetime PTSD, current psychotropic medication use, rs1360780 'risk' allele genotype.

***Table S2,*** below, demonstrates intercorrelations among intron 7 sites 3-6, and justifies the use of mean intron 7 methylation in examining correlations with expression and clinical variables.

**Table S2. Intercorrelations among *FKBP5* intron 7 CpG sites 3-6** [a]

| Intron 7 site | Assayed *FKBP5* intron 7 CpG sites | | | | |
|---|---|---|---|---|---|
| | **Site 3** | **Site 4** | **Site 5** | **Site 6** | **Site 3-6 (mean)** |
| **Site 3** | ● | **.200 (.007)** [a] | -.119, *ns* | .116, *ns* | **.469 (<.0005)** |
| **Site 4** | **.200 (.007)** [a] | ● | **.205 (.006)** | **.190 (.011)** | **.580 (<.0005)** |
| **Site 5** | -.119, *ns* | **.205 (.006)** | ● | **.215 (.004)** | .179, ns |
| **Site 6** | .116, *ns* | **.190 (.011)** | **.215 (.004)** | ● | **.792 (<.0005)** |
| **Site 3 - 6 (mean)** | **.469 (<.0005)** | **.580 (<.0005)** | .179, *ns* | **.792 (<.0005)** | ● |

[a]. Partial correlations, controlling for age, sex, BMI, batch, study and control-offpsring group.

**References:**

1. Yehuda R, Daskalakis NP, Bierer LM, Bader HN, Klengel T, Holsboer F, Binder EB. Holocaust Exposure Induced Intergenerational Effects on FKBP5 Methylation. Biological psychiatry. 2016;80:372-380.
2. Klengel T, Mehta D, Anacker C, Rex-Haffner M, Pruessner JC, Pariante CM, Pace TW, Mercer KB, Mayberg HS, Bradley B, Nemeroff CB, Holsboer F, Heim CM, Ressler KJ, Rein T, Binder EB. Allele-specific FKBP5 DNA demethylation mediates gene-childhood trauma interactions. Nat Neurosci. 2013;16:33-41.
3. Binder EB, Salyakina D, Lichtner P, Wochnik GM, Ising M, Putz B, Papiol S, Seaman S, Lucae S, Kohli MA, Nickel T, Kunzel HE, Fuchs B, Majer M, Pfennig A, Kern N, Brunner J, Modell S, Baghai T, Deiml T, Zill P, Bondy B, Rupprecht R, Messer T, Kohnlein O, Dabitz H, Bruckl T, Muller N, Pfister H, Lieb R, Mueller JC, Lohmussaar E, Strom TM, Bettecken T, Meitinger T, Uhr M, Rein T, Holsboer F, Muller-Myhsok B. Polymorphisms in FKBP5 are associated with increased recurrence of depressive episodes and rapid response to antidepressant treatment. Nat Genet. 2004;36:1319-1325.
4. Binder EB, Bradley RG, Liu W, Epstein MP, Deveau TC, Mercer KB, Tang Y, Gillespie CF, Heim CM, Nemeroff CB, Schwartz AC, Cubells JF, Ressler KJ. Association of FKBP5 polymorphisms and childhood abuse with risk of posttraumatic stress disorder symptoms in adults. JAMA. 2008;299:1291-1305.
5. Arloth J, Bogdan R, Weber P, Frishman G, Menke A, Wagner KV, Balsevich G, Schmidt MV, Karbalai N, Czamara D, Altmann A, Trumbach D, Wurst W, Mehta D, Uhr M, Klengel T, Erhardt A, Carey CE, Conley ED, Major Depressive Disorder Working Group of the Psychiatric Genomics C, Ruepp A, Muller-Myhsok B, Hariri AR, Binder EB, Major Depressive Disorder Working Group of the Psychiatric Genomics Consortium PGC. Genetic Differences in the Immediate Transcriptome Response to Stress Predict Risk-Related Brain Function and Psychiatric Disorders. Neuron. 2015;86:1189-1202.
6. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, Hoang CD, Diehn M, Alizadeh AA. Robust enumeration of cell subsets from tissue expression profiles. Nat Methods. 2015;12:453-457.
7. Yehuda R, Yang RK, Guo SL, Makotkine I, Singh B. Relationship between dexamethasone-inhibited lysozyme activity in peripheral mononuclear leukocytes and the cortisol and glucocorticoid receptor response to dexamethasone. J Psychiatr Res. 2003;37:471-477.
8. Yehuda R, Golier JA, Yang RK, Tischler L. Enhanced sensitivity to glucocorticoids in peripheral mononuclear leukocytes in posttraumatic stress disorder. Biol Psychiatry. 2004;55:1110-1116.
9. Parker G, Tupling H, Brown LB. A Parental Bonding Instrument. British Journal of Medical Psychology. 1979;52:1-10.
10. Beck AT, Ward CH, Mendelson M, Mock J, Erbaugh J. An inventory for measuring depression. Arch Gen Psychiatry. 1961;4:561-571.